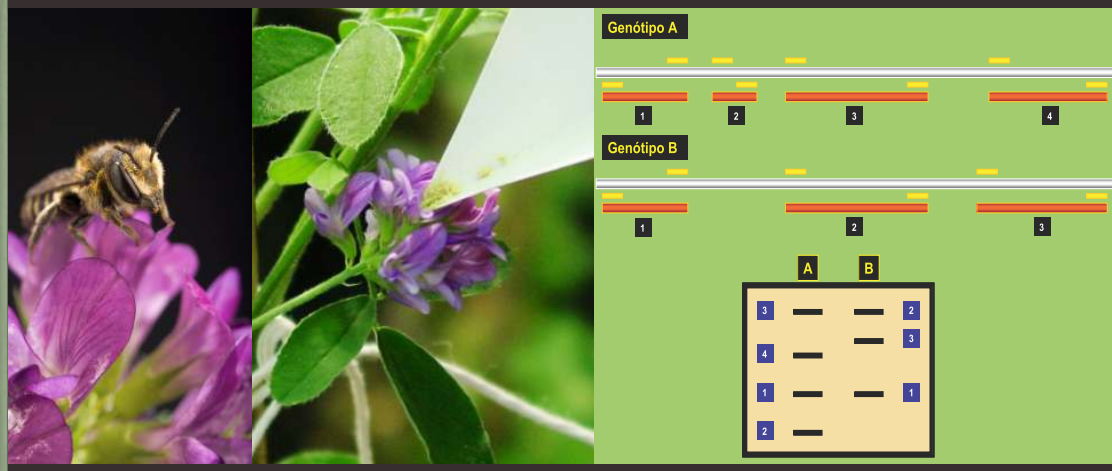
Genetic Improvement of Alfalfa



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Embrapa

Brazilian Agricultural Research Corporation
Embrapa Southeastern Livestock
Ministry of Agriculture and Livestock

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Foreword

Alfalfa, since it is an important forage, was improved to adapt to Brazilian conditions. A group of scientists from Embrapa, Brazilian universities, including Federal University of Lavras, Federal University of Viçosa, State University of Maringá, and Federal University of Rio Grande do Sul, and researchers from the National Agricultural Technology Institute of Argentina participated in this process.

Thus, this work demonstrates the importance of bringing together the competence of scientists from national and foreign universities and research institutions to strengthen the progress of both agriculture and Brazil. The result, therefore, shows the victory of the goodwill of organizations that work to modernize agriculture, with the aim of feeding the Brazilian people. It was also possible to produce surpluses that, through exports, help our economy and, consequently, the development of Brazil.

Since the beginning, Embrapa, with its Decentralized Units coordinated by its Headquarters, has sought partnerships with universities and research institutions to accelerate the modernization of our agriculture. The case of alfalfa is not different and shows how to involve an important group of researchers and professors in a complex endeavor, aiming to fight against primitivism and seek science and technology to leverage this important forage in Brazil.

Eliseu Roberto de Andrade Alves
Former President of Embrapa

Contents

CHAPTER 1

Origin, evolution and domestication of alfalfa

Maurício Marini Köpp

Introduction

Grown in almost all latitudes, alfalfa covers about 32.4 million hectares and is one of the most important forage plants because it combines special characteristics, such as high productivity, high protein content, good palatability, high digestibility, good capacity of fixing atmospheric nitrogen in the soil and low seasonality in forage production.

This forage plant has great value in animal feeding, in the form of hay, silage or dehydrated pellets for cattle and sheep, or incorporated into compound feed for monogastrics or under pasture, especially in Argentina and Australia.

It is very important for the researcher to know the geographic location of the crop's center of genetic variability. Much of a crop's genetic variability is present at its center of origin, or its center of diversity. Other important information is related to the evolution and domestication of the species. Domestication of cultivated crops is a type of evolution, which, however, is directed by man, and nowadays, with the knowledge available and the tools used, it is now called genetic improvement. Knowledge of the different forms and interactions of domestication of cultivated crops allows efficient planning for prospecting and sampling these genetic resources in order to preserve germplasm and/or use it in plant breeding programs.

Over half the domesticated or improved alfalfa varieties and populations come from the intercrossing of *Medicago sativa* ssp. *sativa* and *Medicago sativa* ssp. *falcata*. Because of the allogamy and the autotetraploid structure of the crops grown from this subspecies complex, great genetic diversity is found among populations from different geographic regions and also within these populations.

Genetic resources currently available comprise, on the one hand, officially described varieties and, on the other hand, wild populations, ecotypes and regional populations where the greatest genetic variability is found. The erosion of genetic diversity found essentially in cultivated plants leads to searching wild populations or subspontaneous populations for a source of supplementary variability that can be saved and later exploited by research, especially by genetic improvement. Thus, it is essential to have the greatest possible range of genetic variation for the studied species (center of origin or center of diversity). In a population under natural conditions, genetic diversity is the result of genetic drift, migration and mutations which occur as a function of adaptation to the environment and to chance (evolution). However, the variability of a population in relation to selected traits depends not only on these genetic events as a function of the environment, but also on man's influence (domestication).

Plant evolution

The principle of evolution postulates that species that lived and live on our planet weren't independently created, but they descend from each other, meaning that they are connected through evolutionary bonds. This transformation, called the evolution of species, was presented and satisfactorily explained by Charles Darwin, in his treatise *On the origin of species*, in 1859.

The basis of biological evolution is the existence of variability, in other words the individual differences among organisms of the same species (Allard, 1971). Most of the times, individuals produce a great number of descendents, of which only a few survive until adulthood. This means individuals are selected in nature according to their traits, and often less than 10% of the offspring survive. Individuals having traits which benefit their survival, such as: increased ability to obtain food, better reproductive efficiency and higher tolerance to biotic or abiotic stress, have a greater chance to survive until their reproductive age and pass these beneficial individual characteristics on to their offspring (Diamond, 2002). This happens because all the traits are imprinted into the genes of the individual (Allard, 1971; Grant, 1971). This is Darwin's principle of natural selection whereby traits of individuals tend to be altered over the generations, with the possibility of new species emerging (Ronzelli Júnior, 1996). Based on this theory, all kinship between living beings on Earth could be studied in terms of evolution, which has culminated in one genealogical tree of life, or phytogenetic tree (Martin; Embley, 2004).

All genetic information of living beings is recorded in DNA, the protein that composes genes and chromosomes. During the reproductive process, the replication of these genes undergoes alterations, called genetic mutations (Ronzelli Júnior, 1996; Wendel, 2000). When mutations started to occur in the first living beings on the planet, the process of evolution began through the emergence of the above-mentioned individual variations within the same species. Evolution is thus stimulated by the phenomenon of natural selection, over the hundreds of millennia of geological time (Grant, 1971). The history of the evolution of life is documented in the fossil record found by geologists and evolutionary biologists.

Pressure generated by the environment on living beings represents one of the main causes of evolution (Allard, 1971; Grant, 1971; Ronzelli Júnior, 1996). Natural environments usually have negative and limiting factors, in addition to difficult realities such as predation and competition. Hostile and unstable environments stimulate the evolutionary process, since they strongly select only the survival of the fittest. As a consequence of environmental pressure and

the existence of genetic mutations, life has evolved and specialized, creating the entire range of different biomes and ecosystems which constitute the biosphere (Ronzelli Júnior, 1996).

According to the theory of evolution, current species are descended from other species which have undergone modifications throughout the ages. The ancestors of present species are descendents of predecessors who were different from them, and so on, all the way back to extremely primitive unknown precursor organisms. The theory of evolution argues for a notion of transformation to explain the great diversity of life forms, and has abandoned the obsolete theory called "fixism", which claimed that the number of species is steady and that they do not undergo modifications (Martin; Embley, 2004).

The modern evolutionary (or neo-Darwinian) synthesis

This theory was formulated by several researchers through years of study, taking Darwin's notions on natural selection as a basis and incorporating knowledge of genetics. The most important individual contribution from genetics, extracted from Mendel's studies, replaced the old concept of inheritance through blood mixture with the concept of inheritance through particles, the genes (Mettler; Gregg, 1973).

The modern evolutionary (or neo-Darwinian) synthesis considers, as Darwin did, the population to be the unit of evolution. The population can be defined as a group of individuals of the same species, which occur within the same geographic area at the same time (Freire-Maia, 1988). To better understand this definition, it is important to understand the biological species concept: a group of natural populations, actually or potentially interbreeding and reproductively isolated from other groups of organisms. When we say potentially interbreeding in this definition, it means one species may have populations that do not naturally breed, because they are geographically apart. However, if they are artificially put in contact there will be breeding between the individuals, with fertile descendents and, therefore, they are potentially interbreeding. The biological definition of species is only valid for organisms with sexual reproduction, since in the case of organisms with asexual reproduction similarities among morphological characteristics define the groups as a species (Mettler; Gregg, 1973).

Understanding the genetic variability and phenotypic variability of individuals in one population is also essential for studying evolutionary phenomena, since evolution is actually the transformation of populations over time, or changes in gene frequency of these populations (Smart; Haq, 1997).

Factors which determine changes in gene frequency are called evolutionary factors. Each population has a gene pool which can be altered, subject to evolutionary factors. Evolutionary factors acting on the gene pool of the population can be grouped into two categories (Mettler; Gregg, 1973; Bueno et al., 2006):

- Factors which tend to increase the genetic variability of the population – gene mutation, chromosome mutation and recombination.
- Factors acting on already established gene variability – natural selection and reproductive isolation.

Thus, according to the modern evolutionary (or neo-Darwinian) synthesis, there are four basic processes of evolution: mutation, genetic recombination, natural selection and reproductive isolation. Mutation and recombination are the sources of genetic variability, without which change cannot occur; and are also part of the plant domestication process and will be discussed later on. Natural selection and reproductive isolation guide these variations along adaptive channels.

Natural selection. The basic concept is that favorable heredity traits become more common in successive generations of a population of reproducing organisms, and unfavorable traits become less common. Natural selection acts on the phenotype, or the observable characteristics of an organism, so that individuals with favorable phenotypes are more likely to survive and reproduce than those with less favorable phenotypes. If these phenotypes have a genetic basis, then the genotype associated to the favorable phenotype will increase in frequency in the next generation. Thus, certain traits are preserved due to the selective advantage they confer to their holders, allowing one individual to produce more descendents than individuals without those traits. Eventually, through several interactions of these processes, organisms can develop more and more complex adaptive characteristics. Over time, this process can result in adaptations that specialize organisms for particular ecological niches and can finally result in the emergence of new species.

Reproductive isolation. Reproductive isolation happens when two populations of individuals are prevented from mating and, therefore, from exchanging genes. Isolation mechanisms constitute barriers to gene exchange and may be: prezygotic and postzygotic. Prezygotic mechanisms prevent sexual contact between the species or else make the union of gametes impossible after mating. Thus, the following forms exist:

i) Habitat isolation – this happens when the populations live in the same region, but occupy different habitats. It is very common in plants, because of their sedentary nature.

ii) Temporal isolation – this happens when two populations of individuals can occupy the same area, but their reproductive periods occur in different seasons. Therefore, there is no reproduction, even though physical contact is possible. This form of isolation is very common in plants.

iii) Behavioral (or ethological) isolation – in this form, individuals meet but do not mate due to behavioral differences in chemical signals, color patterns and morphological characteristics by which one individual recognizes a potential partner. These behavioral differences prevent mating rituals and, in consequence, fertilization.

Postzygotic mechanisms prevent the development of hybrids or reduce their fertility or the viability of their descendants, and can be grouped into:

i) Gamete isolation – the exchange of gametes happens, but the male cell does not reach the female cell due to immune reactions or lack of chemical recognition between the gametes.

ii) Isolation due to non-viability of hybrids, also called zygotic isolation – in this case fertilization occurs, but genetic incompatibility prevents normal embryo development.

iii) Isolation due to hybrid sterility, also called postzygotic isolation – in this case the embryo develops, but does not reach adult age or, when it does, it is sterile or eliminated by selection.

iv) Isolation due to non-viability of second-generation hybrids – fertile hybrids are formed, but in their descendants (second-generation hybrids) the embryos are aborted, too weak or sterile.

Origin of cultivated plants

The first references about the origin of cultivated plants are by Alexander von Humboldt and by Alphonse de Candolle in the 19th century (Hawkes, 1967; Zeven; Wet, 1982; Walter et al., 2005). The origin of such species and their connection to the rise of agriculture are closely correlated facts. Studies on the origin of cultivated plants basically consider certain factors such as

locations with greater species diversity, records of the origin of agriculture and archaeological data (Walter et al., 2005; Sereno et al., 2008).

There are areas where the concentration of species of a given genus is remarkable, and such areas are independent from each other. According to Stace (1989), these areas are called centers of genetic diversity, or centers of diversity for the particular genus. According to this author, as the distance between these centers increases, the number of species found decreases. In this sense, the center of diversity does not necessarily represent the area with greatest genetic variability, but simply indicates the geographical area where the largest number of species of that genus is found (Walter et al., 2005).

According to Walter et al. (2005), the concept of center of diversity is widely discussed in phytogeography, taxonomy and biosystematics textbooks, and has evolved in parallel with the concept of center of origin, which is the geographical location where one species originated (Cain, 1951). Origin here should be understood as the emergence of a new form in relation to a preexisting one. However, while center of diversity is a biological fact which can be quantified and limited, center of origin is only an interpretation resulting from deductive thinking (Zohary, 1970). Certainly, each plant species has spread from a given location, but in practical terms finding such a center is no simple task (Cain, 1951), especially for species traditionally cultivated or manipulated by human beings. Besides, some plant species may not necessarily have one single center of origin (Cain, 1951), and therefore it is virtually impossible to indicate the origin of these plants in one precise geographical location (Li, 1974).

Centers of diversity of cultivated plants

According to Walter et al. (2005), cultivated plants originated from the evolution of wild ancestors at locations currently known as centers of origin or centers of diversity, which are specific and more or less restricted geographic areas. There is often one single area for a given genus in which a cultivated plant is included. The number of species in the genus progressively decreases as the distance to the center of diversity increases. Knowledge about centers of diversity and multidisciplinary studies carried out at these locations are essential for understanding the origin of cultivated plants, for their genetic improvement and for conservation of their germplasm.

Studies about centers of diversity began with Russian researcher Nikolai Ivanovich Vavilov in the 1910s to 1930s. Vavilov was concerned about the origin of cultivated plants, and so decided to undertake his studies based on

observations of the distribution of plants existing at that time. For this purpose, he engaged in expeditions to many parts of the world, collecting wild plants that were taxonomically closely related to cultivated species. Vavilov found out that there was frequent coincidence of many unrelated taxa in centers of diversity. The main centers of diversity of cultivated species could be recognized throughout the globe and their number was relatively small. Vavilov (1949 cited by Walter et al., 2005), indicated at least eight centers of origin or of primary and secondary diversity of cultivated plants (Figure 1). Vavilov considered these centers of genetic diversity as centers of origin (where domestication took place) of cultivated species. Nevertheless, some researchers claim the center of origin isn't always the center of greatest genetic diversity of a given species.

More generally, the centers of diversity of cultivated species can be divided into the following regions: 1) Middle East, at the area currently comprising Turkey, Iraq, Syria and Israel, traditionally known as the "Fertile Crescent"; 2) Southern North America, in Mexican territory; 3) Southeastern Asia, comprising parts of India and of China. These centers are geographically separated by deserts, plains or mountains.

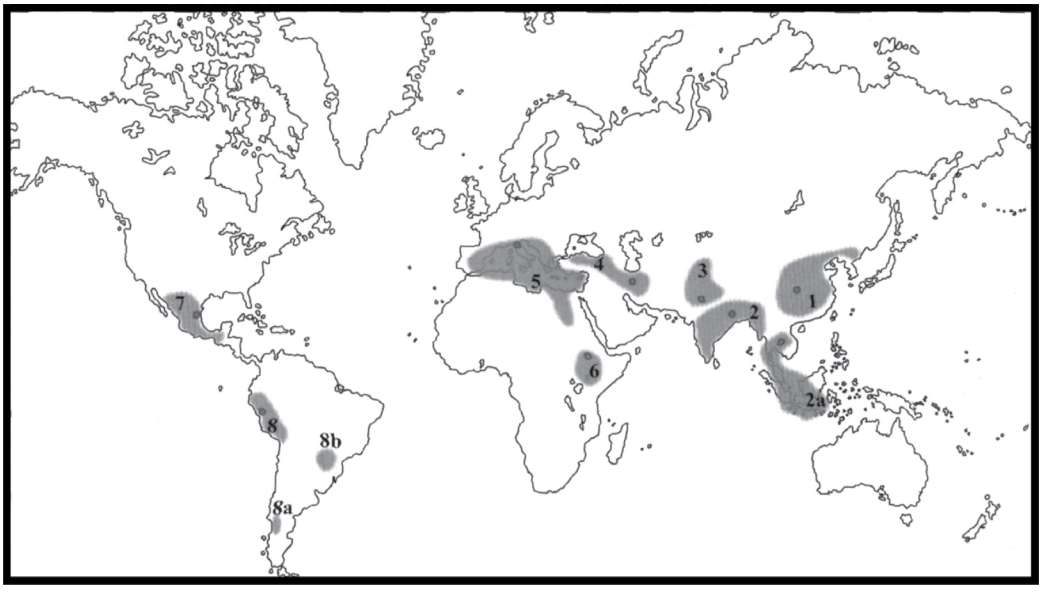

Figure 1. Vavilov's centers of diversity, where agricultural civilizations developed independently. 1) China; 2) India (2a. Indonesia, Malaysia and Oceania); 3) Central Asia; 4) Near East; 5) Mediterranean; 6) Eastern Africa; 7) Mesoamerica; 8) South America (8a. Chile, 8b. Brazil and Paraguay).
Source: Walter et al. (2005).

Usually, one species relates to only one center, but sometimes it may be found in more than one. When this happens, the location of the primary center is the one where the species was domesticated. The secondary center develops from types which migrated from the primary center.

Authors who investigated the so-called "Vavilov centers" found out that areas not explored by the Soviet team also represented centers of diversity, such as Australia, North America and Africa (with the exception of Ethiopia). Therefore, Zhukovsky (1968) subdivided the centers of diversity into 12 areas around the globe (Figure 2).

Currently, three main centers of genetic diversity for cereals and 12 centers of genetic diversity for food plants are recognized. All the centers are located in tropical or subtropical areas, or at least in warm temperate regions. Until quite recently, these 12 centers of origin did not include areas in Brazilian territory. Currently, with the recognition of the importance of plants such as pineapple, peanuts, cocoa and cassava (plants originated in tropical South America), certain areas of Brazil, such as the Amazon for example, now represent important centers of diversity for food plants.

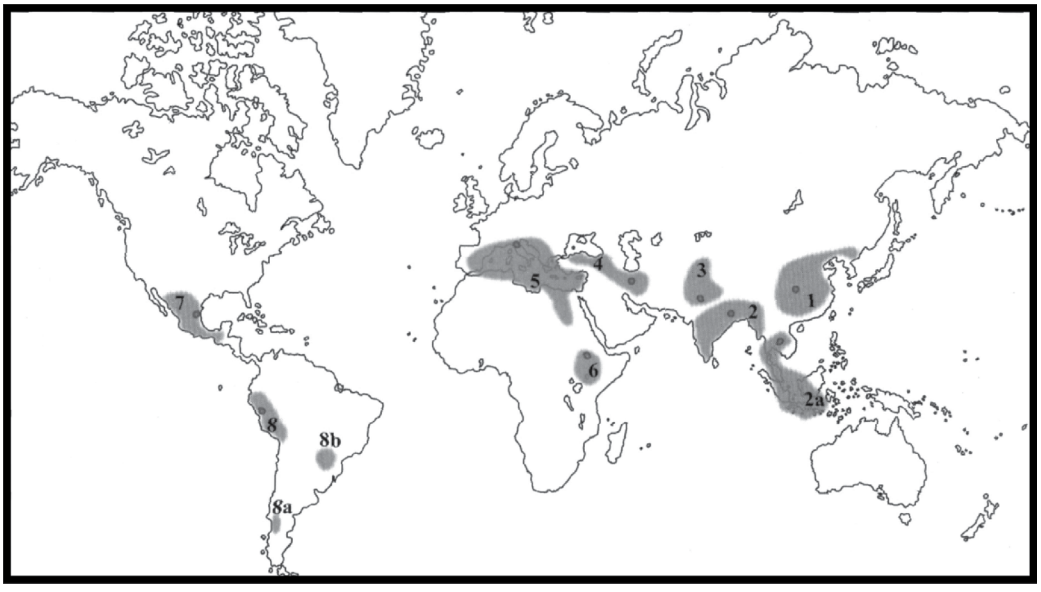

Figure 2. Zhukovsky's centers of diversity. 1) China and Japan; 2) Indochina and Indonesia; 3) Australia; 4) India; 5) Central Asia; 6) Near East; 7) Mediterranean; 8) Africa; 9) Europe and Siberia; 10) South America; 11) Central America and Mexico, and 12) the region of North America.
Source: Zhukovsky (1968) and Walter et al. (2005).

It is very important for the breeder to know the geographic location of the genetic variability of the crop being studied. A large part of a crop's genetic variability is present in its center of origin or in its center of diversity.

Domestication of cultivated plants

Domestication is an evolutionary process carried out by man. This process aims at adapting plants and animals to human needs. Domesticated plants are genetically different from their wild progenitors. A totally domesticated species is completely dependent on man for its survival, and unable to reproduce in nature without human intervention (Harlan, 1992; Evans, 1993; Smart; Haq, 1997).

Modern studies on the origin of agriculture all over the world include archaeological works, which require interaction between specialists from several areas such as historians (archaeologists), anthropologists, botanists and zoologists. Unlike old-time archaeologists, whose ambition included mainly spectacular discoveries which led to valuable pieces for museums, modern archaeology seeks to reveal the ways of life of prehistoric man, what he ate and how he interacted with the environment (Hancock, 2005). Archaeological searches reveal a number of materials, whose assessment requires specialized knowledge from biologists, botanists or zoologists, to determine the organisms from which these materials came. Radiocarbon (carbon-14) dating is obviously carried out to determine the age when the material was used. The most diverse materials are analyzed, such as fruit rinds, flower bracts, leaves, seeds, pollen and bones, in addition to man-made products.

Plant domestication and animal domestication were events occurring at practically the same time in various places. This is why research on animal domestication is a very important factor in archaeological studies about the origin of agriculture (Hancock, 2005). The first domesticated plants, just as the first domesticated animals, already showed characteristics of pre-adaptation to domestication in their wild condition. The first domesticated plants were herbaceous, fast growing and produced profuse seeds (Harlan, 1992; Evans, 1993; Smart; Haq, 1997; Smith, 1998).

In the case of plants, well established criteria for distinguishing between wild and domesticated individuals refer to traits of seeds and of infructescences. Domesticated plant seeds are bigger (have more reserves) and have thinner integuments. Man ended up unconsciously selecting lineages with such characteristics, because individuals with thinner integuments germinate faster than ones with integuments more resistant to decomposition or abrasion

(Smart; Haq, 1997; Smith, 1998). Seeds containing more reserves produce faster growing plantlets and end up competing at an advantage against others coming from smaller seeds, since the former end up being shaded by the latter. Another very important characteristic for distinguishing between cultivated and wild plants is the fact that fruits of the former remain attached to the axis of infructescence, while those of wild plants detach, which facilitates their dispersion. For primitive agricultural man (and also for modern farmers) it is important for the fruits to remain attached, so that there are no losses before harvesting (Harlan, 1992).

Several of the first domesticated plants were probably annual species (Diamond, 2002). Such plants are typically good producers of reproductive organs, such as fruits and seeds. From the point of view of resource allocation ecology, these species are classified as *r*-strategists, meaning they are plants which dedicate great part of the nutrients attained from the environment to producing reproductive structures (the opposed alternative being *K*-strategists, species that massively dedicate resources for building the body of the plant, or for the vegetative parts). *R*-strategist plants like disturbed environments, behaving as opportunistic species which quickly occupy open and eutrophized locations (Pianka, 1970).

Regions and time of establishment of agriculture – basis of domestication

Agriculture may have first originated in the Middle East, in the region known as the Fertile Crescent (Figure 3). It was once believed that the exact location was the valley of the Tigris and the Euphrates rivers. Nowadays however, due to more recent archaeological researches, there is reason to suppose that the beginnings of agriculture took place in mountainous regions near the valley of these rivers and of the Jordan River, in the areas corresponding to the current territories of Iraq and Israel, respectively.

Harlan (1971) proposed three centers of origin for agriculture, defending the hypothesis that agriculture originated in these three locations independently. In the three cases, there would have been a system composed by the center of origin and what the author defined as a *noncenter*, where cultivation and domestication activities are said to have spread over variable areas.

Regarding domestication, Smith (1998) proposed seven areas of the globe which largely correspond to the areas indicated by Harlan (1971) as centers of origin for agriculture. This process is said to have initiated and occurred independently in each of the areas described (Figure 4).

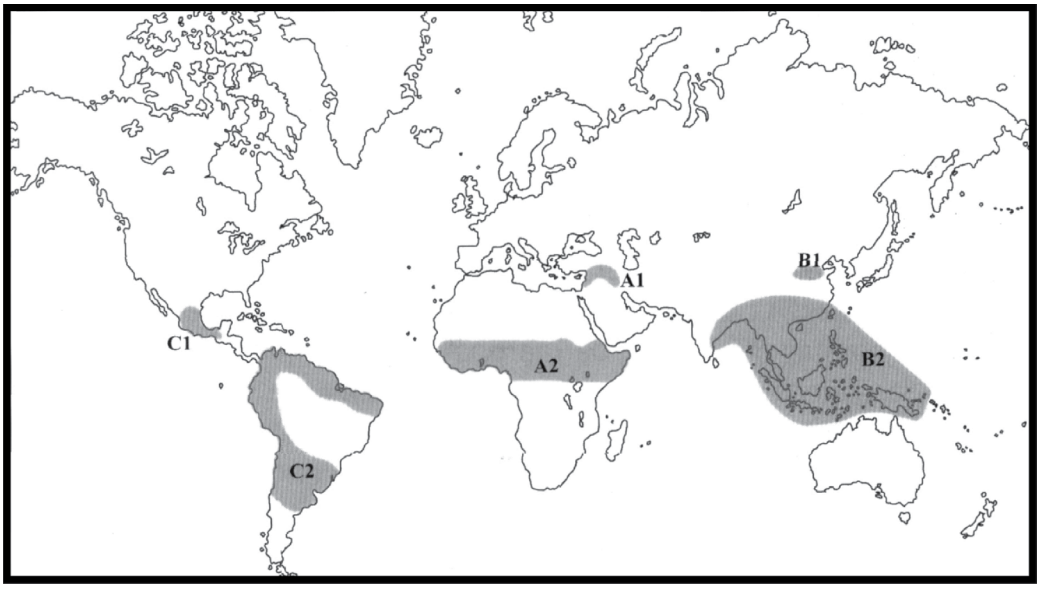

Figure 3. Centers and noncenters of origin of agriculture, according to Harlan (1971). A1) Near East center; A2) African noncenter; B1) Northern China center; B2) Southeastern Asia noncenter and South Pacific noncenter; C1) Mesoamerican center; and C2) South America noncenter.
Source: Walter et al. (2005).

In times before 8000 BC, humans already collected wild cereals in the region. There is evidence that they already cultivated plants and bred domestic animals such as goats and pigs one thousand years later. Deposits from 6750 BC located in Jarmo (Iraq) revealed the presence of wheat and barley seeds, in addition to goat bones. In even older times, 9,600–10,000 years ago, wheat was already cultivated in the Levantine Corridor, a region of the Jordan Valley (currently in Israeli territory). Evidence of wheat cultivating there has been observed in several locations, such as Jericho, Netiv Hagdud and Gilgal. Since these were cultivated plants, their domestication must have occurred even earlier, that is, possibly in 9000 BC, a time that corresponds to the beginning of agricultural activity in the history of civilization. There are those who claim that at around the same time agriculture began in Southeastern Asia, but evidence recovered in that region is very scarce and not very conclusive (Smith, 1998). The reason for the lesser number of findings in India and China is probably due to the weather, which is more moist there than in the Mediterranean zone, which affects the fossilization process (Smart; Haq, 1997).

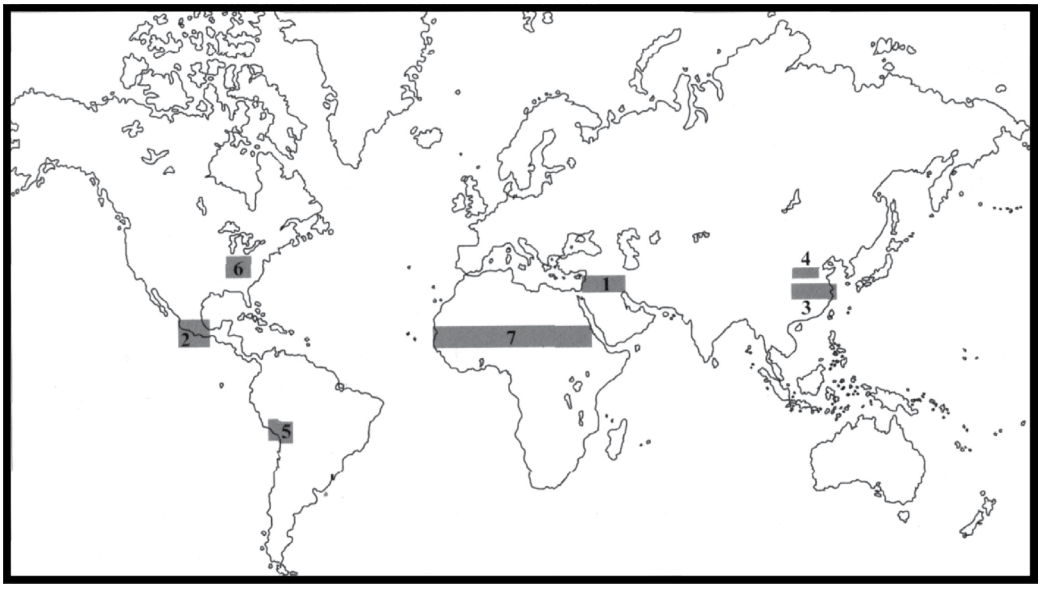

Figure 4. Locations where plant domestication occurred due to the emergence of agriculture. 1) Near East or Fertile Crescent, 9500 BC; 2) Central Mexico,9000 BC; 3) Southern China, 8500 BC; 4) Northern China, 7800 BC; 5) South-Central Andes, 7000 BC; 6) Eastern United States, 4500 BC; 7) Sub-Saharan Africa, 4000 BC.
Source: Walter et al. (2005).

Another relatively well studied area which is a very important center of genetic diversity for useful plants is the southern part of North America, more precisely South-Central Mexico. Archaeological research carried out in the Tehuacán plateaus has revealed that man was already settled there in 10000 BC. The first evidence of cultivated maize plants in that region was found in deposits dating from around 5000 BC. Around that time, squash, avocado and amaranth were also cultivated there. In the immediately following millennia, other plants were cultivated, especially beans. Some plants cultivated by the people living in this location are definitely South-American in origin (pineapple, peanuts and guava), which leads to the conclusion that ancient people from North America had contact with residents from South America at some point.

A very important center of genetic diversity of useful plants in South America is the Andean region, which stretches along the western coast of the continent, from Colombia to central Chile. Agriculture was established in this area much later than in Mexico, and it certainly did not happen before 3000 BC. In these places, beans, pepper, squash and cotton were first cultivated. Later on, potato was domesticated.

Characteristics of domesticated plants

Domesticated species exhibit a number of morphological changes when compared with their wild ancestors. Harlan (1992) and subsequently Smart and Haq (1997), called these changes the “domestication syndrome”. These modifications include seed dormancy loss, increased fruit and seed size, inefficient dispersal mechanisms (indehiscent pods, for example), more compact growth habit, greater plant uniformity, reduction of toxic substances and increased number of seeds per inflorescence.

From a genetic standpoint, evolution is “any change in allele frequencies of the population which aims at making it more adapted” (Allard, 1971; Grant, 1971; Mettler; Gregg, 1973; Freire-Maia, 1988; Wendel, 2000). As mentioned above, there are four basic processes of plant evolution: mutation, recombination, natural selection and reproductive isolation. In the case of plant domestication, plants have been modified to make them more adapted to humans. The main genetic factors involved in the process of plant domestication are mutation, interspecific hybridization, polyploidy and artificial selection.

Mutation. Defined as the sudden change in existing genes, it constitutes the only genetic process to create variability (it creates new alleles). Mutation is divided into different types: genetic, extranuclear and chromosomal. In genetic mutation or point mutation, changes happen in the nitrogenous bases of the DNA. Extranuclear mutations happen in the DNA of cytoplasmic organelles (mitochondria and chloroplasts). In chromosome mutations, changes happen both in structure (deletion, duplication, inversion and translocation) and in chromosome number (aneuploidy and euploidy). The frequency of occurrence of mutations is very low. It is estimated that it takes place at one gene locus per million gametes, i.e., at a frequency of 1:106.

Interspecific hybridization. Crossing or hybridization happens between individuals from different, but related, species. This type of hybridization was very important in the origin of several cultivated species. In several cases, after the crossing of different species, the resulting hybrid was backcrossed with one of the parental species, so that the result is the transfer of some or just one characteristic from one genitor to the other. This phenomenon is called introgression. During the domestication of cultivated species, interspecific hybridization happened naturally. Nowadays, breeders can use it to seek characteristics in related species, or even to create new species.

Polyploidy. This refers to cells or organisms that contain more than two copies of each of their chromosomes. Types of polyploids are divided, according to the number of chromosome sets in their nucleus, into triploids (three

sets, 3x), tetraploids (four sets, 4x), pentaploids (five sets, 5x), hexaploids (six sets, 6x), and so on. A haploid (x) has only one set of chromosomes. Polyploidy has been an important mechanism in the process of domesticating cultivated plants. Polyploid plants are generally more vigorous, with larger fruits and seeds. Some authors suggest that during domestication, polyploid plants, which are stronger and more vigorous, were preferentially selected. Many cultivated species appear to have been unintentionally selected for higher ploidy level.

Polyploids can be divided into two types, according to their origin:

i) Autopolyploids, in which the chromosome sets originate from just one species. In these species, increased size of flowers, fruits and leaves (fruit and ornamental plants) is observed. Autopolyploid species usually have low fertility, due to pairing problems in meiosis. Thus, it is especially important for vegetative propagation species, such as bananas (triploid) and some potato varieties (tetraploid).

ii) Allopolyploids, in which the chromosome sets originate from the crossing of two or more related species. Chromosome duplication of an allopolyploid forms an amphidiploid, which has increased fertility. In comparison to autopolyploidy, allopopolyploidy has had a much bigger impact on the domestication of cultivated plants.

Artificial selection. During plant domestication, the genetic processes described before (mutation, interspecific hybridization, polyploidy) happened mainly in a natural way. The main contribution made by man was artificial selection. It occurs when one individual produces more offspring than another, thus making it relatively more adapted. Selection changes allele frequency (and therefore, genotypic frequency) and is vital for evolution and for domestication. Nature and man do not necessarily want the same phenotypes. Many traits desirable for man are not favored by nature. Selection carried out by man (artificial) can be in the opposite direction of natural selection. Man selects individuals with desirable agricultural characteristics, while nature selects individuals which are more adapted.

Origin, evolution and domestication of alfalfa

According to Shifino-Wittmann (2008), alfalfa is the oldest forage. However, the etiology of the word is questionable, since it may have come from Persian *aspo-asti* (horse food), from Arabic *al-fasfasa* or from Kashmiri *ashwa-bal* (meaning horse power). There is also the theory that the name *lucerne*, used in Europe for alfalfa, was derived from Persian *läjwärd* for lapis

lazuli, referring to the bluish flower of *Medicago sativa* (Russelle, 2001 cited by Shifino-Wittmann, 2008).

It is estimated that alfalfa has been grown in Pakistan since 4000 BC, and since 3000 BC and 2000 BC in Afghanistan and Kashmir. The earliest evidence of the origin of alfalfa dates from 10000 BC and 6000 BC, from wild alfalfa seeds found in samples from Syria and Iran, respectively.

The oldest records of alfalfa utilization date back to 1300 BC, where Turkey currently is (Langer, 1995). It was later included in the list of garden plants of Merodach-Baladan, a contemporary of Hezekiah, king of Judea (Russele, 2001, cited by Shifino-Wittmann, 2008).

Alfalfa had an important role in the advance of civilizations, because it was used to feed horses. Thus, alfalfa was grown and propagated through several parts of the world, but production was centered in the Middle East until about 1200 BC. After that period and thanks to the wars led by Darius in 490 BC, alfalfa reached Greece (Crochemore, 1998; Shifino-Wittmann, 2008), which was the crop's main propagation center to the world, as we will discuss later.

The *Medicago* genus has the Middle-East as its general center of origin (Quiros; Bauchan, 1988), and is said to have differentiated during the Tertiary Era (Lesins; Lesins, 1979). The *Medicago* genus' primary center of origin is in the northwest of Iran and northeast of Turkey (Quiros; Bauchan, 1988; Crochemore, 1998; Shifino-Wittmann, 2008), regions with characteristic cold winters and dry and warm summers, which have well-drained soil with near-neutral pH (Michaud et al., 1988). The ancient, perennial and preferably allogamous forms are said to have had their center of origin on the northern coast of the Mediterranean (Crochemore, 1998; Shifino-Wittmann, 2008).

In the Miocene, the Strait of Gibraltar's intermittent closure through the formation of mountains (Alps, Pyrenees, Apennines, etc.) is said to have temporarily transformed the Mediterranean basin into a hot desert. The creation of this new habitat favored differentiation of colonizing annual species with dormant seeds with short growing cycles, based on pre-existing perennial species. As the species became annual, the autogamous character would have appeared as a key reproductive strategy, due to geographic isolation and lack of pollinators in the new colonized habitats. The Strait of Gibraltar's final opening drove many species to extinction. As these annual species emerged after the end of this geological process, studying them could not effectively contribute to understanding the origin of the genus (Bauchan, 1988; Crochemore, 1998; Quiros; Shifino-Wittmann, 2008).

Regarding the most recent history of the origin, evolution and domestication of alfalfa, it is very important to understand the taxonomy, ploidy, reproduction,

and especially the species in the *Medicago* genus (the *Medicago* complex), since each group within the genus has independent records. The genus comprises a vast number of species and the characterization of these species or of the hybrids originated from them is presented bellow, in addition to taxonomic and genetic information (Crochemore, 1998; Shifino-Wittmann, 2008).

Taxonomy. The *Medicago* genus belongs to division Magnoliophyta, class Magnoliopsida, order Fabales, family Fabaceae. The systematics and phylogeny of the *Medicago* genus result from the work of various authors throughout history: Linnaeus, Urban, Taubert, Ascherson and Graebner, Trabut, Hegi, and Synskaya (Villax, 1963). Nevertheless, it was only from the mid-twentieth century that these species were actually studied, as reported by Michaud et al. (1988) and Quiros and Bauchan (1988). These authors presented important information, especially about the evolution and distribution of *Medicago sativa*. However, there is a lot of information about the species within the genus which can still cause confusion, especially regarding the classification of species (Prosperi et al., 1995).

The *Medicago* genus comprises over 60 species, of which two thirds are annual and one third is perennial (Shifino-Wittmann, 2008). These species are classified into four subgenera (*Medicago*, *Lupularia*, *Orbicularia* and *Spirocarpos*). The *Lupularia* and *Spirocarpos* subgenera include only annual species, the *Medicago* subgenus includes only perennial species, and the *Orbicularia* subgenus includes both annuals and perennials. The *Medicago* subgenus comprises four sections (*Falcago*, *Arborea*, *Marinae* and *Suffruticosae*), classified according to characteristics associated to pods, seeds, pilosity, inflorescences, flower color, vegetative growth and chromosome number. The *Falcago* section comprises four subsections: *Falcatae*, *Rupestres*, *Daghnestanicae* and *Papillosae*.

Cultivated alfalfa is part of the *Falcago* section, *Falcatae* subsection, and consists of the species *M. falcata*, *M. sativa*, *M. glomerata*, *M. glutinosa* and *M. prostrata* (Lesins; Lesins, 1979). The colors of the flowers and shapes of the pods are the most important characteristics for distinguishing between species of this subsection, but characteristics regarding size, longevity, plant morphology, inflorescence morphology and cytology should also be taken into account in distinguishing species (Crochemore, 1998). These species are part of the group called the *Medicago sativa* complex. According to Lesins and Lesins (1979), all species in the *Falcatae* subsection are perennial, are diploid or tetraploid, and have a corolla which is yellow, violet or yellow and violet mixed (variegated). Veins are prominent and pods are straight, sickle-shaped or spiral and thornless.

Interbreeding between diploid and/or tetraploid forms can occur. As there is no barrier to genetic recombination between species from this complex,

some authors have suggested the existence of species and subspecies, which then receive the following denomination: *M. sativa* ssp. *falcata*, *M. sativa* ssp. *sativa*, *M. sativa* ssp. g*lomerata*, *M. sativa* ssp. *glutinosa*, *M. sativa* ssp. *prostrata* (Harlan; Wet, 1971; Quiros; Bauchan, 1988; Crochemore, 1998; Shifino-Wittmann, 2008). The only barrier to gene exchange between species from the *M. sativa* complex is ploidy, which can be bypassed by the production of unreduced diploid gametes (Quiros; Bauchan, 1988).

Genetics. The *Medicago* genus has a basic chromosome number of eight, but a few annual species have a chromosome number of seven: *M. constricta*, *M. praecox*, *M. polymorpha*, *M. rigidula* and *M. murex* (Lesins; Lesins, 1979; Mccoy; Bingham, 1988; Quiros; Bauchan, 1988; Crochemore, 1998).

Three levels of ploidy are found in the different species of the genus: diploid (2n = 2x = 16 or 14), tetraploid (2n = 4x = 32) and hexaploid (2n = 6x = 48), but most species are diploid. It is possible that the basis of the evolution of the genus was diploidy, and that tetraploid species have come from a lack of gamete reduction, which gave rise to very vigorous heterozygous individuals able to colonize other habitats and thus expand the geographical area of the genus (McCoy; Bingham, 1988; Quiros; Bauchan, 1988; Crochemore, 1998). Many alfalfa cultivars originated from the interbreeding of allogamous tetraploid perennial forms of *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* and, therefore, great genetic diversity is found among populations from different geographical regions, and also within these populations (Crochemore, 1998).

Some perennial species such as *M. sativa*, *M. falcata*, *M. prostrata*, *M. papillosa* or *M. arbórea* can have ploidy levels different than 2x, 4x or 6x, with low or no interfertility (Lesins; Lesins, 1979). Two species, *M. rugosa* and *M. scutellata*, have chromosome number 2n = 30 (McCoy; Bingham, 1988; Quiros; Bauchan, 1988). Perennial cultivated species of *Medicago* are allogamous, with perfect flowers and different levels of self-incompatibility; however, it is sometimes possible for selfing to occur in some species (Viands et al., 1988). The alfalfa flower has a membrane which prevents selfing. Since this membrane is only ruptured by pollinating insects, natural interbreeding between species is normally dependent on pollinating action of insects (McCoy; Bingham, 1988). Because of their allogamy, these plants are highly polymorphic (Quiros; Bauchan, 1988; Crochemore, 1998).

The cultivated subspecies *M. sativa* ssp. *sativa* is erect, adapted to mild climate and has bluish purple flowers and spiral pods, with very pronounced autumnal dormancy (Bolton, 1962; Crochemore, 1998; Shifino-Wittmann, 2008). Flowers are small, in numbers of five to fifteen, arranged in open racemes. The fruit of alfalfa is a legume, spiraled in three to five coils, indehiscent; the

number of seeds in the pods is variable, and the color ranges from light yellow to dark brown (Rodriguez; Eroles, 2008).

The existence of diploid and tetraploid forms in *M. sativa* and *M. falcata*, as in other species, suggests that chromosome duplication happened independently in each species (Crochemore, 1998). Hybrid forms between the two levels of ploidy may be due to duplication of diploid hybrids, hybridization of 2n gametes coming from a diploid parent with normal 2n gametes coming from another tetraploid parent, or even to the rare union of two gametes coming from two diploid parents (Stanford et al., 1972; McCoy; Bingham, 1988).

Species of the *Medicago sativa* complex. There are about 60 species of *Medicago* (Shifino-Wittmann, 2008) and only ten are cultivated. Within this complex, some authors adopt a classification into species (Lesins; Lesins, 1979) and others, into subspecies (Gunn et al., 1978; Tutin, 1978; Quiros; Bauchan, 1988). The classification into subspecies has been more widely accepted, since there are no barriers to hybridization among them (Shifino-Wittmann, 2008). The only barrier to gene exchange between species of the *M. sativa* complex is ploidy, but this barrier can be overcome by the production of unreduced diploid gametes (Quiros; Bauchan, 1988). Therefore, Lesins and Lesins (1979) admitted the classification into subspecies regarding heritability of traits, fertility and survival of descendents in experimental conditions.

Perennial cultivated species, with the exception of *M. arborea* and *M. lupulina*, belong to the *Falcago* section, *Falcatae* subsection: *falcata*, *sativa*, *glomerata*, *glutinosa* and *prostrata*. The results of interbreeding possibilities among diploid or tetraploid forms of these species are described by Lesins and Lesins (1979) as a complex of species, called the *Medicago sativa* complex. Most of the current cultivars of alfalfa originated from the interbreeding of perennial allogamous tetraploid forms of *M. sativa* with *M. falcata*. However, all species from the complex can be hybridized with *M. sativa* (Lesins; Lesins, 1979).

Subtle morphological differences resulting from genetic recombination have been used to identify new species or new subspecies. When considering the large variability between species such as *M. sativa*, *M. falcata* and *M. glutinosa*, it is observed that several types of hybrids are produced, because an increased recombination of parental traits takes place (Quiros; Bauchan, 1988).

According to the classification presented by Quiros and Bauchan (1988), there are eight subspecies in the *M. sativa* complex: *sativa* (2n = 32); *coerulea* (2n = 16); *falcata* (2n = 16, 32); *glutinosa* (2n = 32); x *varia* (2n = 32); x *hemicycla* (2n = 16); x *polychroa* (2n = 32) and x *tunetana* (2n = 32). *M. glomerata* (2n = 16) and *M. prostrata* (2n = 16, 32) belong to another closed complex.

Cytological and genetic evidence based on a great number of diploid and tetraploid populations of *M. sativa* and *M. falcata* shows that they have a recent common ancestor. This evidence justifies the interpretation by Gunn et al. (1978) that *M. falcata* is *M. sativa* ssp. *falcata*. Similarly, cytological studies with *M. glutinosa* and *M. sativa* allow the *M. sativa* ssp. *glutinosa* denomination (Quiros; Bauchan, 1988). According to these authors, there are three main subspecies in the complex: *sativa*, *falcata* and *x varia* and a less diversified one *glutinosa*. All these subspecies have endured strong genetic evolution through time and space, because of the strong diversification provided by natural selection and by man.

Characterization regarding the center of diversity of some species or subspecies from the *M. sativa* complex will be addressed more specifically below (Crochemore, 1998). In this classification, ten subspecies, including *glomerata* and *prostrata* – which according to Quiros and Bauchan (1988) belong to a different group – and four hybrids will be discussed (Crochemore, 1998). In this classification *M. sativa* ssp. *coerulea* was also considered as a tetraploid form in the *sativa* subspecies.

Medicago sativa ssp. *falcata*

This subspecies is prostrate, has yellow flowers, fasciculate roots and straight or sickle-shaped pods, sometimes spirally coiled. It is resistant to cold and characterized by winter dormancy. Diploid and tetraploid forms occur, with variable biochemical and morphological characteristics. Based on this variability, diploid forms have received different species or subspecies denominations: *borelis*, *romanica*, *altissima*, *glandulosa*, *quasifalcata*, *difalcata*, *tenderensis* and *erecta*.

The *falcata* subspecies is considered to have come from diploid forms of *M. glomerata* which are said to have colonized vast territories eastward as far as the Caucasus, where they would probably have been ancestors to the diploid forms in the complex. Through reproductive isolation occurring during the Tertiary Era (at Paratethys, which once connected the Black Sea to the Caspian Sea), two ancestral populations were separated: *coerulea* and *falcata* (Quiros; Bauchan, 1988).

To the south, populations of the *coerulea* subspecies lost the carotenoids and populations of the *falcata* subspecies lost the anthocyanins of their flowers, due to selective pressure exerted by competition between pollinators in the new isolated lands. To the north, populations of the *falcata* subspecies developed straight pods due to natural selection. This trait may have been favored by

steppe, the type of vegetation prevailing in this region. In fact, spiral pods disperse more easily in open environments and less easily in steppe regions.

These differentiation processes, that is, geographic isolation and the possibility of transition from diploid to tetraploid level through non-reduction of gametes are probably the mechanisms which allow explaining the evolution of the *M. sativa* complex.

The center of origin of the *falcata* subspecies in the forest steppe regions of Asia and Europe, and it is distributed through areas with similar climates from northern Europe to Siberia (Bolton et al., 1972; Michaud et al., 1988; Small; Jomphe, 1988). It is frequent in steppe regions from the northern coast of the Mediterranean (Bulgaria, Greece, and France) to northern Russia (Prosperi et al., 1995).

The diploid forms spread over regions from western Germany to eastern Siberia, and from the southern coast of the Black Sea to the north of Leningrad [St. Petersburg]. They grow predominantly in the north of Europe (Small; Brookes, 1984). This subspecies is one of the best adapted to cold regions and dry summers (Quiros; Bauchan, 1988; Prosperi et al., 1995). Although tetraploid forms of *M. falcata* are more frequent than diploid ones in the regions of origin (Gunn et al., 1978; Lesins; Lesins, 1979), they appear not to be as widely spread as diploid. *M. falcata* was introduced in Germany and northern France in the 16th century (Bolton et al., 1972; Michaud et al., 1988; Small; Jomphe, 1988).

Medicago sativa ssp. *sativa* and *Medicago sativa* ssp. *coerulea*

The diploid form is called *M. sativa* ssp. *coerulea*, while *M. sativa* ssp. *sativa* is the tetraploid form. These subspecies are characterized by being erect and having violet or blue flowers, taproot and spiral pods. They have little dormancy and have variable tolerance to cold. The distribution of these two forms, diploid and tetraploid, includes the lands surrounding the Mediterranean, the Near East, the Middle East, the Caucasus and Southern and Central Asia, where they are concentrated in the mountains and valleys of Armenia, Anatolia, Iran, Afghanistan, Central Asia and Kashmir.

The center of origin for *M. sativa* is the Near East, Asia Minor, Transcaucasia, Iran and the highlands of Turkmenistan (Michaud et al., 1988). The geographical center most often mentioned is Iran. These regions are characterized by cold winters and dry and hot summers, where the soils are well drained with near-neutral pH. Such regions are said to be the center of origin for some populations that constitute all or part of the basis of certain European varieties. Some authors add a second center: Central Asia (Bolton et al., 1972; Michaud et al.,

1988; Small; Jomphe, 1988), characterized by dry climate and mild winter, where alfalfa plants resistant to certain diseases and insects and with good growth in drought conditions are said to have originated.

Medicago sativa ssp. *glutinosa*

This is a tetraploid species characterized by a corolla with color ranging from bright to creamy yellow. Pods are spiral and covered with glandular hairs. It is adapted to humid subalpine regions of the Caucasus. According to a first hypothesis, the *glutinosa* subspecies was thought to have diploid ancestors. However, these forms have either ceased to exist or not been found yet. Two additional hypotheses suggest that the *glutinosa* subspecies is the result of hybridization of *M. glomerata* and *M. sativa* ssp *falcata*, or that it comes from hybridization of *M. sativa* and *M. falcata*. This is a less probable hypothesis because of the glandular hairs covering the pods of the *glutinosa* subspecies (Lesins; Lesins, 1979).

Medicago glomerata

This species is characterized by bright-yellow flowers and spiral pods covered with glandular hairs. Diploid forms have been found in Southern Europe, in the Alps and in Northern Africa. In these places, tetraploid forms have also been found. The classification as *M. sauva* ssp *glomerata* given by Gunn et al. (1978) is not justified due to the weak fertility between the two subspecies (Quiros; Bauchan, 1988).

Medicago prostrata

Characterized by yellow flowers and spiral pods, this species has diploid and tetraploid forms. Pods are similar to those of the *coerulea* subspecies, but flowers resemble the ones of the *falcata* subspecies. This species originated in dry and rocky coastal regions. It is distributed from eastern Austria and Italy, through the eastern Asian coast as far as Greece (Lesins; Lesins, 1979).

Hybrid subspecies of the *Medicago sativa* complex

Due to the great polymorphism found in flower color and in number of pod spirals, the subspecies below are considered hybrids of *M. sativa* (subspecies *sativa*, *coerulea*, *falcata*, *glutinosa)* and *M. glomerata* (Lesins, 1968).

Medicago sativa ssp. x *hemicycla*

This has a mixed-color corolla 8 mm to 10 mm long. Its pods are sickle-shaped, rarely spiral, with open lumen, 5 mm to 7 mm long, not glandular, and glabrous or slightly pubescent. This subspecies is native to the Caucasus (Gunn et al., 1978) and is possibly the result of intercrossing of the subspecies *falcata* and *coerulea*, as the variability found in artificial hybrids of these two subspecies completely corresponds to the subspecies x *hemicycla* (Lesins; Lesins, 1979).

Medicago sativa ssp. x *varia*

Hybridization between *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* has resulted in very vigorous alfalfas with mixed-color flowers, allowing for great expansion of this crop across northern Europe and North America (Bolton et al., 1972). Hybrids are characterized by their flowers with colors ranging from light yellow to dark green, passing through all shades (from yellow to violet and to brown); and by pod shape, which is more spiral than that of *M. falcata* (Stebler, 1896). According to this same author, this alfalfa is spontaneous in Germany and northern France. However, according to Mayer et al. (1951), it is found all over France, and more abundantly from the Rhône valley to Provence. These populations have intermediate characteristics between the two parental species, which makes their classification difficult. The non-glandular pods have shapes ranging from sickle-shape to spiral (one and a half coil), with open lumen 7 mm to 12 mm wide and 5 mm to 12 mm long. They are characterized by pilosity, which ranges from thick to thin (Gunn et al., 1978).

Medicago sativa ssp. x *tunetana*

The tetraploid form of *M. sativa* ssp. *tunetana* might have originated from tetraploid hybrids of the diploid forms of *M. sativa* ssp. *coerulea* and *M. glomerata* (Quiros; Bauchan, 1988). According to Lesins and Lesins (1979), *M. glomerata* is one of the parents to the *tunetana* subspecies.

Medicago sativa ssp. x *polychroa*

This subspecies has been described as tetraploid, originally coming from interbreeding of the *sativa* and the *glutinosa* subspecies, considering that the variability found in artificial hybridization between these two subspecies completely corresponds to the *polychroa* subspecies. The tetraploid subspecies of the complex differ from their diploid variants by having bigger flowers, pods

and seeds. These wild subspecies have great potential as sources of resistance to diseases, predators and environmental stress (Quiros; Bauchan, 1988). Tetraploid populations are superior to diploids in terms of their leaf size, vigor and forage production. In addition, they are earlier and more stress-resistant (Clement, 1972; Bingham; Saunders, 1974; Dunbier et al., 1975; Arbi et al., 1978).

Propagation of cultivated alfalfa

Alfalfa can be found all over the Middle East, and it was introduced into Greece and into ancient Mesopotamia at around 500 BC. In the second century BC, it reached Italy and was propagated throughout the Roman Empire, especially in Spain, northern Africa and France.

With the barbarian invasion and the fall of the Roman Empire (end of the 4th century), its cultivation disappeared in southern Europe. Alfalfa may have been reintroduced into Spain and France during the Arabic conquests in the 7th and 8th centuries, but in France it was cultivated only from around 1550 (Michaud et al., 1988). Its presence in Holland and Belgium was reported in 1565, in England in 1650, in Germany and Austria in 1750, in Sweden in 1770 and in Russia during the 18th century.

In Germany and in northern France, hybridization of the *sativa* subspecies with the *falcata* subspecies enabled a major evolution for cultivated alfalfa. This hybrid spread throughout Central and Northern Europe, which led alfalfa to move away from its hot and dry habitat into colder regions (Lesins; Lesins, 1979).

With the discovery of Americas in the 16th century, the Portuguese and the Spanish brought it to Mexico and Peru. It probably reached the United States of America (USA) through the Mexican border, and Argentina and Chile through Peru (Basigalup; Hijano, 1995). It was introduced into North America around the mid-19th century, via two routs: 1) in the South, it came from Chile to California and from Mexico to Colorado; 2) in more northern latitudes, it came from northern Europe (Michaud et al., 1988). It was in the USA that alfalfa underwent its most significant expansion, with reports on the evolution of this crop indicating that the first scientific studies were carried out between 1903 and 1915, focusing mainly on the aspect of cold resistance. Later years saw the emergence of *Clavibacter michiganensis* ssp. *insidiosus* [bacterial wilt]. The first records of interest in introducing alfalfa to native field areas of the Northeastern USA are from 1897 to 1909, when Hanson collected in the steppes of Siberia a type of alfalfa adapted to those conditions. These materials were

the basis for the breeding program in Canada's natural pasture region with dry climate. This program gave rise to the release of the Rambler cultivar in 1995, considered a milestone in the evolution of alfalfa cultivation (Heinrichs, 1978).

With the colonization of South and Central America by the Spanish, alfalfa was introduced into Mexico and Peru. From Peru it reached Chile, Argentina and Uruguay, around 1775. It arrived in Brazil around the 19th century, entering through the State of Rio Grande do Sul and spreading to the other states, especially Santa Catarina and Paraná (Nuernberg et al., 1992). In Rio Grande do Sul, it started being cultivated in the valleys of the rivers Caí, Taquari, Jacuí and Uruguai and on the slopes of the mountains in the northeast of the state, where German and Italian immigrant colonies were located (Saibro, 1985). Thus the population currently called crioula alfalfa started as a result of the joint action of natural selection and selection performed by man, since the producers only harvested seeds from four or five-year-old alfalfa fields, selecting the most persistent plants (Oliveira, 1991). Currently in Brazil, there is an increase in the area planted with alfalfa in non-traditional areas such as the Southeast and Midwest, due to the increasing implementation of intensive milk production systems, leading to increased demand for highly nutritional feeds (Rodrigues et al., 2008).

Final considerations

Alfalfa is an herbaceous forage plant, with annual and perennial forms. It is grown mainly in the United States of America and in Argentina. It can be used in many forms as an animal feed (hay, silage, pellets or pasture).

Its center of diversity is the Near East. Due to its large genetic variability, alfalfa has been subdivided according to taxonomic, genetic and reproductive characteristics. The *Medicago* genus has been classified into eight subspecies, which form the so-called "*Medicago sativa* complex". All the subspecies have undergone strong genetic evolution through time and space, because of the great diversification caused by natural selection and by man. The basic chromosome number of the *Medicago* genus is eight and there are three ploidy levels: diploid (2x = 16), tetraploid (4x = 32) and hexaploid (6x = 48), with great predominance of diploid species. There is evolutionary evidence that the tetraploid species originated from a non-reduction of gametes resulting in more flexible individuals more adapted to a greater variety of environments. Cytological and genetic evidence based on a large number of diploid and tetraploid populations shows that they have a recent common ancestor.

The great genetic diversity existing in terms of both its species and its cytogenetic characteristics suggests that alfalfa is one of the oldest crops to have been domesticated by man. Each species independent center of diversity indicates, as reported in this chapter, that alfalfa populations have somehow evolved or coevolved with domestication, and that the barriers between them were crucial for this differentiation.

Regarding its worldwide dissemination, the expansion of the great empires has been the most widely accepted cause for the expansion of cultivation of alfalfa, which was used for animal feeding. More recently, it expanded in Europe, from where it was taken to the Americas by the Spanish and Portuguese conquerors. It is in the USA that its expansion has been most significant. It was introduced to Brazil by colonizers in Rio Grande do Sul, probably through Uruguay and Argentina, where the crop was already largely disseminated.

There is a great reservoir of genetic diversity available for alfalfa, in which strong intraspecific and interspecific variation can be observed. Progress in biotechnology is making possible greater elucidation and understanding of this genus, allowing its resources to be better exploited by genetic breeding programs.

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CHAPTER 2

Morphology of alfalfa

Nora Estela Rodríguez
Sandra Fabiana Eroles

Introduction

Botanical morphology is the science that studies the form of plants, and it includes organography, which describes the form of the different plant organs (Font Quer, 1989).

Based on the work of Teuber and Brick (1988), this chapter aims at describing briefly and in a practical way the organs that constitute the alfalfa plant, including images that illustrate the descriptions in each case. In general, the literature addressing this topic is scarce and not always easy to find, because alfalfa is studied mainly as a forage plant, and as such only aspects linked to forage yield and management for meat and milk production are emphasized.

For greater clarity in the chapter, the organs or structures of alfalfa have been divided into seed, root, crown, stem, leaf, flower and fruit.

Organography of alfalfa

Seeds

The fruit, which in this case is called a legume, gives rise to the seeds. These are generally round-shaped and yellow-colored. However, angular-shaped seeds and seeds with colors ranging from olive green to different shades of brown can be found (Figure 1).

Ripe seeds are 1 mm to 2 mm long, 1 mm to 2 mm wide and 1 mm thick. The seed consists of the funiculus (the stalk attaching the ovule to the ovary wall), the external integument (seed coat or testa), the embryo and the endosperm (Figure 2). The funiculus keeps the seed attached to the fruit; when the funiculus dries, the seed detaches and a scar called a hilum is formed. The seed coat is the outer layer that encloses and protects the seed; in addition, it is responsible for the seed's color. The embryo will give rise to the future plantlet, where the radicle, the hypocotyl, the plumule and the cotyledons are found. The radicle, which emerges during germination through the micropyle, will form the root. Opposite, the hypocotyl will give rise to the aerial (above-ground) part of the plantlet. Meanwhile, the development of the plumule, which is small conical structure made up of embryonic leaves, will give rise to the shoot. The cotyledons, which are thick and fleshy, store most of the reserve tissue for the embryo's development. Finally, the albumen is a reserve tissue which is small in alfalfa, whose primary function is to facilitate the germination process.

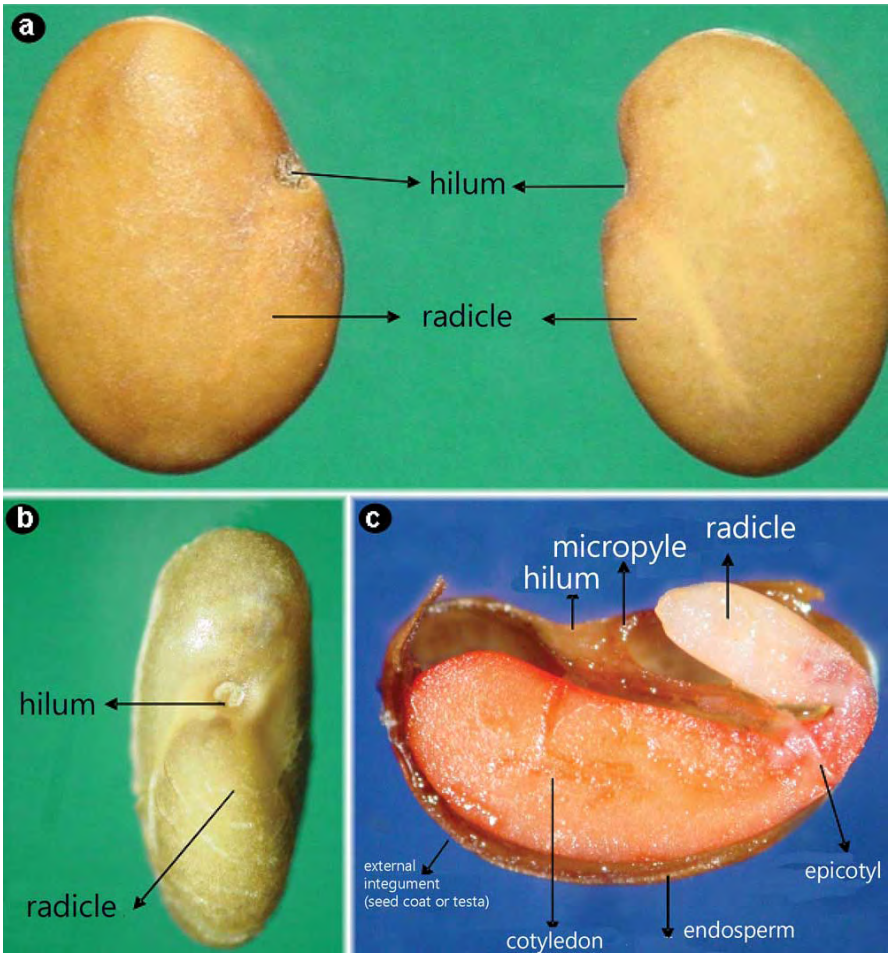
Photos: Nora Estela Rodríguez

Figure 1. Shapes and colors of alfalfa seeds.

Germination and early stages of development

In the germination process, the seed, in contact with moist soil, initiates water absorption and triggers a number of changes including development of a root (starting from the radicle preexisting in the seed) and a small shoot that grows until the cotyledons emerge above the soil surface (Figure 3). These processes are fueled by the seed reserves (Del Pozo Ibañez, 1977).

In order for the seeds to be able to absorb water, the soil must have sufficient moisture. However, the plantlet also needs minimal conditions of aeration for developing, since excessive moisture can paralyze germination by reducing the number of free pores in the soil. Nevertheless, it's common for alfalfa to have "hard seeds", which are incapable of soaking up water even under optimal moisture conditions. This phenomenon, a survival mechanism of the species, is due to increased thickness of the cell walls forming the integument, which constitutes a physical barrier to water absorption. The percentage of hard seeds, which can be high at the time of harvest, decreases over time. The best method to eliminate hard seeds is mechanical scarification, submitting the seeds to the action of an abrasive surface.



Photos: Nora Estela Rodríguez

Figure 2. Parts of the alfalfa seed. Outer section: side view (A) and front view (B). Inner section: cross sectional view (C).

Photos: Nora Estela Rodríguez

Figure 3. Germination of the alfalfa seed: sprouting of the radicle (A) and development of the plantlet, with emergence of the cotyledons (B).

As the development of the aerial (above-ground) part of the plantlet continues, the hypocotyl grows and exposes the cotyledons above the surface of the soil (Figure 4A). Later, the plantlet exhibits first one unifoliolate leaf (Figures 4C, 4B and 4D) and then the trifoliolate leaves, also called “true” leaves (Figure 5). The sequence of development of the alfalfa plant is presented in Figure 6.

Photos: Nora Estela Rodriguez

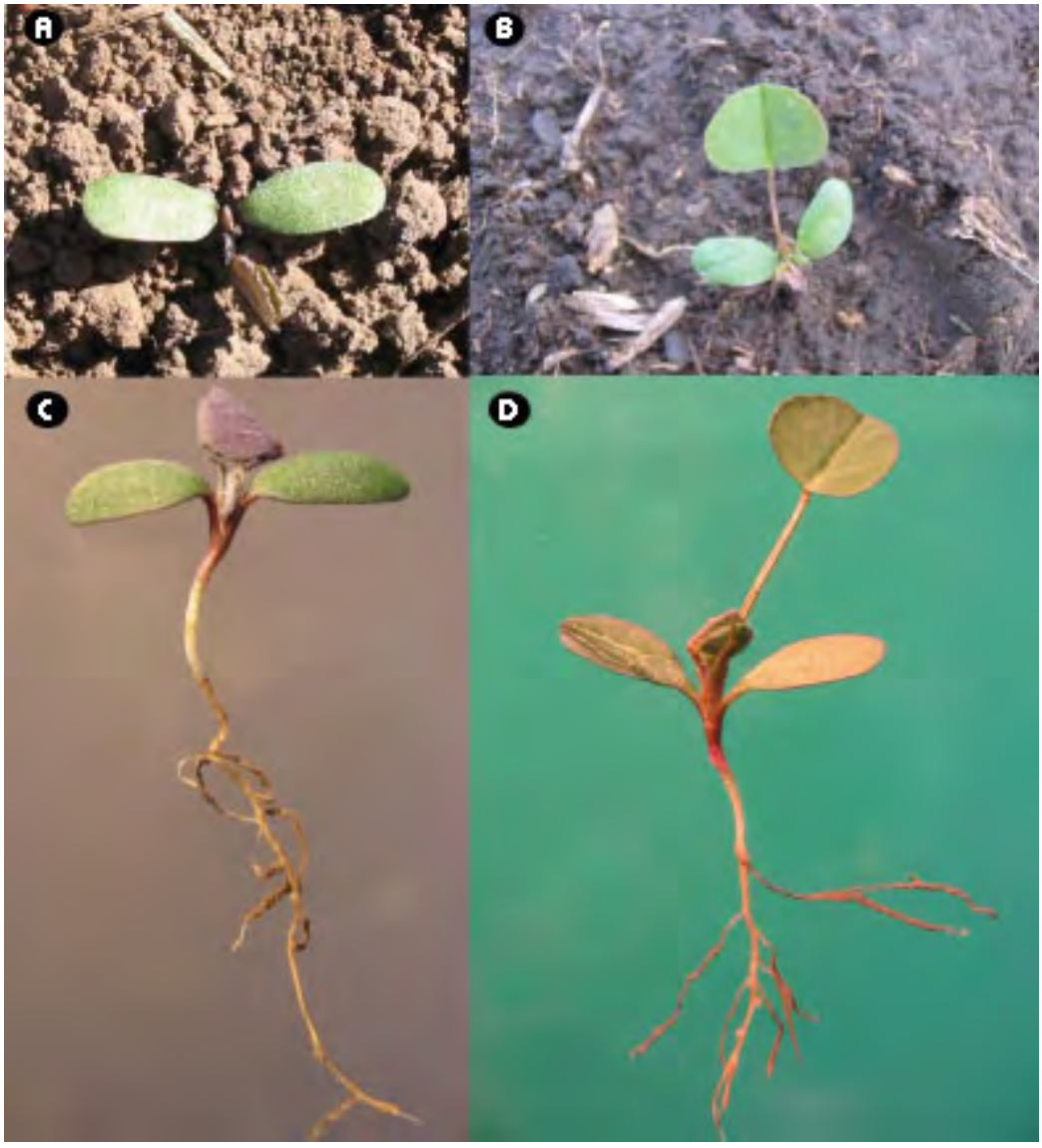
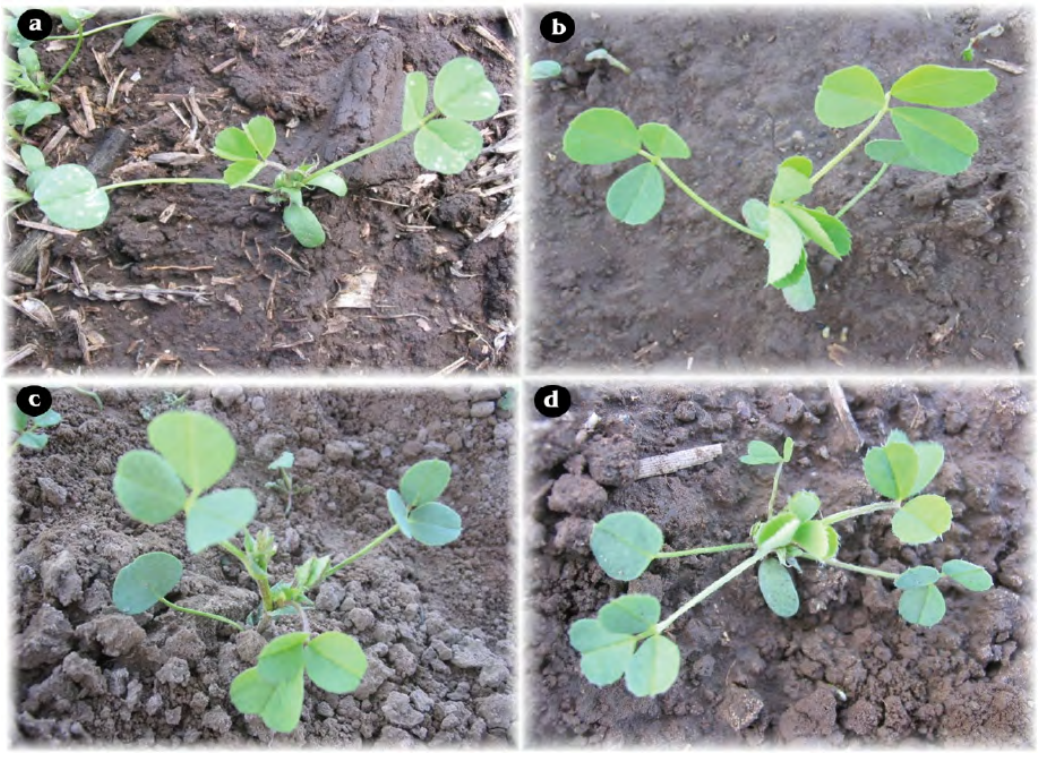


Figure 4. First stages of vegetative development of alfalfa: cotyledon (A and C) and unifoliolate leaf (B and D).



Photos: Nora Estela Rodríguez

Figure 5. First stages of vegetative development of alfalfa, with emergence of one (A), two (B), three (C) and four (D) trifoliolate leaves.

Root

The root system of alfalfa is generally robust and deep, and its main function is to absorb water. If there are no obstacles in the soil profile, the roots can reach 2 m to 5 m of depth with two to four years of life (Figure 7). This enables the plant to absorb water from deep soil layers and gives alfalfa its reputation as a drought-tolerant species.

The main root of the plant emerges next to the hilum and a variable number of secondary or lateral roots may or may not emerge from this main root. The root system can be classified into general types: taproot or main root (axonomorphic root), branched root, rhizomatous root and creeping root (Heinrichs, 1968; Goplen et al., 1980; Pérez de Pereyra; Aguilar de Espinosa et al., 2002). In cultivars that do not have winter rest (WR 8-11), a taproot without many branches is observed most of the times (Figure 8A). Cultivars with intermediate or moderate degree of winter rest (WR 4-7) usually have a large number of secondary roots (Figure 8B), in direct proportion to their increased

Photos: Nora Estela Rodríguez

Figure 6. More advanced periods of the vegetative and reproductive development of alfalfa.

Photo: Nora Estela Rodríguez

Figure 7. Roots of alfalfa cultivated for 2 years, which have reached 1.40 m depth in the soil.

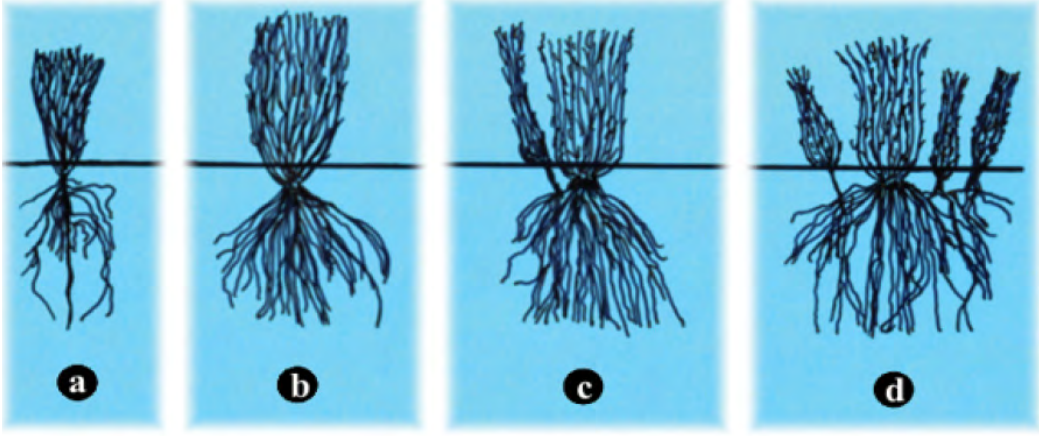


Figure 8. Types of alfalfa roots: taproot (A), branched (B), rhizomatous (C) and creeping (D).

Source: Adapted from Goplen et al. (1980).

level of latency. In cultivars with accentuated winter rest (WR 1-3), side roots have buds giving rise to stems which, after emerging from the soil, will form new shoots. When there are only one or two active buds and the shoots develop at close range from the original plant, these roots are called rhizomatous (Figure 8C); if on the contrary there are several active buds and the sprouts shoots over a relatively long extent, this root is called creeping (Figure 8D). The presence of a taproot is associated with alfalfa cultivars from the *Medicago sativa* species, while the presence of a large number of secondary, rhizomatous or creeping roots is associated with the *M. falcata* and *M. varia* species.

Stem and crown

The primary stem is square in cross section and has stomata and hairs. It has primary and also secondary growth, with the latter giving rise to tissue forming part of the crown. In the herbaceous part, the stem has nodes from which the leaves emerge. The number of stems depends on the age and strength of the plant, and it can reach 20 (Figure 9). Growth of stems is induced by the plant being used (pasture or cutting), or by a new physiological growth cycle (Alfalfa, 2005).

The stems are usually of solid consistency, but in some cases hollow stems can be found, such as in the Argentinean ecotype Saladina. There are also different growth habits, strongly connected to the degree of winter rest. As a general rule, it can be said that crops without winter have upright stems, while crops with intermediate winter rest or crops with accentuated winter rest have semi-upright or semi-creeping stems, respectively.

As the plant grows a set of new stems forms at its base, between the aerial (above-ground) part and the root. This structure is called a crown (Figure 10), and in the grown plant it is formed by perennial stems.

During the crown formation process, Jones (1928) found that if the primary axis – the one related to the cotyledons and the first true leaves – is buried, the crown can develop from the higher area of the axis. Thus, Hayward (1938) determined that the formation of the crown does not depend on buds located in the root.

According to Figure 11, the crown is not a single or unique structure, but a complex area formed by several independent structures (Teuber; Brick, 1988). Although Steward (1926) suggested that the crown may be formed by perennial tissues from the stem, Simonds (1935) concluded that the upper part of the root is also involved in the formation of this structure. Nonetheless, the crown exact morphological delimitation is immaterial, since the drought period, the

Photos: Nora Estela Rodríguez

Figure 9. Alfalfa stems with nodes from which trifoliolate leaves emerge.

cold period, crop practices, attacks by pest and diseases, general strength and age of the plants influence the number and quality of the vegetative parts that may take part in forming crown (Hanson, 1972).

In addition to its morphological formation, it is important to emphasize the crown functional importance as a storage structure for reserve substances and as the location of buds, from which new shoots of the plant will be produced.

Photos: Nora Estela Rodríguez

Figure 10. Early stages of crown formation in alfalfa plants cultivated for 4 months.

Photos: Nora Estela Rodríguez

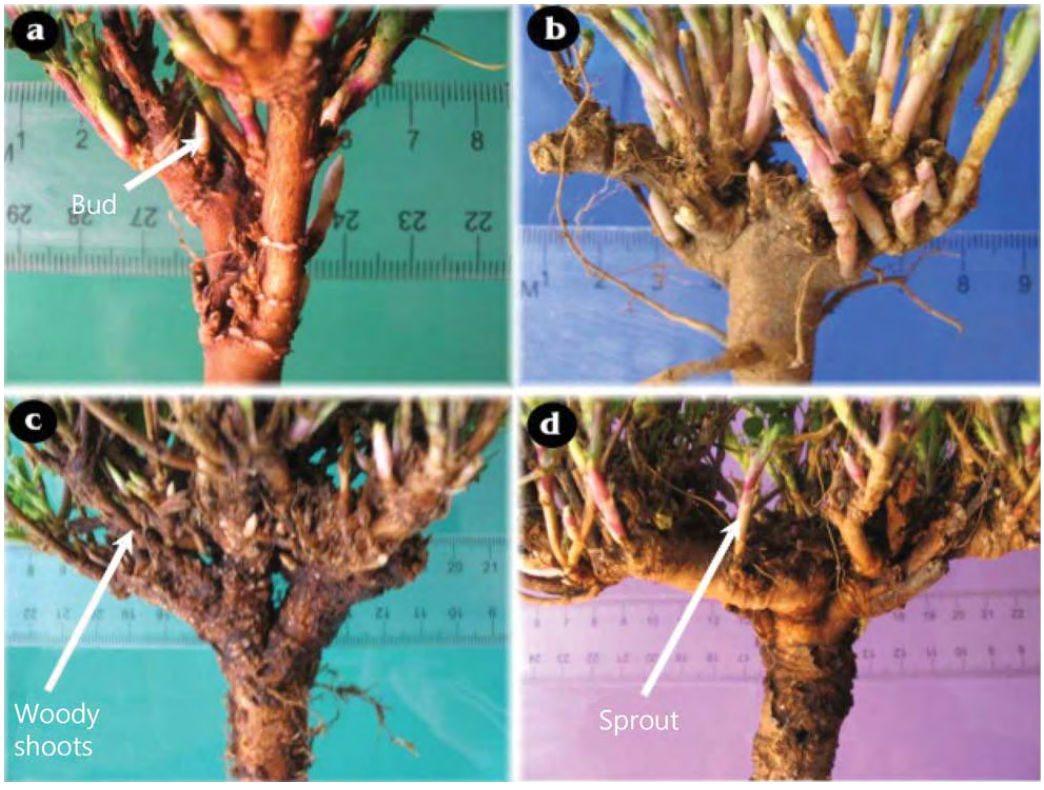
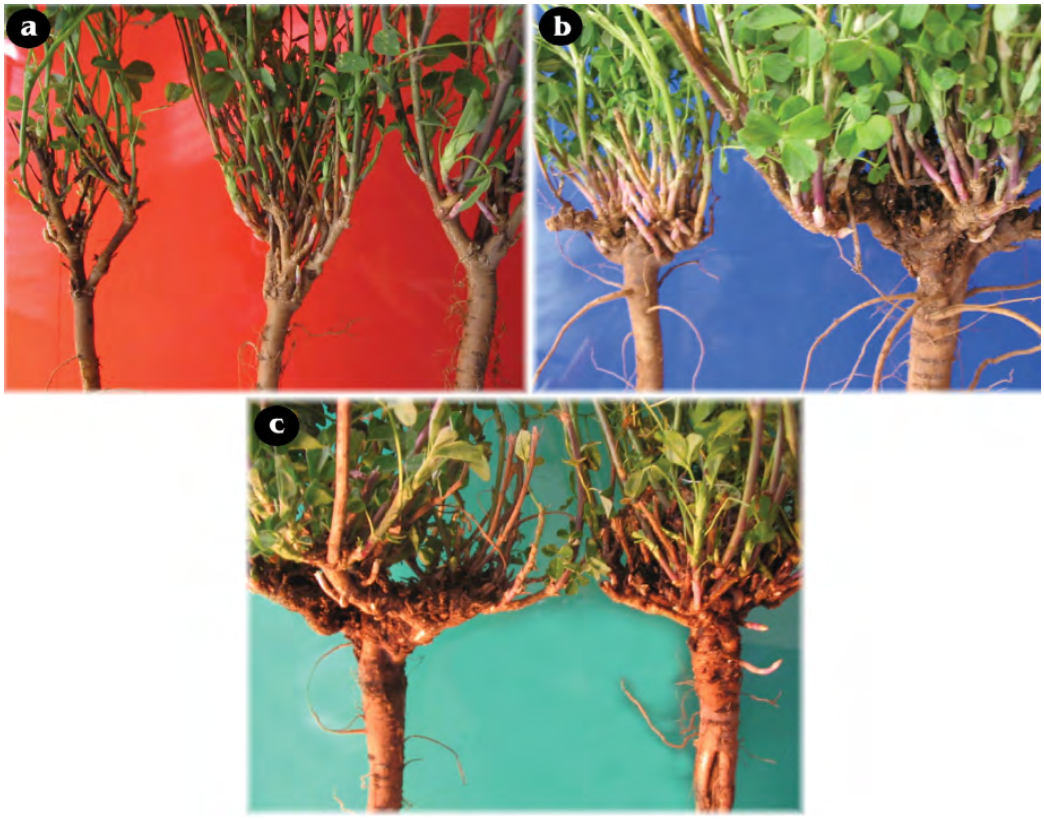


Figure 11. Constitutive parts of crowns already formed in alfalfa at 1 (A), 2 (B), 3 (C) and 4 (D) years of age.



Photos: Nora Estela Rodríguez

Figure 12. Different types and sizes of crowns in alfalfa plants with 1 (A), 2 (B) and 3 (C) years of cultivation.

The cycle of accumulation and utilization of reserve substances is essential for the plant's life, and it influences crop management practices.

The crown size (small, medium, large, etc.) and type (compact or closed, intermediate, open, etc.) depend on genetic and environmental factors (Figure 12). Usually, crops without winter rest have small and compact crowns, while ones with longer winter rest tend to have longer and more open crowns. However, several factors such as plant density, soil type, attacks by pests and diseases, trampling by animals or damage by machinery can significantly influence the crown characteristics.

Leaves

The first leaf of the alfalfa plantlet is unifoliolate and orbicular. The second and subsequent leaves are pinnately compound, imparipinnate and,

most of the times, trifoliolate. The leaves themselves, which are connected to the stem by the petiole, are made up of three petiolate folioles. The folioles are usually oblong or obovate, but they can be found in shapes that range from rounded to obovate-oblong, or even linear (Figure 13).

Leaves originate from the apex of the stem when the plant is grown, but they may also grow from lateral buds on the stems.

Usually, the margins of the folioles are only dentate on the upper third, although this dentate margin can extend as far as the upper half and also include the bottom half (Figure 14). Distribution of dentate margins is related to foliole shape.

The leaves are alternately arranged along the stem axis. Stipules (Figure 15), thin appendages similar to small leaves at the base of the petiole and attached to its sides, are observed during leaf formation. The stipules are usually laciniate (Figure 15A), but there are also entire ones (Figure 15B). Experience indicates that the former are usually found in the leaves of older plants, and the latter exclusively in leaves of younger plants. Thus, it can be empirically inferred that the presence of laciniate stipules or entire stipules is related to the plant's age more than to any other factor.

The foliole midrib (central vein) is prominent and extends the length of the blade; other pinnate lateral veins from it, subdividing and forming a net.

Photos: Nora Estela Rodríguez

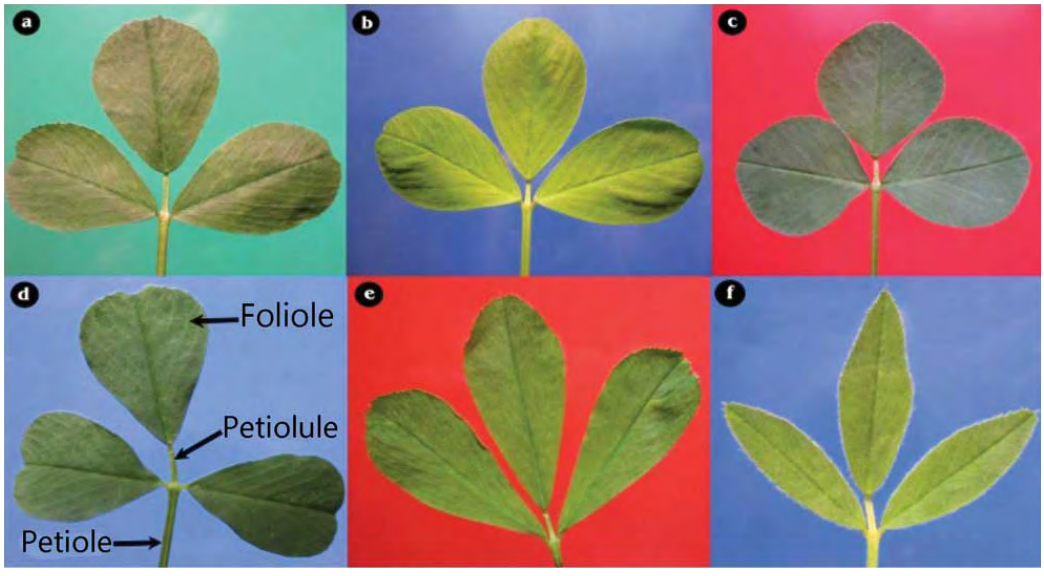
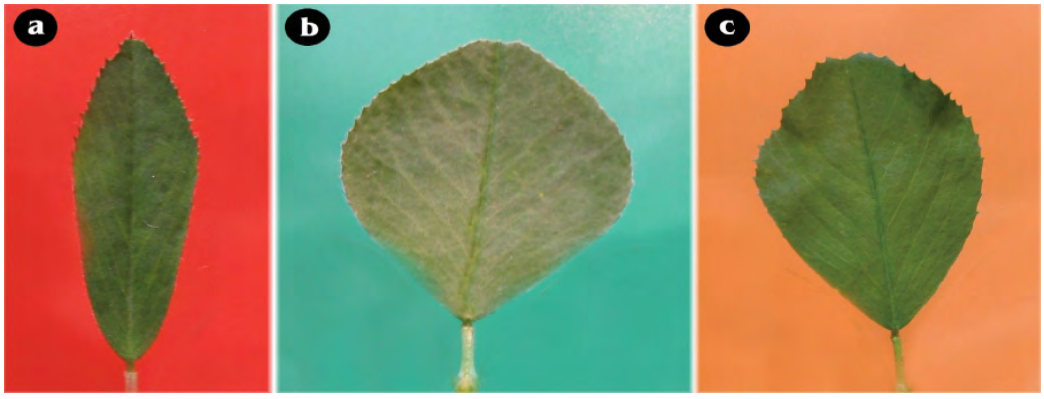
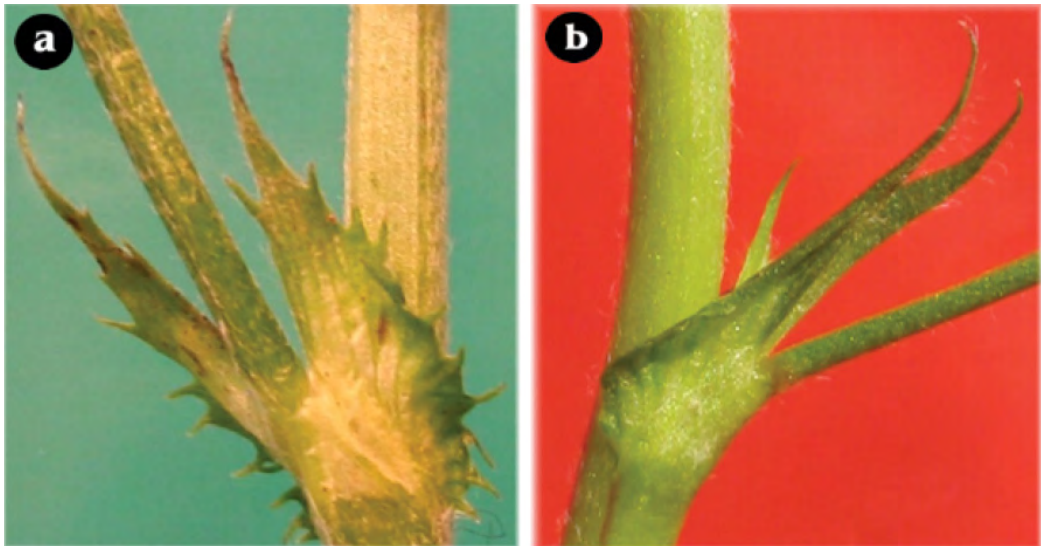


Figure 13. Foliole shapes in trifoliolate alfalfa leaves: obovate (A), oblong (B), rounded (C), cordate (heart-shaped) (D), spatulate (E) and linear (F).



Photos: Nora Estela Rodríguez

Figure 14. Distribution of dentate margin on foliole blade: only on the upper third (A), as far as the upper half (B) and as far as the bottom third (C).



Photos: Nora Estela Rodríguez

Figure 15. Types of stipules observed in alfalfa leaves: laciniate, in a three-year-old plant (A); entire, in a one-year-old plant (B).

The veins are most noticeable on the abaxial (lower) surface of the foliole, which is pubescent. Microscopic observation of the leaf shows that the stomata (openings or pores through which gas exchange in the leaves occurs) are more abundant on the upper surface and at the apex of the foliole.

Although the trifoliolate leaf constitutes the normal situation, leaves with four (tetrafoliolate), five (pentafoliolate) or more folioles can be found, and they are called multifoliolate (Figure 16). Cases of leaves with folioles

Photos: Nora Estela Rodríguez

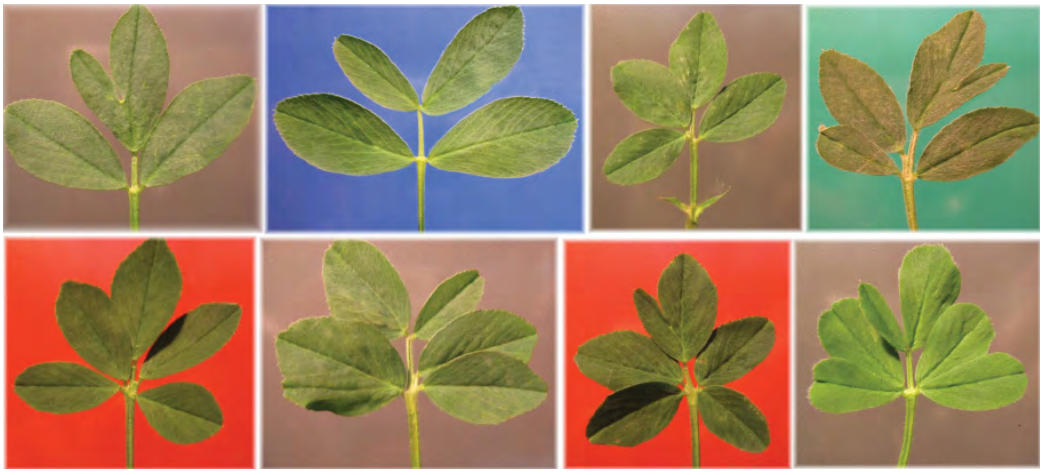


Figure 16. Multifoliolate alfalfa leaves, displaying from four to six folioles.

divided into lobules or with colors different than green (stained or variegate, yellow, etc.) are much rarer.

Flower

The flower develops after the apex of the stem passes from the vegetative to reproductive stage of growth. This change, called transition, initiates with the presence of a protuberance in the axil of the leaf primordium, adjacent to the apex of the stem. From each primordium, one inflorescence originates, in the form of a simple raceme (Figure 17).

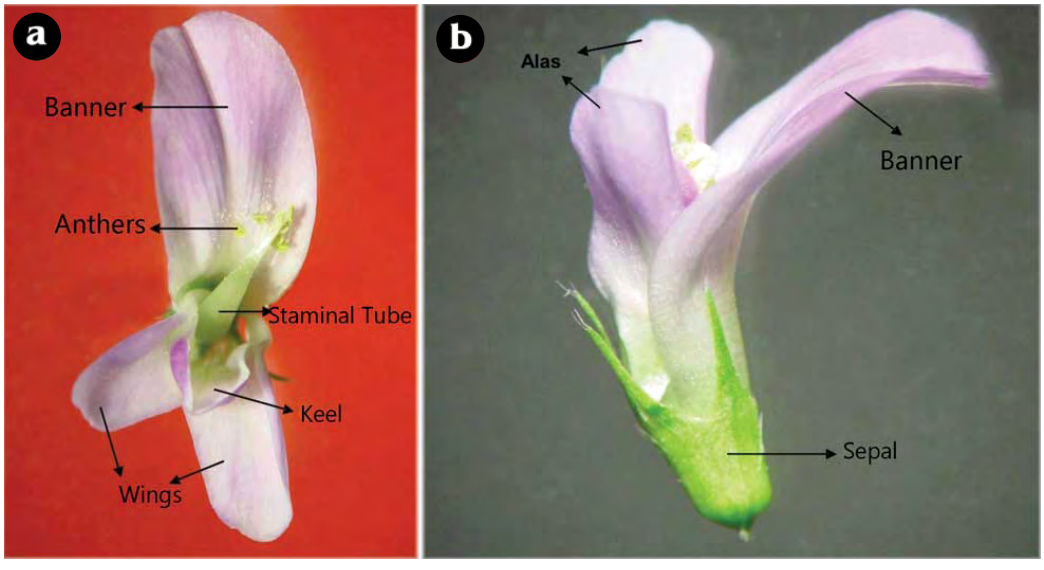
The alfalfa flower is complete, formed by the calyx, the corolla, the stamens and the gynoecium. The flower fits the systematic characteristics of the Papilionoidea subfamily, with a papilionate (butterfly-shaped) corolla (Figure 18).

The calyx is composed of five welded sepals forming a tube; each sepal ends in a lobe or a tooth, which is longer than the calyx tube (Figure 18B). The corolla is formed by five different petals: the vexillum (the standard or banner petal), which is the top petal and the largest one among the five; the lateral wings, which are two smaller petals located on the sides of the standard; and the keel petals, enclosed by the wings and formed by two welded petals, located more internally (Del Pozo Ibañez, 1977) (Figure 18A).

There are ten stamens divided in two groups: one group of nine stamens, connected by the base; and another one formed by the tenth stamen which is free and closer to the standard. This arrangement is called diadelphy, and alfalfa stamens are said to be diadelphous. The filaments of the nine united

Photos: Nora Estela Rodríguez

Figure 17. Alfalfa inflorescence: raceme with flower buds (A) and raceme with two bloomed flowers (B).



Photos: Nora Estela Rodríguez

Figure 18. Structure of the alfalfa flower: top view (A) and side view (B).

stamens have different lengths and the long and short ones alternate where they weld into the tube. The style passes through the inside of this tube, ending in a stigma surrounded by the anthers of the welded stamens. The gynoecium has one carpel, which develops one superior ovary; the gynoecium has well defined style and stigma.

The flower is usually purple, with extremes ranging from light violet to dark purple (Figure 19). It is also possible to find white, yellow or variegate flowers, that is, flowers with mixed colors or in shades that change as the flower develops (Burkart, 1952).

Photos: Nora Estela Rodríguez

Figure 19. Some colors of alfalfa flowers. Bluish (A), light violet (B), light purple (C) and dark purple (D).

Flower development and pollination

The wings, in the corolla, have small hook-like appendages on the base, which keep the staminal column together and rigid; this, in turn, contains the style which is wrapped within it. Thus, pollination is only possible when – as the wings separate through a process called flower tripping – the staminal column releases and exposes the stigma to pollen contact (Figure 20). The sudden movement of releasing the stamina column triggers the opening of the ripe anthers and, therefore, the dissemination of pollen grains.

Several natural mechanisms can trigger flower tripping, such as action of insects and variations in temperature, humidity and wind speed. This mechanism can also be artificially triggered by man, using hand movements or various instruments. The flower can be fertilized by its own pollen (selfing or autogamy) or by pollen from another flower (cross-fertilization or allogamy). Alfalfa is a species with mainly allogamous fertilization, which is favored by natural mechanisms of self-incompatibility and self-sterility (Viands et al., 1988).

Under natural conditions, alfalfa pollination is entomophilous and carried out mainly by the action of bees and beetles. When insects land on the flower to collect nectar or harvest pollen, the pressure they exert on the flower is enough to trigger flower tripping, which causes the staminal column to impact their abdomen. As the insects mentioned successively visit flowers of several

Photos: Nora Estela Rodríguez

Figure 20. Flower development in alfalfa closed flower (A), no separation of wings; and open flower (B), exposing the stigma and the stamens.

plants, their abdomens are always loaded with pollen from different plants, which ensures allogamy. It is estimated that 85% to 95% of developed flowers are fertilized by this mechanism (Del Pozo Ibañez, 1977).

Fruit

The alfalfa fruit is a legume or pod, monocarpellary, dry and indehiscent, usually long and compressed, with the seeds aligned over the ventral suture. Due to its curving, the sheath develops a spiral which usually has one coil by selfing, and three to five coils by cross-fertilization (Figure 21). The coil direction can be dextrogyrate clockwise or anti-clockwise. Each fruit contains a variable number of rounded seeds: two or three from selfing, and nine seeds from cross-fertilization.

Photos: Nora Estela Rodriguez

Figure 21. Points of the alfalfa fruit's evolution, shortly after flower fertilization (top left) until the pod is ripe with several coils (bottom right).

Final considerations

This chapter has attempted to describe the alfalfa plant in a simple way, based mainly on illustration through photos, which provide a clear summary of the shapes and colors that are so difficult to detail.

As a novelty to previous publications on this plant, here we have added a morphological summary to serve as a stepping stone for understanding several aspects of alfalfa cultivation, constituting a starting point for knowledge of this forage plant.

The fundamental structures of the plant have been shown here, specifying in each case the particularities of this species, through which it can be naturally identified.

It should be clarified and emphasized that this study was based mainly on original botanical descriptions. It is also very important to mention that some of the organs described correspond to plants grown the central region of Argentina and thus may be influenced by climate aspects pertaining to that ecosystem.

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Chapter 3

Cytogenetics applied to improvement of alfalfa

Maria Teresa Schifino-Wittmann
Miguel Dall´Agnol

Introduction

Genetic improvement or breeding is a branch of science whose success depends on effective integration with various fields of knowledge, such as botany, entomology, phytopathology and biochemistry. Cytogenetics is very important for breeding, from the initial work of collecting and characterizing germplasm to managing and multiplying this germplasm. For instance, prior determination of chromosome number and study of meiotic behavior are essential when collected material has not yet been cytogenetically characterized. For materials already characterized, cytogenetic information helps and guides future collections, prioritizing certain types. As Sybenga (1997) so well summarized, cytogenetics has two main functions in breeding. First, it provides basic knowledge that every breeder should possess, as well as new information on the material to be collected before, during and after the breeding program. Secondly, cytogenetics provides methods to manipulate the genetic material, through gene transfer, chromosome manipulation, manipulation of ploidy levels or direct manipulation of the genetic system.

This chapter will approach the main cytogenetic aspects of the *Medicago* L. species, emphasizing their applications to genetic improvement of alfalfa.

The *Medicago* genus and the cultivated alfalfa complex

The *Medicago* genus comprises over 60 annual and perennial species, including cultivated alfalfa, tetraploid *M. sativa* L. [or *M. sativa* subsp. *sativa* (L.) L & L.] which belongs to the *M. sativa-falcata* complex, formed both by diploid and tetraploid taxa. Some authors consider the taxa of the complex as a species, but the current trend is to consider them as subspecies. Thus, according to these two positions on taxonomic classification, the main taxa forming the complex are *M. sativa* L., *M. falcata* L. and *M. glomerata* Balb. (according to some authors, syn. *M. glutinosa* M. B.), or *M. sativa* subsp. *sativa* (L.) L. & L., *M. sativa* subsp. *falcata* Arcangeli and *M. sativa* subsp. *glomerata* (Balb.) Rouy. Another species related to cultivated alfalfa is *M. coerulea* Less. ex Nyman, or *M. sativa* ssp. *coerulea* Schmalh.

Despite the great morphological and physiological variation among the various taxa within the complex, there are apparently no restrictions to gene flow when ploidy levels are the same. For example, tetraploidized natural diploids interbreed with natural tetraploids, giving rise to fertile hybrids (Stanford et al., 1972; Quiros; Bauchan, 1988). As will be discussed later on,

this absence of restriction to interbreeding enables a number of cytogenetic manipulations.

The diploid and (rarer) tetraploid forms of *M. sativa* ssp. *falcata*, which are perennial; and diploid, perennial *M. sativa* ssp. *glomerata* have yellow flowers, while bluish-purple flowers occur in the wild, annual, diploid *M. sativa* ssp. *coerulea*, and in the cultivated, perennial, tetraploid *M. sativa* ssp. *sativa* (Langer, 1995).

M. glomerata is said to have been the ancestor to diploid *coerulea* and *falcata*. Meanwhile, cultivated alfalfa probably originated from unreduced gametes of *M. sativa* ssp. *coerulea* (Pfeiffer; Bingham, 1983; McCoy; Bingham, 1988; Quiros; Bauchan, 1988; Langer, 1995).

In the *Medicago* genus, another species is becoming increasingly important, not so much for its forage quality, but mainly for its use in the field of genomics. It is *M. truncatula* Gaertner, a diploid species (2n = 2x = 16) whose genome is being sequenced by several research institutions through an international partnership (Bell et al., 2001; Cannon et al., 2005; Town, 2006). The molecular map, integrated to the genetic and the cytogenetic maps of *M. truncatula*, will contribute to improvement of cultivated alfalfa and its comparison to other species from the genus, as well as to other leguminous species (Paterson et al., 2000; Choi et al., 2004), such as white clover (George et al., 2008).

Medicago Cytogenetics

The majority of *Medicago* species are diploid, 2n = 2x = 16; thus, the basic number x = 8 is predominant in the genus. Exceptions are the annual diploids *M. constricta* Dur., *M. praecox* DC., *M. polymorpha* L., *M. rigidula* (L). All., and *M. rigiduloides* Small., with 2n = 2x = 14, and *M. murex* Willd., with 2n = 2x = 14 and 2n = 2x = 16 cytotypes. Among tetraploids with 2n = 4x = 32, in addition to cultivated alfalfa, there are *M. glutinosa*, *M. arborea* L., *M. dzhawakhetica* Bordz. and *M. schischkinii Sumn*. The diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) ploidy levels occur in *M. sativa* ssp. *falcata*, *M. lupulina* L., *M. papillosa* Boiss. and *M. prostrata* Jacq. There are two hexaploid (2n = 6x = 48) species, *M. saxatilis* M.B. and *M. cancellata* M.B. *M. arborea* has tetraploid and hexaploid cytotypes. Two other species, *M. rugosa* Desr. and *M. scutellata* Mill., have 2n = 30, a rare number in the genus (Lesins; Gillies, 1972; McCoy; Bingham, 1988; Quiros; Bauchan, 1988; Mariani; Falistoco, 1990, 1991; Bauchan, 2009).

Cultivated alfalfa is autotetraploid, that is, it originated from the genome duplication of one ancestor species. Former doubts regarding its autopolyploid or allopolyploid origin were due to observations of meiotic pairing, in which

bivalents predominate. For some time it was accepted that pairing in bivalents indicated an allopolyploid origin. However, in haploids of cultivated alfalfa, bivalents occur, which therefore demonstrates autopolyploid origin (Bingham; Gillies, 1971). The C-banding patterns of the *M. sativa* karyotype also indicate autotetraploid origin (Falistocco et al., 1995). Tetrasomic inheritance characteristic of autopolyploids, for a purple flower factor, was first demonstrated by Stanford (1951) and later confirmed in several other factors, such as isoenzymes (Corts; Martinez, 2000; Quiros, 2000) and DNA markers such as simple sequence repeats and amplified fragment length polymorphism (Julier et al., 2003). Young and induced polyploids have meiotic irregularities as a result of the polyploidization process. However, normally established sexual polyploids have regular meiotic behavior, due to diploidization happening on the chromosomal and genomic level, when the polyploid is reorganized to function as a diploid (Leitch; Bennet, 1997; Soltis; Soltis, 1999). Even in autopolyploids, there is a trend towards quick regularization of chromosome pairing after the polyploidization event (Ramsey; Schemske, 2002). Thus, formation of quadrivalents is not a criterion to allow distinguishing between established autopolyploids and allopolyploids.

For other cultivated plants, there is much more cytogenetic information than on alfalfa and its relatives. The fairly small size (2 µm – 3 µm in somatic cells) and relatively similar morphology of its chromosomes can partly contribute to this (McCoy; Bingham, 1991; Baucham; Hossain, 1997). The vast majority of studies about alfalfa cytogenetics involve counting the number of chromosomes, analyzing karyotypes (both somatic and pachytene) and analyzing meiotic behavior (Lesins, 1957; Stanford; Clement, 1958; Gillies, 1970a, 1970b; Armstrong, 1971; Stanford et al., 1972; Mariani et al., 1978; Schlarbaum et al., 1984; Bauchan; Campbell, 1994; Pupilli et al., 1995). The technique of computerized image analysis has been used as a way to improve visualization and analysis of somatic chromosomes of alfalfa (Bauchan; Campbell, 1994; Bauchan; Hossain, 2001a).

Since 1990, several papers involving C-banding have been published, which have brought more efficiency to chromosome differentiation among and within species. Studies involving C-banding led to the recognition of polymorphism in the constitutive heterochromatin between and within alfalfa populations and genotypes (Bauchan et al., 2002, 2003), and to the identification of the chromosomes of the karyotype (Mariani; Falistocco, 1990, 1991; Falistocco; Falcinelli, 1993; Fallistocco et al., 1995; Bauchan; Hossain, 1997, 2001b) and of extra (B) chromosomes (Hossain; Bauchan, 1999). Using the N-banding technique, Bauchan and Hossain (1998) characterized the chromosomes of the

coerulea and *falcata* subspecies, and were able to differentiate them within hybrids.

In situ hybridization techniques, such as fluorescent *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH), developed in the last decades of the 20th century and which can be ultimately defined as the union of cytogenetics with molecular biology, enabled immense advances – a revolution even – in so-called traditional cytogenetics, giving rise to so-called molecular cytogenetics. Applications made possible by these techniques range from identifying specific sites of various sizes on the chromosome, using FISH; to identifying entire genomes and locating specific chromosomes in hybrids or natural polyploids, with GISH (Guerra, 2004). Excellent examples of various uses of this methodology may be found in the recent study by Puertas and Naranjo (2008).

Bauchan and Hossain (1999) emphasized the discrepancy between cytogenetics of alfalfa and of other important crops, for which the number of cytogenetic studies is much greater. However, these authors suggested that this gap could be filled by applying new molecular cytogenetic techniques and with joint efforts by alfalfa researchers. However, this has not materialized as expected. In cultivated alfalfa, as well as in other species of the genus, there are studies involving *in situ* hybridization techniques, some of which are mentioned below, but they are still few when compared to those addressing other crops.

Calderini et al. (1996) analyzed the ribosomal genes of *M. sativa*, *M. falcata* and *M. coerulea* using FISH with chromomycin A3 staining and with diamino-phenylindole and nucleolus counting, and found that the number of active nucleolus organizer regions (NORs) in *M. sativa* was twice as great as in the diploid species. These authors suggested that no major reorganization or loss of functional rDNA loci had occurred during the evolution of cultivated alfalfa. Using FISH to map rDNA loci in the diploid species *M. glomerata*, *M. sativa* ssp. *coerulea* and *M. sativa* ssp. *falcata*, Falistocco (2000) found that 18S-5.8S-25S rDNA sequences were mapped to two sites corresponding to the secondary constrictions of the nucleolar chromosome pair, and 5S rDNA sequences were distributed in two pairs of sites. The number of rDNA sites in the tetraploid *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* was twice the number found in the in the respective diploid subspecies, which indicated that the distribution of ribosomal genes was maintained during evolution from the primitive diploid to the cultivated tetraploid (Falistocco, 2000). In five natural populations of *M. truncatula*, Falistocco and Falcinelli (2003) observed one

to three loci of genes from subunit 5S, while genes 18S-5.8S-25S were always located in one single locus.

When analyzing the species *Medicago murex* (2n = 14) and *M. lesinsii* E. Small (2n = 16) with FISH and with GISH, Falistocco et al. (2002) found similarity between them in ribosomal gene distribution patterns and suggested that little divergence had occurred between the genomes of the two species after they had separated.

Rosato et al. (2008) used FISH and GISH to analyze three tree species from the *Dendrotelis* section of *Medicago* – tetraploid *M. arborea* and *M. strasseri* Greuter and hexaploid *M. citrina* (Font Quer) Greuter – and one related diploid species from the *Medicago* section, *M. marina* L. Results showed great proximity between the two tetraploids, and that neither would have been involved in the origin of *M. citrina*.

As for the amount of nuclear DNA, based on data from the Royal Botanic Gardens, Kew, determinations have been made for seven species of Medicago. The amounts of nuclear DNA range from 3.90 pg/2C in *M. glutinosa* to 0.95 pg/2C in *M. truncatula*. The 2C value recorded for *M. sativa* ssp. *sativa*, 3.45 pg/2C, is almost twice that of its probable ancestor *M. sativa* ssp. *coerulea*, 1.80 pg/2C.

Recently, there has been an increase in the studies of molecular biology and of genomics of alfalfa and related species (Schifino-Wittmann, 2008), which can be seen in the program of the *41st North American Alfalfa Improvement Conference* (2008).

Application of cytogenetics in improvement of cultivated alfalfa

The major application of cytogenetics directly to alfalfa breeding is the manipulation of ploidy levels, mainly by sexual polyploidization via unreduced gametes (Dall´Agnol; Schifino-Wittmann, 2000). It is the absence of restrictions to gene flow between the various taxa in the complex that ensures the success of this type of genetic manipulation, enabling the transfer of desirable traits.

Induced polyploidization can be performed through somatic methods by using certain substances such as colchicine and nitrous oxide; or through sexual methods, by use of unreduced gametes. The great advantage of sexual versus somatic polyploidization is the conservation of heterozygosity (Schifino-Wittmann; Dall´Agnol, 2003). In various organisms, sexual polyploidization can be more relevant for breeding than somatic polyploidization (Ramanna; Jacobsen, 2003).

Unreduced gametes or 2n gametes, that is, gametes with the somatic number, formed by errors during the meiotic process, normally occur infrequently (less than 1%) in natural populations, but in some genotypes of some plants, such as potatoes, red clover and alfalfa, there is a trend for these gametes to form more often, and that can even be increased by selection (Schifino-Wittmann; Dall´Agnol, 2001, 2003). It is currently accepted that the great majority of natural polyploids, if not all of them, have originated from the union of unreduced gametes (Ramsey; Schemske, 1998). Bilateral sexual polyploidization (union of unreduced male and female gametes) is considered to have been the process that gave rise to cultivated alfalfa (Veronesi et al., 1986; McCoy; Bingham, 1991).

Unreduced gametes resulting from a meiotic process in which reduction of the chromosome number does not occur are mainly formed two ways: by First Division Restitution (FDR) or Second Division Restitution (SDR) meiosis. In FDR, 75% to 80% of heterozygosity is transmitted to the progeny, since in this type of restitution all the heterozygous loci between the centromere and the first crossover will also be heterozygous in the gametes. In SDR, only 40% to 45% of heterozygosity is transmitted to the progeny, since heterozygosity is null between the centromere and the first crossover (Peloquin, 1981). According to Bingham (1980), as there is frequent formation of one chiasma per bivalent in alfalfa, at least 80% of heterozygosity could be transferred by 2n gametes formed by FDR. More rarely, there can be formation of unreduced gametes by a type of non-reduction called indeterminate or by postmeiotic restitution (Ramanna; Jacobsen, 2003). Formation of such gametes can be influenced by the environment and can vary between populations and between individuals of the same species, and differences between populations and between genotypes of alfalfa are well known (Bingham; McCoy, 1979). In several cases, the genes which control the formation of such gametes are known. There are many studies addressing their use in breeding and their role in formation of polyploids (Ramsey; Schemske, 1998; Schifino-Wittmann; Dall´Agnol, 2001; Ramanna; Jacobsen, 2003).

In alfalfa, formation of unreduced pollen occurs by FDR (Vorsa; Bingham, 1979), controlled by the recessive gene *rp*, whose expression is heavily influenced by the environment (McCoy, 1982); and the formation of 2n female gametes occurs by SDR (Pfeiffer; Bingham, 1983; McCoy; Rowe, 1986; Tavoletti et al., 1991a). By means of recurrent selection cycles, Tavoletti et al. (1991b) were able to increase the frequency of 2n pollen and oospheres in diploid alfalfa, and then suggested that production of male and female unreduced gametes is controlled by several genes. Several quantitative trait loci associated

with production of 2n male gametes (Tavoletti et al., 2000) and 2n oospheres were detected by mapping with random amplification of polymorphic DNA and amplified fragment length polymorphism, and there has been suggestion of involvement of at least five genes (Barcaccia et al., 2000). The recessive gene *jp* controls formation of 4n pollen grains ("jumbo"), through lack of cytokinesis at the end of meiosis II. By using plants with *jp* genes it has been possible to recover hybrids of *M. sativa* with *M. dzhawakhetica* or *M. rupestris* M. Bieb. (McCoy; Smith, 1983; Pfeiffer; Bingham, 1983).

The two great advantages of using unreduced gametes in breeding are using them as a bridge for tranfer of genes between different ploidy levels, and maintaining heterozygosity by sexual polyploidization (Ramanna, 1992; Ramsey; Schemske, 1998; Ramanna; Jacobsen, 2003). The possibility of multiallelic interactions in polyploids obtained through sexual polyploidization is much larger than in somatic polyploids, providing better performance.

Sexual polyploidization is an important tool for introgression of traits from wild species into cultivated alfalfa (McCoy; Bingham, 1991; Barcaccia et al., 1998).

According to McCoy and Rowe (1986), unreduced gametes formed by FDR from diploids have 12.5% to 50.0% more heterozygosity than unreduced gametes formed from tetraploids. The same authors reported an increase of 12% to 32% in production by using 2n gametes of the FDR type, compared to the progeny from reduced gametes of somatic tetraploids. Barcaccia et al. (1995) obtained tetraploids via unisexual or bisexual polyploidization, in crossbreeding between producers of 2n gametes, which were superior in several aspects of forage yield to the diploid parents, in the bisexual polyploidization group; and similar to the tetraploids, in the group with unilateral polyploidization. However, these tetraploids showed decreased fertility (Barcaccia et al., 1998), which makes their immediate use in cultivation more difficult (Barcaccia et al., 2003).

One possible use for 2n gametes in alfalfa is the transfer to the tetraploid cultivated species of genes for aluminum tolerance which have been identified in diploid taxa, such as *M. sativa* ssp. *coerulea* (Sledge et al., 2002).

According to Barcaccia et al. (2003), another possible use for unreduced gametes in alfalfa breeding would be by identifying and studying reproductive mutants for formation of unreduced gametes, and through introduction of functional apomixis to alfalfa. For this purpose, it would also be necessary to use selection at the genotype level, with restriction fragment length polymorphism (RFLP) markers and based on the polymerase chain reaction (PCR). However, introducing apomixis into cultivated species has been proving more complex and harder than previously expected (Miles, 2007).

There are studies about alfalfa which look at the haploid level, the triploid level and even higher levels, as reported in the revision by Dall´Agnol and Schifino-Wittmann (2000).

Haploids (2n = "x" = 16). Cultivated alfalfa haploids are actually dihaploids, that is, they derive from polyploids. They are very important for the development of diploid alfalfa, which reproduces through seeds (Bingham; McCoy, 1979). Tetraploid cultivated alfalfa haploids will thus have the same level of ploidy as the wild taxa, such as the *falcata* subspecies, and will be able to interbreed, thus enabling the combination of genes from wild alfalfa and from cultivated alfalfa in hybrids (McCoy; Bingham, 1991). It is possible to perform breeding and selection at this diploid level before restoring the tetraploid level (Bingham, 1971). This procedure, called analytic breeding, consists in reducing the polyploid to its ancestral diploid genome, performing selection at this level and finally re-synthesizing the polyploid based on the diploid components (Chase, 1962, 1964). By backcrossing hybrids of haploid cultivated alfalfa and of the *falcata* subspecies with the haploids and their selections, it was possible to obtain cultivated alfalfa at the diploid level with, for example, better forage yield (Bingham; McCoy, 1979). Cultivated alfalfa at the diploid level is also important for research on breeding methods (McCoy; Bingham, 1991).

Triploids (2n = 3x = 24). Triploid alfalfa plants can be recovered from the progeny of crossings between diploids and tetraploids, and even from crossings between diploids, in this case by the union of one normal gamete to a reduced one (McCoy; Bingham, 1988). In general, triploid production is low, due to the "triploid block", probably caused by unbalance between the proportions of maternal genome and paternal genome in the endosperm (Binek; Bingham, 1970; McCoy; Bingham, 1988, 1991), but there are exceptions which have high triploid frequency, such as the crossing of *M. sativa* with *M. papillosa* (McCoy; Smith, 1984). Triploids are usually sterile males, but with enough female fertility to be used as female parents (McCoy; Bingham, 1991). The main use of triploids is as a bridge for gene transfer between different ploidy levels (Binek; Bingham, 1970; Blake; Bingham, 1986; McCoy; Bingham, 1988).

Hexaploids (2n = 6x = 48) and octaploids (2n = 8x = 64). The advantage of hexaploids and octaploids would be higher mass production, because they have larger leaves than tetraploids. Hexaploids have been obtained through sexual polyploidization in crossings between and within various ploidy levels (Bingham; Saunders, 1974). Some hexaploids were stronger than tetraploid alfalfa, both because of the ploidy level and because of possible effects of

heterosis (in the case of crossing *M. sativa* with *M. falcata*) (Julén, 1944; Bingham; Binek, 1969) and showed less inbreeding depression, when compared to the diploids (Bingham; Binek, 1969). The hexaploids as well as the octaploids showed instability in chromosome number (Yen; Murphy, 1969; McCoy; Bingham, 1991), which may have been caused by frequent meiotic irregularities. Stable hexaploids were obtained by interbreeding *M. sativa* and *M. papillosa* (McCoy, 1989), species having little genomic affinity with *M. sativa*, which only favors formation of bivalents, through preferential intragenomic chromosome pairing. Pupilli et al. (1995) obtained somatic hexaploid hybrids of *M. sativa* ssp. *sativa* with *M. sativa* ssp. *coerulea*, vegetatively vigorous and with forage production equivalent to the most productive parent.

Pentaploids (2n = 5x = 40) and heptaploids (2n = 7x = 56). Plants with these ploidy levels have very low fertility; their use would be important mainly for academic research and they could eventually function as a bridge between ploidy levels (McCoy; Bingham, 1991).

There are collections of trisomics and even of monosomics of several cultivated species. In the case of alfalfa, trisomics at the 2n = 2x + 1 = 17 level can be easily recovered in the offspring of 3x – 2x interbreeding (McCoy; Bingham, 1988), and in some cultivars the natural frequencies of aneuploids can reach 6% (Bingham, 1968). There are no records of monosomics. As McCoy and Bingham (1988) commented, the construction of a complete series of trisomics in alfalfa would be a long term project. So far, there has been very little progress.

Chromosome variations in vitro culture

Schifino-Wittmann (2008) and Bauchan (2009) cited several studies that used strategies ranging micropropagation toin vitro selection, for a number of purposes.

Several cytogenetic alterations can be observed in culture of tissues and cells in vitro, probably caused by the culture medium itself and the culture time. The most common are chromosome duplication and aneuploidy (Bingham et al., 1988). In diploid alfalfa, chromosome duplication was the most frequently observed variant and, in tetraploid alfalfa, aneuploidy and chromosome duplications were the most frequent alterations, as well as certain structural changes (Bingham et al., 1988). Among regenerants, in the case of tetraploid alfalfa, most plants recovered after in vitro culture were also tetraploid (Saunders; Bingham, 1972). In *M. media* Pers., aneuploids also

regenerated after in vitro culture (Nagarajan; Walton, 1987). There may be regeneration of plants from several types of tissues cultured in vitro and after protoplast fusion, and the regeneration capacity of alfalfa based on tissue culture is genetically inherited (Bauchan, 2009).

Somaclonal variation could be an alternative to exploit variability in alfalfa, provided that the desirable variation was separated from the undesirable one, aided by genetic analysis and by breeding (Bingham et al., 1988) and that it was inheritable.

Final considerations

As stated, progress in alfalfa cytogenetics has not matched that of other important cultivated species. There is still much to do, especially in expanding the use of molecular cytogenetic techniques, determining the amount of nuclear DNA and chromosome mapping. Nevertheless, progress in the area of molecular genetics and genomics has been quite significant. If there were integration between two areas, it would be possible for the "queen of forages" to rightfully join the group of well studied important crops at the cytogenetic and genetic levels.

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Chapter 4

Quantitative genetics applied to improvement of alfalfa

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Introduction

One barrier that prevents alfala expansion in Brazil is the lack of cultivars adapted to tropical conditions. In order to understand the real dimension of this problem, it would be sufficient to mention that despite the great demand for new releases from the Brazilian market, the only currently available cultivar is Crioula, a domestic variety with good adaptability and good stability throughout the country (Ferreira et al., 2004).

The development of new cultivars will enable the use of alfalfa in different regions of Brazil, with the consequent increase not only in the alfalfa acreage but also in the availability of high quality feed for intensive milk production systems (Botrel et al., 2001).

The expansion of an introduced exotic species depends on its adaptation to the conditions of the new environment. In this context, cultivars from temperate regions, as is the case of alfalfa, normally have problems adapting to the tropics, since the selective pressure exerted during the breeding process has not included adaptation to a tropical environment (Ferreira; Pereira, 1999).

Breeding methods are a useful tool for developing adapted cultivars starting from exotic materials, assuming there is enough genetic variability in the germplasm of the species. By recombining only selected genotypes, the frequency of favorable alleles in the population is increased and thus it is more likely to achieve effective selection gains in the breeding of the species (Allard, 1971; Rumbaugh et al., 1988; Basigalup, 2007).

In this chapter, the implications of allogamy and autotetraploid inheritance on alfalfa genetic improvement will be addressed. In doing so, some quantitative genetic aspects – such as gamete formation and gamete segregation, gain from selection, response to selection, genetic variance components, heritability, inbreeding, heterosis and inbreeding depression – will be briefly discussed.

Reproductive system

Cultivated alfalfa is a perennial autotetraploid (2n = 4x = 32) species, with perfect flowers and mainly allogamous fertilization. It has self-sterility and self-incompatibility mechanisms that prevent selfing.

Natural pollination is carried out mainly by bees. Because of the pollination control mechanisms, pollinators must visit different flowers, and thus forcing cross-pollination.

Alfalfa is a polymorphic species, with diploid and tetraploid forms. Its basic chromosome number is eight. Since cultivated alfalfa is autotetraploid,

inheritance of traits is complex and has profound consequences on its genetic behavior and on the breeding methods to be used for its improvement.

Quantitative genetics of alfalfa

Biometric methods used by breeders to improve traits related to production and to quality of alfalfa are based on the breeding system and the genetic structure of the species.

Segregation and gamete formation

In discussing this section, two assumptions are made: a) chromosome segregation in alfalfa is at random; and b) the existence of double reduction and preferential pairing, as well as the non-disjunction of chromosomes, is ignored.

Of these factors, only preferential pairing can bring significant deviations to what is expected from theory. However, other phenomena such as preferential pollination of flowers by insects, differential pollen-tube growth rate, incompatibility, sterility and abortion of fertilized ovules, can also cause deviations from the expected results (Busbice et al., 1972).

In a single locus with four alleles (tetraploid), five possible genotypes can be observed: the first, with four dominant alleles (AAAA), is called “quadruplex”; the second, having three dominant alleles (AAAa), is called “triplex”; the third, possessing two dominant alleles (AAaa), is called “duplex”; the fourth, having only one dominant allele (Aaaa), is called “simplex”; and the fifth, with no dominant alleles (aaaa), is called “nulliplex” (Blakeslee et al., 1923).

In the case of complete dominance, the dominant trait can be observed when there is at least one dominant allele; thus, the recessive trait should be observed only under the nulliplex condition. However, in most cases the dominant phenotype in alfalfa is expressed only when two or more dominant alleles are present (Whittington; Bubrage, 1963; Pedersen; Barnes, 1965).

Tetraploid individuals produce diploid gametes. Based on the alleles present at a single locus, these gametes may have different structure and different probabilities of segregation, as shown in Table 1. For example, AAAA individuals only produce AA gametes, with probability equal to 1, unlike AAaa genotypes which can produce three types of gametes: AA with probability 1/6, Aa with probability 4/6 and aa with probability 1/6.

Based on these probabilities, it is possible to obtain the number of individuals to assess from a cross, in order to detect specific genotypes. For

Table 1. Probability of gamete segregation at a single locus by tetraploid individuals with different genotypic constitution (chromosome segregation).

Genotype of the individual	Diploid gametes		
	AA	Aa	aa
AAAA	1	0	0
AAAa	1/2	1/2	0
AAaa	1/6	4/6	1/6
Aaaa	0	1/2	1/2
aaaa	0	0	1

instance, families produced by self-fertilization (selfing) of a duplex individual (AAaa) will produce nulliplex individuals with probability of 1/36 (1/6 x 1/6). If instead of selfing, a test cross is carried out, the probability of obtaining the nulliplex individual is 1/6 (probability of obtaining the aa gamete produced by the duplex individual). Therefore, to identify a nulliplex individual with a 95% level of confidence, 107 self-fertilized offspring should be analyzed, while with the test cross it would be necessary to assess only 17 progeny to achieve the same probability.

The analysis of the evolution of the genetic structure of a tetraploid population under different mating systems is essencial to understand the breeding behavior of a tetraploid species, especially those aspects referred to genetic equilibrium.

Genetic equilibrium in an autotetraploid population can be estimated by comparing the gametic frequencies produced by such population over two or more generations. In this context, when the gametic ratio of the population does not change from one generation to another, the population is in genetic equilibrium. As an example, it can be considered the case in which the initial population has the following structure: 0.13 AAAA: 0.16 AAAa: 0.06 AAaa: 0.08 Aaaa: 0.57 aaaa (Table 2). In doing the calculations, random mating will be assumed. The genotypic ratio of the offspring is given by the square of parent gametic ratio, that is: genotypic ratio of offspring = [gametic ratio of parents]^2.

Thus, probability of each gamete in the parental population (P_0) is: $P(AA)_0 = 0.22$; $P(Aa)_0 = 0.16$ and $P(aa)_0 = 0.62$.

Table 2. Frequency of parental genotypes and frequency of diploid gametes produced by those parental genotypes in a population of autotetraploid plants.

Genotype of the parents	Frequency	Diploid gametes of the parents		
		AA	Aa	aa
AAAA	0.13	0.13	0.00	0.00
AAAa	0.16	0.08	0.08	0.00
AAaa	0.06	0.01	0.04	0.01
Aaaa	0.08	0.00	0.04	0.04
aaaa	0.57	0.00	0.00	0.57
Result	1.00	0.22	0.16	0.62

Therefore, the genotypic ratio in the offspring will be = $(0.22\ AA + 0.16\ Aa + 0.62\ aa)^2$, resulting in this proportion of individuals 0.0484 AAAA: 0.0704 AAAa: 0.2984 AAaa: 0.1984 Aaaa: 0.3844 aaaa.

The next generation of the previous population will have the gametic proportion (P_1) that is shown in Table 3.

That is:

$P(AA)_1 = 0.1333$; $P(Aa)_1 = 0.3333$ and $P(aa)_1 = 0.5333$.

As a consequence, since $P(AA)_0 \neq P(AA)_1$; $P(Aa)_0 \neq P(Aa)_1$ and $P(aa)_0 \neq P(aa)_1$.

Table 3. Frequency of offspring genotypes and frequency of diploid gametes produced by this offspring in the population of autotetraploid plants derived from the parental population described in Table 2.

Offspring genotype	Frequency	Offspring diploid gametes		
		AA	Aa	aa
AAAA	0.0484	0.0484	0.0000	0.0000
AAAa	0.0704	0.0352	0.0352	0.0000
AAaa	0.2984	0.0497	0.1989	0.0497
Aaaa	0.1984	0.0000	0.0992	0.0992
aaaa	0.3844	0.0000	0.0000	0.3844
Total	1.0000	0.1333	0.3333	0.5333

It is concluded that the population in this example was not in equilibrium and that genetic equilibrium was not achieved after one generation of random mating. While the latter is a distinctive condition of autotetraploid populations, one generation of random mating is sufficient to reach equilibrium in diploid populations.

For an autotetraploid population at Hardy-Weinberg equilibrium and under random mating, assuming the gametic frequencies of A and a are represented by $f(A) = p$ and $f(a) = q$, the frequency of the five possible genotypes is given by the equation $(p + q)^4$. For the precious example, in which $f(A) = p = 0.3$ and $f(a) = q = 0.7$, the resulting genotypic frequencies are shown in Table 4.

All of the above has practical implications in the alfalfa breeding. As an example, the assessment of the offspring derived from the cross of two tetrallelic individuals can be considered, as shown in Table 5.

Table 4. Genotypic frequency in a population of autotetraploid plants at Hardy-Weinberg equilibrium.

Genotypes	Frequency	Genotypic frequency in equilibrium
AAAA	p^4	0.0081
AAAa	$4p^3q$	0.0756
AAaa	$6p^2q^2$	0.2646
Aaaa	$4pq^3$	0.4116
Aaaa	q^4	0.2401

Table 5. Possible gametes produced by two different tetrallelic alfalfa parental individuals (A_1-A_4 and A_5-A_8).

Individuals	$A_1\ A_2\ A_3\ A_4$	x	$A_5\ A_6\ A_7\ A_8$
Possible gametes	$A_1\ A_2$		$A_5\ A_6$
	$A_1\ A_3$		$A_5\ A_7$
	$A_1\ A_4$		$A_5\ A_8$
	$A_2\ A_3$		$A_6\ A_7$
	$A_2\ A_4$		$A_6\ A_8$
	$A_3\ A_4$		$A_7\ A_8$

There are 36 possibile genotypes that can be obtained in the F_1 generation. However, all possible genotypes are tetrallelic. Therefore, if the breeder is searching for just a monoallelic combination (i.e., an individual with only one type of allele), such genotype will not be found after only one generation of random mating F_1 plants. This is another distinctive autotetraploid characteristic: all possible genotypes from a cross (monoallelic, diallelic, triallelic and tetrallelic) are produced only after two generations of random mating (Table 6).

As presented in Table 6, the frequency of the monoallelic class is very low, which constitutes another autotetraploid characteristic. In addition, it can be noticed that equilibrium among these different classes is only obtained after four generations of random mating.

Table 6. Genetic frequency and genotypic structure of an autotetraploid population along different generations.

Generation	Structure and frequencies			
	Monoallelic	Diallelic	Triallelic	Tetrallelic
F_1	0.000	0.000	0.000	1.000
F_2	0.000	0.037	0.426	0.537
F_3	0.001	0.074	0.474	0.450
F_4	0.002	0.106	0.492	0.410

Source: Adapted from Busbice et al. (1972).

Gains from selection

To develop superior genotypes, it is necessary to combine a number of favorable traits that allow not only higher yields *per se* but also other characteristics related to the satisfaction of market quality requirements. Thereby, selection based on only one or just a few traits seems inadequate, since it is going to lead to a final product that will be superior only on the selected traits (Cruz; Regazzi, 1997). This is very important in alfalfa improvement as a feed, because the final goal is not only to increase forage yield but mainly to improve forage quality and animal intake.

Response to selection

The need to perform selection for several traits in order to simultaneously improve all of them requires the use of a selection criteria based not purely on one trait or on indirect selection alone. Hill (1971) compared response to selection between diploid and autotetraploid populations, concluding that response was faster in the former that in the latter.

To illustrate and to compare the gain from selection between diploid and and in autotetraploid populations, an example in which the allele to be selected is dominant (A > a) and the gene under consideration is in equilibrium in both populations is analyzed.

If $f(A) = p$ and $f(a) = q$, then $P(A) = p$ and $P(a) = q$, in which $p + q = 1$.

The predicted genotypic ratio is given by the equation $(p + q)^4$ and it will be:

$(p + q)^4 = p^4\ AAAA:\ 4p^3q\ AAAa:\ 6p^2q^2\ AAaa:\ 4pq^3\ Aaaa:\ q^4\ aaaa$

Since selection will eliminate the aaaa genotype, then the frequency of p becomes p', and the effect from selection (Δp) will be:

$\Delta p = p' - p$,

in which

$$p' = \frac{p^4 + 4p^3q + 6p^2q^2 + 4pq^3}{4(p^4 + 4p^3q + 6p^2q^2 + 4pq^3)} = \frac{p}{1 - q^4}$$

Thus, selection gain will be:

$$\Delta p = p' - p = \frac{p}{1 - q^4} - p = \frac{pq^4}{1 - q^4}$$

According to the previous equation, Δp, which expresses the frequency variation for the selected allele, is a function of its initial frequency.

In the case of a diploid population, Δp is estimated by the following equation:

$$\Delta p = p' - p = \frac{p}{1 - q^2} - p = \frac{pq^2}{1 - q^2}$$

Figure 1 shows the frequency variation for allele A, as a response to selection, relative to its initial frequency (p) in both diploid and autotetraploid populations. It can be noticed that the rate of the variation in the tetraploid population is much slower than in the diploid population, which explains the longer time usually required to promote genetic changes in tetraploid species.

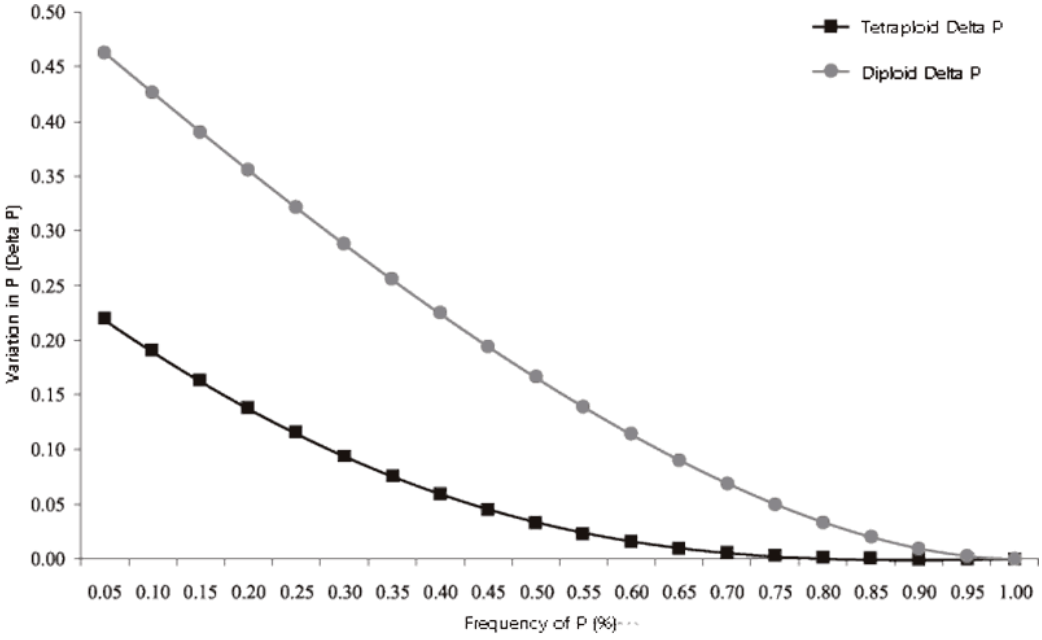


Figure 1. Response to selection as variation of allelic frequency relative to the initial frequency of the allele (*p*) in both diploid and tetraploid populations.
Source: Hill (1971).

Components of genetic variance and heritability

Success in improving any trait necessarily requires that the trait is inheritable and that there is sufficient genetic variation in the population that makes selection possible. In this section, to study the inheritance and the variation of quantitative traits it will be considered the basic model $P = G + E$, in which the phenotypic value of an individual (P), results from the action of the genotype (G) and the influence of the environment (E). Similarly, the phenotypic variance (σ^2_P) is the result of the genotypic variance (σ^2_g) and the environmental variance (σ^2_E).

Kempthorne (1955) demonstrated that in autotetraploid populations, as in the case of alfalfa, genotypic variance (σ^2_g) can be decomposed in:

σ^2_G: genotypic variance of the population
σ^2_A: additive variance
σ^2_D: digenic variance
σ^2_T: trigenic variance
σ^2_Q: quadrigenic variance

These variances are obtained from the genotypic value $V(G)$ which, for a given individual, is given by the equation:

$$V(G) = A_i A_j A_k A_l = \mu + \alpha_i + \alpha_j + \alpha_k + \alpha_l + \beta_{ij} + \beta_{ik} + \beta_{il} + \beta_{jk} + \beta_{jl} + \beta_{kl} + \gamma_{ijk} + \gamma_{ijl} + \gamma_{jkl} + \delta_{ijkl}$$

While A_i, A_j, A_k and A_l are the alleles from a particular locus; μ is the mean of the population at genetic equilibrium.

The other terms in the equation express the effects that cause deviations of each individual from the population mean (μ). In the equation, the variance components σ^2_A, σ^2_D, σ^2_T and σ^2_Q are represented by α, β, γ and δ, respectively.

According to Rumbaugh et al. (1988), additive individual (α_{i-l}) effects in tetraploids are the same as additive effects in diploid models. Likewise, digenic effects (β_{ij-kl}) are analogous to the heterotic effects in diploid organisms. However, trigenic ($\gamma_{ijk-jkl}$) and quadrigenic (δ_{ijkl}) effects have no analogy in diploid models.

In alfalfa, the estimation of the genetic variance based on covariance between relatives has been proposed, as well as a procedure for estimating genetic variance components (Levings; Dudley, 1963). For the latter, it was suggested the use of a partial diallel cross design together with parent-offspring regression and the estimation of genotypic variance among clones. This scheme was used by Dudley et al. (1969) to calculate the variance components related to dry matter and plant size in alfalfa. It was concluded that while trigenic and quadrigenic effects had a relative importance, additive and digenic effects were the most and the least important, respectively.

The establishment of genetic models is very important for estimating genetic variance and covariance components, as well as interactions with the environment. These parameters, in turn, have great implications on the estimation of heritability, both in broad and narrow sense.

Only the phenotypic value of an individual can be directly measured; however, it is just the genetic value will influence over the next generation. Therefore, it is necessary to estimate the proportion of the total variability existing in the population which is genetic in nature.

Heritability expresses the proportion of genetic variation relative to phenotypic variation, i.e., the relationship between genetic variance and phenotypic variance in the selection units (Cruz, 2005). The previous concept implies that heritability is a function of the type of selection that is being performed, whether it is selection among families, selection among individuals, stratified mass selection, or any other type of selection. Heritability also depends on the experimental design and the estimation method that are used, the trait under study, and the genetic diversity of the population, among other factors.

Heritability can be calculated in a broad or in a narrow sense, taking into account genetic variance or just additive variance, respectively. Heritability in broad sense can be estimated by the following equation:

$$h_b^2 = \frac{\sigma_g^2}{\sigma_p^2}$$

where:
h_b^2 = heritability in broad sense
σ_g^2 = genetic variance of the selection unit
σ_p^2 = phenotypic variance selection unit

Kehr and Gardner (1960), using progenies from a polycross and clones of the parental genotypes, estimated heritability through the following equation:

$$h^2 = \frac{4\sigma_{px}^2 + 2COV_{op}}{2\sigma_c^2}$$

in which:
h^2 = heritability
σ_{px}^2 = variance among progenies from polycross
COV_{op} = covariance between parental clones and progenies from polycross
σ_c^2 = phenotypic variance among clones

Once the heritability value was calculated, the gain from selection can be estimated. The possibility to predict the gain from a given selection strategy constitutes one of the main contributions of quantitative genetics to breeding. The use of this information allows not only to more effectively conduct the improvement program, but also to predict the progress (gain) from selection.

Gain from selection (GS) can be estimated by the equation:

$$GS = SD \times h^2$$

where h^2 is the heritability values and *SD* is the selection differential, i.e. the difference between the mean of the selected population and the mean of the original population. For instance, if the mean yield of the original is 15 ton ha^{-1} $year^{-1}$ and the mean yield of the selected population is 18 ton ha^{-1} $year^{-1}$, the *SD* would be 3 ton ha^{-1} $year^{-1}$; and if h^2 is 0.50, then the GS will be:

$GS = 3 \times 0.5 = 1.5$ t ha^{-1} $year^{-1}$

Inbreeding

Inbreeding is the phenomenon which happens as a result of mating related individuals – in other words, sharing common ancestors. The inbreeding

coefficient, represented by *F*, refers to the probability that the alleles of a gene in an individual are identical by descent – that is, the alleles could have been derived by replication of an allele found in a common ancestor.

The main effect of inbreeding is to increase the frequency of homozygotes at all loci in the population. However, in the absence of selection, inbreeding alone does not change allele frequencies; it only alters the arrangement of alleles in the genotypes of the population. Thus, under no selection, this allele reorganization is just a short-lived change: homozygote frequency will decrease as soon as the mating system changes.

According to Wrigth (1922), inbreeding results from the union of identical gametes and it is expressed by the correlation between the values of the gametes that form the progeny from a population.

Considering a particular locus of an individual X which produces a gamete ab, the value of the inbreeding coefficient (*Fx*) for that individual will be given by $Fx = P(a \equiv b)$, where ≡ means being identical by descent. Thus, the inbreeding coefficient for a particular individual is equivalent to the probability to which this individual will produce gametes that are identical by descent.

If individual X is crossed to an individual Y, which produces gametes "ef", their offspring (F_1) will have the following inbreeding coefficient:

$$F_{1xy} = 1/6\ [P(a \equiv b) + P(a \equiv e) + P(a \equiv f) + P(b \equiv e) + P(b \equiv f) + P(e \equiv f)]$$

If r_{xy} is defined as the probability for a random allele from X to be identical by descent to a random allele from Y, then:

$$P(a \equiv e) = P(a \equiv f) = P(b \equiv e) = P(b \equiv f) = r_{xy}$$

Thus:

$$F_{1xy} = 1/6\ (4r_{xy} + F_x + F_y) = 2/3\ r_{xy} + 1/6\ (F_x + F_y)$$

Therefore, in an autotetraploid the progeny can be inbred either when the parents are related or when they are inbred. When parents are not related, the offspring always inherits 1/3 of the parental mean of inbreeding. This is a consequence of the diploid gametes produced by autotetraploids. In this context, if it is assumed that X is an inbred individual with its four alleles identical by descent ($a \equiv b \equiv c \equiv d$), Y is not inbred ($e \neq f \neq g \neq h$), and X and Y are not related, the progeny (Z), from the union of gametes "ab" and "ef" will have the following genotypic constitution: $a \equiv b \neq e \neq f$. If *F* is the probability of alleles being identical by descent, then:

$$F_z = 1/6\ [P(a \equiv b) + P(a \equiv e) + P(a \equiv f) + P(b \equiv e) + P(b \equiv f) + P(e \equiv f)] =$$

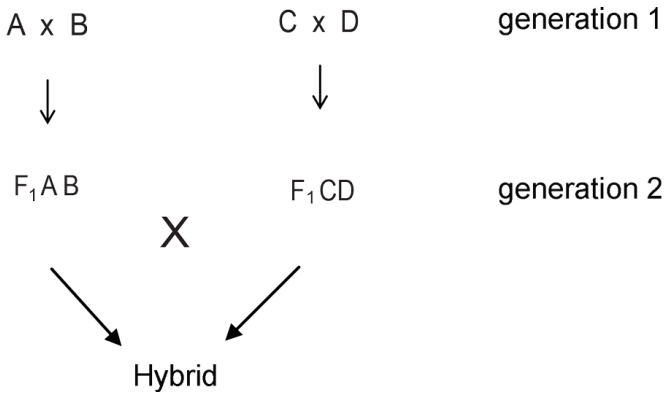
$$= 1/6\ (1 + 0 + 0 + 0 + 0 + 0) = 1/6$$

Since the mean inbreeding of the parents is (1 + 0) / 2 = ½, and one third of this mean is equal to 1/6, which is another way to estimate the inbreeding value of the offspring from crossing ab x ef parents.

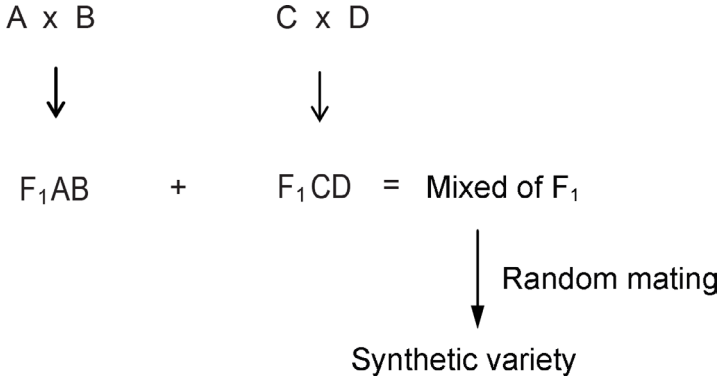
The above has practical consequences for alfalfa breeding and the development of hybrids and synthetic varieties, because the use of parents which are not hybrid nor inbred produce non-hybrid progenies.

The inbreeding coefficient can be used to compare different breeding methods for producing alfalfa varieties. To illustrate this comparison, let us consider three hypothetical breeding schemes involving four parental individuals (A, B, C and D):

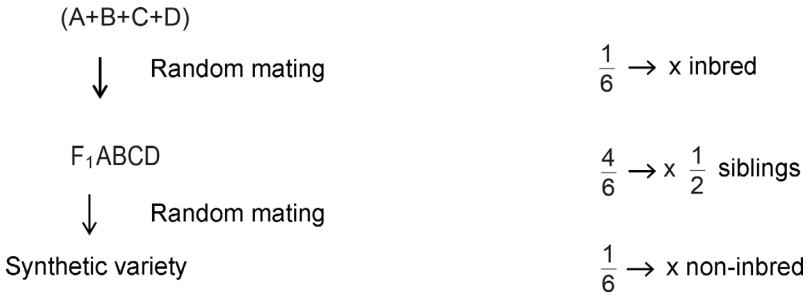
1) Production of hybrid through double crossing in two generations:



2) Production of a synthetic variety through random mating of two F_1 individuals originated from different parents:



3) Production of a synthetic variety through random mating of the four parents:



Assuming that A, B, C and D are not related and that they have a similar degree of inbreeding, identified as F_0, probabilities for gamete formation would be as follows:

- For case 1 (hybrids), tha gametes from parent A (a_1 a_2 a_3 a_4) and B (b_1 b_2 b_3 b_4), having four alleles each, will have the structure and frequencies shown in Table 7.

In the A X B cross, 36 different possible genotypes will be generated. The same will occur for the C X D cross. If the hybrid were produced by crossing parents with two alleles each, as for example a_1a_2 b_1b_2 x c_1c_2 d_1d_2, then the probability for the different gametes would be as shown in Table 8.

Table 7. Probability of occurrence of the different gametes produced by the unrelated parents A and B.

Gametes from A	Frequency	Gametes from B	Frequency
a_1a_2	1/6	b_1b_2	1/6
a_1a_3	1/6	b_1b_3	1/6
a_1a_4	1/6	b_1b_4	1/6
a_2a_3	1/6	b_2b_3	1/6
a_2a_4	1/6	b_2b_4	1/6
a_3a_4	1/6	b_3b_4	1/6

Table 8. Probability of occurrence of the different gametes produced by four unrelated parents (A, B, C and D) each one having two alleles (1 and 2).

Gametes from A and B	Frequency	Gametes from C and D	Frequency
a_1a_1	1/6	c_1c_1	1/6
a_1a_2	1/6	c_1c_2	1/6
a_2a_2	1/6	c_2c_2	1/6
b_1b_1	1/6	d_1d_1	1/6
b_1b_2	1/6	d_1d_2	1/6
b_2b_2	1/6	d_2d_2	1/6

Since

$P(a_i \equiv a_{i'}) = F_A = F_0$

$P(b_i \equiv b_{i'}) = F_B = F_0$

$P(c_i \equiv c_i) = F_c = F_0$

$P(d_i \equiv d_i) = F_d = F_0$

$P(a_i \equiv b_i) = P(a_i \equiv c_i) = P(a_i \equiv d_i) = P(b_i \equiv c_i) = P(b_i \equiv d_i) = P(c_i \equiv d_i) = 0.$

and considering the four possible genotypes that may receive two alleles from the same parent (i.e., $a_1\ a_2\ c_1\ c_2$; $a_1\ a_2\ d_1\ d_2$; $b_1\ b_2\ c_1\ c_2$ e $b_1\ b_2\ d_1\ d_2$), then the value of F in each case can be calculated. For example, for the case of $a_1\ a_2\ c_1\ c_2$, it would be:

$$F = 1/6\ [P(a_1 \equiv a_2) + P(a_1 \equiv c_1) + P(a_1 \equiv c_2) + P(a_2 \equiv c_1) + P(a_2 \equiv c_2) + P(c_1 \equiv c_2)]$$

$$F = 1/6\ (F_A + F_c) = 1/6\ (2F_0) = 1/3\ F_0.$$

Likewise, the coefficient of inbreeding for the 16 genotypes which received two alleles from the same parent ($a_1\ a_2\ c_1\ d_1$, $a_1\ a_2\ c_1\ d_2$, $a_1\ a_2\ c_2\ d_1$, $a_1\ a_2\ c_2\ d_2$, ... , $a_2\ b_2\ d_1\ d_2$) is obtained the same way. For example, for $a_1\ a_2\ c_1\ d_1$ it will be:

$$F = 1/6\ [P(a_1 \equiv a_2) + P(a_1 \equiv c_1) + P(a_1 \equiv d_1) + P(a_2 \equiv c_1) + P(a_2 \equiv d_1) + P(c_1 \equiv d_1)]$$

$$F = 1/6\ (F_A) = 1/6\ F_0.$$

For the remaining genotypes, the value of F will be zero.

Therefore, the mean of the inbreeding coefficient for the hybrid produced by this cross will be given by:

$$\frac{4}{36} \times \frac{1}{3} F_0 + \frac{16}{36} \times \frac{1}{6} F_0 + \frac{16}{36} \times 0\ F_0 = \frac{1}{9} F_0$$

Since it is expected that the other possible crosses be originated by individuals bearing the same characteristics as the ones presented before, it can be inferred that the mean of inbreeding must be repeated in all these crosses; thus, the inbreeding coefficient in the resulting hybrid will be given by 1/9 F_0.

- For cases 2 (synthetic variety from random mating on two F_1 individuals originated from different parents) and 3 (synthetic variety from random mating of the four parents), the value of *F* can be estimated using the same logic as in the previous case, being as a consequence:

$$F = \frac{1}{24} + \frac{17}{72} F + s\left[\frac{13}{17} + \frac{5}{24} F_0 + s\left(\frac{1}{12} + \frac{1}{4} F_0\right)\right]$$

  where *s* represents the frequency of self-fertilization (selfing) within the crosses.

Based on the results from all previous equations, it can be concluded that the coefficient of inbreeding is lower in hybrids than in synthetic varieties. Of course, this is an expected outcome since crosses between related parents were not allowed during the analyzed examples. In the same way, it can be observed that in synthetic varieties developed either by one or two random mating generations the coefficients of inbreeding will have similar values.

Heterosis and inbreeding depression

Alfalfa is very susceptible to inbreeding depression. Tysdal et al. (1942) estimated a 78% reduction in forage yield and 92% in seed production after eight generations of self-fertilization. On the other hand, heterosis also occurs in alfalfa. Demarly (1963) stated that simple, triple (3-way cross) and double hybrids produced 38%, 39% and 45% more forage than the original populations.

Rotili (1970) reported that after three generations of self-fertilization together with selection for vigor, the inbreeding depression in the progenies was significantly reduced. This is attributed to the assumptions that selection makes possible to either maintain heterozygosity or increase the frequency of favorable genes and unknown gene combinations.

Determining the effective degree of inbreeding within the breeding program, in order to favor genetic gains from the parental population, is one of the important objectives for alfalfa breeders. This can be possible when the greatest expression of heterosis in hybrids and synthetic varieties is obtained by the combination of inbreeding and selection.

Demarly (1963) stated that the genome of a tetraploid individual can be characterized by the relative proportion of tetragenic, trigenic, digenic,

simplex and nulliplex genetic constitutions. In this context, the study proposed to estimate the relative proportion of each structure in every generation within a controlled mating system. Complementarily, it was concluded that the initial genetic constitution is very important in explaining heterosis and inbreeding depression.

Dudley (1964) reported that from all possible simple and double mating combinations between quadruplex, triplex, duplex, simplex or nulliplex individuals, in addition to their S_1, S_2, S_3 and their homozygote progenies, maximum heterosis from both dominance and overdominance will be found not only in simple parental crosses, but also in progeny crosses; on the other hand, the effect of selection among crosses will be not very important.

Several mathematical models have been proposed for explaining inbreeding depression and heterosis in autotetraploids. One of these models, suggested by Busbice and Wilsie (1966), the genotypic structures at one locus are given by the proportion of T_0 (quadruplex), T_1 (triplex), T_2 (duplex), T_3 (simplex) and T_4 (nulliplex) constitutions, with frequencies P_0, P_1, P_2, P_3, and P_4, respectively. Thus, the genotypic value for one single locus or for a specific chromossome segment will be given by the individual value of the alleles, as well as by the value of two, three and four alleles, as follows:

$$GV_{T0} = GV_{iiii} = i + i + i + i + 6(ii) + 4(iii) + 1(iiii) = 4(i) + 6(ii) + 4(iii) + 1(iiii).$$

Thus:

$$GV_{T1} = GV_{iiij} = [3i + 1j] + [3(ii) + 3(ij)] + [1(iii) + 3(iij)] + (iiij),$$

$$GV_{T2} = GV_{iijj} = [2i + 2j] + [1(ii) + 4(ij) + 1(jj)] + [2\ (iij) + (2(ijj)] + 1(iijj),$$

$$GV_{T3} = GV_{iijk} = [2i + j + k] + [1(ii) + 2(ij) + 2(ik) + (jk)] + [1(iij) + 1(iik) + 2(ijk)] + 1(iijk)$$

and

$$GV_{T4} = GV_{ijkl} = [i + j + k + l] + [1(ij) + 1(ik) + 1(il) + 1(jk) + 1(jl) + 1(kl)] + [1(ijk) + + 1(ijl) + 1(ikl) + 1(jkl)] + 1(ijkl),$$

where

GV = genotypic value of the structure
i, *j*, *k*, *l* = additive values of each allele, separately
ii, *ij*, *ik*, *il*, *jk*, *jl* and *kl* = values of the first-order interactions
iii, *ijl*, *il* and *jkl* = values of the second-order interactions
iiii, *iiij*, *iijj* and *ijkl* = values of the third-order interactions

According to Busbice and Wilsie (1966), the mean of the population is given by the total of additive and interaction values of genes over all loci and all individuals in a given population. Then, they proposed that all the additive

values of the genes are equal to the population mean (assuming inbreeding to homozygosity and no selection), which is represented by the term A. Since heterosis results from heterogenic interaction between non-identical alleles, they suggested the estimation of the average genotypic values as follows:

$GV_{iiii} = A$

$GV_{iiij} = A + ij$

$GV_{iijj} = A + ij$

$GV_{iijk} = A + ij + ik + jk + ijk$

$GV_{ijkl} = A + ij + ik + il + jk + jl + kl + ijk + ijl + ikl + jkl + ijkl.$

The genotypic value of the population (GV_{pop}) will be given by:

$$GV_{pop} = A + (P_1 + P_2 + 3P_3 + 6P_4)\ (ij) + (P_3 + P_4)\ (ijk) + P_4\ (ijkl),$$

where:

ij, *ijk* and *ijkl* = nonallelic interactions of first, second and third order, respectively.

Based on the genotypic values, it can be verified that genotypes with tetragenic and trigenic structures are more important in the expression of heterosis in alfalfa. Busbice and Wilsie (1966) stated that the proportion of the different structures is affected by the generation of inbreeding, and that these changes could be calculated as the sum of all structures in the theoretical genotypic formations of the inbred progenies. By considering each of the genotypic structures separately, they were able to associate the loss of interactions between non-identical alleles to yield, as well as to the inbreeding coefficient. These authors also observed that inbreeding depression in alfalfa is related to the rate by which first-order interactions are lost from tetragenic and trigenic loci. The effect of losing interactions from digenic loci is not sufficiently fast to explain inbreeding depression.

The previous genetic model and the one presented by Gallais (1967) have provided certain insight on inbreeding, selection and hybridization in alfalfa, even though only for carefully planned experiments. Alfalfa is very sensitive to inbreeding, so that any process which increases inbreeding in the population will consequently lead to a reduction of heterosis together with the emergence of inbreeding depression.

Using a double-cross scheme, Bingham (1979) proposed to cross at least four selected and unrelated cultivars in order to reach maximum heterosis. At the third generation of random mating, 50% of the individuals in the resulting population should be in theory the product of double crossing, thus representing maximum heterosis.

Autotetraploid model: implications on breeding

The topics on quantitative genetics previously presented have implications on alfalfa genetic improvement. In alfalfa, the production of all possible genotypes from a given cross makes necessary to allow at least two generations of random mating, and not only one generation as is the case for diploids. The frequency of extreme genotypes (like nulliplex or quadruplex) in the population is low; therefore, if the breeder is looking for such genotypes, it will be necessary to assess a large number of individuals in order to increase the probability for detecting them.

Another important characteristic of autotetraploids is that they reach gametic equilibrium in an asymptotic way, because the diploid nature of their gametes does not allow the production of all possible genotypes in just one generation of random mating, as is the case for diploids. Generally, equilibrium is reached after four or five generations of random mating (Busbice et al., 1972).

The sensibility of alfalfa to inbreeding has an impact on predicting the yield of synthetic varieties in advanced generations (Busbice; Gurgis, 1976). Thus, breeders should always consider that: 1) self-fertilization followed by selection as a breeding method can be a problem, making the production of pure lines and the development of inbred lines for obtaining hybrids non-practical; and 2) the use of non-related and non-inbred parents should be always taken into account for producing non-inbred progenies with lower vigor and yield reduction.

In case of selection for increasing resistance to pests and diseases, which is usually conditioned by either one or a few genes, response to selection is fast until the frequency of such gene reaches 0.5; after that, response to selection becomes slow and difficult to verify. This is due to the fact that if the frequency of a dominant allele is 0.5, then nearly 93% of the individuals in the population will express this phenotype (Rodriguez, 1986).

Final considerations

The existence of self-incompatibility and self-sterility mechanisms in alfalfa favors cross-pollination. However, the autotetraploid nature of alfalfa has deep implications on the genetic behavior and the genotypic structure of the populations. Effects on segregation and gamete formation, estimation of variance components, gain from selection and production of at least two generations of random mating to obtain all possible genotypes from a cross, are

particularly important. Additionally, alfalfa manifests a pronounced inbreeding depression, which conditions the breeding methods to be used and highlights the importance of using unrelated parents.

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CHAPTER 5

Alfalfa diseases

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Daniel Horacio Basigalup

Introduction

Productivity and endurance of alfalfa are influenced by several factors of the abiotic (salinity, acidity, droughts, floods, toxic aluminum levels, etc.) and biotic kinds. This last group, which includes diseases, has a very important role in the limitations to the crop. According to Stuteville and Erwin (1990), diseases are the result of interactions of susceptible hosts, virulent pathogens and environmental conditions that predispose to the disease.

Diseases cause two kinds of economic losses: direct losses and indirect losses. Direct losses involve decrease in productivity caused by plant mortality or decrease in vigour, and reduction in forage quality due to leaf spots or by defoliation. Indirect losses include decrease in nutritional value of forage by loss and by degradation of chemical compounds with high nutritional value – such as proteins, sugars, lipids and vitamins –, presence of mycotoxins, decrease in nodulation and consequent decrease in N_2 fixation, increased susceptibility to attacks of insects, and proliferation of aggressive weeds – such as *Sorghum halepense*, *Cyperus rotundus* and *Cynodon dactylon*.

In the United States of America, about 50 pathogenic agents that harm alfalfa have been identified, highlighting fungi, nematodes, viruses and mycoplasma (Graham et al., 1979). Only part of this universe of pathogens – for their severity, distribution and frequency of attacks – is responsible for the economic losses in the producing areas. In Argentina, around 25 diseases which can affect the crop were identified, but with different degrees of importance in this country (Ostazeski; Hijano, 1986; Hijano; Perez Fernandez, 1995).

In this chapter, the main diseases found in alfalfa crops in Argentina and in Brazil will be described, including the causal agents, symptomatology and main control measures.

Main alfalfa diseases

Alfalfa diseases are caused by a broad band of phytopathogens, including fungi, bacteria, viruses, phytoplasma and nematodes. Within this group of organisms, fungi are responsible for most economically important diseases. Complete approaches to all the diseases which affect the crop can be found in Graham et al. (1979), Leath et al. (1988) and Stuteville and Erwin (1990).

There are two large groups of fungal diseases, which are different by region of the plant they colonize: root and crown diseases, and leaf diseases (stems and leaves). It is important to highlight that some pathogens which attack

mainly the root and the crown, such as *Colletotrichum trifolli*, *Rhizoctonia solani* and *Verticillium albo atrum*, can also cause injuries to foliage.

In this chapter, in addition to the main diseases of fungal origin, other etiologically different pathologies that can cause economic damage to the alfalfa crop will be described.

Fungal root and crown diseases

Pathogens from this group, by completely destroying the tissues of the crown and of the root, reduce the capacity of absorption and anchorage of the plant, the N_2 symbiotic fixation and the storage of reserves. Generally, these diseases have slow development, but it is accelerated under stress conditions. In some cases, the pathogens affect mainly xylem – the path for water transportation inside the plant – and cause wilt, with evident signals in the foliage.

For pathogens on which there is a greater degree of information, this chapter also details the biological cycles and possible control measures.

Among the most significant root and crown diseases, the following can be included: *Phytophthora megasperma* Drechs f. sp. *medicaginis* [phytophthora root rot], *Fusarium oxysporum* Schl f. sp. *medicaginis* (Weimer) Syn & Hans [fusarium wilt], *Xylaria* spp. [xylaria root rot], the complex formed by *Pythium* spp., *Phoma* spp., *Colletotrichum trifolii* Bain & Essary, *Fusarium oxysporum* Schlecht. f. sp. *medicaginis*, *F. solani* (Mart.) Sacc., *F. roseum* Link. Ex Fr. and *Rhizoctonia solani* Kühn [crown and root rot complex], *Colletotrichum trifolii* Bain & Essary [anthracnose, southern anthracnose], *Rhizoctonia croccorum* (Pers ex Fr) (sin *R. violacea* Tul and C. Tul) [violet root rot], *Sclerotinia trifoliorum* Ricks [sclerotinia], *Sclerotium rolfsii* Sacc [sclerotium blight], *Verticillium albo-atrum* Reinke & Berth [verticillium wilt], *Phomopsis* spp., and *Rhizoctonia solani* Kuhn. [*rhizoctonia* root canker, black root canker].

Phytophthora root rot

Causal agent: *Phytophthora megasperma* Drechs f. sp. *medicaginis*. It is a soil fungus that survives for long periods (in the form of oospores) and is capable of infecting alfalfa even after several years of rotation with other crops. Although the infection can occur at any time of year, the manifestation of the signs of the disease and the greatest damage is observed mainly in the spring and in wet autumns.

Predisposing conditions to the disease. Soils with low fertility, with high clay and/or silt content, with low drainage and slow percolation during periods of heavy rain, favor the movement of oospores, dissemination organs of the

pathogen. In other cases, flooding produced by inefficient systematization of irrigated areas also favors the emergence of the pathogen.

Symptoms. death of plantlets during rooting stage (damping off) by necrosis of the root and or of the basis of the stem. In grown plants, the characteristic signs are located in the roots, where dusky injuries with diffuse margins and usually located at the lateral roots insertion are observed. These injuries primarily cause death of the rhizoids and, in the end, death of the main root, at the level where the soil drainage is interrupted. When cross sections are made on the root, coloration ranging from yellow to light brown is observed in the cortical tissues and in the xylem (Figure 1). Foliage of affected plants acquires reddish-brown color and shows evident delay in sprouting right after cutting or after grazing and, in the more advanced stages of the disease, foliage wilts and finally dies.

Disease management. The most economic and efficient form of control is through the use of resistant cultivars. In heavy soils, or which have serious antecedents of *Phytophthora*, treating seeds with fungicides (metaxyl or mefenoxan) can confer additional protection to plantlets, preventing damping off and favoring a better establishment of the crop. In soils with low fertility, phosphorus and sulfur fertilization during crop implantation stimulates fast and vigorous growth of alfalfa, which contributes for the strength of the alfalfa plantation. Choosing areas

Photos: Jorge Omar Gieco

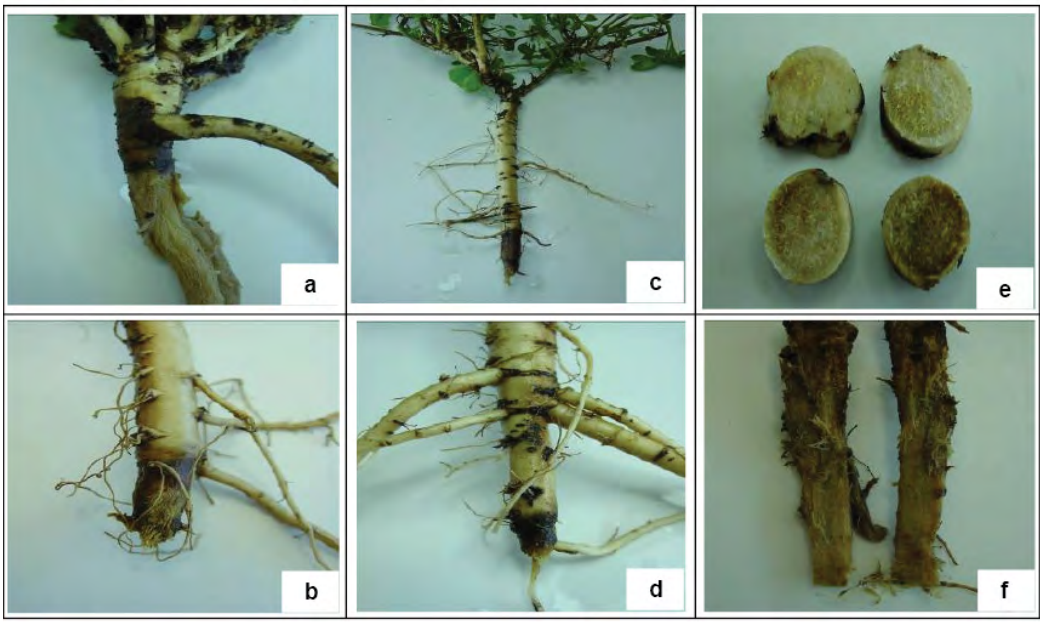

Figure 1. Signs of *Phytophthora megasperma* f. sp. *medicaginis*: characteristic injuries to the roots (A, B, C and D); cross and longitudinal sections of diseased roots (E and F).

with good drainage or performing crop treatments which facilitate infiltration or elimination of excessive water (soils plowed with subsoiler, drainage channels, etc.) can contribute for mitigating – but no eliminating – the problem.

In Figure 2, the biological cycle of the causal agent for *Phytophthora* root rot and the integrated control measures, as well as their application times, are described.

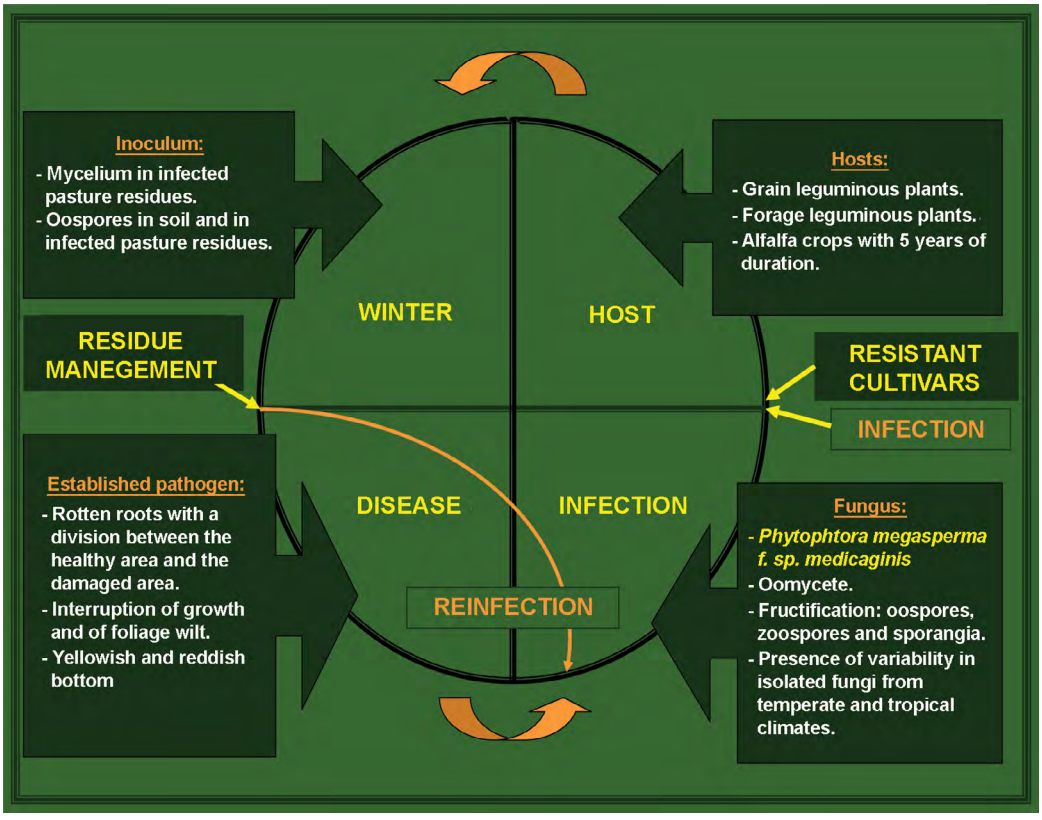

Figure 2. Biological cycle of *Phytophthora megasperma* Drechs. f. sp. *medicaginis*.

Fusarium wilt

Causal agent: *Fusarium oxysporum* Schl f. sp. *medicaginis* (Weimer) Syn & Hans. This fungus survives in the soil in form of chlamydospores, and in form of mycelium in remains of plant tissues, and can remain in the soils for many years without loss to its infection capacity.

Predisposing conditions to the disease. Unlike what is recommended for disease caused by *Phytophthora*, loose soils with good drainage, with moderate water content, constitute the ideal conditions for the pathogen; in addition, high temperatures during summer favor its development and dissemination. Injuries to the root caused by soil insects or by nematodes are an entry path for the pathogen, increasing the occurrence of the disease.

Symptoms. Foliage of plants severely affected by the disease is yellowish green to dusky. Short stalks, rare basal resprouts and evident decrease in speed of resprouting after cutting or pasture are also observed. If a cross section is performed to the root, dusky coloration shaped as a ring, originated from the necrosis of vascular tissues is observed and, as the disease advances, necrosis can affect all radicular tissues (Figure 3A). In the alfalfa plantation, the infection scatters irregularly, spreading in foci or big stains.

Disease management. The main control method is the use of resistant cultivars.

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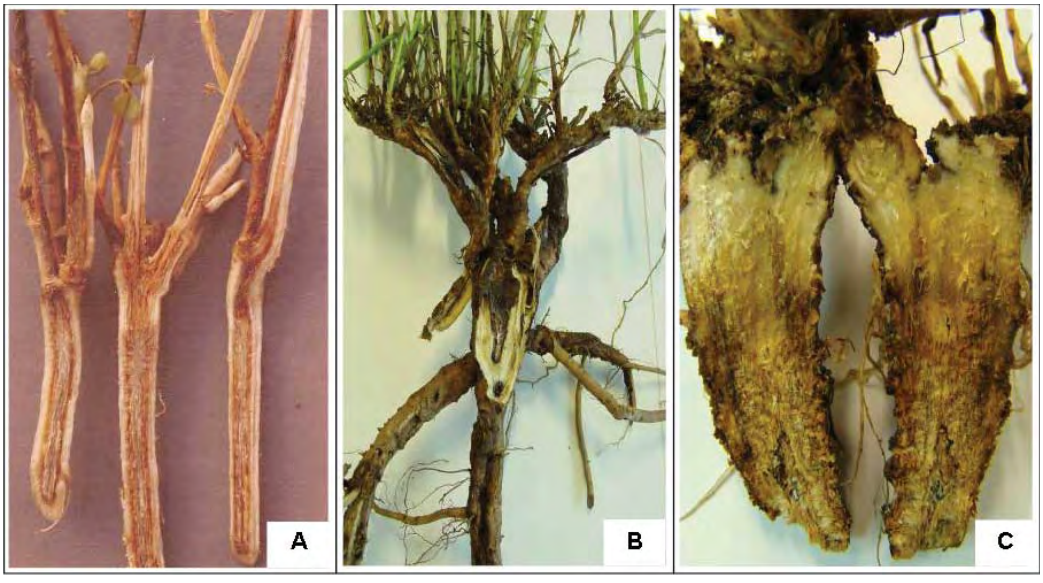

Figure 3. *Fusarium oxysporum* f. sp. *medicaginis* [Fusarium wilt]: cross section of roots and crowns, with completely necrosed xylem (A); *Xylaria* spp. [xylaria root rot], crown and upper root, with injury characteristic to the disease, in the center, in white, we see the mycelium of the pathogen (B); dry rot, characteristic to the disease, affecting the middle portion of the root (C).

Xylaria root rot

Causal agent: *Xylaria* spp. The first reported occurrence of this disease in Argentina was in 1985, and its frequency in alfalfa plantations at the time was assessed as ranging between 22% and 42% (Hijano; Huergo, 1985; Ostazeski; Hijano, 1986). Even if infection happens in the first year of life of the plant, causing necrosis in lateral roots, signals are usually visible after the second or third year of cultivation. In Argentina, the disease causes important damages throughout the country (Itria; Basigalup, 1984).

Predisposing conditions to the disease. The age of the plant (over two years) is one of the factors determining increased sensibility to the pathogen. Mechanical damage caused by low cuttings or by injuries produced to the crown by trampling during grazing favors the penetration of *Xylaria* spp.

Symptoms. Even though it does not show signs in foliage, the absence of resprouting or its delay indicates the presence of the disease. Typical signs are located in the root and in the crown, where it is possible to observe characteristic cork-like dry rotting (canker) (Figures 3B and 3C), with absence of lateral roots. Once the disease has settled, canker slowly grows and acquires a light dusky to greyish coloration. As the disease advances, the canker increases in size and ends up detaching from the root. Finally, the affected plants die and are completely taken over by the fungus mycelium, which in the end acquires olive green to black coloration.

Disease management. There are currently no cultivars of alfalfa with resistance levels to the pathogen. Given the lack of sources of genetic resistance and selection protocols, the only tool available for increasing the number of resistant plants in the population of alfalfa is the identification of plants free of signs of the disease in the alfalfa plantations which are more than three years old, and their later interbreeding. In this context, breeding programs including local selection of plants offer considerable advantage in relation to introduced cultivars, in which the disease does not exist. Rotation with non-host crops (grass crops and or *Melilotus* spp.), for three to four years can mitigate the presence of the pathogen.

Crown and root rot complex

Causal agent. It is a complex formed by fungi from several species, among which we can name *Pythium* spp., *Phoma* spp., *Colletotrichum trifolii* Bain & Essary, *Fusarium oxysporum* Schlecht. f. sp. *medicaginis*, *F. solani* (Mart.) Sacc., *F. roseum* Link. Ex Fr. and *Rhizoctonia solani* Kühn. Other organisms – both pathogenic (bacteria and nematodes) and saprophytic – which synergistically

interact with the environment to produce the crown and root rot usually join that group of fungi. In an evaluation carried out in four-year-old alfalfa plantations located in different points of the Argentinean pampas, Hijano et al. (1986) estimated the disease incidence ranged between 12% and 30%.

Predisposing conditions to the disease. Both the presence of injuries in the crown or in the root by several reasons (weevils, frequent low cuttings, animal trampling, etc.) and stress conditions that can affect the plant (leaf diseases, nutritional deficiency, etc.) favor the propagation of the disease.

Symptoms. The disease develops slowly and starts with the emergence of necrosed areas in the crown (dusky); then, it spreads to the cortical tissue of the root. As the disease advances, the necrosis expands through the crown and causes the number of basal shoots and the plant's vigor to decrease (Figure 4). Even so, presence of signs in foliage is not found, but the lack or delay of basal shoots indicates the presence of the disease. In affected plants that are more than three years old, it is common to find cavities in the upper roots or in the crown.

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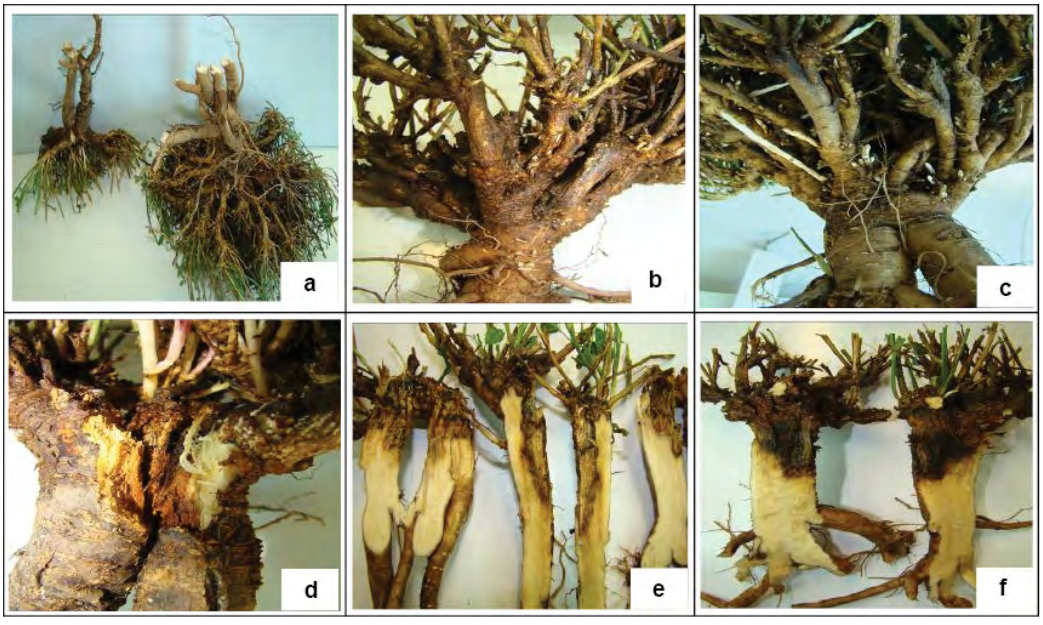

Figure 4. Crown and root rot complex: contrast between a healthy plant (right) and one affected by the disease (left) (A); crown affected by the disease, with evident lack of shoots (B); healthy crown, with active sprouting (C); external view of the injury (D); longitudinal sections of crowns and roots of plants belonging to two alfalfa plantations with two and with 4 years, respectively (E and F).

Disease management. As it is impossible to define effective selection protocols that address the entire complex of pathogens, there are no resistant cultivars. Anyway, the use of varieties with genetic resistance to some of the indicated pathogen agents – such as *Fusarium*, *Phoma* and *C. trifolii* – can contribute to mitigate the development of the disease. Adopting cultivation practices which avoid injuries to the crown (for example, not performing very low cuttings with unsharp knives, performing cuts or pasture respecting the reserve accumulation cycles, not performing grazing over very wet soils, etc.) decreases the entry paths for the pathogens and, consequently, reduces the propagation of the disease.

Anthracnose

Causal agent: *Colletotrichum trifolii* Bain & Essary. This fungus survives from one year to the next in the stem, crowns and in dead plant remains, in the form of acervuli. Although in the United States of America and in Australia three strains of the pathogen (called 1, 2 and 4) have already been found, in Argentina only one strain has been identified (Yang et al., 2008).

Predisposing conditions to the disease. High temperature and relative humidity favor the attack by the pathogen; therefore, it is frequent to find the first affected plants after the first cut in the spring. The greater incidence of the disease is verified in the summer and humid autumns. Until the alfalfa plantation is used, the development of the leaves provides enough shadow to increase humidity conditions in the bottom of the aerial (above-ground) part of the plant, which facilitates spore germination and latter penetration of the pathogen into the plants. Under these conditions damages can be very severe, to the point where complete necrosis of the stems and of the crown is produced.

Symptoms. In the lower third of the stems, elliptical dusky injuries with dark margins are observed, in which there are frequently black spots which are the fruiting bodies of the fungus acervuli (Figure 5). The affected stems become cane-shaped as they show signs of water deficiency and, as the infection advances, wilt completely, but keep the dry leaves attached. In advanced stages of the disease, the crown shows necrosed areas and acquires a bluish black coloration. In some cases, when the crown infection is too severe, it can cause death of the plants, with no evidence of signs in the aerial (above-ground) part. During the establishment period of the alfalfa plantation, the pathogen can also cause death of the plantlets (damping off).

Disease management. Using resistant cultivars is the most effective form of control. Correct management of the pasture, with cuts and grazing at the beginning of blooming, can decrease the propagation of the disease. The same

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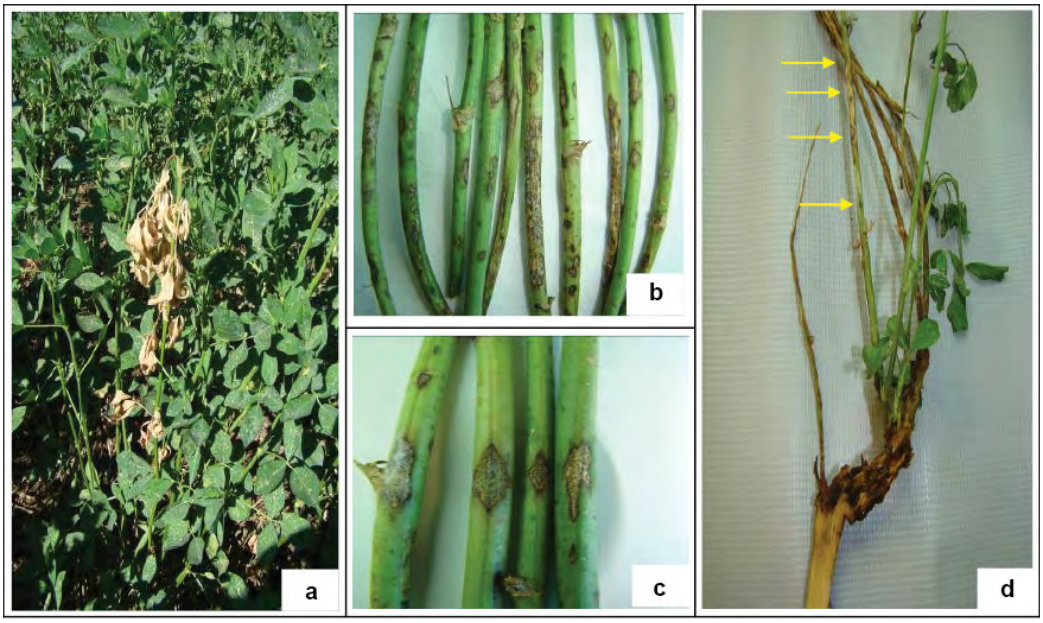

Figure 5. *Colletotrichum trifolii*: stem dead by *Colletotrichum trifolii* with the typical cane-shaped bend (A); details of the injuries to alfalfa stems, with the characteristic globular acervuli (B and C); longitudinal section of the crown and of the root of a diseased plant (arrows indicate typical diamond-shaped injuries) (D).

happens if the cuts and grazing are anticipated when environmental humidity is high. Eliminating plant remains in the area and rotating with grass crops over no less than two or three years can reduce the amount of inoculum available in future infections. In Figure 6 the biological cycle of the pathogenic agent for anthracnose can be observed, as well as integrated control measures and their application times.

Violet root rot

Causal agent: *Rhizoctonia croccorum (Pers ex Fr) (sin R. violacea Tul and C. Tul).*

The fungus can survive in soil for over 20 years. Currently, the disease does not carry the importance it had in Argentina in the early decades of the twentieth century, but it is still possible to detect it on occasion.

Predisposing conditions to the disease. Although conditions of high humidity favor the rapid spread of the fungus, it is also capable of causing damage in a wide range of environments, even producing significant damage in semiarid areas. The use of susceptible species that precede the culture

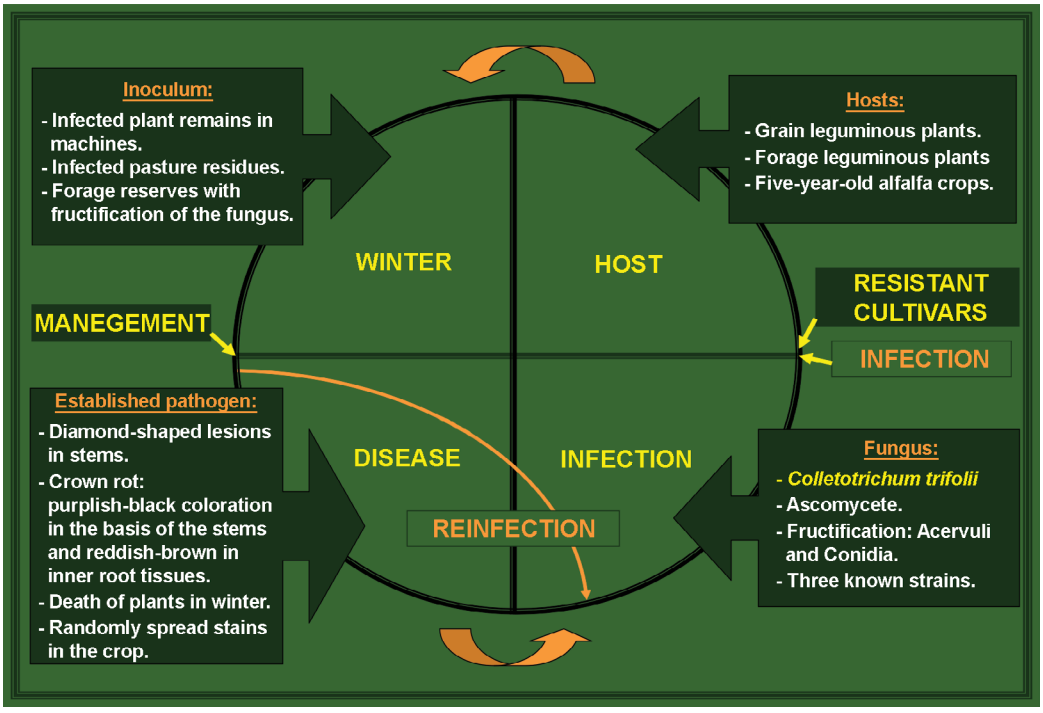

Figure 6. Biological cycle of *Colletotrichum trifolii*.

implantation, such as *Trifolium* spp. [clover] and *Lotus corniculatus* [birdsfoot trefoil], favor the rapid appearance and detection of the problem.

Symptoms. The disease manifests as common rot, in which the mycelium of the pathogen takes over the entire root region. The fungus hyphae form a violet-colored compact mass which externally enwraps the root (Figures 7A and 7B); the inner part of this mass turns white and disintegrates. Over the necrosed tissues it is possible to observe little black sclerotia. The foliage of the infected plant wilts and then loses its color; in the beginning it turns yellowish, then brown and finally it dries out, standing in contrast to the green color of the surrounding healthy plants. In the field, the advance of the pathogen is irregular, and big circles and stains of dead plants are observed.

Disease management. There are no resistant varieties. Including grass crops in the following plantation can contribute to reduce the incidence of the disease in problematic soils.

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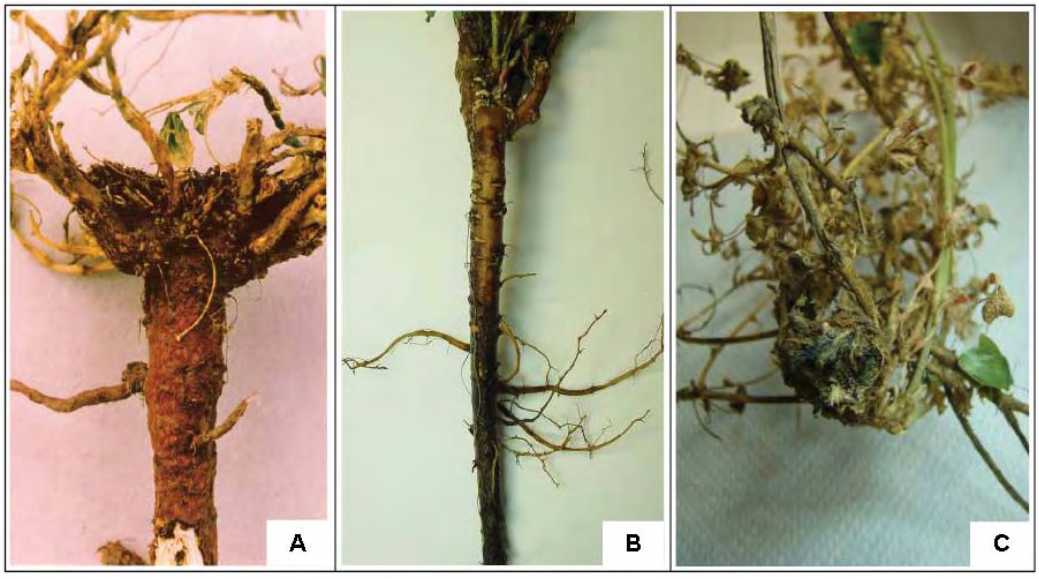


Figure 7. *Rhizoctonia croccorum* (sin *R. violacea* Tul and C. Tul). Plant affected by *Rhizoctonia croccorum*, with the main root covered by the pathogen hyphae (A); middle part of the main root necrosed by *Rhizoctonia* (B); blight caused by *Sclerotinia*: rot resembles the type caused by *Sclerotinia* (C).

Sclerotinia

Causal agent: *Sclerotinia trifoliorum Ricks.*

Symptoms. In the initial phase it is possible to observe discoloration; then the tissues of the infected root become yellowish and subsequently degenerate in a soft dark dusky decay (Figure 7C).

Predisposing conditions to the disease. Damages are more severe at the end of autumn – when soil humidity increases – and when infection happens at the plantlet stage, causing high mortality level in the plant population. As plants grow, they become less susceptible and damage is only observed in isolated individuals (Hijano, 1979). Under high humidity conditions, it is possible to observe the fungus mycelium as a cotton-like mass which grows in the basis of the stems and in the crown of the infected root. In the rest of the dead plant tissues, dark hard specks can be seen with naked eye: they are the sclerotia, resistance structures of the pathogen.

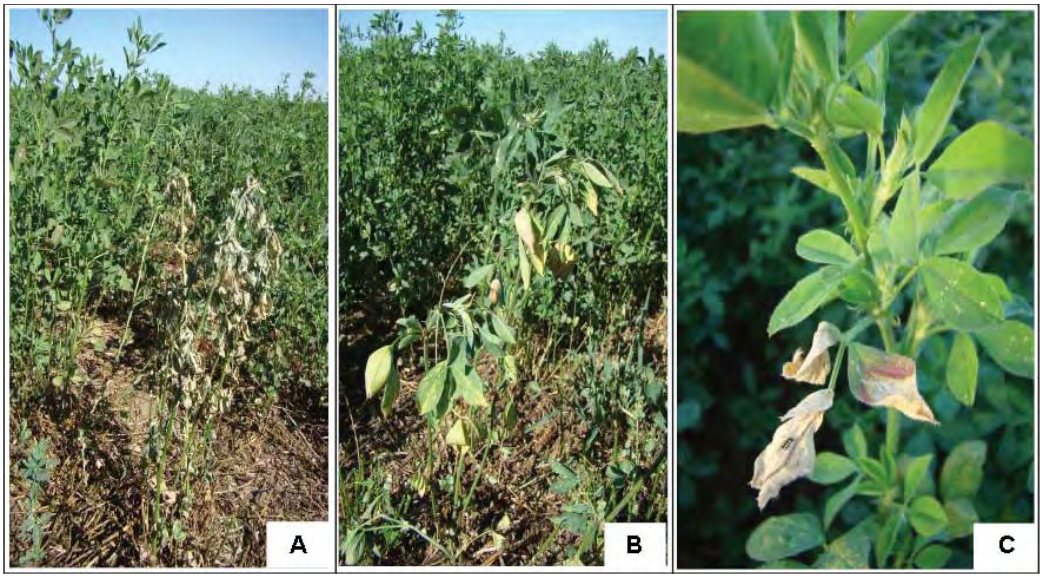
Disease management. It is recommended to eliminate remains from the alfalfa pasture and to perform rotation with grass crops for a period not shorter than two or three years; that will reduce the amount of inoculum available.

Sclerotium blight

Causal agent: *Sclerotium rolfsii* Sacc. It is a polyphagous pathogen that attacks several plant species, but is more frequent in tropical and subtropical regions. This pathogen can survive for many months in mycelium form in plant remains and several years in sclerotia form.

Symptoms. Plants affected by the pathogen show signs of water stress, dried out light brown pending leaves and stems (Figure 8A). The pathogen causes a wet rot to the crown and the basis of the stems; that rot ends up with the necrosis of the affected parts. Under high environmental humidity conditions, the fungus develops a whitish mycelium and, above it, it is possible to observe dusky globular sclerotia. The disease spreads irregularly over the area, evolving as stains that grow so long as the environmental conditions are favorable to its proliferation. In an essay for detection and for frequency of alfalfa pests and diseases in Castelar (Argentina), Basigalup and Hijano (1986) indicated that wilt caused by *S. rolfsii* was responsible for 1% of diagnosed plant deaths.

Predisposing conditions to the disease. Long periods of hot and wet weather followed by water stress lead to increased mortality in alfalfa crops.



Photos: Jorge Omar Gieco

Figure 8. Wilt caused by *Sclerotium rolfsii*: affected plant (right) and healthy plant (left) (A). *Verticillium albo-atrum*: plant with signs of wilt by *Verticillium* (B); foliole of a plant affected with the characteristic V-shaped stain, surrounded by a reddish halo, together with dried out folioles and leaves with incipient chlorosis (C).

Disease management. Using tolerant cultivars, eliminating plant remains from pasture and rotating with grass crops for periods longer than two or three years can mitigate the problem. It is recommended, when rotating crops, to avoid including legume crops which are susceptible to the disease, such as peanuts.

Verticillium wilt

Causal agent: *Verticillium albo-atrum* Reinke & Berth. This fungus spreads through seeds and plant remains of alfalfa. It has a broad spectrum of host legumes, including an important number of weeds.

Symptoms. The leaves of infected plants acquire general yellow color and foliole margins are covered by characteristic V-shaped stains, composed of a central grey necrosed area surrounded by a chlorotic margin. Even after completely necrosed, leaves continue attached to the stems, which remain green even though the fungus inhibits their growth (Figure 8B and 8C). In cross-section, the root has an orangish to light dusky color, which corresponds to the vascular tissues colonized by the pathogen. As the infection advances and the fungus takes over the crown and other organs, the plant eventually perishes. Damages are especially severe on irrigated lots.

Disease management. Using resistant cultivars is the most effective control method for the disease. Some effective practices to reduce the damages caused by this disease are crop rotations, especially using grass crops, in addition to a strict control of host weeds.

Phomopsis spp.

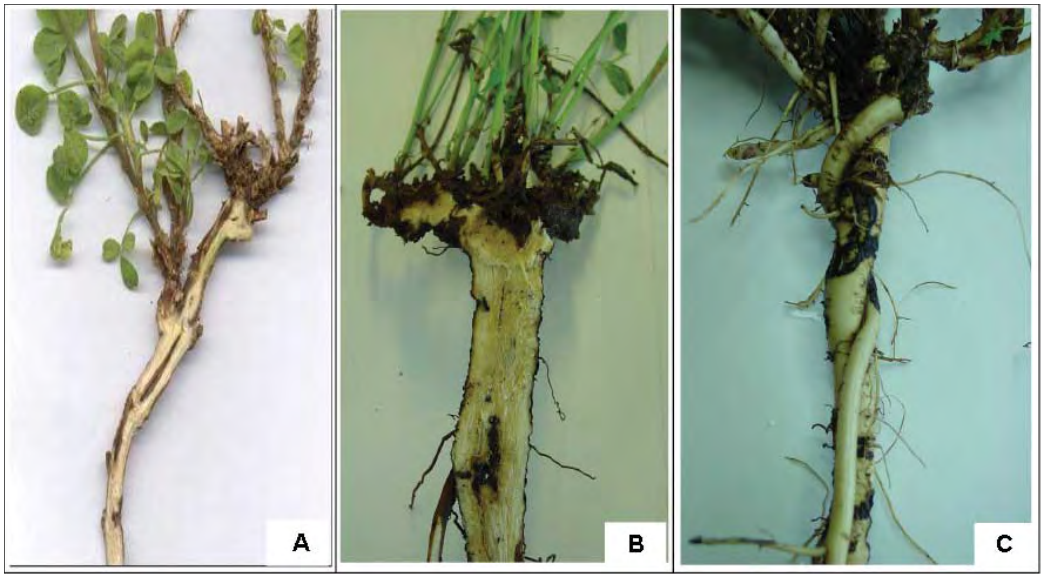
Causal agent: *Phomopsis* spp.

Symptoms. The pathogen causes injuries to xylem walls and partial or total crown necrosis (Figure 9A). In the aerial (above-ground) part, it is possible to observe cane-shaped bent stems. In Argentina, this pathogen represents potential danger, since it has been detected in *Trifolium pratense* L. [red clover] pastures and in *Glycine max* (L.) Merrill. [soybean] and *Helianthus annuus* L. [common sunflower] crops of the Diamante Department in the Province of Entre Ríos (Formento; Verzegnassi, 2001).

Disease management. Rotation with grass crops is recommended.

Rhizoctonia root canker

Causal agent: *Rhizoctonia solani* Kuhn. This pathogen has been sporadically diagnosed in plantation areas with irrigation and during high temperature periods (Hijano; Perez Fernández, 1995).



Photos: Jorge Omar Gieco

Figure 9. Rot by *Phomopsis*: injuries to the xylem of an alfalfa plant affected by *Phomopsis* spp. (A); root canker caused by *Rhizoctonia solani*: canker affecting the crown (B); and cankers affecting young roots (C).

Symptoms. It causes cankers on roots, in the form of dark and deep injuries, with uprisen edges located on the insertion point of the radicles. These injuries accrete and end up decomposing the main root (Figure 9B and 9C).

Disease management. Rotation with grass crops is recommended.

Fungal leaf diseases

These diseases do not cause death of the plant *per se*, but lead to yield or forage quality losses by reducing its photosynthetic capacity. Even when they do not cause a high level of defoliation, they can significantly reduce the non-structural carbohydrates and protein levels in forage. Severe defoliations, especially in autumn, can lead to general stress of the plant and make them vulnerable to attacks from other pathogen agents, adding to stand reduction during winter.

Usually, leaf diseases are especially harmful in springs and fresh and humid autumns; several of these pathogens are very frequently found infecting the same alfalfa leaf. As a rule, cultivars without winter rests (WR 8-10) originally developed for dry environments are more susceptible. Nevertheless, in recent years breeding programs, especially in Argentina, achieved cultivars without winter rest that had better resistance to leaf diseases.

Among the diseases caused by the most important pathogens in this group, based on frequency and severity of the damage caused, the following can be named: *Pseudopeziza medicaginis* (Lib.) Sacc [alfalfa leaf spot], *Leptosphaerulina briosiana* (Poll) Graham & Luttrell [leaf spot], *Uromyces striatus* Schroet [alfalfa rust], *Phoma medicaginis* Malbr & Roum var. *medicaginis* Boerema [spring black stem and leaf spot], *Cercospora medicaginis* Ellis & Everh [summer black stem and leaf spot of alfalfa, *Cercospora* leaf spot], *Leptotrochila medicaginis* (Fckl.) Schüepp. [yellow leaf blotch], *Peronospora trifoliorum* De bary [downy mildew] and *Stemphylium botryosum* Wallr [*Stemphylium* leaf spot].

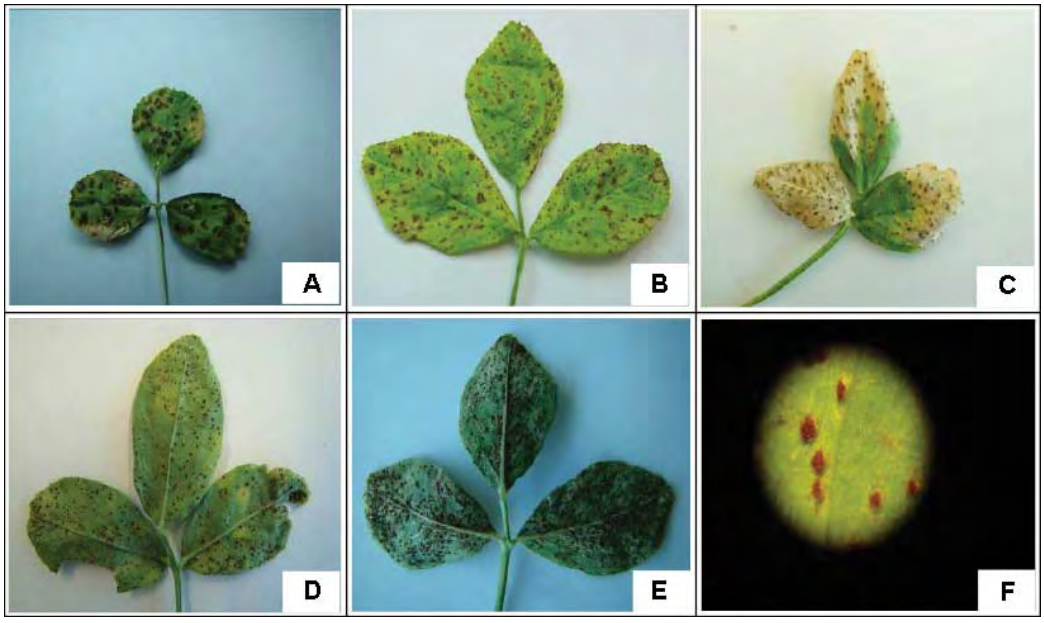
Alfalfa leaf spot

Causal agent: *Pseudopeziza medicaginis* (Lib.) Sacc. This pathogen, considered one of the most damaging for the alfalfa leaf, survives on dead leaves and causes secondary infections when environmental conditions are favorable for the germination of their spores (ascospores). It is more frequent in irrigated areas, where losses in forage yield exceeding 40% have been registered (Morgan; Parberry, 1977).

Predisposing conditions to the disease. Prolonged periods of cold and wet weather, especially during spring and autumn, constitute ideal conditions for the development of *P. medicaginis* (Lib.) Sacc. Inadequate use of irrigation, by sprinkling or flooding, as well as delay in cutting or pasture, which, by effect of shading, increases humidity in the lower part of the plant, favors the propagation of *P. medicaginis*.

Symptoms. The characteristic sign of the disease is the emergence of brown or black small (2 mm to 3 mm in diameter) spots, round and with entire or serrate margins, evenly distributed on the folioles (Figure 10A). Over the foliole set, the oldest spots develop hazel structures that correspond to the fungal fruiting bodies (apothecia). The ascospores produced by these fruiting bodies are scattered by the wind or by raindrops and infect new plants on plantations, starting from the lower leaves. If environmental conditions are favorable, virtually the entire foliage ends up being affected and severe defoliation happens throughout the pasture.

Disease management. Although there are a few cultivars on the market with moderate resistance to the pathogen, their effectivity as a control measure is not very high. As a palliative measure, it is recommended not to delay cuttings or utilization by animals, trying to respect the physiological cycles of reserve accumulation of the plant. In case of very wet weather, it may be necessary to anticipate utilization of the alfalfa plantation, to avoid quality or yield losses of the forage by defoliation. This procedure can noticeably reduce the amount



Photos: Jorge Omar Gieco

Figure 10. *Pseudopeziza medicaginis*: leaf with characteristic signs (A); *Leptosphaerulina briosiana*: eye-shaped injuries (B); leaf with advanced stage of the disease (C); *Uromyces striatus* Schroet: blotches in the lower part of the leaf (D); leaf severely attacked by *Uromyces striatus* Schroet (E); increased blotch (F).

of inoculum for later infections. Applying systemic fungicides in early crop implantation can be effective. In Figure 11, the biological cycle of *P. medicaginis* is presented, as well as integrated control measures and their application times.

Lepto leaf spot

Causal agent: *Leptosphaerulina briosiana* (Poll) Graham & Luttrell. This pathogen has spread over alfalfa cultivation areas in Argentina because of the massive use of cultivars without winter rest, especially susceptible to this disease.

Predisposing conditions to the disease. Long periods of cold and wet weather are the ideal conditions for the development and propagation of *L. briosiana*.

Symptoms. Injuries usually start on the leaves with small dark spots; they soon grow, until they reach 1 mm to 3 mm in diameter, surrounded by a dark brown margin which is in turn surrounded by a yellow halo, which confers its characteristic eye-like appearance (Figure 10B and 10C). As the disease

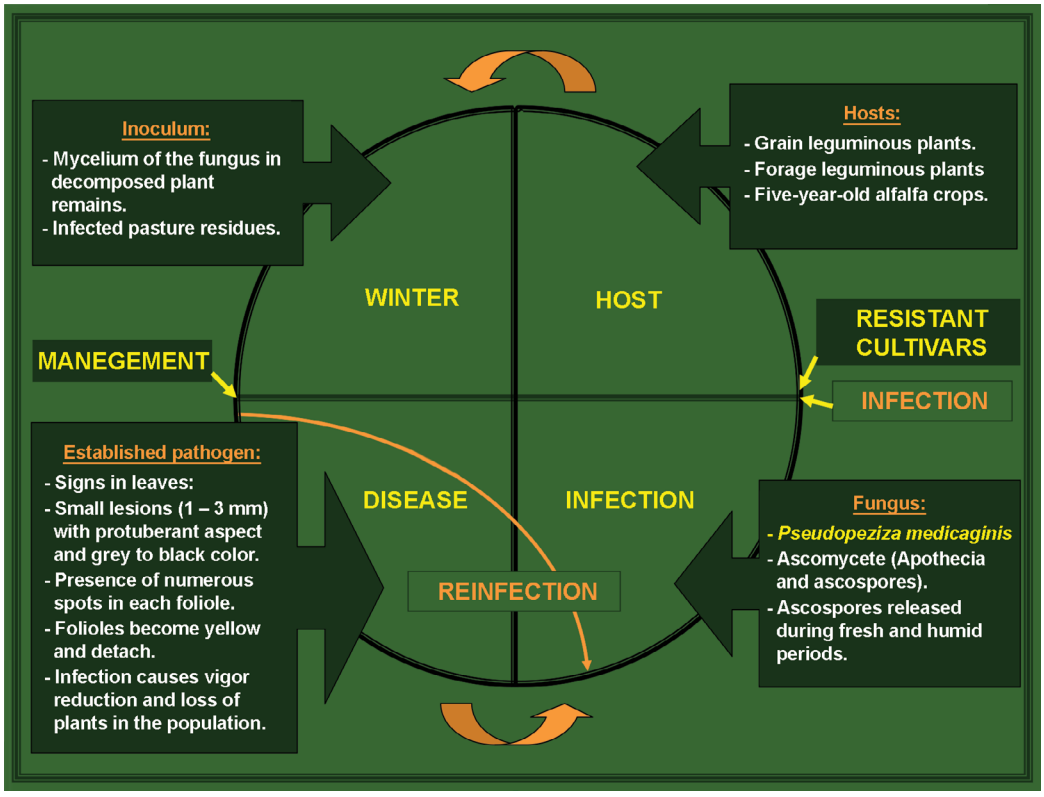


Figure 11. *Pseudopeziza medicaginis* biological cycle.

advances, injuries cover the whole foliole until it finally detaches. Very severe attacks, with total defoliation, have been observed.

Disease management. The same considerations presented for *P. medicaginis* apply. In Figure 12, the biological cycle of *L. briosiana* is presented, as well as integrated control measures and their application times.

Alfalfa rust

Causal agent: *Uromyces striatus* Schroet. It is a pathogen with several strains already identified. In addition to alfalfa, it infects other leguminous species belonging to the *Medicago* and *Trifolium* genera as well as weeds from the *Euphorbia* genus. It forms uredospores, which can survive for several months in dry weather.

Predisposing conditions to the disease. Hot and wet weather favors the emergence and proliferation of the pathogen, especially at the end of summer and during autumn.

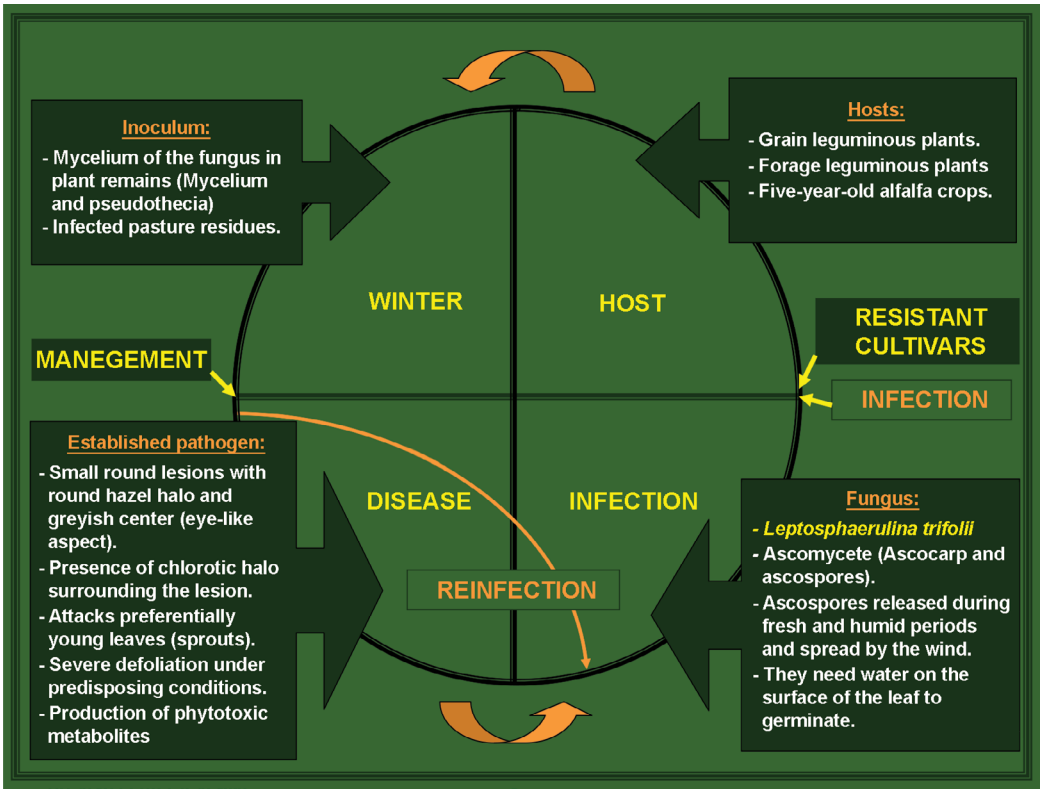


Figure 12. Biological cycle of *Leptosphaerulina trifolii*.

Symptoms. Occurrence of small rounded maroon blotches on both sides of the leaf (Figure 10D, 10E and 10F), which break through the epidermis, is a distinct element for diagnosing the disease. The uredospores detach easily from these blotches and, being carried by the wind, can infect other alfalfa plots located several kilometers away. Leaves covered by the blotches bend and in the end detach, causing total defoliating in conditions that are very favorable to the pathogen. In severe attacks, it is possible to observe elliptical blotches developing on the stems.

Disease management. The same recommendations described before for other leaf diseases apply. Figure 13 shows the biological cycle of the pathogenic agent, as well as integrated control measures and their application times.

Spring black stem and leaf spot

Causal agent: *Phoma medicaginis* Malbr & Roum var. *medicaginis* Boerema. This pathogen can survive for several months on plant remains as pycnidia, and later infect leaves and stems in environmental conditions which favor their germination.

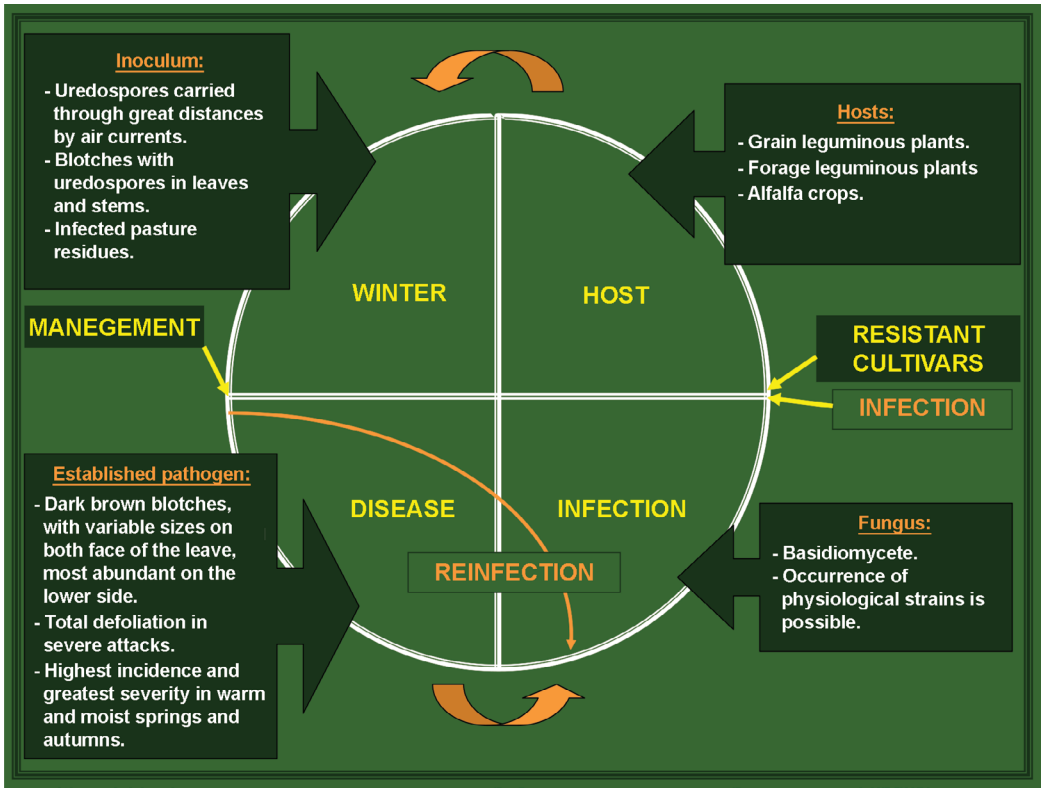
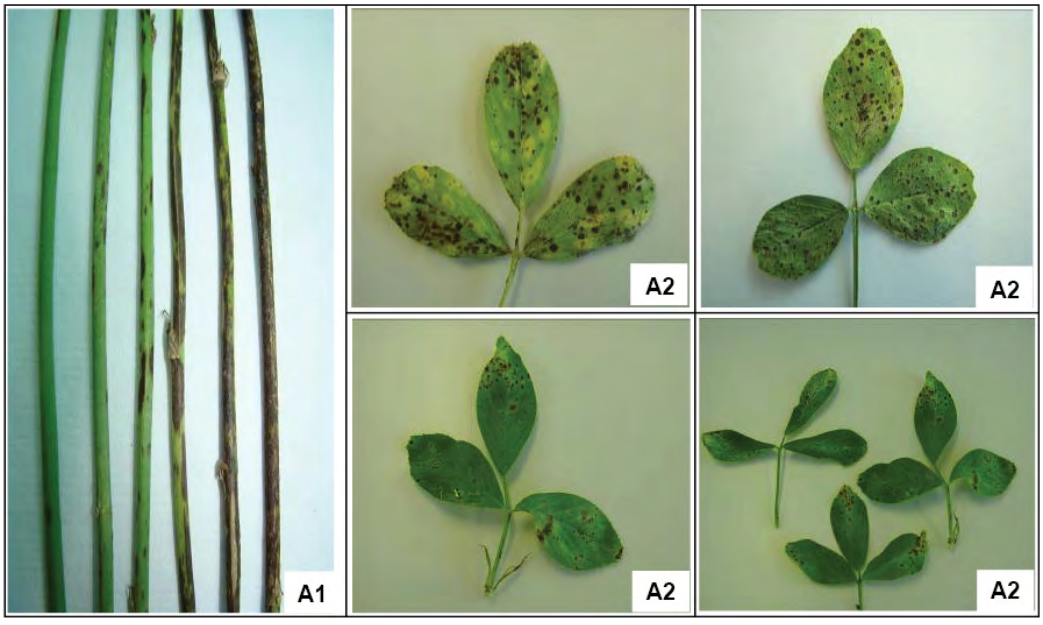


Figure 13. Biological cycle of *Uromyces striatus*.

Predisposing conditions to the disease. Cold and wet springs and autumns favor emergence and propagation of the disease. Humidity is necessary for spore scattering and germination.

Symptoms. The disease starts with dark brown spots on the leaves which coalesce as damage increases, affecting a large surface of the folioles. On stems these initially dark spots are individual, but as they unite they take over large sectors of the stem basis, conferring the characteristic black color which names the disease (Figure 14). Diseased leaves turn yellowish and, in the end, detach from the stem. Under very favorable conditions, the pathogen can also colonize the pods and the crown.

Disease management. Control measures are the same indicated for the leaf diseases mentioned before. Also, rotation with non-host crops (grass crops and *Melilotus* spp.) for a minimum period of two to three years can be added. In Figure 15, the biological cycle of *Phoma medicaginis* Malbr & Roum var. *medicaginis* Boerema, as well as integrated control measures and their application times can be found.



Photos: Jorge Omar Gieco

Figure 14. *Phoma medicaginis* var. *medicaginis*: increasing severity on stems (A1); leaf symptoms (A2).

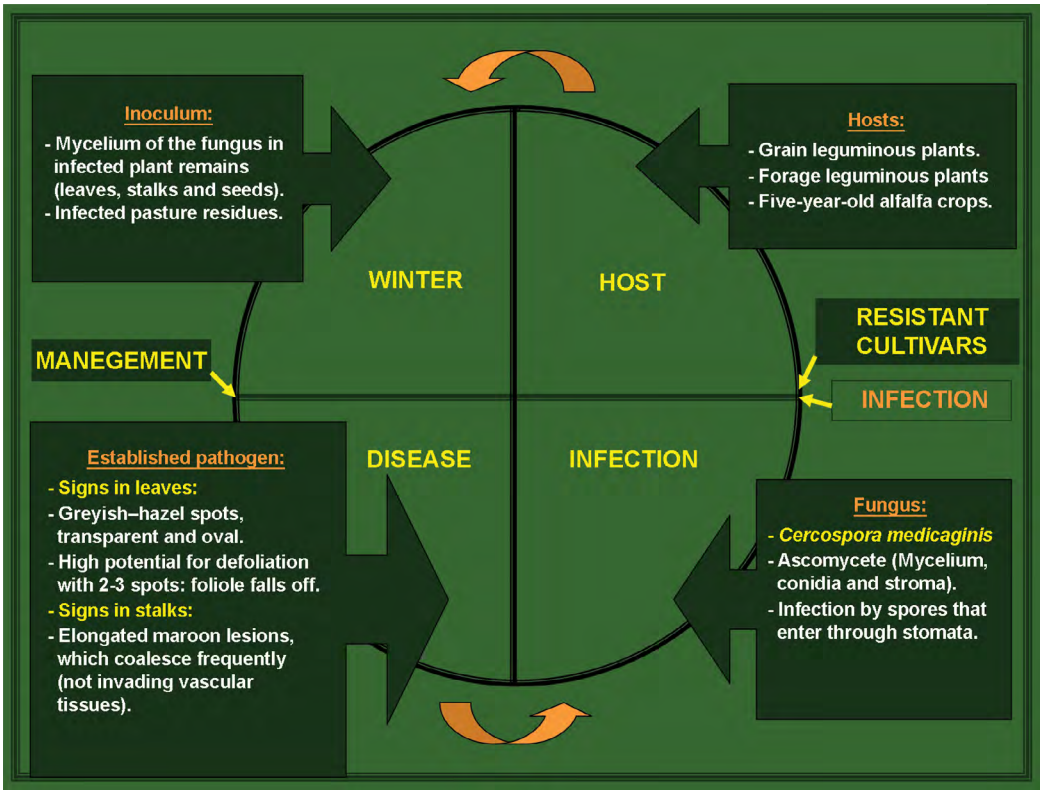


Figure 15. Biological cycle of *Phoma medicaginis*.

Summer black stem

Causal agent: *Cercospora medicaginis* Ellis & Everth. The fungus goes through winter as a mycelium on affected stems but, in order to fructify, it requires high temperature and humidity.

Predisposing conditions to the disease. Hot and wet summers favor the emergence and propagation of the pathogen. Under these conditions, delayed cutting or grazing can intensify the disease.

Symptoms. Damage becomes evident first on lower leaves and then on upper ones, with brown or hazel rounded or elliptical spots with diffuse margins. As the disease advances, these stains converge and are surrounded by a large irregular chlorotic halo. When the fungus fructifies, grey injuries are observed in the central region (Figures 16A and 16B). On the basis of the stem, dark stains similar to the previously described disease are produced.

Disease management. The same recommendations listed for *P. medicaginis* var. *medicaginis* apply. In Figure 17 the biological cycle of the pathogenic agent and the integrated control measures with their application times are described.

Photos: Jorge Omar Gieco

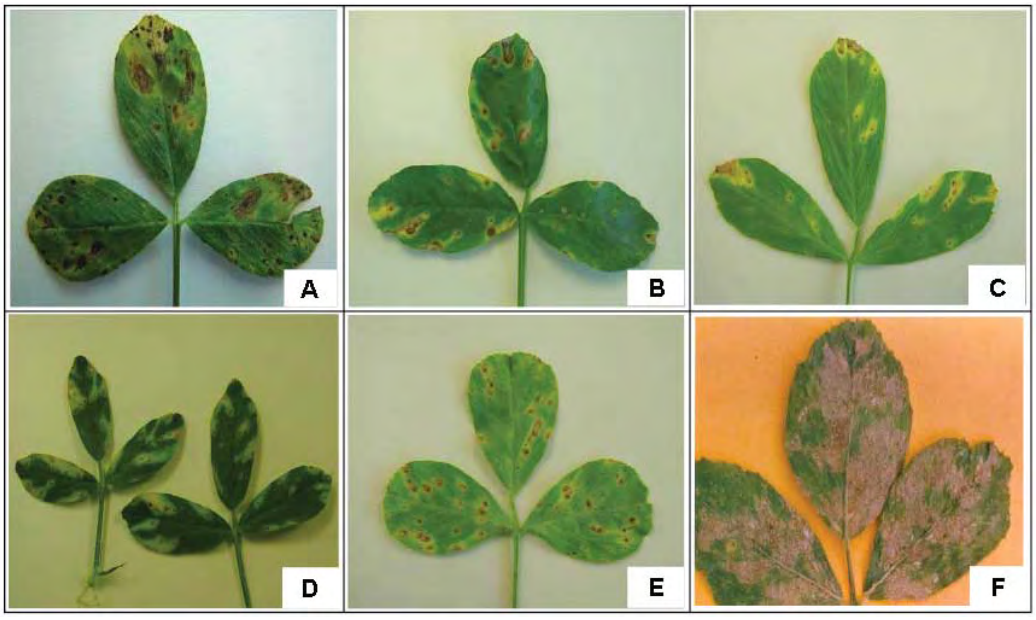


Figure 16. *Cercospora medicaginis*: signs on leaves (A and B). *Leptotrochila medicaginis* (Fckl.) Schüepp.: growing severity of the disease (C, D and E). *Peronospora trifoliorum* De bary: characteristic efflorescence on the abaxial side of a leaf (F).

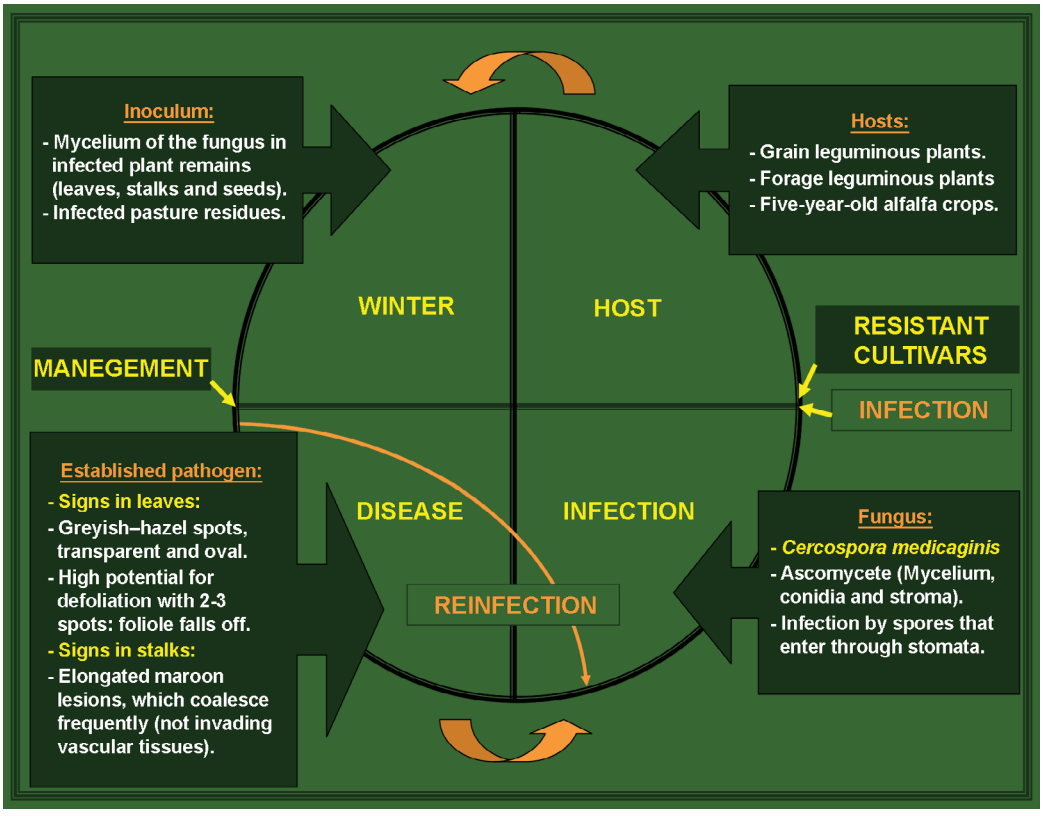


Figure 17. Biological cycle of *Cercospora medicaginis*.

Yellow leaf blotch

Causal agent: *Leptotrochila medicaginis* (Fckl.) Schüepp. By the end of summer and beginning of autumn the fungus forms, over dead leaves, its fruiting bodies (apothecia) which, after hibernation, will release the ascospores in the next spring, when these will take charge of starting the infection. In the United States of America, losses in foliage by 40% in the beginning of the flowering process, and by 80% in pod formation have been estimated (Semeniuk, 1979).

Predisposing conditions to the disease. Cold and wet springs and autumns, or periods of abundant rain followed by cloudy days, favor the development and spreading of the pathogen. Delaying use of the alfalfa crop (for cutting or pasture) also increases disease incidence.

Symptoms. Damages to the plant start on the folioles with small yellowish spots that grow until they take over much of the leaf, usually following the route of the veins and form yellow V-shaped stains, establishing a pale brown area

in the central region (Figures 16C, 16D and 16E). Under favorable conditions, defoliation happens.

Disease management. There are no resistant or tolerant varieties. Other control measures that can be applied are the same indicated for other leaf diseases, as explained before.

Downy mildew

Causal agent: *Peronospora trifoliorum* De bary. This fungus survives winter on living plant tissues, but only fructifies in dark and highly humid conditions; wind and rain are the main dissemination agents. Because it is a recalcitrant parasite, it does not develop in in vitro cultivation.

Predisposing conditions to the disease. Cold and wet springs and autumns favor the emergence and proliferation of the pathogen.

Symptoms. *Peronospora trifoliorum* can cause two types of infection: local and systemic. When infection is local, the folioles have chlorotic colorless sectors that later turn grey, on their opposite side, due to the concentration of reproductive structures (conidiophores) of the fungus (Figure 16F). When infection is systemic, the pathogen takes over stems, buds and complete leaves. Infected stems become larger in diameter and have shorter internodes, frequently producing terminal branched sprouts with rosette-like overlayered leaves. Margins of completely infected leaves bend downwards. In recently planted alfalfa crops and under very favorable conditions, *P. trifoliorum* can cause death of the plantlets (damping off).

Disease management. There are some American cultivars which are tolerant to the pathogen. Treating seeds with systemic fungicides (as metalaxyl) can be useful for implantation in infected areas. If the disease has reached high infestation level, early cutting or pasture contributes to avoid important losses to quality and or to forage yield, noticeably reducing the amount of inoculum in later infections. In Figure 18, the biological cycle of the pathogen is described, as well as the integrated control measures and their times of application.

Stemphylium leaf spot

Causal agent: *Stemphylium botryosum* Wallr. Two variations of the pathogen, distinguished by the different injuries they cause to the leaf and differentiated by the environmental temperature at the time of infection, have been identified. The “high temperature” (HT) biotype is predominant in infections occurring in the summer; while the “low temperature” (LT) biotype manifests in spring and late autumn.

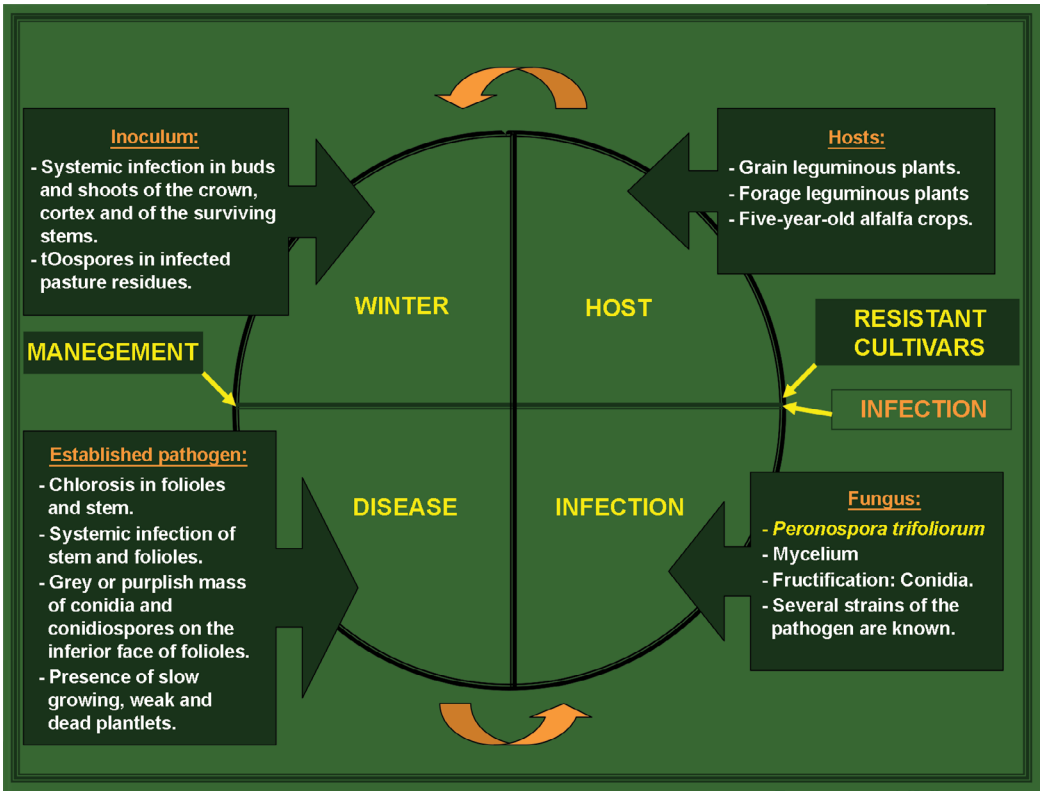


Figure 18. Biological Cycle of *Peronospora trifoliorum*.

Predisposing conditions to the disease. High temperatures in the summer, together with high relative humidity, favor proliferations of the HT biotype. As for the LT biotype, low or moderate temperatures in spring and high humidity predispose to the disease development.

Symptoms. The HT biotype produces oval hazel injuries with diffuse margins accompanied by a light yellow halo. Injuries increase with the progress of the disease, with addition of characteristic concentric rings which thus cover much of the foliole. Under conditions that favor the disease, affected folioles turn yellowish and soon detach from the stem. In this biotype, black coalescent injuries are commonly produced on stems (Figures 19A, 19B and 19C). The LT biotype produces small (3 mm to 4 mm) light grey injuries with irregular margins and surrounded by a thin bright dark brown edge. Pathogen sporulation is confined to the inside of the injury (Figures 19D, 19E and 19F). In severe attacks of the disease, this biotype causes a reduction in forage quality but defoliation is very rare. In the Paraná region (Entre Ríos, Argentina), Formento and Verzagnassi (2001) detected the presence of the pathogen in alfalfa areas

Photos: Jorge Omar Gieco

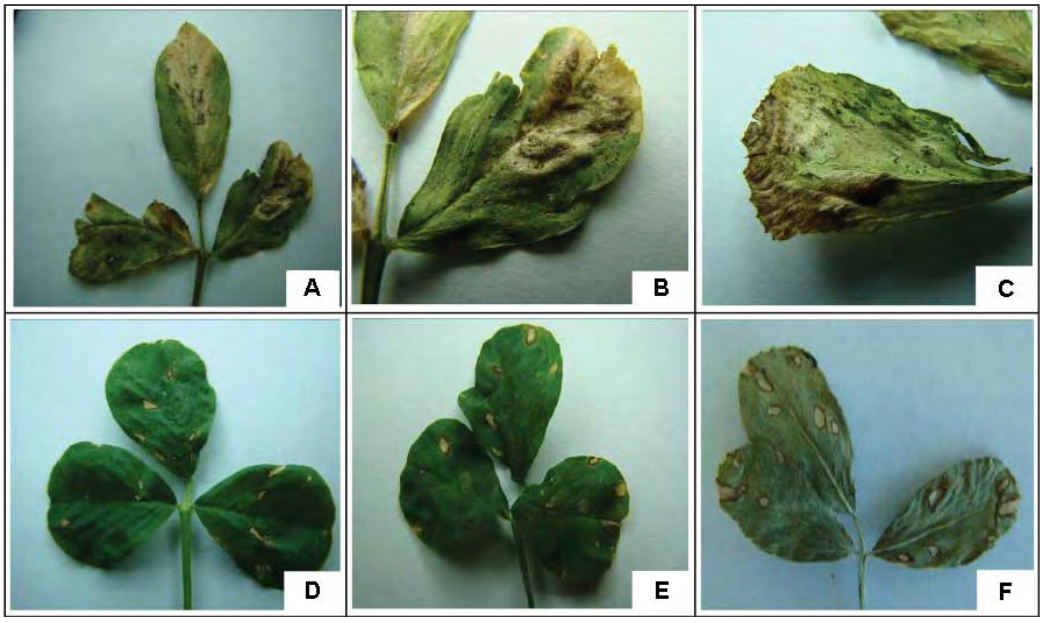


Figure 19. *Stemphylium botryosum*: characteristic injuries of the high temperature biotype (A, B and C);low temperature biotype (D, E and F).

of the Agricultural Experimental Station of Manfredi, at the National Institute of Agricultural Technology, in Córdoba, Argentina. The HT biotype manifests in the summer and at the beginning of October and is the most harmful; the LT biotype, however, is observed in autumn and at the beginning of winter, causing less important damages.

Disease management. The damages caused by the disease to the alfalfa plantation are mitigated by early cutting or pasture. Using tolerant cultivars is an effective control measure. Currently, there already are tolerant cultivars in Argentina. In Figure 20, the biological cycle of *S. botryosum* is observed, as well as integrated control measures and their application times.

Diseases caused by viruses and phytoplasma

Alfalfa mosaic virus (AMV)

Causal agent: *Alfalfa mosaic virus* (AMV). The viral particles take two forms: one like a bacillus (bacilliform) and the other cane-shaped (spheroid). Actually, it is a viral complex composed of several AMV strains with differences

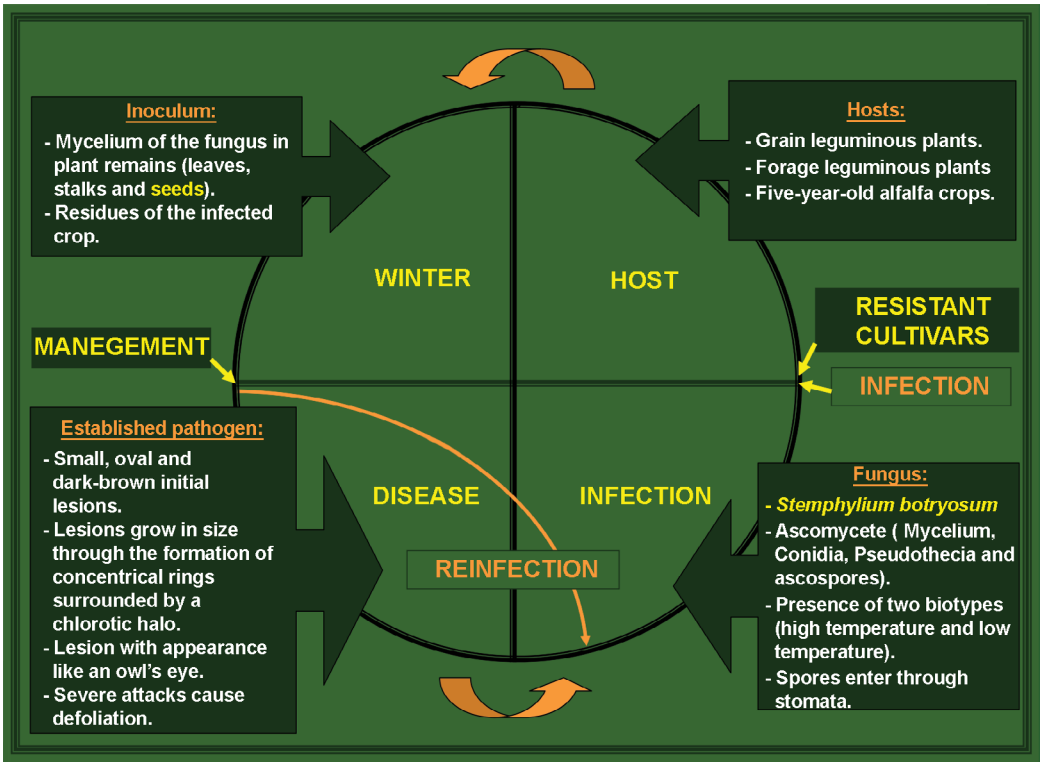


Figure 20. Biological cycle of *Stemphylium botryosum*.

to their infection power and other characteristics. This complex can infect over 200 species of plants, but apparently alfalfa is the favorite host for most strains in the complex (Graham et al., 1979). The pathogen is transmitted by several insects that are disease vectors, but transmission can also occur through seeds and pollen.

Predisposing conditions to the disease. Although it is supposed that AMV can be transmitted by all aphid species that attack alfalfa, *Acyrthosiphon pisum* Harris [pea aphid] is its most important vector. Consequently, conditions that favor insect proliferation also help spreading the disease. In recent years, a growing presence of *Thrips* spp., *Frankliniella* spp. and *Caliotrips* spp., which could also be vectors of AMV, has been observed.

Disease management. Controlling the vector insects is the only preventive measure for disease control. Currently, there are no resistant cultivars of alfalfa, but there are transgenic plants available that are resistant to this virosis.

Alfalfa witches' broom

Causal agent: phytoplasma. According to international taxonomy, this phytoplasma belongs to group 16S rDNA Ash yellows (*Candidatus Phytoplasma fraxini)*. Phytoplasmas (prokaryotes with no cell walls) are phytopathogenic organisms that lodge into the phloem and are transmitted by insects, especially *Homoptera Cicadellidae* [idiocerine leafhopper], which feed from the conductive vessels of the plant (Conci et al., 2005).

Predisposing conditions to the disease. Arid and semiarid climates appear to favor the development of the disease, especially in plots intended for seed production.

Symptoms. There is great propagation of short and thin stems, leaves very reduced in size, generalized nanism, chlorosis and flower abortion (Figures 21B, 21D and 21E); in some cases, substitution of the inflorescence by vegetative structures is observed (Stuteville; Erwin, 1990). In cold periods with adequate humidity, the affected plants can show indication of recovery, but the signs return once the temperature and/or the water deficiency rises. Evidently, forage yield and seed production decrease in diseased plants. As the years go by, the number of infected plants in the area increases.

Photos: Jorge Omar Gieco

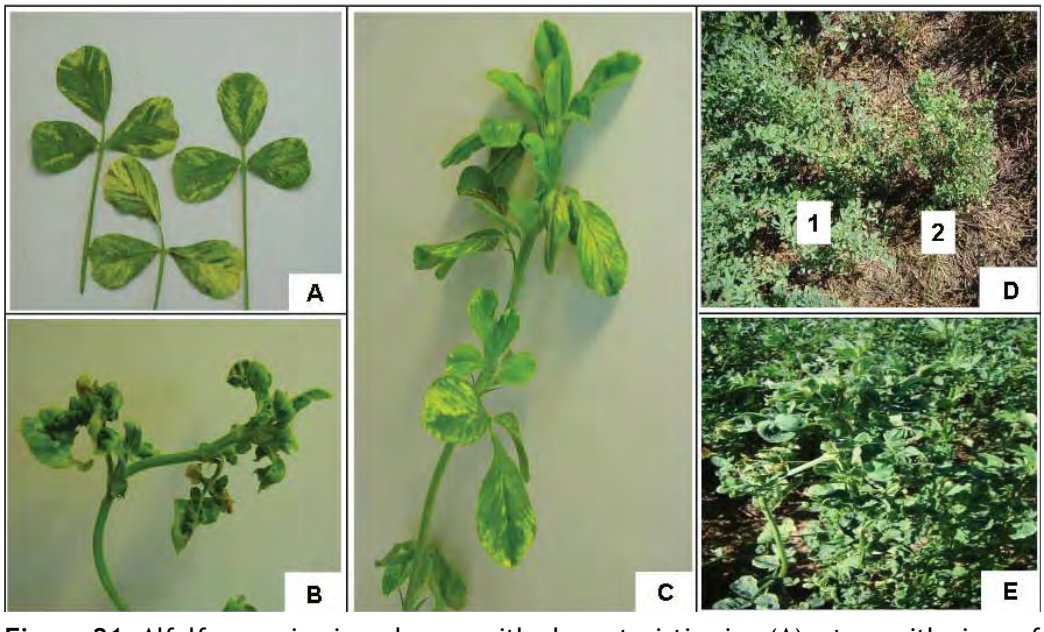


Figure 21. Alfalfa mosaic virus: leaves with characteristic sign (A), stem with signs of alfalfa mosaic virus (C). *Candidatus Phytoplasma fraxini*: sprout severely affected by the virus (B), diseased plant (D1) and healthy plant (D2). Folioles completely deformed by the virosis (E).

Disease management. Immediate removal of diseased plants and control of vector insects appear to be the only effective measures to mitigate the disease dispersion. There are no resistant cultivars.

Diseases caused by nematodes

Alfalfa stem nematode

Causal agent: *Ditylenchus dipsaci* (Kühn Filipjev). This nematode penetrates the plant through the shoots developing in the root crown and later takes over the growing stems.

Symptoms. Affected tissues thicken and lose color, while nodes bloat and internodes shorten. If the infection advances, growing stems thicken and eventually darken and die. During the early decades of the 20th century, the pathogen was considered the most important alfalfa phytosanitary problem in Argentina, and promoted many efforts for obtaining resistant cultivars. However, after the 1950s, its damages have been minor and sporadic.

Disease management. There are currently several resistant cultivars in market, all of American origin. In Figure 22 the biological cycle of *D. dipsaci* is described, as well as integrated control measures and their times of application.

Root-lesion nematode

Causal agent: *Pratylenchus* spp. It invades and destroys secondary roots, and causes dark lesions to the main root (Figure 23E). These lesions constitute an entry door for other pathogenic microorganisms, which worsen the condition of the affected plant.

Symptoms. When infection by this nematode is important, roots turn brown along the length and their growth becomes slow. Within this context, the only sign of infection in the aerial (above-ground) part of the plant is the enlarged development of foliage. Although there are no hard references of the damage caused by the disease, it is common to find *Pratylenchus* spp. in soil samples analyses.

Disease management. It is recommended to rotate cultures with grass crops.

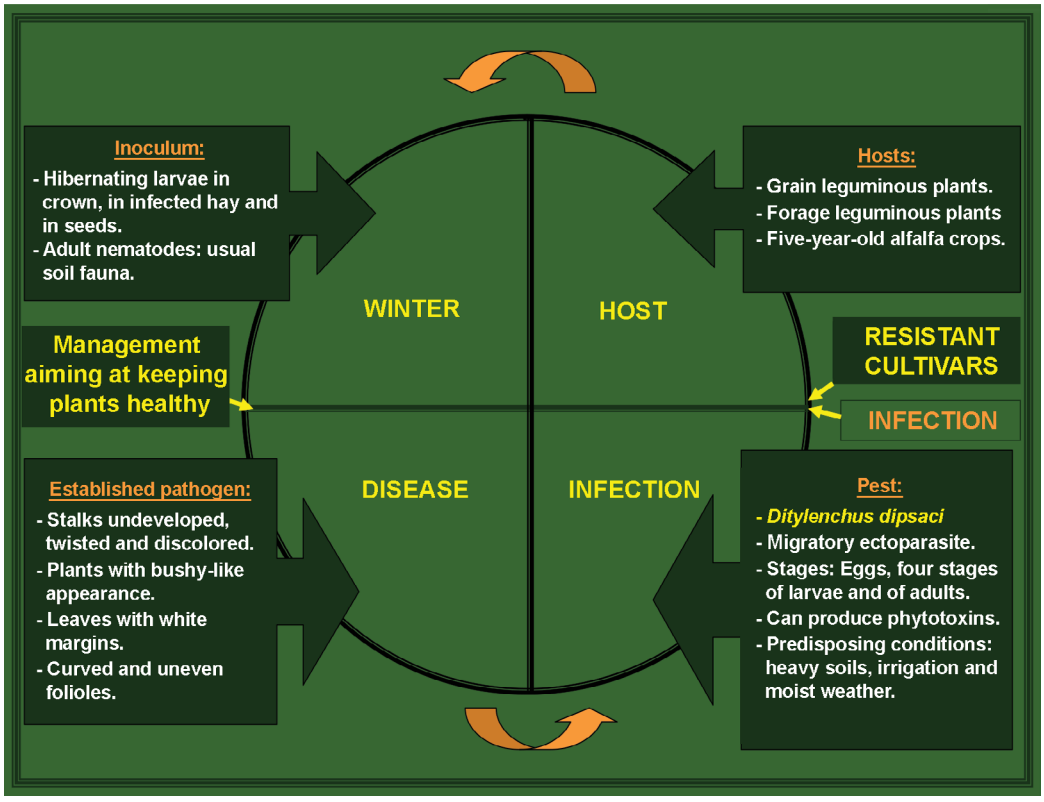


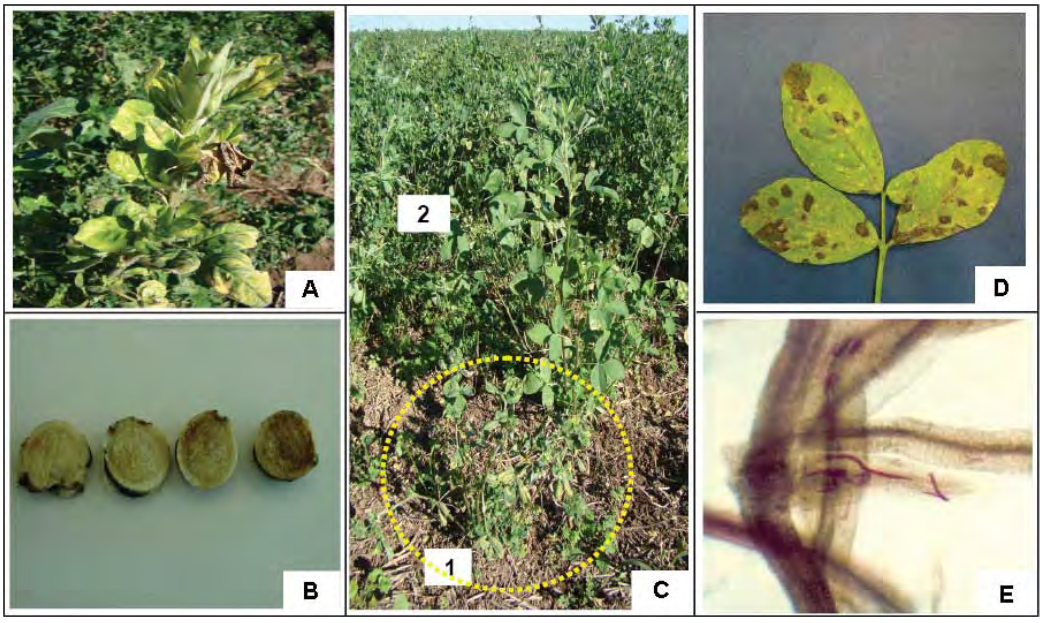
Figure 22. Biological cycle of *Ditylenchus dipsaci*.

Diseases caused by bacteria

Bacterial wilt

Causal agent: *Clavibacter michiganense ssp. insidiosum (McCull.) Davis, [synonyms: Corynebacterium insidiosum (McCull.) Jones, Corynebacterium michiganense ssp. insidiosum (McCull.) Carlson & Vidaver].*

Symptoms. Infected plants are separately distributed in the alfalfa plantation, are easy to identify by their yellowish color and have slow growth and short size. Folioles, which are also reduced in size, have a corrugated and upward-bent appearance. Infected plants are easy to identify after cutting, because resprouting is slow, with formation of short and thin stems, yellowish-green foliage and deformed folioles. Root signs can be observed in cross-section: the conductive vessels have color ranging from yellowish to dark brown (Figures 23A and 23B).



Photos: Jorge Omar Gieco

Figure 23. *Clavibacter michiganense* ssp. *insidiosum*: chlorotic apex of infected plant (A); cross-sections of diseased plant roots (B). Infected plant (C1), healthy plant (C2). *Xanthomonas campestris* pv. a*lfafae* (Riker, Dye): signs on leaves (D). *Pratylenchus* spp. (E).

Predisposing conditions to the disease. Bacteria survive in plant remains found in soils. They penetrate the alfalfa plants and infect them through injuries to the roots, the crown or the stems caused by insects, nematodes or tools used for cutting. They enter the parenchyma, multiply within the cells and pass on to the conductive vessels (xylem and phloem), systemically spreading through the plant. Signs observed and death of the plant are due to the accumulation of phytotoxic compounds produced by the bacterium and to the obstruction of the conductive vessels by mucilaginous substances, metabolic products of these microorganisms.

Disease control. Resistant cultivars are recommended. Damages produced are diluted by early cuttings or pasture when the first signs of the disease are observed, cutting first grown alfalfa plantations and, soon after, washing and disinfecting the knives of forage harvesters before cutting new and young alfalfa plantations (one to two years old). Cutting should be avoided when the environment has high relative humidity. In Figure 24 the biological cycle of *C. michiganense* ssp. *insidiosum* is detailed, as well as integrated control measures and their application times.

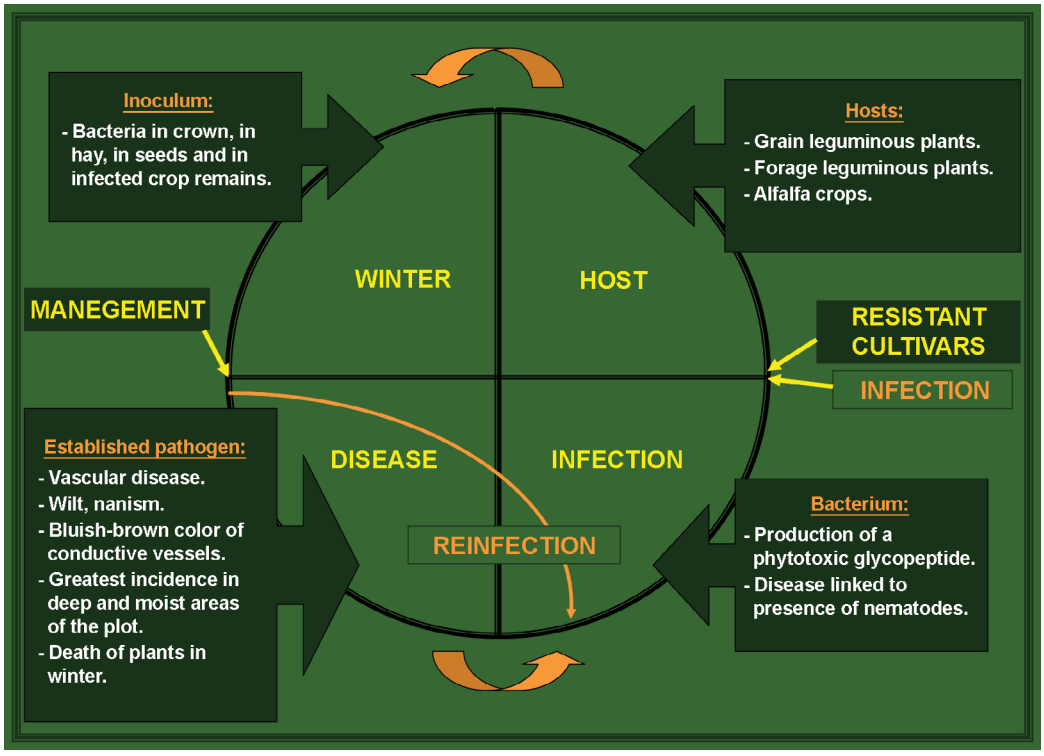


Figure 24. Biological cycle of *Clavibacter michiganense* ssp. *insidiosum*.

Bacterial leaf spot

Causal agent: *Xanthomonas campestris* pv. *alfafae* (Riker, Dye) [synonym *Xanthomonas alfalfa* (Riker)].

Symptoms. When the bacterium attacks during plantlet stage, it causes growth reduction (nanism) and, in case of severe infection and high temperature, death (damping off). In leaves, chlorotic diffuse areas with a rounded thin watery secretion are observed. These secretions are more abundant and prominent on the abaxial side of the plant. Under unfavorable conditions for proliferation of the bacteria, in resistant or tolerant cultivars, lesions are kept small, dry out and necrose. When leaf spots of bacterial origin present a chlorotic halo, they may be confused with lesions produced by fungal pathogens, such as *Pseudopeziza medicaginis* or *Cercospora medicaginis*. Under conditions that favor the disease, the spots expand and coalesce, forming relatively large lesions with irregular margins and bright aspect due to the dry secretion on the surface. In these cases, it is common to observe intense defoliation of the alfalfa plantation. On stems, it is possible to observe small

greyish lesions with secretion on the surface. These lesions lengthen, coalesce and form longitudinal lesions which can comprise several internodes (Figures 23C1 and 23C2) and over time acquire a dark brown color similar to the one caused by *Cercospora medicaginis*.

Predisposing conditions to the disease. This bacterium can survive in soils, in plant remains and in residues that accompany stored alfalfa seeds. The bacterium is spread by wind and rain, and enters the plant through the stomata and through injuries. Hot and rainy climate favors disease development. However, it also appears under hot, dry and windy climate conditions, since the wind carries sand particles that hurt leaves and stems, facilitating bacterial penetration.

Disease control. There are no resistant cultivars. It is possible to weaken the disease by cutting or pasturing in advance, when its first signs appear.

Bacterial stem blight

Causal agent: *Pseudomonas syringae* pv. *syringae* van Hall [synonym *Pseudomonas medicaginis* (Sackett)].

Symptoms. There is nanism, infected stems are shorter, thinner and more fragile than normal. Stem lesions have watery aspect, with color ranging from yellow to olive green; lesions frequently start developing from the joint of the leaf to the stem, darkening in time until they become black. Affected leaves become light yellow, with a watery secretion on the surface.

Predisposing conditions to the disease. Occurrence of low temperatures in spring and damage by late frosts favor the development of the infection. This bacterium survives in plant remains found in the ground and penetrates stems through injuries and epidermal damages produced by frosts.

Disease control. There are no resistant cultivars. Cleaning and disinfecting cutting equipment helps decreasing bacterium incidence.

Final considerations

Alfalfa diseases can cause important productivity losses, both to quantity and to quality of forage. In many cases, the diseases are determining factor for the low persistency of the crop. However, it is important to consider that persistency of an alfalfa plantation is a complex characteristic, to which diseases have an important role, but are not the only intervening factor.

This chapter described the main diseases found in alfalfa crops in Argentina and in Brazil, including causal agents, symptomatology and available control

measures. We emphasize that using fungicides on alfalfa is usually uneconomic and not a routine practice in disease control. In this context, complementing control measures in crop management with the choice of cultivars which are resistant to the greatest possible number of pathogenic agents is a fundamental tool to reach productive and persistent alfalfa cultivations.

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CHAPTER 6

Genetic improvement for disease resistance in alfalfa

Jorge Omar Gieco
Daniel Horacio Basigalup

Introduction

Using resistant cultivars is the most efficient and economic way to manage diseases in alfalfa. Host genetic resistance of the host allows to minimize or to eliminate disease losses and to reduce costs of chemical treatments; these are the cornerstones of integrated management of plant diseases. Disease genetic resistance has its own characteristics, which are decisive when prioritizing the traits to be incorporated in alfalfa breeding programs. In these aspects, the following five factors are highlighted:

a) Specificity: host resistance genes of the host act only on the pathogen, preventing disease establishment or limiting its progress but not affecting other organisms.

b) Stability: in general, genetic resistance tends to remain over time, especially quantitative resistance. Since the latter does not perform strong selection pressure on the pathogen, it allows a harmonic coexistence between the plant and the pathogen and thus minimizing the level of damage to the host. On the contrary, vertical resistance is usually less stable because since the pathogen is unable to infect, it is forced to develop mutation strategies to become a virulent agent fror infecting the host.

c) Favorable environmental impact: genetic resistance, unlike chemical control, does not negatively affect the environment, being the non-contaminating technology for excellence. Utilized in conjunction with adequate chemical control and the use of small areas planted to susceptible cultivars, it reduces selection pressure on the pathogen and decreases the development of new physiological strains that may, in time, break resistance.

d) Cost reduction: high cost of chemical treatments (fungicides, bactericides, etc.) usually prevent their use in the control of alfalfa diseases; therefore, production losses can be important and affect crop profitability. Additionally, if agrochemicals were employed, the time required for their degradation may not coincide with the frequency in which alfalfa must be cut or grazed, complicating not only crop management but also affecting forage quality and animal performance. Since genetic resistance is present in the plant throughout its life cycle, and so continually protecting the crop, it reduces the need for other control methods and thus increases economic gains.

e) Compatibility with other management methods: genetic resistance is totally compatible with other control methods and is one of the fundamental components of the integrated disease management system. Nonetheless, it is unlikely for one cultivar to be resistant to all pathogens and their races. Consequently, it is often necessary to complement genetic, chemical and cultural control strategies.

Epidemiological terminology in plant-pathogen relationship

The different manifestations of host-pathogen interaction can be defined through different concepts which apply to characterize one or the other. The pathogen effect on the host is described by many terms, such as: a) incidence: number of infected individuals per area or sample unit assessed; b) severity: estimated amount of disease produced by the pathogen; c) prevalence: presence of the disease over a continuous period; d) virulence: capacity of an isolated pathogen to produce the disease; and e) aggressiveness: amount of disease in the host per isolated amount of pathogen. The reactions of the host when attacked by the pathogen are defined in the following terms: a) resistance: capacity of the plant to prevent the pathogen-host relationship from establishing (vertical resistance) or to limit pathogen proliferation after the relationship has settled (horizontal resistance); b) immunity: total absence of the disease, given that it is impossible for the pathogen to establish into the host; c) tolerance: ability of the host to continue producing economically, even under infection levels capable of causing damage to susceptible individuals (the genetic basis of tolerance is quantitative); d) susceptibility: inability of the host to limit the pathogen infection and colonization, suffering therefore significant economic losses; and e) escape: lack of synchronization between the periods of greatest host susceptibility and highest density of pathogen population.

Resistance

Basic concepts

Genetic resistance is the result of the complex interaction among host resistance genes, pathogen virulence and avirulence genes, and environment. In the specific case of viral and bacterial diseases, the vectors (insects, nematodes, etc.) also have a decisive participation. The environment has a significant role

in disease manifestation and severity. In general, high humidity and elevated temperature favor infection and faster spreading of fungal and bacterial pathogens. However, in some cases, alternating periods of high humidity and drought periods increase the susceptibility of alfalfa to *Verticillium albo atrum*, *Fusarium solani*, *Sclerotium rolfsii* and *Pseudomonas syringae* pv. *medicaginis*. In the case of *Phytophthora megasperma* f. sp. *medicaginis*, flooding or high soil moisture even for a few days significantly favor infection of alfalfa plants.

Figure 1 illustrates the different interactions among the pathosystem components, which result in two possible final situations: a) compatibility, that represents pathogen establishment and disease development in susceptible genotypes; and b) incompatibility, which results in the absence of disease in resistant genotypes.

Types of resistance

Vertical resistance. Also known as race-specific resistance, it is the result of specific and precise interaction, gene for gene, between the host and the different races of the pathogen. Given that the resistance reaction is usually conditioned by a gene with greater effect, it is also called "monogenic" or

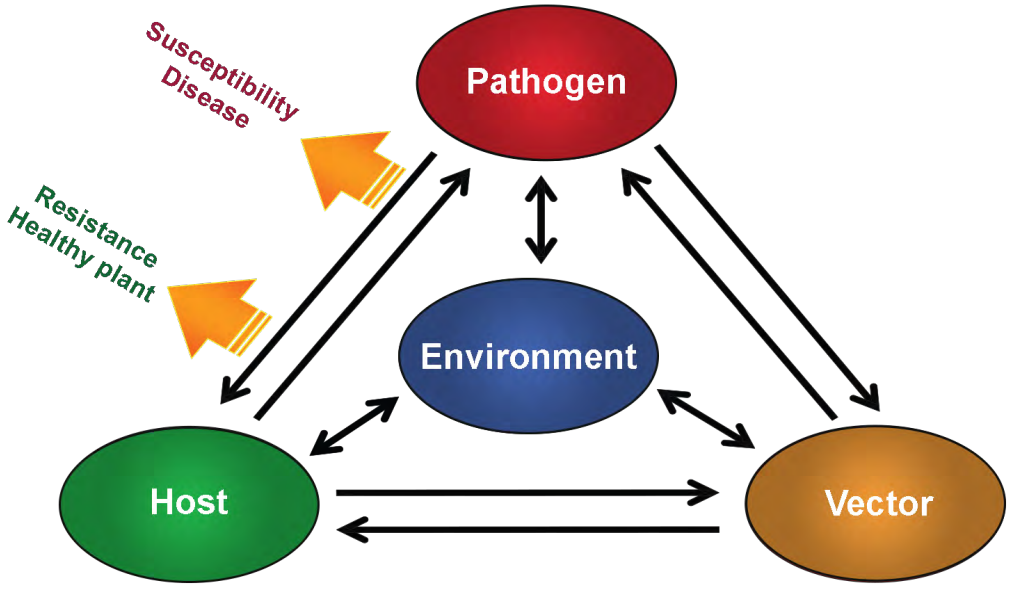

Figure 1. Different types of interaction in plant pathosystems.

"qualitative". In this type of resistance, incompatibility in the host-pathogen interaction results in the impossibility of infection and complete absence of the disease (immune response). However, this situation is often overcome by the development of new strains of the pathogen, which makes that vertical resistance be also named as "short-term resistance".

In Figure 2 are represented the frequencies of resistant and susceptible individuals originated from a cross between a resistant (10% severity) and a susceptible (70% severity) parent based on the different gene actions that may take place in vertical resistance. Individuals in the segregating population are grouped into discrete classes (resistant or susceptible). To facilitate the estimations, the diploid species *Medicago truncatula* was taken as a model, and monogenic resistance and Mendelian segregation were assumed. In this context, different possible situations deriving from gene action are calculated, ranging from completely dominant to recessive resistance (in which RR and Rr individuals are susceptible and rr individuals are resistant, segregating in 3:1 susceptible:resistant ratio), including additive resistance (in which the heterozygote has a moderately resistant or moderately susceptible phenotype), incomplete dominance (in which the heterozygote is more resistant than the parental average), and overdominance (in which the heterozygote is more resistant than the dominant homozygote).

Among the advantages of using vertical resistance, it can be mentioned the production virtually lesion-free plants (important for forage quality), the easy introduction of this type of resistance into the breeding process, and the fast response to selection. However, as it has already been highlighted, the main disadvantage underlies in the high selection pressure that plant population exerts on the pathogen, which accelerates the development of new virulent races that break resistance.

Horizontal resistance. In this case, there is no specificity in the host-pathogen relationship. As a result, there is infection and establishment of the disease but without causing economic damages. Usually, a varied range of disease severity is observed among genotypes of a given cultivar but most of them do not suffer severe damage. Several genes control the expression of this type of resistance, being each one responsible for just a part of it. The environment plays a significant role in the expression of horizontal resistance, even to an extent that the level of resistance can vary as a function of the environmental conditions. Crosses between resistant and susceptible individuals originate a segregating population of genotypes with continuous resistance variation, resulting in a normal distribution of resistance types, from highly resistant to highly susceptible (Figure 3). Since many genes are involved in the expression

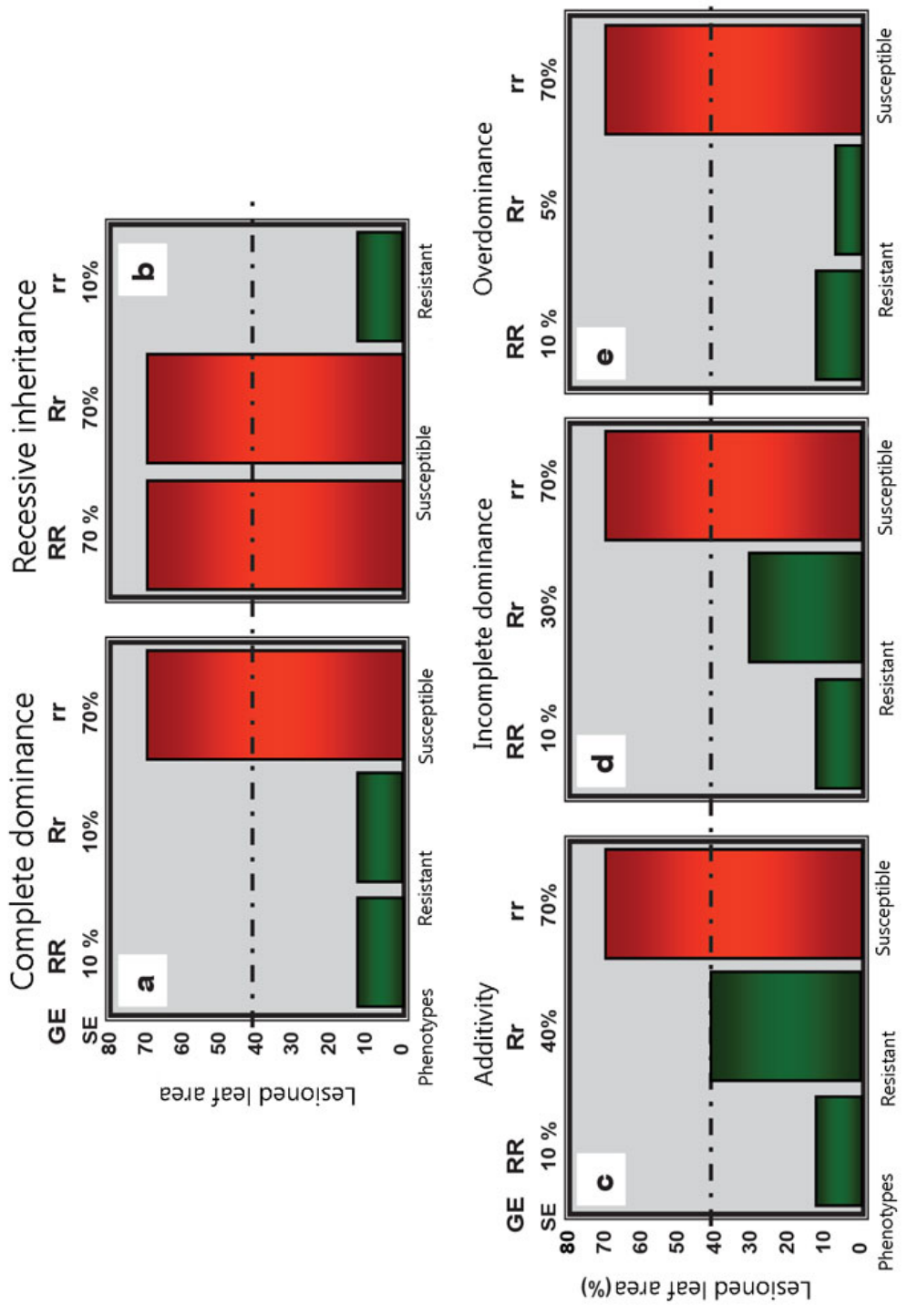

Figure 2. Different types of gene actions in monogenic resistance: complete dominance (A), recessive inheritance (B), additivity (C), incomplete dominance (D) and overdominance (E). GE = genotypes, SE = disease severity (%).

	Resistant parent		Susceptible parent
	AABBCC	**x**	**aabbcc**
Disease severity	10%	↓	70%
F1 Hybrid		**AaBbCc**	
		40%	
Selfing		@	
		↓	

F2 Generation

Phenotype	Frequency	Number of alleles
10%	1	6 (AABBCC)
20%	5	5
30%	15	4
40%	20	3
50%	15	2
60%	5	1
70%	1	0 (aabbcc)

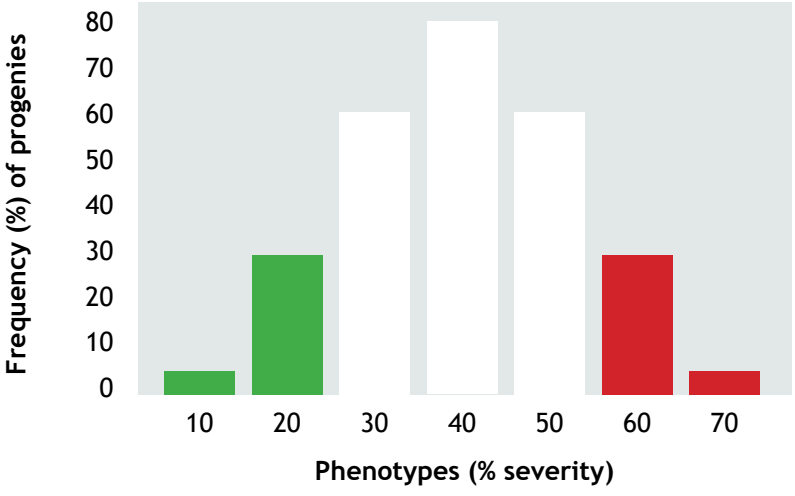

Figure 3. Scheme of horizontal or polygenic resistance.

of horizontal resistance, it is also known as “quantitative”, “polygenic” or “nonspecific” resistance. When just two to five genes are conditioning the resistance response, it is named “oligogenic” (Pataky; Carson, 2004).

The main advantage of this type of resistance is durability, given the lack of high selection pressure on the pathogen. By allowing the pathogen to establish in the host, equilibrium of the pathosystem over time is also favored. Although the infection takes place, proliferation of the pathogen is usually limited to levels that do not economically endanger the host. The disadvantage of horizontal resistance is that its quantitative genetic basis makes its introduction into the breeding population to be slower and harder. However, the use of a molecular marker assisted selection approach can minimize this problem, provided that quantitative trait loci (QTLs) or quantitative resistance loci (QRLs) are identified and available.

A comparison between vertical and horizontal resistances is represented in Figure 4, including complete and incomplete types of vertical resistance. In the case of complete vertical resistance, since the effect of the gene that controls resistance is total, there is an incompatibility reaction between the plant and the pathogen, resulting in lack of infection. In contrast, in incomplete vertical resistance, it is assumed the presence of modifiying genes that may attenuate the effect of the major resistance gene; therefore, there is a small degree of infection even in the resistant genotypes (estimated as less than 5% of disease severity). Differential interaction between pathogen races and host resistance genes is represented in the incompatible reactions of pathogen races R1, R3, R4, R5 and R7, with 90%–100% lesion-free leaves. In contrast, compatible reactions involve races R2 and R6, producing susceptible phenotypes with little or no lesion-free leaf areas. In the particular pathosystem depicted in Figure 4, and assuming dominant resistant, the genetic composition for the pathogen would be: Avr1–Vir2–Avr3–Avr4–Avr5–Vir6–Avr7 and for the host would be R_1–rr2–R_3–R_5–rr6–R_7. In case of horizontal resistance, it can be observed lack of interaction between the host and the virulence genes in the different races of the pathogen. The dashed line represents the ideal level of lesion-free leaf area in the host, represented by the average value of 50%. Finally, “basal” or “natural” horizontal resistance, found in many populations, is represented by a value close to 20% of lesion-free leaves, since it is a usual resistance level acquired during the co-evolution between the host and the pathogen.

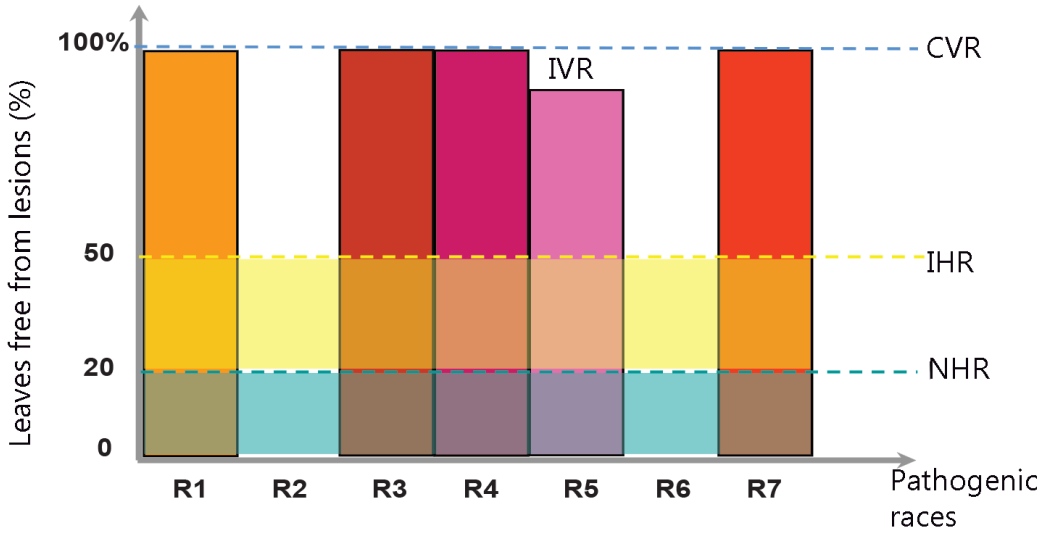

Figure 4. Comparative graph of vertical resistance and horizontal resistance in a plant pathosystem. CVR = complete vertical resistance, IVR = incomplete vertical resistance, IHR = ideal (level of) horizontal resistance, NHR = natural horizontal resistance.

Traditional breeding for disease resistance

Development of resistant cultivars is generally considered a priority in any alfalfa breeding program. Therefore, it is necessary to consider some fundamental aspects for carrying out an efficient work. Among them, the nature of alfalfa regarding autotetraploid inheritance and allogamy are particularly important. These two aspects contribute to the high genetic variability that is generally present in a synthetic variety (population concept). Practical assessment of the resistance level to a given pathogen is expressed by the frequency or the percentage of resistant individuals based on a categorical resistance classes scale. Table 1 summarizes the resistance classes for the characterization of cultivars defined by the *North American Alfalfa Improvement Conference* (NAAIC) through specific standard tests (North American Alfalfa Improvement Conference, 2005; National Alfalfa & Forage Alliance, 2013).

Another important aspect in breeding for disease resistance is to know the pathogen, especially regarding its biological cycle and culture conditions that favor growing, inoculum production and host infection. The latter is necessary for establishing an adequate protocol for each particular disease. In the case that a pathogen produces physiological races, it is necessary to use pure cultures for identifying resistant individuals in the host population.

Table 1. Resistance classes used for characterizing alfalfa cultivars.

Resistant Plants (%)	Resistance classes	Abbreviation
0–5	Susceptible	S
6–14	Moderately susceptible[1]	MS
15–30	Moderately resistant	MR
31–50	Resistant	R
> 50	Highly resistant	AR

[1] Synonym of low resistance (BR).

In some cases it may be important to identify sources of resistance to a given disease in order to develop resistant cultivars. One practical approach for that is to assess germplasm from areas where the disease is endemic and has co-evolved with alfalfa; then, the resistant genes are introduced into the elite breeding material by crossing and selection. For instance, the *Medicago sativa* var. *falcata* is generally a good source of resistance to leaf diseases and to *Verticillium albo-atrum*.

In most cases, disease resistance in alfalfa is conditioned by a few genes with variable degrees of dominance (Elgin Junior et al., 1988). This is the case for *Colletotrichum trifolii*, *Phytophthora megasperma*, *Peronospora trifoliorum* and *Alfalfa mosaic virus* (AMV). In some other cases, resistance inheritance is quantitative, as in *Leptosphaerulina briosiana* and *Verticillium albo-atrum* (Kehr, 1970; Kehr et al., 1972).

Another aspect that has been observed in alfalfa is that resistance genes to different diseases are not usually linked and act independently. In addition, the production of physiological pathogenic races is not significant for most alfalfa pathogens, with the so far exceptions of *C. trifolii* (Welty; Mueller, 1979) and *Peronospora trifoliorum* (Stuteville, 1973).

Breeding methods

Predominance of monogenic inheritance and its subsequent fast progress of selection explain the reason why recurrent phenotypic selection (RPS) has been the most successful method used for developing disease resistant cultivars in alfalfa. Based on adequate selection protocols for identifying resistant genotypes, RPS enables the combination of high selection intensity and short generation interval. As illustrated in Figure 5, desirable individuals are selected

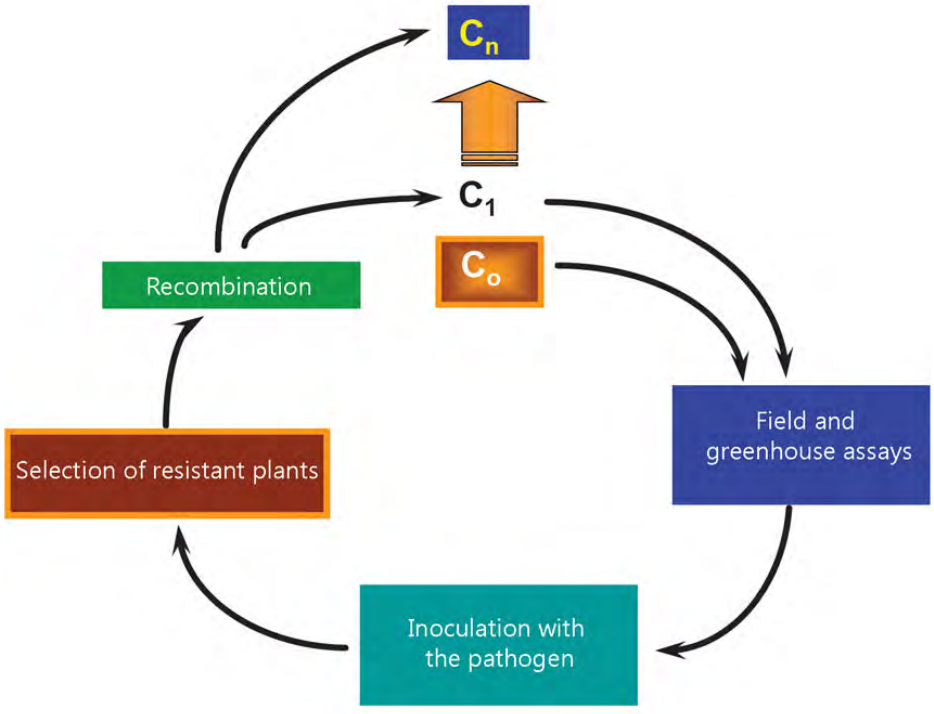

Figure 5. Scheme of the use of recurrent phenotypic selection for obtaining disease resistant alfalfa populations. C = selection cycles.

by their phenotypic characteristics and then polycrossed to produce the next generation, repeating this process in a cyclical (recurrent) way (Fehr, 1987). As many selection cycles are performed as necessary until the desired level of resistance is achieved. The goal of each cycle is to increase the frequency of alleles which confer resistance to one disease (independent selection level) or to several diseases (tandem selection), as depicted in Figure 6.

Another method used in developing resistant cultivars is the complementary crossing of cultivars (CCC), also known as "strain crosses" (Elgin Junior et al., 1983), which aims to combine resistance genes to two or more diseases coming from two or more different sources (cultivars or populations) into one population. For instance, supposing that cultivar I is resistant to disease A and that cultivar II is resistant to disease B, and that in both cases resistance is conditioned by a dominant gene with gene frequency of 0.5, the resulting population from crossing I x II will have, at equilibrium, 46.7% of individuals with both dominant

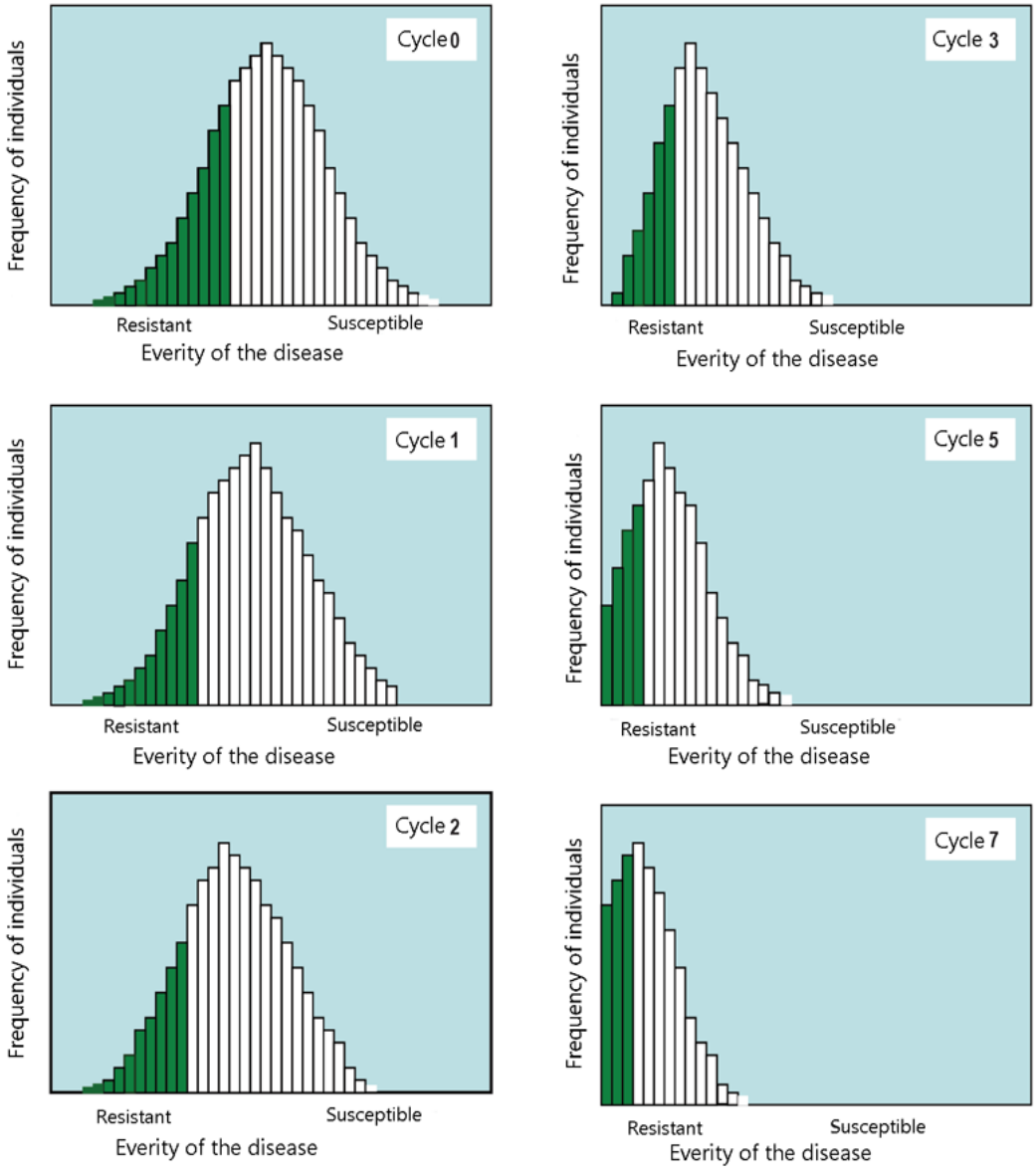

Figure 6. Increase in frequency of resistant individuals in an alfalfa population submitted to seven cycles of recurrent phenotypic selection.
Source: Adapted from Pataky and Carson (2004).

genes, 43.3% with only one dominant gene, and 10.0% with none (Busbice et al., 1972) This is based on the equation (1/4 A + 3/4a)4 (1/4B + 3/4b)4. Cultivar or strain crossing was used by Elgin Junior et al. (1983) to develop alfalfa populations with multiple pest resistance.

Backcrossing has also been used to improve disease resistance in the development of commercial alfalfa cultivars (Peaden et al., 1976; Murphy; Lowe, 1996). Considered a rather conservative breeding procedure, the method is generally used to correct susceptibility to diseases in an otherwise highly valuable agronomic material. In order to avoid consanguinity and inbreeding depression, it is advisable to use several non-related recurrent parents in each backcrossing cycle. Stanford and Houston (1954) suggested utilize 100 to 300 unrelated plants from the recurrent population. Not widely used until a few years ago, the method is now increasingly important for the introgression of trangenes into elite breeding populations.

Molecular techniques for identifying disease resistance

Bases of plant-pathogen interaction

Interaction between host resistance genes and pathogen virulence/ avirulence genes was independently studied in the USA by Flor (1942, 1971) and in the Netherlands by Oort (1994). Flor utilized the *Melampsora lini - Linum usitatissimum* [flax] pathosystem as a model for his studies, and his results gave rise to the hypothesis known as “gene for gene”. In this model, compatibility or incompatibility reactions between the host and the pathogen are the result of the interaction among the corresponding genes from each organism. For instance, host resistance genes (named R) are responsible for triggering plant reaction aimed to prevent pathogen development. If R genes were not present, pathogen recognition (virulence genes) and subsequent activation of resistance mechanism will not take place, resulting in infection and disease development. This reaction is so specific that every R gene recognizes only one race of the pathogen but is unable to recognize other races. It was later discovered that pathogens, besides the virulence genes, have another group of genes, named avirulence genes (*Avr*), which complementarily interact with R and are also involved in the activation of the defense mechanisms. In this case, the absence of the *Avr* gene in the pathogen enables disease development, even when the host has the corresponding R gene (Figure 7). It is highlighted that the resistance reaction in the host only takes place when the product from the pathogen *Avr* gene of the pathogen is detected in the host cell membrane by the corresponding R gene (Staskawicz et al., 1995; Crute; Pink, 1996).

As it was mentioned before, pathogen-host interaction results in two opposed responses: a) *susceptibility*, when the host, in the absence of R genes, does not recognize the pathogen and therefore disease is established;

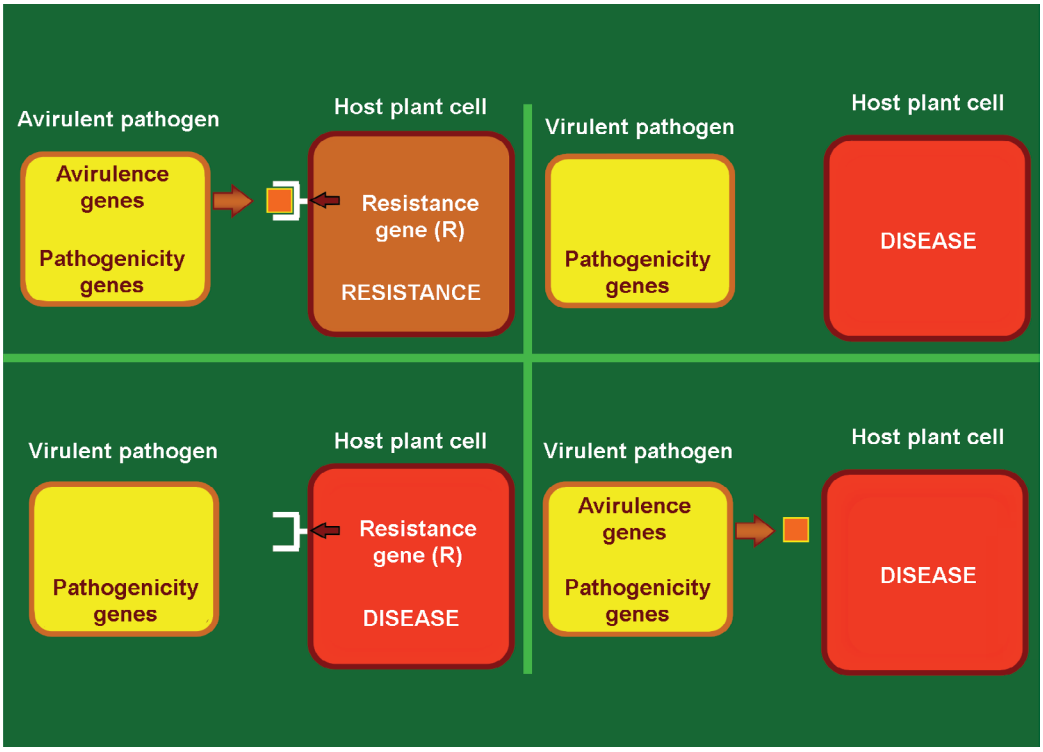

Figure 7. Gene for gene interactions that explain plant resistance and susceptibility to pathogens.
Source: Adapted from Staskawicz et al. (1995).

and b) *resistance*, when the pathogen *Avr* genes (elicitors) are recognized by the host R genes (race-specific receptors), triggering host defense reactions (hypersensitivity reactions) that prevent disease establishment (Figure 8). Further knowledge derived from these basic studies enabled the isolation (gene cloning) and later characterization of R and *Avr* genes in the several pathosystems.

Resistance genes

Resistance (R) genes act basically in two key steps into the pathogen-host relationship: a) recognition of the pathogen, in a receptor-ligand (elicitor) interaction and b) signaling, in which gene products take part in the processes of translating the incompatibility signals.

According to the protein of the receptor function they codify, Baker et al. (1997) grouped resistance genes into five classes:

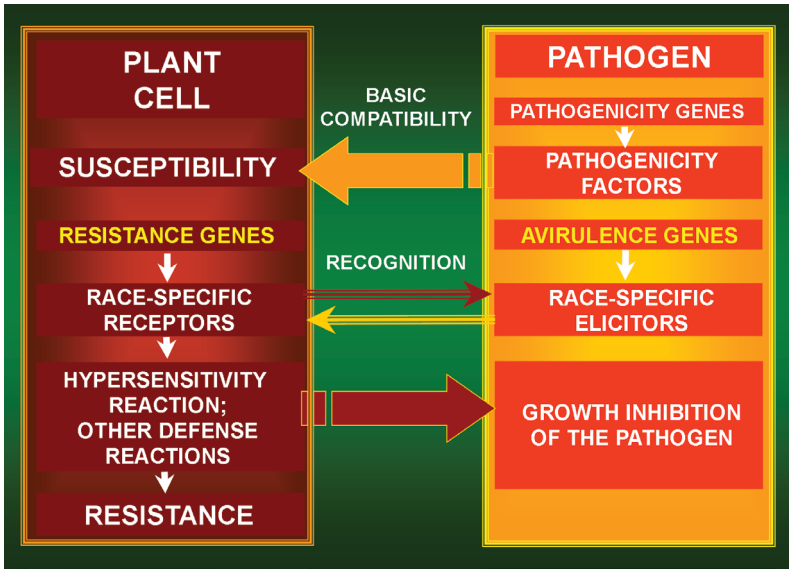

Figure 8. Basic model explaining the reactions resulting from plant-pathogen interaction.
Source: Adapted from de Wit (1992).

Class 1: genes that codify proteins similar to cytoplasmatic receptors, possessing leucine-rich repeats (LRR) sequences and a nucleotide binding site (NBS). To this group belong genes *RPS2*, *RPP5* and *RPM1* genes *Arabidopsis*, *Prf* and *I2* from tomato, *N* from tobacco and *L6* and *M* from flax.

Class 2: genes that codify kinase-type proteins, such as the *Pto* gene from tomato, which are homologous to other kinases from mammals and drosophilae. The *Prf* gene (class 1) is needed for the correct function of the *Pto* gene.

Class 3: genes that codify transmembrane receptors, like *Cf2* and *Cf9* from tomato (with large extracytoplasmic LRR domains) and *HS1pro-1* from beet (LRR transmembrane protein).

Class 4: genes that codify proteins composed by one transmembrane receptor with extracellular LRR domain and intracellular kinase domain. For example, the structure of the *Xa21* gene from rice suggests the existence of an evolutionary gap between the *Cf* gene and the *Pto* kinase.

Class 5: genes that codify reduced reductase NADPH-dependent forms, as the *Hm1* gene which inactivates the toxin produced by race1 of *Cochliobolus carbonum* (Figure 9).

Cloning R genes enabled to characterize not only host resistance mechanisms but also changes responsible for variation in specificity. Resistance genes that have been cloned in several plant species – such as alfalfa,

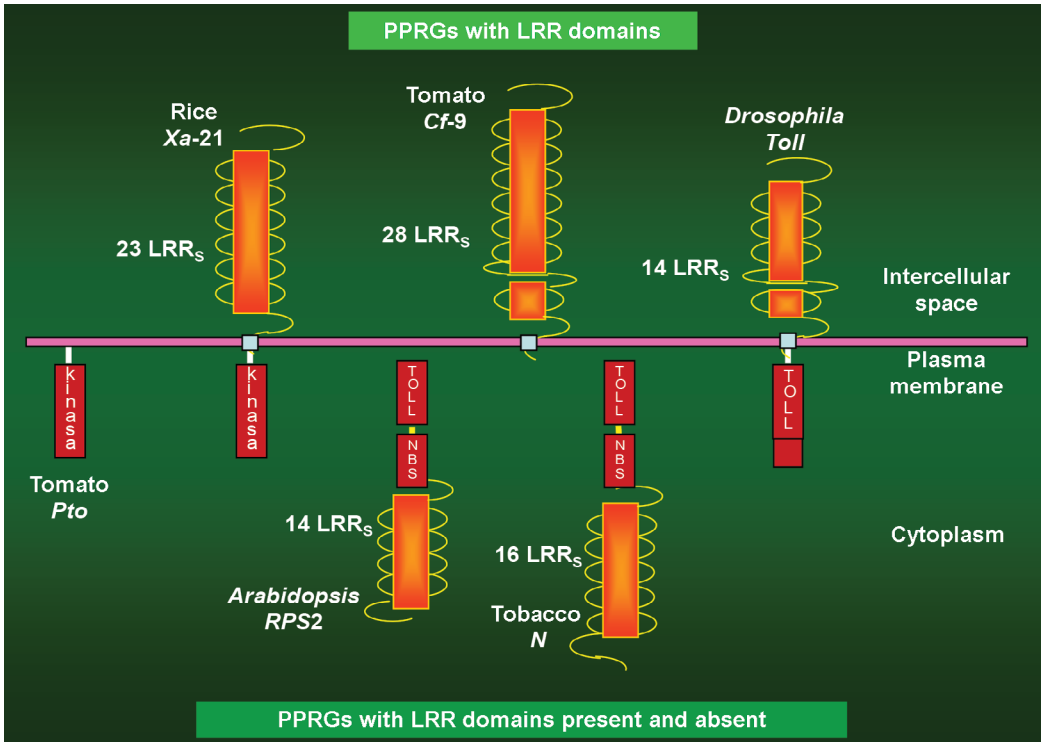

Figure 9. Products of the resistance genes. PPRG = pathogenesis-related proteins deriving from resistance genes; LRR = leucine-rich repeats.
Source: Adapted from Hammond-Kosack et al. (1996).

Arabidopsis spp., tomato, tobacco, flax, beet, rice and maize –, are listed in Table 2. Specific *Avr* genes and the pathogens over which the R genes act are also included. Additionally, the structural characterization of the *R* genes is also provided. The latter is generally divided into the following groups: a) leucine-zipper, with sites that bind to nucleotides and to leucine-rich repeats; b) tyrosine-zipper, with sites that bind to nucleotides and to tyrosine-rich repeats; c) sites that bind to nucleotides and to leucine-rich repeats; d) kinase-type proteins; e) leucine-rich repeats and kinase-type proteins; 6) reductase-type proteins.

Avirulence genes

Avr genes are present in pathogens and have two basic functions: a) to recognize host condition, through the direct or indirect interaction between the *R* gene (dominant or semi-dominant) and the dominant *Avr* gene (Staskawicz

Table 2. Identification and molecular structure of resistance genes (*R*) cloned from different plant species, the pathogens on which they act and the corresponding avirulence genes (*Avr*) from those pathogens.

R gene	Species	Pathogen	Avr gene	Structure
RCT1	*Medicago truncatula*	*Colletotrichum trifolii*	Unknown	TIR-NBS-LRR
RPS2	*Arabidopsis*	*Pseudomonas syringae pv. tomato*	avrRpt2	LZ-NBS-LRR
RPM1	*Arabidopsis*	*Pseudomonas syringae pv. maculicola*	avrRpm1, avrB	LZ-NBS-LRR
Prf	Tomato	*Pseudomonas syringae pv. tomato*	avrPto	LZ-NBS-LRR
N	Tobacco	*tobacco mosaic virus*	TMV Replicase	TIR-NBS-LRR
L6	Flax	*Melampsora lini*	AL6	TIR-NBS-LRR
M	Flax	*Melampsora lini*	AM	TIR-NBS-LRR
RPP5	*Arabidopsis*	*Peronospora parasitica*	avrPp5	TIR-NBS-LRR
l2	Tomato	*Fusarium oxysporum*	Unknown	NBS-LRR
Pto	Tomato	*Pseudomonas syringae pv. tomato*	avrPto	Kinase-type protein
Cf-9	Tomato	*Cladosporium fulvum*	Avr9	LRR-TM
Cf-2	Tomato	*Cladosporium fulvum*	Avr2	LRR-TM
HS1pro-1	Sugar beet	*Heterodera schachtii*	Unknown	LRR-TM
Xa21	Rice	*Xanthomonas oryzae pv. Oryzae*	Unknown	LRR, kinase-type protein
Hm1	Maize	*Cochiobolus carbonum (race 1)*	None	Reductase-type toxin

Source: Bent (1996).

et al., 1995); and b) to determine the infection capacity of the pathogen, resulting from the interaction between the pathogenicity genes and the *Avr*

Table 3. List of some avirulence genes (*Avr*) cloned from different pathogenic fungi species.

Pathogen	Avr	Disease
Bremia lactucae	*PWL2*	Downy mildew of lettuce
Melampsora lini	*AL6*	Flax rust
Magnaporthe grisea	*AVR2-YAMO*	Rice blast
Rhynchosporium secalis	*avrRrs1*	Barley leaf scald
Cladosporium fulvum	*Avr4*; *Avr9*	Tomato leaf mold

genes of the pathogen (Wit, 1992). Table 3 lists the *Avr* genes which have been cloned and characterized from several pathogenic fungi.

R genes cloning

To clone plant resistance genes, two methods have been used so far: insertional mutagenesis and positional mapping (based on genetic maps).

Insertional mutagenesis (transposon tagging). This method is based on the fact that inserting a transposable element (transposon) within a gene causes its inactivation. After insertion, the transposon is used as a probe to detect the gene alteration through restriction fragment length polymorphisms (RFLPs) molecular markers. Insertional mutagenesis has been very successfully used for isolating R genes from different plant species, such as tobacco, tomato, maize and flax.

Positional cloning (chromosome walking). The starting point of this methodology is information regarding genetic mapping of R genes. The initial requirement is the availability of flanking molecular markers strongly linked to the R gene to be cloned (fine mapping) or to the chromosome position of another previously cloned R gene. Based on this information, DNA of the chromosome containing the R gene is cut with two complementary cutting restriction enzymes and the fragments produced are cloned into DNA vectors, such as yeast artificial chromosome or bacterial artificial chromosome; as a consequence, two libraries generally named 1 and 2, are formed (Figure 10A). This method was named “chromosome walking” because it consists in moving (“walking”) along the chromosome until a gene of interest is found. Finally, to make sure the identification of the desired gene, the cloned gene is inserted into a mutant null for a given resistance, aiming at assessing through

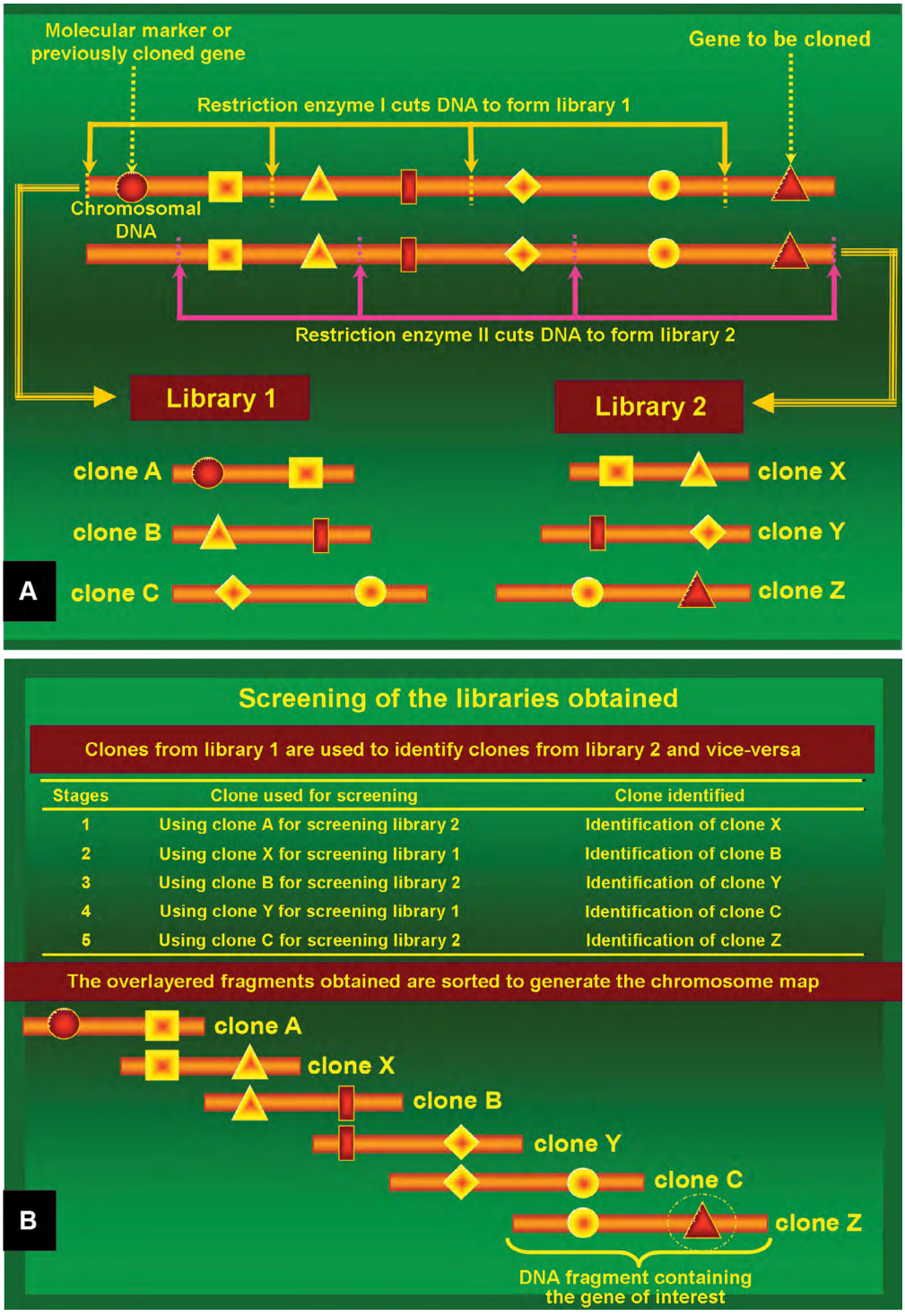

Figure 10. Stages involved in cloning genes via the positional cloning method. Cutting with restriction enzymes to create two genomic libraries (A); reciprocal screening of the libraries with the clones created (B).

transgenesis the functional complementarity of the isolated gene. In this case, when the transgenic plant built is confronted with the pathogen, there will only be resistance if the isolated gene introduced is correct; otherwise, the control transgenic plant which has the mutant resistance gene (inactive) will develop the disease, since it is unable to recognize the pathogen. Through this technology, R genes were cloned into *Arabidopsis thaliana*, tomato, maize and alfalfa. The *RCT1* gene cloned from *Medicago truncatula*, which confers wide resistance to three races of *Colletotrichum trifolii*, has proved to be highly efficient in preventing the development of anthracnose in the transformed alfalfa plants (Yang et al., 2008). Kiss et al. (2006) isolated and characterized the *fms1* gene, which confers resistance to *Fusarium oxysporum* f. sp. *medicaginis*. This gene has great potential for being introduced into alfalfa cultivars via transgenesis.

Isolating R genes offers a new and very interesting perspective for plant breeding aiming at disease resistance. Characterization of a large number of R genes, based on preserved amino acid sequences, will enable breeders to identify desirable genotypes using specific DNA probes, instead of performing phenotypic detection of resistant and susceptible individuals in the presence of the pathogen. This method will also facilitate identification and subsequent introgression of resistance genes from wild species, leaving aside difficulties from interspecific hybridization or the use of distinct polyploidy levels. Cloned R genes can be incorporated into alfalfa through transgenesis.

Likewise, having several R genes available will allow pyramiding them in a given cultivar, granting the plant with simultaneous resistance to several pathogens or with a more stable resistance to a particular pathogen (several races). It is also possible to co-transform the plants with *Avr* and R genes, which will allow the creation of a host being resistant to a wide range of pathogens. Figure 11 illustrates an example, considering genes *avr9* (*Avr*) and *Cf9* (R). The system works as follows: a pathogen, upon contact with the host, produces non-specific elicitors that activate the inducible promoter of the *avr9* gene; the latter then attaches to a kinase-type receptor (product of the *Cf9* gene) and triggers the hypersensitivity reaction (Wit, 1992).

Transgenic plants with R genes expression

Interaction of elicitors from the pathogen with receptors from the host results in the inhibition on the first and in resistance on the second. As illustrated in Figure 12, the virulent pathogen produces enzymes (proteases, cellulases, cutinases, xylanases, etc.) that degrade host cell walls and membranes, initiating the way for the infection process. However, the pathogen also may

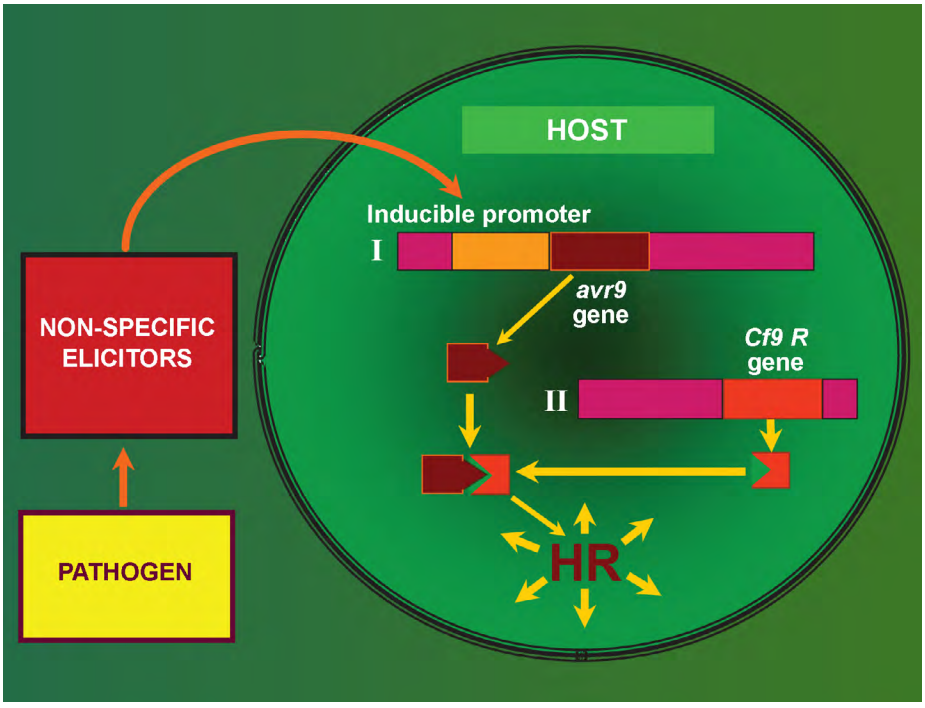

Figure 11. Co-transformation model with avirulence genes (*avr9*) and resistant *R* genes (*Cf9*) to trigger the hypersensitivity reaction (HR) after it has been induced by non-specific elicitors of several pathogens.

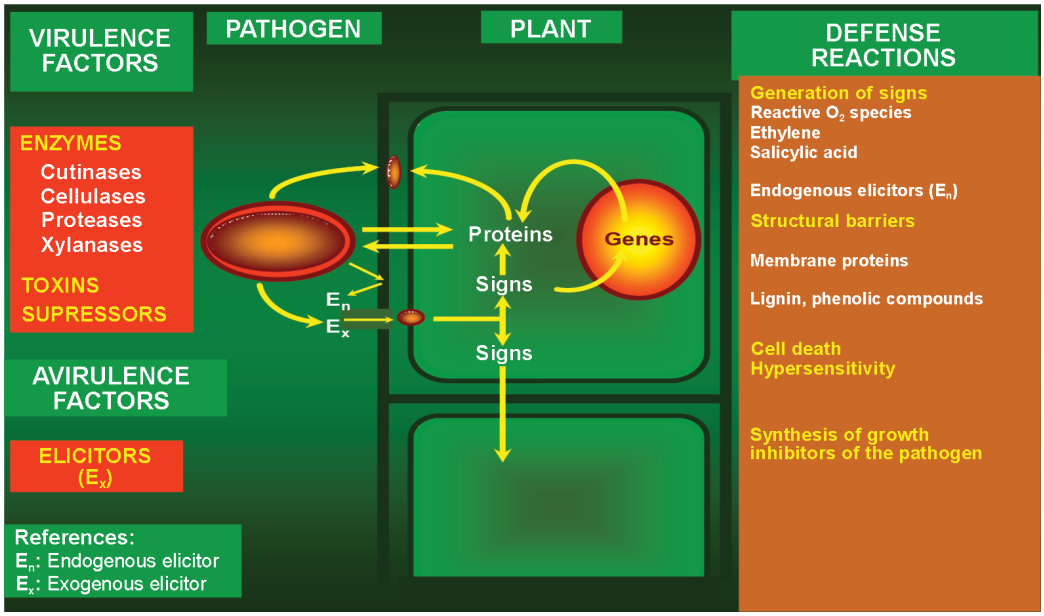

Figure 12. Brief of the reactions involved in plant-pathogen relationship.

have avirulence factors that when detected by the plant R gene (receptors), trigger intercellular signals (involving reactive forms of oxygen, salicylic acid, jasmonates, ethylene and other endogenous elicitors) and synthesis of several compounds (callose, membrane proteins, cutin, suberin, phenols, waxes, etc.), forming structural barriers against penetration by the pathogen. For instance, reactive forms of oxygen produce cellular death in the penetration site of the pathogen, causing hypersensitivity reactions. There are also resistance genes that prevent pathogen establishment through the synthesis of special proteins (like 1,3 ß-glucanasis, chitinases, proteinases and polygalacturonases inhibitors, permatins, thionines, etc.) or the synthesis of phenolic compounds (as phytoalexins). Some of these R genes have been successfully cloned and used for producing transgenic alfalfa plants with resistance to pathogenic fungi.

Some species – as alfalfa, barley, rice, tobacco and beans – had different types of resistant genes that were cloned and used to develop individuals with resistance to several pathogens. Currently, many studies seek to obtain transgenic alfalfa plants with production of glucanases, which grant non-specific resistance to several fungal pathogens (Wit, 1992; KISS et al., 2006).

Using molecular markers to map quantitative resistance genes

The methodology of QTL (quantitative trait loci) or QRL (quantitative resistance loci) mapping consists basically in testing a large number of molecular markers, spread throughout the genome, for detecting polymorphism associated to R genes. Individuals from a segregating population (F2, backcrossing, recombinant inbred lines, etc.) generated by crossing divergent (resistant x susceptible) parents for a given disease, are genotypically characterized with molecular markers and phenotypically (resistant or susceptible) through inoculation with the pathogen. The genotype of each individual is classified according to its band pattern in an agarose gel resulting from each molecular marker. Based on mean and variance estimations, multiple comparissons are performed and the significant differences are interpreted as an indication of association between the molecular marker and the resistance QTL (Young, 1996). Figure 13 shows a single linkage group with four RFLP marker loci (left). Individuals from the population are analyzed by their genotype (markers) at each locus level, using the band pattern to determine segregation of parental alleles in the progenies (center). For each marker locus, individuals are divided into classes according to their molecular genotype (two homozygous and one heterozygous parental classes); later, the mean and variance are calculated for each class (right). In the example, a significant difference in genotype (bands)

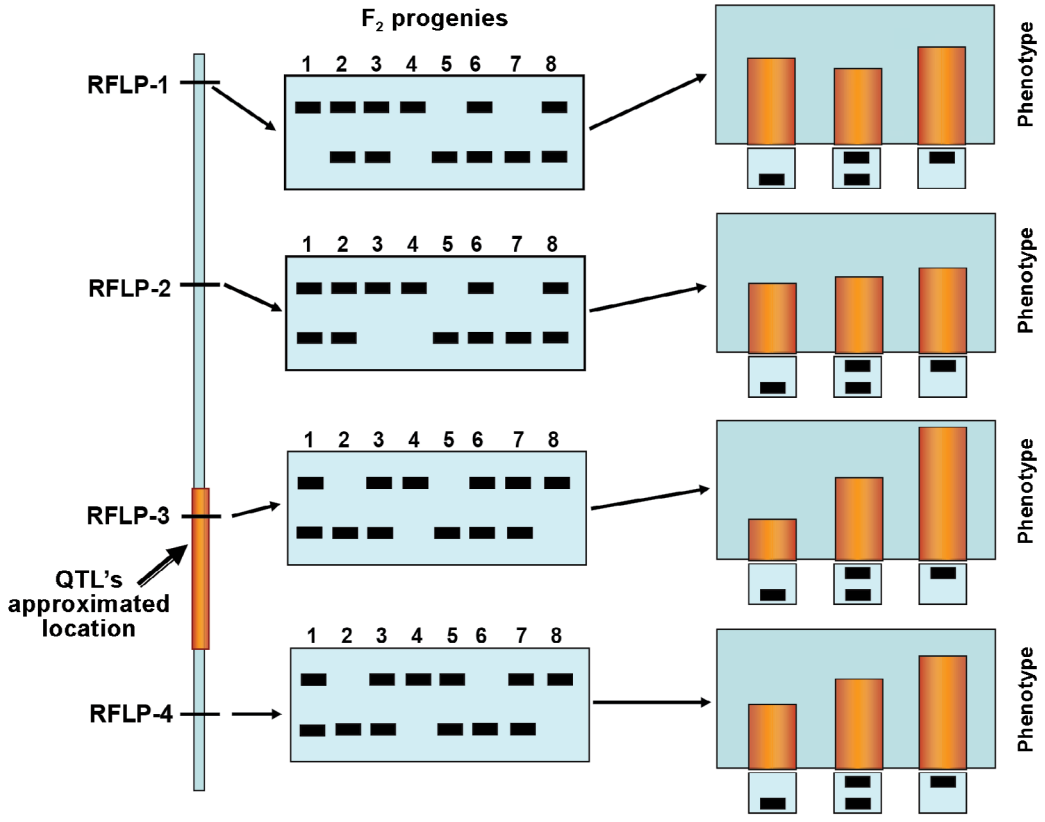

Figure 13. Conceptual basis of QTL mapping in an F2 population. RFLP = restriction fragment length polymorphism.
Source: Adapted from Young (1996).

is related to minor differences in phenotype (resistance) for RFLP markers 1 and 2, indicating low probability of association between these two markers and the resistance QTL. On the contrary, significant phenotype differences among genotypic classes defined by RFLP markers 3 and 4 indicate that the resistance QTL is very likely to be located in between these two markers, showing more proximity to RFLP 3 for exhibiting greater difference between the phenotypic classes.

Even though the methodology is conceptually clear and simple in concept, there are limitations for its use. Among them, the most prominent in alfalfa is the lack of highly saturated genetic maps with a large number of neutral and functional markers. Since the distance between markers (saturation) limits the power of the methodology for detecting QTLs, the smaller the distance between markers, the larger the power of QTL detection will be and vice-versa.

Another limiting factor arises from the impossibility to identify the individual effect of each QTL down when there are multiple QTLs in the same linkage group.

Despite these limitations, which will certainly be overcome in time and with the development of new statistical programs, the method is very powerful and useful for the genetic improvement of the "disease resistance" trait. The advantages become evident in the case of traits that are complex and have high environmental influence. In fact, one of the greatest virtues of this procedure is the possibility of precisely quantifying "gene for gene" interactions (dominance, additivity, epistasis, etc.) and genotype x environment interactions. However, the success of the methodology depends on the adequate planning of the experiments, which will allow the accurate phenotype classification in the segregating population. In this sense, knowing the pathogen and the inoculation and disease assessment techniques, as well as the correct description and characterization of the environments where the plant populations deriving from the breeding program will be used, are crucial and determining aspects of the QTL detection power for resistance.

In alfalfa, utilizing the approach of QTL identification through molecular markers, genes for resistance to a few economically important diseases

Table 4. Quantitative trait loci (QTLs) identified in alfalfa with molecular markers.

Pathogen	QTL location	Phenotypic variation for explained resistance
	Medicago sativa	
Phytophthora megasperma f. sp. *medicaginis*	Linkage groups 2, 14 and 18	6–15%
Colletotrichum trifolii	Linkage groups 8 6 QTLs race 1 4 QTLs race 4 4 QTLs race 2 Linkage groups 4 1 QTL race 1	52–63%
Peronospora trifoliorum	1 QTL	-
	Medicago truncatula	
Colletotrichum trifolii	1 QTL races 1, 2 and 4 (8 genes)	-

such as *Phytophthora megasperma* f. sp. *medicaginis* (Musial et al., 2005), *Colletotrichum trifolii* (Yang et al., 2008) and *Peronospora trifoliorum* (Obert et al., 2000), as summarized in Table 4.

Pyramiding marker-assisted resistance genes

Selection assisted by molecular markers (Figure 14) can be very useful for developing alfalfa populations or cultivars with resistance to several diseases. If there were molecular markers linked to genes of resistance to different pathogens – or races of the same pathogen – it would be possible to accumulate or (pyramid) such genes into individual genotypes or into synthetic populations employing a backcross scheme assisted by molecular markers.

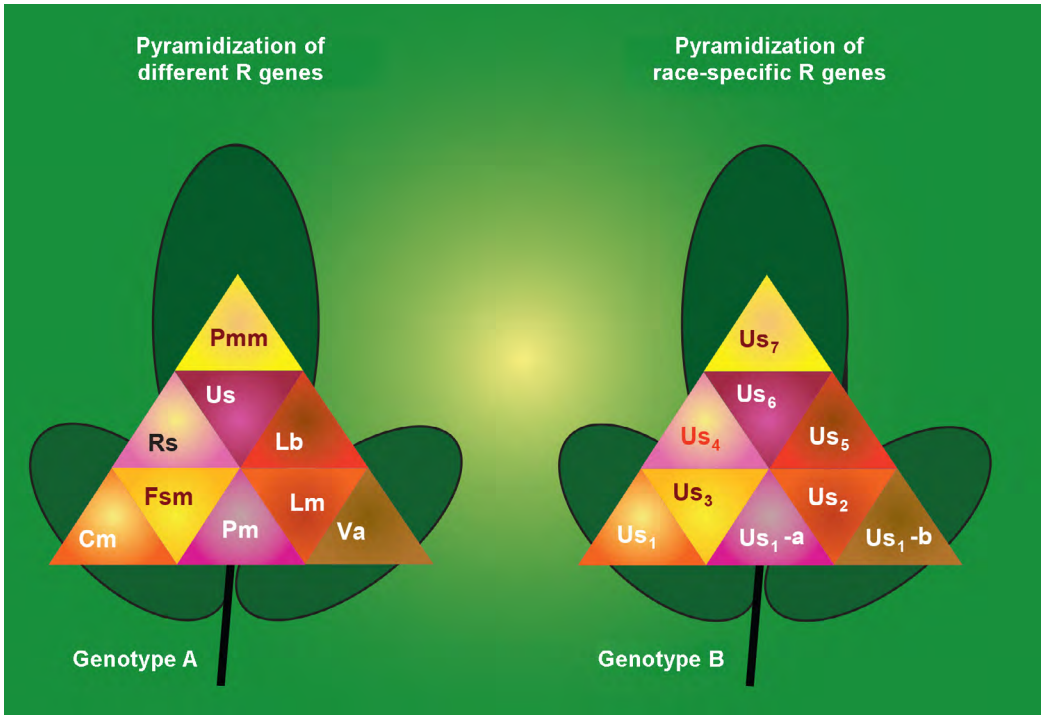

Figure 14. Pyramiding disease resistance genes in alfalfa. Genotype A: accumulating genes of resistance to different pathogens. Genotype B: accumulating genes of race-specific resistance to *Uromyces striatus* [alfalfa rust]. Legends: Pmm: *Phytophthora megasperma f*. sp. *medicaginis*, Us: *Uromyces striatus*,Rs: *Rhizoctonia croccorum*, Lb: *Leptosphaerulina briosiana*, Fsm: *Fusarium solani*, Lm: *Leptotrochila medicaginis*, Pm: *Phoma medicaginis*, Va: *Verticillium albo-atrum*, Cm: *Cercospora medicaginis*, Us1-Us7: genes of resistance to different races of *Uromyces striatus*.

Final considerations

Using resistant cultivars is without doubt the most efficient way to manage alfalfa diseases. In this chapter, the available resistance types and the way in which they are inherited, as well as the most efficient methods to develop resistant cultivars using conventional breeding methods, are described. More recently, the development of molecular techniques presents a new and extremely interesting perspective for alfalfa disease resistance improvement. The utilization of molecular markers for the implementation of an assisted selection scheme as well as for mapping resistance genes, can significantly increase selection efficiency. Likewise, transgenesis allows the development of genotypes resistant to diseases that are hard to manage via conventional methodologies, by enabling the incorporation of genetic variation not naturally present in alfalfa. Complementation of all these techniques will contribute to greatly improve the sanitary status of alfalfa in the near future.

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Chapter 7

Alfalfa pests

Vanda Helena Paes Bueno
Alessandra de Carvalho Silva
Fernando Daniel Fava

Introduction

Alfalfa occurs in all geographic areas of the world, particularly in temperate zones, where it was largely introduced by man during the migration processes and due to the domestication of animals. In Brazil, its occurrence is limited to some regions, where it was initially used for producing hay for horse breeding, although it is currently also used in cattle feeding.

Alfalfa is a legume that attracts several insects, which use it directly for food or oviposition (egg laying), as pests, or as shelter to prey on or to parasite other insects present in the crop, such as predators and parasitoids. In the United States of America, for example, over 100 species of insects are found in alfalfa, but only about 30 of them occasionally cause economic damage.

The most important pests of the alfalfa crop are mentioned in this chapter. These pests occur in several regions where the plant is cultivated, including Brazil.

Alfalfa pests: recognition, ecology and damages

Insects present in the alfalfa crop in Brazil find favorable conditions to establish, and that leads them into causing economic damage if not managed, becoming pests. Recognizing the main pests of the alfalfa crop can be done using the identification key (Figure 1) and the characteristics that are most easily visualized.

Aphids (Order Hemiptera – Suborder Homoptera)

Among the main alfalfa pests, aphids constitute the most economically important, for their high reproductive potential and for their consequent damages to the crop. They are insects which in hot, tropical or subtropical locations such as Brazil, reproduce through thelytokous parthenogenesis, that is, not involving male participation, giving rise to females only. There are two types of females: apterous, in charge of reproduction inside the colony, and winged, which have the function of looking for new plants or new locations to disseminate the colony (Figure 2). According to Gallo et al. (2002), winged females are only found in colonies with many individuals.

In Brazil, four species of aphids which cause damages to the alfalfa crop are found, and each one prefers a given weather condition. Some of these species have biotypes, that is, individuals that have characteristics which differentiate them from the others of the species, but that cannot be deemed

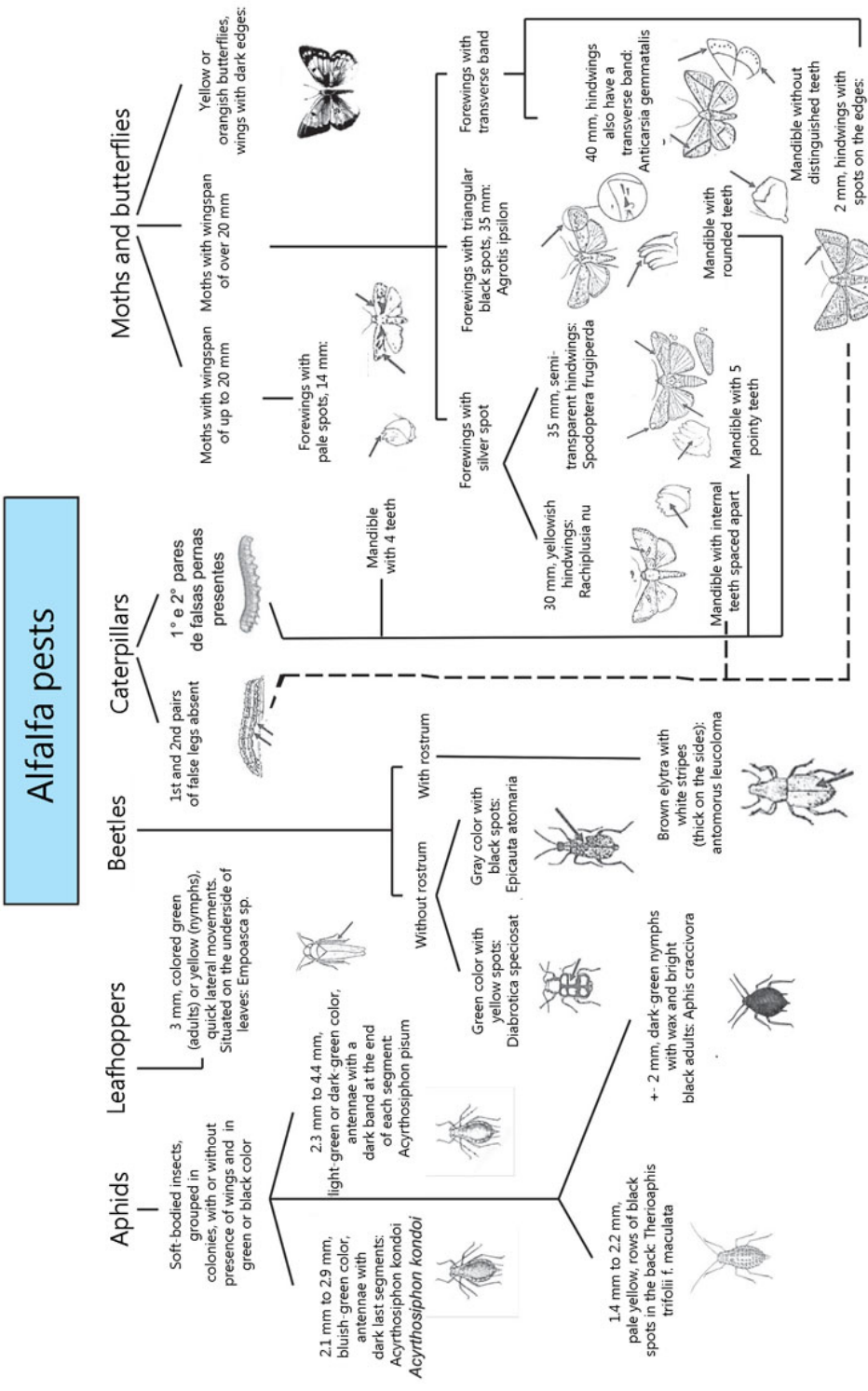


Figure 1. Key to identifying pests in the alfalfa crop.
Source: Adapted from Zucchi et al. (1993).

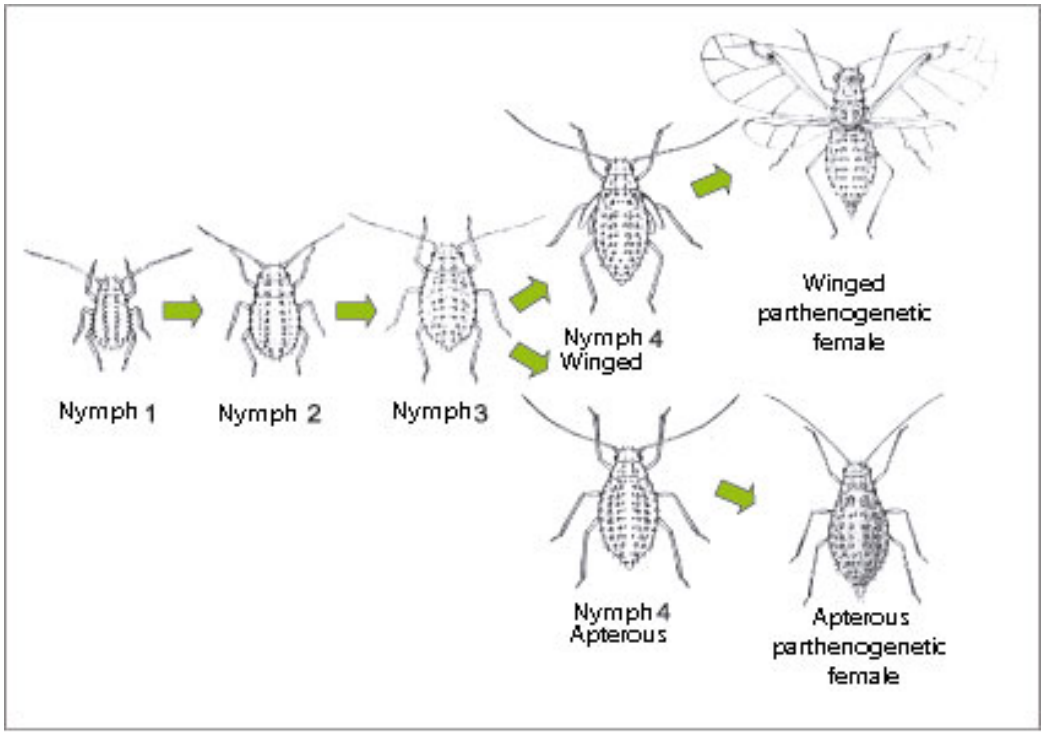


Figure 2. Life cycle of aphids.
Source: Bueno and Carvalho (2008).

as belonging to another species. Biotypes usually have greater resistance to insecticides normally applied for controlling their species, which makes it more difficult to manage them in the crop.

Recognition and particularities of the main species

a) *Therioaphis trifolii* (Monell, 1882) form *maculata* [spotted alfalfa aphid, SAA]

This aphid is known by some authors as a biotype of *Therioaphis trifolii*; however, many choose not to use the expression "form" or apply the name *Therioaphis maculata* (Caver, 1978). With green color, it is easily differentiated from the other alfalfa aphids, for having rows of dark spots in the dorsal region, from which little hairs come out (Figure 3A). It develops well under hot and dry weather conditions; population peaks are related to temperatures around 25 ºC, as long as weekly precipitation is less than 50 mm (Carvalho et al., 1996). According Mendes et al. (2000), when this temperature is associated with high precipitation, there are usually no population peaks.

Photos: Fernando Daniel Fava

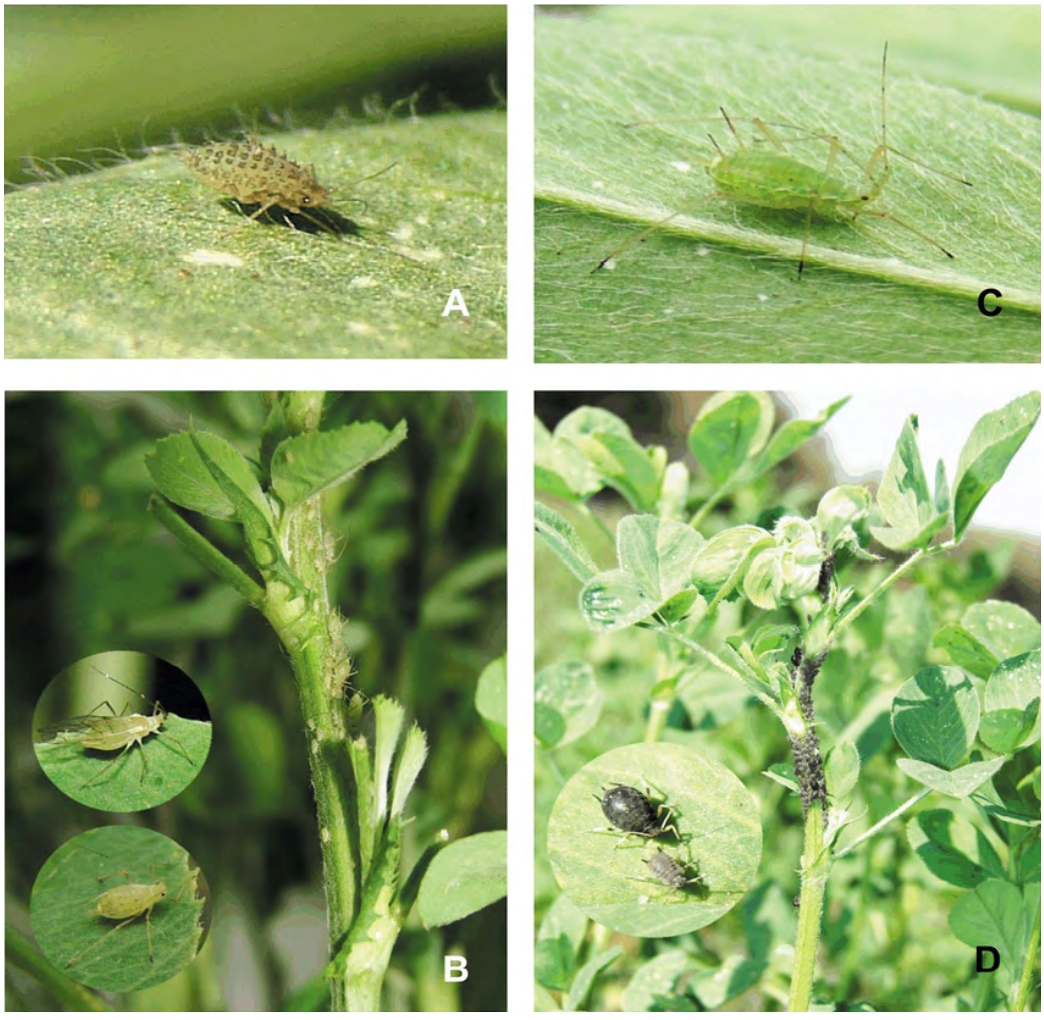


Figure 3. Most common aphid species in alfalfa cultivated in Brazil: *Therioaphis trifolii* f. *maculata* (A), *Acyrthosiphon kondoi* (B), *Acyrthosiphon pisum* (C) and *Aphis craccivora* (D).

Between 1960 and 1980, seven biotypes of *T. trifolii* form *maculata* were reported in the USA (Nielson; Lehman, 1980).

b) *Acyrthosiphon kondoi* Shinji, 1938 [bluegreen aphid]

This species has a bluish-green color and the winged individuals have a brown spot in the thorax. They have clear the first three antennal segments, and the others become gradually darker until the last, which is black and smaller (Figure 3B).

In most regions where *A. kondoi* is found, it develops during spring and autumn, favored by mild temperatures. Even though it is also present in winter, its cycle is much slower then. The largest populations of *A. kondoi* are related to the absence of rainfall and temperatures ranging between 16 ºC and 22 ºC (Carvalho et al., 1996; Mendes et al., 2000). In addition to high temperatures being unfavorable to the reproduction and development of *A. kondoi*, they also favor LT50 – time needed for mortality of 50% of the population (Kodet et al., 1982).

c) *Acyrthosiphon pisum* Harris, 1776 [pea aphid]

This aphid has bright green color and antennas with dark spots at the end of each segment (Figure 3C). Its legs are long and the cornicle, quite tapered.

Acyrthosiphon pisum occurs in locations and times of mild temperature (from 16 ºC to 18 ºC) and tends to be present in winter (July to August), when we also find occurrence of low precipitation, characteristic to Southeastern Brazil (Carvalho et al., 1996; Botrel et al., 2001; Viana et al., 2004). Nevertheless, *A. pisum* is sometimes more tolerant to high temperatures than *A. kondoi*, so that in the municipality of Lavras, Minas Gerais state, Brazil, two population peaks of the pest have been recorded, one in June (18 ºC) and the other in February (23 ºC) (Mendes et al., 2000).

According to Berberet et al. (1983), when temperature approaches 26 ºC or when it crosses that limit, populations of the two species of *Acyrthosiphon* decrease, which causes an increase in the number of *T. trifolii* f. *maculata* in the crop.

Five biotypes of *A. pisum* have already been observed in several plants (Frazer, 1972), some of them with different colors, such as pink and red.

d) *Aphis craccivora* Koch, 1854 [cowpea aphid]

This aphid's nymphs are dark green and adults are bright black with white legs, thus differentiating themselves from other alfalfa aphid species. *Aphis craccivora* forms very dense colonies in plant stems (Figure 3D).

Although this species is related to prolonged drought and high temperature periods, Mendes et al. (2000) observed a population peak in April (18 ºC) in Lavras, MG.

Location and damages to the crop

Nymphs and adults of *T. trifolii* f. *maculata* suck the sap from leaves and stems and are numerous in the lower part of the plant and in the bottom lobe of leaves. The species *A. kondoi* feeds near the terminal part of the plants, lodged on the stem and on the leaves and prefers apical shoots (Aragón; Imwinkelried,

2007). Nymphs of *A. pisum*, on the other hand, frequently live hiding in coiled leaves and thus go unnoticed, while *Aphis craccivora* forms very dense colonies in plant stems (Carvalho et al., 1996).

Aphids damage the plants because they suck the sap and inject toxic saliva, causing severe and generalized bleaching in leaves. Thus, young plants can be killed, atrophy or defoliate, and others can have their growth delayed. Attacks by aphids also cause retention in plant growth and deformation and wrinkling of leaves and shoots (Figure 4). In addition, these insects secrete large quantity of honeydew, a sweetish substance resulting from the excess of sap sucked by the hemiptera, where a black fungi named *Capnodium* spp. [sooty mold] grows, causing damage to photosynthesis and, consequently, to hay quality (Bueno; Carvalho Silva, 2008).

Severe attacks by *A. pisum* cause the leaves to turn yellow and coil up, as well as the stems to shorten, notably reducing forage yield. Leaves become smaller in attacked plants and alfalfa becomes more sensitive to the attack at the beginning of resprouting (Kalvelage, 1990).

Photo: Fernando Daniel Fava

Figure 4. Damages caused by attack of the aphid *Aphis craccivora* to the alfalfa crop.

Another serious consequence of aphid attacks to alfalfa is the fact that many species act as vectors for important viroses which limit plant production. *T. trifolii* f. *maculata* is capable of transmitting alfalfa mosaic viroses (Blackman; Eastop, 1984) and *Red clover vein mosaic virus* (Souza-Silva et al., 1998). The aphid *A. craccivora* is considered to be the vector for two of the most important alfalfa viruses: mosaic and enation (Leclant et al., 1973). Since this aphid can produce many winged individuals and has great flight capacity, its role in spreading these viruses must be considered during pest management (Souza-Silva et al., 1998).

Caterpillars (Order Lepidoptera)

Occurrence of caterpillars in alfalfa can also be quite important, because these insects consume leaves, decreasing production of plant mass, the most interesting product of the plant for farmers and breeders. There are several species of caterpillars present in the crop, even though the vast majority occurs mainly in other crops and only on occasion in alfalfa.

Colias lesbia pyrrhothea Hübner, 1823 [lesbia clouded yellow] is the only one to which alfalfa is the main host; its caterpillars feed on leaves, flowers and thin stems of alfalfa (Gallo et al., 2002); they consume the areas between the leaf veins, giving them a "skeletal" aspect because the veins remain intact. The most severe damages are related to areas where cutting was recent – plants less than 15 cm tall (Summers et al., 1981). In Argentina, this species can complete seven to eight generations per year, but the greatest damages are caused by two or three of these generations (Aragón, 1993). Adults have visible sexual dimorphism and the colors range from white to orangish, passing through several shades of yellow (Figure 5).

Damages caused by *Anticarsia gemmatalis* Hübner, 1818 [velvetbean caterpillar] (Figure 6) start when the caterpillar is still newly hatched, and it scrapes the leaves causing the formation of clear stains; as they grow, caterpillars become more voracious and destroy the leaves completely, and can also damage terminal stalks (Gallo et al., 2002). In Brazil, the *A. gemmatalis* caterpillar has been observed in alfalfa crops in the municipality of Piracicaba, São Paulo state, but without causing meaningful damage (Oliveira et al., 1986).

Spodoptera frugiperda (Smith, 1797) [fall armyworm] (Figure 7) consumes new leaves; in severe attacks to alfalfa, up to 300 caterpillars per square meter can be found (Aragón; Imwinkelried, 1995). However, just as *Mocis latipes* (Gueneé, 1852) [striped grass looper] (Figure 8A and 8B), these caterpillars are more common in grassy crops, from which they can migrate in large quantities

Photos: Fernando Daniel Fava

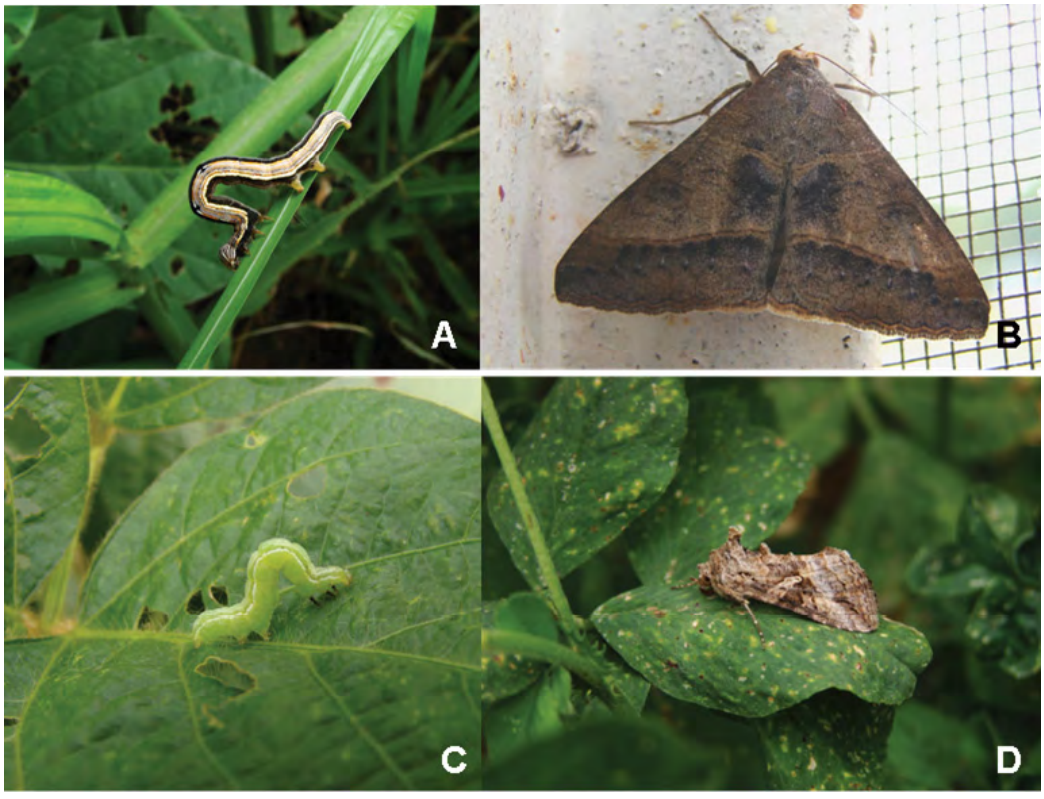
Figure 5. Young (A) and male and female adult (B) phases of *Colias lesbia pyrrhothea*.

Photos: Fernando Daniel Fava

Figure 6. Young (A) and adult (B) phases of *Anticarsia gemmatalis*.

Photos: Fernando Daniel Fava

Figure 7. Young (A) and adult (B) phases of *Spodoptera frugiperda*.



Photos: Fernando Daniel Fava

Figure 8. Young (A) and adult (B) phases of *Mocis latipes*; young (C) and adult (D) phases of *Rachiplusia nu*.

to other crops, such as alfalfa (Gallo et al., 2002). This can characterize a problem in areas destined to cattle breeding and in other crop areas, next to pastures. *M. latipes* is recognized for moving as if it was measuring palms, as well as *Rachiplusia nu* (Gueneé, 1852) (Figures 8C and 8D), another species that can also attack alfalfa.

Agrotis ipsilon (Hufnagel, 1767) [black cutworm] (Figure 9C) causes cutting of sprouts and young shoots at soil level. They are nocturnal caterpillars and, during the day, they remain curled-up and sheltered in the soil (Arágon; Imwinkelried, 1995). In Brazil the attack from these caterpillars with cutting of alfalfa plantlets close to the soil has been observed in the municipality of Piracicaba, São Paulo state (Oliveira et al., 1986). According to Arágon (1985) in Argentina, severe attacks by *A. ipsilon* are occasional. However, they indicate that this species together with other noctuidae with similar feeding behavior can cause infestations of 80 to 100 caterpillars per square meter during dry

Photos: Fernando Daniel Fava

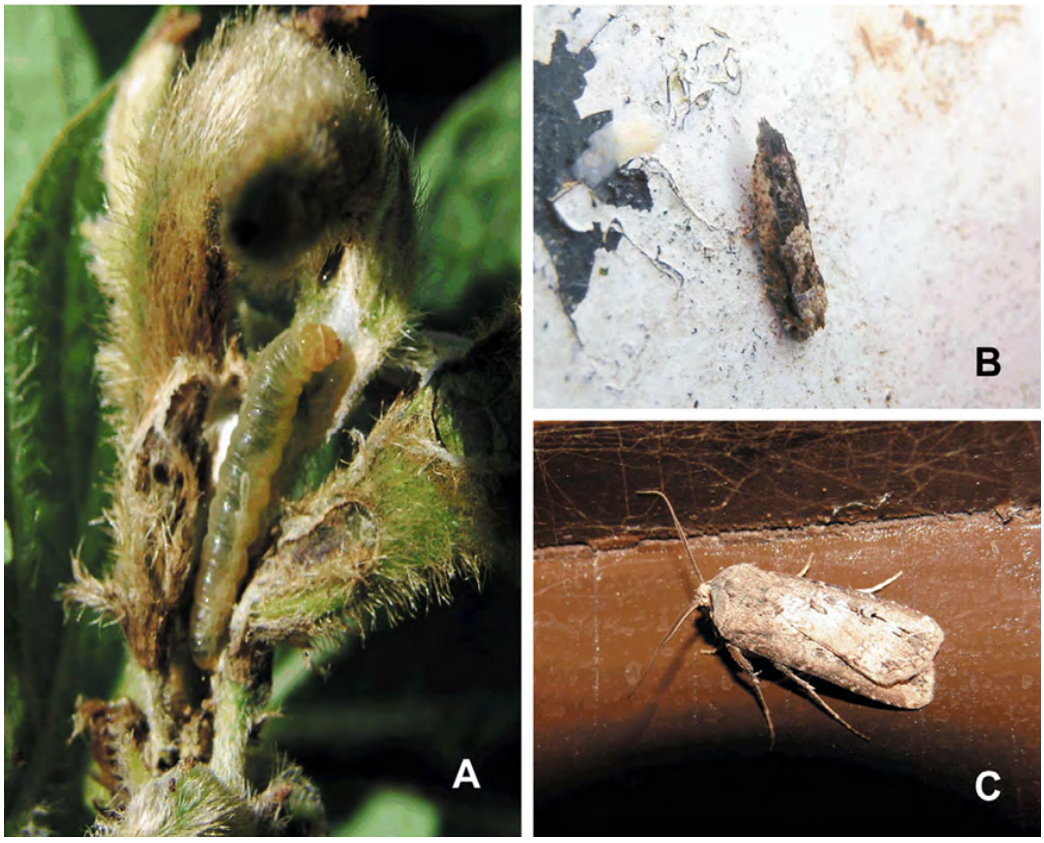


Figure 9. Young (A) and adult (B) phases of *Epinotia aporema*; adult of *Agrotis ipsilon* (C).

springs, in alfalfa with three or more years of development. This situation can also seriously affect the crop and its recovery capacity can be exhausted.

Unlike other caterpillars occurring in alfalfa, *Epinotia aporema* (Walls, 1914) [bud borer] (Figures 9A and 9B) has a gelatinous aspect at first. Little caterpillars unite leaves or flowers at the end of the plant with a silk thread, and feed on them. The greatest loss results from the attack to stems, where they open up passages, causing the branches and leaves at the end of the plant to dry out (Gallo et al., 2002). In Brazil, occurrence of this caterpillar and damage to alfalfa has been reported in the region of the municipality of Bandeirantes, Paraná state (Evangelista; Bueno, 1999).

Beetles (Order Coleoptera)

Beetles can damage roots, as do larvae of *Naupactus leucoloma* Boheman, 1830 [whitefringed beetle] or *Pantomorus leucoloma* (Boheman, 1840)

[whitefringed weevil Curculionidae] (Figure 10) and of *Diabrotica speciosa* (Germar, 1824) [cucurbit beetle Chrysomelidae] (Figure 11) or the aerial (above-ground) part of the plant, as is the case of the adults mentioned before and of *Epicauta atomaria* (Germar, 1821) [blister beetle Meloidae] (Figure 11B).

Damages to alfalfa roots, in addition to decreasing crop productivity and longevity, constitute entry paths for fungi, such as *Fusarium* spp. and *Phoma* spp., which contribute to increasing the damages caused. Damages caused by adults that feed on the leaves are greater in alfalfa plantations in establishment phase (Bueno; Carvalho Silva, 2008).

Young phases of *D. speciosa* are known as corn rootworms and attack the root growing regions, causing death of newly emerged plants; its importance is increased in direct seeding areas, with dark soils, rich in organic matter and moist-irrigated (Gallo et al., 2002). In adult phase, these beetles feed on tenderer leaves, making small holes in the leaf blades, decreasing the photosynthetic area and consequently, production.

Photos: Fernando Daniel Fava (A) and Ariel S. Odorizzi (B and C)

Figure 10. *Naupactus leucoloma* beetle (A) and its damages to roots (B and C).

Photos: Fernando Daniel Fava

Figure 11. *Diabrotica speciosa* (A) *Epicauta* sp. (B) beetles.

Epicauta atomaria destroys the plant's leaves, which end up reduced to their veins (Gallo et al., 2002). Its presence in alfalfa plantations is credited to the proximity to soy, bean or pigeon pea crops. In larval stage, it can cause plantlet death, but in established alfalfa plantations damages are smaller.

Green leaf hopper (Hemiptera: Cicadellidae) – *Empoasca* sp.

These are small sucking insects, 3 mm long, with quick movements (Figure 12). Adults are green and nymphs, smaller, are greenish yellow. Egg laying is endophytic and normally carried out along leaf veins. Yong forms have the habit of moving sideways and can be easily found on the undersides of leaves (Gallo et al., 2002).

Adults as well as nymphs of *Empoasca* sp. feed on the plant, causing economic losses. Sap sucking causes deformation of leaves and damages plant development, due to the toxigenic action associated to the insect feeding; signs are very similar to the ones from virosis, that is, plants become yellowish with reduced growth and leaves acquire coiled or arched down edges (Gallo et al., 2002).

According to Bambara and Watson (2007), *Empoasca fabae* (Harris) is a pest of occasional importance in alfalfa in the USA and it begins to appear in the crop when weather is hot and dry. In Brazil, Viana et al. (2004) found *Empoasca* sp., associated to aphid *A. pisum*, in the entire area (280 m^2) of alfalfa experiment in the municipality of Sete Lagoas, Minas Gerais state.

Photos: Fernando Daniel Fava

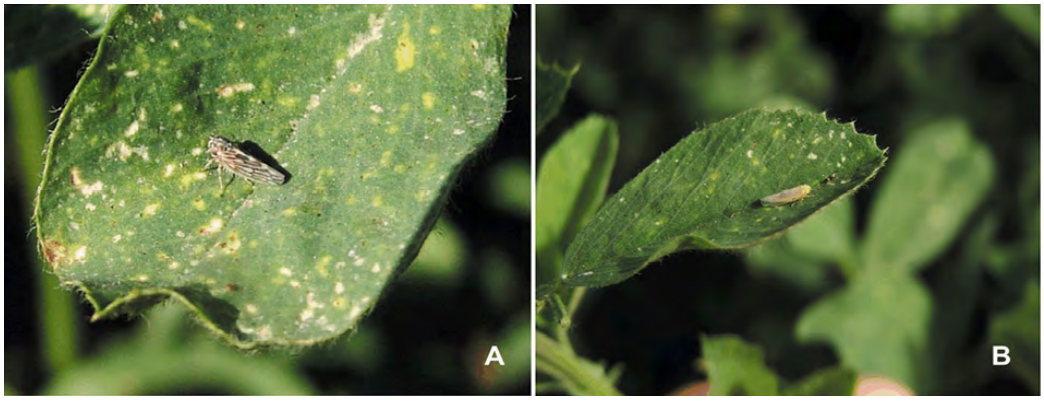


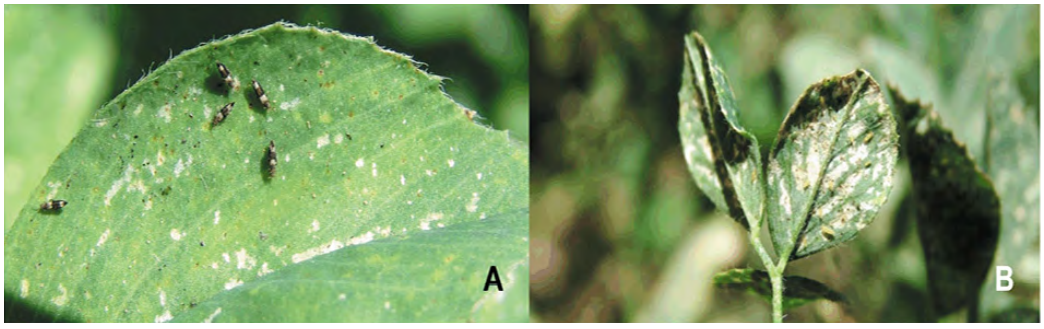
Figure 12. Adult *Empoasca* sp. sucking on alfalfa leaves leaving signs of toxic saliva in the leaves.

Thrips (Order Thysanoptera)

The thrips are very small insects (0.5 mm to 13 mm), with thin body and two pairs of long and narrow fringed wings. In the USA, due to the high population of these insects in alfalfa plantations and to the easily identifiable lesions, some species can be considered pests of the crop. However, in Brazil its occurrence has only been reported by Afonso (2008), who has considered it to be a pest insect to alfalfa grown in Rio Grande do Sul state. In Argentina, the most frequently found species is *Caliothrips phaseoli* (Hood, 1912) [American bean thrips] (Figure 13). The most important damages happen during emergence and establishment of the crop, causing plantlet death. Because of that, when infestations by this insect are severe, partial or total decrease in plant population can be observed (Arágon; Imwinkelried, 2007). According to Summers et al. (2006), species with greater occurrence in the USA are *Caliothrips fasciatus* (Pergande, 1895) [bean thrips], *Thrips tabaci* (Linderman, 1888) [onion thrips] and *Frankliniella occidentalis* (Pergande, 1895) [western flower thrips]; the first two of these species are more aggressive and important for the alfalfa crop in that country.

These species cause direct losses by feeding on the plants, or indirect by acting as vectors to plant viruses. During feeding, the thrips scrape leaf tissue and suck the overflowing sap (Figures 13A and 13B), causing tissues around the lesion to get deformed and grow unevenly, acquiring a wrinkled appearance.

Some times, the thrips population is not harmful to cultivated plants and can contribute to the fixation of natural enemies and other alfalfa pests, to which they serve as complementary feed.



Photos: Fernando Daniel Fava

Figure 13. *Caliothrips phaseoli*: adults (A); nymphs (B); symptoms shown by alfalfa leaves attacked by the pest.

Mites

Even though they are not a primarily important pest to alfalfa, in Brazil the mites have been more and more frequent in crops in Rio Grande do Sul state. Species that occur in Brazil have not been identified yet, but in the USA *Tetranychus urticae* (Kock, 1836) [two-spotted spider mite] is the most common species, especially in water stress conditions (Summers et al., 2006). Spontaneous growth plants can be the main hosts, from which acari migrate to alfalfa.

Colonies of these mites are located on the underside of the leaf, where they also lay their eggs in a web made of silk threads, similar to the ones of a spider (Figure 14A). Nymphs and adults are very small, and the latter are yellowish with dark green spots in the dorsal region, one on each side (Gallo et al., 2002).

As a consequence of acarus feeding, small whitish areas appear in the upper part of the leaves, and they become yellowish over time (Figure 14B); severe damages include leaf drop. Heavily infested plants can atrophy and have yellowish appearance. Reduction in production is greatest when alfalfa is growing or when infestations happen at the beginning of the cutting cycle.

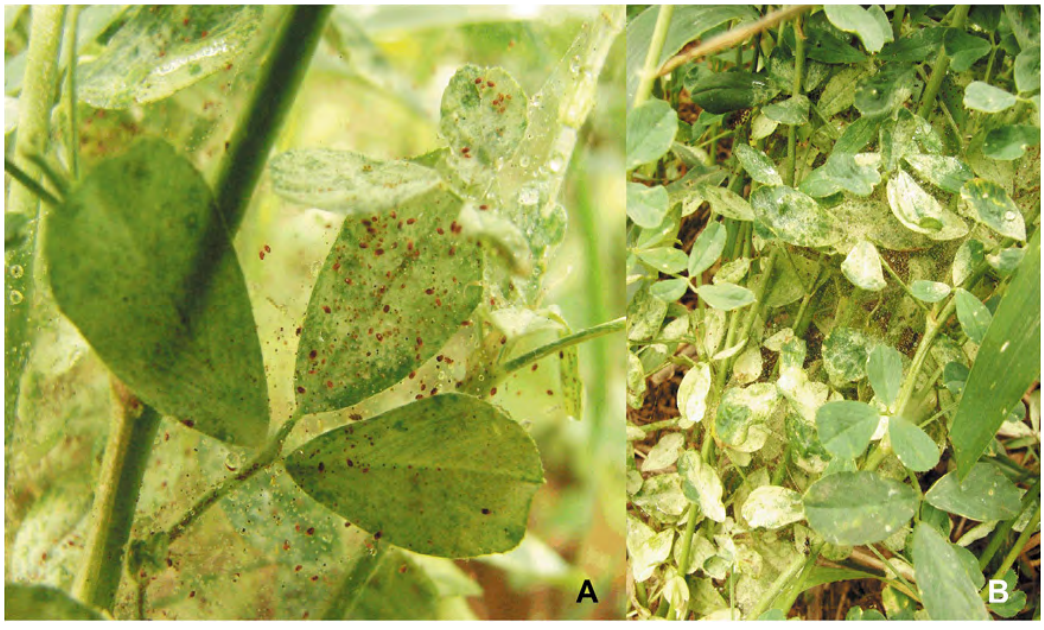


Figure 14. Presence of webs of the *Tetranychus urticae* (A) and symptoms shown by alfalfa plants attacked by the pest (B).

Final considerations

Problems caused by pests to the alfalfa crop take on great importance when factors which facilitate their large scale occurrence, such as weather conditions and susceptible cultivars, are favorable to them. However, recognizing and identifying them can be rather valuable since it enables choosing the best control or management strategy in the crop.

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CHAPTER 8

Genetic improvement for pest resistance in alfalfa

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Alessandra de Carvalho Silva
Jorge Omar Gieco

Introduction

Using alfalfa in animal feeding makes it dangerous to use chemical insecticides for pest management in the cultivation of this forage, due to the remaining of residues in the plant. These residues can also deposit on springs, which can in turn cause contamination of animals through indirect via. Thus other methodologies for pest management in alfalfa should be emphasized to farmers, such as biological management and using resistant cultivars (Bueno; Carvalho Silva, 2008). These methods can allow keeping the pest in levels inferior to the ones that cause economic damages, without harming the environment and causing additional costs. In addition, they have the advantage of being compatible to each other and to other methods, facilitating the combined use within the concept of integrated pest management.

Plant resistance to insects is the relative sum of hereditary qualities presented by the plants, which influence the intensity of damage caused by insects. According to Lara (1991), the resistance of a plant to an insect can many times occur due to changes in behavior or biology of the insect, or simply due to the morphology or reaction presented by the plant without any effect over the insect.

The current availability of molecular techniques, together with information deriving from genome sequencing of the diploid alfalfa species *Medicago truncatula*, offers new and very promising perspectives in the field of developing insect-resistant synthetic alfalfa cultivars. In this section, among other themes, the development of alfalfa transgenics which express endotoxins from *Bacillus thuringiensis* will be addressed, as well as the use of different genomic tools such as resistance genes mapping through molecular markers, association mapping, genome sequencing and development of transgenics which use the information generated based on the use of these techniques.

Thus, the different forms of resistance, when dully managed, can be useful tools to the process of searching for pest management strategies in several economically important crops, as is the case of alfalfa.

Types of plant resistance aiming at pest management

A plant has several ways to resist the attack of a pest, and these mechanisms can favor its development without, however, bringing unfavorable consequences. There are three types of resistance and the plant can have one, two or three types, because the genetic factors that condition them can be independent (Lara, 1991). When the plant has more than one type, it is called multiple resistance.

Nonpreference

Also named antixenosis, nonpreference is the type of resistance that occurs when the cultivar is less used by insects for food, oviposition or shelter than other cultivars in equal conditions, in other words the cultivar leads to a negative response by the insect during the process of selecting the host plant.

Beck (1965) considered that, regarding feeding preferences of both chewing and sucking insects, there are three distinct stages, namely: host orientation, initiation of feeding and maintenance of feeding. There is a chain of stimuli which triggers a chain of responses by the insect, and each positive stimulus corresponds to a negative one; this leads the insect to use the plant or not. Thus, if the repellent negative stimulus excels the attractive one, the insect will not accept the plant; otherwise, the insect heads to the plant and uses it for food or oviposition should the stimuli continue to be positive. According to Gallo et al. (2002), these stimuli can be chemical of physical in nature, and are directed by genetic factors.

According to Lara (1991), insects as a general rule have egg-laying (ovipostion) behavior in substrates which guarantee the development of their larvae or progeny, ensuring survival of the species. However, this behavior depends on stimuli provided by the plant. The fact that an insect deposits eggs more in one plant than in another does not mean that it will consume more food from that substrate; there are even cases in which eggs are not laid in hosts and young insects must then search for the most adequate plants.

In case of sucking insects, such as aphids, most cases of resistance by nonpreference are due to the difficulty of the insect to find the location where it normally feeds. According to Gallo et al. (2002), in susceptible plants, aphids extract sap from the Liberian vessels and, in resistant ones, they extract sap from the epidermis, the subepidermis, the mesophyll and other cells of the phloem, but rarely from the Liberian vessels.

Antibiosis

This form of resistance occurs when the insect feeds normally on the cultivar, but this has an adverse effect over its biology, such as death during immature stage, prolonging of the development period, body size and weight reduction, reduction of fecundity, fertility and of the oviposition period. Nevertheless, effects with high degree of feeding nonpreference can be expressed the same way as the ones presented by antibiosis, because by not feeding much, individuals may consequently show alterations in their biology.

According to Gallo et al. (2002), this type of resistance can be caused by the presence in the plant of chemical substances causing acute or chronic intoxication of the insect, of antimetabolites that make certain essential nutrients unavailable or that act as enzyme inhibitors, of enzymes that inhibit or reduce normal processes of food digestion, and of compounds that interfere on reproduction, or it can be caused by qualitative or quantitative deficiency of plant nutrients for the insect.

To prove antibiosis occurrence, feeding reduction is measured. This measurement can be carried out directly, through the area consumed by chewing insects, or indirectly, through excrements, such as honeydew produced by sucking insects.

Tolerance

This is the type of resistance in which the plant suffers less damage, compared to another one equally infested by insects, without affecting their biology. According to Gallo et al. (2002), this occurs because some cultivars have better capacity to tolerate the pest attack than others due to: a) compensation of the destroyed area through tissue growth or regeneration, or even the quick formation of new leaves, new roots and new tillers; b) lesser extraction of growth hormone from the plants by sucking insects; c) higher strength or larger leaf area; and d) greater hardness of stalks, which reduces the possibility of lodging or breaking when blocking insects attack.

Resistance through tolerance is an advantage, since it decreases selective pressure over herbivores and therefore reduces the chances of breaking resistance through the emergence of insect biotypes or physiological races (Silva et al., 2006). Lara (1991) recalled that maintaining a small population of pest insects in the area contributes for the maintenance of natural enemies. These act as controllers, which increases the chances for natural biological management.

The presence in alfalfa of compounds which trigger responses in herbivore insects was investigated by Maxwell and Painter (1962). These authors observed that auxin levels, as well as levels of certain nutrients, dropped after infestation in tolerant plants. Harvey et al. (1971) found occurrence of resistance by tolerance in the alfalfa cultivar KS6, which remained practically unaltered in presence of aphid *Acyrthosiphon pisum* Harris, while the Cody cultivar was susceptible to the pest, with totally damaged leaf area.

As a plant can have more than one type of resistance, and these can be conditioned by distinct genetic factors, the hypothesis of using them together in breeding programs must be considered, aiming at increasing resistance level and the difficulty of emergence of races or biotypes of insects.

The greatest success of plant resistance to alfalfa pests has been found with aphids. Although the resistance mechanisms of these insects have not been totally elucidated, many studies have shown that resistant plants affect the biology or the behavior of the insect when the plant is used for oviposition (Nielson; Lehman, 1980), and many factors involved, such as antibiosis, nonpreference and tolerance, have already been reported. Knowing the resistance mechanism involved is important because antibiosis can exert selective pressure over aphid populations, resulting in the evolution of biotypes capable of breaking resistance of the original cultivar.

In case of *Therioaphis trifolii* (Monell) form *maculata*, studies showed existence of antibiosis, nonpreference and tolerance, which manifest isolatedly or together in the plant, although reports suggest antibiosis to be the main resistance mechanism. Nielson and Don (1974) assessed the behavior of four biotypes of *T. trifolii* f. *maculata* in resistant clones and in susceptible clones of alfalfa, and revealed that all biotypes had the same behavior in both plants, but that some of these insects were not apt to ingest sap when their stylet met phloem of resistant plants. Differences on the ability of biotypes to ingest sap have been credited to the presence or absence of detoxication mechanisms.

Plant defenses and causes of resistance to insect attacks

Plants do not remain passive when attacked by herbivores, in other words, they have several defense mechanisms, which are appropriate for each location and each causal agent (Pallini et al., 2005). Hence, considering that plants and insects have coexisted for about 350 million years, they have developed very diversified defense and attack mechanisms.

Defense mechanisms of the plant to herbivore attack can be summarized into constitutive defense and induced defense. When the systems are characterized at molecular level, according to Gatehouse (2002), distinction between these two defense mechanisms may not be clear, because plant defensive compounds are frequently the same in a given plant species and involve the expression of the same genes.

Constitutive defense

The plant has constitutive defense when it expresses defense continuously and not depending on the presence or action of herbivores for the defense to be activated. In this type of defense, compounds are synthesized during normal growth and development and, when plants are attacked, they already have

the means for deterrence or death of the herbivore (Paron, 2004). Defense is formed by compounds or substances which, once produced by the plants, can affect the biology, development and reproduction of herbivore insects.

Induced defense

When defense mechanisms against herbivores only manifest after plants are attacked, these mechanisms constitute induced defense. According to Walling (2001), induced defense can be defined as a plastic response, in which carbon and nitrogen are deviated from growth and reproduction processes to provide, for a given period, defenses against pests and diseases. In the first studies involving induced resistance to insects, it was understood as a kind of pseudoresistance, that is, a special situation in which there is temporary reduction of damages by the pest due to conditions of the plant or of the environment (Paron, 2004).

Induced resistance has characteristics with one or more simultaneous effects: low specificity (can reach pests and natural enemies), spatial variation (local, systemic response, action of neighboring plants) and temporal variation (variable time to express and maintain).

Direct induced defense

This occurs when defenses of the attacked plant act on herbivores. Hence, attacked plants can, for instance, have lower nutritional quality to insects in relation to the plants that were not attacked, and thus provide inadequate conditions for populational growth and formation of new populations at the site (Agrawal et al., 1999). Some plants can produce secondary compounds, and alfalfa is one of those since it produces tannins. Compounds like this are toxic and can intensify as the damage caused by the herbivores increases. Thus, plants that have not been attacked would be preferred as oviposition sites in the following generations (Pallini et al., 2005).

Among events related to induced resistance, the increase of activity of enzymes related to plant defense, such as peroxidase and poliphenoloxidase is highlighted (Gomes et al., 2004). Peroxidase is related to the synthesis of lignin and suberine, which increase tissue rigidity, and to the production of quinones and active oxygen, which have antibiotic properties (Stout et al., 1994). Poliphenoloxidase, in addition to being involved in the process of lignification, is also responsible for the oxidative catalysis of phenols and quinones, which are complexed with proteins, decreasing the nutritional quality of the food and making protein digestion more difficult (Felton et al., 1994). Jiang and Miles

(1993) observed that the feeding of *T. trifolii* f. *maculata* induces the activity of poliphenoloxidase in tissues of the alfalfa plant.

Indirect induced defense

It happens when the defenses stimulate attraction and permanence of natural enemies in the plants, by releasing volatile compounds; these natural enemies are predators and parasitoids capable of controlling the herbivore population (Vet; Dicke, 1992). Plants infested by herbivores tend to increase the endogenous concentration of jasmonic acid, responsible for the induction of volatile compounds and for the direct defense against herbivores and pathogens (Thaler et al., 2002). These volatile compounds, mostly monoterpenes and sesquiterpenes, are used as a lead for natural enemies to locate their prey and/or hosts (Pallini et al., 2005).

Herbivores can also take advantage of the communication between plants and natural enemies, avoiding the plants which are already under attack by their competitors (Pallini et al., 1997). The indirect induced resistance system of caterpillars has been well studied (Paré; Tumlinson, 1999) and it has been found that they have specific elicitors in their oral secretion, capable of inducing emission of volatile compounds used by natural enemies; however, artificially injured plants do not produce these volatile products. According to Pallini et al. (2005), in case of predatory acari, they are capable of differentiating volatile compounds released by plants attacked by different preys, as well as by herbivores not preyed, and choosing the phytophagous species that will provide them with best reproductive potential.

Resistance causes

Factors leading one genetic material to be more or less infested or damaged than others are in many cases difficult to determine. In general, causes conditioning plant resistance to insects can be divided into three groups, physical, chemical and environmental, reported bellow.

Physical causes. These are represented basically by the color of the plant substrate which, in some cases, affects not only host selection for feeding and oviposition, but also the biology of the insect.

Even though effective cases of resistance caused by color are rare (Gallo et al., 2002), Cartier and Auclair (1964) observed the influence of colors over weight and survival of *A. pisum*, when artificial diets restrained by different color membranes were offered to these insects. According to the results, yellow and orange were the most favorable colors for this species, while green caused

almost 50% of mortality; red and white interfered on individual survival and weight, and blue caused 100% mortality.

In a study with *Empoasca kraemeri* (Ross and Moore) in bean plants, Galwey (1983) found that resistance of the studied materials was associated with several plant characteristics, including color of flowers and seeds, which led him to suggest that resistance was associated to the purple color of flowers and to black or beige seeds. However, there were cultivars with these characteristics that behaved as susceptible and cultivars with different colors that were revealed to be resistant. It is concluded that, although color can be related to resistance, it cannot isolatedly be the cause of resistance, but one characteristic deriving from another factor (chemical, for instance) that is the proper cause.

Morphological causes. These involve every and any plant characteristic, structural or morphological, that negatively acts over insects, that is, which affects the pest to preserve the plant from more severe damages (Lara, 1991). They are the plant characteristics that can affect locomotion, mating, host selection for feeding and for oviposition, ingestion and digestion of food ingested by the insects. These characteristics, according Gallo et al. (2002), can be basically grouped into structural factors and epidermal factors.

- *Structural factors* – are related to the dimension and disposal of plant structures; for example, the length of the maize straw, greatest leaf compactness, well closed rice husks and the position of ears in the plants. According to Pallini et al. (2005), presence of domatia in the plant can also help its defense providing shelter to natural enemies, such as predatory acari, against unfavorable weather conditions and for reproduction and, thus, increase predation action by these natural enemies as well as their reproductive potential.
- *Factors related to the epidermis* – include hardness, pilosity, texture, waxiness and presence of domatia and of nectaries.

Thicker cuticles or harder ones are linked to silica and lignin deposition. They can reduce feeding of insects, especially of the sucking type, complicate or prevent penetration by mining insects and endophytic oviposition, acting mainly on small insects and in their first instars.

More even or wrinkled texture can affect oviposition. Pilosity, in turn, can directly affect the insect, interfering in oviposition, feeding and locomotion, by means of exudates secreted by glandular trichomes. These exudates on the other hand can reduce oviposition and locomotion, in addition to causing occlusion of the mouth apparatus of the insect. Alfalfa is among the plants having resistance caused by presence of trichomes.

While pilosity is a factor of resistance to some insects, such as *Empoasca fascialis* (Jacobi) in cotton plants and *Empoasca fabae* (Harris) in soybean plants, it can also be a susceptibility factor for others such as for oviposition of *Heliothis* species in maize and cotton plants (Lara, 1991; Gallo et al., 2002).

Cultivars of alfalfa or other species from the genus *Medicago* with presence of simple hairs are less preferred by *E. fabae* for oviposition, although nymphs hatch out and develop without obstacles. However, presence of glandular hairs can restrict oviposition and cause mortality by arresting of nymphs in the first instars, and so there is an inverse relationship between presence of glandular hairs and oviposition, according to Brewer et al. (1986).

Chemical causes. These are represented by the chemical substances acting over the behavior or the metabolism of the insect through the nutritional improprieties of the plant. Chemical defenses of the plant are related to the secondary substances and the allelochemicals (Karban; Baldwin, 1997), which can be found in one or more parts of the plant, and their concentrations vary with plant age (Pallini et al., 2005).

Alteration in insect behavior happens mainly during host selection for feeding and for oviposition, attracting or repelling the insects, and resulting in resistance through nonpreference. Still, resistance through antibiosis can happen by means of the effect of ingestion of toxic metabolites and enzyme or reproductive inhibitors by the insect, and of the qualitative or quantitative deficiency of nutrients in the plant on which the insect has fed, as explained before.

To get to know volatile organic compounds of alfalfa cultivars resistant to *E. fabae*, Ranger et al. (2005) collected substances from the stalks and leaves of cultivar G98A, resistant, and from cultivar Ranger, susceptible. Results showed that significantly more *E. fabae* were oriented in the direction of the volatile compounds of cultivar Ranger than of cultivar G98A, even though they were the same volatile products in both cultivars, only in different quantities. Thus it was concluded that, instead of producing repellent volatile compounds, the resistant cultivar decreased insect attraction in its direction by producing less quantity of attractive compounds.

The plant can also be resistant or less suitable to the insect due to abiotic factors (nutrients, light, moist, CO_2). For example, fertilization can influence the quality of plant tissues as food for the pests. According to Borkert et al. (1987), nitrogen fertilization affects *Spodoptera frugiperda* (Smith) and potassium fertilization reduces infestation by *Diatraea saccharalis* in Poaceae.

In susceptible alfalfa cultivars (P3 and Crioula), the individual concentration of minerals (Mg, P and S) in the plant tissues has been negatively

related to the population of *Acyrthosiphon* spp., to *T. trifolii* f. *maculata* and *Aphis craccivora* Koch. This suggests that minerals have affected the biology of the aphids; however, there has been no correlation between these minerals and the population of aphids in the resistant cultivar, CUF 101 (Silva et al., 2005). A positive correlation between the ratio C:N has also been found, as well as a negative correlation between the N content and the aphid populations, which suggests a possible relationship between alkaloids and alfalfa aphids. In a study carried out by Van Emden (1966), aphids responded negatively to fertilization with N in 36% of the cases.

Silicon, in turn, has been the instrument of many recent researches, aiming at plant protection. Although silicon is not considered an essential element for plants, its addition via silicate solution confers resistance induction in plants, especially in Poaceae, to sucking insects, such as aphids from wheat (Basagli et al., 2003), sorghum (Carvalho et al., 1999) and maize (Goussain et al., 2002). Once absorbed, silicon accumulates and polymerizes into epidermal cells, forming a silicon-cuticle double layer, which confers resistance to penetration by aphid stylets. Nevertheless, silicon has also shown positive effects in controlling chewing insects, such as *S. frugiperda* (Goussain et al., 2002).

For aphid *A. pisum*, common in alfalfa, many factors have been attributed as cause of plant resistance: the acidic condition of the plant, high rates of sugar, nitrogen and amino acids, in addition to high temperature (Bournoville et al., 2000). In case of Curculionidae beetles of alfalfa, the association of boron with the decrease in feeding and oviposition has been reported.

Environmental causes. As for weather factors, it has been observed that temperatures under 15 ºC can reduce alfalfa resistance to three species of aphids: *T. trifolii* f. *maculata*, *Acyrthosiphon kondoi* Shinji, 1938 and *A. pisum*. This is particularly important for those species which occur more frequently in regions of lower temperatures, where the extension of the cold period can favor resistance break (Summers, 1998).

Biotechnology applied to improvement for pest resistance in alfalfa

Transgenic cultivars resistant to insects

Microorganism *B. thuringiensis* (*Bt*) is a gram-positive, spore forming soil bacterium (Figure 1). When resources are limited, *Bt* vegetative cells sporulate and during this process the synthesis of a protein crystal commonly

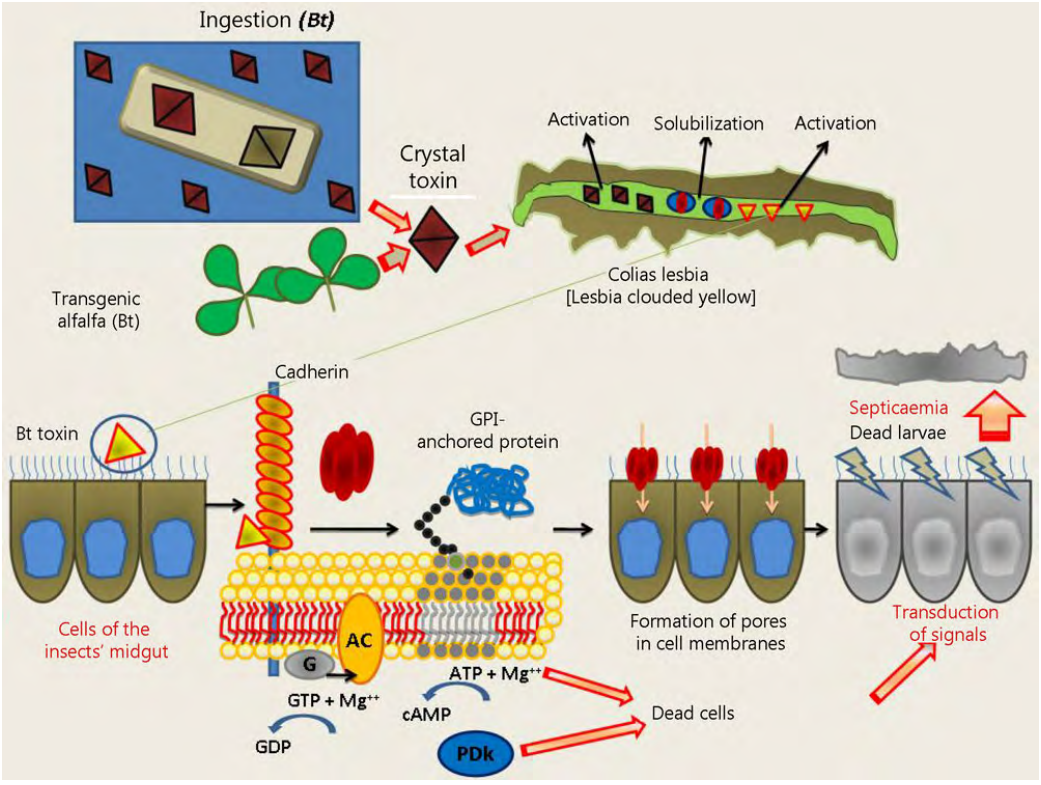


Figure 1. Mode of action of *Cry* toxins of *Bacillus thuringiensis* in Lepdopterans.

called endotoxin happens. The proteins of this crystal are named *Cry* for their crystalline structure, and have been known for decades because they show insecticide activity against certain groups of insects. Even though insecticide formulations with *Bt* toxin basis have been used for many years, it was the development and marketing of transgenic cultivars resistant to insects, called *Bt* crops – expressing *Cry* toxins – that revolutionized the history of agriculture. The benefits of this technology include high specificity and high efficiency, reduction of agrotoxic (insecticides) application with consequent economic and environmental positive impact, and productivity increase of genetically transformed crops.

Although *Cry* toxins have wide commercial use, the particularities of their modes of action are still the object of controversy. This process (Figure 1) includes ingestion of *Cry* protein by a susceptible insect, solubilization in the high pH of its midgut and processing of the protoxin in its latter form of active toxin in the gastric juice of the insect. The toxin nucleus travels through the peritrophic matrix and binds to specific receptors – named cadherins – in the

external membrane of the intestinal brush cells of the insect. Binding of the toxin to the cadherins results in activation of a path for oncotic cell death and/or oligomer formation. These oligomers bind to GPI-anchored proteins which tend to concentrate in the plasmatic membrane region named lipid rafts. Toxin accumulation results in formation of oligomers in these membranes, occurrence of cell osmotic shock and, finally, death of the insect.

Vesicles in the external border of cell membranes of insect intestines (brushes) were identified as the primary action site of *Bt* toxins in several insect species. The activated toxin binds to specific receptors located in the apical brush border of the columnar intestinal cells. There are several receptor or binding proteins for the toxin. 12 to 180 KDa glycoproteins have been identified. In case of Lepidoptera *Manduca sexta* (Linnaeus), a 210 KDa membrane protein is the receptor for the *Cry* IA(b) protein, and a 120 KDa aminopeptidase is the receptor for the *Cry* IA(c) toxin (Figure 2).

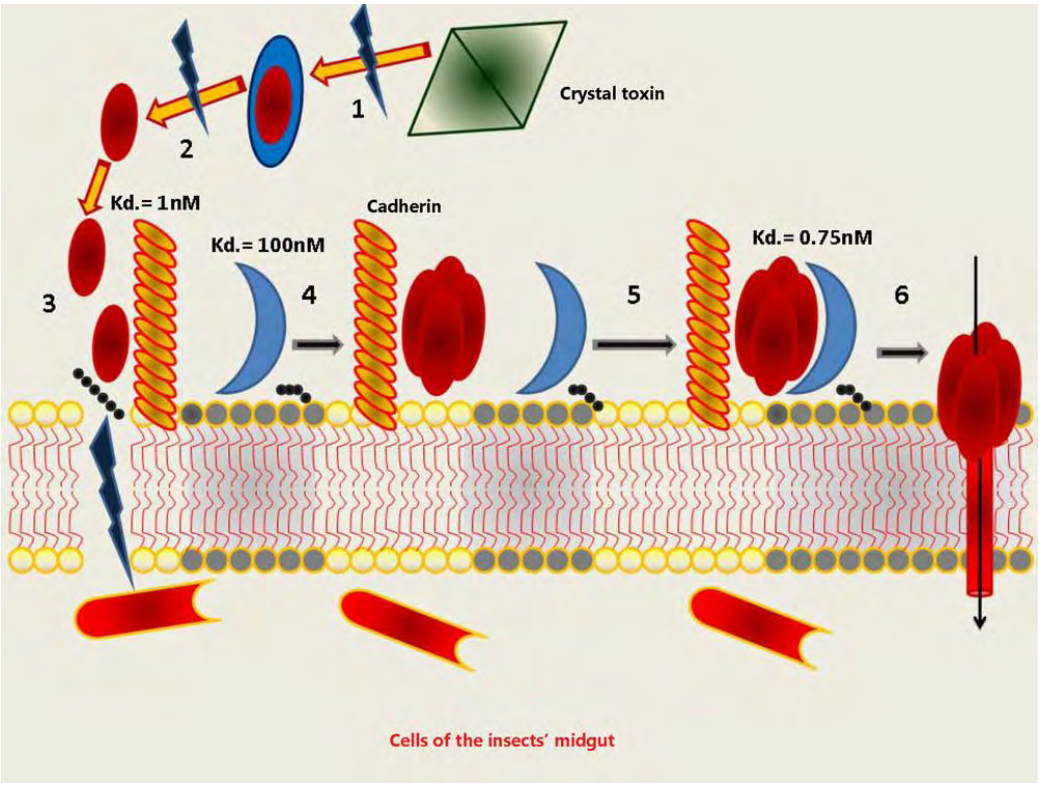


Figure 2. Scheme of the series of events occurring in activation and mode of action of *Bacillus thuringiensis Cry* toxins: 1) solubilization, 2) processing, 3) binding to cadherin-like receptor, 4) pre-pore formation, 5) binding to aminopeptidase receptor, 6) binding to the intestinal membrane of the insect.

Once bound to the receptor, the toxin binds irreversibly to the plasmatic membrane of the cell, triggering the formation of lesions in the membrane. There is a positive correlation between the toxin activity and its capacity to irreversibly bind to intestinal receptors of the insect. Toxicity of the protein crystal correlates to the number of receptors, more than to their degree of affinity (Soberón; Bravo, 2008).

Toxicity of the δ-endotoxin of *Bt* is linked to the helix organization present in the l domain of the molecular structure of the toxin.

After the helices bind to the epithelial cells of the midgut, two events can follow:

a) The helices can penetrate the apical membrane to form an ion channel. Pore formation in the apical membrane of columnar cells activates ion flow. The pores are selective to K, permeable to cations and anions and to solutes, as saccharose, independently of their charge (+ or − signal).

b) The action of *Bt* toxins lead to the termination of the K pump, which produces inflammation of intestinal columnar cells and to osmotic lysis. Interruption of intestinal integrity leads to insect death by starvation or septicaemia. The midgut of Lepdopteran insects has high pH, which facilitates K+ escape through the pores formed. Formation of this selective channel of cations destroys membrane potentials and this results in necrosis of the midgut. In addition, degeneration of the peritrophic membrane and of the epithelium is produced and, finally, septicaemia caused by bacteria occurs.

Differences in the level of solubilization of different toxin types can explain the differences in toxicity of several proteins. In this sense, the decrease in solubility of *Bt* toxins could be a potential mechanism for insect resistance to them. Based on their specificity for certain groups of insects and on their homology of amino acid sequences, *Cry*-type proteins have been gathered into five groups: a) Type I: the genes responsible for *Cry*1-type proteins codify 130 kDa proteins and are particularly active against Lepdopterans, b) Type II: genes that codify 70 Kda proteins, which have specificity against Lepdopterans and Dipterans, c) Type III: genes that codify 70 Kda proteins specific for Coleopteran larvae, d) Type IV: genes that codify *Cry*-type proteins specific for Dipteran larvae and e) Type V: genes that codify proteins effective against Lepdopterans and Coleopteran larvae (Table 1).

The δ-endotoxins produced by *B. thuringiensis* configure a family of related proteins; approximately 140 genes responsible for codifying these toxins have been described (Crickmore et al., 1998).

Table 1. Different groups of *Cry*-type toxins produced by *Bacillus thuringiensis*.

Gene	Crystal form	Protein size (KDa)	Insecticide activity
CryI: Subgroups A(a), A(b), A(c), B,C,D,E,F and G	Bipyramidal	130–138	Lepidopteran larvae
CryII: Subgroups A, B and C	Cuboidal	69–71	Lepidopteran and dipteran
CryIII: Subgroups A, B and C	Flat or irregular	73–74	Coleopterans
CryIV: Subgroups A, B, C and D	Bipyramidal	73–134	Coleopterans
CryV-IX	Various	35–129	Various

The proteins enclosed in the crystals are toxic to different groups of insects, which led to the use of extracts of this bacterium as insecticide. In addition to *Cry*-type proteins, the crystals also contain other toxins called cytolysins (*Cyt* toxins). Hence, *B. thuringiensis* produces other toxins which act synergically with toxins present in the crystals. Among them we can quote toxins that are secreted such as hemolysins, enterotoxins and phospholipases. In natural conditions, all these factors act to facilitate death of larvae of the insect and the development of bacteria within it.

Some strains of *B. thuringiensis*, in vegetative growth stage, produce toxins known as vegetative insecticidal proteins (VIPs). VIPs do not form crystals. When secreted, they act together with *Cry* and *Cyt*-type toxins. The gene for toxin VIP3A codifies an 88 KDa protein that is produced during vegetative growth and that is not processed. This toxin has activity against several Lepdopterans, among them: *Agrotis ipsilon* (Hufnagel), *S. frugiperda*, *Spodoptera exigua* (Hübner) and *Helicoverpa zea* (Boddie). When the susceptible insect consumes lethal quantities of VIP3A, signs of paralysis and of lysis of intestinal cells are observed, similarly to what is observed in intoxications produced by *Cry*-type proteins.

Production of transgenic plants which express *Bt* genes

The basic requirements to obtain transgenic plants resistant to insects are the following:

a) A receiver genome (susceptible elite genotype).

b) A candidate gene (δ-endotoxins, protease inhibitor, chitinases, etc.).

c) An appropriate vector to transport the gene and enable its insertion into the receiver genome.

d) Availability of adequate transformation protocols that allow the correct integration of the gene to the nuclear DNA of the receiver genotype.

e) An efficient tissue culture system that ensures regeneration of viable transgenic plants based in transformed calli.

f) Addition of high expression promoters to ensure adequate levels of expression of the gene and of its corresponding active protein.

g) Protocols destined to identify the transformed cells in a quick and precise way.

h) Genetic and molecular characterization of the transgenic plants obtained, aiming at verifying the gene presence, the number of copies, the stability and the level of gene expression.

i) Bioassays to determine efficiency in management of pest-insects.

j) Assays in greenhouses and in the field to assess biosecurity of the event created, absence of allergens and environmental impact assessment.

A great number of vectors have been developed for transferring the genes responsible for *Bt* d-endotoxins into crops. The system normally used involves the following components: a) a marker gene for resistance to antibiotics or to herbicides (i.e. *phosphoincithrin*), b) a replication site, and c) a multiple cloning site with several restriction sites for DNA insertion. Exogenous DNA (insert) is inserted into the vector using restriction enzymes that recognize a specific DNA sequence and cut it.

Construction of the DNA sequence for incorporation into vectors undergoes several changes, for instance, in case of *Bt*, the promoter sequence is converted from adenine-thymine-rich (typical of bacteria) to guanine-cytosine-rich (typical of higher plants), to increase toxin expression. Other changes are made to the third codon, thereby minimizing changes in the amino acid sequence and ensuring an increase of 10 to 100 times in the expression of *Bt* toxin. To ensure expression of the *Bt* gene in higher plants, a recognizable promoter and a terminator sequence must bracket the *Bt* gene. The most used constitutive promoters include the one from cauliflower mosaic virus (*CaMV*35S) and ubiquitin, while the tissue specific promoter usually employed is the one from PEPC (phosphoenolpyruvate carboxylase). The size of the vectors ranges from 5,000 to 11,000 base-pairs depending on the *Bt* gene and the promoter

incorporated into the vector. Genetic transformation of plant tissues aiming at obtaining transgenic plants with *Bt* genes is successfully achieved by using transgeny methodologies via *Agrobacterium* and biolistics.

The first transgenic tobacco plants with Bt were produced in 1987 (Barton et al., 1987). These plants expressed *Bt* toxin genes (*Cry*1A) under the control of constitutive promoters. The expression level of *Bt* toxins in the plants was quite low, resulting in no more than 20% mortality in *Manduca sexta* [tobacco hornworm] larvae. To solve this problem, it was decided to truncate the extremity of the gene to be introduced. Plants transformed with truncated gene expressed toxin dosage of about 0.02% of total leaf soluble protein.

Currently, a number of additional genetic manipulations are effected aiming at ensuring high toxin expression in plant tissues and minimizing emergence of resistant insects in *Bt* transgenic plants (Tabashnik et al., 2003a, 2003b). These changes are basically gene truncation and the use of different promoters and sequences of fusion proteins.

Bioassays to assess the insecticide power efficiency of transgenic plants

To asses the efficiency of management of pest-insects by transgenics several assays using live insects (bioassays) are carried out. Insects have the possibility of feeding on the events created with *Bt* genes and on the control plants (not genetically transformed). These tests are named “free choice test”. No choice tests, in which insects are confined to the transgenic plants and the delay in growth and percentage of insect mortality are observed, are also performed. Nonetheless, final data recorded in events with *Bt* genes are always compared to the ones from the nontransformed control.

Strategies to prevent emergence of resistant insects

Bellow we mention strategies which, applied together, will allow avoiding or minimizing emergence of insects resistant to *Bt* transgenic cultivars.

a) Obtaining transgenics which express high dosage of *Cry*-type protein in all tissues.

Expression of optimal levels of toxin crystals will allow the efficient control of all target insects, minimizing the emergence of resistant insects. In most insects, resistance appears in heterozygous state, but obtaining transgenics with *Bt* genes which express high dosages of protein crystals will eliminate most of these insects, avoiding transmission of resistance to future generations of pest-insects.

b) Creating refuge areas, that is, locations with nontransgenic crops, where susceptible insects can continue to reproduce, whenever a technology including *Bt* genes is used.

Constructing refuges with materials free of *Bt* genes, which will enable heterozygous resistant insects to join homozygous susceptible ones coming from the refuge areas. The progeny of these matings between insects will be susceptible to the *Bt* gene expressed in the transgenic crop (Sims, 1996; Munkvold; Hellmich, 2002).

c) Combined use of various insecticide proteins or *Cry* gene pyramidization in a given genetic material.

Permanent management, after an event with *Bt* genes has been released in the field, is one of the most efficient management practices, because this management allows detecting possible emergence of resistance in target insects in advance, treating the problem previously and assessing the real severity and distribution of resistance in areas cultivated with events containing *Bt* genes, and also combining strategies to eliminate resistant insects.

Production of transgenic crops expressing more than one protein crystal or *Bt* gene pyramidization is a fundamental factor to reduce emergence of resistant insects (Figure 3). Expression of two protein crystals with different modes of action in the same genotype will enable to manage insects resistant to one toxin by the other toxin. Emergence of insects with combined resistance to both toxins is a rare event, nonetheless pyramiding three *Cry*-type toxins, in addition to rotating them in the different events created, can minimize the emergence of resistant insects (Zhao et al., 2003). Another positive aspect of pyramidization of *Cry*-type genes is that it enables increasing the total concentration of *Bt* proteins in the tissue of transgenic plants, which maximizes survival of resistant heterozygous individuals. The toxicity of *Bt* d-endotoxin is linked to the organization of helices in the l domain of the molecular structure of the toxin (Figure 3).

d) Efficient utilization of integrated pest management and capacitating technicians and farmers for its correct application.

e) Permanent management to detect the possible emergence of resistant insects.

f) Assessing the effects of crops expressing *Bt* genes over beneficial insects (predators).

Up to the present, negative effects of events containing *Bt* genes on predator insects have not been detected. In potato crops with *Bt* genes in the state of Oregon, USA, there were no changes observed in spiders and

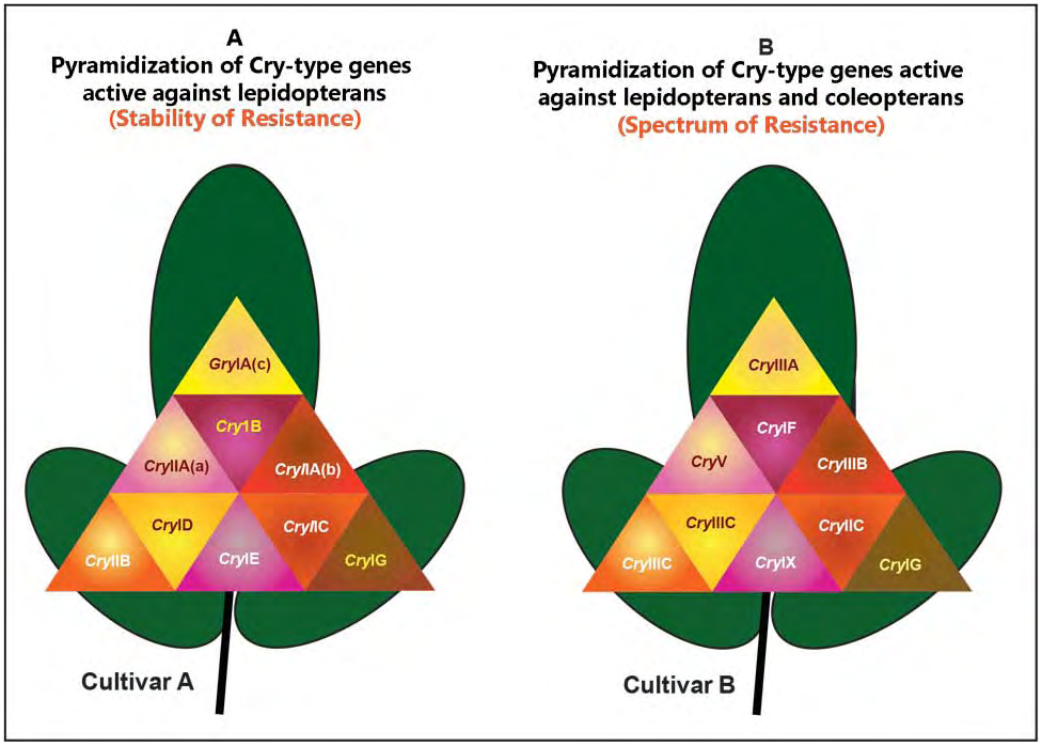


Figure 3. Accumulation of *Cry*-type genes in alfalfa cultivars. Accumulation of different *Cry*-type genes active against lepidopterans, which grants them stability of resistance (A); Accumulation of *Cry*-type genes active against lepidopterans and coleopterans, which provides broader spectrum of resistance (B).

bedbugs that preyed on genera *Nabis* and *Geocoris*. On the other hand, in these transgenic crops, natural enemies were more efficient to manage aphids than in crops without *Bt* genes (Ferré; Rie, 2002; Gould, 2003).

Use of genomic tools objectifying resistance to insects in alfalfa

Presence of mechanisms of defense to insects in alfalfa was reported in the 1990s (Gieco et al., 1994, 1996; Gieco; Basigalup, 1997). These authors identified alfalfa genotypes with different resistance mechanisms (antibiosis, tolerance and antixenosis) to *Acyrtosiphon kondoi*; as well as the coexistence of resistance mechanisms in the same genotype (i.e. antibiosis and antixenosis).

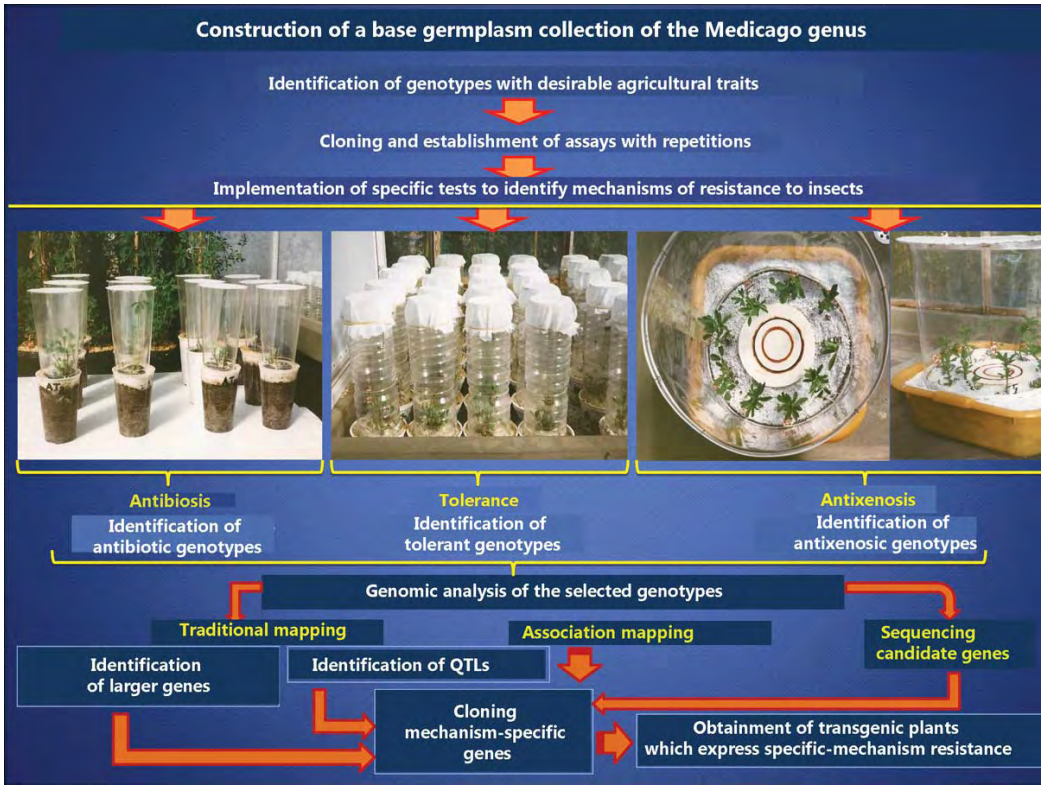
Building a vast germplasm base of the genus *Medicago*, in addition to a wide availability of genomic tools, will enable the development of synthetic alfalfa products that combine mechanisms of resistance to pest-insects. The existence of different species in the genus *Medicago* with resistance to pest-insects was

reported by Chandra et al. (2006). These authors identified, by performing a screening of germplasm, lineages which belonged to species *Medicago laciniata*, *Medicago rugosa*, *Medicago scutellata*, *Medicago muricoleptis* and *Medicago tenoreana* resistant to *Hypera postica*.

The strategy to follow consists in developing a germplasm collection with broad genetic basis, including the largest possible number of species from the *Medicago* genus and the greatest possible representativity of genetic materials from each species. The next step will be the agricultural characterization of these materials, aiming at identifying the accessions with insect resistance. For that purpose, assays with repetitions must be carried out, cloning the genotypes to obtain more precision in identifying resistant genotypes. After identifying the resistant genotypes, specific tests to identify the resistance mechanisms present in these genotypes (antibiosis, tolerance, antixenosis or different combinations of them) must be programmed. The next step will be the genomic analysis aiming at identifying the genes involved in the determination of resistance mechanisms. There are different alternatives to finally lead to obtaining transgenic alfalfa genotypes which express genes of resistance to specific mechanisms. Traditional mapping through strategies to identify the largest genes or quantitative trait loci (QTLs), depending on qualitative or quantitative inheritance of the resistance trait, is based on the development of structured mapping populations, resulting from the biparental crossing of a resistant individual with a susceptible one. Association mapping, in which there are no structured populations developed *a priori*, combines information from neutral and functional molecular markers to identify the genes or QTLs present in the germplasm accessions. Finally, information from the sequencing of *Medicago truncatula*, which enabled the identification of numerous candidate genes, can be successfully used to develop alfalfa genotypes resistant to insects (Figure 4).

Obtaining alfalfa plants resistant to insects can also be done by using basic biotechnological techniques, such as cultivation of anthers, cultivation of immature embryos, protoplast fusion and utilization of somaclonal variation. These techniques are very useful when we intend to transfer resistance genes from species close from the genus *Medicago* to *Medicago sativa* via sexual crossing (Sutrisno, 2001).

Techniques for mapping simple genes and QTLs were described in chapters 6 and 10. In the present chapter, we will explain the methodology for mapping by association to identify genes of resistance to pest-insects in alfalfa.



Photos: Jorge Omar Gieco

Figure 4. Stages involved in developing alfalfa genotypes resistant to pest-insects with the help of biotechnology. QTLs = quantitative trait loci.

Association mapping

Phenotypic variation of several complex traits of interest to agriculture is determined by the many genes of quantitative inheritance (QTLs), the interaction between QTLs, the environment and the interactions between QTLs and environments.

Binding analysis and association mapping are the tools most often used to subdivide the complex traits into their component parts (Figure 5). Binding analysis allows locating QTLs at 10 cM to 20 cM (centiMorgans) intervals, basically due to the limited number of recombinant events which occur during the development of mapping populations and to the limited number of individuals forming these populations. This limited number of individuals is due to the high cost derived from the multiplication and evaluation of such individuals in assays with repetitions. Despite these difficulties, a large number of QTLs has been mapped in different plant species using this methodology. A significantly lower number of QTLs has been cloned.

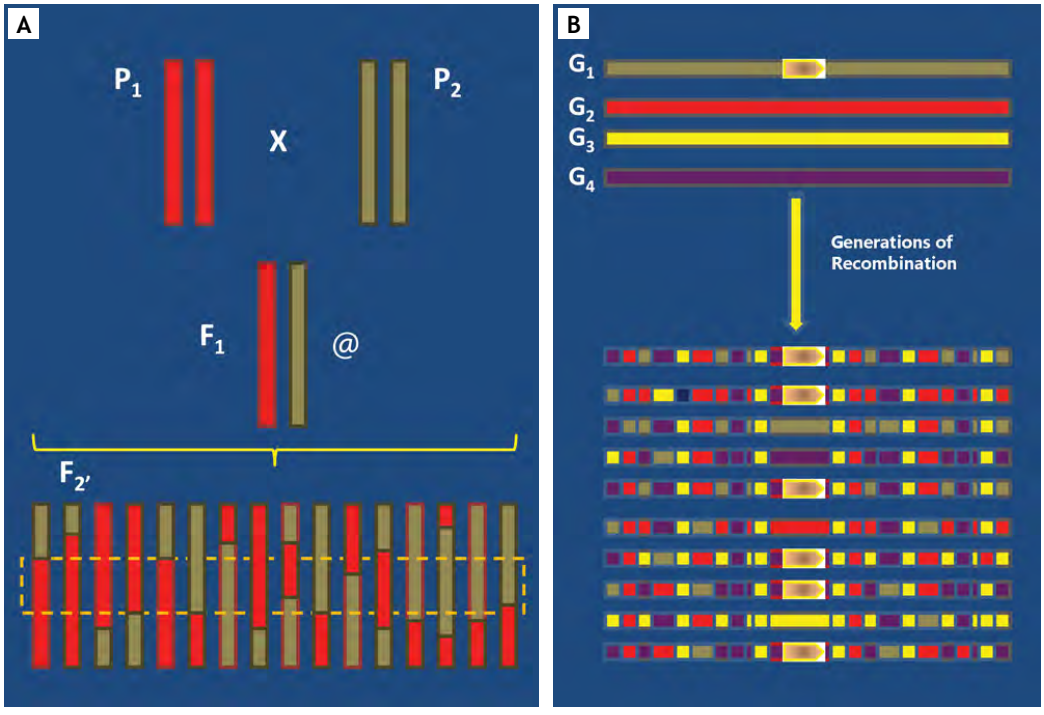


Figure 5. Comparative scheme of binding analysis using structured populations. Quantitative trait loci analysis (A); association mapping analysis using genetically different germplasm collections (B).
Source: Adapted from Zhu et al. (2008).

The methodology for association mapping, also named linkage disequilibrium mapping, has arisen as a new genomic methodology aiming at resolving problems derived from the complex genetic variation at DNA sequences level, through the utilization of information coming from evolutive and recombination events produced over time in the population being studied. This methodology has the following comparative advantages in relation to traditional mapping: a) increased resolution of maps produced, b) reduced working time, and c) increased number of identified alleles. The methodology has gained ground basically because of various factors, such as the development of more automated and more efficient genotyping methodologies, the interest in identifying a large number of superior alleles and the development of new statistical packages (Figure 6).

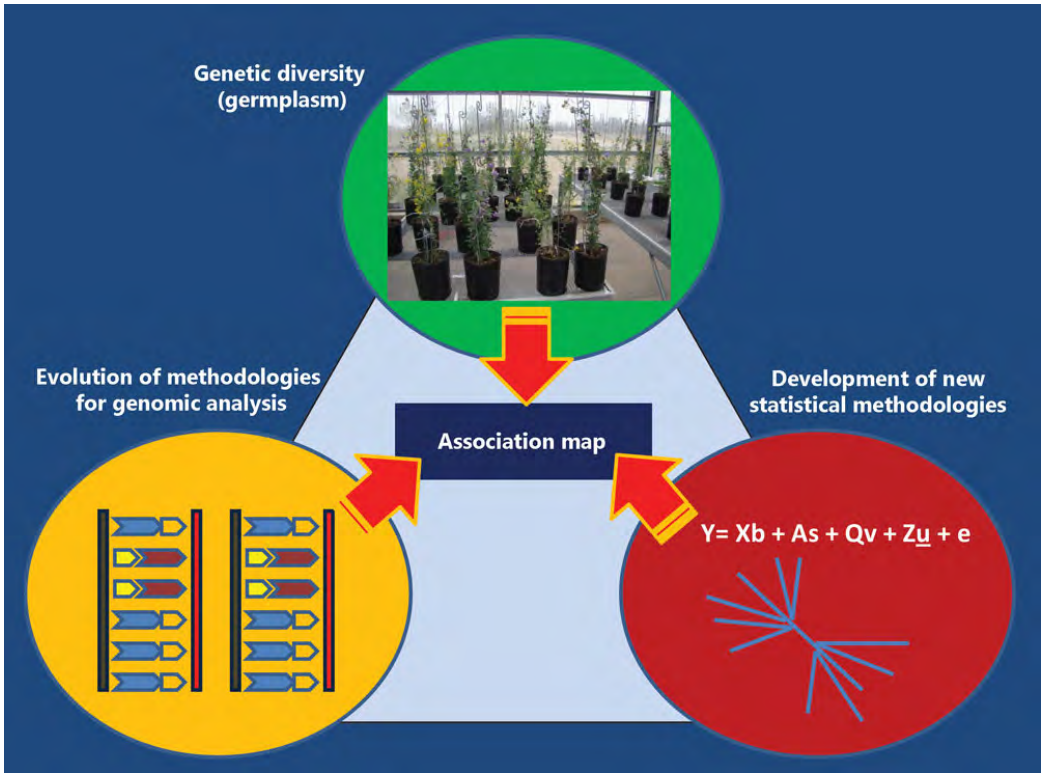


Photo: Jorge Omar Gieco

Figure 6. Main components of the association mapping technique.

There are two ways to perform association mapping:

a) Association mapping based on candidate genes. This variation is based on using the polymorphism of previously selected candidate genes as responsible for the phenotypic variation of various traits of interest.

b) Association mapping through exhaustive genome-wide screening or genomic screening. As indicated by its name, this type of mapping is based on the search of genetic variability throughout the genome aiming at finding signals of association between this variability and several complex traits.

Development of new technologies for genomic analysis (genotyping and sequencing) has significantly reduced the cost per sample analyzed with molecular markers, especially for the detection of single nucleotide polymorphisms (SNPs). When an association mapping methodology based on candidate genes is used, it is usually necessary to rely on additional information on biochemical or metabolic routes and on regulatory stages leading to the

final expression of variation in the trait. Genome sequencing and annotation of model species has provided vital information on genetic sequences of candidate genes that can be used in association studies.

On the other hand, the whole genome screening methodology has developed together with genomic technology, enabling the identification of several thousand SNPs through resequencing a core set of genetically diverse lines in a large number of samples. Thus, it was possible to perform wide association analyses of complex traits along the plant genome (Figure 7).

Association mapping uses genetic diversity present in natural populations to the potential purpose of decomposing the variation existing in complex traits into their individual genes or into nucleotide sequences. Analyzing the traditional linkage in experimental populations coming from biparental crossings provides relevant information about traits tending to be specific of them or of genetically related populations, while results from association mapping are more applicable to a wide germplasm base. The ability to map QTLs into line

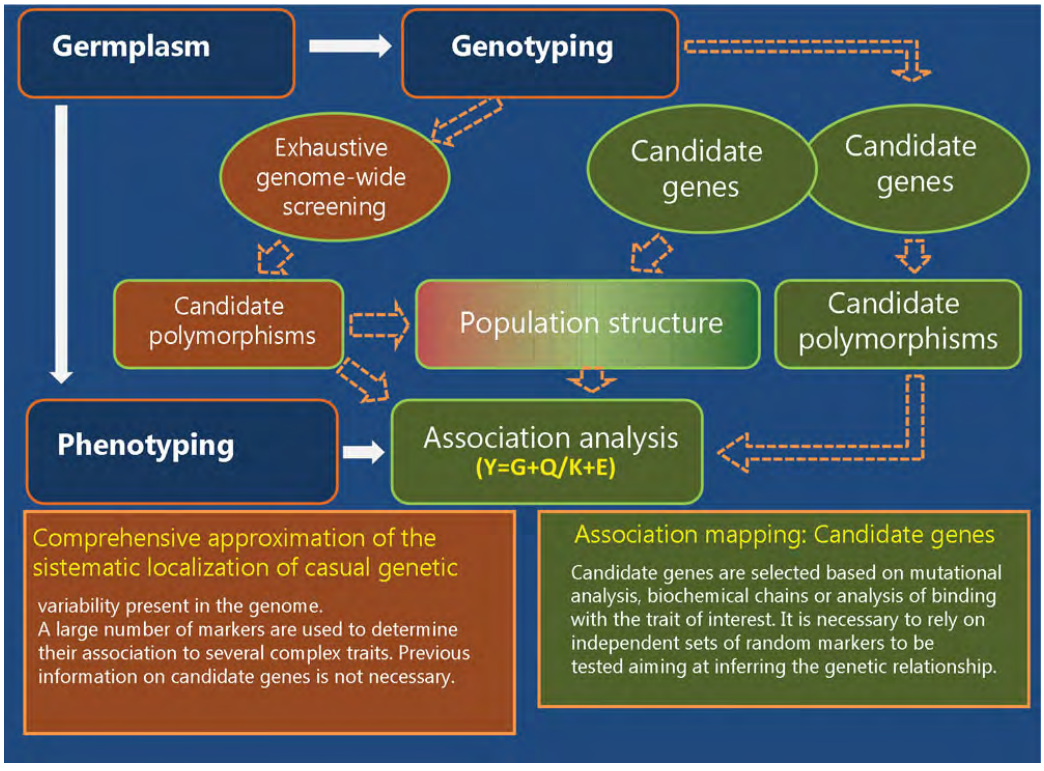


Figure 7. Diagram of alternative methodologies of association mapping: whole genome screening and candidate genes.

collections, local races or samples from natural populations has great potential for improving a particular trait. Advanced backcrossings for introgression of QTLs (AB-QTL) and for introgression of libraries (IL) are strategies to screen exotic germplasm for new alleles aiming at improving productivity, adaptation, quality and nutritional value of crops. Association mapping is a complementary technology to AB-QTL and to IL and, in turn, acts as an additional tool to extensively assess functional diversity of large scale crops.

Alfalfa cultivars resistant to the most common pests

According to Puterka et al. (1992), selecting resistant cultivars and assessing the resistance characteristics must be carried out with care because the selected cultivars are geographically restricted and because aphid populations have great biotype variation. Silva et al. (2006) added that local studies on aphid biology in different cultivars are necessary to provide information about managing these pests and about their susceptibility in tropical conditions.

Results of research with resistant alfalfa cultivars in other countries

Even though there is difference in the resistance shown by cultivars in different places, those which are deemed satisfactory in production and resistance in other countries can be tested in Brazil.

Aphids. The effect of aphid attack (Figure 8) over the growth and development of alfalfa is the result of a complex interaction between the number of insects, time and duration of the infestation and the plant response, as observed by Summers and Coviello (1984) in *A. kondoi*. Damage caused by this species of aphid increases as the period of pest presence extends, but tolerance to attack increases with the growth of the plants (Bishop et al., 1982).

The Lahontan cultivar was the first one resistant to *T. trifolii* f. *maculata* for use in breeding programs. It was observed that in resistant cultivars this species of aphid prefers feeding on the petiole and stalks instead of on mature leaves. In tests, around 50% of aphids moved from cotyledons and unifoliate leaves to petioles in the Lahontan cultivar within 72 h to 96 h, while in susceptible cultivars less than 10% were found in leaf petioles (Berberet et al., 1991).

Some authors, such as Barnes (1963) and Webster et al. (1968) report cultivars in the USA which showed resistance to alfalfa pest aphids and, despite the time elapsed, some of them are still used, such as *A. pisum*, resistant: Kanza and T3-12, susceptible: Ranger, Caliverde and Moapa 69; for *T. trifolii* f. *maculata*, resistant: Moapa, Kanza and Mesa-Sirsa, susceptible: Ranger, Team

Photos: Fernando Daniel Fava

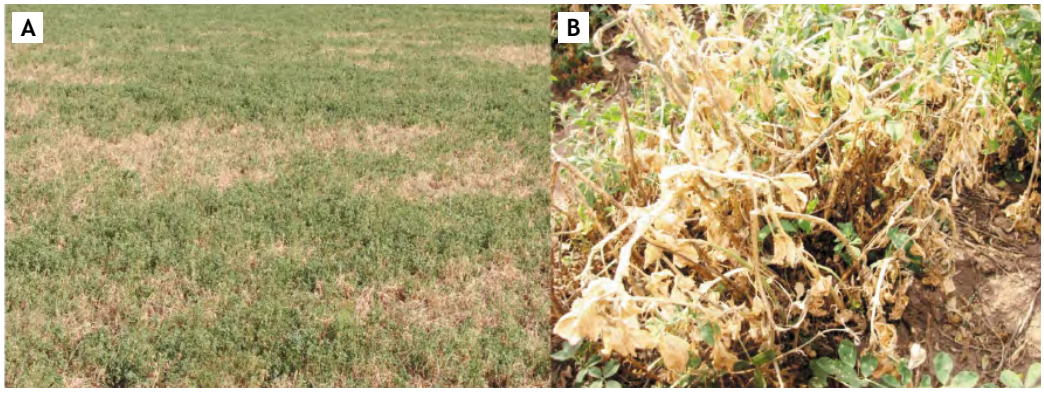


Figure 8. Damages caused by aphids to the alfalfa crop (A) and detail of the damage (B), in Argentina.

and Caliverde; for *E. fabae*, resistant: Rhizoma, Rambler, Teton, MSA-CW3AN3 and KS13, with intermediary resistance: Alfa, DuPuits and Glacier, susceptible: African, Hairy Peruvian, Indian, Moapa, Sonora, Ranger and Lahontan; for Curculionidae *Hypera postica* (Gyllenhal), resistant: Team and Arc, susceptible: Ranger and Vernal.

Among the 272 cultivars recommended for alfalfa cultivation in 2004–2005 in California, USA (University of California, 2009), many showed some resistance to the three main alfalfa aphids: *T. trifolii* f. *maculata* (212 cultivars), *A. kondoi* (246) and *A. pisum* (107). These cultivars were assorted into highly resistant, resistant and moderately resistant. Among them, 100 cultivars are resistant to the three aphids together.

Empoasca fabae. Glandular hairs present in the plants prevent small insects from crawling over their surface. Some of these hairs contain toxic substances and others merely contain sticky substances which capture these insects. This characteristic has been used to develop resistance to *Empoasca sp*. However, most alfalfa plants have no pubescence or only have scarce hairs spread over the surface of the stem and the leaf.

According to McCaslin and Whalen (2009), the initial findings about the interference of hairs in the emergence of *Empoasca sp*. suggested that the main mechanism of resistance to this pest was the imprisonment of the insects onto the sticky extremities of glandular hairs. Nevertheless, many plants with this trait have no resistance to such insects, indicating that not all hairs are efficient in providing resistance. According these authors, trapping does not appear to be an important mechanism of resistance to *Empoasca sp*. in alfalfa, because the growth and survival of nymphs and adults in the plants

with glandular hairs was significantly lesser than in plants from conventional varieties, which indicates that resistance is given by antibiosis. Antibiosis is related to some chemical substance from the glandular exudate, more effective over immature *Empoasca* sp., that is, nymphs. Studies also suggested evidences of nonpreference for oviposition by females of *Empoasca* sp. in resistant alfalfa plants, in other words, plants with glandular hairs present, because females avoid laying their eggs in plants with glandular hairs, when there is another plant available. However, according to Lefko et al. (1998), the population of *Empoasca* sp. necessary to cause economic damage to alfalfa is twice as big in resistant varieties, in comparison to susceptible ones. This was the first evidence of tolerance as a mechanism of resistance to *Empoasca* sp. in alfalfa plants.

The results of this research show the complex nature of resistance to *Empoasca* sp. in alfalfa crops, since antibiosis, nonpreference and tolerance seem to be components of the resistance mechanism. This also helps to explain the genetics of resistance and the challenges that alfalfa breeders face in selecting genotypes for resistance to this pest.

Presence of glandular hairs in alfalfa cultivars still has particularities. No difference has been verified in mortality and feeding levels of *E. fabae* when these insects were confined in basal internodes of cultivar G98A (resistant), in comparison to those in which *Empoasca* insects were confined to basal and apical internodes of the Ranger cultivar, susceptible and with no glandular hairs. Hence, Casteel et al. (2006) concluded that young and active glandular thrichomes of the alfalfa cultivar G98A resistant to *E. fabae*, are located in the apical internodes, while senescent glands are found in older internodes, located in the basal part of this cultivar.

To make result standardization even more difficult, studies carried out by Dellinger et al. (2005) showed that the density of *E. fabae* did not differ in cultivars 54H69 (with glandular hairs) and Choice (without glandular hairs) in populations of two locations in Virginia, USA. Thus, even though it is resistant in other locations, cultivar 54H69 cannot be considered resistant in the locations tested, which demonstrates how important the environment is in manifesting resistance.

The influence of weather and soil conditions in the biology and in the expression of resistance of alfalfa to *E. fabae* was tested by Casteel et al. (2006) in low and high levels of luminosity (250 and 1,000 μmol s−1 m−2) and of temperature (17 °C and 30 °C) in cultivars which had glandular thrichomes present (G98A) and which did not have this characteristic (Ranger et al., 2005). Results showed high mortality of *Empoasca fabae* confined to alfalfa G98A in

conditions of high luminosity and high temperature, indicating that in certain regions, the cultivar G98A of *M. sativa* is better protected from this pest than in others, and that temperature influences the resistance level of glandular hairs of alfalfa.

Curculionidae beetles. In the USA, plants with resistance to larval feeding and also with nonpreference for oviposition by Curculionidae beetles *Hypera postica* and *Hypera brunneipennis* (Boheman) have been found, among them cultivars Team, Arc and Liberty (Nielson; Lehman, 1980). Summers (1998) also included another American cultivar, Weevilcheck, to this group.

Alfalfa cultivars resistant to insects in Brazil

In Brazil, aiming at introducing the alfalfa crop to new areas, several assays to estimate adaptability and stability of several accessions have been carried out (Paim, 1994). Tests aimed mainly at productivity and, when pests were included, only aphids were mentioned.

In Zona da Mata region, Minas Gerais state (Cwa climate, average of 18 ºC in colder months and 22 ºC in warmer months, annual precipitation around 1,500 mm), Botrel and Alvim (1997) observed that cultivars Maxidor and Pioneer 555 were the most susceptible ones to attacks by pests and diseases, while cultivar Monarca displayed tolerance and cultivar Crioula was moderately susceptible (Table 2). The authors added that cultivar Monarca did not stand out in the agricultural patterns assessed, but it can be used in alfalfa genetic breeding programs aiming at obtaining characteristics of pest resistance. Botrel et al. (2001), following Hijano's (1994) classification, reported the level of resistance of 30 cultivars also evaluated in Zona da Mata, Minas Gerais (Table 2), where during rain season the incidence of *A. pisum* was not observed in 70% of the cultivars and, in dry season, only 23% were deemed resistant.

In the bushy region of Zona Metalúrgica, a region of Minas Gerais (Aw climate, minimum temperature 16.4 ºC and maximum of 28.8 ºC, annual precipitation of 1,568 mm) cultivars Crioula and P 30 stood out from the rest; in dry season, 32% of the 28 tested cultivars displayed resistance to insect attacks and, in rain season, 43% of them did (Viana et al., 2004).

In the municipality of São Carlos, São Paulo state, the number of *Acyrthosiphon* spp. in the resistant cultivar CUF 101 was as high as in the susceptible cultivars, demonstrating that, apparently, *Acyrthosiphon* spp. breaks the characteristic resistance of this cultivar (Silva et al., 2005). The existence of *A. pisum* and *A. kondoi* biotypes was also reported by Zarrabi et al. (1995) e Bournoville et al. (2000) respectively.

Table 2. Alfalfa cultivars with resistance to pest attack, in different parts of Brazil.

Cultivar	Location	Resistance[1]	Author
Alfa 200	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Alto	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
Auracana	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
Aurora	Zona da Mata, MG	TDS[5]	Botrel and Alvim (1997)
BR 1	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
BR 3	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
BR 4	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Costera	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Crioula	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
	Central Region, GO	R (1.00)[4]	Heinemann et al. (2006)
Crioula CRA	Central Region, GO	R (1.04)[4]	Heinemann et al. (2006)
Crioula Honda	Central Region, GO	R (1.04)[4]	Heinemann et al. (2006)
Crioula importada	Central Region, GO	R (1.00)[4]	Heinemann et al. (2006)
CUF 101	Zona da Mata, MG	TDS[5]	Botrel and Alvim (1997)
5312	Central Region, GO	R (1.00)[4]	Heinemann et al. (2006)
5454	Central Region, GO	R (1.09)[4]	Heinemann et al. (2006)
54H55	Central Region, GO	R (1.09)[4]	Heinemann et al. (2006)
58N58	Central Region, GO	R (1.04)[4]	Heinemann et al. (2006)
El Grande	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Esmeralda	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
F 708	Central Region, GO	R (1.09)[4]	Heinemann et al. (2006)
Flórida 77	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
Fortineira	Zona da Mata, MG	TDS[5]	Botrel and Alvim (1997)
ICI 990	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Maricopa	Zona da Mata, MG	TDS[5]	Botre and Alvim (1997)
	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Monarca	Zona da Mata, MG	TDRS[6]	Botre and Alvim (1997)
	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)

Continued...

Table 2. Continued.

Cultivar	Location	Resistance[1]	Author
P 30	Central Region, GO	R (1.00)[4]	Heinemann et al. (2006)
	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
P 5888	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Perla SPINTA	Central Region, GO	R (1.14)[4]	Heinemann et al. (2006)
Saladina	Zona da Mata, MG	TDS[5]	Botrel and Alvim (1997)
Sutter	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
SW 8112 A	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
SW 8210 A	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
SW 9210 A	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
Valley Plus	Zona da Mata, MG	RRS[2]	Botrel et al. (2001)
WL 516	Zona da Mata, MG	RRDS[3]	Botrel et al. (2001)
WL 605	Zona da Mata / MG	TDS[5]	Botrel and Alvim (1997)
XA 132	Central Region, GO	R (1.14)[4]	Heinemann et al. (2006)

[1] Specially to *Acyrthosiphon pisum*.

[2] RRS = resistant in rain season.

[3] RRDS = resistant in rain and dry seasons.

[4] R = resistant. Scores used: 1 = absence of damage, 2 = presence of damaged leaves and 3 = generalized presence of damaged leaves with visible loss to the plants.

[5] TDS = tolerant in dry season.

[6] TDRS = tolerant in dry and rain seasons.

Silva et al. (2006) tested resistance and susceptibility of four alfalfa cultivars deemed resistant (CUF 101, Baker, Mesa-Sirsa and Lahontan), two susceptible cultivars (ARC and Caliverde) and the Crioula cultivar against *T. trifolii* f. *maculata* in the municipality of Ribeirão Preto, São Paulo state (Aw climate, high-altitude tropical, with average annual temperature of 26 ºC, annual precipitation of 1,430 mm and altitude of 546 m). The authors found that all the resistant cultivars, except Lahontan, had fewer aphids than the others. The highest density of *T. trifolii* f. *maculata* was found in the Crioula cultivar. The surviving aphids from resistant cultivars, with the exception of Lahontan, were fewer, had lower fecundity and lower longevity than the ones from susceptible cultivars. Results indicated that resistance to the tropical biotype of this aphid species is given by antibiosis, mainly in cultivars Baker

and Mesa-Sirsa, confirming the results obtained with other cultivars from temperate climate (Ruggle; Gutierrez, 1995).

The Crioula cultivar, on the other hand, is possibly tolerant to *T. maculata*, because even though it was heavily infested in laboratory conditions, it showed less visible damage (chlorosis) than cultivars ARC and Caliverde, deemed susceptible.

With the growth of milk production observed in recent years in Goiás state, the application of tests to assess alfalfa plantation conditions in this state became necessary. Heinemann et al. (2006) evaluated 21 cultivars of this legume in the municipality of Anápolis, Goiás state (Aw climate, average annual temperature of 22.3 ºC, precipitation of 1,610 mm and altitude 1,017 m), using scores from 1 to 3 (1 = absence of damages, 2 = presence of damaged leaves and 3 = generalized presence of damaged leaves with visible loss) to classify the damage caused by *A. pisum*. The group deemed resistant to pests, with scores ranging between 1.00 and 1.14 was formed by twelve cultivars – P 30, imported Crioula, Crioula, 5312, Crioula Honda, Crioula CRA, 58N58, F 708, 54H55, 5454, XA 132 and Perla (Table 2), representing 57% of the amount tested. The second group was formed by cultivars which had good pest tolerance and scores between 1.19 and 1.38, with nine cultivars deemed moderately resistant: SW 8200, SW 8210, Vitória, 58N57, SW 9500, SW 7400, SW 9301, SW 7403 and SW 14.

Final considerations

Even though alfalfa resistance to pests is a phenomenon that can occur often among cultivars available to the farmer, it is still not very clear and/or exploited in Brazil. Due mainly to the extension of agricultural and breeding boundaries, many experiments involving a more sensible assessment of pest resistance are still necessary for the many parts of the country, both to the areas where the alfalfa crop is already settled and to those in which there is interest in introducing the crop.

Currently, great part of the cultivars is tested only for assessing agricultural production. However, the diverse weather conditions in which alfalfa is grown around the world cause the emergence of biotypes of the insects that live on it, changing plant-pest relationship. Plant resistance can thus be broken and they can start undergoing damages again. Many techniques have been proposed to prevent the emergence of pest biotypes, among them multiple resistance, and that must be taken into account when recommending a resistant cultivar.

The advances of genomics have enabled obtaining insect-resistant transgenic alfalfas, via the expression of *Cry*-type genes from the bacterium *B. thuringiensis*. Pyramiding *Cry*-type genes will enable the efficient management of pests affecting the alfalfa crop, such as caterpillars and *Diaprepes abbreviatus* (Curculionidae), with reduced environmental impact. It will also enable significant reduction of the production cost of this legume and better stability of the resistance obtained. In addition, using genomic analysis techniques, such as association mapping, will make the exhaustive genomic screening of several alfalfa populations and of related species of the genus *Medicago* possible, with neutral and functional molecular markers, aiming at detecting genomic regions and/or genes associated to resistance to different pest insects.

It also becomes necessary to standardize the resistance levels of the evaluated plants, so that research results can be compared and thus obtaining the behavior variation of cultivars for different regions or countries where alfalfa is grown.

Hence, a greater knowledge about alfalfa pests and the possibilities for using resistant alfalfa varieties can provide information on the most adequate strategies to manage such pests, adding to more sustainable cultivation systems.

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CHAPTER 9

Genetic improvement of alfalfa

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Introduction

Alfalfa (*Medicago sativa* L.) is a perennial plant, with perfect flowers and mainly cross-pollinated. It has a remarkable genetic variability, enriched by introgression of the related species that form the "*Medicago sativa* complex" (Quirós; Bauchan, 1988). The species that constitute this complex have a basic number of eight chromosomes (x = 8), presenting diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) forms.

The autotetraploid nature of alfalfa has deep implications on breeding, which can be summarized as follows (Busbice et al., 1972): a) total range of genotypes is achieved after at least two generations of random mating; in practice, this means that if the objective is to achieve all possible genotypes, it is necessary to allow occurrence of at least two generations of random mating and to assess a large number of individuals; b) gametic equilibrium is achieved asymptotically since, unlike diploids, autotetraploids only lose two thirds of gametic disequilibrium every generation of random mating, because the diploid nature of their gametes prevents free combination of all alleles in only one generation – in practice, it is assumed that gametic equilibrium in alfalfa is reached with four generations of random mating; and c) the diploid nature of the gametes can allow a large degree of inbreeding, which increases the probability of consanguinity.

Alfalfa is very sensitive to inbreeding, which quickly manifests into loss of strength, low forage yield and scarce seed production. This makes it very difficult to go further than the second generation of self-fertilizations. Consequently, obtaining "pure lineages" or developing inbred lines for hybrid formation are impracticable methods. Obtaining varieties for high forage yield is achieved through crossings of divergent parents.

When selection is for only one dominant gene, the speed of response will only depend on the initial frequency of such gene: for values < 0.5, response is usually fast, and for values > 0.5, response becomes slow and not very noticeable; if the frequency is 0.5, 93% of the individuals from a tetraploid population in equilibrium will express the dominant phenotype (Rodríguez, 1986). If the frequency of the dominant gene is too low or if the trait to improve is conditioned by a recessive gene, only the desirable genotypes should be selected, since including undesirable genotypes (escapes) can notoriously delay the progress of selection (Busbice et al., 1972).

Alfalfa has self-incompatibility and self-sterility mechanisms which favor allogamy (Viands et al., 1988). Incompatibility refers to the impossibility for the male gamete and the female gamete – assuming they are both functional –

to be fertilized after pollination. According to Barnes et al. (1972), self-incompatibility prevents self-fertilization, whether by pollen and stigma interactions (sporophytic control), by reactions between pollen and style (gametophytic control), by syngamy failures inside the embryonic sack or by interactions of the pollen tube and the ovule inside the ovary. This last characteristic is typical to alfalfa, in which pollen tubes originated from its own pollen (selfing) grow more slowly than the ones originated by cross-fertilization pollen; nonetheless, self-incompatibility is only partially efficient to prevent self-fertilization. In addition, a high percentage of abortions of fertilized ovules also occurs in alfalfa, which would not be self-incompatibility (since there has been fertilization), but as self-sterility. This last term should be used when the impossibility of fruit and seed formation after self-fertilization is discussed.

Another mechanism that can be used for pollination control is male sterility, either genetic (Childers; Mclennan, 1960) or cytoplasmatic (Davis; Greenblatt, 1967). According to McLennan and Childers (1964), genetic male sterility would be conditioned by one single recessive nuclear gene (*ms*3). The cytoplasmatic male sterility system in alfalfa is characterized by the incomplete development of pollen or by the abortion of pollen grains, after the end of meiosis. According to Barnes et al. (1972), A-type plants (cytoplasmatic male-sterile) are relatively easy to detect. On the other hand, B-type plants (non-fertility-restoring) are harder to identify because it is necessary to resort to observing F_1 progeny from male-sterile x male-fertile crosses: if the progeny shows complete or partial male sterility, it is possible that the pollinator has fertile cytoplasm and non-fertility-restoring genes for fertility; on the contrary, if the progeny is fertile, the pollinator can be deemed R-type (fertility restorer). All these mechanisms are important for developing commercial hybrids, as it will be discussed later on.

An advantage in alfalfa is the ease for cloning individuals based on rooting of stem pieces inserted into an inert medium (vermiculite, sand, etc.). If stems are vigorous and have active growth, a high percentage of rooting is achieved. Using clones can facilitate assessing genotypes for some quantitative traits, determining the magnitude of the genotypic variance and the genotype x environment interaction.

Implementing an alfalfa breeding program

In addition to being familiar with the concepts presented in the previous section, the breeder must carefully analyze a number of issues that will impact the results and the efficiency of the work. It is essential to set the goals of the

program, which must be clear and reachable within a reasonable term, and be defined as a function of the available infrastructure (Rodríguez, 1983, 1986). The vast majority of alfalfa breeding programs aims at developing cultivars with high forage yield, high persistence and multiple pest and disease resistance. It might also be important to improve forage quality or adaptation to acidic, salty or dry environments. Seed yield is not usually a priority for many breeders, but it is an important trait for seed producers, because it has a direct incidence on the production cost. In countries where alfalfa is used under direct grazing, decreasing the potential for bloat could be an important breeding objective.

Obviously, one fundamental requirement to initiate a breeding program is the existence of adequate genetic variance for the traits to improve. Traditional methods use the genetic variability that is naturally present in the species or in wild relatives. If there is none, it must be created either by the use of biotechnological techniques (mutation, somaclonal variation, transgenesis, genetic manipulation, etc.). In the case of alfalfa the employment of mutagenic agents, commonly utilized in past decades, has fallen into disuse at present times; on the other hand, the employment of biotechnology becomes more and more important. Transgenesis has already successfully been used for developing commercial cultivars tolerant to glyphosate (Van Deynze et al., 2008) or for obtaining experimental populations resistant to Lepidoptera (Rios et al., 2007). Genetic manipulation was so far utilized for the development of experimental lines that express condensed tannins (Gruber et al., 2001) or have reduced lignin content (Undersander et al., 2009).

Later on, the breeder must define the breeding method and the selection units to employ, the size of the population to conduct, the intensity of selection to perform and the expected gain from selection to expect. It is important to estimate the genetic effects of selection and the magnitude of the genotype x environment interaction. Sadly, in most cases, this information rarely exists. The availability of experimental fields, greenhouses, laboratories and human and financial resources must be adequate so that the proposed goals can be achieved. It is also important to rely on appropriate seed production areas in order to increase the advanced populations and to produce the breeder seed of the new cultivars.

For the success of the genetic improvement program, it is critical to start with adapted, stable, high-yielding parental genetic material. All breeding programs aiming at obtaining commercial cultivars base their active germplasm bank on *élite* varieties with high forage yield, good persistence, multiple insect and disease resistance and appropriate fall dormancy rates. If necessary, exotic germplasm might be used as a source of rare alleles. In that regard, the

definition of a core collection – which consists in assembling a relatively short number of accessions that represent most of the variability present in a large germplasm collection, could be very useful. Therefore, to assess these fewer accessions can guide and facilitate the search for favorable alleles on traits having costly or complex evaluation. Basigalup et al. (1995) have identified an alfalfa core collection from the US perennial *Medicago* collection.

Breeding methods

The breeding methods define the way to conduct the selection, the selection units to be used and the subsequent management to be performed on the selected genotypes. There are several breeding methods that can be utilized for alfalfa. Based on mating systems and selection units (Rumbaugh et al., 1988) classified those methods into two large groups (Table 1): a) interpopulational mating, based on the concept of open breeding populations,

Table 1. Classification of the breeding methods most commonly used in alfalfa, according to mating systems (A and B) and selection units (*a* and *b*).

A- Interpopulation breeding
- 1- Population formation (strain building)
- 2- Synthetic varieties or synthetics
- 3- Backcrossing
- 4- Complementary cultivar (strain) crossing
- 5- Hybrids

B- Intrapopulation breeding
- a- Individual plant selection
  - 1- Mass selection or phenotypic recurrent selection
  - 2- Clonal evaluation
  - 3- Progeny testing
    - i) Open-pollinated
    - ii) Selfed (S_1)
    - iii) Topcross
    - iv) Polycross
    - v) Diallel crosses
- b- Family selection
  - 1- Half-sib families
  - 2- Full-sib families
  - 3- Within family selection
  - 4- Combined selection

Source: Adapted from Rumbaugh et al. (1988).

in which gene flow between populations is allowed and cross-pollination can be either natural (bees) or manual; and b) intrapopulation mating, which seeks to increase the frequency of favorable alleles within the same plant population. In this latter group, selection units can be individual plants, families of plants (full sibs or half sibs) or their combinations.

It is important to point out that the methods from Table 1 do not necessarily exclude each other and that they can be complementary, depending on the available resources and on the goals of the improvement program. For instance, mass selection or phenotypic recurrent selection can be followed by progeny testing or clonal evaluation of the selected genotypes.

Population formation is actually a general term including any form of strain building through crossing plants from different sources and selecting in subsequent generations. While minimizing inbreeding, the ultimate objective is to increase the frequency of favorable alleles for particular traits in the resulting population (Tysdal et al., 1942).

Backcrossing is used in alfalfa to repair some deficiency, usually susceptibility to a pest or disease, in otherwise agronomic valuable materials. To decrease the risk of inbreeding, it is advisable to employ several non-related recurrent parents in each backcross cycle (Stanford; Houston, 1954).

Clonal evaluation can help identifying superior genotypes when the traits to improve have low or moderate heritability and the genotype x environment interaction is high. However, it is being less and less used for its high cost and the amount of additional work it requires. In addition, cloning can affect the normal development, which may complicate assessing root traits.

Practically all commercial alfalfa cultivars in the world are synthetic varieties (also known as synthetics), which were defined by Busbice (1969) as the product of random mating between several parents, so that all possible crosses among them have equal probability of occurrence. Thus, formation of a synthetic variety starts with selection of individuals based on one or more traits; these selected parental plants constitute the Syn 0 generation. Subsequent generations (Syn 1, Syn 2 and so on) are the result of random mating of plants from seed produced in the precious generation. By following this procedure, it is possible to fix the traits that were selected for as well as preserving a considerable level of genetic variability, depending on the number of non-related parents in Syn 0 (Busbice, 1970). In this sense, it is common to arbitrarily distinguish between “narrow base” (< 100 parents) and “broad base” (> 100 parents) synthetics. As a result of the seed increase process, farmers usually plant advanced generations (Syn 3 to 5) of a synthetic variety. Kehr et al. (1961) demonstrated that the productivity of synthetics decrease

as the number of generations increases, especially between Syn 1 and Syn 2, until genetic equilibrium is reached; therefore, it is advisable to assess the productive potential of a synthetic variety in a Syn 2 or Syn 3 generation. Generally, broad base synthetics are more stable than narrow base sysnthetics.

Two breeding methods widely used in alfalfa are complementary crossing of cultivars (CCC) and phenotypic recurrent selection (PRS). The resulting populations from any of both can be used either as commercial varieties or as a breeding source for further improvement (Busbice et al., 1972). Regarding CCC, Busbice et al. (1972) demonstrated that if two cultivars having dominant genes at different loci at a frequency of 0.5 are crossed, the resulting population will have, at equilibrium, 46.7% of individuals with both dominant genes, 43.3% with only one dominant gene, and 10.0% with none. Cultivar (strain) crossing was used by Elgin Junior et al. (1983) to develop alfalfa populations with multiple pest resistance. On the other hand, and considering the remarkable amount of heterosis obtained when crossing unrelated and non-inbred parental cultivars, CCC may be a very important alternative for developing cultivars with higher forage yield (Bingham, 1983; Hill Junior, 1983).

In turn, PRS consists in a cyclic process of selecting desirable individuals by their phenotypes and later interbreeding them to produce the next generation. It can be considered as a refinement of mass selection because the pollen source is limited to only the selected individuals. Thus, by performing as many selection and interbreeding cycles as needed, the frequency of desirable alleles is increased in the population (Dudley et al., 1963; Eberhart et al., 1967; Hanson et al., 1972). To favor genetic advance and avoid inbreeding, it is important to evaluate populations of considerable size and to employ a quite large number of parents in each inbreeding generation; in the case of alfalfa, the minimum number of 75 individuals was suggested for each cycle (Aalders, 1966; Hill Junior et al., 1969). Even though PRS is usually more efficient to improve qualitative traits with high heritability, this method has also been successful to increase productivity of forage and other quantitative traits in some other forage species (Twamley, 1974).

Inbreeding depression and hybrid vigor manifested by alfalfa have stimulated the development of hybrid cultivars (Tysdal et al., 1942; Burkart, 1947). However, the development of alfalfa inbred lines is virtually impossible, due to the significant inbreeding depression shown by the species. As an alternative, Tysdal et al. (1942) proposed the use of vegetative propagation (clones) of four self-sterile plants, selected also for combinig ability; then, by allowing two of these clones to intercross in one field and the other two in a different field, two simple F_1 hybridswould be produced. Finally, by mixing the

same proportion of seed from each F_1 population and sowing it in another field, most of the harvested seed would be double cross hybrids. However, since there is no complete control of pollination, it is debatable whether this final product can be considered a complete hybrid or not.

The idea of employing male genetic sterility, which is only expressed in the homozygous recessive genotype and requires vegetative propagation of the male-sterile plants, has been considered uneconomic and not very practical (Viands et al., 1988). At the end of the 1960s, the employment of cytoplasmatic male sterility was suggested for developing commercial hybrids (Davis; Greenblatt, 1967; Bradner; Childers, 1968). Barnes et al. (1972) considered this method of pollination control as the most efficient and suggested a scheme to produce three-way hybrids: an A male-sterile clone (ms) is crossed to a B male-fertile clone (non-restoring-maintaining) and then this F_1 AB (ms) is crossed to a C clone, which is the parental pollen source and can be either a B or R (fertility-restoring) line. The way to obtain hybrid seed is to alternate in the field groups of each line and use bees as pollinators. However, the significant lower seed yield observed in the male-sterile lines, attributed to the physical isolation of male-fertile and male-sterile lines and to bee preference, had a major economic impact and restricted hybrid production by using this method (Viands et al., 1988).

In recent years, Dairyland Seed Co. (USA) released a few commercial alfalfa hybrids, using a procedure very similar to the one described by Sun et al. (2001). Since during the final step for producing the three-way [(AB) x C] hybrid, the seed is harvested bulk, it is claimed that there is a minimum of 75% of hybrid seed; thus, the hybrid composition is not the same throughout the production years. In addition, the tetraploid inheritance in alfalfa can complicate the use of fertility-restoring nuclear genes (Childers; Barnes, 1972). As an alternative to capitalize heterosis in alfalfa, Brummer (1999) proposed firstly the development of two independent heterotic groups, and secondly the combination of both in a seed production field; since half the progeny would be the result of interpopulation mating and the other half the result of intrapopulation crosses, the final product would be a “semi-hybrid”.

Within intrapopulational methods, the main difference is based on the selection units: individual plant selection or family selection. While in the first case only the best individuals are selected (either by phenotype or by progeny testing) and intercrossed; in the second case, the best families or the superior progenies (not parental genotypes) are selected and intercrossed to produce the next generation. Family selection, whether half-sib or full-sib, is more effective than mass selection for improving traits with heritability and high

genotype x environment interaction (Rumbaugh et al., 1988). Regarding within family selection, only the best individuals within each family are selected and intercrossed to produce the next generation. Finally, combined selection> complements both selections among and within families.

The use of progeny testing aims to identify superior genotypes through the evaluation of their offspring. Since these tests require more resources and an extra generation for the evaluation of the parents and more resources, they are almost exclusively recommended to improve low heritability traits with significant genotype x environment interaction. It is important to promote simultaneous blooming in the materials to evaluate and to use bees for pollination if large numbers of plants are used. Utilization of open-pollinated and polycross progeny tests allow the estimation of general combining ability for every evaluated individual. On the other hand, topcross and diallelcrosses make possible to estimate both general and specific combining ability. Considering the extra amount of work the two latter require, in particular diallel crosses, it is advisable to perform them in only advanced stages of the improvement program, when it is possible to rely on a small number of clones and when determining the specific combining ability is relevant. Using reciprocal crossings in diallel crosses also provides information on the maternal effects over the genetic control of some traits. Selfed (S_1) progeny tests can be very useful when working with traits in which additive genetic effects are important. At present, not too many commercial breeding programs use progeny tests; however, Basigalup et al. (2004) successfully used polycross testing to identify superior parental genotypes in the development of bloat tolerant alfalfa cultivar.

Selection for more than one trait

The most common situation that breeders face during the development of a cultivar is the need to improve more than one trait simultaneously. For that, some breeding methods, collectively known as “selection for multiple traits” were outlined. There are basically three procedures (Busbice et al., 1972; Rumbaugh et al., 1988), which are described as follows.

Independent levels of selection

In this method, also known as “independent culling”, a separate level to achieve is set for each trait to improve and then only the selection units that simultaneously are above those levels are retained for intercrossing (Rumbaugh et al., 1988). In order to avoid inbreeding, it is important that the source

population be large enough. An alternative to the previous scheme, used specially for developing populations with multiple insect and disease resistance, is the one called "successive ellimination". In this method, survivors to pest A are tested for pest B; the, survivors to pest B are screened for pest C, and so on. By using any of these methods, selection intensity for individual traits decreases when the size of the source population and the number of individuals to be retained for intercrossing are kept constant; however, if the traits are equally important and are genetically independent, the overall progress will be greater than the one obtained with selection for each trait individually (Rumbaugh et al., 1988).

Tandem selection

Every trait is separately improved, one at a time for one or more generations, in a tandem program, until the preset level for each one is achieved. The desirable level to achieve and the number of selection cycles can be different for each trait.

Index selection

Selection units are assessed for several traits, according to a previous scale set for each one. These values, weighed by their genetic or economic importance, are then integrated into a final score. Individuals or progenies which reach the highest levels of this weighed index are used to produce the next generation. In general, this process is more convenient when we are working with high heritability traits and when there is a relatively high genetic correlation among traits. Harris (1963, 1964) suggested the evaluation of large samples – not less than 1,000 individuals – to minimize errors in estimating the gains expected from the use of index selection.

Comparing the three procedures, Hazel and Lush (1942) concluded that index selection is more efficient than independent levels, and that in turn the latter is more efficient than tandem selection. Index selection is even better when heritabilities and genetic correlations among traits are high and environmental correlations are low (Rumbaugh et al., 1988). The advantage of index selection increases as the number of traits increases, but decreases when the traits have different importance or when the selection intensity is augmented. On the other hand, tandem selection is less demanding in resources and infrastructure. Therefore, the choice among methods should be based not only on the outlined technical issues but also on the available resources and the nature of the traits to improve.

Biotechnology applied to alfalfa improvement

Conventional genetic improvement uses the genetic variability already existing and is based on sexual reproduction, which narrows crossings to plants from the same species or from related species. In some cases, obtaining new cultivars through conventional techniques can be inefficient, both because of the time required and the slow progress achieved; in other cases, it can be directly impossible, because of the lack of genetic variability. In this context, biotechnology can facilitate plant breeding by overcoming the limitations of conventional methods (Ríos et al., 2007). In chapter 10, some of the biotechnological techniques applied to the genetic improvement of alfalfa are described.

Molecular markers

Molecular markers can be used for purposes as diverse as constructing genetic maps, characterizing the genetic variability of populations, defining heterotic groups, detecting codifying areas for quantitative trait loci (QTLs), assisted selection and characterizing cultivars through fingerprinting (Ferreira; Grattapaglia, 1998; Martin, 2002). In the genus *Medicago*, markers such as restriction fragment length polymorphism (RFLP) (Kidwell et al., 1994), random amplification of polymorphic DNA (RAPD) (Barcaccia, 1994; Brummer et al., 1995) and microsatellites or single sequence repeats (SSR) have been used, in addition to others (Diwan et al., 1997). In case of alfalfa, some of these markers have been used to develop a genetic map (Brummer et al., 1993), to map QTLs (Alarcón Zuñiga et al., 2004), to characterize the genetic variability among populations (Bonafede et al., 1999) and to study heterosis (Riday; Brummer, 2004). At INTA Manfredi (Argentina) microsatellites are being used to assist the selection for leaf disease resistance (Gieco et al., 2007).

Genetic transformation

Introducing transgenes into the alfalfa genome can be very useful when the objective is to improve traits for which there is no conventional genetic variability. In this sense, obtaining transgenic plants with resistance to insects and diseases, tolerance to herbicides, better forage quality or tolerance to abiotic stresses (drought, salinity, acidity, cold or heat) could have a major impact. A further step would be to improve more complicated traits, like nutritional quality, reduction of allergic compounds or the use of alfalfa for bioremediation or as a bioreactor to produce vaccines, enzymes, etc. A third

step would be to improve metabolic processes for manipulating plant structure, photosynthetic patterns, leaf senescence, and others.

At INTA Castelar (Argentina), Ríos et al. (2007) obtained transgenic experimental alfalfa plants highly resistant to Lepdoptera through the expression of Bt (*Bacillus thuringiensis*) genes. By using genetic manipulation, they also developed alfalfa plants overexpressing glucanase and chitinase genes for resistance to pathogenic fungi, and plants expressing condensed tannins to control bloat and to increase the proportion of by-pass protein. In addition, they reported as well the production of alfalfa plants expressing viral antigens that could control foot and mouth disease, bovine viral diarrhea and bovine rotavirus. In the USA, the company *Forage Genetics* has released several glyphosate-resistant (RR technology) alfalfa cultivars (Van Deynze et al., 2004).

Incorporation of transgenes

Another practical implication of autotetraploidy on alfalfa improvement is referred to transgene introgression into agronomic superior populations for the development of commercial cultivars. The typical transgenic variety of a diploid species has a single copy of a transgene coming from a unique transformation event, which is present in heterozygous condition in just one unique location in the genome (example: A-). The way to incorporate this genetic material (To) into improvement programs is to backcross it to elite lines and then self-fertilize these plants until homozygosity (AA) is reached; if these homozygous lines are used to produce F_1 hybrids or cultivars, all the plants resulting from these crossings will have the transgenic phenotype (A- or AA).

In alfalfa, because of its autotetraploid condition, obtaining relatively high levels (> 90%) of transgene incorporation requires the interbreeding of parental genotypes which have the transgene in duplex (AA--), triplex (AAA-) or quadruplex (AAAA) condition. Such individuals can be developed through phenotypic recurrent selection cycles, requiring in addition the use of laboratory techniques precise enough to help discriminating this type of plants. Consequently, it is also necessary the use of progeny tests that allow identification of individuals having the desirable genotypes. However, besides the extra work this procedure implies, selection and progeny tests increase the risk of generating significant inbreeding depression or genetic drift (Samac; Temple, 2004). An alternative to overcome these difficulties and to maximize expression of transgenic traits in autopolyploids is the method suggested by McCaslin et al. (2002). This method is based on molecular marker assisted selection and the use of multihomogenic plants, term that describes

the presence of more than one copy of a particular transgene in several independent loci along the genome of an individual plant. Plants that have at least one copy of a given transgene coming from two independent events are named “dihomogenic”. For instance, a plant which has a transgene in simplex condition in locus A and duplex in locus B is called “dihomogenic 1,2”, resulting in genotype A---BB--. These dihomogenic plants are then used in a genotypic recurrent selection process to introgress the transgenic trait of interest into the original material. The method described has been successfully used to develop glyphosate-resistant (RR) alfalfa transgenic populations (Temple et al., 2002). In this particular case, four experimental lines were used, each one containing one simple copy of the transgene coming from four independent events (A, B, C and D). For each one, the position of insertion was determined through a technology of event-specific polymerase chain, in which a primer binds to the flanker sequence of region 5’ or 3’ of the transgene, while the other primer binds to the corresponding flanker region of the plant genome. Hence, thousands of transgenic plants could be assessed, until the dihomogenic genotypes were first detected and then subsequently used as parents in each cycle of the genotypic recurrent process. In addition, a computer model to predict the purity and the inheritance of the dominant transgene for an autotetraploid system with two independent transformation events was developed (McCaslin et al., 2002).

Improvement for resistance to pests and diseases

To improve the level of resistance to pests and diseases, it is essential to identify and keep the plants from the population which has the resistance genes. Then, these plants are interbred to obtain the seed which will give rise to the new population with a higher frequency of resistant alleles. In such a process, it is critical to use a large enough number of plants to minimize inbreeding and to preserve genetic variability for other desirable traits (Elgin Junior et al., 1988). It is also important to know the characteristics of the pathogen, such as growth requirements, life cycle, hosts, geographical distribution, existence of races or biotypes, and economic importance.

To increase the efficiency of the breeding process it is necessary to develop selection protocols, which are a combination of laboratory, greenhouse and field procedures for the adequate identification of the resistant genotypes. The purpose is to generate the best environmental conditions that favor optimal pathogen development and plant symptoms characterization. In addition to allowing the evaluation of a large number of genotypes in a short period of time, an ideal protocol must be simple, stable, reproducible, objective,

effective and precise. It is also very important the existence of a high correlation between genotype behaviour under the selection conditions and later under field conditions. Complementarily, it is convenient to determine if genotype resistance is stable throughout environments and different stages of development (seedling, juvenile or adult plants).

The *North American Alfalfa Improvement Conference* (NAAIC) published a complete series of standardized tests set to characterize resistance levels to the main insects and diseases of the crop. For each pest, pathogen growth requirements, test conditions and resistant /susceptible controls are specified (North American Alfalfa Improvement Conference, 2005).

Resistance to diseases

Phenotypic recurrent selection has been the method most successfully used for developing alfalfa cultivars resistant to diseases. Another useful procedure is the complementary crossing of cultivars (Elgin Junior et al., 1983). Backcrossing has been used in a few cases (Murphy; Lowe, 1966; Peaden et al., 1976), but at present is rarely performed; however, it has some potential for the introgression of transgenes into elite populations. In most cases, resistance to diseases is conditioned by one or a few genes with a variable degree of dominance (Elgin Junior et al., 1988); as a consequence, response to selection is expected to be usually quite fast, depending on the gene frequencies. Another favorable aspect of alfalfa is that genes for resistance to one disease are not usually linked to the ones for another. The appearance of new pathogenic races in alfalfa is insignificant for most pathogens, recording just a few exceptions for *Colletotrichum trifolii* (Welty; Mueller, 1979) and *Peronospora trifoliorum* (Stuteville, 1973).

Resistance to insects

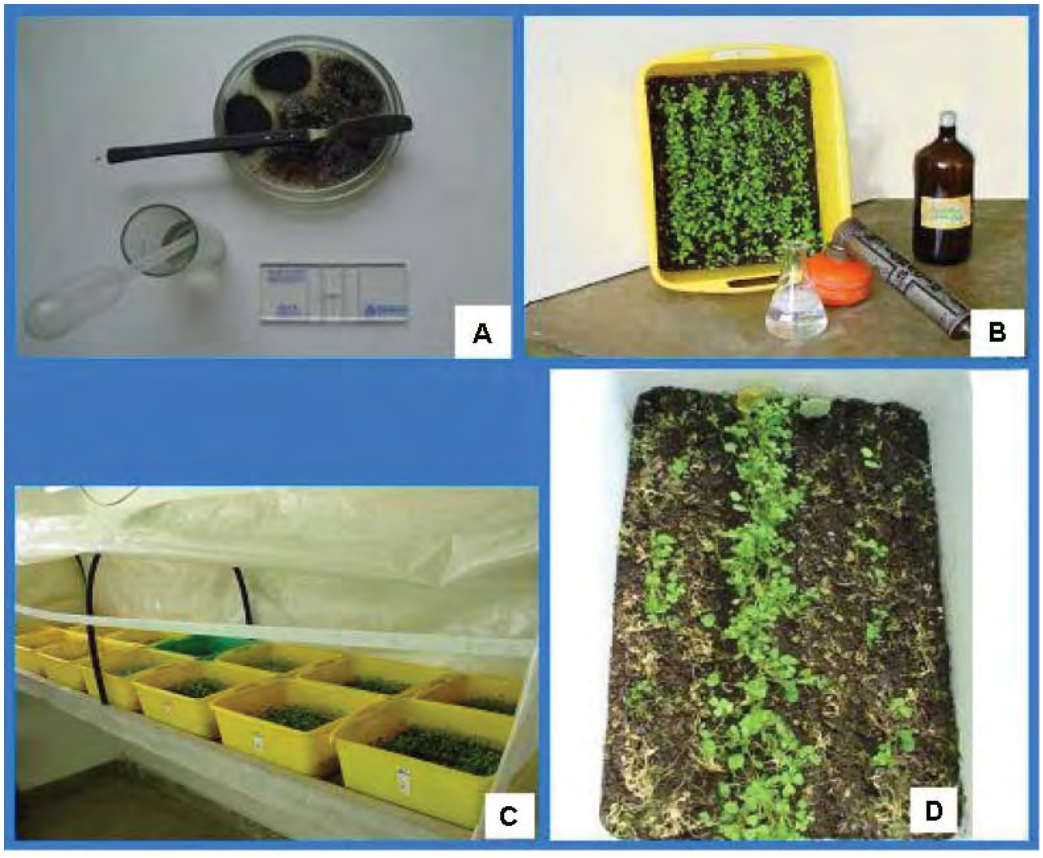
Resistance to insects is usually conditioned by a number of complex insect x host x environment interactions. Sorensen et al. (1988) assorted plant resistance into five categories: high resistance, intermediate resistance, low resistance, susceptibility and high susceptibility. They also defined four additional functional resistance categories: a) pseudoresistance, which is a temporary resistance in potentially susceptible plants and that includes escape, evasion and induced resistance; b) hypersensitivity, which is a fast response by the plant expressed through the premature necrosis of the affected tissue; c) adult plant resistance; and d) juvenile or seedling resistance.

In turn, Painter (1951) classified plant resistance mechanisms to insects as antibiosis (plant compounds that affect insect life), tolerance (plant ability to stand high insect populations with no economic damage) and nonpreference (insect rejection of plants for oviposition, feeding or shelter). Later, Kogan and Ortman (1978) proposed to replace nonpreference for the term antixenosis, referring to plant characteristics (color, hairs, wax, lignin, etc.) that affect insect behavior. Phenotypic recurrent selection is the most utilized breeding method for developing alfalfa cultivars with insect resistance. When multiple resistance is required, many breeders use tandem selection or independent culling. Complementary crossing of cultivars has also been successfully utilized by some breeding programs (Sorensen et al., 1988).

Alfalfa improvement program in Argentina

The INTA Manfredi alfalfa genetic improvement program aims at obtaining commercial cultivars adapted to the main alfalfa areas of Argentina. It is focused on developing varieties with fall dormancy (FD) rates 6 to 10, having high forage yield, good persistence and high multiple aphid and disease resistance. Breeding process starts with selection of genotypes that were able to survive for several years in the field under grazing or cutting conditions either on commercial or experimental stands. Selected genotypes are transplanted to breeding nurseries and evaluated for several agronomic traits. Complementary crossing of cultivars are also used as a way of generating new genetic combinations for subsequent selection and evaluation. Following NAAIC protocols, phenotypic recurrent selection is utilized to select for resistance to anthracnose (*Colletotrichum trifolii* Bain & Essary) (Figure 1), Phytophthora root rot (*Phytophthora megasperma* Drechs. f .sp. *medicaginis*) (Figure 2), blue alfalfa aphid (*Acyrthosiphon kondoi* Shinji) (Figure 3) and spotted alfalfa aphid (*Terioaphis trifolii* Monnell) (Figure 3).

When performing genotype field selection, only those vigorous, healthy plants with solid crowns are retained. Individuals affected by corky root (*Xylaria* spp.), crown and root rot complex (*Fusarium* spp., *Phoma* spp., *Rhizoctonia* spp., *Colletotrichum* spp., etc.), or leaf diseases are eliminated. Selected plants grouped by FD rates, are transplanted at INTA Manfredi into pollination cages with honey bees (*Apis mellifera*) and cross-pollinated to produce the breeder seed (Syn 1) of the new synthetics. About 100 synthetics are originated every year. Every fall, breeder seed of approximately 95 synthetics is sent to INTA San Juan to produce the basic seed (Syn 2). INTA San Juan is located in Western Argentina and possesses more adequate environmental conditions for producing

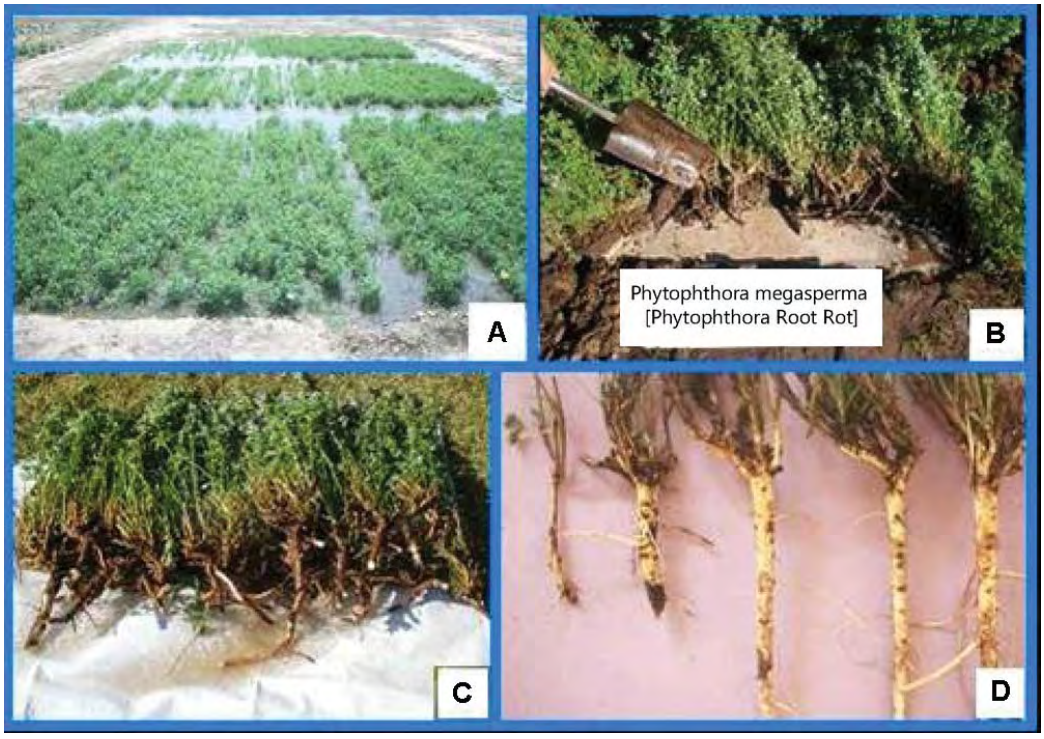


Photos: Daniel Horacio Basigalup and Ariel Sebastian Odorizzi

Figure 1. Selection protocol for resistance to *Colletotrichum trifolli*: fungus in a growth media and spore counting for inoculum preparation (A); spray of seedlings with spore suspension (B); moist chamber (24 to 72 hours) to favor infection (C); and identification of resistant genotypes (R) (D).
Source: Adapted from NAAIC (2005).

higher yield of quality seed. Evaluation of forage yield and persistence of the advanced synthetics (Syn 2 or Syn 3) is performed under cutting conditions in an internal network, including three years of assays in six locations (four under rainfed and two under irrigation conditions). The outstanding synthetics in all locations are characterized and registered as new cultivars. Tables 2 and 3 summarize the levels of forage production that these cultivars have achieved in the evaluation of INTA Alfalfa Cultivars Network.

Since 1987, INTA's alfalfa breeding program is being carried out under a joint venture with different Argentine private seed companies. By financing the activities and paying royalties to INTA, companies have the exclusive license for cultivar multiplication and commercialization. In the period of 2004–2008, in



Photos: Daniel Horacio Basigalup and Ariel Sebastian Odorizzi

Figure 2. Selection protocol for resistance to *Phytophthora megasperma*: growing plants on infested soil under high humidity (A); plant extraction (B); root cleaning (C); and classification of plants into susceptible (first three plants from the left) and resistant (last two on the right) categories according to root symptoms (D).
Source: Adapted from NAAIC (2005).

association with Produsem SA, the program has released five cultivars: ProINTA Luján (FD 6), ProINTA Carmina (FD 8), ProINTA Patricia (FD 7), ProINTA Super Monarca (FD 8) and ProINTA Mora (FD 9). Some agronomic evaluation data of these cultivars are presented in Tables 2 and 3. Since 2010, in association with Palo Verde SRL, three more cultivars were released: Pulmari PV INTA (FD 6), Traful PV INTA (FD 9) and Limay PV INTA (FD 9).

Improvement for specific traits

In addition to breeding for higher forage yelds and multiple pest resistance, it is also necessary sometimes to improve other important traits. In Argentina, three of such traits are reduced bloat potential and tolerance to salty or acidic soils. In this section, selection procedures for these three traits will be briefly discussed.

Photos: Daniel Horacio Basigalup and Ariel Sebastian Odorizzi

Figure 3. Selection protocol for resistance to *Acyrthosiphon kondoi* and *Terioaphis maculata*: raising aphids on alfalfa stem in a growth chamber (A); seedling growing and aphid sprinkling on seedlings under controlled conditions (B and C); and identification of resistant (R) and susceptible (S) genotypes (D).
Source: Adapted from NAAIC (2005).

Bloat

Based on comparisons between forage legume species that do and do not cause foamy bloat, Howarth (1988) concluded that the development of a bloat safe/tolerant alfalfa could be obtained by: a) synthesis of condensed tannins in leaves and stems through gene manipulation or transgenesis; or b) selection of genotypes with lower initial rate of dry matter disappearance (IRDMD) in the rumen. In the first approach, the antifoaming activity of condensed tannins prevents formation of stable foam in the rumen, so that the gas produced by microbial fermentation is not retained as micro-bubbles and can be expelled by eructation (McMahon et al., 2000; Gruber et al., 2001). The second approach is based on the so called the "cell rupture theory" (Howarth et al., 1978), which established that plants with lower IRDMD have thicker cell walls that delay cell

Table 2. Accumulated forage yield (t ha^{-1}) of moderately dormant (FD 6-7) alfalfa cultivars in four locations of Argentinian Pampas Region. Data are from INTA's national alfalfa cultivar evaluation network. Forage production was evaluated under rainfed conditions.

Cultivar	Marcos Juárez	Manfredi	Rafaela	Anguil	Σ
	2002–2006	2002–2005	2002–2006	2002–2006	
ProINTA Patricia	110.56 a[(1)]	47.39 a	67.99 a	29.66 a	255.60
ProINTA Luján	107.69 a	45.07 a	49.40 a	32.27 a	234.43
WL 442	103.36 a	42.02 b	58.39 a	26.23 a	230.00
Candombe	102.81 a	39.35 b	55.26 a	30.29 a	230.00
5683	91.16 b	43.19 b	56.64 a	29.33 a	220.32
Gala	96.57 b	38.81 b	49.40 a	27.68 a	212.46
Victoria SP INTA	87.74 b	42.09 b	45.63 a	25.11 a	200.57
Tango	88.16 b	34.33 c	48.62 a	28.75 a	199.86
S 711	78.11 c	33.22 c	41.96 a	27.28 a	180.57
Key II	63.05 d	30.67 c	33.07 b	22.32 a	149.11
Mean	**92.92**	**39.61**	**51.17**	**27.89**	**211.26**
CV (%)	**5.5**	**8.3**	**21.1**	**14.2**	

[(1)] Values followed by the same letter do not significantly differ (DGC α = 0.05);
DM = dry matter; VC = variation coefficient.
Source: Di Rienzo et al. (2001) and Spada (2006).

disruption by rumen microflora and then slow down the rate of gas production from fermentation to levels that do not exceed the required threshold to cause bloat (Howarth et al., 1982a, 1982b).

Using the second approach, INTA Manfredi initiated in 1991 a breeding program to develop a bloat tolerant alfalfa cultivar. The in situ procedure of the "modified nylon bag technique" (Howarth et al., 1982a) was used to identify plants with lower IRDMD after 4 hours in the rumen of fistulated steers. The improvement method combined phenotypic and genotypic (polycross progenies tests) recurrent selection (Basigalup et al., 2004). In each selection cycle,

Table 3. Accumulated forage yield (t ha^{-1}) of nondormant (FD 8-9) alfalfa cultivars in four locations of Argentinian Pampas Region. Data are from INTA's national alfalfa cultivar evaluation network. Forage production was evaluated under rainfed conditions.

Cultivar	Marcos Juárez	Manfredi	Rafaela	Anguil	Σ
	2004–2008	2004–2007	2004–2008	2004–2008	
Cautiva	88.20 a[(1)]	29.99 b	70.50 a	15.61 b	204.30
ProINTA S. Monarca	91.34 a	32.85 b	62.44 a	16.19 b	202.82
Bacana	88.65 a	31.90 b	66.09 a	15.00 b	201.64
ProINTA Mora	88.39 a	32.40 b	59.49 b	19.04 a	199.32
5939	80.98 b	33.43 b	61.91 a	17.27 a	193.59
Baralfa 9242	83.36 b	33.13 b	62.02 a	14.17 b	192.68
Monarca SP INTA	82.50 b	27.78 b	63.23 a	18.01 a	191.52
969	84.72 a	30.67 b	61.50 a	14.05 b	190.94
Armona	78.24 b	29.52 b	62.37 a	15.06 b	185.19
Maricopa	79.18 b	30.61 b	61.43 a	12.68 b	183.90
Villa	83.39 b	29.80 b	52.82 b	17.65 a	183.66
Yolo	80.65 b	29.94 b	56.23 b	14.89 b	181.71
Exp. 1048	84.16 b	30.99 b	45.15 b	14.89 b	175.19
Medina	81.62 b	25.62 b	46.69 b	17.37 a	171.30
Franca	76.02 b	23.19 b	44.15 b	20.43 a	163.79
ZZ 809S	75.69 b	25.53 b	46.76 b	13.58 b	161.56
MH RD1-SS	79.01 b	25.76 b	36.02 c	15.91 b	156.70
Bar 814	73.92 b	24.48 b	30.27 c	15.63 b	144.30
Mean	**82.85**	**30.30**	**58.49**	**15.97**	**187.61**
CV (%)	**4.2**	**19.4**	**10.3**	**12.1**	

[(1)] Values followed by the same letter do not differ significantly (DGC α = 0.05);
DM = dry matter; CV = coefficient of variation.
Source: Di Rienzo et al. (2001) and Spada (2008).

between 1,200 and 1,850 genotypes were assessed for IRDMD twice a year: spring and fall. About 3% to 4% of plants with the lowest IRDMD were selected in each cycle and then interbred in pollination cages with honey bees. In 2006, and after three cycles of selection, the nondormant (FD 8), bloat-tolerant cultivar ProINTA Carmina was released to the market. Compared to the original population, ProINTA Carmina reduced the IRDMD by 22.6%. Before release, the new cultivar was extensively tested under grazing conditions at different INTA experimental units between 2003 and 2006 (Basigalup et al., 2007). The trials used a rotational grazing system in which steers were "challenged" to graze alfalfa under highly bloating conditions, i.e. fasting animals and late vegetative stage of plant development. Bloat was estimated on a 0 (no bloat) to 5 (treatment or death) visual scale. In almost all locations, ProINTA Carmina presented higher frequencies of cases with no bloat (0) and lower frequencies of slight to moderate bloat (1−3) than the check cultivar. Overall, the steers grazing on ProINTA Carmina showed less ($p<0.05$) bloat incidence than the control treatment and these differences were consistent over time. In another study (Bernaldez et al., 2009), it was conclucled that ProINTA Carmina reduced bloat occurrence ($p<0.01$) by 25.2% compared to the check variety.

In addition, larger grazing trials were also performed at the farmer level in commercial beef operations using a similar visual scale (1 = slight bloat, 5 = treatment or death) to the previous one in order to estimate degree of severity in only bloates animals. Two of these essays were carried out at the ranch "La Angelita" (Buchardo, Córdoba). The first one was sown in March of 2006 and included two 25-ha paddocks: one planted to ProINTA Carmina and the other to the check variety. In both cases, alfalfa (7.5 kg ha^{-1}) was associated with *Festuca arundinacea* Schreber (3 kg ha^{-1}) and *Bromus catharticus* Vahl. (3 kg ha^{-1}). Each paddock was grazed by 100 steers (280 kg average individual weight) in a rotational grazing for 3.5 months. ProINTA Carmina not only presented higher frequencies of animals with no bloat but also a significant decrease of steers with moderate to severe bloat (Figure 4). The second assay was established in April, 2007, in two 48-ha paddocks seeded with a forage mixture similar to the previous one. Each paddock was grazed by 250 steers (230 kg average individual weight) in a rotational grazing for 2.5 months. Results are depicted in Figure 5 and clearly show the effect of ProINTA Carmina on bloat incidence decrease.

Because of a possible unfavorable effect that the selection for thicker cell walls might have on alfalfa digestibility and animal intake, an assay to evaluate forage quality under cutting conditions was conducted at INTA Manfredi. The goal was to compare digestibility and crude protein (CP) and fiber (ADF and NDF) content between cultivars ProINTA Carmina and Bárbara SP INTA (check)

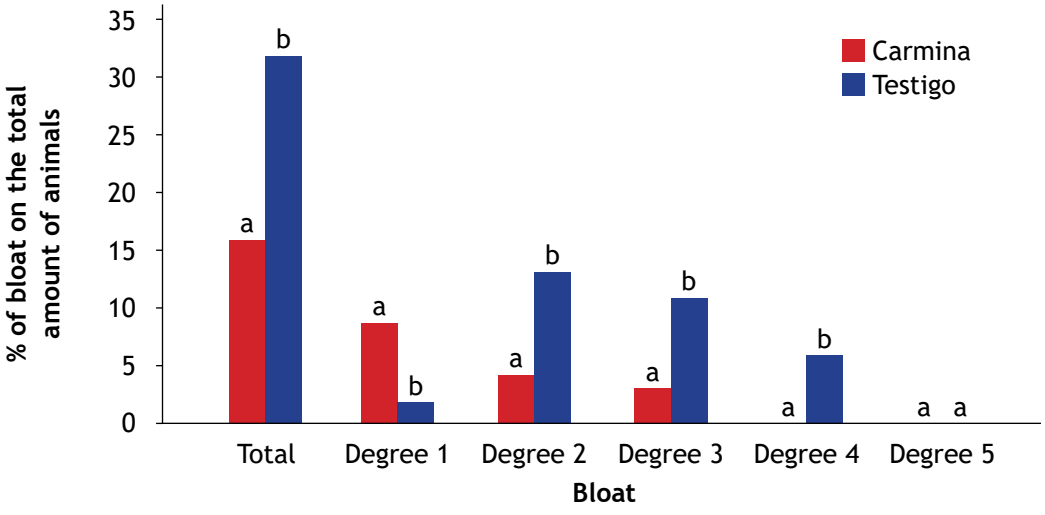


Figure 4. Frequency (%) of bloated steers according to a 1 (slight bloat) to 5 (treatment or death) visual scale used to estimate degree of severity in only bloated steers at Ranch "La Angelita" (Buchardo, Córdoba, Argentina). Trial was conducted for 100 days from October 2006 to February 2007. Bars with the same letter are not significantly different (Kruskal-Wallis, $\alpha = 0.05$).

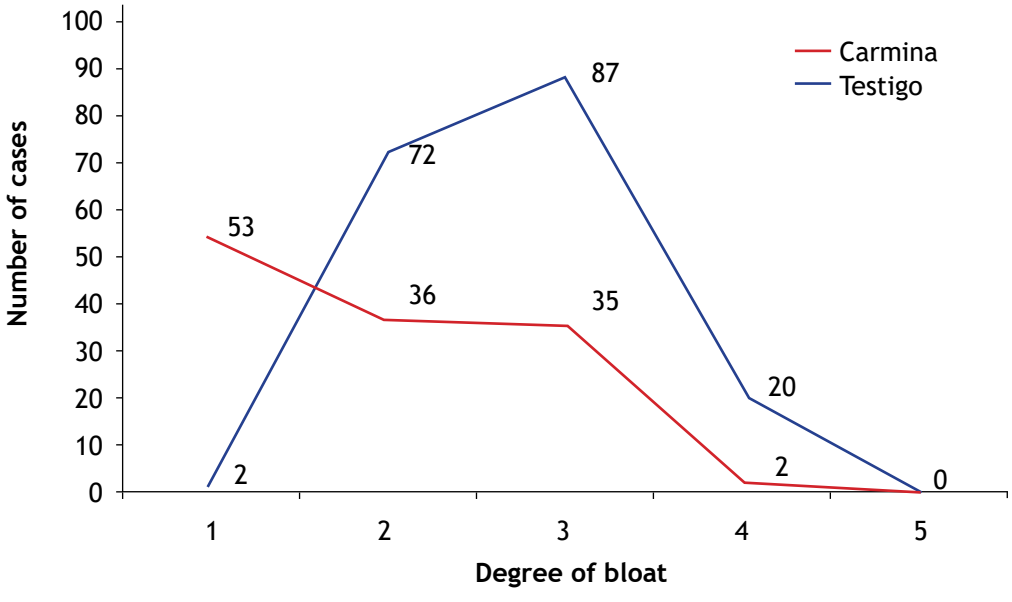


Figure 5. Frequency (%) of bloated steers according to a 1 (slight bloat) to 5 (treatment or death) visual scale used to estimate degree of severity in only bloated steers at Ranch "La Angelita" (Buchardo, Córdoba, Argentina). Trial was conductedfor 75 days during the 2007/2008 growing season.

at three stages of development: late vegetative, late bud and 10% blooming. No differences were detected between cultivars at late vegetative and 10% blooming (Table 4). However, at late bud stage, ProINTA Carmina exhibited lower CP and higher fiber content than the control ($P<0.05$) (Basigalup et al., 2007). This was somewhat expected since selection for lower IRDMD (and then for thicker cell walls) was performed at this stage of development, and therefore could be taken as an indirect indication of response to selection.

Considering all the available evaluation data, it was estimated that ProINTA Carmina was able to decrease bloat incidence by 23.75% (general mean), with a 5%–52% range. Higher fiber content at late bud stage did not impact on animal production (data not shown). Therefore, the cultivar can make an important contribution to diminish bloat under the grazing conditions of Argentina. Nevertheless, in order to achieve higher control efficiency, it is recommended to use ProINTA Carmina along with other prevention measures, such as constant animal supervision, avoiding animal fasting, and not grazing alfalfa at very immature stages.

Tolerance to aluminum and to soil acidity

For many areas of the world, and especially for the tropics, it is important to develop alfalfa cultivars which are tolerant to Al toxicity and acidic soils. Different methodologies have been proposed to identify Al tolerant plants in many species of agricultural importance. Most of those methodologies

Table 4. Comparison of forage quality (average of 12 cuts) between alfalfa cultivars ProINTA Carmina and Bárbara SP INTA at three stages of development. Trial was conducted during the 2002/2003 and 2003/2004 growing season at INTA Manfredi.

Stage of development	Cultivar	IVDMD[1] (%)	NDF (%)	ADF (%)	CP (%)
Late vegetative	Carmina	65.20 a[2]	28.62 a	20.74 a	26.15 a
	Bárbara	66.31 a	28.27 a	19.68 a	26.78 a
Late bud	Carmina	62.00 a	32.70 a	25.60 a	23.78 a
	Bárbara	62.60 a	30.72 b	22.79 b	25.73 b
10% Blooming	Carmina	60.81 a	31.62 a	24.21 a	22.73 a
	Bárbara	61.17 a	31.62 a	23.96 a	23.56 a

[1] IVDMD = in vitro dry matter disappearance; NDF = neutral detergent insoluble fiber (Van Soest); ADF = acid detergent insoluble fiber (Van Soest); CP = crude protein (Kjedahl).

[2] Within each stage, values followed by the same letter do not differ statistically (LSD, $\alpha = 0.05$).

are focused on measuring inhibition of root growth, Al accumulation in the root, or biomass production of plants growing in a solution with variable Al concentrations. In vitro procedures for assessing cell development in a growth media containing Al have also been proposed (Samac; Tesfaye, 2003).

Taylor (1991) proposed assorting the strategies to search plant tolerance to Al into two large groups: a) mechanisms which exclude Al from the root apex; and b) mechanisms which allow the plant to tolerate Al within the cells. Strategies from the first group are based on the fact that tolerant cultivars of some species exude organic acids – such as citrates, oxalates, malates and succinates – which chelate Al and exclude it from the root apex (López-Bucio et al., 2000; Ma, 2000; Ma et al., 2001). Other mechanisms of Al exclusion have also been described, such as the mutant *alr*-104 of *Arabidopsis*, which increases the inflow of H^+ to the root apex, which in turn causes a pH increase in the rhizosphere zone that precipitate Al in the soil solution, making it unavailable for the roots (Degenhardt et al., 1998). In other cases, the presence of mucilaginous compounds around the root apex constitutes a physiscal barrier that prevents contact with Al (Henderson; Ownby, 1991; Li et al., 2000). Among the strategies from the second group, some cases in which Al is sequestrated by organic bonding agents (catechins, phenolic acids, etc.) that form complexes that accumulate into specialized cells of the leaf epidermis (Jensen et al., 2002) or into vacuoles in root tissues (Vasquez et al., 1999) have been characterized. In general, mechanisms from the second group were not as thoroughly studied as the ones from the first group.

For the case of alfalfa, Devine et al. (1976) proposed the use of plastic pots filled with acidic soil and Al toxic concentrations to compare plant growth and then to identify tolerant genotypes. This procedure is simple and allows assessing the plants in juvenile stage, when root development is important for the establishment of the crop; however, acid soils with similar pH can significantly vary in Al saturation thresholds. A variant to the previous method is to grow plants directly on acidic soil; however, its use is limited to only locations where these soils are available. To overcome this problem, Villagracia et al. (2001) proposed to grow plants in sand and to irrigate them with solutions that provide, in addition to all the necessary nutrients, the required values of acid pH and Al toxic concentrations. On the other hand, Voigt and Godwin (1997) indicated that in species with small seeds, like alfalfa, the critical moment is crop establishment: consequently, in order to evaluate germination and first seedling growth on acidic soil with high levels of Al, they proposed to spread a thin layer of acidic soil over a thicker agar layer: the plantlets which reach agar in less time are deemed tolerant. This technique was effectively

employed for evaluating acid tolerance of several legume forage species (Voigt; Mosjidis, 2002).

Growing plants in nutritive solutions aiming at discriminating Al tolerant genotypes has also been proposed for alfalfa (Baligar et al., 2002). In general, plants are first kept in a low pH solution, and then they are placed in another solution with toxic levels of Al; after some time in these conditions, root growth is measured and this value is related to root development of the control treatment (without Al), through the estimation of the Al(+)/Al(-) ratio. The method is quite fast and allows comparing a large number of genotypes in a short time and in a small physical space; however, it has the inconvenience that, when contrasting the treatment/control ration, those plants which naturally grow more slowly than others may be deemed as more tolerant than they actually are. On the other hand, it is difficult to identify the ideal Al concentration in the media, as well as keeping it throughout the whole evaluation or selection period. In addition, root exudates constantly alter the media pH and Al can form complexes with various nutrients, limiting their availability. As an alternative to measuring root growth in nutritive solution, Giaveno and Miranda (2000) proposed to utilize pigments to identify the Al tolerant plants. In short, the method consists of treating the seedling with an acid + Al solution, washing the excess of Al with water, and then coloring the roots with a solution of hematoxylin (0.2%) + $NaIO_3$ or KI (0.02%): tolerant plants do not stain or do it in a very little extent. The main advantages of this method are its high sensibility to detect Al concentrations even before root growth is inhibited and its low cost and non-destructive nature, which enables it to be used in a breeding program. But it has two important drawbacks: a) it measures tolerance in more qualitative than quantitative terms; and b) it does not take into consideration that not all genotypes can exclude Al with the same speed, which can lead to elimination of potentially tolerant plants under different conditions. Overall, it can be concluded that all methods based on evaluation in nutritive solutions (with or without pigments) are effective to identify Al tolerance; however, only in a few cases there is an acceptable correlation between the tolerance observed under experimental conditions and the one detected on acidic soils (Samac; Tesfaye, 2003).

The use of in vitro selection methods is an interesting tool, not only to identify genotypes tolerant to acidity + Al, but also to study the response at cell level. An additional advantage is the possibility of creating somaclonal variants which might contribute to increase genetic variability for the trait (Miller et al., 1992; Foy et al., 1993). Similarly to the use of nutritive solutions, one of the main inconveniences of in vitro techniques is to obtain a culture

media with the appropriate and stable phytotoxic Al concentration throughout the evaluation period. On the other hand, utilization of in vitro selection in improvement programs assumes that the identified Al tolerance at cell level is also expressed later on at plant level. In alfalfa, Parrot and Bouton (1990) observed that tolerance to Al was expressed both in cell cultures and in plants, and that calli from Al tolerant genotypes at cell level displayed better selection gain than those produced without a preliminary selection for Al tolerance. In the same way, Kamp-Glass et al. (1993) proposed for alfalfa the use of a culture media with toxic concentrations of Al to induce calli formation and then the development of tolerant embryos and subsequently tolerant plants.

To developed alfalfa germplasm tolerant to acidic soils with or without toxic Al levels, Dall'Agnol et al. (1996) compared the following evaluation techniques: a) selection in tubes with acid soil (pH_{water} = 4.7; Al_{KCl} = 0.29 cmol kg^{-1}; Ca = 0.283 cmol kg^{-1}; P = 7 kg ha^{-1}); b) selection in tubes with acid soil + a superficial layer of alkalinized and fertilized soil (pH_{water} = 6.5 ; Al_{KCl} = 0.0 cmol kg^{-1}; Ca = 1.80 cmol kg^{-1}; P = 72 kg ha^{-1}); c) divergent selection of calli grown on media with and without Al (Al^{+}/Al^{-} ratio); d) tandem selection combining methods "a" (first step) and "c" (second step); and e) tandem selection combining methods "b" (first step) and "c" (second step). These five populations were then evaluated under greenhouse conditions on three support media: acid soil, soil amended with calcium carbonate, and acid soil + a superficial layer of soil amended with calcium carbonate. On acid soil, most of the experimental populations showed greater root growth and higher forage yield than the original population (without selection), but only population "a" (selected on acid soil alone) exhibited greater development than the others on acid soil + a superficial layer of soil amended with calcium carbonate. On soil amended with calcium carbonate, no population had lesser development than the original population. Inclusion of *in vitro* selection (methods "c", "d" and "e") did not improve tolerance to Al. In terms of success and of time and resources requirements, the authors concluded that direct selection in acid soil was the most effective way to develop alfalfa varieties tolerant to acidity and Al toxicity. Applying direct selection on acid soil, Bouton and Radcliffe (1989) developed the alfalfa germplasm GA-AT, which displayed higher growth and nodulation than the control when both were sown on acidic soil (pH = 4.6) with 32 µg g^{-1} Al concentration (Hartel; Bouton, 1989). However, forage yield of GA-AT on acid soil, compared to the yield on soil amended with calcium carbonate, resulted in unacceptable value from the agricultural point of view, so that the authors concluded that greater tolerance levels to acidity would

be necessary to reach an economically viable yield under acidic soil conditions (Bouton; Radcliffe, 1989).

With the goal of widening the search for genetic variability for acidity tolerance, Bouton (1996) evaluated 200 accessions from the core collection of US perennial *Medicago* germplasm collection using a mix of acid soil + soil amended with calcium carbonate and fertilized with different elements (superficial layer). As tolerance measure, he used the dry weight of roots which were able to penetrate the sub-superficial layer of acid soil in relation to the tolerant control (GA-AT). Assuming the core collection is an adequate representation of the total variability existing in a large germplasm collection, it was concluded that detecting sources of tolerance to acidity and Al toxicity in the whole collection would be very unlikely. Therefore, the development of alfalfa varieties tolerant to acidity and Al toxicity seems difficult in the near future through conventional breeding. In addition, the autotetraploid inheritance in alfalfa and the restrictions imposed by its extreme sensitivity to inbreeding can mask tolerance expression. In this context, the employment of biotechnological techniques offers interesting possibilities for solving the problem. Sledge et al. (2002), using RFLP analysis in F_2 populations and in backcrosses, were able to identify QTLs related to Al tolerance in diploid alfalfas; this would obviously facilitate selection and achievement of tolerant varieties in cultivated alfalfa. Developing transgenic constructions which increase expression of tolerance genes induced by Al presence or that increase production of organic acids which exclude Al from the root apex also constitutes an alternative way for the future. Tesfaye et al. (2001) reported production of transgenic alfalfa plants that overexpressed the enzyme malate dehydrogenase in the root apex, which increased organic acids exudation by seven times, decreasing Al concentration within alfalfa cells. In a later study, Tesfaye et al. (2003) indicated that the higher quantities of organic acids exudated by transgenic alfalfa roots positively influenced not only diversity and activity of the rhizosphere microflora, but also the availability of macronutrients and micronutrients for the plants.

Salinity tolerance

According to Smith (1994), three phases of development under salinity conditions can be identified in alfalfa: a) germination, which includes from seed hydration until cotyledons emergence; b) seedling development, which takes about 20–40 days and comprises from hypocotyl elongation and cotyledons expansion until the beginning of secondary stems development; and c) mature

plant, which goes from secondary stems development to forage harvesting and later regrowth. Based on several studies which analyzed alfalfa germination under salinity conditions, both the percentage germination and the germination rate are decreased at salt concentrations ≥150 mM of NaCl, and none or very little germination is observed in salt-osmotic stress levels between 300 and 500 mM of NaCl. There are relatively few studies regarding the response of alfalfa to salinity during crop emergence and establishment. McKimmie and Dobrenz (1987) observed that around 75% of the emerged seedlings survived for two weeks when flood irrigated with water containing 243 mM of NaCl, and that only 13% survived to a concentration of 289 mM of NaCl. Salinity symptoms are basically the same in seedling and in mature plants: a) under low stress (< 100 mM of NaCl), only above-ground biomass yield is reduced (less and shorter stems); b) under intermediate stress, there is a growth reduction accompanied by foliolate discoloration in young plants, which is associated to greater leaf and stem succulence (Smith, 1994) or to dark-green/bluish-green color along with an increase in leaf:stem ratio in older leaves (Hoffman et al., 1975); and c) under high stress, leaves show necrosis in the margins or chlorosis, followed by older leaves drop (Smith, 1994).

In alfalfa, McKimie and Dobrenz (1991) detected phenotypic variation for plant survival and plant growth under salty conditions. In tolerant genotypes, Na and Cl contents were lower in leaves and stems and tended to accumulate in roots. In general, salinity tolerance appears to be related to lower ionic concentrations (Na^+, Cl^-) in leaves (Rogers, 1998), to a higher cell water potential, and to more vigorous plant growth, which can be useful to dilute ion accumulations (Kapulnik et al., 1989; McKimmie; Dobrenz, 1991). Salt exclusion can occur primarily in roots, which would ensure lower salt concentration in inner tissues compared to soil content (Noble, 1983). According to Talibart et al. (1994), adaptation to osmotic stress seems to be regulated by at least two osmoprotectants compounds.

Screening techniques at cell level based on comparisons between check and stressed cells have been proposed. These procedures would allow not only early salt tolerance detection but also to study the reaction to other stress factors. In this context, Shabala et al. (1998) suggested to measure chlorophyll fluorescence and the use of the bioelectrical technique. The latter, characterizing the response to low intensity electrical pulses, estimates at cell level the reaction of plants to a given stress situation. Both techniques are fast and nondestructive, which makes them appropriate for being used in genetic improvement programs. The bioelectrical technique can be an alternative to

the estimation of salinity tolerance through forage yield measurements in adult plants, which has larger time and space requirements.

Usually, breeding programs for developing salt tolerant materials have focused on genotype selection at germination, emergence and seedling stages (McKimmie; Dobrenz, 1987; Al-Khatib et al., 1992). Nowadays, the importance of incorporating adult plant selection is increasingly recognized, as a way to also improve forage yield (Johnson et al., 1991). In any case, selection protocol to induce salt stress must represent as truly as possible the environment in which the improved material is intended to be used. Noble et al. (1984) developed salt tolerant alfalfa populations based on the percentage of leaf damage. Two phenotypic recurrent selection cycles were enough to significantly increase tolerance without sacrificing yield under non-saline conditions. Estimated trait heritability was reasonably good (h2 = 0.41). Other authors also released tolerant materials, which displayed variable germination and plant survival degrees under controlled (greenhouse) conditions. As examples, germplasms AZ-90NDC-ST (Johnson et al., 1991), AZ-97MEC and AZ-97MEC-ST (Al-Doss; Smith, 1998), and the cultivar Salado (Downes, 2000) can be named. In Argentina, cultivars Salinera INTA (Ochoa, 1980) and Trinidad 87 (Ochoa; Anzardi, 1996) were selected for salt tolerance under natural conditions. Since 2006, INTA Manfredi is conducting, in collaboration with INTA Santiago del Estero (North-Western Argentina), a phenotypic recurrent selection program for developing salt tolerant alfalfa populations. Through field evaluations under moderate to high salty soils, the genotypes able not only to germinate and survive but also to produce a significant amount of forage are selected and cross-pollinated.

The use of molecular techiniques, as early proposed by Winicov (1998), offers a tremendous potential for developing tolerant materials. Worldwide, there are several biotechnological programs in that regard. In Argentina, researchers at the Universidad Nacional del Litoral were able to clone sunflower (*Helianthus annus* L.) genes from the HD-Zip family (like *Hahb*-4) that confer tolerance to salt and drought (Chan; González, 1994; Chan et al., 1998; Dezar et al., 2005a, 2005b). This gene is currently intended to be introduced into alfalfa for generating transgenic tolerant genotypes.

Final considerations

The vast knowledge generated on autotetraploid inheritance and on mechanisms which favor cross-pollination is the basis for defining the most effective breeding methods to be used in alfalfa improvement. As a consequence, and taking advantage of the large genetic variability naturally present in

the *Medicago sativa* complex, an enormous number of cultivars adapted to extremely different environments were developed worldwide. Breeding was highly effective in obtaining cultivars with multiple resistance to economically important insects and diseases. More recently, the application of molecular techniques to alfalfa breeding has opened interesting perspectives for improving traits which are more difficult to approach by conventional procedures, such as herbicide tolerance, resistance to Lepdoptera, reduced lignin content, tolerance to abiotic stresses and synthesis of condensed tannins. In Argentina, an active joint-venture program between INTA and private seed companies has released since 1987 a significant number of adapted cultivars with high yield potential and multiple pest resistance. In addition, a nondormant bloat-tolerant cultivar was also released.

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CHAPTER 10

Biotechnology applied to genetic improvement of alfalfa

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Introduction

Biotechnology is the field of biological sciences that deals with genome analysis and genetic manipulation of living organisms with technological-productive purposes. Genomic analysis covers all the technologies that allow to characterize, in a molecular form, the variability present in the sequences of deoxyribonucleic acid (DNA) of living organisms, such as molecular markers (DNA and protein), the sequencing of these DNA molecules and the search for candidate genes. Genetic manipulation, on the other hand, comprises genetic transformation, that is, the introduction of foreign DNA fragments into a living organism that is being handled, in order to obtain expression of genes contained in these inserted fragments. Genetic transformation is performed through several techniques developed by genetic engineering applied to microorganisms, animals and plants. So far, biotechnology in microorganisms is more advanced, however plant biotechnology is in continuous progress and comprises several research lines, including genetic transformation, the use of molecular markers, tissue culture, somaclonal variation, obtainment of doubled haploids and embryos recovery.

Genome analyses

Molecular markers

There are two types of markers used in genetic studies and plant breeding: morphological and molecular. The first ones are controlled by genes linked to visually identifiable features of the plant, such as nanism, chlorophyll deficiency, coloration of petals, leaf shape and length of the aristae. Its main disadvantage is the low number of such markers identified and available in different species, which significantly limits the probability of finding associations between them and genes of agricultural interest. In addition to this limitation, many of them are linked to lethal genes, such as albinism (O ‘Wadt; Gieco, 1997).

Molecular markers refer to a phenotype or a molecular pattern, derived of both genes expressed as from proteins or from isoenzymes, or of specific DNA segments belonging to coding or non-coding regions of the genome of an individual (Ferreira; Grattapaglia, 1996). There are two types of molecular markers: a) those based on the gene product, such as isozymes and proteins, and b) those based on DNA fragments, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphisms (RAPD), amplified fragment length polymorphisms (AFLP), microsatellites or single sequence

repeats (SSR), sequence characterized amplified regions (SCARs), internal single sequence repeats (ISSRs) and single nucleotide polymorphisms (SNPs). According to Garcia (1997), the advantages of molecular markers can be summarized in the following points: a) the whole plant is not necessary to determine genotype; b) it is possible to detect the entire allelic variation of a population; c) since most markers are codominant, it is possible to differentiate between heterozygous and homozygous individuals; d) markers are phenotypically null, that is, they have no effect in the individual's morphology or physiology; e) they are not epistatic, or do not affect each other; and f) they are not affected by the environment.

The location of molecular markers linked to genes of agricultural interest is conceptually similar to the signaling process of a highway, but in reverse, since it consists in first placing the signals (markers), determining the degree of physical association between the markers (binding), and finally estimating the distance (genetic mapping) between the molecular markers and the genes that control the trait. This is the result of a complex process that includes various cosegregation analyses between the studied characteristic (phenotype) and the molecular markers (genotype). The estimated distances are expressed in centimorgans or recombination units.

Molecular-marker-assisted selection

As suggested by its name, marker-assisted selection is the implementation of a scheme for selecting individuals aided by molecular techniques. The identification of a DNA segment (marker) bound to a gene of agricultural interest allows its use in an indirect selection system based on flanker markers or cosegregants, which can be visualized as a polymorphic band in an agarose or polyacrylamide gel (Figure 1). The polymorphism of the marker can be expressed by the presence or absence of bands in a gel, as in the case of dominant RADP and AFLP markers, or by the presence of two bands with different molecular weights, as in the case of codominant RFLP markers and microsatellites. In the case of dominant markers, only one allele is visualized, since the other is deemed "null" (Figure 1A). However, the advantage of codominant markers – which allow visualization of two alleles – is the fact that there is identification of heterozygote individuals in the segregating generation (F_2-F_5) or in F_1 of a crossing (Figure 1B).

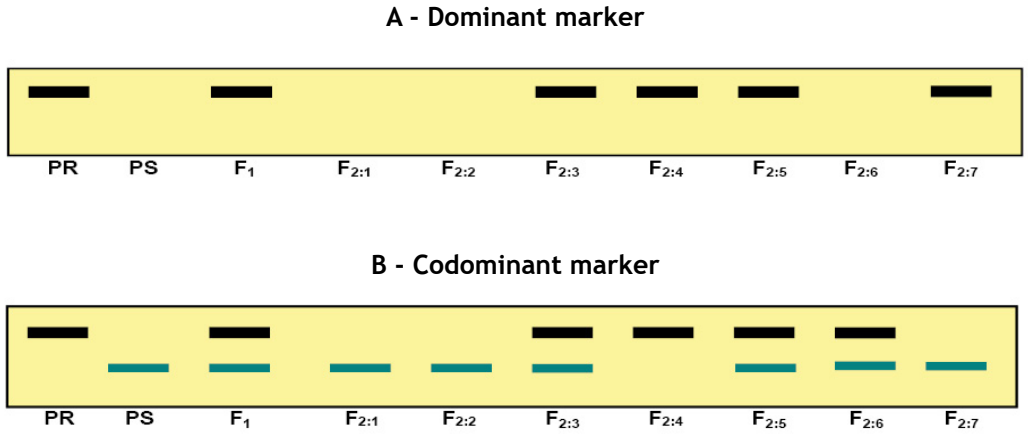

Figure 1. Diagram depicting the segregation of a molecular marker (bands) in gel. Segregation of a dominant marker in a mapping population derived from crossing of two contrasting parents for the trait to be mapped (A); the same previous situation with a codominant marker, in which individuals F1, F2:3, F2:5 and F2:6 are heterozygous. RP = resistant parent; SP = susceptible parent; F1 = Hybrid (R x S); F2 = progeny resulting from selfing of the F1 generation (B).

Genetic maps of the *Medicago* genus

The construction of genetic maps offers many possibilities for the improvement of plant species, because it allows the complete analysis of the genome, the decomposition of complex traits into their Mendelian components, the localization of the regions controlling traits of agricultural importance and quantification of the relative contribution of these regions to the final determination of the character in question.

In the case of the genus *Medicago*, genetic maps were developed in diploid species *Medicago truncatula* (Brummer et al., 1993; Thoquet et al., 2002) which, given the low complexity of their genome, are used as models for several genomic studies. To construct this genetic map of the diploid species, an F_2 population of 124 individuals resulting from the crossing of two contrasting inbred lines, one of the cultivar Vemalong and other from population DZA315, was used. The map included various types of markers (RAPDs, AFLPs, isozymes and expressed genes) covering 1225 cM (470 kb/cM), assorted into eight linkage groups (2n = 16). Molecular markers are uniformly distributed in the map (Figure 2). It is important to emphasize that the eight linkage groups show homology to their respective groups of *Medicago sativa*, and a high degree of similarity is found between the genomes.

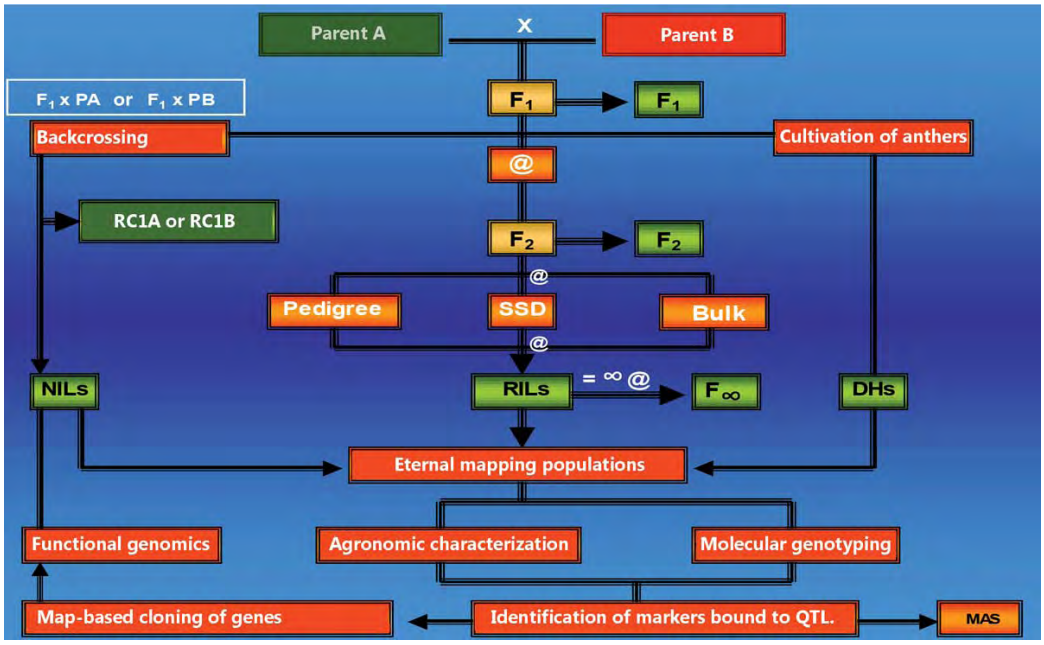

Figure 2. Diagram showing the different types of mapping populations and method to generate the populations. @ = selfing; X = crossing; F1 = hybrid generation; F2 = segregating progenies derived from selfing of F1; RILs = recombinant inbreed lines (until F6-F7); F∞ = progenies derived from successive selfing generations; RC1A = backcrossing population derived from crossing F1 with parent A; RC1B = backcrossing population derived from crossing F1 with parent B; NILs = near inbreed isogenic lines; DHs = population derived from doubled haploid varieties; SSD = single seed descendence; Bulk = population method; QTL = genes responsible for quantitative traits; MAS = molecular markers assisted selection.

Genetic maps in cultivated or tetraploid alfalfa have also been constructed (Brouwer; Osborn, 1999; Julier et al., 2003). Two populations from backcrossing of 101 individuals, obtained from the crossing of Blazer XL 17 (B_{17}) with Peruvian 13 (P_{13}) according to the scheme: backcrossing I – F_1 x B_{17} (for chromosomes A and B) and backcrossing II – F_1 x P_{13} (for chromosomes C and D), were used. In the work, 82 single dose restriction fragments were employed. In the future, the saturation of these genetic maps with a large number of markers will significantly facilitate detection and subsequent localization of genes of agricultural interest. Once identified, these genes can be applied in synthetic populations through molecular marker assisted selection schemes.

Genetic mapping populations

Construction of genetic maps is made in a segregating population or mapping population. These populations can be structured as follows (Coelho, 2000; Singh; Prassana, 2008): F_2 generation, recombinant inbreed lines – RILs, near inbreed isogenic lines – NILs, synthesized until the F_6-F_7, advanced segregating generations (F∞) backcrossings and populations deriving from doubled haploid lines (Figure 2). It is important to remember that all populations derive from the initial crossing of two contrasting individuals for the trait being studied. In the particular case of detecting genes for resistance to biotic (pests and diseases) and abiotic (drought, salinity, etc.) stresses, the ideal mapping population will be a cross between a resistant or tolerant individual with a completely susceptible one. In this context, it is possible to analyze Mendelian cosegregation of resistance and of the markers which are used.

The genetic structure of a species, its reproductive mode (autogamy or allogamy) and/or its tolerance to inbreeding strongly determine the type of mapping population to be used. Particularly in the case of alfalfa which, in addition to being autotetraploid, manifests marked inbreeding depression, among the possible populations structured for mapping, the most commonly used is F_1, the product of crossing two heterozygous genotypes. There is also the possibility of using populations derived from doubled haploid lines. This population is obtained based on the crossing of alfalfa clones or individual plants contrasting for the trait under study, which generates the F_1 population. Later, haploid pollen from the F_1 generation is cultivated in vitro in specific culture media and with colchicine, in order to double the genetic content. Backcross populations are obtained by crossing the F_1 generation with one of the contrasting parents.

It is important to make clear that for the model species *M. truncatula*, mapping populations, described in Figure 2, are easier to build, granting the methodology more flexibility and more effectiveness. In this context, it is possible to develop for this species a subgroup of mapping populations that can be indefinitely multiplied through successive generations, by which characteristic they are called eternal, such as recombinant inbreed lines (RIL), near isogenic lines (NIL), infinite F (F∞) and double haploid (DH) populations. This, in addition to the reduced complexity of the diploid genome, allowed the positional cloning of genes and, subsequently, transforming tetraploid alfalfa plants, in a breeding strategy assisted by molecular techniques (Yang et al., 2008).

The minimum number of segregating individuals employed in the mapping population depends on the inheritance of the trait studied. For qualitative traits controlled by one to three genes, the number of individuals in the population should not be less than 100. As for the case of quantitative traits in

which expression is controlled by several genes, populations must exceed 200 individuals. Clearly, the number of segregating individuals assessed is quite important in the statistical analyses for the construction of the genetic map because less than the minimum recommended number affects the Mendelian segregation of the marker and therefore hinders the mapping of the gene or the genes in question.

Mapping strategies

Regarding the mapping strategies available for detecting genes of agricultural interest, the most commonly used are: a) traditional mapping, in which a large number of markers is the basis to perform genotypic characterization of all individuals of the population (Edwards et al., 1987) and b) selective genotyping by analyzing grouped DNA samples (bulk segregant analysis – BSA), proposed as a quick way to identify markers linked to a gene of disease resistance in plants (Michelmore et al., 1991; Miklas et al., 1996). The first strategy requires more labor and more time, but on the other hand it increases the probability of detecting genes with a smaller effect. On the opposite direction, the second strategy, BSA, which consists in forming and later analyzing bulks or extreme groups (R bulk = resistant and S bulk = susceptible), each one constituted of few individuals, allows fast detection of target genes, simplifying the work of mapping. Although bulk analysis significantly accelerates the process of identifying resistance genes, it greatly favors the probability of detecting genes of greater effect at the expense of decreased effect genes.

In more detail, the goal of the BSA technique is detecting the differences between two groups of DNA samples originated in a segregating population. These samples (bulks) are formed by mixing equal amounts of DNA of the selected individuals for the phenotypic trait being studied. The aim is having, in each bulk, individuals with identical genotypes for the genomic region of interest (target region) and a mixture of genotypes for regions not linked to the target region. Thus, these two bulks are polymorphic for the chosen region, but monomorphic for the other regions. Figure 3 shows the scheme to use BSA for mapping disease resistance genes. Selection of individuals to construct the bulks is performed through identification of extreme phenotypes of the disease (R and S) in a segregating population. DNA from both the parent lines and from the bulks is analyzed with a given number of molecular markers, with later selection of those in which bands are present in one sample and absent in the other, and verifying, at the same time, the parental origin of the marker. Hence it is evidenced preliminarily the genetic linkage between the polymorphic

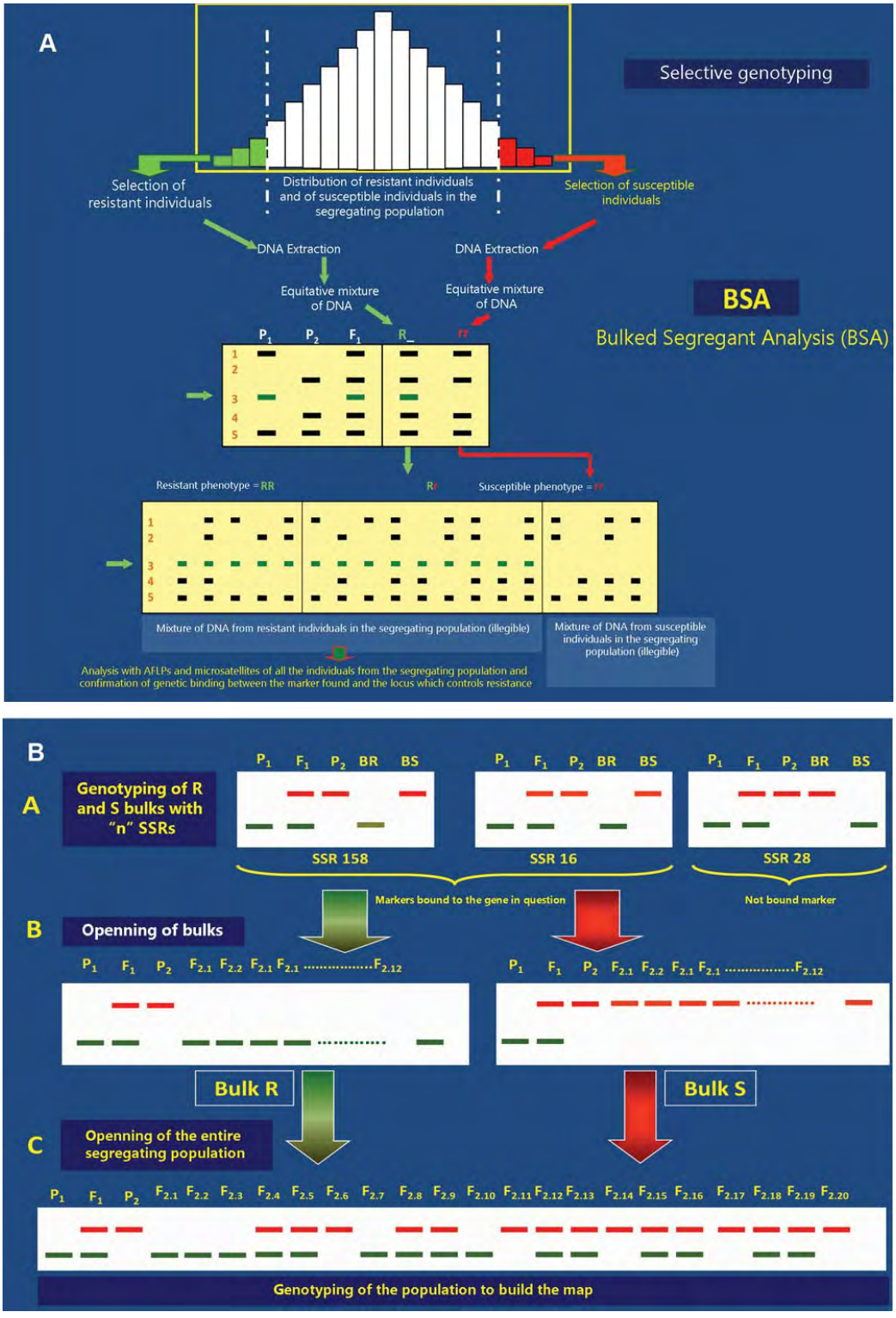

Figure 3. Diagrammatic representation of the use of the bulk segregant analysis (BSA) technique, for mapping disease resistance genes (dominant marker) (A). Adapted from Ferreira and Gratapaglia (1996). BSA example for a codominant marker (microsatellites) (B).

marker and the target locus. Later, this linkage is confirmed through analysis of cosegregation between the marker and the resistance degree in all individuals of the segregating population. Once the linkage has been confirmed, it is possible to calculate the recombination frequency between the marker and the target locus.

The use of this technique is not limited to detecting disease resistance genes. It is also possible to apply it in the identification of genes with major effect which condition several traits of agricultural interest in alfalfa, such as resistance to pests and to abiotic stress (drought, cold, salinity, acidity, etc.) and forage quality.

Molecular markers used in gene identification

The markers often used in gene identification are microsatellites and AFLPs. Among the main advantages offered by these markers for genomic analysis are the capacity of recognizing polymorphism among the genotypes, high reproducibility and wide coverage of the genome.

Microsatellites are simple DNA sequences (≤ 6 base pairs) which repeat side by side or in tandem along the genome (Tautz et al., 1986; Litt; Luty, 1989). Currently, among molecular markers, microsatellites are considered among the most important ones for several superior species (Wang et al., 1994). The analysis of the molecular polymorphism of microsatellites is based on the polymerase chain reaction (PCR), as described in Figure 4. In plants, microsatellites are usually highly informative, locus-specific and codominant (Lagercrantz et al., 1993; Wu; Tanksley, 1994; Liu et al., 1996; Provan et al., 1996). Given their multiallelic nature, microsatellites also display great potential for studies of evolution and genetic kinship (Buchanan et al., 1994). Their main disadvantage is the uneven distribution along chromosomes, because these markers tend to concentrate in pericentromeric and telomeric regions, leaving important empty spaces free of markers in the chromosome arms of the markers (Röder et al., 1995). The way to overcome this inconvenience is to combine the use of AFLPs or of other molecular markers which have random distribution in the genome and which cover the chromosome regions that the microsatellites do not fill.

In most plant species, microsatellites have a high level of polymorphism in relation to other types of molecular markers (Röder et al., 1995, 1998; Bryan et al., 1997). However, in the case of alfalfa and other polyploid species, in which the genome size is quite large, these techniques are slow and expensive. In addition, only between 30% and 50% of primers developed to amplify microsatellite sequences are functional and appropriate for genetic studies

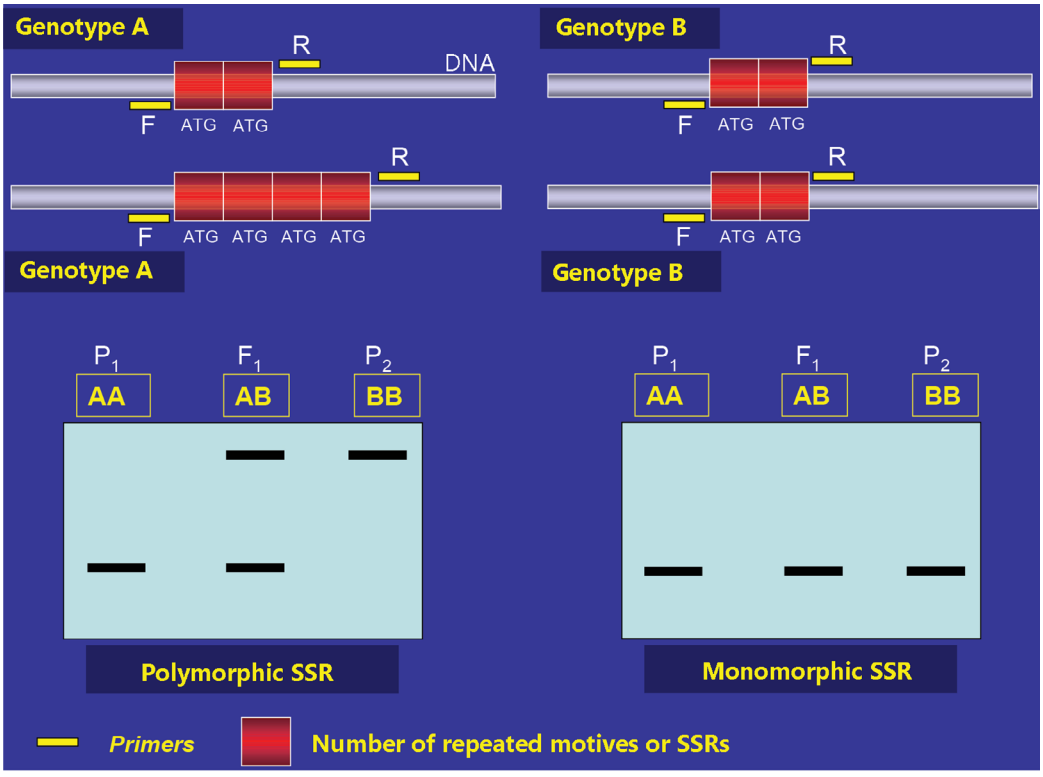

Figure 4. Diagram of molecular polymorphism between two parental genotypes (A and B) and their F1 generation, identified by microsatellites or SSRs. F = primer forward; R = primer reverse used for DNA amplification.

(Röder et al., 1995; Bryan et al., 1997). Nevertheless, there are few studies which indicate the successful use of microsatellites to detect resistance genes in several crops (Brunelli, 1999; Ogliari, 1999; Chantret et al., 2000).

AFLPs are dominant markers with random distribution in genome. These polymorphisms are identified by using PCR, which consists of selective amplification of restriction fragments obtained through digestion of genomic DNA. The development of AFLPs comprises three stages: a) cutting DNA in restriction sites and binding of oligonucleotides, named adapters, b) selective amplification of the set of restriction fragments created and c) separation of amplified fragments in a gel for later analysis (Figure 5). Amplification of restriction fragments via PCR is achieved by annealing the primers which contain an adapter and a sequence of restriction site as target sites. Selective amplification, on the other hand, is achieved through the use of specific primers, which are complementary to the nucleotides flanking the restriction sites. Even

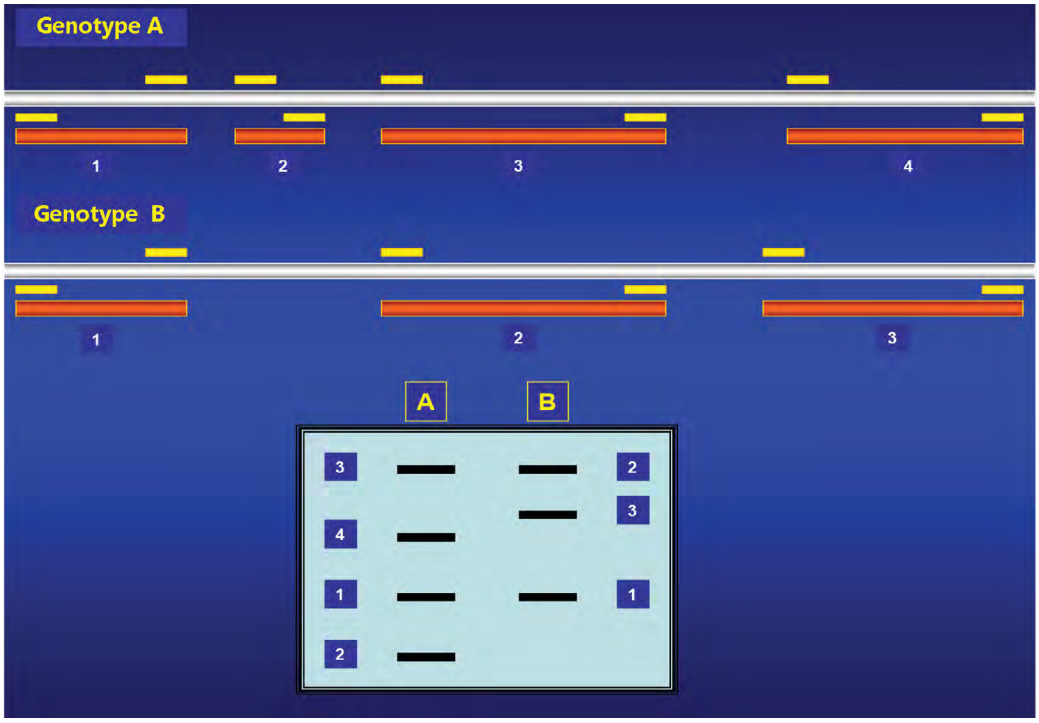

Figure 5. Diagram of molecular polymorphism between two parental genotypes (A and B) identified through markers of amplified fragments length polymorphism.

though the number of fragments that can be analyzed simultaneously depends on the resolution of the detection system used, it is possible to amplify 50 to 100 restriction fragments by using polyacrylamide gels. In recent years, the AFLP methodology became an important tool for fingerprinting DNA of diverse origins and complexities (Vos et al., 1995; Mueller; Wolfenbarger, 1999) and to detect disease and pest resistance genes in some species, as potato (Rouppe et al., 1997) and tomato (Colwyn et al., 1995). In alfalfa, Obert et al. (2000) used AFLPs to identify the genes responsible for resistance to *Peronospora trifoliorum*.

Selection of parents for gene mapping in alfalfa

The following requirements are necessary for correctly choosing the clones or genotypes of alfalfa to use in building mapping populations.

Adequate genetic distance between parental clones. The greater genetic distance between the extreme parents for the trait being studied,

the greater will the possibility to find, among the informative markers, useful polymorphisms for mapping the genes of interest.

Careful phenotypic characterization of the parents. Great part of the success in mapping genes of interest depends on the correct characterization of parental phenotypes, so that they are as contrasting as possible for the studied trait, thus decreasing the probability of existence of distortions in the mendelian segregation of the trait in the mapping population.

Rigorous pollination control. When crossing contrasting parents for obtaining the mapping population, contamination from any other pollen source should be avoided in every possible way, so that the production of undesirable progenies does not invalidate the entire mapping process that follows.

Effective characterization of the segregating progenies. Phenotypic characterization of the progenies deriving from the cross of heterozygous parents (F_1 mapping populations) or from backcrossing constitutes the most critical aspect of the whole process of gene mapping in alfalfa, because it assumes the adequate use of statistical outlines which include a sufficient number of repetitions and of evaluation locations, especially when traits of quantitative inheritance are being studied. Identification and later mapping of genes derive from the analysis of cosegregation between the genotype (band pattern of the marker) and the phenotype (for instance, resistance or susceptibility behaviour of the progenies). Incorrectly measuring of the trait can invalid the entire work and thus make locating the genomic regions impossible or, even worse, lead to identification of ghost or false quantitative trait loci (QTLs).

Cautions in DNA extraction. In order to correctly determine the genotype of individuals from the segregating population, it is necessary to obtain high quality DNA, free from contamination and without a large proportion of degradation caused by nucleases. In the same manner, precision in identifying the material in the field and laboratory, contributes to avoid obtaining undesirable results.

Adequate choice of molecular markers. Markers which reveal the highest level of polymorphism between the parents, which are easy and fast to generate and have the greatest coverage of the genome, should be used. As mentioned before, the most commonly used candidates are microsatellites and AFLPs. It has already been highlighted that the combination of both markers offers good coverage of the genome and increases the probabilities of locating the genes of interest. Hence, primers which are distributed in all chromosomes, in addition to correct genotyping and reproducibility, should be chosen.

Correct choice of the statistical packages for analysis. Statistical analyses performed with software which considers both the genomic characteristics of

the species and the format of the segregating populations significantly increase detection of genes or QTLs of interest. In the particular case of alfalfa, the necessary adjustments will be regarding the ploidy of the species involved.

Mapping genes of resistance or tolerance to biotic stress

When all the concepts explained above are considered, the complete mapping process of resistance genes in alfalfa can be summarized as follows: a) crossing resistant parents (R) x susceptible parents (S) to obtain generation F_1; b) obtaining families of doubled haploid cultivars by in vitro cultivation and treating pollen from F_1 using colchicine; c) inoculation of parents, F_1 generation and families of doubled haploid cultivars with the pathogenic agent being studied, to characterize the plant's reaction to the disease with high precision by using adequate statistical outlines, a sufficient number of repetitions and diversity of locations – in the case of diseases with quantitative resistance, evaluations should be carried out in different environments or locations; d) DNA extraction from parents, F_1 generation and progenies of doubled haploid cultivars; e) genotypic characterization of the materials listed before, through the use of the largest possible number of markers, aiming at increasing the probability of identifying a marker linked to a resistance gene; f) performing statistical analyses that enable confirming the linkage of the marker (DNA fragment) to the resistance gene, calculating the distance between the marker and the gene in centimorgans, and the importance (larger or smaller effect) of such gene in expressing resistance.

This whole process requires a minimum of two or three years of intense work. In return, after the markers linked to the resistance gene are identified, the introduction in a program of marker assisted selection noticeably accelerates the development of resistant populations or cultivars. Figure 6 describes the particular case of the marker linked to a gene of resistance to *Leptotrochila medicaginis* in a schematic form, and shows the cosegregation of the marker and the disease severity. Clearly, when the marker is not linked to the resistance gene, the lack of cosegregation between the marker bands and the phenotype would be observed in the doubled haploid progenies.

Mapping genes related to forage yield in alfalfa

Production of biomass in alfalfa is a complex trait, of quantitative inheritance and with high environmental influence. Robins et al. (2007a) used RFLP and SSR markers to identify 41 of them which were associated to genomic regions responsible for biomass production in a population derived from crossing clones of *M. sativa* and *M. sativa* ssp. *falcata*. Most of the associated

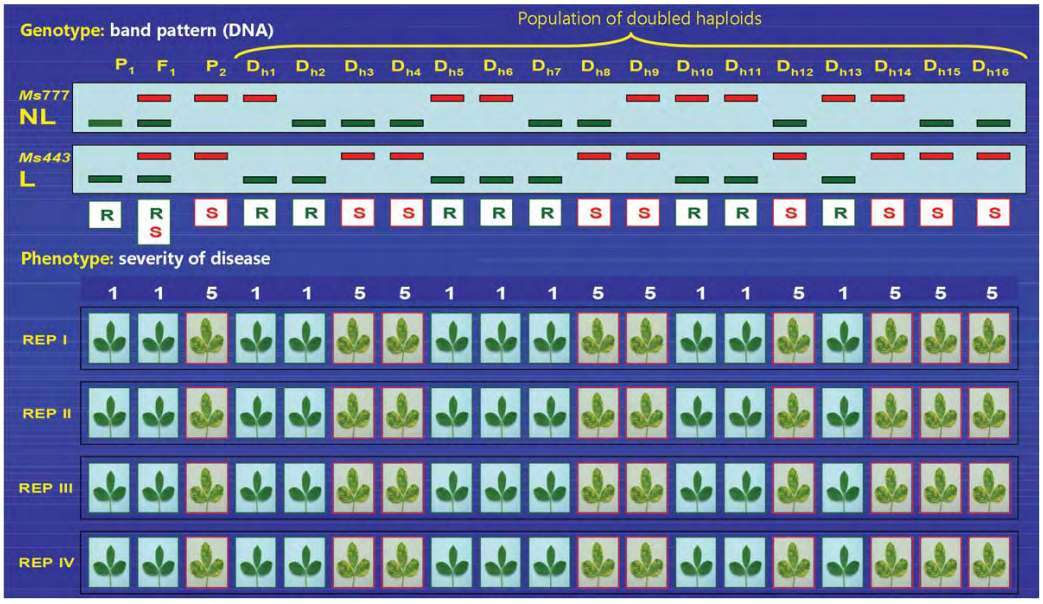

Figure 6. Diagram of cosegregation between the molecular marker and the gene for resistance to *Leptotrochila medicaginis*. Presence of R bands corresponds to phenotype with degree 1 of severity (Resistant = healthy leaf), while presence of S bands corresponds to phenotype of degree 5 of severity (Susceptible = diseased leaf). L = marker linked to resistance gene (*Ms 443*); NL = not-linked marker (*Ms 777*).

markers were identified in linkage groups 5 and 7. Phenotypic characterization was assessed in three environments over 4 years. Seven markers manifested association to biomass production in more than one evaluation period. It was observed that some of the favorable alleles for biomass production had origin in both parents. The QTLs found showed complementary gene effects, which suggests that they participated in the heterotic expression of forage production. In a later study, Robins et al. (2007b) identified some of the QTLs associated to forage yield, to plant height and to speed of resprouting.

Identifying candidate genes

Sequencing regions expressed in the genome – expressed sequence tags (ESTs) – allows identifying candidate genes through the search for homology to genes that are responsible for traits of agricultural interest (Lewin, 1999). The methodology consists in constructing genomic EST libraries and comparing to other libraries available in previously determined sequence banks using the BLAST software (Figure 7).

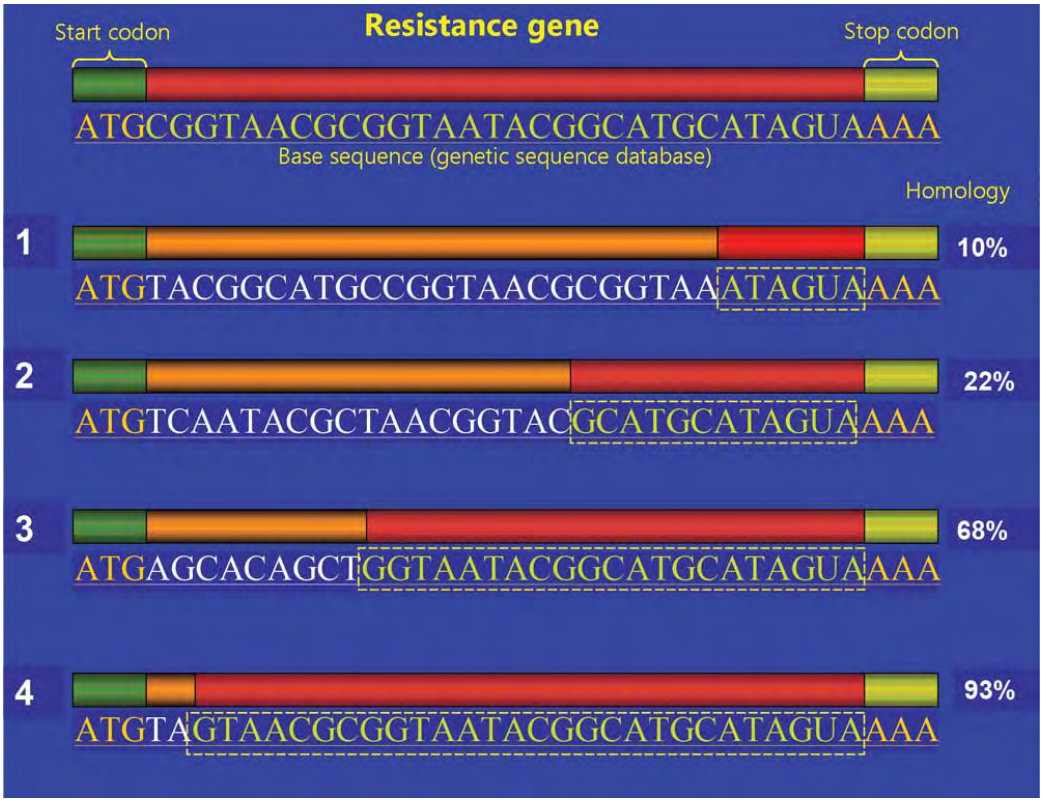

Figure 7. Process of identifying candidate genes. Homology (%) of expressed sequence tags (ESTs) (identified by 1, 2, 3 and 4) of a resistance gene located in a gene databank is estimated by the BLAST informatics package. In this case, EST 4 shows 93% of homology to the gene in question and is a candidate to be the resistance gene.

Thus, the homology percentage of a given EST to a gene involved in some metabolic pathway leading to tolerance to biotic and abiotic stress is estimated. The genes thus identified can later be cloned, inserted into appropriate genetic transformation vectors and used to obtain transgenic plants. The use of this methodology has enabled identifying tolerance genes for several stress factors in alfalfa. Friedberg et al. (2006) isolated a gene responsible for the transcription factor *MsHSFA4*, that has the protection against thermal stress as main function. Similarly, Shi et al. (1997) isolated the gene responsible for the asparagine synthetase, which intervenes in nitrogen assimilation through symbiotic fixation

The most ambitious strategy – in full development at the moment – is the sequencing of regions expressed in the genome of the *Medicago* genus. As a result of this effort, a lot of information is being generated regarding candidate genes

with specific functions for tolerance to biotic and abiotic stresses, as well as for forage quality. Cordero and Skinner (2002), using degenerate primers, isolated and characterized genes analog to resistance genes (R) with nucleotide binding sites. Comparing these genetic sequences has allowed determining the existence in alfalfa of at least 18 genetic families for R genes with nucleotide binding sites, which are useful to obtain transgenic plants with resistance to diseases.

DNA chips

A DNA chip consists of DNA or cDNA oligonucleotides cloned and immobilized in a silicon membrane (microarray or chip). This matrix can later be hybridized with RNA or with DNA, both marked with fluorophores (Figure 8).

This technology is very useful in genetic expression studies, in which around 10,000 individual cDNA clones can be hybridized with total RNA marked with fluorophores, proceeding from control plants and subjected to stress from biotic or abiotic origin. The hybridization level of each point reflects the amount of RNA proceeding from a specific gene present in the total RNA (Figure 9).

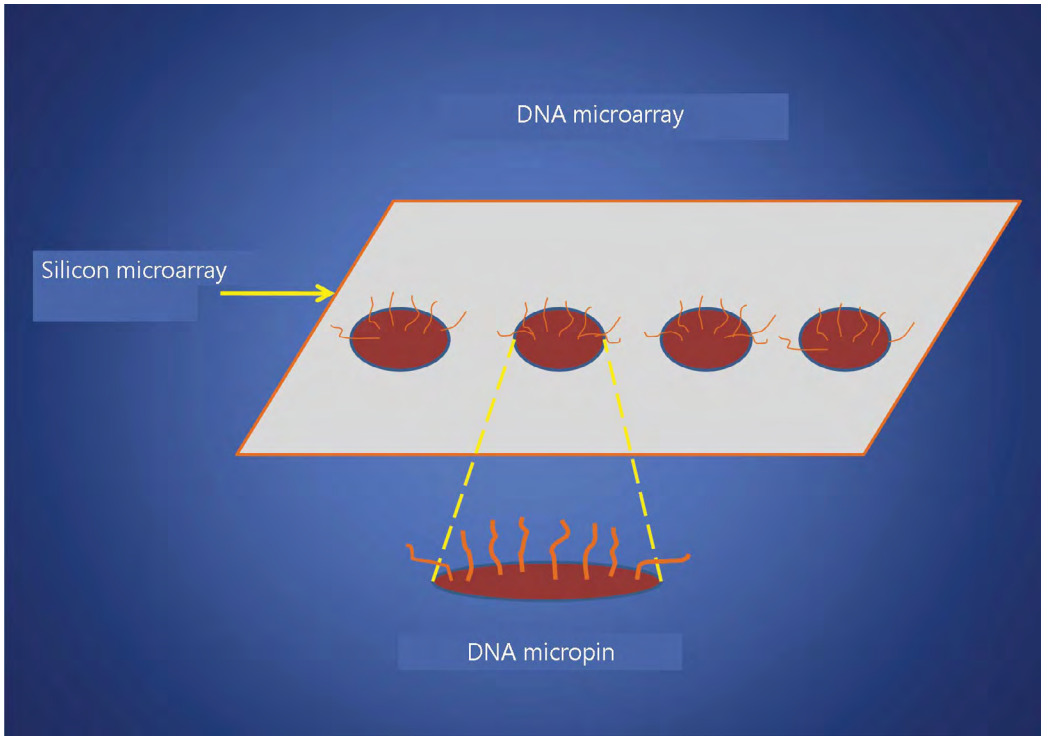

Figure 8. Diagram of a DNA microarray.

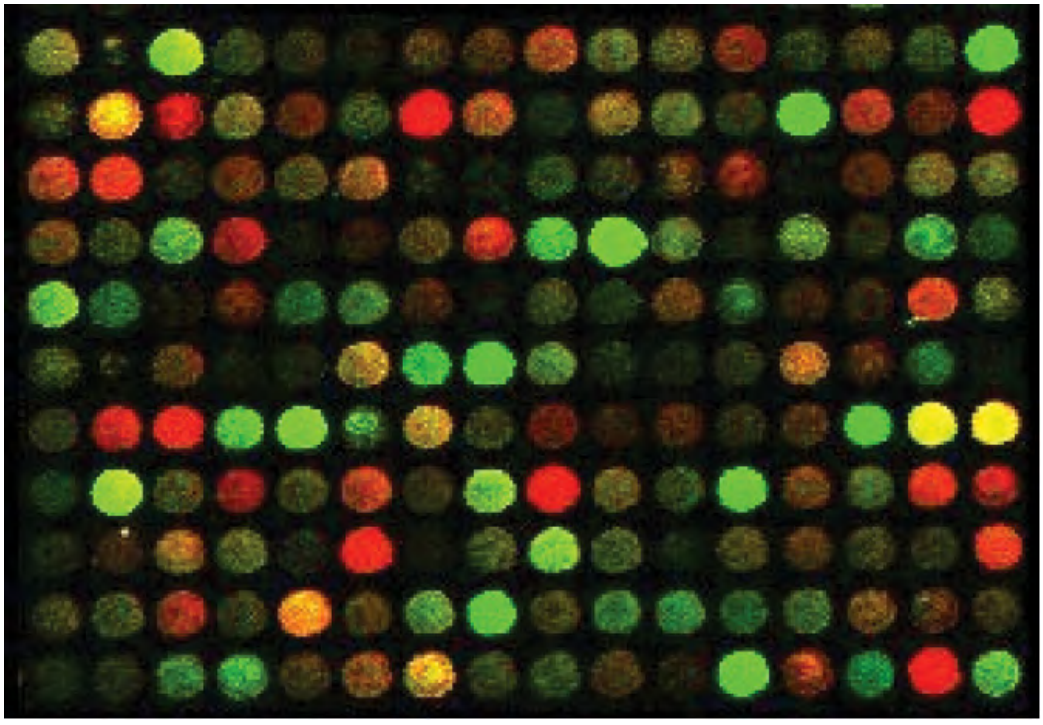

Figure 9. DNA microarray which displays the differential expression of genes immobilized in silico.

The DNA sources for fixation in the microarray are the following: a) cDNA proceeding from reverse RNA transcription, with oligo-dT primers (Figure 10); and b) oligonucleotides previously elaborated and fixated in the chip or synthesized *in situ*.

In a Southern blot experiment, the target DNA is fixated in a nitrocellulose membrane and the probe is a DNA marked in solution. The probe is the "known" DNA used to identify the "unknown" DNA present in the sample analyzed. In the microarray fluorescence assay, the probe (black lines fixated in the silicon surface) is hybridized to the marked target (red lines bound to the probe through hydrogen bridges) (Figure 11). Ultraviolet light is used to excite the fluorescent dye combined with the target nucleic acid. Fluorescence patterns are read by an automated device (scanner) developed to do so, which has an appropriate software. The final patterns of genetic expression are the result of a series of complex statistical analyses (Shi, 2007).

For example, assuming there is interest in determining which are the genes that participate in the reaction of a given alfalfa genotype when faced with a biotic stress, as an attack by *Acyrthosiphon kondoi* Shinji, 1938

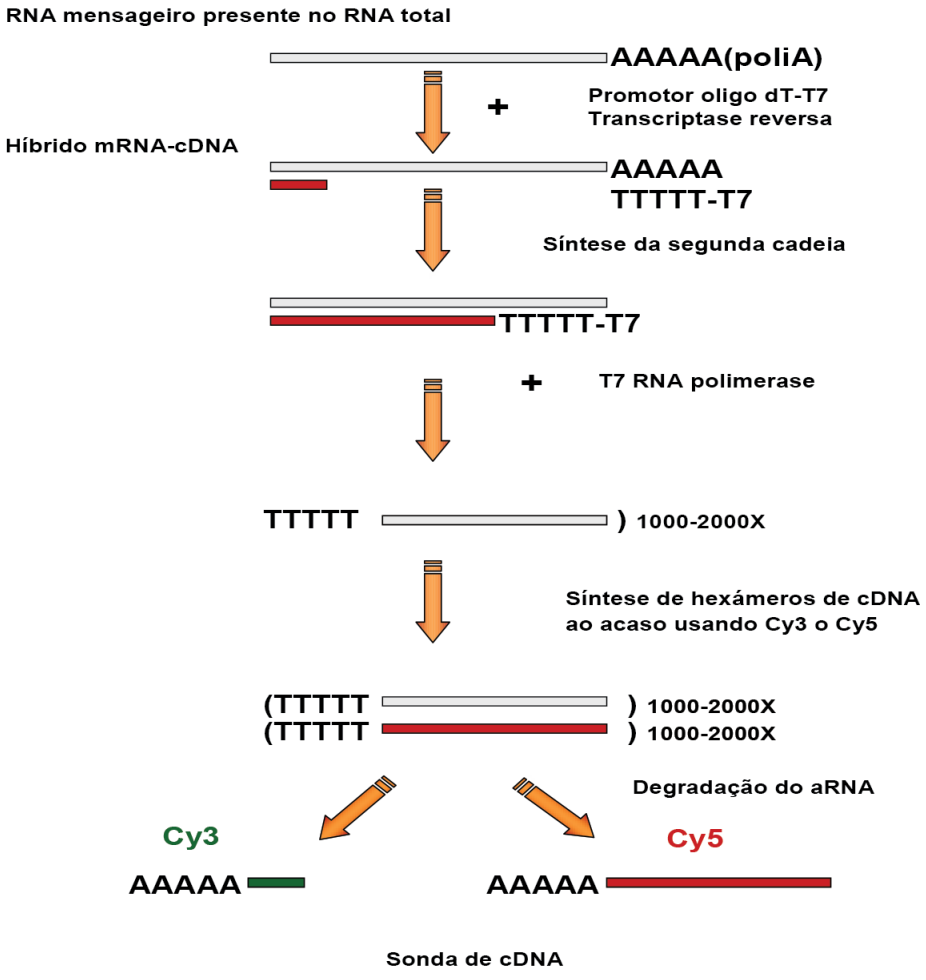

Figure 10. Synthesis of cDNA, to be used as a probe, from RNA, employing reverse transcriptase and oligo-dT primers.

[bluegreen aphid]. Based on the literature, candidate genes from metabolic pathways related to biotic and abiotic stress are selected and the in silico gene immobilization is performed. At the same time, RNA is extracted from control plants challenged with the insect in question. The RNA is later converted in cDNA marked with oligo-dT primers which are extended with reverse-transcriptase-marked nucleotides. The marked cDNA constitutes the target to be determined, since it is unknown. Silicon chips containing around 10,000 unities of cDNA act as in silico fixed probes, over which the RNA obtained from control plants and from plants subjected to biotic stress is hybridized. This is a

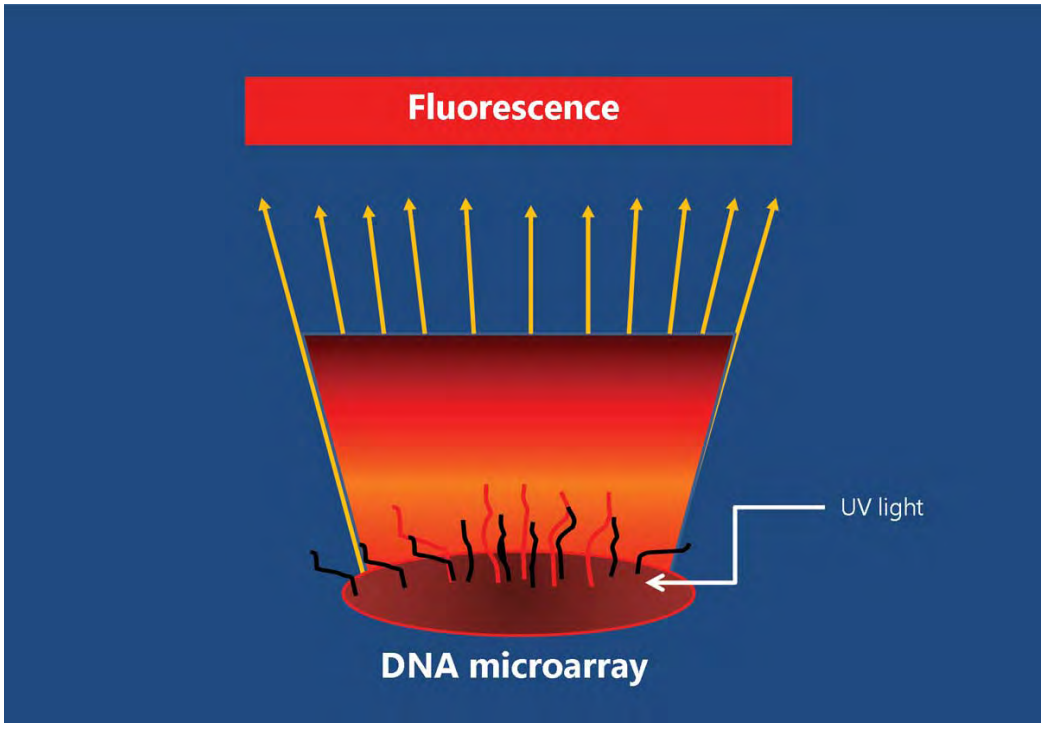

Figure 11. Diagram of detection of a DNA microarray through fluorescence.

highly comprehensive technique, because the expression of about 10,000 genes can be simultaneously analyzed in a single assay. As a result, it is possible to verify what kinds of genes are expressed and what is their level of expression when a plant is affected by a biotic stress, as mentioned in this example. These genes can later be used to develop resistant genotypes, through the production of transgenic plants which overexpress such genes.

DNA chips for the *Medicago* genus

The genome of alfalfa is constituted by about 35,000 to 45,000 genes, distributed in 32 chromosomes. Until recently, due to the technology available, the analyses only allowed studying a few expressed genes simultaneously, which limited the identification of genes of interest for the genetic improvement of alfalfa. A great advance took place in 2005, when the company *Affymetrix* developed the DNA chip for the *Medicago* genus. This chip has probes (in silico fixed DNA probes) of genes from three species: *Medicago truncatula* (around

52,700 genes), *Medicago sativa* (around 1,800 genes) and *Sinorhizobium meliloti*, a nitrogen-fixing bacterium.

The result of comparative studies between species of the *Medicago* genus derived from the sequencing of ESTs in *M. sativa* and *M. truncatula* showed around 95% of homology between them. This high homology between the diploid and the tetraploid species greatly favored the development of genomic studies in alfalfa, enabling the discoveries in the diploid species (*M. truncatula*) to be directly transferred to cultivated alfalfa. Similarly, the primers of microsatellite markers developed for *M. truncatula* are used in genome analysis of *M. sativa*. This will allow detecting genes of interest in tetraploid alfalfa which can be used as probes in the diploid species. As a result of using these DNA chips, it was possible to identify between 24,371 and 28,668 active genes in leaf and root tissues of *M. truncatula* and between 21,526 and 23,202 genes in *M. sativa* (Tesfaye et al., 2009).

With the possibility of verifying the activity of a large number of genes simultaneously, this technology will offer information on the location of genes of agricultural interest, such as the genes responsible for persistence, for forage quality, for disease and pest resistance and for tolerance to abiotic stresses (cold, heat, acidity, salinity, etc.).

Increasing the genetic base through molecular techniques

The genetic resources of a given genus or species constitute the most valuable capital and the basis of any plant genetic improvement program. In them, the breeder redeems genetic variability and reintroduces it for improving the crop. However, as a consequence of applying rigorous selective schemes and of crossings carried out only among superior individuals (elite), in most cultivated species there has been a narrowing of the genetic base, with consequent decrease of genotypic variation and increase of vulnerability to several stress factors. In this context, preserving and characterizing genetic resources of alfalfa *per se* and for using them in breeding programs acquires significant importance. Employing molecular markers to obtain precise characterization of the variability present in the germplasm collection can facilitate the definition of core collections, with the goal of gathering in a small group of accessions (about 10% of the total collection) the greatest genetic variability (≥ 70%) found in the entire collection.

To develop varieties, molecular markers can be used in forming heterotic groups which, together with important agricultural characterizations (such as winter rest, resistance to pests and diseases, and geographical origin), define the

alfalfa cultivars with greatest degree of heterosis. Thus, agriculturally superior genotypes in each contrasting heterotic group will be recombined in several ways and will give rise to synthetic populations with wide genetic base, since preserving variability decreases the risk of inbreeding depression (Figure 12). This breeding scheme is an adaptation of the one originally proposed by Brummer (1999) to capture heterosis and increase the genetic base of alfalfa by employing traditional methods, such as recurrent selection and development of synthetic cultivars.

However, mapping genes of agricultural interest with molecular markers will allow the development of assisted selection schemes to transfer such genes to the new cultivars of alfalfa. This approach, combined with plant transformation, will enable increasing the genetic base of cultivated alfalfa. One of the species that have desirable attributes is *M. sativa* ssp. *falcata*, which has high levels of tolerance to cold and resistance to leaf diseases.

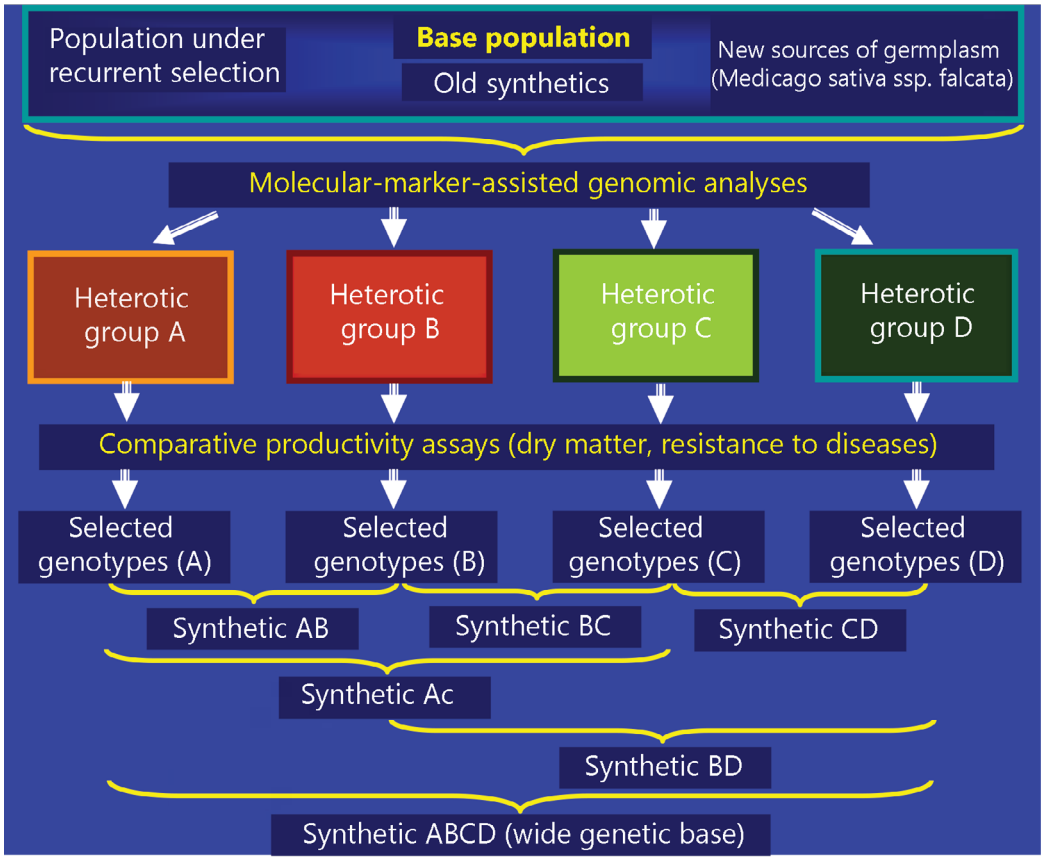

Figure 12. Scheme of molecular-marker-assisted breeding to maximize heterosis and increase the genetic base of cultivated alfalfa.

Overall, the use of biotechnological tools – basically molecular markers and plant transformation – will enable more efficient work of the plant breeder, significantly reducing the time involved in the release to market of new alfalfa cultivars which, currently, through traditional methods, is long and expensive (Figure 13).

Genetic transformation

The universality of the genetic code, given that information within the genes is interpreted the same way in all living organisms, makes genetic transformation or transgeny possible. This biotechnological approach consists in introducing a fragment of exogenous DNA (containing one or more genes) into one organism through methodologies developed by genetic engineering. The success of this approach depends on four critical points: a) transferring the transgene to the interior of the cell, b) stable integration of the transgene to the nuclear or organelle DNA (mitochondria and chloroplasts), c) regeneration

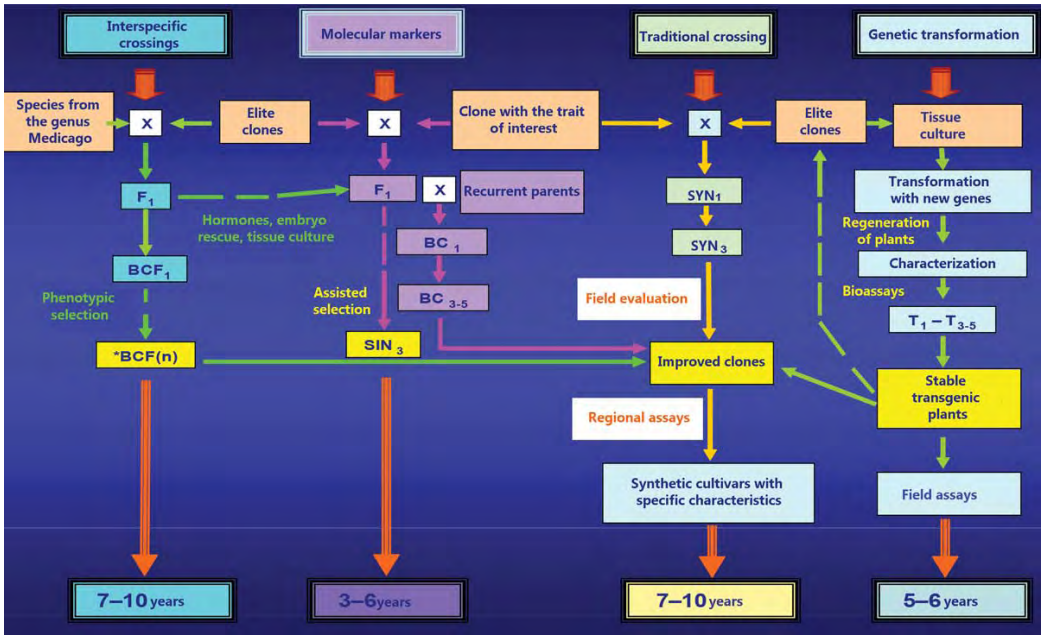

Figure 13. Comparative scheme of the different methodologies aiming at obtaining alfalfa cultivars. F1 = generation 1; BC1 = first backcross generation; BCF(n) = "n" backcross generation; SYN1 = synthetic 1; SYN3 = synthetic 3; T1 = first transgenic generation.

of a completely fertile plant from a transformed cell or group of cells and d) normal and stable expression of the introduced transgene with stable genetic inheritance.

The steps leading to production of a transgenic plant can be summarized as follows: a) identification of the gene of interest, b) construction of the insert and cloning of the transgene, c) transformation, d) selection of the transformed material, e) regeneration of the transformed plants, f) verification of the presence, stability and level of expression of the transgene, and g) confirmation of transgene inheritance. Below, we present brief comments regarding the stages listed before. However, the transformation methods most commonly used in alfalfa will be addressed in more detail in a latter section.

The identification of genes of interest is based on the impact they can have over alfalfa productivity, granting it with disease and pest resistance, tolerance to abiotic stresses and/or to herbicides, improving forage quality, etc. As mentioned in Chapter 6, isolating and cloning these genes is performed through map-based cloning or transposon tagging.

The insert (transgene) is constructed based on techniques from genetic engineering with restriction enzymes, which cut DNA at specific sequences (restriction sites), and ligases, which bind the free ends of the DNA molecules at the cutting points. The insert must be composed of five coding sequences (Figure 14): a) selection gene (allows selecting cells that contain the recombining

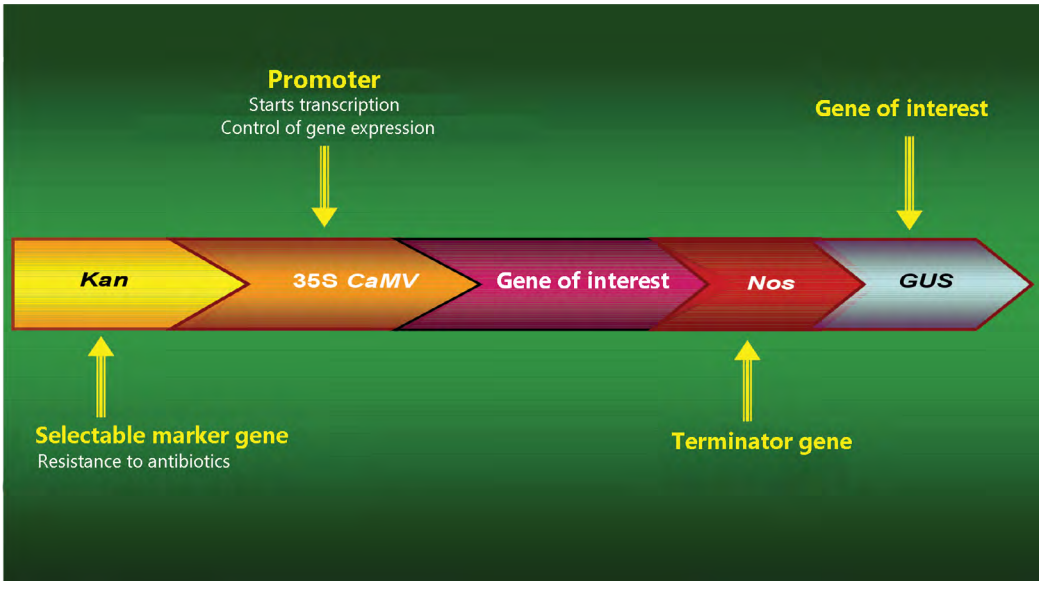

Figure 14. Parts of the insert (transgene).

plasmid in a selective culture medium), b) promoter (DNA sequence which controls the start and the intensity of transcription and the plant tissue in which the transgene will be expressed), c) terminal sequence (it is a messenger RNA transcription, such as nopaline synthase, which determines termination of messenger RNA transcription and addition of a poly(A) tail that stabilizes the fragment and protects it from nuclease degradation), d) reporter gene (allows visualizing transgene expression in transformed cells) and e) gene of interest (it is the coding sequence which we want to express in the modified organism). One of the most commonly used promoters in alfalfa is 35SCaMV [*Cauliflower mosaic virus*], due to the increased power of constitutive expression that ensures transgene expression. In addition to this one, promoter *Blec4* from *Pisum sativum* [pea] has also been used, aiming at directing gene expression in the epidermis and in developing apical tissues (Mandaci; Dobres, 1997). After the insert has been constructed, it is cloned into a multiplication vector for maintenance and later use in genetic transformation methodologies (described in the next section).

Transformed individuals are selected through expression of the reporter gene, included in the insert; this gene codifies proteins naturally present in plant cells, producing an easily identifiable phenotype (Figure 15). The most commonly used reporter gene is the *gus* gene from *Eschericia coli*, which codifies ß-glucuronidase, detected by observation of an indigo blue histochemical reaction that takes place after the contact of the transformed tissue with a specific substrate. Another reporter gene commonly used is the green fluorescent protein isolated from jellyfish, which can be visualized for its fluorescent luminosity in presence of ultraviolet light.

A very important aspect in the process of genetic transformation is the regeneration of fertile individuals, which is based on techniques for cultivating tissues based on an “explant” (plant portion capable of regenerating through in vitro cultivation techniques). In the particular case of alfalfa, the small regeneration capability of genotypes from cultivars currently in the market is one of the main limitations to obtaining transgenic individuals. A way to overcome this is to appeal to some old ecotypes or cultivars which, despite having low agricultural value, have better regeneration capability. After transformed individuals have been obtained in these cultivars, they can transfer the transgene to the elite-material via hybridization. In this process, using molecular markers can make it easier to identify progenies with the transgene region introgressed (see Chapter 6).

To verify the presence, stability and level of expression of the transgene we use some molecular techniques, presented below.

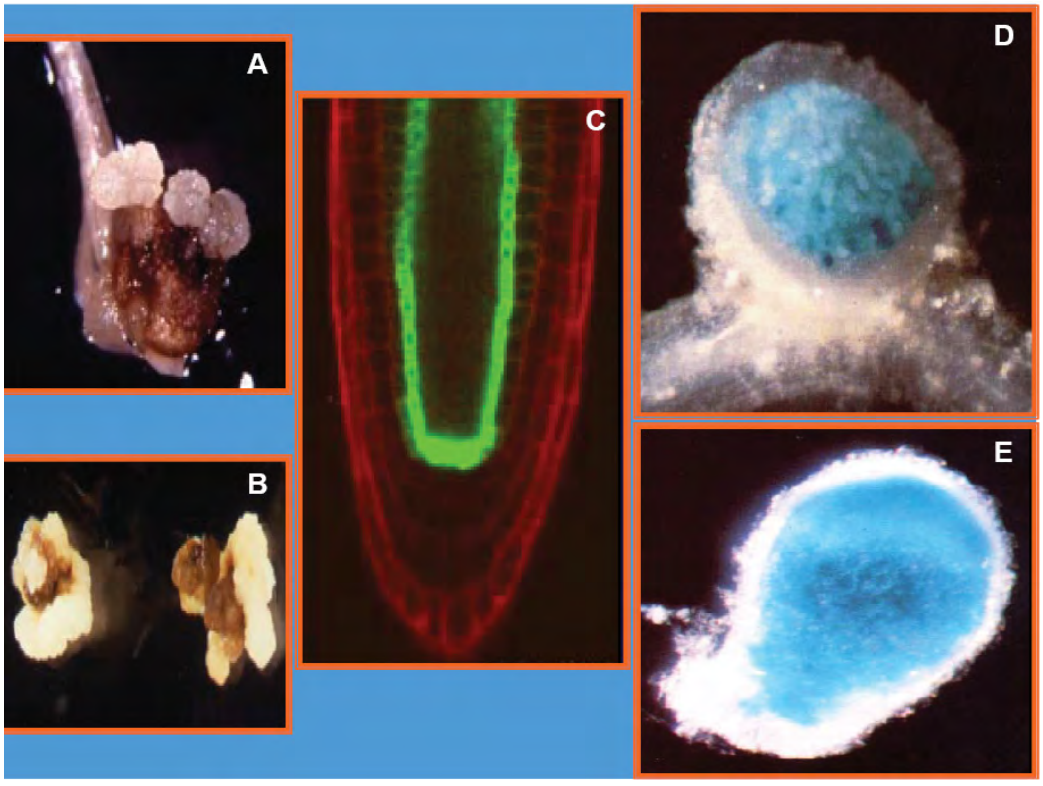

Figure 15. Marker genes used in plant transformation: tolerance to abiotic stress (A); carbon source (mannose) (B); fluorescent protein (C); *gus* gene (D and E).

PCR. It determines transgene presence through amplification with primers having homologous sequences to the transgene. The advantage to this technique is the possibility of performing a quick screening of the regenerated plants; in addition, when used in real time, it also allows determining the number of copies incorporated. The main limitation to PCR is that a positive result only indicates presence of the transgene in the sample assessed, but does not ensure that it is correctly incorporated to the plant genome.

Hybridization through DNA probes (southern blotting). It simultaneously determines presence or absence of the transgene and its integration into the genome (number of copies and number of loci in which integration was produced).

RNA analysis. It includes reverse transcription techniques followed by PCR and RNA hybridization or northern blotting to detect the transcript of interest. The first methodology requires a small amount of material and is very sensitive. In the second methodology, the transgene is used as a probe and the technique

provides information on size, at the same time allowing quantification of the expression.

Elisa and western blotting. The plants that have the transgene, and express it, can be analyzed by immunological techniques which determine the presence of the coded protein, that must later be measured through biochemical methodologies and bioassays.

Finally, confirming the mendelian inheritance of the transgenic events, the final process includes the evaluation of the subsequent generations, obtained through selfing or through crossing with control or nontransformed plants. Correct transgene insertion into the coding regions of the genome of the host plant determines whether it can be expressed and inherited, the same way as any other gene.

"Explant" transformation methodologies

Introducing the gene of interest into an organism different than the one of gene origin is called genetic transformation, which can be performed through the application of several techniques, assorted into two groups: direct and indirect (Andrade, 2003). In the first group, DNA is inserted into the host gene physically and without intermediaries; in the second group, transformation happens with the participation of a living organism (*Agrobacterium*).

Among direct transformation techniques, we quote: a) biolistics: it consists in the bombardment of cells or tissues with particles coated with the exogenous DNA; b) electroporation: it refers to the application of short duration and high voltage electrical pulses to make the cell membrane permeable, allowing the ingress of DNA (Potrykus, 1990; Jones, 1992); c) microinjection: it is the introduction of macromolecules into host cells, with or without cell walls, using micropipettes and microsyringes (Neuhaus; Spangenberg, 1990); d) pollen transformation: consists in sinking the pollen grain in a solution containing the DNA to introduce, followed by using this pollen to fecundate the flowers of the host plant previously emasculated (Potrykus, 1990); and e) transformation via liposomes: it is the introduction of encapsulated exogenous DNA into artificial lipid vesicles in protoplast culture, in presence of agents that promote fusion, such as polyethylene glycol or polyvinyl alcohol, followed by rinsing these cells with a high pH solution saturated with Ca (Andrade, 2003).

In alfalfa transformations, the two most commonly used techniques are *Agrobacterium* and *biolistics*-mediated transformation. The main characteristics of each are described bellow.

***Agrobacterium*-mediated transformation.** This technique uses the natural capacity of bacteria from the *Agrobacterium* genus, *A. tumefaciens* (updated scientific name: *Rhizobium radiobacter*) and *A. rhizogenes* (updated scientific name: *Rhizobium rhizogenes*) – responsible for the pathologies known as "crown gall disease" and "hairy roots", respectively -, of transferring DNA fragments to plant cells. The pathogenic ability of these bacteria is due to the presence of two types of plasmids named tumor inducing (Ti) and root inducing (Ri). During the pathogenesis process of the bacteria, a fragment of these plasmids, called transfer-DNA (T-DNA), is transferred to the plant cell, to which it integrates in stable form to chromosomal DNA. Expression of bacterial genes included in T-DNA induces the synthesis of plant hormones, responsible for cell abnormal proliferation (bacterial tumors), and production of opines, carbon and nitrogen source for bacterial development and reproduction in the plant. Consequently, the methodology of *Agrobacterium*-mediated transformation consists in modifying the Ti plasmid by eliminating the tumor-inducing genes (oncogenes) and the ones which code opine synthesis from it and substituting them by a marker gene and the gene of interest (transgene) (Figure 16). However, using disarmed Ti plasmids (without oncogenes) presents some practical difficulties derived from the size and the difficult insertion of the gene through recombinant DNA techniques. To overcome these difficulties, alternative vector systems (cointegrate and binary) were designed, granting more efficiency to the system.

The most commonly used methodology for *Agrobacterium*-mediated transformation is the so-called "explant" (Horsch et al., 1984), which consists of inoculating a fragment of plant tissue (explant) with the modified bacterium. Just after some time of cultivation and in ideal culture conditions the transfer of modified T-DNA, containing the selection marker and the gene of interest, is produced. Then, the explants are placed in a selective culture medium, in which a specific antibiotic that stops bacterial growth and at the same time acts as selective agent for the transformed explants, is included. Finally, the procedure ends with regeneration of the viable transgenic explants (Díaz et al., 2006).

Integration of the T-DNA into the genomic DNA of the plant happens randomly (by nonhomologous recombination) preferentially in regions with transcriptional activity and with combined participation of plant proteins and bacterial proteins. The *Agrobacterium*-mediated system allows transferring DNA fragments of up to 150 Kb, if vectors as bacterial artificial chromosome and binary vectors are used. Using these vectors enables cloning large-sized DNA fragments and their later transference to the plant genome via *Agrobacterium*, which in turn is very useful in positional gene cloning.

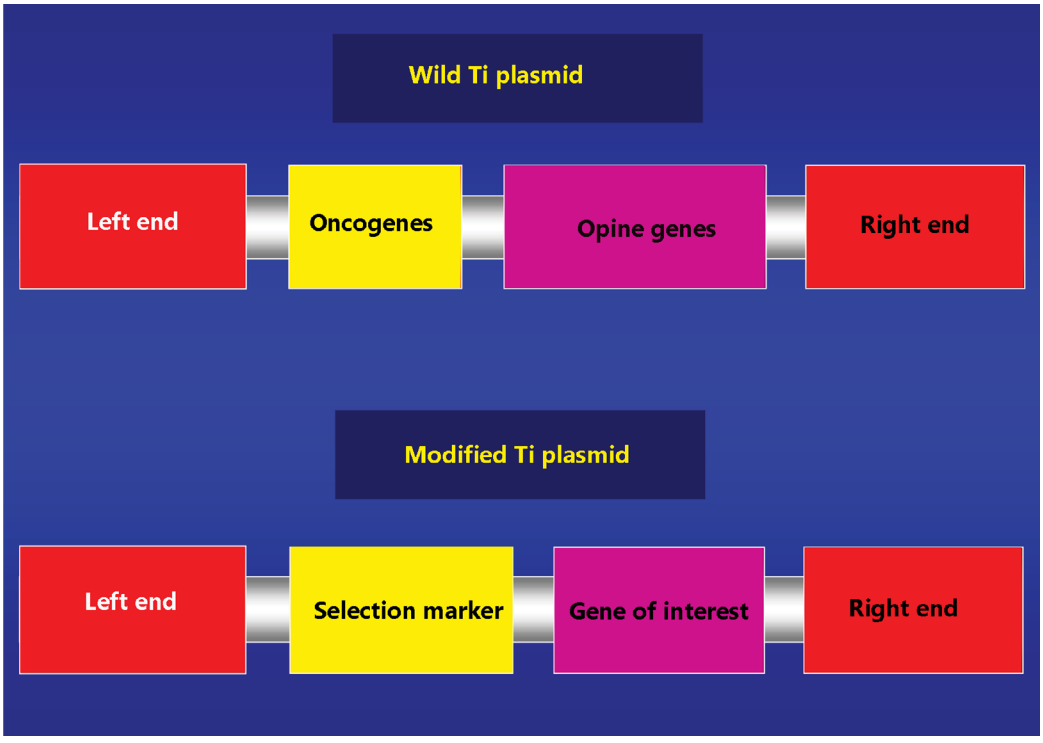

Figure 16. *Agrobacterium* plasmids (wild and modified) structure. Ti = tumor inducing.

Biolistics-mediated (gene gun) transformation. It basically consists in the bombardment of plant cells and tissues with tungsten or gold microparticles (carriers) coated with exogenous DNA. The goal is to overcome the barriers set by cell walls and the plasmatic membrane of plant cells (Sanford, 1990; Andrade, 2003) (Figure 17). The microparticles, with diameter of 0.2 mm to 4.0 mm are coated with DNA and placed in vacuum in a special chamber of the shooting pump called "gene gun" and accelerated through an internal explosion, generally using helium as fuel. These microparticles gain high speed (≥ 1,500 km h^{-1}), but this speed is reduced by a special disc which avoids severe damages to the plant tissue. As a result of the bombardment, the microparticles situate randomly in the organelles of the nucleus and of the cytoplasm, inside the cell, and DNA dissociates from the particles through the action of the cytoplasm, integrating to the genome of transformed cells.

Microparticle acceleration can be done through helium discharge at high pressure, through electrical discharge, through vaporization of a water drop or through chemical explosion with dry gunpowder. The first two methods are the most efficient ones to obtain a transgenic product.

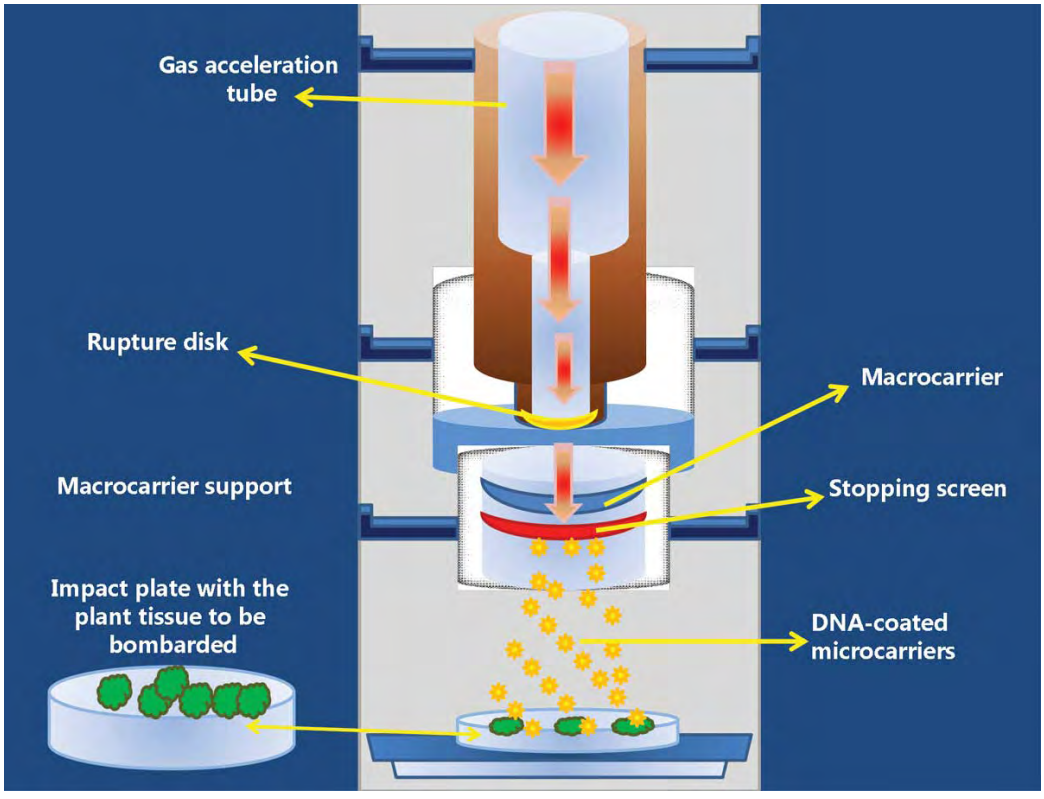

Figure 17. Scheme of the biolistic (gene gun) genetic transformation procedure.

The biolistic method can be applied in any plant tissue or organ, such as embryos, hypocotyls, cotyledons, leaf discs, calli and cell cultures. However, because DNA distributes randomly after the bombardment, it is common to have production of "chimeras", tissues which contain both transformed cells and nontransformed cells. For that reason it is very important to include a reporter gene of easy and quick detection in the insert. Another disadvantage of the technique is the integration of multiple copies of the insert in the genome, in addition to the possibility of insert fragmentation because of the particle impact, which can position genes and vectors in different sites and, depending on their location, this can alter and even silence expression of the introduced transgene (Sanford, 1990).

In Figure 18, the complete process of plant genetic transformation using indirect transformation techniques (mediated by *Agrobacterium*) and direct transformation (biolistics) was schematized;

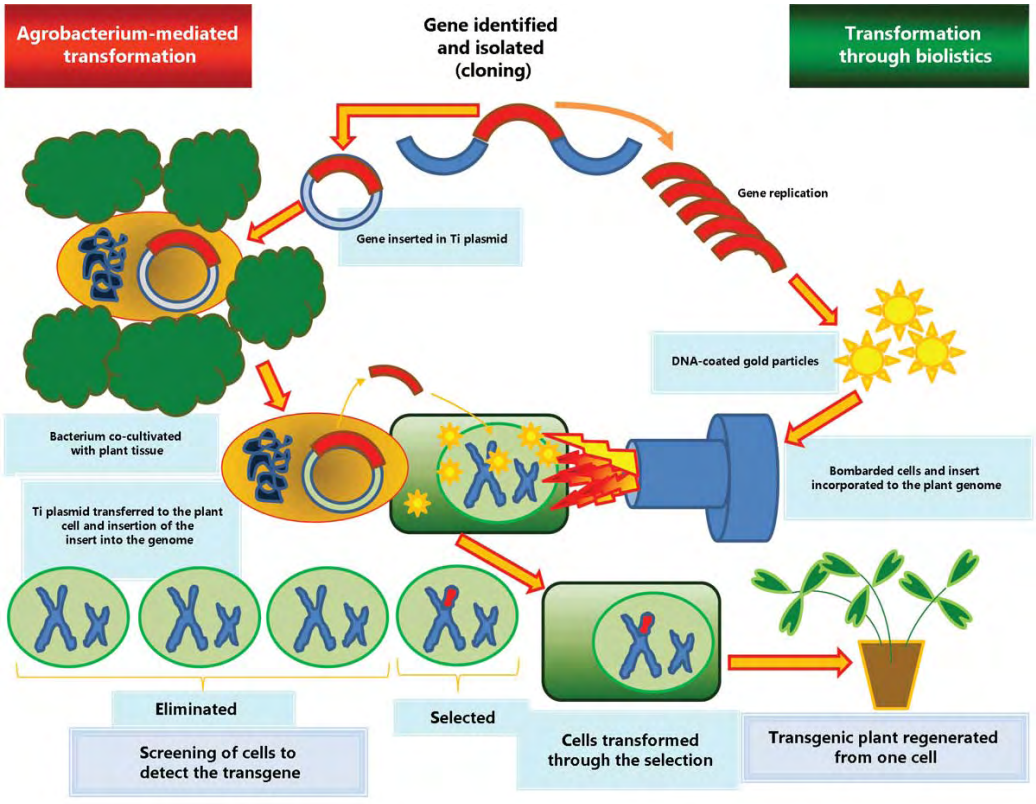

Figure 18. Stages involved in plant genetic transformation, through the indirect technique: *Agrobacterium*-mediated transformation (left) and through the direct technique by biolistic (right).

Transgenic products obtained in alfalfa

In recent years, an important number of transgenic alfalfa cultivars has been developed, but only a few have been commercially released, and only in a few countries. The spectrum of genes worked through this approach is quite broad and comprises, among others, tolerance to herbicide (glyphosate), tolerance to insects (BT) and to pathogenic fungi (chitinases, glucanases, etc.), tolerance to abiotic limitations (acidity, drought, salinity, etc.), forage quality (lower lignin content, synthesis of condensed tannins, etc.) and hay quality (delaying blooming, delaying leaf senescence, etc.). However, the solution to animal health problems (viroses) or to aspects related to the development of byproducts for the chemical industry is also being worked on.

Tolerance or resistance to biotic factors. The Institute for Genetics Ewald A. Favret (IGEAF) [*Instituto de Genética Ewald A. Favret*], at Castelar

Agricultural Station of the National Institute of Agricultural Technology (INTA-Castelar) [*Estación Experimental Agropecuária Castelar – Instituto Nacional de Tecnología Agropecuaria*] in Argentina, has been working on the development of transgenic alfalfa plants with insecticide action to control *Colias lesbia* and other lepidopterans based on genes deriving from *Bacillus thurigiensis*, a bacillus which produces different types of entomotoxins (protein crystals), named *Cry I* (lepidopterans), *Cry II* (lepidopterans and dipterans), *Cry III* (coleopterans) and *Cry IV* (dipterans). Rios et al. (2007) obtained transgenic alfalfa products using the binary vector in which T-DNA contains the selection marker *npt II* and part of the coding sequence of the *Cry IA(b)* gene of *B. thurigiensis* var. *Kurstaki* (strain HD_1), controlled by the double promoter 35S, an enhancer of alfalfa mosaic virus, and the terminator gene T_7. In biological tests with *C. lesbia* larvae, some transgenic products displayed excellent insecticide activity.

General resistance to pathogenic fungi in alfalfa can be obtained through many paths, both by the expression of pathogenesis-related proteins and defense proteins, and by the expression of antifungal compounds. In the first strategy, the most commonly used genes are the ones that code proteins of the glucanases, chitinases and protease inhibitor groups. These proteins limit pathogenic growth and infection progress. Endochitinases are the enzymes which degrade the cell wall of fungi, thus avoiding their proliferation. In transgenic alfalfa plants, overexpression of fungal endochitinase (*ech42*), which comes from the rhizosphere fungus *Trichoderma harzianum* Rifai, granted greater tolerance to fungal pathogens and better rate of nutrient absorption by roots (Tesfaye et al., 2005). At IGEAF, in collaboration with the National University of Luján [*Universidad Nacional de Luján*], in Argentina, transgenic alfalfa plants which expressed genes for chitinase, ß 1,3-glucanase and for a plant defensin were produced. Presence of the selective marker *npt II* and of the gene of interest was confirmed via PCR (Rios et al., 2007). For the second strategy (expression of fungal compounds), however, alfalfa has a special type of phytoalexin called *medicarpina*, whose synthesis is activated in response to attack by pathogenic fungi. The genes responsible for the production of this and other phytoalexins were isolated, cloned and produced in transgenic plants with interesting results (Paiva, 2008). The development of marketable cultivars containing any of the two resistance mechanisms will allow a greater effectivity in the management of several alfalfa diseases which are currently difficult or practically impossible to control (see Chapter 6).

The other focus which has been worked on for some time in the development of alfalfa transgenic products is the search for plants that express the protein

from the protein coat of alfalfa mosaic virus; this protein, acting similarly to an antibody, prevents infection and virus proliferation (Hill et al., 1991).

The most decisive results have been obtained regarding herbicide tolerance. Together, the companies Forage Genetics International and Monsanto developed several cultivars with high levels of tolerance to glyphosate. The transformed plants were obtained in 1998, but the first cultivars were only released to the American market in 2005 (Knipe, 2009) and showed a good behavior (Van Deynze et al., 2004). Alfalfa transgenic products expressing the *Bar* gene are also available; this gene determined tolerance to the herbicide ammonium-glifosinate, used to control perennial weeds difficult to control and parasitic plants, such as Cuscuta (Vlahova et al., 2005). In Argentina, the availability of cultivars tolerant to these herbicides will enable a more efficient control of aggressive weeds, such as *Sorghum halapense*, *Cynodon dactylon*, *Cyperus rotundus*, *Stipa brachychaeta* and *Wedelia glauca*.

Tolerance to abiotic factors. Overexpression of enzymes involved in the synthesis of organic acids increases exudation of such acids in roots, which allows aluminum sequestration and decreases the toxic effects of acid soils for cultivation. Tesfaye et al. (2001) produced transgenic alfalfas which, by overexpressing malate dehydrogenase, secreted seven times more organic acids in their roots than control plants, resulting in greater tolerance to aluminum toxicity. Rosellini et al. (2002) introduced to alfalfa the gene of citrate synthase controlled by the constitutive promoter of *Arabidopsis* act2 or by the tobacco root specific promoter TobRB7 and obtained plants which grew better than the control plants in acid soils with excessive aluminum. Whatever the mechanism used, the development of cultivars tolerant to toxic levels of Al acquires great importance in Brazil, where there are large areas of soil with those characteristics, resulting in limitations for crop expansion.

Another relevant trait to increase the cultivated area of alfalfa is tolerance to drought, which can be achieved through the expression of superoxide dismutase that, by reducing oxidative stress at cell level, has had a positive effect in the productivity rate, regrowth speed and persistency of transgenic plants, both in assays with natural stress in the field and with induced stress in greenhouse (Mckersie et al., 1996).

In addition to these changes, transgenic alfalfas expressing trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase from *Saccharomyces cerevisae* [yeast], have shown tolerance to drought, salinity, thermal stress and cold (Suárez et al., 2009).

Improvement in forage quality. The transgenic products which express cystathionine-Y-synthase from *Arabidopsis thaliana*, have increased methionine

and cysteine levels in alfalfa, to the extent in which it exhibited amounts 32 and 2.6 times greater, respectively, than nontransformed control plants (Avraham et al., 2005). Also, introduction of sunflower genes responsible for rich protein synthesis in amino acids with sulfur and resistant to degradation in rumen (bypass protein) shows promising results (Vlahova et al., 2005).

Genetic manipulation of enzymes which participate in lignin synthesis, O-methyltransferase from caffeic acid and O-methyltransferase from caffeoyl CoA, has enabled obtaining plants that not only have smaller lignin content, but also show different syringyl-to-lignin and guaiacyl-to-lignin proportions in relation to common control plants. Transformed plants had 10% better digestibility than non-transgenic control plants, which significantly increased milk and meat production and greatly decreased production of feces (Knipe, 2009).

Incorporating microbial genes which code the synthesis of alpha-amylases, of phytases and of cellulases to alfalfa has been proposed as a way to improve animal diets and thus increase assimilation efficiency of food consumed by the cattle (Ullah et al., 2002).

Prevention and control of animal pathologies. Condensed tannins (proanthocyanidins), which form the complex of the soluble protein fraction of alfalfa, prevent formation of stable foam in the rumen, avoid production of bloatin animals and also improve the proportion of plant protein absorbed directly in bovine intestine (bypass protein). Alfalfa has condensed tannins in seed coats, indicating that the plant has the complete genes (enzymes) to synthesize such compounds; however, because of regulating factors, not all of these genes are expressed in the canopy of the plant, determining the low rate or the absence of tannins in leaves or in stems. In this sense, IGEAF is working on the development of alfalfa transgenic products which constitutively express the genes involved in the metabolic pathway of condensed tannins, particularly the gene for chalcone synthetase. With this goal, plants with leaves which produced anthocyanins through expression of the *CHS-2* from alfalfa and *CHS-A* from *Petunia hybrida* were obtained (Ríos et al., 2007). These promising results demonstrate the possibility of altering the metabolic flow of great part of the biosynthetic pathway of tannins. There are also other investigation groups who are producing important advances in manipulation of condensed tannins in alfalfa (Gruber et al., 2001).

Another line of work carried out by IGEAF and by the Virology Institute from the Center of Investigation in Veterinary and Agricultural Sciences (CICVyA) [*Centro de Investigación en Ciencias Veterinarias y Agronómicas*] of INTA-Castelar is the expression of antigens from viruses which infect animals,

such as the one from *Aphtae epizooticae* [foot-and-mouth disease] (Wigdorovitz et al., 1999), the one from bovine rotavirus (Wigdorovitz et al., 2004) and the one from bovine viral diarrhea (Chiavenna et al., 2003). The aim is developing vaccines for veterinary use which induce production of specific antibodies in animals fed with forage from transgenic alfalfa. Even though these results are incipient, however promising, it is still necessary to solve some of the problems regarding the insufficient expression of the transgenes (Ríos et al., 2007).

Bioengineering. Production of biodegradable polymers by the expression of genes from *Ralstonia eutropha* and collectively named poly-ß-hydroxybutyrates – *phbA*, *phbB* and *phbC* – (Saruul et al., 2002), can significantly contribute to decreasing environmental contamination caused by using non-degradable plastics.

The development of plants for pharmaceutical use can find in alfalfa an interesting product. For instance, isoflavones are plant estrogens produced by legumes in response to environmental stress. Obtaining transgenic plants which overexpress the coding genes for these compounds (especially ginistein) will enable not only the availability of plants tolerant to environmental stress factors, but also the development of nutritional supplies for human beings, since isoflavones are associated to prevention of several types of cancer, of vascular diseases and of osteoporosis. With the same approach, the expression of genes responsible for synthesizing lactoferrins, proteins present in mammal colostrum which demonstrate strong antimicrobial activity, could have a relevant sanitary impact, regarding the synthesis of human lactoferrins (Vlahova et al., 2005).

Final considerations

The use of biotechnology, especially of molecular markers and plant transformation, constitutes a powerful tool which complements the work of traditional breeding. Assisted selection will make the development of alfalfa commercial cultivars with greater heterosis expression and greater resistance to diseases and pests more efficient. Obtaining transformed plants, in addition to broadening the spectrum of the existing genetic variability, also allows addressing problems difficult to solve by traditional breeding methods. In this context, the combination of these techniques anticipates a very auspicious future for the development of plants having more productivity, more persistency, better nutritional quality and more tolerance to biotic and abiotic factors. The alternative use of alfalfa in other fields, such as production of pharmaceuticals, biodegradable plastics and vaccines for veterinary use is also important. An aspect that is no less inherent but that should be considered in all processes

described is the previous analysis of national and international patents involved and the cost of biosafety studies required for releasing transgenic products to the market.

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Chapter 11

Alfalfa cultivars in Brazil

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Introduction

The Brazilian cattle herd, estimated in over 205 million animals, is the largest one in the world, making Brazil the largest beef producer in the world and one of the biggest producers of milk from pasture-fed cattle (IBGE, 2009). Despite the gradual development of intensive production systems, both in pasture and in confinement, extensive production using cultivated pastures is predominant. This grants Brazil a highlighted position in the international market, which has been more and more demanding of beef and milk produced in more natural conditions and using less concentrates and chemicals (Zen et al., 2008). Brazil relies on over 220 million hectares of pasture, from which around 100 million are taken by cultivated forage and the rest is constituted of pasture formed by native and naturalized species (Jorge, 2008).

Brazilian soil and climate conditions allow cattle breeding to be developed all over its territory, with significant importance in the socioeconomic context of the country. Brazilian pastures are spread through different regions and different ecosystems (temperate climate, Cerrado, Semiarid, tropical wet, Pantanal) which have great environmental variability by themselves. Success in implementing pastures in such diverse environments implies the use of forages which have relatively distinct adaptation mechanisms that enable them to overcome the pressures from environmental stresses and to keep a high productivity.

Animal productivity achieved in tropical pastures is still low, when compared to the performance obtained in temperate climate regions where improved forage is used. The low productivity of tropical pastures during "winter" in central Brazil is one of the causes that contribute the most for the low productivity of the herds. This low pasture productivity is responsible for the reduction of the pasture's carrying capacity, for the marked decrease in milk production and for the weight loss of beef cattle, in that period. The inferior performance of tropical pastures can be related to three basic factors: using non-improved species and cultivars; using marginal or low fertility areas and inadequate pasture management (Pereira; Ferreira, 2008). Among these factors, replacing bad nutritional quality and low productive potential forage by improved cultivars constitutes an excellent alternative to obtain an increase in productivity.

Intensification of milk production in pastures constitutes an important goal of the dairy sector to make the activity competitive and economically profitable. This process has been occurring throughout Brazil, notably in the South, Southeast and Midwest regions. Due to market pressures, producers are

looking for obtaining increased productivity per animal and per area, to keep the activity economically viable. For this purpose, it also becomes necessary to reduce the cost of animal feeding, considered the main component of expenses in milk production. In this sense alfalfa, as a forage with high dry matter production and high nutritional quality, can be an alternative feed by decreasing the amount of concentrate and corn silage, expensive components of the dairy activity, used in animal diet. Concentrate feeds represent roughly 60% of the feeding costs for milk cattle and 36% of the total costs. Corn silage represents about 17% of the feeding cost and about 10% of the total cost. Together, concentrate and silage represent about 77% of feeding costs for milk cattle and 47% of the total cost (Tupy et al., 2000). These results show the potential to insert alfalfa into sustainable and competitive systems of milk production in Brazil.

One of the barriers for the expansion of the alfalfa crop in Brazil is the low availability of cultivars adapted to tropical conditions. Currently, the only alfalfa cultivar with good adaptability and good stability in Brazil is the Crioula and there is great demand for new varieties in the market (Ferreira et al., 2004). The development of new alfalfa cultivars, with good adaptability and stability, will enable their cultivation in different regions of Brazil, with consequent increase of the area of exploitation and will thus ensure high quality and high productivity feeds for the intensive milk production systems (Pereira; Ferreira, 2008).

Another gap to solve is the production of seeds of enough quantity and quality (genetic and cultural) to meet the current and potential requirements of its growing market. The development and indication of cultivars adapted through genetic breeding programs is only justified if seeds are made available to the producer in the necessary amount, in due time and with satisfactory quality and fair price. Nowadays, the largest part of cultivated alfalfa in Brazil is from seeds imported from Argentina, Chile, and the United States of America, at the cost of R$ 30.00 per kilogram (Pereira; Ferreira, 2008).

The achievement of alfalfa cultivars adapted to the tropical climate can be addressed by genetic breeding programs through three basic methodologies: a) breeding of the Crioula population; b) introduction and evaluation of cultivars already bred in other countries; and c) achievement of synthetic populations based on recombining promising introduced genotypes. The process that can lead to the fastest results is introducing and evaluating the adaptation of cultivars bred in other countries. This methodology has been adopted by several institutions, aiming at the acceleration of the process of identifying adapted cultivars. At Embrapa Southeastern Livestock, national and introduced

alfalfa cultivars were evaluated. In these evaluations, the materials LE N 4, P 30, Crioula, Barbara SP INTA and P 5730 stood out in dry matter production. LE N 4, P 30 and Crioula were the ones which had less disease infection (Rassini et al., 2007). LE N 4, developed by the company Palo Verde (Argentina) and introduced from the National Institute of Agricultural Technology (INTA, Argentina), has been presented as a relatively promising material, because it has reached dry matter production superior to Crioula. This promising cultivar is being subjected to the test of cultivation and usage value, with experiments being performed at Embrapa Southeastern Livestock (municipality of São Carlos, São Paulo state), at Embrapa Maize & Sorghum (municipality of Sete Lagoas, Minas Gerais state), at Embrapa Cerrados (district of Planaltina, Federal District), at Embrapa Soybean (municipalities of Londrina and Ponta Grossa, Paraná state), at Embrapa Temperate Agriculture (municipality of Pelotas, Rio Grande do Sul state) and at Embrapa Semi-Arid Region (municipality of Petrolina, Pernambuco state), aiming at a future recommendation as an alfalfa cultivar for Brazil, should its good adaptability and good stability be confirmed.

The future alfalfa cultivars will present increased yield and resistance to the several biotic factors. Also, it is desirable to develop cultivars with special characteristics enabling their use under specific environmental conditions and forms of use (cutting and pasture).

Alfalfa breeding for the cutting systems will select plants with high dry matter production, high forage quality, high capacity for fixating nitrogen, good tolerance to pests and diseases, small degree of winter rest and good persistency. For the pasture system, on the other hand, the necessity of incorporating tolerance to trampling and small bloat rate is added to these characteristics (Hijano; Basigalup, 1995).

The development of cultivars adapted to tropical conditions will result in a significant increase in the cultivated area of alfalfa, mainly to be used in intensive milk production systems of regions South, Southeast and Midwest, where the herds with greater nutritional requirements are located.

Alfalfa cultivation

Due to its potential in forage production and its adaptability to several environmental conditions, alfalfa is one of the most important forage species in the world, with over 32 million cultivated hectares (Costa; Monteiro, 1997). The USA, Russia, Canada and Argentina are the main producer countries. Alfalfa has excellent agricultural and qualitative characteristics, such as protein quality, palatability, digestibility, capacity of biological fixation of nitrogen into the soil

and low seasonality of production; in addition, it has high contents of vitamins A, E and K, as well as the majority of minerals required by dairy and beef cattle, especially calcium, potassium, magnesium and phosphor (Ferragine, 2003).

Alfalfa can be supplied to animals in conserved form or in green ground form, or under pasture. The main forms to conserve alfalfa forage are hay (forage stored with moisture content under 20%), silage (forage stored with moisture content over 70%) and pre-dried (forage normally stored in polyethylene bags and with moisture content ranging between 40% and 60%). There are other less commonly used forms, such as pellets (forage dehydrated and compacted into high density cubes). Alfalfa can also be used under pasture and in green form offered in the through. In Argentina, alfalfa is used in large proportions for pasture and, in the USA, in hay form (Rodrigues et al., 2008). In Brazil, despite the most common use of alfalfa being as hay, several researches demonstrate the high potential of this forage when used for pasture (Costa; Saibro, 1994; Vilela, 1994, 2001; Saibro et al., 1998; Botrel et al., 2000; Oliveira, 2000; Oliveira et al., 2001; Ruggieri et al., 2001, 2005; Perez et al., 2002; Ferragine, 2003; Ferragine et al., 2004; Oliveira, 2006; Oliveira; Herling, 2006; Rodrigues et al., 2008).

It is estimated that the current area cultivated with alfalfa in Brazil is 30 thousand hectares, of which 90% is in Paraná and Rio Grande do Sul; this latter state is Brazil's biggest producer (Jorge, 2008). Alfalfa cultivation has been spreading to the Southeast and Midwest regions, in wider and more technified areas. The limiting factors for increasing alfalfa cultivation in Brazil are the lack of knowledge on cultivation techniques, low soil fertility, inadequate management, low availability of seeds and lack of cultivars adapted to tropical conditions (Paim, 1994; Ferreira; Pereira, 1999; Nabinger, 2002; Ferreira et al., 2004; Vasconcelos et al., 2008). In addition to these factors, the lack of knowledge about the control of invasive plants, of pests and of diseases, which occur more commonly in the tropics, significantly contributes for the low cultivation of this forage in Brazil.

Cultivars

Countries with a greater tradition in cultivating alfalfa, such as the USA, Canada and Argentina, have a large number of cultivars available, adapted to the different environments which they were selected for. Brazil, on the other hand, has most of its area cultivated with alfalfa taken from varieties of the Crioula population. The Crioula population results from a joint selection process, performed in Rio Grande do Sul state through introductions from

Uruguay and Argentina. The main varieties known from the Crioula population are: Crioula CRA, Crioula Itapuã, Crioula na Terra, Crioula Nativa, Crioula Ledur, Crioula Roque, Crioula Chile and Crioula UFRGS.

Alfalfa research results, both for cutting and for pasture, in tropical conditions and in subtropical conditions, have demonstrated superiority of the Crioula varieties, producing up to 25 t ha^{-1} $year^{-1}$ of dry matter, with low seasonality, high biological fixation of atmospheric N and efficient water use (Pereira et al., 1998; Oliveira, 2006; Oliveira; Herling, 2006; Rodrigues et al., 2008).

Introducing and evaluating cultivars already improved is an interesting strategy to adopt in breeding programs. Among cultivars introduced from other countries, only Monarca SP INTA, Super Leiteira, Trifecta, WL-325 HQ and WL-525 HQ are registered in the National Cultivar Registry of the Brazilian National Service for Cultivar Protection (Brasil, 2009) and can, therefore, have their seeds marketed in Brazil. However, it must be emphasized that the Crioula cultivar continues to be the most commonly planted in the country, with good adaptability and good stability.

Cultivar adaptability and stability

Phenotypic manifestation is the result of the action of the genotype under influence from the environment. However, when we consider a number of environments, besides the genetic effects and environmental effects, we detect the additional effect provided by the possible interaction between these effects. Evaluating the interaction of genotypes and environments is very important in breeding since, in case it exists, there is a possibility for the cultivar to show better behavior in a given environment – adaptability – and to repeat the performance in other environments – stability (Ferreira et al., 2000).

The causes of interaction have been credited to physiological and or biochemical factors inherent to each cultivated genotype. Since genotypes develop in dynamic systems, where constant changes take place, from sowing to ripeness, they usually have different behavior regarding the response to environmental variations (Cruz; Regazzi, 1994). Most economically important traits, such as production, are polygenic in nature and have their expression influenced by environmental conditions and by the effects of the interaction between genotypes and environments (Allard, 1971).

It has been frequent in breeding programs to evaluate the behavior regarding a group of cultivars faced with environmental variations, considering different locations as environments. However, studies on the interaction

between cultivars and environments do not provide detailed information of the behavior of each cultivar facing the environmental variations. For this purpose, it is necessary to perform adaptability and stability analyses, through which it becomes possible to identify cultivars with predictable behavior and that respond favorably to environmental variations in specific conditions or in broad conditions (Cruz; Regazzi, 1994).

Because of these aspects, the interaction between genotypes and environments is an extremely important factor to consider in plant breeding. For the producers, it is interesting for plants to show maximum expression of their genetic potential, in the form of economically applicable products, such as grains, forage and fruits. For that purpose, environmental conditioning or the use of specific cultivars for each environment becomes necessary, so that the maximum potential is extracted from the cultivars (Pereira et al., 2001). Since standardizing cultivation environments is practically impossible due to the costs involved, the possible solution to keep high crop productivity in diverse environments is to use plants genetically adapted to each location.

The strategy of selecting plants adapted to the specific conditions of cultivation environments has been adopted in breeding programs of important species, such as maize, rice, wheat, bean, soybean and cotton. It is based on this work that it has been possible for breeding programs to release superior cultivars adapted to different soil and climate conditions.

In alfalfa and in other perennial forage species, morphological, physiological and agricultural traits which promote yield, forage quality and plant persistency in production systems are usually sought. The dry matter yield potential of cultivated alfalfa is estimated in up to 25 t ha^{-1} $year^{-1}$; however, in most cases this potential is not reached, due to environmental limitations, considered in broad form (Ferreira; Pereira, 1999).

In tropical regions, the rain system is an important factor interfering in the adaptation of alfalfa, due to its influence on soil moisture and pH (Melton et al., 1988). Alfalfa adapts better to deep, well drained, slightly alkaline soils with high fertility. Soil acidity is usually related to high precipitations, and alkalinity, to low precipitations. Susceptibility to pests and diseases is the main limitation for alfalfa to adapt to a given environment (Paim, 1994; Hijano; Basigalup, 1995). Disease and pest incidence is influenced by intensity of rain and by temperature (Melton et al., 1988) and can occur in leaves, stems, roots and seeds and is usually more frequent under high temperature and humidity conditions, typical of tropical regions. Damages caused by pests and diseases, mainly to leaves, cause an increase in the stem:leaf ratio, with a negative reflex on forage quality because of the increase in fiber content and the decrease in

crude protein content. Susceptibility to pests and diseases can, in many cases, be the main reason of the low persistence of this legume (Bueno; Silva, 2008; Porto, 2008).

Despite the good performance that an alfalfa variety may have regarding dry matter production, it is necessary for it to also demonstrate response to environmental improvement and predictability of behavior. However, because of the effect of the interaction between genotype and environment, many times a variety that is superior in certain environmental conditions may not keep this superiority in another environment. Thus, the detailed study of the behavior of a genotype, when faced with environmental variations, has been very important, for allowing the most efficient recommendation and use of the genetic material available (Cruz; Regazzi, 1994).

Due to differences in adaptation between cultivars (interactions between genotypes and environments), to environmental requirements and to the current stage of alfalfa breeding programs aiming at obtaining "tropical cultivars", the adoption of strategies that enable more speed for evaluating and selecting genotypes becomes necessary. One of the solutions breeders have found to address the need of quickly evaluating materials in diverse environments is the performance of online assays, through a standardized methodology. The work is normally developed in a partnership among researchers from several institutions, which enables the performance of experiments in several environments simultaneously, as well as comparing the results obtained among locations.

In the case of alfalfa, the National Network for the Evaluation of Alfalfa Cultivars (Renacal) [*Rede Nacional de Avaliação de Cultivares de Alfafa*] was organized, with the objective of recommending alfalfa cultivars for different regions in Brazil.

National Network for the Evaluation of Alfalfa Cultivars

In 1994, Embrapa Dairy Cattle, attentive to the growing interest in the alfalfa crop, promoted a meeting among national and foreign researchers, extension professionals, technicians and producers, with the objective of evaluating the potentialities and limitations of alfalfa for milk production in tropical regions. Among the conclusions of the meeting, carrying out researches aiming at adapting alfalfa cultivars to different tropical environments was highlighted as prioritary (Botrel; Alvim, 1994). At this meeting, it was proposed to create a network of experiments to evaluate the adaptation of national varieties and introduced varieties to the different regions of Brazil. Thus, the National Network for the Evaluation of Alfalfa Cultivars was created with

the objective of identifying and recommending cultivars for such regions. The network was designed based on the use of a standardized experimental methodology, allowing performing comparisons between results from experiments carried out in different locations (Botrel; Alvim, 1994).

The Network was conducted from 1995 to 2005, with experiments performed in over twenty locations of the Southeast, Midwest, South and Northeast Brazilian regions. The following research and teaching institutions were part of the Network: Embrapa Dairy Cattle; Embrapa Southeastern Livestock; Foundation College of Agronomy Luiz Meneghel [Fundação da Faculdade de Agronomia Luiz Meneghel], municipality of Bandeirantes, Paraná state; São Paulo Agency of Agribusiness Technology, Sertãozinho Experimental Station [Agência Paulista de Tecnologia do Agronegócio – Estação Experimental de Sertãozinho]; Federal University of Lavras [Universidade Federal de Lavras]; Vale do Rio Doce University [Universidade do Vale do Rio Doce]; Rio Verde Educational Foundation [Fundação de Ensino de Rio Verde], municipality of Rio Verde, Goiás state; Agency for Rural Development of Goiás State [Agência Goiana de Desenvolvimento Rural e Fundiária]; Bahia State Agricultural Development Corporation [Empresa Baiana de Desenvolvimento Agrícola]; Agricultural Research Corporation of Rio de Janeiro State [Empresa de Pesquisa Agropecuária do Rio de Janeiro]; State Agricultural Research Corporation of Minas Gerais [Empresa de Pesquisa Agropecuária de Minas Gerais]; Espírito Santo Research Institute [Instituto Capixaba de Pesquisa], Technical Assistance and Rural Extension [Assistência Técnica e Extensão Rural]; Agronomic Institute of Paraná [Instituto Agronômico do Paraná]; Agricultural Research and Rural Extension Enterprise of Santa Catarina [Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina]; Federal University of Rio Grande do Sul [Universidade Federal do Rio Grande do Sul]; Federal Rural University of Pernambuco [Universidade Federal Rural de Pernambuco]; Federal University of Paraiba [Universidade Federal da Paraíba]; and Federal University of Ceará [Universidade Federal do Ceará].

At each location, the experimental design used in Renacal was the casualized blocks, with four repetitions for three years. The plots were constituted of 5 m long rows with 15 cm spaces (plot area: 10 m x 5.0 m x 0.15 m = 7.5 m^2). Two rows at each side and 0.5 m off each extremity of the plot were considered as borders. Treatments were constituted of 20 alfalfa cultivars. The following characteristics were evaluated: stem, crude protein rate in leaves, stems and in the whole plant, persistence and tolerance to pests and diseases.

In Renacal, in several experiments and under cutting conditions, around 50 alfalfa cultivars were tested, and their adaptation to climate and soil

conditions from various regions of Brazil were evaluated. Results revealed that alfalfa shows good behavior in various environments, with differential response among the tested cultivars. Crioula and P-30 stood out in most environments, indicating that they show a broad band of adaptation and are therefore recommended for cultivation in areas under influence of Atlantic forest, Cerrado and subtropical climate ecosystems.

Evaluation of cultivars

The number of cultivars available for cultivation is directly related to the adoption and the cultivation of alfalfa in each country. The United States of America, Canada, Argentina and Italy have a relatively large number of improved cultivars available, because they have relatively large areas of alfalfa production. Investment on breeding programs for this forage in these countries clearly reflects their respective productivity in the alfalfa crop. In these countries, breeders have developed cultivars with high productive potential and with great resistance to the main causes of biotic and abiotic stress. Another determining factor of investments in breeding this forage is related to the market of seed commerce, which has great representativity in these countries (Wilkinson; Castelli, 2000; Nabinger, 2002).

The alfalfa crop in Brazil is concentrated in the South region, where Rio Grande do Sul state represents around 80% of the planted area. This situation may be related to cultural aspects of the first producers (immigrants), who already knew this forage, and to the occurrence in the region of similar soil and climate conditions to those prevailing in the traditional producer countries. Researchers from this region were also pioneers in Brazil in the goal of identifying adapted cultivars (Bassols et al., 1979; Zimmer et al., 1982; Fischer et al., 1984; Saibro, 1985; Saibro et al., 2001), in addition to studying the establishment and management of alfalfa under the soil and climate conditions of the South of Brazil.

The growing interest for the productive potential of alfalfa in tropical climate regions is due, mainly, to the intensification of milk and beef production systems (Costa; Saibro, 1994; Vilela, 1994; Oliveira, 2000, 2006; Perez et al., 2002; Ferragine, 2003; Perez, 2003; Ferragine et al., 2004; Ruggieri et al., 2005; Oliveira; Herling, 2006; Pereira; Ferreira, 2008). Researches with alfalfa in tropical conditions have been showing that, in addition to high potential for production and high nutritional value, this legume has variability for adaptation to soil and climate conditions of different regions (Botrel et al., 1992, 2000, 2001, 2005; Evangelista et al., 1993, 2000; Botrel; Alvin, 1997; Viana et al., 1998; Ferreira et al., 1999, 2004; Vieira et al., 2000; Uchoa et al., 2000; Ruggieri

et al., 2001; Lédo et al., 2004, 2005; Costa et al., 2006; Heinemann et al., 2006; Rassini et al., 2007; Vasconcelos et al., 2008). Identifying cultivars adapted to tropical conditions is a prioritary goal which aims at using this legume as high quality and high productivity feed in intensive production systems.

Alfalfa cultivars were evaluated for their adaptation to different ecosystems (Cerrados, Atlantic forest, temperate climate, semiarid) by Renacal. Next, alfalfa cultivars indicated for the cutting and the pasture system were detailed.

Cultivars for cutting

In the works performed by Renacal, 50 alfalfa cultivars introduced from other countries, especially from Argentina and from the USA, were evaluated, in addition to cultivars developed from the Crioula population grown initially in Rio Grande do Sul state. The results obtained from the assays of Renacal showed that alfalfa is an excellent forage resource, standing out for its productivity and for forage quality.

The results of the evaluations by Renacal generally showed a major difference in dry matter production according to the location where the cultivars were tested. However, in all environments, cultivars with high productivity were identified (Table 1). The productivity of the best cultivars matches the indices observed in other countries which are traditional producers of this forage. These results indicate the existence of high influence by the genotype and the environment in the evaluated cultivars. The regions where the best productivity was achieved were the municipalities of Bandeirantes (Paraná), Sete Lagoas (Minas Gerais) and Manoel Vitorino (Bahia) and the worst, in Eldorado do Sul (Rio Grande do Sul), Rio Verde (Goiás) and Governador Valadares (Minas Gerais). It is worth reiterating that the productivity achieved reflects the interaction of several factors involved in the productive system and, thus, the occurrence of adverse factors not controlled by the researchers may have contributed for the low productivity in some locations. Another fact is that these results are not representative of historical series of productivity of the cultivars. Therefore, they only provide a preliminary idea of the behavior of the cultivars in each location.

It was the Crioula cultivar that had the best productivity index, which means ranging from 9.0 t ha^{-1} $year^{-1}$ to 21.3 t ha^{-1} $year^{-1}$. This was the most productive cultivar in seven locations. The P-30 cultivar also achieved excellent annual production of dry matter, ranging from 7.9 t ha^{-1} to 22.9 t ha^{-1}. These cultivars had a broad band of adaptation to the various tropical environments in which they were evaluated, confirming the good performance in Brazilian soil and climate conditions. Possibly because of the lack of improved cultivars adapted to tropical

Table 1. Annual forage dry matter (DM) production, achieved by the three most productive alfalfa cultivars, in 14 locations.

Location (municipality and state)	Cultivar	Annual forage dry matter (t ha^{-1})
Coronel Pacheco, MG	Crioula	13.0
	Monarca	11.9
	P-30	11.8
Sete Lagoas, MG	Crioula	20.0
	P-30	19.6
	Rio	16.8
Lavras, MG	Crioula	17.5
	P-30	16.3
	P-5715	13.7
Governador Valadares, MG	Crioula	10.3
	Victoria	10.3
	CY-9313	9.6
Paty do Alferes, RJ	Crioula	14.2
	P-30	14.0
	Maricopa	13.8
Sertãozinho, SP	SW-8210	14.0
	Monarca	13.9
	P-5715	13.7
São Carlos, SP	Crioula	16.4
	P-30	13.3
	WL-516	12.5
Rio Verde, GO	Crioula 1	9.8
	Crioula 2	9.0
	P-30	7.9
Eldorado do Sul, RS	Crioula	9.2
	Rio	8.9
	P-30	8.4

Continued...

Table 1. Continued.

Location (municipality and state)	Cultivar	Annual forage dry matter (t ha^{-1})
Bandeirantes, PR	P-30	22.9
	WL-516	22.8
	Crioula	21.3
Chapecó, SC	Alto	13.9
	BR-3	13.4
	SW-8112	13.0
Areia, PB	XA-132	17.1
	Crioula	15.3
	SW-14	14.4
Pentecoste, CE	SW-9301	15.2
	P-30	14.4
	Victoria	14.4
Manoel Vitorino, BA	Cordobesa	18.9
	P54H55	17.9
	Victoria	17.1

Source: Botrel (2005).

conditions, it is observed that the Crioula cultivar or populations deriving from it are always among the most productive ones, although some introduced cultivars are superior in some environments. Another highlight is the Victoria cultivar, which despite not having the highest production in any location, was distinguished by being one of the three most productive cultivars in two lower latitude states (Ceará and Bahia), where the characteristic climate is tropical semiarid.

Cultivars for pasture

There are few studies on milk production of cattle in alfalfa pastures, especially in tropical climate. Vilela (1994) presented the results of evaluating two management systems for cattle with high potential for milk production: one of them had an alfalfa pasture as the only feed and the other, corn silage and concentrate, in which the animals were kept in total confinement; it was

concluded that using alfalfa pasture as exclusive food for lactating cows was viable, since it had the potential to support three cows per hectare and to provide an average daily milk production of 20.0 kg per cow, achieving 23.6 kg per cow in the beginning of lactation, without compromising the live weight and the reproductive efficiency of the animals. Works carried out at Embrapa Southeast Livestock (Netto et al., 2008a, 2008b) showed that using alfalfa in pasture, as part of the diet of cows fed with corn silage and 5.0 kg of concentrate, at the middle stage of lactation, enabled an average daily production of 25 L of milk per cow. This represents a significant saving in the amount of concentrate generally used daily (8 kg) to achieve this level of production, as well as the possibility of reducing the protein content of the concentrate and the amount of corn silage needed, which contributes for reducing the production cost of milk. Based on this work, Vinholis et al. (2008) observed a reduction in the production cost of milk of 9% and of 15%, when alfalfa participated with 20% or with 40% of the dry matter in the diet, respectively.

In terms of breeding, the content of non-structural carbohydrates accumulated in the roots may indicate persistence and tolerance to grazing (Smith et al., 1989). Tolerance to grazing can also be related to the residual leaf area and to strength of resprouting. Plants which have decumbent stems with tissue accumulation next to soil level can store a larger amount of photoassimilate, increasing resprouting capability and, consequently, tolerance to grazing. Prostrated stems, number of stems, number of crowns, crown area, forage yield, residual leaf area, root weight after defoliation and non-structural carbohydrates concentration are currently the most commonly used variables to evaluate alfalfa cultivars under pasture (Brummer; Bouton, 1991, 1992).

When Perez et al. (2002) evaluated cultivars ABT 805, Crioula Chilena, Crioula Roque and Crioula Ledur in Southern Brazil, they found survival of 90%, 65%, 59% and 55%, respectively. In a study in pasture conditions (continuous and rotational grazing), Ferragine (2003) observed that under continuous grazing, there was death of cultivars Crioula Chilena and CUF 101 and low survival of the other cultivars. Under rotational grazing, production was lesser, but with survival of 44.9%; 34.4%; 28.2%; 27.6% and 24.9% of cultivars ABT-805, Alfagraze, CUF 101, Crioula Chilena and Pioneer 5432, respectively. Despite the low survival in continuous grazing, the Crioula cultivar achieved the best performance in rotational grazing conditions, with annual production of 18.3 $t.ha^{-1}$ of dry matter. Ruggieri et al. (2005) also demonstrated that the Crioula cultivar was the one with the best productivity and the most recommended for pasture, in a study carried out in the municipality of Sertãozinho, São Paulo state, after eight grazing cycles intercalated with resting periods.

Oliveira et al. (2001) tested twelve alfalfa cultivars in pasture conditions for four short term grazing cycles. In this study, cultivars Crioula Chilena and Pioneer 5312 were the ones with better survival (39.9%) and greater keeping of the crown. Productivity (t ha^{-1} of dry matter per cycle) was 2.60 for Crioula Chilena, 1.74 for Pioneer 5312 and 1.74 for Pioneer XAI 32, in rotational grazing. These results indicate that the Crioula cultivar was the most productive in rotational grazing conditions, with high stocking. Oliveira (2006), evaluating 19 alfalfa cultivars in the municipality of São Carlos, São Paulo state, in pasture conditions over 11 months, determined that cultivars Crioula RS, Crioula Chilena and Crioula Itapuã were the ones that stood out the most, with productivity between 20 ha^{-1} $year^{-1}$ and 22 t ha^{-1} $year^{-1}$ of dry matter, quite favorable production seasonality between 35% and 40%, and survival between 80% and 100% (Table 2). When assessing the same 19 cultivars in the municipality of Pirassununga, São Paulo, and in five grazing cycles, Oliveira and Herling (2006) verified that the most productive cultivars in dry matter were Crioula RS with 15.2 t ha^{-1} $year^{-1}$, Amerigraze with 13.9 t ha^{-1} $year^{-1}$ and Crioula Itapuã with 14.0 t ha^{-1}. Regarding the survival rate, there was no variation among the cultivars evaluated, and the mean was 73.5% (Table 2).

Table 2. Annual production of forage dry matter (DM), achieved by the nine most productive alfalfa cultivars in pasture, in two tropical climate municipalities.

Cultivar	São Carlos, São Paulo[1]		Pirassununga, São Paulo[2]	
	DM (t ha^{-1})	Seasonality (%)	DM (t ha^{-1})	Survival (%)
Amerigraze	16.6	34.8	13.9	78.8
Crioula Chilena	21.1	44.0	11.5	57.6
Crioula Itapuã	21.5	39.2	14.0	69.8
Crioula RS	21.8	43.3	15.2	106.6
CUF 101	18.8	36.2	12.0	56.1
Pioneer 5454	18.2	32.0	11.0	55.8
SW 8200	18.0	38.8	13.0	68.6
ZG 9786	18.3	40.3	13.9	80.0
ZG 9797	18.3	38.7	12.0	68.3

[1] Annual average of two seasons: rain season of 2004–2005 and dry season of 2005.

[2] Twelve months production.

Source: Oliveira (2006) and Oliveira and Herling (2006).

Crioula alfalfa

The Crioula population results from a joint selection process carried out by man and nature, in Rio Grande do Sul state, from the introduction and cultivation of alfalfa in the valleys of rivers Caí, Taquari, Jacuí and Uruguay and in the border of the Mountain Range, which started around 1850 (Saibro, 1985; Oliveira et al., 1993; Perez, 2003). In these crops, producers harvested seeds from four to five-year-old alfalfa plantations, which ended up generating the Crioula population. The consequence to this selection process was the development of a population with broad genetic variability and good adaptation to most environments.

Crioula alfalfa is characterized by not presenting leaf drop during its development, which results in greater accumulation of reserves in the roots and crown of the plant. This leaf retention allows intense and strong resprouting and leads to fast recovery of leaf area after the cuts, with good dry matter yield, good seasonal distribution and great persistence. In addition, because it is a cultivar without winter rest, it shows active growth during autumn and winter (Saibro, 1985; Honda; Honda, 1990; Nuernberg et al., 1990). The Crioula population has an upright growth habit, an interesting characteristic for haymaking, to which purpose it has been cultivated the most in Brazil (Perez, 2003), as well as variation of persistent plant types, ideal for pasture (Favero, 2006).

In Brazil, almost the entire area cultivated with alfalfa is taken by the Crioula cultivar or by populations derived from it (Crioula CRA, Crioula Itapuã, Crioula na Terra, Crioula Nativa, Crioula Ledur, Crioula Roque, Crioula Chile, Crioula UFRGS and others).

Results from experiments conducted in several locations have demonstrated that the Crioula cultivar, or populations derived from it, is always among the best performing ones in forage production (Ferreira et al., 1999, 2004; Botrel et al., 2000, 2001, 2005; Oliveira, 2000; Oliveira et al., 2001, 2003, 2004; Lédo et al., 2004, 2005; Heinemann et al., 2006). Pereira et al. (1998) evaluated the performance of seven Crioula cultivars (Crioula CNPGL, 5715, Rio, Crioula original, Flórida 77, Vale Plus and Crioula EEA-UFRGS) for some characteristics of forage importance, such as dry matter production, plant height, percentage of blooming flowers at cutting time and disease incidence. The authors observed that materials originated from the Crioula cultivar displayed superiority for most of the traits evaluated. These studies revealed that Crioula alfalfa has potential of yearly dry matter production of up to 25 $t.ha^{-1}$, with low seasonality, high biological fixation of atmospheric N and good efficiency of water use.

Breeding of Crioula alfalfa

Through an experiment of polycross progenies test, Oliveira et al. (1993) proved that Crioula alfalfa has genetic variability in dry matter yield, plant height, leaf:stem ratio and crude protein content. Due to this genetic variability, Crioula alfalfa has been used as genetic material to obtain derived cultivars. Breeding programs for this population have been carried out by Embrapa Dairy Cattle, by Embrapa Southeast Livestock and by the Federal University of Rio Grande do Sul (Ferreira; Pereira, 1999; Dall'agnol et al., 2007; Rassini et al., 2007).

Embrapa Dairy Cattle started its alfalfa breeding program in 1996, with the goal of obtaining cultivars that were indicated for production in tropical climate and which had certain characteristics, such as combined resistance to the main pests (*Acyrthosiphon pisum* (Harris) [pea aphid], *Acyrthosiphon kondoi* Shinji [blue alfalfa aphid] and *Aphis craccivora* [alfalfa black aphid]) and diseases (*Colletotrichum trifolii* Bain & Essary [anthracnose] and *Leptotrochila medicaginis* (Fckl.) Schüepp [yellow leaf blotch]), persistency, smaller degree of winter rest and high potential for seed and forage production.

To develop a population more adapted to the tropical environment, selection was performed in a population of Crioula alfalfa, in which plants were selected based on characteristics of forage interest. The resulting population was experimentally called "Crioula CNPGL". This material was evaluated in experiments with other populations derived from the original Crioula, aiming at estimating gains from selection and adaptation to tropical conditions (Pereira et al., 1998). When traits of production and percentage of dry matter, disease tolerance and plant height were evaluated in seven cuts, the cultivars Crioula (original), Crioula CNPGL and Crioula EEA-UFRGS showed remarkable behavior in relation to the other genotypes (Table 3). Results showed that the Crioula cultivar has a high percentage of dry matter, better tolerance to disease and higher plants in relation to the other introduced cultivars. The selection process did not change these traits in the derived populations (Crioula CNPGL and Crioula EEA-UFRGS). In dry matter production per area, populations Crioula (original) and Crioula CNPGL stood out from the rest, which indicates that they are better adapted to tropical conditions.

The Crioula CNPGL population is being subjected to the test of cultivation and usage value, aiming at its release as a cultivar for cutting, adapted to tropical conditions. Other studies on alfalfa breeding were carried out with the goal of selecting materials with high seed production, high dry matter production and high persistence (Bassols et al., 1979; Oliveira, 1991; Oliveira et al., 1993; Fão, 1995; Dutra, 1999).

Table 3. Averages of dry matter (DM) percentage, height of the plant (HP), blooming (BLO), disease incidence (DIS) and dry matter production (DMP) in alfalfa cultivars, in seven successive cuts.

Varieties	Characteristics				
	DM (%)	HP (cm)	BLO (%)	DIS[(1)]	DMP (kg ha^{-1})
Crioula CNPGL	25.0	47.7	5.2	2.04	1,131
Cultivar 5715	24.4	37.7	2.3	3.61	830
Cultivar Rio	23.3	39.9	2.8	2.85	833
Crioula (original)	25.1	45.4	6.1	2.62	1,012
Flórida 77	23.7	42.1	3.7	4.57	865
Vale Plus	23.4	37.6	2.2	3.38	758
Crioula EEA-UFRGS	25.9	46.0	10.0	2.38	819

[(1)] Based on scoring, where 1 = resistant and 5 = susceptible.
Source: Pereira and Ferreira (2008).

When Favero (2006) compared alfalfa populations with different capabilities, she observed similarity between Crioula alfalfa and the Alfagraze cultivar (reference as a cultivar for pasture) in allocation of carbohydrates for the root system. This characteristic is associated with plant resistance in pasture and demonstrates that Crioula alfalfa displays variability for breeding aiming at pasture. These results show that Crioula alfalfa, in addition to performing better for cutting, also stands out in pasture. The genetic variability found in Crioula alfalfa allows selecting materials for these two goals.

Final considerations

Some countries, as the USA, Canada and Argentina, have several cultivars adapted to their different available environments. In Brazil, however, most of the area is cultivated with varieties originated from the Crioula population, which has the best productivity indices comparatively to the other cultivars. This population originated from a selection process carried out in Southern Brazil, from introductions performed from Uruguay and Argentina which brought about several varieties usually called Crioula + “variety selection location”. In addition to these varieties, the following cultivars are registered in the National Cultivar Registry [Serviço Nacional de Proteção de Cultivares]: Monarca

SP INTA, Super Leiteira, Trifecta, WL-325 HQ and WL-525 HQ. There are also several other cultivars without official registration which spread nationwide with the most diverse names and which were introduced mainly from Argentina and from the USA.

Regarding the recommendation of cultivars for pasture, there are still no recommended cultivars of national origin. Some studies evaluated the potential of cultivars for pasture, in which the Crioula variety presented good results. Some cultivars, such as ABT-805, Maxigraze and Amerigraze, of foreign origin, are reported in the literature as being tolerant to pasture but did not show good adaptability and good stability in Brazil.

New alfalfa cultivars must be developed, with adaptability and stability to tropical conditions, increased yield and resistance to the diverse abiotic and biotic factors, in addition to characteristics which enable their use in specific conditions of environment and using forms (cutting and pasture).

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CHAPTER 12

Biometric procedures applied to genetic improvement of alfalfa

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Introduction

In this chapter we address several biometric and statistic principles which allow the researcher to analyze experimental data and to generate useful information for an alfalfa breeding program. The use of these procedures in the several stages of a program of such nature will be illustrated and subdivided into three phases: start, in which the goal is to form a base population with broad genetic variability, with characteristics of agricultural interest and good adaptability; middle, in which there is concern about the conduction of segregating families which allow maximizing direct gains, indirect gains or simultaneous gains in important traits; and end, in which the improved genetic material is already available and the aim is to recommend it for broad regions or for specific regions, which makes studies on genotypes x environment interaction essential, as well as studies on adaptability and stability.

Base population formation

One of the main stages of the breeding program is the choice of parents which, after interbreeding, will form the base population in which the researcher will invest efforts searching for productive genetic material, with quality and good adaptation. Several criteria can be applied to choose the parents, especially the performance regarding characteristics of agricultural interest, combination capability and adaptability. Another key factor is the diversity among the group of parents, evaluated with the goal of identifying the hybrid combinations of greatest heterotic effect and greatest heterozygosity, so that in its segregating generations there is greater possibility of recovering superior genotypes. The formation of the base population is very important in the context of management and conservation of alfalfa germplasm, since it provides information on the available resources and helps the localization and interchange of such resources.

Genetic diversity has been evaluated through biometric techniques based on the quantification of heterosis, or through prediction processes. Among the methods based on biometric models, aiming at evaluating parental diversity, the diallel analyses are cited (Cruz, 2005).

Diallel analysis

Diallel analyses are designed for quantifying the genetic variability of the trait and for evaluating the genetic value of parents and the specific capability

and heterosis manifested in specific crossings. In diallels, it is necessary to evaluate the hybrid combinations between the parents. Diallel analysis has been routinely used in genetic improvement to evaluate a small number of parents (around ten). However, when a large number of potential parents is available to use in crossings to form a base population, obtaining experimental material can be impracticable and the study, impossible.

For diallels including only the hybrid combinations, the following statistic model has been adopted:

$$Y_{ij} = m + g_i + g_j + s_{ij} + \varepsilon_{ij},$$

where

Y_{ij} : mean value of hybrid ij (i, j = 1, 2, ... p, $i < j$)

m: general mean

g_i, g_j: effects of the general combination capability (GCC) in the i-th and j-th parents, respectively

s_{ij}: effect of the specific combination capability (SCC) for crossings between parents of orders i and j and

ε_{ij}: average experimental error

Considering, as illustration, an outline involving four parents, we then have the diallel scheme presented in Table 1.

Table 1. Diallel scheme including hybrids F1 of four parents.

Parent	1	2	3	4
1	-	Y_{12}	Y_{13}	Y_{14}
2		-	Y_{23}	Y_{24}
3			-	Y_{34}
4				-

The effects can be estimated through the following formulas:

$$\hat{m} = \frac{2}{p(p-1)} Y_{..}$$

$$\hat{g}_i = \frac{1}{(p-2)} [Y_{i.} - (p-1)\hat{m}] = \frac{1}{(p-2)} Y_{i.} - \frac{2}{p} Y_{..} = \frac{1}{p(p-2)} [pY_{i.} - 2Y_{..}]$$

$$\hat{s}_{ij} = Y_{ij} - (\hat{m} + \hat{g}_i + \hat{g}_j) = Y_{ij} - \frac{1}{(p-2)} (Y_{i.} + Y_{.j}) + \frac{2}{(p-1)(p-2)} Y_{..}$$

and the variance analysis is carried out as the scheme presented in Table 2.

Table 2. Variance analysis scheme for balanced diallels involving only F1 hybrids, according to the methodology proposed by Griffing (1956).

FV	GL	QM	F		E(QM)	
			Steady	Random	Steady	Random
GCC[1]	p-1	QMG	QMG/QMR	QMG/QMS	$\sigma^2_\varepsilon + (p-2)\phi_g$	$\sigma^2_\varepsilon + \sigma^2_s + (p-2)\sigma^2_s$
SCC[2]	p(p-3)/2	QMS	QMS/QMR	QMS/QMR	$\sigma^2_\varepsilon + \phi_s$	$\sigma^2_\varepsilon + \sigma^2_s$
Residue	f	QMR			σ^2_ε	σ^2_ε

[1] GCC = general combination capability. [2] SCC = specific combination capability.

The sum of squares (SS) will be given by

$$SS(GCC) = \sum \hat{g}_i Y_{i.} = \frac{1}{(p-2)} \sum Y^2_{i.} - \frac{4}{p(p-2)} Y^2_{..} \quad \text{and}$$

$$SS(SCC) = \sum_{i<j}\sum \hat{s}_{ij} Y_{ij} = \sum_{i<j}\sum Y^2_{ij} - \frac{1}{(p-2)} \sum_{i<j}\sum Y^2_{ij} (Y_{i.} + Y_{.j}) + \frac{2}{(p-1)(p-2)} Y^2_{..}$$

Based on this analysis, it is possible to evaluate the relative importance of additional genetic effects, expressed by the effects linked to GCC, as well as the ones due to dominance deviations, linked to SCC. This information is useful for establishing the best breeding strategy. When additional effects are marked, greater gains will be predicted, even when simpler breeding strategies are used. The results are also useful to point the parents with best performance and greatest genetic complementarity to be interbred.

Diallel crossings can also be used to obtain the heterotic potential of certain crossings. Madril et al. (2008) evaluated hybrids and parents of nine alfalfa germplasms which have been displaying importance in the formation of North American cultivars. After the hybrids were obtained, the existence of hybrid strength and great potential for continuous gains from recombining the elite materials used were verified.

Bolanõs-Aguilar et al. (2001) obtained a 7 x 7 diallel including the reciprocal in alfalfa to evaluate production of seeds and their components. In the analysis, the methodology described by Griffing (1956) was used. It was verified that GCC explained most of the evaluated traits, while SCC was only significant for the

trait "seed weight". The large effect of the significance of GCC found by the authors suggests that gains can be obtained with successive breeding cycles. The absence of significance of the reciprocal for most characteristics indicated lack of maternal effect, except on the characteristic "seed production per plant", in which the effect of the reciprocal was significant.

Genetic diversity

When a high number of parents are available, previous studies of predictive nature are recommendable, orienting the number and type of crossings in which to concentrate greater effort to obtain hybrids. Since they dispense the previous obtainment of hybrid combinations, predictive methods of diversity among parents have deserved considerable emphasis. Predictive methods are the ones based on differences – morphological, physiological, etc. – shown by parents for determining diversity, which is usually quantified through a dissimilarity measure (for instance, Euclidean distance and Mahalanobis distance). Inferring based on ecogeographical diversity is also an example of a predictive method for heterosis.

In the prediction of genetic diversity, several multivariate methods can be applied. Among them, we can quote principal components analysis and canonical variables analysis, and the agglomerative methods. The choice of the most adequate method has been determined based on the precision the researcher desires, on how easy the analysis is and on the way the data were obtained.

The methods based on principal components or in canonical variables allow studying the diversity in dispersion graphs, in which, usually, two cartesian axes are considered. In these studies, several characteristics are evaluated in a set of genotypes which, through statistical procedures, are summarized in few components (or canonical variables) and given by linear combinations of the original traits, independent of each other and with decreasing discrimination capacity, so that the first components (or canonical variables) explain the maximum of the variation existing in the original data.

Agglomerative methods differ from the others because they depend fundamentally on previously estimated dissimilarity measures, such as Euclidean distance or the generalized Mahalanobis distance, among others.

Grouping analysis

Grouping analysis aims at assorting, through some classification criterium, the parents (or any other type of sampling unit) in several groups, so that there is homogeneity within the group and heterogeneity between groups.

Alternatively, grouping analysis techniques have the goal of dividing an original group of observations into several groups, according to some similarity or dissimilarity criterium (Cruz et al., 2004).

In grouping analysis, several issues emerge. Thus, the final number of groups desired is questioned, as well as the adequacy of the participation achieved and the type of similarity measure to use. Regarding the number of groups desired, what is usually done is using several numbers of groups and, by some optimization criterium, selecting the most convenient one. To evaluate partition adequacy, it is common to use discriminant analysis and, regarding similarity measures, several are cited, but the most commonly employed in improvement are Euclidean and Mahalanobis distances for quantitative variables and Jaccard or Nei and Li indices for binary variables resulting from molecular markers studies.

The grouping process comprises basically two stages. The first one is related to estimating the similarity (or dissimilarity) measure between the parents and the second, to the adoption of the grouping technique for formation of the groups.

Dissimilarity measures

Genetic diversity studies aiming at identifying parents for hybridization have been carried out based on information on quantitative traits or on molecular markers.

In the case of quantitative traits, Average Euclidean distance ($d_{ii'}$) or generalized Mahalanobis distance ($D^2_{ii'}$) has been used to express genetic diversity. Although the latter is preferred, it can only be estimated when the residual covariances matrix is available, structured based on experimental assays with repetitions.

Generally, if X_{ij} is the observation in the i-th parent (i = 1, 2, ..., p), referring to the j-th trait (j = 1, 2, ..., n) studied, the Euclidean distance between two parents i and i' is defined through the expression

$$d_{ii'} = \sqrt{\sum_j (X_{ij} - X_{i'j})^2}$$

The generalized Mahalanobis distance is defined through

$$D^2_{ii'} = \delta' \Sigma^{-1} \delta,$$

where

δ: vector of deviations between the average values of parents in relation to the variances studies and

Σ: residual variances and covariances matrix, obtained from previous analyses, according to an appropriate statistical model.

To illustrate the example involving the evaluation of 20 cultivars will be considered regarding seven traits: height, dry matter production, dry matter percentage, crude protein, neutral detergent insoluble fiber, in vitro digestibility of dry matter and crude fiber, whose data are presented in Table 3.

Table 3. Evaluation of 20 alfalfa cultivars regarding seven phenotypic traits.

Genotype	Block	HEI[(1)]	DMP	DM	CP	NDF	IVD	CF
1	1	80.0	4,164.75	96.30	25.57	63.81	74.32	1.81
1	2	88.6	4,312.50	96.19	28.46	64.15	75.36	1.89
2	1	88.6	4,057.75	94.65	20.43	45.68	65.94	1.72
2	2	85.4	4,929.17	95.81	19.07	52.50	66.09	1.80
3	1	82.4	4,701.58	92.15	20.61	65.23	76.92	1.82
3	2	78.4	4,426.33	94.22	20.25	64.19	76.80	1.78
4	1	66.0	2,967.00	92.12	19.74	49.78	65.49	0.94
4	2	64.6	2,672.58	92.98	16.41	68.59	63.25	0.97
5	1	66.6	2,362.92	94.23	18.06	48.21	66.82	1.34
5	2	61.2	2,754.25	93.01	19.25	53.10	62.91	0.87
6	1	63.8	3,180.75	91.93	19.03	54.15	62.40	1.02
6	2	66.8	2,797.67	94.51	20.53	48.98	72.63	0.84
7	1	57.0	1,697.25	93.16	17.81	51.36	68.22	0.94
7	2	51.4	2,393.42	93.18	18.13	60.79	63.91	0.96
8	1	61.8	2,475.83	96.36	20.69	47.69	62.54	1.18
8	2	60.2	1,889.42	94.40	22.08	42.31	68.08	0.69
9	1	61.4	2,921.33	94.58	19.86	49.37	65.28	1.08
9	2	62.0	3,757.33	93.91	19.68	51.04	65.56	1.04
10	1	70.0	2,506.75	92.18	19.68	47.60	65.83	0.97
10	2	58.2	2,640.50	94.01	20.75	50.26	69.73	1.00
11	1	61.8	3,305.42	95.55	22.44	46.82	65.07	0.93

Continued...

Table 3. Continued.

Genotype	Block	HEI[(1)]	DMP	DM	CP	NDF	IVD	CF
11	2	60.4	3,625.92	92.51	18.59	63.14	28.92	1.03
12	1	54.0	2,679.75	91.50	21.79	45.99	65.83	0.69
12	2	59.2	3,021.58	93.21	19.16	63.78	65.37	1.23
13	1	59.4	2,253.42	96.14	18.15	47.20	60.52	1.29
13	2	64.8	3,225.17	94.42	20.37	50.72	68.64	0.87
14	1	68.6	3,245.33	95.05	20.48	50.13	62.36	1.82
14	2	63.4	2,907.92	92.99	16.74	50.56	68.57	1.26
15	1	63.6	3,622.42	94.87	18.21	53.00	61.74	0.98
15	2	60.2	3,189.08	91.52	19.51	65.62	62.94	1.05
16	1	64.2	2,567.33	93.94	19.58	43.23	65.75	1.03
16	2	60.4	2,664.50	93.63	18.78	49.47	62.80	1.50
17	1	66.6	2,690.75	92.8	17.37	53.02	64.52	1.22
17	2	67.2	2,880.50	95.03	18.60	50.26	62.36	1.02
18	1	67.2	1,520.83	91.67	19.50	48.92	60.54	0.85
18	2	62.8	1,830.50	94.16	19.72	46.58	60.48	0.96
19	1	67.2	2,751.08	96.16	15.15	48.77	60.99	1.31
19	2	63.4	2,942.50	92.64	14.09	59.25	66.55	1.12
20	1	44.8	1,986.00	90.22	12.37	50.12	62.06	1.09
20	2	48.8	1,610.67	90.72	13.69	43.26	70.26	0.69

[(1)] HEI = height; DMP = dry matter production; DM = dry matter percentage; CP = crude protein; NDF = neutral detergent insoluble fiber; IVD = in vitro digestion of dry matter; and CF = stem/leaf.

To study genetic diversity it is necessary, preliminarily, to obtain the estimates of the residual variances and covariances matrix, through variance analyses. Once we have the average values and the Σ matrix, we obtain the 20 x 20 dissimilarity matrix, which can be later subjected to the grouping analysis. This will allow inferring the similar groups and the dissimilar groups.

Grouping techniques

Since it is desirable to have information regarding each pair of parents in the grouping process, the number of estimates of dissimilarity measures

is rather large, and that makes it impracticable to recognize homogeneous groups through the simple visual examination of those estimates. To perform this task, grouping methods are used.

Among the grouping methods most commonly used in plant breeding, we can quote the hierarchical ones and the optimization ones. Their description is presented as follows.

a) Hierarchical methods

In hierarchical methods, parents are grouped through a process which is repeated in several levels, until the dendrogram or tree diagram is established. In this case, there is no concern for the optimal number of groups, since the greatest interest is in the "tree" and in the branches obtained. Delimitations can be established by visual examination of the dendrogram, in which high level change points are evaluated, usually taking them as delimiters of the number of parents for a given group.

Hierarchical methods are also divided into agglomeration methods and divisive methods. Among agglomerative methods, we can quote the single linkage method; the complete linkage method; the average linkage or unweighted pair group method with arithmetic mean (UPGMA), weighed or not; the centroid method, also weighed of not; and the one proposed by Ward (1963). Among the divisive methods, the Edwards and Cavalli-Sforza (1965) is the most commonly known.

For the example being considered, grouping was carried out through UPGMA, based on the Mahalanobis generalized distance, achieving the result shown in Figure 1.

Touil et al. (2008) used the hierarchical method to classify 29 alfalfa populations from the Mediterranean, to evaluate the genetic diversity among these populations, using ISSR (*inter simple sequence repeat*) molecular markers. To calculate the genetic diversity among the various populations, the authors used the index by Rogers and Tanimoto. After the grouping analyses, four groups were formed and related to the origin of the evaluated populations.

In another study aiming at evaluating genetic diversity among alfalfa populations, Segovia-Lerma et al. (2003), through the UPGMA grouping technique were able to separate 30 genotypes belonging to nine groups with well recognized germplasm based on their geographical origin, using 34 AFLP primers for it.

b) Optimization methods

In optimization methods, the set of parents is parted into non-empty and mutually exclusive subgroups through the maximization of some previously

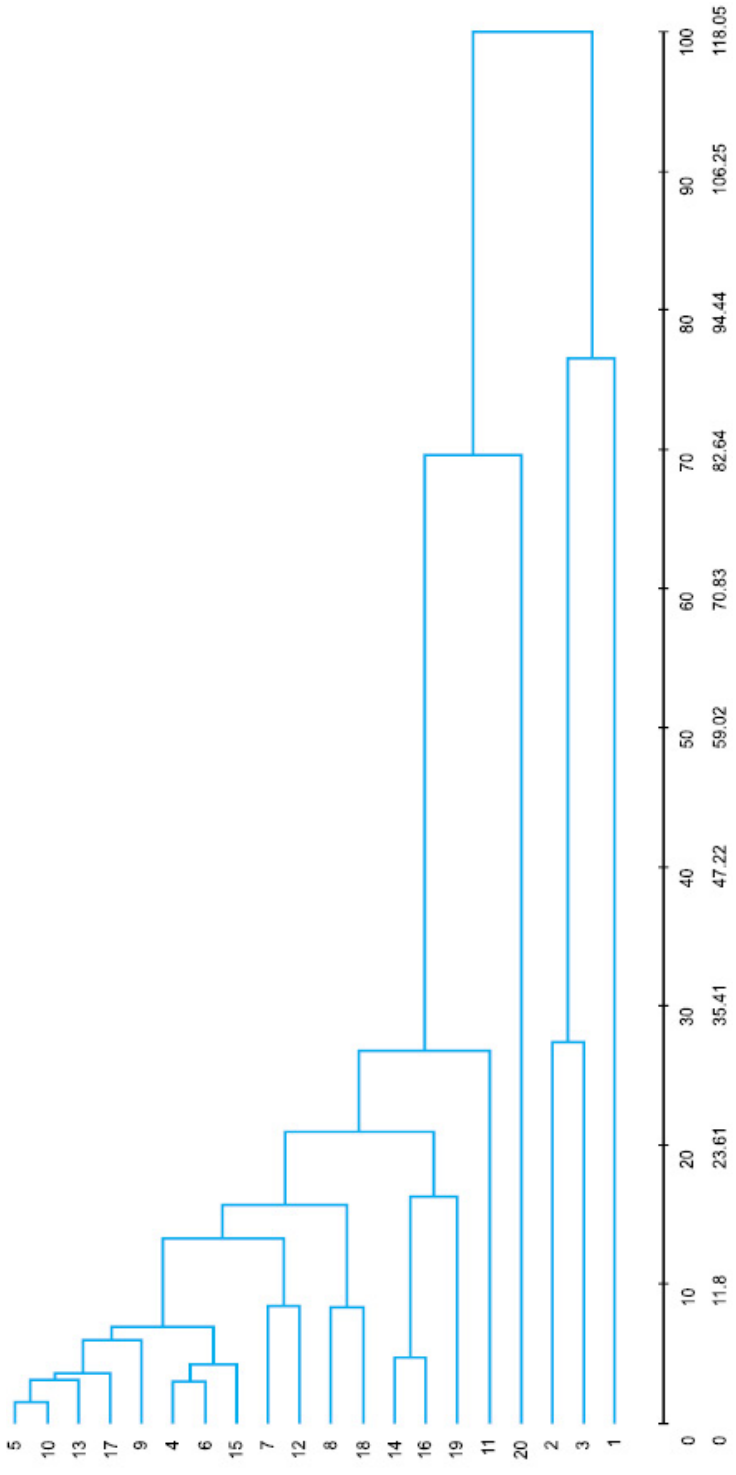

Figure 1. Dendrogram generated through the UPGMA grouping method, based on the Mahalanobis generalized distance.

set measure. One of the optimization methods most commonly employed in genetic improvement is the one proposed by Tocher, cited by Rao (1952).

In the Tocher method, the criterium adopted is that the average of the dissimilarity measures within each group must be smaller than the average distance between any groups. The method requires obtaining the dissimilarity matrix, upon which the most similar pair of parents is identified. These parents will form the initial group. From that, the possibility of including new parents is evaluated, adopting the criterium mentioned above.

For the example being considered, grouping was performed by the Tocher proposal, based on the generalized Mahalanobis distance, and the result obtained is indicated in Table 4.

We find the formation of four groups and that cultivars 1, 2, 3 and 20 are the most diverging ones in relation to the others. There is a group that differs in relation to these cultivars, but which still shows genetic diversity. The choice of cultivars to interbreed must consider the potential regarding the evaluated characteristics and the diversity. In this case, it is recommended to cross good cultivars belonging to different diversity groups.

Canonical variables analysis

This type of analysis requires more refined knowledge about multivariate statistical procedures, but it is easy to interpret and very useful in genetic diversity studies. It is based on generating new variables (named canonical

Table 4. Groups formed by Tocher methodology in an evaluation of 20 alfalfa cultivars.

Group	Cultivar
Ia	5 10 13 6 17 9 4 15 12 16
Ib	8 18
Ic	7
Id	11
Ie	14
IF	19
II	2 3
III	1
IV	20

variables), in which the information of the original measured variables is represented. These canonical variables are independent of each other and estimated so that the most variation is retained in descending order.

Thus, for the example being considered, the analyses allow concluding that with only two canonical variables (CV_1 and CV_2) it is possible to explain 81.2% of the variation found in the original data. These variables are defined through the following equations:

$$CV_1 = 0.177HEI + 0.001DMP + 0.039DM + 0.266CP + 0.063NDF + 0.029IVD + 0.981CF$$
$$CV_2 = 0.072HEI + 0.001DMP + 0.522DM - 0.855CP - 0.247NDF + 0.072IVD + 4.267CF.$$

The graphic analysis of the scores of the cultivars can be performed based on Figure 2. Again we note that cultivars 1, 2, 3 and 20 are the most diverging ones among the others.

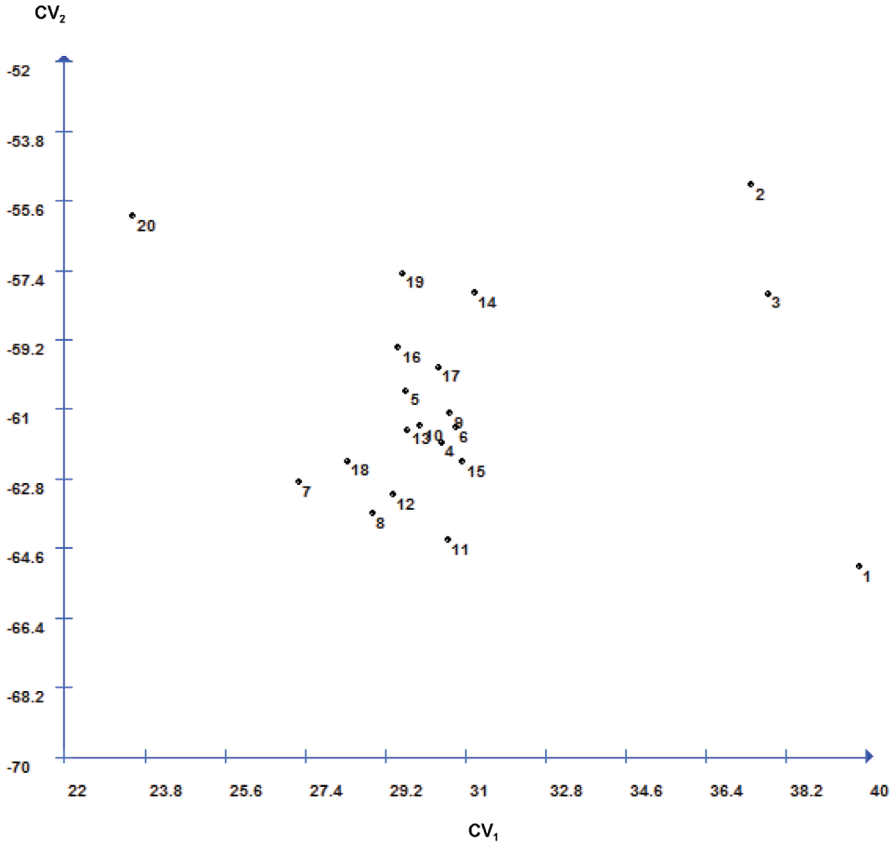

Figure 2. Graphic dispersion of scores of canonical variables obtained based on the linear combination of seven traits evaluated in alfalfa cultivars.

Conducting segregating populations

Predicting gains from selection

One of the great contributions of Quantitative Genetics is the evaluation of gains to be obtained through a given selection strategy. This information allows leading breeding programs, predicting their success, choosing or discarding populations and concentrating efforts in measuring traits of greater importance and greater gain potentiality. In this chapter, we address the prediction of gains obtained from selection in recurrent selection, in which selected individuals from an original population are tested and, themselves or others related to them, are recombined to obtain a new improved population in equilibrium.

To predict the gain from selection (*GS*), we use the expression

$$GS = p\hat{\sigma}_g\, hi,$$

where

p: parental control

$\hat{\sigma}_g$: genetic-additive standard deviation among test unities – normally corresponding to a fraction of the additive variance

h: heritability square root or accuracy of the selection process and

i: selection intensity

Some determinant factors of the gain from selection are: selection differential, selection intensity, parental control, genetic variability, environmental variance and genotypes x environments interaction.

Selection differential and selection intensity. One way to increase the gain from selection is to apply a higher intensity of selection, however, in very intense selection the population can present problems inherent to inbreeding, which is the consequence of crossing related individuals and is closely linked to the reduced size of samples.

Parental control. Parental control defines the similarity between test unities and the improved unities and, consequently, alters genetic covariance and the gain from selection.

Eberhart (1970) reported that parental control in a recurrent selection process can be defined by a function of the kinship relation between the selection unity used to identify superior individuals and the recombination unity used to obtain the improved population.

Genetic variability. Success of the breeding program depends on the existence of variability in the population. There is the concern to ensure wide variability in the work population, through the choice of diverging parents used in interbreedings to form the base population and through high specific capacity of combination.

Genetic variability is kept through adequate matings and proper samplings, so that the effective size of the population is not reduced.

Environmental variation. Phenotypic variation will be close to the genotypic variation when environmental variations are minimal. Thus, the environmental variation influences one of the main determining factors of the gain from selection, that is heritability. This coefficient is directly proportional to the additive genetic variability available in the population and inversely proportional to the phenotypic variation.

Genotypes x environments interaction. The existence of genotypes x environments interactions influences the gain to be achieved from selection. When the breeding program is restricted to a given environmental condition, this interaction is capitalized and, consequently, the fraction of used for predicting the gain is confused with the interaction σ2ga.

To illustrate, it will be considered that the 20 genotypes described in Table 3 constitute, instead of cultivars, half-sibling families derived from a breeding population. The gain achieved from selection of 30% of the best families can be estimated based on the information indicated in Table 5.

Based on the values of the means, of the average squares significance, of the variation coefficients and of the heritability (Table 5), the researcher can infer the genetic potential of the populations, the available variability, the experimental precision and the accuracy of the selective process. In this example, there is no possibility of gains in DM and IVD, since the genetic variability available through the half-sibling families is null. Increasing the genetic variability is recommended, by including new genotypes, by sampling another type of family or by better controlling the environmental influences, among other measures.

It is found that the experimental precision was adequate, that the greatest variation coefficient obtained reached 18% for CF and that there was significant genetic variance to be explored by selection in five out of the seven traits evaluated.

The averages of the evaluated families are presented in Table 6.

Table 5. Results of the variance analysis of seven agricultural traits evaluated in 20 half-sibling families of alfalfa.

FV	GL	QM						
		HEI[(1)]	DMP	DM	CP	NDF	IVD	CF
Block	1	19.04	197.86	0.16	0.17	195.67	0.09	0.05
Treatment	19	177.51**	1,280.33**	3.24[ns]	14.41**	63.17*	64.44[ns]	0.19**
Residue	19	11.51	109.93	2.01	1.98	30.31	45.13	0.04
Mean		65.06	2,953.24	93.71	19.26	52.71	65.11	1.16
VC (%)		5.21	11.23	1.51	7.31	10.44	10.32	18.0
h^2 (%)		93.51	91.41	37.96	86.23	52.00	29.96	77.7

[(1)] HEI = height, DMP = dry matter production, DM = dry matter percentage, CP = crude protein, NDF = neutral detergent insoluble fiber, IVD = *in vitro* digestibility of dry matter and CF = stem/leaf, VC = variation coefficient, h^2 = heritability.
ns,**,* Non-significant and significant, at 1% and 5% probability, by the F test, respectively.

Table 6. Average values of seven characteristics evaluated in 20 alfalfa half-sibling families.

Family	HEI[(1)]	DMP	DM	CP	NDF	IVD	CF
1	84.3*	4,238.63*	96.25	27.02*	63.98*	74.84	1.85*
2	87.0*	4,493.46*	95.23	19.75	49.09	66.02	1.76*
3	80.4*	4,563.96*	93.19	20.43*	64.71*	376.86	1.80*
4	65.3*	2,819.79	92.55	18.08	59.19*	64.37	0.96
5	63.9	2,558.59	93.62	18.66	50.66	64.87	1.11
6	65.3	2,989.21	93.22	19.78	51.57	67.52	0.93
7	54.2	2,045.34	93.17	17.97	56.08*	66.07	0.95
8	61.0	2,182.63	95.38	21.39*	45.00	65.31	0.94
9	61.7	3,339.33*	94.25	19.77	50.21	65.42	1.06
10	64.1	2,573.63	93.10	20.22*	48.93	67.78	0.99
11	61.1	3,465.67*	94.03	20.52*	54.98*	47.00	0.98
12	56.6	2,850.67	92.36	20.48*	54.89	65.6	0.96
13	62.1	2,739.30	95.28	19.26	48.96	64.58	1.08
14	66.0*	3,076.63	94.02	18.61	50.35	65.47	1.54*

Continued...

Table 6. Continued.

Family	HEI[1]	DMP	DM	CP	NDF	IVD	CF
15	61.9	3,405.75*	93.20	18.86	59.31*	62.34	1.02
16	62.3	2,615.92	93.79	19.18	46.35	64.28	1.27*
17	66.9*	2,785.63	93.92	17.99	51.64	63.44	1.12
18	65.0	1,675.67	92.92	19.61	47.75	60.51	0.91
19	65.3	2,846.79	94.40	14.62	54.01	63.77	1.22*
20	46.8	1,798.34	90.47	13.03	46.69	66.16	0.89
$\bar{X}_o$	65.06	2,953.24	93.71	19.26	52.71	65.11	1.16
$\bar{X}_s$[2]	74.98	3,917.79	-	21.67	59.70	-	1.57

[1] HEI = height; DMP = dry matter production; DM = dry matter percentage; CP = crude protein; NDF = neutral detergent insoluble fiber; IVD = in vitro digestibility of dry matter; and CF = stem/leaf.
[2] Average of the six superior families.

The estimates of the gain from selection, considering a breeding method in which the recombination only involves pollen from selected (parental control = 1), for the characteristic HEI, are the following:

$$\hat{\sigma}_g^2 = \frac{QMG - QMR}{r} = \frac{177.51 - 11.51}{2} = 83.00 \quad \text{and}$$

$$h^2 = \frac{\hat{\sigma}_g^2}{(QMG/r)} = \frac{83.00}{(177.51/2)} = 0.9351$$

For the selected percentage of 30%, we have $i = 1.159$ (Cruz, 2005). Hence, the gain from selection is estimated by

$$GS = i\, ph\, \hat{\sigma}_g = (1.159) \times 1 \times \sqrt{0.9351 \times 83.00} = 10.21$$

In percentage terms, we have

$$GS\ (\%) = \frac{GS \times 100}{average} = \frac{10.21 \times 100}{65.06} = 15.70\%$$

An alternative way to estimate the gain from selection is by the formula

$$GS = h^2\, DS = h^2\, (\bar{X}_s - \bar{X}_o) = 0.9351\ (74.98 - 65.06) = 9.28$$

In percentage terms, we have

$$GS\ (\%) = \frac{9.28 \times 100}{65.06} = 14.26\%$$

In Table 7, the estimates of genetic parameters are presented, as well as the predicted gain for variables HEI, DMP, CP, NDF and CF.

The gains from selection range between 6.9% and 29.9%. The two GS estimators, based on selection intensity or on the selection differential, provide similar estimates. It must be highlighted that the expression of GS based on the selection differential requires knowledge of the means of all the evaluated individuals, while the formula based on the knowledge of selection intensity does not require that knowledge; however, it must only be applied when the variable has normal distribution.

Table 7. Estimates of genetic parameters and predicted gain for the evaluated variables.

Trait[(1)]	X_o	X_s	h^2 (%)	GS = h^2DS	GS (%)	GS = $iph\hat{\sigma}_g$	GS (%)
HEI	65.06	74.98	93.52	9.28	14.26	10.21	15.70
DMP	2,953.24	3,917.79	91.41	881.73	29.86	847.69	28.70
CP	19.25	21.67	86.23	2.08	10.80	2.68	13.93
NDF	52.71	59.71	52.00	3.63	6.90	3.30	6.43
CF	1.16	1.57	77.74	0.32	27.14	0.28	24.3

(1) HEI = height; DMP = dry matter production; CP = crude protein; NDF = neutral detergent insoluble fiber; IVD = *in vitro* digestibility of dry matter; and CF = stem/leaf.

Direct and indirect selection of traits

As stated before, the success of a breeding program is primordially based on the existence of genetic variability, which enables selection and, consequently, the achievement of superior genetic materials to the breeder. The fast and efficient utilization of this variability is essential and studies about correlations constitute one of the paths to save time and to reduce efforts.

Estimating correlations is important to establish more adequate strategies for the condition of a breeding program and to evaluate the indirect responses in traits with low heritability or with problems of identification and measurement.

The correlations observed directly are phenotypic. It becomes necessary to distinguish their two causes: genetic and environmental. Genetic correlations are due mainly to pleiotropism and to the genetic changes in disequilibrium situations. Pleiotropism is the phenomenon through which a gene affects simultaneously two or more characteristics, so that if it is segregating it will cause concomitant variation in the characteristics involved. The correlation resulting from pleiotropism expresses the total effect of the segregating genes. Some pleiotropic effects can increase the characteristics, while others can reduce them. In other cases, the effects can increase some characteristics and reduce others, so that pleiotropism does not necessarily cause a correlation that can be detected.

Genetic linkage disequilibrium is the temporary cause of correlation and this can be altered in advanced generations due to breaks in the gene pools resulting from crossovers.

Phenotypic correlations are the ones obtained based on the means of the evaluated traits. When we consider the evaluation of two characteristics, X and Y, in *g* genotypes evaluated in *b* random blocks, we have the following statistical model:

$$X_{ij} \text{ or } Y_{ij} = \mu + g_i + b_j + \varepsilon_{ij}$$

The phenotypic correlation is obtained as follows:

$$r_f = \frac{Cov(\overline{X}, \overline{Y})}{\sqrt{V(\overline{X})\ V(\overline{Y})}}$$

where *X* and *Y* are variables that express the means of the genotypes in relation to traits X and Y, respectively.

To estimate the environmental correlations and the genotypic correlations, we must perform variance analyses and obtain the values of the mean squares (or variances). The mean products (covariances) are calculated using estimates of the mean squares obtained by the variance analysis of the sum of the values of *X* and *Y*, given by

$$Z_{ij} = X_{ij} + Y_{ij}$$

The scheme of variance analyses of variables *X*, *Y* and *X* + *Y* with the mean squares, indispensable for calculating the correlations, is presented in Table 8.

The mean products are obtained considering that

$$V(X + Y) = V(X) + V(Y) + 2\ Cov(X + Y),$$

Table 8. Scheme of variance analyses of traits X, Y and of the sum X + Y, evaluated in random blocks involving g genotypes.

FV	GL	X		Y		X + Y	
		QM	E(QM)	QM	E(QM)	QM	E(QM)
Blocks	r – 1						
Genotypes	G – 1	QMGx	$\sigma_x^2 + r\sigma_{gx}^2$	QMGy	$\sigma_y^2 + r\sigma_{gy}^2$	QMGx+y	$\sigma_{x+y}^2 + \sigma_{g(x+y)}^2$
Residue	(r-1)(g-1)	QMRx	σ_x^2	QMRy	σ_y^2	QMRx+y	σ_{x+y}^2

so that

$$PMG_{x,y} = \frac{QMG_{(x+y)} - QMG_x - QMG_y}{2} \quad \text{and}$$

$$PMR_{x,y} = \frac{QMR_{(x+y)} - QMR_x - QMR_y}{2}$$

The correlations can then be obtained as follows:

Environmental correlation:

$$r_a = \frac{PMR_{xy}}{\sqrt{QMR_x\ QMR_y}}$$

Genotypic correlation:

$$r_g = \frac{\hat{\sigma}_{g(x,y)}}{\sqrt{\hat{\sigma}_{gx}^2\ \hat{\sigma}_{gy}^2}}$$

where

$$\hat{\sigma}_{g(x,y)} = \frac{PMG_{x,y} - PMR_{x,y}}{r}$$

$$\hat{\sigma}_{gx}^2 = \frac{QMG_x - QMR_x}{r}$$

$$\hat{\sigma}_{gy}^2 = \frac{QMG_y - QMR_y}{r}$$

Phenotypic correlation:

Phenotypic correlation can also be obtained based on the mean squares, given by

$$r_f = \frac{PMG_{x,y}}{\sqrt{QMG_x\ QMG_y}}$$

For the example being considered, the correlation estimates obtained are shown in Table 9.

Table 9. Estimates of phenotypic and genotypic (in brackets) correlations, above the diagonal, and environmental, bellow the diagonal, between the combinations of seven characteristics evaluated in alfalfa.

Trait	HEI[(1)]	DMP	DM	CP	NDF	IVD	CF
HEI	1	0.807** (0.868)	0.608** -	0.576** (0.616)	0.416 (0.623)	0.424 -	0.852** (0.996)
DMP	0.059	1	0.463* -	0.493* (0.554)	0.601** (0.801)	0.302 -	0.796** (0.909)
DM	0.058	0.016	1	0.617** -	0.056 -	0.080 -	0.509* -
CP	0.241	0.013	0.139	1	0.359 (0.518)	0.251 -	0.427 (0.580)
NDF	-0.104	0.241	-0.318	-0.606	1	0.323 -	0.423 (0.390)
IVD	0.125	-0.223	0.231	0.480	-0.516	1	0.536* -
CF	0.023	0.215	0.301	-0.276	0.535	-0.350	1

(1) HEI = height; DMP = dry matter production; DM = dry matter percentage; CP = crude protein; NDF = neutral detergent insoluble fiber; IVD = in vitro digestibility of dry matter; and CF = stem/leaf.

**, * Significant, at 1% and 5% probability, by test t, respectively.

Correlated response to selection

The existence of genetic correlation between traits means that selection in one characteristic causes changes in others. Evaluating the direction and

dimension of these changes is essential to have, by the end of a breeding program, genetic materials with superior behavior in a number of characteristics.

Response expected from a trait Y, when selection is applied to a trait X, can be estimated by the following expression:

$$RY(X) = \hat{\beta}_g \, RX = \hat{\beta}_g \, \hat{\beta} \, DS_X$$

where

RX : direct response in trait X, given by $\hat{\beta} DS$;

$\hat{\beta}_g$: regression coefficient that measures variation in genetic values of the trait Y, calculated by

$$\hat{\beta}_g = \frac{Cov(X, Y)}{\hat{\sigma}^2_{gx}} = r_g \frac{\hat{\sigma}_{gy}}{\hat{\sigma}_{gx}}$$

$\hat{\beta}$: regression coefficient that measures variation in the genetic values in the improved population, regarding trait X, with changes caused by phenotypic selection in test unities, calculated by

$$\hat{\beta} = \frac{Cov_g(UM_X \, , \, UT_Y)}{\sigma^2_{fx}}$$

UM and *UT* are unities or individuals from the improved and from the test populations, respectively.

Regarding trait Y, we can estimate the direct gains through the formula

$$RY = p \, i_y \, h_y \, \hat{\sigma}_{gy}$$

and the indirect gains through the formula

$$RY(X) = p \, i_x \, h_x \, \hat{\sigma}_{gy} \, r_{gxy}$$

Therefore, the efficiency of indirect selection in relation to direct selection is given by the following ratio:

$$\frac{RY(X)}{RY} = \frac{p \, i_x \, h_x \, \hat{\sigma}_{gy} \, r_{gxy}}{p \, i_y \, h_y \, \hat{\sigma}_{gy}}$$

With the same selection intensity in traits X and Y, we have

$$\frac{RY(X)}{RY} = \frac{h_x \, r_{gxy}}{h_y}$$

from which we conclude that $RY(X) > RY$ if $r_g h_x > h_y$. Thus, response from indirect selection will be compensatory when the main trait (Y) has low heritability and when an auxiliary trait (X) of easy measurement, high heritability and high correlation to the main trait is available.

Based on the data presented in Table 5, we can obtain the correlated responses shown bellow.

Correlated response in Y, by selection in X

Let's consider the selection of 30% of the studied genotypes. Variable HEI will be called X and variable DMP, Y. Based on the results of the variance analysis presented in Table 5, we have

$$\hat{\sigma}_{gy} = \sqrt{585{,}198.8485} = 764.983;$$

$$h_x^2 = \frac{\hat{\sigma}_{gx}^2}{(QMT_X)/r} = \frac{83.00}{(177.5082/2)} = 0.9351 \text{ then } h_x = 0.9670 \text{ and } r_{gxy} = 0.8685$$

For the selected percentage of 30%, the selection intensity (i) value equals 1.159.

Thus, $RY(X) = 1.159 \times 0.9670 \times 764.983 \times (0.8685) = 744.6145$ assuming $p = 1$.

The direct response in Y is given by

$$RY = i_y h_y \hat{\sigma}_{gy}$$

where:

$$h_y^2 = \frac{\hat{\sigma}_{gy}^2}{(QMT_Y)/r} = \frac{585{,}198.8485}{1{,}280{,}331.3806/2} = 0.9141 \text{ then } h_y = 0.9561$$

thus $RY = 1.159 \times 0.9561 \times 764.983 = 847.6929$.

An alternative way to obtain the direct and correlated response in trait Y is by using the expressions based on selection differentials, obtained based on the means presented in Table 6.

Direct response is given by

$$RY = h_Y^2 DS_Y = h_Y^2 (\bar{Y}_S - \bar{Y}_O) = 0.9141 \ (3{,}917.7983 - 2{,}953.2437) = 881.7346$$

Indirect response is given by

$$RY(X) = h_Y^2 DS_{Y(X)} = h_Y^2 (\bar{Y}_{(X)} - \bar{Y}_O), \quad \text{in which}$$

$\bar{Y}_{(X)}$ is the mean of genotypes for trait Y, whose superiority was identified through the good performance in trait X. For the example at issue, $\bar{Y}_{(X)}$ is the mean of progenies 1, 2, 3, 4, 14 and 17.

Hence, we have

$$\bar{Y}_{(X)} = \frac{4{,}238.63 + 4{,}493.46 + 4{,}563.96 + 2{,}819.79 + 3{,}076.63 + 2{,}785.63}{6} =$$

$$= \frac{21{,}978.1}{6} = 3{,}663.01$$

and thus

$$RY(X) = 0.9141 \times (3663.013 - 2953.2437) = 648{,}8.$$

In some situations, the estimates of correlated responses obtained by the two estimators mentioned present results showing a great deal of discrepancy, disagreeing in dimension and, most surprisingly, in sign. Since the expression based on selection differentials is supported by just the ratio between two traits of the selected genotypes, it seems to be a good option used in breeding studies. In this example, we find that

$$RX(Y) = i_y\, h_y\, \hat{\sigma}_{gx}\, r_{gxy} = 1.159 \times 0.9561 \times \sqrt{83.00} \times (0.8685) = 8.768$$

and

$$RX(Y) = h^2_X\, DS_{X(Y)} = 7.1760.$$

When selecting trait Y, genotypes 1, 2, 3, 9, 11 and 15 were identified as superior. Among these genotypes, only 9, 11 and 15 produce less than the mean of trait X, while the others produce more, and genotypes 1, 2 and 3 were also selected for good performance in both Y and X. Given these facts, we can expect that the correlated response provides substantial gains in both characteristics evaluated.

Recommendation of cultivars

In a given environment, the phenotypic manifestation is the result of the action of the genotype under influence of the environment. However, when a number of environments are considered, we detect, in addition to genetic and environmental effects, an additional effect, given by the interaction between these effects.

Evaluating the interaction of genotypes x environments becomes very important to breeding because, in case it exists, there is the possibility for the

best genotype in one environment not to be so in another. This fact influences gain from selection and makes it difficult to recommend cultivars with broad adaptability. Because of the importance of this interaction, it is the breeder's job to assess its dimension and significance, quantify its effects in breeding techniques and the technology diffusion strategies, and to provide subsidies which allow adopting procedures for to minimize or utilize it.

Even though studies on interactions of genotypes x environments have great importance for breeding, they do not provide detailed information about the behavior of each genotype faced with environmental variations. For this purpose, adaptability and stability analyses are carried out, through which it becomes possible to identify cultivars with predictable behavior and that are responsive to environmental variations, in broad or specific conditions.

There are currently over ten methodologies of adaptability and stability analysis aiming at evaluating groups of genotypic materials tested in a number of environments. These methodologies are based on the existence of interactions and differ from the stability concepts adopted and from certain statistical principles employed. The choice of a method of analysis depends on the experimental data, mainly the ones related to the number of available environments, on the precision required and on the type of information desired. It should also be understood that some methods are alternative, while others are complementary and can be used together.

Eberhart and Russell (1966) suggested performing the adaptability and stability analysis based on a simple linear regression model. By these authors' proposal, the following equation is adopted:

$$Y_{ij} = \beta_{0i} + \beta_{1i} I_j + \delta_{ij} + \bar{\varepsilon}_{ij}$$

where

Y_{ij}: mean of genotype i in environment j

β_{0i}: general mean of genotype i

β_{1i}: linear regression coefficient, that measures the response of the i-th genotype to environmental variation

I_j: codified environmental index $\left(\sum_j I_j = 0\right)$

δ_{ij}: regression deviation and

$\bar{\varepsilon}_{ij}$: average experimental error

By this methodology, both the regression coefficients of phenotypic values for each genotype in relation to the environmental index, and the deviations

of such regression, would provide estimates of stability and adaptability parameters. The concepts involved in this methodology, which are easier to understand, are given bellow.

Adaptability. refers to the capacity of genotypes to utilize the environmental stimulus with advantage. As for adaptability, they are assorted into:

a) Genotypes with general or broad adaptability: in these, β_{1i} equals 1.

b) Genotypes with adaptability specific to favorable environments: in these, β_{1i} is greater than 1.

c) Genotypes with adaptability specific to unfavorable environments: in these, β_{1i} is lesser than 1.

Stability. refers to the capacity of genotypes to display a highly predictable behavior due to the stimulus from the environment. It is evaluated by the variance component attached to the regression deviations σ^2_{di}. The following types of genotypes are found:

a) Genotypes with high stability of predictability: in these, σ^2_{di} equals 0.

b) Genotypes with low stability or predictability: in these, σ^2_{di} is greater than 0.

Eberhart and Russell (1966) considered that the ideal genotype is the one which has a high production average, regression coefficient equal to 1 and regression deviations as small as possible.

Estimation of stability and adaptability parameters

Parameters β_{0i} and β_{1i} are estimated through the following expressions:

$$\hat{\beta}_{0i} = \bar{Y}_{i.} \quad \text{and} \quad \hat{V}(\hat{\beta}_{0i}) = \frac{1}{a}\hat{\sigma}^2_{\varepsilon} \quad \text{and}$$

$$\hat{\beta}_{1i} = \frac{\sum_j Y_{ij} I_j}{\sum_j I_j^2} \quad \text{and} \quad \hat{V}(\hat{\beta}_{1i}) = \frac{1\ \hat{\sigma}^2_{\varepsilon}}{\sum_j I_j^2}$$

where

$$I_j = \frac{1}{g}\sum_i Y_{ij} - \frac{1}{ag}\sum_i \sum_j Y_{ij} \quad \text{and}$$

$$\hat{\sigma}^2_{\varepsilon} = \frac{1}{r}\hat{\sigma}^2 = \frac{QMR}{r}$$

The hypothesis $H_o : \beta_{1i} = 1$ versus $H_a : \beta_{1i} \neq 1$ is evaluated by the statistic t, given by

$$t = \frac{\hat{\beta}_{1i} - 1}{\sqrt{\hat{V}(\hat{\beta}_{1i})}}$$

The stability parameter σ^2_{di} is estimated by the variance analysis method, based on the mean square of the regression deviation for each genotype (MSDi) and on the mean square of the residue, that is:

$$\hat{\sigma}^2_{di} = \sum_j \hat{\delta}^2_{ij} / (a - 2) = \frac{MSD_i - QMR}{r}$$

where

$$MSD_i = \frac{r}{a - 2}\left[\sum_j Y_{ij} - \frac{Y^2_i}{a} - \frac{\left(\sum_j Y_{ij} I_j\right)^2}{\sum_j I^2_j}\right] \qquad \text{(valid for every } i\text{)}$$

Sometimes, it may occur that many genotypes with superior average of yield are presented σ^2_{di} statistically different than zero. However, selection of a few genotypes from the group in which stability (or predictability) is low may be necessary. In these cases, an auxiliary measure of comparison between these genotypes is the determination of coefficient R^2_i, given by

$$R^2_i = \frac{SS(Linear\ regression)_i}{SS(A/G_i)} \times 100$$

To select alfalfa genotypes by adaptability and stability of the "dry matter" trait, Vasconcelos et al. (2008) evaluated 92 genotypes in two periods, rain season and dry season. In this study, the traditional methodology cited by Cruz et al. (2004) was used, in addition to the one by Eberhart and Russell (1966) and the centroid one (Rocha et al., 2005). The result regarding data analysis through the traditional method indicated that genotype WL 612, with the smallest mean square value for environments within the genotype, was the one with less variation in the mean of cuttings in the three environments and with the greatest stability. However, this genotype had a low average of dry matter production, in comparison to the others. According to Cruz et al. (2004), it is very likely that genotypes with smaller mean square of environments within genotypes had a reduced mean. The result of the adaptability and stability analysis, by the Eberhart and Russel methodology, indicated that genotype LE

N 4 was the one presenting a high value of $\hat{\beta}_0$ (or mean equal to 1,834.83 kg of dry matter per cut per ha), and $\hat{\beta}_1$ equal to 1.015, but the estimated regression deviation for this genotype was greater than zero, indicating the material has low stability. Lédo et al. (2005) also found this behavior, reporting that, according to Cruz et al. (2004), it may sometimes occur for several cultivars with high average yield, to present regression deviations statistically different than zero. In this case, cultivars from the group that showed reduced stability can be selected, but using the value of *R2* as an auxiliary measure. Vasconcelos et al. (2008) also highlighted that the Eberhart and Russel methodology could be more efficient if the number of environments was greater. Since the number of environments was three, the number of points used for regression was also three. By the centroid method, 36 out of the 92 genotypes were deemed as having general adaptability, but among them the materials Crioula, LE N 4 and P 30 showed the greatest probabilities (64%, 81% and 75%, respectively) of belonging to class I, that is, of having general adaptability to the environments. The genotypes with best behavior in rain environments were Rocio and Costera SP INTA with 35.7% and 35.0% probabilities of belonging to that class. After evaluation by the three methods, the authors concluded that genotype LE N 4 has general adaptability (centroid method) and high mean of production, and that it is responsive to environmental improvement for dry matter production (Eberhart and Russel method). Genotypes P 5715 and Bárbara SP INTA had, in addition to good adaptability, stability in dry matter production (centroid method). Genotype Bacana had the best adaptability to dry environments (centroid method), with an acceptable mean of dry matter production and of stability (traditional method), and thus constitutes a good option for forage exploitation throughout the year.

When choosing a genotype, it is expected for its initial superiority to last throughout its life. Similarly, it is also expected that the good performance presented in certain structures or integral parts of the individual reflects the potential of the genotype to be used as a whole. The truth of this expectation can be proved by the repeatability coefficient of the characteristic studied. The repeatability coefficient can be obtained when the measurement of a given trait is performed repeatedly to the same individual in time or in space. Generally, this coefficient is useful for breeding, because it allows evaluating the number of necessary measurements to have a good prediction of the real value of the individual. Also, repeatability represents the maximum value that can be reached by heritability.

Different methodologies can be used to obtain the repeatability coefficient. The method presented bellow is based on the variance analysis, according to the model:

$$Y_{ij} = \mu + g_i + a_j + \varepsilon_{ij}$$

where

Y_{ij} : observation regarding the i-th genotype in the j-th environment (time or space)

μ : general mean

g_i : random effect of the i-th genotype under influence of the permanent environment (i = 1, 2, ..., p)

a_j : steady effect of the temporary environment on the j-th measurement (j = 1,2,..., η) and

ε_{ij} : experimental error set by the temporary environmental effects on the j-th measurement of the i-th genotype

The scheme of the variance analysis for the model with two variation factors (g and a) is presented in Table 10.

When we evaluate p genotypes in η repeated measurements, we can estimate the repeatability coefficient by the intraclass correlation obtained from the variance analysis. The repeatability coefficient is given by:

$$r = \hat{\rho} = \frac{C\hat{o}v(Y_{ij}\ ,\ Y_{ij'})}{\sqrt{\hat{V}(Y_{ij})\ \hat{V}(Y_{ij'})}} = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_Y^2} = \frac{\hat{\sigma}_g^2}{\hat{\sigma}^2 + \hat{\sigma}_g^2}$$

The determination or the precision for predicting the real value of the individual based on the mean of η evaluations is given by:

$$R^2 = \frac{\eta\rho}{1 + \rho(\eta - 1)}$$

Table 10. Scheme of the variance analysis for the model with two variation factors.

VF	GL	MS	E(MS)
Genotypes	p-1	MSG	$\sigma^2 + \eta\sigma_g^2$
Environments	a-1	MSE	-
Residue	(p-1)(a-1)	MSR	σ^2

Another interesting calculation is the prediction of the number of measurements (η_0) necessary to achieve a given level of precision (or determination), in the comparison of genotypes, for a given characteristic whose repeatability coefficient *r* is known. The expression to obtain the number of measurements is:

$$\eta_0 = \frac{R^2(1 - r)}{(1 - R^2)r}$$

Low value of repeatability coefficient indicates that there has not been regularity on the repetition of the trait from one measurement to the other; with that we should not perform reductions in the number of measurements performed to save time and labor.

In a study to obtain the repeatability estimates for dry matter production in alfalfa, Souza-Sobrinho et al. (2004) used productivity data obtained in four assays of evaluation of alfalfa carried out in different regions of the state of Minas Gerais. The authors estimated the repeatability coefficient for each of the assays, through the variance analysis method, the principal components method based on the covariance and correlation matrix and through the structural analysis based on the correlation matrix. The average repeatability estimate for dry matter production in the four assays evaluated was 0.59, with average determination coefficient of 0.96. They also concluded that carrying out an average of only four cuts was enough to learn the real genotypic value of the cultivars tested, with 85% reliability.

Ferreira et al. (1999) evaluated 42 cultivars and estimated the repeatability coefficient of the characteristics dry matter production, crude protein content in leaves and in stem and disease tolerance, assessed in rain and dry seasons, in six cuts. The authors found that the repeatability coefficient generally showed low dimension estimates (under 0.4). As for dry matter production, the repeatability coefficient ranged between 0.3195 and 0.4270, the genotypic determination was around 65% and the possibility of reaching prediction of the real value was through seven to nine cuts.

Final considerations

The increase in agricultural productivity associated to nutritional quality of alfalfa can be achieved through improvements in environmental conditions or in the genetic potential of individuals or populations. In many situations, genetic improvement is the only way to achieve this goal, in addition to the advantage of promoting hereditary changes. Due to the existence of great

genetic diversity in alfalfa, it becomes possible to select and recombine genetic forms that are more adapted, more efficient and that have better quality.

Using biometrics during the stages of a breeding program for alfalfa becomes an extremely useful tool for the researcher to make decisions, allowing the gathering of the maximum information on the evaluated experiments. With that, the strategies to conduct the next stages of the breeding program can be planned, increasing the gains achieved and the success in improving the crop.

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