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RESEARCH NOTE

Use of "EP"(Peroxidase) allele in soybean varietal characterization[1]

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ABSTRACT- The aim of this work was to evaluate the use of the molecular biology technique of PCR (Polimerase Chain Reaction) in the characterization of soybean cultivars. The study was performed at the Department of Plant Production, Faculty of Agricultural Sciences/ UNESP and Institute of Bioscience, Botucatu-SP. Fourteen commercial soybean cultivars were used, of which six were selected as positive reaction to peroxidase (BRS 320, BRS 284, BRS 232, BRS 7860RR, BRSMG 760SRR, BRS295RR), four as negative reaction (BRS 326, BRS 8160RR, BRSMG 800A (NutriSoy), BRS Valiosa RR) and four as double reaction (BRSGO 8060, BRS 270RR, FTS Campo Mourão and BRS 239). Thus, the results attained by the traditional biochemical colorimetric test for the 14 cultivars were compared with the conventional PCR assay. For PCR analysis, DNA was extracted from whole seeds and the primers were tested, and subsequently PCR and agarose gel electrophoresis were performed. The combination of primers *prx9* + *prx10* confirmed the use of the PCR reaction to characterize soybean cultivars considered doubtful by conventional colorimetric text.

Index terms: *Glycine max* (L.) Merrill, PCR, DNA, genetic purity.

Uso do alelo "EP" (Peroxidase) na caracterização varietal de soja

RESUMO – O objetivo geral deste trabalho foi avaliar o uso da técnica de PCR (Polimerase Chain Reaction) na caracterização de cultivares de soja. O estudo foi realizado no Departamento de Produção Vegetal da Faculdade de Ciências Agronômicas / UNESP e do Instituto de Biociências de Botucatu-SP. Foram utilizadas quatorze cultivares de soja comerciais, das quais seis foram selecionadas como reação positiva à peroxidase (BRS 320, BRS 284, BRS 232, BRS 7860RR, BRSMG 760SRR, BRS295RR), quatro como reação negativa (BRS 326, BRS 8160RR, BRSMG 800A (NutriSoy), BRS Valiosa RR) e quatro como reação positiva e negativa (BRS 8060, BRS 270RR, FTS Campo Mourão e BRS 239). Assim, as 14 cultivares foram submetidas ao ensaio bioquímico colorimétrico tradicional e os resultados obtidos foram comparados com o ensaio de PCR convencional. Para a análise de PCR, o DNA foi extraído de sementes inteiras, sendo que os *primers* foram testados por PCR e visualizados por eletroforese em gel de agarose. A combinação dos primers *prx9* + *prx10* confirmou a utilização da reação de PCR para caracterizar as cultivares de soja considerada duvidosa por teste convencional colorimétrico.

Temos para indexação: *Glycine max* (L.) Merrill, PCR, DNA, pureza genética.

Introduction

Soybean [*Glycine max* (L.) Merrill] is considered the most important commodity of Brazil's agribusiness. In the growing season of 2013/14 a production of 86.120,8 thousand tons was harvested in a total area of 30.173,1 thousand of hectares. Such area represents an increase of 5,7% over the previous season and accounts for the largest area ever cultivated with soybean in the country (CONAB, 2014).

Public and private breeding companies are seeking for soybean cultivars which are tolerant to diseases, insects and water deficit, among other characteristics. This fact has increased the number of soybean cultivars available in the market to meet these demands (Schuster et al., 2006). Despite the large number of existing cultivars of soybean in Brazil the genetic variation among them is very narrow, since those cultivars originated from

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few ancestors (Miranda et al., 2007; Vieira et al., 2009).

The activities related to the soybean breeding programs are only completed when seeds of new cultivars have been made available to the growers. Thus, it is required to multiply the seeds to increase their volume but also seed quality control must be undertaken in order to ensure the required genetic, physiological, sanitary and physical qualities of the seeds.

The large number of available soybean cultivars and the little genetic difference among them have made it increasingly difficult to assess their genetic purity. This has required the use of modern and efficient laboratory techniques to improve the distinction between the different cultivars.

Buttery and Buzzell (1968) characterized various soybean cultivars into two groups on the basis of the peroxidase activity in the seed coat: peroxidase-positive or negative. For this purpose, two methods were used: a quick assay based on guaiacol reagent and an assay involving polyacrylamide gel electrophoresis. Later, Buzzell and Buttery (1969) demonstrated via genetic analyses that the high and low peroxidase activity in the soybean seed coat are controlled by a major gene, Ep. Such gene, with complete dominance, is responsible for the high activity of the enzyme, and its recessive allele, ep, for the low activity (Steevensz et al., 2013).

The peroxidase enzyme is present in most tissues of living plants, as in soybean seeds, and its activity varies according to the cultivar. The peroxidase assay is a method for soybean varietal identification (Brasil, 2009), so it can be used to detect the presence of a varietal mixture of seeds from other cultivars. Despite the simplicity and low costs the biochemical reaction test to peroxidase involves some subjectivity. Additionally, seeds with moisture content above 12% and with parts of cotyledons in the seed coat cause erroneous interpretations of the test (Gijzen, 1997). In these circumstances and in case of occurrence of two different reactions to peroxidase (both negative and positive) in the same soybean cultivar, the author pointed out the importance of using molecular markers because it enhances the reliability of the results.

Therefore, this work aimed at to evaluate the use of the convenctional Polimerase Chain Reaction technique (PCR) by using specific primers designed for the allelle Ep (peroxidase) in characterization of soybean cultivars and compare those results with the traditional colorimetric method.

Material and Methods

The study was performed at the Laboratory of Seeds Analysis of the Plant Production and Breeding Department from the Faculty of Agricultural Sciences and at the Genetic Laboratory of the Biosciences Institute-UNESP-campus of Botucatu/SP. Fourteen commercial soybean cultivars were used, of which six were selected as positive reaction to peroxidase (BRS 320, BRS 284, BRS 232, BRS 7860RR, BRSMG 760SRR, BRS295RR), four as negative reaction (BRS 326, BRS 8160RR, BRSMG 800A (NutriSoy), BRS Valiosa RR) and four as double reaction (BRSGO 8060, BRS 270RR, FTS Campo Mourão and BRS 239) (França-Neto-Personal communication), as shown in Table 1.

Table 1. Peroxidase reaction in 14 soybean cultivars as described by the obtainers and determined by the colorimetric method.

Cultivars	Obtainers	P.O. Described	P.O. Obtained
BRS 320	EMBRAPA	Positive	100% Positive
BRS 284	EMBRAPA	Positive	100% Positive
BRS 232	EMBRAPA	Positive	98% Positive 2% Negative
BRS 7860RR	EMBRAPA	Positive	98% Positive 2% Negative
BRSMG 760SRR	EMBRAPA	Positive	100% Positive
BRS295RR	EMBRAPA	Positive	100% Positive
BRS 326	EMBRAPA	Negative	98% Negative 2% Positive
BRS 8160RR	EMBRAPA	Negative	100% Negative
BRSMG 800A (Nutrisoy)	EMBRAPA	Negative	100% Negative
BRS Valiosa RR	EMBRAPA	Positive/Negative	99% Negative 1% Positive
BRSGO 8060	EMBRAPA	Negative	100% Negative
BRS 270RR	EMBRAPA	Positive/Negative	36% Positive 64% Negative
FTS Campo Mourão RR	FST	Positive/Negative	46% Positive 54% Negative
BRS 239	EMBRAPA	Positive/Negative	44% Positive 56% Negative

Colorimetric test

In order to confirm the positive or negative reaction to peroxidase, the colorimetric test was performed as described by the Brazilian Rules for Seed Analysis (Brasil, 2009). The seed coat was entirely removed with a razor blade without leaving any fragment of the embryonic axis or cotylenes attached to it. The seed coat portions were placed into 10 x 75-mm test tubes containing ten drops (1.5 mL) of 0.5% guaiacol solution. Following, one or two drops (0.5 mL) of 40-volume of hydrogen peroxide (12% of hydrogen peroxide) were added to the the solution. Two replicates of 50 seeds each were used per cultivar.

PCR reactions and electrophoresis

Samples of 100 seeds were ground to a powder in a basic analytical mill (IKA®, A11 model). Subsequently, a portion of the sample was transferred to 1.5 mL tubes (Eppendorf®) in three biological replicates, and the remaining was transferred to a 50-mL Falcon type tube. The samples were kept in ultra-freezer at a temperature of -80 ºC.

Table 2. DNA concentration determined by Nanodrop.

Samples	DNA Concentration (ng/µL)
1-BRS 326	176.0
2-BRS 8160	154.0
3-BRSMG 800A (NutriSoy)	156.1
4-BRS Valiosa RR	162.7
5-BRSGO 8060	240.5
6-BRS 270RR	187.0
7-BRS 239	166.4
8-FTS Campo Mourão	205.4
9-BRS 320	279.9
10-BRS 7860RR	240.9
11-BRSMG 760SRR	383.9
12-BRS 295RR	462.3
13-BRS 284	138.3
14-BRS 232	290.4

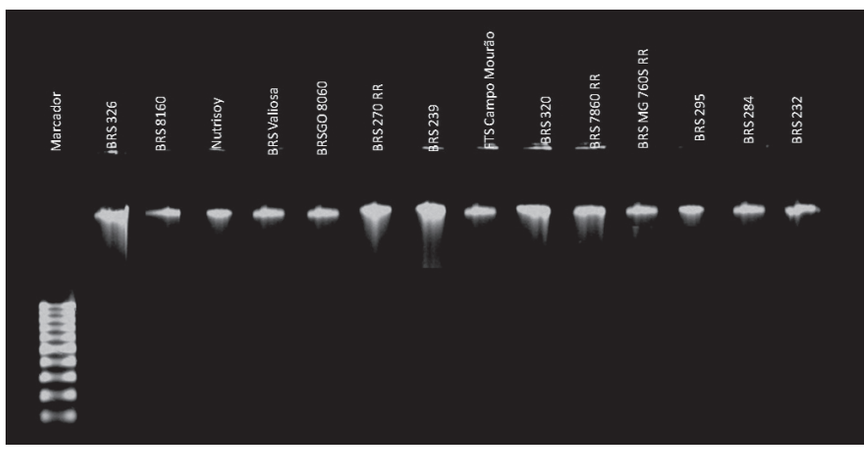

Figure 1. Gel electrophoresis showing the integrity of DNA extracted from seeds of 14 soybean cultivars. (Photo: B. Panoff, 2013).

For the DNA extraction, a commercial kit was used (Invisorb® Spin Plant Mini Kit for 50 samples). DNA was quantified in Nanodrop® (Table 2), and electrophoresis was performed to check the integrity of DNA samples (Figure 1).

The PCR reactions were performed in a thermocycler Eppendorf® Mastercycler Gradient, in 0.5-µL tubes containing 30 ng of template DNA, 2 mM of $MgCl_2$ 1 X Buffer 250 µM mix of dNTPs 0.4 µM of Forward primer 0.4 µM of Reverse primer 1 U Taq DNA Polymerase and autoclaved distilled water were added to complete the reaction for 50 µL. Temperature gradients consisted of initial denaturation of 2 minutes at 94 ºC. Thirty cycles of 1-minute denaturation at 90 ºC, 45 seconds of annealing time at 60 ºC and 1-minute extension at 72 ºC. At the end, 7 minutes of extension at 72 ºC, and the program was closed and set to remain at 10 °C. The primers described by Gijzen (1997) were used in the PCR reaction. The prx 9 (Forward): ATGCATGCAGGTTTTTCAGT with prx 10 (Reverse): TTGCTCGCTTTCTATTGTAT and prx 29 (Forward): ATAAACAATACGTACGTGAT with prx 10 (Reverse): TTGCTCGCTTTCTATTGTAT. According to Gijzen (1997), the primers were designed at the 5' end region surrounding the deletion of 87 bp that occurs in the recessive allele "ep" of the soybean line OX312. The amplified bands were then applied to agarose gels at 1% concentration and coloured with GelRed®. Electrophoresis was performed at 120 V for 30 minutes. Images were taken by the photo documentation system (UV Transilluminator MiniBis®) to view and subsequently analyze the amplified DNA. The results were assessed by observation on the agarose gels. The absence and presence of bands on the gels

were determined for comparison with the results of the traditional peroxidase test previously performed.

Results and Discussion

Among the four cultivars described as peroxidase-negative, 100% of the soybean seeds of cultivars BRS 8160 RR and BRSMG 800A (NutriSoy) had a negative reaction. For cultivars BRS 326 and BRS Valiosa RR, 2% and 1% of the seeds, respectively, were found to be peroxidase-positive, as shown in Table 1.

The test performed with seeds of the six peroxidase-positive varieties, four of them being BRS 320, BRSMG 760SRR, BRS 295RR and BRS 284, indicated that 100% of the seeds had a characteristic reaction, i.e., positive. On the other hand, in the seeds of cultivars BRS 7860RR and BRS 232 it was found 2% of negative contamination.

Considering the cultivars previously described as of both positive and negative reaction to peroxidase, only the seeds of cultivar BRSGO 8060 did not present the described reaction, and 100% of them were found to be peroxidase-negative. Distinct percentages regarding positive and negative reaction were observed in the seeds of the other cultivars. Thus, 64% of the seeds of cultivar BRS 270RR revealed negative reaction and 36% positive reaction, while 56% of the seeds of cultivar BRS 239 indicated negative reaction and 44% positive reaction. Regarding the cultivar FTS Campo Mourão RR, which was found to present equal proportions of positive seeds (50%) and negative seeds (50%), the percentage values were very similar, i.e., 46% of the seeds were peroxidase-positive and 54% peroxidase-negative (Table 1).

As in the cultivars with positive or negative reaction it was found a maximum of 2% of the seeds with different reaction to peroxidase from that described, this is likely due to the presence of seeds of other cultivars. This result is in agreement with that found by Costa et al. (1979), according to which the seeds of only one cultivar among those studied had consistently two reactions. The authors attributed the atypical reaction to varietal mixture or segregation.

According to Zorato and Schuster (2011), the occurrence of up to 1% of the seeds of a given cultivar with opposite reaction to the one described should not be enough to assume it is of dual reaction, because what is expected in peroxidase analyses is the occurrence of only one reaction. These authors also argued that a low and different degree of reaction to peroxidase, below 1%, does not represent per se a mixture of other cultivar.

The seeds of cultivar BRSGO 8060 had a different reaction to the dual reaction contained in the descriptors. In contrast, the seeds of the other cultivars showed consistent behaviour with their descriptions. This aspect was discussed by Costa et al. (1979), and according to these authors soybean cultivars may present significant varietal mixtures over time and generations of cultivation. Regarding this aspect, soybean is an autogamous species and cleistogamy is responsible for the low rate of outcrossing in this species. However, some natural crossing may occur, where bees and "trips" are the main agents involved (Sediyama et al., 2001).

Schuster (2013) mentioned the possibility of soybean cross-fertilization of the order of 1% and up to one meter away despite the fact of being an autogamous species. This dual reaction may also be a consequence of the process of obtaining the seeds in which those considered genetic derive from more than one pure inbread. These authors claim that if the seeds of more than one pure line are peroxidase-positive and seeds of more than one pure line are peroxidase-negative, the seeds of the cultivar would have both positive and negative reactions. This is observed in various Brazilian soybean cultivars whose registration has been made in the past.

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As expected, the combination of primers prx9 + prx29 did not show amplification when the PCR technique was used because they are primers of the forward type without the reverse primer. However, for the primers prx29 + prx10 there was amplification, as well as for the combination of primers prx9 + prx10.

Amplification was not expected in the negative reactions due to a deletion of 87 bp at the 5' end of the gene (Gijzen, 1997), as found in the present research work. Buzzell e Buttery (1969) demonstrated by means of genetic testing, that the high and low peroxidase activity in the seed coat of soybean are controlled by the gene Ep. The Ep gene has complete dominance, produces high activity, and its recessive allele ep and has low activity or lack of activity.

As illustrated in Figure 1, with DNA extracted from the whole seed using a commercial kit and combination of primers prx9 + prx10, the PCR reaction for all negative samples did not reveal amplification and no bands were observed. Seeds of cultivars considered positive showed gel bands, which was not present in the samples considered negative. Various regions were amplified, including those with DNA length of 890 bp, which was the length corresponding to the peroxidase gene (Gijzen, 1997). But with respect to the dual-reaction cultivars,

similarities were observed for the positive cultivars, i.e., they showed amplification of the fragments and various bands.

Thus, this research work shows that it is possible to separate the 14 cultivars by this method into two very distinct groups: one with absence of peroxidase and another with the presence of peroxidase, the latter including doubtful cultivars by the colorimetric method, except for BRSGO 8060, which, although being described as doubtful, had 100% of their seeds negative; however, the bands pattern was similar to the doubtful samples. The cultivars whose seeds were considered doubtful were separated from the seeds with negative reactions. However in general, the intensity of the bands was lower when compared to the cultivars considered positive (Figure 2). To make the method applicable, it is suggested that molecular analysis should be employed only in seed species identified as doubtful when conventional techniques are applied.

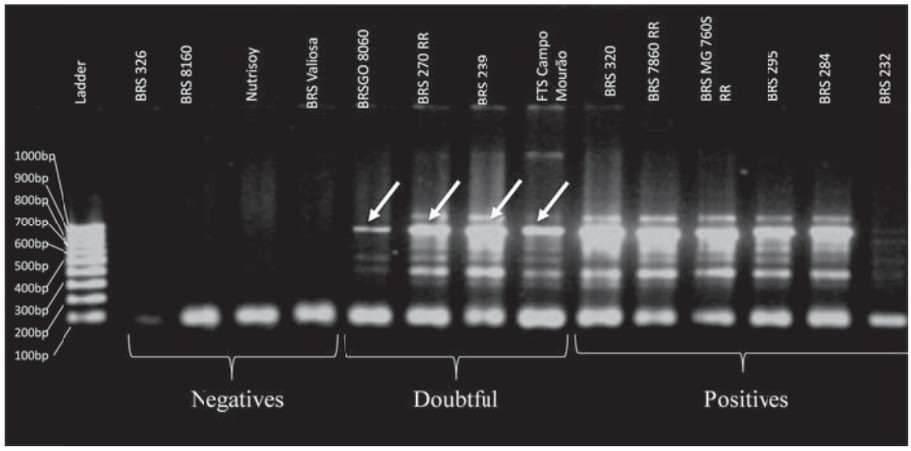

Figure 2. Gel electrophoresis with PCR products of DNA extracted from seeds of 14 soybean cultivars. Arrows indicate the presence of the "Ep" allele in cultivars considered doubtful by the colorimetric method.

Conclusions

The combination of primers prx9 + prx10 confirmed that the use of the PCR reaction is effective to characterize soybean cultivars that are considered doubtful by conventional colorimetric text.

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