Mass Screening Techniques for Selecting Crops Resistant to Disease

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Large figure: Sporulation of cucurbit powdery mildew *(Golovinomyces cichoracearum) on cotyledons of susceptible Cucumis sativus* cv. Stela F1.

Circles: Detail of leaf discs with different degrees of infection
14 days after inoculation with *Golovinomyces cichoracearum*.

Photographs courtesy of A. Lebeda, Palacký University in Olomouc, Czech Republic.

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Chapter 6

In vitro selection for resistance to Fusarium wilt in Banana

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ABSTRACT

Fusarium wilt or Panama disease, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (FOC), is one of the most serious diseases of banana in tropical and subtropical countries. Although there are banana cultivars resistant to the disease, transfer of the resistance trait to susceptible cultivars by traditional cross-breeding is difficult. *In vitro* selections of banana mutants tolerant to race 1 of FOC were carried out with fusaric acid and culture filtrate on multiple bud clumps. Regenerated plants showed tolerance to the disease in the greenhouse, and some of the plants also showed resistance in the field. Although the level of tolerance observed was not sufficiently high, *in vitro* selection by toxic substances extracted from pathogenic fungi resulted in an improvement of plant tolerance to the pathogen. Other selection factors for *in vitro* selection were reviewed, and a protocol for obtaining disease tolerant plants of banana is suggested.

INTRODUCTION

Fusarium wilt or Panama disease is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (FOC). The fungus can survive in the soil for a long period without host plants. Cultural practices for control of this disease, such as fungicide applications or culture rotations, are not economically practicable. Biological control is still in the initial phases of development but does have limited applications (Jaizme-Vega *et al*., 1998; Ting, *et al*., 2003). The only effective control available is based on the use of resistant or tolerant cultivars to the disease (Cordeiro, 1997; Cordeiro and Matos, 2000). Genetic improvement is, thus, indispensable for the sustainability of banana crop production. Genetic improvement by cross-breeding has been intensively conducted for the last three decades, and has produced some results in bananas of the 'Prata' subgroup (*Musa* sp. AAB group). However, the expansion of the disease and the emergence of a new fungal race in the 'Cavendish' subgroup (*Musa* sp. AAA group) still pose a threat to many banana plantations. Efforts to produce improved germplasm must be intensified and a larger number of new varieties must be obtained. Besides, the 'Cavendish' subgroup shows a high level of male and female sterility; thus, improving it by traditional cross-breeding is extremely difficult (Dantas *et al*., 1997). Given these conditions, *in vitro* selection techniques coupled to mutation induction or somaclonal variation could complement conventional breeding methods.

In vitro selection is an *in vitro* cultivation technique under controlled stress conditions that favours or disfavours the growth of a cell or tissue type of interest. Compared with selection in an experimental field, *in vitro* selection can considerably reduce the space needed for improving crops. However, some factors influencing *in vitro* selection may differ from those in field selection, as for instance, resistance to the fungus FOC. In order to select plants resistant to Fusarium wilt, FOC can directly be applied to plants in field selections, but cannot be applied to plantlets *in vitro*. This is because the fungus grows much faster than plant tissues in *in vitro* culture conditions. The fungus quickly dominates the culture media and flasks, and the growth of the plant tissue is impeded by a lack of nutrients or space, rather than due to susceptibility to the disease. That is the biggest problem of *in vitro* selection. Furthermore,

the fungus does not attack *in vitro* tissues equally, thus allowing susceptible plantlets to escape from the selection pressure. To overcome this problem, techniques using several selection factors (agents) have been created, such as fusaric acid, culture filtrates of the pathogenic fungus and other substances that cause similar effects as the fungal attack (Švábová and Lebeda, 2005). In this study, techniques for *in vitro* selection using specific agents are reviewed.

MATERIAL AND METHODS

In vitro selection with fusaric acid

Fusaric acid is the most abundant toxin produced by several species of the genus *Fusarium*, and is responsible for the symptoms of Fusarium wilt disease (Davis, 1969). Once the fungus uses the toxin to destroy vegetable cells and to invade the interior of the plant, plants composed of toxin-resistant cells may block the invasion of the fungus and show tolerance to the disease. *In vitro* selection of mutants tolerant to fusaric acid seems to be a viable strategy for obtaining plants tolerant to Fusarium wilt disease.

The general process for *in vitro* selection is shown in Figure 6.1. We present the methods used and the results obtained in our laboratory. As the first step of *in vitro* selection, multiple bud clumps (see Figure 6.3A) were obtained by cultivating shoot tips of the banana plant cultivars 'Maçã' (*Musa* sp. AAB group) and 'Nanicão' (*Musa* sp. AAA group, 'Cavendish' subgroup) that are susceptible and resistant, respectively, to Fusarium wilt disease caused by race 1 of the fungus FOC. These bud clumps were maintained in a proliferation medium consisting of Murashige and Skoog (MS) medium with salts and vitamins (Murashige and Skoog, 1962), 5 mg/l 6-benzylaminopurine (BAP), 30 g/l sucrose, solidified by 2 g/l phytagel (Sigma Chemical Co., St. Louis, MO, USA). The cultures were maintained in a culture room (28 ± 2°C, 14 hour photoperiod, 56 μmol/m^2/s light intensity). In the second step, concentrations of fusaric acid that inhibit growth of multiple buds were determined. The multiple bud clumps were cultured on the above mentioned proliferation medium supplemented with fusaric acid (Sigma Chemical Co.) at concentrations ranging from 0.05 to 1.6 mM. The fusaric acid was added to the autoclaved culture medium after filter-sterilisation (0.22 or 0.45 μm pore size membrane filter). After one month of culture, the fresh weights of the bud clumps were measured.

The effect of fusaric acid on the growth of multiple bud clumps is shown in Figure 6.2. The growth of both cultivars ('Maçã' and 'Nanicão') was drastically inhibited by 0.1 mM fusaric acid. There was no significant difference in susceptibility to fusaric acid between the disease-susceptible 'Maçã' and disease-tolerant 'Nanicão', suggesting that the degree of disease resistance between these two cultivars cannot be distinguished by fusaric acid.

In the third step of selection, i.e., selection of tolerant Material, we chose 0.1 mM as the appropriate concentration of fusaric acid for the selection of resistant mutants of 'Maçã'. Following treatment with the chemical mutagen ethyl methanesulfonate (EMS) for 2 hours to induce mutations (Matsumoto and Yamaguchi, 1984), multiple bud clumps were transferred into a selection medium, which consisted of proliferation medium supplemented with 0.1 mM fusaric acid. They were cultivated in selection medium for 3 months, with monthly transfers to fresh media. After the selection period, the multiple bud clumps were transferred to the culture medium for rooting (MS salts and vitamins, 0.25 mg/l α-naphthalene acetic acid [NAA], 30 g/l sucrose and 2 g/l phytagel) without fusaric acid, and regenerated plantlets were transferred to polyethylene bags containing 2 l of soil in a greenhouse for acclimatisation (Matsumoto *et al*., 1995).

Tests of tolerance to the disease, which were performed in the fourth and final step of the process, were carried out in a greenhouse by artificially inoculating plantlets (15-20 cm in height) acclimatised for one month or rhizomes (~50 cm in height) from plantlets acclimatised for four months with the fungus FOC. The artificial inoculation was performed by submerging the roots of uprooted plantlets or rhizomes in a spore suspension of the fungus FOC (10^7 conidia/ml). They were then again transplanted

to polyethylene bags with soil previously infested by the fungus FOC (Sun and Su, 1984; Matsumoto *et al*., 1995). The results of the tolerance test on selected 15-20 cm plantlets and rhizomes are shown in Tables 6.1 and 6.2, and in Figure 6.3. At the growth stage of the 15-20 cm plantlets, disease symptoms appeared in as many selected as in unselected plantlets. Thus, the selection did not seem to be effective. However, when rhizomes from 50 cm plantlets were inoculated with the fungus, the selected plantlets showed higher tolerance to the disease than those not selected. These results showed that selection by fusaric acid was effective: however, the acquired tolerance was not visible at the early stages of development of the acclimatised plantlets.

***In vitro* selection with culture filtrate**

The culture filtrate consists of a liquid culture medium in which microorganisms have been cultivated for a certain period of time and eliminated by filtration through a membrane filter. The culture filtrate is expected to contain several metabolic substances produced by the microorganisms, including several toxins besides the fusaric acid that was discussed previously. Microorganisms are usually eliminated by filtration rather than by autoclaving because some metabolic substances are unstable at high temperature. Compared with the use of fusaric acid, selection using culture filtrate may be more efficient for obtaining disease-tolerant plants, because the filtrate contains some toxins that have not yet been identified, but that may be responsible for defining the degree of disease susceptibility or tolerance in the host plants. In some species, *Fusarium*-tolerant plants have already been obtained using culture filtrates as a selection factor (Arcioni *et al*., 1987; Binarova *et al*., 1990; Borrás *et al*., 1997). Therefore, we speculated that the technique could be useful for the assessment of resistance to Fusarium wilt in banana plants as well.

Details of the culture filtrate preparation are described in Matsumoto *et al*. (1999b). Race 1 of the fungus FOC was isolated from a disease-susceptible banana plant that showed symptoms of Fusarium wilt disease, propagated once in potato dextrose agar (PDA) medium and conserved in the refrigerator (4°C). A piece of the fungal colony with the PDA medium was inoculated in 100 ml of Czapek Dox Broth (CZD) medium in a 300 ml Erlenmeyer flask, with a multiple bud clump (~10 × 10 × 10 mm) of a disease-susceptible banana variety (co-cultivation technique). The culture was maintained in a culture room without agitation for 21 days. It was then filtered through a four-layer gauze (or cheesecloth) and centrifuged at 8000 rpm for 20 minutes. The supernatant was finally filtered through a 0.45 µm membrane filter to eliminate the fungus.

Figure 6.4 shows the growth inhibition caused by the culture filtrate on *in vitro* multiple bud clumps of disease-susceptible 'Maçã' and disease-tolerant 'Nanicão' cultivars. Inhibition of growth was higher in 'Maçã' than in 'Nanicão' (Figure 6.4). Since 'Maçã' is a susceptible cultivar and 'Nanicão' a resistant one, we could expect to obtain disease-tolerant plants using culture filtrate for selection. Therefore, in contrast to the experiment using fusaric acid, where no differences in growth inhibition were observed between the two cultivars (Figure 6.2), the use of FOC filtrates seems to be efficient for the selection of tolerance to Fusarium wilt.

To induce mutations, the same treatment was carried out as for selection with fusaric acid. After the mutagenic treatment, the multiple bud clumps were cultivated on proliferation medium (MS, 5 mg/l BAP, 30 g/l sucrose and 2 g/l phytagel) for 5 days, and then transferred to the medium for the first round of selection (MS, 5 mg/l BAP, 10% [v/v] culture filtrate, 30 g/l sucrose and 2 g/l Phytagel). After one month of cultivation, the growing bud clumps were subdivided and transferred to a new medium for the second round of selection (MS, 5 mg/l BAP, 15% [v/v] culture filtrate, 30 g/l sucrose and 2 g/l Phytagel). Another two rounds of selection were undertaken using the same medium. After the successive rounds of selection, the clumps of multiple buds tolerant to the culture filtrate were propagated, and plantlets were regenerated on rooting medium. The plantlets were acclimatised and their tolerance to the fungus was evaluated in the greenhouse as described previously.

Tables 6.3 and 6.4 show the results of the selection using the 10 to 15% (v/v) culture filtrate, after mutations were induced by the chemical mutagen EMS. When acclimatised plantlets (15-20 cm) were artificially infested with FOC and disease symptoms were observed in pseudostem, the selected plantlets did not show any significant increase in disease tolerance. However, symptom observation in rhizomes revealed that the selected plantlets were significantly more tolerant than those not selected (Table 6.3). The selection efficiency was also confirmed by evaluation of pseudostem height and root fresh weight (Table 6.4). Although selection with the culture filtrate was very efficient, the level of tolerance did not reach that of the 'Nanicão' cultivar, which is well-known for its resistance to Fusarium wilt (Table 6.3).

Evaluation methods for *in vitro*-selected plants

The evaluation method for disease resistance is as important as the selection method itself for achieving successful results. As a rule, *in vitro* selection for Fusarium wilt tolerance does not utilise live FOC fungus. Consequently, an evaluation of the selected plants by the fungus itself is indispensable as a final step of the selection process. Such evaluation is carried out on field plants or acclimatised plantlets in the greenhouse. Although field tests are ideal for this purpose (Hwang, 1990; Smith *et al*., 2006), they require a huge experimental area, and plants are frequently damaged by diseases not-targeted or other climatic factors. To overcome these problems, Sun and Su (1984) developed a method for acclimatised plantlets as described in section 2 (*in vitro* selection by fusaric acid). With some modification and adaptation, it was also applied for *ex vitro* selection (Bhagwat and Duncan, 1998; Bermúdez *et al*., 2002; Mak *et al*., 2004). Acclimatised small plantlets in the greenhouse or net-house were infested by the fungus FOC for the evaluation, and symptomless plantlets were selected to be transplanted in the field. This method can be used to analyse a limited number of plantlets and is feasible as a pre-field-selection for tolerance to the disease. Even though the method is very useful, care must be taken in its application, because the disease susceptibility or tolerance of the selected Material somewhat depends on the plantlet stage and evaluation parameter (see Tables 6.1-6.4).

Inoculation of fungus on both acclimatised plantlets and field plants may kill plants that, although susceptible to the disease, might have excellent agronomic characters. More recently, a non-destructive evaluation method for disease resistance was developed using fungal culture filtrate. It was based on concentrated culture-filtrate inoculation onto needle-mediated wounds of middle-aged banana leaves (Companioni *et al*., 2003, 2005). Evaluating seven banana clones, three of which were disease-susceptible and four of which were disease-resistant, the susceptible clones always showed major lesion areas after 48 hours of incubation. This evaluation method is of great interest not only because it is non-destructive but also due to the very short time needed for evaluation.

Protocol for *in vitro* selection of Fusarium wilt-tolerant plants

The *in vitro* selection protocol used in our laboratory is as follows:

Plant material preparation

(1) Small and apparently healthy suckers are collected from a Fusarium wilt-susceptible plant.
(2) Suckers are washed with tap water and cut into $10 \times 10 \times 10$ mm^3 blocks with shoot tip.
(3) The tissue blocks are surface-sterilised in a laminar flow cabinet with 70% alcohol for 15 s, followed by 1% sodium hypochloride with a few drops of Tween 20 for 15 minutes.
(4) External tissues are removed leaving blocks of $5 \times 5 \times 5$ mm3.
(5) They are transferred to banana proliferation medium consisting of MS (Murashige and Skoog, 1962) salts and vitamins, 5 mg/l BAP, 30 g/l sucrose, 2 g/l phytagel and maintained in a culture room (28 ± 2°C, 14 hour photoperiod, 45-60 µmol/m2/s light intensity).
(6) After 3 to 8 months of culture (subcultures each of 30 to 45 days), multiple bud clumps will be obtained.

Production of culture filtrates

(7) FOC fungus is isolated on PDA medium from a disease-susceptible banana plant that shows disease symptoms.
(8) The fungus is proliferated once on the same medium in Petri dishes for two weeks (28 ± 2°C, 45-60 µmol/m2/s of continuous illumination) and stored in a refrigerator (4°C) until use. Pathogenicity of the isolates should be checked using, for instance, the method of Sun and Su (1984).
(9) A piece of the fungal colony (2-3 mm in diameter) and a tissue (~10 mm in diameter) of the banana multiple bud clump from Step 6 are inoculated into 100 ml of CZD medium in a 300 ml Erlenmeyer flask.
(10) The culture is incubated at 28 ± 2°C and 45-60 µmol/m2/s illumination with a 14 hour photoperiod for 21 days without shaking.
(11) The cultured liquid medium is then filtered through a four-layer gauze or cheesecloth and centrifuged (8000 rpm, 20 minutes) to precipitate mycelium and conidia.
(12) The supernatant is passed through a membrane filter (0.45 or 0.22 µm pore size) to remove the remaining fungi. This cultured fungus-free medium is used as the culture filtrate for the following selection. The culture filtrate should be used immediately; it is not recommended to store it.

Mutagen treatment and selection

(13) At least 500 pieces, each ~3 × 3 × 3 mm3, of the banana multiple bud clumps from Step 6 are incubated in a 500 ml Erlenmeyer flask with 200 ml of aqueous solution supplemented with 4% (v/v) dimethylsulphoxide (DMSO) and 0.3% (v/v) EMS.
(14) The flask is kept on a gyratory shaker (100 rpm) for two hours at 28 ± 2°C.
(15) After washing three times with sterile distilled water, the multiple bud clumps are transferred to banana proliferation medium (Step 5) in Petri dishes and cultured for one week.
(16) The multiple bud clumps are then transferred onto the selection medium, which consists of proliferation medium supplemented with 10-15% (v/v) culture filtrate or 0.1 mM fusaric acid.
(17) After 30 to 45 days of culture, growing multiple bud clumps are separated into smaller pieces (3 × 3 × 3 mm3) and transferred to the newly prepared selection medium.
(18) The above process is repeated once more.
(19) The selected multiple bud clumps are propagated on proliferation medium for 2-3 months until multiple shoot formation.
(20) The multiple shoots are separated and transferred to rooting medium composed of MS salts and vitamins, 0.25 mg/l NAA and 30 g/l sucrose solidified by 2 g/l of phytagel.
(21) After 20 to 30 days of culture, regenerated plantlets are transferred to black polyethylene bags or containers with a substrate mixture and acclimatised in a greenhouse equipped with a mist system.
(22) The disease resistance of the acclimatised plantlets is evaluated in the greenhouse and/or in the field.

RESULTS AND DISCUSSION

Perspectives of the *in vitro* selection for disease resistance

The results mentioned in this chapter show the applicability of fusaric acid and culture filtrate in the selection of plants tolerant to the fungus FOC. However, the level of tolerance obtained by both methods was always lower than the tolerance level of 'Nanicão' that was used as a resistant control for the disease. Some of selected lines of 'Maçã' plants showed resistance in the field. At the same time, however, they became more susceptible to another disease, namely Yellow sigatoka disease (Matsumoto *et al*., 1999a). This effect might have been caused by secondary mutations or somaclonal variations induced by the successive rounds of selection, which were carried out with the aim of

avoiding occurrence of escapees and chimeras. Since many of the mycotoxins in the culture filtrates are mutagens (Jimenez *et al*., 1997; Sewram *et al*., 2005; Švábová and Lebeda, 2005), successive selections could induce secondary mutations. Undesirable mutations can be eliminated by new selection steps addressing the correction of the undesired character (Tang and Hwang, 1998). However, to avoid the secondary mutations in the first place, it is advisable to perform a single round or a few successive rounds of selection. The risk of occurrence of escapees and chimeras can be reduced by the use of smaller-sized explants, suspension cells or protoplasts. A reliable protocol of plant regeneration from the cells or protoplasts must become an important tool.

The history of plant breeding for disease resistance has shown that the acquired resistance will always be broken by the emergence of new microbial races. This means that we have to continuously breed new plants with different genes or mechanisms of disease resistance. *In vitro* selections with different selection factors make this strategy possible. Morpurgo *et al*. (1994; Chapter 7 of this book) showed that *in vitro* explants from disease-resistant and disease-susceptible banana cultivars were susceptible to both culture filtrate and fusaric acid irrespective of their known field resistance/susceptibility response. Therefore, they concluded that the use of culture filtrate or fusaric acid was not feasible for selecting a novel resistant genotype of *Musa* to FOC. However, contrary to their observations, the disease tolerances of susceptible banana cultivars were improved after selection with metabolic substances (Matsumoto *et al*., 1995, 1999a; Cardenas *et al*., 2003; Saravanan *et al*., 2003). We could now draw the conclusion that the use of culture filtrates or fusaric acid for selecting a novel genotype of *Musa* resistant to FOC is quite feasible. The disease tolerance mechanism of *in vitro*-selected plants may then be different from that of pre-existing resistant cultivars, since both resistant and susceptible cultivars show equal susceptibility to the metabolic substances. Considering that the mechanisms of tolerance to one selection factor are different from another, tolerances to several selection factors can be accumulated in one genotype by cumulative selection processes (Tang and Hwang, 1998). The *in vitro* selection system may produce new plants that confer several resistance mechanisms regulated by several genes. The acquired tolerance would, then, be a horizontal resistance characteristic and would be difficult to be broken by the emergence of new races of the fungus. It could, therefore, be maintained for a longer period.

Besides pathogen-produced metabolic substances, many other substances or chemicals can be used for *in vitro* selection of tolerance to Fusarium wilt. New selection factors or gene markers can facilitate *in vitro* selections and increase their applicability. Elicitors from fungal cell wall fractions are promising substances, since plant defence responses were induced by the elicitor treatments (De Ascensao and Dubery, 2000). Although we still do not have supporting data, plants selected by a proline analogue, such as hydroxyproline (Matsumoto *et al*., 1987), may over-produce proline and increase the absorption capacity of water from the soil. This could reduce damage caused by Fusarium wilt. Transgenic plants with an introduced glucanase gene have increased tolerance to Fusarium wilt (Mahmood *et al*., 2003). Glucanase over-producing plants may also be obtained by selecting explants that grow on a culture medium containing glucan as a substitute for part of the sucrose.

CONCLUSIONS

Reliable protocols for *in vitro* selection of banana plants resistant or tolerant to diseases are an important tool for the improvement of this crop, particularly in combination with *in vitro* mutagenesis. Our *in vitro* selection protocol based on the use of culture filtrates or fusaric acid for selecting novel genotypes of *Musa* resistant to FOC was shown to be quite feasible. In addition to the substances used in this study, many other substances or chemicals, such as fungal elicitors, may be useful for *in vitro* selection of tolerance to Fusarium wilt in bananas. Gene markers may also facilitate *in vitro* selections and increase their applicability.

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