In vitro selection for *Fusarium* wilt resistance in banana. II. Resistance to culture filtrate of race 1 *Fusarium oxysporum* f. sp. cubense

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Abstract — Introduction. Although there are banana varieties resistant to *Fusarium* wilt disease, transfer of the resistant genes into susceptible varieties by traditional cross-breeding is difficult because of triploidy. However, in vitro mutation breeding combined with toxin-used selection is a promising strategy to select such resistant varieties. The selection of *Fusarium* wilt resistant mutants from a highly susceptible banana variety (Maçã, *Musa* sp., AAB group) was carried out through the effects of fungal culture filtrates on in vitro proliferating shoot meristems. **Materials and methods**. The whitish compact clusters of proliferating shoot meristems, referred to as multiple-bud-clumps, were treated by chemical mutagen (ethylmethanesulphonate). After the mutagen treatment, 117 multiple-bud-clumps resistant to 10 or 15% of the culture filtrates of (*Fo*) were selected. The disease resistance was evaluated by artificial inoculation of the pathogen in a greenhouse condition. **Results and discussion**. Under the greenhouse condition, the plantlets, which were regenerated from the selected multiple-bud-clumps, showed more resistance to the disease than non-selected original ones. Three clones from a first experiment and nine clones from a second-experiment were transplanted to the field of different regions. Different levels of field resistance to the disease were observed. The selection methods developed here should be useful to obtain disease-resistant plants. (© Elsevier, Paris)

Brazil / *Musa* / *Fusarium oxysporum* / fungal diseases / disease resistance / toxins / mutation

Sélection in vitro d'une résistance au wilt du bananier, causé par du fusarium. II. Résistance vis-à-vis d'un filtrat de *Fusarium oxysporum* f. sp. cubense, race 1 (Fo).

Résumé — Introduction. Bien qu'il existe des variétés de bananiers résistants au wilt dû au fusarium (W_f), le transfert, par hybridation, de leurs gènes de résistance à des variétés sensibles est difficile à cause de la triploidie. Cependant, la sélection de mutations in vitro combinée à l'utilisation de toxines est une technique intéressante pour sélectionner de telles variétés résistantes. La sélection de mutants résistants au wilt (W_f) à partir d'une variété de bananier très sensible (Maçã, *Musa* sp., groupe AAB) a été effectuée par étude de la réaction de méristèmes de tiges en prolifération à un filtrat de culture du champignon. **Matériel et méthodes**. Des masses blanchâtres issues de la culture de méristèmes de tige de bananier en prolifération, identifiées comme amas de bourgeons multiples, ont été traitées par un produit mutagène (éthylméthanesulphonate). À la suite de ce traitement, 117 amas de bourgeons multiples résistants à des cultures contenant 10 ou 15 % de filtrat de (*Fo*) ont été sélectionnés. La résistance à la maladie a été évaluée par inoculation artificielle du pathogène à des plantules de bananiers sous serre. **Résultats et discussion**. Les plantules en serre, régénérées à partir des amas de bourgeons multiples sélectionnés, se sont montrées plus résistantes à la maladie que celles issues de matériel non sélectionné pour cette résistance. Trois clones d'un premier lot sélectionné et neuf clones d'un second ont été transférés en champ en différents endroits. Différents niveaux de résistance en champ ont été observés. La méthode de sélection développée dans ces travaux pourrait donc s'avérer utile pour sélectionner des bananiers résistants. (© Elsevier, Paris)

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1. introduction

Fusarium wilt is a serious disease of banana in tropical and subtropical countries. Chemical control of this disease is economically impracticable and its efficient control has been based on the use of resistant variety [1]. Although there are varieties resistant to this disease [2–5], transfer of the resistant genes into susceptible varieties by traditional cross-breeding is difficult because of triploidy and poor seed production. In vitro mutation breeding combined with toxin-used selection is a promising strategy, which has been successful in other crops [6–9].

In vitro mutation breeding requires efficient tissue culture methods to be applied to a large number of plants. Fortunately, rapid micropropagation methods in banana were already established by successive cultures of multiple shoots or buds [10–13]. Somatic embryogeneses should increase much more the efficiency of the mass propagation [14, 15]. On the other hand, it was shown that fusaric acid, a toxin responsible for the wilt symptom of the disease, was useful for the selection of the disease-resistant mutant [16], although it was not host-specific toxin.

Host-specific culture filtrate, which showed a higher toxicity to the disease susceptible variety, could be obtained by co-cultivation technique [17]. In this report, in vitro selection of resistant mutants to *Fusarium* wilt is described through the effects of the culture filtrate on banana multiple-bud-clumps.

2. materials and method

2.1. plant material and culture conditions

Multiple shoots from suckers of Maçã variety (*Musa* sp., AAB group), which is susceptible to race 1 *Fusarium* wilt, and Nanicão variety (AAA group, Cavendish subgroup), which is resistant to the disease, were used in this study. They were monthly subcultured in a 300-mL glass bottle which contained 40 mL Murashige and Skoog (MS) basal medium [18] supplemented with 22.2 µmol 6-benzylaminopurine (BAP), 8 $mg \cdot L^{-1}$ bromocresol purple, 3% sucrose and 0.2% gellan gum (Gelrite: Kelco, Division of Merck and Co., Inc.) [16, 19, 20]. This medium is referred to as the proliferation medium. The whitish compact clusters of proliferating shoot meristems, called multiple-bud-clumps, appeared from the multiple shoots after 6 to 12 months of culture, and were maintained on the same proliferation medium during more than 3 years. The multiple-bud-clumps were cut into pieces of about $3 \times 3 \times 3$ mm^3 and transplanted onto the proliferation medium (30 mL of the medium in 200-mL glass bottle; 5 explants per bottle). The cultures were incubated in a controlled environmental room maintained at 28 ± 2 °C at 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by cool-white fluorescent lights, 14-h photoperiod.

2.2. mutagen treatment and selection of variants resistant to culture filtrate in Maçã banana

To increase mutation frequency, a chemical mutagen treatment was performed. About 600 pieces of multiple-bud-clumps (each one is about $3 \times 3 \times 3$ mm^3 in size) of Maçã variety were incubated in a 500-mL Erlenmeyer flask with 200 mL of the aqueous solution in which contained 0.3% (v/v) ethylmethanesulphonate (EMS) and 4% (v/v) dimethyl sulphoxide (DMSO). They were maintained on a horizontal gyratory shaker (100 rpm) for 2 h at 28 °C [21, 22]. After washing three times with sterile distilled water, the multiple-bud-clumps were transferred onto the proliferation medium. One week after the mutagen treatment, they were transferred onto the selection medium, which was the proliferation medium supplemented with 10% of the fungal culture filtrate. The culture filtrate had previously been prepared by co-cultivation technique as follows:

A piece (approximately $2 \times 2 \times 1$ mm^3) of potato-dextrose-agar medium with a fungal colony of race 1 *Fusarium oxysporum* f. sp. *cubense* was inoculated in 100 mL

Czapek Dox broth (CZD) medium in a 300-mL Erlenmeyer flask, with a Maçã banana multiple-bud-clump (about 10 × 10 × 10 mm^3). The cultures were incubated in the controlled environment room (28 ± 2 °C, 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 14-h photoperiod) for 21 d without shaking. The cultured liquid media were then filtered through four layers of cheesecloth and centrifuged (8000 × *g*, 20 min) to reduce the mycelium and conidia. The supernatant was filtered through a membrane filter (0.45 µm pore size) to remove the fungus completely. This fungus-free medium was used as the culture filtrate for the selection process.

The mutagen-treated multiple-bud-clumps on the selection medium were maintained in the controlled environment room. After 1 month of culture, the growing multiple-bud-clumps were separated into smaller pieces (3 × 3 × 1 mm^3) and transferred again to newly prepared selection medium containing 15% of the culture filtrate. This selection process was repeated a further two times, using 15% of the filtrate, to reduce chimerical mutants and survivors by escape from the selection.

After the successive selections, the growing multiple-bud-clumps were subcultured another three times on the proliferation medium (culture filtrate-free medium). Resistance of the selected multiple-bud-clumps was tested, culturing selected and nonselected multiple-bud-clumps on the medium supplemented with 15% culture filtrate. The frequency of growing multiple-bud-clumps was observed after 1 month of culture.

2.3. tests of race 1 *Fusarium* wilt resistance on plantlets regenerated from the selected multiple-bud-clumps

The multiple-bud-clumps selected by and confirmed for resistance to the culture filtrate were proliferated on the proliferation medium for 1 month, and transferred to regeneration medium composed of MS salts and vitamins, 8 $mg \cdot L^{-1}$ bromocresol purple, 3% sucrose, 0.2% gellan gum and 1.3 µmol α-naphthaleneacetic acid (NAA). After 1 month of culture, regenerated plantlets were transferred to black polyethylene bags containing 2-L soil consisted of a mixture of latossol-type soil, sand and barnyard manure (2:1:1 in volume) in a greenhouse, for acclimation and further growth. After 4 months, when the plants were about 50 cm high, they were uprooted and the soil was washed out. Then, roots and pseudostem were cut off. The rhizomes (2 to 3 cm diameter and 3 to 5 cm high) with a shoot tip and lower part of pseudostem were immersed into fungal solution which contained 10^7 conidia$\cdot mL^{-1}$ of the race 1 *Fusarium oxysporum* f. sp. *cubense* in the liquid proliferation medium. They were then transplanted to 1-L polyethylene bags filled with vermiculite that was previously inoculated by the same fungus. The pre-inoculation of the fungus to vermiculite was carried out as follows:

Conidial suspensions obtained from 14-d-old cultures grown in CZD medium on a shaker (100 rpm, 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 14-h photoperiod, 28 ± 2 °C) were filtered through two layers of cheesecloth, and adjusted to about 10^7 conidia$\cdot mL^{-1}$ by distilled water. Each polyethylene bag with vermiculite was given 10 mL of the conidial solution and maintained in a greenhouse for 10 d, sprinkling daily with distilled water. Then, they were used for the test.

After 1 month of the plantlet cultivation with fungus in the greenhouse, the disease symptoms were evaluated. Root and shoot growths were also observed.

The experimental design was completely randomised. Data were submitted to statistical analyses using *t*-statistics and Fisher's exact test.

3. results and discussion

After the EMS treatment, the multiple-bud-clumps resistant to the culture filtrates were selected. The successive selections increased the surviving multiple-bud-clumps from 11 to 74% in the medium containing 15% culture filtrates (*table I*). Similar results were observed in alfalfa plants, selecting *Fusarium*-resistant calli [23] and cell suspension [8]. It was also observed that the

Table I.
Resistance of in vitro banana selected and nonselected multiple-bud-clumps to 15% culture filtrates in a proliferation medium, after 1 month of culture.

Multiple-bud-clump line	Explants		
	Observed (number)	Survived (%)	Killed (%)
Selected	117	74.4	25.6
Non-selected	45	11.1	88.8

Data showed significance at 1% level by Chi-square statistics.

selected calli or cells regenerated the disease-resistant plants more frequently than non-selected ones. Banana plants were then regenerated from the selected multiple-bud-clumps. The *Fusarium* wilt resistance test was pursued on the regenerated plants under greenhouse conditions. The tests did not show clear differences in the appearance of the disease symptom (vascular discoloration) between the selected plant lines and nonselected one, but showed a certain tendency of the number increasing of symptomless plants in the selected line (*table II*). The resistance increment was confirmed when the pseudostem and root growth were observed. Particularly on root growth, the selected plant lines showed more resistance to race 1 *Fusarium oxysporum* f. sp. *cubense* than nonselected original Maçã plants (*table III*).

After the mutagen treatment of the 600 multiple-bud-clumps, the 117 toxin-resistant clumps were selected, 31 of which regenerated plants and 10 of which showed good level of the disease resistance under greenhouse conditions. It seems that the mutation frequency was extremely high. This result may be explained by the high level of somaclonal variation [24, 25] and mitotic instability [26–29] in micropropagated bananas, besides the mutagen treatment.

As shown above, the plants which were regenerated from the multiple-bud-clumps resistant to the culture filtrates were also

Table II.
Frequency of plants with the symptom of pseudostem and rhizome vascular discoloration caused by race 1 *Fusarium oxysporum* f. sp *cubense* on selected and non-selected Maçã banana plants in greenhouse tests.

Plant line	Explant observed (number)	Pseudostem		Rhizome	
		Without symptom (%)	With symptom (%)	Without symptom (%)	With symptom (%)
Non-selected	34	52.9	47.1	17.6	82.4
Selected	31	58.1	41.9	32.3	67.7
Nanicão[1]	34	97.1	2.9	70.6	29.4

[1] Nanicão was tested as control of race 1 *Fusarium* wilt resistant plant. Comparing between nons-elected and selected Maçã, data showed significance levels of 0.4346 for pseudostems and 0.1406 for rhizomes, by Fisher's exact test. The comparisons between Nanicão and other treatments showed significance levels of less than 0.01.

resistant to the disease in the greenhouse condition. However, this resistance level was not the same as shown on Nanicão plants which were used as a control of the disease-resistant variety (*table II*). The disease-resistance mechanism of Nanicão may be different from that of selected plants. Beckman [30] related that the main mechanism of the disease resistance should be early production of gel and tyloses that inhibited fungus translocation. However, we selected plants resistant to fungal toxin, which was probably not the main response of the host to pathogen. The toxin-resistant plants selected here may then have acquired partial resistance to the disease by delaying the fungus growth and giving sufficient time to gel and/or tylose production. We need a field test if the acquired disease-resistance maintains in further stages of the plants. Shoot tips were re-isolated from some of the selected symptomless plantlets. The fungus-free plants were regenerated and acclimated under greenhouse condition. Three clone lines of the 10 selected plant lines were transplanted to the fields of the Embrapa - Cassava and Tropical Fruit (Cruz das Almas, Bahia State, Brazil). Although it was a preliminary evaluation, they showed a certain level of the disease resistance (Silva, pers. comm.). Detail evaluation is now being carried out, after multiplying to more than 100 plants from each clone.

The experiments were repeated, and more than 200 plants of nine clones were newly transplanted to the field of different regions (Brasilia District, Mato Grosso State and Bahia State) to be evaluated and propagated. Different levels of the disease resistance were observed. One of them showed clear field resistance to the disease (*figure 1*).

Although the selection methods must still be improved, they should be useful to obtain disease-resistant plants.

Table III.
Comparisons of the root and shoot growth between selected and nonselected Maçã banana plants infected by race 1 *Fusarium oxysporum* f. sp. *cubense* in greenhouse tests.

Plant ligne	Pseudostem (mm high)	Root (mg fresh weight)
Non-selected	26.42 ± 6.251	460.71 ± 109.96
Selected	43.63 ± 10.83	1077.27 ± 313.43
Significance level[2]	0.1613	0.0536

[1] : mean ± standard error.
[2] : by *t*-statistic, two-sided significance level.

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Figure 1.
Fusarium wilt resistant plant observed in the experimental field of Mato Grosso State, Brazil, showing original Maçã variety (front left) and selected disease-resistant plant (back right). The banana plants were transplanted to the same place where the plants of the disease-susceptible variety previously died by *Fusarium* wilt.

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Selección in vitro de una resistencia al wilt del banano, causado por fusarium. II. Resistencia frente a un filtrado de *Fusarium oxysporum* f. sp. cubense, raza 1 (Fo).

Resumen — Introducción. Aunque existan variedades de bananos resistentes al wilt causado por fusarium (W_f), resulta difícil la transferencia, mediante hibridación, de sus genes de resistencia a variedades sensibles debido a la triploidia. Con todo, la selección de mutaciones in vitro combinada con el uso de toxinas es una técnica interesante para seleccionar semejantes variedades resistentes. Se realizó la selección de mutantes resistentes al wilt (W_f) a partir de una variedad de banano muy sensible (Maçã, *Musa* sp., AAB grupo) mediante un estudio de la reacción de meristemos de tallos en proliferación a un filtrado de cultivo del hongo. **Material y métodos**. Masas blancuzcas oriundas del cultivo de meristemos de tallo de banano en proliferación, identificados como masas de yemas múltiples, fueron tratadas con un producto mutageno (etilmetanosulfonato). A continuación de este tratamiento, se seleccionaron 117 masas de yemas múltiples resistentes a cultivos conteniendo 10 o 15% de filtrado de (*Fo*). La resistencia a la enfermedad se evaluó por inoculación artificial del patógeno a plántulas de bananos en invernadero. **Resultados y discusión**. Las plántulas en invernadero, regeneradas a partir de las masas de yemas múltiples seleccionadas, demostraron ser más resistentes a la enfermedad que las oriundas del material no seleccionado para esta resistencia. Tres clones de un primer lote seleccionado y nueve clones de un segundo fueron transferidos en campo en distintos sitios. Se observaron distintos niveles de resistencia en campo. Por lo tanto, el método de selección desarrollado en estos trabajos podría resultar útil para seleccionar bananos resistentes. (© Elsevier, Paris)

Brasil / *Musa* / *Fusarium oxysporum* / enfermedades fungosas / resistencia a la enfermedad / toxinas / mutación