In vitro selection for *Fusarium* wilt resistance in banana I. Co-cultivation technique to produce culture filtrate of race 1 *Fusarium oxysporum* f. sp. *cubense*

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In vitro selection for *Fusarium* wilt resistance in banana. I. Co-cultivation technique to produce culture filtrate of race 1 *Fusarium oxysporum* f. sp. *cubense*.

Abstract — Introduction. In vitro mutation breeding combined with toxin-used selection is a promising strategy to obtain disease resistant plants in species of which sexual propagation is limited. Co-cultivation technique to produce effective crude filtrates, i.e., mixed toxin, of fungus culture was developed for in vitro selection of banana mutants. **Materials and methods**. Race 1 *Fusarium oxysporum* f. sp. *cubense* were co-cultivated with banana tissue cultures which were whitish compact clusters of proliferating shoot meristems, referred as multiple-bud-clump. Crude filtrates of the cultivated medium were obtained passing it through a 0.45 µm pore size membrane. Multiple-bud-clumps of resistant and susceptible banana varieties were cultivated on the medium supplemented with the filtrates to evaluate toxin activity. **Results and discussion**. Crude filtrates produced by the co-cultivation showed higher specificity of toxic activities on banana than that produced by sole-cultivation. The banana variety used for obtaining the multiple-bud-clump was highly important for the co-cultivation technique. The co-cultivation of the fungus with a multiple-bud-clump of Maçã variety, which is susceptible to the *Fusarium* disease, produced highly toxic filtrates to susceptible variety (Maçã) and less toxic to resistant variety (Nanicão). The results showed the crude filtrates should be useful to in vitro selection of disease resistant plants. (© Elsevier, Paris)

Brazil / *Musa* (bananas) / disease resistance / *Fusarium oxysporum* / toxins / in vitro selection

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Sélection in vitro d'une résistance au wilt du bananier, causé par du fusarium. I. Production de filtrat de *Fusarium oxysporum* f. sp. *cubense*, race 1, par coculture.

Résumé — Introduction. La sélection de mutations in vitro combinée à l'utilisation d'une toxine déterminée est une technique intéressante pour produire des plants résistants aux maladies, chez les espèces à reproduction sexuée difficile. La technique de coculture utilisée pour produire des filtrats bruts efficaces à partir de la culture d'un champignon a été développée pour être exploitée lors de la sélection in vitro de mutants chez le bananier. **Matériel et méthodes**. Du *Fusarium oxysporum* f. sp. *cubense*, race 1, a été cocultivé avec des masses compactes et blanchâtres issues de la culture de méristèmes de tige de bananier en prolifération, identifiées comme des amas de bourgeons multiples. Des filtrats bruts du milieu de culture ont été obtenus par filtrage au travers d'une membrane à pores de 0,45 µm. Des amas de bourgeons multiples de variétés de bananier résistantes et sensibles au wilt ont été cultivés sur un milieu contenant les filtrats obtenus afin d'évaluer la toxicité des toxines. **Résultats et discussion**. Les filtrats bruts obtenus à partir de coculture ont montré une spécificité de la toxicité du champignon sur les tissus du bananier plus élevée que celle observée à partir de filtrats issus de culture individuelle. La variété de bananier utilisée pour l'obtention des amas de bourgeons multiples influe fortement sur la technique de coculture. La coculture du champignon avec des amas de bourgeons multiples de la variété Maçã sensible au fusarium a produit des filtrats qui ont fortement affecté cette variété sensible (Maçã) et moins nui à la variété résistante (Nanicão). Les résultats ont montré que les filtrats bruts devraient être utilisés pour la sélection in vitro de plants résistants à la maladie du wilt. (© Elsevier, Paris)

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1. introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (FOC), is one of the most important diseases of banana in tropical and subtropical countries. Chemical control of this disease is economically impracticable. Traditional cross breeding to obtain the disease resistant plants is difficult in banana because the most of the widely cultivated varieties are triploids and have poor seed production. Quite a few bred hybrids of restricted variety combinations were obtained after more than 30 years of intensive works under the worldwide breeding programs [1]. In vitro mutation breeding combined with toxin-used selection is a promising strategy that has been successful for *Fusarium* disease tolerance of other crops [2–6] and *Mycosphaerella* disease tolerance in banana [7]. Chemically defined toxins or undefined toxin mixtures, i.e., culture filtrates, are used for these purposes. However, in banana, the culture filtrate of FOC did not show selectivity on plant tissues of resistant and susceptible varieties [8]. No co-relation between culture filtrate tolerance and disease tolerance has previously been observed [9].

In this paper, the co-cultivation technique to obtain effective culture filtrates is described; it will be able to be used in further in vitro mutation breeding.

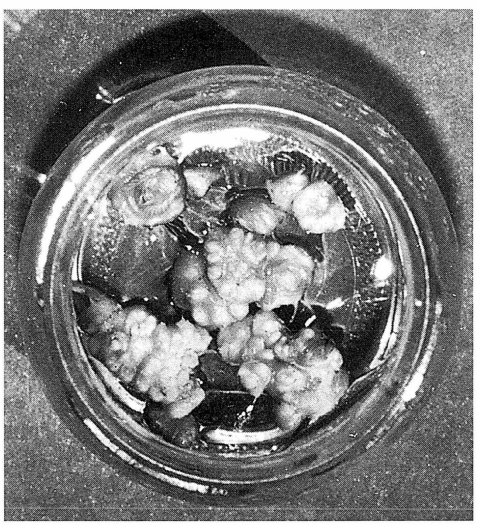

Figure 1.
Aspect of banana multiple-bud-clumps.

2. materials and methods

2.1. plant material and culture conditions

Whitish compact clusters of proliferating shoot meristems, previously referred as protocorm-like bodies [11, 12], and here referred as multiple-bud-clumps (*figure 1*), were obtained from micropropagated banana shoots. 'Nanicão' banana variety (*Musa* sp., AAA, Cavendish subgroup), which is resistant to race 1 *Fusarium* wilt, and 'Maçã' variety (AAB, "Silk"), which is susceptible to the disease, were used. They were established from suckers and micropropagated for more than 3 years, with monthly subculturing in a 300-mL glass bottle which contained 40 mL Murashige and Skoog (MS) basal medium [10] 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.) [11, 12]. The pH of the medium was adjusted to 5.8 before autoclaving. This medium is hereafter referred to as the proliferation medium. The cultures were incubated in a controlled environment room maintained at 28 ± 2 °C and 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by cool-white fluorescent lights, 14-h photoperiod.

2.2. fungus preparation

The pathogen, FOC race 1, was supplied from "Empresa Capixaba de Pesquisa Agropecuária", Vitória-ES, Brazil, where the fungus was isolated from a diseased Maçã banana plant. To prevent a change of the virulence of the isolate, the fungus was proliferated only once on potato-dextrose-agar medium in petri dishes in an incubator (28 ± 2 °C, continuous illumination of 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 2 weeks. Then it was stored in a refrigerator (4 °C) until use.

The phytopathogenicity of the isolate was checked on banana in vivo according to the method of Sun and Su [13], except for their soil (substract) preparation method. The soil consisted of a mixture of latossol-type soil, sand and barnyard manure (2:1:1, in volume). Black polyethylene bags (1 L) were used as containers. To increase the

fungus concentration in soil and to reduce infection escape, the fungus was cultured for 10 d in the liquid MS basal medium supplemented with 22.2 µmol BAP and 3% sucrose, and was then inoculated to the steam-sterilized soil (10 mL of the medium per 1 L soil). The MS basal medium, which was the same as the plant proliferation medium without bromocresol purple and gellan gum, was used here, instead of a specific fungus culture medium, to preventing unexpected effects on the banana plantlets.

The soil was sprinkled daily with distilled water. After 1 week of the pre-treatment, the greenhouse-acclimated plantlets, which had originated from in vitro culture [11], were inoculated with fungus and transplanted to the soil as documented by Sun and Su [13].

The fungal colony stocks, which confirmed their phytopathogenicity in Maçã and not in Nanicão, were used in the following studies.

2.3. co-cultivation of fungus and plant multiple-bud-clump on the culture filtrate production

To obtain the culture filtrate, Czapek Dox broth (CZD) medium was utilized. A piece (approximately $2 \times 2 \times 1$ mm^3) of potato-dextrose-agar medium with fungal colony was inoculated in 100 mL of CZD medium in a 300-mL erlenmeyer flask, with or without a multiple-bud-clump (about $10 \times 10 \times 10$ mm^3) of Maçã or Nanicão variety. The cultures were incubated in the controlled environment room (28 ± 2 °C, 56 µmol·m^{-2}·s^{-1}, 14-h photoperiod) for 21 d without shaking. The cultured liquid media were then filtered through four layers of cheesecloth and centrifuged (8 000 × 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.

On bioassays of the crude culture filtrates, the proliferation medium was supplemented with 0, 5, 10, 15 and 20% of these filtrates, and multiple-bud-clumps of Maçã and Nanicão were cultured on the media for 1 month in the controlled environment room. The toxic activity of the filtrate was evaluated by the reduction of the multiple-bud-clump growth.

Moreover, to distinguish the culture filtrate effects from the CZD medium toxicity, the multiple-bud-clump growth in proliferation medium supplemented with different concentrations of CZD medium was also analyzed.

The experimental design was completely randomized. All data were collected as averages of at least 25 explants (repetition) per treatment and submitted to statistical analyses using *t*-statistics and regression analyses (Statpak Computer program).

3. results and discussion

In the co-cultivation technique, aspect of the cultured banana tissue is very important. Whitish compact clusters of proliferating meristems or multiple-bud-clumps should be used (*figure 1*). When we used normal micropropagated shoots instead of multiple-bud-clumps, the tissue produced polyphenols, and the oxidized polyphenols reduced fungus growth and toxin production (data not shown).

When the culture filtrate concentration increased, the multiple-bud-clump growth of both Nanicão and Maçã decreased (*figure 2*). But, the reduced growth ratio differed depending on the varieties and the culture filtrate production methods. Nanicão, which is resistant to race 1 *Fusarium* wilt, was more susceptible to the culture filtrate produced by sole-cultivation than that of co-cultivation (*figure 2a*). In contrast, Maçã which is susceptible to race 1 was more susceptible to the co-cultivated culture filtrate than sole-cultivated one (*figure 2b*). It means that the co-cultivated culture filtrate permits Nanicão type tissue to grow more intensively than Maçã type tissue.

Aiming to verify effects of plant variety of the co-cultivating multiple-bud-clump for culture filtrate production, the co-cultivation with Maçã multiple-bud-clump was

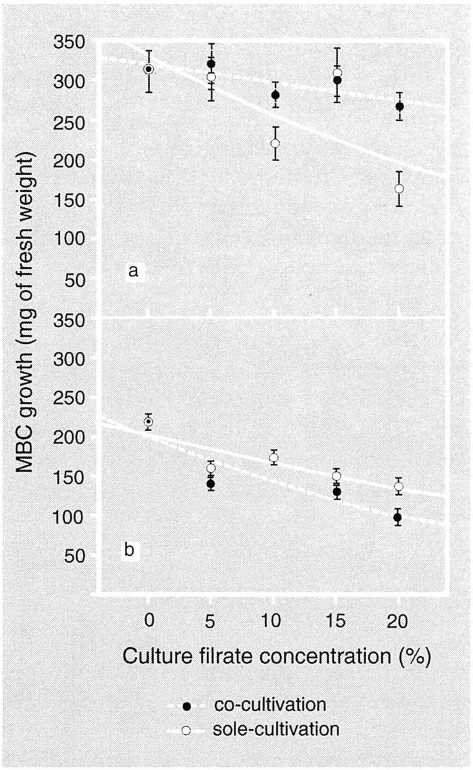

Figure 2.
Inhibition of multiple-bud-clump (MBC) growth cultivated on the media with culture filtrates produced by co-or sole-cultivation technique.
a - Nanicão multiple-bud-clump growth;
b - Maçã multiple-bud-clump growth.
The vertical bars show standard errors of means.

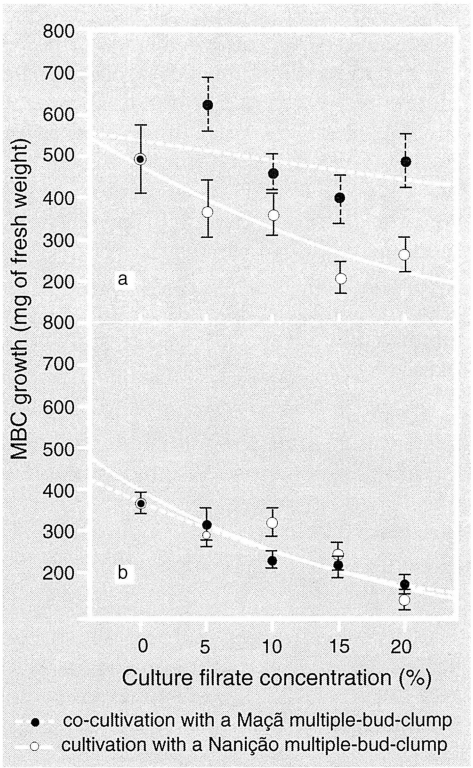

Figure 3.
Inhibition of multiple-bud-clump (MBC) growths cultivated on the media with culture filtrates produced by co-cultivation of fungus with a Maçã or Nanicão multiple-bud-clump.
a - Nanicão multiple-bud-clump growth;
b - Maçã multiple-bud-clump growth.
The vertical bars show standard errors of means.

compared that with Nanicão multiple-bud-clump (*figure 3*). On the Nanicão multiple-bud-clump growth, the culture filtrate co-cultivated with a Maçã multiple-bud-clump showed a less toxic activity than that co-cultivated with Nanicão multiple-bud-clump (*figure 3a*). It was no difference on the Maçã multiple-bud-clump growth, except 10% culture filtrates (*figure 3b*). In this concentration, the culture filtrate co-cultivated with a Maçã multiple-bud-clump showed more efficacy than that co-cultivated with a Nanicão multiple-bud-clump.

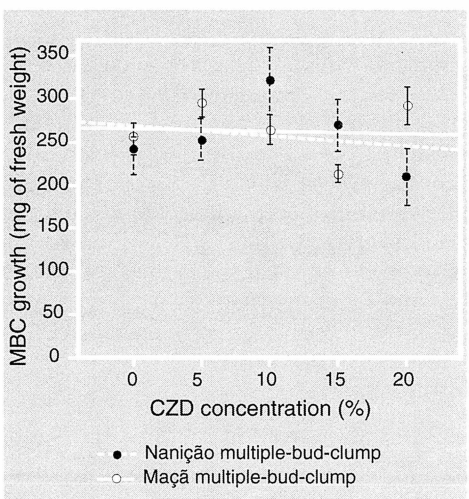

Figure 4.
Effects of Czapek Dox broth (CZD) medium on multiple-bud-clump (MBC) growth.
The vertical bars show standard errors of means.

The CZD medium by itself did not show any significant inhibitory effect on multiple-bud-clump growth (*figure 4*). This result indicates that the growth inhibition caused by the filtrates is due to toxins produced by the fungus, but not by the CZD medium itself.

In the review of Novak [8], Morpurgo (personal communication) related effects of *Fusarium* culture filtrates on shoot tips of banana, showing, however, no differences in toxicity between susceptible (Pisang Mas) and tolerant (SH-3362) varieties. In our experiments, similar results were obtained when the culture filtrates were produced by the sole-cultivation. However, when the culture filtrates were produced by the co-cultivation, Nanicão which is resistant to the disease was more resistant to the filtrates than Maçã, encouraging the use of

culture filtrates for obtaining variants resistant to the disease. Higher production of some toxins may be occurred by the host-pathogen reaction, otherwise, new host-specific products (toxins, enzymes or other metabolites) may be induced, although they are not identified, yet.

Using the above related co-cultivation technique, culture filtrate resistant multiple-bud-clumps were selected after mutagen (ethylmethane sulphonate) treatment. The selected multiple-bud-clumps regenerated a higher number of plants resistant to race 1 *Fusarium* wilt than these of nonselected multiple-bud-clumps, in greenhouse condition (in preparation for publication).

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Selección in vitro de una resistencia al wilt del plátano, provocado por *Fusarium*. 1. Producción de filtrado de *Fusarium oxysporum* f. sp. *cubense*, raza 1, por cocultivo.

Resumen — Introducción. La selección de mutaciones in vitro combinada con la utilización de una toxina determinada es una técnica interesante para producir plantas resistentes a las enfermedades, en las especies de difícil reproducción sexuada. La técnica de cocultivo utilizada para producir resultados de filtración brutos eficaces a partir del cultivo de un hongo fue desarrollada para ser explotada cuando la selección in vitro de mutantes en el plátano. **Material y métodos**. *Fusarium oxysporum* f. sp. *cubense,* raza 1, fue cocultivado con masas compactas y blanquinosas oriundas del cultivo de meristemos de tallo de plátano en proliferación, identificadas como cúmulos de yemas múltiples. Se lograron resultados de filtración brutos del medio de cultivo mediante resultados de filtración a través de una membrana de poros de 0,45 µm. Se cultivaron cúmulos de yemas múltiples de variedades de plátano resistentes y sensibles al wilt en medio conteniendo los resultados de filtración logrados para evaluar la toxicidad de las toxinas. **Resultados y discusión**. Los resultados de filtración brutos logrados a partir de cocultivo mostraron una especificidad de la toxicidad del hongo en los tejidos del plátano más elevada que la observada a partir de resultados de filtración oriundos de cultivo individual. La variedad de plátano utilizada para la obtención de los cúmulos de yemas múltiples influye fuertemente sobre la técnica de cocultivo. El cocultivo del hongo con cúmulos de yemas múltiples de la variedad Maçã sensible al fusarium produjo los resultados de filtración que afectaron fuertemente esta variedad sensible (Maçã) y perjudicó menos la variedad resistente (Nanicão). Los resultados mostraron que los resultados de filtración brutos deberían utilizarse para la selección in vitro de plantas resistentes a la enfermedad del wilt.

Brasil / *Musa* (bananos) / resistencia a la enfermedad / *Fusarium oxysporum* / toxinas / selección in vitro