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Rapid screening of *Musa* species for resistance to Fusarium wilt in an *in vitro* bioassay

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Abstract In order to accelerate breeding and selection for disease resistance to Fusarium wilt, it is important to develop bioassays which can differentiate between resistant and susceptible cultivars efficiently. Currently, the most commonly used early bioassay for screening *Musa* genotypes against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a pot system, followed by a hydroponic system. This paper investigated the utility of *in vitro* inoculation of rooted banana plantlets grown on modified medium as a reliable and rapid bioassay for resistance to *Foc*. Using a scale of 0 to 6 for disease severity measurement, the mean final disease severities of cultivars expressing different levels of disease reaction were significantly different ($P \leq 0.05$). Twenty-four days after inoculation with *Foc* tropical race 4 at 10^6 conidia ml^{-1}, the plantlets of two susceptible cultivars had higher final disease severities than that of four resistant cultivars. Compared with 'Guangfen No.1', 'Brazil Xiangjiao' is highly susceptible to tropical race 4 and its mean final disease severity was the highest (5.27). The plantlets of moderately resistant cultivar 'Formosana' had a mean final disease severity (3.53) lower than that of 'Guangfen No.1' (4.33) but higher than that of resistant cultivars: 'Nongke No.1', GCTCV-119, and 'Dongguan Dajiao' (1.87, 1.73, and1.53, respectively). Promising resistant clones acquired through non-conventional breeding techniques such as *in vitro* selection, genetic transformation, and protoplast fusion could be screened by the *in vitro* bioassay directly. Since there is no acclimatization stage for plantlets used in the bioassay, it helps to improve banana breeding efficiency.

Keywords Banana · Breeding · *Fusarium oxysporum* f. sp. *cubense* · Panama disease · Plantain

Abbreviations

Foc	*Fusarium oxysporum* f. sp. *cubense*
MIS	Medium for interaction system
MS	Murashige and Skoog medium

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Introduction

Banana and plantain (*Musa* spp.) are important tropical and subtropical fruits around the world, and also staple food in developing countries. Global banana production is now increasingly threatened by a number of pests and diseases, of which Fusarium

wilt has become the major constraint. Fusarium wilt of banana, caused by the soil-inhabiting fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is recognized as one of the most destructive diseases of banana worldwide. Three races (1, 2, and 4) of *Foc* affect edible banana cultivars, while race 3 only affects *Heliconia* (Waite 1963). Race 4 not only attacks Cavendish cultivars but also cultivars susceptible to race 1 and 2. Strains of race 4 are separated into subtropical race 4 and tropical race 4. Although they affect many of the same cultivars, tropical race 4 attacks plants in the tropics, but subtropical race 4 only affects plants in the areas with pronounced winters (Ploetz 2006). The vascular pathogen penetrates the plant root system and eventually blocks the xylem vessels. External symptoms of the disease include initial yellowing of the leaf margins of older leaves, before the yellowing progresses from the oldest to the youngest leaves. Leaves gradually collapse to form a 'skirt' of dead leaves around the pseudostem and the plant eventually dies.

At present, there are no economically viable biological, chemical or cultural measures of controlling Fusarium wilt in an infected field (Ploetz 2006; Buddenhagen 2009). It is widely accepted that the breeding and selection for disease tolerance or resistance is the most effective and sustainable management option (Buddenhagen 2009). Although the field performance of genotypes remains the benchmark for evaluating host plant resistance (Daniells et al. 1995), field screening for resistance to Fusarium wilt depends on the presence of environmental conditions conducive to disease development, and is time-consuming and expensive (Vakili 1965). In order to accelerate progress in banana breeding programs for resistance to Fusarium wilt, it is important to develop bioassays that can differentiate between resistant and susceptible cultivars efficiently and accurately.

So far, the most commonly used early bioassay is a pot system (Matsumoto et al. 1995; Subramaniam et al. 2006; Weber et al. 2007; Smith et al. 2008), followed by a hydroponic system (De Ascensao and Dubery 2000; Groenewald et al. 2006; Van den Berg et al. 2007). Not all authors have indicated the age of tissue-cultured banana plants used in the two systems. Smith et al. (2008) concluded that 8 week-old plants (10 to 15 cm tall) were more favorable for consistent infection than the plants less than 10 cm in the pot system, while plants used in the hydroponic system by Groenewald et al. (2006) were much smaller (5 cm tall). For these bioassays, three different inoculation methods were used: roots dipped into a conidial suspension, potting mix inoculated with millet grains colonized by *Foc*, and potting mix or liquid medium inoculated with a conidial suspension of *Foc*. In most of the reports, disease development was evaluated 7 to 8 weeks (Matsumoto et al. 1995; Smith et al. 2008) and 6 weeks (De Ascensao and Dubery 2000; Groenewald et al. 2006; Van den Berg et al. 2007) after inoculation in the pot system and hydroponic system, respectively. All the bioassays reported were conducted in greenhouses. Companioni et al. (2003) also reported the application of culture filtrates of *Foc* on banana leaves and developed a procedure for evaluating resistance at the individual leaf level. The objective of this study was to investigate the utility of *in vitro* inoculation of rooted banana plantlets grown on modified medium as a reliable and rapid bioassay for resistance to *Foc*.

Materials and methods

Plant material and inoculum preparation

Six banana cultivars that express different levels of disease reaction were tested (Table 1). Suckers of these cultivars were micropropagated using shoot-tip meristem culture (Wong 1986; Arinaitwe et al. 1999). One isolate of *Foc* tropical race 4 (VCG 01213, ACC 31282) was procured from the Agricultural Culture Collection of China, which had been collected from a diseased Cavendish banana plant in Hainan Province in 2005. For pathogenicity testing, the culture was transferred to Armstrong's *Fusarium* medium (Booth 1977) and incubated on a rotary shaker at 150 rpm and at 25±2°C for 5 days. After incubation, the sporulation medium was poured through 4 layers of cheesecloth to separate spores from mycelia. Conidia concentration in the suspension was determined with a haemocytometer, and adjusted with sterile distilled water to 10^4, 10^5, or 10^6 conidia ml^{-1}.

In vitro inoculation of plantlets in Erlenmeyer flasks

Rooted plantlets which had been cultured on rooting medium (Wu et al. 2005) for 2 or 3 weeks were

Table 1 Description of *Musa* cultivars evaluated using an *in vitro* bioassay developed for rapid screening of germplasm for resistance to Fusarium wilt

Cultivars	Origin	Reaction	Reference
Brazil Xiangjiao (*Musa* AAA Cavendish subgroup)	Brazil	Highly susceptible	–
GCTCV-119 (*Musa* AAA Cavendish subgroup) [y]	Taiwan	Highly resistant	(Hwang and Ko 2004)
Formosana (*Musa* AAA Cavendish subgroup) [y]	Taiwan	Moderately resistant	(Hwang and Ko 2004)
Nongke No.1 (*Musa* AAA Cavendish subgroup) [z]	Guangdong, China	Resistant	(Liu et al. 2007)
Guangfen No.1 (*Musa* ABB group)	Guangdong, China	Susceptible	(Huang 2005)
Dongguan Dajiao (*Musa* ABB group)	Guangdong, China	Highly resistant	–

[y] GCTCV-119 and 'Formosana' were obtained from National Field Genebank for Banana at the Fruit Tree Research Institute, Guangdong Academy of Agricultural Sciences

[z] 'Nongke No.1' is a Fusarium wilt resistant clone recently selected by Liu et al. (2007) from 'Brazil Xiangjiao'

transferred to 150 ml Erlenmeyer flasks containing modified medium for interaction system (MIS), one plantlet in each flask. MIS consisted of half-strength MS (Murashige and Skoog 1962) salts and 6 g agar l^{-1}. After growing on MIS for 1 to 2 weeks, plantlets for *in vitro* inoculation were selected where: the height of the pseudostem was 4.5 to 5.0 cm, and the plantlet had more than two fully expanded leaves and three white roots. Before *in vitro* inoculation, 5 mm diameter filter paper discs were soaked in a conidial suspension of *Foc*. Then each plantlet was inoculated by putting one disc on the surface of the MIS medium. Cultures were maintained at 25±2°C under a 12 h photoperiod day^{-1} with 50 $\mu mol m^{-2} s^{-1}$ from cool white fluorescent lamps. 'Brazil Xiangjiao', 'Nongke No.1', and 'Guangfen No.1' were inoculated with tropical race 4 spore suspensions of 10^4, 10^5, and 10^6 conidia ml^{-1}, respectively to determine a suitable inoculum concentration. For inoculum concentration study and all subsequent experiments, each treatment included five plantlets and was repeated three times. Control plantlets were prepared in the same manner but grown on inoculum-free MIS.

In vitro disease severity rating

Observations on disease development were made from 4 to 24 days after inoculation. Disease severity was rated on a scale of 0 to 6 where 0 indicated there was discolouration of the root system; 1 indicated that, although the smaller leaves at the base of pseudostem wilted, there was no discolouration of the pseudostem; 2 indicated≤1/2 the height of the pseudostem was discoloured; 3 indicated>1/2 the height of the pseudostem was discoloured and (or) there was discolouration of the leaf stalk; 4 indicated≤50% of the leaves wilted or yellowed; 5 indicated>50% of the leaves wilted or yellowed, and 6 indicated the whole plantlet was wilted.

Results

Disease development after *in vitro* inoculation

The visible "symptoms" of *in vitro* inoculated plantlets are shown in Fig. 1b to f. For susceptible cultivar 'Brazil Xiangjiao' (*Musa* AAA Cavendish subgroup), most plantlets died within 4 weeks after inoculation with tropical race 4 at 10^6 conidia ml^{-1}.

Effect of inoculum concentration on disease development

As shown in Figs. 2 and 3, for susceptible cultivars 'Brazil Xiangjiao' and 'Guangfen No.1', disease progressed more rapidly and the mean final disease severities increased from 4.0, 2.93 to 5.53 and 4.2, respectively when inoculum concentration was increased from 10^4 to 10^6 conidia ml^{-1}, but the difference between 10^5 and 10^6 conidia ml^{-1} was not significant according to Student-Newman-Keuls test ($P<0.05$). For the resistant cultivar 'Nongke No.1', mean final disease severities of the plantlets in all treatments were consistently low (1.93, 2.0, and 1.93, respectively), irrespective of inoculum concen-

Fig. 1 'Brazil Xiangjiao' (*Musa* AAA Cavendish subgroup) plantlet grown on inoculum-free medium for interaction system (MIS) for 30 days (**a**), and development of Fusarium wilt on 'Brazil Xiangjiao' plantlets after inoculation with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 at 10^6 conidia ml^{-1} (**b** to **f**). There was discolouration of the root system 3 days after inoculation (**b**); smaller leaves at the base of pseudostem began to wilt 4 days after inoculation (**c**); the pseudostem discolored and discolouration progressed up the pseudostem (**d**); the upper leaves of plantlets wilted or yellowed from the oldest to the youngest leaves 16 to 24 days after inoculation (**e**); and finally the whole plantlet was completely wilted (**f**)

tration. The results suggested that a high concentration of spore suspension (10^5 to 10^6 conidia ml^{-1}) could differentiate between resistant and susceptible cultivars more efficiently, and 10^6 conidia ml^{-1} was used in all subsequent inoculations.

Evaluation of bioassay established for resistance screening

Twenty-four days after inoculation with *Foc* tropical race 4 at 10^6 conidia ml^{-1}, the plantlets of susceptible cultivars 'Brazil Xiangjiao' and 'Guangfen No.1' had higher final disease severities than that of resistant cultivars 'Formosana', 'Nongke No.1', GCTCV-119, and 'Dongguan Dajiao' (Table 1, Table 2). Compared with 'Guangfen No.1', 'Brazil Xiangjiao' is highly susceptible to tropical race 4 and its mean final disease severity was the highest (5.27) recorded in this trial. The plantlets of moderately resistant cultivar 'Formosana' had a mean final disease severity (3.53), which was lower than that of 'Guangfen No.1' (4.33) but higher than the three highly resistant cultivars 'Nongke No.1', GCTCV-119 and 'Dongguan Dajiao' (1.87, 1.73, and1.53, respectively). Moreover, the final disease severity of cultivars tested was also consistent with field incidence of Fusarium wilt

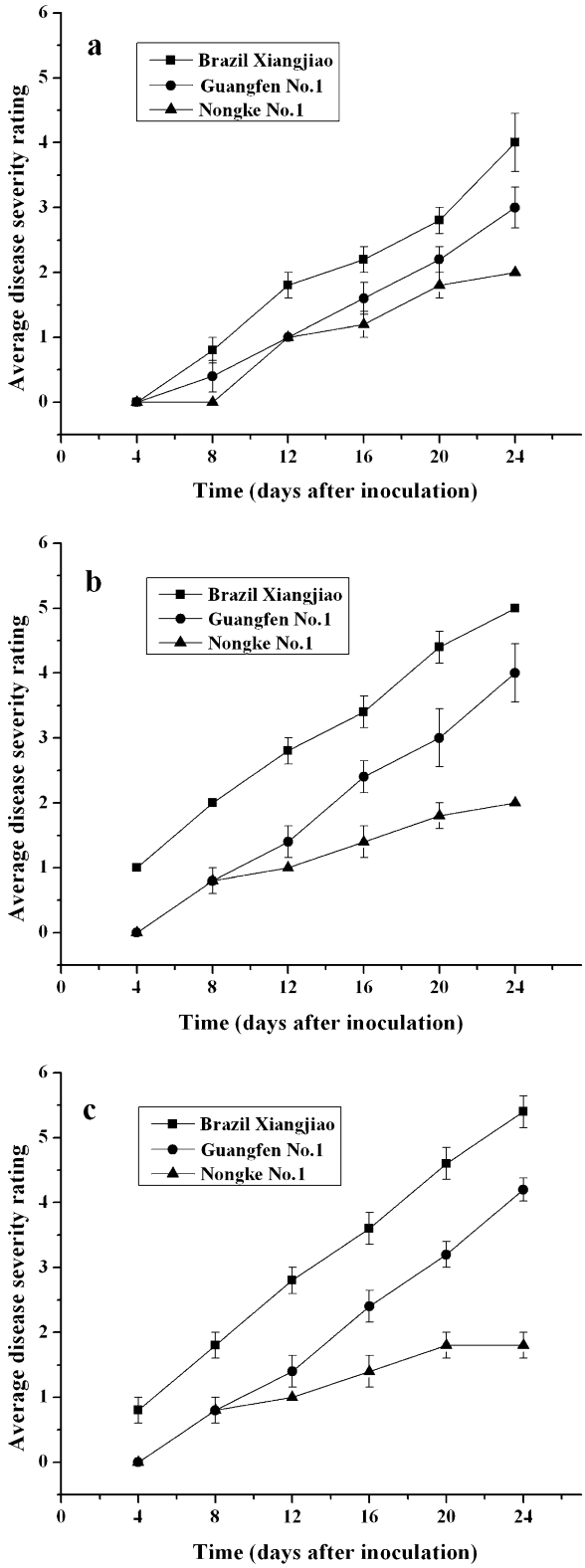

Fig. 2 Temporal development of Fusarium wilt on 'Brazil Xiangjiao' (*Musa* AAA Cavendish subgroup), 'Nongke No.1' (*Musa* AAA Cavendish subgroup), and 'Guangfen No.1' (*Musa* ABB group) plantlets inoculated with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 at 10^4 (**a**), 10^5 (**b**), and 10^6 (**c**) conidia ml^{-1}, respectively. For each cultivar, each data point represents the mean observed disease severity value, and the *vertical bars* represent the standard error of the mean

reported previously by Huang et al. (2005) and Liu et al. (2007) when inoculated with a spore suspension of *Foc* tropical race 4 at 10^6 conidia ml^{-1} (Table 2).

Discussion

To our knowledge, no studies had been conducted to evaluate an *in vitro* bioassay for screening of *Musa* cultivars for resistance to Fusarium wilt prior to this report. Based on these results, a bioassay that is fast, space-effective and accurate can be a new tool for high-throughput screening of *Musa* spp. against the disease.

One critical aspect of this system is *in vitro* inoculation of plantlets on modified medium without

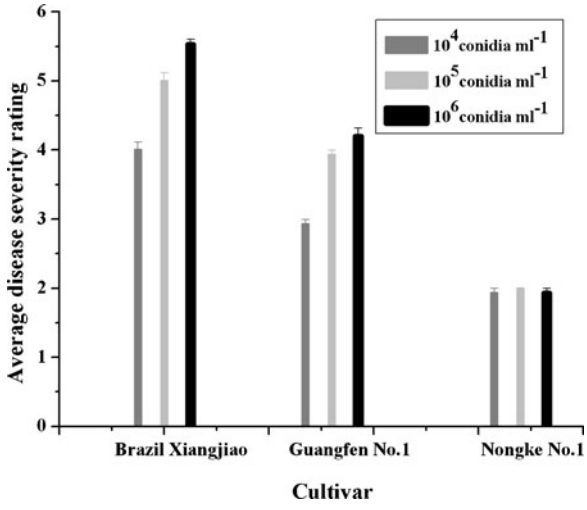

Fig. 3 Effect of inoculum concentration on the final disease severity of 'Brazil Xiangjiao' (*Musa* AAA Cavendish subgroup), 'Nongke No.1' (*Musa* AAA Cavendish subgroup) and 'Guangfen No.1' (*Musa* ABB group) plantlets 24 days after inoculation with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 at 10^4, 10^5, and 10^6 conidia ml^{-1}, respectively. For each cultivar, each *vertical bar* represents the standard error of the mean

Table 2 *Musa* cultivars tested: field incidence of Fusarium wilt and final disease severity assessed on *in vitro* plantlets 24 days after inoculation with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 at 10^6 conidia ml^{-1}

Cultivars	Field incidence of Fusarium wilt (%)	Reference	Average disease rating of plantlets [z]
Brazil Xiangjiao (*Musa* AAA Cavendish subgroup)	60–100	(Huang et al. 2005)	5.27 a
GCTCV-119 (*Musa* AAA Cavendish subgroup)	9.1	(Huang et al. 2005)	1.73 d
Formosana (*Musa* AAA Cavendish subgroup)	35.3	(Huang et al. 2005)	3.53 c
Nongke No.1 (*Musa* AAA Cavendish subgroup)	5–10	(Liu et al. 2007)	1.87 d
Guangfen No.1 (*Musa* ABB group)	–	–	4.33 b
Dongguan Dajiao (*Musa* ABB group)	0	(Huang et al. 2005)	1.53 d

[z] Mean values followed by the same letter are not significantly different ($P \leq 0.05$) by Student-Newman-Keuls test

a carbon source. Media commonly used for the isolation, growth and sporulation of *Foc*, such as potato dextrose agar, potato sucrose agar and Armstrong medium, are carbohydrate-rich; and carbon has been shown to be the first limiting substrate of *Foc* growth in sterilized soil (Couteaudier and Alabouvette 1990). Therefore, growth of *Foc* can be limited by removing sucrose from MS. In addition, considering that rooted plantlets with leaves were able to photosynthesize, the use of MIS in this bioassay can guarantee the normal growth of plantlets (Fig. 1a). In this bioassay, disease development on *in vitro* inoculated plantlets was not equal to the classical and conspicuous external symptoms of Fusarium wilt observed in the field environment, but yellowing and wilting progressed similarly from the oldest to the youngest leaves. Using a scale of 0 to 6 for disease severity measurement, the mean final disease severities of *in vitro* inoculated plantlets of six cultivars expressing different levels of disease reaction were significantly different ($P \leq 0.05$). Of course, genotypes identified as resistant based on the procedure described in this paper have to be evaluated under field conditions as the final confirmatory test.

Compared with the pot and hydroponic systems, the bioassay described in this study is a totally closed system; moreover, conditions of culture growing area, especially temperature, are stable. *In vitro* inoculated plantlets were maintained at 25°C, the optimal temperature for growth of *Foc* (Groenewald et al. 2006). In previous reports on pot and hydroponic systems of screening for Fusarium wilt resistance, the exact temperature range of the greenhouse is not indicated (Matsumoto et al. 1995; De Ascensao and Dubery 2000; Groenewald et al. 2006; Subramaniam et al. 2006; Weber et al. 2007; Smith et al. 2008) or varies from 18 to 25°C (Van den Berg et al. 2007). Since temperature is important in progress of *Foc* invasion and symptom development in banana (Beckman and Halmos 1962), the *in vitro* bioassay is favourable for consistent infection by *Foc*. Additionally, the procedure described in this paper allows a fast resistance diagnosis of 3 to 4 weeks after inoculation with *Foc* tropical race 4 at 10^6 conidia ml^{-1}.

At present, attempts at developing new banana genotypes resistant to Fusarium wilt using conventional breeding techniques face significant hurdles mainly because most cultivars of *Musa* AAA Cavendish subgroup are totally sterile and seedless. Thus, non-conventional approaches such as *in vitro* selection (Bhagwat and Duncan 1998a, 1998b; Matsumoto et al. 1995, 1999), genetic transformation, and protoplast fusion (Matsumoto et al. 2002) receive more attention and several resistant clones have been acquired through somaclonal variation (Hwang and Ko 2004; Liu et al. 2007). Promising resistant clones acquired through non-conventional breeding techniques could be screened using the *in vitro* bioassay directly. Since there is no acclimatization stage for plantlets used in the bioassay, it helps to improve banana breeding efficiency. Considering banana germplasm is often exchanged or released as *in vitro* conserved plantlets, the bioassay would also have application in assessment of newly introduced accessions. Similar early evaluation methods have been developed for rapid screening of *Musa* species for resistance to black leaf streak (Twizeyimana et al. 2007) and *Xanthomonas* wilt (Tripathi et al. 2008), respectively.

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