Single Gene Resistance to *Fusarium oxysporum* f. sp. *cubense* Race 4 in the Wild Banana *Musa acuminata* subsp. *malaccensis*

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Abstract

Fusarium wilt of banana, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most destructive diseases of banana. A particularly virulent strain of the pathogen, tropical race 4 (TR4), presents an emerging threat to banana producing regions throughout the world. No commercially acceptable banana cultivar is resistant to TR4 and, as with all strains of the Fusarium wilt pathogen, there is no effective chemical control. Genetic resistance to TR4 has been observed in the diploid wild banana *Musa acuminata* subsp. *malaccensis,* which has consequently received attention as a potential source of Fusarium resistance genes. The aim of this research was to determine the pattern of inheritance of the resistance trait by screening plants for resistance to Foc subtropical race 4 (SR4) and TR4. Our results showed that the F1 progeny of self-fertilized *malaccensis* plants challenged in pot trials against SR4 (VCGs 0120, 0129, 01211) and TR4 (VCG 01213/16) segregated for resistance according to a Mendelian ratio of 3:1 which is consistent with a single dominant gene hypothesis.

INTRODUCTION

Fusarium wilt of banana, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most destructive diseases of banana. The initial external symptoms of Fusarium wilt are pale green streaks on the base of the petiole and a red-brown discoloration of the xylem vessels below the epidermis of the petiole (Stover, 1962). This is frequently accompanied by a longitudinal splitting of the lower part of the pseudostem just above the level of the soil. The disease is characterized by the premature marginal chlorosis of the oldest leaves which advances towards the midrib. Necrotic patches develop along the leaf margins, followed by the wilt and collapse of the leaves at the base of the petiole (Robinson, 1996). As the disease advances, progressively younger leaves are affected until the entire canopy is desiccated. By this stage, faint brown longitudinal streaking is usually observable under the epidermis of the sheathing leaf bases that form the pseudostem (Stover, 1962; Ploetz, 1994). The first internal symptom is a red-brown discoloration of the feeder roots. Subsequently, discoloration of the xylem tissue becomes evident in the rhizome and is most pronounced where the stele joins the cortex (Stover, 1962). The red-brown discoloration of the xylem vessels advances into the pseudostem which, when cut in longitudinal or transverse section, presents highly

diagnostic symptoms (Stover, 1962). Ultimately, the disease results in the death of the plant, typically soon after flowering (Moore et al., 1995; Robinson, 1996).

Currently, the various Foc pathotypes are crudely grouped into four 'races' based on the host cultivar affected. Race 1 causes disease in 'Gros Michel' (AAA) and cultivars with the AAB genome including 'Lady Finger', 'Silk', and 'Apple'; race 2 affects race 1 susceptible cultivars plus 'Bluggoe' (ABB) and closely related (ABB genome) cooking bananas; race 3 is no longer considered part of the Foc race structure as it is pathogenic to *Heliconia* species, not banana; and race 4 affects race 1 and 2 susceptible cultivars plus Cavendish. Race 4 is further divided into 'tropical race 4' (TR4) and 'subtropical race 4' (SR4) with TR4 isolates causing disease on Cavendish in both tropical and subtropical conditions, and SR4 isolates causing disease on Cavendish only in subtropical conditions (Ploetz and Pegg, 2000; Stover and Buddenhagen, 1986; Su et al., 1986; Waite, 1963). TR4 is considered a serious threat to banana producing regions throughout the world as there are currently no commercially acceptable TR4-resistant replacements for the Cavendish cultivar and, as with all strains of the Fusarium wilt pathogen, there is no effective chemical control.

It is widely agreed that the most promising disease management strategy involves the introduction of genetic resistance into commercial cultivars through conventional breeding or genetic transformation. The genetic bases of resistance to Fusarium wilt, particularly to race 1 in banana has been investigated amongst diploid banana lines (Sasali et al. 2013). In this current study in order to find a source of genetic resistance, we have looked to wild banana progenitors located near the pathosystem's centre of origin in South East Asia (Vakili, 1965; Ploetz and Pegg, 1997). As a result of this exploration, a small number of wild *Musa acuminata* subsp. *malaccensis* banana plants were discovered in Sumatra with putative resistance to TR4 (I.W. Buddenhagen, pers. commun.). The aim of this research was to determine the pattern of inheritance of the resistance trait by screening a segregating population of *M. acuminata* subsp. *malaccensis* for resistance to SR4 and TR4.

MATERIALS AND METHODS

Plant Material

Three *M. acuminata* subsp. *malaccensis* plants (accessions D, E, and F) with putative resistance to Foc race 4 and three putatively susceptible plants (accessions A, B, and C) were multiplied up in tissue culture to generate multiple clones of each. A selection of these plants, A (susceptible), D (resistant), E (resistant), and F (resistant), were planted in the field and self-pollinated. The resulting seeds were embryo rescued and each plant multiplied up in tissue culture in order to generate sufficient replicates for screening purposes. A total of 13 replicates of each plant were generated; three for screening against each of the three SR4 Foc isolates and four replicates for screening against TR4.

Growth Conditions

Screening against SR4 was conducted in subtropical conditions in South East Queensland, Australia (The University of Queensland glasshouse facility in Brisbane) and against TR4 in tropical conditions near Darwin in the Northern Territory of Australia (Berrimah Farm TR4 quarantine research facility). Tissue cultured plants were deflasked according to Daniells and Smith (1991) and planted into seedling trays containing steam-

sterilized University of California mix (Baker, 1957). For the initial three weeks, the plants were hardened-off under clear plastic covers either in a glasshouse covered by 70% shade-cloth or, for the TR4 trial, in a shade house. The plants were watered daily and misted twice daily to maintain high levels of humidity. After the hardening-off period, the plants were transplanted into 140 mm ANOVA pots containing steam-sterilized UC mix. Plants were arranged in a randomized block to reduce positional effects from sunlight, temperature, and humidity and were watered as needed for the duration of the experiment.

Fusarium Isolates

One TR4 and three SR4 isolates were used in this experiment to screen for resistance (Table 1). Monoconidial filter paper cultures of each isolate, which had been stored at -80°C, were recovered onto one-quarter-strength potato dextrose agar amended with streptomycin (PDA/S) and incubated at 27°C until fungal growth was observed. The isolates were then subcultured onto carnation leaf agar amended with streptomycin (CLA/S) and maintained in the dark at room temperature (Burgess and Liddell, 1983).

Preparation of Inoculum

Inoculum was prepared according to Smith et al. (2008) with modifications summarized below. Japanese millet (*Echinochloa esculenta*) was rinsed in tap water and soaked overnight in distilled water. After soaking, the water was poured off through a sieve and the millet was rinsed again in distilled water to remove leached carbohydrate. Plastic tubs (500 mL) containing approximately 100 g of soaked millet were autoclaved for 20 min at 121°C at a pressure of 120 kPa. The millet was then rinsed in distilled water, strained and autoclaved again for 20 min. After the millet cooled to room temperature, each tub was inoculated with mycelial blocks from one of the Foc isolates. Millet tubs were then stored in the dark at room temperature for 14 days. Tubs were gently shaken daily to distribute the Foc evenly. Millet for negative controls was prepared as described above, but was not inoculated with Foc.

Inoculation of Plants

The inoculation of plants was performed at 22 weeks after deflasking. A 2 cm layer of UC mix was added to 200 mm ANOVA pots. Thirty grams of Foc-colonized millet was then added to each pot followed by an additional 2 cm layer of UC mix before repotting the plants. Plants were inoculated in a randomized order with the exception of negative controls which were processed first in order to avoid cross contamination.

Assessment of Symptoms

At 22 weeks after inoculation, disease symptoms were rated by assessing the yellowing of foliage, pseudostem splitting, changes in new leaves, and petiole collapse according to the International Network for the Improvement of Banana and Plantain (INIBAP) guidelines (Orjeda, 1998). Transverse sections of the rhizome were made and vascular discoloration was assessed on a scale of 1-6 as follows: 1) no discoloration, 2) isolated points of discoloration, 3) less than one-third discoloration, 4) between one-third and two-thirds discoloration, 5) more than two-thirds discoloration, and 6) complete discoloration.

Re-isolation of Pathogen

Samples of root, rhizome and pseudostem tissue were harvested from each accession. Samples were surface sterilized in 10% bleach and plated onto one-quarter-strength PDA/S. Where *Fusarium* was isolated, it was identified by PCR using the techniques described by Fraser-Smith et al. (2014) that target the *SIX8* gene to distinguish between SR4 and TR4.

RESULTS AND DISCUSSION

At 22 weeks after inoculation assessment of the parent plants A, B, and C showed that they were susceptible to all four Foc isolates tested (VCG 0120, 0129, 01211, and 01213/16), whereas the parent plants D, E, and F were resistant. Susceptibility in these plants is supported by the development of highly characteristic external and internal symptoms of Fusarium wilt in the susceptible plants and the subsequent re-isolation of Foc from the symptomatic root, rhizome, and vascular tissues of the pseudostem of these plants. The observed symptoms were highly characteristic of Fusarium wilt and included: longitudinal splitting of the base of the pseudostem above the soil line; extensive marginal chlorosis and necrosis of the leaves progressively advancing towards the midrib and increasing in severity in older leaves; collapse of leaves at the petiole base; extensive desiccation of the canopy; discoloration of the internal tissues of the rhizome; reddish-brown discoloration of the vascular tissue of some roots; and reddish-brown discoloration of the vascular tissue of the pseudostem.

Resistance in D, E, and F is supported by the absence of symptoms in those plants 22 weeks after inoculation. Plants exhibited uniformly green leaves with no marginal chlorosis, except for the normal senescence of the oldest leaves. Pseudostems exhibited no splitting and, upon internal examination, the vascular tissue of the pseudostem and the internal tissues of the rhizome were white to slightly off-white in color. The only exception to this was in some resistant plants inoculated with TR4 which exhibited minor brown flecking in the rhizome. However, intensive sampling of this tissue revealed that *Fusarium* was not present in this tissue and thus the flecking may be attributable to a host defense response. Interestingly, some regions of tan to purplish-brown discoloration of adventitious and lateral roots were observed in resistant plants and Foc was subsequently recovered from these roots, demonstrating that the pathogen is capable of gaining entry into the roots of resistant plants. This finding is consistent with infection process studies which showed GFP-transformed Foc (VCG 0120) in the root cortex of the resistant plants (Fraser-Smith, pers. commun.). It can thus be inferred that pre-colonization host-pathogen signaling mechanisms and defenses at the level of the root epidermis are not of primary importance in the determination of resistance in this population of *M. acuminata* subsp. *malaccensis*. In light of the finding that Foc was isolated from the rhizome and pseudostem of all susceptible plants sampled, and that Foc was never isolated from the rhizome or pseudostem of resistant plants, it appears that the pathogen is able to gain access to the root, but is not invading the rhizome in the resistant plants. It may therefore be inferred that the primary defense mechanism has the effect of excluding Foc from the rhizome in these resistant lines, but not the roots. This may be occurring at any level between the hypodermis and the rhizome including: the endodermis, parenchyma of the stele, or xylem.

Three or four replicates of each plant were screened against each Foc SR4 isolate and TR4 isolate respectively and all replicates exhibited consistent results. When screened against VCG 0120, the progeny of the resistant parent F were all resistant, whereas the

progeny of the resistant parent E segregated for resistance according to a ratio of 3.375 : 1 (R : S). The progeny of the resistant parent D segregated for resistance according to a 3 : 1 ratio of (R : S). The progeny of the susceptible parent A were all susceptible (Table 2). The results are consistent with a Mendelian single dominant gene hypothesis.

When an overlapping set of progeny was screened against VCGs 0129, 01211, and 01213/16, the same pattern of resistance that was seen for VCG 0120 was observed. The progeny of the resistant parent F were all resistant, whereas the progeny of the resistant parent E segregated for resistance according to a ratio of 4.67 : 1 (R : S). The progeny of the resistant parent D segregated for resistance according to a 4 : 1 ratio of (R : S). The progeny of the susceptible parent A were all susceptible (Table 3). Three or four replicates of each plant were screened against each of the SR4 and TR4 isolates respectively, and all replicates exhibited consistent results with the exception of a small number of plants screened against TR4. In four of 34 putatively resistant accessions, one of the four replicate clones exhibited symptoms of Fusarium wilt. The cause of this is not known and further tests will be conducted to confirm the results. Of the plants inoculated with sterile millet as a control, none developed symptoms.

All plants, both resistant and susceptible, that were inoculated with Foc did however exhibit a brown to black non-necrotic discoloration of the margins of the older leaves. Although superficially similar to Fusarium wilt symptoms, this reaction was distinguishable from Fusarium wilt symptomology by its earlier, more rapid onset and its more limited distribution over the leaf. The discoloration developed between 4 and 10 days post inoculation after which time it progressed no further. This discoloration was predominantly limited to within 15 mm of the leaf margins and was relatively uniform in width. Unlike typical Fusarium wilt symptoms, it was not preceded by chlorosis and was only associated with chlorosis in susceptible plants after Fusarium wilt symptoms developed later. Additionally, this discoloration was distinguishable from the marginal necrosis of Fusarium wilt by the absence of desiccation in the discolored leaf tissue. The discoloration appeared on all leaves simultaneously, but did not subsequently develop on leaves which emerged after inoculation. The dark brown discoloration of the margins of the leaves of all plants inoculated with Foc appears to be a response to either the pathogen itself or a product of the pathogen's metabolism present in the millet inoculum. The absence of the response in controls inoculated with sterile millet indicates that the reaction is not caused by the millet alone. If the response is a reaction to the live pathogen, it is possible that it is either the result of damage caused to the roots by the pathogen, or a consequence of a defense response by the host.

ACKNOWLEDGEMENTS

Literature Cited

Baker, K. 1957. The U.C. system for producing healthy container-grown plants. California Agricultural Experiment Station Manual 40: 155-160.

Burgess, L.W. and Liddell, C.M. 1983. Laboratory Manual for Fusarium Research. Sydney: Department of Plant Pathology and Agricultural Entomology, University of Sydney.

Daniells, J. and Smith, M. 1991. Post-flask management of tissue-cultured bananas, p. 1-8. Canberra: Australian Centre for International Agricultural Research. ACIAR Technical Reports No. 18. Canberra.

Fraser-Smith, S., Czislowski, E., Meldrum, R.A., Zander, M., O'Neill, W., Balali, G.R. and Aitken, E.A.B. 2014. Sequence variation in the putative effector gene *SIX8* facilitates molecular differentiation of *Fusarium oxysporum* f. sp. *cubense*. Plant Pathol. 63:1044-1052.

Moore, N.Y., Bentley, S., Pegg, K.G., and Jones, D.R. 1995. Fusarium wilt of banana. *Musa* Disease Fact Sheet No. 5. INIBAP, Montpellier. http://www.musalit.org/viewPdf.php?file=IN960048_eng.pdf&id=13798

Orjeda, G. 1998. Evaluation of *Musa* germplasm for resistance to Sigatoka diseases and Fusarium wilt. INIBAP Technical Guidelines No. 3. INIBAP, Montpellier. http://www.musalit.org/viewPdf.php?file=UP990670_eng.pdf&id=14000

Orjeda G. 1998. Evaluation of Musa germplasm for resistance to Sigatoka diseases and Fusarium wilt. INIBAP Technical Guidelines 3. International Plant Genetic Resources Institute, Rome, Italy; International Network for the Improvement of Banana and Plantain, Montpellier, France; ACP-EU Technical Centre for Agricultural and Rural Cooperation, Wageningen, The Netherlands.

Ploetz, R.C. 1994. Panama disease: return of the first banana menace. Int. J. Pest Manage 40:326-336.

Ploetz, R.C., and Pegg, K.G. 1997. Fusarium wilt of banana and Wallace's line: Was the disease originally restricted to his Indo-Malayan region? Australas. Plant Pathol. 26:239-249.

Ploetz, R.C. and Pegg, K.G. 2000. Fusarium wilt. p. 143-159 In: D.R. Jones (ed.), Diseases of Banana, Abaca and Enset. CABI Publishing, Wallingford.

Robinson, J.C. 1996. Bananas and plantains. CAB International, Wallingford.

Ssali, R.T., Kiggundu, A., Lorenzen, J., Karamura, E., Tushemereirwe, W. and Viljoen, A. 2013. Inheritance of resistance to *Fusarium oxysporum* f.sp. *cubense* race 1 in bananas. Euphytica 194:425-430.

Smith, L.J., Smith, M.K., Tree, D., O'Keefe, D. and Galea, V.J. 2008. Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by *Fusarium oxysporum* f.sp. *cubense*. Australas. Plant Pathol. 37:171-179.

Stover, R.H. 1962. Fusarial Wilt (Panama Disease) of Bananas and Other *Musa* Species. The Commonwealth Mycological Institute, Key, Surrey.

Stover, R.H. and Buddenhagen, I.W. 1986. Banana breeding – polyploidy, disease resistance and productivity. Fruits 41:175–91.

Su, H.J., Hwang, S.C., and Ko, W.H., 1986. Fusarial wilt of Cavendish bananas in Taiwan. Plant Dis. 70:814–8.

Vakili, N.G. 1965. Fusarial wilt resistance in seedlings and mature plants of *Musa* species. Phytopathology 55:135-140.

Waite, B.H. 1963. Wilt of *Heliconia* spp. caused by *Fusarium oxysporum* f. sp. *cubense* race 3. Trop. Agr. 40:299–305.

Tables

Table 1. *Fusarium oxysporum* f. sp. *cubense* isolates used to screen *Musa acuminata* subsp. *malaccensis* plants for resistance to Fusarium wilt.

Accession No.	VCG	Race
22615	0120	SR4
23518	0129	SR4
23707	01211	SR4
19285	01213/16	TR4

Table 2. Resistance of *Musa acuminata* subsp. *malaccensis* progeny to *Fusarium oxysporum* f. sp. *cubense* VCG 0120 (SR4). Assessment was conducted 22 weeks after inoculation with Foc. Three replicates of each F1 plant were screened.

Parent	Resistance or susceptibility of parent (R/S)	No. of asymptomatic F1 plants	No. of symptomatic F1 plants	Ratio of asymptomatic : symptomatic plants
A	S	0	34	0 : 1
D	R	9	3	3 : 1
E	R	27	8	3.375 : 1
F	R	21	0	1 : 0

Table 3. Resistance of *Musa acuminata* subsp. *malaccensis* progeny to *Fusarium oxysporum* f. sp. *cubense* VCG 01213/16 (TR4) and VCGs 0129 and 01211 (SR4). Assessment was conducted 22 weeks after inoculation with Foc. Three replicates of each F1 plant were screened against each VCG strain.

Parent	Resistance or susceptibility of parent (R/S)	No. of asymptomatic F1 plants	No. of symptomatic F1 plants	Ratio of asymptomatic: symptomatic plants
A	S	0	8	0 : 1
D	R	12	3	4 : 1
E	R	14	3	4.67 : 1
F	R	8	0	1 : 0