Continued analyses of the effect of silicon on Fusarium wilt on banana

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The University of Queensland

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BA09057

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This report describes continuation of our studies to determine the effects of silicon amendments on Fusarium wilt in banana and of on-going studies of the host-pathogen interactions of the fungus that causes Fusarium wilt on banana

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Contents

Media Summary

Fusarium wilt causes large losses in banana production worldwide. In Australia Ladyfinger and Ducasse production is affected by race 1 strains of the Fusarium wilt fungus whereas Cavendish production until recently was only affected in south east Queensland and northern NSW where subtropical race 4 of the fungus is present. However in the Northern Territory, Cavendish has succumbed to tropical race 4 of the Fusarium wilt fungus. There is increasing concern that this particularly aggressive strain could devastate Cavendish production in Northern Queensland if accidently introduced to that region.

In our investigations to look at the interaction of the Fusarium wilt fungus with banana and to assess possible control methods, we have examined the potential use of silicon supplements. We previously showed that when silicon is applied either in tissue culture or as a supplement to small pot plants that subsequent Fusarium wilt disease levels were reduced. In this current study we investigated where the silicon was deposited in the plant tissues when added as a supplement. We found that the silicon was taken up and deposited throughout the root tissue but that the highest levels were in the zone known as the inner cortex. It is possibly in this zone where the plant is required to exhibit its greatest defence against the invading Fusarium wilt fungus.

We have also determined that certain pathogenicity genes previously identified in the strains of the Fusarium wilt fungus that attack tomato are also present in strains that attack banana. Significantly these pathogenicity genes are absent in the non-pathogenic strains we assessed providing further evidence that they are important in causing disease. We have also noted a variation in these pathogenicity genes within the strains that attack banana allowing us to distinguishes race 1 and race 4 isolates and further analysis is ongoing that should allow us to distinguish tropical (TR4) and non-tropical (ST4) strains of race 4. This will provide a very useful tool for molecular diagnostics and also lead to a better understanding of the mechanism of pathogenicity of Fusarium wilt which should ultimately allow for better control strategies.

In our tests on the wild banana line Malaccensis that exhibits resistance to Fusarium wilt we have discovered that the resistance is effective against all known pathogenic races of the Fusarium wilt fungus on banana. Work is continuing which will allow us to develop a marker for this resistance which can ultimately be used in a banana breeding programme with the aim of producing commercially acceptable Fusarium wilt resistant banana plants.

Technical Summary

Fusarium wilt caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*), causes large losses in banana production worldwide. In Australia, Ladyfinger and Ducasse production is affected by *Foc* race 1 strains whereas Cavendish production until recently was only affected in south east Queensland and northern NSW where subtropical race 4 of *Foc* (ST4) is present. However in the Northern Territory, Cavendish has succumbed to tropical race 4 of *Foc* (TR4). There is increasing concern that this particularly aggressive strain could devastate Cavendish production in Northern Queensland if accidently introduced to that region.

In our investigations to look at the interaction of the *Foc* with banana and to assess possible control methods, we have examined the potential use of silicon supplements. We previously showed that when silicon is applied either in tissue culture or as a supplement to small pot plants that subsequent Fusarium wilt disease levels were reduced. In this current study we investigated where the silicon was deposited in the plant tissues when added as a supplement. We found that the silicon was taken up and deposited throughout the root tissue but that the highest levels were in the inner cortex. It is possibly in this zone where the plant is required to exhibit its greatest defence against the invading Fusarium wilt fungus.

We have also determined that the ***S**ecreted **i**n **X**ylem* (*SIX*) genes, previously identified in *Fusarium oxysporum* f.sp*. lycopersici,* are also present in *Fusarium oxysporum* f.sp. *cubense.* and most significantly that these pathogenicity genes are absent in the non-pathogenic strains assessed. We have also noted a variation in these *SIX* genes that distinguishes race 1 and race 4 isolates and further analysis is ongoing that should distinguish tropical (TR4) and non-tropical (ST4) strains of race 4. This will provided a very useful tool for molecular diagnostics and also lead to a better understanding of the mechanism of pathogenicity of *Foc* which should ultimately allow for better control strategies.

In our tests on the Fusarium resistant banana diploid line *Musa acuminata* subspecies *Malaccensis* we have discovered that the resistance is effective against all known pathogenic races of *Foc* and is controlled by a single dominant gene. Work is continuing on this to identify molecular markers which will assistant in banana breeding programmes.

Introduction

Fusarium wilt

As reported in HAL final Report BA09057 (*Analyses of the effect of silicon on Fusarium wilt on banana*) Fusarium wilt, caused by the soil borne fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*), is responsible for large losses in banana production worldwide. In Australia, Ladyfinger and Ducasse banana growers are impacted by the effects of race 1 of *Foc*, where the fungus causes vascular wilt leading to death of the plants. Cavendish can also be affected by *Foc* by what is known as subtropical race 4 (ST4) and also tropical race 4 (TR4). *Foc* ST4 occurs in SE Qld and northern NSW where it has for several years caused problems with banana production. Until recently Cavendish plantations in tropical regions have remained free of Fusarium wilt. However since the late 1990s, *Foc* TR4 has been a problem in the Northern Territory where Cavendish plantations have succumbed to large losses. The threat that *Foc* TR4 poses to the Australian banana industry is huge and the importance of keeping it out of the main production area in north Queensland cannot be underestimated. It should be noted that *Foc* TR4 strains are genetically quite distinct from strains described as *Foc* ST4 (Bentley *et al.* 1998; Gerlach *et al.* 2000). Although both can cause disease on Cavendish, *Foc* TR4 is considered to be more aggressive (Buddenhagen 2009). Previous impacts and potential future threats of Fusarium wilt to the worldwide banana industry have been reviewed by Ploetz and Pegg (2000).

Fusarium oxysporum is long-lived in the soil either as spores or as vegetative mycelia (Stover &Waite 1954) making it difficult to eradicate (Pegg *et al.* 1996). Resistance to *Foc* TR4 is present in some wild diploid lines such as *Musa acuminata* subsp. *malaccensis* (Peraza-Echeverria *et al.* 2009) but to date it has not been introduced successfully into a viable commercial banana cultivar. In the meanwhile, alternative control measures including nutrient amendments and application of biological control agents may offer some hope in reducing disease levels and slowing disease spread. Our studies have shown that silicon applications can reduce the levels of Fusarium wilt (Forsyth 2006).

Silicon in plants

Silicon has been associated with increased resistance to both abiotic and biotic stresses in several plant species including resistance to various fungal, insect and bacterial pathogens (Bélanger *et al*. 2003; Diogo & Wydra 2007; Epstein 1994; Fauteux *et al*. 2005; Kvedaras *et al*. 2007). Silicon is thought to either create a physical barrier, preventing fungal penetration, or induce systemic acquired resistance (SAR), and so enhance resistance (Mohandas *et al.* 2004; Schneider & Ullrich 1994).

In previous HAL and ABGC funded projects (BA07012 and BA 09057), pathogenicity tests, plant defence gene expression studies and initial histological observations, all indicated that silicon applications enhanced resistance to Fusarium and that the defence gene up-regulation seemed to only occur in the presence of the fungus.

We are continuing our work with silicon and in this report we describe our research on identifying where silicon is deposited in the plant tissue, more specifically in the root and we discuss how this might lead to enhanced resistance to *Foc.*

Comparative analysis of Fusarium races on banana

As mentioned above, isolates of *Foc* ST4 and *Foc* TR4 are genetically distinct from each other, yet isolates of both of these "race 4" types are capable of attacking Cavendish as well

as Ladyfinger banana plants, whereas isolates of *Foc* race 1 cannot cause disease on Cavendish but will affect Ladyfinger. Recent studies on *Fusarium oxysporum,* and in particular of *Fol* (*Fusarium oxysporum* f.sp*. lycopersici)* the strain that attacks tomato, identified a suite of genes known as *SIX* (**S**ecreted **i**n **X**ylem) genes (Rep *et al.* 2004; Houterman *et al*. 2009). These *SIX* genes have been associated with pathogenicity by *Fusarium oxysporum* in tomato (Rep *et al*. 2004) and in cotton (Chakrabarti *et al*.2011). Our initial studies showed that at least some the *SIX* genes or their homologues, were present in *Foc* and that there were differences between the races of *Foc* with regards to the complement of *SIX* genes. Differences in the *SIX* gene complementation between the *Foc* races offers the possibility of an effective molecular diagnostic tool. Diagnostics in *Fusarium oxysporum* has been notoriously difficult. Most molecular diagnostics are based on phylogenetic analyses reflecting evolutionary distances between strains, races, species etc. In *Fusarium oxysporum* the difficulties in associating races with evolutionary trends may well have been due to the ability of *Fusarium oxysporum* to acquire pathogenicity factors such as the *SIX* genes by horizontal transfer (see Ma *et al.* 2010). If this is the case, then the most effective molecular diagnostic would in fact be the pathogenicity genes themselves.

To further understand the role of *SIX* genes in *Foc*, that is to determine if they are important in pathogenicity and/or if they can be a useful tool for molecular diagnostics, we screened a number of isolates of *Foc* across different VCGs (vegetative compatibility groupings). VCG analysis is a well established method for distinguishing different genetic groupings within *Fusarium oxysporum* and in other fungal species as well (Correll *et al.* 1986). Also to confirm initial PCR results showing the presence of *SIX* genes, we have conducted slot-blot hybridisation analysis using radioactively labelled probes of the *SIX* genes: *SIX 1*, *SIX7* and *SIX8.* This method is robust in determining the presence or absence of specific genes.

Identification and utilisation of genetic resistance to Fusarium wilt in the wild diploid line Malaccensis

Our previous studies showed that single gene resistance to *Foc* putatively existed in a wild diploid line of banana *Musa acuminata* subsp *malaccensis* (BA9057). When tested against an isolate belonging to VCG 0120 (*Foc* ST4) progeny of certain genotypes of this wild diploid banana segregated 3:1 for resistance to susceptibility, indicative of a single dominant gene for resistance. To determine if the resistance was also effective against isolates representing different VCGs, those of *Foc* race 1 (VCG0125), and another VCG of *Foc* ST4 (VCG01221), we have conducted pathogenicity pot trials at The University of Queensland in Brisbane. Trials using an isolate of *Foc* TR4 are on-going and being carried out in Northern Territory courtesy of Northern Territory DPI.

Materials and Methods

Analysis of the mechanism of silicon in reducing Fusarium wilt

Tissue culture banana plants sourced from DEEDI Nambour were treated with monosilicic acid during tissue culture where 0, 5,10, 15 and 20 g L^{-1} amorphous silicon dioxide (SiO_2) powder (Sigma) was added to nutrient medium (Murashige and Skoog, 1962) and adjusted to pH 6. After 4 weeks both lateral and proximal roots were harvested and assessed for silicon content and distribution. Roots were prepared and then examined using Scanning Electron Microscopy Energy Dispersive X-ray analysis (SEM-EDS) to determine location of silicon deposition; for details of methods see Jones *et al*. (2012 in Prep).

Comparative analysis of Fusarium races on banana

Isolates of *Foc* were selected to represent a range of VCGs which includes isolates from *Foc* race 1, *Foc* ST4 and *Foc* TR4 as well as control samples of *Fol* (see Table 1). All isolates were taken from storage and grown on nutrient media in Petri plates. After approximately 10 days, mycelia were scraped from the plates and DNA extracted using DNeasy Plant Maxi 111 Kit (Qiagen, Valencia, USA).

As part of a CRCNPB PhD funded scholarship PCR primers were designed based on sequences of *SIX* genes available on the Genbank database. Sequences of *SIX1*, *SIX7* and *SIX8* genes were used; details of primers are provided in Meldrum *et al.* (2012).

Radioactively labelled probes of the *SIX1*, *SIX7* and *SIX8* genes were prepared by initially amplifying 260, 610, and 250 bp fragments of each gene respectively, verifying by sequencing analysis and then labelled using P^{32}. The *TEF-1a* gene fragment (650bp) was used as a positive control. Probes were then hybridised to nylon membranes (Amersham Hybond-N+) previously slot-blotted and fixed with 5ug of DNA from each of *Foc* isolates. Further details are provided in Meldrum *et al.* (2012).

Identification and utilisation of genetic resistance to Fusarium wilt in the wild diploid line Malaccensis

Tissue-cultured plants of *M. acuminata* subsp. *malaccensis* of known resistance or susceptibility to VCG 0120 (ST4) were deflasked according to Daniells and Smith (1991) and grown in University of California (UC) potting mix (Baker 1957) and hardened off for 3 weeks under high humidity. The plants were then transplanted into 14 cm pots containing steam-sterilised UC mix, and maintained in a temperature-controlled glasshouse.

Based on previous pathogenicity tests with *Foc* ST4 (VCG0120) the Malaccensis line 850 was considered to be homozygous resistant, Malaccensis lines 851 and 852 were considered heterozygous for resistant and 845 was consider homozygous susceptible. Progeny (self fertilised) of all four of these lines were tested against *Foc* isolates from VCG 125 (*Foc* race 1) and VCG 1211 (*Foc* ST4). All progeny lines had been maintained in tissue culture and at 16 weeks after deflasking, the plants were moved to two separate glasshouses at The University of Queensland for inoculation.

Foc isolates BRIP W95-170 (VCG 125, *Foc* race 1) and BRIP 23707 (VCG 1211, *Foc* ST4) were recovered from storage and grown on potato dextrose agar prior to transferring to steam sterilised millet. For each isolate, the plant inoculations were done by covering the bottom of each 25 cm pots with a layer of steam-sterilised UC mix, then spreading a layer (30 g) of the *Foc*-colonised millet evenly on top followed by a 4 cm layer of steam-sterilized UC mix. The whole root ball of the transplanted plant was then placed into the pot and the pot was then filled with more steam-sterilised UC mix.

In order to minimize any risk of cross-contamination between the plants inoculated with the two different isolates, inoculations were performed in separate glasshouses with dedicated watering equipment, disinfectant foot baths and hand disinfectant.

Results

Analysis of the mechanism of silicon in reducing Fusarium wilt

Analysis using SEM-EDS indicated that silicon was detected to varying degrees in all young and old root tissue and that concentrations in mature roots were greater than young roots. It was evident that silicon concentration in root cell walls was greatest in the inner cortex, followed by the epidermal tissue and then the endodermis. Previous studies showed that following application of monosilicic acid, resistance to Fusarium wilt was enhanced (BA09057). Studies are on-going to determine if the accumulation of silicon in the cortex is significant for host defence against Fusarium wilt.

Comparative analysis of Fusarium races on banana

The results of the PCR analyses* and slot-blot hybridisation analysis are shown in Table 1. These results indicate the *SIX1* gene (or homologue) is present in all the *Foc* isolates tested but was absent in the three non-pathogenic *Fusarium oxysporum* isolates tested. Whereas *SIX7* and *SIX8* genes (or homologues) were present only in the race 4 isolates ie both *Foc* ST4 and *Foc* TR4. As expected *SIX1, SIX 7, and SIX8* were present in the *Fol* isolate (positive control) and *Tef1*α was in all isolates. For further details see Meldrum *et al.* (2012).

Table 1. Summary of PCR and slot-blot hybridisation analyses using *SIX* gene primers and radioactively labelled probes respectively, on DNA of representative isolates of a range of VCGs covering different *Foc* races, three isolates of non-pathogenic *Fusarium oxysporum* and one isolate of *Fusarium oxysporum* f.sp *lycopersici* (positive control for *SIX1*, *SIX 7* and *SIX8*). Primers and radioactive labelled probes of the *Tef1* α gene were used as a positive control for the PCR and slot-blot hybridisation analyses respectively.

* The PCR analysis was carried out as part of CRCNPB / NT DPI funded PhD project

			PCR analysis*				Slot-blot analysis			
	VCG	No. isol's	*SIX 1*	*SIX7*	*SIX 8*	*Tef 1* α	*SIX 1*	*SIX7*	*SIX 8*	*Tef 1* α
Foc ST4	0120	2	√	√	√	√	√	√	√	√
Foc race 1	0124	2	√	x	x	√	√	√	√	√
Foc race 1	0124/5	2	√	x	x	√	√	x	x	√
Foc race 1	0125	2	√	x	x	√	√	x	x	√
Foc race 2	0128	2	√	x	x	√	√	x	x	√
Foc ST4	0129	2	√	√	√	√	√	√	√	√
Foc ST4	01211	1	√	√	√	√	√	√	√	√
Foc TR4	01213/16	2	√	√	√	√	√	√	√	√
F.oxysporum non path		3	x	x	x	√	x	x	x	√
Fol		1	√	√	√	√	√	√	√	√

***Identification and utilisation of genetic resistance to Fusarium wilt in the wild* diploid line Malaccensis**

Previous studies showed that when progeny of the Malaccensis lines 851 and 852 were tested with an isolate of VCG120 (ie *Foc* ST4), the progeny segregated in a 3:1 ratio for resistance to susceptible which is indicative of inheritance via a single dominant gene for resistance. Whereas line 850 bred true for resistance indicating that it was homozygous for this resistance and line 845 was susceptible and all progeny were susceptible, indicating homozygous recessive. In this study the progeny of the Malaccensis lines 845, 850, 851 and 852 were tested against a *Foc* race 1 isolate belonging to VCG0125 and a *Foc* ST4 isolate belonging to VCG 1211. The results in Table 2 indicate that the segregation for resistance to susceptibility on the progeny was consistent with previous segregation patterns when these populations were tested with a *Foc* ST4 isolate belonging to VCG0120.

Table 2. Number of plants of *Musa acuminata* subsp. *malaccensis* showing resistance and susceptibility as assessed 16 weeks after inoculation with isolates of VCG 01211 (*Foc* ST4) and VCG 0125 (*Foc* Race 1).

Malaccensis parent line		VCG 01211		VCG 0125	
	Genotype as tested with VCG0120	**Resistant**	**Susceptible**	**Resistant**	**Susceptible**
845	rr	0	9	0	8
850	RR	11	0	9	0
851	Rr	10	3	9	3
852	Rr	5	1	4	1

Discussion

This report covers the work undertaken with the interim funding provided to support ongoing student research on the application of silicon to control Fusarium wilt in banana and in the host-pathogen interaction of *Fusarium oxysporum* on banana in general.

Our results show that silicon when applied as a supplement in tissue culture is taken up and deposited across the root but that it is mostly confined to the inner cortex. Results from this student research will contribute to the ongoing accumulation of data relating to the use of silicon in banana. Work has now started (BA10020 Banana Plant Protection Program) on field trials using silicon treated tissue culture plants to determine if in the field, results reflect what we have observed in pot trials *ie* that silicon application can reduce the levels of Fusarium wilt. If these field trails produce positive results recommendations to apply silicon in tissue culture will be made to industry.

Our results show that the putative pathogenicity genes *SIX1*, *SIX7* and *SIX8* are present in *Foc* and not in non-pathogenic *Fusarium oxysporum.* This provides further support for the findings of Rep *et al.* (2004) and Chakrabarti *et al.* (2011) that these genes are associated with pathogenicity in Fusarium wilt. The presence of *SIX7* and *SIX8* in the race 4 type *Foc* isolates (ie both ST4 and TR4) but not the race 1 type isolates raises several possibilities.

These genes or their homologues offer potential useful sites for molecular diagnostics and on-going work shows the potential for also discriminating between *Foc* ST4 and *Foc* TR4 which would be critical for biosecurity should *Foc* TR4 become more widespread in Australia. An understanding of the function of these genes and assessment of their relevance in pathogenicity on banana would also lead to potential targets for control whether via chemical or genetic means.

The mechanism of resistance in the Malaccensis plants is not known but from these results it appears to be controlled by a single dominant gene which is effective across all known pathogenic races of *Foc ie* both race 1 and race 4 isolates. Intriguingly, the Malaccensis plants that appear to lack this resistance to *Foc* ST4 are also susceptible to *Foc* race 1 isolates. This suggests that there is a common effector across all pathogenic strains of *Foc* which is recognised by the resistance mechanism in Malaccensis. This potential parallels the mechanism of resistance and pathogenicity observed in tomato-*Fol* interactions (Houterman *et al.* 2009). Work is continuing to determine how this resistance mechanism might work and if, and how it may be interacting with the putative pathogenicity genes in *Foc*.

Technology Transfer

This work has been collaboration between The University of Queensland and Queensland and Agri-Science Qld, Dept. of Employment, Economic Development & Innovation, Queensland with interactions with the NT Department of Primary Industries. The research findings will feed directly back to banana tissue culture labs with the aim of producing more resistant banana plants.

Aspects of this work have been recently published in *Australasian Plant Pathology* (Meldrum *et al.* 2012) with another paper soon to be submitted to *Annals of Botany* (Jones et al.).

This project has supported the consumable costs involved in two on-going PhD projects (Kevan Jones, Sam Fraser-Smith) at The University of Queensland and one Honours project (Gloria Goh) as well as interactions with the PhD project of Rachel Meldrum who has been principally supported by the CRC for National Plant Biosecuriy and NT DPI.

As well as the direct research outcomes benefitting the industry, these four young scientists mentioned above are developing their skills in banana pathology as a consequence of this support.

Recommendations

We recommend that the application of silicon should be tested in field trials to determine if application in tissue culture does have a lasting effect in reducing Fusarium wilt in the field. It is anticipated that such trials will be carried out as part of the Banana Plant Protection Programme. If proven to be effective in the field, then tests to determine the best form of silicon use in tissue culture should be carried out.

Research should continue to determine if the *SIX* genes are important in banana –Fusarium interactions. Their potential for targets of molecular diagnostics to distinguish between *Foc* strains should and will be investigated further as part of the Banana Plant Protection Programme.

The Malaccensis line is a valuable resource for resistance to Fusarium wilt, confirmation of this resistance, and with liaison with the international Promusa consortium should allow mapping of this gene which can then be used as a tool in banana breeding programmes in selecting for Fusarium resistant cultivars.

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