Silicon in banana plants: uptake, distribution and interaction with the disease fusarium wilt

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Abstract

Banana cultivation worldwide is under threat from a wide variety of pathogens and negative environmental factors. Most cultivated banana plants are vegetatively propagated, resulting in a dearth of breeding and a genetic bottleneck. This has led to enhanced susceptibility to a number of lethal plant diseases. Novel solutions are being pursued to enhance the innate defences of the banana plant in an effort to combat these diseases.

Of all current banana diseases, Fusarium wilt poses the greatest overall threat. Fusarium wilt, sometimes known as Panama disease, is caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). A complex grouping of polyphyletic fungal strains, collectively referred to as races, is responsible for causing disease in banana. Race 1 of *Foc* caused the collapse of the global ‘Gros Michel’ trade industry in the mid-20th century. The industry recovered by substituting ‘Cavendish’ cultivars for ‘Gros Michel’, but a new race (race 4) is now threatening ‘Cavendish’ production.

Breeding and transgenics programmes for developing *Foc* resistant banana cultivars are in progress, but advancement is slow and durable resistance cannot be guaranteed. In the interim, innovative control strategies for *Foc* are being sought. These strategies involve the development of new cultural controls or soil amendments and are intended to inhibit fungal inoculum in the soil or to upregulate innate plant defences. The research in this thesis focusses on the poorly studied plant nutrient, silicon, and its potential for controlling fusarium wilt of banana.

Silicon is classified as a "quasi-essential" element: not fulfilling the strict requirements for essentiality, but still playing an active role in the plant. Despite its prevalence in the environment and content within plants, it remains one of the least studied and understood plant nutrients. Adding silicon to plants as a fertiliser makes them more tolerant to various biotic (pathogens) and abiotic (environmental) factors. This protective effect is not consistent between species and can vary within species. The mechanism by which silicon enhances innate plant defences is poorly understood. Anecdotal reports of silicon
improving banana tolerance to both pathogens and environmental factors have been reported and subsequently investigated in the scientific literature.

For this thesis, the research objectives were to investigate the location and deposition of silicon within roots and shoots of banana plants; investigate silicon absorption through the roots and uptake dynamics; to determine if silicon enhances tolerance to Foc; observe the beneficial effects of silicon in the tissue culture phase of banana cultivation, and; examine how soil distribution of silicon influences uptake and resistance to Foc. All research took place on the ‘Cavendish’ “Williams” cultivar and subtropical race 4 of Foc as these are most relevant for Australian banana production.

The location of silicon in the roots of banana plants was determined using scanning electron microscopy coupled with energy dispersive X-ray microanalysis. No physical discrete deposits of silicon (aka phytoliths) were detected. Silicon was localised within or on the cell walls of root tissue and followed an increasing concentration gradient from the outer epidermis to the inner endodermis. This form of deposition is unique compared to other reported crops and suggests a potential physiological role for silicon in the roots. Silicon was also detected in the protoplasts of intact cells, raising the possibility of a bioactive role for silicon in planta.

For the shoots, silicon was present in or on the cell walls at low concentrations for most tissues investigated. Discrete deposits were detected in fibrous bundles of the vascular tissue predominantly in the leaf lamina and occasionally in the leaf midrib, petiole and pseudostem. This is consistent with previous reports on phytoliths in banana, and like the roots, suggests a physiological role for silicon. Amorphous silicon dioxide was found to be the most effective at increasing gross silicon content in the plant when uptake was tested. Xylem sap silicon was constant when plants were supplied with monosilicic acid, but varied considerably when plants were supplied with amorphous silicon dioxide.

Silicon treatment was found to enhance banana plant tolerance to the expression of fusarium wilt symptoms. Internal symptoms in 4 month old plants, 14 weeks post inoculation were approximately 30% lower compared to untreated, inoculated controls. Decreasing disease symptoms were correlated with an improved biochemical defence
response in the form of enhanced phenolic production coupled with transmission electron micrographic evidence of electron dense globules associated with an improved plant defence response. Silicon was shown to be neither fungitoxic nor fungistatic, implying silicon treatment either directly or indirectly enhances innate plant defences against Foc.

Split root results indicated that uneven distribution of silicon in the soil has the potential to affect the silicon-mediated defence response. Silicon decreased symptoms of fusarium wilt when evenly available to the plant, but increased symptoms when only available to half the root system at high concentrations. This highlights the complex relationship between the soil, plant nutrition and fungal infection.

Finally, silicon was found to be beneficial in the multiplication and post-rooting phase of tissue culture micropropagation. Adding silicon to the multiplication media caused an increase in up to 50% of the number of explants produced during proliferation. Using silicon when deflasking during the post-rooting phase, plantlets were more tolerant of environmental conditions such as water deficit and slightly more tolerant of Foc.

In conclusion, silicon was found to be a beneficial nutrient in multiple areas of banana cultivation. Based on the evidence, silicon is recommended for use in banana cultivation. Large-scale trials are necessary to determine how well this effect translates to the field. The next avenue of research would be to determine the most cost-effective method for applying cheap, high-purity, plant-available silicon to cultivated banana plants.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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List of Abbreviations

**Cultivar (cv.)** - Cultivated variety

**EDS/EDX** - Energy-dispersive x-ray spectroscopy

**Fo** - *Fusarium oxysporum*
  - **Foc** - *Fusarium oxysporum f. sp. cubense*
  - **Foa** - *Fusarium oxysporum f. sp. albenidis*
  - **Forl** - *Fusarium oxysporum f. sp. radicis-lycopersici*

**f. sp.** - Forma specialis or formae speciales (plural)

**qRT-PCR** - Quantitative real-time polymerase chain reaction

**SAR** – Systemic acquired resistance

**SCS** - Soilless cultivation system

**SEM** - Scanning electron microscopy

**Si** – Silicon (elemental silicon unless otherwise specified)

**TEM** - Transmission electron microscopy

**UHQ water** - Ultrapure water (ultra-high quality)

**VCG** – Vegetative compatibility grouping

**ZAF correction** - $Z =$ atomic number correction; $A =$ absorption correction; $F =$ characteristic fluorescence correction
Chapter 1 - Introduction

Overview
Fusarium wilt of banana (often called Panama disease) is a fungal plant disease causing severe banana crop losses worldwide (Pegg et al., 1996). In most cases, fusarium wilt results in complete loss of a plantation. Fusarium wilt on “Gros Michel” banana plantations was one of the most devastating agricultural diseases of the 20th century (Ploetz, 2006). In banana crops, genetic resistance is the most reliable form of defence against fusarium wilt, but in cases where this is impossible or difficult to achieve, alternative strategies are being investigated.

Genetic resistance to fusarium wilt occurs in wild banana species, but introducing it to cultivated varieties is progressing slowly due to the challenges associated with breeding seedless plants. For these reasons, novel treatment strategies are being investigated for Foc. These alternatives involve cultural techniques or soil amendments, such as the addition of beneficial soil bacteria or improved fertiliser regimes. In most cases this does not render plants immune to fusarium wilt, but anything that ameliorates fusarium wilt symptoms is still advantageous for the grower. Many alternative strategies have been proposed: the focus of this research was soil nutrition, specifically the use of elemental silicon.

Throughout this thesis, the terms ‘resistance’ and ‘tolerance’ will be used in the context of Foc infection on banana. To prevent ambiguity, resistance is henceforth defined as the total absence of disease symptoms on a banana cultivar exposed to Foc. Conversely, tolerance refers to plants expressing disease symptoms when exposed to Foc, but to a lesser extent than under normal susceptible conditions. For a discussion of these terms, see Politowski & Browning (1978).

The banana plant
Banana plants are perennial, parthenocarpic polyploids of the genus Musa (Price, 1995b). The plant itself is large and herbaceous, with a pseudostem composed of tightly packed
leaves arranged in sheaths (Jones, 2000b). Plants grow collectively in clumps, producing bunches of fruit then dying back to be replaced by clonal suckers from the rhizome. Reproduction by seed happens in wild banana plants, but only rarely in cultivated varieties (cultivars). The centre of diversity and origin for most banana species is Southeast Asia (Price, 1995b). Banana plants were important in early agrarian societies, although most likely for plant fibres and not fruit (Lentfer, 2009). Wild plants of the Musaceae family are found ranging from the Pacific to West Africa, concentrated mostly in southeast Asia and New Guinea (Jones, 2000b). New Guinea and the Solomon Islands are considered the epicentre of banana diversity (Jones, 2000b).

Most edible banana cultivars are hybrids of the wild diploid plants Musa acuminata (AA) and Musa balbisiana (BB) (Simmonds, 1962). Banana genetic nomenclature lists the cultivar name plus the origin of each chromosome set, e.g. ‘Cavendish’ (AAA) has three chromosomes from M. acuminata and Ladyfinger (AAB) has two sets of M. acuminata chromosomes and one set from M. balbisiana (Jones, 2000b; Simmonds, 1962). M. acuminata plants evolved in the tropical rainforest regions of Southeast Asia whereas M. balbisiana evolved in monsoonal areas of northern Southeast Asia and southern Asia (Ploetz et al., 2007; Simmonds, 1962). Natural hybridisation most likely occurred when their distributions overlapped (Ploetz et al., 2007; Simmonds, 1962).

While there is a substantial diversity of wild banana species, only a small number of them are cultivated for trade (Price, 1995b). Ploetz et al. (2007) estimates there are between 300 and 1000 edible cultivars grown globally, the majority for domestic consumption. The major banana edible cultivars can be divided into five categories based on their use: 1) dessert ‘Cavendish’ bananas (AAA); 2) dessert bananas (AAB); 3) plantains (AAB); 4) Bluggoe and other cooking bananas (ABB) and 5) East African cooking and beer bananas (AAA) (Buddenhagen, 1990). Botanically, the banana fruit is a berry and is eaten as either a sweet dessert banana and or a starchy plantain (Collins et al., 2004).

The banana fruit is consumed in various ways including fresh, fried, cooked, baked and brewed to make “banana beer”, an economically and socially important beverage in Uganda and other African regions (Gaidashova et al., 2005). Banana fruits are highly nutritious and are valued for their high potassium concentration. The leaves can also be
used in cooking along with the flower hearts (Collins et al., 2004). Banana fruit are one of the most carbon efficient crops, with each kilogram of fruit equating to 480 g of CO\textsubscript{2} equivalent released (Berners-Lee, 2010).

Most banana plants are grown for domestic consumption. This frequently takes the form of smallholder subsistence agriculture, particularly in Africa, where consumption per person can reach up to 200-250 kg a year (Jones, 2000b). Intercropping banana plants is common in Africa and Central America, often with maize (Zea mays), yam (Dioscorea sp.), cassava (Manihot esculenta), rice (Oryza sativa), coffee (Coffea arabica), avocado (Persea Americana), coconut (Cocos nucifera) and sugarcane (Saccharum officinarum) (Lahav, 1995). Principal commercial and subsistence production areas are in Latin-America, the Caribbean, Africa and the Asian-Pacific region (Jones, 2000b).

Banana is a valued perennial crop in tropical regions as the fruit is produced year round (Jones, 2000b). The banana fruit has likely been consumed in South-East Asia for thousands of years. Bananas have been known in the west for hundreds of years, but industrial cultivation for Western consumption only began on a large scale in the 20\textsuperscript{th} century. The first recorded instance of a banana in England was 1633, when an apothecary named Thomas Johnson displayed a bunch of banana fruit in his shop window (Pavord, 2005).

Banana fruits have been a staple trade commodity for the last century. In the 20\textsuperscript{th} century, enormous tracts of land were dedicated to growing bananas for the export trade to Western countries, particularly the United States of America. The United Fruit Company grew vast quantities for trade in South America (Koeppel, 2008). At the time, the dominant cultivar was ‘Gros Michel’, which was replaced by ‘Cavendish’ after most ‘Gros Michel’ plantations succumbed to fusarium wilt.

Banana production in Australia is entirely domestic (Collins et al., 2004) with production as of 2011 at 202 751 tonnes (FAOSTAT, 2013). Approximately 95% of bananas produced in Australia are ‘Cavendish’ (AAA), with the remaining 5% a combination of Ladyfinger (AAB), Goldfinger (AAAB) and various dessert and plantain cultivars (Pegg et al., 1996). Banana plants are susceptible to various diseases, the most significant including: Sigatoka
leaf spots - *Mycosphaerella* sp. (Marín et al., 2003), freckle – *Phyllosticta* sp. (Jones, 2000a), Moko - *Pseudomonas solanacearum* (Fegan and Prior, 2006), banana bunchy top virus (Harish et al., 2009) and fusarium wilt – *Fusarium oxysporum* f. sp. *cubense* (Pegg et al., 1996).

*Fusarium wilt*

Fusarium wilt of banana is a plant disease caused by the soilborne ascomycete fungus *Fusarium oxysporum* Schlechtend: Fr. formae specialis (f.sp.) *cubense* (E.F. Smith) Snyder and Hansen (henceforth: *Foc*) (Snyder and Hansen, 1940). Fusarium wilt is not unique to banana and can be found in a wide variety of agricultural and ornamental plants, including tomato (Lagopodi et al., 2002), flax (Olivain et al., 2003), cotton (Rodríguez-Gálvez and Mendgen, 1995), banana (Pegg et al., 1996), melon (De Cal et al., 2009), and beans (Alves-Santos et al., 2002). In each of these cases, fusarium wilt is caused by a distinct *formae speciales* of *Fusarium oxysporum*.

*Foc* has no recorded sexual stage, although mating type (MAT) genes are present, suggesting the possibility of a cryptic teleomorph (Klein and Correll, 2002; Kerényi et al., 2004). *Foc* can survive as a saprophyte on dead and decaying organic matter and has been reported surviving asymptotically in the living roots of several diverse genera including *Paspalum, Panicum, Ixophorus, Commelina, Chloris, Euphorbia, Tridax and Cyanthilium* (Jeger et al., 1995; Waite and Dunlap, 1953; Hennessy et al., 2005). Non-pathogenic or endophytic strains of *F. oxysporum* (*Fo*) that colonise plants, but cause no symptoms, have also been described (Lievens et al., 2008; Lemanceau and Alabouvette, 1991). Non-pathogenic *Foc* have been isolated from Australian banana fields (Forsyth, 2006).

The fungus *Fo* produces three different spore types: chlamydospores, macroconidia and microconidia (Marois, 1990). Chlamydospores are structurally-reinforced resting spores that can remain dormant in soil for many years, whereupon they will germinate to either infect susceptible plants, or grow saprophytically to produce more resting chlamydospores (Marois, 1990). Microconidia are small spores produced abundantly in culture, and are thought to play a role in the secondary phase of *Fo* pathogenesis, as spores are moved
around the xylem in planta (Marois, 1990). Macroconidia are usually produced on surface lesions and can function similarly to microconidia, or produce chlamydospores (Marois, 1990).

*Fusarium oxysporum* is considered a species complex, as the asexual nature of lineages within the *Fusarium* genus makes species demarcation difficult (Michielse and Rep, 2009). Not all isolates of *Foc* are equally pathogenic on the same plants. Pathogenic isolates are grouped into categories known as “races”, denoting their pathogenicity on specific banana cultivars and related species (Pegg et al., 1996). Four main races of *Foc* are currently described, summarised in Table 1-1:

**Table 1-1.** Race specificity of *Foc* on various banana cultivars and related species. Adapted from (Bentley et al., 1998; Daly and Walduck, 2006; Stover, 1990).

<table>
<thead>
<tr>
<th>Race 1</th>
<th>Race 2</th>
<th>Race 3*</th>
<th>Race 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Affected cultivars</strong></td>
<td>Ladyfinger, ‘Gros Michel’, Silk, Pisang Awak, Silk, Pome</td>
<td>Bluggoe</td>
<td><em>Heliconia</em> sp. ‘Cavendish’, All race 1 affected cultivars</td>
</tr>
</tbody>
</table>

*Race 3 does not cause disease on banana plants and is often excluded from the race classification system.

Race 4 of *Foc* is further divided between sub-tropical race 4 and tropical race 4 (Ploetz, 2006). Race 4 had originally been noted on subtropical plantations, but was detected in the 1990s in tropical plantations. These latter incidences were shown to be caused by unique strains of *Foc* distinct from existing race 4 strains, leading to the new categorisation of tropical race 4. This relatively new race is considered the greatest overall threat to continued ‘Cavendish’ production and has already caused significant losses in Malaysia,
China, the Philippines, Indonesia and in the northern territory of Australia (Ploetz, 2006; Ploetz and Pegg, 2000).

Belonging to a race does not imply genetic relatedness: a race is simply a collection of isolates that are pathogenic on a differential set of cultivars (Bentley et al., 1998). In addition to races, *Foc* can also be described by vegetative compatibility grouping (VCG). A VCG is a distinct population where individual fungi can form heterokaryons with each other, implying they are phylogenetically related (Jeger et al., 1995; Lievens et al., 2008). There are currently 24 described VCGs for *Foc*. In the literature, a combination of race and VCG is often used to prevent confusion.

The taxonomy of *Foc* is complex as pathogenesis on banana plants appears to have evolved independently several times in *Fo* (Fourie et al., 2009). Changes in *Foc* genetics are driven by host/pathogen coevolution, parasexuality, heterokaryosis and sexual recombination (Fourie et al., 2009). Recent research has begun to reclassify *Foc* by the presence and absence of putative pathogenicity genes. These secreted-in-xylem (SIX) genes have been found to describe pathogenicity on cultivars more accurately than race and VCG alone (Meldrum et al., 2012). Emerging research on *Fo* implicates horizontal gene transfer via the movement of lineage-specific genomic regions or 'disposable' chromosomes between different fungal species and even genera (Ma et al., 2010).

Symptoms caused by *Foc* on banana include external wilting, yellowing and necrosis of the oldest leaf margins, splitting of the pseudostem base and discolouration of the vascular tissue (Figure 1-1) and rhizome (Rishbeth, 1955; Daly and Walduck, 2006). The symptoms are a result of *Foc* colonising the vascular tissue, usually referred to the secondary phase of infection. A combination of fungal obstructions combined with induced plant defences, such as tyloses and gums, block the flow of water in the xylem, leading to wilting and eventual plant death (Stover, 1972a). The fungus has been successfully, though not always consistently, isolated from all parts of a diseased plant with the exception of the fruit (Daly and Walduck, 2006).
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Figure 1-1. Transversely cut pseudostem of a mature ‘Ladyfinger’ banana plant colonised by *Foc*. The plant was expressing external symptoms such as leaf yellowing and wilting. The pseudostem was cut in half with a cane knife. Note the reddish-brown discolouration (arrow) of the vascular tissue which is a diagnostic symptom of banana plants with fusarium wilt

The first case of *Foc* causing disease was reported in 1874 in Brisbane, Australia (Pegg et al., 1996). From then onwards, different races of *Foc* have been described in banana growing regions around the world. The largest epiphytotic occurred during the first half of the 20th century, where what was later described as race 1 of *Foc* caused the near complete collapse the then dominant cultivar ‘Gros Michel’ (AAA) (Ploetz and Pegg, 2000).

The banana industry recovered by introducing the resistant cultivar ‘Cavendish’ (AAA) (Jeger et al., 1995) where plantations maintained resistance to *Foc* until the 1990s when they began to succumb to fusarium wilt symptoms (Ploetz, 2006).

*Foc* is challenging to manage once established in the field; fungicide application, flood fallowing, crop rotation and organic amendments are sometimes effective, but rarely
provide long-term control (Pegg et al., 1996). Soil fumigation can provide temporary control, although fungal inoculum often increases to levels greater than pre-fumigation, as *Foc* recolonises the area. *Fo* is a pioneer fungus, and will usually be the first to recolonise soil after it has been sterilised (Marois, 1990). Fungicidal pseudostem injections have given erratic results (Ploetz and Pegg, 2000). Good quarantine practices like rapid destruction of infected plants and strict import quarantine can slow the spread of *Foc* (Hennessy et al., 2005). Laws prohibiting the unauthorised spread of banana material have impeded the spread of *Foc* within Australia (Pegg et al., 1996).

A notable phenomenon in the spread of fusarium wilt is the existence of suppressive soils (Stover, 1990). There are frequent reports of fusarium infested soil in which banana plants grow without any apparent symptoms. This has been reported as far back as 1930 in Central America (Stover, 1990). The specifics of suppressive soil are still not understood, but are thought to be a combination of rhizospheric microbial activity, soil structure, water relations and possibly arthropods (Stover, 1990; Alabouvette et al., 1993; Marois, 1990). Interactions between all these variables inhibits development of disease, despite presence of the pathogen. Recreating suppressive soils with composts, bio-fertilisers and the addition of plant debris have produced promising results (Klein et al., 2013; Shen et al., 2013) but are still not fully sufficient in controlling *Foc*.

The chief cause of inoculum dispersal for *Foc* is the movement of diseased suckers and infested soil. To limit dispersal, current best practice is to produce disease-free banana material through tissue culture. Plants produced in tissue culture are less fit than their field-derived counterparts, experiencing developmental distortions such as: poor cuticle development, reduced photosynthesis and inhibited stomatal development (Nowak, 1998). Tissue culture plantlets experience increased susceptibility to *Foc* for up to 24 months in the field after planting compared with plants propagated vegetatively from the field (Smith et al., 1998). Despite these limitations, tissue culture plantlets are still favoured for initiating plantations as new plants are sterile and free of pathogenic bacteria and fungi.
**Fusarium wilt and banana**

As of writing, the following races are present in Australia: race 1, sub-tropical race 4 and tropical race 4. Of all the races of Foc, tropical race 4 has the greatest potential to affect global banana production (Ploetz, 2006). Currently present only in the Northern Territory of Australia and in parts of Asia, tropical race 4 is expected to spread (Ploetz and Pegg, 2000; Ploetz, 2006). ‘Cavendish’ banana cultivation in Australia will then become unviable.

The emergence of fusarium wilt on banana was the driving force for concentrated breeding programmes for banana (Buddenhagen, 1990). Peraza-Echeverria et al. (2009) identified two putative resistance genes from the wild diploid banana *Musa acuminata* ssp. *malaccensis*, designated RGC2 and RGC5. These genes are being investigated as a means to enhance ‘Cavendish’ resistance to Foc. Breeding into established elite banana cultivars like ‘Cavendish’ is difficult due to the lack of viable seeds (Pillay et al., 2011). New varieties must be generated from progenitor parental banana plants, which is time-consuming, laborious and often results in commercially unacceptable varieties. Genetic resistance is also not necessarily permanent; the possibility exists that other races of Foc would evolve to overcome any novel resistance genes, especially if single gene resistance was deployed.

Apart from resistance genes, novel transgenic approaches are being investigated. Paul et al. (2011) generated transgenic Lady Finger banana plants expressing apoptosis-inhibiting genes which lead to enhanced tolerance to fusarium wilt caused by race 1 of Foc. Apoptosis is a part of programmed cell death that occurs normally in plant growth and development. It has been hypothesised that apoptosis is beneficial to necrotrophs. Like all described Fo, Foc is a hemibiotroph and is capable of engaging in necrotrophy during pathogenesis. Inhibiting apoptosis should therefore negatively interfere with Foc infection. Dickman et al. (2001) demonstrated this with anti-apoptotic animal genes expressed in tobacco, rendering them more resistant to a range of necrotrophic fungi. Transgenic expression of single plant defence, tolerance and resistance genes like this are a promising avenue for engendering banana resistance to Foc.

Switching to a new banana cultivar introduces many technical problems for growers. Banana ripening and processing infrastructure is geared to deal with ‘Cavendish’ and any
change of cultivar will require a significant financial investment to replace ripening and transport facilities (Thompson and Burden, 1995; Pegg et al., 1996). ‘Cavendish’ is especially favoured because it has enhanced growth and vigour compared to other cultivars (Perrier et al., 2009).

For the above reasons, novel methods for inhibiting Foc or promoting banana defences are being sought, so that ‘Cavendish’ production can be sustained for as long as possible. Current endeavours include using endophytic bacteria and non-pathogenic Fo to enhance plant defences (Jie et al., 2009). For example, the fungus Trichoderma harzianum has been used effectively to control fusarium wilt in melons (Bernal-Vicente et al., 2009). Biological control of Foc can take the form of: competition (changing the microbial community to inhibit the growth and activity of Foc), antibiosis (production of fungitoxic compounds by microbes) and parasitism (encourage microbes that attack/inhibit Foc) (Marois, 1990). This can involve colonising plants in tissue culture with beneficial microbes that upregulate the defences of the plant (Ting et al., 2008; Nel et al., 2006).

Mineral nutrients have a long history in modulating the effects of plant pathogens on cultivated crops. The application of silicon, which confers increased disease resistance in some plants, has been proposed as a viable alternative to conventional control techniques. This in turn has led to the proposal of using silicon as a fertiliser (Bond and McAuliffe, 2003). Silicon use may be a valuable tool in controlling fusarium wilt.

The element silicon

Silicon is the second most abundant element in the soil after oxygen. Rarely occurring as a free element, it is mostly found in the soil as silicon dioxide (Richmond and Sussman, 2003). Plants absorb silicon from the soil at differing rates depending on genotype, its concentration in the soil and environmental conditions, resulting in plant dry weights of silicon ranging from 0.1 to 10% (Epstein, 1994). Horsetails (Equisetum hyemale) are the highest described silicon accumulating vascular plants with up to 25% plant weight being silicon (Gierlinger et al., 2008). In rice (Oryza sativa), silicon is present at higher concentrations than the essential macronutrients nitrogen, phosphorus and potassium.
Most other elements are absorbed in ionic form, but silicon, like boron, is noncharged on absorption (Miwa et al., 2009).

There are 17 described essential plant elements: the macronutrients C, H, O, Ca, K, Mg, N, S and P, and the micronutrients, Cl, B, Cu, Fe, Mn, Mo, Ni and Zn (Pilon-Smits et al., 2009). Additionally, there are what are referred to as beneficial elements. These do not fit the definition of essentiality in plants, but are nonetheless capable of promoting growth or enhancing other biotic factors in plants. Five elements are currently considered to impart additional beneficial effects: Al, Co, Na, Se and S (Pilon-Smits et al., 2009). Silicon belongs to a separate classification between essential and beneficial known as quasi-essential. Elemental silicon is a metalloid, a division in the periodic table that includes boron, germanium, arsenic, antimony and tellurium. Metalloids have properties that are a mixture of metals and nonmetals and range from essential in the case of boron, to highly toxic with arsenic (Bienert et al., 2007).

The silicon biogeochemical cycle is a complicated process. It involves the cycle of silicon from the ocean to land and back again, and from living organisms to the soil. Microbial communities are associated with silicon cycling in the soil and the oceans. Bacterial biofilms forming on both amorphous and crystalline silicon formations can greatly accelerate dissolution of silicon into the soil solution (Brehm et al., 2005). Mineral or elemental silicon is not biologically available to plants. Silicon is often present in the soil at high concentrations but often not in plant available form (Richmond and Sussman, 2003). Silicon is absorbed from the soil solution exclusively as silicic acid or Si(OH)₄ (Raven, 1982). The average silicic acid concentration in the soil solution ranges between 0.1 - 0.6 mM (Epstein, 1994).

All plants are divided into three categories based on their capacity for silicon uptake: accumulators, passive accumulators and rejecters (Ma and Takahashi, 2002a). When grown in nutrient solutions containing silicon, plants defined as accumulators will deplete silicon in the nutrient solution, passive accumulators will maintain constant silicon concentration, and rejecters will cause an increase in silicon concentration of the solution as water is absorbed and silicon remains behind (Carnerio et al., 2010). Although defined as rejecters, such plants still absorb silicon in low amounts. Using ²⁸Si-nuclear magnetic
resonance spectroscopy, Mitani et al. (2005a) demonstrated that silicon transported in the xylem of rice was exclusively monosilicic acid. A similar experiment in wheat (Triticum aestivum) achieved the same result, although they also found disilicic acid in the xylem (Casey et al., 2003). Xylem sap concentrations in rice can reach up to 12 times the external concentration of silicon within 30 minutes of being supplied (Mitani et al., 2005a). In wheat it can reach 8 mM (Casey et al., 2003). McCully et al. (2010) question whether the low pH (~4) maintained in the apoplast by proton extrusion may be involved in preventing silicic acid polymerisation.

Absorption of silicon occurs from the soil solution and is transported radially into the root through an energy dependent transport system in the symplast and passively through the apoplast of the cortical cells (Mitani and Ma, 2005). From there, silicon is loaded into the xylem via active transport or passive diffusion and is transported to deposit sites in shoot and sometimes root tissue. This process generally follows the movement of water through plant during transpiration.

Varying rates of silicon uptake in the plant kingdom are due to the functionality and density of silicon transporters in plant roots and shoots (Mitani and Ma, 2005). Silicon uptake is under metabolic control in wheat (Rains et al., 2006) and rice (Ma et al., 2006). Several silicon transporter genes have been described in rice, including LSI1 a silicon influx transporter gene (Mitani et al., 2008), LSI2 a silicon efflux transporter gene (Ma et al., 2007) and LSI6 a shoot distribution transporter gene (Yamaji et al., 2008). LSI6 encodes an aquaporin of the nodulin-26 intrinsic protein II subgroup. It is localised at the xylem parenchyma cells of the leaf and is responsible for unloading silicon from the xylem. Yamaji et al. (2008) demonstrated that the leaf deposition of silicon in rice is mediated by LSI6 in a symplastic fashion. LSI1 and LSI2 have polar localisation, with LSI1 (influx) being distal to the stele, and LSI2 (efflux) being proximal (Mitani et al., 2008; Mitani et al., 2009b). Similar transporter genes have been discovered in barley and maize, and are most likely present in other silicon accumulators (Mitani et al., 2009).

Monosilicic acid undergoes polymerisation to amorphous silicon dioxide (SiO$_2$) once it reaches a concentration of around 2 mM (Mitani et al., 2005a). Polymerised SiO$_2$ comprises the bulk of silicon content in plants and is thought to remain inert, as silicon
dioxide is poorly water soluble (Kim et al., 2002; Dietrich et al., 2003). This stored silicon can be found in many forms. Plants from the Gramineae family store silicon as silica bodies and in specialised silica cells (Dietrich et al., 2003). In rice a silica-cuticle and silica-cellulose double layer in the stem and leaves are observed, as well as deposition occurring in bulliform and dumbbell cells (Mitani et al., 2005a; Bonnett, 1972).

Silicon-mediated defence
The mechanism by which silicon is beneficial for plants is not entirely understood (Ghanmi et al., 2004). Under optimum conditions, silicon does not affect plant growth or development (Henriet et al., 2006; Epstein, 1994). Beneficial effects only manifest under adverse biotic and abiotic conditions (Ma, 2004). This has led to the assignment of quasi-essential status to silicon in the literature (Epstein, 1999). Despite this, silicon has been shown to improve resistance in many plants to various fungal, insect and bacterial pathogens (Table 1-2) (Bélanger et al., 2003; Diogo and Wydra, 2007; Epstein, 1994; Fauteux et al., 2005; Kvedaras et al., 2007; Bekker et al., 2006). The effect on disease resistance is not limited to high silicon-accumulators, and has been described in low accumulators such as pea (Pisum sativum) and Arabidopsis thaliana (Dann and Muir, 2002; Ghanmi et al., 2004).
Table 1-2. Example studies demonstrating the protective effect of silicon against a variety of pathogens.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Pathogen/Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bell pepper</td>
<td>Phytophthora blight&lt;br&gt; <em>Capsicum annuum</em></td>
<td>Phytophthora capsici &lt;br&gt; (French-Monar et al., 2010)</td>
</tr>
<tr>
<td>Tomato</td>
<td>Fusarium crown and root rot&lt;br&gt; <em>Solanum lycopersicum</em></td>
<td>Fusarium oxysporum f. sp.&lt;br&gt; radicis-lycopersici &lt;br&gt; (Huang et al., 2011)</td>
</tr>
<tr>
<td>Wheat</td>
<td>Bacterial leaf streak&lt;br&gt; <em>Triticum aestivum</em></td>
<td>Xanthomonas translucens pv. undulosa &lt;br&gt; (Silva et al., 2010)</td>
</tr>
<tr>
<td>Avocado</td>
<td>Root rot&lt;br&gt; <em>Persea americana</em></td>
<td>Phytophthora cinnamomi &lt;br&gt; (Bekker et al., 2006)</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>Downy mildew&lt;br&gt; <em>Pennisetum glaucum</em></td>
<td>Sclerospora graminicola &lt;br&gt; (Deepak et al., 2008)</td>
</tr>
<tr>
<td>Cucumber</td>
<td>Powdery mildew&lt;br&gt; <em>Cucumis sativus</em></td>
<td>Sphaerotheca fuliginea &lt;br&gt; (Fawe et al., 1998)</td>
</tr>
<tr>
<td>Pea</td>
<td>Fungal leaf spot&lt;br&gt; <em>Pisum sativum</em></td>
<td>Mycosphaerella pinodes &lt;br&gt; (Dann and Muir, 2002)</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Powdery mildew&lt;br&gt; <em>Arabidopsis thaliana</em></td>
<td>Erysiphe cichoracearum &lt;br&gt; (Ghanmi et al., 2004)</td>
</tr>
</tbody>
</table>

Silicon also enhances tolerance to negative abiotic factors. Deposited silicon in various parts of the plant provide reinforced structural support lending greater resistance to physical stresses such as wind, temperature, drought etc (Jones and Milne, 1963). Maintaining an erect status helps to improve light interception and photosynthesis, reducing the impact of mechanical stresses such as wind and decreasing the chance of collapse due to vascular infestation (Takahashi, 1995). Silicon is also reported to reduce the negative effects of salinity, drought and cold, though the mechanisms are not fully understood (Liang et al., 2007). Deposition of silicon within the epidermis prevents...
unnecessary transpiration, thereby decreasing water loss (Mitani et al., 2005). Silicon has also been shown to alleviate the symptoms associated with heavy metal toxicities (Richmond and Sussman, 2003). For example, maize seedlings grown in hydroponics amended with silicon and cadmium showed less symptoms of cadmium induced growth inhibition (Vaculíka et al., 2009).

The accumulation of silicon in plant plays an important role in herbivory defence. Both vertebrate and invertebrate herbivores suffer adverse effects when feeding on silica-rich plants (Massey et al., 2007). Grasses that accumulate silicon are less digestible to insects and amorphous silicon deposits in plant tissue increase mandible wear, thus decreasing insect fitness (Massey and Hartley, 2009; Hunt et al., 2008). Grazing rabbits have been shown to avoid feeding on silicon-treated winter wheat (Cotterill et al., 2007). Keeping and Kvedaras (2008) have also produced evidence that sap-sucking insects such as aphids do not feed as often on silicon treated plants. Massey et al. (2007) demonstrated that silicon uptake can be induced in response to insect herbivory and not with mechanical damage. In their study, grasses subjected to mechanical defoliation showed no increase in foliar silicon, but feeding by voles or locusts caused increases of up to and over 400%. This evidence suggests that silicon is being actively deposited at sites of herbivory to deter herbivores, although whole plant studies of silicon redistribution in this context are lacking.

The existence of silicon deficiency symptoms has been contentious, as it is experimentally difficult to grow plants in environments totally devoid of silicon (Epstein, 1994). Several studies have investigated the gene expression of plants treated with and without silicon under normal conditions. Brunings et al. (2009) studied the gene expression of silicon treated rice using a microarray and found differential regulation of 221 genes compared to the untreated control including some transcription factors. In wheat, Chain et al. (2009) demonstrated a comparable differential response with 47 genes of varying function. Conversely, Fauteux et al. (2006) performed a similar experiment with Arabidopsis thaliana, a dicot, and found only two genes that were differentially expressed. Whether this indicates a fundamental difference between dicots and monocots, or specific to Arabidopsis thaliana, is unknown.
**Silicon in humans and animals**
As with plants, silicon is considered a quasi-essential element in humans, with evidence building for a role in human physiology particularly bone health (Robberecht et al., 2009; Jugdaohsingh et al., 2004). Although a daily intake standard has not been experimentally established, Robberecht et al. (2009) suggests an approximate 10-25 mg/day for the average person. Human dietary silicon needs are met by eating plant material rich in silicon. Silicon can alter the palatability of foods, although most cultivars do not deposit significant silicon in edible portions. There is also limited evidence that silicon rich foods can lead to oesophageal cancers, although the mechanism is not well understood (Parry et al., 1984). In livestock, it is essential for chickens, horses and possibly trout (Jugdaohsingh, 2007). Conversely, high levels of silicon in fodder crops can be detrimental for ruminant animals (Van Soest, 2006).

Silicon is a key element in the oceanic biosystem (Treguer and De La Rocha, 2013). The most significant silicon accumulators in the marine environment are the many species of diatom which absorb silicon to form a silicified outer wall called a frustule (Martin-Jézéquel, 2000). The ancient remains of these frustules constitute diatomaceous earth, a rich terrestrial source of silicon which is increasingly being applied as a fertiliser in agricultural systems. Silicon is important in microbiological systems as well. The ciliophoran protist, *Maryna umbrellata* has been shown to store amorphous silicon dioxide and then excrete it to form a surface silicon cover of its resting cyst (Foissner et al., 2009). It is likely that other microbes, particularly soilborne ones, make use of silicon.

**Possible silicon mechanisms**
The exact mechanism by which silicon acts in plants has not been elucidated, although several theories have been proposed. Currently there is no major consensus, as cause and effect are difficult to establish. For reviews, see (Guntzer et al., 2012; Raven, 1982; Richmond and Sussman, 2003). The earliest and most common theory ascribed to the beneficial effects of silicon within plants was that of the physical barrier.

Several researchers have postulated that a silicon barrier enhances resistance to pathogens by preventing fungal penetration and increasing resistance to enzymatic
degradation (Ma, 2004; Stein et al., 1993). Rice shows increased resistance to rice blast (*Magnaporthe grisea*) when treated with silicon (Datnoff et al., 2001). Kim et al (2002) posit that this enhanced resistance is due to the silicification of the epidermal cells inhibiting pathogen penetration. Experiments by Hayasaka et al. (2008) show that increasing polymerised silicon in rice leaf tissue is directly correlated with decreasing appressorial penetration of the rice blast fungus. Silicon is also deposited in the roots, primarily at the endodermis surrounding the stele, which may provide a physical barrier against vascular pathogens penetrating into the vascular tissue (Lux *et al.*, 2003). Despite the association with fungal penetration sites and silicification, there is little evidence that silicon is actively deposited by the plant in response to pathogen penetration although silicon may be unintentionally deposited by the act of uncontrolled transpiration through sites of penetration (Kunoh and Ishizaki, 1975; Fauteux et al., 2005).

Similar to the physical barrier theory, silicon may act as a substitute for plant-produced defence compounds such as lignin (Hatfield and Fukushima, 2005). Deposition of lignin is often induced in response to pathogen ingress (Fang and Ma, 2006). Lignin is energetically costly to produce whereas deposition of silicon requires only enough energy to transport it from the soil to the point of pathogen penetration. Raven (1982) has calculated the energetic cost of incorporating lignin into cell walls as 27 times that of incorporating silicon. Silicon may also act as a reinforcing agent of lignin. Bélanger *et al.* (2003) speculated that silicon deposition in cell walls is due to the affinity of the element with lignin precursor molecules, and it would therefore accumulate in cell walls during lignin biosynthesis.

The possibility that silicon in the soil solution, or in the plant itself, is directly harmful to fungi has been commonly theorised. At least two studies have been done on the potential antifungal activity of exogenous potassium silicate applied to fungal cultures (Li *et al.*, 2009; Kaiser *et al.*, 2005). Both showed decreased fungal growth and fungal malformation in cultures treated with soluble silicon, but were unable to exclude the role of possible pH effects as soluble silicon solution is highly alkaline (pH > 11).

The beneficial effects of silicon have been linked to the upregulation of defence genes and the production of phytoalexins and other plant based defence compounds (Fawe *et al.*, 2006).
Silicon application may therefore be inducing systemic acquired resistance (SAR), in a method similar to the way plants pre-treated with biological or chemical elicitors show enhanced resistance (Mohandas et al., 2004; Schneider and Ullrich, 1994). SAR is an induced, plant-wide response activated by the detection of biotic elicitors (usually from a pathogen) that causes a generalised defence response. This can include: the hypersensitive response, strengthening of the cell wall, oxidative bursts, expression of defence genes and the accumulation of pathogenesis-related proteins (Conrath et al., 2002; Durrant and Dong, 2004). SAR by definition remains active for a long period, even after the inducer or elicitor has degraded, especially in monocots like banana (Tally et al., 1999).

The SAR pathway is related to specific resistance gene defences within the plant (Hammerschmidt, 1999). SAR is defined as working on a broad range of pathogens, involves the upregulation of pathogenesis-related proteins and is mediated via the salicylic acid pathway. Generally this involves the expression of antibiotic/antifungal proteins and structural reinforcement of tissue to prevent pathogen ingress, usually often by using lignin and callose deposition (Hammerschmidt, 1999). In peas, silicon was shown to enhance the expression of two pathogenesis-related genes, chitinase and β-1,3-glucanase, when exposed to the foliar leaf pathogen* Mycosphaerella pinodes* (Dann and Muir, 2002). When rice plants were challenged with the rice blast fungus, they differentially regulated 60% less genes in plants that had been treated with silicon compared to the untreated silicon control plants (Brunings et al., 2009). This suggests that silicon potentially ameliorates the metabolic stress associated with fungal infection instead of directly inhibiting pathogenesis itself.

The beneficial effects of silicon are not necessarily limited to physical deposition. Isa et al. (2010) show that rice still experiences beneficial physiological effects like enhanced growth even when a silicon uptake mutant (*lsi1*) is grown in place of wild type rice. The mutant still absorbs silicon from the soil solution, but it is not loaded as efficiently to the xylem. Isa et al. (2010) further theorises that silicon concentration in the roots was causing enhanced growth by a theoretical root-to-shoot signalling process. The biochemical role of silicon in defence may require a constant supply of silicon to function (Sun et al., 2010). Halting the supply of silicon in the soil solution tends to stop the prophylactic effect of
silicon, regardless of the presence of polymerised silicon (Fauteux et al., 2005; Datnoff et al., 2001).

Silicon can ameliorate the effects of abiotic factors, such as wind, light and salinity, which in turn leads to enhanced tolerance to pathogens. Greater light interception would mean more resources are available for a plant to contribute to plant defence. This is usually due to silicon improving plant stature and decreasing the effects of mutual leaf shading (Pilon-Smits et al., 2009). Similarly, if silicon is acting as a substitute for lignin, the same freeing of resource may be occurring. A microarray using Arabidopsis thaliana showed inoculation with the powdery mildew fungus (Erysiphe cichoracearum) caused less downregulation of primary metabolism genes in silicon treated plants compared to untreated plants (Fauteux et al., 2006). It is possible that the alleviation of negative abiotic factors frees up plant resources, allowing them to mount a more effective defence against pathogens.

Silicon and banana plants
The banana plant is a silicon accumulator (Henriet et al., 2006). The plants are known to produce phytoliths (discrete silicon deposits) in the leaves and pseudostem, which persist in the soil long after plant death, leading to their use in archaeological studies to track the cultivation and consumption of banana (Lentfer, 2009). When silicon is present in the soil solution at high concentrations, banana plants absorb it passively but switch to active uptake via theoretical transporters when concentrations are low (Henriet et al., 2006). Banana plants have been used as a model to study biogeochemical silicon cycling by comparing $^{28}$Si and $^{29}$Si isotope absorption (Opfergelt et al., 2006).

Silicon is not normally considered agronomically important when investigating and preparing soils for banana cultivation. In a survey of soils used in sugarcane cultivation, Berthelsen et al. (2001) found that 85% of Australian soils in the Atherton Tablelands, Mulgrave, South Johnstone, Babinda, Mourilyan, Mossman and Tully areas contained marginal soil silicon concentrations (defined as 20 mg/kg Si). These areas are coexistent with tropical banana cultivation in Australia, indicating the potential for silicon deficiency in banana farming areas.
Silicon treatments and fertilisers
Like all plant nutrients, silicon must be delivered to the plant when it is optimal for uptake. The simplest method of delivery is soil fertilisation, where silicon fertiliser is incorporated into the soil. The commonest applied silicon is calcium silicate (Rezende et al., 2009; Silva et al., 2010) and potassium silicate (Rezende et al., 2009). Silicon foliar sprays have been investigated and in some cases shown effectiveness in controlling foliar pathogens (Bowen et al., 1992). Fruit sprays have shown effectiveness at preventing post-harvest diseases, although whether this is due to direct fungitoxic/fungistatic effects or through enhancing innate plant defences is unknown (Li et al., 2009; Bi et al., 2006).

A less conventional application method of silicon is injection: using a syringe to deliver silicon directly into the plant. Trunk injection of phosphonic acid is successfully used in the avocado industry to treat Phytophthora root rot (Pegg, 1990). Trunk injection involves drilling a small hole in the trunk of an avocado tree and using a pressurised offset hypodermic syringe to inject the solution. The same method has also been successfully adapted for silicon injection in avocado (Anderson et al., 2005). Unlike avocado, banana plants are herbaceous and lacking in bark, which would make pseudostem injection significantly easier.

Mixing solid silicon into the soil prior to planting and subsequent broadcast fertilisation through the life of the plant would likely be the most cost effective method of silicon application. In Japan, rice farmers make frequent use of silica slag, a cheap by-product of steel production (Ma and Takahashi, 2002b). Plant by-products produced during cultivation and harvest are also a possibility for silicon fertilisation. Examples include: rice hulls, rice straw, sugarcane trash and mill mud (a by-product of sugar production). Berthelsen et al. (2003) evaluated mill ash, mud ash, cement, cement building board, rock dust, thermophosphate, soluble potassium silicate and calcium silicates in a trial for the Australian sugarcane industry. All but rock dust lead to an increase in sugarcane yield. The availability of cheap silicon in Australia needs to be investigated carefully, as does their effectiveness, as some industrial silicon by-products can cause phytotoxicity (Côté-Beaulie et al., 2009).
For liquid applications of silicon to the plant, the only applicable forms are potassium silicate and sodium silicate, commonly known as water glass. Soil drenching is feasible and has been demonstrated in avocado to be effective against *Phytophthora cinnamomi* (Bekker et al., 2006). There are limitations to liquid applications, though. If used with an irrigation system, the possibility of irrigation lines becoming silicified and blocked needs to be investigated and the risk quantified. Liquid silicates are also highly alkaline and may have adverse effects on soil pH.

Prior work and precedent

Previous work at The University of Queensland demonstrated silicon has the capacity to suppress *Foc* in banana (Forsyth, 2006). Banana plants treated with weekly drenches of potassium silicate were more tolerant of fusarium wilt symptoms compared to plants not treated with silicon. The expression of five putative defence genes was studied using quantitative real-time PCR (qRT-PCR). Silicon treatment caused the upregulation of: proteinase inhibitor, phenylalanine ammonia lyase, peroxidase and endochitinase genes in response to inoculation with *Foc* (Forsyth, 2006).

The last two years have seen reports in the literature of silicon augmenting the defence response of banana plants. Fortunato et al. (2012a) et al. showed for the first time that silicon enhances tolerance of Grand Nain and Maçã banana cultivars to *Foc* race 1. Additionally, silicon has also been shown to enhance tolerance to the fungal leaf spot disease Black Sigatoka (*Mycosphaerella fijiensis*). Kablan et al. (2012) demonstrated silicon reduces the disease progress of Black Sigatoka on the ‘Grand Nain AAA ‘Cavendish’” cultivar of banana.

Vermeire et al. (2011) also demonstrated a 50% reduction in visible root necrosis fourteen days after inoculation of banana plants treated and not treated with silicon with *Cylindrocladium spathiphylli*. This reduction was temporary, and disease symptoms appeared to return to standard levels after another seven days. This suggests silicon may also only provide temporary protection or that the effect is mostly cosmetic. This is a limitation with most silicon studies as disease tolerance is difficult to quantify in an agronomic context unless variables such as yield are investigated.
Aim and scope

The overall aims of the research presented in this thesis were to: a) investigate the involvement of silicon in the banana defence response to *Foc* subtropical race 4, b) determine the magnitude of this response, c) uncover mechanisms by which silicon is mediating a defence response *in planta*, d) investigate the use of silicon in tissue culturing of banana plantlets, and e) study how soil distribution of silicon influences uptake and pathogen resistance. In an applied sense, the goal is to determine whether silicon would make an appropriate fertiliser for banana growers needing to combat *Foc*.

This introductory chapter reviewed the literature and summarised the current overall understanding of silicon, banana and *Foc* biology. The following chapters detail five independent studies that comprise original research for this thesis:

- **Chapter 2** explores the basic biology of silicon in the root systems of banana. The location of silicon deposits in root cells was characterised and discussed in a disease/pathogenesis context.

- **Chapter 3** continues the emphasis on fundamental silicon biology, focussing on the shoot and pseudostem tissues. Silicon content in the leaves and pseudostem were investigated and silicon soil uptake dynamics were investigated.

- **Chapter 4** covers the relationship between *Foc* pathogenesis, silicon and banana plants. The infection process of the fungus was characterised through electron microscopy and the effects of silicon were observed.

- **Chapter 5** further explores *Foc* pathogenesis in relation to silicon soil dynamics and determines whether plants require a uniform distribution of silicon in the soil to express silicon-mediated defence.

- **Chapter 6** investigates the potential for using silicon in the three phases of tissue culture: multiplication, rooting and deflasking.
Chapter 7 discusses the previous chapters in a research context with an emphasis on potential future work and applications.
Chapter 2 - Silicon distribution in ‘Cavendish’ banana roots

Abstract:

Background and Aims: Silicon is the least understood of all plant nutrients. Many aspects of silicon bioactivity in planta are unknown or only theorised. In this study, the physical distribution of silicon in banana roots was investigated to assist in further understanding plant/silicon biology. Results are interpreted in a disease resistance context.

Methods: Banana plants of Musa acuminata (AAA group) cv. ‘Cavendish’ “Williams” were grown in 5, 10, 15 or 20 g/kg amorphous silicon dioxide (SiO₂) amended potting mix or tissue culture media. After 4 and 6 weeks respectively, root segments from both systems were harvested and analysed as follows: silicon distribution was mapped using scanning electron microscopy energy dispersive spectroscopy (SEM-EDS) and relative amounts determined using standardless semi-quantitative X-ray analysis. Frozen-hydrated root cells were analysed with cryo-SEM-EDS to investigate soluble silicon.

Key Results: Silicon was detected in all young and mature root tissue and followed a distinct distribution. Semi-quantitative analysis illustrated a trend in cell wall content, with silicon concentration increasing from the epidermis, peaking in the inner cortex then decreasing through the endodermis to the stele. Concentrations in mature roots were comparatively greater than young roots. Silicon was present in frozen-hydrated protoplasts and cell walls. Gross silicon shoot content did not correlate with maximum uptake in roots.

Conclusions: These data highlight the inner cortex as a site for the greatest deposition of silicon in young and old banana roots. Silicon concentration was highest in the oldest roots. The amount of silicon supplied did not correlate with silicon uptake in roots or shoots, suggesting a complex relationship between water uptake, growth media chemistry and active uptake mechanisms. The silicon detected in the protoplast may represent soluble silicon flux through the root or possible cell-silicon homeostasis. The activity of soluble silicon in planta needs to be explored in future work.
Introduction:

The importance of elemental silicon in plant biology has long been recognised, with various studies confirming its role in plant health (Epstein, 2009; Ma, 2004; Epstein, 1999). Silicon addition enhances plant tolerance to diseases and abiotic stresses but maintains a quasi-essential status as a nutrient, as silicon deficiency is not a widely recognised condition (Epstein, 1999; Wiese et al., 2007).

Plants absorb silicon from the soil as silicic acid (Si(OH)$_4$) via two pathways/mechanisms: passively through mass flow or actively via metabolically-driven transporters (Mitani et al., 2008; Raven, 1982). The rate of uptake varies greatly between plant species. Groupings of plants are defined as either silicon accumulators, intermediates or excluders, depending on their rate of uptake (Ma and Takahashi, 2002a).

Silicon transporter proteins have been recently described in rice (Oryza sativa), barley (Hordeum vulgare) and maize (Zea mays) and are likely present but undescribed in many other species (Mitani et al., 2009; Mitani et al., 2008; Yamaji et al., 2008; Ma et al., 2007). In rice, silicon is taken up by LSI1 at the exodermis (root hypodermis with a casparian strip) and then released into the apoplast by LSI2 and subsequently transported to the stele for xylem loading by LSI1 and LSI2 at the endodermis (Mitani et al., 2009a). Conversely, in maize and barley, silicon is transported symplastically to be released to the stele by LSI2 homologues (Mitani et al., 2009a).

Silicic acid is found in both the transpiration stream and in cell protoplasts of plants but is ultimately deposited as polymerised amorphous silica gel (SiO$_2$·nH$_2$O) at terminal transpiration points in the shoot (Bauer et al., 2011). These deposits can occur at three sites: the cell wall, the cell lumen and in the extracellular spaces (Sangster et al., 2001). Total polymerised silicon content of plants can range from 0.1 to 10% dry weight, depending on genotype, soil concentration and environmental conditions (Epstein, 1994).

Polymerised silicon can be found in a wide variety of shoot tissues, often with uneven distribution due to the variable rate of transpiration in plants (Epstein, 1999). The
distribution of deposited silicon is both species and age dependent (Parry and Kelso, 1975). Root silicon deposition is not as well characterised as shoots, with most research on roots undertaken in species from the Poaceae family (Sangster, 1978). The endodermis was identified as the major point of silicon deposition for all plants analysed: either contained within the cell wall or as discrete aggregates extending from the wall (Sangster, 1978). Silicon has also been identified in the protoplast of root cells, presumably in soluble form (Hodson and Sangster, 1989).

There are two possible ways silicon can enhance disease tolerance in planta: physical and biochemical. Polymerised silicon incorporated into cells and cell walls can provide a physical barrier against pathogen penetration in some pathosystems (Bauer et al., 2011; Epstein, 1994). The biochemistry of soluble silicon is less understood, but is capable of inducing the biosynthesis of defence compounds and secondary metabolites (Epstein, 2009). Whether silicon does this by direct elicitation or an involvement in plant signalling is currently unknown.

The banana plant (Musa acuminata x balbisiana) is classified as a silicon accumulator, with silicon present in the leaves, pseudostem and roots (Henriet et al., 2006). Banana cultivars are susceptible to a wide range of pathogens and abiotic factors (Jeger et al., 1995; Stover, 1972a; Wardlaw, 1935; Stover, 1972b; Brake et al., 1995). Because of this, silicon is being investigated for possible use in banana plant protection programmes. Polymerised silicon deposits in the form of phytoliths are common in banana shoot tissue (Tomlinson, 1969) but have not been recorded in roots. Silicon shoot uptake occurs via both mass flow and active uptake, with active uptake dominating when soil silicon concentrations are low (Henriet et al., 2006). How this affects silicon concentration in the roots is unknown.

This study describes the analysis of silicon distribution in banana roots using scanning electron microscopy coupled with energy dispersive spectroscopy (SEM-EDS) and cryo-SEM-EDS (cSEM-EDS). SEM-EDS allows for rapid tissue-specific localisation of silicon deposition and semi-quantitative comparisons of silicon concentrations, although this is limited to tissue that can survive SEM processing (e.g. freeze-drying). Conversely,
processing for cSEM-EDS maintains the cell protoplast *in-situ* and allows elemental analysis of frozen hydrated tissue in natural hydrated distributions.

The objectives of this study were to (1) map the location of silicon in root segments from plants grown in tissue culture and from plants grown in potting mix, (2) identify any conserved regions of silicon deposition between the two, and (3) to correlate the results with bulk silicon concentration in shoot tissue. Both types of root segment were investigated as silicon deposition differs depending on the age of the tissue and the type of system it is grown in (Henriet et al., 2006; Motomura et al., 2006). Results are discussed in the context of silicon biology with a focus on disease management and fusarium wilt control.
Methods & Materials

Plant culture

*Musa acuminata* (AAA group) cv. 'Cavendish' "Williams" banana plants were used for all experiments. Plantlets were taken from stock tissue culture at The University of Queensland, Brisbane, Australia. Growing conditions for tissue culture were: illumination on a day-night cycle of 16 hours light at 5000 lux with approximate day-night temperatures of 26 - 22°C. For glasshouse conditions: plants were grown at facilities located in Brisbane, Queensland, Australia (Latitude: 27°29'45.36"S, longitude: 153° 0'35.81"E) with a mean day-night temperature of 27.8 - 20.5°C during the course of the experiment.

Proximal banana root SEM-EDS

Two regions of the root system were analysed: proximal (root segment 10-15 mm from the root tip) and distal (root tissue 10-15 mm from the corm/rhizome). Proximal roots represent youngest root tissue and distal roots the oldest root tissue. All roots analysed were primary roots; lateral roots were not investigated. To investigate silicon content in proximal roots, an *in-vitro* tissue culture system was devised using banana plantlets subcultured into Murashige and Skoog (MS) medium in 120 cm tubs (Murashige and Skoog, 1962). Nutrient medium consisted of 4.43 g/L MS (Austratec), 1% Phytagel™ (Sigma-Aldrich), 30 g/L sucrose (Chem-Supply) plus separate amendments of 0, 5, 10, 15 and 20 g/L amorphous silicon dioxide (SiO₂) powder (Sigma-Aldrich). Final working media were adjusted to pH 6.0 with dilute sodium hydroxide. Roots from subcultured plants grew exclusively on the surface of the media due to the high concentration of Phytagel™. This allowed roots to be harvested with minimal interference. Plantlets were cultured for 4 weeks under tissue culture conditions, then harvested. At harvest, proximal root samples of approximately 3mm diameter were excised 10-15 mm from the root tip with a razor blade and immediately frozen on a liquid nitrogen-cooled metal block.

For scanning electron microscope-energy dispersive X-ray analysis (SEM-EDS), frozen proximal root sections were freeze-fractured transversely on a cooled metal block with a cooled razor blade and freeze dried for 5 days at -30°C and before being returned to room temperature. Samples were mounted on stubs in preparation for SEM. Microanalysis was
performed on a JEOL JSM-6460 LA SEM in low vacuum mode, equipped with an integrated JEOL hyper mini-cup energy dispersive X-ray spectrometer. The EDS system had a polymer ultra-thin window (UTW), a SiLi crystal with a nominal 133 eV resolution, and integrated JEOL Analysis Station software (V3.8).

Uncoated samples were analysed under low vacuum at 15 kV accelerating voltage. Imaging was performed using backscattered electrons to highlight elemental differences. Spot size was kept constant between samples with dead time ranging between 10 and 20% and live time of 60 seconds. Initial spot analyses were performed to confirm the presence of silicon, followed by X-ray mapping and sequential spot analysis measuring elemental presence in the cell walls of epidermal, hypodermal, cortical, endodermal and stele tissue (Figure 2-1). For sequential spot analysis, seven roots were analysed per treatment group, with each root analysed three times in different regions. X-ray count data were converted to weight percentage (wt%) through standardless quantitation with a ZAF correction. This allowed for semi-quantitative analysis: i.e. wt% values can only be interpreted comparatively and not as absolute amounts or measurements.

![Image of banana roots](image)

**Figure 2-1.** Backscattered scanning electron micrographs of transversely fractured, freeze-dried proximal (A) and distal (B) banana roots demonstrating the cell types/tissues investigated in this study. Abbreviations: E, epidermis; H, hypodermis; OC, outer cortex; IC, inner cortex; A (small), aerenchyma; EN, endodermis; S, stele.
Proximal banana root cSEM-EDS

For cSEM-EDS, roots from the 10 g/L silicon treatment group were harvested as per conventional SEM and frozen by plunging in liquid nitrogen slush. Frozen hydrated roots were fractured transversely, etched and lightly platinum coated on an Oxford CT1500 cryo stage attached to a Phillips LaB6 XL30 high vacuum scanning electron microscope with EDAX brand EDS. The EDS system had a polymer super ultra-thin window, SiLi crystal with a nominal 128 eV resolution and EDASX Phoenix software (V3.3). As fracturing does not produce surfaces ideal for X-ray analysis, care was taken to select regions that fractured as cleanly as possible. X-ray analysis was performed as for regular SEM-EDS, with the addition of sequential spot analysis targeting the intact protoplasm.

Distal banana root SEM-EDS

To investigate the silicon content of distal roots, banana plantlets from tissue culture were deflasked into potting trays containing University of California (UC) mix “C” with fertiliser regime II (Matkin and Chandler, 1979) and hardened under conditions of decreasing humidity and increasing light for a month. Plants were potted up to 20 cm pots containing UC mix amended with 5, 10, 15 and 20 g/kg of amorphous silicon dioxide powder (Sigma-Aldrich). Plants were grown for a further 6 weeks then carefully uprooted and rinsed with water. Healthy, living roots (no discolouration apparent) were harvested approximately 10 mm from the base of the rhizome. Samples were thoroughly rinsed in distilled water, cut to 5-10 mm length with a razor blade, instantly frozen and processed for SEM-EDS as above except roots were not fractured as this generally caused the root to shatter.

Gross banana shoot silicon content

To test for silicon uptake in the shoots, the leaf and pseudostem of plants used for proximal root analysis and distal root analysis were harvested at the same time that root samples were taken. Samples were dried in an oven at 60°C for 7 days then analysed for silicon content using the autoclave induced digestion/silicomolybdous blue colourimetric (AID-SBC) method with modifications as per Taber et al. (2002) (Appendix 2). Briefly, samples were ground to a powder and 0.1 g of material added to a 5mL autoclave resistant polyethylene tube. Material was wetted with 5 mL of 35% hydrogen peroxide, 3.5 mL of 50% sodium hydroxide was added, lightly capped and autoclaved for an hour. The
resulting liquid was filtered through grade 541 quantitative hardened ashless filter paper (Whatman) into a tube and made up to 20 mL with water.

A 1 mL aliquot of the digested plant tissue solution was analysed as follows: solution was added to 5 mL of 16% acetic acid followed by 1 ml of ammonium molybdate (54 g/L), left for 5 minutes, then 0.5 mL of 20% tartaric acid and 0.5 mL of reducing solution. The reducing solution comprised 2 g of Na₂SO₃ in 20 mL water and 0.4 g of 1-amino-2-naphthol-4-sulfonic acid in 25 mL water combined with 25 g of NaHSO₃ in 200 mL water, mixed and brought to a final volume of 250 mL with water. Absorbance of the resulting blue-coloured solution was measured after 30 minutes at 650 nm on a Hitachi U-2800 UV-Visible double beam spectrophotometer. Blanks consisted of all reagents using UHQ water as the sample, made fresh for each sample run. Absolute silicon values were determined with a standard curve derived from serial dilutions of 1 g/L silicon standard solution (Sigma-Aldrich). Each sample run was calibrated by running a sample spiked with a known quantity of silicon dioxide.

**Differential gross silicon content of banana roots**

To determine whether the silicon content of roots increases over time, the root systems of 4-month-old banana plants maintained under glasshouse conditions were investigated. The only silicon supplied was that normally present in the UC mix. Roots of lengths greater than or equal to 16 cm were separated from the rest of the root ball, thoroughly stripped of potting mix, triple-rinsed in distilled water then cut in half at 8 cm from the root tip (representing a proximal half and distal half). Roots were oven-dried at 60°C for 7 days analysed with AID-SBC as above. Each cut half was analysed separately, then the proximal half compared with the distal half.

**Data analysis**

Statistical analysis was performed in GraphPad Prism version 5.03 for Windows. For proximal and distal root analysis, a one-way ANOVA was performed with Tukey’s post-test to confirm differences within treatments between cell types. To compare between treatment groups, an area under curve (AUC) value was computed for each replicate to give an approximation of total silicon concentration and then compared with one-way
ANOVA with Tukey’s post-test. For root distance analysis, a Student’s t-test was performed. For all tests, significance was set at (p < 0.05) and all error reported as standard error of the mean (SE). Post-processing of all images performed in either ImageJ version 1.44p for Windows or Photoshop CS2 Version 9, Adobe.
Results:

**Proximal root SEM-EDS**

Proximal roots viewed under SEM displayed morphological characteristics as previously described for plants in Musaceae (Tomlinson, 1969; Price, 1995a). Roots were all approximately 2mm in diameter. Freeze-drying caused the collapse of most cell protoplasts but left the cell wall intact (Figure 2-1). The following tissues were present: epidermis with root hairs, hypodermis, a large indeterminate cortex, endodermis, and finally the stele (Figure 2-1). Condensed remains of vacuolar, cytoplasmic and possibly apoplastic contents remained visible in micrographs as randomly distributed, high brightness crystals. Performing EDS at 15 kV, elements of up to atomic mass 26 (Fe) were consistently detectable. A summary of elements detected is presented in Table 2-1.

**Table 2-1.** Elements commonly detected in cell walls of banana roots from all treatment groups.

<table>
<thead>
<tr>
<th>Root type</th>
<th>Always present</th>
<th>Mostly present</th>
<th>Rarely present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>C, O, P, K, Cl, S</td>
<td>Al, Na, Mg, Si</td>
<td>Ca, Fe*</td>
</tr>
<tr>
<td>Distal</td>
<td>C, O, P, K, Cl</td>
<td>Al, Na, Mg, Si, Ca, S</td>
<td>Fe*</td>
</tr>
</tbody>
</table>

Note: Always present: detected in every sample. Mostly present: detected in at least 50% of samples. Rarely present: detected in less than 50% of samples. Accelerating voltage for analysis was 15 kV, therefore elements above the atomic weight of Fe were not reliably detected. *Fe was possibly an environmental contaminant, e.g. dust.

X-ray mapping of transverse proximal root sections revealed silicon in all tissue, with the highest comparative concentration in the cortex and lowest concentration in the stele (Figure 2-2). There were no strong concentrations of silicon that would indicate discrete aggregates (they would appear as a collection of white pixels). Sequential spot analysis of cell walls demonstrated an increasing silicon concentration gradient from the epidermis to the inner cortex and decreasing from the endodermis to the stele (Table 2-2). Total silicon
approximations (area-under-curve) did not differ significantly between treatments (Table 2-2) suggesting that total silicon concentration in proximal roots did not change with increasing concentrations of silicon.

Figure 2-2. Scanning electron micrograph (A) and background-subtracted x-ray map (B) of silicon distribution in a transversely fractured freeze-dried proximal banana root section. Silicon concentration per pixel is represented via a colour-scale, from black (lowest) to white (highest). Silicon is present in the cortex in highest concentration and is noticeably absent from the stele. No aggregates present (which would appear as clusters of white pixels) suggesting silicon is associated with cell walls.
Table 2-2. Semi-quantitative silicon distribution (wt%) in cell walls of transversely fractured freeze-dried proximal banana root sections analysed by SEM-EDS. Peak values with regards to cell type for each treatment are underlined. Letters represent statistically significant differences within cell tissue group (for cell type, i.e. within column) and between treatment groups (for area under curve). Error is standard error and n = 21 per treatment group (p < 0.05).

<table>
<thead>
<tr>
<th>Silicon treatment (application g/kg)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue type</td>
<td>15</td>
<td>10</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>I - Epidermis</td>
<td>0.20 ± 0.02 a</td>
<td>0.18 ± 0.01 ab</td>
<td>0.23 ± 0.02 ab</td>
<td>0.12 ± 0.01 a</td>
</tr>
<tr>
<td>II - Hypodermis</td>
<td>0.25 ± 0.02 ab</td>
<td>0.31 ± 0.03 abc</td>
<td>0.24 ± 0.03 ab</td>
<td>0.22 ± 0.02 ab</td>
</tr>
<tr>
<td>III - Outer cortex</td>
<td>0.40 ± 0.05 c</td>
<td>0.34 ± 0.04 bc</td>
<td>0.33 ± 0.04 ac</td>
<td>0.31 ± 0.03 b</td>
</tr>
<tr>
<td>IV - Cortex</td>
<td>0.47 ± 0.05 c</td>
<td>0.43 ± 0.04 c</td>
<td>0.45 ± 0.05 c</td>
<td>0.53 ± 0.04 c</td>
</tr>
<tr>
<td>V - Inner cortex</td>
<td>0.39 ± 0.04 bc</td>
<td>0.41 ± 0.05 bc</td>
<td>0.36 ± 0.05 ac</td>
<td>0.59 ± 0.05 c</td>
</tr>
<tr>
<td>VI - Endodermis</td>
<td>0.24 ± 0.03 ab</td>
<td>0.27 ± 0.03 ab</td>
<td>0.25 ± 0.03 a</td>
<td>0.30 ± 0.03 b</td>
</tr>
<tr>
<td>VII - Stele</td>
<td>0.10 ± 0.01 a</td>
<td>0.12 ± 0.01 d</td>
<td>0.10 ± 0.01 b</td>
<td>0.12 ± 0.02 c</td>
</tr>
<tr>
<td>Area under curve</td>
<td>1.90 ± 0.14 a</td>
<td>1.86 ± 0.15 a</td>
<td>1.80 ± 0.16 a</td>
<td>2.06 ± 0.09 a</td>
</tr>
</tbody>
</table>

Transversely cryo-fractured roots viewed using cSEM-EDS showed intact cell walls with cytoplasmic contents condensing into characteristic ripples during sublimation (Figure 2-4). Spot analysis results for cSEM-EDS were similar to freeze-dried samples, with an increasing silicon concentration gradient from epidermis to cortex and decreasing into the stele significant (Table 2-3). Spot analysis values for cell protoplasm were of a similar magnitude to cell wall values, but did not follow any sort of trend (Table 2-3).
Figure 2-3. Example secondary electron SEM micrograph of transversely cryo-fractured banana root post-sublimation and platinum coated. Bright white regions are charge artifacts (arrow). Vacuolar and cytoplasmic contents have condensed during ice crystal formation and become evident after sublimation to leave the ripples characteristic of cryoSEM. Abbreviations: CC, cortical cell; EN, endodermis; S, stele; X, xylem.
Table 2-3. Semi-quantitative silicon distribution (wt%) in the cell walls and cell protoplast of transversely fractured proximal frozen banana root sections (from 10 g/kg silicon treatment) analysed by cSEM-EDS.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell wall</th>
<th>Cell protoplast*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Epidermis</td>
<td>0.24 ± 0.04 a</td>
<td>0.24 ± 0.04 a</td>
</tr>
<tr>
<td>II - Hypodermis</td>
<td>0.38 ± 0.14 a</td>
<td>0.49 ± 0.19 a</td>
</tr>
<tr>
<td>III – Outer cortex</td>
<td>0.40 ± 0.07 a</td>
<td>0.57 ± 0.35 a</td>
</tr>
<tr>
<td>IV – Cortex</td>
<td>0.72 ± 0.12 b</td>
<td>0.29 ± 0.06 a</td>
</tr>
<tr>
<td>V – Inner cortex</td>
<td>0.42 ± 0.11 a</td>
<td>0.42 ± 0.12 a</td>
</tr>
<tr>
<td>VI - Endodermis</td>
<td>0.40 ± 0.10 a</td>
<td>0.46 ± 0.16 a</td>
</tr>
<tr>
<td>VII - Stele</td>
<td>0.20 ± 0.06 a</td>
<td>0.29 ± 0.06 a</td>
</tr>
</tbody>
</table>

*Protoplast refers to the whole cell making no distinction between the cytoplasm and vacuole. In the majority of cells vacuole is far larger than the cytoplasm. Peak values for each treatment are underlined. Letters represent statistically significant differences within cell treatment group (p < 0.05). Error is standard error and n = 3 per treatment group.

Distal root SEM-EDS

Distal roots were morphologically similar to proximal roots with the following differences: root diameter was variable, ranging from approximately 4 mm to 8 mm and the cortex had differentiated into distinct outer and inner cortical tissue, divided by aerenchyma (Figure 2-1). Root hairs were either not present or heavily degraded. The epidermis was intact and had not sloughed off as is common in mature banana (Riopel and Steeves, 1964). A summary of elements detected during spot analysis is presented in Table 2-1.
X-ray maps of transverse distal root sections were similar to those for proximal roots, with the highest concentration in the inner cortex (Figure 2-3). No aggregates or physical accumulations were present. Sequential spot analysis was similar to the pattern obtained for the proximal roots, with an increase in concentration from the epidermis to cortex, a statistically significant major peak in the inner cortex, and decreasing concentration through the stele (Table 2-4). Total silicon approximation values (area-under-curve) were significantly lower for the 5 g/kg treatment compared to all other treatments (Table 2-4).

**Figure 2-4.** Scanning electron micrograph (A) and background-subtracted X-ray map of silicon distribution in transversely sectioned freeze-dried distal banana root sections. Silicon concentration per pixel is represented on a colour-scale, with white highest and black lowest. Silicon signal is most intense in the inner cortical region, with much lower signal in the outer cortex, and very little in the stele and the aerenchyma.
Table 2-4. Semi-quantitative silicon distribution (wt%) in cell walls of transversely sectioned freeze-dried distal banana root sections analysed by SEM-EDS

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Epidermis</td>
<td>0.64 ± 0.04 ab</td>
<td>1.25 ± 0.35 ab</td>
<td>1.92 ± 0.21 ab</td>
<td>0.99 ± 0.08 ab</td>
</tr>
<tr>
<td>II - Hypodermis</td>
<td>0.69 ± 0.14 ab</td>
<td>0.93 ± 0.032 ab</td>
<td>1.25 ± 0.25 bc</td>
<td>0.99 ± 0.13 ab</td>
</tr>
<tr>
<td>III - Cortex</td>
<td>0.63 ± 0.11 ab</td>
<td>1.19 ± 0.24 ab</td>
<td>0.80 ± 0.04 cd</td>
<td>0.85 ± 0.11 b</td>
</tr>
<tr>
<td>IV - Cortex</td>
<td>0.65 ± 0.08 ab</td>
<td>1.30 ± 0.19 ab</td>
<td>0.99 ± 0.13 cd</td>
<td>0.75 ± 0.10 b</td>
</tr>
<tr>
<td>V - Cortex</td>
<td>0.98 ± 0.19 ac</td>
<td>1.54 ± 0.26 ac</td>
<td>1.22 ± 0.18 bc</td>
<td>1.00 ± 0.20 ab</td>
</tr>
<tr>
<td>VI - Cortex</td>
<td>1.32 ± 0.17 c</td>
<td>2.62 ± 0.51 c</td>
<td>2.17 ± 0.31 a</td>
<td>1.64 ± 0.36 a</td>
</tr>
<tr>
<td>VII - Endodermis</td>
<td>0.63 ± 0.14 ab</td>
<td>0.74 ± 0.16 ab</td>
<td>0.72 ± 0.12 cd</td>
<td>0.71 ± 0.16 b</td>
</tr>
<tr>
<td>VIII - Stele</td>
<td>0.45 ± 0.08 ab</td>
<td>0.54 ± 0.11 ab</td>
<td>0.63 ± 0.23 cd</td>
<td>0.62 ± 0.16 b</td>
</tr>
<tr>
<td>IX - Stele</td>
<td>0.50 ± 0.07 ab</td>
<td>0.39 ± 0.02 b</td>
<td>0.33 ± 0.08 d</td>
<td>0.37 ± 0.07 b</td>
</tr>
<tr>
<td>X - Stele</td>
<td>0.34 ± 0.06 b</td>
<td>0.47 ± 0.07 ab</td>
<td>0.33 ± 0.04 d</td>
<td>0.51 ± 0.08 b</td>
</tr>
<tr>
<td>Area under curve</td>
<td>7.22 ± 0.53 a</td>
<td>9.72 ± 0.65 b</td>
<td>9.13 ± 0.44 ab</td>
<td>8.11 ± 0.61 ab</td>
</tr>
</tbody>
</table>

Peak values with regards to cell type for each treatment are underlined. Letters represent statistically significant differences within cell treatment group (i.e. within column) and between treatment groups (for area under curve). (p < 0.05) n = 8 per treatment group. Area under curve represents an approximation of total silicon content in treatment, i.e. silicon from epidermis (I) to stele (X).

Banana shoot silicon content
Proximal plant silicon shoot concentration values were at the lower detection limit of the AID-SBC method and were either very low or not present (Table 2-5). This analysis was repeated twice to confirm values, consistently returning negligible silicon amounts. Distal shoot silicon content was lowest for the 20 g/kg treatment with 7.52 mg/g silicon and highest for 15 g/g with 11.84 mg/g silicon (Table 2-5).
Table 2-5. Silicon content of banana leaf and pseudostem tissue as determined by the silicomolybdous blue colourimetric method

<table>
<thead>
<tr>
<th>Silicon applied (g kg⁻¹)</th>
<th>Silicon content (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>15</td>
<td>N/A</td>
</tr>
<tr>
<td>20</td>
<td>N/A</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.15 ± 0.04 a</td>
</tr>
<tr>
<td>10</td>
<td>8.56 ± 0.07 a</td>
</tr>
<tr>
<td>15</td>
<td>11.84 ± 1.81 b</td>
</tr>
<tr>
<td>20</td>
<td>7.52 ± 0.23 a</td>
</tr>
</tbody>
</table>

N/A: samples were at detection limit: values are either zero or extremely low. Each sample run was calibrated by running a sample spiked with a known quantity of silicon dioxide. Letters represent statistically significant difference for Student’s t-test at p <0.05. Error is standard error and n = 3.

Banana root gross silicon content

The roots of pot-grown banana plants analysed for gross silicon content showed a small but significant increase from 0.63 mg/g silicon in the proximal half to 0.67 mg/g silicon in the distal half (Table 2-6).

Table 2-6. Root silicon content of pot-grown banana plants harvested at different lengths as determined by the silicomolybdous blue colourimetric method

<table>
<thead>
<tr>
<th>Distance from root tip</th>
<th>Silicon content (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal (0-8 cm)</td>
<td>0.63 ± 0.01 a</td>
</tr>
<tr>
<td>Distal (8-16 cm)</td>
<td>0.67 ± 0.02 b</td>
</tr>
</tbody>
</table>

Letters represent statistically significant difference for Student’s t-test at p <0.05. Error is standard error and n = 6.
Discussion:

Scanning electron microscopy coupled with energy dispersive X-ray spectroscopy revealed elemental silicon in most banana root tissues, with highest concentrations in the cell walls of the inner cortex (Tables 2-2 and 2-3). No discrete deposits or aggregates (i.e. phytoliths) were found in the roots, in agreement with previous reports on banana roots (Tomlinson, 1969). This lack of phytoliths suggests silicon is either integrated in the cell wall or in close association. This root silicon distribution appears to be unique to banana compared to all previously studied species. The only other plant with reported silicon deposition in the cortex is the mature roots of purple moor grass (Molinia caerulea) (Montgomery and Parry, 1979; Parry and Kelso, 1975).

While silicon was present in the endodermis, it was not highly concentrated, which is in contrast with most studied species in the Poaceae family, such as barley (Hordeum vulgare), oats (Avena sativa) and rice (Oryza sativa) (Bennett, 1982; Parry and Soni, 1972; Parry and Kelso, 1975). The endodermal accumulations in these plants are thought to be due to a build-up of excess soluble silicon at the endodermis as water enters the vascular system through the casparian strip (Parry and Kelso, 1977). This may also be occurring in banana roots, where excess silicon is diverted into cortical cell walls, or the apoplast, instead of the endodermis, resulting in silicification of the cortex. Whether this process occurs passively, or is actively controlled, remains unknown.

No discrete silicon deposits were detected in the epidermis or hypodermis. If silicon provides a physical barrier against pathogen penetration, similar to the cuticle-silicon double layer in rice leaves then the root epidermis is a reasonable place to expect silicon deposition (Hayasaka et al., 2008). Mature banana roots usually shed their epidermis which would make an epidermal silicon layer ultimately ineffective (McCully, 1987). There is limited evidence for dicotyledenous plants accumulating silicon in the root epidermis, which may be characteristic of dicots (Sangster, 1978).

Lateral roots were not investigated in this experiment; tentative analysis indicated they follow a similar pattern of silicification to primary roots (data not shown). Root hairs were also not investigated, as freeze-drying and cryo-fracturing does not produce surfaces
amenable to X-ray analysis. Border cells, detached living cells shed by the root cap in the soil, were also not investigated (Hawes et al., 2000). However, considering their involvement in root disease development, their interaction with silicon should be investigated.

From investigating proximal roots, it is clear that banana plants absorb silicon into the roots. This may have implications for tissue culture production as silicon may help alleviate some of the stress associated with deflasking and glasshouse hardening. Silicon shoot content was negligible, indicating silicon is not translocated to the shoot in appreciable amounts under tissue culture conditions. If shoot uptake is governed by mass flow then the high humidity environment of tissue culture would drastically reduce uptake. Decreased shoot deposition under high humidity conditions has previously been demonstrated in cucumber (*Cucumis sativus*) (Chérif et al., 1992).

Analysis by cSEM-EDS gave greater silicon values than freeze-dried material for all tissue types (Tables 2-2 and 2-3). This was due to the flat, fractured surfaces allowing the electron beam to interact with more material (McCully et al., 2010). The lack of replicates for cSEM-EDS meant that results were not statistically significant whereas freeze drying allowed for analysis of many samples in a reasonable time-frame giving greater statistical reliability.

Silicon was detected in the condensed protoplasm of frozen hydrated cells, but concentration did not follow any trends with cell type. EDS resolution was not sufficient for determining whether silicon was located in the vacuole or cytoplasm. This protoplastic silicon may represent symplastically transported monosilicic acid as it moves temporarily through the cell, or conversely, soluble silicon may be stored in the cell for unknown purposes. Sorghum (*Sorghum bicolor*) has been demonstrated to maintain a protoplastic concentration of silicon (Hodson and Sangster, 1989). Tomato (*Solanum lycopersicum*), a silicon excluder, still has root symplastic silicon content higher than that of the external silicon solution, suggesting the action of active transporters does not necessarily result in physical deposition of silicon (Mitani and Ma, 2005). The dynamics and biochemistry of soluble silicon *in planta* need to be investigated.
Distal roots displayed the same trends for silicon deposition as proximal roots albeit with greater values (Tables 2-4). The comparatively higher amounts of silicon in the distal roots suggests that deposition occurs over time and is greater with age or deposition increases in relation to root size. Aerenchyma were present in all distal roots and showed some silicification but silicon concentration was greatest in the cortical region between the stele and the aerenchyma (Figures 2-1 and 2-3). Values for the epidermis should be interpreted with caution, as the root surface was most likely contaminated with silicon from the potting media.

Distal roots investigated in this study ranged from 40-80 cm in length. It is unknown whether these results can be extrapolated to field-grown banana, as their roots do not fully mature until 90-130 cm from the apex and can reach up to 7.5 m in length (Riopel and Steeves, 1964; Price, 1995a). In general, silicon uptake and deposition studies for plants under field conditions are lacking. However, in a study of field-grown sorghum roots over time, gross silicon content of roots varied considerably and did not follow a linear trend in increasing or decreasing uptake (Lanning and Linko, 1961).

Root distance results suggest that silicon content increases with age. This is seen in other accumulator plants, such as purple moor grass which has low silicon levels in young root tissue and high silicon levels in older root tissue (Parry and Kelso, 1975). Rice and sorghum both show basipetal increase in endodermal silicon content, likely due to passive endodermal deposition occurring over time (Lux et al., 1999; Hodson and Sangster, 1989).

Shoot silicon content did not increase linearly with silicon supply and was lowest at the highest applied rate (Table 2-5). High concentrations of silicon dioxide in the growing media may have generated adverse physiological conditions by altering the soil chemistry, or may have had a directly phytotoxic effect. Alternatively, increased silicon availability may decrease uptake through the lowered expression of transporter genes. This has been demonstrated in rice, with the constitutively expressed \( LSI1 \) transporter gene being downregulated when provided with a constant silicon supply (Ma et al., 2006).

There are several ways silicon deposition in the cortex may be advantageous in banana roots. Root pathogens generally must penetrate cortical cell walls to gain access to the
cytoplasm of individual cells or to progress towards the stele and vascular tissues. Silicified cortical cells may resist fungal infiltration in the same way that the silicified epidermis of rice leaves avoids the penetration of foliar fungal pathogens (Kim et al., 2002). The silicified inner cortex may also provide a physical barrier against vascular pathogens penetrating into the vascular tissue (Lux et al., 2003). Endodermal silicification in sorghum is correlated with resistance to species of Striga, a collection of angiosperm root parasites (Maiti et al., 1984).

Parry and Kelso (1975) theorise that heavy silicification of the endodermis would render it impermeable to water and solutes, effectively sealing the stele off from the cortex. This is a possibility in banana roots, as silicification of the inner cortex appeared to increase with age (Table 2-4). As most uptake of water and nutrients occurs in younger roots, sealing the older tissue with silicon may improve tolerance to drought, by preventing the leakage of water, or impeding the penetration of pathogens.

Silicon may act as a substitute for lignin (Hatfield and Fukushima, 2005). Lignin is energetically intensive to produce whereas silicon deposition requires only enough energy for transport and deposition. Silicon also has an affinity with phenolic lignin precursor molecules, and may simply accumulate in cell walls during lignin biosynthesis (2003; Raven, 1982). The beneficial effects of silicon have been linked to the upregulation of defence genes and the production of phytoalexins and other plant based defence compounds (Epstein, 1994; Epstein, 1999; Ma, 2004). Currently it is not clear whether this is associated with deposited silicon, soluble silicon, or a combination of both. Soluble silicon alone has been demonstrated to reduce Pythium aphanidermatum infection on bitter gourd (Mormodica charantia) (Heine et al., 2007).

Banana roots play an important structural role. The shoots of the plant are large with no woody structural tissue for support. The root system is shallow, rendering them susceptible to lodging, especially during high-wind events (e.g. cyclones). Silicon reinforcement of the roots may improve their capacity for elongation or enhance their structural integrity making them more resistant to lodging (Hattori et al., 2003). Dakora and Nelwamondo (2003) showed that silicon application in cowpea (Vigna unguiculata) influences growth hormone levels in planta, including abscisic acid and cytokinin which in turn impacts root:shoot
ratios. This suggest that silicon is involved in the elicitation of hormone biosynthesis, possibly acting as the elicitor itself.

This study demonstrated silicon to be located primarily in the cortical tissue of banana roots. Analysis showed greatest deposition occurring in the inner cortex. Whether this deposited silicon is beneficial for protection from pathogens or for structural integrity, or both, and what role soluble silicon present in protoplasts plays, requires further study in banana.
Chapter 3 - Shoot silicon uptake and distribution in ‘Cavendish’ banana

Abstract:

*Background and Aims:* Silicon has been shown to enhance plant tolerance to various diseases and environmental factors via an unknown mechanism. The banana plant is a silicon accumulator. The location of silicon in banana plants has only ever been confirmed by the presence of discrete deposits (phytoliths) and bulk analysis. The goal of this research was to use scanning electron microscopy coupled with energy dispersive X-ray microanalysis (SEM-EDS) to determine the location of silicon in the shoots, and investigate any non-discrete deposits of silicon such as silicon in association with cell walls.

*Methods & Materials:* ‘Cavendish’ banana plants were grown under hydroponic conditions amended with 1.66mM silicon or grown in pots with 2 g/kg silicon dioxide. After 1 month, plants were harvested, snap-frozen and analysed with SEM-EDS. Plants were divided into tissue type (young/old leaves, pseudostem, rhizome) and analysed initially via spot analysis and then by elemental mapping. Silicon xylem uptake was investigated in plants grown in pots with various sources of silicon: silicon dioxide, potassium silicate, sodium silicate, calcium silicate and magnesium silicate. Sap silicon content was analysed every week over a month via the silico-blue molybdate test. Whole tissues were digested and analysed for bulk silicon content.

*Key Results:* Discrete silicon deposits were found in the leaves, in rows adjacent to the vascular tissue and sometimes in the pseudostem. The abaxial leaf appeared to have the greatest concentration of deposits. Silicon was detected within or in association with most cell walls analysed. For uptake, calcium silicate, magnesium silicate and silicon dioxide treatment resulted in greater plant silicon content compared to potassium silicate and sodium silicate. Xylem sap silicon content varied considerably in plants amended with solid silicon dioxide but was consistent when silicon was constantly supplied.
Conclusions: The location of deposited silicon in the plant would suggest an important physiological role. Elemental mapping confirmed silicon bodies to be located almost exclusively within fibre bundles in the vascular tissue. The dynamics of plant silicon uptake and how they change over time make testing potential sources of silicon challenging. Understanding the role of soluble silicon in planta needs to be investigated in banana shoot tissue.
Introduction:

Silicon is the second most abundant element in the soil (Epstein, 1994). In plant nutrition, silicon is classified as quasi-essential, as it is only essential in a limited number of species and is beneficial in others (Epstein, 1999). Silicon fertilisation can improve the tolerance of plants to a wide variety of negative biotic and abiotic conditions. Silicon as a plant nutrient has only been lightly studied over the last century, but recent experiments have renewed interest in its use as a fertiliser (Epstein, 1994; Epstein, 1999; Guntzer et al., 2012).

Enhanced tolerance to biotic and abiotic factors ranges from increased growth to improved defence responses to plant pathogens (Guntzer et al., 2012; Ma, 2004). There are no reported adverse side effects to the application of silicon to plants. It is currently unknown whether the improved tolerance effect of silicon is due to a biochemical influence on the plant, or the presence of discrete, polymerised silicon bodies forming a physical barrier. Researching this is a high priority for several crop species, particularly the banana plant.

Banana plants are commonly grown in the tropics and subtropics, and their fruit is a staple in both western diets and in developing countries (Jones, 2000b). Most cultivated banana plants are clonal and genetically uniform, rendering them uniquely susceptible to a wide array of plant diseases, disorders and adverse weather conditions (Jeger et al., 1995). The plant itself is large, herbaceous and will reproduce vegetatively through the production of suckers. New leaves develop at a ground level meristem and grow upward to form pseudostem in a tight roll. The lamina of the leaf is large and oblong with numerous parallel veins extending to the margin (Jones, 2000b).

The banana plant is a silicon accumulator (Henriet et al., 2006). It absorbs silicon from the soil solution in the form of monosilicic acid, which is transported via the flow of water in transpiration through the xylem. This process occurs passively when silicon concentrations are high in the soil solution, but switches to active transport when concentrations are low (Henriet et al., 2006). Monosilicic acid is usually present in the soil solution at concentrations ranging from 0.01 to 1.99 mM (Epstein, 1994). This upper limit is due to automatic condensation of monosilicic acid into silicon dioxide (Raven, 1982).
Active transporters have been described in several species, and are likely present in many more. Silicon transporters recently describe include: LSI1, LSI2 and LSI6 in rice (Ma et al., 2006; Ma et al., 2007; Yamaji et al., 2008). Similar transporters have been described in maize and barley (Mitani et al., 2009). Details of silicon deposition in banana roots are covered in Chapter 2 of this thesis.

Once absorbed into the plant, monosilicic acid polymerises into amorphous silicon dioxide (SiO$_2$) in what was once thought to be a passive process, but has recently been demonstrated to be under the control of shoot silicon distribution transporters, at least in rice (Yamaji et al., 2008). Silicon tends to polymerise in cell walls, cell lumens and intercellular spaces. These polymerised deposits are often of unique shapes depending on their sites of deposition and have been frequently referred to as phytoliths (Lentfer, 2009).

Phytoliths are silicon deposits no longer embedded in plant tissue that retain morphological characteristics that allow them to be identified visually as to belonging to a certain genus or species (Richmond and Sussman, 2003). They are frequently used in archaeology to determine planting sites and diets of ancient humans, as phytoliths will persist in the soil long after the plant as died (Lentfer, 2009; Parr and Sullivan, 2005; Pease and Anderson, 1969). The term phytolith is sometimes used interchangeably with silicon deposits, or silicified cells, although technically a phytolith does not need to be composed of silicon.

The most comprehensive study of silicon uptake in banana was by Henriet et al. (2006). They described the distribution and uptake of silicon in ‘Cavendish’ banana plants grown under hydroponic conditions. Supplied silicon did not affect plant growth or nutrient uptake, but silicon concentration of tissues increased with silicon supplied. Shoot silicon content was greatest in the old leaves in the lamina, with decreasing concentrations in the midrib, petiole and lowest in the pseudostem leaf sheaths (Henriet et al., 2006).

The goal of this study was to expand on the current knowledge of silicon distribution in banana shoots, and attempt to investigate silicon deposition directly in planta via scanning electron microscopy coupled with energy-dispersive spectroscopy (SEM-EDS).
Additionally, the uptake of silicon in pot-conditions and using different sources of silicon were investigated. Sap silicon measurements of banana xylem fluid were also measured to determine silicon uptake dynamics.
Methods & Materials

Plant material and growth conditions
All banana plants used in this study were *Musa acuminateata* (AAA group) cv. ‘Cavendish’ "Williams". Plantlets were obtained from stock tissue culture at The University of Queensland, Brisbane, Australia. All potting media used was University of California potting mix "C" (UC mix C) (Matkin and Chandler, 1979). Plants were maintained in black plastic pots with standard drainage. Glasshouse conditions were average Brisbane temperatures in late spring/early summer with upper temperatures maintained at no more than 30°C by automated ventilation. Plants were daily watered with tapwater until replete and given full strength Thrive (Yates) liquid fertiliser weekly, unless specified otherwise.

Scanning electron microscopy and energy dispersive spectroscopy (SEM-EDS)
To investigate silicon deposition with SEM-EDS, banana plants were grown in either silicon-amended potting mix or in hydroponics with additional silicon. For potted plants, tissue culture plantlets were obtained as above, deflasked and raised for 4 weeks (Appendix 6). Acclimated plantlets were then potted up into 12 cm pots with UC mix C amended with and without 5 g/kg amorphous silicon dioxide powder (Sigma-Aldrich). Pots were maintained in the glasshouse for 4 weeks.

For hydroponics, plantlets were deflasked to plastic trays of perlite containing aerated nutrient media composed of 0.1X Mursashige and Skoog media (Murashige and Skoog, 1962). Silicon was not added during the deflasking process. Otherwise the deflasking process remained the same. After 4 weeks, plantlets were moved to 10 cm diameter polycarbonate tubes containing the same aerated nutrient media, amended with and without 1.66 mM of silicon added as amorphous silicon dioxide powder (Sigma-Aldrich). The tubes were coated in black tape to prevent sunlight from interfering with root development. Plants were maintained for 4 weeks in the glasshouse until harvest.
Harvest and processing for SEM-EDS

To build a profile of silicon distribution in the whole shoot, the following tissues were harvested: rhizome, pseudostem, leaf lamina, leaf petiole and leaf midrib (Figure 3-1). Organs were roughly separated with a knife, then fine samples were sectioned by cutting with a double-edged razorblade. The blade was chilled on a liquid nitrogen-cooled metal block, then used to cut the sample. Sectioned samples were immediately snap-frozen on the same cooled metal block. Samples were stored in a -80°C freezer until processing.

Figure 3-1. Guide to banana plant tissues harvested for analysis: Rhizome, pseudostem, leaf laminar (adaxial and abaxial surface), leaf petiole, leaf midrib. Illustration is of an approximate 3-month-old, pot-grown banana plant.
Frozen samples were freeze-dried for 14 days at \(-30^\circ\text{C}\) before returning to room temperature. Samples were stored under vacuum. Microanalysis was performed on a JEOL JSM-6460 LA SEM in low vacuum mode, equipped with an integrated JEOL hyper mini-cup energy dispersive X-ray spectrometer. The EDS system had a polymer ultra-thin window (UTW), a SiLi crystal with a nominal 133 eV resolution, and integrated JEOL Analysis Station software (V3.8).

Uncoated samples were analysed under low vacuum at 15 kV accelerating voltage. Imaging was performed using backscattered electrons to emphasise elemental differences. Spot size was kept constant between samples with dead time ranging between 10 and 20% and live time of 60 seconds. Initial spot analyses were performed to confirm the presence of silicon, followed by X-ray mapping and sequential spot analysis.

After initial spot analysis, tissues were investigated systematically for discrete silicon deposits. These deposits can be determined visually in backscattered electron mode. If found, the presence of silicon was confirmed through spot analysis. To investigate non-discrete silicon deposition (e.g. cell walls) tissues were analysed systematically, one cell type at a time. Each cell type was analysed in three separate samples from the same plant, in five different plants, making a total of 15 points of analysis. For most tissues, four cells walls were analysed: epidermal, hypodermal and two mesophyll cells. For vascular bundles: fibrous sheath, xylem, phloem and second fibrous sheath. After spot analysis, elemental maps were generated in all tissues to investigate general silicon distribution.

**Xylem silicon content analysis**
Plantlets for xylem analysis were deflasked, hardened then transplanted to 12 cm pots containing potting media as above. Plants were divided into three treatment groups: negative control (no silicon), treatment (amorphous silicon dioxide powder added to media at 2 g/kg rate), and positive control (pots watered daily with 1.66 mM monosilicic acid solution to capacity). Plants were grown for 4 weeks, with a harvest every 7 days. Sixteen plants were grown for each treatment group: four per week per treatment.
Harvesting was performed by cutting the pseudostem approximately 5 cm above the rhizome. The cut was performed with a fresh double-edged razorblade perpendicular to the pseudostem. Milky fluid rapidly accumulated and was discarded via pipette. After 15 minutes, clear xylem exudate had collected. This was removed via pipette. Collection was repeated every 15 minutes for 2 hours to test if silicon concentration in the xylem exudate changed over time. This process was repeated three times for each plant, yielding approximately 1mL of xylem fluid. Silicon content was immediately determined as below.

Silicon uptake analysis
To investigate uptake of silicon present in shoot tissue, banana plants were grown on media amended with different sources of silicon (Table 3-1). This included amorphous silicon dioxide, potassium silicate, sodium silicate, calcium silicate and magnesium silicate. Each treatment was added at a rate to ensure they all received equal moles of elemental silicon. Five replicate plants were grown for each treatment.

Table 3-1. Amounts of silicon added to potting mix to ensure equal amounts (moles) of elemental silicon compared to the standard of silicon dioxide (2 g/kg).

<table>
<thead>
<tr>
<th>Type of silicon</th>
<th>Rate added g or mL per kg potting media</th>
</tr>
</thead>
<tbody>
<tr>
<td>No silicon (control)</td>
<td>0</td>
</tr>
<tr>
<td>Silicon dioxide</td>
<td>2.00</td>
</tr>
<tr>
<td>Potassium silicate (liquid)</td>
<td>5.13</td>
</tr>
<tr>
<td>Sodium silicate (liquid)</td>
<td>4.07</td>
</tr>
<tr>
<td>Calcium silicate (insoluble)</td>
<td>5.73</td>
</tr>
<tr>
<td>Magnesium silicate (insoluble)</td>
<td>3.155</td>
</tr>
</tbody>
</table>

Silicon content determination
Silicon content was determined by the autoclave induced digestion/silicomolybdous blue colourimetric (AID-SBC) method (Appendix 2). Xylem extract did not require digestion (AID) and was analysed immediately after harvest.
Data analysis
Statistical analysis was performed in GraphPad Prism version 5.03 for Windows. Graphs were generated in Microsoft Office Excel 2010. For shoot silicon content of plants treated with various sources of silicon a one-way ANOVA was performed with Tukey’s post-test to confirm differences between means. Post-processing of all images performed in either ImageJ version 1.44p for Windows or Photoshop CS2 Version 9, Adobe.
Results:

Banana shoot elemental analysis
Of the following phenotypes measured: height, leaf number, leaf size, no differences were apparent in plants treated with and without silicon (data now shown). There was no apparent difference in silicon distribution for plants grown in pots or in hydroponics. All data presented henceforth are from plants grown in pots. Silicon was detected in the cell walls of the leaf midrib, petiole, lamina, pseudostem and rhizome (Table 3-2). Silicon was consistently absent from the vascular tissues of the leaf lamina, petiole and pseudostem (Table 3-2). Comparing young and old tissues, silicon became more reliably detected in older midrib and leaf lamina tissue (Table 3-2) suggesting an increase in silicon concentration over time.

Table 3-2. Summary of silicon detection in individual tissues. See (Tomlinson, 1969) for a full description of cell types. Mature = oldest expanded green leaf (includes petiole, midrib lamina). Young = newest expanded green leaf. Pseudostem (as collective leaf sheaths) and rhizome were same age.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Cell type</th>
<th>Silicon detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midrib (abaxial)</td>
<td>Epidermis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Hypodermis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Mesophyll 1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Mesophyll 2</td>
<td>+</td>
</tr>
<tr>
<td>Midrib (adaxial)</td>
<td>Epidermis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Hypodermis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Mesophyll 1</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Mesophyll 2</td>
<td>+</td>
</tr>
<tr>
<td>Midrib (vascular)</td>
<td>Fibrous sheath</td>
<td>-</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Xylem</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phloem</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibrous sheath</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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</tbody>
</table>

| Petiole (abaxial) | Epidermis | + | - |
|-------------------|------------|
|                   | Hypodermis | + | - |
|                   | Mesophyll 1| + | - |
|                   | Mesophyll 2| + | - |

| Petiole (attached lamina) | Epidermis | + | + |
|---------------------------|------------|
|                           | Hypodermis | + | + |
|                           | Mesophyll 1| + | - |
|                           | Mesophyll 2| + | - |

| Petiole (right) | Epidermis | + | + |
|-----------------|------------|
|                 | Hypodermis | + | + |
|                 | Mesophyll 1| + | + |
|                 | Mesophyll 2| + | + |

| Petiole (left)  | Epidermis | + | - |
|-----------------|------------|
|                 | Hypodermis | + | - |
|                 | Mesophyll 1| + | - |
|                 | Mesophyll 2| + | - |

<p>| Petiole (vascular) | Fibrous sheath | - | - |
|--------------------|----------------|
|                    | 1              |   |   |</p>
<table>
<thead>
<tr>
<th>Lamina (vascular)</th>
<th>Epidermis</th>
<th>++</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophyll 1</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mesophyll 2</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fibrous sheath</td>
<td>-</td>
<td>+</td>
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<tr>
<td>1</td>
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<tr>
<td>Xylem</td>
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<td>Phloem</td>
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<tr>
<td>Fibrous sheath</td>
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<td>2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lamina (adaxial)</td>
<td>Epidermis</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hypodermis</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Mesophyll 1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Mesophyll 2</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>Epidermis</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Hypodermis</td>
<td>++</td>
<td>-</td>
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<tr>
<td>Mesophyll 1</td>
<td>++</td>
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<tr>
<td>Mesophyll 2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Hypodermis</td>
<td>++</td>
<td>-</td>
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</tr>
<tr>
<td>Mesophyll 1</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mesophyll 2</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pseudostem (out 1)*</td>
<td>Epidermis</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Hypodermis</td>
<td>++</td>
<td></td>
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<tr>
<td>Mesophyll 1</td>
<td>++</td>
<td></td>
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<tr>
<td>Mesophyll 2</td>
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<tr>
<td>Pseudostem (in 1)*</td>
<td>Epidermis</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hypodermis</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>Tissue Type</td>
<td>Epidermis</td>
<td>Hypodermis</td>
<td>Mesophyll 1</td>
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<tr>
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<td>-----------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Pseudostem (out 2)</strong>*</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Pseudostem (in 2)</strong>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Pseudostem (out 3)</strong>*</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Pseudostem (in 3)</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Rhizome</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* Pseudostem numbering refers to leaf sheath number (1 = outermost sheath; 3 = innermost sheath)
++ silicon consistently detected in every sample tested (n = 15/15)

+ silicon signal was detected in more than half samples tested (n = 8/15)

- no silicon detected above background in more than half samples tested

Elemental mapping via SEM-EDS generally conformed to the data in Table 3-2 (Figure 3-2). Discrete deposits, visible as collections of white pixels, were occasionally detected in the rhizome, pseudostem and petiole (Figure 3-2) and frequently observed in the leaf lamina (Figure 3-2), with highest concentrations in the abaxial surface compared to the adaxial surface. In all cases the deposits were found in association with the vascular bundle, located within the fibrous bundle cells at either end of the bundle (Figure 3-2).
Figure 3-2. Backscattered scanning electron micrographs (A) and corresponding energy dispersive x-ray microanalysis maps (B) of banana tissue. Banana plants were snap-frozen in liquid nitrogen, freeze-dried and then viewed uncoated at low vacuum. Analysis was performed at accelerating voltage of 15kV at low-magnification. Background subtracted map (B) illustrates detected silicon signal with colour representing intensity. Collections of white pixels are discrete silicon deposits. Panels: i = rhizome; ii =
pseudostem; iii = petiole; iv = midrib; v = adaxial leaf lamina; vi = abaxial leaf lamina; vii = leaf lamina cross section.

Banana shoot silicon content

The shoot silicon content of banana plants varied depending on the silicon treatment supplied (Figure 3-3). Untreated control plants had the lowest silicon concentration of 0.4% and amorphous silicon dioxide treated plants had the highest concentration of 2% (Figure 3-3). Calcium silicate and magnesium silicate gave the next highest amount of shoot silicon with around 1.7 and 1.6% respectively. Sodium and potassium silicate resulted in the lowest increase in silicon around 1% (Figure 3-3).

Figure 3-3. Shoot silicon of banana plants treated with different forms of silicon. Shoot included all leaves, the pseudostem and the rhizome. Silicon dioxide, calcium silicate and magnesium silicates were added as solids, the rest were in liquid form. Plants were harvested after 30 days and silicon content as a percentage of total shoot mass determined by the autoclave induced digestion silicomolybdous blue colourimetric method. Error bars are standard error and different letters represent statistically significant differences. n = 5.
Xylem sap silicon content

For measuring xylem sap silicon content, sufficient sap was produced for analysis (Figure 3-4). Negative controls of nutrient solution had no detectable silicon in the xylem sap (Figure 3-5). Positive controls (supplied daily with 1.66 mM silicon) maintained sap silicon content of 0.2% for the first two weeks, slowly rising to 0.3% by week 4 (Figure 3-5). The plants treated with amorphous silicon dioxide had a more varied xylem sap silicon content. Initial concentrations in week 1 and 2 varied between 0.7 and 0.8% before dropping to 0.4% in week 3 and rising again to 0.9% in week 4 (Figure 3-5).

Figure 3-4. Banana pseudostem 15 minutes after severing the top, showing accumulation of xylem fluid (arrow). For xylem sap collection the first runoff was removed via pipette to clear cellular debris and phloem contaminants and then subsequent fluids (approximately 0.25 mL) were collected.
Figure 3-5. Silicon concentration of the xylem exudate as a percentage of total xylem fluids for banana plants receiving different silicon treatments. Xylem sap was harvested from fresh plants every week for a month. Sap was immediately tested for silicon content and converted to a percentage (%) of total fluid. Solid silicon was supplied as amorphous silicon dioxide powder at a rate of 2 g/kg potting mix. Constant silicon was supplied by daily watering with 1.66 mM monosilicic acid, composed of water and dissolved amorphous silicon dioxide. Bars represent standard error. n = 4 for each time point.

The plants treated with silicon dioxide powder produced consistently less xylem fluid compared to the positive and negative control (data not shown). If samples were left to sit at room temperature instead of immediate processing, the xylem sap extract would turn from clear to brown. The sap from the silicon dioxide treated plants turned a darker shade of brown at a faster rate (data not shown).
Discussion:

Silicon was observed in banana shoot tissues both as discrete bodies and in association with cell walls (Table 3.2; Figure 3.2). This is the first known report of silicon in banana shoot cell walls for banana but the presence of discrete silicon bodies is in agreement with previous descriptions of Musaceae (Tomlinson, 1969). Tomlinson (1969) states that silica is common in all shoot tissues, mostly located in leaf lamina in stegmata cells that contain a trough-like silica body. Hotomura (2004) et al. theorises if silica deposition followed water uptake, then silicon would be concentrated in the mesophyll, where water transpiration occurs, not the epidermis. This was consistent with observations in banana.

In this study, silicon was found to be mostly in the form of discrete bodies in the vascular system of leaves and the pseudostem (Figure 3.2). The deposited silicon bodies were of a similar nature to those reported to be found in orchids (Phalaenopsis sp.) (Shou, 1995). The bodies were organised in a linear fashion along veins in the leaves. However, not all vascular bundles were silicified, and others were not. Older leaves appeared to have more silicified vascular bundles compared to younger leaves (data not shown) which is consistent with the observation that banana leaf silicification increases over time.

Silicon was shown to be in association with cell walls. This is consistent with root data from Chapter 2 where silicon was found exclusively in association with cell walls. From the data, it is unclear whether this means silicon is embedded on or within the walls, or silicon from the cytoplasm or vacuole condensed on the cell wall during processing for SEM-EDS. Determining this would require analysing the tissue using cryo-SEM-EDS, but mature banana shoot tissue is difficult to work with when frozen. Large, fluid filled vacuoles coupled with hard, inelastic cell walls makes clean sectioning extremely challenging. Very small samples would need to be carefully cryo-sectioned to obtain workable material.

When comparing the silicification of banana to other plants, bamboo (Sasa veitchii) is most appropriate, as it is closer in habit and size to banana than any other plant studied for silicon deposition (Lux et al., 2003). Bamboo plants contain silica cells at the abaxial end of the vascular bundle similar to those found in banana and orchids, as well as deposits in
the epidermis (Hutomura et al., 2004). Using SEM-EDS, Hotomura et al. (2004) also found silicon in the cell walls of most tissues, and established that silicification in bamboo increased with age.

Silicon enhances resistance to mechanical forces associated with wind in rice (Savant et al., 1996). Banana plants, being large and herbaceous are susceptible to cyclones in Australia and other tropical/subtropical regions. It is imperative to determine whether structural reinforcement via silicon in the shoot tissue imparts mechanical tolerance to wind in banana plants. Furthermore, understanding how silicon is absorbed and deposited in planta will assist in understanding the overall role silicon plays in plant biology.

For silicon absorption, the amount of silicon absorbed was dependent on the source of silicon supplied (Figure 3-3). This highlights the complex chemistry that occurs in the soil solution when siliceous materials are supplied: not all silicates will provide an equal amount of plant available silicon. Magnesium silicate for example, while high in silicon, is completely water insoluble (Weast et al., 1985). This may affect its ability to convert to monosilicic acid. Amorphous silicon dioxide treatment resulted in the greatest silicon uptake (Figure 3-3). This was expected, as it most readily converts to monosilicic acid (Raven, 1982). Additionally, supplied silicon can also interfere with expression of silicon transporters. Expression of LSI2 is downregulated when exogenous silicon is supplied in maize and barley (Mitani et al., 2009a).

The path silicon follows during uptake and distribution in planta needs to be studied. Elemental germanium is a silicon analogue; plants grown in the presence of germanium will absorb it along the same pathways as per silicon (Rains et al., 2006). This makes radioactively tagged or isotopic germanium (Ge-68) a useful tool for studying silicon uptake in live plants (Nikolic et al., 2007). Elemental germanium can be also used as a simple visual tag for silicon uptake. In rice, absorption of germanium in place of silicon causes low-level toxicity leading to brown spotting on leaves (Richmond and Sussman, 2003). This allows for study of silicon uptake but is also a useful tool for rapid visual screening of mutants that absorb more or less silicon.
An often ignored area of silicon research is the use of silicon solubilisers: substances that increase the availability of silicon in the soil or enhance overall uptake (Ranganathan et al., 2006). This was first described by Parry (1975) with poly-2-vinyl-pyridine 1-oxide (PVO) on rice, which enhanced silicon uptake even in the absence of exogenously supplied silicon, likely due enhanced uptake of silicon contaminants in the growth environment. Further studies have identified more silicon solubilisers, including: histidine, imidazole, glutamic acid, glycine and glutamine (Voleti et al., 2008). These solubilisers should be investigated in conjunction with various sources of silicon.

The xylem silicon vertical flux results for the plants treated with silicon dioxide followed an unexpectedly nonlinear trend (Figure 3-5). It was thought that a constant supply of plant available monosilicic acid would greatly increase the xylem fluid silicon concentration, but this was not the case. The plants with less available silicon dioxide had the highest rate of silicon. This suggests that banana plants are capable of preventing silicon from being transported through the xylem via exclusion, or that vertical flux varies considerably throughout the day. This is consistent with the observations of Henriët et al. (2006) that silicon uptake in banana is active (via transporters) at low concentrations and passive (via transpiration) at high concentrations. This has implications for soil management of silicon in the field.

Silicon transport in the xylem is not fully understood. Silicon exists in the xylem of accumulator plants as monosilicic acid at concentrations far above the threshold of ~2 mM that results in automatic polymerisation to silicon dioxide (Mitani et al., 2005a; Casey et al., 2003). The current theory is that these concentrations exist only transiently, and are not present long enough to result in polymerisation. Raw xylem fluid will not prevent polymerisation of monosilicic acid (Mitani et al., 2005a).

A constant supply of plant available silicon may be necessary for silicon to exert beneficial effects. If that is the case, total plant silicon content is not a good representation of silicon available to the plant. Silicon absorption, if it follows transpiration of water, can be expected to change seasonally: higher rates of deposition in summer and lower deposition in winter. Season variation of silicon absorption has been observed in bamboo (Motomura et al., 2002).
The enhanced browning of xylem sap from silicon treated plants was an unexpected but potentially interesting result. The rapid browning in silicon treated plants may be a defence response induced by cutting, perhaps as a herbivory response. This may represent the increased storage of premade phenolics, which are integral in the constitutive defence of banana against pathogens and mechanical wounding (Beckman and Mueller, 1970). Whether these phenolics were released intentionally or unintentionally is unclear.

In conclusion, understanding the location, uptake and deposition of silicon in planta will help explain how silicon functions as a beneficial nutrient. Deposited silicon most likely plays a structural role in banana plant shoots and may assist in preventing pathogen foliar pathogen penetration toward the vascular system. Finally, the role of soluble silicon in shoot tissue needs to be investigated.
Chapter 4 - Silicon enhances tolerance of banana to fusarium wilt

Abstract:

Background and Aims: Silicon is being investigated as a possible soil amendment for making banana plants more tolerant of the soil borne fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The addition of elemental silicon has been shown in other plant pathosystems to up-regulate defences causing plants to experience less disease symptoms. In this study, the protective effect of silicon was investigated in ‘Cavendish’ banana plants by a pot experiment. Possible mechanisms for silicon function were investigated via electron microscopy and enzyme assays.

Methods: Banana plants inoculated with *Foc* and treated with 2 g/kg amorphous silicon dioxide powder were investigated in a pot trial and their total phenolics and lignin were quantified. Banana plantlets were cultured in tubs containing Murashige & Skoog medium under sterile conditions. These plantlets were inoculated with *Foc* spore suspension onto the root surface. Roots were harvested after 3, 6 and 12 days post inoculation and investigated with transmission electron microscopy. An enzyme assay was also performed to identify levels of phenolics and lignin during fungal infection.

Key Results: Fusarium wilt internal symptoms in banana were significantly lower in the silicon treated plants. Transmission electron microscopy data indicated that biochemical responses were occurring intracellularly, appearing to delay fungal colonisation. The enzyme assay demonstrated a significant increase in both phenolics and lignin in silicon treated plants after inoculation.

Conclusions: Silicon enhances tolerance to Fusarium wilt in ‘Cavendish’ banana plants. Plants treated with silicon still develop symptoms, but at a slower rate compared to untreated plants. Experimental data strongly implies that silicon is modulating a defence response via an unknown signalling pathway, resulting in presumed upregulation and downregulation of genes involved in plant defence and metabolism.
Introduction:

The global banana industry currently faces a panphytotic outbreak of fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Similar outbreaks have occurred in the past. The worst outbreak took place in the first half of the 20th century where what was later to be known as race 1 of *Foc* caused the near universal collapse of the banana cultivar ‘Gros Michel’ (Ploetz and Pegg, 2000). The banana industry recovered by introducing the race 1 resistant cultivar ‘Cavendish’ (Jeger et al., 1995). ‘Cavendish’ resistance proved temporary, and by the late 1990s new races of *Foc* were causing severe disease in banana plantations (Pegg et al., 1996).

Fusarium wilt symptoms begin internally as the fungus colonizes roots at the site of infection. Internal discolouration is visible within the xylem of colonised roots. Over a variable period of time, the fungus spreads to the rhizome and then into xylem tissue of the pseudostem (Daly and Walduck, 2006; Beckman, 1990). The exact timing of fungal infection has not been established, with plant death occurring from anywhere from a few months up to two years depending on environmental conditions (Jeger et al., 1995).

External symptoms are characterised by wilting, yellowing and necrosis of the leaf margin, drooping, splitting of the pseudostem and eventual collapse and death of the plant (Rishbeth, 1955; Daly and Walduck, 2006). These symptoms can appear at any point during the infection process, but rapidly worsen as the fungus colonises the pseudostem. A combination of fungal obstructions and induced plant defences such as tyloses and gums block the flow of water in the xylem cells, leading to the development of wilt symptoms (Stover, 1972a).

Although long sought after, there are no effective alternative controls for fusarium wilt (Pegg et al., 1996). Fungicides such as methyl bromide, carbendazim and potassium phosphonate can temporarily control *Foc* (Ploetz and Pegg, 2000), but it is rarely cost effective and the fungus will almost always recolonise treated soil (Ploetz, 2006). Quarantine practices can slow the spread of *Foc* (Hennessy et al., 2005). The only way to truly overcome an *Foc* infestation is to plant a resistant cultivar (Buddenhagen, 1990).
The infection process of *Foc* in banana has not been fully characterised. The primary determinative phase (PDP) (Beckman, 1987), the phase in which *Foc* penetrates and colonises roots, has not been studied as extensively as vascular colonisation, the secondary determinative phase (SDP). PDP has been characterised to an extent in several susceptible species including: *Arabidopsis thaliana* (Czymmek et al., 2007), tomato (Lagopodi et al., 2002), cotton, (Rodríguez-Gálvez and Mendgen, 1995) and flax (Olivain et al., 2003). The intensity of defence responses during PDP are thought to be one of the primary factors determining whether *Foc* will successfully cause disease or not.

One of the most important defence compounds present in banana are phenolics (de Ascensao and Dubery, 2003). The term phenolics is used to describe a wide variety of phytochemicals present in most plants, which are produced by plants when challenged by a pathogen (Matern et al., 1995). Phenolic defences are frequently associated with rapid accumulation near the infection sites (Matern et al., 1995). Phenolics are often divided into two categories: the phytoanticipins which are present constitutively in cells as a preformed defence, and the phytoalexins that are produced in response to pathogen penetration (Nicholson and Hammerschmidt, 1992). In the Goldfinger cultivar of banana, *p*-coumaric, ferulic, sinapic and vanillic acid phenolics have all been detected (de Ascensao and Dubery, 2003). Lignin is a phenolic polymer, often produced as a structural reinforcement in plant pathogen defence (Bhuiyan et al., 2009). Phenolic and lignin levels can increase when exposed to pathogenic elicitors within hours and even minutes (de Ascensao and Dubery, 2003).

Understanding how the banana plant defends itself against *Foc* is necessary to develop novel control strategies. Although genetic resistance is being developed, alternative treatments focusing on soil amendments and biological control are being investigated out of necessity. Research in other plants has implicated elemental silicon as a possible positive mediator of plant defences against pathogens (Guntzer et al., 2012).

Silicon rarely occurs as free element and is usually found in the soil as silicon dioxide or a silicate and is the second most abundant element in the soil after oxygen (Richmond and Sussman, 2003). The silicon content of plants ranges from 0.1 to 10% dry weight,
depending on genotype, soil concentration and environmental conditions (Epstein, 1994). Silicon is absorbed from the soil via a combination of passive flow and active transporters (Mitani et al., 2009). The mechanism by which silicon is beneficial for plants is not understood (Ghanmi et al., 2004). Under optimum conditions, silicon does not affect plant growth or development (Henriet et al., 2006; Epstein, 1994).

Silicon is involved in mediating plant defences. Whether this is due to deposited silicon creating a physical barrier, or soluble silicon modulating plant signalling pathways is unknown. Several researchers have postulated that a silicon barrier enhances resistance by preventing fungal penetration and increasing resistance to enzymatic degradation (Ma, 2004; Stein et al., 1993). Rice (Oryza sativa) plants supplied with silicon showed increased tolerance to rice blast (Magnaporthe grisea) which Datnoff et al. (2001) demonstrated was due to silicification of the epidermis. It was further demonstrated that there was a decrease in appressorial penetration due to silicification of rice leaves (Hayasaka et al., 2008). If plant root tissue becomes sufficiently silicified, it may also resist fungal penetration in a similar fashion.

The possibility that silicon in the soil solution or within the plant itself is harmful to fungi has been theorised but not fully explored. Two studies have been undertaken on the potential antifungal activity of exogenously supplied silicon to fungal cultures (Li et al., 2009; Kaiser et al., 2005). Both showed decreased fungal growth and fungal malformation in cultures treated with soluble silicon, but were unable to exclude possible pH effects as soluble silicon is highly alkaline (pH > 11).

The beneficial effects of silicon have been linked not only to the production of phytoalexins and other plant based defence compounds, but also to the upregulation of defence genes (Epstein, 1994; Epstein, 1999; Ma, 2004). Previous work at The University of Queensland has demonstrated silicon has the capacity to suppress Foc in banana by upregulating defence genes (Forsyth, 2006). Calcium silicate applications have been recently shown to suppress Foc symptom development in the susceptible “Maçã” variety of banana (Fortunato et al., 2012a). Kablan et al. (2012) demonstrated that silicon treatment enhances resistance of banana to black Sigatoka, a fungal foliar disease.
Due to the variable nature of silicon effects on plants, silicon application experiments combined with pathogenicity tests are necessary to establish whether silicon is beneficial in the *Foc* banana pathosystem. Previous research in silicon has favoured the use of silicates in trials e.g., potassium silicate (K$_2$SiO$_3$), sodium silicate (Na$_2$SiO$_3$) although acidic cation exchangers can be used to remove the K and Na ions (Henriet et al., 2006; Kablan et al., 2012). The possibility of these conjugate ions (K$^+$/Na$^+$) interfering with experimental results could not be ruled out. Recent studies have begun to use variants of silicon dioxide in an effort to overcome this (Hayasaka et al., 2008). The majority of experimentation in this project will make use of amorphous silicon dioxide (SiO$_2$) which is readily converted to monosilicic acid (Raven, 1982).

The goal of this research was to determine if silicon treatment enhances the tolerance of banana plants to *Foc* infection, and if so, by what magnitude. Additionally, components of the predicted silicon-mediated defence response in banana were characterised in an effort to help understand the mechanism by which silicon works. A pot trial was established to determine the effectiveness of silicon on bananas, and to investigate silicon mechanisms. A system of inoculating and harvesting roots of banana plants grown on agar was developed to minimise disturbance of the roots during processing, therefore allowing an accurate analysis of biochemical activity taking place in the roots. The infection process of *Foc* was partially characterised using TEM.
Chapter 4 | Silicon enhances tolerance to *Foc*

**Methods & Materials**

*Pot Trial*

‘Cavendish’ banana plants from tissue culture stocks at The University of Queensland were deflasked into seedling trays containing University of California II (UC) mix and hardened in enclosed plastic trays for 4 weeks until acclimated. Plants were then transferred to 20cm pots in UC mix, with half of the pots containing silicon as amorphous silicon dioxide power (Sigma-Aldrich) at a rate of 2 g/kg potting mix. To avoid disturbing the root system during inoculation, three polycarbonate plastic tubes of 10mm diameter were inserted into the potting mix in a triangular formation around the base of the plants (Appendix 6).

All growth took place in The University of Queensland glasshouse facilities, with daily watering until replete and temperatures consistent with Brisbane, Qld, Australia from Oct 2009 to Mar 2010. Once in 20 cm pots, plants were fertilised every 14 days with full strength Thrive (Yates) liquid fertiliser.

Inoculum was prepared on millet grains as per Smith et al. (2008). Briefly, untreated millet (*Echinochloa esculenta*) grains were soaked in tap water overnight and strained. Grains were placed into 10 cm sealable tubs, autoclaved, rinsed with sterile distilled water and autoclaved again. Once cooled, each tub was inoculated using sterile technique with plugs from three *Foc* subtropical race 4 isolates (VCG 0120: BRIP no: 22615, 24322 and 23598). Multiple isolates were used to ensure pathogenicity was intact. Tubs were left in the dark at room temperature for 2 weeks to promote colonisation of the grains and were shaken daily to avoid clumping.

Plants were inoculated 2 months after deflasking (1 month hardening, 1 month growth in glasshouse) with the inoculated millet grains. Two days before inoculation, the polycarbonate tubes were removed, leaving a void in the potting mix, which was temporarily covered until inoculation. Inoculum was removed from tubs, and inserted directly into the voided potting mix at a rate of 8 g/kg potting mix. Each void was then filled in with potting mix (Appendix 6).
Treated plants were subsequently grown in the glasshouse for 14 weeks. External symptoms were recorded weekly. Plant height and fresh weights were both analysed via a two-way ANOVA with Tukey’s post-test. Plants were harvested at 14 weeks and internal symptoms were described using a modified version of the INIBAP guidelines (Orjeda, 1998). Briefly, 0 = no discolouration; 1 = minor flecking in the rhizome; 2 = less than 1/3 rhizome discolouration; 3 = less than 2/3 rhizome discolouration; 4 = greater than 2/3 rhizome discolouration; 5 = total discolouration; 6 = total discolouration plus necrosis/rotting. This data was converted to a disease severity index as per Sun et al., (2010) and compared using a Student’s T-test. Briefly, the disease index was calculated as follows:

Disease index(%)={ \( \frac{N_1 + 2N_2 + 3N_3 + 4N_4 + 5N_5 + 6N_6}{6N_t} \) ×100

Where \( N_1 \) to \( N_6 \) equal the number of scored plants in the modified INIBAP system described above and \( N_t \) equals the total number of plants scored. Finally, \( Foc \) was reisolated from diseased tissue to confirm infection.

**Plate trial**

Petri dishes were prepared with full strength potato dextrose agar amended with amorphous silicon dioxide powder (Sigma-Aldrich) at rates of 1 g/L, 2 g/L and 4 g/L. Each plate was inoculated with a plug of \( Foc \) (BRIP no: 22615) from carnation leaf agar (CLA) plates. Plates were subsequently incubated in darkness for 4 days at a constant temperature of 28°C. Colony diameter and spore counts were measured. For spore counts, plates were flooded with sterile distilled water and aliquots were measured using a haemocytometer. Results were subjected to a one-way ANOVA test with Tukey’s post-test to confirm differences between means.

**Soilless inoculation and TEM trial**

A soilless cultivation system (SCS) was developed to facilitate inoculation and harvesting of roots. Plastic round tissue culture tubs of 190 mm diameter were obtained for tissue culture. Growing media for banana plantlets was 1% Murashige & Skoog media (Sigma-
Aldrich) with the addition of 2% Phytagel™ (Sigma-Aldrich). The high concentration of Phytagel™ was used to prevent roots from penetrating the media, and forcing them to grow exclusively on the surface. When required, silicon was added as 1.66 mM monosilic acid.

Tissue cultured ‘Cavendish’ “Williams” banana plantlets were obtained from The University of Queensland tissue culture facility. Healthy, green plantlets were selected and subcultured onto the media and sealed. Tubs were placed into a temperature controlled light room with an illumination cycle of 10 hours at 1000 lux, and 14 hours of darkness and constant temperature of 26°C. Plantlets were grown for 4 weeks wherein leaves had fully emerged and primary roots were beginning to differentiate into secondary roots.

Spore suspension inoculum was prepared by subculturing square plugs of Foc sub-tropical race 4 (BRIP: 22615) colonised agar into 1/4 strength potato sucrose broth growing media. This medium has been previously established experimentally to produce greater spore numbers with this particular isolate compared to potato dextrose broth (data not shown). After 5 days of growth, the liquid was strained through cheesecloth to remove mycelia. The spore suspension was diluted with sterile distilled water to achieve a concentration of 1x10⁶ spores per ml. The protective effect of silicon is independent of inoculum concentrations in the tomato/Fusarium oxysporum f. sp. radicis-lycopersici pathosystem (Huang et al., 2011), therefore only one level of spore concentration was used throughout the experiment.

Inoculation and harvest for TEM

Roots were inoculated in the zone of extension (approximately 5mm behind the root tip) of around five primary roots per plant. To inoculate, the spore suspension was mixed with red food dye and pipetted directly on the root surface under sterile conditions. The dye marked the root surface, allowing reference back to the point of inoculation in subsequent days. Uninoculated controls were mock-inoculated with sterile distilled water mixed with red food dye. To test root extension, the distance the root tip progressed from the point of inoculation was measured with a ruler.
Roots were harvested at 3, 6 and 12 days post inoculation to observe progress of the fungus over time. *Fo* pathogenesis is temporally complex, but important defence events are known to occur in the first week following inoculation (Benhamou and Lafontaine, 1995). To harvest for TEM, a 500 μL drop of fixative made up of 2.5% glutaraldehyde in phosphate buffer (7.4 pH) with 0.7% caffeine was pipetted on each inoculation site. Caffeine was added as it assists in the visual detection of phenolics in electron micrographs (Mueller and Greenwood, 1978). Root sections 5 mm length encompassing each inoculation site were excised transversely and then placed in a vial of fixative in preparation for processing.

Processing was performed in a Pelco microwave processing system (Pelco International, Clovis, CA, USA). Ideal processing for banana roots colonised by *Foc* were established prior to this experiment. For the full protocol, see Appendix 3. After processing, fixed and embedded samples were sectioned on a Leica EM UC6 Ultramicrotome. Thick sections (~1 μm) were taken using a glass knife, the sections were then stained with toluidine blue and viewed with a BX-61 Olympus light microscope fitted with a digital camera/optical capture system. General features of *Foc* infection were apparent at this point and were noted.

For viewing on the TEM, thin sections of approximate 70 nm width were cut using a Diatome diamond knife on an ultramicrotome. Sections were transferred to copper grids which were subsequently post stained with uranyl acetate and lead citrate (see Appendix 4). Samples were visualised at 80 kV accelerating voltage on a JEOL 1010 transmission electron microscope (JEOL, Ltd, Akishima, Tokyo, Japan) with a SIS Megaview III Slowscan Camera and Image Capture System (Olympus, Pennsylvania, USA).

*Phenolic and lignin analysis*

Total soluble phenolics and gross lignin were analysed at 3 dpi (days post inoculation) and 6 dpi in plants inoculated with *Foc*. ‘Cavendish’ banana plants from tissue culture stocks at The University of Queensland were deflasked into seedling trays containing University of California II (UC) mix and hardened in enclosed plastic trays for 4 weeks until acclimated. *Foc* colonised millet was prepared as above. Plants were potted up into 10 cm pots with
inoculation tubes (see Appendix 6). After 4 days, the plants were inoculated with infested millet, prepared as above, at a rate of 8 g/kg potting mix. At 3 dpi and 6 dpi, plants were carefully uprooted, rinsed in distilled water then snap frozen by plunging in liquid nitrogen. This was performed as rapidly as possible. The concentration of phenolics and lignin were assessed as per Whan (2009) who modified the phenolics procedure from Payet et al. (2006) and the lignin procedure from Barber and Ride (1988).

**Phenolic quantification procedure**

Total soluble phenolics were analysed using the Folin-Ciocalteau (FC) reagent method. Snap frozen roots were individually ground to a fine powder using a liquid nitrogen cooled mortar and pestle. Approximately 1 g of powder was transferred to a 15 mL Falcon tube (BD Biosciences, USA) and 10 mL/g of extraction solvent consisting of 60% v/v methanol in sterile distilled water was added. The resulting mixture was vortexed and placed in a dark cupboard for 4 hours. After resting, the tubes were vortexed again, filtered through Miracloth and 1.5 mL aliquots transferred to microcentrifuge tubes. The extract was centrifuged at maximum speed for 5 minutes and the supernatant transferred to fresh 1.5 mL tubes before being diluted with equal amounts of sterile distilled water. The solid matter that remained was preserved for lignin analysis. Gallic acid (Sigma-Aldrich) was used to construct a standard curve for analysis. A 2 mg/mL stock solution of gallic acid was prepared by dissolving 0.2 g of gallic acid in 10 mL of ethanol and diluting to 100 mL with sterile distilled water. Serial dilutions were performed with sterile distilled water to give final concentrations of 0.2, 0.1, 0.05 and 0.0 mg/mL.

Spectrophotometric assays were performed in plastic cuvettes using a double beam spectrophotometer (Hitachi U2800). Controls runs were performed by making a mock aliquot with all reagents bar FC reagent. To each cuvette the following was added: 30 μL of extracts, 150 μL of FC reagent (10% v/v) or sterile distilled water for controls, and 120 μL of sodium carbonate solution (7.5% w/v). Samples were gently mixed then incubated in the dark for 2 hours at room temperature. After this, the absorbance of each sample was measured at 725 nm. Results were expressed as mg GA/g dried weight.
Lignin quantification procedure
Gross lignin quantification was done via the thioglycollic acid (TGA) method. The solid plant residue remaining after extraction for phenolics analysis was resuspended in 1.5 ml of sterile distilled water, centrifuged at 12000 g for 5 minutes and the resultant supernatant discarded. The washed pellet was oven dried at 65°C in 200 μl plastic tubes before 1.5 ml of a 1:10 solution of thioglycollic acid to 2 N HCL was added. The homogenized residue was boiled for 4 hours in a hot water bath and subsequently cooled on ice for 10 minutes. Tubes were centrifuged at 12000 g for 5 minutes and the supernatant discarded. The remaining precipitate was washed with 1.5 ml of sterile distilled water, centrifuged at 10000 g for 10 minutes, supernatant discarded and 1.5 ml of 0.5 N NaOH was added.

The reactions were placed on a rotary shaker at room temperature for 24 hours. After this, tubes were centrifuged at 10000 g for ten minutes and the supernatant transferred to fresh tubes. To the extract was added 200 μl of concentrated HCl and placed in a cold room set at 4°C for 4 hours to precipitate ligninthioglycollic acid (LTGA). The suspension was then centrifuged at 10000 g for 10 minutes, the precipitate retained and dissolved in 2 ml of 0.05 N NaOH. The absorbance of each sample was read using a spectrophotometer (Hitachi) at 280 nm. A sample curve was constructed as per the phenolics procedure using lignin alkali, 2-hydroxypropyl ether (Sigma-Aldrich) as a lignin standard. Readings were expressed as percentage (%) lignin content/g dry weight.

Data analysis
Graphs were generated in Microsoft Office Excel 2010. One-way and two-way ANOVAs and Student’s T-tests were performed in Graphpad Prism version 6 for Windows. For all tests, significance was set at (p < 0.05) and all error reported as standard error of the mean (SE). Post-processing of images was consistent across images and performed in either ImageJ version 1.44p for Windows or Photoshop CS2 Version 9, Adobe.
Results:

*Pot trial disease assessment*
Fusarium wilt symptoms developed over a period of 3 months, with initial wilting symptoms appearing after approximately 3 weeks. Plants rapidly recovered from the initial wilt and then gradually declined until full Fusarium wilt symptoms were apparent. Symptoms were not uniform and varied considerably, although all inoculated plants showed some signs of *Foc* colonisation, confirmed by re-isolation and growth on plates (data not shown). No symptoms were apparent in the uninoculated controls and no fungus was isolated from the roots.

Overall, silicon treated plants inoculated with *Foc* had significantly less internal discolouration than untreated plants, with a greater number of rating 2 plants and total absence of rating 6 compared to the control (Figure 4-1). The calculated disease severity ratings were 75.55% for untreated plants and 47.77% for silicon treated plants. The difference was statistically significant. For external symptoms, there was no significant difference between silicon treated and untreated for leaf discolouration splitting, with both inoculated silicon treated and untreated plants having approximately four fully expanded green leaves, two to three yellow leaves and seven to eight necrotic leaves. For height, silicon treated inoculated plants were significantly taller than all other treatments (Figure 4-2). All inoculated plants showed a significant decrease in weight compared to uninoculated (Figure 4-3).
Figure 4-1. Symptoms of fusarium wilt 14 weeks after inoculation as measured by internal discolouration of the rhizome for banana plants treated with or without silicon. Uninoculated control plants (treated with and without silicon) displayed no discolouration. All inoculated plants displayed some degree of discolouration. Internal discolouration is measured on the INIBAP (International Network for the Improvement of Banana and Plantain) scale for internal symptoms from 0-6 of increasing disease symptoms. 0 = no discolouration; 1 = minor flecking in the rhizome; 2 = less than 1/3 rhizome discolouration; 3 = less than 2/3 rhizome discolouration; 4 = greater than 2/3 rhizome discolouration; 5 = total discolouration; 6 = total discolouration plus necrosis/rotting.
Figure 4-2. Mean height in (mm) of *Fusarium oxysporum* f. sp. *cubense* inoculated and uninoculated banana plants treated with 2 g/kg amorphous silicon dioxide (+Silicon) and without (-Silicon). Heights were measured 14 weeks after inoculation with a ruler from soil level to the top of the petiole of the youngest, fully expanded leaf. Different letters denote statistically significant difference within treatment groups (between inoculated and uninoculated. Bars represent standard error (p < 0.05, n = 15).
Figure 4-3. Shoot and root mean fresh weights in grams (g) of Fusarium oxysporum f. sp. cubense inoculated and uninoculated banana plants treated with 2 g/kg amorphous silicon dioxide (silicon) and without (control). Plants were uprooted 14 weeks after inoculation, carefully rinsed with distilled water and all potting mix removed. Shoots and roots were thoroughly dried with paper towels and immediately weighed on digital scales. Bars represent standard error, n = 15.

Plate trial
Growing Foc on plates amended with silicon at all three rates tested caused no obvious phenotypic changes (Figure 4-4). There was no statistically significant difference in mean diameter of Foc colonies for all treatment groups (Table 4-2). The addition of amorphous silicon dioxide did not significantly alter the pH of media (Table 4-2). Number of conidia recovered from each plate by washing with sterile distilled water did not change between treatments or compared to control (Table 4-2).
Figure 4-4. Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) fungus after 4 days on potato dextrose agar (PDA) amended with silicon at the rates $A = 0$ g/L, $B = 1$ g/L, $C = 2$ g/L and $D = 4$ g/L. There were no phenotypic differences in *Foc* when exposed to increasing amounts of silicon. $n = 5$.

Table 4-1. Mean diameter of cultures of *Fusarium oxysporum* f. sp. *cubense* grown on PDA plates amended with varying amounts of amorphous silicon dioxide. The pH values are of each media before pouring. Error is standard error, $n = 5$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean diameter (cm)</th>
<th>Spore count ($10^4$/mL)</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.95 ± 0.23 a</td>
<td>154 ± 8 a</td>
<td>5.37 ± 0.04 a</td>
</tr>
<tr>
<td>1 g/L silicon dioxide</td>
<td>6.96 ± 0.27 a</td>
<td>180 ± 14 a</td>
<td>5.43 ± 0.03 a</td>
</tr>
<tr>
<td>2 g/L silicon dioxide</td>
<td>6.81 ± 0.29 a</td>
<td>145 ± 5 a</td>
<td>5.46 ± 0.05 a</td>
</tr>
<tr>
<td>4 g/L silicon dioxide</td>
<td>6.92 ± 0.15 a</td>
<td>172 ± 10 a</td>
<td>5.42 ± 0.01 a</td>
</tr>
</tbody>
</table>

**Soilless Foc inoculation TEM trial**

**Uninoculated**

Uninoculated roots were generally cream coloured with a profusion of root hairs in the extension zone. Root extension did not differ between control and silicon treated uninoculated roots (Figure 4-5). When viewed via TEM, cells were intact and orderly with no signs of processing artefacts or deformation (data not shown). What appeared to be detached cells were frequently seen in proximity to the epidermis. No cellular damage or
disorganisation was observed. There was no apparent difference between silicon treated and non-treated roots.

**Figure 4-5.** Root extension (mm) of primary roots after inoculation with *Fusarium oxysporum* f. sp. *cubense*. Banana roots growing in specialised soilless cultivation tubs were inoculated with a drop of spore suspension containing red food dye. At 3 days post inoculation the amount of growth that occurred from the point of inoculation (stained red) was measured with a ruler. This point was subsequently marked, and at 7 dpi the distance extended from that point was measured. Different letters denote statistical significance. Bars represent standard error, n = 20 per treatment.
Inoculated 3dpi

For external root symptoms, surface discolouration of the root was visible at 3 dpi, manifesting as reddish-to-dark brown patches. Mycelia had entirely colonised the elongation zone of the root and was beginning to colonise media around the root. Root extension had slowed (Figure 4-5).

General observations for 3 dpi are as follows. In all cases, hyphae had massed externally to the epidermis (Figure 4-6). Often this was in contact with the root hairs and epidermal cells. Generally there was no evidence of direct penetration: no hyphae were identified actively penetrating the epidermis. Epidermal cells were frequently colonised.
Figure 4-6. Transverse root section showing example of fungal density at 3 days post inoculation in the rhizosphere, i.e. beyond the epidermis. (A) Hyphae were frequently observed massed beyond the epidermis but rarely in direct contact. Note the vacuolation occurring in most hyphal cells (*). This is usually indicative of chitinase activity. (B) Hyphal activity in direct contact with the epidermis. Note the hyphae growing in the grooves between epidermal cells (EC). Fibrous material was present in association with hyphae.
(arrow) suggesting degradation of cell walls. Abbreviations: H, hyphae; EC, epidermal cell; RH, root hair; BC, border cell.

Hyphae external and in the epidermis frequently showed evidence of severe vacuolation (Figure 4-6). Vacuolation manifests as large, slightly opaque ovals appearing in hyphal cytoplasm. This is in stark contrast to *Foc* observed under non-pathogenic conditions (data not shown). Vacuolation was only observed in hyphae either external to the root, or in the process of penetrating through the epidermis and occasionally hypodermis.

In inoculated, silicon untreated roots, hyphae were present intracellular in the epidermis and occasionally hypodermis, but were almost exclusively intercellular when reaching the cortex (Figure 4-7). Very few direct penetration events were visually observed in any of the cell types. From the results, it was unclear where *Foc* was penetrating to gain access to the root. Hyphae were never observed in the stele or the xylem.
Figure 4.7. Examples of fungal penetration/defence response in inoculated control roots (without silicon). A) Epidermal and hypodermal response of control root at 3 days post inoculation. Little evidence of defence responses (accumulation of osmiophilic compounds) can be seen. B) Inner cortex defence response in control 3 days post inoculation. Note the accumulation of electron dense granules (arrow). C) The furthest hyphae were detected was when they had penetrated into the intercellular spaces of the
inner cortex. Abbreviations: H, hyphae; EC, epidermal cell; HC, hypodermal cell; RH, root hair; CC, cortical cell; IS, intercellular space.

Silicon treated roots showed similar degrees of fungal colonisation, but also displayed evidence of enhanced biochemical and physical defence responses (Figure 4-8). This included the distinctive buckling of cells sometimes seen in root defence against pathogens (Olivain et al., 2003). Buckling, also called cell collapse, is characterised by the complete collapse of cells followed by the rapid influx of electron dense material, presumably phenolics or phytoalexins (Figure 4-8, arrows).
Figure 4-8. Examples of fungal penetration/defence response in 3 days post inoculation silicon treated roots. A&B) Two instances of defence responses occurring at the epidermis, hypodermis and outer cortex. Hyphae appear to colonise the epidermis with little plant response. Cellular buckling (arrows), sometimes called cellular collapse is occurring in the hypodermis and some cortical cells. Intercellular spaces are also filling with electron dense material (arrowheads). Abbreviations: EC, epidermal cell; HC, hypodermal cell; CC, cortical cell; H, hypha.
Electron dense accumulations were often seen in the hypodermis and sometimes the outer cortex (Figure 4-8). The roots of silicon treated plants often experienced a unique response visible as the accumulation of electron dense globules (Figure 4-9). These globules were found in root hairs and occasionally in the hypodermis and cortex (Figure 4-9). Globules ranged in size from approximately 4000 nm to 2000 nm and were roughly spherical in all cases. As they were not observed in contact with or in proximity to any malformed or dead hyphae they were assumed to be not fungitoxic. The globules were occasionally observed outside cells (Figure 4-10). Globules were possibly observed in the endodermis, but not confirmed (data not shown).
Figure 4-9. Examples of uncharacterised electron-dense globules (arrows) in roots of silicon treated banana plants grown in SCS at 3 dpi. A) Globules present in a root hair extending from the epidermis. B) Border cell or detached epidermal cell in the rhizosphere. C) Globule present in a collapsed/buckled cortical cell in the inner cortex. Abbreviations: RS, rhizosphere; RH, root hair; BC, border cell; CC, cortical cell.
Figure 4-10. Examples of electron-dense globules observed external to the root in the rhizosphere. A) Several globules in association with hyphae near a border cell or detached epidermal cell. B) Another border cell with associated globules and hyphae. Abbreviations: H, hyphae; BC, border cell; RS, rhizosphere.
After observing several samples to determine how widely symptoms varied, a system was established to quantify severity of pathogenicity (see Methods & Materials). Each root section viewed was assigned a score ranging from A to D based on how far hyphae had penetrated into the root. All roots were divided into categories summarised in Table 4-1:

**Table 4-2.** Rating system devised after TEM observations to categorise stages of pathogenicity in banana roots colonised with *Foc* at 3 dpi.

<table>
<thead>
<tr>
<th>Score</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>hyphae present in the endodermis or inner cortex</td>
</tr>
<tr>
<td>B</td>
<td>hyphae present in the outer cortex and hypodermis coupled with a low defence response in the hypodermis (lack of buckling, little accumulation of electron dense compounds)</td>
</tr>
<tr>
<td>C</td>
<td>same as B but hyphae restricted to the hypodermis</td>
</tr>
<tr>
<td>D</td>
<td>hyphae in hypodermis only coupled with a high defence response (cell collapse/buckling, concentration of electron dense material)</td>
</tr>
</tbody>
</table>

Twelve individual root sections were observed for control and silicon treated plants then scored according to Table 4-2 (Figure 4-11). This was converted to a severity index as described in the methods. Silicon treated roots had a greater proportion of roots with the highest defence response and least fungal ingress (Figure 4-11). Silicon roots had a significantly lower severity index at 19.23% compared to 46.15% in the control plants. Instances of “D”, hyphae in hypodermis only coupled with a high defence response, were only observed in silicon treated roots at 3 dpi.
Figure 4-11. Internal symptoms of roots of *Fusarium oxysporum* f. sp. *cubense* inoculated banana plants treated with and without silicon (control) at 3 days post inoculation. Severity of infection was inferred based on physical progression of the mycelia coupled with the intensity of the defence response observed. Legend indicates the extent of hyphal ingress: A = hyphae present in the endodermis or inner cortex; B = hyphae present in the outer cortex and hypodermis coupled with a low defence response in they hypodermis (lack of buckling, little accumulation of electro dense compounds); C = same as B but hyphae restricted to the hypodermis; D = hyphae in hypodermis only coupled with a high defence response (cell collapse/buckling, concentration of electron dense material

Inoculated 6dpi

For external root symptoms, patches of surface discolouration were uniformly brown, with some roots showing necrosis. Mycelia had colonised the media in a large area around the roots. Root extension had practically ceased (Figure 4-5). Upon sectioning, reddish discoloration was often visible inside the root, in a continuous patch from the epidermis toward the stele.

External hyphae were observed to be massing directly outside the root (Figure 4-12). Unlike 3 dpi, hyphal massing was not uniform, with some samples showing large numbers of hyphae, and others showing decreased numbers compared to 3 dpi (compare Figures 4-12 to 4-6). When quantified, silicon treated roots displayed a higher proportion of low fungal density observations (Figure 4-12).
Figure 4-12. Transmission electron micrographs of *Foc* inoculated banana plantlets grown in SCS at 6 dpi. Hyphal density in the rhizosphere and on the epidermis varied between root samples. Each root was categorised into low, medium or high categories based on a visual assessment of the density of hyphae. (A) Low fungal density. (B) Medium fungal density. (C) High fungal density. Note the vacuolation of hypha (arrows). H, hypha; EC, epidermal cell. (D) Percentage (%) of sampled roots displaying either A, B, or C levels of fungal density. Different letters (a,b) denote statistically significant difference (p < 0.05, n = 12).
Observable defence responses were less consistent at 6 dpi. Buckling as a defence response did not occur as smoothly or regularly (Figure 4-13). Buckling was now occurring in the hypodermis, all areas of the cortex and the endodermis (Figure 4-13). External hyphae were still showing severe vacuolation, but such was not present at all in hyphae in internal banana tissues (Figure 4-12).

**Figure 4-13.** Quantification of cellular buckling as a defence response in the roots of *Fusarium oxysporum* f. sp. *cubense* inoculated banana plants at 6 dpi. Buckling is the structured collapse of plant root cells in response to a pathogen followed by the rapid influx of phenolics and/or phytoalexins. From root exterior to the stele: hypodermis -> outer cortex -> inner cortex -> endodermis. The frequency of buckling was rated on a scale of 0%, 25%, 50%, 75% and 100% depending on the approximate number of cells effected. No buckling was observed in uninoculated roots. Bars represent standard error (p < 0.05, n = 12, number of roots observed).

Globules were found in approximately half of both silicon and control samples, located in the same places observed at 3 dpi. These globules were occasionally visible in xylem parenchyma cells through all other tissues. Hyphae had progressed as far as the xylem, including in the xylem parenchyma cells (Figure 4-14). Internal symptoms were quantified in a similar fashion as to those at 3 dpi, but reworked to account for further progress of *Foc*
Control roots had a severity index of 56.92% and silicon roots had an index of 35.38%. This difference was statistically significant.

**Figure 4-14.** Hyphae in root stele parenchyma cells of -Si banana plants at 6 days post inoculation showing plasma membrane disorganisation (arrows). Hyphae (H) are located intercellularly. Note the inclusion of electron dense material in the intercellular spaces (arrowheads) beginning at the cell wall and eventually filling the whole space. Abbreviations: H, hypha; XP, xylem parenchyma cell.
Figure 4-15. Internal symptoms of roots of *Fusarium oxysporum* f. sp. *cubense* inoculated banana plants grown in soilless cultivation system treated with silicon (+Silicon) and without (-Silicon) at 6 dpi. Severity of infection was inferred based on physical progression of the mycelia. Legend indicates the extent of hyphal ingress: A = epidermis; B = outer cortex; C = inner cortex; D = stele; E = xylem vessel.

**Inoculated 12 dpi**

At 12 dpi, the fungus had totally colonised the surface of the media and roots were showing signs of extensive necrosis. Roots sampled for TEM did not survive processing and were unable to be investigated. Plantlets were experiencing classic fusarium wilt symptoms, with leaf yellowing/necrosis and wilting both manifesting. This suggests fungal infection is greatly accelerated when using this method of inoculation.
Lignin and phenolic concentration
Total quantified phenolics and lignin remained approximately the same for uninoculated regardless of silicon treatment (Figure 4-16). Once inoculated with *Foc*, lignin content double by 3 dpi and nearly tripled by 6 dpi, compared to the uninoculated controls (Figure 4-16). There was no difference between –Silicon and +Silicon treatments. The phenolic concentration of -Silicon treated plants doubled by 3 dpi and tripled by 6 dpi compared to uninoculated controls, in a progression similar to lignin (Figure 4-16). For +Silicon treated plants, phenolic concentration more than tripled by 3 dpi, reaching a concentration equivalent to –Silicon plants at 6 dpi (Figure 4-16).
Figure 4-16. Phenolic quantitation at 3 days post inoculation and 6 days post inoculation in *Fusarium oxysporum* f. sp. *cubense* inoculated and uninoculated banana plants treated with and without silicon. Total soluble phenolics were determined using the Folin-Ciocalteau reagent method. Phenolic amount is expressed as milligrams of gallic acid equivalent per gram of dry weight. Error bars represent standard error, n = 10. Lignin quantitation at 3 dpi and 6 dpi in *Foc* inoculated and uninoculated banana plants treated with and without silicon. Total lignin content was determined using the thioglycollic acid (TGA) method. Lignin amount is expressed as % lignin per gram of dry weight. Bars
represent standard error, \((p < 0.05, n = 10)\). Letters represent statistically significant differences between inoculated and uninoculated treatments at each time point.
Discussion:

Silicon was demonstrated to enhance tolerance of young ‘Cavendish’ banana plants to fusarium wilt. Micrographic and biochemical data indicate that silicon is modulating plant defence responses in a way suggesting an association with plant signalling. This is consistent with recent advances in silicon research, which supports a multivariate role for silicon in inducing plant defence (Guntzer et al., 2012).

The steps involved in *Foc* infection of banana remain partially uncharacterised. The initial stages follow the same general process: root recognition, root surface attachment and colonisation, penetration, colonisation of the root cortex followed by growth into the xylem (Michielse and Rep, 2009). TEM results obtained would appear to support this general process. This initial colonisation is often referred to as the primary determinative phase of fungal infection. The secondary phase involves the subsequent colonisation of the plant from the xylem.

Hyphae were observed growing in grooves (where two cells meet) along the epidermis during root surface attachment (Figure 4-8), which is characteristic of pathogenic *Fusarium* species (Michielse and Rep, 2009). Hyphae were seen in various stages of colonisation of the cortex, although very few cell wall penetration events were noted (data not shown). As disease development is assumed to be a direct result of xylem colonisation, it is unclear how important cortical colonisation is to successful *Foc* pathogenesis and whether cortical aerenchyma are involved.

Aerenchyma are hollow gas channels present in plants adapted to growing in waterlogged or hypoxic root environments. The hollow spaces provide a path for continuous O$_2$ diffusion from shoots to roots (Aguilar et al., 2000). Aerenchyma are only present in mature banana roots, and ‘Cavendish’ roots have 10% cortex volume as aerenchyma (Aguilar et al., 1999). Aguilar et al. (2000) hypothesise that anoxia of the stele predisposes banana roots to *Foc* infection, as production of plant defence compounds are inhibited during low oxygen conditions (Aguilar et al., 2000). Aerenchyma may provide easy
passage for *Foc* to colonise the plant out of the stele, and potentially avoid any hypoxia or defence compounds that are produced.

Colonisation of the cortex may be an important step in reaching the xylem. Schneider (1990) theorises that leakage of solutes from the cell into intercellular spaces can provide *Foc* with a limited source of nourishment. Controlling root cell turgor during periods of rapid shifts in osmotic potential (like root growth or rain) requires the import and export of inorganic solutes and ions like K+ and Cl- or sugar alcohols from the symplasm to the apoplast (Schneider, 1990). *Foc* could theoretically metabolise these solutes, leading to enhanced growth and greater capacity for invading the xylem (Schneider, 1990).

After primary pathogenesis has occurred, secondary pathogenesis is initiated when the fungus moves from initial xylem colonisation to invade the entire plant. Beckman (1990) postulates that *Foc* switches to low hyphal growth during the secondary phase of pathogenesis and instead produces large numbers of microconidia which travel through the xylem. If this is the case, then preventing the spread of conidia is paramount in preventing the advancement of disease symptoms. Normally, this is achieved *in planta* by the induction of gels and tyloses in the vascular tissue. Gels and tyloses are generalised vascular defences that assist in sealing off damaged or diseased xylem vessels (Beckman, 1990). In *Foc* inoculated banana plants, Beckman (1990) saw the development of vascular gels by 3 days and tyloses by 10 days post inoculation. Production of too many gels and tyloses may actually exacerbate wilt symptoms, by limiting the movement of water in the plant.

This study did not investigate specific events in the secondary determinative phase of *Foc* pathogenesis, only in the primary phase. This is important to consider as silicon-mediated disease resistance can occur early during infection, but disappear later as the pathogen continues colonisation, albeit at a slower rate (Huang et al., 2011). In the tomato/*Fusarium oxysporum* f. sp. *radicis-lycopersici* pathosystem, treatment with silicon caused a decrease in symptoms 4 weeks after inoculation by delaying disease onset (Huang et al., 2011). Infection still occurred but at a slower rate, i.e. symptoms took longer to manifest.
How silicon enhanced tolerance in this experiment was not clear, but results implicate part of the biochemical defence pathway. Electron micrographs indicated a greater incidence of electron-dense material accumulating in cells in response to fungal infection in silicon treated roots compared to nontreated. No examples of deposited silicon inhibiting fungal ingress were observed.

If silicon is influencing the biochemical defence pathway, then it must be directly or indirectly regulating gene expression. Precedent for this exists. Fauteux et al. (2006) found 4000 differentially regulated genes in *Arabidopsis thaliana* treated with the powdery mildew fungus. Upregulation of defence genes occurred in the silicon treated plants compared to inoculated control, and downregulation of metabolic genes were attenuated in silicon inoculated. This was also observed in wheat challenged with *Blumeria graminis* f. sp. *tritici* (Chain et al., 2009).

The unidentified electron-dense globules observed in silicon treated 3 dpi roots (Figure 4-9) and in both silicon treated and untreated roots at 6 dpi suggest a biochemical response against *Foc* infection. Visually, they are very similar to structures identified in a study on *Forl* in tomato. Benhamou and Lafontaine (1995) treated tomato plants with biotic elicitors, e.g. chitosan, to induce a heightened defence response against *Forl*. In uninoculated, elicitor treated plants, they saw electron dense globules manifesting in the vacuole and cytoplasm of cortical cells and vascular parenchyma cells. Fully characterising the nature of the globules in TEM would require the use of immune-gold staining (Benhamou and Lafontaine, 1995).

Although the use of electron microscopy allows for visual identification of events during fungal infection, it cannot show how silicon is physically interacting within the cell. Using complementary spectroscopic techniques in conjunction with microscopy (such as electron energy loss spectroscopy (EELS) for transmission electron microscopy and energy-dispersive x-ray spectroscopy (EDS) for scanning electron microscopy) will allow for identification of silicon involvement at specific sites of pathogenesis, such as penetration of cell walls and lignin formation.
In addition to these spectroscopic techniques, spectral maps of plant tissue can be formed. Two techniques are available including infra-red microscopy (IR) or an SEM with EDS mapping capabilities. Silicon deposition within root tissue can be fully explored via the construction of a spectral map. Emphasis will be on whether inoculation with *Foc* causes deposition of silicon, or whether silicon is moved in response.

The production and deposition of both phenolics and lignin are involved in plant defence responses against pathogens (Fang and Ma, 2006). Both phenolics and lignin are upregulated in the rice-*Magnaporthea grisea* pathosystem when rice is treated with silicon (Datnoff & Rodrigues, 2005; Sun et al., 2010). Avocado plants challenged with *Phytophthora cinnamomi* showed a root phenolic content doubling when plants were treated with silicon (Bekker et al., 2006). There was no change in leaf phenolic content. The Goldfinger cultivar of banana, which is partially resistant to fusarium wilt, has a greater capacity for phenolic metabolism compared to ‘Cavendish’ banana plants (de Ascensao and Dubery, 2003). Phenolic metabolism and storage may be a predictor for enhanced plant defence in banana cultivars.

Most silicon research has focussed on silicon-enhanced defence against pathogenic microbes: little attention has been paid the effects on beneficial non-pathogenic microbes. These microbes can grow in the rhizosphere or colonise the plant non-pathogenically as an endophyte. They are considered beneficial when they have a positive influence on plant growth or habit. For example, a free-living chitinolytic bacteria that grows in association with banana roots has the dual effect of antagonising pathogenic fungi, and enhancing plant resistance through the production of chitin monomers during fungal antagonism. If silicon enhances plant defence against pathogens, it may cause an upregulation of plant defences against beneficial microbes. This could theoretically limit or counteract the beneficial effects of silicon.

Pot trials do not accurately reflect conditions in the field, so field trials are necessary when testing soil amendments. Differing levels of naturally occurring silicon in the soil mean that field trial results will vary significantly. Exploring both silicon treatments for new plantlets and for established plants is very important. Mixing treatments into the soil for new plantlets is fairly easy, whereas a liquid drench (or possibly injection) is more feasible for
established plants. For this reason, field trials of silicon will need to be performed. Once the efficacy of silicon has been established, the next step is to determine the cheapest and most efficient method of providing banana plants with available silicon. There are many things to consider, including: silicon type, method of application, soil pH and soil silicon content. There are a large variety of silicon materials available, including: silicates (including sodium silicate, potassium silicate, magnesium silicate and aluminium silicate) amorphous silicon dioxide, concrete powder, crushed drywall and various plant based materials (e.g. rice hulls and sugarcane trash).

A more appropriate soil free system for inoculation and study should be developed. Gunning and Cahill et al. (2009) developed a soilless cultivation system (SCS) for *Lupinus angustifolius* to be used in association with *Phytophthora cinnamomi*. It was composed of a two-part polycarbonate tray system maintained in a base container by a holding rack. Seeds were germinated between the two trays, so that the roots would grow down the inside. The trays were connected together by clips, allowing them to be separated at will. The two trays were coated with filter paper, which facilitated the absorption of nutrient solution up the inside of the trays to the growing seedlings. This prevented any light reaching the roots, but still allowed access whenever necessary. A setup similar to this would be appropriate for studying banana root/pathogen interactions without the artifacts introduced by tissue culturing. The development of a good, standardised *in vitro* bioassay for *Foc* infection on banana is also a high priority (Wu et al., 2010). Currently, most research is performed in pots or hydroponics, which is both time intensive and wasteful of space.

In conclusion, silicon is emerging as a viable tool in managing and mitigating pathogens and disease in the field. Enough research has emerged indicating the efficacy of silicon at enhancing plant defences, therefore the focus of research should shift toward the most efficient and cost-effective ways of supplying silicon in the field.

In this study, silicon was found in the root cortical region despite the absence of polymerised silicon bodies (Chapter 2). Silicon was found both in the cell wall, and potentially in the cytoplasm. Although this silicon-reinforcement of the cell walls may impede the progress of *Foc* during the primary determinative phase of the infection
process, it most likely upregulated biochemical defences as well. Also, *Foc* can infect through root injuries and damaged areas, e.g. nematode damage, mechanical stress (Ploetz and Pegg, 2000; Jeger et al., 1995). If silicon treatment simply enhanced physical defences, then wound-infection would likely overcome the beneficial influence of silicon.
Chapter 5 - Banana and silicon soil relations

Abstract

Background and Aims: Fertilising with silicon causes various beneficial effects in plants. Absorption, uptake and distribution dynamics of silicon from the soil into plant tissue is not well understood. Silicon is transported exclusively through the xylem, meaning silicon is absorbed from the soil and deposited in a linear fashion along with water uptake. For this reason, spatial distribution of silicon in the soil becomes very important. Silicon is thought to induce plant-wide protective effects, but it is unknown whether silicon is acting as a messenger or is influencing biochemical activity. For the latter to occur, silicon must be distributed in all relevant tissues throughout the plant. The aim of this research was to determine whether silicon distribution in the soil affects silicon uptake and banana resistance to fusarium wilt caused by the fungal root pathogen, Fusarium oxysporum f. sp. cubense (Foc).

Methods: A banana split root system was established by growing ‘Cavendish' banana plants in two separate 20 cm pots containing half the root system each. Each split pot system was treated with silicon (+Si) and without (-Si) in the following combinations: -Si/-Si, +Si/+Si, +Si/-Si, +Si/-Si, with */* representing a single split root system. Upon inoculation with Foc, only one pot in each split system was inoculated. After 3 months, external and internal disease symptoms were recorded. Symptoms were analysed using a visual assessment scale and by scanning diseased tissue, digitising and quantifying the results via software. These two methods were compared.

Key Results: For split root internal discolouration, +Si/+Si treatments had lower symptoms compared to -Si/-Si. The -Si/+Si and +Si/-Si split pot plants were not significantly different from each other and displayed greater symptoms compared to -Si/-Si and +Si/+Si. Results were the same using visual assessment and scanning/digitisation. For external symptoms and height, there was no difference between treatments. All treatment groups showed an increase in shoot silicon in inoculated plants compared to uninoculated plants.
**Conclusions:** The interaction between silicon/banana/\textit{Foc} is more complex than originally thought. High levels of silicon in the soil may predispose banana plants to \textit{Foc} infection, although it is not clear if this was an artifact of the split rooting procedure. Regardless, the lack of difference between -Si/+Si and +Si/-Si suggests that silicon does not exert a localised effect and is indeed acting systemically by a still undetermined mechanism.
Introduction:

Silicon is a quasi-essential plant nutrient (Epstein, 1999). Once thought to be inert, a consensus has now been reached amongst researchers that silicon positively influences the defence pathways of plants (Datnoff et al., 2007; Epstein, 2009). In addition to enhancing plant defences, silicon fertilisation has been shown to improve plant resilience to a wide range of environmental factors (Guntzer et al., 2012). Despite the firm correlation between silicon and enhanced plant defence, evidence for a silicon mode of action is lacking.

Most soils contain silicon and siliceous minerals, but silicon is only absorbed from the soil solution in the form of monosilicic acid (Raven, 1982). Monosilicic acid is transported throughout the plant following the stream of transpiration, until it reaches a termination point and polymerises into silicon dioxide, forming solid deposits alternatively known as silicon bodies or phytoliths (Lentfer, 2009; Mitani and Ma, 2005). The deposition of these bodies has been studied in several plants, but the activity of monosilicic acid in planta is poorly understood (Ma and Takahashi, 2002a). Physical bodies are thought to provide enhanced resistance to penetration from pathogens and to improve the physical stature of some crops, but this is not enough evidence to explain the other beneficial effects of silicon (Datnoff et al., 2001).

Different soils contain different types of silicon. Levels of silicon in the soil solution are maintained by the weathering and dissolution of rocks and minerals (Raven, 1982). This process is assisted by the soil microbiota and from plant rhizospheric exudates that accelerate the dissolution of silicon (Hinsinger et al., 2001; Hirota et al., 2010). Despite being abundant in the soil, global silicon distribution varies considerably. Tropical soils are susceptible to silicon depletion due to high temperatures, humidity and rainfall (Berthelsen et al., 2001). Silicon is weathered away and aluminosilicate clay minerals are dissolved, leaching silicon until source silicate materials are exhausted. Long term crop production can rapidly strip the soil of plant available silicon, especially in crops where large amounts of biomass are removed from the field.
Like most elemental nutrients, silicon is not evenly distributed through soil. Crop plants are rarely fertilised with silicon and are therefore reliant on silicon already present in the soil or returned by ecosystem services in the biogeochemical silicon cycle (Raven, 1982). Consequently, due to uneven distribution of silicon in the soil, not every plant growing in a field will have equal access to silicon. Plants with large root systems will be exposed to regions of soil with varying silicon concentrations. Silicon uptake and deposition in plants follows the transpirational flow from root to xylem to shoot; therefore uneven soil distribution of silicon may lead to uneven distribution and translocation in the plant. This may have an impact on enhanced plant tolerance.

Silicon fertilisation is being investigated for use in banana cultivation. Banana plants are grown perennially in clonal monocultures where they experience a wide variety of biotic and abiotic factors such as frost, high temperatures, drought, salinity and wind (Jones, 2000b; Jeger et al., 1995). Banana crop improvement through breeding is difficult, as most edible banana cultivars are seedless or have very few seeds (Buddenhagen, 1990). Cultivation of banana ranges from small-scale subsistence growers to industrial-scale plantations grown on a wide variety of soils. Banana plants thrive in tropical and subtropical environments and are grown on a large assortment of different soils (Jones, 2000b). The silicon content of these soils varies and in Australia the silicon content of soils remains mostly unquantified.

Despite being subject to a number of different pathogens, the primary threat to global banana cultivation is from fusarium wilt, a disease caused by several races of the soilborne fungus, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Ploetz, 2006). Symptoms of fusarium wilt first manifest as leaf yellowing, followed by wilting and splitting of the pseudostem base (Rishbeth, 1955). After a varied period of wilting, plant death almost always occurs. The infection process of the fungus is not fully characterised (see Chapter 4). Generally, spores or hyphae of *Foc* come in contact with a banana root, the hyphae then penetrate to the xylem and grow up the vascular system to the shoots, causing severe wilting (Ploetz and Pegg, 2000).

During fungal infection, internal symptoms are marked by red to brown discolouration in the root vascular tissue, the rhizome and the shoot vasculature (Jeger et al., 1995).
Discolouration of the rhizome can be used as a proxy for disease development: discolouration and necrosis of the rhizome is indicative of how far infection has progressed. This internal discolouration is the standard for measuring not only disease progression, but also host resistance (Orjeda, 1998).

Host genetic resistance is the best way of inhibiting *Foc* infection. Genetic resistance is being developed for the most common banana cultivars, but progress is slow (Buddenhagen, 1990). Treating plants with silicon may allow more time for this process to occur. Fortunato et al. (2012a) were the first to demonstrate in the literature that silicon decreases symptoms of *Foc* race 1 in banana. Further evidence suggests this enhanced tolerance to *Foc* is via biochemical defence pathway (Fortunato et al., 2014; Fortunato et al., 2012b). Anecdotal evidence for silicon enhancing tolerance to fusarium wilt has also been reported. Despite this, there are many facets of the interaction between soil silicon, the banana plant and *Foc* that are not well understood.

The primary objective of this study was to investigate how silicon distribution in the soil influences absorption and distribution *in planta*, and whether this influences the protective effect that silicon exerts upon the plant against *Foc*. In an attempt to mimic the varied soil conditions that field-grown plants encounter, banana plants were grown in a split pot system with half the root system in one pot and half in another. This allowed for control over which part of the root system was inoculated with *Foc* and which part was treated with silicon. If silicon uptake induces a plant-wide defence response, then it will not matter where the plant is inoculated and where the silicon is supplied. If it only provides localised protection, then pots treated with silicon and inoculated with *Foc* will show different disease symptoms compared to those without silicon.
Methods & Materials

Split root setup

‘Cavendish’ "Williams" banana plantlets were obtained from The University of Queensland tissue culture facility (Appendix 3). Plantlets were deflasked into seedling flats containing University of California potting media "C" (UC mix) and hardened under conditions of decreasing humidity and increasing light over 4 weeks in a glasshouse. For glasshouse conditions: plants were grown at facilities located in Brisbane, Queensland, Australia (Latitude: 27°29'45.36"S, longitude: 153° 0'35.81"E) with a mean day-night temperature of 29.4 - 20.7°C during the course of the experiment. After this, plantlets were transferred to 12 cm pots containing UC mix. Plants grew for a further 4 weeks and were then transferred to the split root system.

For each treatment, 15 plants were set up in a split pot system. Each split pot system consisted of two 20 cm pots linked together to provide a place for two halves of the root system. The rhizome was contained within a 10 cm square pot with the bottom removed (Figure 5-1). To facilitate inoculation without disturbing the root system, three polycarbonate tubes were inserted into each 20 cm pots in a triangular formation (Appendix 6). At inoculation, they were removed and inoculum was introduced to the voids in the potting mix left behind. Non-split control plants were prepared as above in single 20 cm pots to compare any confounding effects generated by the split root system.
Figure 5-1. Demonstration of ‘Cavendish’ "Williams" banana plants grown under split root conditions. Plants are approximately 6 weeks old. Note the plastic tubing for inoculation.

There were four treatment groups, summarised in Table 5-1:

Table 5-1. Summary of individual pot treatments used in split-pot system. Asterisk (*) indicates pot was inoculated. Pots without an asterisk were not inoculated.

<table>
<thead>
<tr>
<th>Label</th>
<th>Pot 1 (inoculated)</th>
<th>Pot 2 (uninoculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[-Si*</td>
<td>-Si]</td>
<td>0 g/kg</td>
</tr>
<tr>
<td>[+Si*</td>
<td>+Si]</td>
<td>5 g/kg</td>
</tr>
<tr>
<td>[+Si*</td>
<td>-Si]</td>
<td>10 g/kg</td>
</tr>
<tr>
<td>[-Si*</td>
<td>+Si]</td>
<td>0 g/kg</td>
</tr>
</tbody>
</table>

Plants were watered daily until replete. Each pot of the split and non-split root systems were fertilised every 2 weeks with Thrive (Yates) liquid fertilisers in place of watering.
Inoculum preparation and inoculation

Inoculations were performed via millet infested with \textit{Foc}, with methodology adapted from Smith et al. (2008). Isolates of \textit{Foc} were obtained from the Fusarium collection at The University of Queensland and were recovered and grown on 1/4 strength PDA plates. Millet (\textit{Echinochloa esculenta}) seeds were rinsed with water, soaked overnight in distilled water, sieved, autoclaved, rinsed again, autoclaved and allowed to cool. Plugs of \textit{Foc} colonised agar were introduced to the tubs of millet under sterile conditions. Millet was left to colonise the tubs for 2 weeks and were shaken daily to prevent settling.

The inoculum was subsequently introduced to the plants which had 4 weeks previously been established into the split root system. Inoculation was conducted by removing the polycarbonate tubes and introducing millet at a rate of 15 g/tube. Millet was covered with UC mix. Plants were left to grow for a further 3 months until symptoms had developed. Uninoculated controls were inoculated with millet prepared as above without introducing the fungus.

Harvest for disease analysis

Plants were harvested for disease analysis at 3 months post inoculation. General visual observations were made and plant heights measured with a ruler. Leaf discolouration was determined by counting the total number of leaves and dividing by number of diseased leaves to achieve a ratio. A healthy leaf was determined to be any leaf that was at least 75\% green. Anything less was considered diseased, whether it was chlorotic or necrotic.

For internal discolouration, the shoot was cut vertically with a sharp knife and the rhizome carefully removed with a knife. The rhizome was then sectioned equally into four parts vertically with a razor blade to expose any diseased tissue (Figure 5-2). Internal discolouration of the rhizome was rated initially using a scale adapted from the INIBAP system for scoring fusarium wilt symptoms (Orjeda, 1998). The rhizome was scored from 0 – 6, each number representing a level of discolouration in the rhizome, summarised in Table 5-2:
Figure 5-2. Scanned image of an inoculated rhizome from a control plant. Rhizome was cut from the plant with a knife at 3 months post inoculation and carefully cut with a razor blade vertically into four equally sized sections. This image was taken on a Canon CanoScan LIDE 110 flatbed scanner. Discolouration of the rhizome was calculated as a percentage using ImageJ software.
Table 5-2. Rating scale adapted from INIBAP (Orjeda, 1998) for visual determination of fusarium wilt symptoms in banana based on degree of internal discolouration in the rhizome.

<table>
<thead>
<tr>
<th>Score</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no discolouration</td>
</tr>
<tr>
<td>1</td>
<td>minor flecking in the rhizome</td>
</tr>
<tr>
<td>2</td>
<td>less than 1/3 rhizome discolouration</td>
</tr>
<tr>
<td>3</td>
<td>less than 2/3 rhizome discolouration</td>
</tr>
<tr>
<td>4</td>
<td>greater than 2/3 rhizome discolouration</td>
</tr>
<tr>
<td>5</td>
<td>total discolouration</td>
</tr>
<tr>
<td>6</td>
<td>total discolouration plus necrosis/rotting</td>
</tr>
</tbody>
</table>

This data was converted to a disease severity index as per Sun et al., (2010) and compared using a one-way ANOVA. Briefly, the disease index was calculated as follows:

Disease index(%)={(1×N_1+2×N_2+3×N_3+4×N_4+5×N_5+6×N_6)/6N_t}×100

Where N_1 to N_6 equal the number of scored plants in the modified INIBAP system described above and N_t equals the total number of plants scored.

Internal discolouration was concurrently determined by pixel counting the disease rhizome tissue. Each individual section of the cut rhizome was placed on a flatbed scanner (Canon CanoScan LIDE 110) in a row of 4 and scanned at high resolution 2400x4800 dots per inch (Figure 5-2). Each image was uploaded to ImageJ version 1.44p for Windows image processing software for pixel counting. The border of each rhizome was manually set and total pixel count established. Number of diseased/discoloured pixels was determined. The pixel counts were then divided to give a percentage of diseased tissue.
Data analysis

All analysis was done in GraphPad Prism version 5.03 for Windows. Internal rhizome discolouration based on scanning and digitisation data and the disease severity index of the INIBAP data were analysed by a one-way ANOVA with a post hoc Bonferroni’s Multiple Comparison Test. For height and healthy leaf determination, uninoculated and inoculated treatments were compared to each other with a Student’s t-test. For all tests (p<0.05) and error is presented as standard error.

Results:

Split root inoculation: Foc internal symptoms

Split root uninoculated banana control plants showed no physiological or phenotypic differences compared to non-split controls, i.e. plants in individual pots (data not shown). All inoculated plants regardless of silicon treatment, developed typical fusarium wilt symptoms of varying intensity. External symptoms manifested at approximately 4 weeks post inoculation, with leaf yellowing, mild wilting and pseudostem splitting. By 14 weeks post inoculation, internal symptoms were apparent with reddish-brown discolouration of the rhizome.

The disease severity indices were as follows: [-Si* | -Si] = 41.11%; [+Si* | +Si] = 33.33%; [+Si* | -Si] = 72.22%; [-Si* | +Si] = 76.66%. Split root plants uniformly amended with silicon [+Si* | +Si] demonstrated a statistically significant decrease for internal symptoms (measured via the INIBAP system) compared to plants not treated with silicon [-Si* | -Si] (Figure 5-3). There was no significant difference in symptoms between the two inoculated split treatments [+Si* | -Si] and [-Si* | +Si] (Figure 5-3). Compared to [-Si* | -Si], both [+Si* | -Si] and [-Si* | +Si] treatments produced significantly worse internal symptoms as measured by discolouration (Figure 5-3).
Figure 5-3. Internal rhizome discoloration of 3 month post inoculation ‘Cavendish’ banana plants inoculated with *Foc* in a split pot system. Square brackets [ ] indicate which pots were treated with silicon (+Si) and without (-Si). Asterisk (*) signifies inoculated pot was inoculated. Disease intensity is measured by a scale based on the INIBAP system.

Comparing photo digitisation and scanning, results were in consensus with the INIBAP method in that both showed similar statistical differences between treatments (Figure 5-4). The silicon treated plants [+Si* | +Si] had significantly less symptoms compared to the untreated controls [-Si* | -Si] (Figure 5-4). The two split treatments, [+Si* | -Si] and [-Si* | +Si], were not significantly different from each other and had worse symptoms compared to the inoculated control (Figure 5-4).
Figure 5-4. Internal rhizome discoloration of 3 month post inoculation ‘Cavendish’ banana plants grown in split-root systems and inoculated with *Foc*. Disease intensity was calculated by photographing exposed rhizome, digitising and scanning with pixel-counting software to determine the exact percentage of diseased tissue. Different letters indicate statistical significance. Bars represent standard error. Asterisk (*) indicates pot was inoculated. n = 15, each replicate being the mean of 4 rhizome sections.

**Split root inoculation: Foc external symptoms**

For external symptoms at 14 weeks post inoculation, inoculation with *Foc* caused a significant increase in plant height for all combined treatments except for silicon (Figure 5-5). Inoculation caused a dramatic decrease in the healthiness of leaves for all treatment groups compared to uninoculated, but there was no difference between groups (Figure 5-6).
Figure 5-5. Heights of *Fusarium oxysporum* f. sp. *cubense* inoculated and uninoculated plants ‘Cavendish’ banana plants grown in split root systems measured before harvest at 3 months post inoculation. There were no statistically significant differences within treatment groups. Square brackets [ ] indicate which pots were treated with silicon (+Si) and without (-Si). Asterisk (*) signifies inoculated pot was inoculated. Bars represent standard error (p < 0.05, n = 15).
Figure 5-6. Ratio of healthy leaves to total leaves of *Fusarium oxysporum* f. sp. *cubense* inoculated and uninoculated ‘Cavendish’ banana plants measured before harvest at 3 months post inoculation. Healthy leaves were considered on visual inspection to be leaves at least 75% green. Leaves were not counted as healthy if greater than ¼ of leaf surface area was chlorotic or necrotic. Different letters indicate statistical significance. Square brackets [ ] indicate which pots were treated with silicon (+Si) and without (-Si). (*) signifies which pot contains inoculum. Bars represent standard error (p < 0.05, n = 15).

**Shoot silicon concentration**
Silicon content of untreated plants was around 5 mg/g suggesting the presence of extra silicon in the UC potting mix, most likely from the sand. For the other treatment groups, silicon content varied between 15 and 25 mg/g (Figure 5-7). Inoculation caused a slight, but significant increase in silicon content for all groups (Figure 5-7).
Figure 5-7. Silicon content of banana shoots at harvest 3 months post inoculation. Shoots were harvested at the same time as rhizome discolouration was measured. Silicon was determined by the AID-SBC method (Appendix 2). Different letters indicate statistically significant differences between inoculated and uninoculated plants within treatment groups. Bars represent standard error. Square brackets [ ] indicate which pots were treated with silicon (+Si) and without (-Si). Asterisk (*) indicates pot was inoculated. n = 15.
Discussion:

Although treatment with silicon decreased symptoms of *Foc* as expected, the increase in symptoms of [+Si* | -Si] and [-Si* | +Si] plants compared to inoculated control [-Si* | -Si] was unexpected (Figures 5-3, 5-4). Silicon when present in both pots lead to a decrease in symptoms, but when only in one pot there was an increase in symptoms compared to the control [-Si* | -Si] control. Silicon was supplied as amorphous silicon dioxide powder at a rate of 10 g/kg for each plant. For the silicon treatment this meant there was 5 g/kg in each pot. For the [+Si* | -Si] and [-Si* | +Si] treatments, this was 10 g/kg in a single pot. This suggests that high concentrations of silicon dioxide in this form enhances fusarium wilt symptoms. There are several possible explanations for this.

Silicon dioxide can act as a desiccant. It is possible that the high silicon load decreased plant available water placing extra stress on the plants and inhibiting their ability to defend themselves against *Foc*. Water deficit has been suggested to predispose banana plants to *Foc* infection (Shivas et al., 1995). Similarly, silicon in the soil may be interfering with nutrient uptake in the plant. Soil chemistry is complex and adding high amounts of silicon can disrupt nutrient uptake by binding to plant available nutrients (Raven, 1982). Plants may require a constant supply of silicon to see a protective effect (Sun et al., 2010). The large dose of silicon provided (10 g/kg) may have rapidly polymerised as water was progressively added over the experiment, rendering itself unavailable for uptake.

Another possibility is there was a large defence response occurring in the [-Si* | +Si] and [+Si* | -Si] split plants and this counterintuitively caused the enhanced symptoms. Diseased tissue in inoculated banana plants is a combination of necrotised tissue attacked by the fungus, and plant cells undergoing apoptosis and other defence responses mediated by the plant (Paul et al., 2011). A heightened defence response may exacerbate *Foc* infection, as more apoptotic cells provide sustenance for the necrotrophic phase of the fungus (Paul et al., 2011). The possibility exists that upregulated plant defences could enhance *Foc* pathogenesis. For example, when *Fusarium oxysporum* senses plant phytoalexins it can upregulate the production of mycotoxins in response (Michielse and Rep, 2009). Although silicon is most frequently associated with an increase in plant
tolerance to pathogens and disease, there have been reports of it enhancing symptoms. This has been most commonly noted as isolated incidents involving viruses and enhanced viral symptoms (Datnoff et al., 2007).

Determining internal discolouration through scanning and digitisation was more reliable and easily repeatable than using the INIBAP visual assessment scale (Orjeda, 1998). Results were similar through either method, with a statistically significant decrease in symptoms for silicon treated plants compared to control and an increase in symptoms for the [+Si* | -Si] and [-Si* | +Si] plants (Figures 5-3, 5-4). The INIBAP scale (Orjeda, 1998), while susceptible to individual bias, still has utility in field analysis. If working from a lab, the use of scanning and digitisation is recommended for future use if possible.

Rhizome and pseudostem vascular discolouration is normally used as a proxy for Foc disease progression. Although this is cheap, straightforward and easy to do in the field it is only an approximate indicator of the passage and presence of Foc. Another potential method for quantifying Foc is with quantitative real-time PCR (qRT-PCR). Lievens et al. (2006) demonstrated this with Fusarium solani and Verticillium species pathogenic on tomato. Using qRT-PCR with Foc specific primers will both, a) confirm the presence of the pathogen, and b) give a quantitative value for fungal biomass, giving a better indication of Foc infection.

This study highlighted the complex interaction of variables that occur when looking at a plant, a pathogen and the soil in combination. When selecting a silicon fertiliser, care should be taken with how much is applied at any one time, as too much may have an indirect detrimental effect.
Chapter 6 - Silicon use in banana micropropagation

Abstract:

Background and Aims: Micropropagation (or tissue culture) is current best practice for producing healthy, uniform, pathogen-free planting material. Vegetative propagation is simpler but the benefits of micropropagation outweigh the costs. Despite this, micropropagation has several limitations. Plantlets produced in tissue culture are not climatically adapted and must be hardened before planting in the field. This process is costly and time-consuming and leads to plants more susceptible to disease in the first few years of cultivation. The element silicon was investigated as a means for lessening the impact of these problems. The effect of silicon on the three primary phases of micropropagation were investigated: multiplication, rooting and deflasking. The effects of allowing improved gas exchange through venting of the culture tube and decreased carbon sources in the rooting phase were also investigated.

Methods: Silicon amendment during the multiplication phase (where shoot proliferation is encouraged) was tested by adding 1.66 mM silicon to multiplication media when subculturing. The rooting phase (when root production is encouraged in preparation for deflasking) was tested by amending rooting media with silicon under several conditions including control, no sucrose, low sucrose and vented. For the deflasking phase (where rooted shoots are acclimated to non-tissue culture conditions), plantlets were treated with or without silicon in tissue culture, then deflasked into cell trays with or without silicon also containing the soilborne fungal root pathogen Fusarium oxysporum f. sp. cubense (Foc).

Key Results: Silicon was not beneficial in the rooting phase, although it may increase shoot biomass under vented conditions. Silicon did not ameliorate the effects of low or no sucrose conditions. Disease symptoms in inoculated, deflasked plantlets decreased when supplied with silicon. Shoot proliferation increased significantly in silicon supplied plants during the multiplication phase.
Conclusions: Silicon is recommended for use in the multiplication phase of micropropagation, and warrants further research in other phases, especially deflasking. Enhancing plants produced during deflasking will result in overall more productive banana plants, leading to an increase in tolerance to disease and negative environmental factors.
Introduction:

The banana plant (*Musa acuminata x balbisiana*) is grown around the world as both an economically significant trade crop and forms the basis of many subsistence farming systems. Subsistence farming and small-scale commercial cultivation involve a wide selection of cultivars, but the primary cultivar in world trade is ‘Cavendish’, *Musa acuminata* (AAA group). ‘Cavendish’ banana fruits, like those from most edible cultivars, are seedless and must be vegetatively propagated. This is traditionally done by rhizome splitting and transplantation of suckers from mature plants growing in the field (Israeli et al., 1995). This practice is still favoured on smallholding farms, but for industrial scale cultivation, plants are usually prepared via tissue culture (Israeli et al., 1995). Most cultivars derived from tissue culture are agronomically equivalent to plants from suckers, if not superior (Niere et al., 2014; Robinson & Galan Sauco, 2010).

Tissue culture (or micropropagation) is the production of vegetatively propagated, clonal plantlets under totally sterile conditions. The process results in very large numbers of genetically similar, microbe-free plantlets of uniform size. There are several related methods of micropropagation, but in all cases they involve the induction of shoot proliferation from plant meristem or callus tissue under sterile conditions (Israeli et al., 1995). Because of the technical nature involved, micropropagation requires a dedicated facility with clean rooms, laminar flow cabinets and qualified staff to operate them.

Micropropagation involves five phases: meristem initiation, subculture, root initiation, hardening and establishment (Israeli et al., 1995). Briefly, a tissue culture line is initiated from material obtained from the field. Corm tissue is harvested, sterilised and aseptically introducing to growth media. This tissue will eventually produce many shoots which can be split and introduced to new growth media. Once the line is established, plantlets are subcultured on an approximate 4-6 week cycle. Each subculture cycle is performed on media that encourages shoot proliferation, resulting in increased plantlet number. The number of plantlets produced each cycle can vary considerably.
When banana plants are required for field planting, they are subcultured from multiplication media to root initiation media for approximately a month then deflasked and hardened. During the multiplication and rooting phases, plantlets are grown in a low light environment at 100% humidity. Plantlets cannot survive an immediate transition from this environment to the highly variable climatic conditions of the field. Therefore they are hardened after the rooting phase. Hardening involves the gradual increase of available light, and decrease of humidity to acclimate the plants to field conditions. Usually they are further hardened in a glasshouse in pots then finally planted in the field.

When produced under sterile conditions from clean, virus-indexed motherstock, plantlets will most likely be free of fungal and bacterial pathogens (Israeli et al., 1995). This is essential for growers, as banana plants suffer from several pathogens vectored by planting material, such as the fungus *Fusarium oxysporum* f.sp. *cubense* (Foc), the causative agent of fusarium wilt. This fungus is a major threat to both the global banana industry and subsistence growers (Pegg et al., 1996). The process of tissue culturing will eliminate the majority of fungal and bacterial pathogens, but viruses that display no obvious phenotypic symptoms can still be present. Micropropagated banana plantlets must therefore be virus indexed to prevent the spread of virus-contaminated planting material (Singh et al., 2011).

Micropropagation has several disadvantages. Plantlets derived from tissue culture can be more susceptible to some biotic factors in the field: possibly due to the absence of beneficial and commensal microorganisms that are normally present in vegetatively propagated plants (Smith et al., 1998). Micropropagated plantlets are initially ill-adapted to growth in the field. Growing in tubes with overwhelming access to carbon, near maximum humidity and high nutrient availability causes the plants to adapt to a low transpiration/photosynthesising habit (Jackson et al., 1991). Even after hardening, micropropagated plants can be more susceptible to disease under field conditions compared to vegetatively propagated plants (Smith et al., 2008).

Although micropropagation follows a well-established protocol, there are several areas that can be investigated for enhancement. Improvement in micropropagation involves fine-tuning the composition of media to maximise plant growth and proliferation, or altering equipment and techniques to produce better adjusted plants that require less hardening.
For example, the simple addition of activated charcoal to micropropagation media can improve overall cell growth and development (Thomas, 2008).

Micropropagation can lead to morphological defects in plantlets known as vitrification or hyperhydricity. Plantlets develop abnormally, with excessive storage of water and low lignification leading to structurally weak plants. These plantlets are especially susceptible to the stresses associated with deflasking and usually do not survive the process (Rossetto et al., 1992). Vitrification can be a serious problem in some plants, but does not frequently occur in banana (Israeli et al., 1995). Aeration of tissue culture vessels (also known as venting) can alleviate vitrification symptoms by preventing the accumulation of gaseous hormones and increasing transpiration and photosynthesis via improved gas exchange (Jackson et al., 1991). Aeration can be achieved simply by drilling holes in tissue culture containers and sealing them with superfine mesh or filter paper to allow sterile gas exchange (Rossetto et al., 1992).

Micropropagated plants are grown with a carbon source in the media (usually sucrose), negating the necessity of photosynthesis in culture. In banana micropropagation, this involves a sucrose concentration of up to 30 g/L (Vuylsteke, 1989). While this results in enhanced biomass and greater shoot proliferation in vitro, plantlets struggle to adapt to low carbon conditions during the hardening process (Daniells and Smith, 1991). If micropropagation could be adapted to work under low carbon, high light conditions, this may encourage the development of photosynthesising plantlets in vitro, which would decrease the time required for hardening.

Micropropagated plantlets depend entirely on the nutrients provided in tissue culture media. The ideal culture media composition for banana micropropagation was studied extensively by Vuylsteke (1989). The foundation of most micropropagation compositions is Murashige and Skoog media (Appendix 1) plus sucrose, with the addition of optional vitamins, hormones and other beneficial compounds. For example, activated charcoal is added to media to inhibit light stimuli from interfering with root development (Kaçar et al., 2010). A possible additive worth investigating is the element silicon. Most recommended media formulations do not include added silicon, although some is always present from environmental contamination.
Silicon has been demonstrated to play an active role in the nutrition of monocots and some dicot plants (Epstein, 1999; Epstein, 1994; Epstein, 2009). Silicon is involved in alleviating plant stress and enhancing defence responses (Datnoff et al., 2007). The mechanism by which this occurs is under debate, but the effects are well characterised. Banana plants have been established to benefit from silicon, but beneficial effects have been rarely studied. Silicon was recently demonstrated to enhance banana tolerance to the foliar pathogen *Mycosphaerella fijiensis* (Kablan et al., 2012) and the fungal root pathogen *Foc* (Fortunato et al., 2012a).

All phases of micropropagation involve a variety of plant stresses, and it is hypothesised that silicon treatment will alleviate micropropagation-related stress leading to better adapted plants *ex vitro*. In this study, silicon was investigated as an additive for both tissue culture media and potting media (for deflasking) in three separate experiments: Experiment 1 considered the use of silicon in the rooting phase, plus several other factors including low sucrose and venting, and whether these factors interacted with each other; Experiment 2 involved the deflasking phase and used *Foc* to test for increased disease tolerance, and; Experiment 3 investigated silicon use in the multiplication phase. Enhancement of growth factors, such as weight and height, were used as markers for increased abiotic tolerance and disease resistance was measured as an indicator of biotic tolerance.
Methods & Materials

Plant material and common methods

*Musa accuminata* cv. ‘Cavendish’ "Williams" (AAA) banana plants were used in all experiments as this cultivar is most common in Australia. Plantlets were originally obtained from the Nambour Department of Primary Industries tissue culture facility in Queensland, Australia. Once established, plantlets were subcultured on a 4 week multiplication cycle in a sterile tissue culture facility with a temperature range of 26°C max/22°C min. For a guide to subculturing technique, see Israeli et al. (1995). Illumination was provided by fluorescent tubes at approximately 5000 lux on a 12 hour day/night cycle. All experiments were conducted under these conditions unless otherwise stated.

For the multiplication phase, plantlets were subdivided and subcultured under sterile conditions to polycarbonate plastic tubs containing banana multiplication media (Austratec) adjusted to pH 6 (see Appendix 1 for full media composition). For the rooting phase, plantlets were subcultured under sterile conditions into containers of 1X Murashige and Skoog media (Sigma-Aldrich) solidified with 0.3% Phytogel™ (Sigma-Aldrich) and adjusted to pH 6 (Appendix 1).

Silicon content was analysed as follows: harvested plant samples were placed in brown paper bags and oven-dried at 60°C for 7 days then subsequently analysed with a modified version of the autoclave induced digestion/silicomolybdous blue colourimetric (AID-SBC) with a spectrophotometer (Hitachi U-2800). For detailed AID-SBC methodology see Appendix 2.

Effects of silicon on the rooting phase

The effects of silicon on the rooting phase were tested under four conditions: 1) control, 2) no sucrose, 3) low sucrose, and 4) vented. Plantlets were subcultured to 15 cm diameter polycarbonate tubs to which sucrose was added at a rate of 30 g/L for the control and vented, 5 g/L for the low sucrose treatment, and 0g/L for no sucrose. Each treatment group was divided in half and treated with silicon or without. Each treatment group contained 20 plantlets: 10 with silicon and 10 without.
Silicon was added as powdered amorphous silicon dioxide (Sigma-Aldrich) to achieve a concentration of 1.66 mM monosilicic acid (approximately 0.1 g/L). Vented containers were constructed following the methodology of Rossetto et al. (1992): polycarbonate plastic tubes of 5cm diameter were vented by drilling 9 mm diameter holes in each screw lid with a metal drill bit then covering the holes with a cut piece of filter paper (Whatman Grade 1) on the lid top and bottom with autoclave-resistant tape (Figure 6-1).

Figure 6-1. Photo of vented polycarbonate tissue culture containers used for banana micropropagation: A – fully vented container with top layer of filter paper visible and secured with autoclave tape. B – Underside of lid showing second layer of filter paper and autoclave tape. C – Drilled vent without filter paper.

After initial subculturing, plantlets were maintained under tissue culture conditions as described above. The plantlets were harvested at 4 weeks by removal from the tub followed by careful rinsing with deionised water to remove adhered media. The following characteristics were measured: silicon content, dry mass, number of leaves, number of roots, number of stomata on the abaxial leaf surface, height, root length, water content.
Stomata count was determined with the nail-polish impression method. Each leaf was painted with clear nail-varnish, allowed to dry and the impression removed by peeling with tweezers. Peels were then observed under an Olympus BX60 binocular microscope and number of stomata counted. Three fields-of-view were counted at x400 magnification and averaged to give mean number of stomata. Both the abaxial and the adaxial surfaces were investigated. All data were analysed by a one-way or two-way ANOVA with a post hoc Bonferroni’s Multiple Comparison Test (p < 0.05).

**Effects of silicon on the deflasking and post-flask phase**

To investigate the deflasking phase and test disease tolerance, plantlets were subcultured to tubs containing rooting media amended with or without 1.66 mM silicon (see above). Plantlets were treated with silicon both in tissue culture and during deflasking to see if silicon treatment in tissue culture has any kind of priming effect, i.e. if silicon treatment in tissue culture still provides a benefit after treatment stops in deflasking. At 4 weeks, rooted plantlets were divided into four categories detailing their silicon treatment *in vitro* and *ex vitro*, respectively: control/control, silicon/control, control/silicon and silicon/silicon.

Plantlets were deflasked to 30-cell trays filled with University of California "C" potting media (Matkin and Chandler, 1979) amended with or without 2 g/kg amorphous silicon dioxide powder (Sigma-Aldrich). Plants were further divided into uninoculated and inoculated treatments. Infested millet was used as the source of inoculum (Smith et al., 2008). Briefly, millet grains (*Echinochloa esculenta*) were rinsed once with tap water then soaked overnight in a covered container. Thereafter, grains were rinsed in distilled water, and approximately 150 g of soaked millet was placed into individual tubs, autoclaved, rinsed then autoclaved again. Total tubs were divided in half and designated as either for inoculum or control treatments.

For inoculum, the millet was inoculated with plugs from carnation-leaf agar plates containing *Foc* subtropical race 4 VCG 0120, BRIP no: 22615, 24322 and 23598. The three isolates of the same VCG were mixed together to ensure pathogenicity. Inoculated millet was stored in a darkened cupboard for 2 weeks and shaken daily to homogenise inoculum and prevent clumping. Uninoculated control millet was stored in a separate room.
and treated in the same fashion. After 2 weeks, inoculations were performed by mixing the millet in with potting media at a rate of 20 g/kg. Plantlets were deflasked directly into the potting mix with *Foc*-colonised millet, or sterile millet. Care was taken to ensure roots remained intact during deflasking.

The plantlets were transferred to a growth cabinet set initially at 22°C for 1 week and then 30°C for the remaining 3 weeks. Illumination was provided with a 15 kW sodium arc lamp at approximately 12 000 lux. Deflasked plantlets were under 75% shade cloth for 7 days, followed by 25% shade cloth for 14 days, then full light for the remaining 7 days. Trays were watered daily until replete and daily misted with a spray-bottle for the first two weeks.

After 4 weeks, plantlets were harvested and disease symptoms were recorded per a modified version of the INIBAP guidelines for internal discolouration (Orjeda, 1998). Diseased rhizomes were carefully dissected with a fresh razor blade to expose the rhizome, which ranged from off-white (no disease) to dark brown/orange (fully necrotic). Diseased rhizomes were scored on a rank from 1-6 with 0 being no disease and 6 fully diseased: 0 = no discolouration, 1 = minor flecking in the rhizome, 2 = less than 1/3 rhizome discolouration, 3 = less than 2/3 rhizome discolouration, 4 = greater than 2/3 rhizome discolouration, 5 = total discolouration, 6 = total discolouration plus necrosis and rotting.

This data were converted to a disease severity index as per Sun et al., (2010) and compared using a Student’s T-test. Briefly, the disease index was calculated as follows:

\[
\text{Disease index} \% = \frac{(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6)}{6N_t} \times 100
\]

Where \( N_1 \) to \( N_6 \) equal the number of scored plants in the modified INIBAP system described above and \( N_t \) equals the total number of plants scored. The results were analysed by a one-way ANOVA with a post hoc Bonferroni's Multiple Comparison Test (\( p<0.05 \)).

Leaf discolouration was estimated by calculating the approximate percentage discolouration of each fully expanded leaf on the plantlet. Approximation was done by
measuring the amount of discolouration with a ruler and dividing by the total length of the leaf. Discolouration tended to begin at the leaf tip and move along a straight front toward the petiole, making measurements fairly straightforward. The total ratio was summed for each plant and analysed as a percentage. Finally, the following physical characteristics were measured: silicon content (as above), wet and dry root and shoot masses and height. Data were analysed by a one-way ANOVA with a post hoc Bonferroni's Multiple Comparison Test (p<0.05).

Effects of silicon on the multiplication phase
To test the effect of silicon on the multiplication phase, regular subculture cycles were adapted by culturing half of the multiplied plantlets on regular media and the other half on media amended with 1.66 mM silicon. Multiplication media was purchased premade (Austratec) and made up with pH adjusted to 6. Silicon was added as amorphous silicon dioxide powder to a concentration of 1.66 mM before pH was adjusted then autoclaved. Subculture efficiency was measured by counting the number of explants and diving by the number of initiator explants, giving a ratio of increase. Plantlets that died during multiplication were counted separately. Four multiplication cycles were studied over a period of four months. Monthly ratio increases were compared between control and added silicon using a Chi-squared test for significance (p < 0.05).

Overall data analysis
All statistical analysis and graphing were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.
Results:

Silicon in the rooting phase
The effects of silicon on the tissue culture rooting phase were tested under the following conditions: control, no sucrose, low sucrose and vented. Adding silicon in tissue culture did not significantly affect the following characteristics when compared to plants treated without silicon: shoot weight (Figure 6-2a), root weight (Figure 6-2b), root:shoot ratio (Figure 6-2c), number of leaves (Figure 6-2d), number of roots (Figure 6-2 e), number of stomata (Figure 6-2f), height (Figure 6-2g), maximum root length (Figure 6-2h) and total water content (Figure 6-2i).

The no and low sucrose treatments resulted in plantlets that were pale green and less turgid when compared to controls (data not shown). No and low sucrose resulted in decreased shoot and root weight compared to the control (Figure 6-2a,b). The shoot:root ratio increased significantly in low and no sucrose treatments compared to control (Figure 6-2c), indicating an increase in shoot mass at the expense of root mass. Silicon did not appear to ameliorate the effects of decreased sucrose (Figure 6-2).
Figure 6-2. Measurements of vegetative statistics for banana plantlets generated in the rooting phase of micropropagation. All measurements were taken after 28 days. The eight treatment groups were control (30 g/L sucrose), no sucrose (0 g/L sucrose), low sucrose (5 g/L sucrose) and vented (tubes were aerated; 30 g/L sucrose); each then being further divided into no silicon and plus 1.66 mM silicon (+Si) treatments. Each data set was analysed by a two-way ANOVA with a post hoc Bonferroni’s Multiple Comparison Test, (p < 0.05, n = 10). Different letters indicate statistical significance between silicon treatments. Bars represent standard error. Panels: (a) Banana explant shoot dry weight in grams; (b) Banana explant root dry weight in grams; (c) Root:shoot ratio of dry banana explant tissue; (d) Mean number of leaves of banana explants; (e) Mean number of roots of banana explants; (f) Mean number of stomata on abaxial leaf surface; (g) Mean height
of banana explants in centimetres; (h) Mean of longest intact root from each banana explant in centimetres; (i) Total water content (wet mass minus dry mass) of banana explants in grams.

The shoot silicon content of controls was the same regardless of added silicon (Figure 6-3). Comparatively, low and no sucrose treatments resulted in an approximately 3-4 fold increase in silicon (Figure 6-3). The vented tubes also saw a significant increase in shoot silicon content when silicon was added (Figure 6-3).

![Silicon Shoot Content Graph](image_url)

**Figure 6-3.** Silicon shoot content of banana explants grown in tissue culture on rooting media. Silicon was quantified with the autoclave induced digestion/silicomolybdous blue colourimetric method. All measurements were taken after 28 days. The treatment groups were control (30 g/L sucrose), no sucrose (0 g/L sucrose), low sucrose (5 g/L sucrose) and vented (tubes were aerated). Each of these treatment groups was further divided into no silicon and plus silicon (+Si). Data were analysed by a two-way ANOVA with a post hoc Bonferroni's Multiple Comparison Test, (p <0 .05, n = 10). Different letters indicate statistical significant. Bars represent standard error.
Silicon in the deflasking and post-flask phase

The shoot silicon content of uninoculated plants did not differ significantly between treatments (Figure 6-4). When plants were inoculated with *Foc*, the subsequent shoot silicon content increased across all amendment treatments, i.e. C/C, C/Si, Si/C, Si/Si compared with plants not inoculated with *Foc*. There was no significant difference in final leaf discolouration (Figure 6-5). Internal discolouration was significantly lower in the Si/Si and C/Si treatments compared to the C/C and Si/C treatments (Figure 6-6). Calculated disease severity indices were: C/C = 72.34%; Si/C = 54.98%; C/Si = 43.22%; Si/Si = 31.22%.

**Figure 6-4.** Silicon shoot content of banana plants either uninoculated or inoculated with *Fusarium oxysporum* f. sp. *cubense*. Silicon was quantified with the autoclave induced digestion/silicomolybdous blue colourimetric method. */* is in-vitro pre-treatment and post-treatment respectively. C = Control, Si = Silicon. Pre-treatment silicon was added to tissue culture as 1.66 mM monosilicic acid. Post-treatment silicon was mixed in with potting media at a rate of 2 g/kg amorphous silicon dioxide. Data were analysed by a two-way
ANOVA with a post hoc Bonferroni’s Multiple Comparison Test, \( p < 0.05, n = 7 \). Different letters indicate statistical significant. Bars represent standard error.
Figure 6-5. Leaf disease symptoms of banana plants inoculated with *Fusarium oxysporum* f. sp. *cubense*. Leaf disease is expressed as a percentage of leaf discolouration (chlorosis and necrosis) compared to healthy leaf tissue, expressed as a mean for all plants. */* is in-vitro pre-treatment and post-treatment respectively. C = Control, Si = Silicon. Data was analysed by a one-way ANOVA with a post hoc Bonferroni's Multiple Comparison Test, \( p < 0.05, n = 7 \). Different letters indicate statistical significant. Bars represent standard error.
Figure 6-6. Internal disease symptoms of banana plants inoculated with *Fusarium oxysporum* f. sp. *cubense*. Internal disease symptoms are based off the INIBAP scale of discolouration on a scale from 0 to 6. A brief summary is 0 = no discolouration, 1 = minor flecking in the rhizome, 2 = less than 1/3 rhizome discolouration, 3 = less than 2/3 rhizome discolouration, 4 = greater than 2/3 rhizome discolouration, 5 = total discolouration, 6 = total discolouration plus necrosis and rotting. */* is in-vitro pre-treatment and post-treatment respectively. C = Control, Si = Silicon.

Inoculated plants had decreased shoot weight for all treatment groups (Figure 6-7a). A similar decrease occurred in root dry weight, with the largest decrease in Si/Si (Figure 6-7b). There was a significant interaction with the dry shoot/root ratio. The shoot/root ratio of uninoculated control plants (C/C) was higher in uninoculated plants compared to inoculated, with the reverse in Si/Si treated plants (Figure 6-7c). The total water content (fresh weight – dry weight) was significantly lower between uninoculated and inoculated plants for all treatment groups except Si/Si (Figure 6-7d). Similarly, all treatment groups experienced a significant decrease in height when inoculated, except for those that received silicon both pre and post deflasking (Si/Si) (Figure 6-7e).
Figure 6-7. Measurements of vegetative statistics for banana plants deflasked after the rooting phase and grown for 28 days. Plants were either uninoculated or inoculated with \textit{Foc}-infested millet grains. Treatments */* refer to silicon treatments in tissue culture or post tissue culture. Silicon (Si) was supplied in tissue culture as 1.66 mM monosilic acid. Post tissue culture it was supplied as amorphous silicon dioxide at a rate of 2 g/kg potting mix. C = no added silicon. Panels: (a) banana dry shoot weight. (b) banana dry root weight. (c) dry weight shoot to root ratio. (d) total water content of shoots and roots combined. (e) banana plant heights. Different letters represent statistically significant effect between inoculated and uninoculated (p < 0.05).
During the sodium hydroxide extraction phase of the silicon testing process, digested shoot tissue changed colours depending on whether they were inoculated or uninoculated with *Foc* (Figure 6-8). This phenomenon has not been previously described. The uninoculated tissue was dark green; the inoculated tissue was dark brown. This colour was consistent across samples.

**Figure 6-8.** Photograph of digested banana shoot tissue during preparation for determination of silicon concentration. A) inoculated samples. B) uninoculated samples. Dried plant tissue was ground to a fine powder and added to concentrated sodium hydroxide. After bubbling, the digested solution uniformly changed colour to either brown for inoculated (A) or green for uninoculated (B).
Silicon in the multiplication phase

Adding silicon to the media of the multiplication phase caused an increase at a statistically significant level in the number of explants generated (Figure 6-9). There was no difference in the number of dead plantlets; increase in explants was due to enhanced shoot proliferation (data not shown).

Figure 6-9. Proportional increase in banana explants during the multiplication phase after one month of subculturing. Plantlets were subdivided onto multiplication media either amended with 1.66 mM monosilicic acid (Silicon) or without (Control). Silicon was added during media preparation as amorphous silicon dioxide. Proportional increase was calculated by dividing the number of explants at subculturing by the number of explants used at the beginning of a cycle. Bars represent standard error. Asterisks indicate a statistically significant difference between silicon and control plants in each month.
Discussion:

Improving micropropagation outcomes involve either, a) increasing the cost-effectiveness of production, or b) enhancing the general vigour of plants when transplanted to the field. In this study, silicon was demonstrated to improve multiplication frequency (Figure 6-9) and to improve tolerance to Foc in deflasked plantlets (Figures 6-5, 6-6). Improving multiplication frequency will produce more plantlets at a cheaper cost and improving the disease tolerance of deflasked plants will allow for easier establishment of new banana plantations.

Silicon usage was investigated in this study at several stages of banana micropropagation. Silicon addition in the rooting phase did not have any significant effects on the characteristics measured in this study. Silicon treatment also did not significantly affect any morphological characteristics. There was no difference in shoot silicon content for control plants compared to a significant difference in vented plants (Figure 6-3). Silicon shoot deposition is thought to be mostly driven by transpiration (Henriet et al., 2006). Vented containers likely experienced an increase in transpiration, although this was not experimentally measured.

The low and no sucrose treatments also contained significantly higher amounts of shoot silicon (Figure 6-3). These plants had very poorly developed root systems (Figure 6-2b). Banana roots have been demonstrated to contain silicon (see Chapter 2) therefore the lack of a developed root system may have caused an increase in shoot silicon. Conversely, the lack of roots may have permitted unfettered uptake of silicon into the shoot via transpiration.

Many of the non-silicon treated groups still had silicon present in the shoots (Figure 6-3). Silicon is an omnipresent contaminant, and it is likely that trace amounts of silicon were present in the media and the water used during the tissue culture process. It is extremely difficult to generate an environment where silicon is completely absent (Epstein, 1994). The focus of this study was not on silicon essentiality, but whether silicon supplementation was beneficial.
The lack of an increase in dry weight and other vital characteristics in the vented containers was unexpected. Improved transpiration should lead to higher rates of photosynthesis and carbon deposition and an increase in stomatal density (Rossetto et al., 1992; Jackson et al., 1991). The high levels of sucrose in the media may have inhibited photosynthesis. In addition, venting would prevent the build-up of ergastic gaseous hormones which can inhibit growth and root formation (Jackson et al., 1991). The tubes may not have been sufficiently filtered. A relatively conservative 9mm hole was drilled to prevent drying out of the media, as a larger hole may have caused too much evaporation (Figure 6-1), but at the end of the experiment, there was no appreciable decrease in media water content (data not shown). Future experiments should therefore increase the size or number of vents.

Silicon does not appear to ameliorate low and no sucrose conditions in the multiplication phase. Absence of sucrose caused several interesting phenomena, including a substantial shift away from root biomass to shoot (Figure 6-2c). Despite the lack of transpiration, silicon treated plants had a far greater shoot silicon content compared to plantlets that did not receive additional silicon (Figure 6-3). This could represent stress-response induced preferential uptake of silicon, which has been previously theorised by Ma (2004). Conversely, if the root system acts as a silicon sink, the lack of root biomass may have resulted in an excessive transfer of silicon to the shoots.

Reagent grade sucrose is the commonest sugar used as a carbon source in plant micropropagation. But less pure sucrose can be cheaply and easily obtained, even if from unconventional sources (Buah et al., 2011). Sugarcane is a silicon accumulator, and unprocessed sugarcane juice, which is sometimes used in Africa, may even be a significant source of silicon (Keeping et al., 2009). That would make sugarcane juice a source of both sucrose, silicon plus other plant-growth promoting phytohormones (Buah et al., 2011).

Silicon was found to enhance the tolerance of banana plantlets to Foc. Shoot silicon content was consistently higher in diseased tissue compared to uninoculated (Figure 6-4). From the results it is unclear whether this is an induced response to infection. Fungal
infection resulted in overall decreased root growth which may translate to more silicon being translocated to the shoots. Root silicon dynamics remain mostly unexplored, especially in a plant disease context and require much further study. Interestingly, post-treatment with silicon appeared to increase dry root weight. This could be due to actual deposited silicon enhancing mass, or somehow regulating increased root growth.

Both internal discolouration and leaf symptoms suggest that pre-treatment of tissue culture with silicon is less effective than post-treatment (Figures 6-5). This is consistent with studies indicating a constant supply of silicon is necessary to induce tolerance to biotic and abiotic conditions (Sun et al., 2010). Results suggested that the combination of both pre-treatment and post-treatment with silicon (Si/Si) was most effective overall. Sun et al. (2010) demonstrated this with the rice-\textit{Magnaporthe grisea} pathosystem. A single dose of silicon provided after pathogen inoculation was more effective than a dose of silicon provided before inoculation. A continuous supply of silicon is generally more effective.

The addition of silicon to multiplication media increased the number of divided banana plantlets in the multiplication phase of micropropagation in three out of the four month-long periods assessed (Figure 6-9). This was due to increased shoot proliferation and not survival of plantlets in culture (data not shown). The mechanism behind increased proliferation remains unknown. Silicon may be alleviating in-culture stress effects, although this was not apparent in the other experiments. Another possibility is that silicon is involved in plant signalling or hormone production/sensing. Silicon is known to influence gene expression by a still yet to be determined mechanism (Fauteux et al., 2006).

Only 0.1 g/L of silicon was required to induce a protective effect on the plant. Therefore determining cheap sources of silicon should be a major concern, especially in developing countries with nascent tissue culture programmes. Potential sources include: diatomaceous earth, concrete dust, steel slag, etc (Berthelsen et al., 2003). It is also possible that glass containers could provide a trace amount of silicon. Additionally, there are several biotic sources, such as rice hulls or sugarcane trash that could be used.

The unexpected change in colour during the silicon testing process (Figure 6-8) may be useful as a diagnostic tool for confirming infection with \textit{Foc}. What caused this colour
change is unknown, but may be related to the production of plant defence compounds or the expression of fungal toxins during pathogenesis. Currently, confirmation of infection involves either PCR or recovering fungi on plates. Each process is time consuming. A simple biochemical test could prove to be diagnostically useful, although reliability would need to be established.

Venting may provide benefits under hardening and post-hardening conditions which could lead to better, more easily adapted plantlets. Venting is usually only performed with species that are especially susceptible to hyperhydricity, which is not an issue with most banana cultivars (Rossetto et al., 1992; Israeli et al., 1995). Enhanced multiplication dry weights have been previously demonstrated in both aerated and temporary immersion liquid culture (Alvard et al., 1993). This was mostly due to a supply of oxygen to the roots, which is largely curtailed in solid media (Alvard et al., 1993). The combination of venting and silicon needs to be further explored. Silicon-induced resistance to fusarium wilt is an interesting prospect, but requires considerable research before recommending use in the field.

Overall, silicon addition in tissue culture was found to be most effective in the multiplication phase, deflasking phase and post-deflasking hardening phase. Silicon was not tested in the meristem initiation phase and should be investigated. The addition of silicon may still be worthwhile in the rooting phase, depending on the construction of the tissue culture tubes.
Chapter 7 - Discussion & Conclusion

Silicon treatment partially enhanced tolerance to fusarium wilt in ‘Cavendish’ "Williams" banana plants. Partial tolerance was expected as silicon treatment rarely imparts total resistance (Datnoff et al., 2007; Fortunato et al., 2012a). For now, complete resistance to Foc is only possible through the introduction of Foc resistance genes to banana. As for silicon, Epstein (1994) argues that it should be considered an essential nutrient. In that context, added silicon should not be interpreted as enhancing tolerance to fusarium wilt, but correcting a silicon deficiency that caused increased susceptibility to fusarium wilt. Regardless, silicon should be examined for use in banana standard practice fertiliser regimes, as partial resistance is still useful in controlling Foc.

Although a conclusive role for silicon in planta was not established in this study, hypotheses for future research can be generated. Deposited silicon has a protective mechanical effect, but it is becoming clear that silicon influences biochemical activity in planta. Deposited silicon is assumed to be inert after polymerisation—therefore it is likely that soluble silicon is the mechanism influencing plant biochemistry. The basic model for silicon absorption involves soluble silicon travelling temporarily through the plant during translocation from the roots through the xylem where it is finally deposited at predetermined sites in the shoot tissue (Raven, 1982). Whether this silicon temporarily exerts a beneficial effect in the plant, or soluble silicon in the symplast is biologically active, needs to be investigated.

Polymerised silicon in the xylem has never been recorded despite high concentrations of monosilicic acid present during transpiration (Mitani et al., 2005a). Once monosilicic acid concentration passes a threshold of ~2 mM at neutral conditions, it automatically polymerises (Raven, 1982). Low levels of soluble silicon in the cell symplast have generally not been investigated and it is possible that this soluble silicon is exerting a biologically active effect. If monosilicic acid was involved in signalling or other cellular process, then it would only need to be present in the symplast at low concentrations.
If soluble silicon was modulating plant defences, then it must either: a) be sensed as part of a signalling pathway, b) influence already established signalling pathways, or c) create an environment better suited to hormone sensing or production. Silicon has been shown to indirectly influence hormone expression. In an experiment on rice inoculated with the rice blast fungus, Brunings et al. (2009) demonstrated that rice plants treated without silicon upregulated auxin-related genes, unlike silicon treated plants, which showed no upregulation. Silicon has also been implicated in the regulation of transpiration and photosynthesis (Ueno and Agarie, 2005) although again it is unclear if this was due to the direct action of silicon or a secondary signalling effect involving hormones.

If silicon were involved in signalling, it would explain how silicon treatment causes a wide variety of nonspecific beneficial effects in plants (Liang et al., 2007; Ma, 2004). Many plant defence pathways are regulated by hormone expression, such as induced system resistance, which is mediated by the jasmonate/ethylene pathway (Hammerschmidt, 1999). How silicon influences these pathways should be investigated.

Despite the evidence of silicon being involved in biochemical plant defence, there are other possible theories for the mechanism behind silicon-mediated defence. Tolerance to negative abiotic factors would theoretically make field-grown plants more resilient to pathogens, as resources otherwise used for ameliorating the effects of negative environmental conditions are freed for use in bolstering defences. Goto et al. (2003) speculates that deposited silicon provides a UV protective effect by dispersing or scattering incoming UV rays, which would theoretically lead to an increase in overall plant vigour. Furthermore, Bekker et al. (2006) demonstrated an improvement in root density and canopy condition of avocado trees treated with silicon under field conditions.

A rarely explored area of silicon-mediated resistance is whether the pathogen itself can make use of silicon. Any soilborne fungus lives and evolves in a silicon rich environment. Fungi may utilise silicon in a similar fashion to plants. Many microorganisms are already known to use silicon, including bacteria (Hirota et al., 2010) and diatoms (Martin-Jézéquel, 2000). If soil fungi do make use of silicon, then careful consideration of soil ecology needs to be made when fertilising with silicon. The use of inorganic nutrients by Foc is not well understood. With nitrogen, high levels of NH₄ can increase symptoms and NO₃ can
decrease them (Schneider, 1990). Whether silicon rhizosphere influences \textit{Foc} growth is unknown.

Brunings et al. (2009) hypothesise that silicon (either polymerised or soluble) could block the targets of pathogen effectors, effectively preventing access and interference with plant signal transduction pathways. This would prevent the induction of pathogenesis and inhibit development of symptoms. Silicification of cell walls, cell apoplasts or intercellular spaces could prevent or slow the movement and absorption of effector molecules, inhibiting fungal infection.

Silicon can enhance tolerance to heavy metal contamination in several different species (Guntzer et al., 2012). This occurs through a number of different mechanisms, summarised by Pilon-Smits et al. (2009). Silicon can: 1) reduce metal availability in the growth medium/soil, 2) regulate heavy metal uptake and transport, 3) modulate the cation binding capacity in the cell wall, 4) induce production and distribution of antioxidants to counteract the effects of heavy metal uptake, and 5) co-precipitate with heavy metal ions followed by vacuole sequestration. All of these involve the amelioration of heavy metal toxicity symptoms which would lead to an overall increase in plant vigour and defence.

Other inorganic elements are involved in plant defence. Elemental sulphur is the only inorganic phytoalexin, playing a major role in defence against vascular pathogens in some plant species (Hammerschmidt, 2003). For example, sulphur is involved in the defence responses of cacao \textit{(Theobroma cacao)} to \textit{Verticillium dahliae} and french beans \textit{(Phaseolus vulgaris)} to \textit{Fusarium oxysporum} (Williams and Cooper, 2003). In these cases, sulphur is absorbed and stored in the xylem and xylem-associated tissues where it persists for upwards of 60 days (Williams and Cooper, 2003; Hammerschmidt, 2003). If silicon interacts with sulphur, it may enhance or mediate the phytoalexin effect.

Elemental boron shares some similarities with silicon: they are both: a) metalloids, b) absorbed in a similar fashion, c) involved in cell wall strengthening and, d) transported by a combination of transpiration and transporter proteins (Miwa et al., 2009). Boron, like silicon, is also poorly understood. Boron influences plant defences through the indirect
regulation of lignin and phenol production and cell-wall reinforcement (Stangoulis and Graham, 2007). Based on their similarities, it would be worth silicon and boron together.

Similar to boron, there are several similarities between the activity of calcium and silicon in planta. Both are biomineralised in the plant: for calcium as calcium carbonate, calcium phosphate and calcium oxalate and amorphous silicon dioxide for silicon (Bauer et al., 2011). Both are presumed to be nonsoluble once they have polymerised and therefore immobile. Both tend to accumulate in older tissue (Rahman and Punja, 2007). They are both associated with cell walls: calcium has a known role in maintaining cell membrane stability by enhancing gel formation in the middle lamella (Legge et al., 1982) and silicon is frequently found in association with cell walls. Soil treatment and foliar sprays are both effective at enhancing plant defences. Both require continuous, available supply in the soil to have a beneficial effect in the plant.

Calcium plays a known role in enhancing plant tolerance to pathogens. Calcium is second only to nitrogen in the management of plant diseases with inorganic nutrients (Rahman and Punja, 2007). Free calcium is present as Ca\(^{2+}\) ions and is involved in response to general pathogen stimuli, usually sensed by the calcium-binding messenger protein calmodulin. Calmodulin-dependant sensing of Ca\(^{2+}\) ions is an integral part of cell signalling for many organisms, not just plants. The flux of Ca\(^{2+}\) from intercellular spaces into the cytoplasm is sensed and modulates regulatory sequences or downstream signalling leading to gene expression (Reddy et al., 2011).

Calcium acts as a secondary messenger in the regulation of phytoalexin synthesis and a calcium deficiency leads to enhanced susceptibility to plant pathogens (Rahman and Punja, 2007). Based on the similarities between calcium and silicon, it would not be surprising if there was a relationship between calcium and a putative silicon signalling pathway. Silicon treatment has been shown to decrease expression of putative calcium signalling pathway genes in rice plants challenged with rice blast (Brunings et al., 2009). Monosilicic acid may act in a similar fashion to Ca\(^{2+}\) ions. This would necessitate the existence of a silicon-sensing protein, which has not currently been described.
When plants are tested for mineral composition, there appears to be an exclusive relationship between silicon and calcium. In silicon-rich monocot species, the calcium content is usually low; in calcium-rich dicot species, silicon levels are low (Ma and Takahashi, 2002c). Silicon uptake is more prevalent in evolutionary basal plants (Ma and Takahashi, 2002c). The highest silicon content is found in the Equisetaceae, a genus of plants often referred to as living fossils (Gierlinger et al., 2008). Other early or primitive plants appear to be silicon accumulators, such as the “fern ally” Selaginella emmeliana (Dengler and Lin, 1980).

The negative correlation between calcium and silicon suggests a possible relationship or shared functionality. If they are both involved in signalling, it is feasible that silicon signalling evolved initially and was later supplanted or co-opted by calcium signalling. It is possible that silicon transiently complexes with proteins when present in the symplast, similar to calcium and calmodulin. Live, in situ study of dynamic silicon transport and movement should be a research priority.

Uncovering and characterising silicon-binding proteins is a challenging prospect. The study of calcium signalling was greatly advanced by the development and use of calcium binding fluorescent proteins, which allowed for detailed studies of the movement of calcium in and out of suspended cell cultures using fluorescent microscopy (Bouché et al., 2005). Currently, no fluorescent tag for silicon exists. This prevents the observation of silicon movement and translocation in real time. Options for live imaging silicon include x-ray fluorescence utilising synchrotron radiation (Wang et al., 2013) and Raman spectroscopy (Gierlinger et al., 2008).

There are still many unanswered questions as to why plants deposit silicon. Blueberry plants (Vaccinium corymbosus) accumulate large amounts of silicon in their leaves but shed them in the Autumn, necessitating seasonal re-uptake of silicon (Morikawa and Saigusa, 2004). Similarly, banana roots, which were demonstrated to absorb silicon in Chapter 2, only live for approximately 6 months before senescence (Price, 1995a). This discarded silicon will eventually be cycled back into the soil, but it is not obvious why plants accumulate silicon in short-lived tissues. Raven (1982) suggests that the metabolic cost of excluding silicon may be higher than allowing silicon to be passively absorbed.
The possibility of silicon redistribution \textit{in planta} needs to be studied. The current consensus is that absorbed monosilicic acid in the plant is irreversibly polymerised to amorphous silicon dioxide, i.e. silicon deposition in tissue is permanent. If the silicon-mediated defence response is controlled by soluble silicon, this would explain why the protective effect of silicon diminishes when soil available silicon drops.

Although the role deposited silicon plays in enhancing defences against plant pathogens is well established, there is also evidence that silicon content is directly correlated with resistance to herbivory (Massey and Hartley, 2009; Hunt et al., 2008). This resistance to herbivory is thought to be due to a reduction in palatability for the herbivore. Massey and Hartley (2009) found that organisms feeding on silicon-rich material plant material did not adapt with age and theorised that organisms have a limited capacity for developing a tolerance to silicon-rich plant tissue. This suggests that silicon deposition provides plants with an evolutionary advantage against herbivores, and it would be interesting to compare silicon deposition with the presence of anti-herbivore toxins to see if there is a negative correlation.

Based on the research gathered in this thesis, silicon is recommended for use in banana cultivation. The next phase of research should involve field trials to establish the effectiveness of silicon in Australian soils. The Australian sugarcane industry has performed a trial of this nature (Berthelsen et al., 2003) and it would act as a good template for a future banana industry trial. A banana field trial should consider the following recommendations: the purest form of plant available silicon should be used (e.g. amorphous silicon dioxide or fertigation with monosilicic acid) to rule out any influence from conjugate elements, e.g. potassium in potassium silicate. Silicon uptake dynamics in the plant need to be measured over time, to determine if they experience seasonal variation or influences from the environment. Microbe interactions with silicon in the soil, whether they increase or decrease the availability of silicon, need to be investigated (Brehm et al., 2005).

Silicon soil levels need to be quantified, especially in Australia. Methods exist for quantifying total soil silicon content, but there is no accepted standard for determining the
plant available silicon in soils. This is important, as high silicon content does not always correlate with plant availability, and growers need reliable information on the amount of plant available silicon in products they use. Buck et al. (2011) attempted to rectify this by testing the efficacy of various silicon extractors. They found that the Na$_2$CO$_3$ + NH$_4$NO$_3$ method was best for solid fertilisers, and the total Si (HCl + HF) method for liquid fertilisers. The extracted silicon can then be determined using the ammonium molybdate complex colorimetric test.

Fertilising banana plants presents several unique challenges. Established plantations are usually not ploughed unless being replanted, therefore solid fertilisers can only be incorporated at planting (Lahav, 1995). The banana root system is shallow and extensive, making broadcast fertilisation inefficient (Lahav, 1995). For comparative purposes, the average rate of silicon application in rice cultivation is 900 kg silicon/ha/year (Datnoff et al., 2001). In banana, shed leaves and decaying pseudostems (known as trash) are often left around the bases of the plant during cultivation (Figure 7-1). This trash decomposes in the soil liberating silicon and other nutrients which becomes available to the plant. Phytoliths in the trash are composed of amorphous silicon dioxide, and unlike crystalline silicon dioxide, are highly soluble (Fraysse et al., 2006). Trash supply is practically continuous in mature banana plantations (Delvaux, 1995). Although a theoretically ample supply of silicon, trash can also be a substrate for root and foliar pathogens, and these considerations must be weighed before deciding on trash retention.
Figure 7-1. Example of trash retention around the bases of banana plants at a plantation in northern New South Wales, Australia.

Guntzer et al. (2012) recommends the return of trash to the soils wherever possible to prevent depletion of bio-available silicon in the soil. Exportation of banana fruit at harvest will eventually deplete the soil of silicon if none is externally applied (Guntzer et al., 2012). This decrease in plant available silicon may be contributing to unexplained cases of yield decline in banana and other crops.

Silicon should be investigated in the context of sustainable agriculture. Fungicides work in certain plant/fungal pathosystems, but their use can have adverse environmental consequences and can accelerate the emergence of fungicide-resistant pathogenic strains, Mass silicon fertilisation as a substitute for fungicide use has only been established for a few species, mostly in rice (Guntzer et al., 2012). Wollastonite (CaSiO₃) is a common silicate fertiliser along with the silicon-rich slag residue from steel blast furnaces (Datnoff et al., 2001). All silicon fertilisers need to be tested before use in the
field. Organic silicon compounds such as methyl silanols can be phytotoxic (Côté-Beaulie et al., 2009) making them impractical as a fertiliser. Continuous application is recommended for silicon (Bekker et al., 2006). Most forms of fertilisable silicon are non-toxic and easily stored.

Most silicon research focuses on supplying silicon to the soil, but foliar and fruit silicon applications warrant further attention. The current model for silicon/plant interactions has silicon absorbed from the soil through the roots where it exerts a plant wide protective effect (Guntzer et al., 2012). Whether foliar applications results in silicon flowing into the soil and being absorbed through the roots is unclear. A more intriguing possibility is that silicon is locally influencing gene/hormone expression in the leaves and fruit. The other possibility is that silicon is directly inhibitory or toxic to pathogenic microbes. Some studies have shown that silicon can inhibit fungal growth in vitro, but overall results are mixed (Bi et al., 2006). It remains unclear if this is due to the silicon itself, or other confounding factors such as pH or nutrient binding.

Foliar application of silicon as a substitute for fungicide has had mixed results (Rezende et al., 2009). Rezende et al. (2009) suggest that plants that do not deposit silicon in the shoots could benefit the most from foliar application. Generally, it seems that foliar application does not cause an upregulation of defences (unlike root application) and therefore the silicon compounds used are causing some sort of localised fungitoxic or fungistatic effect on pathogenic microbes (Rezende et al., 2009). While soluble silicon may play a role in postharvest treatment of fruits and vegetables as argued by Li et al. (2009) it is not likely that elemental silicon plays a direct fungitoxic role in plant/pathogen interactions. No causal mechanism has been described. It is possible that soluble silicon supplied directly to fruit and vegetables is absorbed and utilised to enhance biochemical defences, as plant tissue is active and respiring even when detached from the plant. Preliminary testing was performed in the course of this research on banana fruits and Colletotrichum musae, but results were inconclusive (data not shown).

The potential for using silicon extends beyond banana cultivation. Silicon is being investigated in the floriculture industry for improving the quality of cut flowers during growth, harvest and maintenance after sale (Kamenidou et al., 2009). Seed treatment of
silicon for preservation purposes is another possibility. Deepak et al. (2008) treated pearl millet (*Pennisetum glaucum*) with silicon dioxide dissolved in water by soaking the seeds at 26°C on a rotary shake for 3 hours. Treatment with 10 mM silicon enhanced both germination rate of the seeds and overall vigour of the plants (Deepak et al., 2008). Seed priming alone also enhanced pearl millet tolerance to subsequent downy mildew (*Sclerospora graminicola*) infections in germinated plants (Deepak et al., 2008).

Banana plants will continue to be a useful model for studying the effects of silicon. Order Zingiberales, of which banana belong, are a sister lineage to Poales (D’Hont et al., 2012) which contains the majority of studied silicon accumulator plants. The banana genome was published in late 2012. D’Hont et al. (2012) sequenced a double-haploid *Musa acuminata* subspecies *malaccensis* DH-Pahang plant, identifying approximately 36,542 protein-coding genes. As more silicon-related genes are described in the Poales, related genes can be identified from the banana genome.

Uncovering the genetic components of silicon uptake and transport will allow for their manipulation in banana plant genetic enhancement and breeding programmes. Altering expression of *LSI1* and *LSI2* gene homologues, via transgenics or breeding can lead to increased or decreased silicon deposition (Mitani et al., 2009a). At this stage, it is not known whether increased silicon deposition will lead to enhanced silicon-mediated resistance.

Within Zingiberales, It would be interesting to investigate species related to banana in the family Zingiberaceae, such as ginger (*Zingiber officinale*), to see if there are similar patterns of silicon distribution in the shoots of roots. Silicon in banana shoot tissue is present in discrete accumulations predominantly in the vascular tissue of stem material. This is consistent with phytolith observations made in the past (Lentfer, 2009). Based on current knowledge, this type of deposition is unique to banana.

The focus of silicon research for the last century has been on the monocots, particularly the crop species in the Poaceae. Dicots have been generally ignored due to their low shoot silicon contents, as silicon-mediated resistance was originally thought to be directly
correlated with amount of deposited silicon. More research is implicating a biochemically-active role for low levels of monosilicic acid in planta, levels of which occur in dicot plants.

In this work, and other recent publications, silicon has been demonstrated to enhance plant defences against both Foc and Mycosphaerella fijiensis (Kablan et al., 2012; Fortunato et al., 2012b). It seems likely that this enhanced defence would extend to other pathogens, and judging by precedence in other plants, extend to enhanced tolerance of negative environmental factors. Future research needs to concentrate on two areas: 1) does silicon consistently work in the field, and 2) by what pathway is silicon enhancing biochemical defence in planta.

Silicon treatment as a way to ameliorate drought stress should be studied, especially when banana cultivation is viewed in the context of accelerating climate change (Guntzer et al., 2012; Cook et al., 2013). Silicon reduces transpiration in leaves of rice (Ueno and Agarie, 2005). This reduction in transpiration is thought to be due to silicification of the epidermis preventing cuticular evaporation, but silicon is also involved in stomatal guard cells and regulating response times for opening and closing (Ueno and Agarie, 2005).

In conclusion, the quasi-essential status of silicon should be seriously reconsidered. The next logical step is to investigate silicon treatment in banana plants grown under field conditions. The protective effects of silicon will likely manifest more strongly in plants subjected to the unique combination of biotic and abiotic stresses present in the field.
## 1. Detailed composition of tissue culture media

All values (mg/L)

**Multiplication media:**

*Murashige and Skoog basal medium with additives*

Final working concentration = 36.36 g/L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Nitrate</td>
<td>1650</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>6.2</td>
</tr>
<tr>
<td>Calcium Chloride, Anhydrous</td>
<td>332.2</td>
</tr>
<tr>
<td>Cobalt Chloride•6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Cupric Sulfate•5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Na₂EDTA•2H₂O</td>
<td>37.26</td>
</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>27.85</td>
</tr>
<tr>
<td>Magnesium Sulfate, Anhydrous</td>
<td>180.74</td>
</tr>
<tr>
<td>Manganese Sulfate•H₂O</td>
<td>16.9</td>
</tr>
<tr>
<td>Molybdcic Acid (Sodium Salt)•2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Potassium Iodide</td>
<td>0.83</td>
</tr>
<tr>
<td>Potassium Nitrate</td>
<td>1900</td>
</tr>
<tr>
<td>Potassium Phosphate Monobasic</td>
<td>170</td>
</tr>
<tr>
<td>Zinc Sulfate•7H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td>L-Ascorbic Acid</td>
<td>20</td>
</tr>
<tr>
<td>6-Benzylaminopurine (BA)</td>
<td>4.5</td>
</tr>
<tr>
<td>Gellan Gum – CultureGel, Biotech</td>
<td>2 000</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Indole-3-acetic Acid</td>
<td>0.175</td>
</tr>
<tr>
<td>Nicotinic Acid (Free Acid)</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Pyridoxine•HCl 0.5  
Sucrose 30 000  
Thiamine•HCl 0.4

Rooting media: 
*Murashige and Skoog basal medium with Gamborg’s vitamins*

Final working concentration = 10 g/L

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>1650.0</td>
</tr>
<tr>
<td>Boric acid</td>
<td>6.2</td>
</tr>
<tr>
<td>Calcium chloride anhydrous</td>
<td>332.2</td>
</tr>
<tr>
<td>Cobalt chloride • 6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Cupric sulfate • 5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Na₂-EDTA</td>
<td>37.26</td>
</tr>
<tr>
<td>Ferrous sulfate • 7H₂O</td>
<td>27.8</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>180.7</td>
</tr>
<tr>
<td>Manganese sulfate • H₂O</td>
<td>16.9</td>
</tr>
<tr>
<td>Molybdic acid (sodium salt) • 2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.83</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>1900.0</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>170.0</td>
</tr>
<tr>
<td>Zinc sulfate • 7H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>100.0</td>
</tr>
<tr>
<td>Nictotinic acid (free acid)</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxine • HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Thiamine • HCl</td>
<td>10.0</td>
</tr>
</tbody>
</table>

2. *Silico-blue analysis method for quantifying silicon*

A spectrophotometric method for determining gross silicon amounts from either tissue or aqueous solution. Adapted from (Elliott and Snyder, 1991; Taber et al., 2002).
Samples were ground to a powder and 0.1 g of material added to a 5 ml autoclave resistant polyethylene tube. Material was wetted with 5 ml of 35% hydrogen peroxide, 3.5 ml of 50% sodium hydroxide was added, lightly capped and autoclaved for an hour. Resulting liquid was filtered through grade 541 quantitative hardened ashless filter paper (Whatman) into a tube then made up to 20 ml with water.

A 1 ml aliquot of the digested plant tissue solution was analysed as follows: solution was added to 5 ml of 16% acetic acid followed by 1 ml of ammonium molybdate (54 g l\(^{-1}\)), left for five minutes, then 0.5 ml of 20% tartaric acid and 0.5 ml of reducing solution. The reducing solution comprised 2 g of Na\(_2\)SO\(_3\) in 200 ml water and 0.4 g of 1-amino-2-naphthol-4-sulfonic acid in 25 ml water combined with 25 g of NaHSO\(_3\) in 200 ml water, mixed and brought to 250 ml with water. Absorbance of the resulting blue-coloured solution was measured after 30 minutes at 650 nm on a Hitachi U-2800 UV-Visible double beam spectrophotometer. Blanks consisted of all reagents using RO water as the sample, made fresh for each sample run. Absolute silicon values were determined with a standard curve derived from serial dilutions of 1 g L\(^{-1}\) silicon standard solution (Sigma-Aldrich).

### 3. Tissue culture conditions at The University of Queensland

The tissue culture facility at The University of Queensland is located on level 5 of the John Hines building, St Lucia campus, Queensland, Australia. Temperature is kept constant at 26°C. Illumination is by wall-mounted fluorescent tubes and is approximately 5000 lux on each rack. Day/night cycle is 11 hours light/13 hours darkness. Tubes are maintained equidistant from light sources to ensure maximum light interception.

### 4. TEM Protocol and procedure for banana roots

Plant tissue is notoriously difficult to work with when processing for TEM. For this reason, a unique protocol was developed to maximise the efficiency of fixation and resin infiltration.
of banana roots. The protocol was refined in a series of trial-and-error experiments on banana roots (data not shown). Samples were processed with the Pelco Biowave® microwave and a nearby fume hood. Microwave power (in Watts) was varied depending on what was required for each step. Samples were either under vacuum or not. Each step is summarised below in Table 8-1.

Table 8-1. TEM protocol from fixation to resin polymerisation. Each line or step represents the removal of old solution and substitution with fresh solution. (m = minutes)

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Watts</th>
<th>Time</th>
<th>Vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fixation</td>
<td>glut</td>
<td>150</td>
<td>2m on/2m off/2m on</td>
<td>on</td>
</tr>
<tr>
<td></td>
<td>glut</td>
<td>150</td>
<td>2m on/2m off/2m on</td>
<td>on</td>
</tr>
<tr>
<td>2. Buffer rinse</td>
<td>buffer</td>
<td>-</td>
<td>fume hood</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>-</td>
<td>fume hood</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td>80</td>
<td>40s</td>
<td>on</td>
</tr>
<tr>
<td>3. Osmium</td>
<td>osmium</td>
<td>80</td>
<td>2m on/2m off/2m on</td>
<td>on</td>
</tr>
<tr>
<td></td>
<td>osmium</td>
<td>80</td>
<td>2m on/2m off/2m on</td>
<td>on</td>
</tr>
<tr>
<td>4. Water</td>
<td>water</td>
<td>-</td>
<td>fume hood</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>80</td>
<td>40s</td>
<td>on</td>
</tr>
<tr>
<td>5. Dehydration</td>
<td>acetone 30%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 40%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 50%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 60%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 70%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 80%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 90%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 100%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>acetone 100%</td>
<td>250</td>
<td>1m on/1m off/1m on</td>
<td>off</td>
</tr>
</tbody>
</table>
6. Resin

<table>
<thead>
<tr>
<th>Resin Ratio</th>
<th>Duration</th>
<th>Time on</th>
<th>Time off</th>
</tr>
</thead>
<tbody>
<tr>
<td>resin:acetone 1:3</td>
<td>250</td>
<td>3m</td>
<td>on</td>
</tr>
<tr>
<td>resin:acetone 1:2</td>
<td>250</td>
<td>3m</td>
<td>on</td>
</tr>
<tr>
<td>resin:acetone 1:1</td>
<td>250</td>
<td>3m</td>
<td>on</td>
</tr>
<tr>
<td>resin:acetone 2:1</td>
<td>250</td>
<td>3m</td>
<td>on</td>
</tr>
<tr>
<td>resin:acetone 3:1</td>
<td>250</td>
<td>3m</td>
<td>on</td>
</tr>
<tr>
<td>100% resin</td>
<td>-</td>
<td>24h (on rotator)</td>
<td>-</td>
</tr>
<tr>
<td>100% resin</td>
<td>250</td>
<td>15m on/15m on*</td>
<td>hi-vac</td>
</tr>
</tbody>
</table>

7. Polymerisation

Fully infiltrated samples are placed in latex moulds and polymerised in an oven for 48 hours at 60°C

*15 minutes on, break vacuum, 15 minutes on again

- glut = 2.5% gluteraldehyde in 0.1 M phosphate buffer (6.8pH) with 0.7% caffeine
- buffer = 0.1 M phosphate buffer (6.8pH)
- osmium = 4% osmium tetroxide solution
- water = UHQ water
- resin = Spurr’s resin (Spurr, 1969)
- hi-vac = separate high vacuum unit

Biological samples are usually post-stained with heavy metals to enhance contrast in the electron microscope. Grids with samples are first stained with uranyl acetate then lead citrate. Grids were placed face down in droplets of 5% uranyl acetate in 50% ethanol for 2 minutes then thoroughly rinsed in UHQ water. Excess water is removed with filter paper. Grids were then placed face down in droplets of Reynolds lead citrate solution for 1 minute then rinsed again in UHQ water. Grids are allowed to thoroughly dry before placing in the electron microscope.
5. Deflasking procedure for banana plantlets in tissue culture

The process of deflasking tissue cultured banana plants is designed to ease the transition from the high-humidity, high-carbon, hermetically-sealed environment of tissue culture containers, to field conditions. This process must occur gradually, or plants will adapt fast enough. General procedure as follows:

1) Mature, rooted plantlets are carefully removed from tissue culture containers and rinsed in buckets of water to remove all tissue culture media adhering to roots. The roots may be trimmed at this stage.

2) Plantlets are potted into cell trays containing UC potting mix and covered with a plastic vented cell tray cover. The vents on the cover are closed, and the inside is misted to encourage high levels of humidity.

3) Trays are placed into a glasshouse with up to 75% shade cloth coverage.

4) For the first week, plantlets are kept at low light conditions with the shade cloth and are misted daily (twice-daily during summer).

5) After one week, shade cloth intensity is decreased to 50%, and the vents on the plastic cover are opened. Misting continues daily.

6) After two weeks, shade cloth is reduced to 25% and the plastic cover is removed. Daily overhead watering begins at this stage (twice-daily during summer).

7) After three weeks, shade cloth is removed entirely.

8) After one month, plantlets are ready for potting up.
6. Tube inoculation method for introducing *Foc* to banana plants in pots

When studying pathogenesis, it is usually necessary to artificially inoculate plants with the pathogen of interest. Ideally, inoculation conditions should mimic field conditions as much as possible. This is relatively straightforward for foliar pathogens but becomes far more complicated with root and soilborne pathogens. In the banana/*Foc* pathosystem, there is currently no internationally accepted standard way of inoculating plants. There are three general ways of inoculating: spore drench, spore dip and introducing fungal colonised organic matter.

At The University of Queensland we tend towards spores for soilless culture (e.g. hydroponics) and introducing fungal colonised millet for plants grown in pots. The millet preparation procedure is described in Chapter 4. Usually the colonised millet is mixed into the potting mix when plants are being potted up into larger pots. While this virtually guarantees infection and development of symptoms, it does not accurately mimic field conditions, as the process of potting up causes considerable damage to the root system, most likely predisposing it to *Foc* infection.

For that reason, we developed a less disruptive inoculation technique. The goal was to introduce inoculum into the potting mix without uprooting the plants. To achieve this, when plants were potted up, two ~10 cm long polypropylene plastic irrigation tubes were inserted into the pots while mix was added. When plants were ready for inoculation, the tubes were removed to produce two cylindrical voids in the soil. Inoculum was added to these voids at the desired rate, then covered with potting mix. To avoid root injury predisposition, tubes were removed 24 hours before inoculation. This method allowed for introduction of inoculum without seriously damaging the roots.
Chapter 9 References


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