Assessing the potential of silicon in the control of Fusarium wilt in banana

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This report describes work carried out to assess the influence of silicon amendments in conferring resistance to Fusarium wilt in banana

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Media Summary

Fusarium wilt causes large economic losses to the banana growers of the subtropics in Australia. It also poses the greatest threat to tropical banana production should the highly virulent tropical race 4 strain of this fungus (which is currently present in Northern Territory) arrive in the main production area in Tully, Queensland. Paradoxically, tissue culture banana plants which are produced to eliminate the spread of diseases including Fusarium wilt, are actually more susceptible to Fusarium wilt when first planted out than plants not derived from tissue culture. One of the reasons postulated for this increased susceptibility is lack of beneficial microbes.

Silicon applications have previously been implicated in enhanced resistance to plant disease. In this study, two types of silicon formulations (potassium silicate and sodium silicate) were applied to banana plants both in tissue culture and as pot plants in the glasshouse. These plants were then challenged with the Fusarium wilt fungus (subtropical race 4, the strain that occurs in SE Queensland). The potassium silicate treatment was the most effective in reducing initial disease symptoms. Silicon applied in tissue culture did have a lasting effect in reducing Fusarium wilt when plants were later challenged with the fungus. However there was no apparent difference between silicon and non-silicon treatments in the final disease assessments taken at ten weeks after inoculation; this was thought to reflect the high inoculum level and ideal growing conditions for Fusarium which would not necessarily reflect conditions in the field. Experiments should now be undertaken in field conditions to determine the best protocol for silicon application. When the fungus was observed at the microscopic level its growth was retarded when observed in the plants treated with potassium silicate. Further research is required, but these results give a useful indication that silicon treatment, even when applied only in tissue culture, may reduce the affects of Fusarium wilt.

Technical Summary

Fusarium wilt caused by *Fusarium oxysporum* f.sp. cubense (*Foc*), is responsible for large economic losses to the banana growers of the subtropics in Australia. It also poses the greatest threat to the main production area of Tully should the tropical race 4 (TR4) strain which is currently present in NT arrive in that region. Paradoxically, tissue culture banana plants which are produced to eliminate the spread of diseases including Fusarium wilt, are actually more susceptible to Fusarium wilt when first planted out, than plants not derived from tissue culture. One of the reasons postulated for this increased susceptibility is lack of beneficial microbes. Our earlier studies indicated that silicon applied in tissue culture can render plants more resistant to Fusarium wilt when subsequently challenged as pot plants.

In this project we assessed the different silicon formulations, potassium silicate (Kasil) and sodium silicate, with potassium sulphate used in control treatments, to determine if resistance to Fusarium wilt could be enhanced. We also investigated mechanisms by which silicon may be acting, by assessing the plants treated with silicon and *Foc* (subtropical race 4) at the molecular and histological level.

Treatment with potassium silicate did enhance resistance when plants were assessed for early disease development (leaf chlorosis). However, final disease assessments did not show any significant difference between potassium silicate and control treatments. The pot trial test undertaken was considered too harsh and a subsequent test is being carried out using lower inoculum levels; this should more truly reflect field situations. Sodium silicate returned inconsistent results and also indications of phytotoxicity and will not be used in further assays. Mineral analysis of silicon treated banana plants confirmed uptake of silicon into the banana pseudostem.

The effects of silicon were examined through changes in gene expression identified by reverse transcriptase-quantitative polymerase chain reaction. The results suggest that silicon, as well as other plant nutrients; increase the plant response to the pathogen in the early stages of infection. Further analysis is required to determine if priming of defence gene activation is occurring.

The infection process of *Foc* on Cavendish banana was studied in the elongation zone of roots using transmission electron microscopy and comparisons made between plants that were treated with potassium silicate with those not receiving a nutrient amendment. In non-silicon treated plants, penetration occurred randomly, with hyphae mostly penetrating the epidermis intracellularly. Hyphae penetrated further intracellularly through the hypodermis and into the cortex where it grew both intercellularly and intracellularly. Defence responses (such as occlusion of intercellular spaces, wall appositions, and wall thickening) were concentrated mostly in the hypodermis at 3 days post inoculation. In potassium silicate treated plants, *Foc* hyphae external to the root appeared swollen, vacuolated and had an indistinct cell wall; these are all symptoms of exposure to chitinase and/or lectin. By 7 days post inoculation, hyphae were growing intercellularly in the cortex in a vertical direction. Penetration of the stele was never observed. A definite mode of action by silicon was not elucidated. Results suggest that silicon is initially slowing down the infection process

Introduction

Fusarium wilt

Fusarium wilt, caused by the soil borne fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*), devastated world-wide banana production in the 1950s of the then cultivar of trade, Gros Michel. The commercial production survived through the adoption of the apparent Fusarium wilt-resistant cultivar Cavendish. However in the 1980s, strains of the Fusarium wilt fungus that could attack Cavendish were noted in subtropical growing regions including SE Qld and NSW. These Cavendish-attacking strains were designated Race 4 whereas the term Race 1 was given to those that attacked Gros Michel which included Lady Finger cultivars. In the 1990s, strains were observed that could attack Cavendish in tropical regions and were found to be very aggressive; the term Tropical Race 4 (TR4) was coined. It is TR4 that now poses the greatest threat to the banana industry world wide including that of the main production area in Australia in the Tully region.

As a soil borne pathogen, *Foc* does not spread rapidly. The world wide epidemic in the mid 20th century can be attributed to the wide distribution of diseased bits and suckers from which new plantations were established. For this reason and for attempts to limit spread of viruses as well, most new plantings are now initiated from plants propagated in tissue culture. However, plants derived from tissue culture are more susceptible to Fusarium wilt when planted out into the field compared with plants derived form bits and suckers. For this reason research has been on-going to improve the resilience of tissue culture derived plants.

Fusarium oxysporum is long-lived in the soil, surviving as a saprophyte on root debris or as long lasting resting spores. Once established it is next to impossible to eradicate. So far resistance to TR4 is not an option and so cultural control methods are being investigated.

Silicon in plants

Despite its current non-essential status, silicon has been shown to improve resistance in particular plants to various fungal, insect and bacterial pathogens (Bélanger *et al.*, 2003; Diogo & Wydra, 2007; Epstein, 1994; Fauteux *et al.*, 2005; Kvedaras *et al.*, 2007). Studies have shown that silicon application on banana plants both in tissue culture and pot trials (post tissue culture) can induce resistance to *Foc* (Forsyth, 2006).

Deposited silicon in plant tissue provides structural support for the plant, such as stiffening of stem tissue in oats (Jones & Milne, 1963). Erect status helps to improve light interception and photosynthesis, reduce the impact of mechanical stresses such as wind, or decrease the chance of collapsing due to vascular infestation (Takahashi, 1995). Silicon is also known to reduce the negative effects of salinity, drought and cold (Liang *et al.*, 2007). It is possible that the alleviation of negative abiotic factors frees up plant resources, allowing them to mount a more effective defence against pathogens (Smith *et al.*, 1998).

Others have postulated that silicon deposition increases resistance by creation of a physical barrier, preventing fungal penetration and resisting enzymatic degradation (Ma, 2004; Stein *et al.*, 1993). For example, rice (*Oryza sativa*) shows increased resistance to rice blast (*Magnaporthe grisea*) when treated with silicon due to a barrier

in the epidermis (Datnoff *et al.*, 2001). Silicon is also deposited in the roots, primarily at the endodermis surrounding the stele, which may provide a physical barrier against vascular pathogens (Lux *et al.*, 2003). Additionally, Fauteux *et al*. (2005) theorised that silicon is deposited passively at wound sites by the act of transpiration.

Silicon application may induce systemic acquired resistance (SAR), in a method similar to the way plants pre-treated with biotic elicitors (such as endophytic bacteria) or chemical elicitors (such as salicylic acid) show increased resistance (Mohandas *et al.*, 2004; Schneider & Ullrich, 1994). SAR is a generalised plant-wide response to an elicitor that includes the hypersensitive response, strengthening of the cell wall, the oxidative burst, expression of defence genes and the accumulation of pathogenesis-related proteins (Conrath *et al.*, 2002; Durrant & Dong, 2004). SAR by definition remains active for a long time, even after the inducer or elicitor has degraded, especially in monocots such as banana (Tally *et al.*, 1999).

Priming, a subset of SAR, is defined as the enhanced capacity for rapid activation of cellular responses and defence mechanisms when challenged with a pathogen (i.e. defence responses are activated faster in the presence of a pathogen). When a plant is primed for defence, morphological changes related to the defence response are generally not seen until pathogen challenge occurs (Steiner & Schönbeck, 1995). Priming therefore has less of a metabolic cost than constitutive defence expression. Silicon may be inducing priming in plants and therefore would be a very efficient means of protecting plants against pathogens such as *Foc*.

In our studies at the University of Queensland in collaboration with DPI&F we showed that silicon application applied in tissue culture did seem to have a lasting effect and increased resistance to Fusarium wilt when the banana plants were later challenged with *Foc*. Continual application of silicon to pot plants also enhanced resistance to *Foc*.

In this study we applied different formulations of silicon to Cavendish banana plants both in tissue culture and post tissue culture and then challenged the plants with *Foc* (sub tropical race 4) in the pot trials in the glasshouse. Samples were taken at the early stage of infection for histological and molecular analysis in order to elucidate the mechanism by which silicon is enhancing the plant's defence mechanism. Comparisons were made between different silicon formulations (potassium silicate and sodium silicate) with potassium sulphate used as a control for the potassium. Shoot mineral analysis was conducted to confirm the silicon uptake.

Materials and Methods

Plant Material

Tissue cultured *Musa acuminata* Cavendish cv. Williams banana plantlets were obtained from the Department of Primary Industries & Fisheries facility in Nambour, in small tubs containing approximately 12 plants per tub. Plants had been subcultured three times at intervals of 4 weeks. Each plantlet was cultured on Murashige-Skoog (MS) media (Murashige & Skoog, 1962), in plastic containers (to limit silicon contamination from glassware). The plantlets were divided into four groups and were cultured with the following amendments to the media: no addition, sodium silicate (NaSi – Sigma-Aldrich) at 3.92mL/L, potassium silicate (KSi - Sigma-Aldrich) at 5.06mL/L or potassium sulphate ($KS0_4$ - LabServ) at 1.34 g/L. Plantlets were deflasked according to Daniels and Smith (1991) planted into seedling cell trays containing standard University of California (UC) mix (Barker *et al.*, 1998). Trays were then transported to a custom built humidity chamber in a glasshouse covered by a 70% shade-cloth. Trays were watered daily and misted twice daily to maintain high levels of humidity. Plants were organised in a randomised block to decrease potential positional effects from sunlight, temperature and humidity.

After twenty days, once the plantlets were sufficiently hardened they were transferred to the Crop Development Facility at the DPI&F station in Redlands, Queensland for potting up. Each plant was potted up into individual 10 cm pots containing UC mix. Pots were then transferred to a climate controlled glasshouse maintained at 24°C at night and 27-30°C during the day. Each pot received a weekly drench depending on treatment group consisting of 50mL of either: water, NaSi at 3.92mL/L, KSi at 5.06mL/L or $KS0_4$ at 1.34g/L. Plants were hand-watered twice daily for the duration of the experiment except when they were drench-treated. Drenching was not performed in the weeks when plants were inoculated.

	Treatment plants post tissue culture							
Treatment of plants in tissue culture	Sodium silicate		Potassium silicate		Potassium sulphate		Control	
Sodium silicate	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc
Potassium silicate	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc
Potassium sulphate	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc
Control	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc	With Foc	No Foc

Table 1 The experimental design of nutrient treatments and inoculation treatments. The replication was spread over two experiments performed 3 weeks apart. Sampling for defence gene activation and histological assays occurred at 3 days, 7 days in the original and in the repeat experiment. Plants were assessed for external disease symptoms at 4 weeks and for internal symptoms at 10 weeks post inoculation. The replicate number of plants for each treatment x time point assay was six for each of the two repeat experiments.

Fungal Inoculum

Three pathogenic isolates (Table 2) of *Fusarium oxysporum* f. sp. *cubense* subtropical race 4 were used in this experiment. Freeze-dried filter paper cultures of each isolate stored at -80°C were recovered onto potato dextrose agar + 100ug/mL streptomycin plates (PDA) and maintained on carnation leaf agar + 100ug/mL streptomycin plates (CLA) in an incubator at 27°C (Burgess & Liddell, 1983).

BRIP number	**VCG**
22615	0120
24218	01220
23598	0120

Table 2. Isolates of *Fusarium oxysporum* f. sp. *cubense* subtropical race 4 used in this experiment. BRIP refers to the number assigned to each isolate by the Queensland Plant Pathology Herbarium Collection. Vegetative compatibility group (VCG) refers to which genetic grouping each isolate belongs to.

Millet Inoculation

Millet inoculum was prepared as per Smith *et al.* (2008) with alterations summarised below: untreated jap millet (*Echinochloa esculenta)* was rinsed in tap water then soaked overnight in distilled water. Approximately 200g of soaked millet grains were strained through a sieve into 500mL tubs and autoclaved for 20 minutes at 121°C and at a pressure of 120kPa. Millet grains were rinsed, strained and autoclaved again for 20 minutes. Once millet tubs reached room temperature they were inoculated with agar plugs from CLA plates containing the isolates BRIP numbers 22615, 24218 and 23598. The three isolates were combined to counteract the potential loss of virulence which arises from continuous subculturing. Millet tubs were then stored in the dark at room temperature for 8 days. Tubs were gently shaken daily to maximise exposure to inoculum and to prevent settling. Plants were inoculated by re-potting into the same pot with fresh UC mix combined with approximately 30g of inoculated millet.

Disease Assessment of Wilt Symptoms

Four weeks post inoculation, external disease symptoms were rated by determining the percentage leaf yellowing occurring in inoculated plants. Scale ranged from 0% (healthy) to 100% (completely necrotic).

At 10 weeks post inoculation, internal disease symptoms were rated by determining the scale of vascular discolouration within the rhizome of the plant, according to INIBAP (International Network for the Improvement of Banana and Plantain) guidelines (Orjeda, 1998). Rhizomes were cut into quarters and rated. Scale was as follows: 1) completely clear, no discolouration; 2) isolated points of discoloration; 3) one third discolouration; 4) between one and two thirds discolouration; 5) greater than two thirds discolouration; and 6) total discolouration. *Foc* was reisolated from diseased tissue by taking vascular strands and diseased rhizome tissue, surface sterilising with 2.5% sodium hypochlorite (bleach), rinsing in sterile distilled water and plated onto PDA+S plates. After 5 days, cultures were inspected microscopically for *Foc*.

RNA Extractions and RT-PCR

Sampling occurred at 3 and 7 days post inoculation (dpi). Six plants of each treatment were removed from the soil and a selection of all the root types was removed. The roots were washed and flash frozen in liquid nitrogen before being stored at -80^{o}C. Six plants were pooled into two samples of three plants each.

Samples that were treated with the same treatment *in vitro* and in the glasshouse were examined for gene expression. With the repeated experiment there were four biological replicates for each treatment and inoculation. Defence gene expression at two time points, 3 and 7 days, were examined. Root samples were ground to a fine powder in liquid nitrogen to prevent thawing. Total RNA was extracted using RNeasy Plant RNA extraction kit (Qiagen, Valencia, USA). Initial lysis buffer was amended with polyethylene glycol and polyvinylpyrrolidone (Sigma, St Louis, USA) to reduce phenolic interference (Forsyth, 2006; Gehrig *et al.*, 2000). Integrity and quality of the RNA checked with a nanodrop ND-1000 spectrophotometer (Biolab, Melbourne, AUS). RNA was cleaned up, DNase treated, and cDNA was synthesised with SuperScript III (Invitrogen, Carlsbad, USA).

Reverse transcriptase quantitative PCR (RT-qPCR) was used to detect the expression of four defence genes in the samples converted to cDNA. The genes and corresponding primers were previously identified by Forsyth (2006) (see Table 3). The genes examined were: an osmotin-like gene (OSM), phenylalanine ammonia lyase (PAL), an endochitinase (ENDO), and a peroxidase (POX). The PCR was performed on a rotor-gene 3000 (Corbett Research, Sydney AUS). Each tube contained 20µl reaction with 10µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.4µl forward primer, 0.4µl reverse primer, 8.2µl of water, and 1µl sample. Cycling conditions were based on recommended cycling program for ABI instruments beginning with 50^{o}C for 2 mins hold, 95^{o}C for 2 mins hold followed by 40 cycles of: 95^{o}C for 15 seconds and 60^{o}C for 30 seconds.

Defence Gene Primer	**Forward/Reverse**	**Sequence 5'-3'**
Actin	Forward	TTGCTGGAAGAGACCTGA
Actin	Reverse	TGAGTGATGGCTGGAAGA
Endochitinase	Forward	GTCACCACCAACATCATCAA
Endochitinase	Reverse	CCAGCAAGTCGCAGTACCTC
Osmotin	Forward	ACTGTTGCAACTCCGGCAT
Osmotin	Reverse	AAGGTGCTCGTCTGATCGTC
PAL	Forward	CCATCGGCAAACTCATGTTC
PAL	Reverse	GTCCAAGCTCGGGTTTCTTC
Peroxidase	Forward	CGGTAGGATCCAAAGAAAGC
Peroxidase	Reverse	TTCAGAGCATCGGATCAAGG

Table 3: Primers identified by Forsyth (2006) used to amplify the listed defence genes for real time PCR.

The gene expression was measured by noting the number of cycles at which the amplification of each sample passed a fluorescence threshold of 0.05 (Ct). Each sample was run in duplicate. Two controls were included; controls containing no template were included in each run and controls where no reverse transcriptase was included in the synthesis of cDNA. To test the efficiency of each primer a sample of

pooled control treated samples were diluted by a factor of 10 in a dilution series and run in triplicate.

Harvesting Samples for Microscopy

Samples for microscopic investigation were taken at 3 and 7 days post inoculation. Three day samples were taken from the zone of elongation, just following the root cap and zone of mitosis (Figure 1). Previous work by Toh (2003) demonstrated that banana plants inoculated with *Foc* colonised millet show hyphal massing near the root cap and the zone of mitosis. Seven day samples were taken approximately from the zone of maturation. It was expected that colonisation of the vascular tissue would have occurred by 7 days, and hyphae would be present in the xylem vessels (but not in the cortex) in root tissue distant from the region of penetration.

Root tissue for sectioning was selected randomly from roots that had been washed under running water until completely free of potting media. Roots to be sectioned transversely were immersed in a droplet of water and then cut free-hand to an approximate thickness of 1mm with a double-sided razor blade. Transverse sections that were too large for TEM processing (generally larger than 1mm diameter) were cut in half perpendicular to the plane of sectioning. Smallest sized sections are preferred for processing, as it maximises chance of complete fixative penetration and resin infiltration (Hawes & Satiat-Jeunemaitre, 2001). Approximately 60 sections were taken per treatment group and time point.

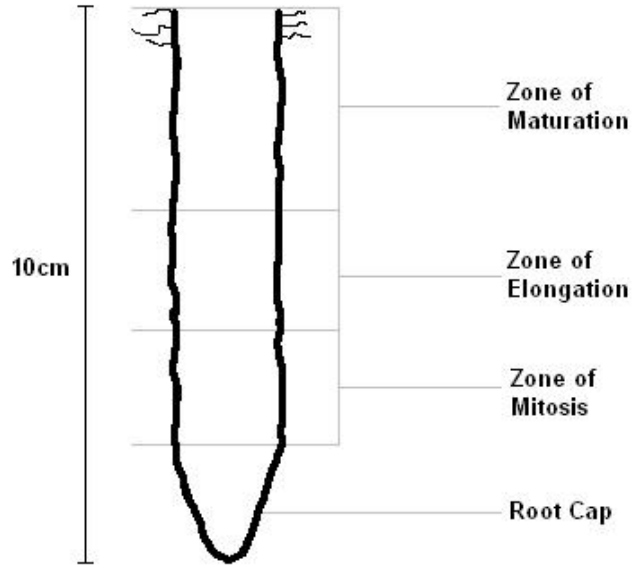

Figure 1. Diagram of a primary banana root demonstrating the four main zones of a growing root. Samples for 3dpi were taken from the zone of mitosis. Samples for 7dpi were taken from the zone of maturation. Note that root hairs are present in the zone of maturation only.

TEM Processing and Microscopy

Fresh sections were immediately transferred to vials of 2.5% glutaraldehyde fixative in phosphate buffer (pH6.8) amended with 0.7% caffeine and stored in a refrigerator. Caffeine was added to preserve the natural state of phenolics within cells, as fixatives tend to react with phenolics, causing them to leach from storage vacuoles into the cytoplasm (Mueller & Greenwood, 1978). Samples were processed using a Biowave

Microwave (Pelco) (Giberson & Demaree Jr, 1995). A generalised microwave protocol was adapted to suit banana (R. Webb & J. Whan, personal communication) as follows: i) Samples transferred to vials containing fresh fixative (refer to *Harvest* section) and microwaved twice for 2 minutes on/off/on at 150 watts with vacuum on; ii) rinsed in phosphate buffer three times and microwaved for 40 seconds at 80 watts with vacuum on; iii) immersed in 1% osmium tetroxide (OsO_4) and then microwaved for 2 minutes on/off/on at 80 watts with vacuum on; iv) washed twice with deionised water then microwaved for 40 seconds at 80 watts with vacuum on; v) serial dehydration with acetone at 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% (x2), each microwaved for 1 minute on/off/on at 250 watts with vacuum off per step; vi) resin infiltration with Spurr's medium grade resin (Spurr, 1969) by immersion in resin:solvent mixes of 1:3, 1:2, 1:1, 2:1 and 3:1 respectively and microwaved at each step for 3 minutes at 250 watts with vacuum on; vii) final infiltration by immersion in 100% resin and microwaving for 15 minutes (x2) at 250 watts with vacuum on and then left on a rotator overnight at room temperature and pressure; viii) infiltrated samples poured into silicone block moulds and polymerized in an oven for two days at 60°C. Fifteen blocks (containing two samples per block) were obtained per treatment group/time point.

Polymerised resin blocks were trimmed with a double-edged razor blade and cut with a glass knife. Semi-thick (300nm) sections were taken using a glass knife made from a glass knife-maker (Reichert-Jung) on a UC Ultracut 6 ultra-microtome (Leica) and post-stained with toluidine blue in 5% borax solution. Sections were visualised with bright-field using a BX6061 microscope (Olympus) with a DP70 digital camera (Olympus). Thin (80nm) sections were cut using a 35° ultra diamond knife (Diatome) on the same ultra-microtome, treated with chloroform vapour (to remove creases in sections) and transferred to 80-mesh copper grids (ProSciTech). Grids were post-stained with 5% uranyl acetate in 50% ethanol and then Reynolds lead citrate (Reynolds, 1963) and were visualised using a JEM1010 Transmission Electron Microscope (JEOL) operating at an accelerating voltage of 80kV. TEM images were captured using a Megaview III digital soft imaging system. All electron and light micrographs were imported into Adobe Photoshop CS2 for contrast and brightness adjustment. For each treatment group, 20-30 sections from six individual plants were viewed.

Mineral Analysis of Banana Tissue

Banana pseudostem tissue was collected for mineral analysis, from samples taken at 7dpi. For each sample, roots were removed from the rhizome and all remaining tissues (pseudostem, leaves) were placed into brown paper bags for desiccation. Samples were placed in a drying oven maintained at 60°C for five days. Plant material was pooled, with two plants per sample (total of three samples per treatment group). Dried banana tissue was ground in a mechanical grinder and submitted to the Analytical Services Unit of the School of Land Crop and Food Science at the University of Queensland for mineral analysis by microwave digestion using the USEPA 3052 method (Kingston & Walter, 1992) summarised as follows: i) 500mg of sample material is weighed out into a Teflon vessel along with 10mL of water, 5mL of concentrated nitric acid, 4mLof hydrofluoric acid and 2mL of concentrated hydrochloric acid; ii) samples are pre-digested for 16 hours then submitted to closed vessel digestion using a CEM MDS2000 microwave digestor at 68% power for 20 minutes; iii) digests are made up to 50mL volume with the addition of 4mL of

saturated boric acid; iv) digests are analysed using a Varian Vista Pro ICPOES instrument running at 1200 watts forward power (D. Appleton, personal communication). The following elements were analysed: aluminium (Al), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulphur (S), silicon (Si) and zinc (Zn).

Measurement of pH

A 250g representative sample of potting mix was taken from six pots for each uninoculated treatment group 14 weeks after inoculation (to compensate for any possible effects that millet had on the potting mix). Potting mix was taken from several locations within the pot to form a homogenous sample. Each sample was mixed with 250mL of distilled water, mixed thoroughly and allowed to settle for five minutes. Measurements of pH were taken on a pH meter at room temperature.

Statistical Analysis

All data requiring statistical analysis was imported into Microsoft Office Excel and analysed using the standard data analysis package. All statistical tests performed were either Student t-tests or two sample paired tests when samples were not independent.

Results

External Symptoms at 4 Weeks Post Inoculation

The 4 week external disease assessments showed the beginnings of wilt symptoms in all inoculated plants, including splitting at the base of pseudostems, deformed leaf initials, and chlorotic leaves beginning with the oldest. Leaf yellowing was rated on a scale from 0% (no yellowing) to 100% (all leaves totally chlorotic) (Figure 2). Non-*Foc* treated control plants showed negligible amounts of yellowing. Only potassium silicate treated plants had significantly less leaf yellowing than water treatment ($p < 0.05$). There was no significant difference between plants with different nutrient treatments applied in the glasshouse. However, there was a significant difference associated with the treatment whilst in tissue culture. Plants treated with potassium silicate whilst in tissue culture and subsequently inoculated with *Foc* showed less symptoms of chlorosis than plants subject to potassium sulphate or sodium silicate tissue culture nutrient treatments (Figure 3).

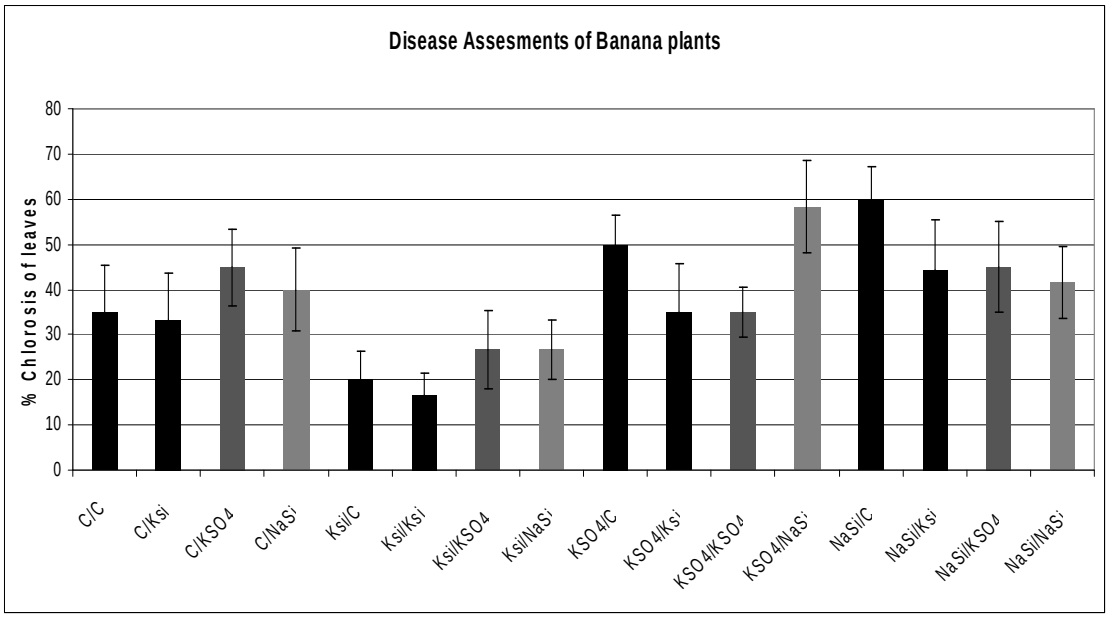

Figure 2: External disease assessment of Cavendish plants 4 weeks after inoculation with *Fusarium oxysporum f.sp. cubense* . Plants were treated with either: Murashuge and Skoog (MS) media only (C), potassium silicate (KSi), potassium sulphate (KSO4) or sodium silicate (NaSi) *in vitro* then with either water (C,), potassium silicate (KSi, , potassium sulphate (KSO4,) or sodium silicate (NaSi,) in the glasshouse. Error bars indicate standard error, n = 6, samples taken from experiment 1. Chlorosis was not observed on any of the non *Foc* treated plants at this stage.

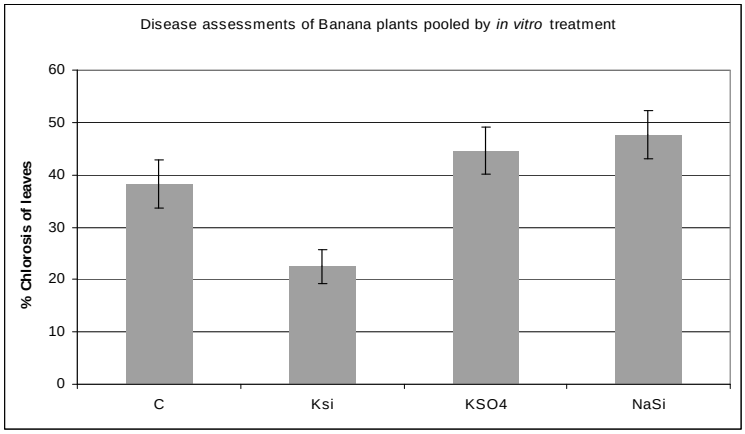

Figure 3: Disease assessment of banana plants 4 weeks post *Fusarium oxysporum f.sp. cubense* infection. The data were pooled showing *in vitro* treatment including all of the glasshouse treatments. Plants grown on Murashuge and Skoog (MS) media only or MS amended with, potassium silicate, potassium sulphate or sodium silicate. Error bars represent standard error, n = 6.

Internal Symptoms at 10 Weeks Post Inoculation

No strong correlation between treatment and the level of disease was observed for internal symptoms at 10 weeks post inoculation (Figure 4). Pooling of data by *in vitro* treatment showed a general trend to lower levels of disease in the control and potassium silicate treated plants but this was not statistically significant (Figure 5).

The external characteristics of the plant were also recorded. There appeared to be no difference between the height of the pseudostem correlated with treatment groups nor in the number of leaves the plant of different treatments (data not shown).

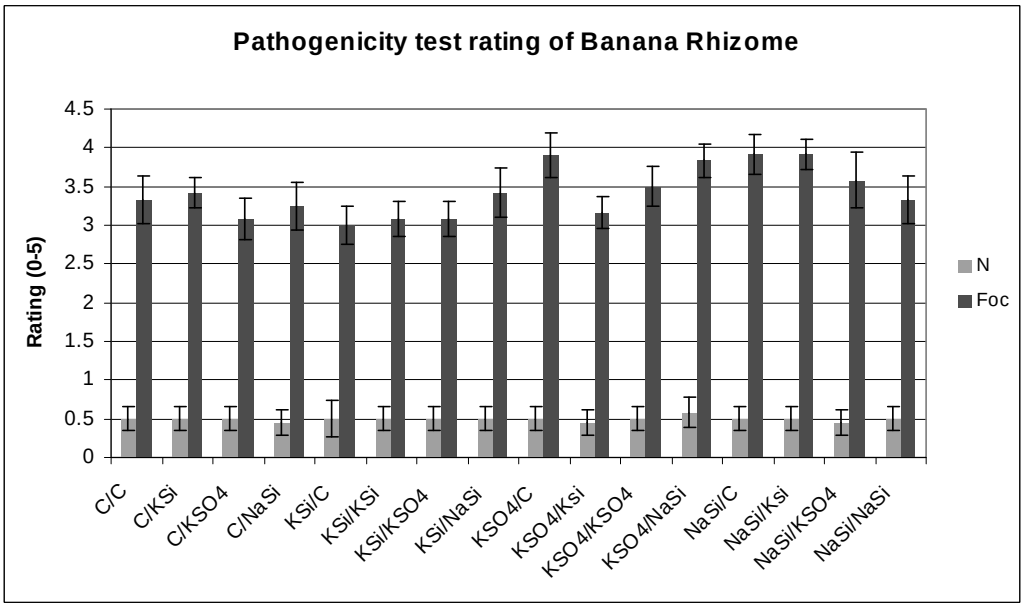

Figure 4: Pathogenicity test at 10 weeks post inoculation. Discolouration of the rhizome on a scale of 1-6 based on INIBAP rating system. Plants were treated with either: Murashuge and Skoog (MS) media only (C), potassium silicate (KSi), potassium sulphate (KSO4) or sodium silicate (NaSi) *in vitro* then with either: water (C), potassium silicate (KSi), potassium sulphate (KSO4) or sodium silicate (NaSi) in the glasshouse. Foc (dark coloured colums) relates to plants inoculated with millet infested with *F.oxysporum* f.sp. *cubense* and N (light coloured columns) to those treated with sterile millet only. Error bars represent standard error, n = 12.

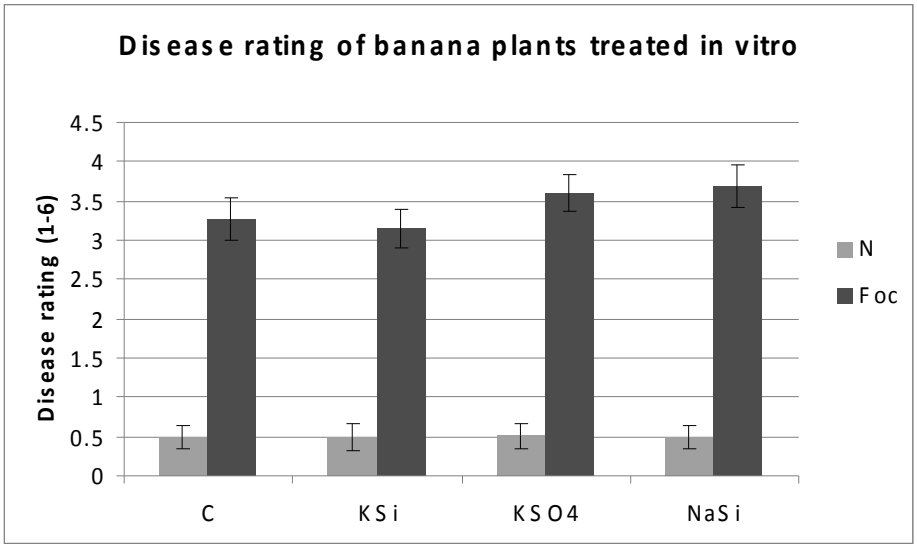

Figure 5: Pathogenicity test at 10 weeks post inoculation. All plants treated with the same treatment *in vitro* pooled together despite glasshouse treatment. Disease rating of rhizome discolouration on a scale of 1-6 based on the INIBAP rating system (Orjeda, 1998). Error bars represent standard error, n = 12.

Defence Gene Expression

Defence gene expression levels assessed 3 days following treatment with control millet (ie no *Foc*) are shown in Figure 6. Data are shown for plants subject to the same nutrient treatment *in vitro* and in the glasshouse. Expression levels are relative to the control non-nutrient supplemented plants ie where plants were grown on MS media only *in vitro* and treated with water in the glasshouse. The expression of the defence genes in the control treatment is considered to be 1 (Livak & Schmittgen, 2001).

Plants treated with potassium silicate did not show any significant difference in the induction of each of the defence genes compared with the non-nutrient supplemented control. Potassium sulphate treated plants showed an increased expression of the phenylalanine ammonia lyase (*PAL*) gene compared to plants treated with water. Plants treated with sodium silicate showed a large standard error in gene expression levels; with only osmotin expression level showing consistent up regulation.

Defence gene expression levels assessed 3 days following treatment with *Foc* are shown in Figure 7. The standard error is large in all treatments however there was a trend towards an increased induction of osmotin and *PAL* compared to controls. What is apparent in the comparison between *Foc* treated (Figure 7) and non-*Foc* treated plants (Figure 6), is that the potassium silicate treatment seems to only induce defence gene expression in the presence of *Foc*.

The gene expression was also examined at 7 days post inoculation. The standard error was high however the overall trend was for an increased defence gene expression level (data not shown).

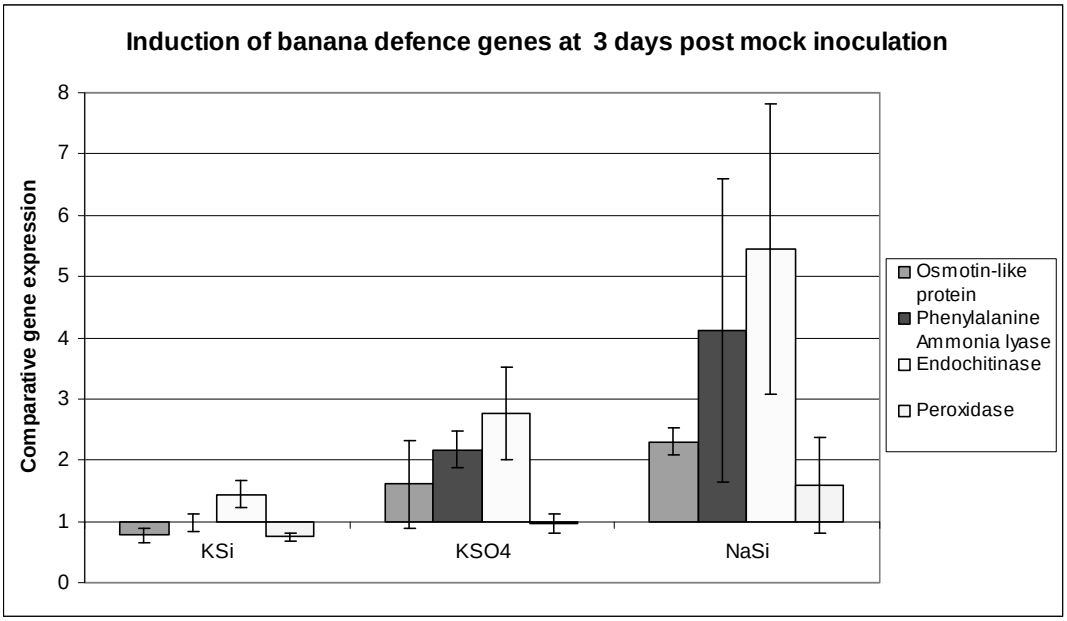

Figure 6: The expression of banana defence genes 3 days after mock inoculation. Nutrient treatments occurred *in vitro* and in the glasshouse. Expression normalized to actin and standardised to untreated control (grown on MS media and water treatment in the glasshouse) which has gene expression level of 1. Error bars the standard error between the four biological replicates.

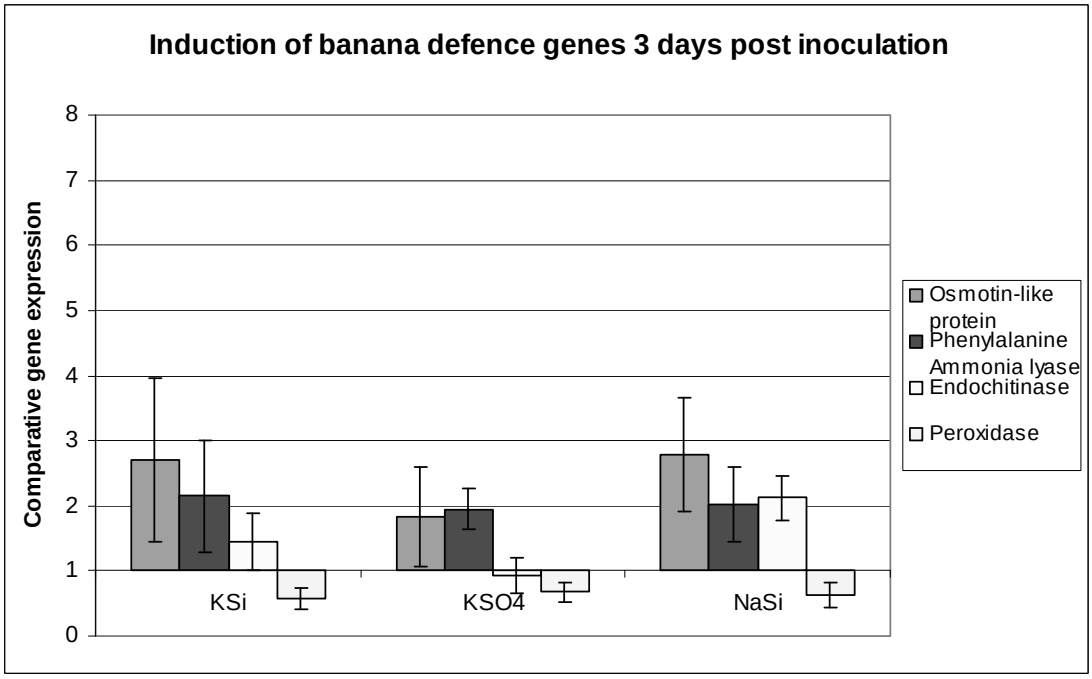

Figure 7: The expression of banana defence genes 3 days after inoculation with *Fusarium oxysporum* f.sp. *cubense*. Nutrient treatments occurred *in vitro* and in the glasshouse. Expression normalized to actin and standardised to inoculated untreated control (grown on MS media and water treatment in the glasshouse) which has gene expression level of 1. Error bars the standard error between the four biological replicates.

Histological Analysis - Three days following Foc inoculation

Control (non- nutrient supplemented) plants

Three days post inoculation of non-nutrient supplemented plants, *Foc* colonisation of the root had occurred, with fungal density generally very high in the epidermis **(**Figure 8). Fungi massed on the surface and within the epidermal/hypodermal/top cortical layers. Penetration occurred without the formation of an appresorium, but with the formation of a blunt penetration peg directly from hyphae **(**Figure 9), although where wounding occurred it was often colonised by hyphae (Figure 10).

Thickening of hypodermal and cortical cells occurred, along with occlusion of intercellular spaces, forming a barrier against encroaching hyphae. Penetration appeared to occur in the hypodermis, as *Foc* appeared to remove epidermal cells and grew between them, often engulfed in fibrillar material of either fungal or plant origin. Detached epidermal cells often remained in close proximity to the root and also appeared intact, with little internal cell disorganisation. Hyphae were growing both intercellularly and intracellularly in epidermal, hypodermal and cortical tissue (Figure 11). Intercellular growth was predominantly along the longitudinal axis of the root as evidenced by cross section whereas surface growth was often seen to be toward the stele.

Defence responses (such as wall appositions and wall thickening) were proportional to the level of infection. Hyphae commonly grew within the grooves of epidermal cells and penetrated epidermal tissue with little plant cellular response apart from loss of turgor. Within the hypodermis, intense cellular responses such as the accumulation of phenolics and wall appositions were present. Hypodermal cells became thin and misshapen, adopting a buckle shape which could trap hyphae. The most centripetal any hyphae were observed at 3dpi was near the endodermis, usually intercellularly . Disorganisation of cortical cells was present to varying degrees in all samples, both in the direct presence of hyphae and without.

Intercellular spaces in the hypodermal region are occasionally full of an electron opaque substance (has an affinity for the osmium tetroxide stain or lead citrate) in water treated plants, but in inoculated plants they become fully occluded . Lateral roots and root initials were frequently present, but did not show much fungal colonisation although they were not studied in depth.

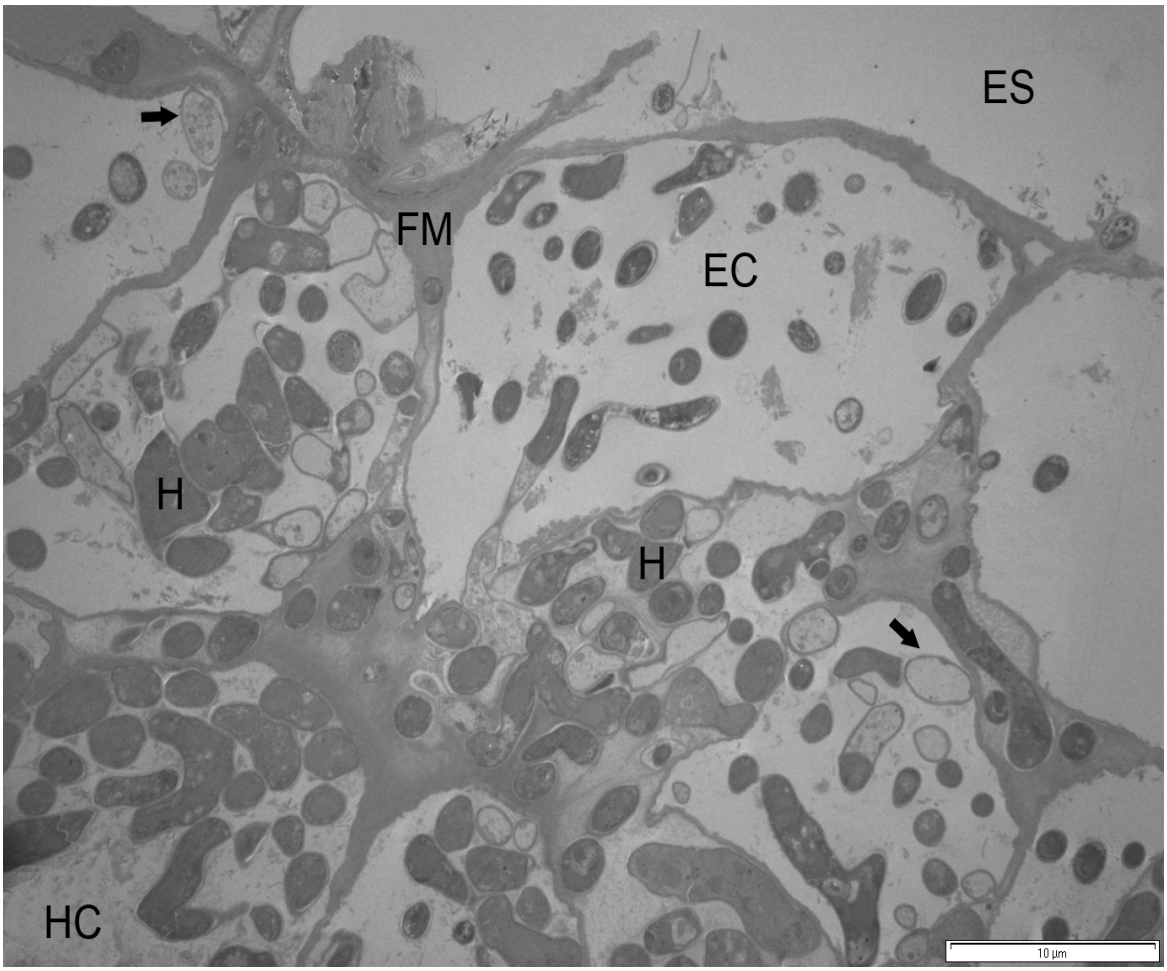

Figure 8. Electron micrograph of extensive fungal colonisation of epidermal and hypodermal cells in water treated banana root at 3 days post inoculation with *Foc* x2500. Multiple hyphae (H) can be seen growing intercellularly, embedded in a thick fibrillar matrix (FM). Hyphae are growing either vertically (circular hyphae) or longitudinally (cigar shaped hyphae). Fungi growing intracellularly are occasionally moribund (arrow). ES – extracellular space; EC – epidermal cell; FM – fibrillar material; H – hyphae; HC – hypodermal cell.

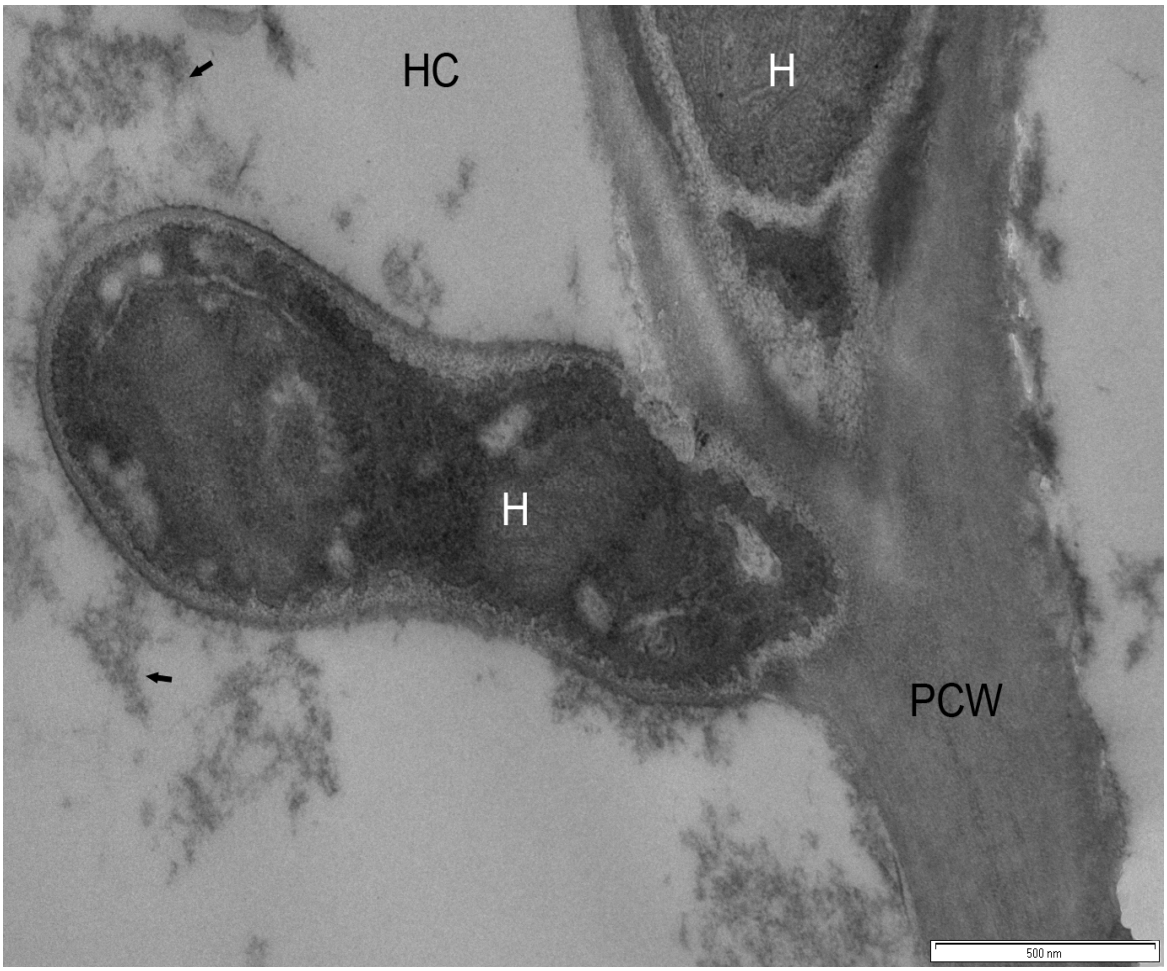

Figure 9 Electron micrograph of *Foc* hyphae penetrating a wall bordering a hypodermal and cortical cell at 3 days post inoculation with *Foc* x50 000. A single hypha (H) can be seen here forming a blunt penetration peg in a plant cell wall (PCW). The region immediately adjacent to the penetration peg in the plant cell wall is showing discolouration and disorganisation. Note the accumulation of electron opaque material in association with the hyphae (arrows). Another hypha (H) is present within the intercellular space adjacent to the plant cell wall. HC – hypodermal cell; H – hyphae; PCW – plant cell wall.

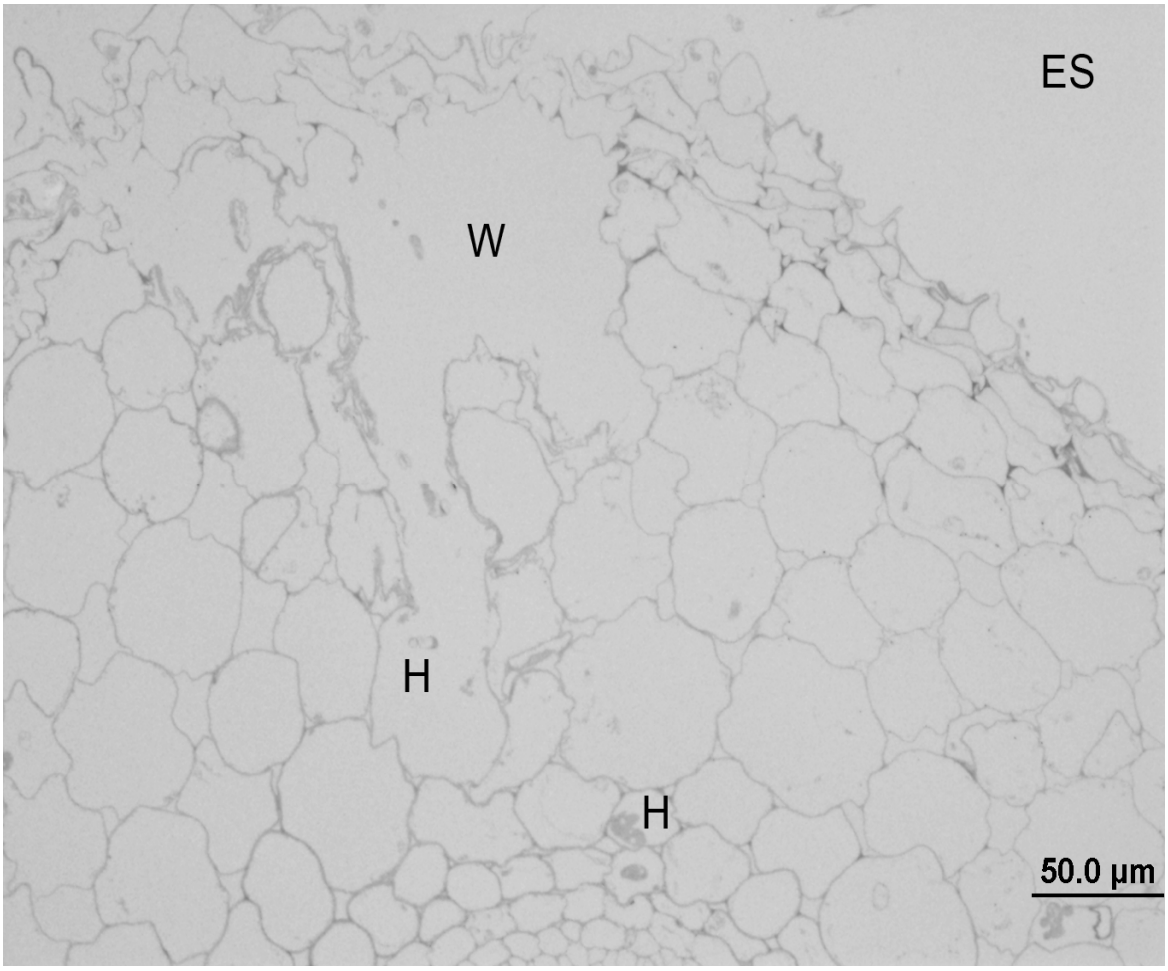

Figure 10. Light micrograph of hyphal colonisation in a wounded banana root at 3 days post inoculation with *Foc* x200. Section of an untreated plant at 3 dpi showing hyphal (H) colonisation of a wound (W). The hyphae appear to have penetrated as far as the endodermis. W – wound; H – hyphae; ES – extracellular space.

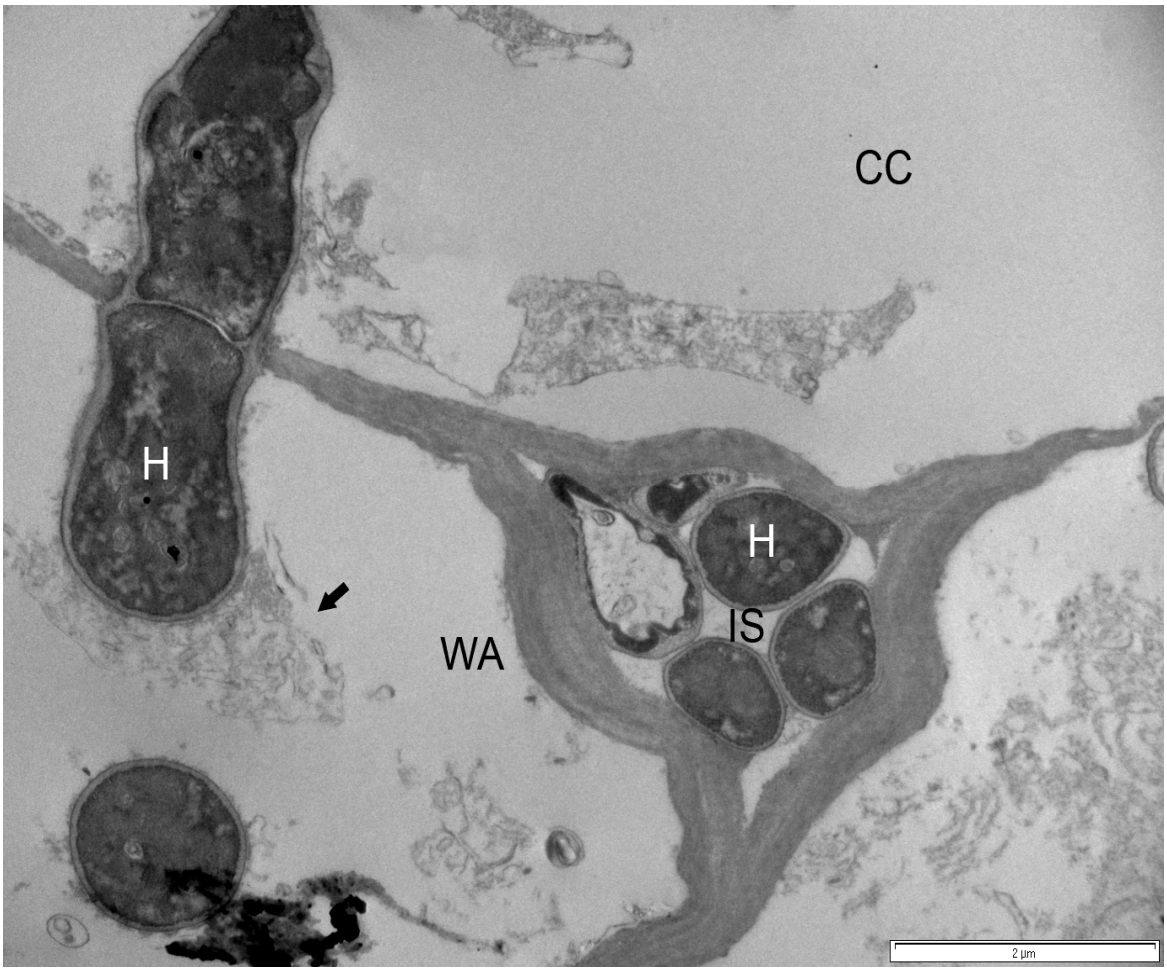

Figure 11. Electron micrograph of *Foc* hyphae growing intercellularly and intracellulary within the cortex of a water treated banana root at 3 days post inoculation x15 000**.** Hyphae growing within the intercellular space (IS) are growing vertically, adhered to the cell wall. Wall appositions (WA) are forming within the cell opposite the hyphae. Hyphae are capable of directly penetrating plant cell walls, and develop septa in line with the cell wall. Hyphae growing intracellularly are found in association with an electron opaque material (arrow). H – hyphae; WA – wall apposition; IS – intercellular space; CC – cortical cell.

Nutrient-supplemented plants

In potassium silicate treated plants, the external hyphal diameter was significantly larger than for water treated plants. Hyphae were generally only found external to the root, not in contact with epidermal cells (Figure 12). Hyphae were highly vacuolated, swollen and had alterations in their cell wall (Figure 13, 14). Both external and internal average hyphal diameter, measured from six individual samples, ranged from 1.38±0.07µm in water treated plants to 2.41±0.43µm in potassium silicate treated plants, significantly different ($p < 0.05$) with $n = 8$.

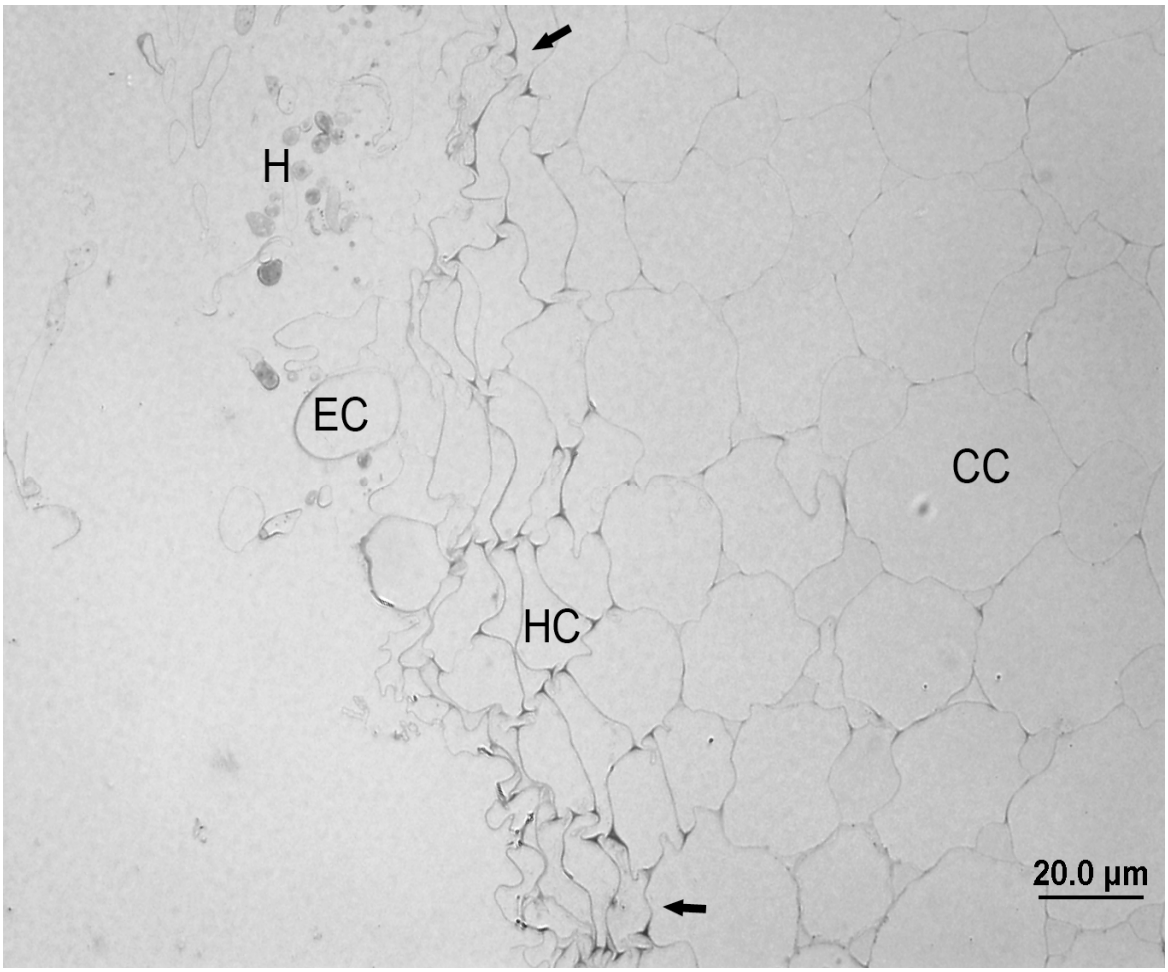

Figure 12. Light micrograph of *Foc* hyphae growing in association with epidermal cells in Potassium silicate treated banana root at 3 days post inoculation x400. Hyphae (H) in Potassium silicate treated plants at 3dpi are generally found external to the epidermis, in association with detached epidermal cells (EC). Defence responses often manifested in the hypodermis, and the cortex: thickening of cell walls, and complete occlusion of intercellular spaces (between arrows). H – hyphae; EC – epidermal cell; HC – hypodermal cell; CC – cortical cell.

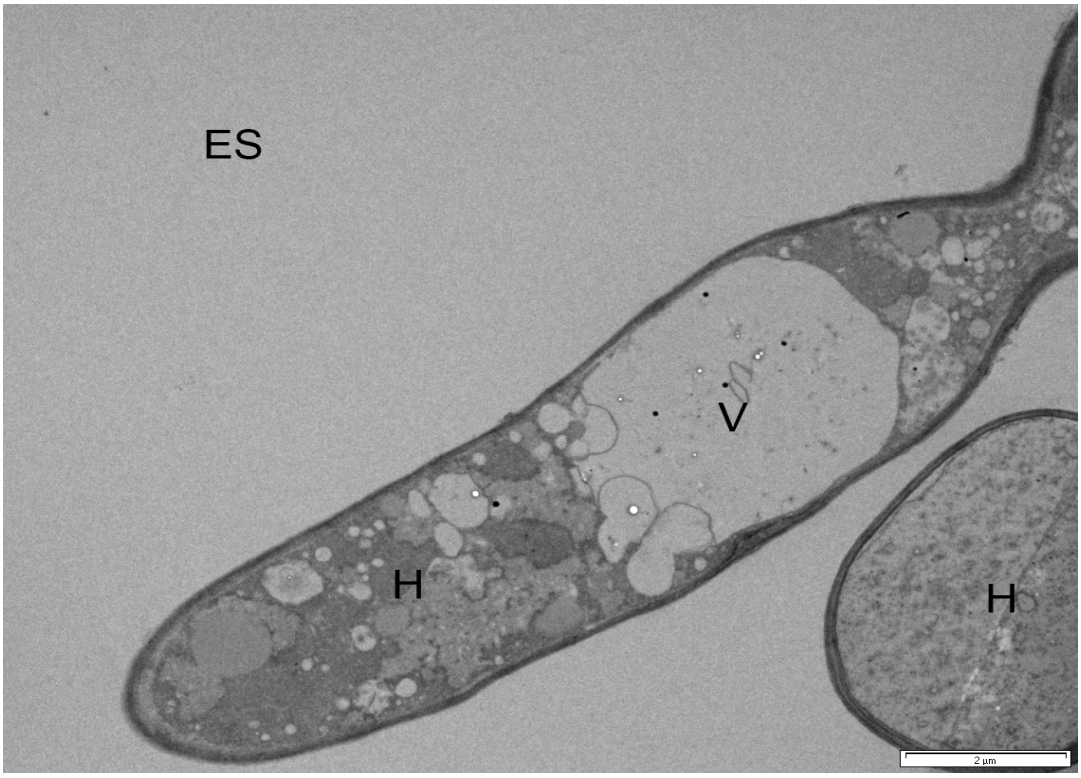

Figure 2. Electron micrograph of an hyphae of *Foc* growing in association with the root of an inoculated, potassium silicate treated banana plant at 3 days post inoculation x8000. Hyphae (H) are swollen, have a reduced cell wall and considerable vacuolation (V). ES – extracellular space; H – hyphae; V – vacuole.

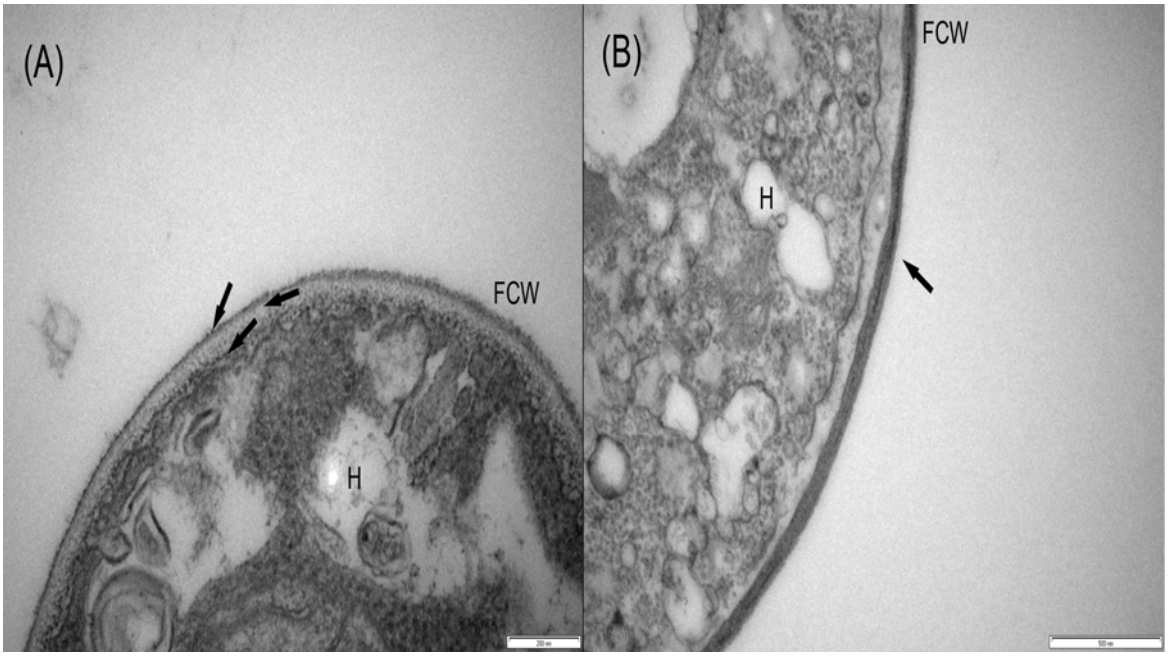

Figure 3. Electron micrograph comparing the fungal cell walls of hyphae in water treated and potassium silicate treated banana roots at 3 days post inoculation x50 000. The fungal cell wall (FCW) of hyphae growing in association with water treated plants (A) showed three distinct layers (arrows): an electron opaque layer (top), a lighter, more diffuse layer (middle) and another electron opaque layer (bottom). Conversely, hyphae in the Potassium silicate treated plants (B) showed a reduction in the thickness of the FCW with the three layers becoming indistinct. FCW – fungal cell wall; H – hypha.

Histological Analysis - Seven days following Foc inoculation

Control (non- nutrient supplemented) plants

After 7 days, defence responses were present in the form of wall appositions although less surface colonisation was occurring. Hyphae were occasionally found in association with the endodermis, but never in the stele or xylem. Hyphae were also seen to be occasionally growing intracellularly, with no obvious detriment to the cell, although hyphae appeared to accumulate an electron opaque substance in association with their cell wall. The fungus appeared to be growing mostly intercellularly within in the cortex (Figure 15).

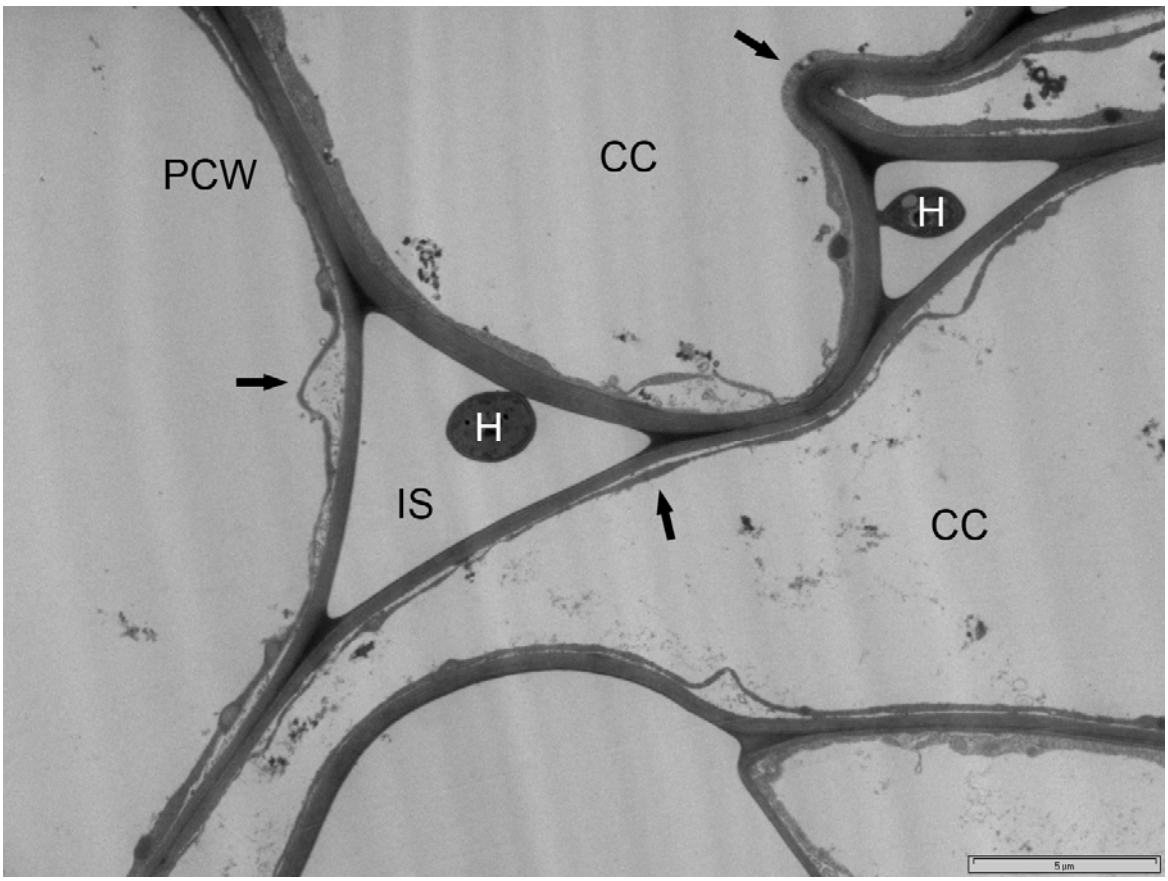

Figure 15. Electron micrograph of intercellular growth of *Foc* between cortical cells in the root of an untreated banana plant at 7 days post inoculation x4000. Hyphae are growing vertically within the intercellular space. Note the accumulation of material (arrows) opposite the hyphae along the plant cell wall. CC – cortical cell; H – hyphae; PCW – plant cell wall; IS – intercellular space.

Nutrient-supplemented plants

Potassium silicate treated samples no longer showed vacuolation, swelling and their cell walls had regained their distinct three-layered shape. Similar to water treated plants, hyphae which had penetrated intracellularly were found in association with an electron opaque substance (Figure 16). This substance was found more frequently and with greater density in Potassium silicate treated plants. Intercellular fungi were frequent (Figure 17). Growth was predominantly along the longitudinal axis of the root as evidenced by the hyphae resembling circles in cross section.

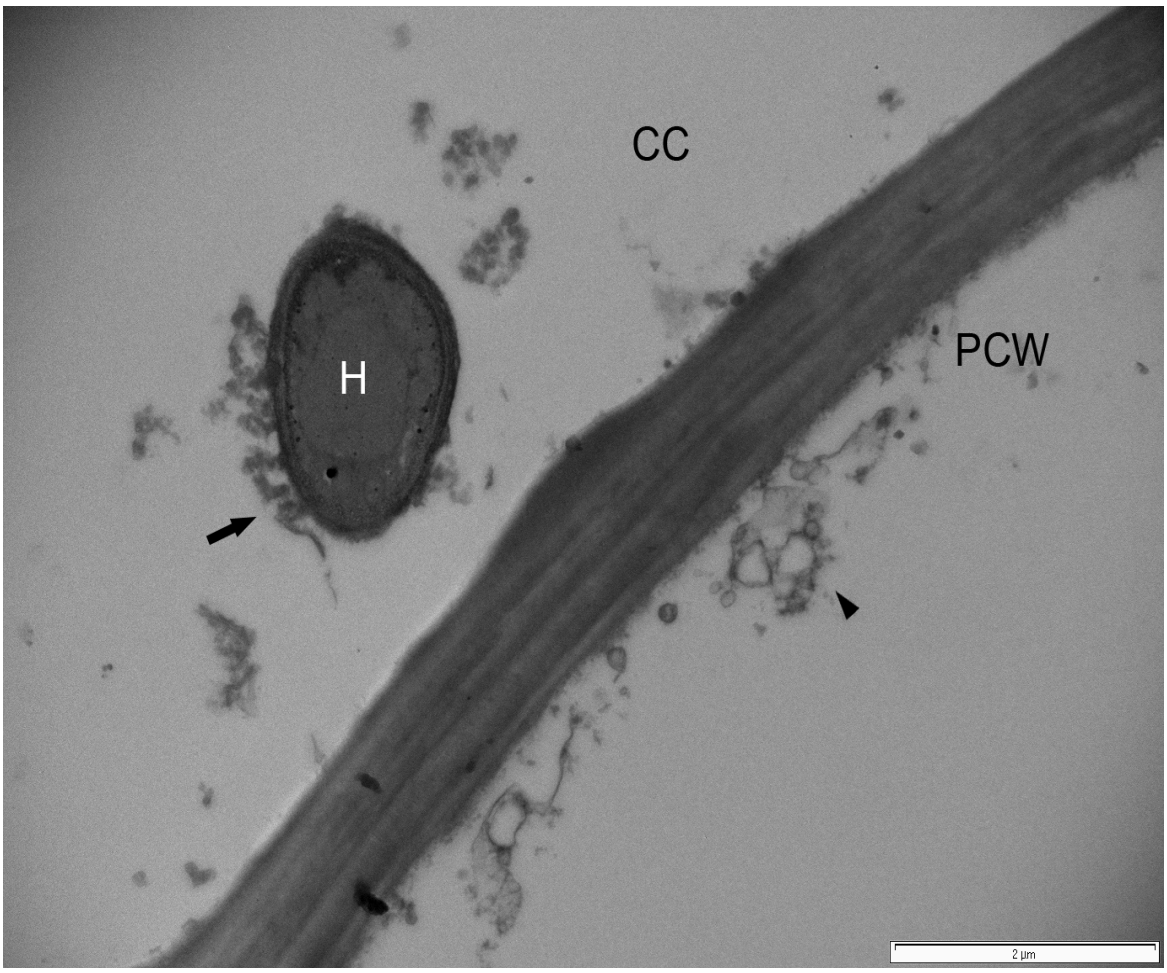

Figure 16. Electron micrograph of hypha of Foc growing intracellularly within the cortex of a Potassium silicate treated banana root at 7 days post inoculation x15 000. The hypha (H) has penetrated the cell, and has subsequently become surrounded by a dense, electron opaque material (arrow). Vesicles (arrowhead) can be seen aggregating on the opposite side of the plant cell wall (PCW). H – hyphae; CC – cortical cell; PCW – plant cell wall.

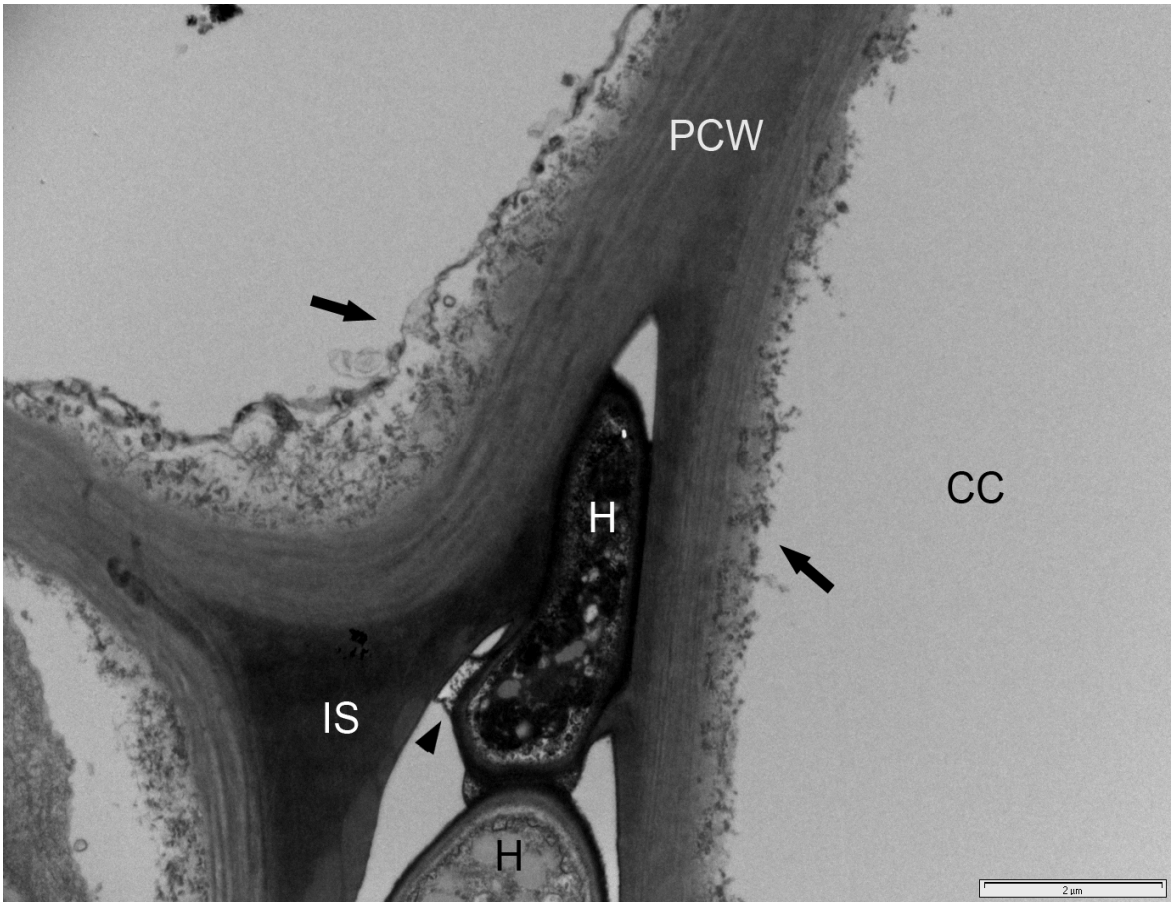

Figure 17. Electron micrograph of intercellular growth of *Foc* in the cortex of a Potassium silicate treated banana root at 7 days post inoculation **x10 000.** Hyphae (H) can be seen growing along the walls of an intercellular space between cortical cells (CC). A large amount of electron opaque material is being deposited (arrows) along the internal walls of the cortical cells adjacent to the hyphae. A mucilaginous substance is seen in association with hyphae (arrowhead). CC – cortical cell; PCW – plant cell wall; IS – intercellular space; H – hyphae.

Mineral Analysis

Results of analysis by microwave digestion of plants results for silicon (Figure 18), potassium (Figure 19), sulphur (Figure 20), sodium (Figure 21), and magnesium (Figure 22) are reported (aluminium, calcium, manganese, phosphorus, copper and zinc did not return pertinent results).

Silicon content in water and potassium sulphate treated plants was approximately 0.7% of dry weight, whereas sodium silicate and potassium silicate treated plants were 1.5% dry weight (Figure 18). Potassium dry weight content ranged from approximately 4.5% in water and sodium silicate treated plants, and 7.5% in potassium sulphate and potassium silicate treated plants (Figure 19). Sulphur content in water, sodium silicate and potassium silicate treated plants were approximately equal 0.25% dry weight, whereas potassium sulphate treated plants contained 0.325% (Figure 20). Sodium content in uninoculated plants ranged from approximately 600mg/kg in water and potassium sulphate treated plants, to 350mg in potassium silicate treated plants, and upwards of 1000mg/kg in sodium silicate treated plants. After inoculation, sodium content dropped significantly ($p < 0.05$) in all plants except for potassium silicate, where it remained relatively equal (Figure 21). Magnesium

content in potassium sulphate treated plants and potassium silicate treated plants was approximately 0.30% dry weight, which was significantly less ($p < 0.05$) than water treated plants which contained 0.45% dry weight (Figure 22).

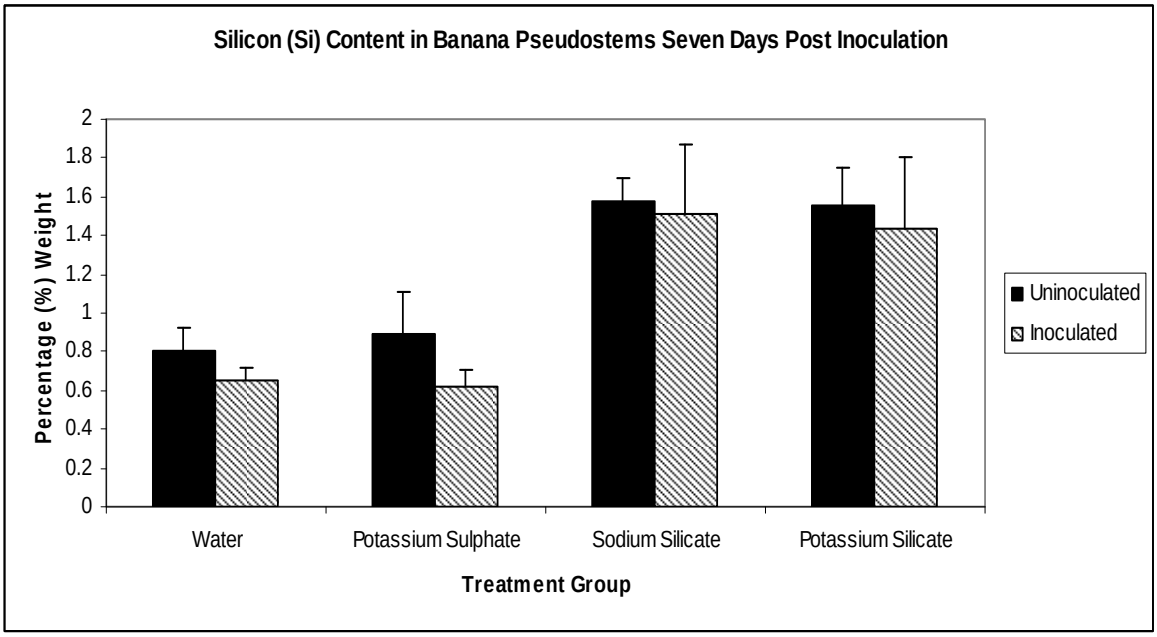

Figure 18. Mineral analysis results for the silicon content of banana pseudostems seven days after inoculation with *Foc*. Both potassium silicate and sodium silicate took up similar amounts of silicon into the pseudostem. Silicon increase was significant compared to water treatment ($p < 0.05$). Error bars represent standard error (S.E). n = 3 (2 replicates pooled per sample)

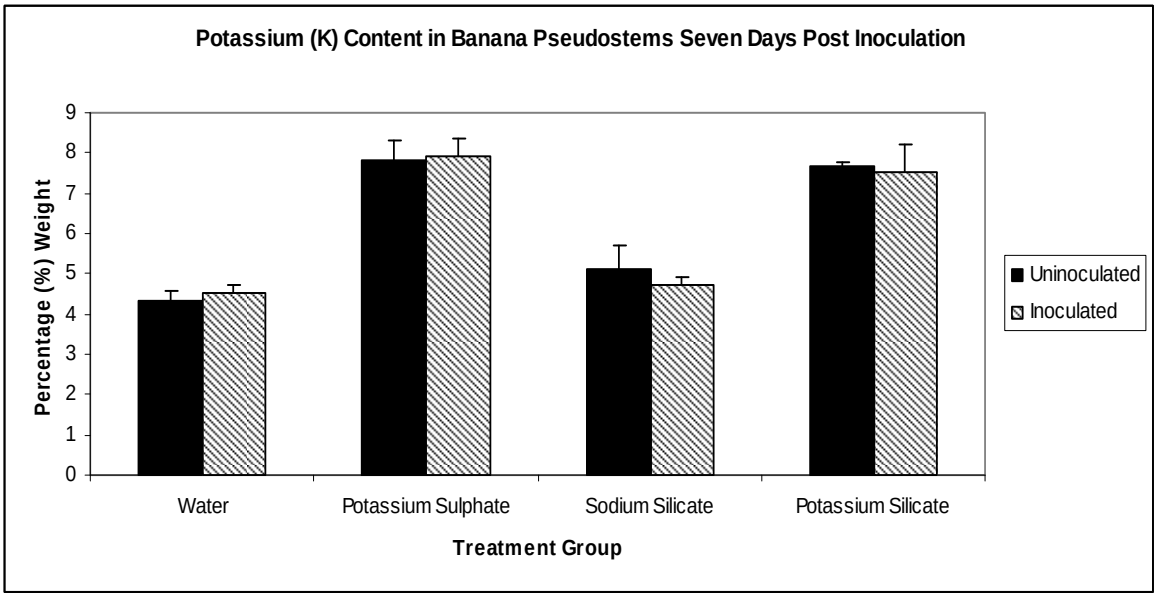

Figure 19. Mineral analysis results for potassium content of banana pseudostems seven days after inoculation with *Foc*. Potassium levels in potassium silicate and potassium sulphate plants were approximately equal, and both were significantly higher compared to water treatment ($p< 0.05$). Error bars represent standard error (S.E). n = 3 (2 replicates pooled per sample)

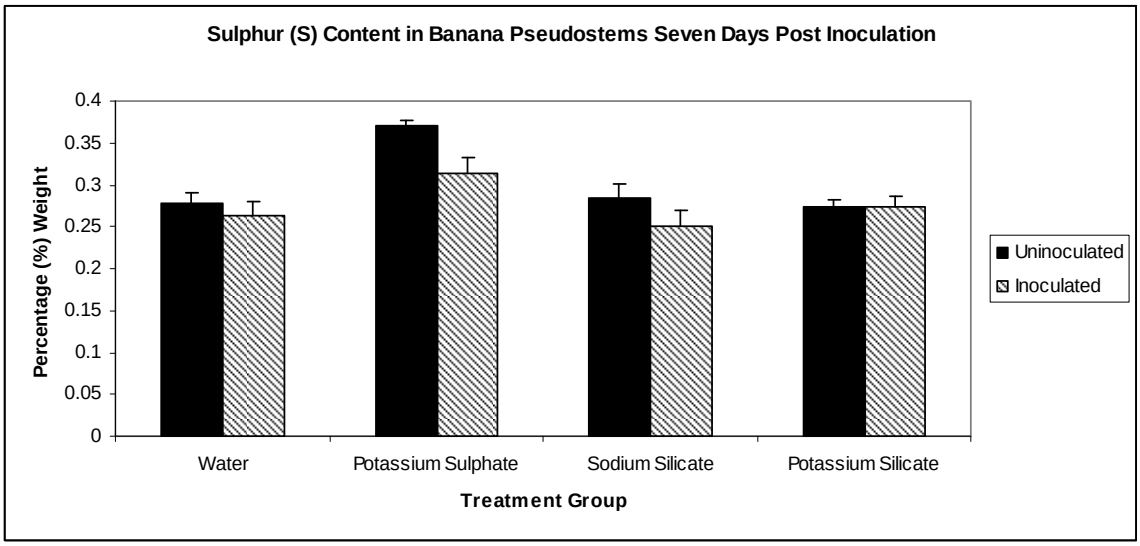

Figure 20. Mineral analysis results for sulphur content of banana pseudostems seven days after inoculation with *Foc*. The addition of potassium sulphate results in a significant increase in the amount of sulphur present in treated plants compared to water treatment. Error bars represent standard error (S.E). n = 3 (2 replicates pooled per sample)

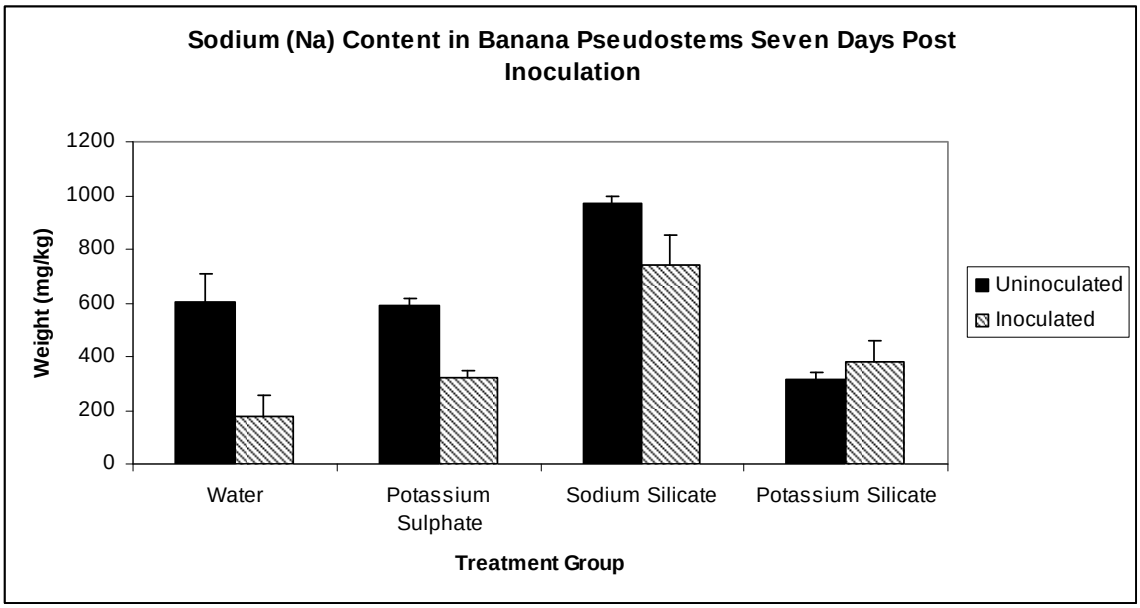

Figure 21. Mineral analysis results for sodium content of banana pseudostems seven days after inoculation with ***Foc*.** Treatment with sodium silicate significantly increased the sodium content of plants compared to water treatment ($p<0.05$). Error bars represent standard error (S.E). n = 3 (2 replicates pooled per sample).

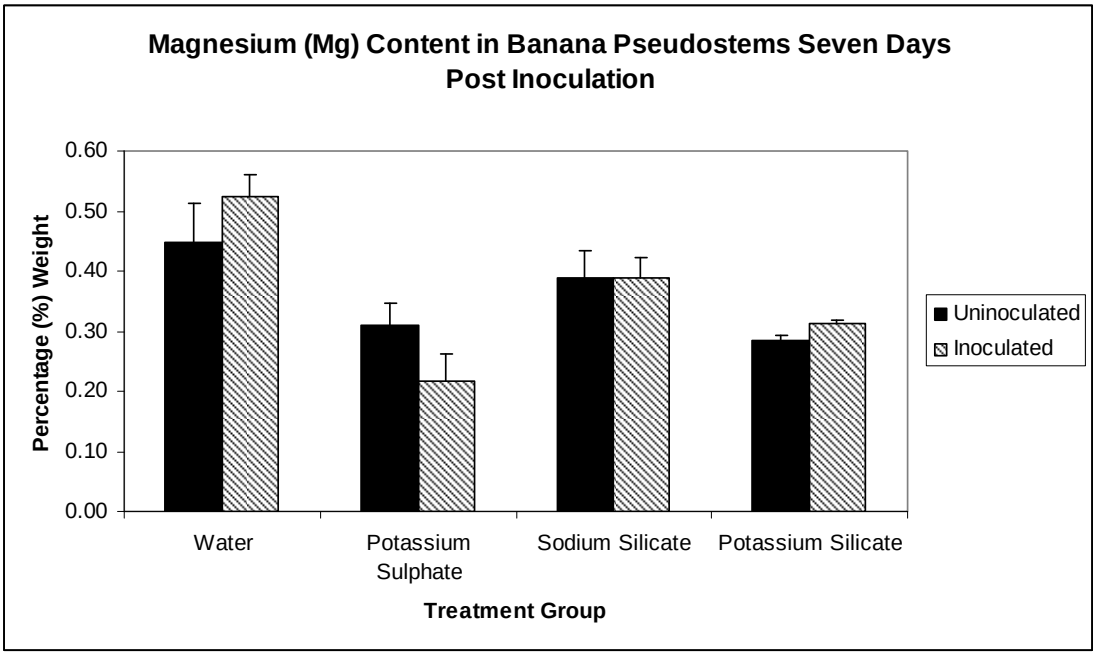

Figure 22. Mineral analysis results for magnesium content of banana pseudostems seven days after inoculation with *Foc*. Samples treated with potassium (potassium silicate and potassium sulphate) have a reduced level of magnesium compared to water treatment (control). Error bars represent standard error (S.E). n = 3 (2 replicates pooled per samples).

Measurement of pH

Potting medium pH exhibited a fairly consistent trend, with water treated and potassium sulphate samples being slightly but significantly ($p < 0.05$) more acidic than sodium silicate and potassium silicate treated plants (Figure 23)

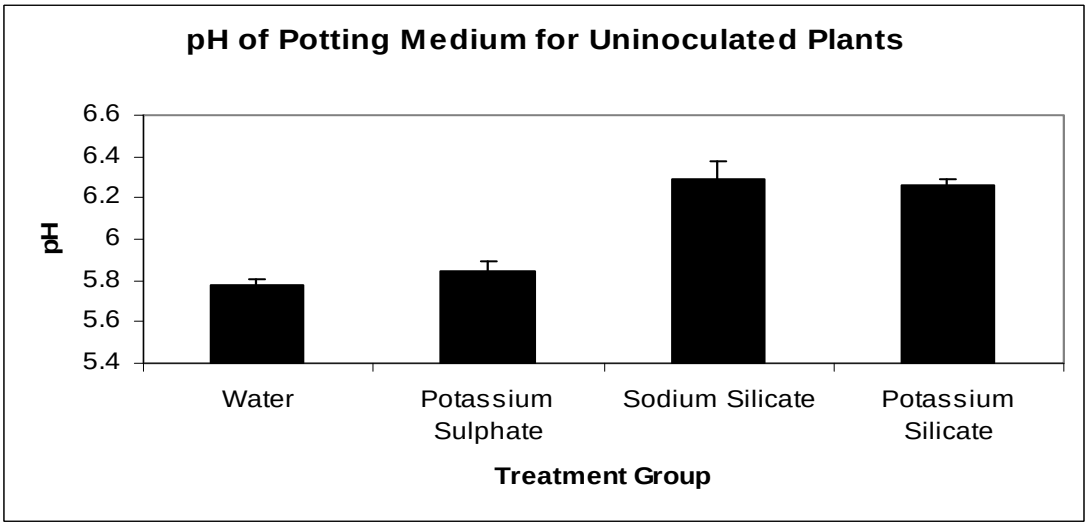

Figure 23. The pH of soil solution taken from pots after 14 weeks of drenching**.** Pots contained uninoculated (control) millet. Silicon treatments were significantly less acidic than water and potassium sulphate ($p < 0.01$). Error bars represent standard error (S.E). n = 6.

Discussion

Disease Assessment

Treatment with nutrient solutions had a different effect on disease development at different times during the experiment. The external symptoms of chlorosis at 4 weeks post inoculation were indicative of the development of disease (Orjeda, 1998). The *in vitro* treatment with potassium silicate resulted in reduced disease development. This is in line with current research expounding the protective effects that silicon may have in plants under biological stress (Fawe *et al.*, 2001). These results also conflict with research by Chérif *et al* (1994) and Samuels *et al*. (1991) that saw the effects of silicon decline once silicon application stoped. In this experiment *in vitro* treatment had a greater influence on disease than glasshouse treatment, which was applied at the time of inoculation. This suggests that the mechanism is not a silicification of epidermal cells, but rather a systemic defence mechanism within the plant. The evidence for a silicon induced systemic response is established in the current literature (Fauteux *et al.*, 2005; Fawe *et al.*, 2001). Some literature suggests that all silicon is polymerised into the leaves and endodermis of roots. However the continuing affects of silicon weeks after removal from the nutrient media suggests that the silicon is still available within the plant.

Sodium silicate treatment showed greater disease levels in the first 4 weeks post inoculation compared with controls. While it would be expected that silicon would have a positive effect on the health of the plant, it may be disadvantaged by the negative effects of sodium (Epstein & Bloom, 2004). Sodium can affect plants by acting as a salt, changing the osmotic potential resulting in plant stress and at high concentrations can be toxic (Epstein & Bloom, 2004). The sodium silicate glasshouse treated plants contained high levels of sodium approximately 800 (*Foc*) - 900 (uninoculated) mg/kg. Sodium silicate caused toxicity in the tissue culture phase and may lead to disadvantaged plants that are unable to respond to the pathogen as effectively as a control plant or potassium silicate treated plant.

The lack of difference in internal symptoms at 10 weeks post inoculation was not surprising. Inoculated plants showed very intense colonisation with associated symptoms 4 weeks after inoculation, including intense vascular discolouration up to the petiole in some cases (data not shown). All plants then appeared to recover for the next 6 weeks, with discolouration being limited to the roots and the rhizome. The INIBAP scale for evaluating discolouration was not sensitive enough to determine small differences in rhizome discolouration. Performing a range of pathogenicity tests, earlier in inoculation (e.g. 4 weeks) and later (e.g. 6 months) would help in elucidating any short or long-term effects silicon may be exerting.

Being in a climate controlled glasshouse, with a regular watering regime, plants were not subjected to varying temperature, humidity or water, all things which can influence virulence of *Fusarium oxysporum* (Beckman, 1987). The benefits of silicon are more readily seen when plants are subjected to a variety of biotic and abiotic stresses (Ma, 2004).

Aerenchyma cells were occasionally observed in all treatments groups. Aerenchyma provide a continuous longitudinal path for gas diffusion in roots, and are usually formed in response to soil anaerobiosis (Webb & Armstrong, 1983). Aerenchyma are

formed constitutively in banana as radially widened lysigenous spaces in the cortex, and have been previously postulated to increase susceptibility to *Foc* (Aguilar *et al.*, 1999). Lysigenous tissue (induced necrosis) may provide a source of nourishment for the fungus. This increased presence of aerenchyma may have contributed to the increased disease symptoms expressed in inoculated sodium silicate and potassium sulphate treated plants compared to water treatment. Aerenchyma provides an unobstructed path for hyphae to grow to almost near the rhizome. This may have obscured any beneficial effects that potassium sulphate or sodium silicate were exerting upon the plant. The influence aerenchyma have on *Foc* pathogenesis, particularly during the primary determinative phase of infection should be the focus of more research.

Histological assays

Histological studies revealed that hyphae in association with the roots of potassium silicate treated plants at 3 days post inoculation showed an indistinct cell wall, swelling, and extensive vacuolation . These are signs of either exposure to chitinase or lectins (Ciopraga *et al.*, 1999; Di Pietro *et al.*, 2003; Iseli *et al.*, 1993). The increased expression of chitinases due to silicon treatment has been previously demonstrated in the roots of cucumber (*Cucumis sativus*) inoculated with several different *Pythium spp.* (Chérif *et al.*, 1994). If chitinases had weakened the fungal cell wall, this may have interfered with the ability of the fungus to penetrate plant cell walls, explaining why the fungus was only rarely observed within cells in potassium silicate treated plants.

The secretion of an unknown substance into the rhizosphere with apparent anti-fungal capabilities, suggests the possibility of potentiation or priming by Potassium silicate, as the plant was more readily able to produce defence compounds as *Foc* was growing in association with the root.

Fawe *et al.* (1998) demonstrated that silicon application during plant growth induced the accumulation of fungitoxic metabolites (phytoalexins) in cucumber when challenged with powdery mildew. As hyphal deformation was not readily observed in sodium silicate treated plants, this response cannot be solely attributed to silicon. Swelling, vacuolation and cell wall alterations were apparent in hyphae that had reached the endodermis (in all treatment groups), but not enough samples were observed to reach a conclusion.

Silicon deposited in cell walls my help plant cells resist enzymatic degradation which in turn allows plant defences to be mobilised faster. Results comparing *Pythium aphanidermatum* infection in tomato (*Lycopersicon esculentum*) and bitter gourd (*Mormodica charantia*) treated with and without silicon correlate improved resistance with higher concentrations of root symplastic silicon, as opposed to cell wall bound silicon (Heine *et al.*, 2007). This hints at a still unknown biochemical, or cellular role for silicon.

The differences between fungal growth in 7dpi and 3dpi samples is likely due to a change in *Foc* pathogenesis over time (the fungus is switching to predominantly intercellular growth) or the fact that different root tissue was sectioned at 7dpi compared to 3dpi. The more frequent wall appositions and fungal-associated electron opaque material observed in Potassium silicate treated samples suggest potassium

silicate is still exerting an effect at 7dpi. These results again were not correlated to either sodium silicate or potassium sulphate treated plants.

The gene expression results also hint at this argument through the demonstration of 'priming' or 'potentiation'. Priming is the faster and greater activation of defence response only after contact with the pathogen. While it is difficult to identify if the activation of PAL and peroxidise was any faster, it was certainly stronger than in the plants that had been treated with water. This priming phenomenon is closely linked with SAR and has shown to increase cell wall strengthening and the regulation of defence genes (Conrath *et al.*, 2002). It has been postulated that silicon may act as a post elicitation messenger of intracellular signals (Fauteux *et al.*, 2005). Further detailed molecular analysis may confirm this.

Banana has been shown to produce phenolics in response to various pathogens (Valette *et al.*, 1998). Mace (1963) previously established that cells in banana roots contain scattered phenol-containing parenchyma cells in the cortex and stele. Phenols are known to play an important role in induced plant defence, often as a precursor (Beckman & Mueller, 1970; Nicholson & Hammerschmidt, 1992). When challenged with a pathogen, plants will reinforce their cell walls in an attempt to resist penetration often involving the rapid synthesis of phenolic compounds (Sagi, 2003). As phenolics play a fundamental role in banana defence responses, cataloguing total phenolic content via HPLC in silicon treated plants would likely yield a result.

As a caveat, Chérif *et al.* (1994) used the cucumber-*Pythium* pathosystem to demonstrate that silicon treated plants show earlier and far greater activity of peroxidase and polyphenolperoxidase (which act upon phenolics, often converting them to a fungitoxic state) (Chérif *et al.*, 1994; Thipyapong *et al.*, 1995) compared to non-silicon treated plants. Total phenol content, however, remained the same, suggesting the state of phenolics is more important for improved defence. Similar results are found in the rice (*Oryza sativa*)/rice blast (*Magnaporthe grisea*) pathosystem, although increases in lignin were seen (Kunzheng *et al.*, 2008).

Mineral Analysis

The technique for mineral analysis (USEPA method 3052) did not discriminate between polymerised silicon and free silicon within the xylem or phloem. More research needs to be done on the state and mobility of silicon within the plant, something that has recently been studied (Matichenkov *et al.*, 2008). Silicon content within the phloem remains relatively unstudied.

Potassium silicate and potassium sulphate both took up similar amount of K^+ and yet did not show similar results, suggesting that potassium is at least not entirely responsible changes seen in potassium silicate treated plants. The possibility exists that potassium is having an effect in soil solution, e.g. either increasing or decreasing fungal growth, both of which have been reported (Fageria *et al.*, 2007). High soil K^+ has been correlated with reduced disease expression specifically in banana with *Foc* (Rishbeth, 1957). Treatment with potassium interfered with uptake of magnesium (Figure 30), which is in agreement with similar analysis work performed by Forsyth (2006). Whether this change in magnesium influences the virulence of *Foc* remains undetermined.

Although potassium sulphate was used as a control for potassium silicate, to determine whether the conjugate K^+ ion was causing any difference, it is impossible to rule out the influence of the S^+ ion in potassium sulphate. Mineral analysis data

clearly shows an increased amount of sulphur in $KS0_4$ treated plants. This confounding effect is a common problem in nutrient substitution experiments (Schneider, 1990). The conjugate salt for potassium sulphate ($-S0_4$) would likely have an effect on plant nutrition, making interpretation of results difficult. Sulphur has been reported to cause induced resistance (Haneklaus *et al.*, 2007).

Eliminating the need for control solutions for potassium silicate would be preferable. This can be done by normalising fertiliser solutions so that each treatment group receives the same amount of potassium, as demonstrated by (Chérif *et al.*, 1994). This requires careful preparation of nutrient solutions. Varying levels of silicon should be investigated, along with other silicon conjugates, such as calcium silicate.

pH Levels

The fact that both potassium silicate and sodium silicate shared the same pH and potassium sulphate and water shared the same pH suggests the possibility that silicon is the reason for lowered pH. Banana has been shown to actively weather inaccessible silicate material in the soil so that silicon can be absorbed (Hinsinger *et al.*, 2001). A more acidic pH in the non-silicon treated plants suggests that the plants are altering pH so they can take up more silicon. Generally, Si is less available at higher pH (Datnoff *et al.*, 2007). The other possibility is that sodium silicate and potassium silicate solutions (which are highly alkaline at a pH of approximately 11.5) raised the pH of the media potassium sulphate. Some fungal genes have been shown to be pH dependent, with gene expression increasing as ambient pH becomes more acidic (for review see (Di Pietro *et al.*, 2003). Lowering of external pH may interfere with fungal virulence factors such as cell wall degrading enzymes (Rollins & Dickman, 2001).

Limitations

For disease symptoms to be expressed, the pathogen, the environment and the plant must all interact in the correct fashion, i.e. the disease triangle (Parker & Gilbert, 2004). The millet inoculation method used in this experiment not only introduced a large amount of actively growing mycelium but also provided a "saprophytic haven" where the fungus could grow and continuously challenge the plant. While this is similar to conditions plants experience in the field, the quantity of millet inoculum was likely too high, overwhelming the plant. Regardless, millet inoculation is still more efficient than traditional spore dip (Smith *et al.*, 2008). A system should be developed where millet can be introduced without disturbing the root system such as a pot with a removable base which can be opened and replaced with millet inoculum. A very small amount of inoculum added to a pot would be a more realistic recreation of field conditions. Like many plant pathogen interactions, *Foc* infection is quantitative not qualitative (Stover, 1962). Therefore a high amount of inoculum may still overcome tolerant or even resistant plant varieties.

Processing and fixation for electron microscopy can produce artefacts (Mollenhauer, 1993). Necrotic tissue and fine fungal structures often do not survive TEM processing intact. Dirt and dust landing on grids produce large image artefacts in micrographs. Other techniques allow for faster processing, but lower resolution. Root hairs did not survive processing and were only rarely observed. Root hairs are important in some *Fusarium oxysporum* interactions, such as tomato (Lagopodi *et al.*, 2002). It would be useful to compare such data with controls, but use a different preparation technique such as cryofixation (Saga, 2005).

Adding caffeine to preserve phenolics in their natural state had benefits and disadvantages. While in most cases it helped to fix phenolics, especially in the specialised phenolic containing cells of banana (Beckman & Mueller, 1970) it made them more difficult to infiltrate with resin and subsequently section. This led to tears and holes in the resin for thin (80nm) sections for TEM. In silicon (potassium silicate and sodium silicate) treated samples, epidermal cells often contained regular chains of small holes in the resin, always in close contact with the cell wall. The possibility exists that these holes were phenolic depositions that did not survive processing intact. To counteract this, future TEM work with caffeine in banana should allow for a much longer infiltration time (even with the assistance of microwave infiltration). This will maximise the chance for preserved phenolics to become infiltrated with resin.

Technology Transfer

This study has shown that potassium silicate applied in banana tissue culture has the potential to reduce the symptoms of Fusarium wilt when these plants are deployed in the field. The histological studies have implied that the silicon treatment is inducing antifungal compounds which are inhibiting the Fusarium external to the root tissue. This needs further analysis but this is a useful step towards deploying a pre-treatment of banana plants that could be used in tissue culture which would render the material more resistant in the field.

This work has been collaboration between the University of Queensland and the Dept of Primary Industries and Fisheries Queensland (Nambour and the Queensland Agricultural Biotechnology Centre). The research findings will feed directly back to the DPI&F banana tissue culture lab with the aim of producing more resistant banana plants. The combination of silicon treatment along with possible beneficial endophytic bacteria (as assessed in concurrent studies in the project led by Ms Sharon Hamill) will lead to improved control of Fusarium wilt.

The results of the this work will be presented at the Banana Industry Congress in the Gold Coast in June 2009 by way of an

- oral presentation by Dr Elizabeth Aitken entitled "Can silicon play a role in reducing Fusarium wilt in banana?" and
- a poster presentation and field day exhibit by Mr Kevan Jones entitled "The Infection Process of *Fusarium oxysporum* f. sp. *cubense* subtropical race 4"

In relation to this study two Honours thesis have been submitted and awarded, First Class Hons in both cases, at the University of Queensland in 2008

- Kevan Jones. "How silicon affects the infection process of *Fusarium oxysporum* f. sp. *cubense* on banana: an ultrastructural investigation." Mr Jones is now continuing this research as a PhD study with a living allowance scholarship from the University of Queensland
- Rachael Woods "Examination of silicon mediated resistance in banana to *Fusarium oxysporum f. sp. cubense* through defence gene expression"

Recommendations

Studying the effects of silicon are complicated by the diverse beneficial effects it provides, and how those effects differ wildly between plant species (Richmond & Sussman, 2003). Future silicon research need to focus on two distinct outcomes: i) determining the mode of action silicon is exerting; and ii) establishing the viability of silicon as an amendment for potting/culture media or as a fertiliser for field crops. Continuing genetic work, such as the isolation of silicon transporters (Ma *et al.*, 2004; Ma *et al.*, 2006) and determination of silicon state and content within plants (Matichenkov *et al.*, 2008) will help to develop a model for silicon mode of action.

Large scale field trials in different soil types, at varying temperatures and rainfall with various types of silicon applications need to be performed to establish viability of silicon as a fertiliser, and whether the benefits of use outweigh the cost. The benefits of silicon application may not be seen equally under all field conditions.

A goal of this experiment was to replicate field conditions as accurately as possible. The millet inoculation was used for this reason. To determine the mode of action of silicon, it would be far more constructive to perform reductionist experiments. As an example, performing an *in vitro* root assay, by placing sterilised sectioned roots (from banana plants treated with and without silicon) on an agar plate with *Foc* inoculum and testing how fast vascular colonisation occurs. Whilst this is not an accurate representation of *Foc* infection in the field, it allows for a rapid and easily reproducible exploration of silicon-mediated effects.

It would be interesting to combine silicon amendments with a competitive biotic agent such as *Pseudomonas fluorescens* (which has been demonstrated to reduce *Foc* severity) to see if they provide synergistic benefits to banana (Mohandas *et al.*, 2004). Or alternatively, combine silicon with abiotic agents such as fungicides including benomyl and propiconozole (Deepak *et al.*, 2008).

More microscopy needs to be done to fully characterise the infection process. Immuno-flourescent staining or GFP expression (Hawes & Satiat-Jeunemaitre, 2001) coupled with scanning electron microscopy (SEM) can be used to explore the larger scale processes involved. Video microscopy of the infection process, as suggested by Lagopodi *et al.* (2002) and put into practice by Czymmek *et al.* (2007) allows for real time characterisation with negligible interference. Stains for compounds such as lignin (phloroglucinol) callose and suberin can easily be performed in conjunction with light microscopy (Spence, 2001).

Futher TEM work is possible, such as EELS (electron-energy loss spectroscopy) to determine elemental composition of ultrastructural objects (e.g. look at silicon content embedded within cell walls) to see if silicon is associated with penetration events, i.e. silicon being deposited. Immuno-gold labelling using enzymes specific for substances known to play a role in the plant defence response (such as phenolics (Bendayan & Benhamou, 1987)) or in fungal pathogensis (such as chitin (Rodrigues *et al.*, 2003)) would allow for confirmation of the predicted presence of phenolics or chintinases as seen in micrographs.

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