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ACCUMULATION OF TOTAL PHENOLICS DUE TO SILICON APPLICATION IN ROOTS OF AVOCADO TREES INFECTED WITH *Phytophthora cinnamomi*

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Accumulation of phenols and phenolic polymers in *Persea americana* Mill. roots exposed to the pathogen *Phytophthora cinnamomi (Pc)*, and treated with potassium silicate was investigated during a field trial. The reported data indicates that potassium silicate application to avocado trees infected with *Pc* increase total phenolic content of root tissue. The trials consisted of three applications (Si x 3) during March 2005 and January 2006. Following elicitation, the conjugated and non-conjugated phenolic metabolites were found to be induced. Significantly higher crude phenolic concentrations are reported in Si x 3 during March and May 2006 when compared to potassium phosphonate (Avoguard ®) and three silicon applications resulted in higher glucoside bound phenolic acid concentrations compared to the untreated control. The integration of the phenolic into the cell wall was found to vary according to the season. However, results indicate that potassium silicate application leads to lower cell wall bound phenols. Data analysis by HPLC separation revealed that all treatments samples contained 3,4-hydroxibenzoic and vanillic acid. The presence of syringic acid could be related to the application of silicon.The study provides further indication that phenolic compounds are affected by silicon application. Data from this study does not excluded the fact that various mechanical resistance mechanisms could also play an important role and some synergism could take place in the plant's defence system. The results of this study provide further evidence for application of silicon as an alternative control measure for *P. cinnamomi* root rot of avocado.

Keywords: Silicon, *Phytophthora cinnamomi*, Avocado, *Persea americana*, Root rot

ACUMULACIÓN DE FENÓLICOS TOTALES ATRIBUIBLE A LA APLICACIÓN DE SILICIO EN RAÍCES DE ÁRBOLES DE AGUACATE INFECTADOS CON *Phytophthora cinnamomi*

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La acumulación de fenoles y polímeros fenólicos en *Persea americana* Mill. detectados en raíces expuestas al patógeno *Phytophthora cinnamomi* (Pc), y tratadas con silicato de potasio fueron investigados durante un ensayo de campo. Los datos informados demuestran que la aplicación de silicato de potasio

en árboles de aguacate infectados con Pc aumentó el contenido fenólico total del tejido radical. Los ensayos consistieron en tres aplicaciones (Six3) durante marzo de 2005 y enero de 2006. Después de elicitar información, se encontró que metabolitos fenólicos conjugados y no conjugados fueron inducidos. Significativamente las concentraciones fenólicas crudas más altas son informadas en aplicaciones de Si x 3 durante marzo y mayo 2006 en comparación con fosfonato de potasio (Avoguard ®) y tres aplicaciones de silicio resultaron en concentraciones más altas de ácido fenólico ligado a glucosa comparado con el control sin tratar.

Se detectó que la incorporación de los fenoles a la pared celular varió de acuerdo a la estación del año. Sin embargo, los resultados indican que la aplicación de silicato de potasio induce a una menor unión de fenoles en la pared celular. Los análisis de datos por la separación de HPLC revelaron que todas las muestras de tratamientos contenían 3,4 - ácido hydroxibenzoico y vanillico. La presencia del ácido siríngico podría estar relacionada con la aplicación de silicio. El estudio provee la señal adicional de que los compuestos fenólicos se ven afectados por la aplicación de silicio. Los datos de este estudio no excluyen el hecho de que diversos mecanismos de resistencia mecánicos también podrían tener un papel importante y algo de sinergia podría darse en el sistema de defensa de la planta. Los resultados de este estudio suministran pruebas adicionales para la aplicación de silicio como medida alternativa de control para la pudrición de raíces de palto causada por *P.cinnamomi*.

Palabras claves: Silicio, *Phytophthora cinnamomi*, Aguacate, *Persea americana*, Putrefacción de la raíz

Introduction

Due to the threat of infection, plants have evolved a multitude of chemicals and structures that are incorporated into their tissue for the purpose of protection. These defences can repel, deter, or intoxicate including resin-covered or fibrous foliage, resin-filled ducts and cavities, lignified or phenol-impregnated cell walls, and cells containing phenols or hormone analogues (Chérif *et al*., 1994)

Various antimicrobial compounds which are synthesized by plants after infection have been discovered. Most phenolic compounds are phenolic phenyl-propanoids that are products of the shikimic acid pathway. Non-pathogenic fungi induce such high levels of toxic compounds in the host, that their establishment is prevented, while pathogenic fungi either induce only non-toxic compounds or quickly degrade the phytoalexins (Macheix *et al*., 1990; De Ascensao and Dubery, 2003). Rapid and early accumulation of phenolic compounds at infection sites is a characteristic of phenolic-based defence responses. This accumulation of toxic phenols may result in effective isolation of the pathogen at the original site of entrance (De Ascensao and Dubery, 2003).

The current study was initiated to determine whether the application of potassium silicate to avocado trees might increase the phenolic concentration in avocado tissue. This will confirm the hypothesis that silicon increases the phenolic

concentration of host tissues, resulting in the inhibition of *Phytophthora* root rot in avocados.

Materials and Methods

Experimental Layout

An avocado orchard (latitude 23° 43' 60S; longitude 30°10'0E) at an altitude of 847m was selected in the Tzaneen area, South Africa. Trees consisted of thirteen-year-old "Hass" on "Duke7" seedling rootstocks planted at a density of 204trees.ha^{-1} (7 x 7m spacing). Trees were on a southern facing slope. The trial layout consisted of 50 trees (n) with 10 trees randomly assigned per treatment, and organised in a randomised block design.

T*reatments*

Treatments consisted of a soil drench with a 20 litre solution of 20ml.l^{-1} soluble potassium silicate (20.7% silicon dioxide) (Bekker *et al*., 2005) per tree either once, twice or three times in a growing season. Trees injected with potassium phosphonate (Avoguard®) were incorporated as a standard fungicide treatment. Untreated trees served as controls. Data was collected from January 2005 to July 2006. Root and leaf samples were taken every second month on the northern side of the tree, and fruit samples were taken at harvest.

Extraction and Quantification of Total Phenolic Compounds

Samples were freeze dried for 120h. The dried material was ground with an IKA® A11 basic grinder (IKA Werke, GMBH & Co., KG, D-79219 Staufen) to a fine powder. One millilitre of a cold mixture of methanol: acetone: water (7:7:1, v:v:v) solution was added to 0.05g powdered plant sample, ultrasonified for 5min and centrifuged as described by Regnier (1994) to obtain crude extracts. Crude samples were stored at 4℃ until extraction. Extraction of Non-Conjugated Phenolic Acids, Glycoside-Bound Phenolic Acids, Ester-Bound Phenolic Acids and Cell Wall-Bound Phenolic Acids were done according to the method used by De Ascensao and Dubery (2003). A gallic acid equivalent calibration curve ($y = 0.013x + 0.0177$, $R^2 = 0.9982$) was used to determine the amount of each fraction contained in the sample material. The targeted extract values are representative of the relative amount of each fraction in the crude extract. This is in agreement with phenolic acid functionality (Zhou *et al*., 2004).

Quantification of Phenolics by the Folin-Ciocalteau Method

The concentration of phenolic compounds in the various extracts was determined using the folin-ciocalteau reagent. Quantification of phenolic concentrations in the extracts was done as described by Bekker (2007).

Extracted phenolic fractions were analysed by means of reverse phase - high performance liquid chromatography (RP-HPLC) (Hewlett Packard Agilent 1100 series) with DAD detection (diode array detector, 280, 325, 340nm) as described by Bekker (2007).

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Mean differences were separated according to Duncan's multiple range test ($P < 0.05$).

Results and Discussion

Although high concentrations were obtained in the crude extracts, crude extract values do not reflect the combined values of the four other phenolic acid fractions extracted with more specific hydrolysis reactions. This is because phenols are bound to large molecules in the cell cytoplasm, and by hydrolysis, these molecules are split, resulting in the relevant concentrations being measured.

During the harvesting period (July 2005 & 2006), no significant differences were seen between any treatments with regards to crude phenolic concentrations. For the period of March 2005 to January 2006, three silicon applications (Si x 3) per season resulted in significantly higher total phenolic concentrations in root tissue compared to the control (Figure 1). From March to May 2006, the control treatment (133.66µg.l^{-1}; 109.08µg.l^{-1}) resulted in higher crude phenolic levels compared to Si x 3 (94.61µg.l^{-1}; 67.98µg.l^{-1}). Although this data does not correlate with any of the parameters of the phenological model proposed by Kaiser (1993), it is proposed that the lower metabolic rates observed in plants were due to lowered physiological activity in the plant leading to sub-optimal photosynthesis. Although Si x 3 resulted in significantly higher phenolic concentrations in avocado roots only during March and May 2006 (94.61µg.l^{-1}; 67.98µg.l^{-1}) compared to potassium phosphonate (Avoguard®) (49.07µg.l^{-1}; 59.46µg.l^{-1}), Si x 3 are statistically comparable to potassium phosphonate application.

Glucoside bound phenolic acid concentrations (Figure 2) for Si x 3 differed significantly from the control for the period January to May 2006. Significant differences between these two treatments prior to Jan 2006 were only detected during May 2005.

Three silicon applications per season resulted in significantly lower cell wall bound phenolic acid concentrations in avocado roots (Figure. 3) compared to the control treatment during May and Sept 2005, and March and May 2006. This trend was negated during January 2006 when a significantly higher cell wall bound phenolic concentration was obtained in Si x 3 (0.71µg.l^{-1}) than the control (0.36µg.l^{-1}). Although the trend was not consistent as regards Si x 3, the potassium phosphonate (Avoguard®) treatment did not differ from the control throughout the tested period. Three silicon treatments per season resulted in significantly lower cell wall bound phenols during May, Jul and Sept 2005 and May 2006 compared to Si x 1, with higher concentrations obtained in Si x 3 only during Jan 2006 (0.71µg.l^{-1} vs. 0.38µg.l^{-1}). No significant difference was obtained between Si x 1 and Si x 3, except during Jan 2006, when Si x 3 (0.71µg.l^{-1}) resulted in higher cell wall bound phenols compared to Si x 2 (0.35µg.l^{-1}). Results indicate that potassium silicate application leads to lower cell wall bound phenolics. Their results indicated that silicon accumulation was subsequent to phenol appearance in infected tissue, challenging the physical barrier-hypothesis that silicon accumulation in plant cell walls in close contact with the pathogen confers resistance to fungal penetration by physical means.

No significant differences were seen between treatments for ester bound phenolic concentrations throughout the duration of the trial (results not shown).

Non-conjugated phenolic concentrations did not differ significantly between treatments during March, Jul and Sept 2005, and Jan and March 2006. Three silicon applications per season (1.62µg.l^{-1}) and potassium phosphonate (Avoguard®) (2.44µg.l^{-1}) resulted in significantly lower non-conjugated phenol

concentrations compared to that of the control (2.80µg.l^{-1}) only during Nov 2005 (Figure 4), while the concentrations between Si x 3 and potassium phosphonate (Avoguard®) were statistically similar.

Silicon application to diseased plants has been shown to control disease development and spread with various degrees of success (Epstein, 1999). It is generally accepted that plants with higher silica content are more resistant to phytopathogenic fungi than those plants with a lower content (Ishiguro, 2001). After silicon is taken up by a plant, it goes through a silicification process, and is either deposited in the cell wall, cell lumen, or intercellular spaces (Epstein, 1999). Electron microscopy and dispersive x-ray analysis led Samuels *et al.* (1991) and Chérif *et al*. (1992) to conclude that enhanced defence reactions in the cucumber plant to fungal attack appear to be the result of silicon present in the plants' transpiration stream, and not because it becomes bound to the plant cell wall. Although Menzies *et al*. (1991) deemed the possibility of silicification of cell walls as not to be completely discarded, silicon is more likely to affect signalling between the host and pathogen, resulting in more rapid activation of a hosts' defence mechanisms. Heath (1981) reported that silicon accumulation as a response to infection is not limited to silicon accumulating plants (Epstein, 1999). Heath and Stumpf (1986) suggested the high levels of wall-associated phenolics in silicon-depleted tissue to result in faster inhibition of fungal enzymes involved in fungal-penetrating peg formation. Results from the current study indicate that potassium silicate application to avocado trees leads to higher crude extract phenolic concentrations but lower cell wall bound phenolics compared to the control. Silicon accumulation was subsequent to phenol appearance in infected tissue, challenging the physical barrier-hypothesis that silicon accumulation in plant cell walls in close contact with the pathogen confers resistance to fungal penetration by physical means.

Silicon application to avocado trees resulted in fewer identifiable phenols in avocado roots compared to the control and potassium phosphonate treatments. HPLC separation of hydrolysed phenolic acids extracted from roots revealed that all non-conjugated phenolic acid samples contain 3,4-hydroxibenzoic acid. The hydrolysed glucoside bound samples of both the potassium phosphonate and control treatments also contained 3,4-hydroxibenzoic acid and vanillic acid. The control treatment contained syringic acid in the hydrolysed glucoside bound extract.

Conclusion

The accumulation of phenols and phenolic polymers in *Persea americana* roots exposed to cell wall derived elicitors from the pathogen *P. cinnamomi*, and treated with water soluble potassium silicate, was investigated. These findings support the hypothesis that silicon application results in heightened resistance against *P. cinnamomi* infection via an elevation of phenolic levels in the roots. Although crude phenolic concentrations differed between treatments and no clear deduction may be made concerning the effect of potassium silicate on the phenolic content of avocado roots in the presence of *P. cinnamomi*, it is clear that similar or higher crude phenolic concentrations are obtained in avocado roots with three silicate applications per season compared to potassium phosphonate treated trees. This was also true for glucoside bound phenolic concentrations in

roots from trees treated three time per season with potassium silicon (Si x 3) compared to potassium phosphonate treated trees.

In this study the potassium silicate application lead to lower cell wall bound phenolics. The possibility that silicon replaces phenol-binding molecules is not fully understood. However, this study indicates that the accumulation of silicon was subsequent to phenol appearance in infected tissue; challenging the physical barrier and conferring to the cell wall in close contact with the pathogen some resistance to fungal penetration by physical means. The future search on the use of silicon therefore can be upheld with this strategy to control plant disease in general and avocado root diseases in particular.

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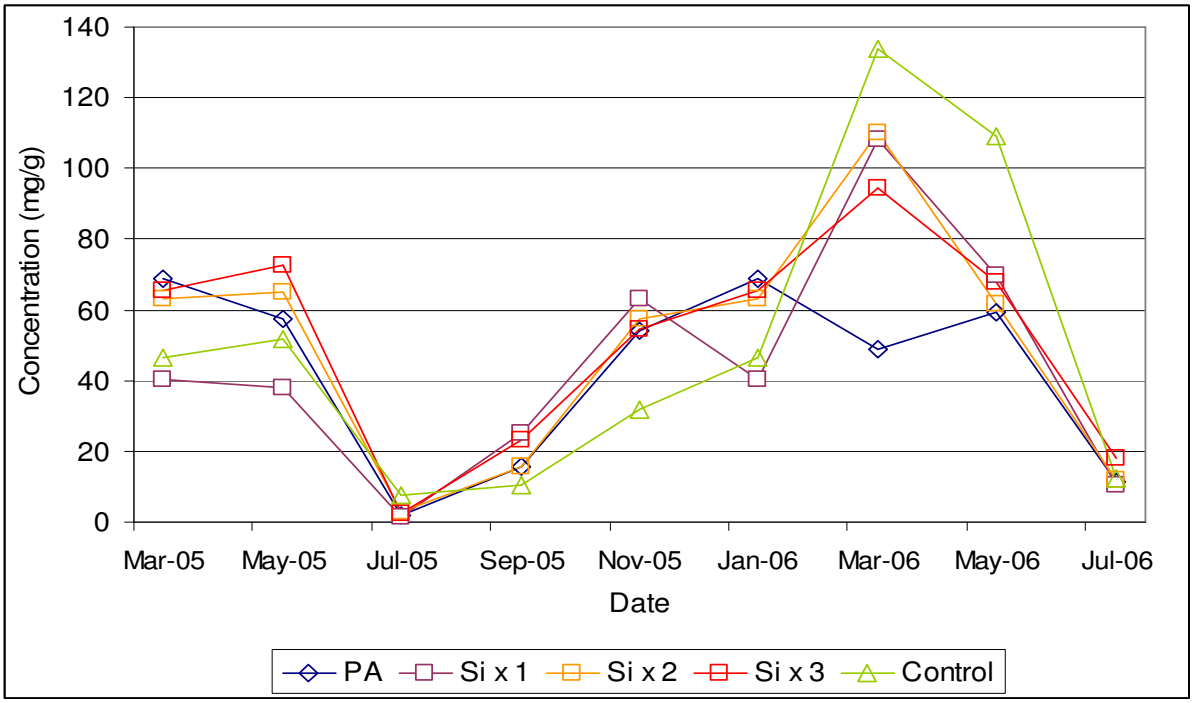

Figure 1: Total soluble phenolic content of avocado roots recovered over a period of 18 months in *P. cinnamomi* infected trees, which were either untreated (controls) or treated with potassium silicate as a soil drench. Treatments consisted of either one (Si x 1), two (Si x 2) or three (Si x 3) potassium silicate applications per season; trees injected with potassium phosphonate (PA) and trees receiving no treatment as a control treatment. Values in table with different symbols indicate significant differences at a 95% level of significance (student t-test). Phenolic concentration expressed as mg gallic acid equivalent per gram of dry weight.

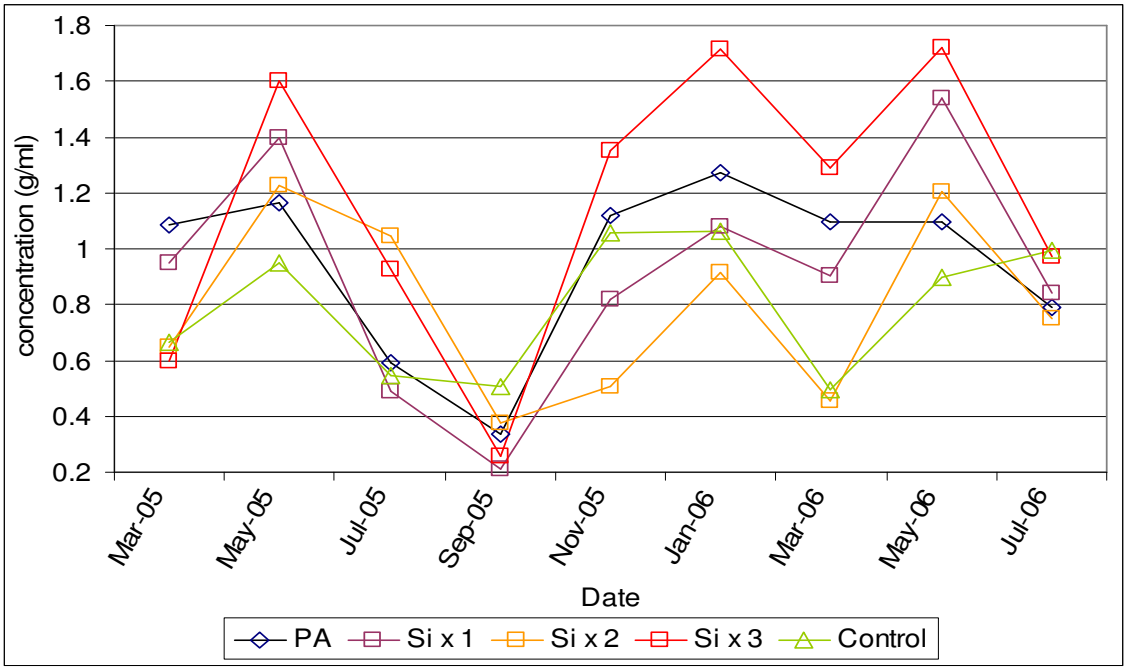

Figure 2: Total concentration of glucoside bound phenolic acid after hydrolysis of avocado roots recovered over a period of 18 months in *P. cinnamomi* infected trees, which were either untreated (controls) or treated with potassium silicate as a soil drench. Treatments consisted of either one (Si x 1), two (Si x 2) or three (Si x 3) potassium silicate applications per season; trees injected with potassium phosphonate (PA) and trees receiving no treatment as a control treatment. Values in table with different symbols indicate significant differences at a 95% level of significance (student t-test). Phenolic concentration expressed as mg gallic acid equivalent per gram of dry weight.

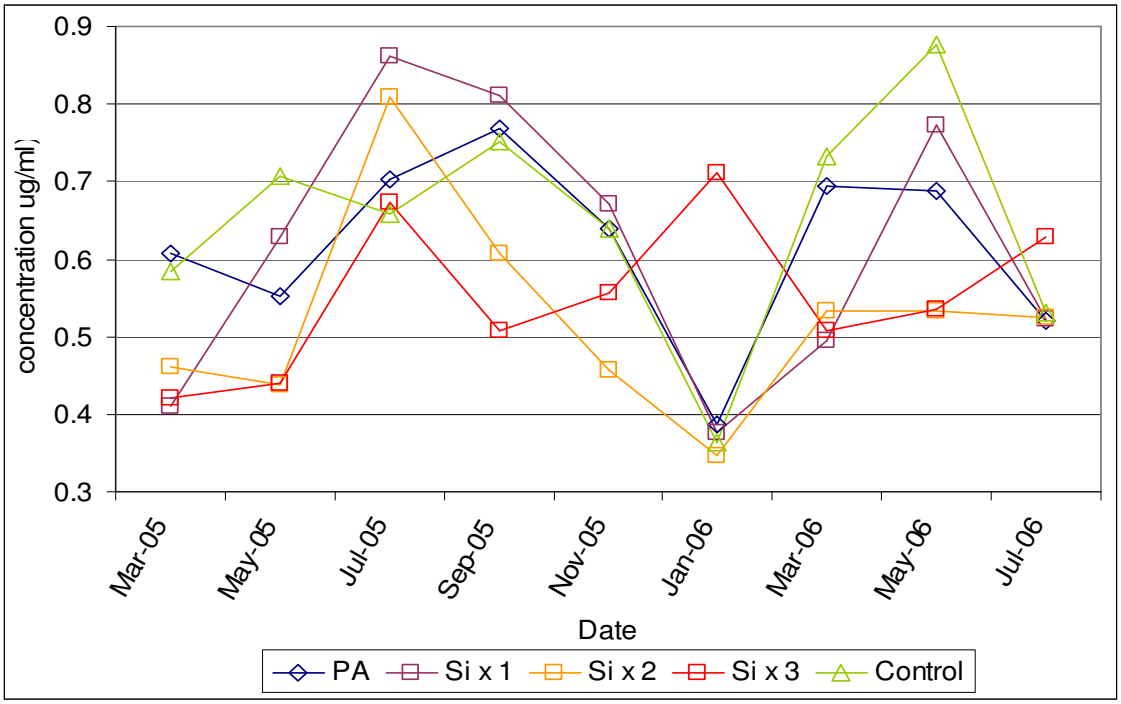

Figure 3: Total concentration of cell wall bound phenolic acid after hydrolysis of avocado roots recovered over a period of 18 months in *P. cinnamomi* infected trees, which were either untreated (controls) or treated with potassium silicate as a soil drench. Treatments consisted of either one (Si x 1), two (Si x 2) or three (Si x 3) potassium silicate applications per season; trees injected with potassium phosphonate (PA) and trees receiving no treatment as a control treatment. Values in table with different symbols indicate significant differences at a 95% level of significance (student t-test). Phenolic concentration expressed as mg gallic acid equivalent per gram of dry weight.

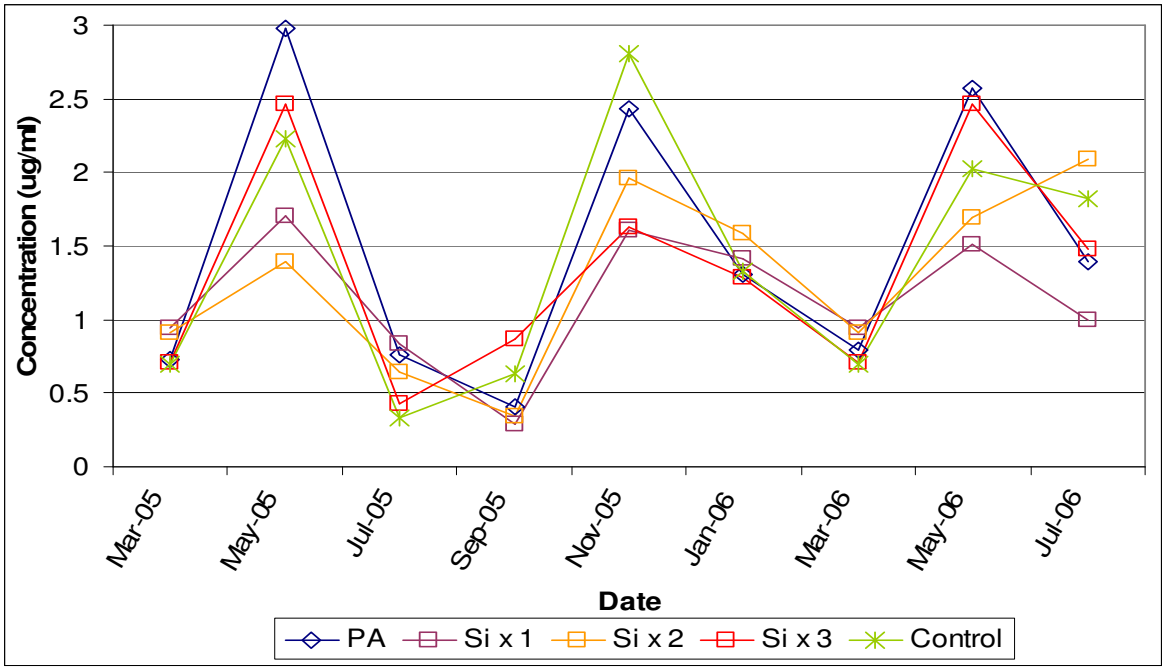

Figure 4: Total concentration of non-conjugated phenolics acid after hydrolysis of avocado roots recovered over a period of 18 months in *P. cinnamomi* infected trees, which were either untreated (controls) or treated with potassium silicate as a soil drench. Treatments consisted of either one (Si x 1), two (Si x 2) or three (Si x 3) potassium silicate applications per season; trees injected with potassium phosphonate (PA) and trees receiving no treatment as a control treatment. Values in table with different symbols indicate significant differences at a 95% level of significance (student t-test). Phenolic concentration expressed as mg gallic acid equivalent per gram of dry weight.