Effect of calcium soil amendments on phenolic compounds and soft rot resistance in potato tubers

Elizabeth Ngadze[a,b], Teresa, A. Coutinho[a,c], David Icishahayo[b], Jacquie, E. van der Waals[a*]

[a]Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa
[b]Department of Crop Science, University of Zimbabwe, P.O. Box MP167, Mount Pleasant Harare, Zimbabwe[c]
Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

*Corresponding author. Tel.: +27 012 420 4065, Fax: +27 12 420 4588
Email: Jacquie.vdwaals@up.ac.za

Abstract

Nutrients such as boron, nitrogen and calcium stimulate the production of phenols. This research focuses on the role of calcium in increasing phenol metabolism in potato peels and the ensuing tuber resistance to soft rot pathogens was investigated. Two field experiments were conducted at the University of Zimbabwe campus plots in 2008 and 2009 summer seasons. Sprouted tubers of cv. BP1 were planted in plots treated with different fertilizer combinations. The treatments were: 1) compound S (7N: 21P: 8K) + ammonium nitrate (34 %N) 2) compound D (7N: 14P: 7K) + calcium nitrate (19 Ca: 15.5N) 3) compound S + calcium nitrate and 4) compound D + ammonium nitrate. The harvested progeny tubers were inoculated with *Pectobacterium carotovorum* subsp. *brasiliense*. Calcium amendment increased the activities of enzymes (phenylalanine ammonia-lyase, polyphenol oxidase and peroxidases) involved in the metabolism of phenolics and total soluble phenols. Calcium amendment significantly reduced (P < 0.05) the maceration effect of *Pectobacterium carotovorum* subsp. *brasiliense* in tuber tissues. Chlorogenic acid, caffeic acid and calcium, but not ferulic acid content were significantly higher (P < 0.05) in plants grown in calcium treated plots. Calcium positively and significantly correlated with polyphenol oxidase (PPO), phenylalanine (PAL) and peroxidase (POD), while chlorogenic and caffeic acid showed a positive relationship with POD and PPO. Calcium amendment significantly reduced maceration symptoms caused by the bacteria (P < 0.05), resulting in smaller decayed zone diameters on inoculated tubers from calcium-treated plots. This study shows that soil amendments of calcium increase concentration of calcium, caffeic and chlorogenic acid in tuber peels and also

reduces maceration effect of pectinolytic pathogens. Reduced maceration could be due to increased levels of caffeic and chlorogenic acid which have antimicrobial properties.

1. Introduction

In Zimbabwe, potato growers face the challenge of significant post harvest losses of potato tubers (20 – 60 %) due to soft rot (Manzira, 2010). The causal agents were identified as *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), *Pectobacterium atrosepticum* (*Pa*) and *Dickeya dadantii* (*Dd*) (Ngadze et al., 2010, Ngadze et al., 2012b)*.* The pathogens produce large quantities of pectolytic enzymes which macerate plant tissues (Collmer and Keen, 1986). *Pectobacterium* spp. secrete pectinases that induce polyphenol oxidase activity in the hosts, which subsequently oxidises phenols, forming a black margin around the infection site. The margin restricts the pathogen from spreading (Lovrekovich et al., 1967). Most of the potato cultivars grown in Zimbabwe have some level of susceptibility to soft rot pathogens (Ngadze et al., 2012a).

Calcium is an essential nutrient and considered one of the most important nutrients associated with plant defense (Datnoff et al., 2007). It has been linked to interactions between plant pathogenic bacteria and their host plants. Calcium confers some resistance to pests and diseases in plants via its influence on growth pattern, anatomy, morphology and chemical composition of the plant. Increased plant calcium has been shown to enhance resistance to plant tissue macerating bacterial phytopathogens (Pèrombelon and Kelman, 1980; McGuire and Kelman, 1984; 1986, Schöber et al., 1999). Calcium increased resistance of witloof to soft rot pathogens, while high concentrations of nitrogen increased susceptibility of the same plants to soft rot pathogens (Schöber et al. 1999). Calcium is involved in eliciting signal transduction pathways and in membrane and cell wall integrity (McGuire and Kelman, 1984; Busse and Palta, 2006; Datnoff et al., 2007). It also enhances the structural integrity of cell walls and membranes. Adjustments in mineral nutrition could therefore reduce disease severity (Marschner, 1995; Datnoff et al., 2007).

Metabolism of phenolics in plants has been associated with injuries, thermal stress, and tolerance against exposure to UV rays, biotic stress and ozone. The role of Ca^{2+} in phenolic metabolism has been described by several authors; Castañeda and Pérez (1996) demonstrated a direct role of calcium in the synthesis of phenols. Ruiz et al. (2003) observed that foliar application of 10 µM of $CaCl_2$ increased PAL activity and caused subsequent accumulation of phenols, increasing resistance of citrus to infection caused by *Alternaria alternata*. Given the essential role played by phenol metabolism in many resistance responses to different stresses, rapid and effective manipulation of the metabolic process could enhance resistance in plants to adverse conditions. The objective of this study was to determine the effect of calcium soil amendments in increasing calcium levels in the plant and the subsequent effect thereof on metabolism of phenolic compounds.

2. Materials and methods

2.1 Experimental site

Two experiments were conducted at the University of Zimbabwe (UZ) campus in the 2008 (experiment 1) and 2009 (experiment 2) summer seasons (August - December). The UZ campus is situated in Harare (17º50´ South and 31º30´ East) at an altitude of 1500 m above sea level. The area is characterized by fersiallitic red clay soils with more than 40% clay and receives an annual rainfall of 800 - 1000 mm. Average maximum temperatures during the growing season ranged from 20 to 25ºC. The fields were planted to Brassicae prior to the experiments. Compound S [7N: 21P: 8K] and compound D [7N: 14P: 7K] fertilizers were used in this experiment because compound S is the fertilizer recommended for growing potato in Zimbabwe, but some smallholder farmers opt for compound D which is cheaper. The crop was hoe weeded when necessary and pests were controlled with carbaryl used at the recommended rate.

2.2 Experimental design

The experiment was laid out as a randomized complete block design with four treatments. The treatments were: 1) compound S [7N: 21P: 8K] + ammonium nitrate [34.5 % N]; 2) compound D [7N: 14P: 7K] + calcium nitrate [19Ca: 15.5N]; 3) compound S + calcium nitrate and 4) compound D + ammonium nitrate. The combination of fertilisers in these treatments ensured that calcium was present only in

treatments 2 and 3. The different fertilizer treatments were applied to the appropriate plots. Three blocks were used in the experiment and the treatments were replicated three times in each block for each year (that is in experiments 1 and 2). Certified potato seed of cultivar BP1 was used in the experiment.

2.3 Agronomic practices

Compounds D (7N: 14P: 7K) and S (7N: 21P: 8K) were applied as basal fertilizers at a rate of 1000 kg ha^{-1} in the relevant treatments. Calcium nitrate at a rate of 250 kg ha^{-1} was mixed with basal fertilizer for treatments 3 and 4. The first application of calcium nitrate was applied after opening the furrows, and then slightly covered with soil before planting the tubers at a depth of 10cm. Ammonium nitrate and the second calcium nitrate application were applied as top dressing at a rate of 250 kg ha^{-1} 6 weeks after crop emergence. The fertilizer was placed about 5 cm away from the plants to avoid scorching. The fields were irrigated when necessary and 300 mm of water was applied to the field as a supplement for the whole season. The total amount of precipitation was 650 mm measured for the whole season.

2.4 Plant mineral analysis

2.4.1 Extraction and quantification of total Ca^{2+}

For determination of calcium in the leaf tissue, the top fully expanded leaf was collected from each of 10 plants per treatment at the flowering stage and these were pooled for each treatment. For tuber analysis, five tubers were randomly selected at harvest from each treatment per block and taken as the representative sample. Total Ca^{2+} in the peels was determined by atomic-absorption spectrophotometry (Hocking and Pate 1977), after digestion of dry and milled material with 12 N H_2SO_4 and H_2O_2. The content of calcium was expressed as m mol g^{-1} dry weight (DW).

2.4.2 Extraction of phenolic compounds

Five daughter tubers from each treatment were randomly selected at harvest. The tubers were washed under running water and peeled with a potato peeler. Tuber peels were freeze-dried for five days and then ground to a fine powder. Two hundred milligrams of this fine powder was passed through a 1 mm sieve and placed in a 1.5 ml micro centrifuge tube for extractions. Aliquots of 1 ml of a cold mixture of methanol: acetone: ultra-pure water (7:7:1; v:v:v) were added, vortexed and

ultrasonified for 5 min. After sonification, samples were shaken for 20 min at 160 rpm while on ice. Samples were centrifuged for 5 min at 5 000 rpm and the supernatant of each sample was transferred to a 20 ml centrifuge tube. This process was repeated three times on the same sample and supernatants finally evaporated in a laminar flow cabinet at room temperature. The residue was dissolved in 1 ml sterile, ultra-pure water. Finally, samples were filtered through 0.45 μm, 25 mm, Ascrodise, GHP, syringe filters (Separations, South Africa). Samples were stored at 4 ºC until analysis using reverse phase – high performance liquid chromatography (RP – HPLC).

2.4.3 Reverse Phase – High Performance Liquid Chromatography

For identification and quantitative analysis of samples, 10 μl of purified extract per sample was analyzed using RP - HPLC (Hewlett Packard Agilent 1100 series) with a UV diode array detector, at 325 and 340 nm. Separation was achieved on a Gemini 3 μ, C18, 110A (Phenomenex®) reverse phase column (250 mm length, 5 μm particle size, 4.6 mm inner diameter). A gradient elution was performed with water (pH 2.6 adjusted with H_3PO_4) and acetonitrile (ACN) and consisted of 0 min, 7% ACN; 0 – 20 min, 20% ACN; 20 – 28 min, 23% ACN; 28 – 40 min, 27% ACN; 40 – 45 min, 29% ACN; 45 – 47 min, 33% ACN; 47 – 50 min, 80% ACN. Solvent flow rate was 0.7 ml min^{-1}. Identification of the phenolic compounds was done by comparing their retention times and UV apex spectrum to those of standards: chlorogenic acid, caffeic acid and ferulic acid. The column was re-equilibrated for 10 min, after each run.

2.5 Enzymatic analysis

The extraction of phenylammonia lyase (PAL) in the leaves was carried out following the method proposed by Lister et al. (1996). Five plants were randomly selected from each treatment per plot at 10 WACE and 2 young leaves were taken from each plant. The leaves were pooled to make a composite sample. PAL activity was assayed according to the method described by McCallum and Walker (1990), and determined from the yield of cinnamic acid, estimated from absorbance at A_{290} in the presence and absence of L-phenylalanine. The procedure was repeated three times for each sample.

The extraction of polyphenol oxidase (PPO) in the leaves was performed at 10 WACE, five plants were randomly selected from each treatment per plot and 2 young leaves were taken from each plant. The leaves were pooled to make a composite

sample. The assay was carried out according to the method described by Thypyapong et al. (1995) and activity assayed as described by Nicoli et al. (1991), measured by the change in assay mixture at 30ºC at A_{370}. Activity was based on the enzymatic oxidation of caffeic acid. The procedure was repeated three times for each sample.

Peroxidase (POD) extraction and determination were according to the method described by Zhang et al. (2008). The sampling procedure is similar to that of PAL and PPO. The procedure was repeated three times for each sample. Controls contained catalase from bovine liver (Sigma-Aldrich).

In all the cases, samples were boiled and assayed to determine whether the reactions were enzymatic. Protein content in the sample extracts was estimated using the method described by Bradford (1976) and bovine serum was used as a standard.

2.6 Bacterial culture

A type strain of *P. c.* subsp. *brasiliense* (ATCC BAA-419 *Pcb* Strain 371, American Type Culture Collection) was used in this study. The culture was grown for 24 hours at 25˚C in a shaken culture of 25 ml Luria Bertani (LB) broth (pH 7.0) supplemented with 0.1 % pectin from citrus fruits (Sigma) and 0.1 % lyophilized potato cell sap. After centrifugation at 5000 rpm for 5 minutes at 4˚C, the bacteria pellet was washed with sterile water, centrifuged again and re-suspended in sterile water before adjusting optical density ($OD_{600 - }$0.1) using a spectrophotometer.

2.7 Maceration of potato tuber tissue by bacteria

Eight randomly selected progeny tubers from each treatment were washed thoroughly under running tap water and surface-disinfected by dipping in 70% ethanol for 5 min, before being cut longitudinally into halves (giving 16 halves). Each tuber half was inoculated with 10 mm diameter filter paper discs (Whatman's No. 1), pre-soaked for 10 min in *Pcb* bacterial suspension. For the control, filter paper discs were soaked in sterile distilled water for 10 min and placed on tuber halves. One filter paper disc was placed in the pith and four around the edges of each tuber half. The experimental design was a Randomized Complete Block Design (RCBD) with four replicates per treatment (4 half tubers per treatment). After inoculation and incubation at 25˚C for 24 h the filter paper discs were removed and the diameter of each rotting zone was measured.

2.8 Statistical analysis

All the data was subjected to analysis of variance (ANOVA) to determine the significance of treatment means using the GenStat statistical package (GenStat, 2002 Release 6.1, Lawes Agricultural Trust, Rothamstead) (Payne, 2003). Means were separated using Fisher's protected Least Significant Difference (LSD) at P = 0.05.

3. Results

3.1 Plant mineral analysis

3.1.1 Extraction and quantification of total Ca^{2+}

The concentration of calcium in both the leaves and tuber peels was significantly higher (P < 0.05) in plants grown in the plots amended with calcium nitrate. The amount of calcium in the leaves was almost double that which was recorded for the tubers in both experiments 1 and 2 (Table 1).

3.1.2 Phenolic compounds

Tuber peels from all treatments contained low levels of ferullic acid and there were no significant differences in the concentrations of this compound between treatments. However chlorogenic and caffeic acid concentrations were significantly higher (P < 0.05) in tuber peels from the calcium treatments than the controls. The concentration of caffeic acid was higher than that of chlorogenic acid in plots where $Ca(NO_3)_2$ was applied in combination with either compound D or S for both experiments 1 and 2 (Table 2). Calcium correlated positively and significantly with increased chlorogenic and caffeic acid concentrations in both the 2008 and 2009 seasons (Table 3).

3.1.3 Enzymatic analysis

Concentrations of POD, PAL and PPO were significantly higher in the calcium treated plants in both experiments 1 and 2. The concentration of PPO in the calcium treated plants was more than double that which was recorded in plants not treated with calcium (Table 2). Calcium amendments significantly correlated with increases in POD, PPO and PAL concentrations (P < 0.001) in both seasons (Table 3). Chlorogenic and caffeic acid significant and positively correlated with POD and PPO in seasons 1 and 2 (Table 4).

Table 1. Concentration of chlorogenic, caffeic and ferulic acid in thetable 3 tuber peels, and calcium in foliage and peels measured in plants grown in the field in 2008 and 2009.

Treatment	Experiment 1 (2008)						Experiment 2 (2009)					
	Calcium in foliage (m mol g^{-1} DW)	Calcium in peel (m mol g^{-1} DW)	Rotting zone diameter (mm)	Chlorogenic acid (mg g^{-1} FW)	Caffeic Acid (mg g^{-1} FW)	Ferulic Acid (mg g^{-1} FW)	Calcium in foliage (m mol g^{-1} DW)	Calcium in peel (m mol g^{-1} DW)	Rotting zone diameter (mm)	Chlorogenic acid (mg g^{-1} FW)	Caffeic Acid (mg g^{-1} FW)	Ferulic Acid (mg g^{-1} FW)
CompD+ AN	2.595a (2.7)	0.956a	29.90b	1.341a	1.940a	1.158	2.584a (2.2)	1.189a	30.00b	1.850a	3.380a	1.231
CompS+CaN	3.011b (1.6)	1.943b	20.10a	2.200b	3.400b	1.582	3.427b (1.6)	2.133b	16.90a	2.325b	4.340b	1.423
CompD+CaN	2.967b (1.3)	2.245b	19.70a	2.135b	3.526b	1.712	3.322b (1.7)	1.978b	18.20a	2.520b	4.450b	1.612
CompS+AN	2.753a (2.3)	1.175a	26.30b	1.570a	2.187a	1.234	2.695a (2.3)	1.179a	28.50b	1.784a	3.540a	1.193
P-Value	0.003	<0.001	<0.001	0.002	0.001	0.127	0.002	<0.001	<0.001	0.002	0.003	0.623
SED	0.249	0.357	0.479	0.231	0.315	0.628	0.232	0.396	1.174	0.326	0.128	0.251
LSD $_{(0.05)}$	0.498	0.714	0.954	0.462	0.628	NS	0.464	0.792	2.348	0.652	0.256	NS

Means followed by the same letter in a column are not significantly different at LSD (0.05).

AN – Ammonium Nitrate, CaN – Calcium Nitrate, CompD – Compound D fertilizer, CompS – Compound S fertilizer.

Table 2. Effect of calcium treatment on concentration of PPO, POD and PAL in potato leaves recorded in 2008 and 2009 in the field.

Treatment	Experiment 1 (2008)			Experiment 2 (2009)		
	POD	PPO	PAL	POD	PPO	PAL
CompD+ AN	2.57a	2.95a	1.20a	2.73a	1.73a	1.10a
CompS+CaN	3.35b	5.92b	1.98b	3.92b	5.02b	1.87b
CompD+CaN	3.92b	6.23b	1.69b	4.00b	4.95b	1.79b
CompS+AN	2.31a	2.78a	1.11a	2.25a	2.15a	1.15a
P-Value	0.003	< 0.001	0.002	< 0.001	< 0.001	0.003
SED	0.375	0.675	0.132	0.575	0.575	0.163
LSD $_{(0.05)}$	0.74	1.350	0.33	1.155	1.155	0.33

PAL: μmol cinnamic acid produced mg^{-1} protein min^{-1}; PPO: μmol caffeic acid oxidized mg^{-1} protein^{-1}; POD: μmol guaiacol oxidized mg^{-1} protein min^{-1}.

POD – Peroxidase, PPO – Polyphenol oxidase, PAL – Phenylalanine ammonia lyase.

Table 3. Correlation coefficients between chlorogenic, caffeic acid, POD, PPO, PAL and maceration with calcium soil amendment.

	2008	2009
Chlorogenic	0.988 ***	0.982 ***
Caffeic	0.697 **	0.660 **
POD	0.954 ***	0.966 ***
PPO	0.979 ***	0.975 ***
PAL	0.635 **	0.682 **
Maceration	-0.972 ***	-0.958 ***

POD – Peroxidase, PPO – Polyphenol oxidase, PAL – Phenylalanine ammonia lyase.

Table 4. Correlation coefficients between chlorogenic and caffeic acids with POD, PPO and PAL.

	2008			2009		
	POD	PPO	PAL	POD	PPO	PAL
Chlorogenic	0.929^{***}	0.982^{***}	0.559^{ns}	0.918^{***}	0.988^{***}	0.559^{ns}
Caffeic	0.621^{*}	0.660^{**}	0.451^{ns}	0.599^{*}	0.645^{**}	0.451^{ns}

POD – Peroxidase, PPO – Polyphenol oxidase, PAL – Phenylalanine ammonia lyase.

$*P < 0.05$, $**P < 0.01$, ***;$P < 0.001$, ns: not significant.

3.2 Maceration of potato tuber tissue by bacteria

Addition of calcium nitrate to the soil significantly reduced (P < 0.05) the maceration symptoms caused by *Pcb*. Significantly smaller (P < 0.05) rotting zone diameters were recorded on tubers harvested from plots treated with $Ca(NO_3)_2$ in both seasons (Table 1). Calcium significantly negatively correlated with maceration (P < 0.001) in both experiments 1 and 2 (Table 3).

4. DISCUSSION

Calcium is an essential mineral that has been shown to be important in many physiological processes, such as plant defense. A deficiency of calcium in the plant can create conditions favourable for pathogen infection (Rahman and Punja, 2007). Calcium has been implicated in the interaction between plant pathogenic bacteria and their hosts. Increased plant calcium has been shown to enhance resistance to plant tissue macerating bacterial phytopathogens (Pérombelon and Kelman, 1980; McGuire and Kelman, 1984; 1986, Schöber et al. 1999). Calcium also plays any important role in signaling and plant cell wall formation. It is involved in cross-linking pectin, the main substrate of plant cell wall degrading enzymes and forms calcium pectate which cannot be degraded by pectinolytic enzymes (Demarty et al.1984)

In this study the concentration of calcium was higher in the leaves than in the tubers, which was not surprising because stems and leaves are richer in calcium compounds than underground storage organs. This is related to the inability of phloem to transport calcium from the leaves to the root system (Demarty et al., 1984). This higher foliar accumulation of calcium was due to the fact that after absorption by the root cells, calcium is transported through the xylem towards the shoot and accumulates in areas where transpiration is greatest (Bharti et al., 1996, Ruiz and Romero, 1998; Ruiz et al., 1999). Calcium content was also significantly higher in the peels of tubers harvested from the calcium treated plots. This was most likely due to the fact that the second batch of calcium nitrate was applied when the tuber roots had been formed. Placement of calcium is important for enhancing calcium uptake by the tuber as the main root does not supply the tuber with calcium (Palta, 2010). Addition of calcium to the tuber and stolon areas can result in a threefold increase in calcium concentration in the tuber peel and medullary tissue (Kratzke and Palta 1986). Resistance to blackleg and tuber soft rot may be related to calcium concentration in the tubers, but results

varied between cultivars tested over 3 years of field experimentation (Pagel and Heitefuss, 1989).

Low calcium content in plants harvested from plots top dressed with ammonium nitrate could have been attributed to the fact that ammonium competes with other cations for uptake, including calcium. By using ammonium nitrate, calcium levels might have been lower than they would otherwise be in plots treated with ammonium nitrate. Rayar and Van Hai, (1977) reported that high levels of ammonium inhibited uptake and content of calcium and magnesium in soybean seedlings. A high calcium content in host tissues has been correlated with increased resistance to several diseases (Bateman and Lumsden, 1965; Bateman and Millar, 1966; Forster and Echandi, 1975; McGuire and Kelman, 1983; 1984). Plants grown in nutrient conditions of high Ca^{2+} content showed resistance to soft rot diseases (McGuire and Kelman, 1984). Calcium nitrate significantly reduced the macerating effect of *P. c.* subsp. *brasiliense* and significantly smaller rotting zone diameters were recorded in inoculated tuber halves with higher calcium content. These results are consistent with the findings of Schöber et al. 1999 who reported resistance of witloof plants in high calcium concentration treatments. The increased resistance in tissues with high levels of calcium has been attributed to decreased maceration owing to calcium deposition in the cell wall pectate and structural enhancement of cell wall integrity (McGuire and Kelman, 1984; 1986; Carpita and Gibeaut, 1993). Excess calcium combines with pectin to form calcium pectate, which is resistant to the action of polygalacturonase (PG) (Demarty et al., 1984).

The ability of *Pc.* subsp. *carotovorum* and other soft rot bacteria species to macerate plant tissue is dependent on the massive production and secretion of plant cell wall-degrading enzymes, especially pectinolytic enzymes such as polygalacturonase (Peh), pectin lyase (Pnl) and isoforms of pectate lyase (Pel) (Collmer and Keen, 1986). These enzymes are crucial for the virulence of *Pectobacterium* species and mutations which affect production and secretion of these enzymes lead to reduced virulence (Pirhonen et al., 1993; Reeves et al., 1994). The reduced rotting zone diameters noted in this experiment could also be attributed to calcium interfering with the production of endopolygalacturonase, an enzyme required in the early stages of infection, which could also in turn lead to reduced virulence. Increased extracellular calcium

inactivates the gene which codes for endopolygalacturonase production but does not affect the production of other cell wall-degrading enzymes (Flego et al., 1997). Inactivation of a single gene encoding a particular pectic enzyme can drastically reduce virulence (Flego et al., 1997). Reduced maceration recorded in tubers harvested from the calcium treated plots could also have been due to high concentrations of chlorogenic acid and caffeic acid in these tubers. Chlorogenic, caffeic and ferulic acid are the three main phenolic compounds found in potato tubers. They have antibacterial effects and inhibit growth of soft rot bacteria (Ghanekar et al., 1984; Kumar et al., 1991). Chlorogenic acid is produced as a defense in potatoes in response to infection or injury (Ghanekar et al., 1984). Caffeic acid is known to inhibit the growth of soft rot bacteria (Kumar et al., 1991). The combination of caffeic acid and chlorogenic acid may significantly inhibit infection by soft rot pathogens (Ghanekar et al., 1984; Kumar et al., 1991).

The concentration of chlorogenic acid was lower than that of caffeic acid. This was not surprising since the mother tubers were inoculated before planting. The progeny tubers may have carried latent infection from the field and chlorogenic acid is the storage form of caffeic acid and can be converted to caffeic acid during stress conditions (Ghanekar et al., 1984). These findings contradict the findings of Mattila and Hellström (2006) who reported that the concentration of caffeic acid was lower than that of chlorogenic acid in raw and cooked potato peels. The difference between the two experiments was the fact that tubers used in this study may have been impacted by either biotic or abiotic stresses. Chlorogenic and caffeic acid were significantly higher in tubers harvested from the calcium amended plots. The results show that calcium has a positive effect on the production of chlorogenic and caffeic acid which enhance the protection and resistance against soft rot pathogens. Chlorogenic acid is formed in potato tissues in response to infection or injury (Ghanekar et al., 1984).

Foliar PAL was significantly higher in the plots amended with calcium and PAL activity correlated significantly with calcium. These findings are similar to the observations made by Castañeda and Pérez (1996) who recorded an increase in PAL activity in lemon seedlings after addition of calcium. The increase in PAL could be due to a response triggered by a series of transduction signals which result in

increased activity of the enzymes PPO and POD. These results are in agreement with findings of Ruiz et al., 2003 who reported that PPO and POD concentrations increased with the addition of Ca^{2+}. Calcium activates PPO, modifying the conformational state of the enzyme, thereby boosting its activity (Söderhall, 1995). The Ca^{2+} ion is also required for inducing the cross-linking of polygalacturonan chains into a structure recognized by the peroxidase (Penel et al., 1999). The positive relationship between PPO and POD might indicate that calcium stimulates the production of these enzymes.

Several researchers have reported the beneficial effects of calcium in increasing potato resistance against soft rot pathogens (McGuire and Kelman, 1984; 1986; Bain et al., 1996; Flego et al., 1997). This study has confirmed these findings under field conditions in Zimbabwe. This study has shown that calcium plays a significant role in increasing caffeic and chlorogenic acid which are antimicrobial compounds. Calcium soil amendment also increased the concentration of PAL, PPO and POD enzymes involved in plant defense mechanisms. It will thus be beneficial for potato growers to supplement calcium in the field in order to reduce the maceration effect of pectolytic pathogens since calcium improves tuber resistance against the pathogens.

References

Bain, R.A., Millard, P., Pérombelon, M.C.M., 1996. The resistance of potato plants to *Erwinia carotovora* subsp. *atroseptica* in relation to their calcium and magnesium content. Potato Res. 39, 185 - 193.

Bateman, D.F., Lumsden, R.D., 1965. Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani.* Phytopathol. 55, 734 – 738.

Bateman, D.F., Millar, R.L. 1966. Pectic enzymes in tissue degradation. *Ann. Rev. Phytopathol.* 4, 119 – 139.

Bharti N., Singh R.P., Sinha S.K., 1996. Effect of calcium chloride on heavy metal induced alteration in growth and nitrate assimilation of *Sesamun indicum* seedlings. Phytochem. 41, 105 - 109.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248 – 254.

Busse, J.S., Palta, V.P., 2006. Investigating the in vivo calcium transport path to developing potato tuber using 45Ca: a new concept in potato tuber calcium nutrition. Physiol. Plant. 128, 313 – 323.

Carpita, N.C., Gibeaut, D.M., 1993. Structural models of primary cell walls in flowering plants: consitency of molecular structure with the physical properties of the cell walls during growth. Plant J. 3, 1 – 30.

Castañeda P., Pérez L.M., 1996. Calcium ions promote the response of citrus lemon against fungal elicitors or wounding. Phytochem. 42, 595 – 598.

Collmer, A., Keen, N.T., 1986. The role of pectic enzymes in plant pathogenesis. Ann. Rev. Phytopathol. 24, 383 – 409.

Datnoff, L.E, Elmer, W.H., Huber, D.M., 2007. Mineral nutrition and plant disease. APS Press, Minnesota.

Demarty, M., Morvan, C., Thellier, M.B., 1984. Calcium and the cell wall. Plant, Cell and Env. 7, 441 – 448.

Flego, D., Pirhonen, M., Saarilahti, H., Palva, T.K., Palva, E.T., 1997. Control of virulence gene expression by plant calcium in the phytopathogen Erwinia carotovora. Mol. Microbiol. 25, 831 – 838.

Forster R.L., Echandi, E., 1975. Influence of calcium nutrition on bacterial canker of resistant and susceptible *Lycopersicon* spp. Phytopathol. 65, *84 – 85*.

Ghanekar, A.S., Padwal-Desai, S.R., Nadkarni, G.B., 1984. The involvement of phenolics and phytoalexins in resistance of potato to soft rot. Potato Res. 27, 189 – 199.

Hocking, P.J., Pate J.S., 1977. Mobilization of minerals to developing seeds of legumes. Ann. Bot. 41, 1259 – 1278.

Kratzke, M.G., Palta, J.P., 1986. Calcium accumulation in potato tubers: Role of basal roots. Hortsci. 21, 1022 – 1024.

Kumar, A., Pundhir, V.S., Gupta, K.C., 1991. The role of phenols in potato tuber resistance against soft rot by *Erwinia carotovora* ssp. *carotovora*. Potato Res. 34, 9 – 16.

Lovrekovich, L., Lovrekovich, H., Stahmann, M.A., 1967. Inhibition of phenol oxidation by *Erwinia carotovora* in potato tuber tissue and its significance in disease resiastance. Phytopathol. 57, 737 – 742.

Lister C.E., Lancaster J.E., Walker J.R.L., 1996. Phenylalanine ammonia-lyase activity and the relationship to anthocyanin and flavonoid levels in New Zealand's grown apple cultivars. J. Am. Soc. Hort. Sci. 121, 281 – 285.

Marschner, H., 1995. Mineral nutrition of higher plants, 2nd ed. Academic Press, San Diego.

Mattila, P., Hellström, J. 2006. Phenolic acids in potatoes, vegetables, and some of their products. Food Comp. and Anal. 20, 152 – 160.

Manzira, C., 2010. Potato production handbook. Potato Seed Association. Zimbabwe Publishers, Harare.

McCallum J.A., Walker J.R.L., 1990. Phenolic biosynthesis during grain development in wheat: changes in phenylalanine ammonia-lyase activity and soluble phenolic content. J. Cereal Sci. 11, 35 – 49.

McGuire, R.G., Kelman, A., 1983. Relationship between calcium levels in potato tubers and Erwinia soft rot. Phytopathol. 72, 1138 (abstract).

McGuire, R.G., Kelman, A., 1984. Reduced severity of Erwinia soft ror in potato tubers with increased calcium content. Phytopathol. 74, 1250 – 1256.

McGuire R.G., Kelman, A., 1986. Calcium in potato tuber cell walls in relation to tissue maceration by *Eca*. Phytopathol. 76, 401 – 406.

Ngadze, E., Coutinho, T.A., Icishahayo, D., van der Waals, J.E., 2012a. Role of polyphenol oxidase peroxidise, phenylalanine ammonia lyase, chlorogenic acid and total soluble. Plant Dis. 96, 186 - 192.

Ngadze, E., Coutinho, T.A., van der Waals, J.E., 2010. First report of soft rot of potatoes caused by *Dickeya dadantii* in Zimbabwe. Plant Dis. 94, 1263.

Ngadze, E., Brady, C.L., Coutinho, T.A., van der Waals, J.E., 2012b. Pectinolytic bacteria associated with potato soft rot and blackleg in South Africa and Zimbabwe. Eur. J. Plant. Pathol. 134, 533 - 549.

Nicoli M.C., Elizale B.E., Pitotti A., Lerici C.R., 1991. Effect of sugar and maillard reaction products on polyphenol oxidase and peroxidase activity in food. J. Food Biochem. 15, 169 – 184.

Pagel, W., Heitefuss, R., 1989. Calcium content and cell wall polygalacturonans in potato tubers of cultivars with different susceptibilities to *Erwinia carotovora* subsp. *atroseptica*. Physiol. Mol. Plant P. 35, 11 – 21.

Palta, J.P., 2010. Improving potato tuber quality and production by targeted calcium nutrition: the discovery of tuber roots leading to a new concept in potato nutrition. Potato Res. 53, 267 – 275.

Penel C., van Cutsem P., Greppin H., 1999. Interactions of a plant peroxidase with oligogalacturonides in the presence of calcium ions. Phytochemistry 51, 193 – 198.

Pérombelon, M.C.M., Kelman, A., 1980. Ecology of soft rot *Erwinias*. Ann. Rev. Phytopathol. 18, 361 – 67.

Pirhonen, M., Flego, D., Heikinheimo, R., Palva, E.T., 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant. *Erwinia carotovora*. EMBO. J. 12, 2467 – 2476.

Rahman, M., Punja, Z.K., 2007. Calcium and plant disease. In mineral nutrition and plant disease, eds. Datnoff, L.E, Elmer, W.H. and Huber, D.M., APS Press, Minnesota.

Rayar, A.J, Van Hai, T., 1977. Effect of ammonium on uptake of phosphorus, potassium, calcium and magnesium by intact soybean plants. Plant Soil 48, 81 – 87.

Reeves, P.J., Douglas, P., Salmond, G.P.C., 1994. Beta-lactamase topology probe analysis of the peptidase, and six other out protein components of the *Erwinia carotovora* general secretion pathway apparatus. Mol. Microbiol. 12, 445 – 457.

Ruiz, J.M., Rivero, R.M., Garcia, P.C., Baghour, M., Romero, L., 1999. Role of $CaCl_2$ in nitrate assimilation in leaves and root of tobacco plants (*Nicotiana tabacum* L.). Plant Sci. 141, 107 – 115.

Ruiz, J.M., Romero, L., 1998. Calcium impact on phosphorus and its main bioindicators: response in the roots and leaves of tobacco. J. Plant Nutr. 21, 2273 – 2285.

Schöber, B.M., Vermeulen, T., 1999. The role of calcium and nitrogen in susceptibility to witloof for soft rot coliforms. Eur J. Plant Pathol. 105, 341 – 49.

Söderhall I., 1995. Properties of carrot polyphenol oxidase. Phytochem. 39, 33 – 38.

Thypyapong P., Hunt M.D. and Steffens J.C., 1995. Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. Phytochem. 40, 673 – 676.

Zhang, S., Zhang, F., Hua, B., 2008. Enhancement of PAL, PPO and POD in cucumber seedlings by *Bemisia tabaci* (Gennadius) (Hemiptera: leyrodidae) infestation. Agri. Sci. in China. 7, 82 - 87.