CHAPTER 4

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ROLE OF POLYPHENOL OXIDASE, PEROXIDASE, PHENYLALANINE AMMONIA LYASE, CHLOROGENIC ACID AND TOTAL SOLUBLE PHENOLS IN RESISTANCE OF POTATOES TO SOFT ROT

Abstract

Pectobacterium atrosepticum (*Pa*), *P. carotovorum* subsp. *brasiliensis* (*Pcb*) and *Dickeya* spp. cause soft rot of potato (*Solanum tuberosum*) worldwide. Plants respond to bacterial invasion by activating defense responses associated with accumulation of several enzymes and inhibitors, which prevent pathogen infection. This study focused on the role of polyphenol oxidase (PPO), Peroxidase (POD), phenylalanine ammonia lyase (PAL), chlorogenic acid and total soluble phenols in imparting resistance to soft rot pathogens. Seven and 11 varieties grown by farmers in South Africa and Zimbabwe, respectively, were used in the study. The results showed significantly higher ($P < 0.001$) enzyme activity of PPO and PAL as well as higher concentrations of chlorogenic acid and total soluble phenols in Vanderplank, Pentland Dell, M69/11, Romano, M59/20 and Mondial$_{Zw}$. PAL activity increased significantly with time in all varieties and the highest activity was recorded 8 h after cutting. The resistance of the varieties was correlated with high PPO and PAL enzyme activity as well as increased concentrations of chlorogenic acid and total soluble phenols. PPO, POD and PAL activities increased significantly in wounded and inoculated tubers. These findings show that PAL, PPO, POD, chlorogenic acid and total soluble phenols play a role in imparting resistance to potato soft rot infection.

4.1 INTRODUCTION

Tuber soft rot of potatoes (*Solanum tuberosum* L.) caused by *Pectobacterium atrosepticum,* subspecies of *P. carotovorum* and *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (Samson *et al.*, 2005) leads to significant economic losses in agriculture worldwide (Stevenson *et al.*, 2001). The disease affects the crop both in the field and in storage. After invading host tissue, the bacterial population increases to a critical density, above which a large amount of cell wall degrading pectolytic

enzymes are produced resulting in tissue maceration and granular rot of infected tubers. Rotted tissue is initially white or cream coloured, but brown pigments subsequently appear due to the oxidation of diseased tissue (Thipathi and Verma, 1975). The major virulence factors of *Pectobacterium* and *Dickeya* spp. are isoforms of pectate lyase (PL) enzymes that degrade plant cell wall pectin into unsaturated oligogalacturonates (OG) eliciting plant defense responses (Ryan, 1988). The virulence of soft rot bacteria appears to be influenced by temperature and humidity in conjunction with other factors. These other factors include intrinsic differences in the resistance of individual potato varieties to the pathogen, ability of the pathogen to multiply at the site of inoculation and differences in aggressiveness of bacterial isolates (Peltzer and Sivasithaparam 1985).

Disease resistance in plants is associated with activation of a wide array of defense responses that slow down or halt infection at certain stages of the host-pathogen interaction. The defense mechanisms include preexisting physical and chemical barriers that interfere with pathogen establishment. Other methods of protection rely on inducible defense responses in the form of enzymes that are activated upon infection (Vanitha *et al.*, 2009). The interaction between the pathogen and the host plant induces some changes in cell metabolism; primarily activity of enzymes, particularly phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), lipoxygenase (LOX), superoxide dismutase (SOD) and β-1,3 glucanase (Fukasawa-Akada *et al.*, 1996; Hammerschmidt *et al.*, 1982; Mauch *et al.*, 1988, Ohta *et al.*, 1991; Thipathi and Verma1975; Thipyapong and Steffens 1992). PAL is the primary enzyme in the phenylpropanoid pathway, which leads to the conversion of L-phenylalanine to trans-cinnamic acid with the elimination of ammonia. It is the key enzyme in the synthesis of several defense-related secondary compounds such as phenols and lignin (Hemm *et al.*, 2004). PPO is a nuclear encoded enzyme that catalyzes the oxygen-dependent oxidation of phenols to quinones. PPO levels in a plant increase when a plant is wounded or infected (Vanitha *et al.*, 2009).

Although tuber soft rot causes considerable economic losses in Zimbabwe and South Africa, potato varieties grown in the two countries have not been assessed for resistance to the pathogens that cause the disease. The objectives of this study were (i) to determine the concentrations of PPO, PAL, POD, chlorogenic acid and total soluble phenols in potato tubers and (ii) to establish the relationship between the defense-related enzymes PPO, PAL, chlorogenic acid and total soluble phenols and resistance in potato tuber tissue to *P. atrosepticum (Pa), P. carotovorum* subsp. *brasiliensis (Pcb)* and *Dickeya dadantii* (*Dd*).

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Experiments were carried out to evaluate potato varieties for tolerance to soft rot pathogens. The seed was sourced from various potato seed companies. The seed of eleven varieties from Zimbabwe and seven from South Africa (Table 1) were evaluated in the experiment. The seed was harvested and stored for a month at 4˚C prior to the experiments. Seed tubers from each of the 18 varieties were used for the PPO, POD, PAL, chlorogenic acid and total soluble phenol assays. Each experiment was repeated twice.

4.2.2 Bacterial cultures

Bacterial strains of *P. atrosepticum* (LMG 2386^T, Belgian Coordinated Collections of Microorganisms), *Pectobacterium carotovorum*. subsp. *brasiliensis* (ATCC BAA-419 *Pcb* Strain 371, American Type Culture Collection) and *D. dadantii* (*Erwinia chrysanthemi* 3937, Scottish Crop Research Institute) were used in the study. *Pcb* and *Dd* were used as they have been shown to be the dominant soft-rotting bacteria of potatoes in South Africa (van der Merwe *et al*., 2010) and Zimbabwe (Ngadze *et al*., 2010), respectively. It has always been assumed that *P. atrosepticum* is the only causal agent of potato blackleg in Zimbabwe (unpublished data) and this is the reason for its inclusion in this study.

The bacterial cultures were grown for 24 h 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 5,000 rpm for 5 min (4˚C) the bacteria were washed with sterile water, centrifuged again and re-suspended in sterile water before adjusting for the appropriate density of 6 x 10^8 CFU mL^{-1} (OD_{600} – 0.6).

4.2.3 Maceration of potato tuber tissue by bacteria and viability of cells after inoculation

Eight randomly selected seed tubers from each variety 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), that had been soaked for 10 min in a bacterial suspension of *P. atrosepticum, P. carotovorum* subsp. *brasiliensis* and *D. dadantii,* respectively. 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 variety per pathogen (4 half tubers per variety per pathogen). 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. The tubers halves were incubated for a further 24 h and the rotting zone diameter was measured again.

Cell viability of the inoculated half tubers was determined after measuring the rotting zone diameters at 48 h. Each tuber half was shaken in 49 ml sterile distilled water for 1 h at 20˚C in order to generate osmotic stress, and then stained for 90 min in a solution containing 50 mg $litre^{-1}$ neutral red dissolved in 0.2 M Na-phosphate buffer (pH 7.5) containing 0.8 M KNO_3. The tuber halves were rinsed in distilled water, after which the absorbed dye was extracted twice from the tuber tissue in 96 % ethanol for 10 min each. The final volume of dye from each tuber half was adjusted to 50 ml by adding 0.01 M sulphuric acid, before determination of optical density at OD_{535} according to the method by Wegener

(2002). Cell viability was determined by comparing the amount of neutral red retained by the inoculated tubers to that retained by the control tubers and the experiment was repeated.

4.2.4 Inoculation of tubers

Tubers of selected cultivars were surface sterilized by submersion in 1% sodium hypochlorite for 10 min and each potato inoculated with 10 µl of a 48 h culture of *P. carotovorum* subsp. *brasiliensis* grown in Luria-Bertani broth. Holes of a fixed depth were stabbed into the potatoes using a sterile pipette tip, filled with inoculum and sealed with Vaseline. Three tubers of each cultivar were inoculated and wounded before the enzyme assays. The inoculated potato was then wrapped in a plastic bag and incubated at 25ºC for 8 h. The inoculated tubers were assayed for PPO (Jockusch, 1966), POD (Zhang *et al.*, 2008) and PAL (Okey *et al.*, 1997) activity at 8 h after inoculation.

4.2.5 Polyphenol oxidase assay

PPO activity was measured according to the method described by Jockusch (1966) with minor modifications and 10 tubers of each variety were used for the assay. One cylinder, about 20 mm long was cut from each of the potato tubers using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder using a scalpel and discarded. The tissue cylinders were pooled and ground in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 5 % polyvinylpolypyrrolidone (wt/vol). The homogenate was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer and 1 ml of 0.1 M catechol (Sigma). The mixture was aliquoted into three portions for measurement of PPO activity. In the control the tuber extract was replaced with 1 ml of sodium phosphate buffer. The absorbance at 546 nm was measured for 4 min at 20 s intervals and the values per minute calculated. The results were presented as U μl^{-1} min^{-1} and the experiment was repeated.

4.2.6 Peroxidase assay

POD activity was measured according to the method described by Zhang *et al*., (2008) with minor modifications and 10 tubers of each variety were used for the assay. One cylinder, about 20 mm long was cut from each of the potato tubers using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder using a scalpel and discarded. The tissue cylinders were pooled and ground in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 5 % polyvinylpolypyrrolidone (wt/vol). The homogenate was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer, 1 ml guaiacol (Sigma), and 1 ml of 2 % H_2O_2 (vol/vol). The mixture was aliquoted into three portions for measurement of POD activity. In the control the tuber extract was replaced with 1 ml of sodium phosphate buffer. The absorbance at 470 nm was measured for 4 min at 20 s intervals and the values per minute calculated. The results were presented as U μl^{-1} min^{-1} and the experiment was repeated.

4.2.7 Phenylalanine ammonia lyase assay

PAL activity was assayed following the method of Okey *et al*. (1997) with minor modifications. Three 20 mm long cylinders were cut longitudinally from each of 10 tubers per variety using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder and discarded. PAL activity was measured at 3 time intervals of 0, 2 and 8 h after sectioning and one cylinder per tuber was used at each time interval. The cylinders for each time interval were pooled and homogenized in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of a buffer containing 50 mM Tris, 15 mM 2-mercaptoethanol and 5 % polyvinylpolypyrrolidone (Sigma) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was mixed with 2 ml 0.05 M borate buffer (pH 8.8) and 1 ml 0.02 M L-phenylalanine. The samples were incubated at 30˚C for 1 h. In the control the extract was replaced

with 1 ml borate buffer. The reaction was stopped by adding 0.2 ml of 6 M trichloroacetic acid (TCA). The supernatant was aliquoted into 3 portions for measurement. One activity unit was defined as a change in absorbance of 0.01 at 290 nm h^{-1} g^{-1} protein. The results are presented as µg PAL g^{-1} tissue and the experiment was repeated.

4.2.8 Total soluble phenols determination

A 5 mm diameter cork borer was used to excise two 20 mm long tissue samples from each of 10 potato tubers per variety. The potato cylinders were left at room temperature for 24 h. After 24 h, the cylinders were pooled to make a composite sample for each variety. The cylinders were ground in liquid nitrogen using a mortar and pestle, and 1 g of the tissue homogenate was mixed with 4 ml of methanol. The suspension was shaken for 1 h and centrifuged at 5,000 rpm for 10 min. The supernatant was removed, and the extraction procedure was repeated. The amount of total soluble phenols present in the extracts was determined using Folin-Ciocalteau reagent (Sigma) according to Cahill and McComb (1992). The supernatant was aliquoted into 3 portions for measurement. The concentration of total soluble phenols was calculated as g p-coumaric acid per kg tissue (Sigma) and the experiment was repeated.

4.2.9 Chlorogenic acid assay

Two 5 mm diameter cores, 20 mm long, were excised from 10 tubers per variety. The tissue samples were pooled and freeze-dried. The freeze-dried samples were assayed for chlorogenic acid, using the sodium nitrite method according to Griffiths *et al.* (1992). Standards were prepared from commercially available chlorogenic acid (Sigma). The procedure was repeated three times. The concentration of chlorogenic acid was expressed as g kg^{-1} fresh weight.

4.2.10 Statistical analyses

Data were explored for normality using the Anderson-Darling test ($P < 0.05$) and plotted for residuals using Minitab Release 12.22 (1998) to check assumptions for the Analysis of Variance (ANOVA) (the assumptions checked were independence, homogeneity and normality). The ANOVAs and Standard Errors of Difference (SED) were generated using GenStat Discovery Edition 2 (2006). All significant means were separated using the Least Significant Difference ($P < 0.05$). The error bars on the figures represent SED. The dendrogram was created using the software, Minitab Release 12.22, 1998. Hierarchical cluster analysis was performed using means of rotting zone diameters, PAL, POD, PPO, chlorogenic acid, total soluble phenols and cell viability per variety across the three pathogens evaluated. These values were used in generating the clusters using the complete linkage method and Squared Euclidian distance. The data are expressed as the average of two independent experiments.

4.3 RESULTS

4.3.1 Maceration of potato tuber tissue by bacteria and viability of cells after inoculation

There was a significant interaction ($P \leq 0.001$) of variety x subspecies at both 24 and 48 h of incubation with *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis* and *D. dadantii* (Table 2). The largest rotting zone diameter for *D. dadantii*, 24 h after inoculation was 15.8 mm, recorded in BP1. The smallest rotting zones for the same pathogen and time of incubation were recorded in M69/11, M59/20, $Mondial_{Zw}$ and Up-to-Date. After 48 h of incubation with *D. dadantii*, significantly smaller rotting zone diameters ($P < 0.05$) were recorded in Vanderplank, M69/11 and $Mondial_{Zw}$. Viability of cells 48 h after incubating tubers with *D. dadantii* ranged from 1.4% recorded for Up-to-Date and M62/51 to 81.4% recorded in M69/11 (Table 2).

Caren recorded the largest rotting zone diameter of 14.85 mm for *P. atrosepticum* at 24 h, while BP1 recorded the smallest diameter of 8.6 mm for the same pathogen and incubation time. The largest rotting zone diameter 48 h after inoculation with *P. atrosepticum* was 29.17 mm, recorded in both

Caren and Amethyst. The highest percentage of viable cells after inoculation with *P. atrosepticum* was recorded in Romano (92.6%) and the lowest value of 1.7% was recorded in M62/51.

The largest rotting zone diameter for *P. carotovorum* subsp. *brasiliensis*, 24 h after inoculation was 15.36 mm, recorded in Maradona. The smallest rotting zone for the same pathogen was recorded in BP1, which was 8.6 mm. After 48 h of incubation with *P. carotovorum* subsp. *brasiliensis*, Montclare had the largest rotting zone diameter of 28.2 mm. The smallest rotting zone was recorded in M69/11 (13.88 mm). The viability of cells inoculated with *P. carotovorum* subsp. *brasiliensis* ranged from 1.7% in Up-to-Date and Montclare to 82.3% in M69/11. *Dickeya dadantii* was the most virulent pathogen because 10 out of the 18 varieties tested recorded a cell viability of less than 10 % for *D. dadantii*, compared to 5 out of 18 and 4 out of 18 recorded for *P. atrosepicum* and *P. carotovorum* subsp. *brasiliensis,* respectively (Table 2).

4.3.2 Polyphenol oxidase assay

Significant differences in PPO enzyme activity ($P < 0.05$) were recorded across the varieties. PPO activity ranged from 1.111 U μl^{-1} min^{-1} for variety M62/51 to 1.94 U μl^{-1} min^{-1} for Vanderplank. Varieties that recorded significantly high concentrations of PPO ($P < 0.05$) were Pentland Dell, Vanderplank, M69/11, Romano, $Mondial_{Zw}$ and M59/20 (Fig. 1). PPO increased significantly ($P < 0.05$) in wounded and inoculated tubers of M69/11, Romano, Amethyst and M62/51 compared to the healthy (Table 3). PPO significantly correlated with rotting zone diameters at 24 h ($r = -0.624$, $P < 0.05$), 48 h ($r = -0.75$, $P < 0.01$) and viability of cell ($r = -.081$, $P < 0.05$) (Table 4). In addition PPO also significantly correlated with chlorogenic acid ($r = -0.654$, $P < 0.01$) and total soluble phenols ($r = -0.855$, $P < 0.01$). However PPO did not significantly correlate with PAL and POD.

4.3.3 Peroxidase assay

There was no significant difference in POD activity in healthy tubers of all cultivars. However POD activity increased significantly ($P < 0.05$) in wounded and inoculated tubers of Romano, Amethyst, M69/11 and M62/51 (Table 3). POD was significantly ($P < 0.05$) correlated to viability of cell ($r = -0.534$, $P < 0.05$ (Table 4).

4.3.4 Phenylalanine ammonia lyase assay

Enzyme activity of PAL increased significantly ($P < 0.05$) with time and the highest enzyme concentrations in all varieties were recorded at 8 hours after sectioning (Fig. 2). At the initial stage (0 h after sectioning), M59/20 recorded the highest enzyme activity of 1.716 µg g^{-1}. This value remained unchanged 2 hours after cutting the tubers. The lowest value recorded immediately after cutting the tubers was 1.115 µg g^{-1} in Amethyst. At 2 h after sectioning the tubers, the highest significant enzyme activity ($P < 0.05$) was recorded in $Mondial_{Zw}$ and Montclare, and the lowest in Up-to-Date, Amethyst and BP1. At 8 hours the highest enzyme activity was recorded in Montclare, which also showed the highest increase in enzyme activity over time (Fig. 2). PAL increased significantly ($P < 0.05$) in wounded and inoculated tubers of M69/11, Romano, Amethyst and M62/51 compared to the healthy (Table 4). Neither PAL at 0 h nor PAL at 2 h was significantly correlated with rotting zone diameters after 24 and 48 h after inoculation. However PAL at 8 h was significantly correlated with rotting zone diameter after 48 h ($r = -0.536$, $P < 0.05$ and viability ($r = -0.694$, $P < 0.05$) (Table 3).

4.3.5 Total soluble phenols and chlorogenic acid assays

The highest concentrations of total soluble phenols in the Zimbabwean varieties were recorded in M62/51, M69/18 and Montclare while Amethyst and Maradona recorded the lowest (Fig. 3A). A significantly high ($P < 0.05$) concentration of chlorogenic acid was recorded in Up-to-Date and the lowest was recorded in Buffelspoort, BP1 and Caren, for varieties grown in South Africa. The highest concentration of total soluble phenols was recorded in Up-to-Date, while BP1 contained the lowest

concentration of the same compound (Fig. 3A). Total soluble phenols significantly correlated with rotting zone diameters after 24 h ($r = -0.791$, $P < 0.05$), after 48 h ($r = -0.648$, $P < 0.05$) and viability ($r = -0.947$, $P < 0.01$) (Table 4).

4.3.6 Chlorogenic acid

For the varieties grown in Zimbabwe, the highest concentrations of chlorogenic acid were recorded in M59/20, M69/11, Mondial and Romano while the lowest were recorded in Amethyst and Maradona. As for the cultivars grown in South Africa, Vanderplank and Pentland Dell recorded significantly high concentrations of chlorogenic acid and the lowest was recorded in BP1. Chlorogenic acid significantly correlated with rotting zone diameter after 24 h ($r = -0.68$, $P < 0.01$), 48 h ($r = -0.600$, $P < 0.01$) and viability ($r = -0.579$, $P < 0.05$) (Table 4).

4.3.7 Cluster analysis

The varieties were divided into 5 clusters according to tolerance and susceptibility to the three pathogens. The resistance / susceptibility levels were based on the rotting zone diameters that developed after 24 and 48 h after inoculation and cell viability 48 h after inoculation. The varieties were divided into 5 clusters. Vanderplank, M69/11, Romano, M59/20, $Mondial_{Zw}$ and Pentland Dell were grouped in cluster 1, which is composed of resistant varieties (Fig. 4). These varieties recorded rotting zone diameters of less than 20 mm for the three pathogens 48 h after incubation and viability of cells was more than 70% (Table 2). The members of cluster 1 also recorded significantly higher concentrations of PPO, PAL, chlorogenic acid and total soluble phenols (Figs. 1, 2, and 3A and B). $Mondial_{SA}$, Maradona and Buffelspoort fell in cluster 2, while M69/18, Montclare, 157/91 and 157/79/91 were grouped in cluster 3. Clusters 2 and 3 contained varieties with intermediate resistance. BP1 and Amethyst fell into cluster 4. Cluster 5 contained Caren, Up-to-Date and M62/51. Varieties in clusters 4 and 5, with the exception of Up-to-Date, also recorded significantly lower levels of PPO, PAL, chlorogenic acid and total soluble phenols (Figs. 1, 2, and 3A and B) and were highly

susceptible to the three pathogens. These varieties recorded significantly the largest ($P < 0.05$) rotting zone diameters and the lowest viability (less than 10 %) (Table 2, Fig. 4). Mondial, a variety grown in both countries fell into two different classes. $Mondial_{Zw}$ fell into cluster 1 resistant varieties), while $Mondial_{SA}$ fell into cluster 2 (intermediate resistance). $Mondial_{Zw}$ recorded significantly higher ($P < 0.05$) PPO and PAL activity than $Mondial_{SA}$. Viability of cells as well as concentrations of chlorogenic acid and total soluble phenols was notably higher in $Mondial_{Zw}$.

4.4 DISCUSSION

In our study tuber resistance, expressed as rotting zone diameter and viability of cells after inoculating tubers with *Dd, Pa* and *Pcb*, was determined in 7 and 11 potato varieties grown in South Africa and Zimbabwe, respectively. Our results clearly show that there is a wide range of susceptibility to soft rot in potato varieties currently grown in both countries. These measurements do, however, have some limitations, since the inoculation method challenges the pith directly and bypasses the periderm, which is a physical barrier to entry of pathogens. However, other researchers found that immersing mechanically harvested tubers in a suspension of *P. atrosepticum* and injecting bacterial suspension into the tubers had no effect on the ranking of clones for susceptibility (Tzeng *et al.*, 1990). This may suggest that the periderm does not play a major role in determining resistance to tuber soft rot (Peltzer & Sivasithaparam 1985).

Laboratory assessment for tolerance must be linked with field data and the use of varieties of known susceptibility is therefore essential. In the field varieties such as Up-to-Date and Amethyst are considered susceptible to many diseases and in storage they also tend to rot at a faster rate than others (unpublished data) this is the main reason why they were included in the study. The mean rotting zone diameters of these varieties was more than 20 mm and the viability of cells after inoculation was less than 10 %, compared to no rotting and 100% cell viability recorded for the controls, thus confirming these field observations. Romano and M59/20, considered by growers to be

tolerant to most diseases, had mean rotting zone diameters of less than 20 mm and cell viability above 70 % compared to no rotting and cell viability of 100 % recorded for the controls. However, clear cut classes of resistance and susceptibility could not be established, confirming findings of other researchers who observed similar trends in their evaluation of susceptibility of different potato varieties to soft rot (Allefs *et al.*, 1995; Lapwood and Read 1985). The virulence of the three pathogens evaluated was different. *D. dadantii* was the most virulent pathogen and this could be explained by the fact that it produces five major and at least four secondary pectate lyase (Pels), whereas *P.carotovorum* subsp. *brasiliensis* and *P. atrosepticum* secrete four and three major Pels respectively. The secondary Pels are only produced *in planta*, have low enzymatic activity and are important in host specificity (Kelemu and Collmer, 1993). Although the production of pectinases is essential for pathogenicity, not all isoenzymes are required in all situations. This phenomenon could also explain the response of BP1 to the different pathogens evaluated in the experiment. *D. dadantii* was more virulent on BP1 compared to the other pathogens.

The difference in the behaviour of Mondial grown in the two countries could be attributed to macro- and micro-environmental variation and different agronomic practices between the two countries. The significant effect of the environment and agronomic practices on tuber tolerance to soft rots was reported by Wegener (2002) and Kumar *et al.* (1991), respectively. Contradictory results on ranking resistance of tubers to soft rot were also reported by Bourne *et al.* (1981) and Lapwood and Read (1985) when they evaluated the variety Desiree grown under different environmental conditions.

Metabolic changes in plants resulting from wounding or pathogen invasion have been reported in numerous host-pathogen systems (Okey *et al.*, 1997). In this study, enzyme activities of PPO, POD and PAL as well as concentrations of chlorogenic acid and total soluble phenols were found to differ significantly in resistant and susceptible varieties. Activities of these three enzymes also increased significantly in wounded and inoculated tubers. The potato varieties differed in three major aspects.

First, PPO activity was notably higher in the varieties that also exhibited high PAL activity (except Montclare). Secondly, PAL activity in all varieties increased significantly 8 h after cutting the tubers and after inoculating with *Pcb*. Other researchers have also recorded peaks in enzyme activity around 4 and 8 h after wounding or inoculating plants (Pereira *et al.*, 1999; Zhang *et al.*, 2008) and this was followed by a sharp drop in enzyme activity around 10 h (Ryan, 1988). This is because the accumulation of phenolic compounds occurs before polymerization thereof by peroxidase into lignin (Pereira *et al.*, 1999). Thirdly, concentrations of total soluble phenols and chlorogenic acid were considerably higher in some of the varieties. The results confirm the findings of Lewis *et al.* (1998), who recorded different concentrations of chlorogenic acid in different varieties. They also reported that chlorogenic acid was more concentrated in the tuber tissues than in the rest of the plant.

It was interesting to note that potato varieties that showed enhanced PPO activity also recorded a higher concentration of total soluble phenols and chlorogenic acid. These varieties (except Up-to-Date) exhibited on average higher tolerance to soft rot. After 48 h the extent of rotting was less in some of the varieties tested, particularly those with higher PPO activity. Of the 18 varieties tested, Pentland Dell, Mondial$_{ZW}$, Romano, Vanderplank, M59/20 and M69/11, with high concentrations of PPO, chlorogenic acid and total soluble phenols in tuber tissue were most resistant. These resistant varieties also had the highest number of viable cells and lowest extent of decay at 24 and 48 h after inoculation with the three pathogens. It appears that these resistant varieties produce more secondary metabolites involved in plant defense mechanisms than the other varieties tested. However, there are numerous components involved in tolerance to soft rot pathogens (Lyon and McGill, 1989) and each disease interaction can rarely be generalized as representative of host defense response in diverse plant species (Hammerschmidt *et al.*, 1982; Nicholson and Hammerschmidt, 1992). This was demonstrated by Up-to-Date, which was susceptible and prone to maceration by pectolytic enzymes despite having a high concentration of total soluble phenols and chlorogenic acid.

Total soluble phenols together with PPO appear to play a role in tolerance to soft rot since these compounds were present in considerably higher levels in tuber tissue of resistant varieties. This is supported by the work of Li and Steffens (2002), who reported that an over-expression of PPO coincided with a 10-fold increase in PPO enzyme activity, which led to enhanced bacterial disease resistance in transgenic tomatoes. Thipathi and Verma (1975) and Kumar *et al*. (1991) also observed that resistant potato cultivars demonstrated a high PPO activity and contained large amounts of phenolic compounds. PPO is important in the initial stage of plant defense where membrane damage causes release of phenols, such as chlorogenic acid. Phenol lyases catalyse the oxidation of these compounds. Phenols and their oxidative products were shown to inhibit *P. carotovorum* (Ghaneka *et al*., 1984; Lyon and McGill, 1989; Weber *et al*., 1996) and their cell wall-degrading enzyme activity of the bacteria (Lyon *et al*., 1992; Lyon and McGill, 1989). Phenolic compounds also function as precursors in the synthesis of lignin (Lyon *et al*., 1992) and suberin (Eslelie *et al*., 1986; Riley and Kolattukudy, 1975), which are involved in the formation of physical barriers that can block the spread of *Pectobacterium* spp. (Fox *et al*., 1971). In addition, PPO is known to catalyze the oxidation of phenolics to free radicals, which can react with biological molecules, thus creating an unfavorable environment for pathogen development (Jockusch, 1966). Phenolic compounds and PPO alone do not guarantee resistance against *Pectobacterium*, as can be seen in Montclare, which was severely macerated by the pathogen despite having a high concentration of total soluble phenols. This result supports the findings of Lojkowska and Holubowska (1992), who reported a low level of tolerance in Polish potato cultivars that had a high content of phenolic compounds and PPO. High densities of pectobacteria can inhibit and reverse phenol oxidation (Lovrekovich *et al*., 1967).

PPO, POD and PAL activity increased significantly in wounded and inoculated potato tubers. This is supported by the findings of Okey *et al*., (1997), who reported significantly high enzyme activities in inoculated and wounded cocoa clones. The researchers also reported that clones with higher enzyme activities exhibited resistance to *Phytophthora palmivora* infection. The increase in POD activity was not surprising because POD is involved in the oxidative polymerization of hydroxycinnamyl alcohols to

yield lignin (Vance and Sherwood, 1980) and crosslinking isodityrosine bridges in cell walls (Fry, 1982). These compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms.

PAL through its formation of cinnamic acid, an important compound in the biosynthesis of phenolics and lignin, also plays a role in plant defense systems (Fukasawa-Akada *et al.*, 1996). For PAL to be effective it must be available in potato tuber tissues in high concentrations at the initial stages of infection. This was shown in Montclare, which was severely macerated by pectolytic enzymes despite the fact that it had the highest increase in enzyme activity 8 h after cutting the tuber. The slow buildup of PAL allows the bacteria to multiply within host tissues, and reach the critical population density required for the production of pectic enzymes.

The significant correlation of viability with PPO, POD, PAL after 8 h of wounding, chlorogenic acid and total soluble phenols reflects the importance of the enzyme in resistance expression. However these findings contradict the findings of Lojkowska and Holubowska (1992), who reported that there was no correlation between soft rot resistance and enzyme activities. The difference in response to soft rot resistance between Polish and Zimbabwean or South African cultivars could be due to differences in the conditions under which the experiments were carried out. Factors such physiological state, size and maturity of the tubers can affect the response of the tubers to pathogen infection (Marquez-Villavicencio et al., 2011). These researchers also reported that soft rot susceptibility could be correlated with tuber weight, small tubers being more resistant to soft rot. They also observed differences in responses among tubers of the same cultivar harvested from different fields.

The importance of PPO, POD, chlorogenic acid and total soluble phenols in defense mechanisms against *Pectobacterium* cannot be underestimated. The cluster analysis performed on all tested variables grouped the varieties into three categories namely resistant, intermediate and susceptible, confirming field observations. All the varieties that fell in the resistant group had high enzyme activity,

while those in the susceptible category had low activity. These experiments clearly show a positive correlation between amounts of PPO, POD, PAL and the level of degradation by pectic enzymes.

The findings indicate that PPO, POD, PAL, chlorogenic acid and total soluble phenols play a role in disease resistance against *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis* and *D. dadantii.* Potato varieties with a high content of these compounds in tuber tissue can exhibit tolerance to pathogen attack. Of the varieties tested, Vanderplank and Pentland Dell are the South African varieties that are most resistant to soft rot bacteria, while Caren and Up-to-Date are the most susceptible. Zimbabwean varieties M69/11, Romano and M59/20 were identified as the most resistant, while Montclare and M62/51 were the most susceptible.

4.5 REFERENCES

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Table 4.1. Potato varieties used in the study

Variety	Country where grown
Bp[x]	South Africa
Buffespoort[x]	South Africa
Caren[x]	South Africa
Mondial[x]	South Africa
Pentland Dell[x]	South Africa
Up-to-Date[x]	South Africa
Vanderplank[x]	South Africa
157/79/91[y]	Zimbabwe
157/91[y]	Zimbabwe
Amethyst[x]	Zimbabwe
M59/20[y]	Zimbabwe
M6/18[y]	Zimbabwe
M62/51[y]	Zimbabwe
M69/11[y]	Zimbabwe
Maradona[x]	Zimbabwe
Mondial[z]	Zimbabwe
Montclare[x]	Zimbabwe
Romano[x]	Zimbabwe

[x] Cultivated commercially, originated from Holland

[y] New release, bred in Zimbabwe

[z] Experimental, imported from South Africa

Table 4.2. Viability of cells and rotting zone diameter (mm) after incubating at 20˚Cpotato half tubers inoculated with *Pectobacterium* species and *Dickeya dadanti*

		Dickeya dadantii			*Pectobacterium atrosepticum*			*P. carotovorum subsp. brasiliensis*		
Country	Variety	Extent of Decay (mm)		Viability	Extent of Decay (mm)		Viability	Extent of Decay (mm)		Viability
		24h	48h	(%)	24h	48h	(%)	24h	48h	(%)
South African varieties	BP 1	15.80e	25.47d	2.00a	8.60a	16.73b	44.00b	8.60a	19.03c	58.50b
	Buffelspoort	14.13d	24.93c	3.10a	14.05e	19.00c	55.10c	14.05e	16.57b	43.90b
	Caren	14.60d	28.83d	1.60a	14.85f	29.17f	7.10a	14.85f	25.07e	2.90a
	MondialSA	12.05b	17.23b	32.50b	12.40d	18.50c	54.30c	12.40e	12.65d	68.20c
	Pentland Dell	12.27b	18.23d	75.50b	12.65d	18.50c	75.50d	12.65d	16.67b	79.80d
	Up-to-Date	10.80a	28.67d	1.40a	11.95c	28.43d	2.90a	11.95c	22.40e	1.70a
	Vanderplank	13.70c	15.93a	79.80c	10.10b	18.93c	76.40d	10.10b	15.67a	76.10d
	157/79/91	13.47d	26.43c	5.80a	11.88c	20.47d	29.70a	12.81d	22.51d	50.40b
	157/91	13.47d	26.43c	5.80a	11.88c	20.47d	28.80a	12.18d	21.08d	46.80b
	Amethyst	14.60e	28.83d	1.80a	14.00e	29.17f	9.40b	14.85f	10.07a	2.80a
	M59/20	8.96a	17.60b	79.50c	8.80a	11.24a	87.30d	8.96a	14.00a	70.60d
	M62/51	10.80b	28.67d	1.40a	12.40d	28.43f	1.70a	11.95c	22.40d	12.50a
Zimbabwean varieties	M69/11	9.36a	14.72a	81.40c	9.36a	12.44a	90.90d	10.92b	13.88a	82.30d
	M69/18	12.05c	28.23d	4.10a	11.40c	18.50c	33.80b	11.40b	15.65b	62.60c
	Maradona	13.84d	17.60b	1.50a	10.20b	12.68a	52.10c	15.36f	20.44c	52.10b
	MondialZw	9.08a	13.68a	79.20b	10.12b	12.68a	88.60d	9.32a	13.93a	73.20d
	Montclare	13.25d	29.80d	1.50a	12.50d	23.52e	8.10a	13.20c	28.20e	1.70a
	Romano	10.36a	17.08b	81.00c	10.36b	12.32a	92.60d	12.36d	16.08b	80.90d
	P Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	SED	0.286	1.174	15.22	0.386	0.678	15.22	0.289	0.479	15.22
	LSD (0.05)	0.579	2.348	30.59	0.772	1.346	30.59	0.590	0.952	30.59

[y] Data are expressed as the average of two independent experiments

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

Table 4.3. Polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidise (POD) activity in health, wounded and inoculated tuber tissues[z]

Variety	PPO activity (A_{546}) nm min^{-1} g^{-1} fresh mass			POD activity (A_{470}) nm U min^{-1} μl^{-1}			PAL activity (A_{290}) nm cinnamic acid min^{-1} g^{-1} fresh mass		
	Healthy	Wounded	Inoculated	Healthy	Wounded	Inoculated	Healthy	Wounded	Inoculated
M69/11	1.852	2.154	2.209	0.451	0.979	1.200	1.521	1.975	2.133
Romano	1.810	2.218	2.553	0.481	0.832	0.895	1.450	1.785	1.906
Amethyst	1.120	1.500	1.556	0.342	0.773	0.772	1.115	1.612	1.632
M62/51	1.113	1.497	1.624	0.265	0.567	0.662	1.162	1.599	1.674

LSD ($P < 0.05$) cultivar x treatment 0.380. Values are means of 3 replicates. Assessments were made 8 h after wounding or inoculation

Table 4.4. Correleration coefficients for the enzyme activities and the extension of rotting on tuber halves (first two rows) and viability of cells in the tuber slices (third row)[y]

Assay	PAL 0 h	PAL 2 h	PAL 8 h	PPO	POD	Chlorogenic acid	Total soluble phenols
After 24 h	-0.261^{ns}	-0.351^{ns}	-0.391^{ns}	-0.75^{**}	-0.197^{ns}	-0.68^{**}	-0.791^{*}
After 48 h	-0.201^{ns}	-0.291^{ns}	-0.536^{*}	-0.624^{*}	-0.435^{ns}	-0.600^{**}	-0.648^{*}
Cell viability	-0.283^{ns}	-0.54^{ns}	-0.694^{*}	-0.817^{*}	-0.534^{*}	-0.579^{*}	-0.947^{**}

[y]PAL = phenylalanine ammonia lyase; PPO = polyphenol oxidase; POD = peroxidase

[z] * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; ns = not significant

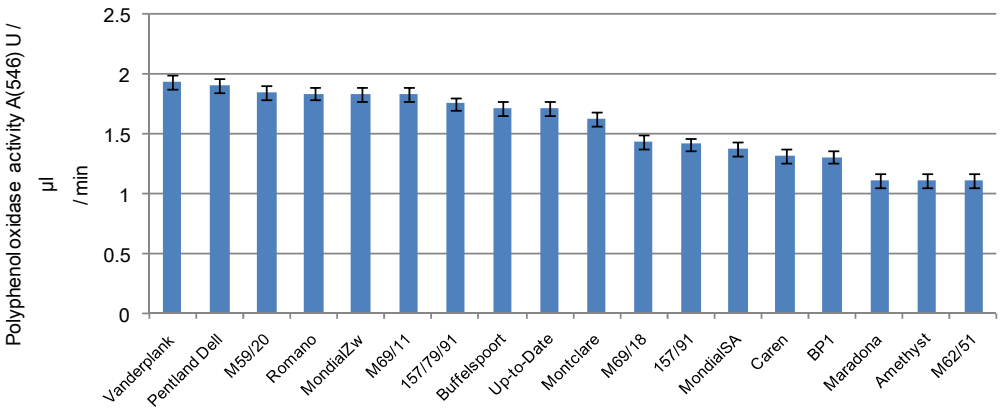


Figure 4.1. PPO activity measured in tuber tissue of the various cultivars. The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.

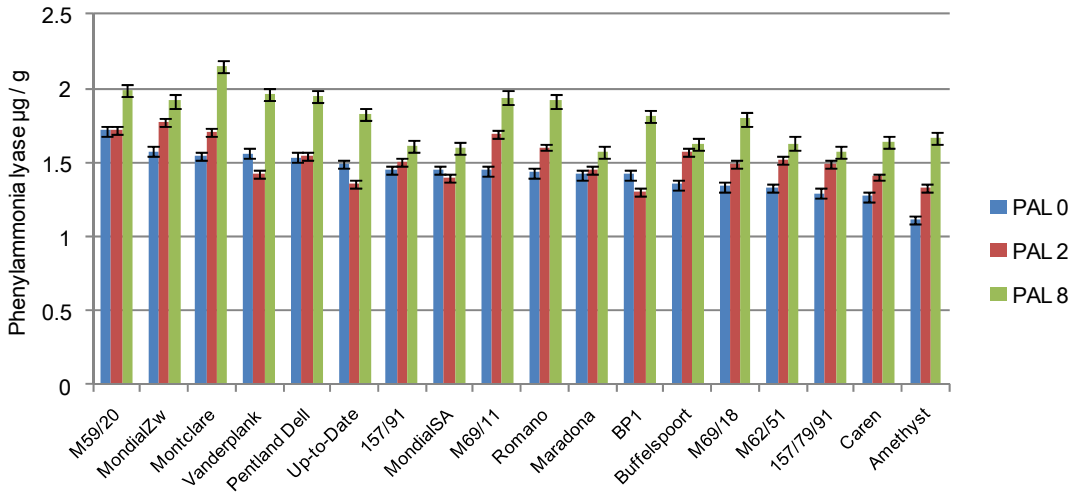
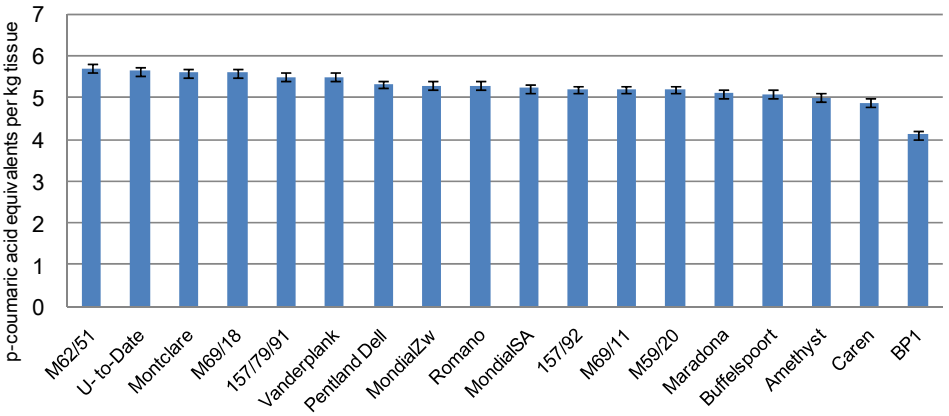
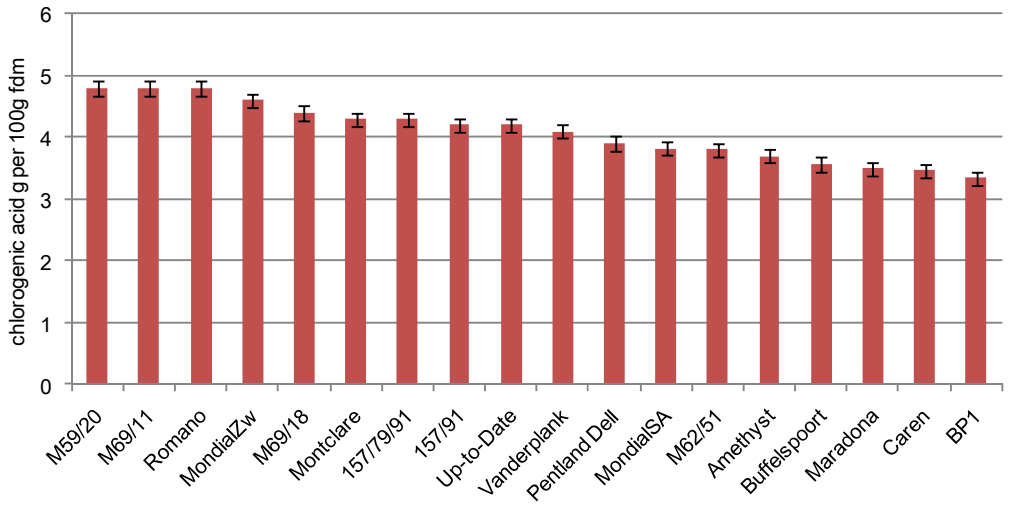


Figure 4.2. PAL activity measured in tuber tissue of the various cultivars at 0, 2 and 8 h after sectioning the tubers. The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.



A



B

Figure 4.3. Concentration of total soluble phenols presented as p-coumaric acid equivalents per kg tissue (A) chlorogenic acid (B) 24 hrs after sectioning the tubers of the different cultivars ($P < 0.05$). The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.

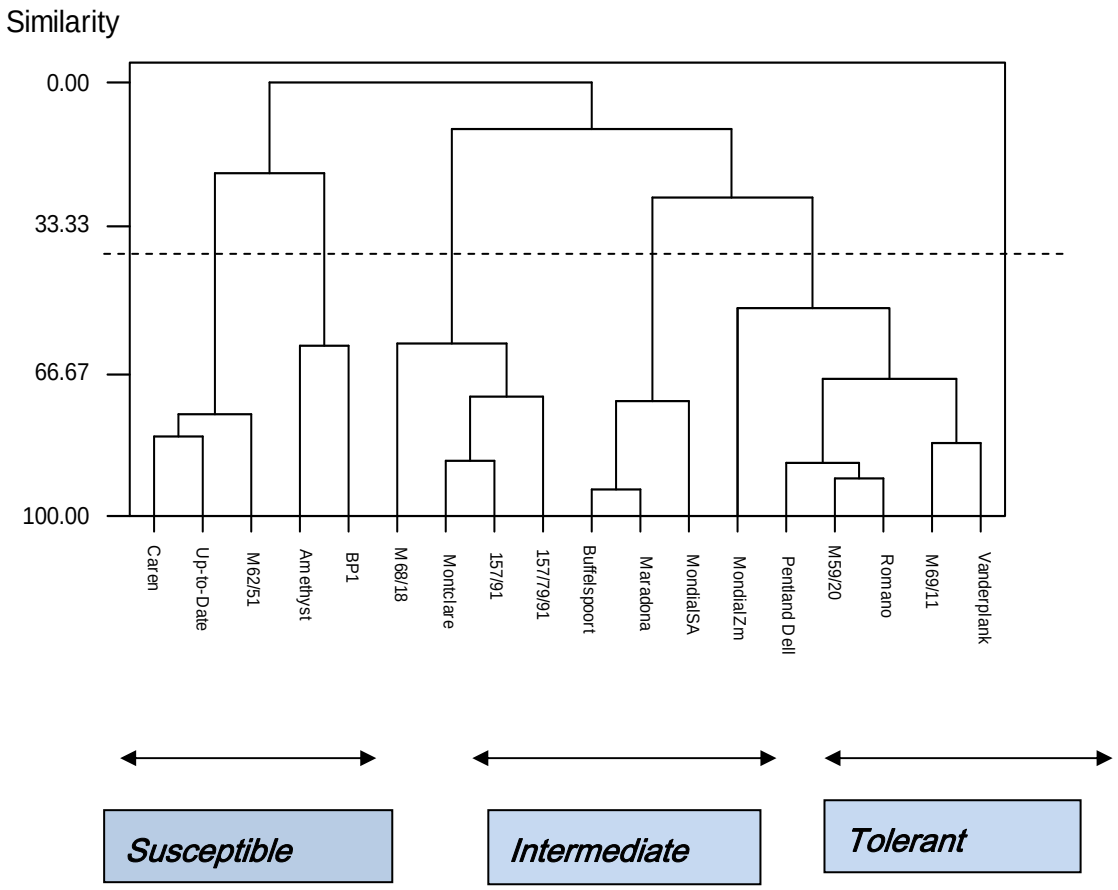


Figure 4.4. Dendrogram for hierarchical clustering of 18 potato cultivars. The dendrogram was created using the software, Minitab Release 12.22, 1998. The dendrogram was cut at the 57.05 % similarity level, producing five subgroups. The data from means of rotting zone diameters, phenylalanine ammonia lyase, peroxidise, polyphenol oxidase, chlorogenic acid, total soluble phenols and cell viability were used in the generation of the dendrogram. The data are expressed as the average of two independent experiments with three replicates.