CHAPTER 7

RELATIONSHIP BETWEEN CALCIUM NUTRITION, TISSUE CALCIUM CONTENT AND SUSCEPTIBILITY OF SUNFLOWER TO *SCLEROTINIA MINOR* WILT.

7.1 Summary

Calcium has been implicated as a factor in the resistance of plant tissue to soft rot diseases. Experiments were made to test whether differences in calcium levels within and between sunflower lines were responsible for differences in incidence of wilt caused by *Sclerotinia minor*. Growth of *S. minor* on agar media was not affected by calcium concentrations over the range 125-5000μM although at higher concentrations harvested mycelia was heavier. This was attributed to the formation and accumulation of insoluble crystals, most probably calcium oxalate, that adhered to the hyphae.

Sunflower were grown in both sand and solution culture and supplied with nutrient solutions differing in calcium concentration. Increasing concentrations of calcium chloride increased levels of tissue calcium but reduced resistance to *S. minor* as assessed by the rate at which lesions developed. At 10 and 20mM concentrations lesions developed on stems in advance of fungal hyphae. This was attributed to enhanced sensitivity of the tissue to toxins released by the fungus. Other parameters of plant growth such as height, leaf number, and root and stem base dry weight were not significantly affected ($P>0.05$) by calcium chloride in the range 0.125 to 20mM.

In contrast, when seedlings were supplied with calcium nitrate the increased tissue calcium levels were associated with an increase in resistance to *S. minor*. This effect was repeatable using sunflower hybrids and inbreds. In direct comparisons with calcium chloride fed plants it was found that lesions on nitrate-fed plants tended to darken and cease elongating sooner while on chloride-fed plants lesions remained pale and elongated longer. It is speculated that this effect may have been due inhibition of polyphenol oxidases by the oxalate and chloride anions.

Susceptibility to *Sclerotinia minor* was also affected by growing plants in solutions containing other members of the alkaline earth group. Barium at the concentration used was toxic to seedlings. Seedlings of the two sunflower hybrids being tested showed differential tolerance to strontium. Seedlings of Suncross 40R were stunted while

seedlings of Hysun 44 were not adversely affected. Plants supplied with strontium were more resistant, in terms of lesion size, than plants supplied with calcium which, in turn, were more resistant than plants supplied with magnesium.

No correlation was found between calcium content and disease incidence in field grown plots of eight inbred sunflower lines. Nor were correlations found for sodium, magnesium, or sulphur. However, there were significant positive correlations between phosphate content of roots and the susceptibility of the eight inbred lines at both field sites. Addition of superphosphate in another two trials increased disease incidence but addition of diammonium phosphate, which was used at one site only, did not.

7.2 Introduction

Calcium is one of the commonest elements in the earth's crust. Depending on the mineralogy and ontogeny of different soils they may provide plants with different levels of available calcium. This is important because calcium has a number of very important physiological functions in living cells. The calcium cation (Ca^{2+}) forms a large number of stable complexes with organic molecules due to its ionic radius (99 picometers), divalent nature and presence of an outer '*d*' orbital (Hepler and Wayne, 1985). The majority of calcium is bound by electrostatic bridges to component molecules of the middle lamella and cell wall or sequestered in organelles (Clarkson, 1984). In the middle lamella, calcium ions bind with the carboxyl groups of nonesterfied galacturonosyl residues in neighbouring pectic chains to form stable and rigid cross-linking.

Calcium is essential for cell membrane stability. Membrane fluidity is reduced as Ca^{2+} forms bridges between neighbouring anionic phospholipids or between these and cytoskeletal elements as well as binding to and compressing gycerolipids (Leshem, 1992). In contrast to the membrane levels of calcium in the 10^{-3}M range the cystolic concentrations are maintained at low levels (10^{-6} - 10^{-7}M) in order to prevent the chelation of inorganic phosphate moieties (Roux and Slocum, 1982). Maintenance of this gradient requires an active process that has been examined as a possible driving force for signal transduction across the cell membrane. The cystolic protein calmodulin acts as a Ca^{2+} sensor and readily binds with up to four ions. The Ca^{2+}:calmodulin complex can then activate a number of enzymes including Ca^{2+}-dependent protein kinase C, Quinate:NAD oxido-reductase, phospholipase A_2, Ca^{2+}-ATPase and others (Leshem, 1992). Calcium may also act as a messenger in the action of auxin, gibberellin and cytokinin plant hormones (Elliott, 1986).

Calcium has also been implicated in the delay of senescence of plant tissues. This activity has been attributed to the role of calcium in regulating ethylene production and responses to abscisic acid in plants (Yang and Hoffman, 1984; Owen, Hetherington and Wellburn, 1987). The Ca^{2+}:calmodulin complex has been implicated in the regulation of the activity of abscisic acid (De Silva, Cox, Hetherington and Mansfield, 1985; Bailly, Corbineau and Côme, 1992). The activity of methyl jasmonate, which has recently gained recognition as being a plant hormone or growth regulator controlling senescence, has been shown to influenced by the presence of calcium (Bailly *et al.*, 1992).

The importance of calcium in normal cellular physiology suggests that it should also have an important influence on the reaction of plants to disease. Calcium is involved in the synthesis of structures that decrease pathogen penetration. For example, the synthesis of callose in soybean cells is a calcium dependent process (Kohle, Jeblick, Poten, Blaschek and Kauss, 1985). Calcium can enhance plasmalemma stability and reduce the effect of toxins that act to destabilise it as measured by increased permeability (Doupnik, 1968). The degree of calcium cross-bridges between pectic molecules in the middle lamella affects its susceptibility to maceration through the action of hydrolytic enzymes. Calcium may be involved in the elicitation of phytoalexins. Application of Ca^{2+} antagonists or calcium-free media indicate that calcium may have a regulatory role in the expression of the phytoalexins rishitin and lubimin in potato (Zook, Rush and Kuc, 1987) and glyceollin in soybean (Stäb and Ebel, 1987).

Two previous studies have suggested a role for calcium in influencing the susceptibility of sunflower to *Sclerotinia sclerotiorum*. Orellana, Foy and Fleming (1975) showed that alleviation of aluminium toxicity by addition of calcium carbonate reduced the susceptibility of sunflower. The effect of calcium nutrition in reducing leaf symptoms produced by *S. sclerotiorum* on sunflower and pumpkin (*Cucurbita pepo* L.) have been reported (Chrominski, Abia and Smith, 1987). The experiments reported in this chapter were initiated to test the effect of calcium nutrition on the susceptibility of sunflower to sclerotinia wilt caused by *S. minor* and whether the differences in susceptibility of sunflower lines could be related to differences in calcium content.

7.3 Experimental

7.31 General Materials and method

i. Estimation of tissue calcium.

In several of the subsequent experiments the level of calcium in sunflower tissue was

estimated using atomic absorption spectrophotometry (A.A.S.) with a Perkin Elmer Model 290 A.A.S. using an air-acetylene flame to volatilise the samples. An Applied Research Labs Model 3560B I.C.P. Analyser was also used when it became available late in this study for tissue analysis by inductively coupled plasma atomic emission spectrometry (I.C.P.) in an argon plasma. For both these analyses it was necessary to wet-ash or digest tissue.

Plant material was oven dried ($80^{o}C$) for 24h and weighed for dry weight determinations. The material was then ground in a mill to pass through a 1mm aperture screen. The tissues were then returned to the oven for 2h to remove any absorbed moisture before duplicate fractions were weighed and digested using a modification of the sealed chamber digestion protocol published by Anderson and Henderson (1986). It is important to follow this protocol closely as the combination of high temperature, pressure, presence of strong oxidants and organic matter can be explosive.

The wet-ashings (digestions) were performed as follows.

1. Place 0.2g of ground tissue (less used if insufficient tissue) in a 30ml polycarbonate tube with polypropylene screw cap closure,
2. add 2ml of a 7:3(v/v) mixture of perchloric acid ($HClO_4$) and hydrogen peroxide solution (30% H_2O_2), lightly cap and predigest at ambient temperature for 2h,
3. add 1ml hydrogen peroxide solution (30% H_2O_2), tightly cap and place in an oven with fan-forced heat distribution set at $80^{o}C$ for 30min,
4. remove from the oven and allow to cool before opening,
5. add 1ml of hydrogen peroxide solution and digest for another hour
6. solution should be clear or light straw colour, if not, continue to add hydrogen peroxide solution in 1ml aliquots at 30min intervals and continue digestion,
7. and make volume up to 25mL with deionised water.

For A.A.S. the digests were diluted with lanthanum oxide (La_2O_3) stock solution to a final 1% lanthanum concentration in order to reduce interference from phosphates and other anions with which calcium forms stable complexes. Lanthanum was also added at equal concentration to the calcium standard solutions. Atomic absorption spectrometry was performed as suggested by Perkin Elmer. For I.C.P. analysis aliquots of the digests were diluted with 1% HNO_3. Samples were then supplied to the Department of Agronomy, University of New England for analysis.

Estimated elemental concentrations were calculated from the mean of the duplicate digests of each sample and are expressed as $g.kg^{-1}$ tissue dry weight.

ii Sand culture and wilt screening of sunflower

The support medium of coarse river sand was prepared by washing with town water to remove silt, soaking in 0.5M HCl for 6h to dissolve any insoluble calcium aggregates, washing with distilled water until the pH of the wash water reached 6.0, draining and drying. The support medium was then dispensed into 10cm diameter plastic pots. Two seeds of hybrid sunflower were sown 2cm deep in the centre of each pot. This was thinned back to a single seedling per pot after emergence. The sunflower hybrid used, Hysun 44 which was chosen because, (i) of the ready availability of seed, ii) it was a single-cross hybrid between two highly inbred sunflower lines (*pers. comm.*, Mr Alan Scott, Pacific Seeds Pty Ltd), (iii) it was uniform in growth and disease reaction, and (iv) since it is moderately susceptible to sunflower wilt it should have been possible to detect both increases and decreases in resistance due to differences in calcium nutrition The pots were watered with distilled water each day until seedling emergence when nutrient feeding commenced. Hoagland's solutions with iron added as the chelate were prepared as outlined in Table 7.1 and applied by totally immersing pots in the solutions for 15 minutes and then returning to the glasshouse bench to drain.

Treatments were arranged in a completely randomised design on a glasshouse bench. Depending on the experiment, a proportion of plants in each treatment were inoculated while others were taken for growth assessments and calcium analysis. The pot base method was used to inoculate plants at 34 days after sowing. Disease susceptibility was determined by assessing the proportion of plants killed, the time taken for lesion appearance at the stem base and the linear rate of lesion extension. The roots of plants that were harvested for calcium determinations were freed of sand with tap water, then rinsed with 0.05M magnesium sulphate and finally with distilled water. Seedlings were partitioned into root and stem portions, dried at 80°C overnight, dry weights taken and then prepared for calcium determination.

iii Solution culture and wilt screening of sunflower

Solution culture was also used as a means of controlling the availability of nutrients to the plants. All solution culture experiments were conducted in the following manner. Individual culture vessels consisted of 1 litre round plastic food containers used as the nutrient solution reservoirs. These containers were first painted black to prevent light penetration then with a surface coat of silver to help reflect light and reduce heating. The plant platform placed on top of the reservoir consisted of a circular piece of polystyrene bead board of slightly larger diameter than the solution reservoir. In this were cut 6 x 1.5cm holes equidistance apart and 3cm from the circumference. A plastic tube inserted through a central hole was connected to a supply of filtered air which was bubbled through the solution.

Table 7.1 Composition of nutrient solutions used to evaluate of the effect of calcium concentration on susceptibility of sunflower to *Sclerotinia minor*.

	Stock	Nutrients added ($ml.L^{-1}$)					
	Conc.	Ca^{2+} Concentration					
	(H_2O)	125µM	625µM	1250µM	2.5mM	5.0mM	5.0mM
KNO_3	1M	5	5	5	5	5	5
$MgSO_4.7H_2O$	1M	2	2	2	2	2	2
KH_2PO_4	1M	1	1	1	1	1	1
$Ca(NO_3).4H_2O$	1M	0.125	.625	1.25	2.5	5	
$CaCl_2.2H_2O$	1M						5
$NaNO_3$	1M	9.75	8.75	7.5	5	0	10
$Na_2FeE.D.T.A.$	100mM	1	1	1	1	1	1
Micronutrients[1]		1	1	1	1	1	1

1. Micronutrient stock solution consisting of: 2.86g H_3BO_4, 1.81g $MnCl_2.4H_2O$, 0.1g $ZnCl_2$, 0.05g $CuCl_2$, 0.02g $Na_2MoO_4.2H_2O$ $.L^{-1}$ deionised water

Sunflower seedlings were usually raised in trays of sand in the glasshouse. Seedlings were washed from the sand once the cotyledons had fully expanded and the hypocotyls were 3-4cm long. These were then given a final rinse in distilled water before the roots of seedlings were inserted through the holes in the plant platforms. Seedlings were secured in place by wrapping a polyurethane foam collar around the hypocotyls before insertion into the holes. Preliminary experiments showed that necrotic lesions developed on some seedling hypocotyls that were seemingly associated with the polyurethane collars. The problem was alleviated by washing the collars before use in 70% ethanol followed by hot tap water and a final rinse in distilled water. The need to detoxify polyurethane foam supports has also been noted by Wagner and Wilkinson (1991).

The nutrient solutions were changed completely every second day until plants reached the V4 growth stage after which nutrient solutions were changed daily. Plants were inoculated at 30 days after introduction to solution culture by inserting two millet grains colonised with *Sclerotinia minor* UNE#3 between the polyurethane collar and the stem. Lesion length along the stem was measured daily and the linear rate of lesion extension calculated. One seedling from each vessel was selected at random and harvested at the time of inoculation for fresh and dry weight determination and calcium analysis.

7.3.2. Effect of calcium nutrition on growth of **Sclerotinia minor** *in vitro.*

Before examining the effect of alterin; the supply of calcium to sunflower seedling on susceptibility of sunflower plants to *Sclerotinia minor* it was considered prudent to examine whether calcium has any direc effect on growth of the fungus.

Materials and methods

The radial growth of colonies of *S. minor* on agar plates and biomass accumulation in broth cultures were assessed. The medium used in both was based on Hoagland's solution (Hoagland and Arnon, 1950) since this nutrient solution was to be used in later experiments with solution cultured plar ts. Differences in nitrate (NO_3^-) concentration was compensated for by addition of sodium nitrate (Table 7.2). Sucrose was used as the carbon source. Agar was added at 20g.L^{-1} to solidify the medium for radial growth assays. pH was adjusted to 6.0 by the addition of 1 N NaOH before autoclaving.

a) Radial growth. To determine the effect of calcium concentration on radial growth rate of colonies, 20 plates were prepared with each of the Ca^{2+} concentrations to be tested. Fifteen mL of media was dispensed into sterile 9cm diameter plastic Petri dishes. A 3mm diameter disk of inoculum taken from the edge of *S. minor* isolate UNE#3 actively growing on potato dextrose agar was transferred, hyphal surface down, to the centre of each Petri dish. Each plate was sealed with Parafilm™ and placed in an incubator at 20°C and darkness. Colony diameter was measured at 3, 6 and 12 days after inoculation by taking the mean of two measurements taken at right angles.

Table 7.2 Composition of nutrient solutions used to evaluate of the effect of calcium concentration on *in vitro* growth of *Sclerotinia minor*.

	Stock Conc. (H_2O)	Nutrients added (ml.L^{-1})				
		Ca^{++} Concentration				
		125μM	625μM	1250μM	2500μM	5000μM
KNO_3	1M	5	5	5	5	5
$MgSO_4.7H_2O$	1M	2	2	2	2	2
KH_2PO_4	1M	1	1	1	1	1
$Ca(NO_3).4H_2O$	1M	0.125	.625	1.25	2.5	5
$NaNO_3$	1M	9.75	8.75	7.5	5	0
Sucrose	-	30	30	30	30	30

b) Biomass accumulation. The medium used was the same as that used in the radial growth except that agar was omitted. 50mL of each Ca^{2+} concentration was dispensed to eight 150mL erlenmeyer flasks and autoclaved. Three 3mm diameter disks of inoculum taken from the edge of *S. minor* isolate UNE#3 actively growing on potato dextrose agar were transferred to each flask. The flasks were placed on an orbital shaker at room temperature (14-22°C) and shaken at 100 revolutions per minute. Mycelium was harvested after 10 days incubation by filtering through pre-weighed (after drying at 60°C 2h) filter papers. The pH of each filtrate was measured after harvesting the mycelia. The mycelium was rinsed with deionised water before the filter paper and mycelium were dried overnight at 60°C and weighed. Mycelial dry weight was calculated as the difference between final weight and initial weight of the filter paper.

Results

Increasing the concentration of calcium (or decreasing the alternative cation sodium) in the growth medium had no significant effect on the radial growth of *Sclerotinia minor* (Figure 7.1). The fungus grew more slowly on the defined medium used in this experiment than that observed on the non-defined media potato dextrose or V-8® juice agar. In the present experiment the radial growth rate was about 5mm.d^{-1} while in Experiment 3.3.3 conducted with V-8 juice agar the rate of growth exceeded 35mm.d^{-1} at a similar incubation temperature.

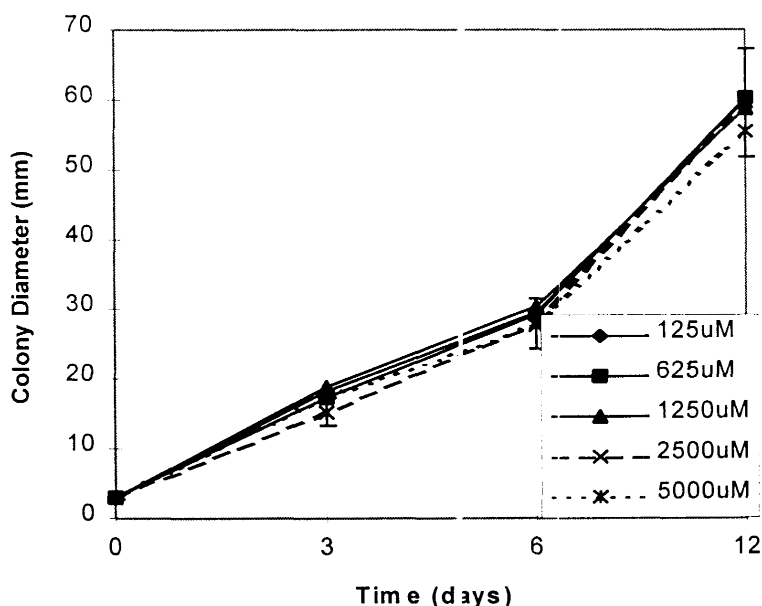

Figure 7.1 Effect of Calcium concentration on radial growth of *Sclerotinia minor* (Bars represent standard errors).

The mycelial dry weight differed significantly when *S. minor* was grown in solutions

containing different concentrations of calcium (and sodium)(Figure 7.2). The dry weight of mycelium grown at 5000μM Ca^{++} was twice that of mycelium harvested from 125, 625 0r 1250 μM Ca^{++}. The pH of the broth from which the mycelia were harvested decreased with increasing calcium concentration. The pH for the 125, 625, 1250, 2500 and 5000 μM Ca^{2+} solutions were 2.94, 2.79, 2.66, 2.62 and 2.50 respectively.

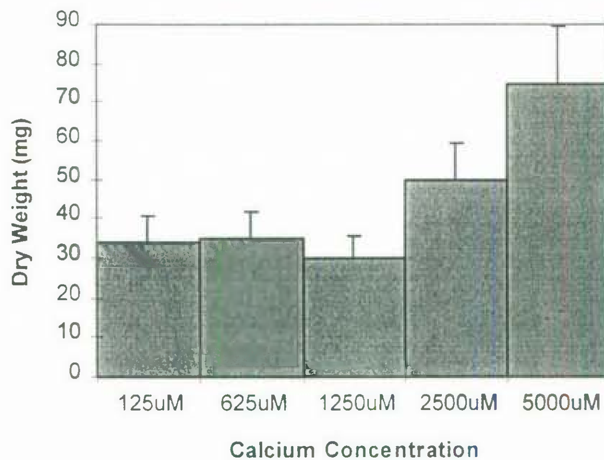

Figure 7.2 Effect of calcium concentration in a nutrient broth on biomass accumulation of *Sclerotinia minor* (Bars represent standard errors).

7.3.3 Effect of calcium concentration on the susceptibility of sunflower to **Sclerotinia minor** *when grown in sand culture.*

There are several ways of modifying the calcium concentration of plant tissues, one of which is to grow plants in a calcium-free medium and add calcium at the desired concentrations. In this experiment sunflower plants were raised in acid washed sand and watered with nutrient solutions differing in calcium concentration. The pot base inoculation method was then used to test whether supplying plants with different concentrations of calcium influenced susceptibility to *Sclerotinia minor*.

Materials and method

Plants of the sunflower hybrid Hysun 44 were gown as described in Section 7.3.1 and supplied with either 0.125, 0.625, 1.25, 2.5 or 5.0 mM Ca^{2+} as calcium nitrate or 5.0mM Ca^{2+} as calcium chloride with nitrate balance provided by addition of sodium nitrate. Twelve plants of each treatment were grown in a completely randomised design on a glasshouse bench. At thirty days after sowing four plants from each treatment were chosen at random by drawing lots and used to determine growth parameters and calcium content of

plant tissue. The remaining plants were inoculated.

Results

Supplying calcium over the range 0.125 to 5mM did not significantly affect (P>0.05) the height or number of leaves on plants (Table 7.3). There were significant differences in the calcium content of stem bases with increased calcium supply (Table 7.3). The relationship between calcium supply and calcium content of roots was different from that of stems. Plants that were supplied with nutrient solutions containing only 0.625mM Ca^{2+} had on average similar contents in roots as plants supplied with 5mM.

The number of plants killed at 28 days after inoculation with *S. minor* was significantly lower (P<0.05) in plants supplied with 5mM calcium nitrate. All other calcium treatments resulted in over 80% mortality. The time taken for the appearance of basal stem lesions also differed significantly between treatments with the delay significantly longer in plants supplied 5mM Ca^{2+} as the nitrate form than with chloride form. The rate of lesion progression along the stem decreased with increased calcium supply except in the case of plants supplied 5mM calcium chloride where lesion progress was significantly quicker than plants fed with 5mM calcium nitrate.

Table 7.3. Effect on supplying increasing concentrations of calcium on growth, calcium content and susceptibility of sunflower to *Sclerotinia minor*.

Parameters		Calcium Supply (mM)						
		0.125 NO_3^-	0.625 NO_3^-	1.25 NO_3^-	2.5 NO_3^-	5.0 NO_3^-	5.0 Cl^-	L.S.D. P=0.05
Height	(cm)	42.4	41.0	40.9	41.3	38.9	41.5	5.28
Leaves	(#)	9	8.[illegible]	8	7.8	8	8	1.5
Stem Ca^{2+}	$(g.kg^{-1})$[1]	10.3	10.3	12.1	15.2	19.9	16.5	3.97
Root Ca^{2+}	$(g.kg^{-1})$	10.1	11.6	11.0	11.7	11.7	10.2	1.68
Mortality	(/8)	8	8	8	7	4	7	4
Delay	(d)	14.1	16.8	13.8	15.9	16.0	12.6	3.11
Rate	$(mm.d^{-1})$	8.24	8.[illegible]8	7.88	7.88	5.98	8.59	2.52

1. Dry weight basis

Lesions on plants supplied with 5mM Ca^{2+} as the nitrate tended to be drier and to darken with age relative to those supplied with chloride which remained pale in colour and continued to elongate (Figure 7.3).

At twenty-one days after inoculation the root systems of plants without basal stem lesions were gently washed free of sand and examined for root lesions to confirm that inoculation had in fact taken place. Lesions and sclerotia were found on all root systems. New roots were growing in contact with newly formed sclerotia. A selection of these new sclerotia were collected, surface sterilised in 70% ethanol for one minute and 1.5% sodium hypochlorite for two minutes, rinsed with sterile water and plated onto potato dextrose agar. In all 100 sclerotia were plated and in every case *Sclerotinia minor* was recovered.

Figure 7.3 Basal stem lesions on plants supplied with 5mM Ca^{2+} as nitrate (left) and as chloride (right).

7.3.4 Effect of calcium chloride concentration on the susceptibility of sunflower to **Sclerotinia minor** *when grown in sand culture.*

In the previous experiment it was shown that the application of calcium to sunflower seedlings in its chloride form was associated with an increase in tissue susceptibility to *Sclerotinia minor*. In this experiment a wider range of $CaCl_2$ concentrations was used to test whether this observation was a general phenomenon.

Materials and methods

The experiment was conducted as described in Experiment 7.3.1 except that the calcium was supplied at either 1.25, 2.5, 5.0, 10.0 or 20.0 mM Ca^{2+} as $CaCl_2.2H_2O$. Nitrogen was supplied as sodium nitrate. Seventeen replicates of each treatment were grown. Twelve plants of each treatment were inoculated at 32 days post-sowing using the pot base method. The remaining 5 plants were analysed for calcium content. The lower 10cm of stem was collected for analysis.

Results

Sunflower seedlings treated with nutrient solutions containing different concentrations of calcium chloride had little observable effect on plant morphology although quantitation of several parameters (Table 7.4) revealed a trend towards reduced plant growth in plants exposed to very high levels of calcium chloride. The concentration of calcium present in the roots and stem bases increased as calcium levels in the nutrient solution increased (Table 7.4) but this trend was not linear.

Table 7.4. Effect of calcium nutrition with different levels of $CaCl_2$ on growth parameters of sunflower seedlings

Calcium	Plant Characteristics					Ca^{2+} Concentration	
Supply	Height	Leaves	Root	Stem Base	Roots	Stem Base	Roots
(mM Ca^{++})	(cm)	(#)	Index[1]	(g)	(g)	($g.kg^{-1}$)	($g.kg^{-1}$)
1.25	44.8	10	3.1	0.67	0.85	16.6	41.2
2.5	44.8	10	3.2	0.66	0.71	21.1	42.9
5.0	42.4	10.4	3.0	0.68	0.77	27.4	50.1
10.0	39.6	9.4	3.0	0.66	0.79	33.6	58.6
20.0	40.1	9.2	2.9	0.59	0.70	39.4	63.5
L.S.D. (P=0.05)	5.55	1.04	0.01	0.167	0.136	12.72	12.99

1. Root Index determined on degree of root ball base occupied by roots (refer 2.7)

An increase in the supply of calcium as calcium chloride appeared to have a marked effect

on the susceptibility of sunflower seedlings to *Sclerotinia minor* (Table 7.5). However, interpretation of these results was complicated by the appearance of stem lesions that were not associated with the presence of *S. minor*.

Table 7.5. Effect of different levels of $CaCl_2$ in the nutrient solution on the susceptibility of sunflower seedlings to *Sclerotinia minor*.

Assessment Parameter		Calcium Supply (mM)					L.S.D.
		1.25	2.5	5.0	10.0	20.0	(P=0.05)
Mortality	(/12)	12	11	11	11	11	-
Delay	(d)	11.9	10.3	10.6	10.1	8.9	1.35
Apparent rate of lesion extension	($mm.d^{-1}$)	9.1	11.8	9.3	24.9	32.9	6.24

The 'lesions' associated with exposure to high concentrations of $CaCl_2$ where characteristic of those caused by *S. minor* in that they were tan, sunken and 'water-soaked'. To determine whether *S. minor* was present in the lesions associated with high concentrations of $CaCl_2$ all stems from plants provided with 10 or 20 mM Ca^{2+} were harvested at 21 days after inoculation, surface sterilised with 1% sodium hypochlorite for 2min, rinsed in sterile distilled water, cut into 1cm lengths and plated onto potato dextrose agar. After incubation at 20°C for 4 days the tissue pieces were inspected for the presence of *S. minor*. Although the lesions on harvested stems ranged from 15 to 24 cm in length *S. minor* was only isolated from within the first 10cm of the stem.

7.3.5 Effect of calcium concentration on the susceptibility of sunflower to **Sclerotinia minor** *when grown in solution culture.*

Solution culture was also used to test the observations that supplying sunflower seedlings with increased concentrations of calcium resulted in reduced susceptibility to *S. minor*.

Materials and methods

Two sunflower hybrids (Hysun 44 and Suncross 40R) were grown in solution culture and supplied with 0.125, 0.625, 1.25, 2.5 or 5.0mM $Ca(NO_3)_2$. Sodium nitrate was used to standardise the concentration of nitrate in the treatments supplied with lower levels of calcium nitrate. Each treatment consisted of five replicate culture vessels. The experiment was repeated once and the results combined for analysis.

Results

Sunflower seedlings supplied with solutions containing 0.125mM Ca^{2+} showed a significant reduction (P<0.05) in shoot growth as determined by fresh and dry weight (Table 7.6) relative to the other calcium concentrations used. The calcium content of plant tissue increased with increased concentration of the element in the nutrient solution.

In both hybrids this increase in tissue calcium was inversely related to the rate of lesion extension (Figure 7.4). Although tissue calcium levels were similar in both hybrids the rate of lesion extension on stems of the hybrid Suncross 40R was greater than that of Hysun 44.

Table 7.6. Effect of calcium concentration in the nutrient solution on the fresh and dry weight of two sunflower hybrids.

Ca Conc.	Hysun 44		Suncross 40R	
(mM)	F. W.[1] (g)	D. W. (g)	F. W. (g)	D.W. (g)
0.125	2.6	0.20	3.4	0.25
0.625	9.4	0.32	9.4	0.66
1.25	11.0	0.39	7.5	0.62
2.5	9.7	0.79	8.0	0.67
5.0	11.9	0.97	8.0	0.71
L.S.D. (P=0.05)	4.00	0.315	4.34	0.351

1. F.W. fresh weight D.W. dry weight

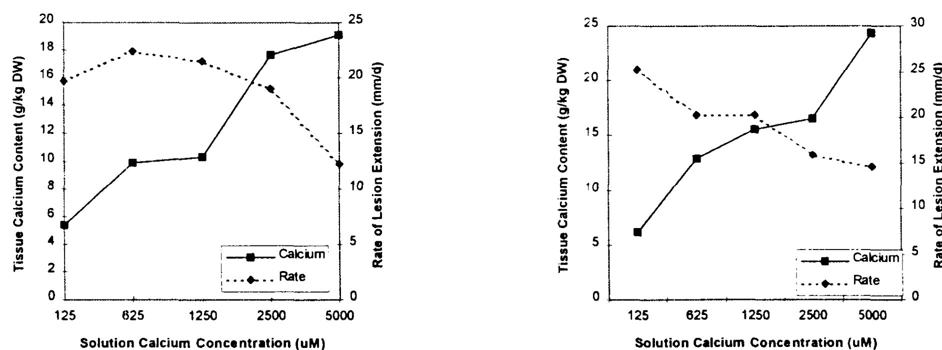

Figure 7.4 Effect of supplying increasing concentrations of calcium on tissue calcium content and rate of lesion extension on two sunflower hybrids, Hysun 44 (left) and Suncross 40R (right).

7.3.6 Effect of alkaline earth elemen's on the susceptibility of sunflower to **Sclerotinia minor** *when grown in solution cultur.*

In the Periodic Table calcium belongs to a group of elements referred to as the alkaline earth elements or alkaline earth metals. These elements include beryllium, magnesium, strontium, barium and radium. In this experiment a study was made on the effect of supplying barium, calcium, magnesium, and strontium to sunflower seedlings on their susceptibility to sclerotinia wilt caused by *Sclerotinia minor*.

Materials and method

The solution culture apparatus was the same as that described previously. Two sunflower hybrids (Hysun 44 and Suncross 40R) were used in this study. The nutrients were supplied as shown in Table 7.7. It should be noted that each of the elements was used as the chloride form as these were available and readily soluble. Ammonium nitrate was used to standardise nitrogen inputs. The solutions also contained a minimum of 1mM Ca^{2+} to provide this element for calcium specific essential functions.

Table 7.7. Composition of nutrient solution used for evaluation of the effect of alkaline earth elements on susceptibility of sunflower to *Sclerotinia minor*.

Compound	Stock Conc.	Nutrients added ($ml.L^{-1}$)					
		$Ca(NO_3)_2$/ $NaNO_3$	$Ca(NO_3)_2$	$Ca(NO_3)_2$/ $MgCl_2$	$Ca(NO_3)_2$/ $CaCl_2$	$Ca(NO_3)_2$/ $SrCl_2$	$Ca(NO_3)_2$/ $BaCl_2$
KNO_3	1M	5	5	5	5	5	5
$MgSO_4.7H_2O$	1M	2	2	2	2	2	2
KH_2PO_4	1M	1	1	1	1	1	1
NH_4NO_3	1M			4	4	4	4
$Ca(NO_3).4H_2O$	1M	1	5	1	1	1	1
$NaNO_3$	1M	8					
$MgCl_2$	1M			4			
$CaCl_2.2H_2O$	1M				4		
$SrCl_2$	1M					4	
$BaCl_2$	1M						4
$Na_2FeE.D.T.A.$	0.1M	1	1	1	1	1	1
Micronutrients[1]		1	1	1	1	1	1

1. As outlined in Table 7.3.

Results

Seedlings of both hybrids supplied with 4mM barium did not grow and were omitted from the analysis. Seedlings supplied with calcium nitrate only were considered as 'control'. Seedlings supplied with 8mM Na^+ plus 1mM Ca^{2+} showed no significant differences ($P>0.05$) in plant growth (as determined by fresh weight) although the rate at which lesions progressed increased in both hybrids This increase in rate of lesion extension was significant ($P<0.05$) in the case of Suncross 40R. The rate of lesion extension was also greater in plants supplied 4mM $CaCl_2$ with nitrogen supplied as ammonium nitrate than in those supplied $Ca(NO_3)_2$ only. However, the effect was not significant at the 5% level. Plants of both hybrids given an excess of magnesium (6mM Mg^{2+} / 1mM Ca^{2+}) had lower fresh weights than the 'controls'. The rates of lesion extension on these plants were the most rapid of all treatments. In contrast, rates of lesion extension were slowest in plants of both hybrids fed with 4mM Sr^{2+}. The two hybrids showed differential sensitivity to strontium nutrition. Seedlings of Suncross 40R were more sensitive to Sr^{2+} and on average were significantly smaller than those exposed to all other treatments except excess magnesium. Seedlings of Hysun 44 were not adversely affected (Figure 7.5) at the concentration of Sr^{2+} used.

Table 7.8 The effect of supplying seedlings of two sunflower hybrids with nutrient solutions differing in sodium and alkaline earth elements on growth and susceptibility to *Sclerotinia minor*.

		Nutrient					L.S.D.
		$Ca(NO_3)_2$/ $NaNO_3$	$Ca(NO_3)_2$ only	$Ca(NO_3)_2$/ $MgCl_2$	$Ca(NO_3)_2$/ $CaCl_2$	$Ca(NO_3)_2$/ $SrCl_2$	(P=0.05)
Hysun 44							
Fresh Weight	(g)	5.59	5.17	3.47	5.28	5.06	2.76
Lesion Rate	($mm.d^{-1}$)	7.92	6.97	13.72	8.03	4.72	4.14
Suncross 40R							
Fresh Weight	(g)	3.85	4.26	3.26	4.05	1.85	1.49
Lesion Rate	($mm.d^{-1}$)	9.08	6.58	9.70	7.44	5.66	1.73

Figure 7.5 Differential sensitivity of sunflower seedlings to Sr^{2+}. Left- Hysun 44 Right- Suncross 40R.

7.3.7 Susceptibility to **Sclerotinia minor** *of eight inbred sunflower lines when grown in solution culture at two calcium concentrations.*

In the previous experiments it was demonstrated that increased calcium supply in either sand or solution culture reduced the rate at which stem lesions caused by *S. minor* developed. To determine whether this relationship was a general phenomenon a number of sunflower inbred lines, for which field susceptibility and calcium content were known, were screened in solution culture at two calcium concentrations.

Materials and method

Eight sunflower inbred lines, RHA801, Pac R2, CM497, CM526, Pac A1, Pac A3, *cms* HA 89 and *cms* HA 124, were grown in solution culture supplied with either 0.625mM or 5.0mM Ca^{2+} as described earlier. A single plant from each culture vessel was harvested at 30 days after transfer to the culture vessels to determine fresh and dry weights and calcium

content. The remaining five seedlings were inoculated with *S. minor*. Each inbred line x calcium level combination was replicated four times. The experiment was repeated three times.

Results

Combined factorial analysis of the data revealed that the concentration of calcium supplied to plants in solution culture had a significant effect on fresh weight, dry weight, calcium content and rate of lesion extension (Table 7.9). The fresh weight, dry weight and rate of lesion of extension also varied among the lines tested. However, the calcium supply x inbred line interaction was not significant for any of the parameters measured.

Table 7.9 Summary of probabilities from factorial analysis of effect of two levels of calcium in the nutrient medium on growth, calcium content and disease susceptibility of eight inbred sunflower lines.

Source	Variable			
	Fresh Wt	Dry Wt	Ca Content	Lesion Rate
Calcium (A)	<0.01	<0.01	<0.01	<0.01
Inbred Line (B)	<0.01	<0.01	>0.1	<0.05
A x B	>0.1	>0.1	>0.1	>0.1

The tissue calcium content was higher for each inbred sunflower line grown in 5mM Ca^{2+} than for plants grown in 0.625mM Ca^{2+}. Calcium content was inversely related to the rate of lesion extension (Figure 7.6). The growth of plants (as determined by dry weight) was greater at the higher calcium concentration except for the inbred lines RHA801 and Pac A1 where growth at the two concentrations did not differ.

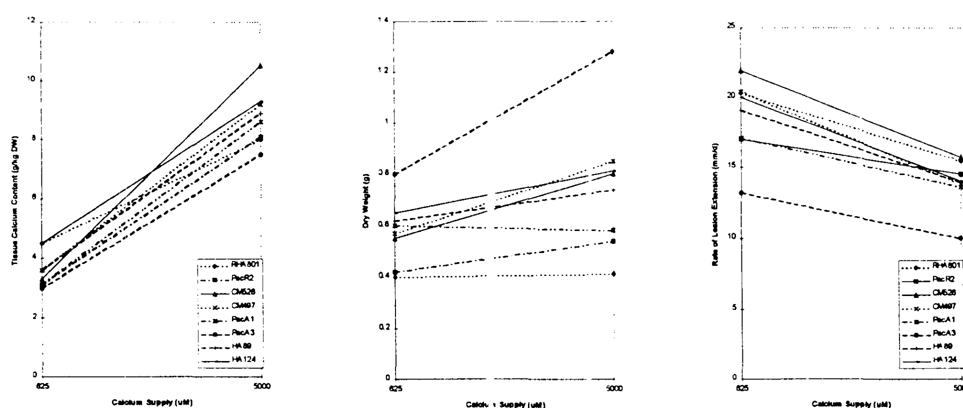

Figure 7.6 Effect of calcium supply to eight sunflower inbred lines on tissue calcium content (left), shoot dry weight (centre) and rate of lesion extension (right). (Legend for shoot dry weight as for the other plots).

7.3.8 Relationship between nutrient levels in field grown sunflowers and susceptibility to Sclerotinia minor.

The experiments described above showed that high calcium availability in the medium in which sunflower plants were grown and high calcium levels in plant tissue were associated with a reduction in the susceptibility of sunflower plants to *Sclerotinia minor*. In this experiment an attempt was made to relate susceptibility of plants to *S. minor* and calcium levels in root and stem tissue of eight inbred sunflower lines grown under field conditions.

Materials and method

In Experiment 6.3.1 sunflower plants grown at two field sites were harvested for root length determinations. Extra plants were taken from these sites to determine the calcium content. Plants were harvested over a period of a few weeks when each inbred line had reached 50% anthesis. To facilitate removal of plants from soil, small earthen dams were constructed around randomly selected plants. These dams were kept filled with water for six hours by which time the soil around the chosen plants was saturated. A hose supplying a stream of water at low pressure was then used to excavate the root systems by washing away the surrounding soil. Whole plants were placed in plastic bags and returned to the laboratory where they were partitioned into root and shoot portions. The roots were immediately washed in tap water followed by deionised water. The basal 20cm of stem was removed and the remainder of the shoot discarded. All material was then dried for 48h at 80°C before being cut into pieces and milled to pass through a 1mm screen. The powders were stored in air tight bottles at 4°C until the inbred line that flowered last was harvested. The material was digested and analysed for calcium, sodium, potassium, magnesium, phosphate and sulphur content by I.C.P. analysis.

Results

The roots extracted in this experiment supported the results obtained by core analysis (Section 6.3.1) and showed that the root volume varied among the different inbred sunflower lines. Photographs of the extracted root systems of representative plants of the different lines are shown in Figure 6.1

Significant differences ($P<0.05$) were found in the levels of each nutrient among the sunflower lines at both sites (Tables 7.10). Potassium was the most abundant cation present in both root and stem tissues from both sites. This result is consistent with the observations of others on different soils which showed that this cation is dominant in sunflower roots and shoots (Robinson, 1970; Blamey *et al.*, 1980). One distinguishing

feature of the present analysis was that the calcium/magnesium ratio of less than 1 in the plant tissue. This reflects the nutrient status of the chocolate earths of the New England area where magnesium is the predominant divalent cation (Appendix 1; Stace *et al.*, 1972). In other reports of elemental composition of sunflower the calcium/magnesium ratio is greater than 1 (Robinson, 1970; Blamey *et al.*, 1980; Seiler, 1986).

The calcium content of the roots or the stem bases was not significantly correlated ($P>0.05$) with cumulative mortality of the same sunflower inbred lines growing in disease screening trials adjacent to both sites. The only significant correlations ($P<0.05$) were positive and were between content of phosphorus in roots and cumulative mortality at both sites and phosphorus content of stem bases and cumulative mortality at the LD92 site (Tables 7.10 and 7.11).

7.3.9. Effect of pre-plant soil amendments with calcium compounds on the incidence of sclerotinia wilt caused by **Sclerotinia minor.**

The previous sand and solution culture experiments showed that high calcium levels in plant tissue was associated with increased resistance to *S. minor*. Punja(1989) reported that application of $Ca(NO_3)_2$ up to $154kg.ha^{-1}$ reduced the incidence of *Sclerotium rolfsii* wilt on sugarbeet by 68%. Two small field trials were established at the LD91 and LD92 field screening sites to test whether application of calcium compounds at a rate equivalent to $200kg\ Ca^{2+}.ha^{-1}$ altered the susceptibility of a sunflower hybrid to *Sclerotinia minor*.

Materials and method

The calcium compounds used at both sites were calcium oxide (CaO), calcium hydroxide ($Ca(OH)_2$), calcium chloride ($CaCl_2.2H_2O$), calcium nitrate ($Ca(NO_3)_2.4H_2O$), calcium carbonate ($CaCO_3$) and superphosphate (22% Ca). Diammonium phosphate (D.A.P.) was included in the second trial. Nitram (NH_4NO_3) was added to mixtures to raise the nitrogen level to that equivalent to $Ca(NO_3)_2.4H_2O$ (ie 11.85% N w/w). The sunflower hybrid used was Pacific Seeds Hysun 33. This is a single cross hybrid which has been shown to very susceptible to *Sclerotinia minor* in the irrigation areas of Victoria, Australia (Porter, Clarke and Woodroofe, 1994).

Table 7.10. Nutrient contents of eight sunflower inbred lines at 50% anthesis when grown at the LD91 field site.

	Root						Stem					
Inbred	Na	K	Mg	Ca	P	S	Na	K	Mg	Ca	P	S
			(g.kg^{-1})						(g.kg^{-1})			
RHA801	5.65	6.26	2.70	2.51	0.36	0.72	0.62	20.9	5.35	3.66	0.58	0.78
Pac R2	3.38	8.63	2.07	3.12	0.62	0.88	1.90	9.61	4.48	2.31	0.36	0.65
CM526	4.90	9.63	3.09	3.79	0.69	1.32	0.59	12.1	7.50	2.80	0.69	0.68
CM497	6.23	10.7	2.04	2.28	0.99	1.00	0.59	12.9	4.59	2.85	0.68	0.61
Pac A1	1.22	4.64	1.93	2.96	0.47	0.66	1.22	12.6	4.75	2.61	0.68	0.43
Pac A3	5.06	11.1	2.77	3.05	0.66	0.64	0.55	10.3	3.79	1.94	0.80	0.52
HA89	5.09	10.3	2.29	3.51	0.53	0.81	0.91	11.5	3.12	2.44	0.43	0.35
HA124	5.55	10.2	3.51	3.67	0.56	0.88	0.32	15.1	4.31	2.98	0.61	0.64
Mean	4.64	8.93	2.55	3.11	0.61	0.86	0.84	13.13	4.74	2.69	0.52	0.58
L.S.D.[1]	2.79	5.04	1.83	0.16	0.07	0.10	1.21	7.04	1.78	0.81	0.61	0.35
r[2]	-0.06	0.66	-0.37	-0.43	0.79	0.11	-0.09	-0.36	-0.36	-0.33	0.05	-0.16
p[3]	ns	*	ns	ns	**	ns	ns	ns	ns	ns	ns	ns

Table 7.11. Nutrient contents of eight sunflower inbred lines at 50% anthesis when grown at the LD92 field site.

	Root						Stem					
Inbred	Na	K	Mg	Ca	P	S	Na	K	Mg	Ca	P	S
			(g.kg^{-1})						(g.kg^{-1})			
RHA801	4.10	9.79	2.79	1.80	0.73	0.49	2.18	6.07	9.71	2.87	0.59	0.68
Pac R2	4.02	7.25	1.31	1.22	0.34	0.37	2.49	5.78	4.65	1.97	0.32	0.54
CM526	5.13	5.12	2.21	1.29	0.67	0.59	3.66	4.53	8.32	1.96	0.60	0.83
CM497	9.56	4.32	1.88	1.67	1.53	0.64	12.4	4.65	12.2	2.47	1.75	0.66
Pac A1	8.78	5.47	1.38	2.06	1.10	0.62	9.52	8.22	5.09	3.18	0.95	0.75
Pac A3	2.43	13.2	2.08	1.86	1.42	0.59	1.52	13.4	7.87	2.14	1.67	1.34
HA89	4.18	10.1	1.11	1.54	1.17	0.43	3.36	11.1	4.23	1.81	1.04	0.46
HA124	4.00	12.3	1.53	1.96	0.76	0.51	1.40	5.84	5.18	2.67	0.45	0.53
Mean	5.28	8.44	1.79	1.68	0.97	0.53	4.57	7.45	7.16	2.38	0.92	0.72
L.S.D.[1]	3.07	5.33	1.58	2.56	0.80	0.64	8.73	9.21	5.09	0.90	1.09	0.53
r[2]	0.22	0.30	-0.44	-0.34	0.76	-0.18	0.37	0.46	-0.16	-0.36	0.73	-0.19
p[3]	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	**	ns

1. L.S.D. P=0.05 2. coefficient of correlation of regression of nutrient level against cumulative mortality at 50% anthesis (from 6.3.1) 3. Level of significance of correlation: ns P>0.10, * 0.10>P>0.05, ** 0.05>P>0.01 and *** P<0.01.

Both trials consisted of randomised complete block designs with 5 replications. Individual plots were 4m square and separated from other plots by 3m on each side. Soil preparation consisted of rotary tilling to produce a fine tilth before hand application of amendments at a rate equivalent to 200 kg $Ca^{2+}.ha^{-1}$ to each plot. This was immediately incorporated by rotary tilling. The plots were sown two weeks later with a tractor-mounted cone seeder. The LD91 site was sown 18 October 1991 and the LD92 site sown 16 January, 1992. Each plot consisted of 6 rows with an inter-row spacing of 0.75m and the inter-plot buffer zones were sown with 3 rows of the sunflower hybrid Pacific Seeds Hysun 35 at a similar row spacing. Three weeks after sowing the plant stand was thinned manually to 6 plants per metre. The herbicide Fusilade 212® (ICI Australia Operations Pty Ltd) was applied once 4 weeks after sowing at $500mLha^{-1}$ in $100Lha^{-1}$ water plus Agral 60 wetting agent with a tractor-mounted boom spray to kill/suppress annual ryegrass that emerged at the LD92 site.

Disease incidence caused by *Sclerotinia minor* was recorded every 4 days by walking through the plots and examining the bases of plants for the appearance of basal stem lesions. Once a lesion was found the plant was marked with spray paint so that it would not be counted in later assessments. The area under the disease progress curves (A.U.D.P.C.) were calculated. Elemental analyses were not conducted due to lack of financial support.

Results

Disease developed well in both sites. In the summer maturing site at LD91 the A.U.D.P.C.'s ranged from 304 to 635 (Figure 7.7) while mortality at 50% anthesis ranged from 27 to 60%. Disease levels were higher in the autumn maturing trial at LD92 with mortalities at anthesis ranging from 74 to 94% and A.U.D.P.C.'s ranging from 4649 to 5352. At the LD91 site disease incidence was highest in plots treated with superphosphate and lowest in plots to which calcium was not added. Addition of calcium nitrate did not cause a significant increase ($P<0.05$) in disease incidence compared to the control plots. The average incidence of *S. minor* wilt at the LD92 site was lowest in plots treated with calcium nitrate and highest in the control treatment and those treated with calcium oxide, calcium chloride and superphosphate.

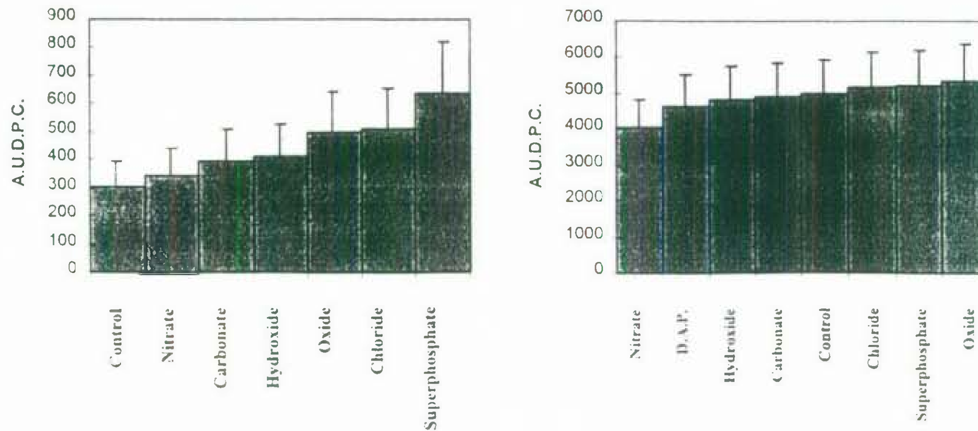

Figure 7.7 Effect of soil amendments with calcium compounds and a phosphate fertiliser (D.A.P.) on incidence of sclerotinia wilt caused by *S. minor* at two field sites; LD91 (left) and LD92 (right).

7.5 Discussion.

The logic behind conducting the research reported in this chapter was threefold;

- increased calcium nutrition has been associated with reduced severity of several diseases (Punja, 1989; Sugar *et al.*, 1991),
- *Sclerotinia* species produce oxalic acid and pectolytic enzymes during pathogenesis. Higher levels of calcium ions in tissues might be expected to protect those tissues from fungal ingress by increased calcium cross-linking of pectic molecules and the removal of oxalate through the production of insoluble calcium oxalate (Volpin and Elad, 1991) and,
- calcium content among sunflower germplasm is known to differ (Blamey, Vermeulen and Chapman, 1980; Seiler, 1986; de la Gaurdia, Alcantara and Fournier, 1990).

Calcium was found not to be toxic to *Sclerotinia minor in vitro* so any effect *in planta* would be expected to be due to alterations in the plant itself or on determinants of pathogenicity. However, dry weight differences were found between mycelia cultured in the presence of different concentrations of calcium. These differences were not anticipated since under macroscopic examination the amount of mycelium seemed very similar. Microscopic examination of the dry mycelium revealed masses of bipyramidal crystals on the hyphae especially of those from cultures grown in high calcium concentrations. It is quite likely that these crystals were of calcium oxalate and that these crystals contributed towards the weight increase of the mycelium. The aqueous insolubility of calcium oxalate would have resulted in the crystals not being removed by the water rinse used to remove

soluble extracellular material from the harvested mycelium. Formation of crystals confirmed as being calcium oxalate by energy-dispersive X-ray analysis were associated with infection of several crucifers by *Rhizoctonia solani* Kühn (Yang, Tewari and Verma, 1993).

Studies of the influence of host nutrition on the susceptibility of plants to disease can be done by several methods. Plants can be grown in directly in nutrient solutions, or in an inert matrix such as sand and supplied with defined nutrient solutions or they can grown in the field culture. Each method has certain advantages and disadvantages. Sand culture is simple, inexpensive and allows good aeration of roots. However, it requires close supervision to avoid desiccation or accumulation of salts. The pot soak method was used in the present study in an attempt to allow solubilisation and removal of excess salts. Sand culture has a long history of use in plant pathological studies (eg Tharp, 1938; Ranney, 1962). Solution culture requires more maintenance as well as close supervision to ensure adequate aeration of roots but it does allow nutrition levels to be quickly altered. Field trials require a thorough knowledge of the complexities of nutrient movements in soils and it is expensive to alter availability of cations on a large scale.

The overall conclusion from the glasshouse experiments conducted was that increased availability of calcium increases the resistance of sunflower to *S. minor*. Orellana *et al.*(1975) demonstrated enhanced resistance to *Sclerotinia sclerotiorum* in sunflower seedlings supplied with calcium carbonate but the experiment was confounded by being conducted in an acidic soil with soluble aluminium at toxic levels. It is unclear whether the enhanced resistance was due to increased pH, increased available calcium or both. Similarly, the results of the experiments reported in this chapter must be qualified by noting that the source of calcium is important. Supplying calcium as the chloride may in fact increase susceptibility of sunflower lines to *S. minor*. Muchovej *et al.* (1980) also found that addition of calcium as $CaCl_2$ increased the incidence of mortality of pepper plants challenged with *Phytophthora capsici*. The mechanism behind this increased susceptibility may be related to the role of the chloride ion in altering either pathogenesis or host resistance. The observation that lesions on plants supplied with calcium chloride remained pale while those fed calcium nitrate darkened with age suggests that the action of host polyphenol oxidases (EC 1.14.18.1 o-diphenol: oxygen oxido reductase, P.P.O.) may have been inhibited. These enzymes play a central role in production of dark pigments (Mayer, 1987) and have been implicated as being actively involved in host resistance response reactions. Halides including chloride have been reported as inhibitors of P.P.O. possibly through interaction with the copper atom at the active site. This interaction is further favoured by low pH (Ben-Shalom, Kahn, Harel and Mayer, 1977). In the acidic environment produced by *Sclerotinia* infections it is likely that excess chloride could

suppress P.P.O. activity. This inhibition would be further enhanced by the presence of oxalate produced by the fungus since this molecule also reduces P.P.O. activity probably through chelation of Cu^{2+} as copper oxalate (Watanabe, Hiraoka, Masada and Sato, 1991; Ferrar and Walker, 1993).

Sodium nitrate was used in many experiments to provide the nitrogen balance. Chrominski *et al.* (1987) also used sodium nitrate to balance nitrate when supplying different levels of calcium nitrate to sunflower plants. The presence of higher levels of sodium in tissue could complicate interpretation of the results. Several root diseases seem to be favoured by saline soils (Bouchibi, van Bruggen and MacDonald, 1990) where sodium competes with calcium, magnesium and potassium for uptake by roots. Once in plant tissue sodium may displace calcium from cell membranes (Cramer, Läuchli and Polito, 1985; Rengel, 1992) resulting in reduced membrane integrity and selective permeability with a final consequence being cell leakage leading to increased root exudations or, in a pathosystem, increased susceptibility. High levels of sodium can be tolerated if sufficient calcium is supplied using compounds including calcium chloride (LaHaye and Epstein, 1969; Cramer, Läuchli and Epstein, 1986). In the present experiments it cannot be discounted that increased susceptibility of plants given lower concentrations of calcium involved an interaction with the higher concentrations of sodium. However, in Experiment 7.3.6 it was shown that disease susceptibility also increased in plants fed calcium chloride with ammonium nitrate used as the nitrogen source.

Elucidation on the actual mechanism/s by which calcium increases resistance of sunflower to sclerotinia wilt will require more detailed physiological studies than were possible in this study. Infections by soft rot pathogens such as *Sclerotinia minor* involve cell dissociation and loss of integrity of cell membranes to produce the 'wet' symptoms therefore any agent that protects tissue from these phenomena would be expected to increase tissue resistance. Calcium fulfils that role. The presence of calcium may even modulate the expression of plant or pathogen produced endo-polygalacturonases as has been shown for *Erwinia carotovora* by Flego *et al.* (1994). These enzymes are considered important in pathogenesis by other soft-rotting pathogens including *Sclerotinia* spp. (Bateman and Beer, 1965).

The effect of growing plants in solutions providing other alkaline earth elements produced conflicting results. Plants provided with excess magnesium were more susceptible than those provided with charge equivalent concentrations of calcium while plants provided with strontium were more resistant. The relationship of magnesium to disease resistance has not been studied as intensively as that of calcium. In apple (*Malus domestica* Borkh.) treatment of fruit with calcium compounds (including chloride) has been shown to reduce

physiological decay and susceptibility to pathogenic attack (Ferguson, 1984; Tobias, Conway, Sams, Gross and Whitaker, 1993). Infiltration with magnesium salts increased the physiological disorder bitter pit (Burmeister and Dilley, 1994). At a cellular level excess Mg^{2+} may compete with Ca^{2+} for anionic binding sites in the apoplast (for example, carboxyl groups of pectic acid) and may interfere with Ca^{2+} channels and the role of calcium as a cellular messenger. Strontium, in contrast, reduced plant susceptibility possibly by occupying the apoplastic binding sites normally occupied by calcium. Infiltration of apple flesh with strontium increased the mechanical strength (Stow, 1989). Differential sensitivity to a continual supply of strontium was demonstrated by the two sunflower hybrids used in the present study. Different plant genera differ in their ability to grow in the presence of strontium (Creger, Holt and Lovelace, 1970). However, the observation made in this chapter of the occurrence of a varietal difference may be first record of this phenomenon in sunflower.

No correlations were found between the calcium content of roots or stems of eight sunflower inbred lines at 50% anthesis and the incidence of sclerotinia wilt. A recent report by Cassells and Walsh (1995) revealed a correlation (r=-0.90) between field susceptibility, calcium content of leaves and the ability of nodal shoots of four somaclones of *Helianthus tuberosus* to elongate on a calcium-free nutrient medium *in vitro*. They could not, however, discount the possibility of calcium carry-over during tissue transfer *in vitro*.

In the present study there were also no correlations between calcium contents of the eight inbred lines grown in solution culture and in the field. This is not surprising given the differences in nutritional status of the field sites and culture solutions. In solution culture the inbred Pac A3 grew vigorously and was more resistant than the other inbreds but under field conditions this inbred is highly susceptible as assessed by disease incidence. This difference may also be a result of different inoculation and assessment procedures. Reaction of solution cultured plants was measured as the rate of lesion progression along inoculated stems. Disease incidence in the field results from root infections.

A significant correlation that was observed among the inbred lines was between phosphorus level of roots and disease incidence. Variation in phosphorus content among sunflower germplasm has been reported (Blamey, Vermeulen and Chapman, 1980; Seiler, 1986) but this is the first indication that there may be a relationship between phosphorus content and susceptibility to sclerotinia wilt. Application of phosphate fertilisers have been associated with increased incidence and severity of a diverse range of diseases including *Septoria nodorum* on wheat (Leath, Scharen, Lund and Dietz-Holmes, 1993), *Peronospora farinosa* Fr. f.sp. *betae* Byf. on sugarbeet (Fargasova and Bojnansky, 1993) and *Verticillium dahliae* Kleb. on lucerne (Isaac, 1957) but in Experiment 7.3.8 additional

phosphorus was not applied so variation in content of this element is a genotypic response to available phosphorus. Phosphorus is such an important element fundamental to almost all, if not all, metabolic processes that it is difficult to suggest why higher tissue levels would render the host more susceptible to *Sclerotinia minor*. During pathogenesis free phosphates released from host cells may act with oxalate produced by the fungus to chelate calcium and cause further cellular disruption.

Application of superphosphate increased the incidence of wilt compared to control in two field trials. The effect was most marked in the trial at the LD91 site were disease incidence was lower than the site than at the LD92 site. Chun and Maric (1989) found that fertilising in general increased the incidence of sclerotinia wilt caused by *Sclerotinia sclerotiorum* but did not separate the effects of nitrogen and potassium from that of phosphorus.

It is concluded that calcium nutrition is important for resistance to sclerotinia wilt so situations where calcium supply is limited should be avoided. The data from this chapter do not support the use of calcium content of host tissue as a selectable characteristic or ideotype for breeding sunflower for resistance to sclerotinia wilt. However, further work should be directed at elucidating the relationship between phosphorus content and resistance.