



Role of Zn Nutritional Status on Infection of *Medicago* Species by *Rhizoctonia solani*

Tania C. Streeter

Submitted for the degree of Doctor of Philosophy
in the Department of Plant Science,
Faculty of Agriculture and Natural Resource Sciences
The University of Adelaide

June 1998

Table of Contents

Abstract	vii
Declaration	xi
Publications	xii
Acknowledgments	xiii
 CHAPTER 1	
General Introduction	1
 CHAPTER 2	
Literature Review	5
2.1 Zinc	5
2.1.1 Zinc in Plants - Historical Perspective	5
2.1.2 Areas of Zinc Deficiency	6
2.1.3 Zinc in the Soil	6
2.1.4 Causes of Zinc Deficiency	7
2.1.4.a Soil Conditions	7
2.1.4.b Interaction with Other Elements	9
2.1.4.c Genotypic Differences	10
2.1.5 Zinc Deficiency Symptoms in Plants	12
2.1.6 Plant Uptake of Zinc	13
2.1.7 Transport of Zinc Within Plants	14
2.1.7.a Xylem	14
2.1.7.b Phloem	14
2.1.8 Zinc Distribution Within Plants	15
2.1.9. Zinc Function in Plants	16
2.1.9.a Detoxification of the Activated Oxygen Species	16
2.1.9.b Protein Metabolism	17
2.1.9.c Carbohydrate Metabolism	18
2.1.9.d Cell Division	19
2.1.9.e Membrane Integrity and Permeability	19
2.1.9.f Growth Factor Metabolism	20
2.2 Rhizoctonia Root Rot	21
2.2.1 Introduction	21
2.2.2 Distribution	22
2.2.3 Characteristics of <i>Rhizoctonia</i> Species	22
2.2.4 Infection Process	24
2.2.5 Symptoms	25
2.2.6 Controlling <i>R. solani</i>	26
2.2.6.a Soil Disturbance	28
2.2.6.b Rotations	28
2.2.6.c Chemical Control	29
2.2.6.d Disease Suppressive Soils	29
2.2.6.e Host Resistance/Tolerance	29
2.2.6.f Fertiliser Application	30
2.3 Zinc Nutrition and Disease	31
2.3 Objectives of this Study	32

CHAPTER 3

Preliminary Experiments: Establishing Techniques	33
3.1 Introduction	33
3.2 Experiment 1	34
3.2.1 Aim	34
3.2.2 Materials and Methods	34
3.2.2.a Experimental Design.	34
3.2.2.b <i>R. solani</i> Inoculum	36
3.2.2.c <i>Rhizobium meliloti</i> Inoculum	36
3.2.2.d Soil	36
3.2.2.e Basal Nutrients	37
3.2.2.f Seed	37
3.2.2.g Procedure	37
3.2.3 Results	39
3.2.3.a Visual Observations	39
3.2.3.b Plant Growth Measurements	41
3.2.4 Discussion	46
3.2.5 Conclusion	48
3.3 Experiment 2	48
3.3.1 Aim	48
3.3.2 Materials and Methods	49
3.3.2.a Experimental Design	49
3.3.2.b Soil	49
3.3.2.c Basal Nutrients	49
3.3.2.d Seed	49
3.3.2.e Procedure	49
3.3.3 Results	50
3.3.4 Discussion	51
3.4 Experiment 3	52
3.4.1 Aim	52
3.4.2 Materials and Methods	52
3.4.2.a Experimental Design	52
3.4.2.b Soil	52
3.4.2.c Basal Nutrients	53
3.4.2.d Seed	53
3.4.2.5 Procedure	53
3.4.3 Results	54
3.4.4 Discussion	58
3.5 Experiment 4	59
3.5.1 Aim	59
3.5.2 Materials and Methods	60
3.5.2.a Experimental Design	60
3.5.2.b Soil	60
3.5.2.c Basal Nutrients	60
3.5.2.d Seed	60
3.5.2.e Procedure	60
3.5.3 Results	61
3.5.4 Discussion	62
3.6 Conclusion	62

CHAPTER 4	
Effect of Infection by <i>R. solani</i> on the Nutritional Status of <i>Medicago</i> species	64
4.1 Introduction	64
4.2 Pot Experiment	65
4.2.1 Aim	65
4.2.2 Materials and Methods	66
4.2.2.a Experimental Design	66
4.2.2.b <i>R. solani</i> Inoculum	66
4.2.2.c Soil	66
4.2.2.d Basal Nutrients	66
4.2.2.e Seed	66
4.2.2.f Procedure	66
4.2.3 Results	67
4.2.4 Discussion	73
4.3 Field Survey	77
4.3.1 Aim	77
4.3.2 Materials and Methods	77
4.3.2.a Site Description and History	77
4.3.2.b Plant Sampling	78
4.3.3 Results	79
4.3.3 Discussion	81
4.4 Conclusion	83
CHAPTER 5	84
Effect of Zn Supply on Infection by <i>R. solani</i> of <i>Medicago truncatula</i> genotype Caliph	84
5.1 Introduction	84
5.2 Pot Experiment 1	85
5.2.1 Aim	85
5.2.2 Materials and Methods	85
5.2.2.a Experimental Design	85
5.2.2.b <i>R. solani</i> Inoculum	86
5.2.2.c Soil	86
5.2.2.d Basal Nutrients	86
5.2.2.e Seed	86
5.2.2.f Procedure	86
5.2.3 Results	87
5.2.4 Discussion	92
5.3 Pot Experiment 2	93
5.3.1 Aim	93
5.3.2 Materials and Methods	94
5.3.2.a Experimental Design	94
5.3.2.b <i>R. solani</i> Inoculum	94
5.3.2.c Soil	94
5.3.2.d Basal Nutrients	94
5.3.2.e Seed	95
5.3.2.f Procedure	95
5.3.3 Results	98
5.3.4 Discussion	108
5.4 Pot Experiment 3	112
5.4.1 Aim	112
5.4.2 Materials and Methods	112
5.4.3 Results	113
5.4.4 Discussion	116
5.5 Conclusion	116

CHAPTER 6

Effect of Soil Zn Application on Growth of <i>Medicago</i> species.- Field Observations	118
6.1 Introduction	118
6.2 Effect of Residual Soil Zn Application	120
6.2.1 Aim	120
6.2.2 Methods	121
6.2.2.a Site History	121
6.2.2.b Procedure	122
6.2.3 Results	122
6.2.4 Discussion	128
6.3 Field Observations on Zn Response in Medics	129
6.3.1 Methods	129
6.3.1.a Site Description	129
6.3.1.b Genotypes	129
6.3.1.c Procedure	131
6.3.1.d Sampling	131
6.3.2 Results	132
6.3.3 Discussion	143
6.4 Conclusion	145

CHAPTER 7

Genotypic Differences in Growth of <i>Medicago</i> species in Soil of Low Zn status	147
7.1 Introduction	147
7.2 Aim	149
7.3 Materials and Methods	150
7.3.1 Experimental Design	150
7.3.2 Soil	150
7.3.3 Basal Nutrients	150
7.3.4 Seed	150
7.3.5 Procedure	150
7.4 Results	153
7.5 Discussion	166

CHAPTER 8

Effect of Zn Nutritional Status on Infection by <i>R. solani</i> in <i>Medicago</i> species. Differing in Zn Efficiency	172
8.1 Introduction	172
8.2 Aim	173
8.3 Methods	173
8.3.1 Experimental Design	173
8.3.2 Soil	173
8.3.3 Basal Nutrients	173
8.3.4 Seed	173
8.3.5 Procedure	174
8.4 Results	175
8.5 Discussion	186

CHAPTER 9	190
General Discussion	190
9.1 Improving Tolerance of <i>Medicago</i> species to <i>Rhizoctonia solani</i>	190
9.1.1 Zn Application	191
9.1.2 Genotypic Variation	191
9.1.3 Seed Quality	192
9.2 Mechanisms for Increased Tolerance	192
9.3 The Potential for Future Breeding of Zn Efficiency in Medics	193
9.3.1 Genetic Diversity for the Zn Efficiency trait in Medics	194
9.3.2 Agronomic and Economic Reasons for Pursuing a Breeding Solution to Zn Deficiency in Medics	195
9.4 How Does the Zn- <i>R. solani</i> Interaction Relate to the "Medic Decline" Currently Occurring Across the State?	196
CHAPTER 10	199
Conclusions	199
Appendix 1	201
Appendix 2	202
Appendix 3	203
Appendix 4	204
Appendix 5	208
Appendix 6	210
Appendix 7	211
Appendix 8	212
REFERENCES	218

Abstract

A decline in the quality of medic pastures has been observed across Australia which may be due in part to a causal relationship between Zn deficiency and increasing severity of *Rhizoctonia solani* infection. Zinc deficiency is widespread in Australia and with the use of minimum till farming practices, the incidence of infection by *R. solani* has increased on Zn-deficient soil. The primary aim of this thesis was to determine if Zn-deficient medics are more sensitive to infection by *R. solani*.

To determine if a relationship between Zn nutrition and infection by *R. solani* existed in medics, a field survey was performed in 1994. The paddock was infected with *R. solani* and Zn had been previously applied with varying rates of application. Since Zn fertiliser has a residual effect in the soil, and *R. solani* was present, this paddock was ideal for a survey. The concentration of Zn in the medic shoots was inversely related to the severity of disease.

To further understand the effects of *R. solani* on the nutritional status of medics, *Medicago truncatula* genotype Caliph was grown in a non-nutrient-limiting controlled environment with three levels of *R. solani* in the soil (0, 1 and 8 propagules/kg soil). Plants were harvested at 14, 28, 42 and 56 days. With increasing levels of *R. solani*, no change occurred in dry matter accumulation at any harvest time, nor was there any change in the concentrations of nine out of ten elements analysed in the shoot. Manganese was the only element to become lower in the shoot with increasing levels of *R. solani*. At this level of *R. solani*, the pruning effect of *R. solani* does not have a significant effect on the capacity for maintaining adequate levels of nutrients in the plant, when grown in non-nutrient-limiting conditions.

To determine the effect of *R. solani* (AG8) on the growth of medics with declining levels of Zn, a factorial experiment with five levels of Zn fertiliser (0, 0.05, 0.1, 0.3 and 0.9 mg Zn/kg)

and six levels of *R. solani* inoculum (0, 1, 2, 4, 8 and 16 propagules/kg soil) was performed. This experiment demonstrated for the first time an increase in tolerance to *R. solani* with Zn application in medics. Although the disease score on the root system declined with increasing level Zn fertiliser, the number of infection points on the root system was higher, indicating the presence of more pathogen on the roots of medics supplied with Zn.

A subsequent factorial experiment of four inoculum levels (0, 1, 8 and 32 propagules/kg soil) and two Zn levels (0 and 0.9 mg Zn/kg) was conducted to investigate the effect of infection by *R. solani* (AG8) on the root system in more detail. Compared with Zn-deficient medics, medics fertilised with Zn not only had a higher number of infection points on the root system as a result of infection by *R. solani*, but more *R. solani* DNA was extracted from the root systems. However, the addition of Zn allowed medics to reach maximum yield potential despite the heavier infection with *R. solani*. Medics also maintained a stable concentration in the shoot of all analysed nutrients when Zn was applied, while Zn-deficient plants had a reduction in nutrient concentration with severe levels of *R. solani*. In contrast to this, Zn-deficient plants in the presence of a low amount of *R. solani* (1 propagule/kg soil) increased concentration of most nutrients in the shoot. This occurred in parallel with an increase in fine root length and surface area, which was not observed in medics supplied with Zn. Overall, the results indicate Zn application does not directly inhibit the fungus or reduce its pathogenicity but does strongly increase root growth. The net result is that Zn increases tolerance to the pruning effects of the fungus, and this is most likely due to compensation in nutrient and water uptake from a greater mass of uninfected root axes.

A field experiment was performed to determine if residual levels of Zn applied three years prior to sampling were beneficial to a medic pasture. Zinc fertiliser was applied in the surface soil (0-0.05m) and in the sub-soil (0.05-0.40m) in combination with N and P fertiliser in 1993. Caliph medic was sampled in 1996 with a response to Zn fertiliser observed in concentration of shoot and in seed. A higher concentration of Zn occurred in seed when Zn was applied to the top soil (as granulated fertiliser), compared to the sub-soil (as a liquid

solution). This is most probably due to the slow-release nature of granulated Zn. Although a response to Zn occurred, all plants were deficient in Zn. Zinc fertiliser should therefore be applied at higher concentrations in the first instance or applied more frequently.

Field and pot experiments were performed to determine if Zn-efficient medics could be identified. Twelve genotypes were sown in the field on Zn-deficient soil with two levels of Zn (0 and 8 kg Zn/ha), while 19 genotypes were planted in a controlled environment with four levels of applied Zn (0, 0.1, 0.3 and 0.9 mg Zn/kg soil). Overall, four genotypes from *M. tornata* were identified as Zn-efficient (Rivoli, SA20248, SA20278 and SA20521). Rivoli was grown in both experiments and ranked Zn-efficient in each. Although Rivoli did not have a high yield potential, yield potential was reached at the lowest level of Zn in both the field and pot experiments. The remaining three Zn-efficient genotypes were high yielding under Zn deficiency but maximum yield occurred under conditions of non-limiting Zn only. The effect of seed size was also studied in the field on two of genotypes planted using small and large seed. Although differences in seed size influenced final yield, the Zn efficiency ranking was not affected.

The final experiment was designed to determine if Zn-efficient genotypes have a higher tolerance to *R. solani* under Zn-deficient conditions than Zn-inefficient genotypes. Five genotypes, two Zn-efficient (SA20278 and SA20521), one moderately Zn-efficient (Caliph) and two Zn-inefficient (SA18289 and Z1064) were grown under Zn-deficient conditions in a controlled environment, and seed collected. Since the seed was low in Zn, Mg, Na, K and P it had a lower nutritional value than seed used in previous experiments. This seed was used in the subsequent experiment. A factorial experiment with five genotypes, four levels of *R. solani* (0, 1, 8 and 32 propagules/kg soil) and two Zn applications (0 and 0.9 mg Zn/kg) was conducted. All genotypes generally had a lower dry matter accumulation with increasing levels of *R. solani* at both Zn treatments. Unlike the remaining genotypes, Zn-efficient genotypes maintained a stable concentration of all nutrients in the shoot under Zn-deficient conditions, indicating a higher tolerance to the pathogen. The use of low quality seed resulted

in a reduction in tolerance to *R. solani* and a lower Zn-efficiency ranking in all genotypes.

This thesis identifies a strong relationship between Zn nutrition of medics and severity of disease by *R. solani* (AG8) which may therefore be a major factor contributing to the current decline in medic pastures observed in Australia. The effect of seed nutritional value had a profound impact on the growth of medics and may also be a key factor in poor medic growth.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person except where reference has been made in the text.

I consent to a copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Tania Streeter

June, 1998

Publications

- Streeter, T.C.**, Graham, R.D., Lake, A.W.H. and Rengel, Z. (1998) Potential for breeding *Medicago* spp. for Zn efficiency to increase tolerance to *Rhizoctonia solani*. Proc. Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition (in press)
- Streeter, T.C.**, Cooke, J.A., Neate, S.M. and Graham, R.D. (1997) Effect of Zn application on infection by *R. solani* on wheat and medics. Australasian Plant Pathology Society, 11th Biennial Conference, Perth, p70.
- Streeter, T.C.**, Lake, A.W.H., Neate, S.M., Rengel, Z. and Graham, R.D. (1997) Effect of Zn application in medics on infection by *Rhizoctonia solani*. Farming Systems Developments, Adelaide, March 18-20, pp. 77-78.
- Streeter, T.C.** (1996) The tolerance of annual medics to *Rhizoctonia solani* (AG8) in relation to zinc in Australia. Rhizoctonia Newsletter, 1(2): 3.

Acknowledgments

I would like to express my appreciation to the following people for their assistance:

- to my main supervisor, Professor Robin Graham, for his help and comments throughout the course of my studies and for providing support and encouragement when needed the most;
- to my second supervisor, Dr. Zdenko Rengel, for providing additional comments (in record time) despite moving to Western Australia;
- to co-supervisors, Dr. Stephen Neate and Mr. Andrew Lake, for assistance and guidance when required;
- the Grains and Research Development Corporation for funding this research;
- to the Medic Breeding Unit at SARDI, in particular Mr. Jake Howie, for providing seed and advice at any time, and for assistance with field trials;
- to the ICP laboratory, in particular Mrs. Teresa Fowles for processing samples for analysis;
- to Mr. Jim Lewis, for his advice and patience in explaining field trial design and for sowing and spraying field trials;
- to Mr. Noel Pederson for use of root scanning equipment;
- to Dr. Kasia Siwek for analysis of *R. solani* DNA;
- to the members of the Crop Nutrition Group for interesting discussions and continual support during my studies;
- to Mrs. Lorraine McMahon, for bringing order to the group;
- to my family for understanding and supporting me during my studies;
- to Ben, for his continual love and support.



CHAPTER 1

General Introduction

“It is our will and pleasure that you do immediately upon your landing after taking measures for securing yourself and the people who accompany you from any attacks or interruptions of the natives...proceed to the cultivation of the land...and with all convenient speed transmit a report of the actual state and quality of the soil...and the most effectual means of improving and cultivating the same” (George III, 1787 cited in Shaw, 1967).

Since King George gave these instructions to Governor Phillip before establishing the penal settlement at Botany Bay, New South Wales, improving Australian agriculture has been of utmost importance. With an abundance of unfarmed land, Australian agriculture was originally exploitive (Donald, 1967) with settlers developing new land rather than attempting to increase crop yields on a per hectare basis. On newly developed land in the mid 1800's, average wheat yields were slightly less than 1000 kg/ha. As a result of nutrient depletion, average wheat yields in Australia had dropped to less than 500 kg/ha by 1900 (Crawford *et al.*, 1989). Over the next four decades, yields increased as a result of phosphatic fertiliser use, fallowing, and new cultivars, but degradation in soil structure and erosion as a result of fallowing became common problems faced by farmers.

By 1945, a pasture ley between crops was shown to improve soil structure (Stephens *et al.*, 1945) and the value of annual medics had been established (Trumble, 1939). This led to the development of the classic Australian ley farming system. In ley farming, cereal crops are alternated with pasture leys based on annual legumes, principally medics. The legumes self-regenerate from the softening of hard seed set in previous pasture years. A good legume pasture increases soil fertility through nitrogen fixation and also provides high quality feed

for stock. Legume pastures also have the advantage of acting as a break between cereal crops, particularly for diseases. Since the introduction of pasture legumes into cropping rotations, cereal yields and stock numbers increased rapidly through to the early 1960's (Sheaffer and Lake, 1998).

In the latter half of the 1970's a decline in the quality of pasture leys in traditional regenerating systems began to emerge and has continued (Reeves and Ewing, 1993). This decline was indicated by a reduction in legume content and by declining seed reserves in pastures. Although medics are not the only legumes affected, their pre-eminent role in these ley systems has meant that they have been the major focus of this decline, which is hence referred to as the "medic decline" (A. Lake, pers. comm.).

Medic decline is a result of a range of biological, management and economic factors (Carter *et al.*, 1982). The main problem is believed to be due to density of medic regeneration (Wheeler, 1987), since medics rely on seed set during previous ley seasons. Hence, any factors which reduce medic growth and seed set in the pasture phase of the rotation can have a compound effect over many years. More frequent cropping therefore results in a reduction in the amount of medic seed capable of regenerating. Economic factors have also contributed to the decline of many medic pastures. These include the reduction in profitability of sheep enterprises (Vere and Muir, 1986), the increase in fertiliser price - for example, super-phosphate rose from \$18/t to \$130/t in the mid 1980's (French, 1987) and is now \$390/t- and the "hidden" benefits for growing medics in a ley farming system, which occur indirectly through increased yield and quality of the cereal crop (Sheaffer and Lake, 1998).

Other factors involved in the "medic decline" include: pests such as various aphid species, Sitona weevil, and red-legged earth mite; phytotoxins from cereal straw which influence germination, emergence, establishment and productivity of medics (Leigh *et al.*, 1995); poor grazing management; plant diseases; and decline in the use of fertiliser, particularly phosphate, and by default Zn contained naturally in the phosphate. It has been hypothesised

that severity of Zn deficiency in Australia has increased as a result of increased use of high analysis phosphatic fertilisers from being used, which do not contain significant amounts of Zn as an impurity (Ozanne *et al.*, 1965). Of particular interest to this study is the effect of declining Zn fertiliser on medics and the increase in plant disease, notably *Rhizoctonia solani*.

R. solani is a world-wide root disease, infecting Australian pastures and crops since at least the 1920's when it was first recorded (Samuel, 1928). The incidence of *Rhizoctonia* infection, or bare patch disease as it is commonly known, increased in areas with sandy soil since the late 1970's in parallel with increased use of minimum tillage methods. Reduced soil disturbance, for example, direct drilling versus conventional cultivation, has the advantage of reduced soil erosion and reduced burial of seed too deep for successful emergence (Carter and Challis, 1987). However, minimum tillage methods are also associated with increased *Rhizoctonia* severity (Dubé *et al.*, 1987).

Like *Rhizoctonia* disease, Zn deficiency is widespread in calcareous and/or siliceous sands affecting cereal plants from 90% of farms in the Mallee region in South Australia (Graham *et al.*, 1990).

Since these two perceived problems, an increase in *Rhizoctonia* bare patch and an increase in Zn deficiency, occurred simultaneously, they may be causally linked. This relationship was first hypothesised over fifty years ago when Millikan (1938) observed less disease in plots receiving 17-35 kg ZnSO₄/ha when root diseases were severe in the Mallee region of Victoria. This work was not continued until Thongbai *et al.* (1993a; 1993b) demonstrated in wheat that Zn application of 2.5 kg Zn/ha reduced the area affected by bare patch from 42% to 21% of the total crop area, while also overcoming Zn deficiency and increasing grain yield from 1.0 to 2.8 t/ha. This study also demonstrated that in the initial stages of the interaction, disease did not increase Zn deficiency, but rather, Zn sufficiency suppressed disease severity. A causal link between Zn nutrition and *Rhizoctonia* root rot severity was firmly established

for the first time.

Medics, like the cereals, are susceptible to both *Rhizoctonia* root rot and Zn deficiency. The severity of disease and Zn deficiency appears to have increased since the late 1970's and at least in part are likely factors in the medic decline that is being observed. Since these factors have occurred gradually over a longer period they have been overshadowed by other problems such as the medic aphids. The primary hypothesis of this project is that Zn deficiency decreases the tolerance, or increases the susceptibility, of *Medicago* species to the fungus *Rhizoctonia solani*, predisposing the plants to an increase in bare patch disease.

The objectives of the project were:

- to investigate if medics in Zn-deficient soil are more susceptible/tolerant to *Rhizoctonia* root rot than when Zn is sufficient;
- to determine if *Medicago* species differ in their sensitivity to Zn deficiency; and
- to investigate across a range of medic cultivars if their susceptibility/tolerance to *Rhizoctonia* in Zn-deficient soil is a function of their sensitivity to Zn deficiency.

CHAPTER 2

Literature Review

A review of literature regarding Zn nutrition in plants, *Rhizoctonia solani* and the interactions between Zn nutrition and the disease is presented. The description begins with a general review of Zn deficiency in Australia and the role of Zn in the plant. This is followed by a review on *R. solani*, its mode of infection and possible mechanisms of control. The review then covers the effect of Zn interactions on disease control.

2.1 Zinc

2.1.1 Zinc in Plants - Historical Perspective

The importance of Zn was first recognised in 1869 by J. Raulin, who demonstrated that Zn stimulated the growth of *Aspergillus niger*. This was followed by the work of K.A. Timiryazev in 1872 when he demonstrated that Zn not only affected growth and development, but also alleviated chlorosis in maize (Shkolnik, 1984). Zinc was finally established as an essential nutrient in 1928, almost 60 years later, when Sommer and Lipman (1926) confirmed the requirement of Zn for growth in buckwheat, bean and barley plants. In 1932 the first widespread commercial use of Zn salts in the field occurred and the use of Zn as a valuable fertiliser and spray material was accepted (Camp, 1945). Since then much work has been performed studying the physiological role of Zn, with the conclusion that Zn is an essential micronutrient involved in protein synthesis and maintaining the integrity and permeability of biological membranes. Even though the importance of Zn in biological systems is now recognised, Zn deficiency remains the most common micronutrient deficiency world-wide (Sillanpää, 1990) resulting not only in significant losses in crop production, but also in Zn deficiencies in animals and humans (Graham and Welch, 1996).

2.1.2 Areas of Zinc Deficiency

Zinc deficiencies have been reported throughout the world, with tropical and mediterranean regions most affected (Leon *et al.*, 1985). Lindsay (1972) reported the majority of states in the United States experienced low soil Zn levels, along with Australia, Israel and 10 of 15 European countries studied. Zinc deficiency has been reported in six countries in Africa (Kang and Osiname, 1985) and also throughout Asia (Katyal and Vlek, 1985).

In Australia, much of the soil used for cropping is severely Zn-deficient. It was first recognised as a problem by Millikan (1938) in the grey clay soils of the Wimmera district in Victoria. Other areas include soils such as the terra rossas, sandy podsolics, solodised solonetz, groundwater rendzinas and calcareous sands of the South-East of South Australia (Blackburn, 1964, cited in Donald and Prescott, 1975), the podzolic, lateritic podzolic, yellow earths and calcareous sands of the South-West of Western Australia, and the black earths of Queensland and New South Wales (Donald and Prescott, 1975). More recently, Hannam (1990) reported soils of the Murray Mallee and Eyre Peninsula in South Australia to be low in Zn, while it has been claimed by Welch *et al.* (1991) that the western wheat belt in Western Australia is the largest single area of Zn deficiency in the world.

2.1.3 Zinc in the Soil

According to Goldschmidt (1954) the average Zn concentration in the earth's crust is 80 mg/kg, ranging from 10-300 mg/kg (Swaine, 1955). Zinc content varies depending on the type of rock; igneous rocks, such as basalt, contain 100 mg/kg Zn on average; granite contains only 40 mg/kg. In comparison, sedimentary rocks vary more widely, with shale containing 95 mg/kg while limestone and sandstone average 20 and 16 mg/kg respectively (Lindsay, 1972).

The total amount of Zn in soils is considered to be uniform throughout the soil profile, but the amount of extractable Zn decreases with depth (Lindsay, 1972). Zinc does not move down the

profile as it is readily fixed and immobilised on soil surfaces, so the increase in Zn availability in the upper soil layers is due to organic matter and bio-residues which contain and release Zn during decomposition (Brennan and McGrath, 1988).

Adsorption of Zn is an important factor determining the availability of Zn in soils. Two adsorption mechanisms are proposed which depend on the pH of soil. In low pH soils, adsorption of Zn is related to cation exchange sites, while in alkaline soil adsorption is considered to be chemisorption which is influenced by organic ligands (Kabata-Pendias and Pendias, 1992). Since adsorption of Zn^{2+} is reduced by competing cations in acidic soils, an increase in the mobility of Zn results (Kabata-Pendias and Pendias, 1992). However, with every unit increase in pH value of the soil, Zn^{2+} activity theoretically decreases 100-fold resulting in low Zn mobility in alkaline soil (Lindsay, 1972).

2.1.4 Causes of Zinc Deficiency

Many factors contribute to Zn deficiency in plants, either separately or in conjunction with other factors. The main causes are a result of soil type and conditions, interactions of other elements with Zn and differential responses of plant species and genotypes.

2.1.4.a Soil Conditions

The earth crust has a stable Zn content, however the Zn content of individual soils may vary (Harmsen and Vlek, 1985). For this reason, sandy soils tend to be Zn-deficient as quartz has a low Zn content of approximately 10-30 ppm (Lindsay, 1972). However, the available micronutrient content in the soil is more dynamic, depending on soil and climatic conditions and changing with time and depth in the soil profile (Harmsen and Vlek, 1985).

Acidic soil may be low in available Zn even if total Zn content is high. This is due to Zn^{2+} mobility increasing in low pH soils, as mentioned in the previous section, so that when it is released by weathered minerals Zn is rapidly leached from the profile (Harmsen and Vlek,

1985). In alkaline soils, Zn deficiency may also occur due to decreased Zn^{2+} activity in the soil solution. An example of this can be seen in calcareous soil with high total Zn content. As the pH increases, available Zn concentration is reduced as adsorption of Zn by carbonate occurs. Overall, Zn deficiency occurs more frequently on alkaline than on acid soil (Thorne, 1957).

The most predictable occurrences of Zn deficiency are in areas where the surface soil has been removed. This may occur as a result of erosion, often accelerated by heavy tillage from farmers. Cutting (ie levelling) the soil results in lower organic matter and a higher pH in the exposed subsoil (Lindsay, 1972).

Soils with restricted root zones: hardpans, high water tables and compacted areas, and soils at low temperatures tend to be deficient in Zn (Lucas and Knezek, 1972 cited in Lindsay, 1972). Low soil temperatures restrict microbial activity and the establishment of plant root systems and this then restricts the release and uptake of Zn respectively from organic matter (Lindsay, 1972). This can be observed in annuals where Zn deficiency symptoms occur in cool winter months and may disappear with warmer conditions (Brennan *et al.*, 1993).

As a result of the reaction of Zn with free sulphide or with sesquioxides, Zn deficiency is associated with flooded soils (Takkar and Walker, 1993). This is a result of a decrease in the redox potential of the soil causing insoluble sulphides of Zn to form, thus decreasing Zn availability. Providing the redox potential of the soil does not decrease greatly, availability of Zn may increase under flooded conditions as occluded iron and aluminium oxides dissolve (IRRI, 1970; Ponnampetuma, 1972).

High levels of available soil phosphorus and nitrogen have also been implicated in contributing to Zn deficiency (Lindsay, 1972), although the effect of nitrogen has been reported to be less severe. The mechanism of their effects on Zn has yet to be fully determined and is discussed in the next section.

To summarise, Zn deficiency may be associated with many soil types, including sandy, acidic and alkaline soils, soils with the top soil removed, have a restricted root zone, low temperature, low organic matter, are flooded, or have high available phosphorus and nitrogen concentrations.

2.1.4.b Interaction with Other Elements

Several anions, including HPO_4^{2-} , H_2PO_4^- , NO_3^- and SO_4^{2-} , have been shown to inhibit Zn uptake and absorption, resulting in Zn deficiency (Ozanne *et al.*, 1965; Rashid *et al.*, 1976; Leece, 1978; Panavasasivam and Axley, 1982). Zinc deficiency may result from the interactions of Zn with other elements, with some functions of Zn replaced by other dissimilar elements (Brown *et al.*, 1993). With Cd and Zn, both antagonism and synergism have been observed between the two elements in the uptake-transport processes. The ratio of Cd to Zn in the growth medium is believed to control what reaction will occur between the cations (Kabata-Pendias and Pendias, 1992) and probably whether there is a growth response to Zn.

Other elements to interact with Zn include Cu, Fe, P, N, Ca, Mg and Mn (Kabata-Pendias and Pendias, 1992). Zinc and Cu interact antagonistically whereby the uptake of one element is competitively inhibited by the other. This suggests the same carrier site/channel in absorption mechanism for both elements (Graham, 1981). This should not be surprising as Cu is located next to Zn on the periodic table and has a similar radius (hydrated radii 0.42 and 0.43 nm for Cu^{2+} and Zn^{2+} respectively) (Kochian, 1993). Copper and Zn may also interact antagonistically as Cu affects the redistribution of Zn within plants (Loneragan and Webb, 1993). This interaction is common in southern Australia where many sandy soils are deficient in both Zn and Cu.

Manganese also inhibits uptake of Zn, but it is less competitive than Cu as its hydrated ionic radius is slightly larger (Lindsay, 1972). Zinc and Fe antagonism and synergism have also been observed (Graham *et al.*, 1987). The competition between Zn^{2+} and Fe^{2+} in the uptake

processes and the interference in chelation processes during the uptake and translocation of Fe from the roots to tops are two possible mechanisms for Zn-Fe antagonism. Synergism between the two elements is reported to be linked with P supply (Kabata-Pendias and Pendias, 1992).

The Zn-P interaction remains complex, with P able to decrease or increase Zn concentrations in plant shoots. Four mechanisms have been postulated for the depression in Zn absorption from the soil by P: 1) P suppresses root infection by VAM which are responsible for significant Zn uptake in plants; 2) cations added with P inhibit Zn absorption from solution; 3) H⁺ ions generated by P inhibit Zn absorption from solution; and 4) P enhances the sorption of Zn to soil components (Loneragan and Webb, 1993). Two effects that P application may have on enhancing Zn concentrations within plants include: 1) an increase in the acidity of the root environment; and 2) an inadvertent increase in Zn supply from Zn contamination of the P fertilisers (Loneragan and Webb, 1993). There is no direct evidence which suggests P induces Zn deficiency by inhibiting Zn transport from roots to shoots, however under high Zn supply P may immobilise Zn in the roots through formation of Zn phytate (Loneragan and Webb, 1993).

Nitrogen may both reduce and enhance the effect of Zn deficiency (Loneragan and Webb, 1993). The strongest interactions have resulted from the effects of N in promoting plant growth and to altering the pH of the root environment. The Zn-N interaction has been described by Kabata-Pendias and Pendias (1992) as a secondary "dilution" effect due to the increase in biomass with heavy nitrogen treatment.

2.1.4.c Genotypic Differences

Crops differ in their ability to extract Zn from soil. For example, lucerne was shown to extract adequate amounts of Zn from soil on which grasses suffered a deficiency (Hoagland, 1944 cited in Shkolnik, 1984). It has also been shown that bean, maize, and cotton are all sensitive to Zn deficiency compared to wheat, oat or pea (Marschner, 1995). Differences between species in Zn acquisition are believed to be related to inherent differences in rhizosphere pH,

root exudation, or root colonisation with VA mycorrhizas (Thompson, 1990; Marschner, 1995).

Different sensitivities to Zn-deficient soils can be observed not only among plant species, but also among varieties of the same species. Examples of differences in Zn sensitivity can be seen within genotypes of bean (Ambler and Brown, 1969), maize and sorghum (Ramani and Kannan, 1985), potato (Sharma and Grewal, 1990), tomato (Parker *et al.*, 1992), millet (Takkar *et al.*, 1988), and wheat (Graham, 1991), with studies on cereals dominating the literature (Graham and Rengel, 1993; Rengel and Graham, 1995b). To date, no studies on Zn efficiency in annual medics have been performed.

Agronomically, Zn efficiency has been defined as the ability of a cultivar to grow and yield well in soils too deficient in Zn for a standard cultivar of that species (Graham *et al.*, 1992), using the equation:

$$\text{Zn efficiency} = \frac{\text{Shoot dry weight in Zn - deficient soil}}{\text{Shoot dry weight in Zn supplemented soil}} * 100\%$$

This definition adjusts for differences in yield potential among all cultivars, and does not imply a mechanism involved. In most cases, genotypic differences in Zn efficiency have proved to be due to increased root ability to extract Zn, to make greater contact with soil Zn, or to increase Zn availability in the soil, and not a lower Zn requirement within the plant (Graham *et al.*, 1992). Efficiency definitions based on differences in internal Zn requirement of the plant are rarely appropriate.

An increase in the extraction of Zn from the soil has been shown to occur in wheat by the release of Zn-mobilising phytosiderophores from the roots of cereals (Cakmak *et al.*, 1996b). However, Erenoglu *et al.* (1996) observed an imperfect correlation between the capacity to exude phytosiderophores and the level of tolerance to Zn deficiency, indicating that mechanisms other than phytosiderophore exudation are important in the expression of Zn efficiency in cereals. Other physiological mechanisms important for Zn efficiency include a greater contact with soil Zn by enhancement of root growth. It has been demonstrated that Zn-

efficient wheats have longer, thinner roots and have a greater proportion of thinner roots in the total biomass early in the growth period compared with Zn-inefficient wheat (Dong *et al.*, 1995). The authors postulated that long, thin roots may be one of the contributing, though non-specific, characters associated with Zn-efficient cultivars. Since the concentration of Zn did not differ in leaves of cereals with varying Zn efficiency rankings, Graham and Rengel (1993) have also suggested differential Zn utilisation and/or compartmentation in leaf cells may be involved in Zn efficiency. Zinc-efficient cultivars may have a higher proportion of their Zn in a mobile and physiologically active form to participate in metabolic reactions and to maintain the activity of Zn-requiring enzymes at a high level. Direct evidence for this comes from a comparison of rye and wheat where rye has a higher Zn efficiency than wheat cultivars. Cakmak *et al.* (1997b) found that under severely Zn-deficient conditions, and at similar leaf Zn concentrations, rye maintained a higher activity of Zn-containing superoxide dismutase than wheat.

2.1.5 Zinc Deficiency Symptoms in Plants

In most crops and pasture plants the levels of Zn range from 10 to 100 mg/kg, with the critical deficiency concentration in plant tissues between 10 and 20 mg/kg dry weight (Lindsay, 1972; Kabata-Pendias and Pendias, 1992).

The first visual sign of Zn deficiency is interveinal chlorosis in older leaves (Lindsay, 1972). Further Zn deficiency symptoms are more noticeable during the earlier stages of growth, in particular in annuals, and include stunted growth, decreased internode and petiole elongation and stunting of young leaves (Longnecker and Robson, 1993). The small malformed leaves caused by Zn deficiency are known as "little leaf" and result in the classic "rosette" symptom in young growth of dicotyledons and "fan shaped stem" in monocotyledons (Brennan *et al.*, 1993). Root systems may also be much smaller in Zn-deficient plants (Brennan *et al.*, 1993).

2.1.6 Plant Uptake of Zinc

The form in which Zn is taken up by the roots has not yet been precisely defined, although predominating uptake as a divalent cation (Zn^{2+}) is generally accepted (Marschner, 1995). Chelating agents, which increase the solubilisation of Zn from soils, facilitate more rapid diffusion of Zn to plant roots. Previous work suggested that Zn dissociates outside the root and the complexed form is not taken up (Halvorson and Lindsay, 1977). However, Reid *et al.* (1996) found the low Zn activities used in chelate-buffered solutions were insufficient to supply plants with levels of Zn adequate for growth and suggested Zn may be taken up in chelated form across the cell membrane.

A linear increase in net Zn uptake has been shown to occur in barley (Welch and Norvell, 1993) and wheat (Rengel and Graham, 1996) with an increase in solution Zn^{2+} activity. This is consistent with recent suggestions on passive movement of Zn through a non-selective divalent cation channel (Kochian, 1993). However, Zn uptake may still be dependent on metabolism since low temperature and metabolic inhibitors have been shown to decrease uptake (Bowen, 1969; Giordano *et al.*, 1974; Reid *et al.*, 1996). Reid *et al.* (1996) proposed a proton-linked carrier system, which exchanges 2 H^+ for one Zn^{2+} regulated by ATPase, to account for the lack of response in Zn influx when membrane potentials were clamped. Since high- and low-affinity uptake of Zn occurs depending on the concentration of Zn available in the solution, they also suggested that more than one type of channel is involved in Zn uptake.

In graminaceous species, under Zn-deficient conditions, a specific adaptive uptake mechanism operates. This mechanism involves the release of non-proteinogenic amino acids, known as phytosiderophores. Phytosiderophores are chelating agents which are effective at mobilising Zn^{2+} compounds. This mechanism has been suggested to occur as a result of Zn-deficiency-induced iron deficiency (Walter *et al.*, 1994) where the release of phytosiderophores is repressed in Zn-deficient plants after foliar spray of iron.

2.1.7 Transport of Zinc Within Plants

Zinc was measured in xylem sap in concentrations ranging from 2-85 μM (White *et al.*, 1981), while in phloem sap levels were 3 and 170 μM (Robson and Pitman, 1983). Older work suggested that Zn is transported only in the xylem and is immobile in the phloem (Orphanos, 1975), while more recent studies demonstrate intermediate phloem mobility (Pearson and Rengel, 1995a; 1995b). This earlier conclusion of phloem immobility appears incorrect and with improved techniques more complex transport processes are emerging, as described below.

2.1.7.a Xylem

Zinc absorbed by the roots is rapidly transported to the shoots, with transport from roots to shoots in wheat detected within 30 minutes (Pearson and Rengel, 1995a). With Zn accumulating in expanding leaves and not older leaves, it has been postulated that Zn transport from the roots into the leaves is primarily determined by the relative transpiration rate of the leaves (Obata and Kitagishi, 1980; Pearson and Rengel, 1995a). It has also been suggested that transport of Zn in the transpiration stream is not simply a passive process, with Reay (1987) demonstrating the flux of Zn to be five times greater than that of silicon which exhibits passive movement. This conclusion about Zn may be explained by phloem transport of Zn, rather than metabolic control of xylem transport.

2.1.7.b Phloem

Although it has been shown that plants are able to translocate Zn in their phloem tissue (Loneragan *et al.*, 1987), very little research has been conducted. In split-root experiments, the transport of Zn has been observed from root zones supplied with Zn to root zones with no external Zn supply (Webb and Loneragan, 1990) and from root zones exposed to ^{65}Zn to root zones not exposed (Pearson and Rengel, 1995a). Since transfer of ^{65}Zn in the unexposed roots occurred shortly after accumulation within the wheat crown, Pearson and Rengel (1995a) suggested loading of Zn from the xylem to the phloem occurs in the crown.

In the study by Webb and Loneragan (1990), Zn was shown to have high mobility in the plant. Here the supply of Zn was adequate to the shoots even though Zn was omitted from the external environment of half the root system. However, the authors proposed that translocation of Zn to the root system not supplied Zn was inadequate to maintain all root functions, suggesting Zn has intermediate mobility. Retranslocation of Zn in plants may then be considered variable, depending on the Zn supply to the plant and favouring upward movement to shoots (Webb and Loneragan, 1990; Longnecker and Robson, 1993).

2.1.8 Zinc Distribution Within Plants

Zinc distribution in plants is affected by the level of Zn supply and plant age. At low levels of supply, Zn concentrations are higher in the growing tissue than in mature tissue (Longnecker and Robson, 1993), while at toxic levels, a large proportion of the total Zn taken up accumulates in the root system (White *et al.*, 1979). Changes in Zn concentration with plant age have also been reported, with concentration highest in very young seedlings and reducing with age.

Considerable fluctuations in Zn level have been observed within plants during vegetative growth (Scheffer *et al.*, 1978 cited in Kabata-Pendias and Pendias, 1984). In their study, the highest Zn content of barley leaves, sheaths and internodes was always during the phase of intensive growth. However, the variation in the Zn content of wheat was small with the content increasing throughout the growth period. It has been calculated that up to 75% of total Zn taken up is in the tops of young plants, whereas in old plants 20-30% occurs in the top (Baumeister and Ernst, 1978 cited in Kabata-Pendias and Pendias, 1992).

Using the radioisotope ^{65}Zn , Polar (1976) demonstrated that Zn concentration in root cells was highest in the water-soluble Zn fraction. This is probably due to the association of Zn with enzymes and low-molecular-weight organic compounds. The water-soluble Zn fraction is usually considered to be the physiologically active fraction and is regarded as a better indicator of

Zn status than is total Zn content (Brown *et al.*, 1993). The cell-wall debris contained the next largest fraction.

In shoots Zn has been reported to be concentrated primarily in chloroplasts, cell membranes and vacuole fluids (Tinker, 1981). Various cell wall compounds (e.g. lignin, cellulose, hemicellulose) possess a high binding affinity for Zn, indicating that the cell wall may play a role in controlling free Zn activity (Brown *et al.*, 1993). However, the significance of cell wall binding of Zn is controversial, particularly as the total binding capacity of the cell wall may be too small to be physiologically significant (Brown *et al.*, 1993).

Zinc content and concentration in seeds vary significantly (Longnecker and Robson, 1993), influencing the performance of a particular genotype under Zn-deficient conditions (Graham and Rengel, 1993). Its concentration is highest in the embryo, supporting the suggestion of Milthorpe and Moorby (1979) that Zn is required by seedlings for maximum growth during emergence. Zinc also plays an important role in the reproductive process, being in high concentration in the protoplasm of the oosphere and synergids of the female gametophyte (Shkolnik, 1984). Pollen grain development under Zn-deficient conditions in both wheat and maize is abnormal (Sharma *et al.*, 1987).

2.1.9. Zinc Function in Plants

Zinc is an essential element to various aspects of plant physiology and biochemistry, playing a major role as constituent and activator of more than 300 enzymes (Vallee and Auld, 1990). Zinc is also involved in hormone biosynthesis, structural stability of organelles, cytochrome c synthesis, protein synthesis, and stability and integrity of the root cell membrane (Kochian, 1993).

2.1.9.a Detoxification of the Activated Oxygen Species

Zinc plays a key role in preventing the destruction of cellular components by controlling both generation as well as detoxification of free oxygen radicals. A number of activated oxygen

species may form in living cells, with the superoxide radical ($O_2^{\cdot-}$) most prominent. It forms during several different biochemical reactions in the cytosol (Kellogg and Fridovich, 1975), the cell wall (Mader and Amberg-Fisher, 1982), mitochondria (Takeshige and Minakami, 1979) and chloroplasts (McRae and Thompson, 1983). The destruction caused by $O_2^{\cdot-}$ to cellular components is mainly a result of its derivatives which are much more reactive. These derivatives include the hydroxyl radical $OH^{\cdot}$, hydrogen peroxide (H_2O_2) and singlet oxygen $^1O_2$ (Cakmak and Marschner, 1988a). They are known to be major mediators of peroxidative damage to various cellular components such as membrane fatty acids, proteins, nucleic acids and chlorophyll (Cakmak and Marschner, 1988a).

To prevent an accumulation of $O_2^{\cdot-}$ within the cell, the enzyme superoxide dismutase (SOD) converts $O_2^{\cdot-}$ into H_2O_2 , which is subsequently converted by catalase into H_2O and O_2 (Cakmak and Marschner, 1988a; 1988c). Zinc is therefore essential in preventing destruction of cellular components as it is required by SOD and catalase for their enzymatic activity. Furthermore, the use of NADPH by oxidases is strongly inhibited when Zn^{2+} is present (Chvapil, 1973; Jeffery, 1983).

Under Zn-deficient conditions, Cakmak and Marschner (1988a; 1988c) observed a reduction in SOD activity before a depression in growth rate. An increase in NADPH oxidation and NADPH-dependent superoxide radical generation in microsomal membranes and in the cytosol also occurred along with a reduction in catalase activity. Cakmak and Marschner (1988a) hypothesised that catalase activity may be inactivated by superoxide radicals or as a result of depressed catalase synthesis due to impaired protein synthesis.

2.1.9.b Protein Metabolism

Zinc plays a role in maintenance of structural integrity of RNA and ribosomes (Sherstnev *et al.*, 1970 cited by Praske and Plocke, 1971; Shkolnik, 1984; Kitagishi *et al.*, 1987). In Zn-deficient plants, although protein composition tends to remain unaltered, the amount of protein synthesised is dramatically reduced, while amides and amino acids accumulate (Cakmak and

Marschner, 1990; Brown *et al.*, 1993). Zinc is also required for the activity of RNA polymerase and protection of ribosomal RNA from attack by ribonuclease (Brown *et al.*, 1993). The effect of Zn deficiency on the degree of protein synthesis is therefore attributed to an impairment of synthesis and structural integrity of RNA and ribosomes as well as higher activity of RNase (Cakmak *et al.*, 1989; Brown *et al.*, 1993). Zinc deficiency has a greater effect on the biosynthesis of RNA compared with enhancement of RNase activity (Brown *et al.*, 1993).

Recently, Zn-dependent protein molecules, known as Zn-metalloproteins, have been identified and are involved in DNA replication, transcription and therefore regulation of gene expression (Coleman, 1992; Vallee and Falchuk, 1993). With transcription, Zn forms a tetrahedral complex with amino acid residues of the polypeptide chain (Marschner, 1995). The polypeptide chain then forms a "finger" (loop) of 11-13 amino acid residues which bind to the specific DNA sequences. This is known as the Zn-finger motif.

About 45 different proteins which contain the Zn finger-motif have been identified (Coleman, 1992). The first protein discovered was TFIIA (Hanas *et al.*, 1983). The TFIIA protein contains 2-3 mole of Zn per mol and activates transcription of the 5S RNA gene (Vallee *et al.*, 1991; Rhodes and Klug, 1993). Zinc-finger proteins require a protein with one or multiple repeats of about 30 amino acids, conservation of both two cysteine and two histidine residues and their spacing, contain two aromatic residues and leucine, and have a 3-dimensional structure resembling a finger (Vallee *et al.*, 1991).

2.1.9.c Carbohydrate Metabolism

Many Zn-dependent enzymes are involved in carbohydrate metabolism, including carbonic anhydrase (CA), fructose 1,6-bisphosphatase, and aldolase (Marschner, 1995). These enzymes are located in chloroplasts and cytoplasm and decrease in activity with Zn deficiency (Shrotri *et al.*, 1983). This decrease is correlated with a reduction in the rate of photosynthesis and an accumulation of sugars and starch in the leaves (Sharma *et al.*, 1982).

Several mechanisms contribute to the decline in net photosynthesis under Zn deficiency, including decreased activity of CA and other photosynthetic enzymes, decreased chlorophyll content and impaired chloroplast structure. However, Zn deficiency results in impaired photosynthesis in all plant species whereas the relative importance of CA to the photosynthetic process varies greatly between C3 and C4 plants (Marschner, 1995). It seems doubtful then that the primary role of Zn in photosynthesis is through its function in CA. Other photosynthetic enzymes are also reduced when Zn is deficient. For example, Jyung *et al.* (1972) observed a reduction in the activity of ribulose 1,5-bisphosphate carboxylase (Rubisco), an enzyme which catalyses the initial step of photosynthetic CO₂ fixation. Here Zn may stabilise carbonate formation in a manner similar to Mg²⁺ in the enzyme-CO₂-Mg²⁺ complex (Brown *et al.*, 1993). With regard to chlorophyll content and chloroplast structure, a reduction in photosynthesis under Zn deficiency can be a result of a drastic decrease in chlorophyll content, and abnormal structure of both the mesophyll and bundle sheath (Brown *et al.*, 1993).

2.1.9.d Cell Division

Fujii (1954) proposed a direct role for Zn in mitosis after Zn was detected in the nucleolus. Later, Falchuk *et al.* (1977) stated that Zn is essential for cell division. It has also been suggested that, as a result of the importance of Zn in protein synthesis, high concentrations of Zn may be required by meristematic tissue, where cell division as well as synthesis of nucleic acid and protein is actively taking place (Brown *et al.*, 1993). This would explain the high Zn requirement in young seedlings as mentioned in section 2.1.8.

2.1.9.e Membrane Integrity and Permeability

Zinc is required in animal and plant cells to maintain integrity and control permeability of membranes (Marschner, 1995). This is achieved through its interaction with phospholipids and sulfhydryl groups of membrane protein (Chvapil, 1973; Welch *et al.*, 1982; Cakmak and Marschner, 1988b; Brown *et al.*, 1993). The first direct effect of Zn on the integrity of plant

cell membranes was shown by Welch *et al.* (1982) who demonstrated increased root cell membrane permeability for ^{32}P in Zn-deficient wheat plants. Welch *et al.* (1982) hypothesised that the loss of membrane integrity in root cells leads to the accumulation of toxic P concentrations in older leaves as nutrient solution P non-selectively entered the afflicted root cells by mass flow and accumulated in the most actively transpiring older leaves.

In Zn-deficient cotton roots, Cakmak and Marschner (1988b) also demonstrated an increase in plasma membrane permeability directly. This was indicated by a 3-, 5- and 2.5-fold increase in root cell leakage of K^+ , NO_3^- and organic carbon compounds, respectively, when compared to Zn-sufficient plants. This membrane leakage decreased with resupply of Zn to the deficient plants. Higher levels of O_2^- and NADPH-oxidase activity also occurred in Zn-deficient plants with both parameters decreasing with resupply of Zn.

The major role of Zn in maintaining membrane integrity and permeability appears to be related to protection of membrane lipids and proteins against oxidation (Cakmak and Marschner, 1988b; Brown *et al.*, 1993). As previously mentioned, NADPH oxidation is inhibited by Zn^{2+} (Chvapil, 1973; Jeffery, 1983) and this action is of great importance for the integrity of biological membranes. This is a result of NADPH-derived free radicals being involved in the peroxidation of unsaturated fatty acids in microsomal, mitochondrial, nuclear and plasmalemmal membranes (Cakmak and Marschner, 1988a; 1988c). Cakmak and Marschner (1990) have since demonstrated an increase in the permeability of the plasma membrane in Zn-deficient plants as a result of an increase in toxic oxygen free radicals and an impairment in the detoxification system against these lipid oxidants.

2.1.9.f Growth Factor Metabolism

In Zn-deficient plants, free and bound auxin concentrations are reduced before a reduction in plant growth and other Zn deficiency symptoms are observed (Sherstnev *et al.*, 1970 cited by Shkolnik, 1984; Cakmak *et al.*, 1989). A decrease in tryptophan concentration also occurs under Zn deficiency with a reduction in its synthesis from indole and serine (Singh, 1981). It

was therefore concluded that under Zn deficiency the enzyme tryptophan synthetase, which catalyses the synthesis of tryptophan from indole and serine, has reduced activity.

However, there is evidence against a role for Zn in tryptophan synthesis, with many studies observing an increase in tryptophan under Zn-deficient conditions rather than a decrease (Takaki and Kusushizaki, 1970; Cakmak *et al.*, 1989). This difference in tryptophan levels under Zn-deficient conditions was suggested by Cakmak *et al.* (1989) to be a result of the methods used. In the studies where a lower level of tryptophan in Zn-deficient plants was observed, the tryptophan was measured colorimetrically, whereas in the studies that found higher levels, tryptophan was measured using HPLC.

It has subsequently been suggested that the reduced IAA level in Zn-deficient plants is not a result of impaired tryptophan synthesis but is rather due to impaired conversion of tryptophan to IAA (Brown *et al.*, 1993). Suge *et al.* (1986), working with maize, barley and oats, showed that under Zn-deficient conditions concentration of gibberellin-like substances was reduced. This was followed in 1987 by a proposal implying a role for gibberellins in IAA synthesis (Law, 1987). It was proposed that D-tryptophan was the precursor of IAA and not L-tryptophan, and that the rate of transformation from the L- into the D-form is enhanced by gibberellins (Cakmak *et al.*, 1989). It may therefore be possible that gibberellin, not auxin, is the growth regulator initially affected by low Zn, and that the low auxin content observed is a consequence of a low gibberellin content. The exact role Zn plays in IAA synthesis however has to be determined.

2.2 *Rhizoctonia* Root Rot

2.2.1 Introduction

Rhizoctonia solani occurs world-wide, and is a destructive and versatile pathogen. It is responsible for causing a larger number of diseases, on a wider range of plants, under more diverse environmental conditions than any other plant-pathogen species (Baker, 1970). It

causes seed decay, damping off, stem cankers, root rots, fruit decay and foliage disease in over 250 plant species (Wong *et al.*, 1985). As a result of this, it is probably the most extensively studied of the *Rhizoctonia* species.

The first record of *R. solani* infecting cereals is dated in the 1920's (Samuel, 1928) and was considered at the time "not of great economic importance" (Samuel and Garrett, 1932). However, in the last decade the incidence of disease of cereals caused by *R. solani* has risen, resulting in loss of crops; the amount of damage is now of economic importance, not only in Australia, but also in many other parts of the world.

2.2.2 Distribution

R. solani has an extensive host range, including cereals, legumes, rapeseed, grasses and other pasture species (MacNish, 1983). It is responsible for causing brown patch of turf grasses in New Zealand and in North Carolina (Wong *et al.*, 1985), stunting of clovers in Ontario, barley stunt disorder in Scotland (Dillon-Weston and Garrett, 1943), seed rots, seedling damping-off, and bare patch in Australia, Canada and in England (Samuel and Garrett, 1932; Benedict and Mountain, 1956), crater disease of cereals in South Africa (Scott *et al.*, 1979) and root rot of white clover in Western Australia (Wong *et al.*, 1985). It has also been reported in the Pacific North West USA, in Washington and Idaho (Weller *et al.*, 1986), Oregon (Pumphrey *et al.*, 1987) and Georgia (Rothrock and Hargrove, 1988).

2.2.3 Characteristics of *Rhizoctonia* Species

Rhizoctonia is a genus of basidiomycetous imperfect fungi with the following characteristics: 1) branching near the distal septum of cells in young, vegetative hyphae; 2) formation of a septum in the branch near the point of origin; 3) constriction of branch hyphae at the point of origin; 4) presence of the dolipore septal apparatus; 5) absence of clamp connections; 6) absence of conidia; 7) sclerotial tissue not differentiated into rind and medulla; and 8) absence

of rhizomorphs (Parmeter Jr. and Whitney, 1970; Ogoshi, 1987). Most pathogenic *Rhizoctonia* isolates fall into 3 major groups: 1) multinucleate *Rhizoctonia* which have three or more nuclei per cell, larger hyphae (6-10 μm diameter) and teleomorph in the genus *Thanatephorus* Donk; 2) binucleate *Rhizoctonia* which have two nuclei per cell (rarely one or three), smaller hyphae (4-7 μm diameter) and teleomorph often in the genus *Ceratobasidium* Rogers; and 3) includes *R. oryzae* and *R. zae* which are multinucleate and have teleomorphs in the genus *Waitea* Warcup and Talbot.

Identification of *Rhizoctonia* spp. using conventional taxonomic criteria is difficult as a result of high levels of phenotypic variation and inability to produce the teleomorph of most strains in culture (Vilgalys and Cubeta, 1994). To improve taxonomy and understanding of *Rhizoctonia* spp. the development of anastomosis groups (AG) was suggested in 1937 by Schultz and later developed by Richter and Schneider in 1953, Watanabe and Matsuda in 1966 and Parmeter *et al.* in 1970 (Anderson, 1982). According to Vilgalys and Cubeta (1994) an AG represents a group of closely related isolates capable of recognising each other and able to undergo hyphal fusion. In a typical anastomosis reaction, pairings between isolates belonging to the same AG lead to hyphal fusion. The mechanism by which an isolate recognises hyphae from the same AG is currently unknown. If fusion of hyphae between different AG occurs, five to six cells on either side of the fusion cells become vacuolated and die. This is referred to as the killing reaction (Flentje and Stretton, 1964) and appears as a cleared zone at the junction of the two colonies on agar plates (Anderson, 1982). The killing reaction is thought to be a somatic incompatibility reaction, limiting outbreeding and reducing the spread of cytoplasmic genetic elements, including viruses, between isolates in nature (Caten, 1972) and is used as an aid to AG determination.

Recognising variation within certain anastomosis groups, Ogoshi (1987) developed intraspecific groups (ISG) for more specific identification. An ISG is a subgroup of isolates within an AG that anastomose more frequently with each other, or possess other unique

distinguishing characteristics, including host range specificity, nutrient utilisation, and genetic differences based on molecular markers (Vilgalys and Cubeta, 1994).

Since Ogoshi (1987) proposed the term ISG, some confusion in the literature with regard to its use has occurred. Recognising this, Ogoshi (1996) recommends the term ISG be discontinued and the more accurate term “subgroup” used in its place.

2.2.4 Infection Process

R. solani is mainly located in the top 10 cm of soil (Papavizas, 1970; Neate, 1987) and survives between crops as sclerotia on plant debris or within plant debris particles as aggregations of monilioid cells (Sumner, 1996). In Australia, Neate (1987) mainly isolated *R. solani* (AG 8) from plant debris particles containing monilioid cells, while sclerotia were rarely found.

Initiation of mycelial growth is temperature- and moisture-dependent and is not restricted by dormancy or maturity requirements (Sherwood, 1970). Mycelial growth may then occur toward the plant, with plant exudates acting as a stimulant. This was first demonstrated by Kerr (1956) with hyphal aggregations on plant roots which were inside cellophane bags. Stimulation of mycelial growth by plant exudates has since been demonstrated in a number of other systems and correlates to the age of the plant (Kerr and Flentje, 1957; de Silva and Wood, 1964). Younger plants, with higher concentrations of amino acids, carbohydrates, phenols and organic acids present in the exudates compared to older plants, stimulate hyphal growth to a greater extent (de Silva and Wood, 1964; Reddy, 1980).

Once hyphae come into contact with the plant root they grow over the surface before attachment occurs (Keijer, 1996). Hyphae that are not attached to the root are round in shape and easily washed off. When attachment occurs, hyphae become flattened, are surrounded by a mucilaginous sheath and are not as easily detached. Once attachment has occurred, hyphae

start to follow the anticlinal epidermal cell walls, a process described as directed growth (Dodman and Flentje, 1970). The topology of the surface and enhanced exudates is believed to be the cause of directed growth (Armentrout *et al.*, 1987).

The next stage in the infection process is the formation of T-shaped branches which result in simple branched appressoria-like structures with swollen hyphal tips, or tightly packed dome-shaped infection cushions with swollen hyphal tips (Keijer, 1996). The swollen hyphal tips then form thin infection pegs allowing the pathogen to exert maximal hydrostatic pressure on the surface, resulting in penetration of the plant surface (Sneh *et al.*, 1989). Penetration may be both mechanical and enzymatic, with *R. solani* producing extracellular enzymes, probably excreted locally from the tip of the infection peg (Fukutomi and Takada, 1979). Extracellular enzymes secreted by *R. solani* include pectinase, pectin lyase, pectin methylesterase, cellulase, and phosphatase, while evidence exists for the secretion of DNase, RNase, alpha-amylase, chitinase, betaglucanase, xylanase, protease, urease, and at least seven more enzymes that have yet to be identified (Bertagnolli *et al.*, 1996; Weinhold and Sinclair, 1996).

Colonisation by AGs 2, 3 and 4 is characterised by cell wall degradation and cell damage caused by extracellular enzymes, in advance of the hyphae (Weinhold and Sinclair, 1996). Advancing hyphae grow rapidly in the cells with extensive branching, as well as into the cortex, endodermis and then the vascular tissue. In hypocotyls severely infected with AG2-1, (Yang *et al.*, 1992) observed disintegration of the whole cortex while the endodermis, phloem and xylem parenchyma cells were infected by the fungus. The xylem elements remained uncolonised, presumably due to cell wall lignification.

2.2.5 Symptoms

Symptoms of *R. solani* root rot may be observed above and below ground. Above ground symptoms range from those associated with poor nutrition, particularly reddened stem base and leaf sheaths, moisture stress and stunting at about the four-leaf stage. This stunting forms

well-defined patches (see Photo 1) that become more noticeable as the plants age and a hollow space is left in the crop (Samuel and Garrett, 1932). If infection is severe enough, seedlings in a patch may die before the end of the season, having attained a height considerably less than those outside the patch (Samuel and Garrett, 1932).

Patch size ranges from less than 30 cm in diameter to large, irregular areas covering up to 40% of a hectare (Samuel and Garrett, 1932; MacNish, 1985a). In Australia, patches are caused by *R. solani* AG 8, with each patch a result of a single isolate from a single infection focus (MacNish, 1996). Patches have been observed to persist in the same spot in a field over several years and may enlarge, while in other paddocks, they decline and disappear (Samuel and Garrett, 1932; MacNish, 1985a). The change in shape between seasons is due to differential growth rates, tillage effects, coalescing of patches, and changes in soil suppressiveness (MacNish, 1996).

Roots tend to show characteristic symptoms with the pathogen causing brown sunken lesions on the root cortex. If the disease is severe, the cortex may also rot away exposing and breaking the stele to form the characteristic “spear tip” (Samuel and Garrett, 1932). By killing root tips, and other parts of the root system, lateral roots develop which are in turn attacked by the fungus. This results in a highly-branched root system in infected plants (see Photo 2). If the attack on the host is not severe, fresh roots may form, allowing plants to recover (Samuel and Garrett, 1932).

2.2.6 Controlling *R. solani*

R. solani may be controlled in a number of ways, including soil disturbance, rotations, chemical control, biocontrol agents, herbicides, host resistance and fertiliser application. No technique is completely satisfactory and a combination of methods is recommended for best results (MacNish and Neate, 1996).



Photo 1: A well defined patch in a medic pasture due to infection on the roots by *R. solani*.

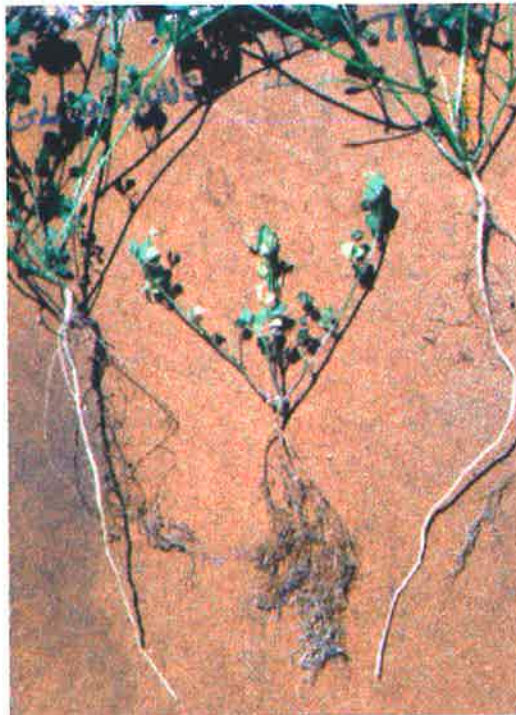


Photo 2: Infection by *R. solani* on the root system of medic plants. The plant in the middle was sampled from the centre of the patch in Photo 1. The other plants were sampled from either side, outside of the patch.

2.2.6.a Soil Disturbance

The increase in incidence of bare patch disease in Australia over the past decade has mainly been attributed to reduced tillage in cereal crop production (Neate *et al.*, 1988). Reduced tillage methods allow grazing of the annual pasture of grasses and legumes which are regenerated by autumn rains, and also offer the advantage of reduced soil erosion and a reduction in soil preparation costs (Rovira, 1986). In contrast to these advantages, a number of studies (field- and laboratory-based) have shown an increase in disease with less soil disturbance (Neate, 1984; McDonald and Rovira, 1985; MacNish, 1985b; Rovira, 1986; MacNish and Fang, 1987; Pumphrey *et al.*, 1987). It has also been demonstrated that the distribution of *R. solani* propagules in the soil is associated with particulate organic matter, as previously mentioned. From this, (Rovira, 1986) proposed that during cultivation there is a fragmentation of organic matter, reducing propagule size and affecting the pathogenicity of hyphae that have been ramified through the soil.

In the past, *R. solani* has been associated mainly with grey, calcareous sands, and to a lesser extent, with neutral to acid siliceous sands (Rovira, 1986). However, with reduced tillage systems now being adopted, *R. solani* has been reported on a larger range of soil types, including clay loams and red-brown earths (MacNish and Neate, 1996). With the trend away from cultivation, *R. solani* is becoming a serious, economic problem in Australian agriculture and management strategies need to be developed.

2.2.6.b Rotations

Contradictory results have been observed with crop rotations and chemical "fallows". Roget *et al.* (1987) reported that killing pasture with herbicides to achieve a chemical "fallow" 3-6 weeks prior to sowing reduced bare patch and increased yield up to 83% (1 t/ha) compared with sowing into a pasture killed immediately prior to sowing. However, the results of a study by MacNish and Fang (1987) suggest no reduction in root rot after a short chemical fallow in Western Australia.

Previous crop has also been shown to have a minor influence on bare patch (Rovira, 1986), with a higher patch area occurring in wheat after the annual grass-medic pasture compared with wheat after medic, peas or wheat (Rovira, 1986). This was suggested to be a result of the more fibrous root system of grasses supplying the pathogen with more substrate.

2.2.6.c Chemical Control

To date, chemical control has been ineffective, with incidence and severity of disease rarely reduced (Cotterill *et al.*, 1990; Cotterill, 1991).

2.2.6.d Disease Suppressive Soils

Natural suppression of *R. solani* has been demonstrated in Australia where the levels of the pathogen have increased for a number of years (4-5 years and 7-9 years; Roget (1995) and MacNish (1988) respectively) and then declined to negligible levels over the following years. This decline in *R. solani* has also been observed in the glasshouse as a result of general microbial suppressiveness (Wiseman *et al.*, 1996).

2.2.6.e Host Resistance/Tolerance

In Australia, small differences in response to *R. solani* have been observed within the cereal germplasm (Neate, 1989), but as discussed by MacNish and Neate (1996), these differences are too small to warrant use in alternative rotations, or be useful to breeders. However, an increasing number of plant species has provided germplasm with high resistance (and/or tolerance) to diseases caused by *R. solani* (Panella and Ruppel, 1996).

Host resistance to *R. solani* has been shown to occur due to differences in epicuticular waxes and/or a thicker cuticle which act as mechanical barriers to infection in resistant varieties of rice (Massaquoi and Rush, 1987), rapeseed, mustard (Yang *et al.*, 1992) and bean (Stockwell and Hanchey, 1985). An enzymatic mechanism for resistance can be seen in bean (Bugbee, 1993) and sugar beet (Lafitte *et al.*, 1984) where inhibitors for a number of extracellular enzymes

produced by *R. solani* are found in higher concentrations in resistant genotypes compared with susceptible genotypes.

A large number of medic genotypes have been screened for disease resistance, with no genotype exhibiting resistance when a highly pathogenic strain was used (Kulik and Dery, 1995; Kulik *et al.*, 1995). This study did however demonstrate a high degree of variation between genotype response to infection by *R. solani* strains of lower pathogenicity, but even at the lower level of disease pressure no genotype was completely resistant to the pathogen.

2.2.6.f Fertiliser Application

Since poor fertility and low soil nitrate have been shown to aggravate the disease (MacNish, 1985b), increasing soil fertility by applications of superphosphate and ammonium sulphate fertiliser and increasing available nitrogen by improving legume content of pastures is considered an effective management strategy. Ammonium sulphate has been shown to act as a "satisfactory control" of bare patch with a reduction in the incidence of the disease with N application (MacNish, 1985b). However, Pumphrey *et al.* (1987) found neither N nor ammonium sulphate application to have any significant effect.

Recent studies have shown low soil Zn content to increase severity of *R. solani* in cereals (Thongbai *et al.*, 1993a). In this study, field trials demonstrated Zn applications of 2.5 kg Zn/ha reduced the area of bare patch from 42 to 21% and increased grain yield from 1 to 2.8 t/ha. Interaction between nutrient deficiency and pathogen may occur in one of two ways: 1) the disease may damage the root system, thereby decreasing the absorbing surface leading to deficiency of the limiting nutrients in the soil; or 2) nutrient deficiency may adversely affect a biochemical pathway responsible for resistance, increasing disease severity directly (Graham, 1983). In the study by Thongbai *et al.* (1993b), laboratory-based experiments further demonstrated that *R. solani* did not exaggerate Zn deficiency, but rather that Zn sufficiency suppressed disease severity.

2.3 Zinc Nutrition and Disease

The effect of Zn nutrition on the incidence of disease has been extensively reviewed by Graham (1983), Huber (1989) and Graham and Webb (1991). Both promotive and inhibitory effects of Zn on disease have been observed in the past, with promotive effects most likely due to Zn inhibiting some other element (Graham and Webb, 1991).

Zinc nutrition may be beneficial to the plant with regard to disease control by directly affecting the pathogen or through a change in the physiology of the plant. According to Graham and Webb (1991), many studies showing a reduction in disease with Zn application are probably related to toxic effects of Zn directly on the pathogen. The majority of the studies are on artificial media and are probably at Zn levels toxic to plants.

One of the earliest reports on the effect of Zn on disease was on root rot diseases of wheat (Millikan, 1938). Wheat grown without added Zn had a higher incidence of root rot than wheat grown with 35 kg/ha of ZnSO₄. The next study on the Zn and disease interaction was by Bolle-Jones and Hilton (1956). They produced Zn-deficient rubber trees (*Hevea brasiliensis*) and demonstrated more severe infection with *Oidium heveae* compared with Zn-sufficient trees. This was presumably a result of increased leakage of sugars onto the leaf surface with Zn deficiency (Marschner, 1995).

The importance of the cells internal concentration of Zn in the root in determining infection by a vesicular-arbuscular mycorrhizae (*Glomus mosseae*) was demonstrated by Gildon and Tinker (1983). Using a split-root system, translocation of Zn from one half of the root system was shown to be effective in suppressing the level of infection in onion root grown without Zn in the external medium.

Zinc application has also been shown to depress upward movement of *Fusarium graminearum* through the stele of wheat, with only an increase in tissue Zn concentration from 7.8 to 8.6 mg/kg dry matter required (Sparrow and Graham, 1988). In a following study, Grewal *et al.*

(1996) suggest disease suppression is due to the protective role of Zn in the structural and functional integrity of cell plasma membranes. However, they also mention that direct toxicity of high soil Zn on *F. graminearum* cannot be ruled out. Grewal *et al.* (1996) further demonstrated disease to be more severe in Zn-inefficient wheat genotypes (Songlen and Durati) compared with the Zn-efficient cultivar (Excalibur). This is a result of Zn-efficient genotypes being able to extract more soil Zn under deficiency, as discussed in section 2.1.4.c.

With respect to *R. solani*, field experiments and pot experiments have confirmed a relationship between the severity of disease and the Zn nutrition of wheat (Thongbai *et al.*, 1993a; 1993b). This decrease, however, is confined to the concentration range where the Zn supply limits plant growth (Thongbai *et al.*, 1993a; 1993b) and has already been discussed.

2.3 Objectives of this Study

The work by Thongbai *et al.* (1993a; 1993b) suggests a possible mechanism for the medic decline currently occurring in Australia (see Chapter 1). This then leads to the hypothesis that Zn deficiency increases the sensitivity of *Medicago* species to infection by *R. solani*, thereby lowering pasture production. This study was carried out to determine if Zn deficiency does increase medic sensitivity to infection by the pathogen, with the objectives outlined in Chapter 1.

1. The experiments were specifically designed to determine the following:

- the effect of infection by *R. solani* on medic growth when grown under non-limiting conditions and under Zn deficiency;
- response of *Medicago* species to Zn-deficient conditions in the field and in a controlled environment;
- the Zn efficiency ranking of a range of medic genotypes;
- the effect of infection by *R. solani* on Zn-efficient and Zn-inefficient medic genotypes grown under Zn-deficient conditions.

Preliminary Experiments: Establishing Techniques

3.1 Introduction

Much research has been focused on infection by *R. solani* and on the effect of Zn deficiency on cereal crops in controlled environments. Annual medics, however, have been poorly studied. The one study on the response to Zn in annual medics was performed on four medic genotypes adapted to Mediterranean environments (Materon and Ryan, 1995), while Kulik and Dery (1995) screened 27 annual medics for resistance to *R. solani*. Neither study is relevant for the situation in Australia, with Materon and Ryan (1995) using cold-tolerant medic genotypes and Kulik and Dery (1995) using an isolate of *R. solani* that does not cause significant damage in Australia. At the time of this research, no work had been done with medics on the interaction between Zn nutritional status and infection by *R. solani*.

Since no published work existed for medics on either Zn deficiency or *R. solani* infection in Australia, preliminary experiments were required to determine the ideal growing conditions before any detailed experiments could be performed in a controlled environment. This would ensure *R. solani* and Zn deficiency to be the only factors limiting medic growth. This chapter describes a set of four experiments, performed in series, to determine ideal growing conditions.

The first experiment consists of three parts performed at the same time, designed with three aims in mind. The first aim was to determine if Zn deficiency and *R. solani* infection could be induced in medics, and how to supply N to medics for maximum growth; secondly, to determine what environmental conditions are suitable for growing medics while studying Zn deficiency, and finally to determine if a suitable Zn-deficient sand could be found. The

second experiment was designed to improve the nutrient status of medics by adjusting the basal nutrients added to the sand. Nutrient levels were further adjusted in the third experiment, the time of harvest determined and a standard genotype selected for studies on Zn deficiency. The fourth experiment was designed to reduce variation by increasing plant number per pot.

3.2 Experiment 1

3.2.1 Aim

Part 1a

There were two main aims: firstly, to determine if Zn deficiency and *R. solani* infection could be induced in a known Zn-deficient sand, and secondly, to compare the growth of medics inoculated with *Rhizobium meliloti* with uninoculated medics supplied with NH_4NO_3 with and without Zn fertiliser.

Part 1b

The aim was to ensure that the day-length and temperature were suitable for inducing Zn deficiency in medics.

Part 1c

The aim was to compare medic growth with and without Zn supply in Laffer sand, a known Zn-deficient sand for wheat, with Mt Compass sand, a potential Zn-deficient sand.

3.2.2 Materials and Methods

3.2.2.a Experimental Design.

Part 1a

To test the first aim two species, *M. scutellata* genotype Robinson and *M. truncatula* genotype Paraggio, were grown at two Zn levels, 0 and 0.3 mg Zn/kg soil and two *R. solani* levels, 0 and 8 propagules/kg soil in a factorial design in three replicates. These genotypes

were selected as Robinson was shown to be the most Zn-efficient and Paraggio the least Zn-efficient out of 10 genotypes screened in a preliminary experiment in the glasshouse by Dr R.J. Hannam (pers. comm.). During the growing period, 4 mL of solution containing 75 mMoles NH_4NO_3 were supplied weekly to all plants.

To test the second aim, results from the experiment described above were utilised, excluding the addition of *R. solani* (ie both experiments shared the same control). Instead, a treatment with *Rhizobium meliloti* as the primary N source was added to compare with NH_4NO_3 . That is, two species, *M. scutellata* genotype Robinson and *M. truncatula* genotype Paraggio, were grown at two Zn levels, 0 and 0.3 mg Zn/kg soil, and two sources of N, *Rhizobium meliloti* and NH_4NO_3 in three replicates.

Part 1b

M. scutellata genotype Robinson was grown in a factorial design combining two Zn levels (0 and 0.3 mg Zn/kg) in four different growth environments, in three replicates. The four growth environments consisted of two controlled growth rooms, a semi-controlled laboratory and a glasshouse. The first controlled environment, growth chamber A, was used in Part 1a. In this chamber, light was supplied by banks of high-intensity metal-halide lights which gave a light flux density of photosynthetically active radiation of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$, set on an 8/16 h, 15/10°C day/night cycle. The second controlled environment, growth chamber B, was similar to growth chamber A but was set on a 20/15°C day/night cycle. In the glasshouse, natural light conditions were used with temperature exceeding 30°C during the day. The semi-controlled environment consisted of four high-intensity, metal-halide lights set up in the laboratory emitting a light flux density of photosynthetically active radiation of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$, set on an 8/16 h day/night cycle. Due to air-conditioning problems, temperature during the day ranged from 23-32°C. No temperatures were measured during the night cycle.

Part 1c

M. scutellata genotype Robinson was grown in a factorial design combining two Zn levels, 0 and 0.3 mg Zn/kg, and two different soils, Laffer and Mt Compass, in three replicates. Plants were grown in growth chamber A.

3.2.2.b *R. solani* Inoculum

Inoculum of *R. solani* was prepared using a modified version of the protocol described by McDonald and Rovira (1985). White millet seeds, 1 mm in diameter on average, were soaked in reverse osmosis (RO) water overnight, drained, and autoclaved for 1 h at 121°C and 100 kPa in a 250 mL flask on two consecutive days. A pure culture of *R. solani* anastomosis group 8 (S21) was added (35 mm³), taken from the edge of colonies actively growing on potato dextrose agar (Warcup, 1955). This group was selected as it has been observed to infect medics in Australia (Ludbrook *et al.*, 1953). The inoculated millet was incubated at 25°C for 3 weeks (shaken weekly) to ensure complete colonisation by the fungus. The millet was then air dried in a laminar flow cabinet and stored in a freezer for subsequent use. For adequate infection, as determined by Neate *et al.* (1988), 8 millet seeds (propagules) were added to one kilogram of soil.

3.2.2.c *Rhizobium meliloti* Inoculum

Rhizobium meliloti was provided by Mr. R. Ballard, from the South Australian Research and Development Institute (SARDI). Strains WSM688 and CC169G were incubated on 10 mL yeast mannitol agar slopes in 20 mL McCartney bottles for 5 days (see Appendix 1 for recipe).

3.2.2.d Soil

A Zn-deficient, siliceous soil (Laffer sand) which was removed from a naturally vegetated area at Tintinara in the southeast of South Australia was used. It has been classified by Moore *et al.* (1983) as great group Quartzisamment according to the USDA 1975 Taxonomy. Mount Compass sand was collected from a quarry at Mt Compass in South Australia. Soils

were rinsed three times with RO water to remove organic matter and leach soluble salts, and air dried before use.

3.2.2.e Basal Nutrients

A basal nutrient solution was prepared containing essential nutrients, excluding Zn for Zn-deficient treatments, and added to the soil using RO water that had been passed through a Milli-Q filtration system (Milli-Q water). The basal nutrients include (in mg/kg): 350 NH_4NO_3 ; 150 KH_2PO_4 ; 120 K_2SO_4 ; 90 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 7 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.5 $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$; 1 $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$; 0.15 $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$; 5 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 1 H_3BO_3 ; 0.7 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.3 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. CaCO_3 (0.3%) was mixed thoroughly with the dry soil before basal nutrients were added.

3.2.2.f Seed

All medic seeds were supplied scarified, by the SARDI Medic Breeding Unit. *M. scutellata* genotype Robinson and *M. truncatula* genotype Paraggio were sown in this experiment and their characteristics are presented in Table 3.1.

Table 3.1 Seed characteristics for Experiment 1.

Genotype	Weight (mg/dry seed)	Zn concentration (mg/kg)	Zn content (μg Zn/seed)
Robinson	18	46	0.84
Paraggio	4	41	0.18

Seeds were surface-sterilised prior to germination by rinsing in 70% ethanol for 1 minute. They were then rinsed five times with Milli-Q water, soaked for 5 minutes in an aerated solution of 4% sodium hypochlorite, rinsed 10 times in Milli-Q water, and germinated for 24 hours on filter paper at 25°C.

3.2.2.g Procedure

Pots were 19.5 cm high and 7 cm wide and lined with clear plastic prior to potting. Dry soil was mixed with the basal nutrient solutions and then potted in 1 kg pots. For Zn-treated soil,

Zn solution was added to pots separately, and mixed through the soil. For treatments inoculated with *R. solani* (Part 1a), half the propagules were placed on top of the first 400 g of soil in the pot and the other half on top of the subsequent 400 g. All pots in Part 1a were watered to 10% moisture content by weight, covered with aluminium foil to reduce evaporation, and incubated for two weeks in a controlled environment (McDonald and Rovira, 1985). After two weeks, two germinated medic seeds were planted in each pot and water added to 12% moisture content. Pots were not incubated for Part 1b or 1c.

After planting, the soil surface was covered with acid-washed black plastic beads in all experiments to reduce water loss and prevent algal growth (see Appendix 2 for acid washing procedure). For part 1b, pots were then placed in the four growth rooms previously described, while pots were placed in growth chamber A for part 1a and 1c. All pots were watered to 12% moisture content by weight every second day.

After 4 days growth, plants were thinned to 1 plant/pot. Ten millilitres of Milli-Q water was added to the 20 mL McCartney bottles with the agar slopes and shaken gently to suspend *Rhizobium meliloti* cells in water, and 0.2 mL of each strain was added to the base of seedlings with the *Rhizobium meliloti* treatment in Part 1a and to all plants in Part 1b and 1c. Plants not inoculated with *Rhizobium meliloti* in Part 1a, were supplied with 4 mL of 75 mM NH_4NO_3 solution weekly, near the base of the plant.

Plants were harvested 28 days after planting and shoot and root dry weight recorded. All plant tops were digested according to Zarcinas (1984) and analysed for Zn concentration with an Inductively Coupled Plasma Optical-Emission Spectrometer (ICP). In addition, roots in part 1a were scored visually for disease using a modified version of the root rot scale developed by Sweetingham and MacNish (1992). The disease score consists of a 0-5 scale with 0.5 increments and is inversely related to root length. A score of 0 represents no infection, while 5 represents severe truncation of the root system.

3.2.3 Results

3.2.3.a Visual Observations

Part 1a

Two weeks after planting all Paraggio plants, even those supplied with *Rhizobium meliloti*, appeared N-deficient, with reddening of stalks and undersides of leaves, cupping of leaves and chlorosis around the margins of old leaves (Weir and Cresswell, 1994). Symptoms were more severe with NH_4NO_3 as the N source compared with other treatments. To rectify this problem the NH_4NO_3 solution was supplied twice a week to all plants not inoculated with *Rhizobium meliloti* for the remainder of the experiment. Three days after increased N application, N deficiency symptoms were no longer visible in NH_4NO_3 -treated plants. Plants inoculated with *Rhizobium meliloti* exhibited N deficiency symptoms up until day 21.

Differences between Zn treatments were noticeable by reduced growth in the zero treatment at 14 days after planting. By day 28, classic symptoms of Zn deficiency were observed in all zero Zn treatments (Photos 3 and 4): small, pale necrotic spots extending from the middle of the leaf to the margins on the younger leaves (Weir and Cresswell, 1994) with symptoms more pronounced in Robinson. No visible differences were observed between controls and *R. solani* treatments during the growing period when comparing plants within Zn treatments.

A visible difference in growth due to N source occurred by day 21, with plants inoculated with *Rhizobium meliloti* exhibiting higher shoot growth. This difference was greater in Robinson than Paraggio.

Part 1b

At 16 days after sowing, plants grown in the glasshouse had well developed Zn deficiency symptoms. These symptoms were later observed on plants grown in the other rooms by day 21. At day 26, plants in the glasshouse supplied with Zn had started to flower.



Photo 3: *Medicago scutellata* genotype Robinson grown with and without Zn, in Laffer sand for 28 days.



Photo 4: Close up of Zn deficiency symptoms in *Medicago scutellata* genotype Robinson grown without Zn application in Laffer sand for 28 days.

Part 1c

By day 14, plants in Mt Compass sand had reduced growth and were showing signs of Fe deficiency: interveinal chlorosis on young leaves while older leaves remained dark green (Weir and Cresswell, 1994). No visible difference between Zn treatments were observed in Mt Compass sand. To overcome Fe deficiency, 0.7 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in solution, was applied to the soil surface of each pot of Mt Compass sand. Symptoms of Fe deficiency had not disappeared by day 21; however, a visible difference between Zn treatments was noticeable. By day 28, classic Zn deficiency symptoms had developed in plants grown without the addition of Zn in both Laffer and Mt Compass sand.

*3.2.3.b Plant Growth Measurements**Part 1a*

The interaction between Zn application, genotype and inoculation with *R. solani* did not have a significant effect on shoot or root dry weight, on the concentration of Zn in the shoot, or on the disease score on the roots ($p \geq 0.7$ in all cases; data not shown). However, the main effect of genotype did have a significant effect on shoot and root dry weight ($p \leq 0.009$ in all cases). In all cases, Robinson maintained a significantly higher shoot and root growth than Paraggio (Fig. 3.1), with the highest shoot dry weight recorded for Robinson almost 3.5 times greater than the highest measured for Paraggio. The two-way interaction between genotype and Zn application had a significant effect on the concentration of Zn in the shoot, with Robinson having a higher concentration of Zn than Paraggio at both Zn treatments (Fig. 3.2). Robinson also had an increase in concentration of Zn in the shoot with Zn application, whereas no change occurred in Zn concentration in Paraggio with the addition of Zn.

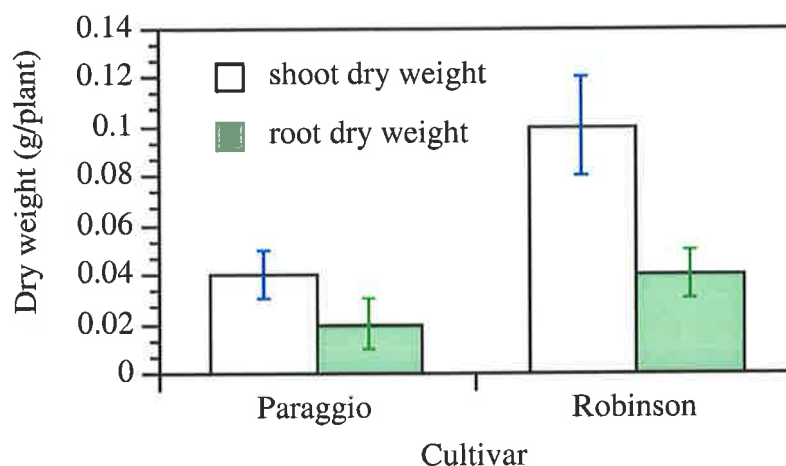


Figure 3.1 The shoot and root dry weight of *M. truncatula* genotype Paraggio and *M. scutellata* genotype Robinson (main effect of genotype). Plants were grown in Laffer sand for 28 days. Vertical bars represent $\pm$ standard error ($n=12$).

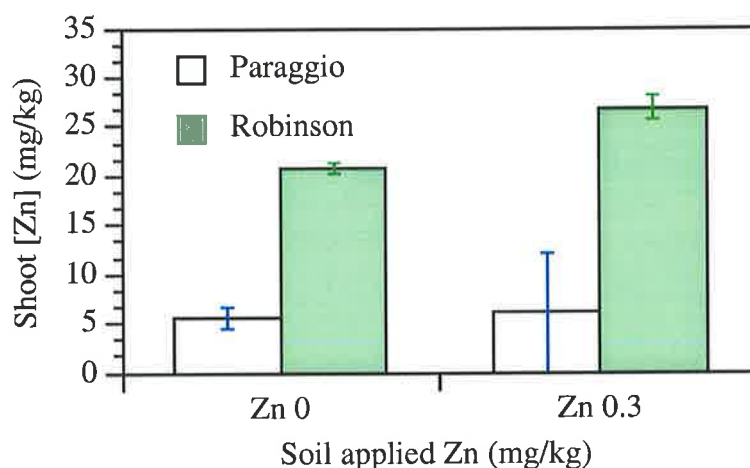


Figure 3.2 Effect of Zn application on the concentration of Zn in the shoot of *M. truncatula* genotype Paraggio and *M. scutellata* genotype Robinson (data are means of replicates and *R. solani* treatment). Plants were grown in Laffer sand for 28 days. Vertical bars represent $\pm$ standard error ($n=6$).

No significant difference in disease score as a result of infection by *R. solani* on root systems occurred between genotypes and no change in the degree of infection occurred with Zn application. The addition of *R. solani* did however result in a significant effect on the disease score: 0 with no disease and 3.5 in the presence of *R. solani* ($p \leq 0.0001$).

The interaction between Zn application, genotype and N source had a significant effect on shoot and root dry weight, but not on the concentration of Zn in the shoot ($p \leq 0.02$, 0.07 and

0.8, respectively). In all treatments, shoot dry weight increased with inoculation of *Rhizobium meliloti* (Fig. 3.3), with the effect more pronounced in Robinson. Root dry weight increased in Robinson, with inoculation by *Rhizobium meliloti* at both Zn treatments, but an increase was only observed in Paraggio at 0 mg Zn/kg soil (Fig. 3.3). Although the concentration of Zn in the shoot was not significantly affected by the three-way interaction, the two-way interactions between Zn application and N source and between genotype and Zn application were significant ($p \leq 0.03$ in both cases; Fig. 3.3). Zinc deficiency was more severe in both species with *Rhizobium meliloti* as the source of N compared with NH_4NO_3 .

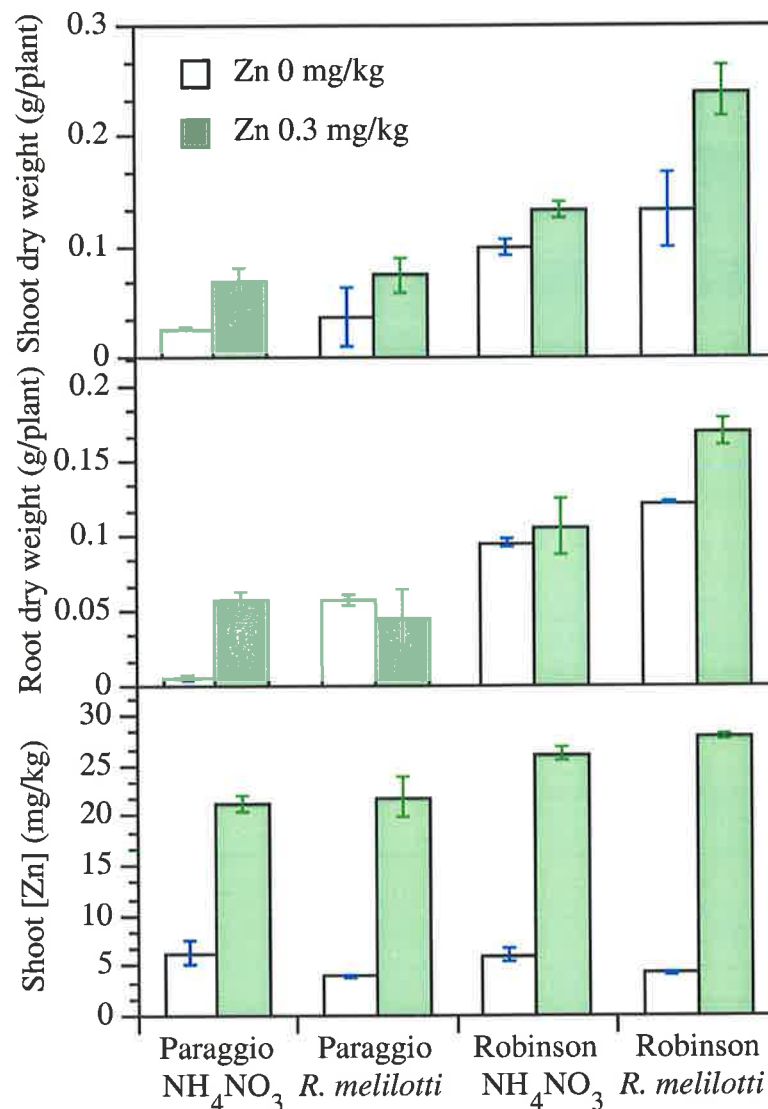


Figure 3.3 Effect of Zn application on the shoot and root dry weight and concentration of Zn in the shoot of *M. truncatula* genotype Paraggio and *M. scutellata* genotype Robinson. Plants were grown in Laffer sand for 28 days with two sources of N. Vertical bars represent $\pm$ standard error (n=3).

Part 1b

The interaction between growth environment and Zn application had a significant effect on the shoot and root dry weight and on the concentration of Zn in the shoot (Fig. 3.4). The difference in growth between growth environments was greater when Zn was not limiting growth. When Zn was applied to the soil, the highest shoot growth occurred when plants were grown in the glasshouse, followed by growth chamber B, growth chamber A, and the semi-controlled environment. When Zn was not applied, plants in growth chamber B had the highest shoot growth, followed by those grown in the glasshouse, growth chamber A and the semi-controlled environment.

The effect of soil Zn application on root dry matter did not follow the same trend as shoot dry weight in the various growing rooms. At both Zn applications, mean root growth was higher in plants grown in growth chamber B, followed by growth chamber A, the glasshouse, and then the semi-controlled environment (Fig. 3.4).

The concentration of Zn in the shoot was in the range for normal growth in all growth rooms when Zn was applied to the soil, with plants in growth chamber A maintaining a lower Zn concentration in shoots than plants in other rooms (Fig. 3.4).

Part 1c

A significant interaction occurred between Zn application and soil type on the shoot dry weight and on the concentration of Zn in the shoot (Fig. 3.5). Zinc application increased shoot growth in both sands, with the effect more pronounced in Laffer sand. Laffer sand produced the highest shoot dry weight, at both Zn treatments while plants grown in Mt Compass sand had the highest concentration of Zn in the shoots. Severe Zn deficiency occurred in both soils (Table 3.2). Root dry weight was not significantly influenced by the interaction between Zn and soil type, but the main effect of soil type was significant, with root dry weight higher in Laffer compared with Mt Compass sand (Fig. 3.5).

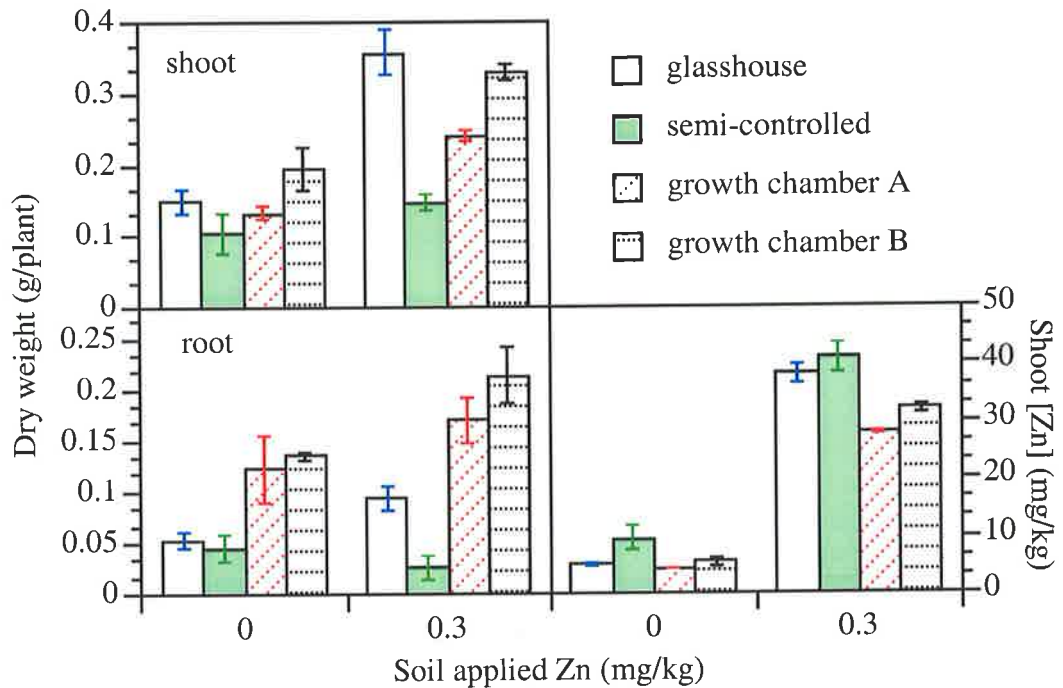


Figure 3.4 Effect of Zn application on shoot and root growth and concentration of Zn in the shoot of *M. scutellata* genotype Robinson. Plants were grown in Laffer sand in four environments for 28 days. Vertical bars represent $\pm$ standard error (n=3).

Nutrient analysis of the shoots (Table 3.2) confirmed Fe deficiency in Mt Compass sand, with a lower concentration of Fe measured in the Zn-deficient treatment. Many nutrients were in high concentrations with Mn in the toxicity range in Mt Compass sand when Zn was applied, B in the toxicity range in all treatments, and P in the toxicity range in Zn-deficient Laffer sand (Table 3.2). Calcium and K levels were also higher than the requirement for normal growth in all treatments, but toxicity had not occurred.

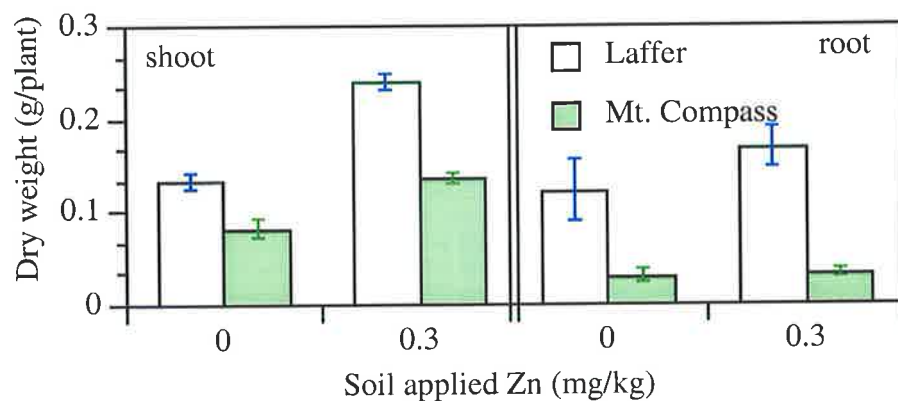


Figure 3.5 Effect of Zn application on shoot and root growth and concentration of Zn in the shoot of *M. scutellata* genotype Robinson. Plants were grown in Laffer and Mt Compass sand for 28 days. Vertical bars represent $\pm$ standard error (n=3).

Table 3.2 Mean nutrient analysis from shoots of Robinson grown in Laffer and Mt Compass sand, with and without Zn supply, compared with the requirements for normal growth in medics as outlined in Weir and Cresswell (1994).

Element	Laffer sand (mg Zn/kg)		Mt Compass sand (mg Zn/kg)		Normal Growth Requirements
	0	0.3	0	0.3	
	mg/kg shoot dry weight				
Fe	67	55	30	39	>35
Mn	110	176	189	646	35-250
B	56	77	74	95	25-50
Cu	10	10	10	11	6-25
Zn	4	28	6	58	25-60
	g/kg shoot dry weight				
Ca	19	20	21	22	8-12
Mg	5	4	4	4	2-6
K	35	41	41	68	15-40
P	10	8	7	6	3-5
S	4	4	4	3	3-6

3.2.4 Discussion

These experiments demonstrated that Zn deficiency can be achieved in Laffer sand, with growth reduction and classic Zn deficiency symptoms occurring in two medic species. Severe Zn deficiency was achieved, with concentration of Zn in the shoots consistently less than 10 mg/kg, much lower than the 25 to 60 mg Zn/kg recommended for normal growth (Weir and Cresswell, 1994).

Genotypic differences occurred in yield potential, with Robinson maintaining a much higher yield than Paraggio. The larger seed size of Robinson, and therefore the greater nutrient reserves, allow Robinson the advantage of a “starter fertiliser” and therefore increasing

seedling vigour. This will also affect the sensitivity of the plant to Zn-deficient conditions and resistance to infection by *R. solani*. This is discussed in detail in Chapters 5 and 6.

Infection by *R. solani* (AG8) of two *Medicago* species was also achieved in this experiment. Since no reduction in growth occurred in either genotype with infection, some other factor, most probably low N availability, was limiting growth to a greater extent.

The use of *Rhizobium meliloti* as the N source not only increased yield up to 50%, but also resulted in more severe Zn deficiency than the NH_4NO_3 solution. The advantage in supplying *Rhizobium meliloti* to medics is that it allows N to be obtained as required. Symptoms of N deficiency are expected to occur with inoculation of *Rhizobium meliloti* in the first 14 days as nodules are yet to become fully operational (R. Ballard, pers. comm.). However, N deficiency was observed up to day 21. This may be due to N levels in the rhizosphere being high enough to limit nodulation when *Rhizobium meliloti* was added 4 days after planting. To prevent this from reoccurring, *Rhizobium meliloti* was added after 7 days of growth in later experiments, to allow N levels to decline slightly.

The growing conditions to which plants were subjected had a significant effect on plant growth. Plants in the glasshouse maintained a higher growth rate when Zn was not limiting than plants in any other environment tested. The higher growth rate resulted in plants flowering by day 26 when supplied with Zn and senescence when deficient in Zn. Under ideal growing conditions, Robinson takes 80 days to flower in the field (Boyce, 1995), but under the extreme conditions of the glasshouse, flowering occurred in one third of that time. Growing plants in glasshouse conditions are therefore not suitable for studies on Zn deficiency at the Waite Campus over summer.

Plants grown in the semi-controlled environment appear to be limited by other factors, possibly high temperatures and high light intensity, with no response in yield with Zn application. This environment is also not suited for Zn deficiency studies.

The controlled environments (growth chambers A and B) are more ideal for Zn deficiency experiments. In these rooms, Zn was a limiting factor causing a reduction in growth rate. This ensures a response to Zn is observed, flowering does not occur and the Zn deficiency treatment survives. Since the growth rate and concentration of Zn was lower in the shoots of Zn-deficient plants in growth chamber A, this room was considered to be more suitable for studies on Zn deficiency.

Zinc deficiency was also induced in both Laffer and Mt Compass sand. If Laffer sand were to be used in future experiments, lower concentrations of B, Ca and P would need to be applied. Mount Compass sand would require an increase in the amount of Fe, along with lower levels of Mn, B, K and P. Since Laffer sand is costly and time consuming to obtain, Mt Compass sand was used in subsequent experiments, with a modified basal nutrient solution.

3.2.5 Conclusion

From these experiments, it can be concluded that Zn deficiency, as well as infection by *R. solani*, AG 8, can be achieved in medic in controlled conditions. Subsequent experiments were performed in growth chamber A (light flux density of photosynthetically active radiation of $450 \mu\text{E m}^{-2}\text{s}^{-1}$, set on an 8/16 h, 15/10°C day/night cycle) with plants inoculated with *Rhizobium meliloti* 7 days after planting. Mt Compass sand was used, with adjustment of the basal solution.

3.3 Experiment 2

3.3.1 Aim

The aim of this experiment was to alter the basal nutrient solution added to the soil to achieve non-limiting nutrient levels.

3.3.2 Materials and Methods

3.3.2.a Experimental Design

Genotypes Robinson and Paraggio were grown at two Zn levels (0 and 0.3 mg Zn/kg) in Mt Compass sand with an adjusted basal nutrient solution in three replicates. Levels of B, Mn, K and P were reduced, and Fe was increased. Plants were grown for 28 days in growth chamber A.

3.3.2.b Soil

Mount Compass sand was used, leached of soluble salts in RO water and air-dried as outlined in Experiment 1.

3.3.2.c Basal Nutrients

A modified nutrient solution was applied to Mt Compass soil prior to potting. The basal nutrients included (in mg/kg): 350 NH_4NO_3 ; 85 KH_2PO_4 ; 3.5 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 90 K_2SO_4 ; 90 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 3.5 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.5 $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$; 1 $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$; 0.15 $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$; 5 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.4 H_3BO_3 ; and 0.3 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. CaCO_3 (0.3%) was mixed thoroughly with the dry soil before the solution was added.

3.3.2.d Seed

Seeds of *M. scutellata* genotype Robinson and *M. truncatula* genotype Paraggio (Table 3.1) were surface-sterilised and germinated as described in Section 3.2.2.g.

3.3.2.e Procedure

Soil mixing and potting was performed as described in Section 3.2.2.h, with two germinated seeds per pot. Plants were thinned to one per pot 4 days after planting. After planting, the soil surface was covered in acid-washed, black plastic beads, and the soil watered to 12% water content. Pots were then placed in growth chamber A, inoculated with *Rhizobium meliloti* after 7 days growth and watered every second day.

After 28 days, plants were harvested, shoot and root dry weight determined and the shoots analysed for Zn concentration by ICP analysis as described in Section 3.2.2.h.

3.3.3 Results

Visual Observations

After 21 days, differences between Zn treatments were observed, with the effects more pronounced in Robinson than in Paraggio. No symptoms of Fe deficiency were observed during the growing period. At the end of the experiment, infection was observed on the roots, with symptoms characteristic of *R. solani* despite no inoculation with the pathogen.

Plant Growth Measurements

A significant interaction between genotype and Zn application occurred with shoot and root dry weight (Fig. 3.6). Robinson maintained a higher shoot dry weight at both Zn treatments, with a greater increase in response to Zn application than Paraggio (Fig. 3.6). Root dry weight was lower for Robinson deficient in Zn compared with genotype Paraggio, but with Zn application, Robinson maintained a higher root growth.

An adequate supply of Fe, B and K in the shoots occurred; however, P was still above the normal range. Manganese was deficient along with Zn at both levels of Zn application (Table 3.3).

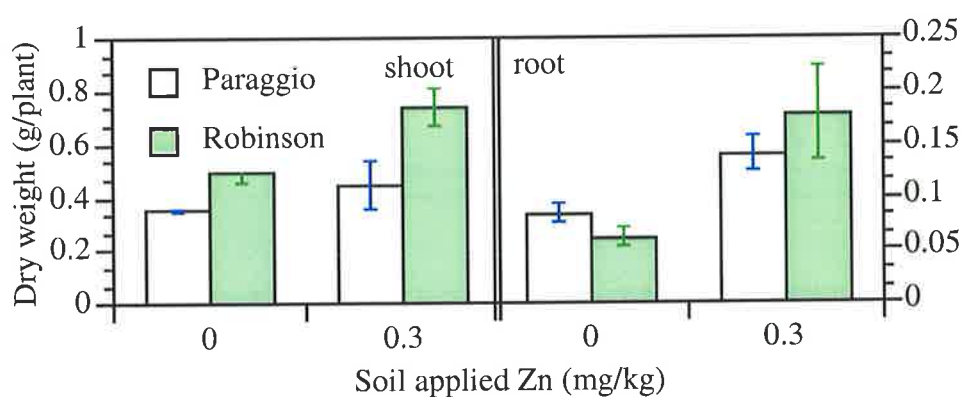


Figure 3.6 Effect of Zn application on shoot and root growth of *M. truncatula* genotype Paraggio and *M. scutellata* genotype Robinson. Plants were grown in Mt Compass sand with a modified nutrient solution for 28 days. Vertical bars represent $\pm$ standard error ($n=3$).

Table 3.3 Effect of Zn application on mean nutrient concentration from shoots of *M. scutellata* genotype Robinson and *M. truncatula* genotype Paraggio. Plants were grown in Mt Compass sand with adjusted basal nutrients for 28 days. See Table 3.2 for normal growth requirements in medics.

Element	Robinson (mg/kg)		Paraggio (mg/kg)	
	0 mg Zn/kg	0.3 mg Zn/kg	0 mg Zn/kg	0.3 mg Zn/kg
	mg/kg shoot dry weight			
Fe	46	52	52	35
Mn	18	21	33	28
B	37	32	46	44
Cu	11	10	14	12
Zn	11	16	14	21
	g/kg shoot dry weight			
Ca	25,741	16	18	16
Mg	4,177	3	5	4,
K	43,234	37	49	48
P	8,617	7	11	9
S	4,092	3	4	3

3.3.4 Discussion

With improvements to the proportion of nutrients in the soil, medics grew well, so the level of Zn applied was insufficient to meet demands. This indicated a need for an increase in Zn application to ensure the availability of an adequate supply of Zn. The lower Zn concentration observed in the Zn applied treatment is most probably a result of a higher dry weight produced due to improved soil nutrient status. When compared with experiment 1 Part a, Robinson and Paraggio were more than four times larger in this experiment.

Shoot Mn concentration was low in both genotypes, while P was high. Levels of Mn, may have been high in the previous experiment due to Fe deficiency inducing Mn toxicity. With the combination of increased Fe and reduced Mn, Mn became limiting. In the next experiment Zn and Mn levels were increased.

The increase in dry weight for both species occurred despite infection on the roots by *R. solani* in Mt Compass sand. *Rhizoctonia solani* has since been reported to be abundant in Mt Compass sand (A. Lake, pers. comm.) and that heating the soil above 60°C would be sufficient to kill the hyphae (S. Neate, pers. comm.). Heat treatment of Mt Compass sand was therefore adopted in subsequent experiments. This altered the availability of most nutrients which needed to be monitored.

3.4 Experiment 3

3.4.1 Aim

The aim of this experiment was to study the effects of soil-applied Zn, ranging from deficiency through to toxicity, in five medic genotypes grown in heat-treated Mt Compass sand, with the basal nutrient solution used in Experiment 2 adjusted further. More specifically, it was designed to determine the degree of variation within *M. truncatula*, in order to select a “standard” genotype for future experiments; and also to select the time of harvest which would allow a significant difference between Zn treatments to be observed. *M. truncatula* is one of the most commonly grown medic species in South Australia, making it the logical species to begin with.

3.4.2 Materials and Methods

3.4.2.a Experimental Design

A factorial design combining five genotypes of *M. truncatula*: (Caliph, Mogul, Paraggio, Parabinga and Ghor) with 5 Zn levels (0, 0.1, 0.3, 0.7 and 2 mg Zn/kg) in five replicates was conducted in pots containing heat-treated Mt Compass sand. Plants were grown in growth chamber A and harvested 28 and 42 days after planting.

3.4.2.b Soil

Mount Compass sand was used, leached of soluble salts in RO water, air-dried as previously described and heat-treated at 80°C for 6 hours on each of two consecutive days.

3.4.2.c Basal Nutrients

The basal nutrients were applied as described in Experiment 2, except for $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ which was increased to 5.5 mg/kg soil.

3.4.2.d Seed

Seed of Caliph, Mogul, Paraggio, Parabinga and Ghor were surface sterilised and germinated as previously described. See Table 3.4 for seed characteristics.

Table 3.4 Seed characteristics for Experiment 3.3

Genotype	Weight (mg/dry seed)	Concentration of Zn (mg/kg)	Zn content ($\mu\text{g Zn/seed}$)
Caliph	3.9	46	0.18
Mogul	3.6	51	0.18
Paraggio	4.0	41	0.16
Parabinga	4.0	53	0.21
Ghor	4.7	62	0.29

3.4.2.5 Procedure

Soil mixing and potting were performed as previously described, with two germinated seeds sown per pot of 1 kg soil. After planting, soil surface was covered in acid-washed black plastic beads, and soil watered to 12% water content. Pots were then placed in growth chamber A, inoculated with *Rhizobium meliloti* strains after 7 days, and thinned to one plant per pot. Watering was done every second day.

After 28 and 42 days, plants were harvested, shoot and root dry weight determined and the shoots and roots analysed for Zn concentration by ICP. Zinc efficiency rankings were calculated for each genotype based on shoot dry matter production from the following equation (Graham and Rengel, 1993):

$$\text{Zn efficiency} = \frac{\text{Shoot dry weight at 0 Zn}}{\text{Shoot dry weight at + Zn}} * 100\%$$

Genotypes were ranked as Zn-efficient, Zn-moderately efficient or Zn-inefficient, according to the technique employed by Rengel and Graham (Rengel and Graham, 1996). The mid-point of the medium-efficiency interval corresponds to the median Zn-efficiency value, while the upper and lower boundaries are constructed by adding to, or subtracting from, the median point the value of the standard error of the genotype effect. Genotypes above or below the medium interval were ranked as Zn-efficient or Zn-inefficient, respectively.

3.4.3 Results

Visual Observation

Symptoms of Zn deficiency were observed when harvested at 28 and 42 days in the 0 and 0.1 mg/kg Zn treatments, with reduced growth more pronounced at the 0 Zn levels.

Plant Growth Measurements

A three-way ANOVA indicated that the interaction between genotype, Zn application and time of harvest did not have a significant effect on shoot or root dry weight or on the shoot/root ratio ($p \geq 0.9$; Fig. 3.7). However, the two-way interactions between genotype and time of harvest and between Zn and time of harvest did have a significant effect on shoot and root dry weight ($p \leq 0.001$ in all cases; Fig. 3.7). Genotype Ghor maintained the highest yield across Zn treatments. Maximum yield occurred in all genotypes with 0.7 mg Zn/kg soil. The main effect of Zn application was the only significant effect on the shoot/root ratio ($p \leq 0.0001$; data not shown), with an increase in the shoot/root ratio with declining Zn.

Zinc treatment at 0.7 mg/kg was used as the +Zn treatment for calculating Zn efficiency. With Zn deficiency, levels 0 and 0.1 both resulted in Zn deficiency and were therefore used to calculate Zn efficiency of the five genotypes at severe Zn deficiency and moderate Zn deficiency, respectively. Zinc-efficiency was determined by comparing the ratio of shoot growth at deficient (0 and 0.1 mg Zn/kg) and sufficient (0.7 mg/kg) levels, as discussed in Chapter 2.

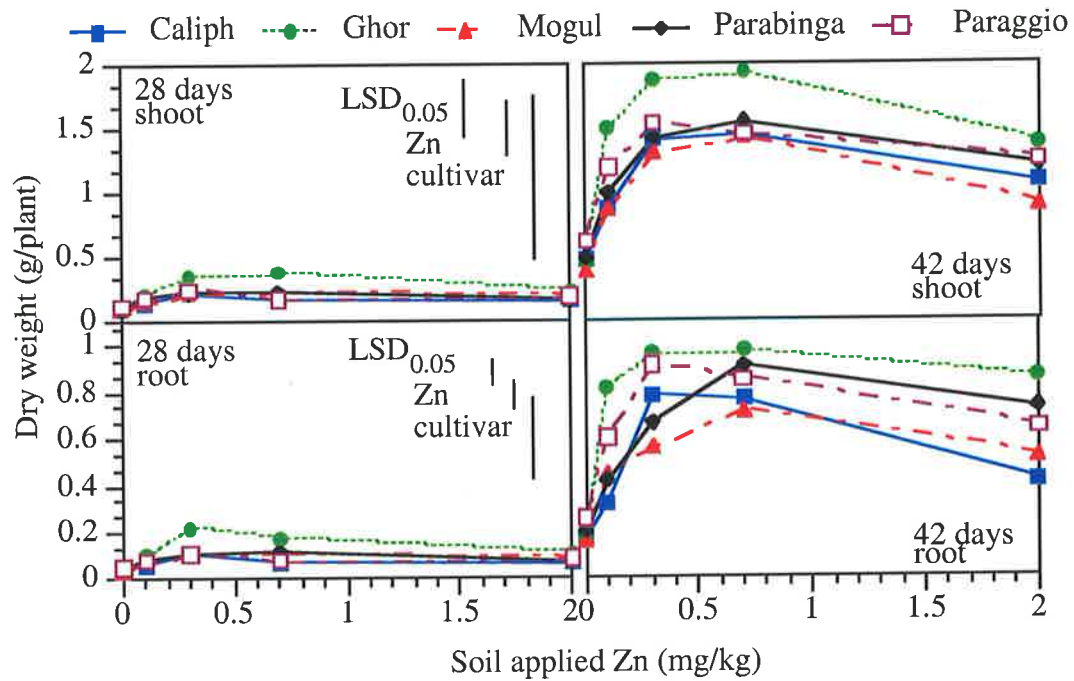


Figure 3.7 Effect of Zn application on the shoot and root dry weight of five *M. truncatula* genotypes. Plants were grown in Mt Compass sand and harvested 28 and 42 days after sowing. Vertical bars represent Fishers Protected LSD for main effects: genotype, Zn application and time of harvest.

The Zn efficiency ranking differed with harvest time, and with the level of Zn deficiency (Fig. 3.8). Paraggio was the only genotype consistently ranked as Zn-efficient, while Parabinga was the only genotype consistently ranked as medium-efficient. Caliph was ranked medium-efficient except at day 28 at 0 Zn where it was Zn-efficient. A similar trend occurred for Mogul except ranking increased from Zn-inefficient to medium-efficient with moderate and severe Zn deficiency, respectively, at day 28. Ranking for Ghor ranged from Zn-inefficient to Zn-efficient.

The interaction between genotype, Zn application and time of harvest was not significant at the $p=0.05$ level, for the concentration of Zn in both the shoot and root (Fig. 3.9). However, the two-way interaction between Zn application and time of harvest was significant in the shoot and root ($p \leq 0.006$ in both cases; Fig. 3.9), with a higher concentration of Zn with Zn application at the earlier harvest. Zinc concentration was higher in roots than shoots for all genotypes (Fig. 3.9).

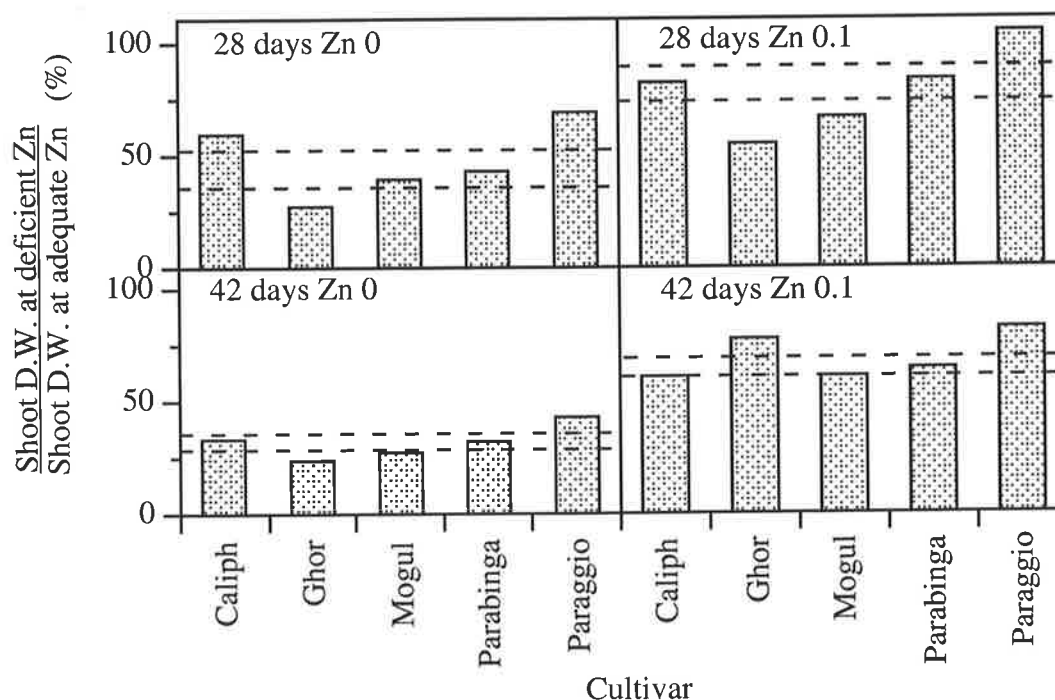


Figure 3.8 The Zn efficiency ranking of five *M. truncatula* genotypes based on the ratio of shoot dry matter production at Zn deficiency (severe, 0 Zn, and moderate, 0.1 mg Zn/kg), and sufficient Zn supply (0.7 mg Zn/kg). Plants were grown in Mt Compass sand for 28 and 42 days. Dashed lines indicate the upper and lower boundaries of the medium efficiency interval, with Zn-efficient ranking above and Zn-inefficient ranking below the medium-efficiency interval.

At 0, 0.1 and 0.3 mg Zn/kg, Zn concentration in the shoots at both harvests were below 25 mg/kg (Fig. 3.9), the level required for normal growth (Weir and Cresswell, 1994). Adequate Zn concentration occurred at 0.7 mg Zn/kg, and toxicity occurred at 2 mg/kg. With toxicity, Zn concentration in the root increased to a greater extent than in the shoots.

Although the interaction between genotype, Zn application and time of harvest did not have a significant effect on the proportion of Zn transported to the shoot, the main effects were significant ($p \leq 0.03$ for all main effects; Fig. 3.10). The proportion of Zn transported to the shoots increased between 0 and 0.7 mg Zn/kg soil. At the highest level of Zn fertiliser, a lower proportion of Zn was transported to the shoots compared with other levels of Zn application. At the later harvest, a higher proportion of Zn was present in the shoot.

Genotype Mogul tended to have the higher amount of Zn transported to the shoot while Ghor had the lowest (Fig. 3.11).

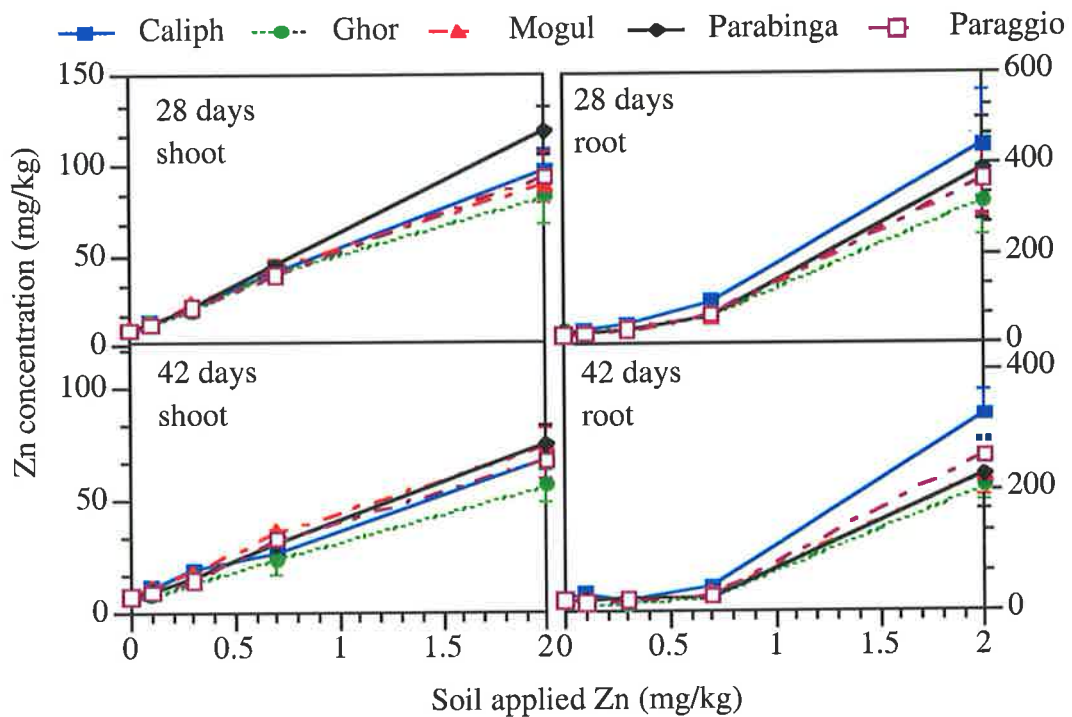


Figure 3.9 Effect of Zn application on Zn concentration in the shoots and roots of five genotypes of *M. truncatula*. Plants were grown in Mt Compass sand for 28 and 42 days. Vertical bars represent $\pm$ standard error of 3 replicates.

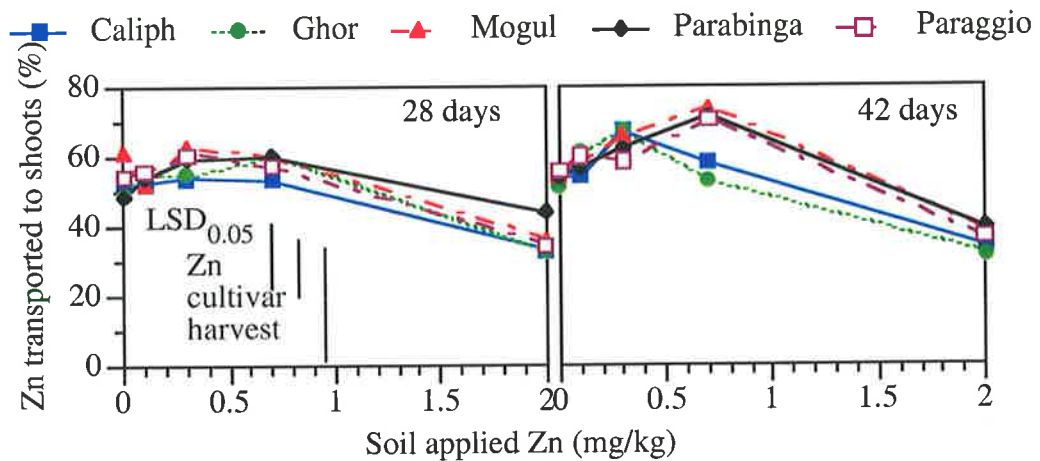


Figure 3.10 Effect of Zn treatment on the relative amount of Zn transported to the shoots of five genotypes from *M. truncatula*, grown in Mt Compass sand for 28 and 42 days. Vertical bars represent Fisher's Protected LSD.

With severe and moderate levels of Zn deficiency, a significant increase in the concentration of Fe, Mn, B, Cu, Mg, K, P and S in the shoots of all genotypes was observed by day 42 (data not shown). In the roots, Mn, B, K, and P were significantly higher in Zn-deficient plants compared with an adequate Zn level (0.7 mg Zn/kg). Concentration of Mg and S were significantly lower in the roots of Zn-deficient plants compared with roots of Zn-adequate plants. When Zn was applied at a toxic level (2 mg Zn/kg), Mn was higher in shoots and roots while Ca and P were in higher concentration in the roots only.

Although most elements increased in concentration in the shoot, only P reached toxic levels (approximately 15,000 mg/kg at day 42) for all genotypes. Phosphorus levels were high with an adequate Zn supply, with genotypes ranging from 5,000 to 6,000 mg/kg in the shoot. As Zn deficiency increased in severity, all other elements remained in the adequate range for normal growth as outlined in Table 3.2.

3.4.4 Discussion

Genotypic differences were not obvious with increasing levels of soil-applied Zn in this study, with all genotypes deficient in Zn at 0, 0.1 and 0.3 mg Zn/kg, sufficient in Zn at 0.7 mg/kg and suffering Zn toxicity at 2 mg/kg at both harvests. Differences in Zn-efficiency between genotypes were more pronounced 42 days after sowing compared with 28 days.

Zinc efficiency ranking varied with the severity of Zn deficiency and age of the plant. For example, at 42 days Ghor was shown to be Zn-inefficient and Zn-efficient at severe and moderate Zn deficiency, respectively. The larger seed size of Ghor would have contributed to Ghor being Zn-efficient when Zn was moderately deficient, however, when Zn was severely deficient the greater seed nutrient reserves were not sufficient to overcome Zn deficiency. Paraggio was ranked Zn-efficient at both levels of Zn deficiency despite a smaller seed size. This suggests some other factor is contributing to Zn efficiency. The requirements for a Zn-efficient genotype are the subject of Chapter 7.

A medic cultivar representative of the average was desired as a 'standard' genotype for future work on the Zn nutrition and *R. solani* interaction. Caliph was selected as it had medium Zn efficiency at day 42, and the seed Zn content and Zn concentration in the shoots were in the middle range compared with the other genotypes.

Zinc deficiency resulted in higher concentrations of P in the shoot. An increase in plasma membrane permeability of root cells under Zn deficiency is involved in P accumulation at low Zn supply. This results in increased passive permeability of nutrients into the plant and has been demonstrated for P, Cl and B (Welch *et al.*, 1982; Graham *et al.*, 1987). It is not surprising that the concentration of other nutrients (eg B, Mg and S) also increased. A reduction in applied P levels was therefore required in subsequent experiments.

The concentration of Fe, Cu and Mn also increased under Zn deficiency. An increase in Fe may be explained by acidification of the rhizosphere or increased release of reductants as a result of Zn deficiency (Brown, 1979; Jolley and Brown, 1991). Zinc deficiency has been shown to stimulate ferric reductase activity in roots of peas and beans and to acidify nutrient solutions, both of which enhance Fe uptake (Jolley and Brown, 1991; Kochian, 1993). Previous reports of Zn deficiency increasing Cu uptake have been explained by antagonism between the two nutrients. Since Zn^{2+} and Cu^{2+} are similar in size, competition for the same membrane transporter occurs (Kochian, 1993). Similarly, Mn uptake can be reduced with increasing Zn supply due to antagonistic interactions.

3.5 Experiment 4

3.5.1 Aim

This experiment was designed to determine if increasing the number of plants per pot would reduce variation and to determine if a decreased level of P fertilisation was adequate for normal growth under Zn-adequate conditions.

3.5.2 Materials and Methods

3.5.2.a *Experimental Design*

A factorial design combining increasing numbers of Caliph plants per pot (n=1, 2 and 3) in three replicates was conducted in pots containing heat-treated Mt Compass sand. Plants were grown in growth chamber A and harvested 42 days after planting.

3.5.2.b *Soil*

Mt Compass sand was used, leached of soluble salts in RO water, air dried, and heat-treated at 80°C for 6 hours on each of two consecutive days as previously described.

3.5.2.c *Basal Nutrients*

The basal nutrients were applied as outlined in experiment 3, with KH_2PO_4 reduced to 57 mg/kg soil.

3.5.2.d *Seed*

Seed of Caliph, used in the previous experiment, were surface sterilised and germinated as previously described. Seed characteristics are presented in Table 3.4.

3.5.2.e *Procedure*

Soil mixing and potting were performed as previously described, with five germinated seeds sown per pot of 1 kg soil. After planting, soil surface was covered in acid-washed black plastic beads and soil watered to 12% water content. Pots were then placed in growth chamber A, inoculated with *Rhizobium meliloti* strains after 7 days and, depending on the treatment, thinned to one, two or three plants per pot. Watering was done every second day.

After 42 days, plants were harvested, shoot and root dry weight determined and the shoots analysed for Zn concentration by ICP analysis. Soil pH was also measured before planting and at the end of the experiment.

3.5.3 Results

After nutrients were added to the soil, the pH prior to planting was measured at 6.38. At the end of the experiment the pH had increased to 8.39.

With an increase in plant number, dry weight per plant was significantly reduced (Fig. 3.11), and less variation was recorded. Although dry weight per plant was reduced, no nutrients were severely limited (Table 3.5). By reducing the amount of KH_2PO_4 added to the soil, P levels were reduced considerably, and were in the range for adequate growth with one and two plants per pot. With three plants, P levels were slightly below the level recommended for adequate growth. A similar situation also occurred with Mn and B.

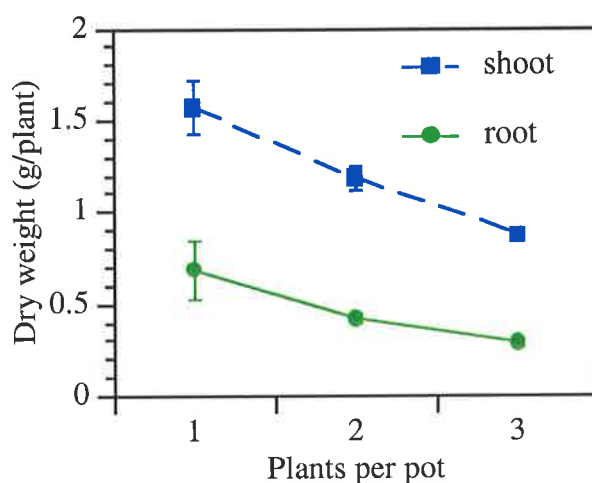


Figure 3.11 Effect of plant number per pot on shoot and root dry weight of *M. truncatula* genotype Caliph, 42 days after planting in Mt Compass sand. Vertical bars represent $\pm$ standard error (n=3).

Table 3.5 Mean nutrient analysis from shoots of *M. truncatula* genotype Caliph grown in Mt Compass sand, with increasing numbers of plants per pot. See Table 3.2 for requirements for normal growth of medics as outlined in Weir and Cresswell (1994).

Element (mg/kg)	Plant number per pot		
	1	2	3
	mg/kg shoot dry weight		
Fe	57	52	48
Mn	41	38	33
B	25	25	24
Cu	9	9	10
Zn	27	28	31
	g/kg shoot dry weight		
Ca	23	20	22
Mg	3	3	3
K	25	20	18
P	3	3	3
S	3	3	3

3.5.4 Discussion

In subsequent experiments, two plants per pot were used. This was decided based on one plant per pot having a higher variation between replicates, while three plants per pot were bordering on nutrient deficiency for P, Mn and B. Since all nutrients measured were in the adequate range with one and two plants, and plants were assumed to have a constant water supply since watering occurred every second day, the reduction in growth per plant in treatments with one to two plants may be due to competition for light.

3.6 Conclusion

From experiments 2, 3 and 4, an adequate basal nutrient solution added to Mt Compass sand was determined. In order for Mt Compass sand to be used in studies on Zn deficiency and on infection by *R. solani*, heat-treatment of the soil is required to destroy any naturally occurring *R. solani*. Zinc deficiency is still achieved in Mt Compass sand after heat-treatment. Genotype

Caliph was selected as the standard genotype to be used in all experiments as it is of medium Zn efficiency. Planting two plants per pot is optimal under conditions tested and results in low variation among replicates.

Effect of Infection by *R. solani* on the Nutritional Status of *Medicago* species

4.1 Introduction

Earlier studies have reported concentrations of nutrients in shoot tissue to be affected by pathogens. Blevins *et al.* (1995) found an increase in leaf concentration of Mg and Ca with infection by cereal cyst nematode, while K and Mg concentration in the root decreased and Ca and P increased. Wall *et al.* (1994) reported a decrease in concentrations of P, K, Fe, Zn, Cu, B, Mn and Mo in the shoots of plants infected with *R. solani*, an increase in concentrations of Ca and Na, and no change in concentrations of Mg and S due to the disease. Despite infection by *R. solani*, all nutrients remained in the adequate range, except P (Wall *et al.*, 1994). Phosphorus became marginally deficient, when a high level of *R. solani* inoculum was added to the soil. Wall *et al.* (1994) suggest that purpling of stem bases, often seen in infected field-grown plants, may be due to P deficiency induced by severe root pruning.

During infection, *R. solani* produces brown sunken lesions on the root cortex which have been shown to reduce Zn uptake in wheat (J. Cooke, unpublished). Nutrient transport from areas above or below the point of infection are not affected initially as the xylem and phloem remain intact. However, if infection is sufficiently severe, the characteristic "spear tip" may form, where the cortex rots away exposing, and finally breaking, the stele. The root on the distal side of the lesion dies and nutrient transport is disrupted. By "pruning" the roots, *R. solani* may reduce nutrient uptake in plants, imposing further stress on the plant.

Pruning the root system physically has been shown to alter the biomass and nutrient composition in shoots, roots, and fruit in a number of studies. Baugher and Miller (1991) observed root pruning in nectarines to decrease fruit N, P, K, Mg, Fe, B and Zn levels, while

Ca levels were increased. Root pruning did not affect nutrient levels in the leaf, but it did reduce fruit diameter and shoot length. Another study demonstrating the effect of root pruning on growth was conducted by Ayling (1989). In this study, half of the root axes in winter wheat were removed at the start of stem elongation with a 13% reduction in shoot production observed one month later and an 8% reduction in grain yield. Ayling (1989) also observed negligible effects of root pruning on the concentration of N, P, K, Mg and Mn in the shoots. Colquorn (1937) found that early removal of wheat roots caused a more severe yield loss than later. This is of particular relevance to the "pruning" effect of *R. solani* as plants are most susceptible to infection during emergence and at the seedling stage.

Before studies on the effect of Zn nutrition on infection by *R. solani* are performed, it is desirable to determine the effect of *R. solani* on the nutritional status of medics in a non-nutrient-limiting environment. To justify a detailed study of the interaction between disease and an environmental factor, it is also desirable to establish evidence of a relationship between the particular disease and the factor under field conditions (Colhoun, 1973). A field survey by Thongbai *et al.* (1993a) demonstrated that of eleven elements analysed, only the Zn concentration in the shoots of wheat showed a significant negative correlation with disease severity. To date, no field survey has been undertaken with medics. This chapter contains the results from one experiment, performed in controlled conditions, and one field survey, both designed to observe the relationship of infection by *R. solani* on the nutritional status of medics.

4.2 Pot Experiment

4.2.1 Aim

The aim of this experiment was to determine if infection by *R. solani* alters the concentration of certain nutrients in genotype Caliph, from 14 to 56 days after sowing.

4.2.2 Materials and Methods

4.2.2.a *Experimental Design*

A factorial design was used, studying the effects of increasing levels of soil inoculation with *R. solani* (0, 1 and 8 propagules/kg soil) at four harvests (14, 28, 42 and 56 days after sowing) in four replicates. Caliph was grown in Mt Compass sand with adequate levels of all nutrients.

4.2.2.b *R. solani Inoculum*

Inoculum was prepared on sterile millet seed as outlined in Chapter 3. For low and high infection 1 and 8 propagules infected with *R. solani* were added to one kg soil. For the control, nil *R. solani*, 8 sterilised propagules were added to one kg soil.

4.2.2.c *Soil*

Mt Compass sand was used, leached of soluble salts in RO water, air-dried, and heat-treated at 80°C for 6 hours on each of two consecutive days as outlined in Chapter 3.

4.2.2.d *Basal Nutrients*

The basal nutrients were applied as outlined in Chapter 3 with 0.9 mg Zn/kg added as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

4.2.2.e *Seed*

Seed of Caliph were surface sterilised and germinated as outlined in Chapter 3. Seed characteristics for Caliph are presented in Table 3.4.

4.2.2.f *Procedure*

Soil mixing and potting were performed as previously described with pots watered to 10% water content, covered and incubated after the soil was inoculated with the appropriate *R. solani* treatment. At the end of two weeks, five germinated seeds were planted per pot.

After planting, the soil surface was covered in acid-washed black plastic beads and soil watered to 12% water content. Pots were then placed in growth chamber A, inoculated with *Rhizobium meliloti* strains after 7 days and thinned to two plants per pot. Pots were watered every second day.

After 14, 28, 42 and 56 days, plants were harvested, shoot and root dry weight determined and nutrient analysis performed on the shoots and roots by ICP analysis. The total amount of nutrient in the shoots and roots was calculated by multiplying the dry weight by the nutrient concentration. To further understand the effect of infection by *R. solani* on the nutritional status, the proportion of total plant nutrient content that was transported to the shoot, expressed as a percentage, was calculated, as well as the total amount of nutrient taken up by the plant per gram of root.

4.2.3 Results

Visual Observations

At day 14, the unifoliate was fully emerged in plants treated with 0 and 1 propagule while it had not yet fully expanded in pots inoculated with 8 propagules. At day 28 and 42, no visible difference in treatments was observed, with all plants appearing healthy. Between day 42 and 56, growth of plants in all pots was reduced, with new leaves smaller in size than normal. Older leaves also began to turn yellow with some eventually senescing. Visual symptoms at day 56 were similar to those of P deficiency.

Plant Growth Measurements

Shoot and root dry weight were not significantly affected by soil inoculation of *R. solani*, at any harvest (Fig. 4.1). However, at day 42, a stepwise reduction in mean shoot dry weight was observed with increasing inoculation (Fig. 4.1).

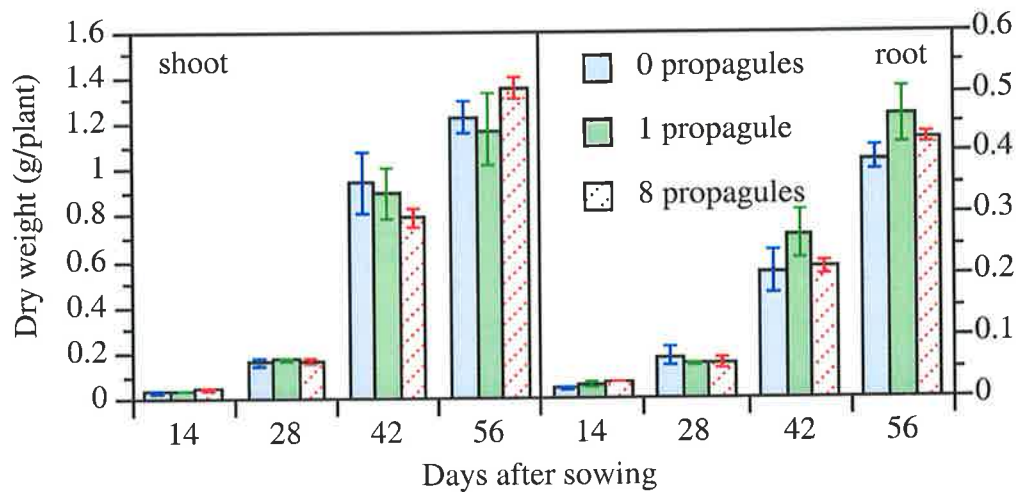


Figure 4.1 Effect of inoculation with *R. solani* AG8 on shoot and root dry weight of Caliph medic grown in Mt Compass sand with adequate nutrients: 14, 28, 42 and 56 days after sowing. Vertical bars represent $\pm$ standard error ($n=4$).

Changes in nutrient concentration in both the shoots and roots occurred with infection by *R. solani* at all harvests along with differences in the amount of nutrient transported to the shoot and the amount taken up per gram of root. Mean data, at each harvest, are presented in Appendix 4. All nutrients remained in the range for normal growth until after day 42. At day 56, B and S were below the range for normal growth in all treatments, whereas P was below the range for normal growth only when a high amount of *R. solani* was present.

Owing to small size of plants 14 days after sowing, the 4 replicates were pooled into two groups for more accurate analysis by ICP. All statistics on nutrient concentration (shoot and root), percentage transported to the shoot, and total uptake, at day 14 were therefore based on two replicates.

Time of harvest had a significant effect on the concentration of most elements in the shoot and root, on the amount of nutrient uptake per unit root and on the proportion of that nutrient transported to the shoot: Mn, B, Cu, Zn, Ca, Mg, K, P, and S ($p \leq 0.05$). Iron concentration in the root and Fe uptake per unit root were not significantly different between harvests, but shoot concentration and proportion transported to the shoots were affected significantly by harvest.

The amount of *R. solani* added to the soil had a significant effect on the concentration of Mn, Ca and P in the shoots (Fig. 4.2). At all harvests, the concentration of Mn in the shoot was significantly lower with increasing inoculum. Generally, P followed a similar pattern but concentration was not significantly different at day 14. Calcium concentration generally increased with increasing inoculum, with the effect most noticeable at day 42.

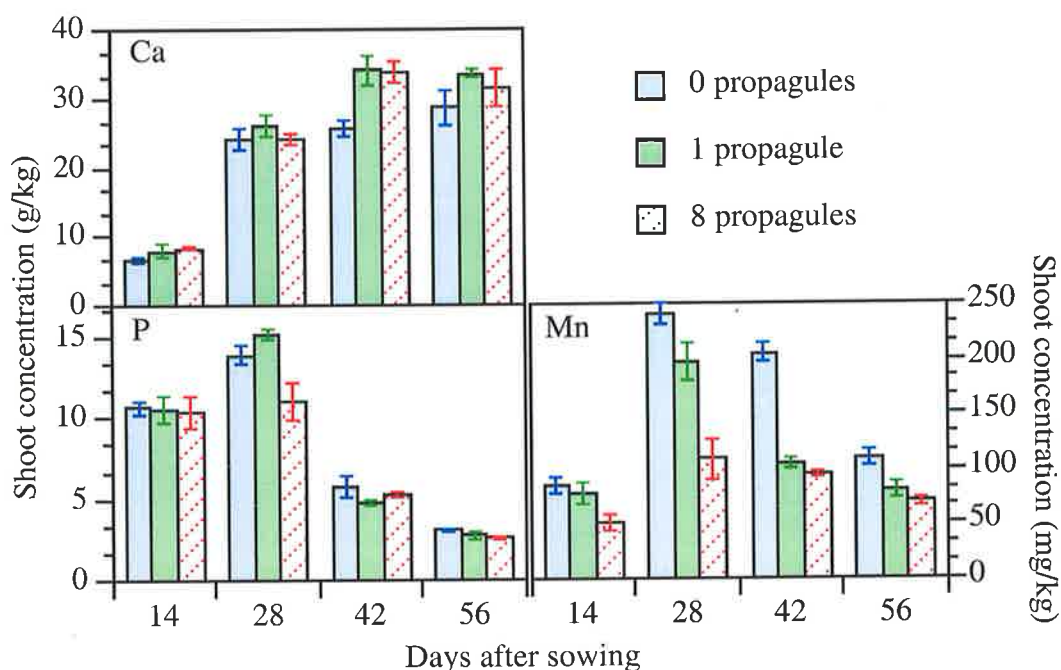


Figure 4.2 Effect of inoculation with *R. solani* AG 8 on the concentration of Mn, Ca and P in the shoot of Caliph medic grown in Mt Compass sand with adequate nutrients: 14, 28, 42 and 56 days after sowing. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at days 28, 42 and 56).

The concentration of Zn in the shoots was not affected by increasing levels of *R. solani* (see Appendix 4). However, the concentration of Zn in the root was reduced significantly at day 14 and 28 and increased significantly at day 42 and 56 with the highest level of added inoculum (Fig. 4.3). No significant change in the amount of Zn uptake per gram root occurred with infection (see Appendix 4), but the proportion of Zn transported to the shoot increased at day 14 and 28 and decreased at day 42 and 56 due to *R. solani* (Fig. 4.3).

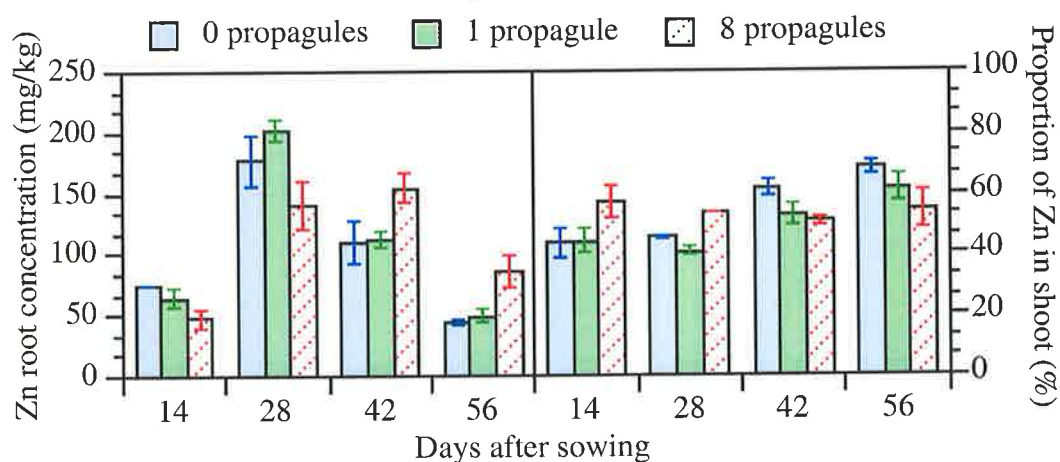


Figure 4.3 Effect of inoculation with *R. solani* AG 8 on the concentration of Zn in the root and the proportion of Zn transported to the shoot for Caliph medic. Plants were grown in Mt Compass sand with adequate nutrients for 14, 28, 42 and 56 days. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at days 28, 42 and 56).

The concentration of Mn in the roots was significantly lower during the first three harvests at the highest level of added inoculum (Fig. 4.4). No difference was observed at the last harvest. This corresponds to a reduction in uptake of Mn during the first three harvests, with no change at day 56 with infection. One point that does not fit this trend occurred at day 28 in the 1 propagule treatment. Here, the Mn concentration increased in the root along with an increase in uptake (Fig. 4.4). This increase was not observed in the shoot (Fig. 4.2).

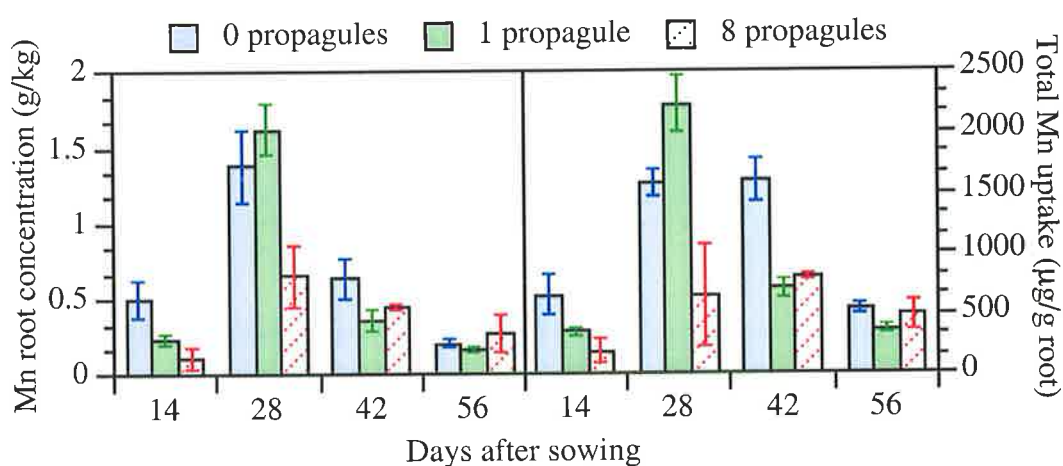


Figure 4.4 Effect of inoculation with *R. solani* AG 8 on the concentration of Mn in the root and on total uptake per gram of root for Caliph medic grown in Mt Compass sand with adequate nutrients: 14, 28, 42 and 56 days after sowing. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at day 28, 42 and 56).

At day 14, K concentration in the root was reduced in the presence of *R. solani* which corresponded with a reduction in total K uptake (Fig. 4.5). The proportion of K in the shoot was also higher with increasing levels of inoculum. At day 28, variation was high in estimating root concentration, with the treatment of 1 propagule resulting in a significantly lower concentration. Again, this corresponded with a reduction in uptake at 1 propagule and an increase in transport to the shoot. By day 42 no significant difference in K concentration was recorded in the shoot in the presence of *R. solani*, but a general decrease in K uptake occurred. Again, uptake was lower when 1 propagule was added to the soil. The proportion of K in the shoot was lower when *R. solani* was added. At the last harvest, root K concentration and transport of K to the shoot were not affected by inoculation but uptake was lower when only 1 propagule was present.

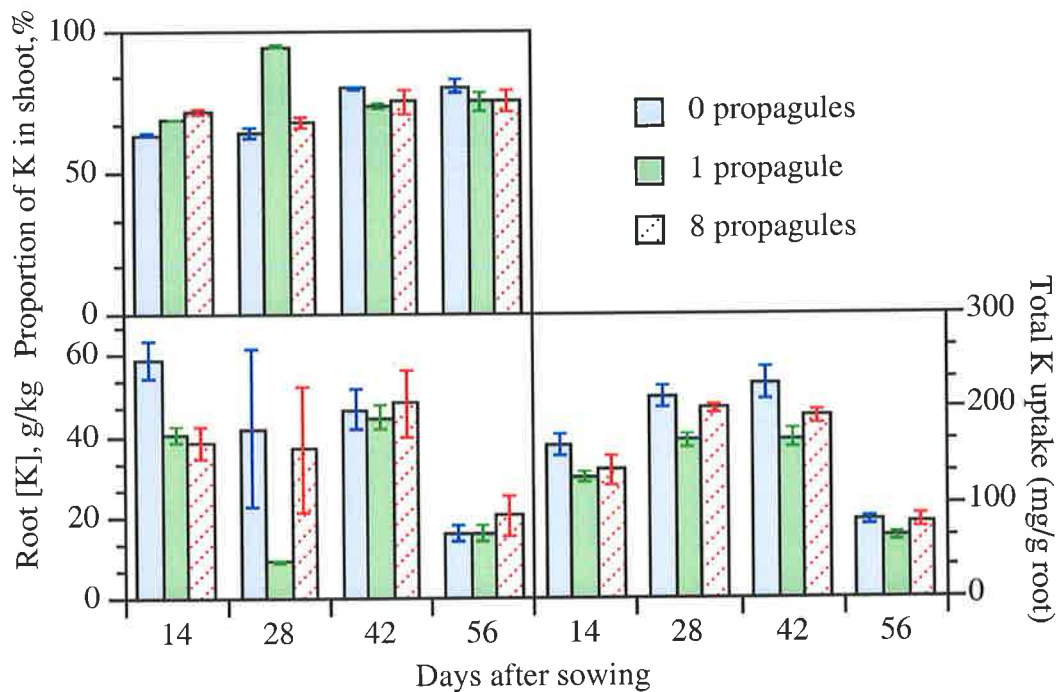


Figure 4.5 Effect of inoculation with *R. solani* AG 8 on the concentration of K in the root and on total uptake per gram of root for Caliph medic. Plants were grown in Mt Compass sand with adequate nutrients for 14, 28, 42 and 56 days. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at days 28, 42 and 56).

No change in shoot P concentration occurred at day 14 due to *R. solani* treatments (Fig. 4.2), but a reduction in root concentration and uptake occurred along with an increase in

transport of P to the shoot (Fig. 4.6). At day 28, plants grown in the presence of only 1 propagule infected with *R. solani* had a higher P concentration in shoots, a higher transport of P to the shoot, a higher uptake rate per unit root, and a lower concentration of P in the root than plants exposed to no fungus and plants exposed to a higher amount of the fungus. At day 28, plants grown in the presence of 8 propagules had a lower shoot P concentration (Fig. 4.2) and a lower level of P uptake per unit root (Fig. 4.6) compared with 0 and 1 propagules/kg soil. However, the proportion of P in the shoot, and root P concentration, did not differ significantly from plants not exposed to the fungus at day 28. By day 42, shoot P concentration, transport to the shoot and P uptake were lower with increasing levels of inoculum, but root P concentration was not affected by *R. solani* (Fig. 4.6). Shoot concentration of P was still lower with increasing inoculum at day 56 along with the proportion in the shoot. Inoculum caused no change in the uptake of P per unit root (Fig. 4.6).

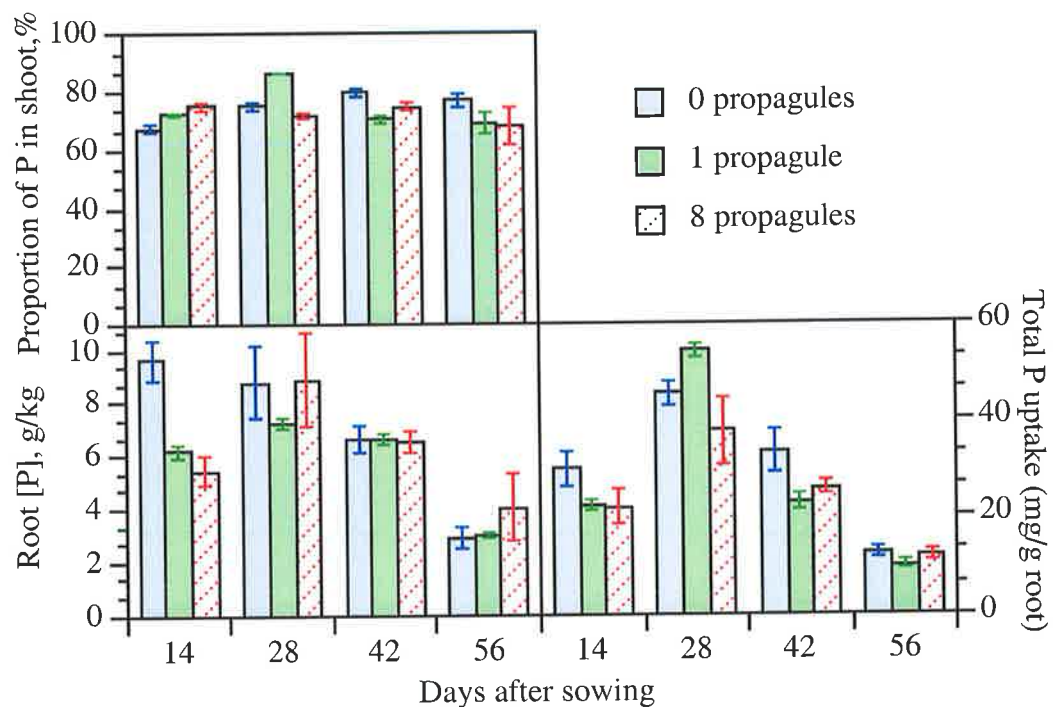


Figure 4.6 Effect of inoculation with *R. solani* AG 8 on the concentration of P in the root, on total uptake per gram of root and on the proportion of P transported to the shoot for Caliph medic. Plants were grown in Mt Compass sand with adequate nutrients for 14, 28, 42 and 56 days. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at days 28, 42 and 56).

The amount of S uptake per unit root was significantly reduced with added inoculum across harvests (Fig. 4.7). No significant change in the S concentration of either shoots or roots occurred and the relative amount of S transported to the shoots was not affected by the addition of *R. solani* (Appendix 4).

The concentration of Cu in the shoots and roots, and uptake of Cu per unit root were not affected by the addition of the fungus (see Appendix 4). Transport of Cu to the shoot was significantly increased at day 14 and 28 with increasing levels of *R. solani*, and were lower at day 42 and 56 (Fig. 4.7).

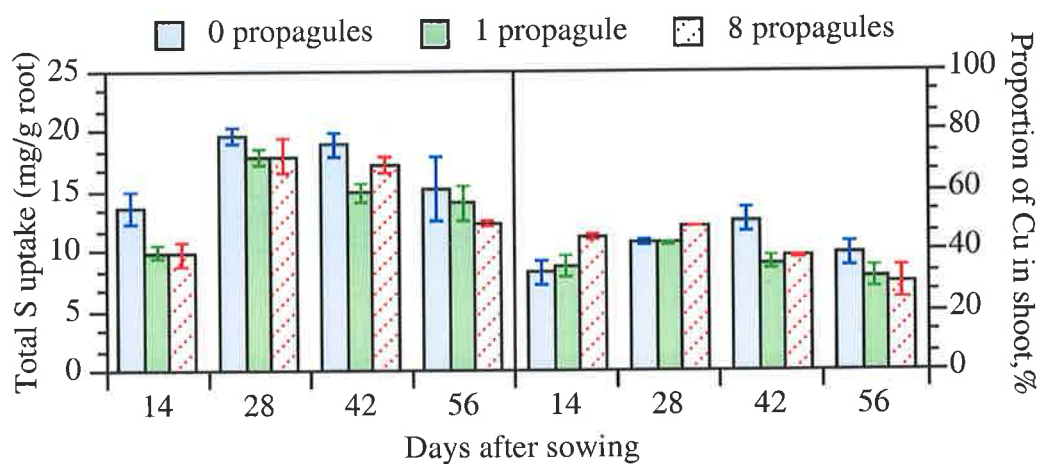


Figure 4.7 Effect of inoculation with *R. solani* AG 8 on total uptake of S per gram of root and on the proportion of Cu transported to the shoot for Caliph medic. Plants were grown in Mt Compass sand with adequate nutrients for 14, 28, 42 and 56 days. Vertical bars represent $\pm$ standard error ($n=2$ at day 14 and $n=4$ at days 28, 42 and 56).

4.2.4 Discussion

In this experiment no significant change in dry weight occurred as levels of *R. solani* increased, even though visual differences were observed at day 14. By day 14, plants were still small (emerged cotyledons to fully emerged unifoliate leaf), with yield loss difficult to detect. The lack of yield reduction also implies that infection was not severe at any level of inoculum. However, changes in nutrient uptake and transport within plants occurred, suggesting that even

with a low degree of infection, the delicate balance between the physiological processes of the root and shoot are disrupted. This experiment demonstrates the complexity involved in maintaining adequate nutrition in plants infected by *R. solani* even at levels of infection too low to affect yield.

The concentration of most nutrients in the shoots remained unaffected by increasing inoculum throughout the experiment. Manganese was the only nutrient to be reduced in concentration in the shoots at all harvests (Fig. 4.2), but even it remained in a range sufficient for normal shoot growth. This reduction in shoot concentration corresponds with reduced uptake by the roots in the presence of *R. solani* and suggests the fungus is reducing the availability of Mn to the plant. Root exudates may provide a food source for *R. solani*, resulting in less root exudates being available for the dissolution of Mn oxides. Alternatively, *R. solani* may be similar to *G. graminis* in its ability to oxidise Mn (Buchhorn, 1988), making it no longer available to the plant. Since Mn is an immobile nutrient in soil and roots largely rely on diffusion for uptake, a large contact surface with the soil is important for Mn uptake. Root pruning by *R. solani* reduces the root surface thus impacting on root uptake. However, Mn deficiency did not have a significant effect on disease severity in wheat (Hall, 1984). Other nutrients are also diffusion-limited, but no reduction across harvests occurred with increasing infection. This suggests either medic is more sensitive to the effects of root pruning on Mn nutrition compared with other nutrients, or Mn oxidation in the soil is enhanced.

Concentrations of B, S and P in the shoot were reduced by day 56, with the concentration of P in the shoot below the requirements for normal growth when a high amount of *R. solani* was present. However, visual symptoms of P deficiency were observed in all treatments, indicating the sufficiency range given in Weir and Cresswell (1994) for normal growth may be too low when whole shoot analysis is done. They did not indicate what vegetative material was used for obtaining the levels reported to be associated with normal growth. The reduction in concentration of B, S and P suggests soil nutrient reserves were either depleted or made unavailable, and plants may have been subjected to deficiencies which limited growth in all

treatments, and hence reduced the response to infection by *R. solani*. This may explain why a decrease that occurred in shoot dry weight at day 42 with increasing inoculum, although not significant, was not exaggerated 14 days later. If this were the case, results from day 56 should be used with caution as *R. solani* may not be the only factor limiting growth and nutrient uptake.

In order to maintain a constant concentration of nutrient in the shoot of inoculated plants, an increase in nutrient uptake and an increase in translocation from uninfected roots is required. Compensation from uninfected root axes could make it difficult to determine the direct effect of the pathogen by observing nutrient concentration in the shoot alone. Although the dry weight and the concentration of most nutrients remained constant in this experiment, the percentage of nutrients transported to the shoot and the amount taken up on a per gram root basis did differ in the presence of *R. solani*, with differing responses often occurring between the low and high pathogen treatment. For example, at day 28 the uptake of K (Fig. 4.5) was lower when 1 propagule was added to the soil compared with both 0 and 8 propagules. Conversely, transport to the shoot was much higher with a low level of inoculum.

For the purpose of future experiments, understanding the effect of *R. solani* on Zn nutrition is essential. The concentration of Zn remained constant in the shoot (Fig. 4.2) with infection by *R. solani*, which is consistent with the findings of Thongbai *et al.* (1993b), but not of Wall *et al.* (1994). Thongbai *et al.* (1993b) grew wheat (*Triticum aestivum* cv. Songlen) in pots containing 0, 2 and 4 propagules in 500 g of soil for 50 days, while Wall *et al.* (1994) grew wheat (*T. aestivum* cv. Condor) in 3 kg of soil with 0, 20 and 40 propagules for 49 days with varied nitrogen supply. In the experiment by Wall *et al.* (1994), Zn concentration was significantly reduced in the shoot from 35 to 28 to 24 mg/kg with increasing inoculum. In both experiments the soil was inoculated 2 weeks prior to planting with sterile millet seed infected with *R. solani* AG8, after it was divided equally between two soil layers. All experiments (including the one presented in this chapter) used the same technique for making and adding inoculum, used similar levels of inoculum per kg soil, and grew plants to a similar age, yet

Wall *et al.* (1994) observed a significant reduction in the concentration of Zn in the shoots which was not observed in the study presented here or study by Thongbai *et al.* (1993b). It would be unlikely that genotypical differences are responsible for the differences observed since two genotypes of wheat are showing variable responses to infection rather than *M. truncatula* cv. Caliph, which is when genotypical differences are greatest.

Another experiment by Thongbai *et al.* (1993b) used sterile wheat bran (levels 0, 1, 2 and 4 wheat bran flakes per 500 g pot) instead of millet seed for inoculum of *R. solani* and again no significant effect was observed on the concentration of Zn in the shoots 23 days after planting. The discrepancy in results suggests a higher level of *R. solani* virulence in the work by Wall *et al.* (1994), but their mean disease scores were 1.8 and 2.8 for 20 and 40 propagules per pot respectively on a 0 to 5 scale, where 0 represents no infection and 5 corresponds to 100% of roots rotted or missing. In the experiment by Thongbai *et al.* (1993b) using wheat bran, 4 flakes per pot resulted in a disease score between 3.5 and 4.5, much higher than Wall *et al.* (1994). These differences in results indicate the variation involved when working with soil pathogens, and the caution required when analysing results.

4.3 Field Survey

4.3.1 Aim

To determine if a correlation exists between Zn concentration in medic shoots and the severity of *R. solani* infection, a field survey was performed in the paddock previously surveyed by Thongbai *et al.* (1993a). This will also determine if a correlation between the shoot concentration of any other nutrient exists with the degree of infection by *R. solani*.

4.3.2 Materials and Methods

4.3.2.a Site Description and History

In August 1994, a paddock was selected at Lameroo, 200 km south-east of Adelaide, South Australia. This was the same site used in the study by Thongbai *et al.* (1993a) in which various levels of soil Zn were applied. The paddock was on siliceous sand, deficient in Zn, and had been part of a long term Zn trial between 1987 and 1991 (Hannam, 1991). In 1987 a randomised block design was established in this paddock with four replicates. Each replicate contained plots, 20 m by 2 m, with treatments of Zn applied at rates of 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 15 and 20 kg Zn/ha (Hannam, 1991). In 1990, 5 untreated plots (with 11 replicates) were supplied with Zn at rates of 0.5, 1, 2.5, 5 and 10 kg Zn/ha to compare effects of residual and freshly applied Zn. The trial was sown to wheat (*Triticum aestivum* cultivar Spear) in 1990. After 1990, medic regeneration occurred and the paddock had since been used for grazing. In 1994 vegetation consisted of *M. littoralis* cultivar Harbinger, *M. truncatula* cultivar Paraggio, rye grass and clover. Since Zn fertiliser has residual effects in the soil for approximately 6 years, and *R. solani* was present, this paddock was considered ideal for a survey.

4.3.2.b Plant Sampling

One month prior to sampling a fence was placed around an area 85 x 110 m to encourage medic growth by preventing sheep grazing on the site. Four sections of the paddock (plots A, B, C and D) were selected to cover a range of Zn treatments previously applied with samples randomly selected within the plots. Since it was not known precisely where the different treatments were applied in the past, random sampling concentrating in an area with a range of Zn treatments was considered to be the best option to ensure soil Zn variability. Plot A covered 700 m² with 40 samples, plot B 270 m² with 10 samples, plot C 380 m² with 15 samples and plot D 400 m² with 15 samples. Each sample consisted of one spade-full (20 by 20 cm and 10 cm deep) of roots and shoots. Soil was lightly shaken off to reduce root damage in the field and plants brought back to the laboratory in plastic bags.

All medic plants were washed of soil, cleaned in millipore water and roots and shoots separated. Maximum root length was measured for all roots and the mean calculated for each sample. Roots were then placed in 70% ethanol for later assessment of mean *R. solani* infection per sample. Roots were classified as either infected with *R. solani* or uninfected, according to the visual symptom of spear tips and/or brown sunken lesions. The degree of *R. solani* infection was assessed using a modified version of the 0-5 scoring system developed by Sweetingham and MacNish (1992). Disease score values range from 0 to 5, where a score of 0 indicates no disease and a score of 5 represents 100% of tap root severely truncated. After scoring for *R. solani*, roots were dried at 80°C for 24 hours and weighed. Mean root dry weight was then calculated for each sample.

Individual plant shoots were dried and weighed, as described above, and mean weight of each sample calculated. All plants in the sample were pooled and analysed for mean nutrient concentrations by ICP.

To confirm the presence of *R. solani*, the fungus was isolated using a method modified from Neate (1984), from twenty randomly selected root samples classified as infected with *R.*

solani. Roots were cut approximately 0.5 cm above, and if possible below, the visual site of infection and placed on weak Czapek Dox plus yeast extract agar medium (NDY/6; see Appendix 3 for recipe) containing 100 mg/l streptomycin sulphate and 100 mg/l tetracycline hydrochloride. Plates were incubated at 15°C and inspected for emerging hyphae using a binocular microscope 48 hours after plating. Actively growing hyphae with *R. solani* characteristics were cut and replated onto full strength NDY agar. Hyphae were then identified as *R. solani* 48 hours later.

4.3.3 Results

No distinct bare patches were observed in the field. From the 80 samples of pasture swards, 1,045 medic plants were examined. Only 161 medic plants had no evidence of *R. solani* on their root system. The distribution of samples infected with *R. solani* did not conform to a binomial distribution (see Appendix 5 for analysis). An estimation of the variance component, expressed as a percentage, was determined for the root length, estimation of disease severity, and shoot and root dry weight for plants within a sample, samples (20 by 20 cm) within an area, and between the four areas (A-D) of the paddock. Variation was highest between plants within a sample for all parameters except root dry weight (Table 4.1).

Table 4.1 Estimation of variance component, expressed as a percentage, for the various parameters measured between plants within a sample, samples within an area of the paddock and between the four areas surveyed at Lameroo in 1994.

Parameters Measured	Estimation of Variance Component (%)		
	Between Plants (n variable)	Between Samples (n=80)	Between Area (n=4)
Root Length	62.0	15.9	22.1
Disease Severity	43.9	23.3	32.8
Shoot Dry Weight	68.9	17.4	68.9
Root Dry Weight	39.1	54.3	6.6

Multiple correlation was performed including all factors measured, with the complete matrix presented in Appendix 6. The correlation coefficients in Table 4.2 were extracted from the complete matrix, demonstrating the association of disease severity with plant number per sample, root length, shoot and root dry weight and nutrient composition in the shoots. Disease severity was significantly correlated with the number of plants per sample, root length, and the concentration of Zn, Mn and Mg in the shoots.

Table 4.2 Correlation coefficients between an estimation of disease severity on the roots determined by the root scoring technique and various plant growth parameter. Values with asterisks are significant at 5% (n=80).

Variable	Correlation Coefficient (r)
Plant number/sample	-0.30 *
Root length	-0.46 *
Shoot dry weight	0.03 ns
Root dry weight	0.12 ns
[Fe]	-0.21 ns
[Mn]	-0.49 *
[B]	0.11 ns
[Cu]	-0.16 ns
[Zn]	-0.47 *
[Ca]	-0.07 ns
[Mg]	0.32 *
[Na]	0.04 ns
[K]	-0.16 ns
[P]	0.04 ns
[S]	-0.17 ns

A curvilinear regression of disease severity with Zn and Mn concentration in shoots was calculated in order to further characterise the relationship (Fig. 4.8), and to demonstrate the variation involved. Zinc concentration ranged from 9 to 69 mg/kg, with 31% of the samples above 25 mg/kg. Manganese concentration ranged from 16 to 94 mg/kg, with 50% of the samples above 35 mg/kg. According to Weir and Cresswell (1994), Zn should be between 25

and 60 mg/kg and Mn between 35 and 250 mg/kg for normal medic growth. No other nutrients were in deficient concentrations in the shoot.

The concentration of Zn in the shoot was also significantly correlated with maximum root length ($r=0.24$), and shoot concentration of Mn, B, Cu, Mg and S ($r=0.43$, -0.22 , 0.24 , -0.41 and 0.31 , respectively; data not shown).

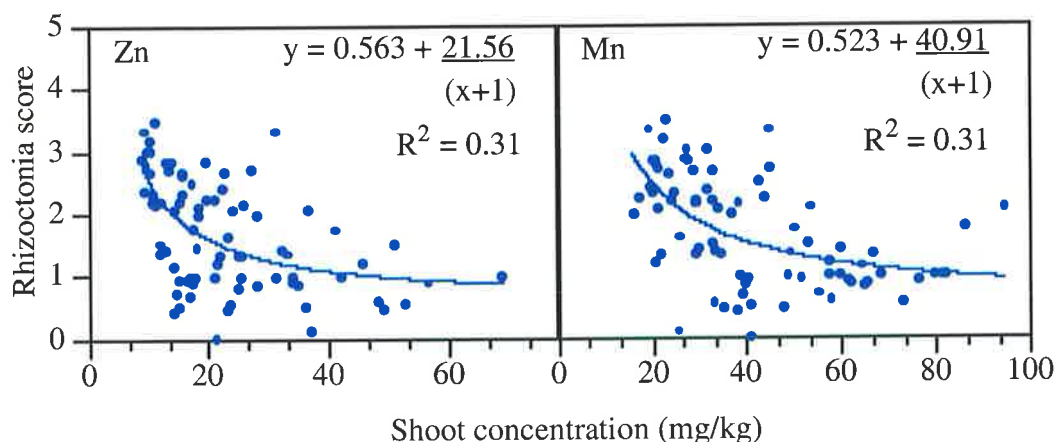


Figure 4.8 Curvilinear regression for Zn and Mn concentration in shoots with disease severity as determined by the root scoring technique (0= no infection; 5= severe infection), $n=80$.

4.3.3 Discussion

The survey was performed during one of the driest seasons in 50 years with only 155 mm of rain between April and October in the Lameroo area compared to an average of 279 mm. Since over 60% of this rain occurred during the June-August period plants may not have suffered severe water stress. Shoot yields were low, less than 0.14 g/plant, and may be a result of intentional grazing by sheep prior to the fence being erected and by unintentional grazing by cattle after the fence was erected. This may have contributed to the lack of correlation between shoot dry weight and other parameters and the high degree of variation observed.

Despite low yields, a range in Zn and Mn concentrations were observed throughout the field with over half the samples deficient in both. *R. solani* appeared to be uniform across the

paddock, with only 15 % of plants sampled not infected by the pathogen. Even with the high incidence of disease, severe infection (disease score >4) was not observed. This might have been due to sampling late in a dry season. Plants with severe root infection would not be able to survive due to water and nutrient deficiency. The correlation between nutrition and disease may have been more pronounced if sampling occurred earlier in the season, as a greater number of roots may have been present with no infection and extreme infection.

This is the first report of a negative relationship between Zn nutritional status and disease severity on medics to *R. solani*. From this survey it is not possible to tell if Zn deficiency exacerbates the effect of disease, or if disease results in Zn deficiency by pruning the root system. Since the same also applies with respect to Mn and disease, it is not possible to say that Zn is the only nutrient involved, as was the case with wheat (Thongbai *et al.*, 1993a). Plants that were Zn-deficient were also deficient in Mn. This is surprising, since Mn concentration has been shown to decrease with increasing Zn supply. Since Zn was the only fertiliser applied at different rates in the past, the increase in concentration of Mn with increasing Zn must be a result of healthier plants (possibly due to less infection as well as more Zn available) capable of extracting more Mn from the Mn-deficient environment. However, it remains possible that a combination of deficiencies may result in a higher incidence of disease.

Shoot Zn concentration was negatively correlated with concentrations of Mg and B, possibly due to increased membrane permeability, with Zn deficiency increasing passive permeability of Mg and B into the plant. Disease severity was also positively related to Mg concentration in the shoot, and this suggests that Mg may only be correlated with disease through its increase in uptake due to Zn deficiency.

Shoot Zn concentration was positively correlated with concentrations of Cu and S, possibly due to similar reasons as for Mn, an increase in plant health with an increase in Zn fertilisation. Although a positive relationship between Zn nutrition and maximum root length occurred, root length should be used with caution since only the top 10 cm layer of soil was sampled. Root

length is an indication of which plants had a high amount of root present in the sampled soil layer. It is not an indication of which plants were able to send roots further into the profile to "escape" the disease and/or increase their water uptake.

4.4 Conclusion

Nearly all observations on effects of root pathogens on root function have been made in controlled conditions. Results from the experiment and field survey presented in this chapter demonstrate the caution that is required in extrapolating from results obtained under controlled conditions to the more complex field situation. In the field the level of infection is spatially variable, and many additional growth limiting are factors present, such as limited availability of water and nutrients.

In both the pot experiment and field survey, concentration of shoot Mn was lower when a high amount of *R. solani* was present. Previous work by Thongbai *et al.* (1993a; 1993b) found a negative relationship between Zn nutrition and the amount of infection of wheat roots by *R. solani* in the field, as well as in experiments under controlled conditions. The effect of Zn deficiency on infection in medics by *R. solani* is the basis of the following chapter. Work by Hall (1984) did not find any relationship between *R. solani* infection and the level of Mn supply in wheat under controlled conditions, and neither did Thongbai *et al.* (1993a) in a field survey.

Effect of Zn Supply on Infection by *R. solani* of *Medicago truncatula* genotype Caliph

5.1 Introduction

Results from the previous chapter demonstrated that infection by *R. solani* caused a decrease in nutrient uptake and transport within the infected plants. Infection by *R. solani* did not cause a corresponding reduction in dry weight or shoot Zn concentration, indicating that the pruning effect of *R. solani* does not necessarily result in Zn deficiency in medics when adequate Zn is supplied. However, the effect of *R. solani* on medics exposed to the additional stress of Zn deficiency has not been demonstrated. Results from the field survey in Chapter 4 demonstrated a negative relationship between Zn nutritional status and severity of disease caused by *R. solani* in medics but it was not possible to separate the cause and effect of infection by *R. solani* and Zn deficiency. In order to understand the link between Zn nutrition and disease severity in medics, it is essential to observe the interaction of independent inoculum and Zn treatments, the latter extending to low levels of available Zn.

A relationship between Zn nutrition and severity of disease by *R. solani* in wheat was demonstrated by Thongbai *et al.* (1993b) with levels of Zn supply ranging from deficiency through to adequacy. In their study, a reduction in disease score caused by Zn application was demonstrated. They suggested that the effect was not the result of fungitoxicity of Zn but, rather, they hypothesised the effect was due to the role of Zn in the integrity or stability of the host plant's membranes (Thongbai *et al.*, 1993b). Zinc deficient plants contain low levels of superoxide dismutase and therefore high levels of superoxide radicals, which lead to peroxidation of membrane lipids, loss of membrane integrity and increased membrane permeability (see 2.1.9 for more detail). In addition, the accumulation of free amino acids and amides occur as a result of inhibition of protein synthesis by Zn deficiency. This may result in

an increase in free amino acids in the root exudate of Zn-deficient plants owing to the leakiness of the membrane. Since *R. solani* is attracted to root exudates (Flentje *et al.*, 1963), the increased root exudation by Zn-deficient plants may act as an attractant for the fungus, thus accelerating the infection process.

The aim of this chapter was to determine the effect of reduced Zn supply to medics on infection by *R. solani*. This was studied in three experiments in controlled conditions. The first experiment used wheatbran inoculum to observe the effect of Zn application to Zn-deficient plants when low levels of *R. solani* were present. The second experiment used millet seed inoculum to achieve a higher level of infection. This experiment also studied the effect of *R. solani* on the root system in more detail. The third experiment compared the effect of *R. solani* on the growth of medics sown with seed containing high and low levels of Zn under Zn-deficient and Zn-sufficient soil conditions.

5.2 Pot Experiment 1

5.2.1 Aim

The aim of this experiment was to determine if Zn deficiency predisposed Caliph medics to infection by *R. solani*.

5.2.2 Materials and Methods

5.2.2.a Experimental Design

A factorial design was used, studying the interactive effects of increasing levels of soil-applied Zn (0, 0.05, 0.1, 0.3 and 0.9 mg Zn/kg) with increasing levels of soil-applied *R. solani* (0, 1, 2, 4, 8 and 16 propagules/kg soil) at 42 days after sowing. Caliph was grown in Mt Compass sand with four replicates.

5.2.2.b *R. solani* Inoculum

Inoculum was prepared on sterile wheatbran flakes as outlined in Chapter 3 for millet seed. Wheatbran flakes were chosen as a lower level of infection can be achieved (Thongbai, 1993) presumably due to the smaller food source immediately available to the fungus. For a range of infection, 1, 2, 4, 8 and 16 flakes (propagules) infected with *R. solani* were added to one kg soil. For the nil *R. solani* control, 16 sterile propagules were added to one kg soil.

5.2.2.c Soil

Mt Compass sand was used, leached of soluble salts in RO water, air dried, and heat-treated at 80°C for 6 hours on each of two consecutive days as outlined in Chapter 3.

5.2.2.d Basal Nutrients

The basal nutrients were applied as outlined in Chapter 3 with Zn added as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ to plus Zn treatments as necessary.

5.2.2.e Seed

Seeds of Caliph medic, used in previous experiments, were surface sterilised and germinated as described in Chapter 3. Seed characteristics are presented in Table 3.4.

5.2.2.f Procedure

Mixing of nutrients through the soil and potting were performed as described in Chapter 3, with pots watered to 10% water content, covered and incubated after the soil was inoculated with the respective *R. solani* treatment. At the end of 2 weeks, five germinated seeds were sown per pot. After sowing, the soil surface was covered in acid-washed black plastic beads and watered by weight to 12% water content. Pots were then placed in growth chamber A, inoculated with *Rhizobium meliloti* strains after 7 days and thinned to two plants per pot. Watering by weight was done every second day.

After 42 days, plants were harvested, and root length was measured using the line-intersection technique of Tennant (1975). On the roots, the number of lesions and spear tips per plant was also counted, along with an estimate of disease score based on a 5-point scale where 0 represents no disease and 5 represents 100% infection using the scale of Sweetingham and MacNish (1992). Shoot and root dry weight were determined, and nutrient analysis performed on the shoots by ICP spectrometry.

5.2.3 Results

Visual Observations

Between 7 and 11 days after planting, lights in the growth chamber did not function due to an electrical fault, resulting in small, pale yellow plants, the plants however continued to be watered. Originally plants were considered to be Fe-deficient, and a small amount of Fe was added to each pot to rectify the problem. After day 11 the growth chamber was functioning normally and the plants recovered. Plants were not as large by day 42 as in previous experiments, but a response to Zn application was still visible. With zero or low levels of applied Zn (0 and 0.05 mg Zn/kg), a response to added *R. solani* was noticed, with a reduction in shoot matter with increasing levels of *R. solani*. This effect with *R. solani* was not seen with higher amounts of applied Zn.

Plant Growth Measurements

A significant interaction occurred between Zn application and inoculum of *R. solani* on shoot dry weight ($p \leq 0.04$; Fig. 5.1). Increasing soil Zn application resulted in no change in shoot dry weight with increasing level of inoculation of *R. solani*. A similar effect occurred with root dry weight ($p \leq 0.24$) and root length ($p \leq 0.08$) (data not shown).

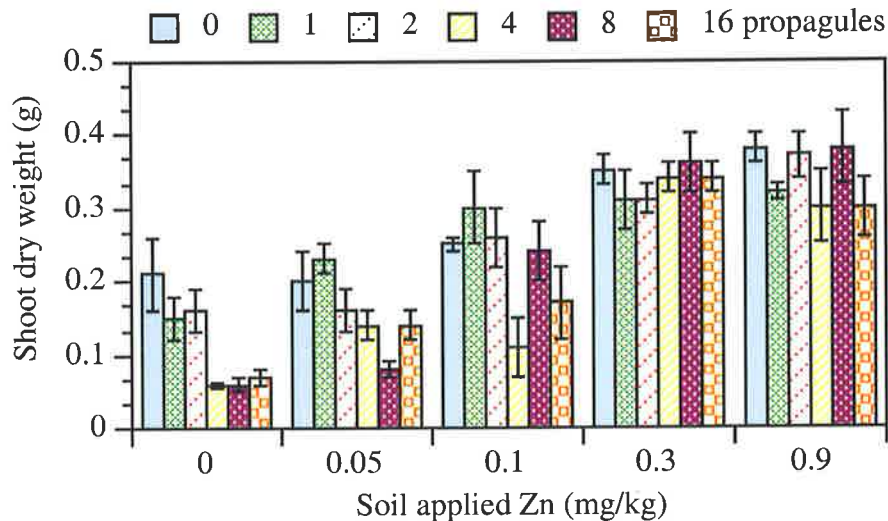


Figure 5.1 Effect of inoculation with *R. solani* AG8 (0-16 wheatbran propagules) on shoot dry weight of genotype Caliph. Plants were grown in Mt Compass sand for 42 days with increasing levels of soil-applied Zn. Vertical bars represent $\pm$ standard error (n=4).

Infection by *R. solani* was highly variable and increasing the number of propagules did not necessarily result in a proportionate increase in the amount of infection. To reduce this effect, the six inoculum levels applied to the soil were pooled into three groups, nil (0 propagules), low (1 and 2 propagules), and high infection (4, 8 and 16 propagules). These groups were selected on the basis of results from shoot dry weight at 0 Zn application (Fig. 5.1).

Figure 5.2 illustrates the effect of pooling data on shoot and root dry weight. The interaction between Zn application and inoculum level is significant for shoot dry weight ($p \leq 0.02$) and significant for root dry weight at the 8% level ($p \leq 0.075$). With increasing levels of Zn, the shoot and root dry weight increased significantly up to 0.3 mg of applied Zn/kg. No further increase in dry weight occurred between 0.3 and 0.9 mg of applied Zn/kg. The effect of *R. solani* was more pronounced on plants deficient in Zn. For comparison, when Zn was not applied to the soil, shoot and root dry weight decreased by 71% and 67% respectively, when a high amount of *R. solani* was present (Fig. 5.2). At 0.9 mg Zn/kg, shoot and root dry weight decreased by only 10% and 14% respectively, when the same amount of *R. solani* was added to the soil, a reduction that was not statistically significant.

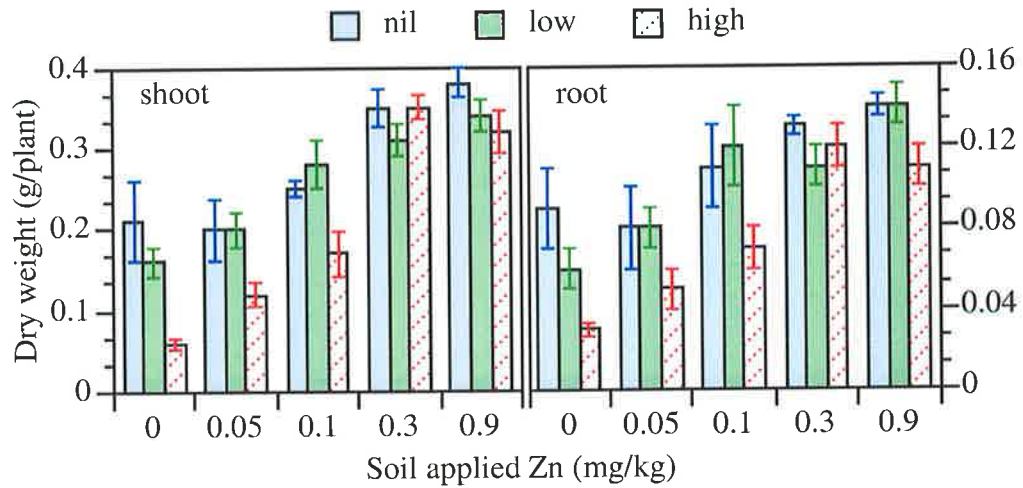


Figure 5.2 Effect of inoculation with *R. solani* AG8 on shoot and root dry weight of genotype Caliph. Plants were grown in Mt Compass sand for 42 days with increasing levels of soil-applied Zn. The low level of *R. solani* represents 1 and 2 propagules and the high level combines 4, 8 and 16 propagules. Vertical bars represent $\pm$ standard error ($n = 4, 8$, and 12 for nil, low and high levels of *R. solani*, respectively).

Root length responded in a similar way to infection (Fig. 5.3), with a reduction in length by 71% at 0 Zn application compared with 22% at 0.9 mg Zn/kg, when a high amount of *R. solani* was added to the soil. The number of lesions and spear tips counted on the root system increased with increasing inoculum and with increasing application of Zn (Fig. 5.4): plants with the larger root systems had a higher number of infection sites.

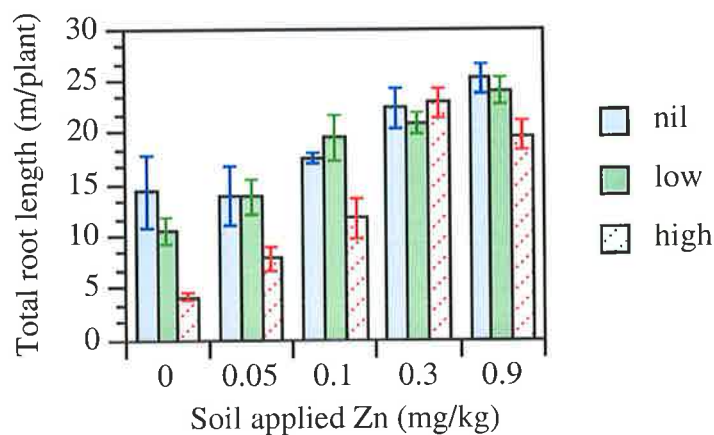


Figure 5.3 Effect of inoculation with *R. solani* AG8 on root length of genotype Caliph with increasing levels of soil-applied Zn. Plants were grown in Mt Compass sand for 42 days. Low level of *R. solani* represents 1 and 2 propagules and high level combines 4, 8 and 16 propagules. Vertical bars represent $\pm$ standard error ($n = 4, 8$, and 12 for nil, low and high levels of *R. solani*, respectively).

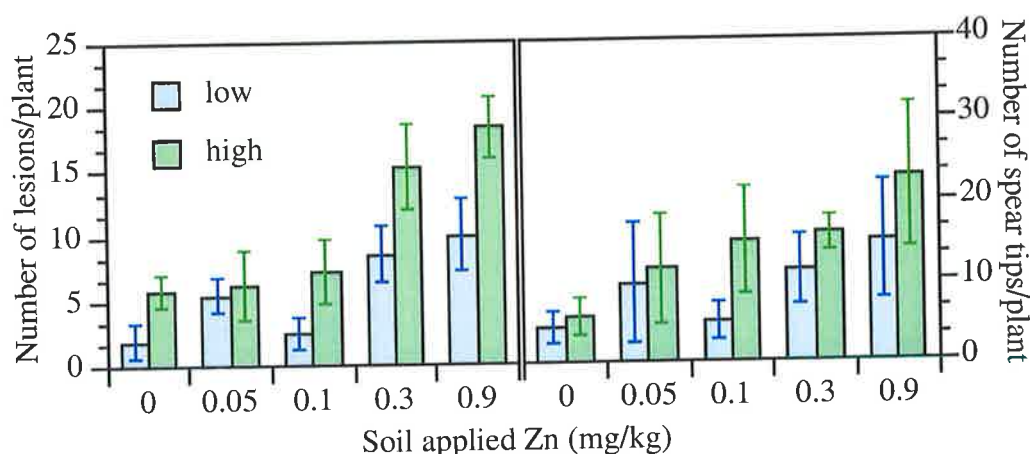


Figure 5.4 Effect of low and high levels of *R. solani* AG8 inoculum on the number of lesions and spear tips present on the root system of genotype Caliph. Plants were grown in Mt Compass sand with increasing levels of soil-applied Zn for 42 days. Low level of *R. solani* represents 1 and 2 propagules and high level combines 4, 8 and 16 propagules. Vertical bars represent $\pm$ standard error ($n = 8$ and 12 for low and high levels of *R. solani*, respectively).

With a low level of *R. solani*, mean disease score was less than 0.5 in all treatments, with no significant effect with increasing levels of Zn (Table 5.1). When a high amount of *R. solani* was present, mean disease score increased to 1.3 when no Zn was applied to the soil. With increasing levels of Zn, disease score was significantly reduced.

Table 5.1 Mean disease severity, as determined by the scoring system of Sweetingham and MacNish (1992), with a low and high level of applied *R. solani* on genotype Caliph, with increasing levels of soil-applied Zn. Low level of *R. solani* represents 1 and 2 propagules/kg and high level combines 4, 8 and 16 propagules/kg. Numbers in parentheses represent the standard error, $n=8$ and 12 for low and high levels of *R. solani*, respectively.

Soil Applied Zn (mg/kg)	<i>R. solani</i> level	
	Low	High
0	0.4 (0.1)	1.3 (0.2)
0.05	0.4 (0.1)	0.9 (0.3)
0.1	0.5 (0.1)	1.2 (0.4)
0.3	0.5 (0.1)	0.8 (0.1)
0.9	0.5 (0.1)	0.7 (0.1)

The concentration of Zn increased significantly in the shoot with increasing levels of applied Zn ($p \leq 0.0001$; Fig. 5.5). Severe Zn deficiency occurred at Zn levels 0 and 0.05 mg/kg soil, moderate Zn deficiency occurred at 0.1 mg Zn/kg, while at 0.3 mg Zn/kg soil plants were approaching the level of Zn in the shoot required for normal growth (25 mg/kg). Adequate Zn levels were achieved at 0.9 mg Zn/kg soil. Infection by *R. solani* did not affect the concentration of Zn in the shoots (Fig. 5.5). Iron, B, Cu, Mg, K, P and S (except B, data not shown) all decreased significantly with increasing Zn application ($p \leq 0.02$ for Cu and $p \leq 0.0001$ for others). Boron was the only analysed element to be affected significantly by infection of *R. solani* ($p \leq 0.0001$; Fig. 5.5). When a high level of *R. solani* was applied to the soil, the amount of B in the shoot increased. The interaction between Zn application and inoculum of *R. solani* was found to be significant for B concentration in the shoots ($p \leq 0.02$) with the difference in B concentration in the shoot between no fungus present and high levels of the fungus more pronounced as Zn deficiency increased in severity.

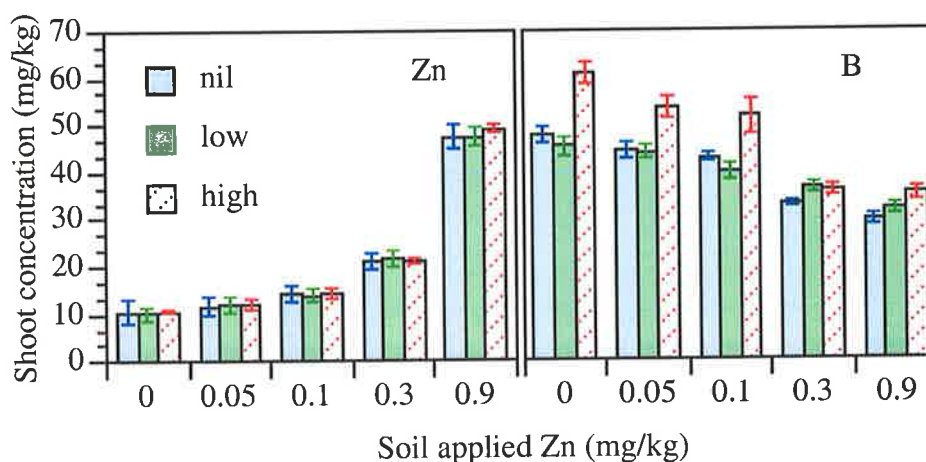


Figure 5.5 Effect of inoculation with *R. solani* AG8 on Zn and B concentration in the shoot for genotype Caliph. Plants were grown in Mt Compass with increasing levels of soil-applied Zn sand for 42 days. Low level of *R. solani* represents 1 and 2 propagules and high level combines 4, 8 and 16 propagules. Vertical bars represent $\pm$ standard error ($n = 4, 8$, and 12 for nil, low and high levels of *R. solani*, respectively).

5.2.4 Discussion

This study represents the first record of Zn application reducing the impact of *R. solani* on the growth of medics. This supports the results from the field survey, reported in the previous chapter, which demonstrated a negative relationship between Zn nutritional status of medics and severity of *R. solani*. The concentration of Zn in the shoots was not reduced with increasing inoculum at any level of applied Zn, indicating Zn deficiency is not enhanced by infection of *R. solani*.

Boron levels were however influenced by the amount of *R. solani* present in the soil, with a high level of *R. solani* corresponding to a high concentration of B in the shoot. Boron increased with increasing severity of Zn deficiency, a phenomenon also observed in Chapter 3 and explained by increased plasma membrane permeability with Zn deficiency, leading to increased passive permeability of B. Boron is transported to the root passively by the mass flow of water to the root and the increase in concentration of B in the presence of a high amount of *R. solani* occurred despite a reduction in root length. An alternative explanation may be a reduction in shoot yield, which occurred at high levels of *R. solani*, is responsible for the higher concentration of B in the shoot as the dilution effect is less. Combination of these two effects was also possible and may explain the higher B concentration in Zn-deficient plants infected with high levels of *R. solani*.

Overall, infection levels were low, with a highest mean value for disease score of 1.3 compared with the defined maximum score of 5. The addition of 8 wheatbran propagules infected with *R. solani* to 1 kg of soil in a study by Thongbai *et al.* (1993b) resulted in a mean disease score of 4.5 in wheat when plants were deficient in Zn. This suggests medics are more tolerant to infection by *R. solani* than wheat, which is supported by field observations. Bare patches, caused by *R. solani*, are readily observed in the field where wheat is grown, but when medics are sown the patches are often not observed (S.M. Neate, pers. comm.).

It was interesting to note that increasing Zn application caused an increase in the number of lesions and spear tips, in contrast to a decrease in disease score. The use of disease score as a measure of disease severity is highly subjective. It attempts to take into account the number of infection sites (lesions and spear tips) and the size of the root system. Scoring roots for disease severity is confounded when other treatments, in this case Zn application, influence root structure as well. The increase in the number of lesions and spear tips suggests Zn application in medic does not directly inhibit the fungus or reduce its pathogenicity, as suggested by Thongbai *et al.* (1993b) for wheat. However, Zn application strongly increased root growth. The larger root system of a Zn-adequate plant may increase tolerance to disease by compensation, absorbing more of the limiting nutrients from uninfected root axes. Compensation may occur by increasing the amount of nutrients removed from the soil, or by altering the movement of nutrients within the plant. More detailed studies on the root system of Zn-deficient and Zn-adequate plants infected with *R. solani* are required, along with studies on the transport of Zn within the plant. The net result is that Zn increases tolerance to the fungus, and not resistance, as previously thought.

Results from this experiment may be exaggerated due to the additional stress seedlings were subjected to at the start of the experiment, that being lack of light. Considering this, and the fact that plants were supplied extra Fe, it was decided to repeat the experiment with higher inoculum levels. This would also allow the interaction between Zn application and infection by *R. solani* to be studied in further detail.

5.3 Pot Experiment 2

5.3.1 Aim

- 1) To study the root geometry of Caliph plants subjected to Zn deficiency and Zn adequacy after exposure to *R. solani* at levels which exceed those achieved in the previous experiment;
- 2) To determine the level of *R. solani* in the root system after 42 days;

3) To determine the effect of infection on the amount of Zn within the plant and on the proportion of Zn present in the shoot at deficient and adequate levels of soil-applied Zn.

5.3.2 Materials and Methods

5.3.2.a *Experimental Design*

A factorial design was used, studying the effects of two levels of soil-applied Zn (0 and 0.9 mg Zn/kg) with increasing levels of soil-applied *R. solani* (0, 1, 8 and 32 propagules/kg soil) at 42 days after sowing. Genotype Caliph was grown in Mt Compass sand with nine replicates.

5.3.2.b *R. solani Inoculum*

Inoculum was prepared on sterile millet seed as outlined in Chapter 3. Millet seed was chosen as a higher level of infection can be achieved (Thongbai, 1993) due to the larger food source immediately available to the fungus. For a range of infection 1, 8 and 32 millet seeds (propagules) infected with *R. solani* were added per one kg soil. For the control, nil *R. solani*, 32 sterilised propagules were added to one kg soil to provide a control for the nutrient contained in the millet seed.

5.3.2.c *Soil*

Mt Compass sand was used, leached of soluble salts in RO water, air-dried, and heat-treated at 80°C for 6 hours on each of two consecutive days as previously described.

5.3.2.d *Basal Nutrients*

The basal nutrients were applied as outlined in Chapter 3, and Zn was added as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

5.3.2.e Seed

Seeds of genotype Caliph, used in the experiment described in the previous chapter, were surface sterilised and germinated as previously described. Seed characteristics are presented in Table 3.4.

5.3.2.f Procedure

Soil mixing and potting were performed as previously described in Chapter 3, with pots watered to 10% water content, covered and incubated after the soil was inoculated with the respective *R. solani* treatment. At the end of 2 weeks, five germinated seeds per pot were planted. After planting, soil surface was covered with acid-washed black plastic beads and soil watered to 12% water content. Pots were then placed in growth chamber, inoculated with *Rhizobium meliloti* strains after 7 days and thinned to two plants per pot. Watering was done every second day.

After 42 days, plants were harvested, with roots from replicates 1 to 3 used to extract and estimate the amount of *R. solani* present in the root system. Replicates 4 to 6 were used for nutrient analysis, and replicates 7 to 9 were used to visually assess damage to the root system by *R. solani*.

Root DNA extraction

Extraction of *R. solani* from the roots was based on the technique described by Raeder and Broda (1985). For the extraction procedure, the total root material (2 roots/pot) was frozen and ground in liquid nitrogen using a mortar and pestle. A sub-sample (0.1 g, with two replicates per sample) was transferred to a 50 mL tube and mixed with alkaline extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaOH, 25 mM EDTA, 0.5% SDS). A 1mL aliquot of the suspension was transferred into an Eppendorf tube. One mL of phenol/chloroform (1:1) was added, mixed and centrifuged at room temperature for 30 min at 10 000 g. Upper aqueous phase (500µL) was placed into a new tube and RNase (0.1 mg/mL) added. After 30 min of incubation at 37°C, 500 µL of chloroform:isoamyl alcohol

mixture (24:1) was added, mixed and centrifuged for 10 min. The upper aqueous phase (450 μ L) was transferred into a new tube together with 1130 μ L of 100% ethanol, mixed by inversion and incubated overnight at -20°C. The following morning the solution was centrifuged for 20 min, the pellet rinsed in 70% ethanol and centrifuged for 10 min. The solution was then decanted and the pellet air-dried. To dissolve the pellet, 160 μ L of TE buffer was added. An aliquot of 80 μ L was then used for slot-blots.

DNA slot-blot assay

DNA slot-blot assay was based on the technique of Matthew *et al.* (1995), using a Bio-Dot slot format micro filtration unit with a Zeta-Probe nylon membrane. DNA samples (80 μ L) were denatured with 20 μ L of 2 M NaOH for 10 min. The reaction was halted with an equal volume of 2 M NH₄OAc and the product loaded into the wells of the slot-blot apparatus. Control samples, with no DNA, were treated in the same manner. The DNA was fixed to the membrane by baking at 80°C for 30 min.

DNA labelling and hybridisation

The clones were digested with *Bam* H1 and run on 1% agarose gel as outlined in Matthew *et al.* (1995). The insert band was cut and purified using Qiaquick gel extraction kit (Qiagen, Pty). The DNA fragment was labelled with [α -³²P]dCTP using random oligonucleotides as a primer (Feinberg and Vogelstein, 1983) and a labelled probe with specific activity of about 1.8×10^8 cpm μ g⁻¹ was generated. The labelled DNA probe was separated from unincorporated nucleotides through a size exclusion resin column (Nucleon QC kit).

Hybridisation of the membranes was performed at 65°C overnight in hybridisation buffer containing 2mL sterile water, 3 mL 5xHSB (3 M NaCl, 100 mM PIPES and 25 mM Na₂EDTA, pH 6.8), 1 mL 10xDenhardt's III solution (2% gelatin, 2% Ficoll, 2% polyvinyl pyrrolidone, 10% SDS and 5% tetrasodium pyrophosphate), 4 mL 25% dextran sulphate and 0.2mL salmon sperm DNA (5 μ g mL⁻¹). After hybridisation, the membranes were washed under low stringency conditions using 2xSSC and 0.1% SDS for 20 min, at 65°C three

times, or under high stringency conditions at 65°C using 2xSSC and 0.1% SDS, then in 1xSSC and 0.1% SDS, and followed by washing in 0.5xSSC and 0.1% DSD for 20 min each. The washed membranes were exposed to a phosphor screen overnight and quantified using a Storm Phosphor-Imager (Molecular Dynamics). These bands were quantified as the concentration of *R. solani* DNA in the root, according to Matthew *et al.* (1995).

Nutrient Analysis

For nutrient analysis, the dry weight of both shoot and roots was measured after roots had been washed from the soil using Milli-Q® water. Nutrient concentrations in the shoots and roots was determined by ICP analysis. The proportion of total plant nutrient content that was present in the shoot was also calculated, expressed as a percentage.

Disease Assessment

The degree of infection on the root system was determined by counting: 1) the total number of root tips present (including spear tips); 2) the total number of lesions and spear tips on the root system; 3) the number of first order laterals present; and 4) the number of first order laterals with either lesions or spear tips present. Roots were also scored for disease severity on a 0 to 5 scale using the technique outlined by Sweetingham and MacNish (1992) where 0 represents no infection and 5 stands for 100% infection. Finally, the length of the tap root was measured with a ruler, while total root length and total root surface area were calculated within a set diameter range by a scanner linked to a computer (see below).

Roots were scanned for root length using the technique outlined in Pederson *et al.* (1994). Roots were washed from the soil and stored in a preservative solution of 70% ethanol and 30% demineralised water. Methyl violet dye (2% in storage solution) was added to provide contrast for the root scanning procedure (Harris and Campbell, 1989). Individual root samples were floated in a shallow water bath consisting of a lower reservoir tray and an upper perforated tray which contained a sheet of blotting paper (Goubran and Richards, 1979). The root systems (2/pot) were floated into position over the blotting paper, with

roots preferably at 90° to one another and no overlaps for scanning accuracy. Roots remained on the paper, in position, once the water was withdrawn. An over-head transparent sheet was placed over each sample and a commercial photocopier adjusted to produce a high contrast image of the root system. Scanning of individual root images and computation of root length and diameters (mean diameter class size $\leq 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, 2,$ and 3 mm) was achieved using a flat-bed optical character recognition scanner set at 300dpi resolution, an IBM-compatible computer, and software developed by Kirchhof (1992).

5.3.3 Results

Visual Observations

Differences in shoot growth were visible from the time of emergence, with plants inoculated with 32 propagules of *R. solani* showing reduced leaf size compared to all other treatments. Three weeks after sowing, Zn deficiency symptoms were also evident on plants without Zn fertiliser.

Plant Growth Measurements

No significant interaction occurred between Zn application and addition of *R. solani* on shoot or root dry weight ($p \leq 0.11$ and 0.28 , respectively; Fig. 5.6). However, shoot and root dry weight increased significantly with Zn application ($p \leq 0.0001$ in both cases), while increasing levels of *R. solani* had no significant effect on dry weight ($p \leq 0.08$ and 0.25 , shoot and roots respectively).

Effect of infection on plant nutrient status

No significant interaction occurred between Zn application and addition of *R. solani* for the concentration of Zn in the shoot or root ($p \leq 0.32$ and 0.22 , respectively). However, the main effect of Zn application significantly increased the concentration of Zn in both the shoot and the root ($p \leq 0.0001$ in both cases; Fig. 5.7). Zinc deficiency occurred when no Zn was applied,

with levels in the shoot below 10 mg/kg at all levels of *R. solani*, while less than 30 mg/kg was present in the root. This compares with between 40 and 50 mg/kg in the shoot and 80 and 150 mg/kg in the root when Zn was applied. Infection by *R. solani* did not affect the concentration of Zn in the shoots ($p \leq 0.35$) but the concentration of Zn in the roots was significantly reduced at the 7% level ($p \leq 0.068$) with increasing levels of *R. solani*.

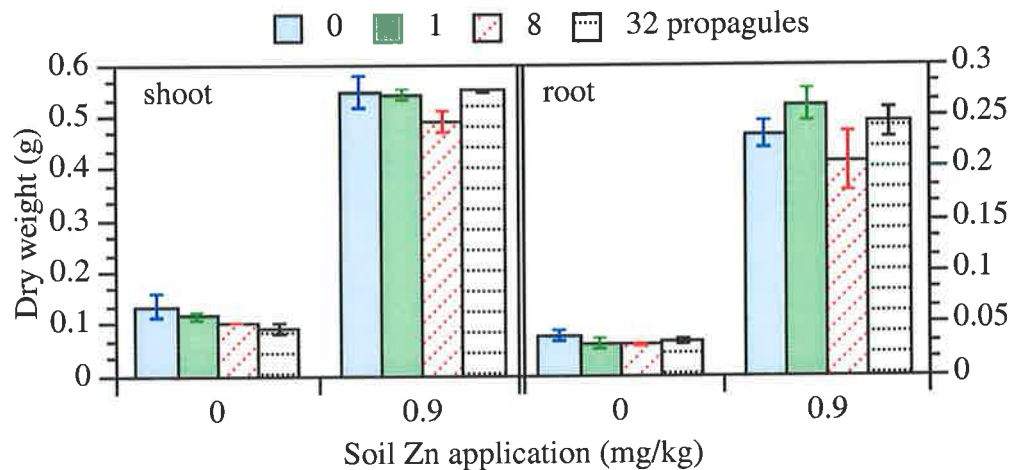


Figure 5.6 Effect of increasing levels of *R. solani* AG8 inoculation on shoot and root dry weight for genotype Caliph at two levels of soil Zn applications, 0 and 0.9 mg/kg. Plants were grown in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, $n=6$.

Increasing levels of *R. solani* also reduced the total amount of Zn taken up by the plant ($p \leq 0.02$), with a significant interaction occurring between Zn application and *R. solani* rate ($p \leq 0.03$; Fig. 5.7). Increasing levels of *R. solani* reduced Zn uptake in both Zn treatments, with the effect more pronounced when plants were supplied with Zn. With regards to the proportion of this Zn transported to the shoot, a significant interaction occurred between Zn and *R. solani* inoculation ($p \leq 0.01$; Fig. 5.7). Under Zn deficiency, the proportion of Zn transported to the shoot, expressed as a percentage of the total amount of Zn present in the plant, decreased with increasing levels of *R. solani*. Conversely, with the addition of Zn, the amount of Zn transported to the shoot increased with increasing levels of *R. solani*. The proportion of Zn transported to the shoot was significantly higher in Zn-deficient plants, except when 32 propagules were present.

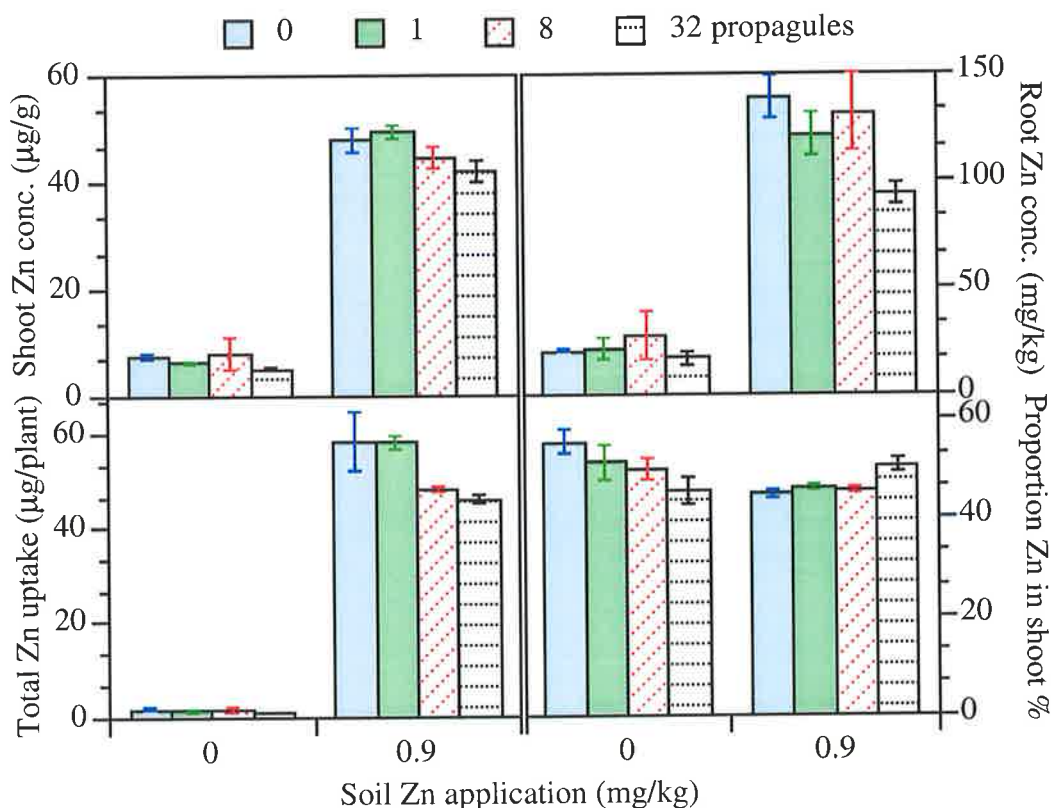


Figure 5.7 Effect of increasing levels of *R. solani* AG8 inoculation on concentration of Zn in the shoot and root, total amount of Zn accumulated in the plant and proportion of this Zn present in the shoot for genotype Caliph at two levels of soil Zn applications, 0 and 0.9 mg/kg. Plants were grown in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, $n=3$.

The interaction between applied Zn and *R. solani* inoculation was significant for the concentration of B (Fig. 5.8), P (Fig. 5.9), Cu, Ca, Mg and S (Fig. 5.10) in the shoot ($p \leq 0.0002$ to 0.05) and for the concentration of Mn (Fig. 5.11), P (Fig. 5.9), and S (Fig. 5.10) in the root ($p \leq 0.0001$ to 0.02). The concentration of these nutrients in the shoots was not affected by increasing levels of *R. solani* inoculum when Zn was applied to the soil. However, when Zn was deficient, nutrient concentration varied depending on the level of infection. For example, the concentration of S in the shoot increased with increasing *R. solani*, while the concentration of Ca in the shoot decreased in the presence of a low amount of *R. solani* compared to the control and then exceeded the control when the highest level of *R. solani* was added to the soil (Fig. 5.10). The reverse occurred for P, Cu and Mg in the shoot. That is, at low levels of *R. solani* (1 and 8 propagules) the concentration in the shoot exceeded the

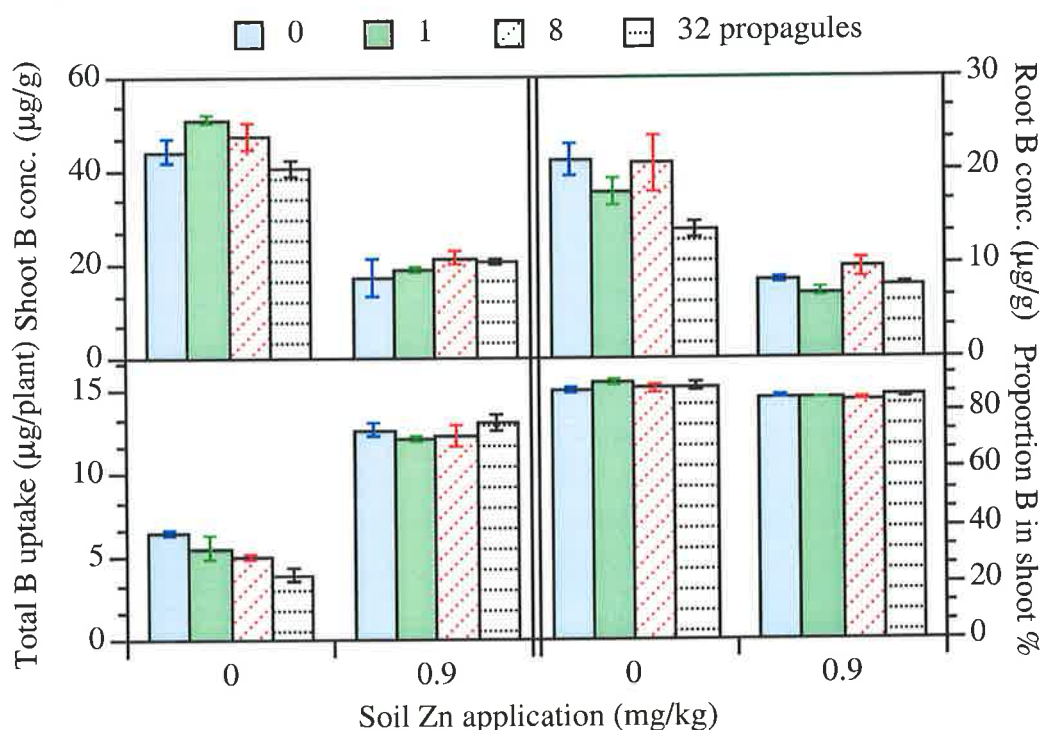


Figure 5.8 Effect of increasing levels of *R. solani* AG8 on the concentration of B in the shoot and root, the total amount of B present in the plant and the proportion of this B present in the shoot for Caliph with two soil Zn applications, 0 and 0.9 mg/kg. Plants were grown in Mt Compass sand during for 42 days. Vertical bars represent $\pm$ standard error, n=3.

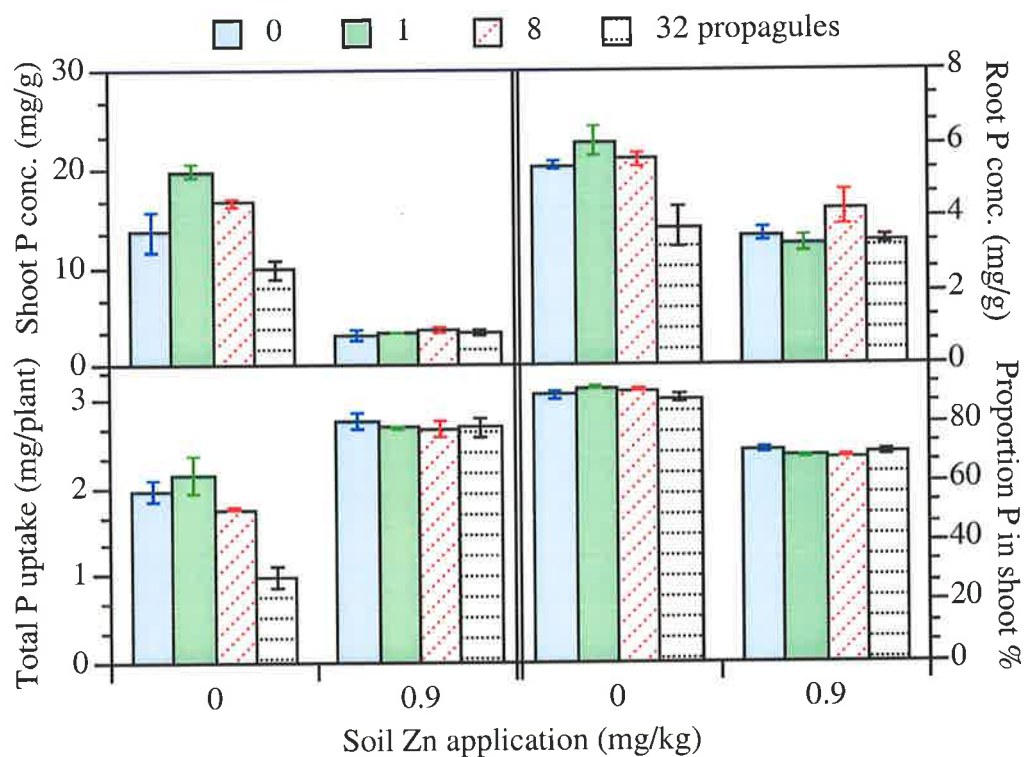


Figure 5.9 Effect of increasing levels of *R. solani* AG8 on concentration of P in the shoot and root, total amount of P present in the plant and proportion of this P present in the shoot for genotype Caliph with two soil Zn applications, 0 and 0.9 mg/kg. Plants were grown in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, n=3.

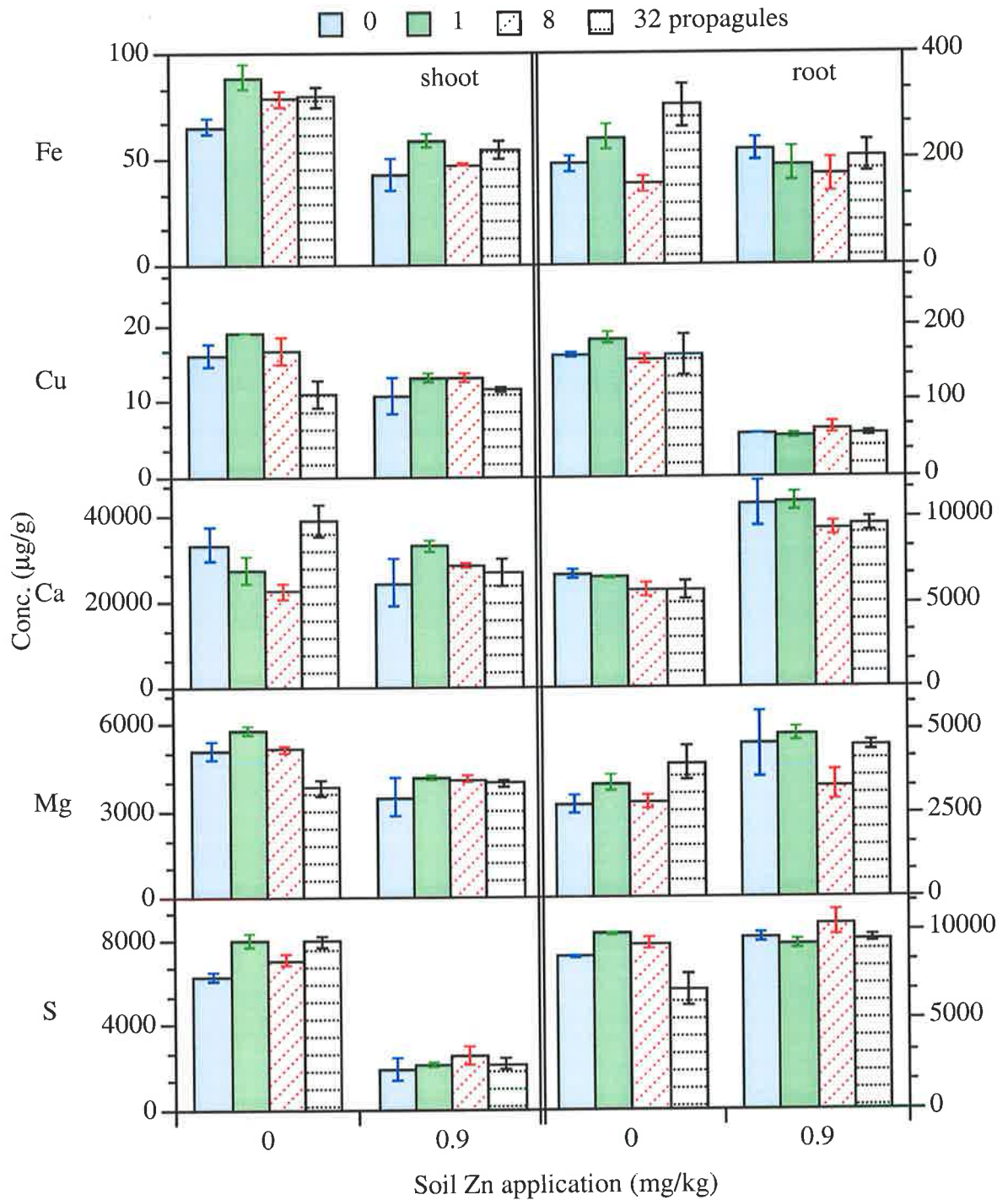


Figure 5.10 Effect of increasing levels of *R. solani* AG8 on concentration of Fe, Cu, Ca, Mg, K and S in the shoot and root for genotype Caliph. Plants were grown with two soil Zn applications, 0 and 0.9 mg/kg, in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, n=3.

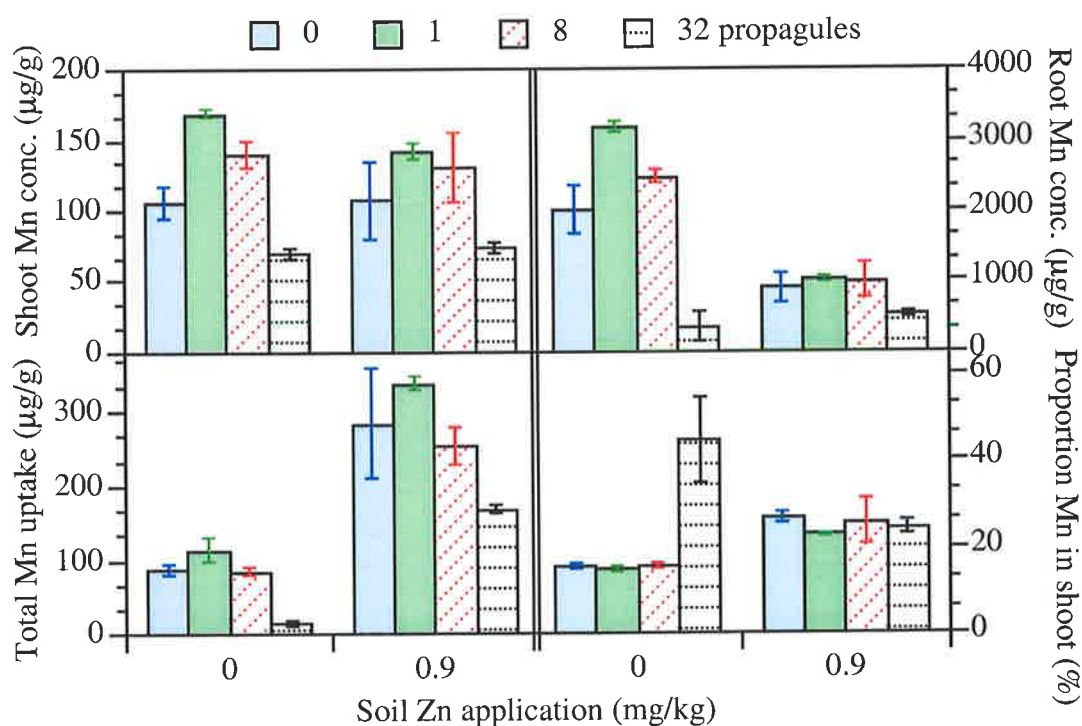


Figure 5.11 Effect of increasing levels of *R. solani* AG8 on concentration of Mn in the shoot and root, total amount of Mn present in the plant and proportion of this Mn present in the shoot for genotype Caliph. Plants were grown with two soil Zn applications, 0 and 0.9 mg/kg, in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, n=3.

control, while at high levels of *R. solani* (32 propagules) the concentration in the shoot was below that of the control. When plants are deficient in Zn, the concentration of Mn, P and S in the root increased in the 1-propagule treatment and then decreased in the 8 and 32 propagule treatment. When Zn was supplied, and with a high amount of *R. solani* (32 propagules), the concentration of Mn decreased in the root while P and S remained similar to the control.

Although the interaction between Zn application and *R. solani* inoculation was not significant for the concentration of Mn and Fe in the shoots or for B, K, Mg and Ca in the roots, the main effect of Zn application was significant. Compared to Zn-sufficient plants, Zn deficiency, increased shoot concentration of Mn and Fe, while in the roots the concentration of B increased and Mg and Ca decreased in Zn-deficient plants.

The main effect of *R. solani* inoculation also resulted in a reduction in the concentration of Mn in the shoots, a reduction of B in the roots and an increase in Fe in both the shoots and roots with increasing infection.

A significant interaction occurred between Zn application and *R. solani* on the total content of B and P in the plant ($p \leq 0.02$; Figs. 5.8 and 5.9). When plants were supplied with an adequate amount of Zn, no change occurred in B and P content with increasing levels of *R. solani*. However, with Zn deficiency, the amount of both elements decreased with increasing *R. solani*. With the addition of 1 propagule, the amount of P in the plant increased slightly whereas the amount of B was decreased. This increase in amount of P corresponded with an increase in the concentration of P in both the shoot and root (Fig. 5.9).

Although no significant interaction for the total Mn content occurred between Zn and *R. solani* application, the main effect of *R. solani* inoculum was significant ($p \leq 0.002$; Fig. 5.11). When 1 propagule was added to the soil, an increase occurred in the amount of Mn present at both levels of applied Zn. With further additions of *R. solani*, the amount of Mn decreased.

The proportions of total P and Mn present in the shoot were significantly affected by the interaction between Zn application and the addition of *R. solani* ($p \leq 0.01$; Figs. 5.9 and 5.11). When no Zn was applied to the soil, the highest level of *R. solani* resulted in a dramatic increase in the amount of Mn taken up and transported to the shoot. No change in the amount of Mn transported to the shoot occurred when Zn was applied to the soil. An increase in the amount of P present in the shoot occurred when 1 and 8 propagules were applied to the soil when deficient in Zn. When Zn was supplied, the amount of P decreased at 1 and 8 propagules of *R. solani*.

Most nutrients remained in the range required for normal growth (see Table 3.4) except B, Ca, P, and S. Boron levels were below the range for normal growth when Zn was applied, Ca levels were above the range for normal growth at both Zn treatments, and levels of P and S were higher than the levels required for normal growth when plants were Zn-deficient, while P was the only nutrient in the toxic range.

Effect of infection on root morphology

The interaction between Zn application and the addition of *R. solani* to the soil had a significant effect on the length of the tap root ($p \leq 0.03$; Fig. 5.12), with a progressive reduction in length with increasing *R. solani* in the absence of added Zn. When Zn was applied, no change in tap root length occurred with increasing levels of *R. solani*. Zinc application and *R. solani* infection had a significant effect on both total root length and total root surface area affecting all root size classes similarly ($p \leq 0.008$ and 0.0001 ; Fig. 5.13). When plants were deficient in Zn, total root length and root surface area increased at low levels of applied *R. solani* (1 propagule) and then decreased with further increase in inoculum density. Total root length and root surface area of Zn-treated plants decreased with increasing levels of *R. solani*. Both total root length and root surface area had a maximum value at mean root diameter 0.4 mm for both Zn treatments. Zinc application and mean diameter range also had a significant effect on total root length and root surface area ($p \leq 0.0001$ in both cases; Fig. 5.13). Although the response between the two Zn treatments was similar, plants supplied with Zn had a more pronounced response. For both total root length and root surface area, no significant interaction occurred between *R. solani* application and mean diameter class ($p \leq 0.6$ in both cases) nor for the three-way interaction between Zn application, *R. solani* application and mean diameter class for root length or root surface area ($p \leq 1.00$ in both cases).

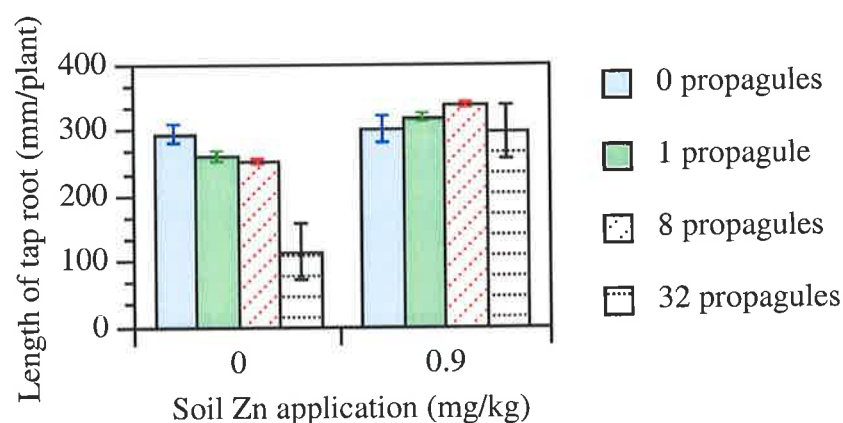


Figure 5.12 Effect of increasing levels of *R. solani* AG8 on length of the tap root for genotype Caliph. Plants were grown with two soil Zn applications, 0 and 0.9 mg/kg, in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, $n=3$.

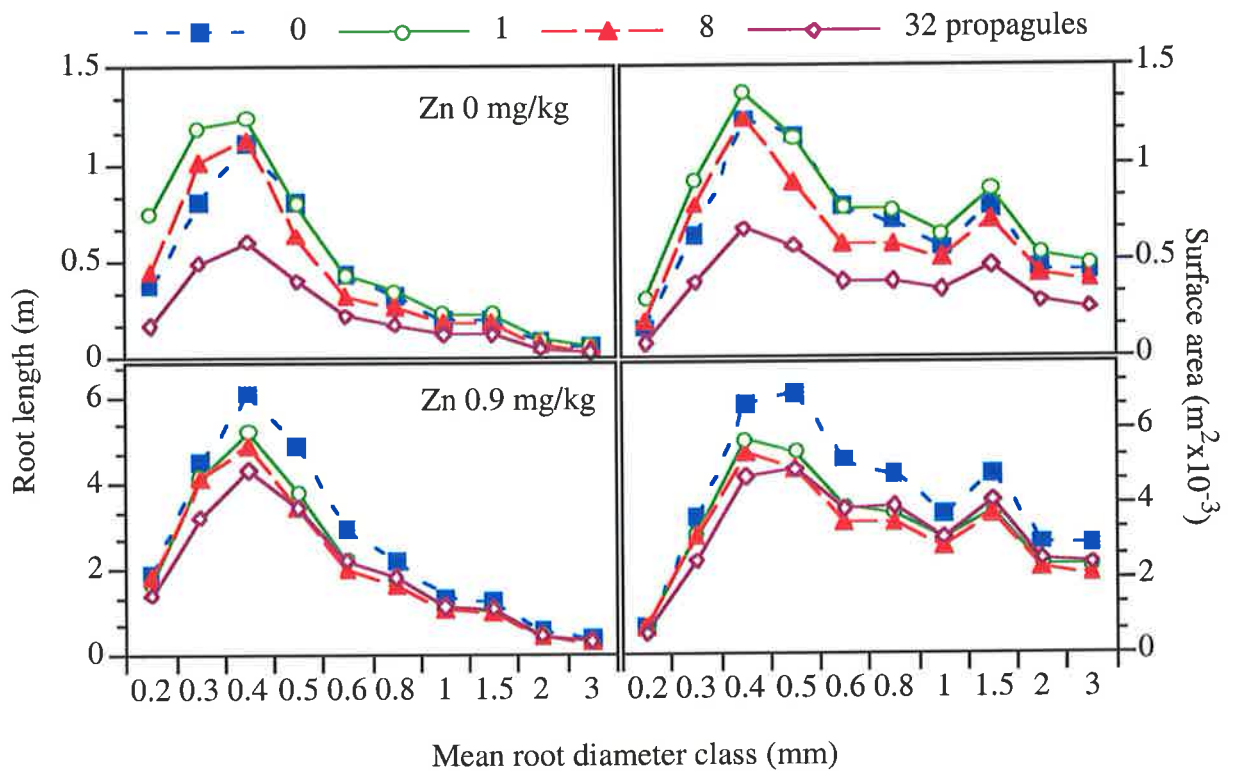


Figure 5.13 Effect of increasing levels of *R. solani* AG8 on root length and root surface area per plant within mean diameter classes for genotype Caliph. Plants were grown with two soil Zn applications, 0 and 0.9 mg/kg, in Mt Compass sand for a 42 days.

Zinc application had a significant effect on the number of root tips per root system, with Zn-deficient plants having fewer root tips (Fig. 5.14). The number of root tips present were not significantly influenced by increasing levels of *R. solani* ($p \leq 0.7$), and the interaction between Zn and *R. solani* was not significant with the number of root tips present ($p \leq 0.3$). However, a significant interaction between Zn application and the addition of *R. solani* occurred with the number of first order laterals on the root system and with the number of first order laterals with visible signs of infection (ie spear tips and lesion; $p \leq 0.001$; Fig. 5.14). Plants deficient in Zn had a reduction in the number of first order laterals present with increasing levels of *R. solani*. The number of first order laterals demonstrating signs of infection increased slightly with increasing levels of *R. solani* when Zn-deficient. When Zn was supplied, no change in the number of first order laterals occurred despite an increase in the number of laterals with signs of infection (Fig. 5.14).

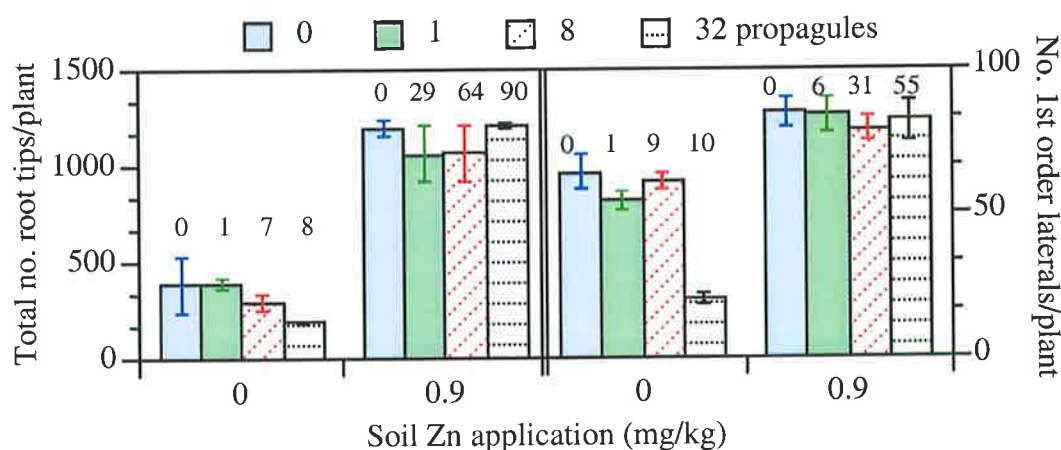


Figure 5.14 Effect of increasing levels of *R. solani* AG8 on number of root tips and first order laterals on the root system of Caliph. Plants were grown with two soil Zn applications, 0 and 0.9 mg/kg, in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error, $n=3$, and numbers above the bars represent the number of root tips present as spear tips and the number of first order laterals with visible signs of infection.

A significant interaction between Zn application and the addition of *R. solani* occurred for the number of spear tips and lesions on the plant as well as disease score ($p \leq 0.0002$, 0.0029, and 0.0359, respectively; Table 5.2). The number of spear tips and lesions generally increased with increasing levels of *R. solani* in both treatments, with a greater increase when plants were supplied with Zn. However, disease score increased with increasing levels of *R. solani* in both Zn treatments, with a greater increase when plants were deficient in Zn.

Table 5.2 Effect of increasing levels of *R. solani* on mean disease severity, as determined by the scoring system of Sweetingham and MacNish (1992), and on the number of spear tips and lesions on the root system of Caliph, with and without Zn application. Numbers in parentheses represent the standard error, $n=3$.

Applied Zn (mg/kg)	Applied <i>R. solani</i> (propagules/kg)	No. spear tips per root system	No. lesions per root system	Disease Score
0	0	0 (0)	0 (0)	0 (0)
	1	1 (0)	2 (0)	0.5 (0)
	8	7 (3)	10 (4)	2 (0.5)
	32	8 (2)	9 (3)	3.5 (0.5)
0.9	0	0 (0)	0 (0)	0 (0)
	1	29 (13)	19 (5)	0.5 (0)
	8	64 (8)	45 (7)	1 (0.5)
	32	90 (13)	68 (16)	1.5 (0.5)

R. solani DNA in roots

Background levels of *R. solani* DNA were detected in the soil (mean 98 ng/g root); this level was subtracted from the data obtained in all treatments. When Zn was not applied to the soil, the amount of *R. solani* DNA increased from 1 to 8 propagules and then decreased at the 32 propagules (Table 5.3). When Zn was applied, the amount of DNA increased with increasing levels of inoculum. From the slot blot, an image was only visible from roots that were supplied with Zn and exposed to the highest level of *R. solani*.

Table 5.3 Effect of increasing levels of *R. solani* on the amount of DNA detected on the root system of Caliph, grown in Mt Compass sand in the presence of *R. solani*, 42 days after sowing. The total amount of *R. solani* DNA per root system was calculated from the mean root dry weight and mean concentration of *R. solani* DNA.

Applied Zn (mg/kg)	Number of propagules	Conc. <i>R. solani</i> DNA (ng/g root)	Total <i>R. solani</i> DNA (ng/root)
0	1	7 (1)	0.2
0	8	47 (3)	1.4
0	32	4 (0)	0.1
0.9	1	15 (1)	3.9
0.9	8	39 (2)	8.0
0.9	32	65 (1)	15.86

5.3.4 Discussion

In-depth studies on the effect of inoculation by *R. solani* on the root system has demonstrated medic supplied with Zn are more tolerant to the pathogen than plants deficient in Zn. This is also supported by a 31% reduction in shoot dry weight due to severe infection by *R. solani* in a Zn-deficient medic compared with a 2% increase when plants were supplied with sufficient Zn.

Infection by *R. solani* did not reduce the concentration of Zn in the shoot at either level of applied Zn, agreeing with results from the previous experiment in this chapter and that of

Thongbai *et al.* (1993b). However, the concentration of Zn was reduced in the root by the addition of *R. solani*, with a 16% decrease in Zn-deficient plants when a high amount of *R. solani* was applied and a 32% reduction in plants supplied with Zn. This shows that high levels of *R. solani* can lower the concentration of Zn by pruning of the root system.

The argument that *R. solani* decreases the amount of Zn plants take up by pruning the root system, resulting in Zn deficiency in wheat was refuted by Thongbai *et al.* (1993b) based on shoot concentration data. Thongbai *et al.* (1993b) stated that "disease did not enhance Zn deficiency, but rather, that Zn deficiency enhanced disease severity". However, using similar techniques Wall *et al.* (1994) observed a reduction in Zn concentration in the shoots of wheat with increasing disease severity when an adequate amount of Zn was applied to the soil. This difference is most likely due to the higher amount of *R. solani* present in the soil in the study by Wall *et al.* (1994) compared with that by Thongbai *et al.* (1993b). A higher level of infection is therefore required to result in a lowering of the concentration of Zn in the shoot of medics. The sole use of shoot analysis may therefore be inappropriate in determining the severity of *R. solani* infection on plants as concentration may be maintained in the shoot at the expense of the root.

Although results from disease score with medics correlate with results by Thongbai *et al.* (1993a; 1993b), the information provided by disease score alone is insufficient to identify the mechanisms by which *R. solani* affects a Zn-deficient plant. For example, in the presence of a low amount of *R. solani* inoculum (1 propagule), Zn-deficient plants appear to compensate for the pruning effect of the fungus by increasing their root length, particularly those of the finer roots (≤ 0.4 mm diameter), and therefore increasing surface area. This will assist in uptake of any nutrients that may become limiting. As the amount of inoculum increases, the root length is reduced due to the dominating effect of pruning by the pathogen. This increase in fine root length and surface area with a low level of *R. solani* did not occur when plants were supplied with an adequate amount of Zn. Instead, with increasing inoculum, root length was reduced to a greater extent. Since pruning of approximately 40% of the root system has been shown to be

required before a reduction in leaf growth is observed in wheat (James *et al.*, 1997), the difference between Zn treatments in medics, is most likely due to the larger number of roots of a Zn-sufficient plant already present to compensate for pruning of other roots. The higher number of roots on a Zn-sufficient medic are more than enough to supply the Zn requirements at the Zn level used.

An increase in disease score on Zn-deficient plants also suggests Zn-adequate plants are infected with less *R. solani* and therefore have greater resistance to disease. However, the smaller root system of a Zn-deficient plant was shown to support a much lower amount of *R. solani* (Table 5.3) compared with the larger root system of a Zn-adequate plant. Increasing the number of propagules applied to the soil did not strictly result in an increase in amount of *R. solani* DNA present in the root system of Zn-deficient plants. This is most probably a factor of timing. If roots were sampled for *R. solani* DNA at an earlier date, a much higher amount of *R. solani* would possibly have been detected. With severe infection in this experiment, many of the roots that were originally colonised by the fungus would have been pruned and rotted off at the time of harvest. The low level of *R. solani* detected in Zn-deficient medics is not indicative of the actual amount of infection the plants were subjected to. Plants supplied with Zn are therefore able to tolerate much higher levels of *R. solani* in the soil compared with Zn-deficient plants. This is also supported by the greater number of lesions and spear tips on roots of Zn-adequate plants.

Zinc was not the only nutrient reduced by the addition of *R. solani*. The concentration of Mn, Cu, Mg, and P in the shoot and Mn, B, P and S in the root were all lower when the plant was infected by a high amount of *R. solani*. This provides further evidence for the pruning effect of *R. solani* resulting in a reduction in nutrient uptake. However, Fe concentration in the shoot, and in the root, increased with high levels of *R. solani* especially in Zn-deficient plants. An increase in Fe may not be related to the addition of *R. solani*, but may be a response to a decrease in the concentration of Mn. Manganese interacts with Fe with the addition of Fe known to ameliorate Mn toxicity (Rorison, 1986). It may therefore be possible that a decrease

in Mn concentration results in an increase in Fe. It is also well documented that Mn hinders Fe translocation to the shoot (Kuo and Mikkelsen, 1981), but in this experiment the proportion of Fe present in the shoot was not altered as the concentration of Mn was decreased at high levels of infection.

Addition of *R. solani* did not always result in a decrease in nutrient concentration. When a low amount of *R. solani* inoculum was present (1 propagule), concentration of many nutrients, including Zn, increased in plants deficient in Zn. This was particularly noticeable for the concentration of Mn, Cu, and P in the shoot and root of Zn-deficient plants. Since shoot growth was reduced in Zn-deficient plants by 23% when a low amount of *R. solani* was added to the soil, the increase in carbohydrates that would be available to the roots may have been used to increase root length and hence surface area, thereby alleviating any potential nutrient deficiencies.

Since nutrients were not limiting, the reduction in shoot growth may have been in response to a direct message produced by the roots as a result of unfavourable soil conditions. Direct "root signals" have been shown to reduce leaf growth in drying soils (Davies *et al.*, 1990), hard soils (Masle and Passioura, 1987) and saline soils (Kefu *et al.*, 1991), with Passioura and Stirzaker (1993) suggesting a hormonal feed-forward response to slow shoot growth and prevent it exceeding the ability of the roots to provide water and nutrients. More recently, Kirkegaard *et al.* (1998) observed high levels of *R. solani* infection to reduce leaf elongation of wheat despite being grown in a pressure chamber to maintain maximum turgor and with increasing levels of P in the soil (P was considered to be the most likely nutrient to demonstrate a reduction due to root pruning). The authors hypothesised that any reduction in leaf elongation was due to hormonal responses to *R. solani*, and not limited water and nutrient supplies due to root pruning. The higher nutrient concentration observed in this experiment may therefore be a combination of increased root surface area available for uptake and reduced demand from the shoot. This effect would be hidden with increasing levels of *R. solani* inoculum as pruning of the root system reduced nutrient uptake further.

Since plants supplied with an adequate amount of Zn did not exhibit a reduction in shoot dry weight, this suggests the levels of *R. solani* used in this experiment were not severe enough to warrant a "root signal" response that would be observed 42 days after sowing. This provides further evidence for increased tolerance to the pathogen when plants were supplied with a sufficient amount of Zn.

5.4 Pot Experiment 3

5.4.1 Aim

The aim of this experiment was to compare the effect of *R. solani* infection on the growth of Caliph derived from seed containing low and high levels of Zn under Zn-deficient and Zn-sufficient conditions.

5.4.2 Materials and Methods

Medicago truncatula, cv. Caliph, grown from seed containing high and low levels of Zn were exposed to four levels of *R. solani* inoculum (0, 1, 8 and 32 propagules/kg soil) with two levels of Zn application (0 and 0.9 mg/kg) in three replicates. This section contains results from two separate experiments. The effect of seed nutrition can be assessed by a direct comparison of the growth of Caliph medic from the previous experiment with that from the experiment presented in Chapter 8. It is not a "true" experiment with pots completely randomised, so should be regarded with appropriate caution. Both experiments however were performed at the same time, using the same soil and basal nutrient solution, with plants exposed to the same growing conditions in the same growth room, and planted, watered and harvested on the same days. Experiments were placed in the growth room on separate tables approximately one metre apart, and randomised within each experiment. Seed from Experiment 5.3 was obtained from the medic breeding unit at SARDI and contained adequate levels of all nutrients (see Table 5.4). Seed from Chapter 8 was produced in a controlled environment under Zn-deficient conditions and therefore had a lower Zn content (Table 5.4).

Since the levels of Mg, Na, K and P were also lower in seed with low Zn, the seed is considered to have a lower nutritional value than that sown in Experiment 5.3. Although low-Zn seed has a higher Mn, B, Ca and S content compared with seed obtained from the medic breeding unit, these levels are considered to be above those considered necessary for normal germination and early seedling growth. The effect of seed nutrition on the shoot dry weight, nutrient composition of the shoots, disease score, root length and root surface area were compared. The Zn efficiency rankings calculated from pots without inoculation of *R. solani* were also determined.

Table 5.4 Nutrient content of Caliph medic seed sown in Experiment 5.3 and Experiment 8.1 in Experiment 8.1.

Exp.	Weight mg/seed	Seed nutrient content ($\mu\text{g}/\text{seed}$)										
		Fe	Mn	B	Cu	Zn	Ca	Mg	Na	K	P	S
5.3	3.9	0.37	0.07	0.07	0.05	0.18	6.4	10.8	0.41	40.6	35.3	12.8
8.1	3.7	0.32	0.12	0.11	0.04	0.13	9.6	8.8	0.12	33.7	27.6	13.7

5.4.3 Results

The interaction between seed quality, Zn application and inoculation with *R. solani* had a significant effect on shoot dry weight of Caliph ($p \leq 0.0001$; Fig. 5.15). Plants derived from seed with low nutrient status had reduced growth compared with plants derived from seed with adequate nutrition. Plants were more sensitive to infection by *R. solani* and exhibited greater reduction in shoot dry weight when grown from seed of lower nutritional value.

The interaction between seed quality, Zn application and inoculation with *R. solani* was also significant for shoot concentration of Zn (Fig. 5.15), Mn, Ca, K and S in the shoot tissue ($p \leq 0.05$ in all cases, except Ca where $p \leq 0.06$; data not shown), and for the number of infection points (spear tips and lesions combined) on the root system (compare Tables 5.2 and 8.4). Although the three-way interaction was not significant for Fe, B, Cu, Mg and P, various combinations of the two-way interactions were (data not shown). There was a significant interaction between seed quality and inoculation on disease score of Caliph as well, with a

higher disease score occurring in plants with reduced seed quality ($p \leq 0.001$). Overall, Caliph derived from seed with poor nutritional status had reduced concentration of Zn, Mn and Ca in the shoot at both levels of Zn application. The concentrations of B, Cu, Mg, K, P and S were also lower in shoots of plants derived from seed with low nutritional value when grown in soil deficient in Zn, but when Zn was applied the concentration of B, Cu, K and P were higher in the shoot of plants derived from seed of poor nutritional value compared with those derived from seed of adequate nutritional value. With Zn application, the concentrations of Mg and S in the shoots were similar in plants derived from both types of seed. With increasing levels of *R. solani* the shoot levels of all nutrients declined, with the decline more pronounced in plants derived from seed of lower nutritional value.

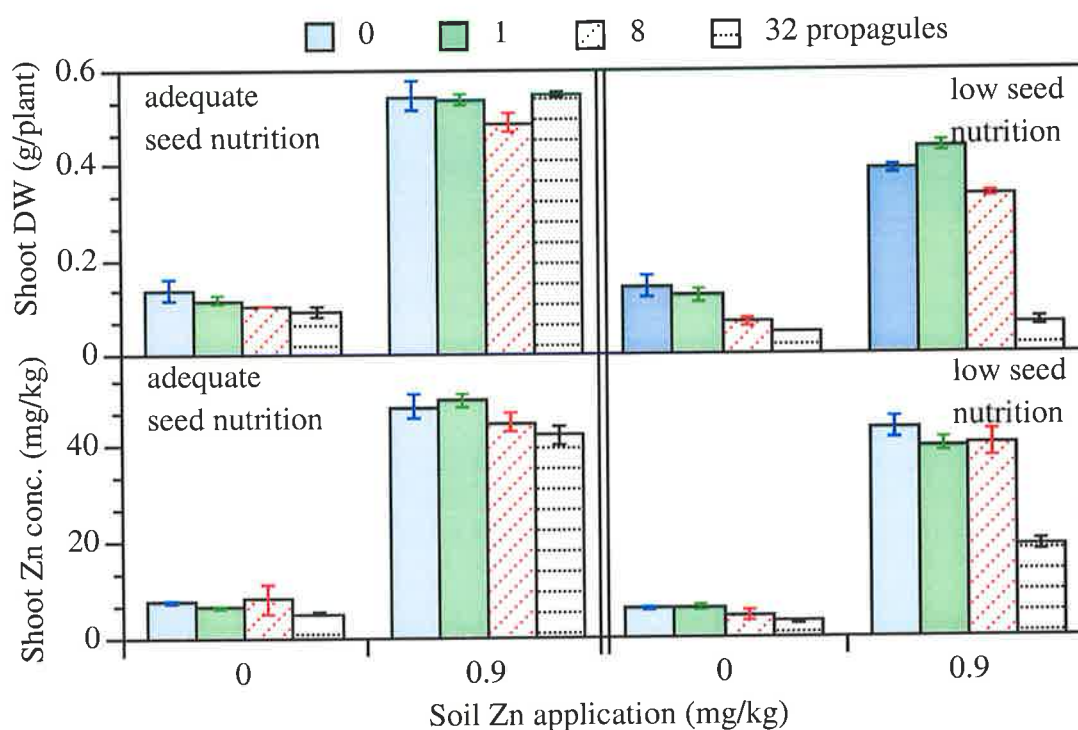


Figure 5.15 Effect of Zn application on the shoot dry weight and shoot Zn concentration of Caliph medic derived from seed of adequate or poor nutritional content. Plants were grown with increasing levels of *R. solani* in Mt Compass sand for 42 days. Vertical bars represent $\pm$ standard error.

The four-way interaction between seed quality, Zn application, inoculation with *R. solani*, and mean root diameter class had a significant effect on root length and root surface area ($p \leq 0.001$ in both cases; Fig. 5.16). When Caliph was deficient in Zn, root length and root surface area

were greater in plants derived from seed with poor nutrition compared to those from seed with adequate nutrition, when *R. solani* was either absent or present at low levels. The addition of Zn reversed this trend, with plants derived from adequate seed having a higher root length and root surface area in all *R. solani* treatments (Fig. 5.16).

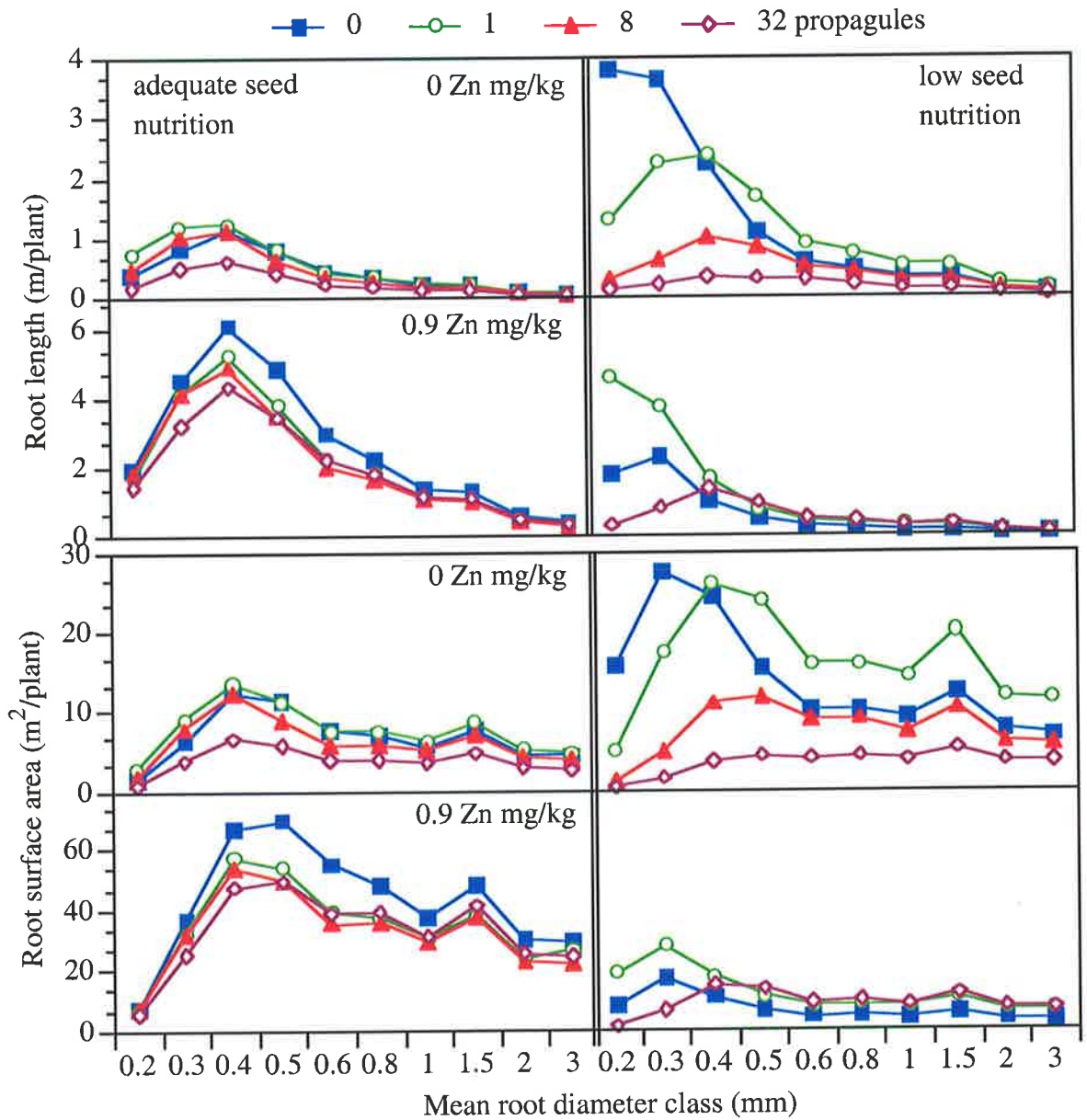


Figure 5.16 Effect of Zn application on root length and root surface area within mean diameter classes for Caliph medic derived from seed with adequate and poor nutritional content. Plants were grown with increasing levels of *R. solani* in Mt Compass sand for 42 days. Vertical bars represent Fisher's Protected Least Significant Difference for the four-way interaction: seed quality x Zn application x inoculation with *R. solani* x mean root diameter class.

5.4.4 Discussion

The effect of pruning the root system by *R. solani* was exacerbated in plants derived from seed with a lower nutritional value. No reduction in growth occurred in Caliph plants derived from seed with high nutrient reserves at high levels of *R. solani*. However, a reduction in the nutritional status of the seed results in a lower tolerance to infection by *R. solani*. This may partly be due to the lower level of nutrients, but it is most probably the level of available amino acids to the developing embryo that has the greatest effect on tolerance to *R. solani*. Due to the limited number of seed, it was not possible to conduct analyses for seed amino acids.

5.5 Conclusion

The addition of Zn has been shown to reduce above-ground symptoms caused by *R. solani*. Contrary to previous work with wheat (Thongbai *et al.*, 1993b), the decrease in shoot growth is not due to lower resistance to the pathogen, but lower tolerance. The present study also demonstrated that the pruning effect of *R. solani* lowers the concentration of Zn in medics with the effect observed in the root system first. The higher level of infection achieved in the second experiment compared with the first may explain the difference observed in concentration of most nutrients in the shoot. The level of *R. solani* inoculum and the quality of the seed sown also affects the concentration of nutrients in the plant. The reduction in shoot dry weight with increasing *R. solani* under Zn-deficient conditions may be exacerbated with the use of seed with low nutritional reserves and should be considered an important factor in future work on the interaction between disease and nutrition.

Plants produced longer roots when fertilised with Zn despite increased amounts of *R. solani* in the root system. In Zn-deficient fields, this should translate to improved pasture growth and improved seed nutrition with Zn fertilisation. Although improved root growth with Zn application leads to more inoculum potential for *R. solani*, an adequate Zn supply will increase

tolerance to *R. solani*, thereby improving root growth further. Improved shoot growth will then follow, along with seed with higher nutritional quality. The higher amount of organic matter in the soil produced by improved root systems of pastures may also promote population growth of other organisms which will compete with *R. solani*. After a number of years, not only will seed nutrition be maintained for the regenerating pasture, but the competition between *R. solani* and the general microbial population will take effect and a reduction in inoculum potential of *R. solani* may result.

Along with Zn fertilisation and maintaining adequate medic seed to increase tolerance to *R. solani*, the use of Zn-efficient medic genotypes should also be considered. Chapters 6 and 7 explore the potential for finding a Zn-efficient medic genotype, while Chapter 8 tests the hypothesis that Zn-efficient medics are more tolerant to infection by *R. solani* under Zn-deficient conditions.

Effect of Soil Zn Application on Growth of *Medicago* species.- Field Observations

6.1 Introduction

Zinc deficiency is more widespread than any other micronutrient deficiency (Sillanpää, 1990); it occurs in cold and warm climates, in acid and alkaline soils and in all soil textures (Graham *et al.*, 1992). In order to overcome Zn deficiency, many factors need to be considered. It is essential that adequate levels of Zn fertiliser be applied to areas naturally low in Zn, with Zn fertilisers mainly applied in a mixture with NPK fertilisers. This may occur either by incorporation during the initial synthesis of the fertiliser, by bulk blending of granular fertilisers, or by granular coating using a binding agent. Although each of the processes results in a decrease in the immediate availability of Zn compared with separate application of Zn, the residual effectiveness of Zn fertiliser increases with insoluble Zn sources becoming more soluble, and therefore more available, over time (Holloway, 1996).

The placement of Zn fertiliser within the soil profile is also an important aspect in overcoming Zn deficiency, with field studies by Holloway (1996) demonstrating an increase in grain yield and concentration of Zn in the grain when Zn was applied to the subsoil, to a depth of 0.40 m, compared with placement of Zn to the topsoil to a depth of 0.05 m. This finding is supported by Nable and Webb's (1993) study that compared fertilised with unfertilised subsoil. They observed that applying Zn fertiliser to the topsoil and withholding Zn from the subsoil resulted in delayed head emergence and lowered grain yield in the Zn-inefficient genotype of wheat (Gatcher). However, the Zn-efficient genotype of wheat (Excalibur) was tolerant to the Zn-deficient subsoil and did not display any difference compared with plants grown in fertilised subsoil. This suggests Zn-efficient genotypes may be used to overcome Zn deficiency when Zn fertiliser is not applied to the subsoil.

Genotypic variation for Zn efficiency has been recognised in many species. This variation is important as it provides one approach to solving plant productivity problems with plants growing more effectively on soils with greater mineral nutrient stresses. This approach has been described as “tailoring the plant to fit the soil” rather than “tailoring the soil to fit the plant” (Foy, 1983). With Zn-efficient plants, more efficient use of available nutrients with fewer fertiliser applications to soils are both practical and feasible.

Not only do Zn-efficient medic genotypes have the potential to overcome Zn deficiency, they may also be able to tolerate disease to a greater extent than Zn-inefficient genotypes on soils deficient in Zn. Grewal *et al.* (1996) demonstrated the Zn-efficient genotype of wheat, Excalibur, was able to “build and maintain resistance” to *Fusarium graminearum* while Zn-inefficient genotypes, Durati and Songlen, required Zn fertilisation to achieve a response comparable to that of the Zn-efficient genotype. Since previous work in Chapter 5 has demonstrated Zn application to increase tolerance of medics to infection by *R. solani*, it may therefore follow that Zn-efficient medics are better able to tolerate disease than Zn-inefficient medics. However, before the interaction between genotypes with varying levels of Zn efficiency can be studied with regards to *R. solani* infection under Zn limiting conditions, Zn efficiency rankings of medic genotypes are required. Studies on genotypic variation for Zn efficiency have mainly been conducted on crops, with cereals dominating the literature. To date, no field trials studying response to Zn application in medics have been performed.

Along with the addition of Zn fertiliser and the use of Zn-efficient genotypes, Zn content of seed sown has been shown to be an important factor in overcoming Zn deficiency. Previous work with Zn-efficient and Zn-inefficient wheat (Excalibur and Gatcher, respectively) demonstrated seed with a higher seed Zn content acts like a starter-fertiliser by improving early vegetative growth compared with a seed containing 65% less Zn (Rengel and Graham, 1995c). These differences extended to the end of the season with grain yield highest for the Zn-efficient genotype derived from seed containing more Zn. More importantly a higher seed Zn content was shown to compensate for differences in Zn efficiency of wheat genotypes (Rengel and

Graham, 1995c). The amount of Zn in the seed therefore requires careful consideration when determining Zn efficiency rankings.

The aims of these field trials were two-fold; firstly to determine if current Zn fertilisation practices are suitable for medics by comparing the residual effect of Zn on soils supplied to the subsoil and the topsoil. Most Zn fertilisers have a residual effect lasting at least 5 years (Mortvedt and Gilkes, 1993), with farmers advised to supply 2 kg Zn/ha to the soil every four to six years in order to correct Zn deficiency in cereals (HiFERT, 1995). However, inputs into pasture systems tend to be less than those for cereals due to the lower economic return (Sheaffer and Lake, 1998), and the residual effect of Zn may not be sufficient to benefit pasture after a minimum of three crops in the rotations. As an experimental site that in the past had a range of Zn treatments was to be sown to medics at Minnipa in 1996, the opportunity was taken to study the response of the medics to various Zn treatments under field conditions.

The second aim of the trial was to determine Zn efficiency rankings of 12 commonly grown medic genotypes (from a range of species) in a field situation. Not only will this allow Zn-efficient medics to be selected for soils deficient in Zn, it will also assist future work in determining the effect of Zn efficiency on tolerance to infection by *R. solani*. Included in the field trial was a comparison of the effect of seed size variation with two of the 12 genotypes. With these two genotypes, the effect of large and small seed weight was compared in response to Zn application.

6.2 Effect of Residual Soil Zn Application

6.2.1 Aim

The aim of this field trial was to determine if Zn applied in 1993 to a cereal crop would still be beneficial to a medic pasture in 1996.

6.2.2 Methods

6.2.2.a Site History

In 1993 a field trial was conducted at site N6 of the Minnipa Research Centre on Upper Eyre Peninsula of South Australia (Holloway, 1996). The trial consisted of a randomised block design with four replicates on solonised brown soil. Treatments consisted of Zn, P, and N fertiliser applied to the topsoil (0-0.05 m) or subsoil (0.05-0.4 m) in various combinations.

More specifically, treatments were:

- 1) Control - ripped to 0.4 m,
- 2) Control - not ripped,
- 3) N+P - applied as granulated solid fertiliser to the top 0.05 m (41 kg N ha⁻¹ (67% as NH₄NO₃, 33% as diammonium phosphate (DAP)) and 15 kg P ha⁻¹ as DAP),
- 4) N+P - applied in water to a depth of 0.4 m (41 kg N ha⁻¹ (83% as NH₄NO₃, 17% as monoammonium phosphate (MAP)) and 15 kg P ha⁻¹ as MAP),
- 5) Zn - applied as solid fertiliser to the topsoil (0-0.05 m; 10 kg Zn ha⁻¹ as ZnSO₄.7H₂O),
- 6) Zn - applied in water to 0.4 m (10 kg ZnSO₄.7H₂O),
- 7) N+P+Zn - nitrogen, phosphorus and zinc applied to the topsoil as granulated fertiliser (10 kg Zn ha⁻¹ as ZnSO₄.7H₂O, 41 kg N ha⁻¹ (67% as NH₄NO₃, 33% as DAP) and 15 kg P ha⁻¹ as DAP),
- 8) N+P+Zn - nitrogen, phosphorus and zinc applied in water as a single application to a depth of 0.4 m (10 kg Zn ha⁻¹ as ZnSO₄.7H₂O, 41 kg N ha⁻¹ (83% as NH₄NO₃, 17% as MAP) and 15 kg P ha⁻¹ as MAP).

Plots were 30 m long, with 5 m spacings between plot centres. Each plot consisted of two adjacent strips sown with a 10-row combine drill, with 0.18 m row spacings. All plots were ripped in the same direction in May 1993 at a speed of 0.9 m/s with a three-tyned Howard "Paraplow" with tyne spacings of 0.45 m. Plots consisted of three ripped strips, each 1.35 m wide. Spray nozzles were attached to the back of each tyne to allow nutrient solutions to be

pumped into the subsoil below 0.05 m at a pressure of 210 kPa. Surface treated plots were also ripped and received water only. The water application rate of 16,700 L ha⁻¹ occurred on all plots. After ripping, fertiliser was applied to the surface-treated plots using a small plot drill and all plots received the same number of passes. In the subsoil treatments, 70% of total N, 50% of total P and 70% of total Zn (totals include basal dressings) were applied in water to the subsoil below 0.05 m.

In 1993 plots were sown to Machete wheat at a sowing rate of 59 kg/ha with a basal dressing of 9 kg N ha⁻¹ and 10 kg P ha⁻¹ as DAP applied with the seed. In 1994 plots were sown to Stirling barley at 60 kg/ha with a basal dressing of 6 kg N/ha and 13 kg P/ha as MAP applied with the seed. In 1995, plots were sown to Machete wheat at a sowing rate of 65 kg/ha with a basal dressing of 50 kg/ha DAP.

6.2.2.b Procedure

In April 1996, plots were sown to *M. truncatula* genotype Caliph, at a sowing rate of 29 kg/ha. One sample per plot (20x20) cm was taken 145 days after sowing. The number of plants per sample was counted, shoot material dried and analysed by ICP. At 188 days after sowing three samples per plot (30x30 cm) were taken and shoot dry weight recorded for a final estimate of dry matter production. Seed yield was determined at the end of the season and analysed for nutrient composition by ICP.

6.2.3 Results

During the first two months after sowing, plants were subjected to below average rainfall (Fig. 6.1). In the following four months (June to September) rainfall was above average providing sufficient water for growth. Plants were sampled in early September and at the end of the growing season in October when rainfall was low.

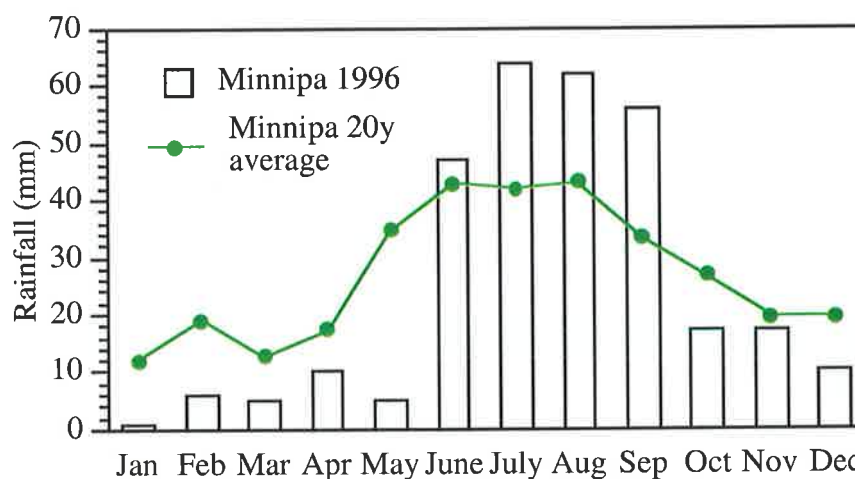


Fig. 6.1 Monthly rainfall data during 1996 and mean rainfall data over the last 20 years at Minnipa, South Australia.

At 145 days after sowing visual differences between type of fertiliser applied were observed with N+P+Zn plots appearing more vigorous than the controls. No visual differences could be seen between surface and subsoil treatments. A two-way ANOVA indicated no significant difference in the number of plants per sample and the interaction between type of fertiliser and depth of placement in the soil profile on the shoot dry weight was also non-significant. However, the main effect, type of fertiliser, had a significant effect on shoot dry weight ($p \leq 0.008$), while depth of placement had no effect (Fig. 6.2). Dry matter increased in the following order (identical letters in parentheses represent no significant difference as determined by Fisher LSD):

$$\text{control (a)} < \text{N+P (ab)} < \text{Zn (bc)} < \text{N+P+Zn (c)}$$

No visual differences were observed at 188 days after sowing between any two treatments. This corresponded with no statistical differences from a two-way ANOVA (Fig. 6.2). The type of fertiliser had a significant effect on seed yield at the 6% level ($p \leq 0.059$), with yield increasing in the following order (similar letters in brackets represent no significant difference as determined by Fisher LSD):

$$\text{N+P (a)} < \text{N+P+Zn (b)} < \text{control (b)} < \text{Zn (b)}$$

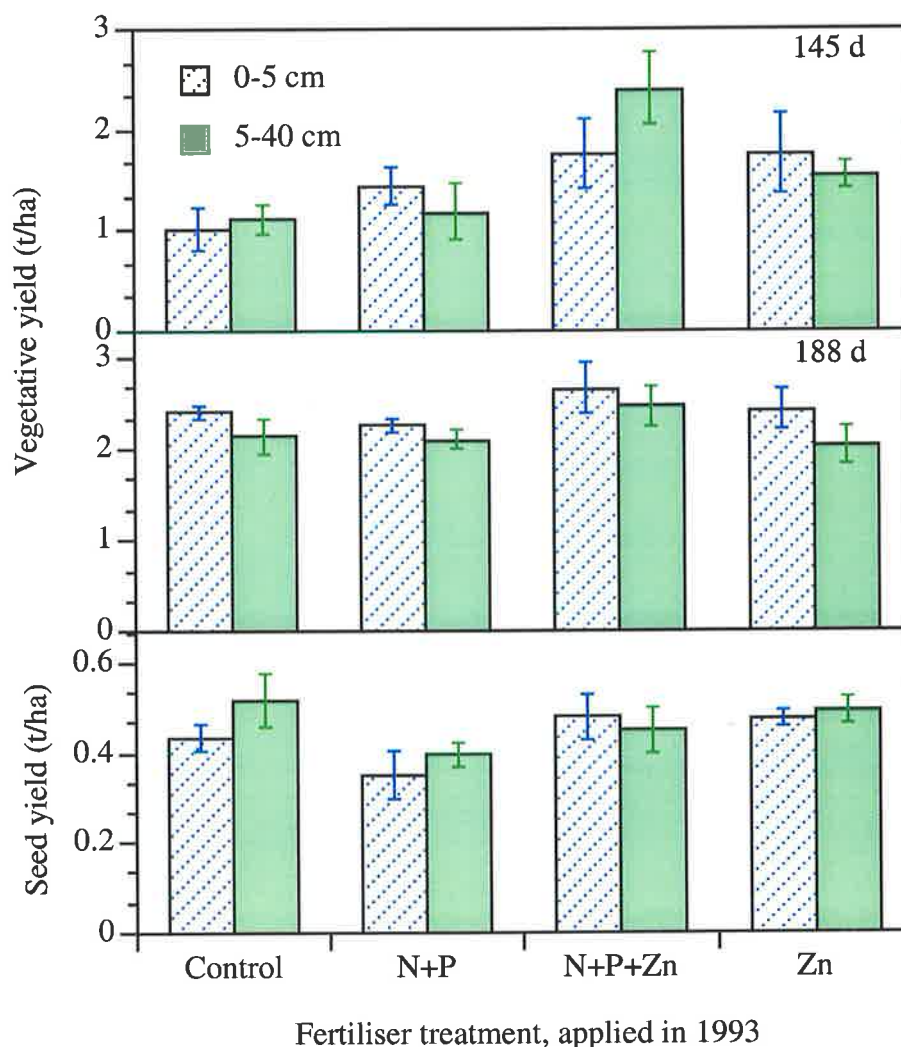


Figure 6.2 Effect of fertiliser applied in 1993 on shoot growth at 145 and 188 days after sowing and on final seed yield of genotype Caliph grown at Minnipa, South Australia, in 1996.

The interaction between type of fertiliser and depth of placement on concentration of Zn in the vegetative tissue at 145 days after sowing was not significant, but the interaction was significant with regards to the concentration of Zn in the seed ($p=0.005$; Fig. 6.3). Zinc concentration in the seed increased with the use of Zn fertiliser, and the increase was higher when the fertiliser was applied to the surface soil (0-5 cm). Seed Zn concentration was higher when Zn was applied with N and P as opposed to Zn on its own (Table 6.1). The type of fertiliser used had a significant effect on concentration of Zn in vegetative tissue ($p=0.0001$), with Zn concentration significantly higher when Zn fertiliser was applied in 1993 (Table 6.1). Depth of fertiliser placement did not have a significant effect on the concentration of Zn in the vegetative tissue.

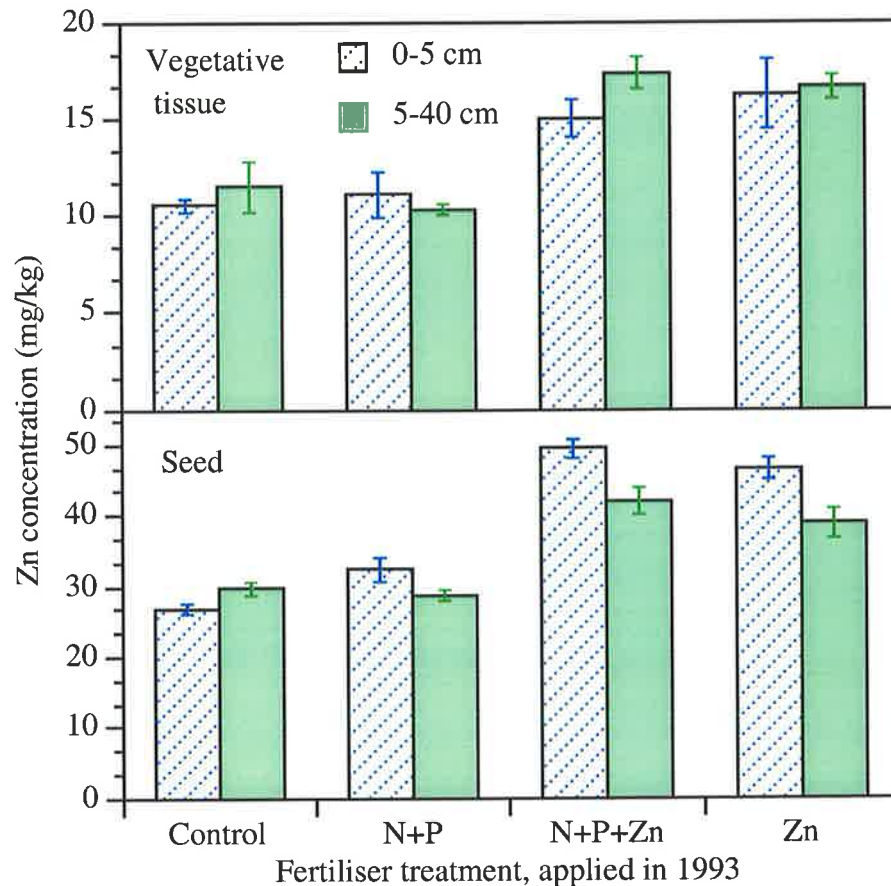


Figure 6.3 Effect of fertiliser applied in 1993 on the concentration of Zn in the shoots at 145 days after sowing and in the seed of genotype Caliph grown at Minnipa, South Australia, in 1996.

The interaction between fertiliser and fertiliser placement in the soil profile did not have a significant effect on the concentration of P in the vegetative tissue at 145 days after sowing nor in the seed (Fig. 6.4). Depth of fertiliser placement also had no significant effect on the concentration of P in the tissue and in the seed. However, fertiliser treatment did have a significant effect on P concentration in vegetative tissue ($p \leq 0.003$) and in the seed ($p \leq 0.018$), with P concentration greatest when P was applied with N (Table 6.1). The next highest concentration of P in vegetative tissue occurred in the control followed by the N+P+Zn treatment and then the treatment with Zn alone.

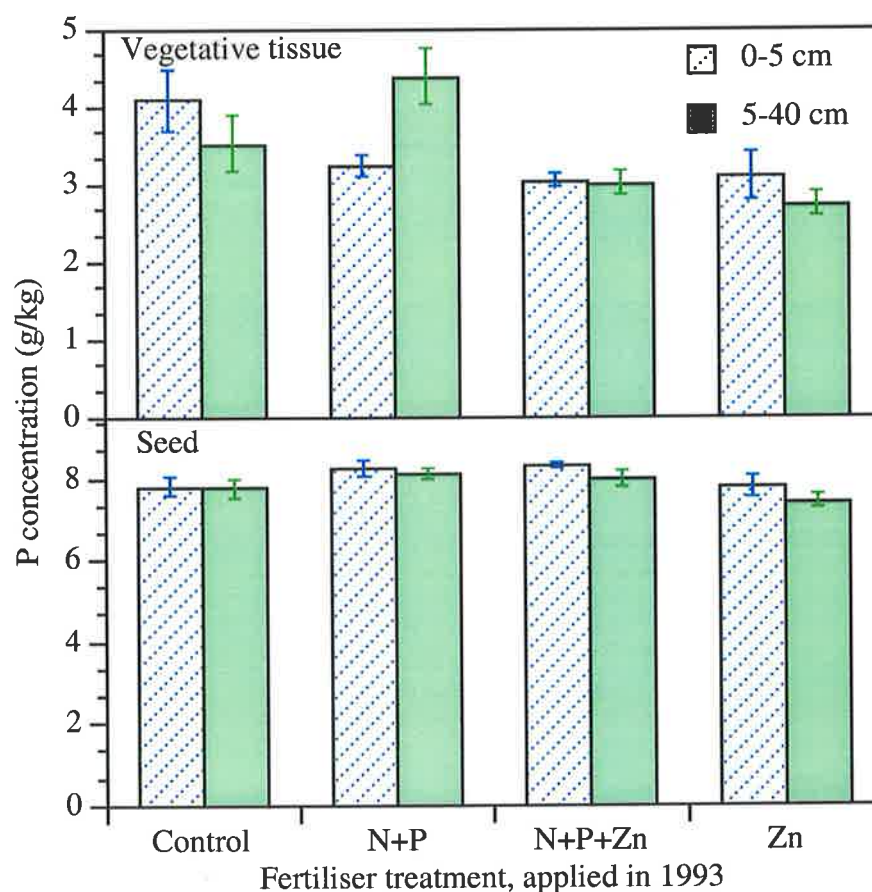


Figure 6.4 Effect of fertiliser applied in 1993 on concentration of P in the shoots at 145 days after sowing and in the seed of genotype Caliph grown at Minnipa, South Australia, 1996.

The interaction between fertiliser treatment and fertiliser placement was not significant for the concentration of any other nutrient analysed in either vegetative tissue or seed. The main effect, fertiliser treatment, had a significant effect on most nutrients analysed (Table 6.1), but fertiliser placement was not significant with any nutrient. The concentrations of Mn and Mg in shoot tissue and seed were highest when N and P fertilisers were applied in 1993, and lowest when Zn fertiliser was applied. The same occurred with shoot tissue concentration of B, Cu and S being highest when N and P fertiliser were used, and lowest when Zn fertiliser was applied. All treatments resulted in plants deficient in Zn and Mn at 145 days after sowing, while B and Ca were at levels above the range for normal growth (Table 6.1).

Table 6.1 Effect of different fertilisers on the nutrient composition of shoots at 145 days after sowing and in seed at the end of the season for genotype Caliph, grown at Minnipa, South Australia in 1996. Identical letters after the concentration value represent no significant difference as determined by Fisher's Protected LSD.

ns - not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Nutrient			Tissue		Fertiliser			
			Control	N+P	Zn	N+P+Zn		
mg/kg shoot dry weight								
Fe	Shoot	ns	316 a	338 a	253 a	253 a		
	Seed	ns	53 a	43 a	46 a	51 a		
Mn	Shoot	**	26 a	29 b	24 a	26 a		
	Seed	*	17.9 ab	18.6 a	17.7 b	17.5 b		
B	Shoot	***	61 a	62 a	56 b	56 b		
	Seed	ns	15 a	15 a	15 a	16 a		
Cu	Shoot	*	7 a	9 b	7 a	7 a		
	Seed	ns	11 a	12 a	13a	12 a		
Zn	Shoot	***	11 a	11 a	16 b	16 b		
	Seed	***	28 a	31 a	43 b	46 c		
g/kg shoot dry weight								
Ca	Shoot	ns	18.3 a	18.6 a	16.5 a	17.5 a		
	Seed	ns	17 a	1.6 a	1.6 a	1.6 a		
Mg	Shoot	**	3.8 ab	3.9 a	3.2 c	3.5 bc		
	Seed	*	2.1 ab	2.2 a	2.0 bc	2.1 c		
K	Shoot	ns	28.7 a	30.5 a	21.4 a	31.5 a		
	Seed	ns	10.3 a	10.6 a	10.1 a	10.6 a		
P	Shoot	**	3.8 a	3.8 a	2.9 b	3.0 b		
	Seed	*	7.8 ab	8.2 b	7.6 a	8.1 b		
S	Shoot	*	3.2 ab	3.5 a	2.9 b	3.0 b		
	Seed	ns	3.0 a	3.0 a	3.0 a	3.0 a		

6.2.4 Discussion

Zinc fertiliser applied in 1993 was sufficient to increase tissue Zn concentration by 5 mg/kg and increase seed concentration by more than 10 mg/kg. However, all medics were deficient in Zn, indicating the residual effect of 10 kg/ha of Zn in either the top- or sub-soil is insufficient for normal medic growth after 3 cereal crops in the rotation. Although the placement of fertiliser did not affect the concentration of Zn in the shoot, it did influence the concentration of Zn in the seed. Zinc applied as granulated fertiliser to the top soil increased the concentration of Zn in the seed more than liquid solution of Zn to the sub-soil. Previously, the concentration of Zn in the seed of cereals sown on the site in the years prior to medics had a higher concentration when liquid fertiliser was applied to the sub-soil (Holloway, 1996). The larger sized particles of Zn in granulated form may therefore act as a slow release fertiliser compared with liquid form, thereby maintaining a greater residual effect of Zn in the top soil. Conversely, Zn may be binding to subsoil clay particles over time.

The levels of Zn in the shoots of medics is of concern as these levels are representative of pastures across the state. However, it is also important to note that no cereal Zn trial in South Australia conducted by the Trace Element group from the University of Adelaide showed a response to Zn application in 1996. This is most probably a result of low rainfall and is discussed in the following experiment.

The increase observed in the concentration of B, Mn, Cu, Mg, P and S when Zn fertiliser was not applied to the soil is most probably a result of increased membrane permeability due to more severe Zn deficiency. This phenomena was also observed, and described in detail, in Chapters 4 and 5.

6.3 Field Observations on Zn Response in Medics

The aim of this experiment was to determine Zn efficiency rankings of 12 medic genotypes, from a range of species, in a field trial. A comparison of the effect of seed size variation was also made with two of the 12 genotypes. With these two genotypes, large and small seed sizes were obtained and compared in response to Zn application.

6.3.1 Methods

6.3.1.a Site Description

The field trial was sown 15 km north of Lameroo, South Australia, on Zn-deficient sandy loam. Twelve genotypes (two of which were sown with both large and small seed sizes) were grown at two Zn levels in four replicates. Plot dimensions were 6 x 0.9 m after sowing.

6.3.1.b Genotypes

Twelve medic genotypes from 6 species were sown. They were: *M. truncatula* cvs. Caliph, Parabinga, Paraggio, Mogul, Ghor; *M. littoralis* cv. Harbinger, Herald; *M. tornata* cvs. Tornafeld and Rivoli; *M. polymorpha* cvs. Santiago; *M. scutellata* cvs. Sava; and *M. rugosa* cvs. Paraponto. Seed was available from a number of sources and was preferentially chosen for the lowest Zn content available for each genotype (Table 6.2). Caliph and Paraggio seed were sieved to create two lots: seed having greater than 2 mm diameter and seed smaller than 1.2 mm diameter. Table 6.3 illustrates the differences in nutrient composition in the large and small seeds of Caliph and Paraggio.

Table 6.2 Seed characteristics of 12 medic genotypes grown in the field at Lameroo, 1996.

Genotype	[Zn] (mg/kg)	Seed dry wt	Zn content
		(mg/seed)	(µg/seed)
Caliph (small seed)	24	3.3	0.08
Caliph (large seed)	35	4.3	0.15
Parabinga	53	4.0	0.16
Paraggio (small seed)	26	2.6	0.07
Paraggio (large seed)	33	4.7	0.16
Mogul	51	4.6	0.18
Ghor	62	4.8	0.29
Harbinger	88	2.3	0.20
Herald	26	1.9	0.05
Tornafeld	42	5.6	0.23
Rivoli	10	3.9	0.04
Santiago	80	2.8	0.22
Sava	56	16.4	0.92
Paraponto	67	10.3	0.69

Table 6.3 Seed nutrient composition for 2 genotypes, Caliph and Paraggio, comparing small (<1.2 mm) and large seeds (>2.0 mm).

Nutrients	Caliph		Paraggio	
	small	large	small	large
	µg/seed			
Fe	0.62	0.71	0.33	0.96
Mn	0.05	0.06	0.04	0.10
B	0.04	0.05	0.03	0.06
Cu	0.04	0.06	0.02	0.07
Zn	0.08	0.15	0.07	0.16
Mg	7.2	9.6	4.8	11.3
Na	0.18	0.29	0.18	0.18
Ca	7.1	5.7	6.4	6.8
K	29	43	23	47
P	23	36	14	31
S	8.3	13.8	7.5	15.7

6.3.1.c Procedure

A trial with a two-factorial randomised split plot design was sown with 12 genotypes and 2 soil Zn levels in June 1996. Zinc was applied as Zn-oxysulphate in the form of small pellets at the rate of 0 and 22 kg/ha. Since Zn comprises 36% of Zn-oxysulphate, this is equivalent to 8 kg Zn/ha. Zinc from pellets is not fully available to the plants at the start of the season, accounting for the relatively high amount of total Zn added to the soil. Prior to sowing, 100 kg/ha of fertiliser (diammonium phosphate plus Cu, Co, Mn and Mo) was added. Fertiliser consisted of the following nutrients (kg/ha): 15 N, 17 P, 1.3 S, 1.2 Cu, 0.14 Co, 2.9 Mn, and 0.14 Mo. Seed was sown at a rate of 400 seeds/m², with seed weight varying significantly between genotypes (Table 6.2). During sowing, *Rhizobium meliloti* was added to the soil with the seed and Zn treatment. Strain WSM826 was added to genotypes of *M. littoralis* and *M. tornata*, while strain WSM688 was added to the remaining species.

6.3.1.d Sampling

Shoots of five plants were removed from all plots containing Caliph and Paraggio at 43, 81 and 117 days after sowing to compare growth of plants derived from large and small seeds. Zinc concentration was also measured in the shoots of Caliph and Paraggio at 43 and 81 days after sowing, and in the seed at the end of the season. A 30x30 cm cut was removed from all plots at 81 days after sowing to compare dry weight and Zn concentration of all genotypes, and a final cut was taken at 117 days after sowing to compare final yield from replicate 1, 2 and 4. At the end of the season, seed was collected from each plot in two 30x30 cm cuts. Pod were threshed to determine seed weight. Zinc efficiency rankings were calculated from the following equation based on shoot dry matter production at day 81 (Graham and Rengel, 1993):

$$\text{Zn efficiency} = \frac{\text{Shoot dry weight at 0 Zn}}{\text{Shoot dry weight at + Zn}} * 100\%$$

Analysis of variance techniques were not sufficient to analyse the trial as the data was unbalanced due to incorrect sowing and missing plots, all beyond the control of the researcher. To overcome unbalanced data, Restricted Maximum Likelihood (RML) was calculated. Using

RML, the importance of treatment effects (genotype and Zn application) was assessed using the Wald statistic which was compared with an asymptotic chi-squared distribution. The standard error of difference (SED) was calculated. Due to the unbalanced nature of the data, the SED was different for each pair of means depending on replication, and conclusions were therefore based on the maximum SED. Genotypes with large and small seeds were analysed together with other genotypes and also separately because extra samples were taken during the season.

6.3.2 Results

Rainfall data from the Lameroo district was collected and compared with the average rainfall between 1994 and 1996 (Fig. 6.5). For the majority of the growing season (June to September) rainfall exceeded the three year average. Rainfall was, however, below the average at the start (April-May) and end of the season (October-November).

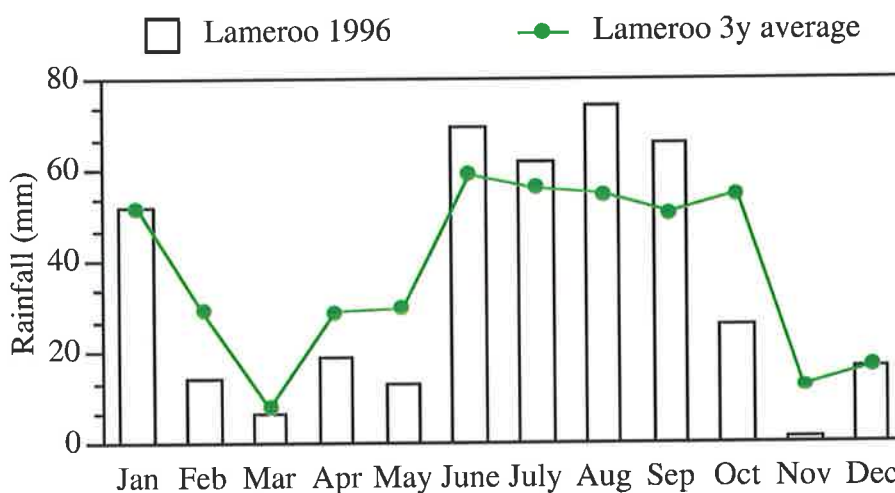


Figure 6.5 Monthly rainfall recorded at the Lameroo district in 1996 compared with the mean rainfall during 1994-1996.

There were clear differences in the growth response of Caliph and Paraggio to sowing small and large sized seed (Fig. 6.6). When large seed of Paraggio was sown, yield at day 43 and day 81 was significantly greater than that from the small seed of Paraggio. However, at day 117, this effect was not observed. It was observed however, at the end of the season in the

seed yield. Zinc application did not have a substantial effect on either large or small seeded Paraggio.

The effect of size of seed sown was not as dramatic for Caliph as the difference in seed weight was less. Dry weight of plants derived from large seed was greater than for plants derived from small seed. This effect on dry weight was more pronounced when no Zn was applied to the soil compared with Zn application (Fig. 6.6). Seed size did not have an effect on Caliph dry weight at day 81, but the addition of Zn increased yield. At day 117 an increase in yield due to the larger seed of Caliph was again seen without Zn but not when Zn was applied. With regards to seed yield, the reverse occurred in Caliph compared to Paraggio with a higher yield recorded in plants derived from small seed at both Zn treatments.

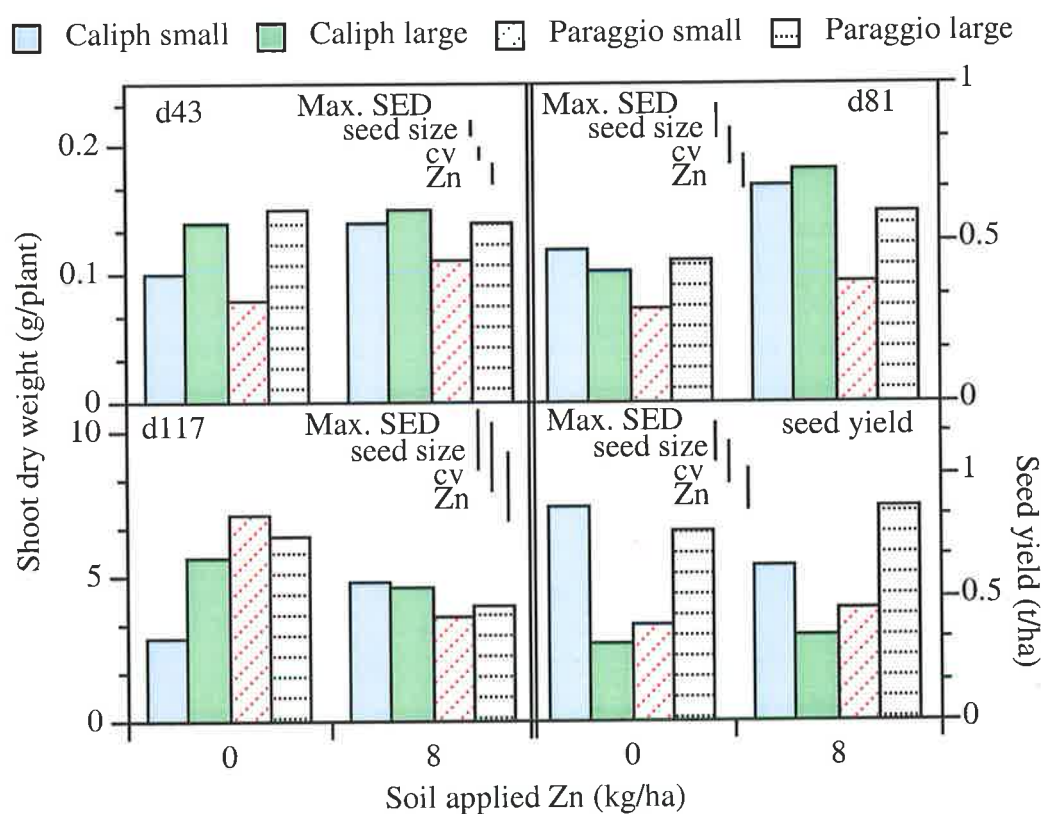


Figure 6.6 Effect of Zn application on shoot dry weight and seed yield for small and large sized seeds of Caliph and Paraggio, grown at Lameroo, 1996. Vertical bars represent maximum standard error of the difference between means for the main effects. Significant interactions, and main effects ($p \leq 0.05$) between seed size, genotype and Zn at the respective harvest include: *d43* seed size and cv x Zn; *d81* Zn: seed x cv and seed x Zn; *d117* cv x Zn; *seed yield* Zn.

The addition of Zn resulted in a significant increase in concentration of Zn in the shoot at 43 and 81 days and also in the seed for both Paraggio and Caliph (Fig. 6.7). At day 43, the interaction between genotype, Zn and seed size also had a significant effect on the concentration of Zn in the shoot with no change in the concentration of Zn between Caliph and Paraggio, with either seed size, when Zn was not applied to the soil. However, when Zn was applied the concentration of Zn was higher in Caliph plants derived from large seed than small seed, while the opposite occurred for Paraggio at day 43. By day 81, only Zn application had a significant effect on the concentration of Zn. This single effect followed through to the end of the season with mean seed Zn concentration of 32 with no Zn compared with 39 mg/kg with Zn application. Although Zn application was the only factor to influence the concentration of Zn in the seed, other factors (genotype and size of seed sown) were shown to affect seed yield (Fig. 6.6).

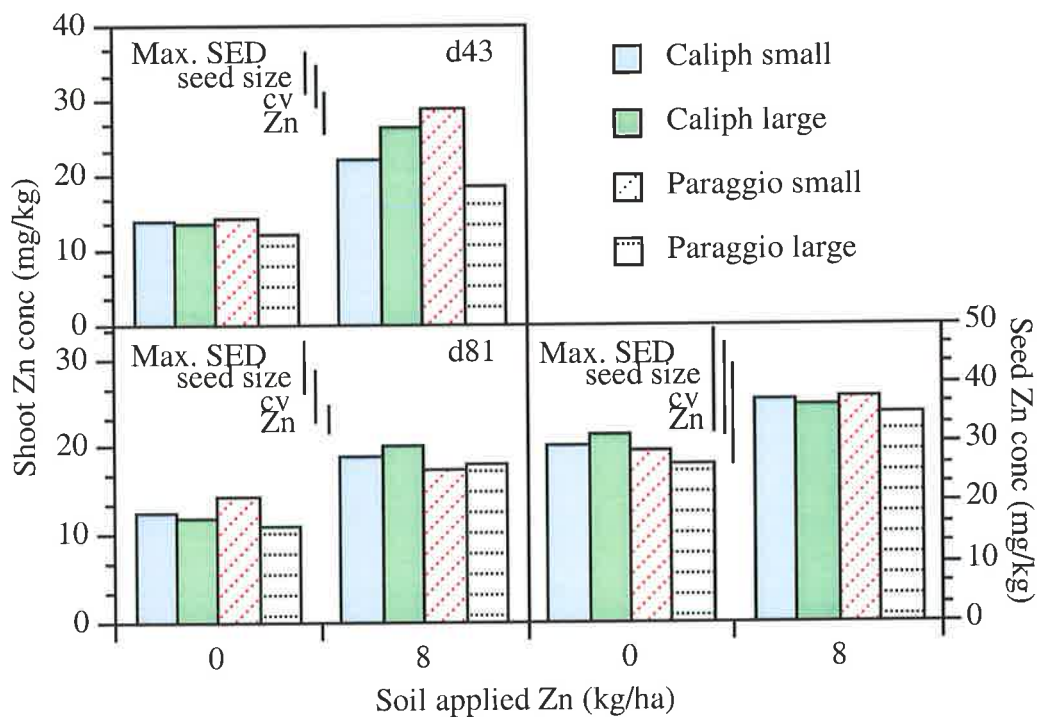


Figure 6.7 Effect of Zn application on the concentration of Zn in the shoot and seed for small and large size seeds of Caliph and Paraggio, grown at Lameroo, 1996. Vertical bars represent maximum standard error of the difference between means for the main effects. Significant interactions and main effects ($p \leq 0.05$) between seed size, genotype and Zn at the respective harvest include: *d43* seed size x cv x Zn; *d81* Zn; *seed* Zn.

In the seed, the concentration of nutrients (excluding Mg) was similar for both genotypes derived from small and large seed (Table 6.4). Magnesium was the only nutrient measured that was present in higher seed concentration for Caliph plants originally derived from large seed, while Mg concentration was lower in seed from Paraggio plants derived from large seed.

Table 6.4 Concentration of nutrients (mg/kg) in the seed of 2 genotypes (*M. truncatula* cv. Caliph and Paraggio) derived from small and large seed grown at Lameroo, 1996. SD represents standard deviation of the seed size effect for Caliph and Paraggio.

Genotype	Seed	Fe	Mn	B	Cu	Zn	Na	Ca	Mg	K	P	S
Caliph	large	91	13	14	11	34	62	1377	2198	11478	7794	2662
	SD	5	1	0	2	4	61	144	16	467	447	91
	small	79	13	15	11	33	70	1093	2164	11375	7714	2534
	SD	9	1	1	2	5	233	251	17	1312	1031	305
Paraggio	large	70	14	14	10	31	54	1162	1917	11987	7077	2517
	SD	8	2	1	1	7	136	189	12	1220	705	240
	small	76	15	13	11	33	61	1178	2181	12144	7655	2695
	SD	7	2	2	1	8	113	228	28	1636	991	348

Genotypic differences occurred among the 12 genotypes in response to Zn application at day 81, with the interaction between genotype and Zn significant for shoot dry weight ($p \leq 0.0003$; Fig. 6.8). However, by day 117 the interaction was no longer significant. Results from day 81 are more likely to be relevant to grazing animals and will be discussed in more detail in section 6.3.3. Nevertheless, the main effects of genotype and Zn application were significant at day 117 ($p \leq 0.004$ and 0.043 , respectively; Fig. 6.8). Differences between genotypes in shoot dry weight were more pronounced 81 days than 117 days after sowing, with a 3-fold difference in weight between genotypes at day 81 compared with a 2-fold difference at day 117 when Zn was not applied. The magnitude of response to Zn application varied between the two harvest days with Sava, Paraponto, Caliph (small seed), Santiago, Tornafeld, Paraggio (small seed) and Herald demonstrating a greater difference between Zn treatments at day 117 compared with

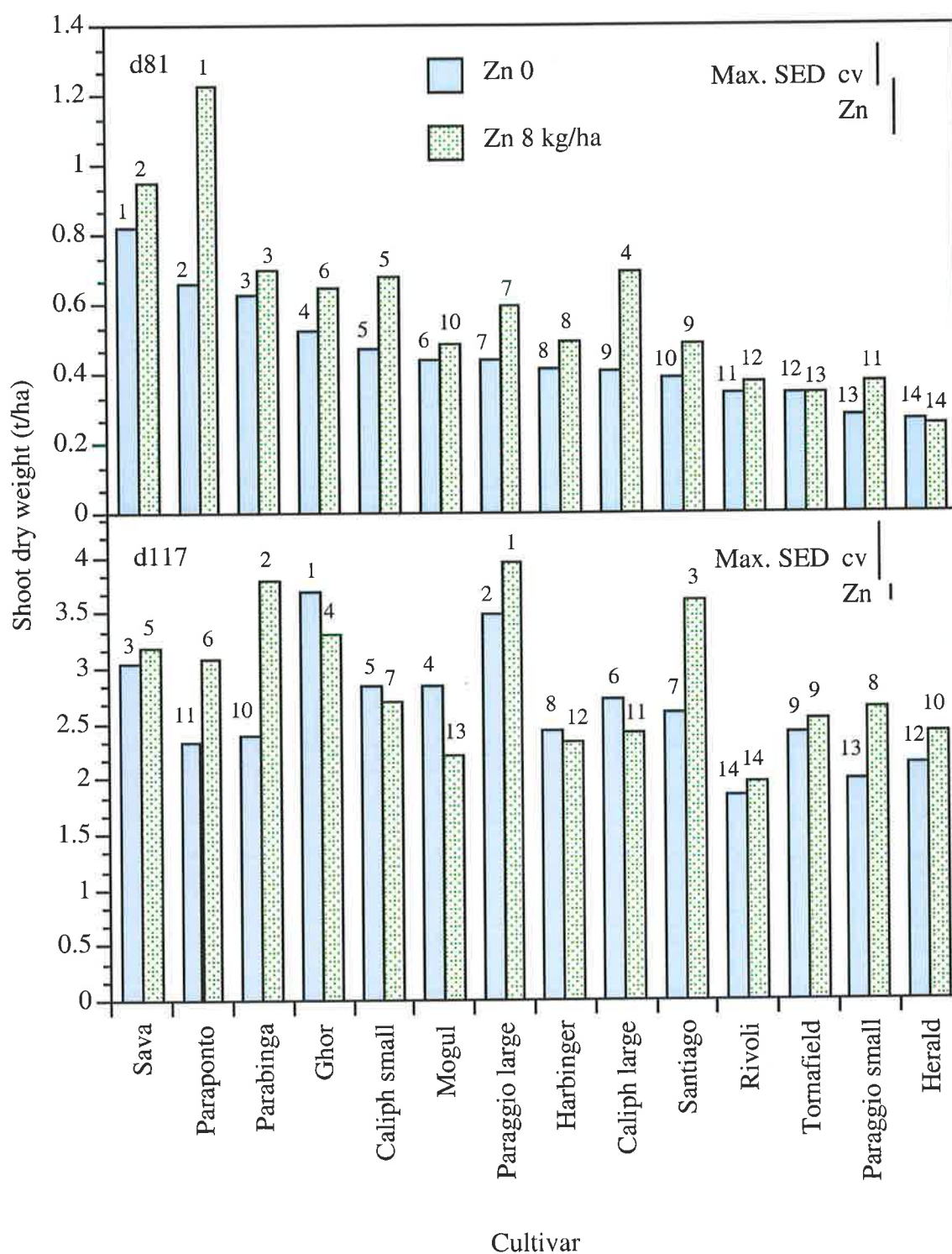


Figure 6.8 Effect of Zn application on the shoot dry weight of 12 medic genotypes (two genotypes, Paraggio and Caliph, sown with small and large seeds) 81 days after sowing at Lameroo in 1996. Numbers above columns represent a ranking for genotypes within each Zn treatment. SED represents the maximum standard error for the genotype and Zn application effect.

day 81 (Fig. 6.8). All other genotypes had smaller differences in shoot dry weight between Zn treatments at day 117 than on day 81. Genotypes that did not exhibit a change in shoot dry weight with Zn application include Rivoli and Harbinger at both harvest days, and Sava, Parabinga, Ghor, Paraggio (large and small seeded), Santiago, Tornafeld and Herald at day 81. A significant increase in dry weight with Zn application was observed in Paraponto, Caliph (large and small seeded) and Mogul at day 81, while Sava, Paraponto, Parabinga, Paraggio (large and small seeded), Santiago, Tornafeld, and Herald increased at day 117. A decrease in weight with Zn application occurred at day 117 in Ghor, Caliph (large and small seeded), and Mogul.

Although the interaction between genotype and Zn application had a significant effect on seed yield ($p \leq 0.012$; Fig. 6.9), most genotypes exhibited only a slight increase in seed yield with Zn application. Parabinga was the only genotype to have a significantly higher seed production with Zn application, while Caliph (small seeded) had a significantly lower seed production.

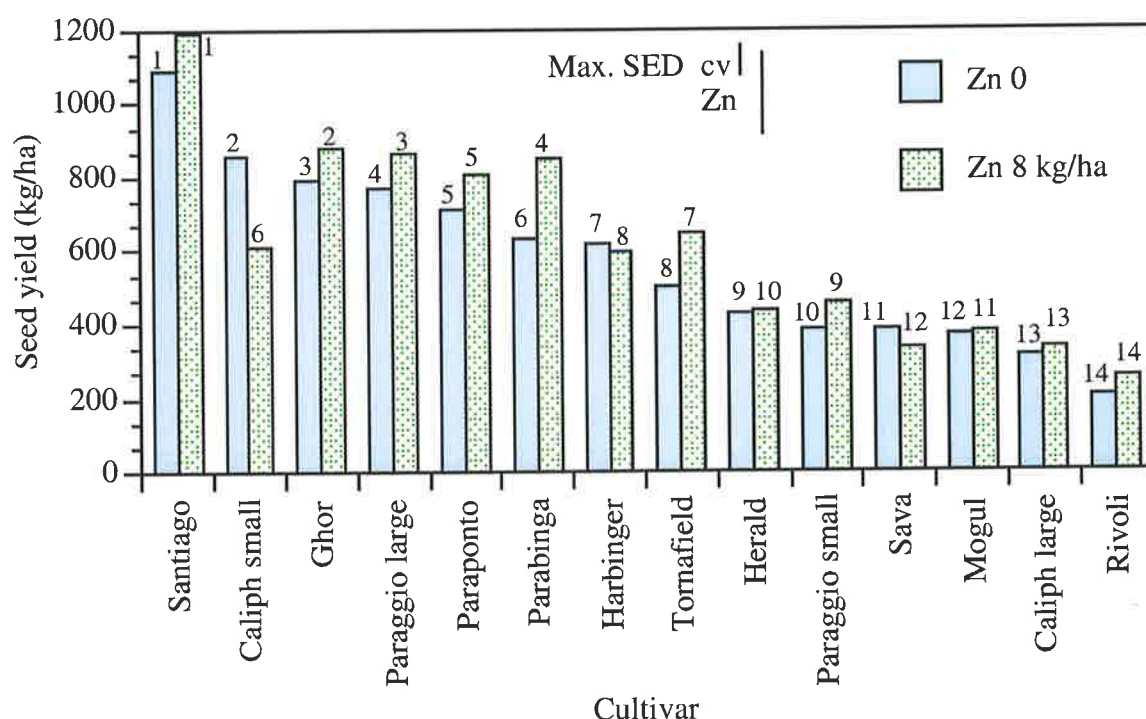


Figure 6.9 Effect of Zn application on seed yield of 12 medic genotypes (two genotypes, Paraggio and Caliph, sown with small and large seeds) grown at Lameroo in 1996. Numbers above columns represent a ranking for genotypes within each Zn treatment, SED represents the maximum standard error for the genotype and Zn application effect.

The concentrations of Zn in the shoot at day 81 and in the seed were significantly greater in all genotypes when Zn fertiliser was applied ($p \leq 0.0001$ in both cases; Fig. 6.10). The interaction between Zn application and genotype did not have a significant effect on the concentration of Zn in the shoot or in the seed. A greater difference in Zn concentration occurred in the shoot compared with seed Zn concentration between Zn treatments: Ghor had a 1.9-fold increase in shoot Zn concentration when Zn was applied compared with a 1.1-fold increase in Tornafeld. The increase in the seed Zn concentration with the addition of Zn ranged from 1.1 for Mogul to 1.4 for Santiago. Genotype had a significant effect on the concentration of Zn in the seed, but not in the shoot. The highest concentration of Zn in the seed occurred in Rivoli at both levels of Zn, while small-seeded Paraggio had the lowest concentration when no Zn fertiliser was applied and Parabinga had the lowest when Zn fertiliser was applied.

The amount of Zn present in the seed (product of Zn concentration and seed weight) was affected by the genotype and by the addition of Zn, with the interaction between genotype and Zn application significant ($p \leq 0.0001$; Fig. 6.11). Sava and Paraponto derived from the largest seed (Table 6.2) had the highest Zn content per seed, while Herald, derived from the smallest seed (Table 6.2), had the lowest amount of Zn in seed produced. All genotypes had a higher amount of Zn present in the seed when supplied with Zn fertiliser (Fig. 6.11).

During the growing season, levels of Fe, Ca, Mo, Mg, Na and K were within the range for normal growth. A significant interaction between genotype and Zn application with Mn concentration in the shoot occurred ($p \leq 0.01$; Table 6.5), with Mn levels sufficient for Herald at both Zn treatments and sufficient for Mogul with Zn application. All other genotypes were marginally deficient in Mn at both levels of Zn fertiliser. Boron levels were significantly affected by genotype and were in the range of normal growth only for Herald and Sava. All other genotypes had shoot B concentrations approaching toxicity, with the highest level of 72 mg/kg in Paraponto. A significant interaction occurred between genotype and Zn application with Cu levels in the tissue at 81 days ($p \leq 0.0001$), with Herald, Mogul, Parabinga, Paraggio

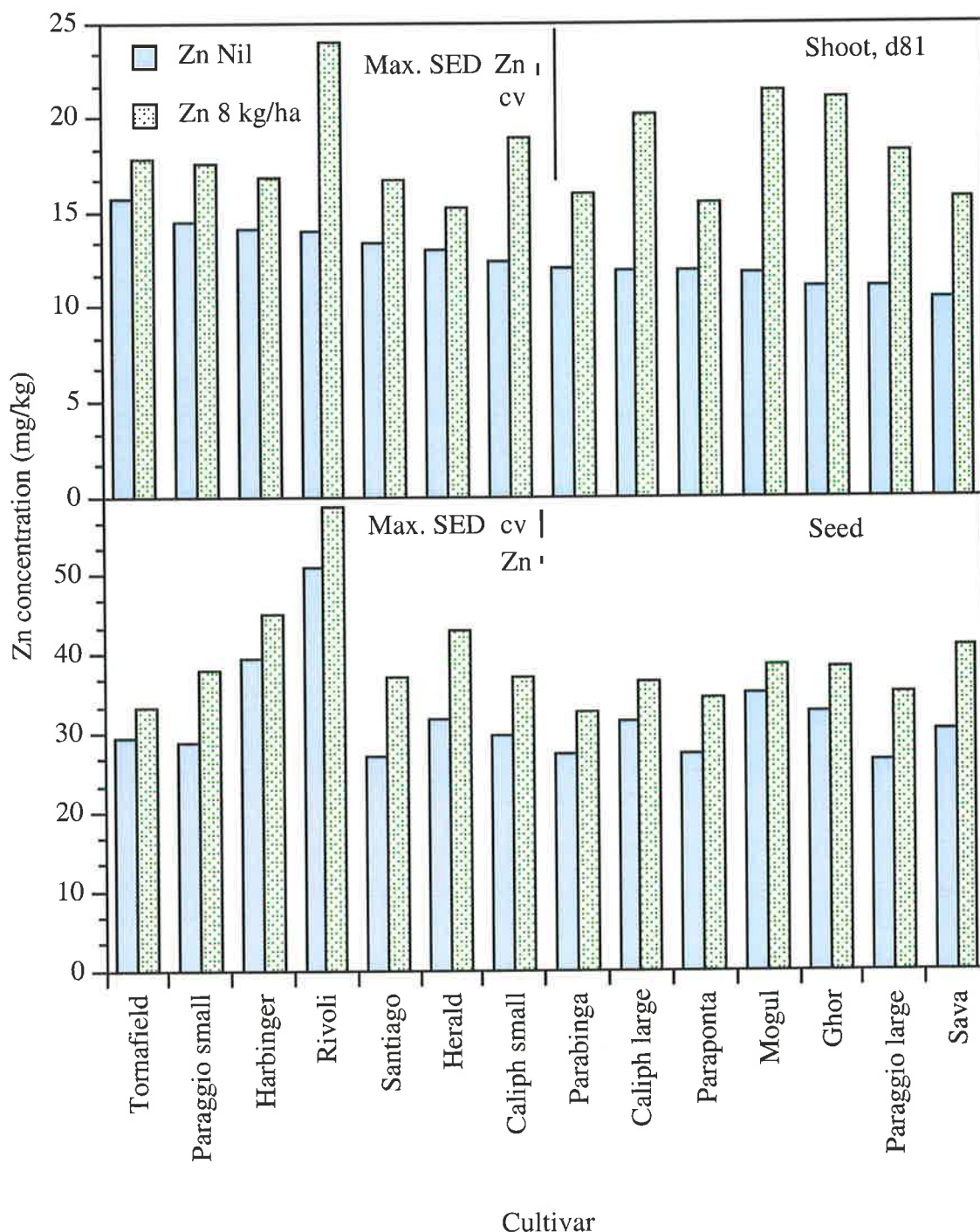


Figure 6.10 Effect of Zn application on concentration of Zn in the shoot at day 81 and in seed of 12 medic genotypes (two genotypes, Paraggio and Caliph, sown with small and large seeds) grown at Lameroo in 1996. SED represents the maximum standard error for the genotype and Zn application effect.

(derived from large and small seed), and Sava marginally Cu deficient at both Zn treatments. Ghor, Paraponto and Santiago were marginally Cu-deficient when Zn was applied, while Caliph (derived from large and small seed) was marginally Cu-deficient when no Zn was

applied. In all other cases, the concentration of Cu was sufficient (Table 6.5). Although the interaction between Zn and genotype was not significant for P concentration, both main effects were significant ($p \leq 0.0003$ in both cases). Shoot concentration of P was slightly higher than required in Caliph (large and small seed), Harbinger, Mogul and Santiago, with the highest P concentration in Mogul at 5,318 mg/kg. This level is not considered toxic. With the addition of Zn fertiliser, the mean concentration of P decreased significantly. Sulphur was the only other nutrient not in the range for normal growth, with Herald, Parabinga, Paraggio (small and large seed), Paraponto, Rivoli, Santiago and Sava below 2,500 mg/kg. The lowest concentration of S occurred in Paraponto at 2,110 mg/kg. Although this level is below the requirements for normal shoot growth, it is not considered deficient (Weir and Cresswell, 1994).

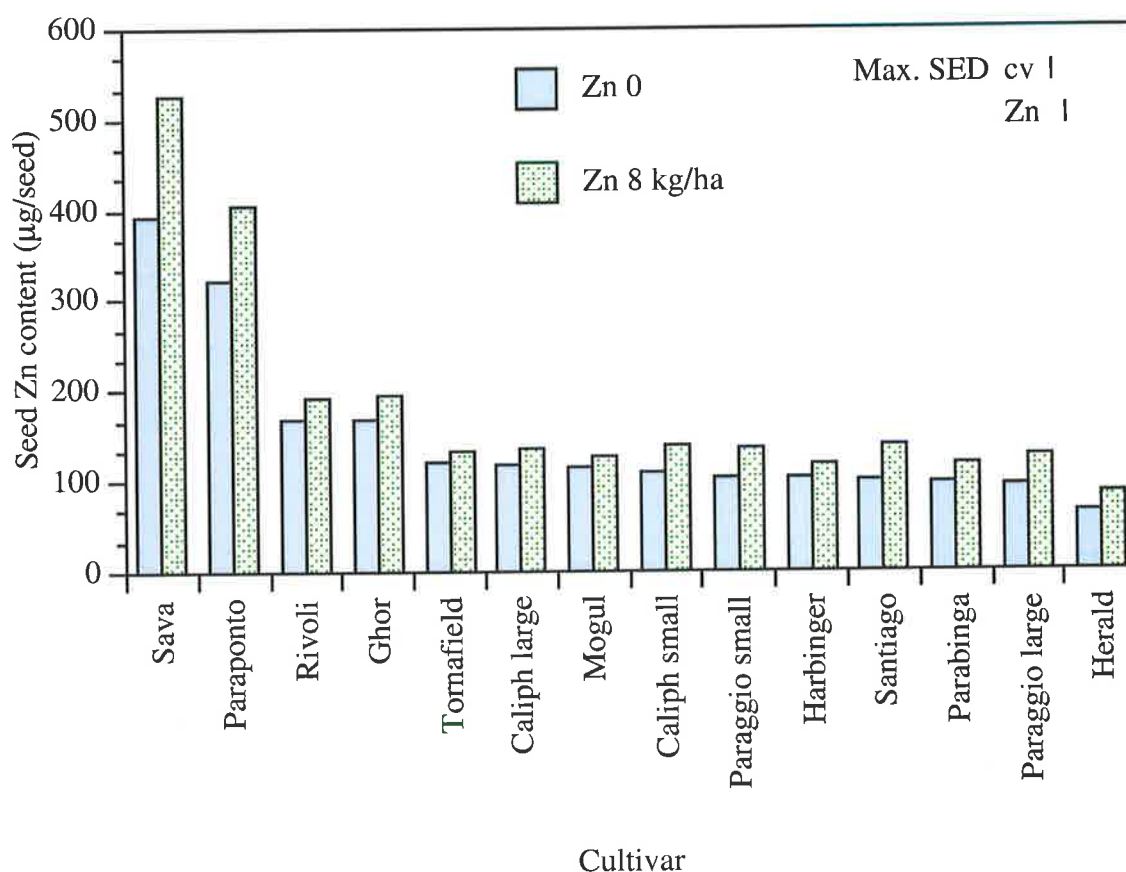


Figure 6.11 Effect of Zn application on content of Zn in the seed of 12 medic genotypes (two genotypes, Paraggio and Caliph, sown with small and large seeds) grown at Lameroo in 1996. SED represents the maximum standard error for the genotype and Zn application effect.

Table 6.5 Effect of Zn application (0 and 8 kg/ha) on the concentration of Mn and Cu (mg/kg) in the shoots of 12 genotypes (Paraggio and Caliph, sown with small and large seeds) grown at Lameroo in 1996. Maximum standard error difference (SED) between the means for genotype effect is 2.6 for Mn and 0.7 for Cu and for Zn effect SED is 4.2 for Mn and 0.9 for Cu.

Genotype	Mn		Cu	
	0	8	0	8
	mg/kg dry weight			
Caliph small seed	23	26	5.2	6.1
Caliph large seed	24	28	6.6	4.7
Parabinga	25	22	4.9	5.7
Paraggio small seed	24	25	5.6	5.4
Paraggio large seed	23	25	4.8	4.8
Mogul	32	36	5.4	5.8
Ghor	25	25	5.2	6.1
Harbinger	29	27	6.5	6.2
Herald	42	35	5.6	4.6
Tornafeld	30	27	9.8	6.7
Rivoli	21	21	7.1	8.5
Santiago	29	27	6.2	5.2
Paraponto	32	29	6.1	5.8
Sava	23	24	5.4	5.7

Zinc efficiency was calculated from the ratio of shoot dry matter produced at day 81 at nil Zn compared with plus Zn (Fig. 6.12). Herald, Tornafeld, Rivoli, Mogul and Parabinga were ranked as Zn-efficient, while Paraggio and Caliph (large and small seed) and Paraponto were ranked as Zn-inefficient. All other genotypes were ranked as moderately Zn-efficient. Since the interaction between genotype and Zn did not have a significant effect on the shoot dry weight at day 117, Zn efficiency was not calculated at this harvest.

When the original weight of seed sown was compared with the weight of the shoot derived from that seed, a significant, positive relationship occurred (Fig. 6.13). Plants derived from larger seeds (for example, Sava and Paraponto) had a higher shoot dry weight at day 81 compared with plants derived from smaller seeds (for example, Herald and Harbinger). This

occurred at both Zn treatments and is supported further by the comparison of shoot dry weight at day 81 for Paraggio derived from large and small seed (Fig. 6.6). Although the growth of plants increased significantly with an increase in weight of the seed they were derived from, the Zn efficiency ranking was not influenced by original seed weight (Fig. 6.13). This is again highlighted by comparing Paraggio (and Caliph) derived from small and large seed which have similar Zn efficiency rankings (Fig. 6.12).

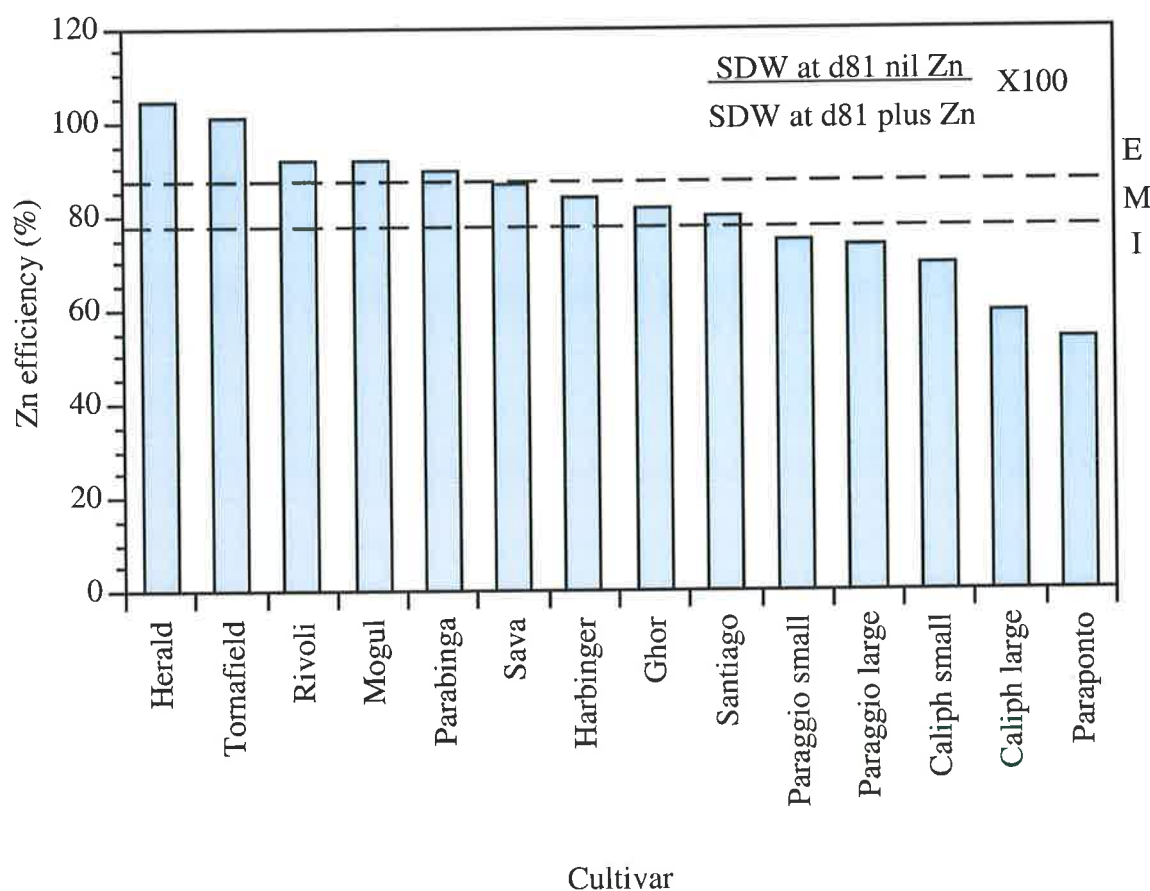


Figure 6.12 The Zn efficiency ranking (E=efficient, M=medium, I=inefficient) of 12 medic genotypes (two genotypes, Paraggio and Caliph, sown with small and large seeds) grown at Lameroo in 1996. The ranking was based on ratio of shoot dry matter production at nil Zn and Zn application (8 kg/ha) 81 days after sowing. The mid-point of the medium efficiency interval corresponds to the median value of the parameter given on the y-axis; the upper and lower boundaries of the medium-efficiency interval were constructed by adding to or subtracting from the median point the value of the standard error of the genotype effect.

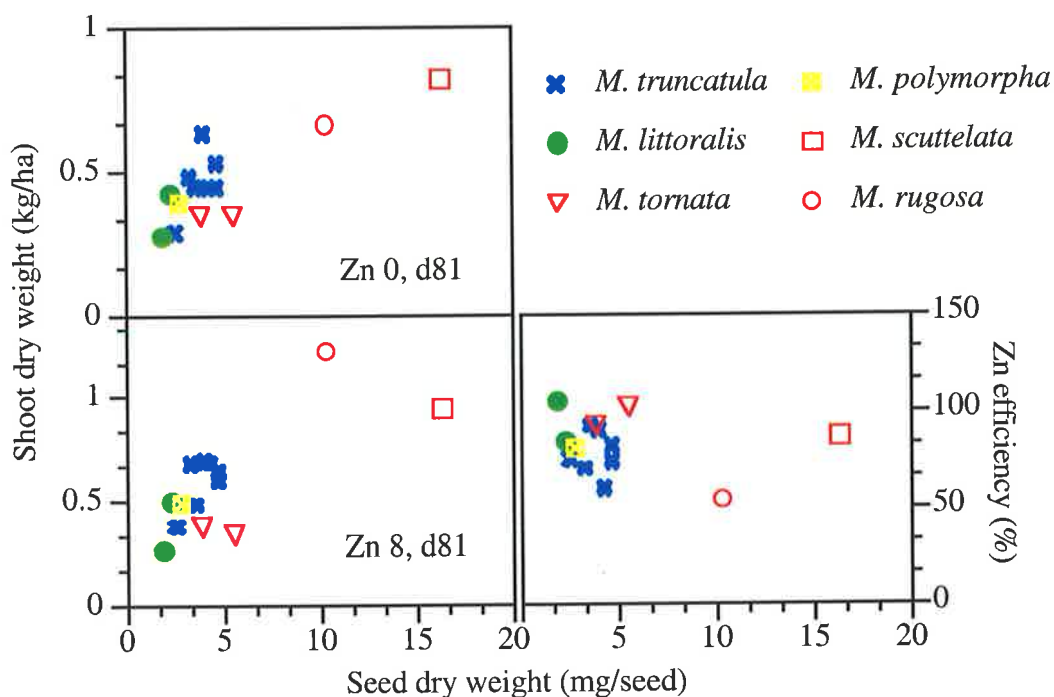


Figure 6.13 Effect of the size of seed sown on the shoot dry weight and Zn efficiency of 12 medic genotypes grown at Lameroo with 0 and 8 kg/ha of Zn in 1996, 81 days after sowing.

6.3.3 Discussion

For the first time, genotypic variation in response to Zn application has been demonstrated in medics in the field. This occurred despite the concentration of Zn in the shoot of all genotypes (excluding Rivoli) being below the requirement for optimal growth. Rivoli was the only genotype to have a concentration of Zn in the shoot in the range for normal growth when Zn fertiliser was applied. Rivoli also had the highest concentration of Zn in the seed at both Zn treatments, which is most probably a product of low seed yield potential. Other genotypes appear to have maintained a higher seed yield at the expense of seed Zn concentration.

Rankings of Zn efficiency were determined from shoot dry weight at 81 days after sowing only, because the interaction between genotype and Zn application was non-significant at 117 days. This, however, was not the main reason for calculating Zn efficiency rankings at day 81 only. Firstly, after day 81 soil moisture levels became limiting with below average rainfall. As

soil moisture is diminished, movement of Zn to the roots is restricted exposing plants to Zn deficiency along with moisture stress. This will then prevent plants from reaching their yield potential and result in a higher Zn efficiency ranking. Secondly, by day 117, difference in growth rates between genotypes occurred, with half the genotypes exhibiting a reduced response to Zn. This suggests that vegetative growth may have been reduced in one or both Zn treatments for some genotypes and energy expenditure would have preferentially gone towards seed production instead of vegetative growth. This is not surprising since flowering times vary considerably among the species sown. For example, Boyce (1995) observed Ghor to flower 77 days after sowing at Parafield in South Australia, while Rivoli flowered after 123 days in the same year. A comparison of Zn efficiency rankings between genotypes at different stages of growth is therefore best done before the complications of the timing of onset of reproductive growth become serious.

The time of flowering will also have an impact on seed yield and seed nutrient composition, particularly under Zn-deficient conditions and a dry season finish. Plants that flower early are less likely to be exposed to dry conditions at the end of the growing season. Therefore, plants that flower later in the growing season have a higher probability of encountering dry soil conditions which will affect Zn nutrition to a greater extent than plants with earlier flowering times.

In comparing the effect of size of seed sown on shoot dry weight and seed yield, it might be expected that the plant derived from the larger seed would have a higher weight and seed yield compared with the plant from the smaller seed. This was partially confirmed in seedlings of *Desmodium paniculatum* by Wulff (1986a) who demonstrated plants derived from larger seed have a longer root system, a larger leaf area and a greater overall seedling dry weight. In the present experiment, Paraggio supported this observation as plants derived from the larger seed consistently produced a higher shoot weight and seed yield than the plants derived from smaller seed at both Zn treatments (Fig. 6.6). However, this did not occur with Caliph. Caliph derived from small seed had a higher shoot weight and seed yield than Caliph derived from

larger seed. The type of response shown by Caliph was also observed in a follow-up study by Wulff (1986b). Wulff observed *D. paniculatum* grown individually from small seed had a higher seed production than those derived from large seeds. Wulff (1986b) suggested that the higher seed production attained by plants originating from smaller seeds was due to a reduction in the size of the seeds produced. Caliph plant derived from small seed may therefore be producing a larger quantity of small seeds than Caliph derived from large seed.

Although differences in seed size (which represent differences in Zn content) did not affect the Zn efficiency of genotypes, it is still preferable for genotypes screened for Zn efficiency to have similar Zn contents in seed. A higher seed Zn content has been shown to minimise differences in Zn efficiency of wheat genotypes in pots (Rengel and Graham, 1995c) when the difference in seed size sown exceeded 65% for both Zn-efficient and Zn-inefficient genotypes. Differences in seed size may not have altered the Zn efficiency ranking of Paraggio and Caliph in the present experiment as the difference between large and small seed size was only 45 and 23%, respectively. If the difference in seed size of medics sown was closer to 65%, differences in Zn efficiency might have occurred.

6.4 Conclusion

Current levels and frequency of Zn application are insufficient to overcome Zn deficiency in medics in low rainfall areas. Although 10 kg Zn/ha does have a residual effect beneficial to medics 3 years after application, the level or frequency, of Zn applied needs to be increased in order to overcome Zn deficiency completely. The addition of 8 kg Zn/ha with the seed, in granulated form, also benefits medics, resulting in significant differences between Zn treatments in the first year of application on a Zn-deficient site. However, fertiliser levels were still too low to increase Zn concentration in the shoots above the critical level for most medic genotypes. An increase in the amount of Zn applied to the soil along with a foliar spray of Zn during the growing season may overcome the effect of Zn deficiency in medics.

Genotypic differences have also been demonstrated in medics and should be more pronounced when higher levels of Zn are applied. From this experiment, Rivoli may be considered the most Zn-efficient. Not only was it ranked as Zn-efficient but it was also able to maintain a high concentration of Zn in the shoot and in the seed when deficient in Zn. Although it was the lowest yielding, Herald is also considered to be Zn-efficient as growth was not reduced when Zn was not applied to the soil. Sava and Paraponto, on the other hand, were the highest yielding but were not ranked as Zn-efficient by the definition used, as yield under Zn-deficient conditions was significantly lower than their yield potential.

More field trials were required to determine which genotypes are Zn-efficient with levels of Zn in the +Zn treatment in the range for normal growth for all genotypes screened. Since field trials are time consuming, a pot experiment was performed next to screen for Zn-efficient genotypes. This also allowed a larger number of genotypes to be screened at once.

Genotypic Differences in Growth of *Medicago* species in Soil of Low Zn status

7.1 Introduction

Results from Chapter 6 demonstrated differences between *Medicago* species in response to Zn fertilisation. As mentioned previously, studies on genotypic variation in expression of the Zn efficiency trait have been performed mainly on cereal crops (see Section 2.1.4.c for review). To date, the only work on the response of different *Medicago* spp. to Zn application has been in a controlled environment on four cold-tolerant varieties selected for the West Asia region where frost is common (Materon and Ryan, 1995). Calculating Zn efficiency from shoot dry matter production recorded by Materon and Ryan (1995) showed *M. noeana* (SA15485) to be the most Zn-efficient, with a Zn efficiency rating of 95%, compared with *M. rigidula* (SA716) the most Zn-inefficient, with a Zn efficiency rating of 53%. For this calculation, Zn efficiency was defined as the yield under Zn limiting conditions as a percentage of that in non-limiting conditions (Graham *et al.*, 1992).

In the experiment described here, four determinants of efficiency are compared, all involving a greater uptake efficiency, with several objectives in mind (see Chapter 2 for review on Zn efficiency). These include relative yield between Zn limiting and non-limiting conditions, shoot dry weight under Zn deficiency, Zn accumulation per gram of root under Zn deficiency, and shoot/root ratio when Zn is limiting compared with that under non-limiting conditions. The four determinants of efficiency are discussed below.

As previously mentioned, Zn efficiency is most commonly calculated as the relative yield under Zn-limiting compared to non-limiting conditions, often expressed as a percentage (Graham *et al.*, 1992). It identifies material that approaches its yield potential without the need for Zn

supplements and is an important tool for plant breeders as it identifies the parental material to donate Zn efficiency in a breeding program.

The second criterion to determine Zn efficiency involves ranking yield under Zn-limiting conditions. This is suggested to be a good efficiency indicator, reflecting "the natural course of evolution" (Rengel and Graham, 1995a) and is of interest to the farmer who desires a high yielding variety in soils low in Zn. This method reflects actual genotypic differences under Zn-deficient conditions, but does not take into account variation in yield potential under non-limiting conditions. Since this technique ignores the fact that genotypes may differ in yield potential, the genotype defined as Zn-efficient by this technique may not be the highest yielding when Zn fertiliser is applied to the soil. It is a combination of these first two determinants that provides much of the information needed in determining Zn-efficient lines of interest to both the plant breeder and the farmer.

Whereas the first two techniques determine which genotypes are Zn-efficient in terms of yield, the third and fourth techniques may indicate specific mechanisms involved in Zn efficiency. The third index used is commonly referred to as Zn-uptake efficiency, or Zn accumulation efficiency, and is calculated from total Zn accumulated by the plant divided by root dry mass. This technique selects genotypes that are able to take up a higher amount of Zn per unit root and is of some use to the plant breeder. A genotype classified as Zn-efficient by the first three indices may be able to maintain growth when available soil Zn is low by increasing the amount of Zn extracted from the soil. An additional strategy for a Zn-efficient genotype is where a genotype has a lower internal Zn requirement, compared with a standard genotype when the soil is low in Zn.

The final criterion, based on changes in the shoot/root ratio from non-limiting to limiting conditions, has recently been used by Rengel and Graham (1995a). Shoot/root ratio may be characteristic of a genotype and those maintaining a high, yet stable, shoot/root ratio as rate of applied Zn decreases, may be classified as Zn-efficient. Genotypes with a high shoot/root ratio

may also be considered as Zn-efficient. For example, an increase in the shoot/root ratio was observed in Zn-efficient wheats, while Zn-inefficient wheats maintained a higher root growth at the expense of shoot growth (Rengel and Graham, 1995a). Shoot/root ratio may therefore be a sensitive parameter for the evaluation of genotypes for their Zn efficiency ranking. Cakmak *et al.* (1996a) demonstrated impaired shoot growth of Zn-deficient wheats to be more distinct in plants exposed to high light intensity, and suggested a decrease in the shoot/root ratio with Zn deficiency, especially in Zn-inefficient wheat genotypes, may be a reflection of Zn deficiency induced photo-oxidative damage in shoots leading to lower shoot growth. Genotypes classified as Zn-efficient by the first two criterion and the fourth criterion may therefore be able to maintain growth in soil of low Zn status, thus sustaining a stable shoot/root ratio.

It is the aim of this chapter to determine Zn-efficiency rankings of commonly grown medic genotypes from a range of species under controlled conditions. The first two Zn efficiency indices will be used to identify Zn-efficient genotypes, while the third and fourth indices will be used to demonstrate possible mechanisms involved in Zn efficiency. Zinc efficiency in medics will be discussed, and recommendations made regarding which genotypes have the potential for use in future breeding programs.

7.2 Aim

The aim of this experiment was to compare, in controlled conditions, the Zn response of *M. noeana*, a Zn-efficient genotype (Materon and Ryan, 1995), with eight genotypes grown in the field trial in Chapter 6 as well as 10 breeding lines (unregistered genotypes) currently being developed by the Medic Breeding Unit at SARDI.

7.3 Materials and Methods

7.3.1 Experimental Design

A factorial design combining 19 genotypes/breeding lines from eight species (*M. truncatula*, *M. tornata*, *M. littoralis*, *M. noeana*, *M. polymorpha*, *M. tornata* x *M. littoralis*, *M. scutellata* and *M. rugosa*, see Table 7.1) and four Zn levels (0, 0.1, 0.3 and 0.9 mg Zn/kg) in three replicates.

7.3.2 Soil

Mt Compass sand leached of soluble salts in RO water, air dried and heat treated at 80°C as outlined in Chapter 3 was used in this experiment.

7.3.3 Basal Nutrients

A basal nutrient solution was applied to the soil as outlined in Chapter 3.

7.3.4 Seed

Seed sown in the field experiment of Chapter 6 was also used in this experiment to reduce variation between the field and pot experiment and allow a direct comparison of Zn efficiency rankings. Seed characteristics are listed in Table 7.1. Seed was surface-sterilised and germinated as outlined in Chapter 3.

7.3.5 Procedure

Soil mixing and potting were performed as outlined in Chapter 3, with four germinated seeds sown per pot of 1 kg soil. After planting, the soil surface was covered in acid-washed, black plastic beads, and the soil mass watered to 12% water content. Pots were then placed in a controlled environment, and thinned to two plants per pot one week later. After thinning, plants were inoculated with the appropriate strain of *Rhizobium meliloti* (see Table 7.1) and watered every second day.

After 42 days, plants were harvested, shoot and root dry weight determined and the shoots and roots analysed separately for Zn concentration by ICP analysis. Zinc efficiency rankings were calculated using the four indices: 1) yield difference in growth with and without Zn application; 2) yield under Zn limiting conditions; 3) Zn accumulation per unit root weight at low available soil Zn; and 4) shoot/root ratio with and without Zn application. Homogeneity of variance was tested for and data found to have a normal distribution. For the first three efficiency rankings, three Zn efficiency classes were constructed using the technique of Rengel and Graham (1995a) where genotypes were ranked as either Zn-efficient, moderately Zn-efficient or Zn-inefficient. The Zn-efficiency classes for the first three techniques were produced by finding the median value of the trait under consideration and creating the medium-efficiency interval as median $\pm$ SE of the genotype effect. Genotypes with data falling above or below this middle interval were classed as Zn-efficient or Zn-inefficient, respectively. For the fourth technique the efficiency ranking was created from shoot/root ratio of 100% - SE of the genotype effect, with genotypes falling above the line considered to be Zn-efficient. Genotypes with data falling below the efficiency interval were classed as Zn-inefficient.

Table 7.1 Seed characteristic of 19 medic genotypes, sown in experiment 7.2 including sub-species where relevant. All plants were inoculated with two strains of *Rhizobium meliloti*, CC169G and the other specific for the species.

Species	Genotype	Seed size (mg)	Seed Zn content (µg/seed)	<i>Rhizobium meliloti</i> strain
<i>M. truncatula</i>				
subsp. truncatula	Caliph	3.9	0.18	WSM688
subsp. truncatula	SA12591	3.7	0.22	WSM688
subsp. truncatula	SA18289	5.5	0.29	WSM688
subsp. truncatula	Z942	3.3	0.16	WSM688
<i>M. tornata</i>				
subsp. spinulosa	Rivoli	3.9	0.04	WSM826
subsp. rugulosa	Tornafeld	5.6	0.23	WSM826
subsp. aculeata	SA20278	8.2	0.36	WSM826
subsp. aculeata	SA20248	6.2	0.28	WSM826
subsp. aculeata	SA20521	6.3	0.29	WSM826
<i>M. littoralis</i>				
subsp. littoralis	Harbinger	2.3	0.20	WSM826
subsp. littoralis	Herald	1.9	0.05	WSM826
<i>M. noeana</i>				
	SA15485	3.4	0.30	WSM688
<i>M. polymorpha</i>				
subsp. brevispina	Santiago	2.8	0.22	WSM688
subsp. brevispina	SA4188	3.9	0.20	WSM688
<i>M. littoralis</i> x <i>M. tornata</i>				
	Z1064	1.9	0.11	WSM826
	Z1065	2.1	0.11	MSUR529
	Z1068	1.5	0.09	MSUR529
<i>M. scutellata</i>				
	Sava	16.4	0.92	WSM688
<i>M. rugosa</i>				
	Paraponto	10.3	0.69	WSM688

7.4 Results

Visual Observations

By 28 days, symptoms of Zn deficiency were observed in plants supplied with no Zn. Plants supplied with Zn at 0.1 mg/kg soil exhibited poor growth but no visible symptoms were observed on any of the leaves. No visual difference between Zn treatments 0.3 and 0.9 mg Zn/kg occurred by 42 days, but differences in growth between genotypes were observed from time of emergence across Zn treatments.

Plant Growth Measurements

Genotypes demonstrated large differences in response to Zn application, with a Zn by cultivar interaction significant for both shoot and root dry weight ($p \leq 0.0001$ and 0.0009 , respectively; Fig. 7.1). An increase in both shoot and root growth occurred with increasing levels of applied Zn, but there was no difference among genotypes in yield obtained at 0.3 and 0.9 mg Zn/kg. When sufficient Zn was applied, Sava had the highest (0.97 g/plant), and Herald the lowest yield (0.21 g/plant). When Zn was moderately deficient (0.1 mg Zn/kg), yield ranged from 0.55 g/plant for SA20521 to 0.17 g/plant for Z1064. With severe Zn deficiency (0 mg Zn/kg) Herald had the lowest yield (0.06 g/plant), while SA20278 had the highest (0.30 g/plant).

The interaction between Zn application and genotype treatments had a significant effect on the shoot/root ratio ($p \leq 0.001$). The shoot/root ratio increased with a decrease in applied Zn for most genotypes, the exceptions being no change between Zn deficiency and Zn adequacy for SA12591, Rivoli, Sava, SA20248, SA15485 and Tornafield (Fig. 7.2). The highest shoot/root ratio of all genotypes tested occurred in Zn-deficient Z1068, while the lowest occurred with sufficient Zn supply in SA20278.

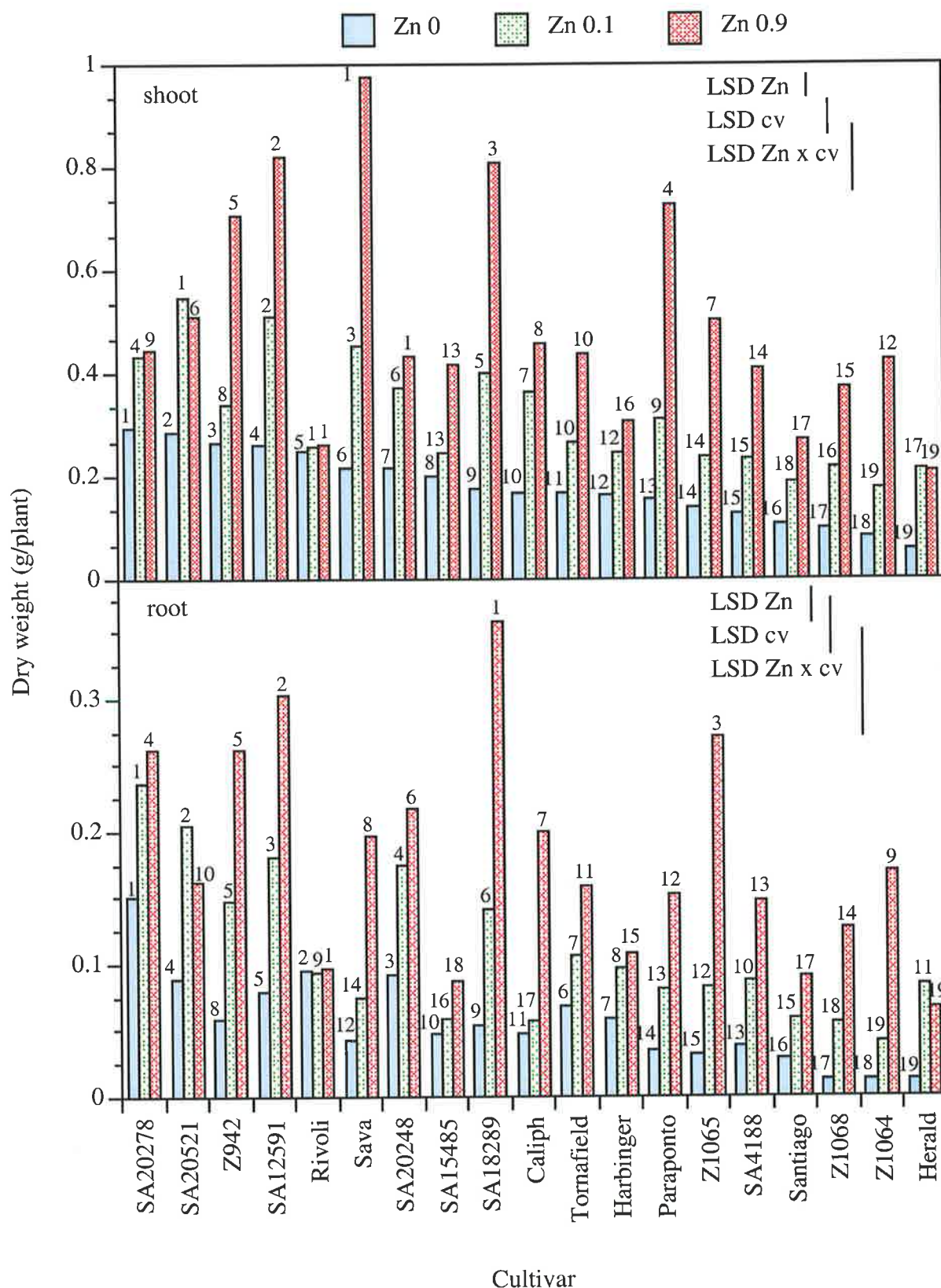


Figure 7.1 Effects of Zn treatment on the shoot and root dry weight of 19 medic genotypes. Data obtained at Zn level 0.3 mg/kg soil were not included as they were not significantly different from those observed at 0.9 mg/kg. Numbers above columns represent a ranking for genotypes within each Zn treatment. LSD represents Fishers Protected Least Significant Difference $\alpha=0.05$ for the main effects and the interaction between Zn application and genotype.

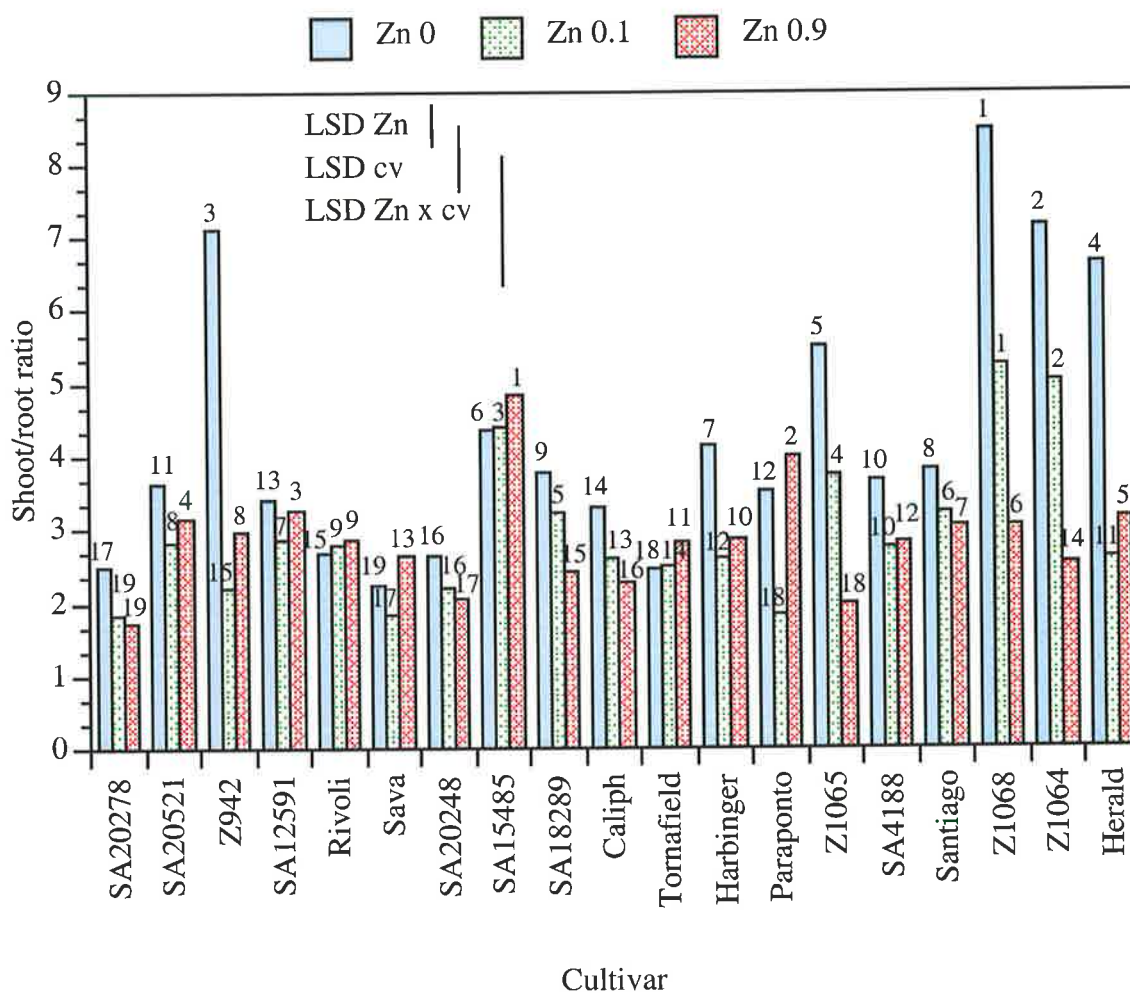


Figure 7.2 Effects of Zn treatment on the shoot/root ratio of 19 medic genotypes grown in Mt. Compass sand for 42 days. Data obtained at Zn level 0.3 mg/kg soil were not included as they were not significantly different from those observed at 0.9 mg/kg. Numbers above columns represent a ranking for genotypes within each Zn treatment. LSD represents Fisher's Protected Least Significant Difference $\alpha=0.05$ for the main effects and the interaction between Zn application and genotype.

Root Zn concentrations were greater than shoot Zn concentrations for all genotypes tested, with the Zn by genotype interaction significant for both tissues ($p \leq 0.001$ in both cases; Fig. 7.3; see Appendix 8 for mean concentrations). Within each Zn treatment the concentration of Zn in the shoot and root varied by a factor of two across genotypes. Severe Zn deficiency occurred in all plants at the 0 Zn treatment, with mean Zn concentration of 10 mg/kg in the shoot and 27 mg/kg in the root. With 0.1 mg Zn/kg in the soil, all genotypes were severely deficient in Zn,

except Rivoli and SA20248 which were only moderately deficient, with shoot Zn concentration of 19 and 20 mg/kg respectively.

The proportion of Zn present in the shoots varied between genotypes and between Zn treatments, with the interaction being significant ($p \leq 0.005$; Fig. 7.4). Under Zn deficiency, an increase in the proportion of Zn transported to the shoot occurred in SA15485 and Herald, a reduction in the proportion of Zn transported to the shoot occurred in SA20521, Z942, SA12591, Tornafeld, and SA4188, while all others had no change in the relative amount of Zn in the shoot.

Total Zn accumulation on a per gram root basis increased with increasing level of applied Zn (Fig. 7.5). The interaction between Zn application and genotype treatment had a significant effect on Zn accumulation per gram root ($p \leq 0.001$), with accumulation ranging from 39 to 107 $\mu\text{g Zn/g root}$ for Caliph and Z1068, respectively, when no Zn was applied to the soil. In the 0.1 mg/kg Zn treatment, uptake ranged from 53 to 109 $\mu\text{g/g root}$ for Z942 and Z1064, respectively, and for 0.3 mg/kg Zn uptake ranged from 52 to 135 $\mu\text{g/g root}$ for SA12591 and Rivoli. When sufficient Zn was applied (0.9 mg/kg), a greater than 3-fold difference occurred between SA18289 and Caliph with respective levels of 118 and 407 $\mu\text{g Zn/g root}$ extracted from the soil.

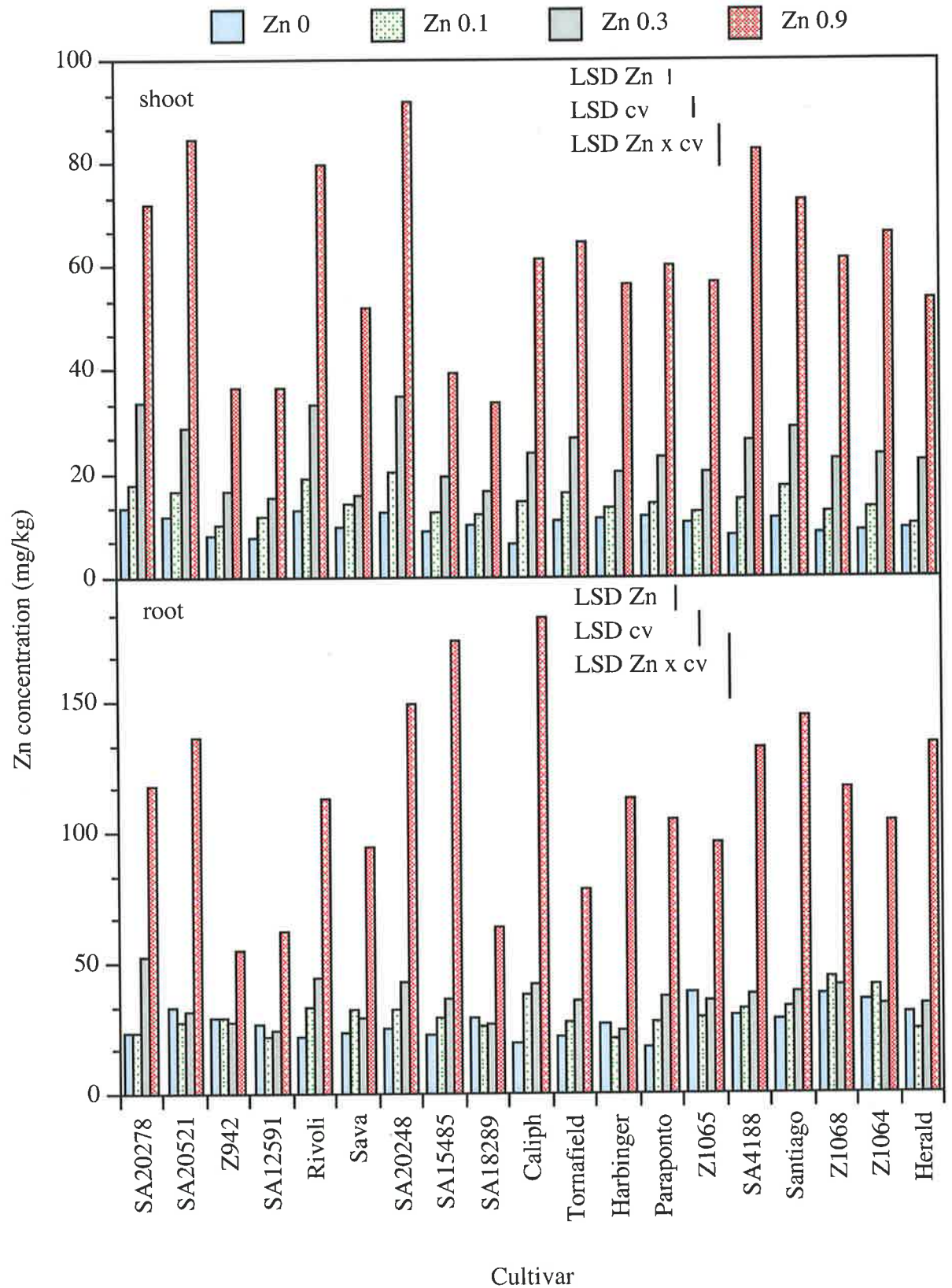


Figure 7.3 Effect of Zn application on concentration of Zn in shoots and roots of 19 medic genotypes grown in Mt. Compass sand for 42 days. LSD represents Fisher's Protected Least Significant Difference $\alpha=0.05$ for the main effects and the interaction between Zn application and genotype.

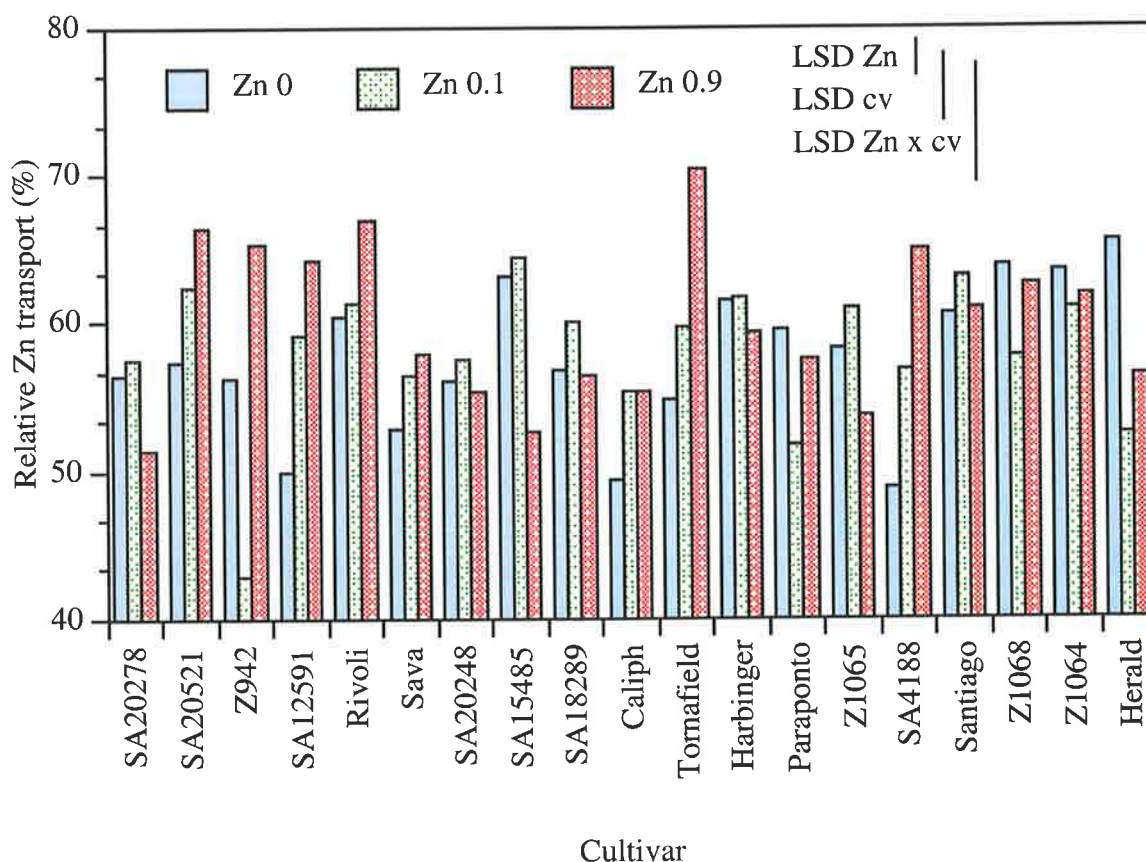


Figure 7.4 Effect of the Zn treatment on the proportion of total plant Zn transported to shoots of 19 medic genotypes grown in Mt. Compass sand for 42 days. LSD represents Fisher's Protected Least Significant Difference $\alpha=0.05$ for the main effects and the interaction between Zn application and genotype.

The level of most other nutrients in the shoots was sufficient for normal growth. Levels of Ca and P were high in all genotypes, with P extending into the toxicity range, as expected, when the plants were deficient in Zn (see Appendix 8). Calcium was high across all treatments, but the level is not considered toxic (Reuter and Robinson, 1997). Magnesium was high in Zn-deficient Caliph, SA12591, SA18289, Tornafeld, SA15485, Santiago, Z1064 and Paraponto, and in all Zn treatments for SA20248, SA20278 and SA20521. Rivoli was high in S across all Zn treatments, whereas Caliph and SA18289 were high in S only at low levels of applied Zn. Boron was marginally deficient in SA12591, SA18289, Z942 and Sava when sufficient Zn was applied (0.3 and 0.9 mg Zn/kg). As Zn supply increased, the concentrations of Fe, B, Cu, Mg, P in the shoot decreased and Mn increased, while K and S were lower with Zn deficiency in some genotypes but higher in other genotypes (see Appendix 8).

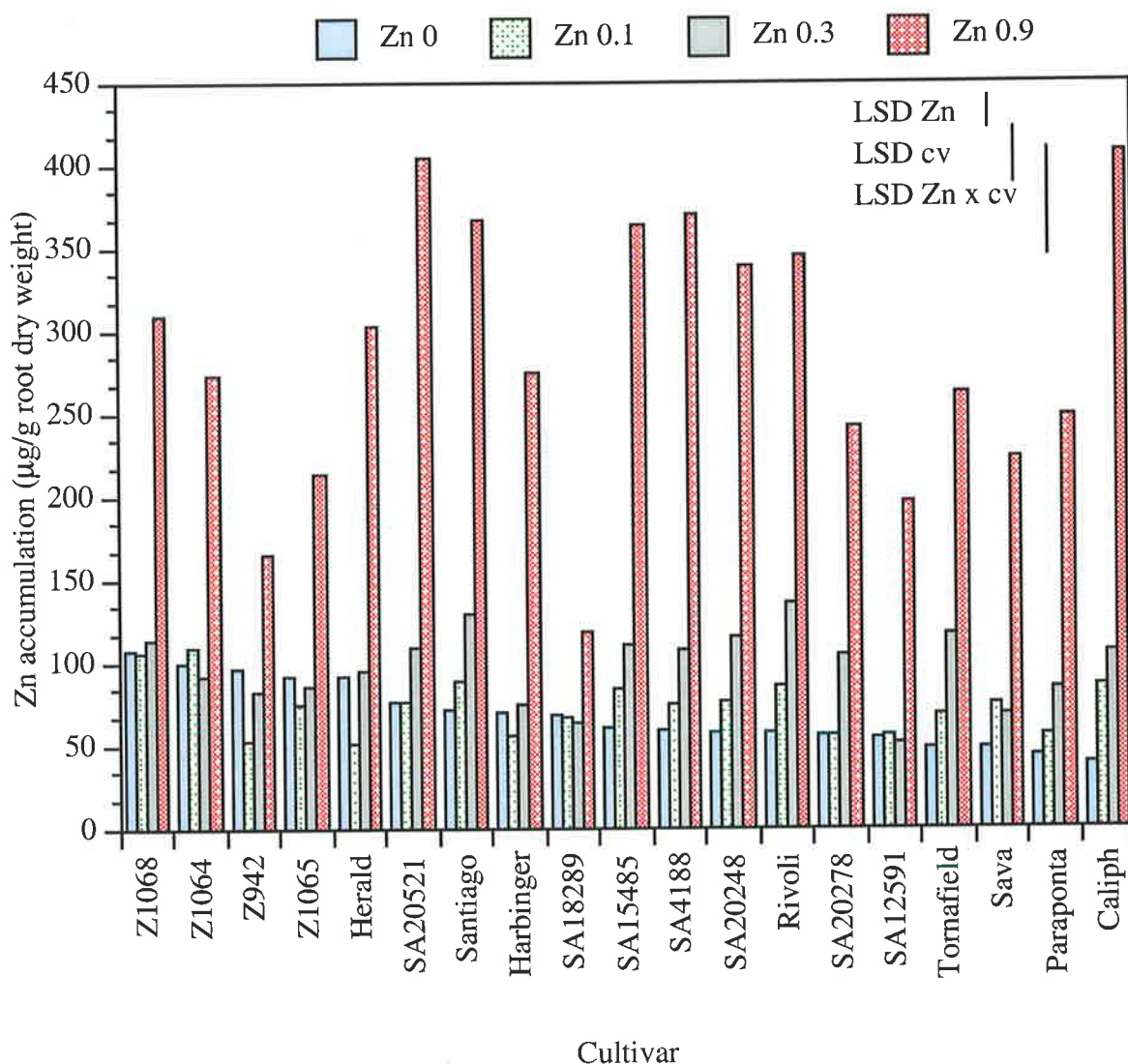


Fig. 7.5 Effect of Zn application on Zn accumulation per gram of root, for 19 medic genotypes grown in Mt. Compass, 42 days after sowing. LSD represents Fisher's Protected Least Significant Difference $\alpha=0.05$ for the main effects and the interaction between Zn application and genotype.

Two expressions of Zn efficiency of medic genotypes were calculated: Zn efficiency (Fig. 7.6); and shoot growth at low levels of Zn fertiliser (Fig. 7.7). Two other Zn efficiency indices were also calculated to determine possible mechanisms involved in Zn efficiency: high Zn accumulation per gram root under Zn deficiency (Fig. 7.8); and stability of shoot/root ratio as Zn status declined (Fig. 7.9). A summary of Zn efficiency rankings from the four indices is presented in Table 7.2.

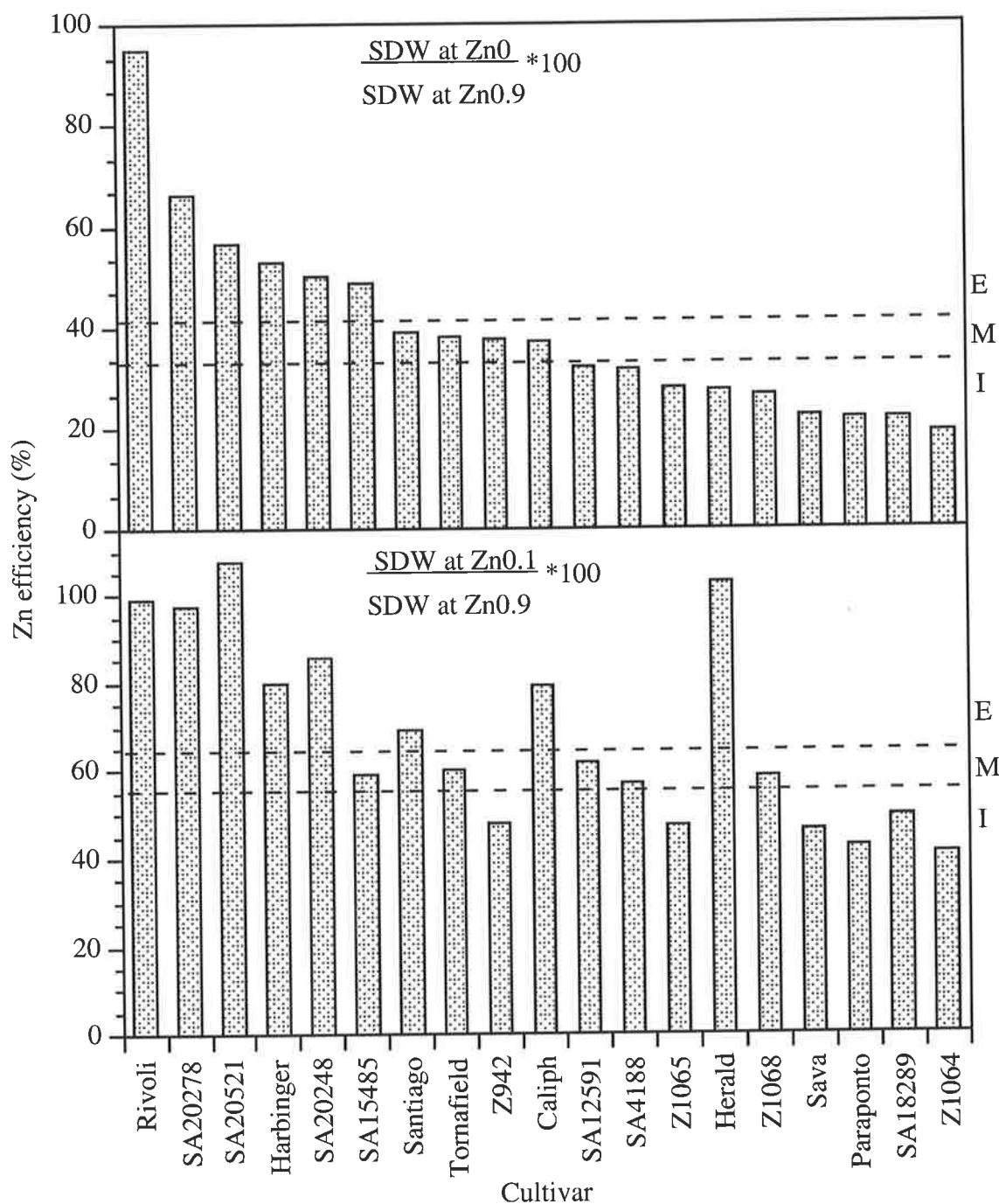


Figure 7.6 The Zn efficiency ranking (E=efficient, M=medium, I=inefficient) of 19 medic genotypes grown in Mt. Compass sand for 42 days. The ranking was based on ratio of shoot dry matter production at severe Zn deficiency (0 mg/kg), or at moderate Zn deficiency (0.1 mg/kg), and sufficient Zn supply (0.9 mg/kg). The mid-point of the medium efficiency interval corresponds to the median value of the parameter given on the y-axis; the upper and lower boundaries of the medium-efficiency interval were constructed by adding to, or subtracting from, the median point the value of the standard error of the genotype effect.

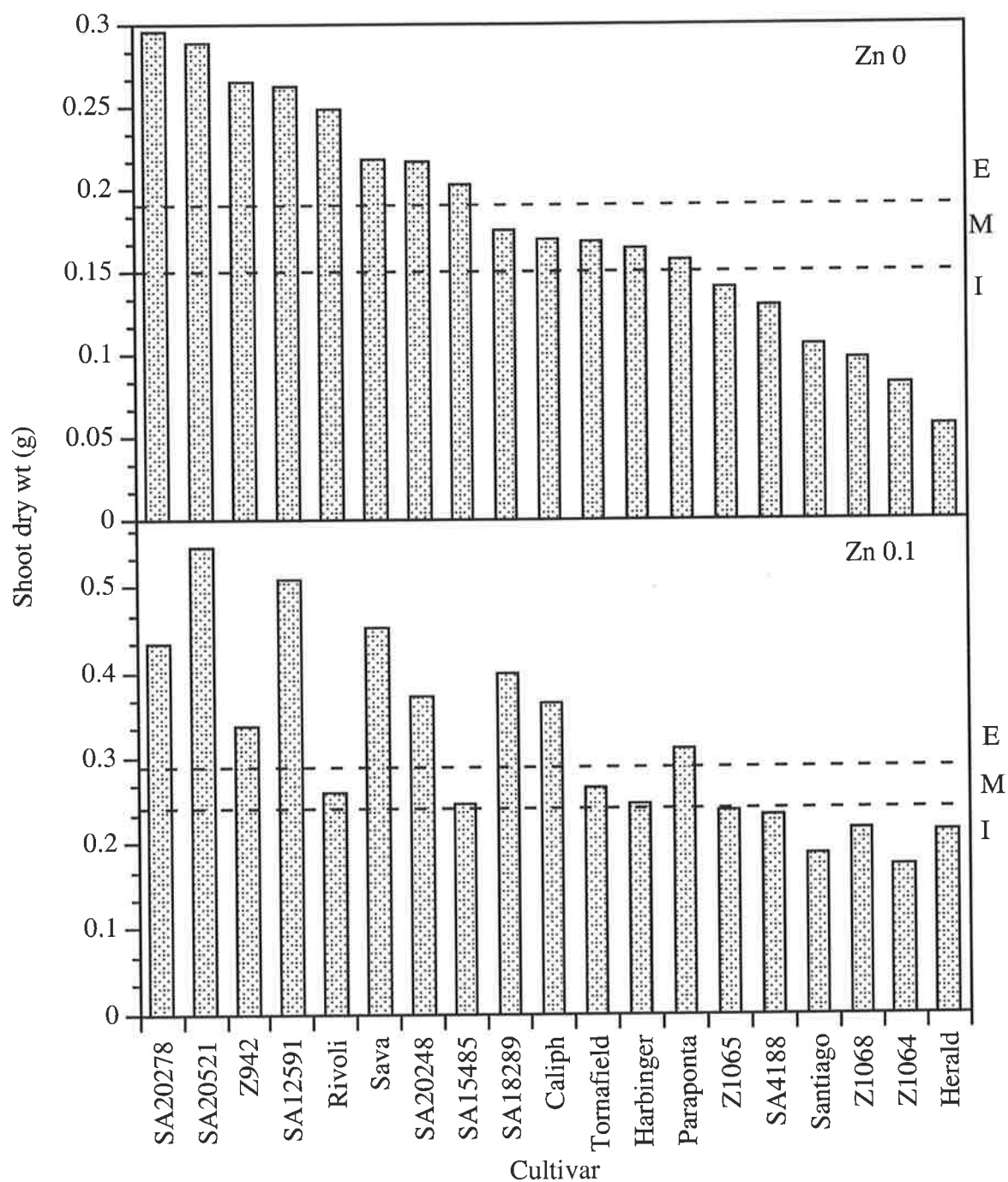


Figure 7.7 The Zn efficiency ranking (E=efficient, M=medium, I=inefficient) of 19 medic genotypes grown in Mt Compass sand for 42 days. The ranking was based on shoot dry matter accumulation (per plant) when severely deficient in Zn (Zn 0) and moderately deficient in Zn (Zn 0.1 mg/kg). Refer to Fig. 7.6 for construction of efficiency intervals.

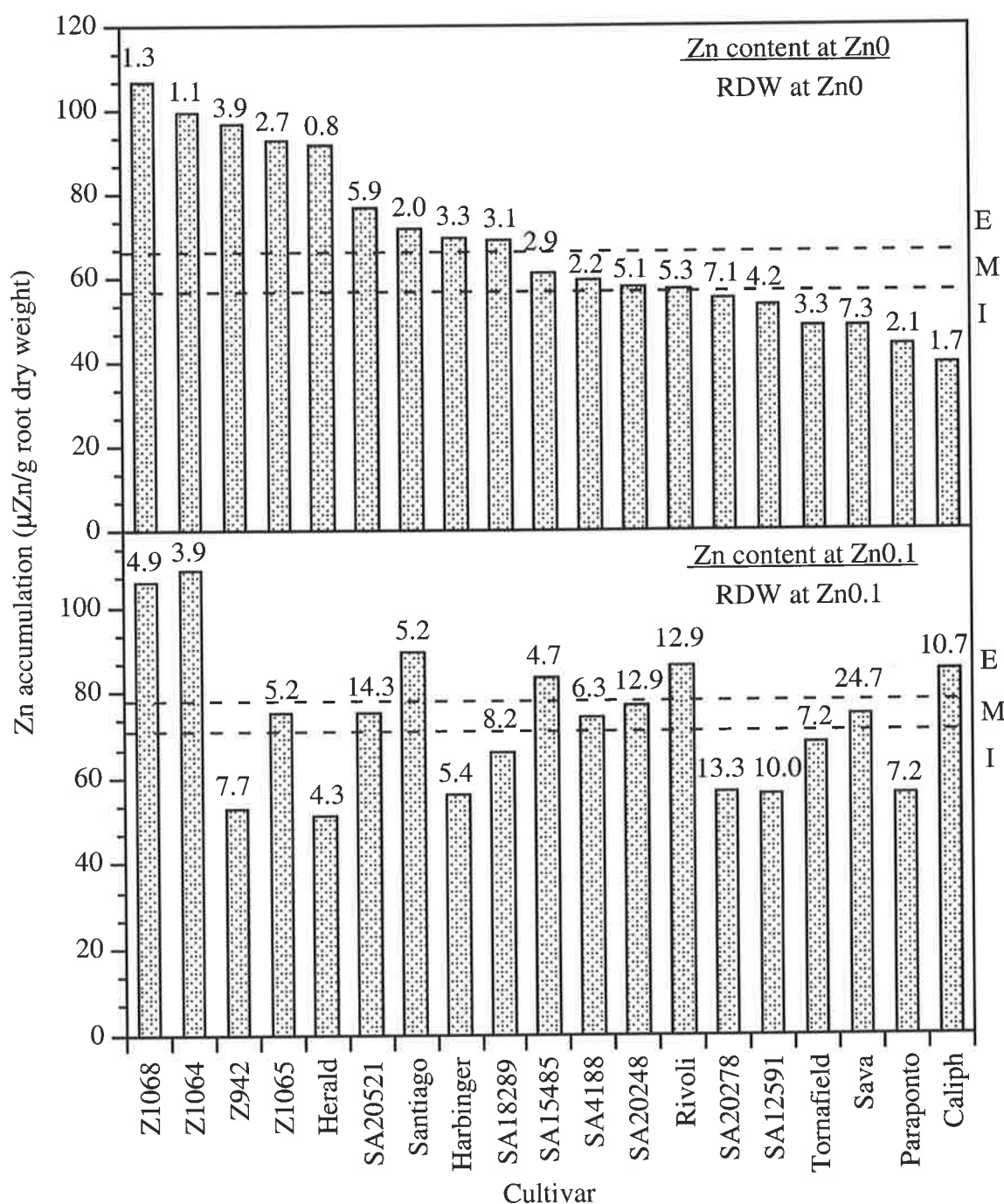


Figure 7.8 The Zn efficiency ranking (E=efficient, M=medium, I=inefficient) of 19 medic genotypes grown in Mt. Compass sand for 42 days. The ranking was based on the total amount of Zn accumulated by the plant (shoot and root) divided by root dry mass at severely deficient Zn (0 mg/kg) or moderately deficient Zn (0.1 mg/kg). Refer to Fig. 7.6 for construction of efficiency intervals. Numbers above bars represent the total amount of Zn accumulated per plant (μg/plant).

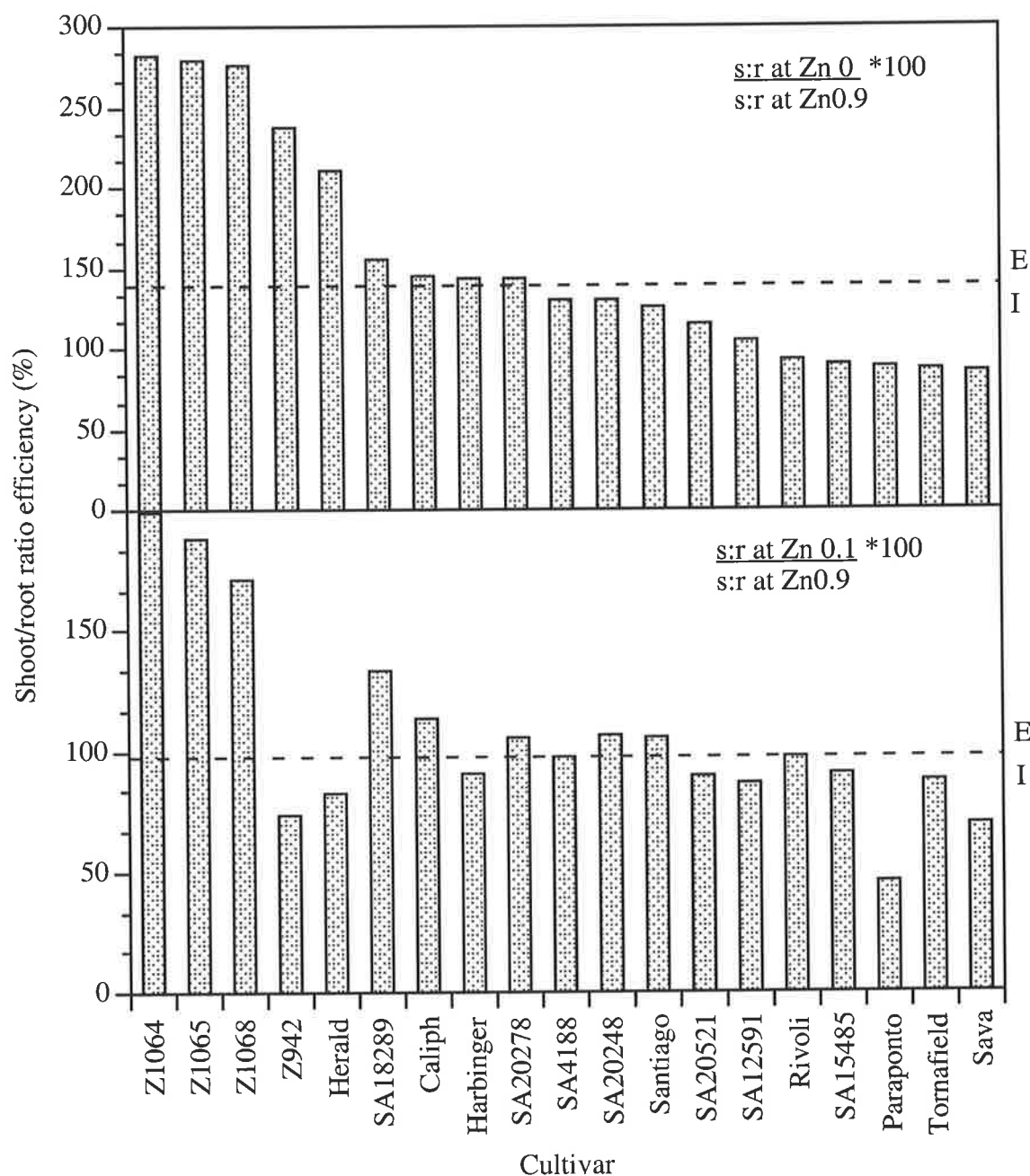


Figure 7.9 The Zn efficiency ranking (E=efficient, I=inefficient) of 19 medic genotypes grown in Mt Compass sand for 42 days. The ranking was based on quotient of shoot/root ratios at severe Zn deficiency (0 mg/kg) and sufficient Zn supply (0.9 mg/kg) and on the quotient of shoot/root ratios at moderate Zn deficiency (0.1 mg/kg) and sufficient Zn supply (0.9 mg/kg). Efficiency ranking was calculated as median - SE with values above the line considered efficient. Inefficient rankings are below the line.

Table 7.2 Summary of Zn efficiency rankings of 19 medic genotypes grown in severe (0 mg/kg) and moderate (0.1 mg/kg) Zn-deficient conditions for 42 days in Mt. Compass sand. Zinc efficiency rankings were defined by four techniques: A) ratio of shoot dry matter production with deficient and sufficient Zn supply (0.9 mg/kg); B) shoot dry matter accumulation at deficient Zn supply; C) total Zn accumulation divided by root dry mass at deficient Zn supply; and D) quotient of shoot/root ratios at deficient and sufficient Zn supply (0.9 mg/kg). Shaded numbers represent Zn-efficient genotypes as classified in Figures 7.6 to 7.9.

Genotype	Applied level of Zn deficiency	A	B	C	D
Caliph	0	10	10	19	7
	0.1	8	7	8	5
SA12591	0	11	4	15	14
	0.1	9	2	17	15
SA18289	0	18	9	9	6
	0.1	14	5	13	4
Z942	0	9	3	3	4
	0.1	15	8	18	17
Rivoli	0	11	5	13	15
	0.1	8	11	4	9
Tornafeld	0	8	11	16	18
	0.1	10	10	12	14
SA20278	0	2	1	14	9
	0.1	4	5	14	8
SA20248	0	5	7	12	11
	0.1	5	6	7	6
SA20521	0	3	2	6	13
	0.1	1	1	8	13
Harbinger	0	4	12	8	8
	0.1	6	13	16	11
Herald	0	14	19	5	5
	0.1	2	17	19	16
SA15485	0	6	8	10	16
	0.1	11	12	6	12
Santiago	0	7	16	7	12
	0.1	8	18	5	7
SA4188	0	12	15	11	10
	0.1	13	15	11	10
Z1064	0	19	18	3	1
	0.1	19	19	1	1
Z1065	0	13	14	4	2
	0.1	16	14	9	2
Z1068	0	15	17	1	3
	0.1	12	16	2	3
Sava	0	16	6	17	19
	0.1	17	2	10	18
Paraponto	0	17	13	18	17
	0.1	18	9	15	19

Table 7.3 Regression analysis (r^2 values) for the four Zn efficiency indices at severe (Zn 0) and moderate (Zn 0.1) deficiency as summarised in Table 7.2. * $p \leq 0.03$.

Zn 0 (mg/kg)					Zn 0.1 (mg/kg)				
	A	B	C	D		A	B	C	D
A	-	0.31*	0.02	0.10	A	-	0.03	0.003	0.002
B		-	0.14	0.16	B		-	0.11	0.13
C			-	0.52*	C			-	0.43*
D				-	D				-

Efficiency rankings varied with the degree of Zn deficiency (Figs. 7.6-7.9), with a significant correlation between the rankings for the first two efficiency indices only at 0 mg Zn supply. Surprisingly, no significant relationship occurred between the first two efficiency rankings under moderate Zn deficiency, even though index A is simply index B divided by the yield potential (Table 7.3). The only other significant relationship occurred between indices C and D at both severe and moderate levels of Zn deficiency (Table 7.3).

Genotypes ranked as Zn-efficient by the first two efficiency indices (Fig. 7.6-7) include Caliph, Rivoli, SA20278, SA20248, SA20521, and SA15485. Caliph was the only genotype considered efficient due to both high Zn accumulation (index C) and maintenance of a stable shoot/root ratio with declining Zn (index D; Table 7.2). Rivoli and SA20521 were also efficient in accumulating a higher amount of Zn per gram root compared with other genotypes (Fig. 7.8), while SA20278 and SA20248 maintained a stable shoot/root ratio with declining Zn (Fig. 7.9). Although Rivoli was ranked as efficient by three indices (A, B and C), this did not occur at the same level of Zn deficiency (Table 7.2). SA15485 was not efficient by either index C or D (Table 7.2).

Seed size had a positive effect on total dry weight and total Zn accumulation at all Zn treatments (Fig. 7.10). However, seed size did not influence the Zn-efficiency ranking.

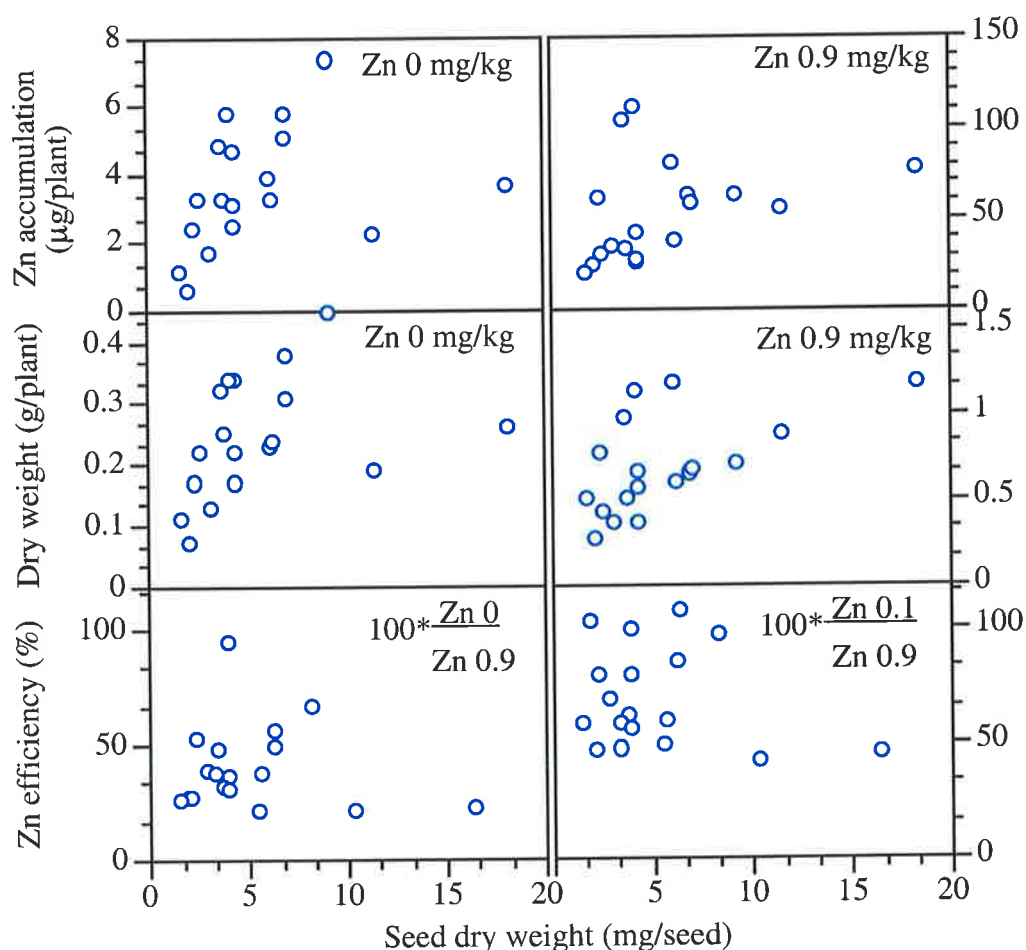


Figure 7.10 Effect of seed size on total dry matter accumulation, total Zn accumulation and Zn efficiency.

7.5 Discussion

The known genetic diversity within *Medicago* species for the Zn efficiency trait has been extended by this study. Genotypes SA20248, SA20278 and SA20521, which were not included in the field study, have been demonstrated to be more Zn-efficient than all species screened in the field study. Overall, genotypes from *M. tornata* and *M. littoralis* were more tolerant to Zn deficiency, demonstrating less response to Zn application than other species, with genotypes from *M. tornata* also high yielding. Surprisingly, genotypes developed from the cross between *M. littoralis* and *M. tornata* were consistently Zn-inefficient despite the parents belonging to species that appear generally Zn-efficient. The *M. tornata* parent used in the cross

with *M. littoralis* was not screened in the present experiment (SA11720), nor was the *M. littoralis* parent. However, the *M. littoralis* parent was a hybrid derived selection closely related to the cultivars Harbinger and Herald. This suggests selection conditions were such that the Zn-efficiency trait(s) present in Harbinger and/or Herald were either lost or that SA11720 was Zn-inefficient. This is possible since the *tornata* sub-species was not screened in this experiment (see Table 7.1). The cross was designed to incorporate Harbinger's resistance to blue-green aphids, spotted aphids and cow-pea aphids into *M. tornata*. Even though *M. tornata* and *M. littoralis* are similar in chromosome morphology (Simon and Simon, 1965), not all of the sub-species of *M. tornata* can be crossed with *M. littoralis*. SA11720 was selected as the parent since it is capable of crossing with *M. littoralis*. Since dry matter production of SA11720 is considered to be low (A. Lake, pers. comm.), future work with the progeny from the cross (ie Z1064, Z1065 and/or Z1068) will include an additional cross with a high yielding genotype of *M. tornata*. From this cross, Zn-efficient traits may enter the genetic pool from which selections will be made.

Genotypes considered the most Zn-efficient at severe and moderate levels of Zn deficiency include: Rivoli, SA20278, SA20248 and SA20521. These genotypes were bred specifically for light, sandy soils in South Australia that are Zn-deficient. It is likely that the Zn efficiency trait (or traits) were retained in the selection process offering an obvious advantage in adaptation to these soils.

The relative transport of Zn to the shoots was not consistently higher in medic genotypes defined as Zn-efficient by index A or B (Fig. 7.4), and a higher rate of Zn accumulation per gram root dry matter tended to occur in Zn-inefficient medic genotypes (Fig. 7.5; Z1064, Z1065 and Z1068). This suggests relative transport of Zn to the shoots and accumulation of Zn per gram root is not a mechanism for Zn-efficiency in the medic genotypes screened. In chickpea, however, the relative transport of Zn to the shoots as well as Zn accumulation per gram root dry matter was higher in Zn-efficient genotypes (Khan *et al.*, 1998). This enabled a higher rate of Zn accumulation and shoot growth compared with Zn-inefficient genotypes.

Compared with Zn-inefficient genotypes, Zn-efficient wheat genotypes also have a greater ability to acquire Zn from a deficient soil (Nable and Webb, 1993) with a higher rate of Zn uptake (Rengel and Graham, 1996) and a superior Zn transport mechanism (Grewal *et al.*, 1996). Such a contrast between medics and both wheat and chickpeas could be explained if, for example, medics proved to be more susceptible to photo-oxidative damage under low Zn conditions than the cereals or chickpeas. Although Zn accumulation per gram root and Zn transport to the shoot were not higher in Zn-efficient medic genotypes, they did however have a higher root dry matter accumulation and total Zn uptake per plant compared with Zn-inefficient genotypes. Zinc efficiency may either be the cause or result of a higher root dry matter production and/or higher accumulation of Zn per plant.

The high root dry matter production of Zn-efficient medic genotypes was connected with maintaining the highest concentration of Zn in the shoot. The concentration of Zn in the roots of Zn-efficient genotypes was not maintained to the same extent as that in the shoot suggesting a genetic control may exist for the concentration of Zn in tissue, with efficient genotypes having increased concentration of Zn in the shoot. Since the proportion of Zn in the shoot did not consistently increase under Zn-deficient conditions in Zn-efficient genotypes, this suggests a higher Zn concentration in the shoot is not maintained at the expense of the root. Since Zn accumulation by medics was not higher in Zn-efficient genotypes on a per gram root basis but only on a per plant basis, root mass may not be a sufficiently sensitive parameter to observe differences in Zn uptake. Other parameters, such as root surface area or volume of root, are therefore required.

Although Zn-efficient medic genotypes maintained a stable shoot/root ratio under Zn deficiency, so did most Zn-inefficient genotypes. This implies that shoot/root ratio is not a good indicator of Zn efficiency in medic genotypes, and this conclusion was supported by the non-significant correlation between Zn-efficient genotypes (index A and B) and shoot/root ratio with Zn deficiency (index D).

Under Zn deficiency, no medic genotype had a decrease in shoot/root ratio, including those genotypes that experienced a reduction in the amount of Zn transported to the shoot. Where a shortage of a nutrient in the outside medium causes reduction in the amount transported to shoots, plants generally adjust the shoot/root ratio to improve the mineral supply (Jackson, 1993). Since the shoots then experience nutrient deficiency, reduced growth results in changes in assimilate partitioning. In medics, Zn deficiency depressed root dry weight to a greater extent than shoot dry weight as the shoot/root ratios of most medic genotypes were higher in the 0 Zn treatment compared with the treatment with 0.9 mg Zn/kg soil (Fig. 7.2). The opposite has been shown to occur in wheat with Rengel and Graham (1995a) reporting a decrease in the shoot/root ratio under Zn deficiency. The authors considered this to be a compensatory mechanism geared toward greater acquisition of a scarce resource from the environment, in this case Zn, by maintaining or increasing root growth at the expense of shoot growth. This response was also reported by Cakmak *et al.* (1996a) and Khan *et al.* (1998). The change in partitioning of dry matter resulted in depressed shoot growth and enhanced root growth in wheat and chickpea (Rengel and Graham, 1995a; Khan *et al.*, 1998).

Rengel and Graham (1995a) also reported the compensatory mechanism to be less obvious for Zn-efficient wheat genotypes while Khan *et al.* (1998) observed a similar occurrence in Zn-efficient chickpea genotypes. In medics however, shoot/root ratio was greatest in Zn-inefficient genotypes. Zinc-inefficient medics may be unable to reduce shoot growth thereby reducing assimilates available to promote root growth. This situation may occur if Zn deficiency in medics has a greater impact on root cells compared to cells in the shoot, or if Zn deficiency inhibits the export of assimilates from the shoot to the roots. The decrease in root dry matter accumulation correlates with work on canola where Grewal *et al.* (1997) observed impaired root growth in Zn-inefficient genotypes in soils of low Zn status.

A genotype classified as Zn-efficient when Zn was severely deficient was not necessarily ranked as Zn-efficient when Zn was moderately deficient, and vice versa. This may be due to different mechanisms of Zn efficiency operating. The ideal soil Zn status required for studies

on Zn deficiency is the level where the greatest difference between Zn-efficient and Zn-inefficient genotypes occurs. A greater range of shoot dry weight under Zn deficiency occurred when plants were supplied with 0 Zn compared with 0.1 mg/kg of Zn. Compared with moderate levels of Zn deficiency, severe Zn deficiency resulted in a higher correlation between index A and B. It is important to maintain Zn availability similar to that observed in the field. In the field experiment described in Chapter 6, the concentration of Zn in the shoot when plants were not supplied with Zn fertiliser was less than 16 mg/kg. These levels correspond with the 0 Zn treatment in the controlled experiment, indicating Zn efficiency rankings for future work should be based on this level of Zn, and not on the results where plants were only moderately deficient in Zn.

Comparing the Zn efficiency ranking determined in the field with those genotypes included in this experiment, Rivoli was the only cultivar consistently classified as Zn-efficient. This occurred at both levels of Zn deficiency in the controlled experiment. Paraponto was the only cultivar to be consistently ranked as Zn-inefficient. All other genotypes had different classifications depending on whether the experiment was performed in the field or in a controlled environment. Herald, however, was classified as efficient in the field trial and also in the pot experiment when Zn was moderately deficient. Plants may be prevented from reaching their yield potential in the field with factors such as pests and diseases, reduced levels of nutrients, insufficient soil moisture, and differences in growth stages, which all impact on the Zn efficiency. Medics grown in the field may not have reached their yield potential due to water deficit towards the end of the season in 1996. As already stated in Chapter 6, dry conditions were responsible for the lack of response to Zn application in cereal field trials (R.D. Graham, pers. comm.). Further work is required to ensure Zn efficiency rankings in controlled experiments correlate with field rankings.

Although yield was affected by the size of the seed, Zn efficiency rankings of medic genotypes were not significantly related to seed size. Similar observations were made in wheat (Cakmak *et al.*, 1994; 1996a) and in chickpeas (Khan *et al.*, 1998). This is useful for future work as

medics have considerable variation in seed size among genotypes, and using similar seed size with similar Zn content for many genotypes at once in a breeding program would be difficult to achieve.

In conclusion, differences in response to Zn supply occurred between genotypes, with SA20248, SA20278 and SA20521 the most efficient in terms of minimal yield difference between low and high Zn levels. These genotypes were also able to yield well in non-limiting conditions. Genotypes considered Zn-inefficient include Z1064, Z1065 and Z1068. Although yield of Rivoli was low at all levels of Zn it was also classified as Zn-efficient in the field and in controlled conditions based on the ratio of shoot dry matter production at Zn-limiting and non-limiting conditions. It may therefore be a useful donor line in a breeding program. Zinc-efficient genotypes did not maintain a high uptake of Zn per gram root, but they were able to maintain a higher uptake of Zn per plant. A constant shoot/root ratio with declining levels of Zn, a higher root mass and a higher concentration of Zn in the shoot were also present in Zn-efficient compared with Zn-inefficient medic genotypes. The effect of seed nutrient composition did not appear to influence the Zn-efficiency ranking, but did play an important role in yield response and Zn accumulation with Zn supply.

Effect of Zn Nutritional Status on Infection by *R. solani* in *Medicago* species. Differing in Zn Efficiency

8.1 Introduction

A link between the Zn nutritional status of medics and tolerance to *R. solani* AG 8 infection was established in Chapters 4 and 5. Medics, deficient in Zn, are more sensitive to infection by *R. solani* AG 8 and the addition of Zn results in increased tolerance to the fungus. Results from Chapter 6 and 7 demonstrated genotypic differences between medic cultivars in their sensitivity to Zn. The question then arises whether Zn-efficient medic cultivars are more tolerant to disease.

Previous work on Mn deficiency has demonstrated increased resistance to the take-all fungus in Mn-efficient cultivars of wheat (Wilhelm *et al.*, 1990; Rengel *et al.*, 1993). Wheat plants grown under Mn-deficient conditions are more susceptible to take-all infection caused by *Gaeumannomyces graminis* var. *tritici* (Ggt) than plants fertilised with Mn (Graham and Rovira, 1984), while Mn-inefficient plants are more sensitive to infection by Ggt compared with Mn-efficient wheats at the same soil Mn status (Wilhelm *et al.*, 1990; Rengel *et al.*, 1993). Rengel *et al.* (1994a; 1994b) suggested Mn-efficient wheat may have a more efficient mechanism for conversion of phenolics to lignin, a trait which appears related to higher resistance to Ggt. It has also been demonstrated that in Zn-deficient soil a Zn-efficient wheat cultivar (Excalibur) has greater resistance to *Fusarium graminearum* than two Zn-inefficient wheat cultivars (Durati and Songlen) (Grewal *et al.*, 1996). Given the established effect of Zn fertiliser on tolerance to *R. solani* in medics, it was therefore desired to determine if medic cultivars ranked as Zn-efficient in Chapter 7 have greater tolerance to *R. solani* infection compared with Zn-inefficient medics when the soil is deficient in Zn.

8.2 Aim

The aim of this experiment was to determine if tolerance of medic to *R. solani* AG 8 is affected by the Zn efficiency of medic genotypes, as determined in Chapter 7, in soil of low Zn status.

8.3 Methods

8.3.1 *Experimental Design*

A factorial design was used combining five genotypes with seed low in Zn (Caliph, SA20521, SA20278, SA18289 and Z1064), at two Zn levels (0 and 0.9 mg Zn/kg) and four levels of *R. solani* (0, 1, 8 and 32 propagules) in three replicates.

8.3.2 *Soil*

Mt Compass sand was used, leached of soluble salts in RO water, air-dried and heat-treated at 80°C as outlined in Chapter 3.

8.3.3 *Basal Nutrients*

A basal nutrient solution was applied to the soil as outlined in Chapter 3.

8.3.4 *Seed*

Seed sown was collected from plants grown in Zn-deficient conditions in a controlled environment. This was done to reduce the effect of seed Zn content which is exacerbated by the differences in seed size between genotypes (see Discussion in Chapter 6). The nutrient content of the seed is presented in Table 8.1. Seed was surface sterilised and germinated as described in Chapter 3.

Table 8.1 Seed characteristics of seed grown in 3 kg pots with 0.1 mg Zn/kg soil.

	Caliph	SA20278	SA20521	SA18289	Z1064
Weight, mg/seed	3.7	7.8	8.1	6.6	2.2
Content, µg/seed					
Fe	0.32	0.26	0.26	0.31	0.11
Mn	0.12	0.20	0.24	0.19	0.06
B	0.11	0.02	0.02	0.02	0.02
Cu	0.04	0.15	0.13	0.07	0.02
Zn	0.13	0.29	0.27	0.26	0.10
Ca	9.61	25.35	26.36	14.49	6.83
Mg	8.76	14.85	15.69	14.26	4.43
Na	0.12	0.40	0.16	0.12	0.10
K	33.72	88.94	80.65	71.18	24.58
P	27.56	29.56	39.07	33.12	10.47
S	13.66	22.90	22.70	23.23	9.96

8.3.5 Procedure

Soil mixing and potting were performed as outlined in Chapter 3, with four germinated seeds sown per pot of 1 kg soil. After planting, the soil surface was covered in acid-washed, black plastic beads, and the soil mass watered to 12% water content. Pots were then placed in a controlled growth room, and one week later thinned to two plants per pot. After thinning, plants were inoculated with the appropriate strain of *Rhizobium meliloti* (see Table 7.1) and watered every second day.

After 42 days, plants were harvested, shoot dry weight determined and shoots analysed for Zn concentration by ICP analysis. Roots were assessed for damage caused by *R. solani* by measuring the length of the tap root, counting the number of sites of infection (lesions and spear tips), and scoring for disease on a 0 to 5 scale where 0 represents no disease and 5 severe truncation of the root system (Sweetingham and MacNish, 1992). Total root length and total root surface area were calculated within a set diameter range by scanning the root system and analysing scans by computer as outlined in Chapter 5.

8.4 Results

Visual Observation

Differences in plant growth were observed from day 1, with plants inoculated with 32 propagules taking longer to emerge than in other treatments (data not shown). Once plants emerged in the high *R. solani* treatment, leaf size was visibly reduced at both Zn treatments. The only plants that did not survive the duration of the experiment were Zn-deficient SA20278. This genotype emerged but died within four days in the presence of the highest level of *R. solani*. Lower growth with 32 propagules of *R. solani* was observed in all genotypes through the entire growing period. When Zn was not applied to the soil, Zn deficiency symptoms were observed on all plants 3 weeks after sowing.

Plant Growth Measurements

A significant three-way interaction for shoot dry weight occurred between genotype, Zn application and the level of *R. solani* added to the soil ($p \leq 0.001$; Fig. 8.1). Addition of Zn increased shoot dry weight in all genotypes when no *R. solani* was present. However, with increasing amounts of *R. solani*, shoot dry weight was reduced at both levels of Zn. With high infection, Zn application did not increase shoot dry weight for Caliph or SA18289. However, Caliph and SA20521 are the most tolerant genotypes to *R. solani* in the presence of Zn, with a reduction in yield only with severe infection (32 propagules). The shoot dry weight of other genotypes decreased in the presence of 8 propagules of *R. solani* when Zn was applied. In soils of low Zn status, SA18289 was more tolerant to *R. solani* than other genotypes with a reduction in yield at 32 propagules only. Other genotypes were sensitive to low levels of *R. solani* under Zn deficiency, resulting in lower growth.

With increasing levels of *R. solani*, the Zn efficiency value decreased to the point of severe infection where the efficiency value increased (Table 8.3). In the absence of *R. solani*, Z1064 has the highest, and SA18289 the lowest Zn efficiency.

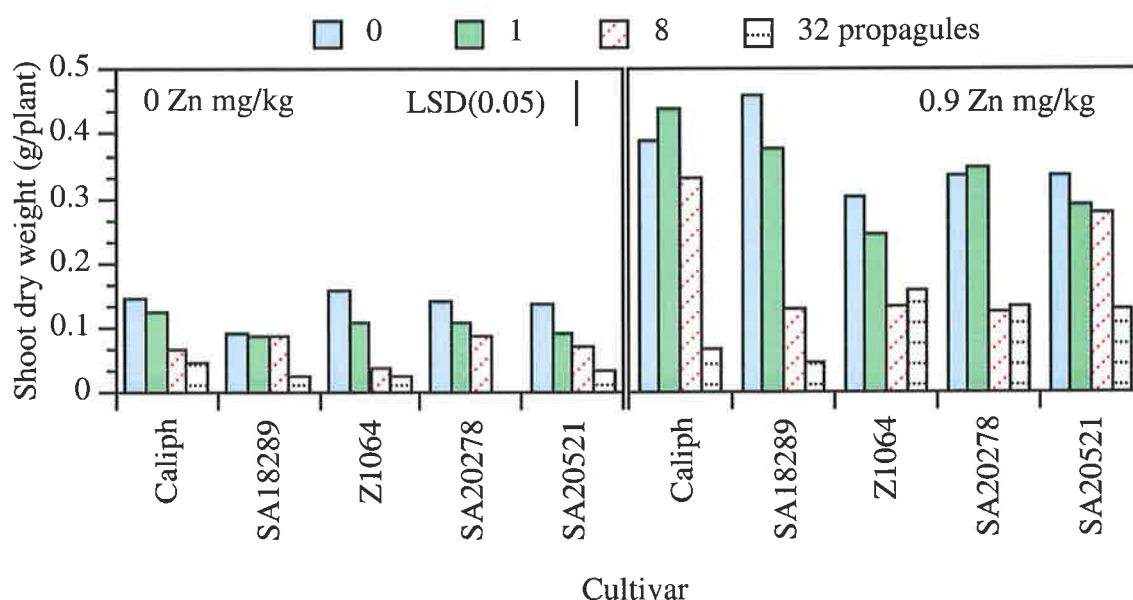


Figure 8.1 Effect of Zn application on shoot dry weight of five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fisher's Protected Least Significant Difference for the three-way interaction between genotype, Zn application and the level of *R. solani* in the soil ($p \leq 0.001$).

Table 8.3 Zinc efficiency ranking of five medic genotypes with increasing levels of *R. solani* in the soil, calculated from: $100 * (\text{shoot weight at 0 Zn} / \text{shoot dry weight at 0.9 Zn})$.

Genotype	number of propagules/kg soil			
	0	1	8	32
Zn Efficiency Rankings (%)				
Caliph	37	28	20	69
SA18289	20	22	66	58
Z1064	52	44	26	16
SA20278	42	31	67	0
SA20521	40	31	25	25

Zinc deficiency occurred in all plants in the 0 Zn treatment: shoot tissue concentrations were between 2 and 9 mg Zn/kg under severe *R. solani* infection (Fig. 8.2). The interaction between genotype, Zn application and addition of *R. solani* had a significant effect on the concentration of Zn in the shoots ($p \leq 0.001$). Despite the addition of Zn to the soil, Caliph was marginally Zn-deficient when 32 propagules were present. Although SA18289, SA20278 and SA20521

had a reduction in Zn concentration in the shoot with the highest level of *R. solani*, these genotypes were not deficient in Zn in the treatment with 0.9 mg Zn/kg soil. Surprisingly, Z1064 had a greater concentration of Zn in the shoot with the highest level of *R. solani* when Zn was not limiting (Fig. 8.2). SA20521 also showed an increase in Zn concentration in the shoot, but this only occurred at low levels of *R. solani*.

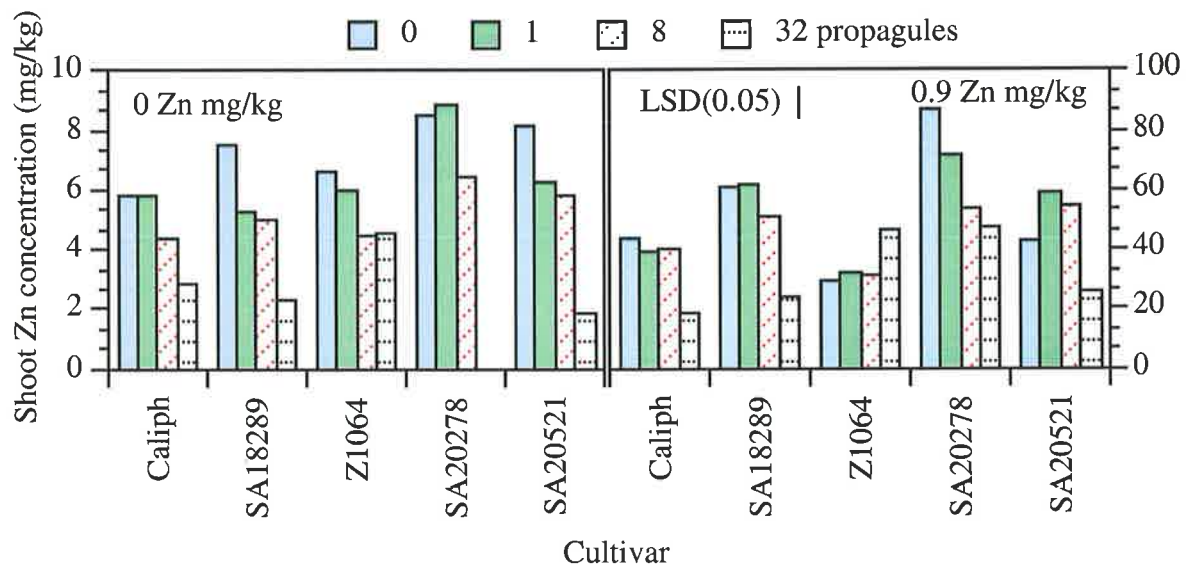


Figure 8.2 Effect of Zn application on shoot Zn concentration of five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fishers Protected Least Significant Difference for the three-way interaction, genotype $\times$ Zn $\times$ *R. solani*. Note differences in y-axis between Zn treatments.

Other nutrients, including Mn, B, Cu, Mg, Na and K, were also influenced significantly by the three-way interaction ($p \leq 0.056$ for Mn and $p \leq 0.005$ for others; Figs. 8.3 and 8.4). Although the three-way interaction was not significant for Ca, P and S ($p \leq 0.2$; Fig. 8.5), all two-way interactions were significant ($p \leq 0.05$ in all cases). Under Zn deficiency, SA20278 and SA20521 maintained a stable concentration of all nutrients (excluding Ca for SA20521) in the presence of 0, 1 and 8 propagules in the soil (Figs. 8.3-8.5). At the highest level of *R. solani*, concentrations of all nutrients in the shoot of Zn-deficient SA20521 had reduced. When deficient in Zn, Caliph and SA18289 had lower concentration of all nutrients with high levels of *R. solani*, while Z1064 had a reduced concentration of Mn, Cu, Mg, K, and Ca (Figs. 8.3-

8.5). Levels of B, Na, S and P remained relatively stable in the shoot of Z1064 compared with other genotypes.

With Zn deficiency, P was approaching toxicity in all genotypes with 0, 1 and 8 propagules/kg soil (Fig. 8.5). Calcium was the only nutrient to be at a level above that required for normal shoot growth of SA18289 and in Z1064 when Zn fertiliser was applied to the soil (Fig. 8.5).

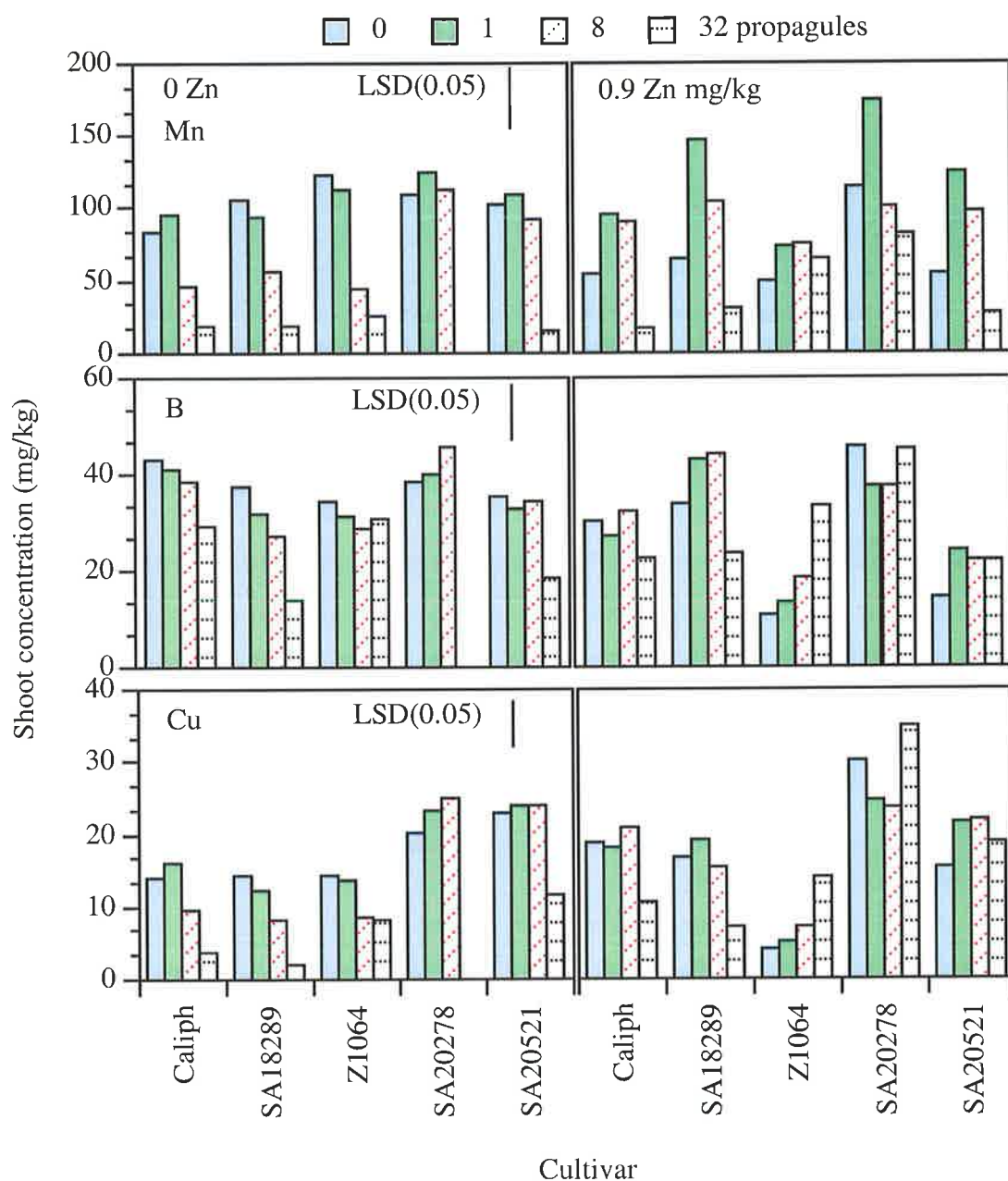


Figure 8.3 Effect of Zn application on shoot concentrations of Mn, B and Cu in five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fisher's Protected Least Significant Difference for the three-way interaction, genotype x Zn x *R. solani*.

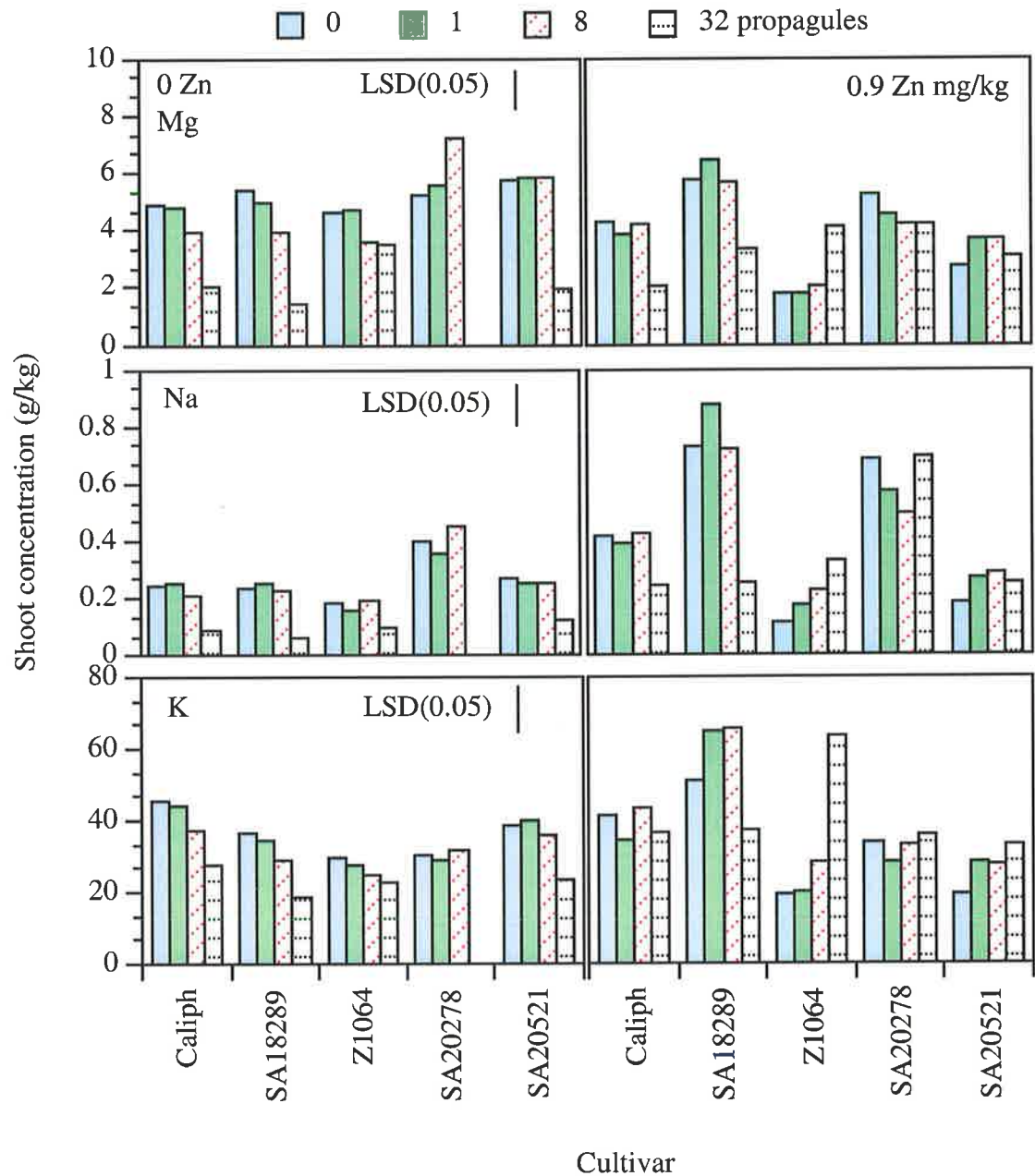


Figure 8.4 Effect of Zn application on shoot concentrations of Mg, Na and K in five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fisher's Protected Least Significant Difference for the three-way interaction, genotype x Zn x *R. solani*.

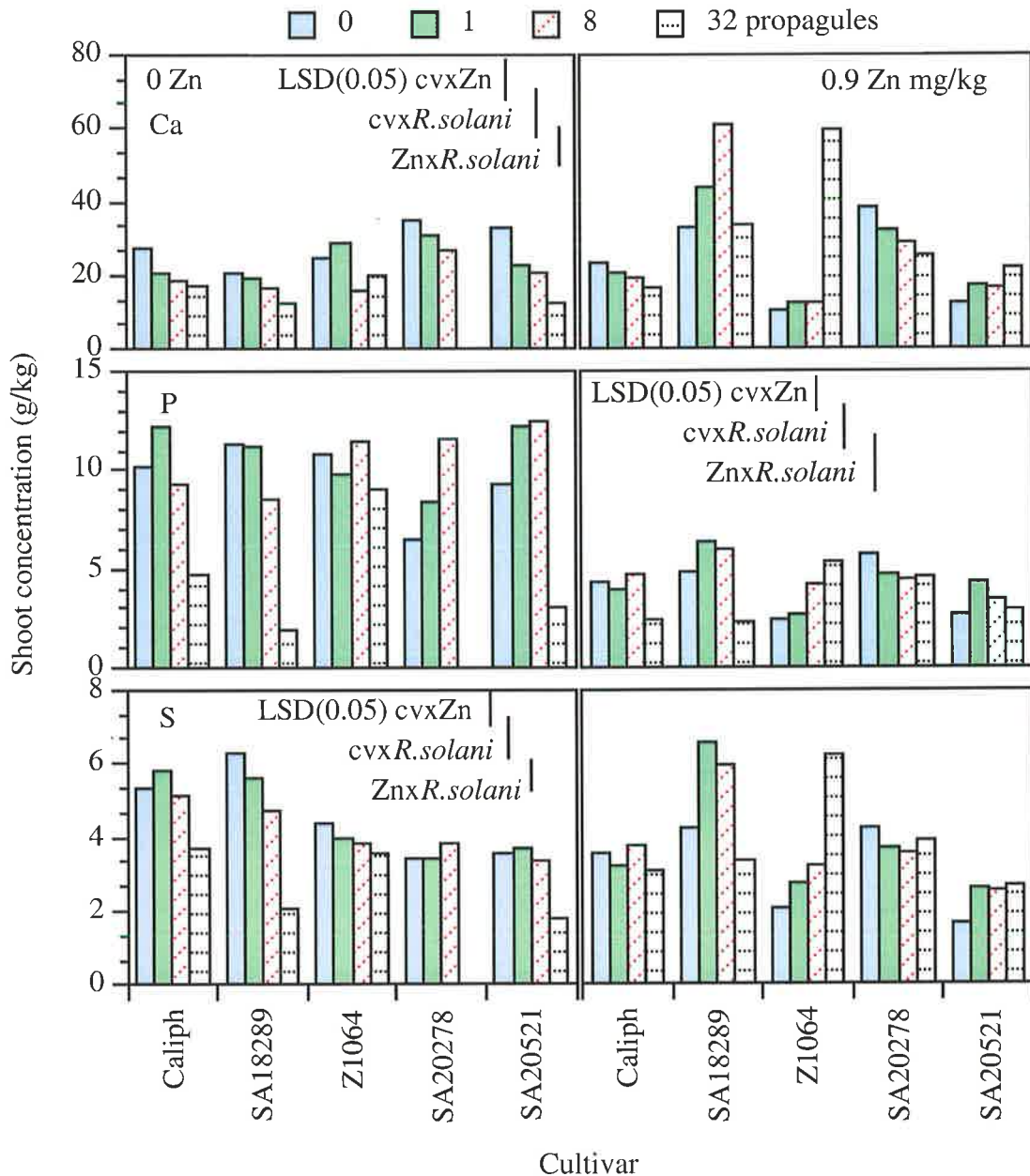


Figure 8.5 Effect of Zn application on shoot concentrations of Ca, P and S in five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fisher's Protected Least Significant Difference for the two-way interactions, genotype x Zn, Zn x *R. solani*, genotype x *R. solani*.

When Zn was not applied to the soil, all genotypes were deficient in Mn in the presence of 32 propagules of *R. solani* (see Table 3.4 for nutrient requirements by Weir and Cresswell, 1994). Deficiency of other nutrients also occurred in Zn-deficient plants with the highest level *R. solani* only: Caliph was deficient in Cu and Mg; SA18289 was deficient in B, Cu, Mg, P and S; and SA20521 was deficient in B, Mg and S. Z1064 was not deficient in any of these

nutrients under Zn deficiency; however, with the addition of Zn to the soil, B, Cu, Mg, P and S were below the range for normal growth when no *R. solani* was applied to Z1064. As the amount of *R. solani* increased in the soil, so did the concentration of all other nutrients in the shoots of Z1064 (Figs. 8.3-8.5).

The three-way interaction between genotype, Zn and *R. solani* did not have a significant effect on the number of infection points per root system or on the disease score ($p \leq 0.45$; Tables 8.4 and 8.5). However, the two-way interaction between Zn and addition of *R. solani* was significant at the 6% level ($p \leq 0.06$), with an increase in the number of infection sites with Zn application and increasing inoculum, while the disease score was significantly decreased by the addition of Zn fertiliser ($p \leq 0.009$) and by increasing levels of *R. solani* ($p \leq 0.001$). No significant difference in the number of infection sites or disease score occurred between genotypes.

Table 8.4 Effect of Zn application on the number of lesions and spear tips on the root system of five medic genotypes with increasing levels of *R. solani*, $n=3$.

Propagules per kg soil	Soil Applied Zn (mg/kg)	
	0	0.9
0	0±0	0±0
1	20±5	34±19
8	45±9	75±15
32	26±4	88±12

Table 8.5 Effect of Zn application on the disease score on the root system using the technique of Sweetingham and MacNish (1992), where 0 represents no infection and 5 severe truncations, with increasing levels of *R. solani*. Since the three-way interaction was not significant, data were averaged for the five genotypes.

Propagules per kg soil	Soil Applied Zn (mg/kg)	
	0	0.9
0	0	0
1	1	1
8	3	2
32	4.5	3.5

The interaction between genotype, Zn application and *R. solani* had a significant effect on the length of the tap root (Fig. 8.6). The addition of Zn increased the length of the tap root when *R. solani* was absent. However, with increasing amounts of *R. solani* in the soil the beneficial effect of Zn fertilisation did not occur. When deficient in Zn, all genotypes had a reduced tap root length with a high level of *R. solani*, and a minimal reduction with 1 propagule for Caliph and SA18289. With the addition of Zn, Caliph and Z1064 did not show a reduction in length in the presence of 8 propagules, while the remaining genotypes had a reduced tap root length at levels exceeding 1 propagule.

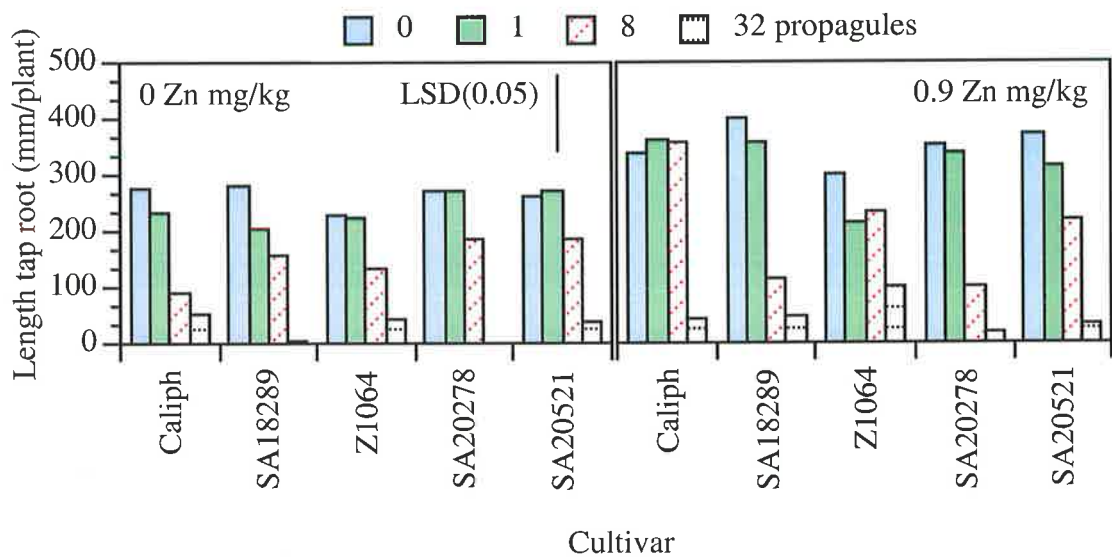


Figure 8.6 Effect of Zn application on length of the tap root in five medic genotypes with increasing levels of *R. solani*, $n=3$. Vertical bar represents Fisher's Protected Least Significant Difference at 5% significance for the three-way interaction, genotype x Zn x *R. solani*.

The effect of increasing levels of *R. solani* at the two levels of Zn application was significantly different for the root system geometries of the five genotypes studied, with SA20278 tending to maintain the largest root system across most treatments while SA18289 had the smallest (Fig. 8.7). The interaction between genotype, Zn, *R. solani* and root diameter class had a significant effect on the root length and root surface area. Note that data for root length and root surface area for Zn-fertilised Caliph, inoculated with 8 propagules *R. solani*, are missing.

Figure 8.7 Effect of increasing inoculation with *R. solani* on root length and root surface area within mean diameter classes for five medic genotypes: Caliph, SA18289, SA20278, SA20521, and Z1064; with two soil Zn applications, 0 and 0.9 mg/kg, in Mt. Compass sand during a 42 day growing period. See Table 8.3 for analysis of variance.

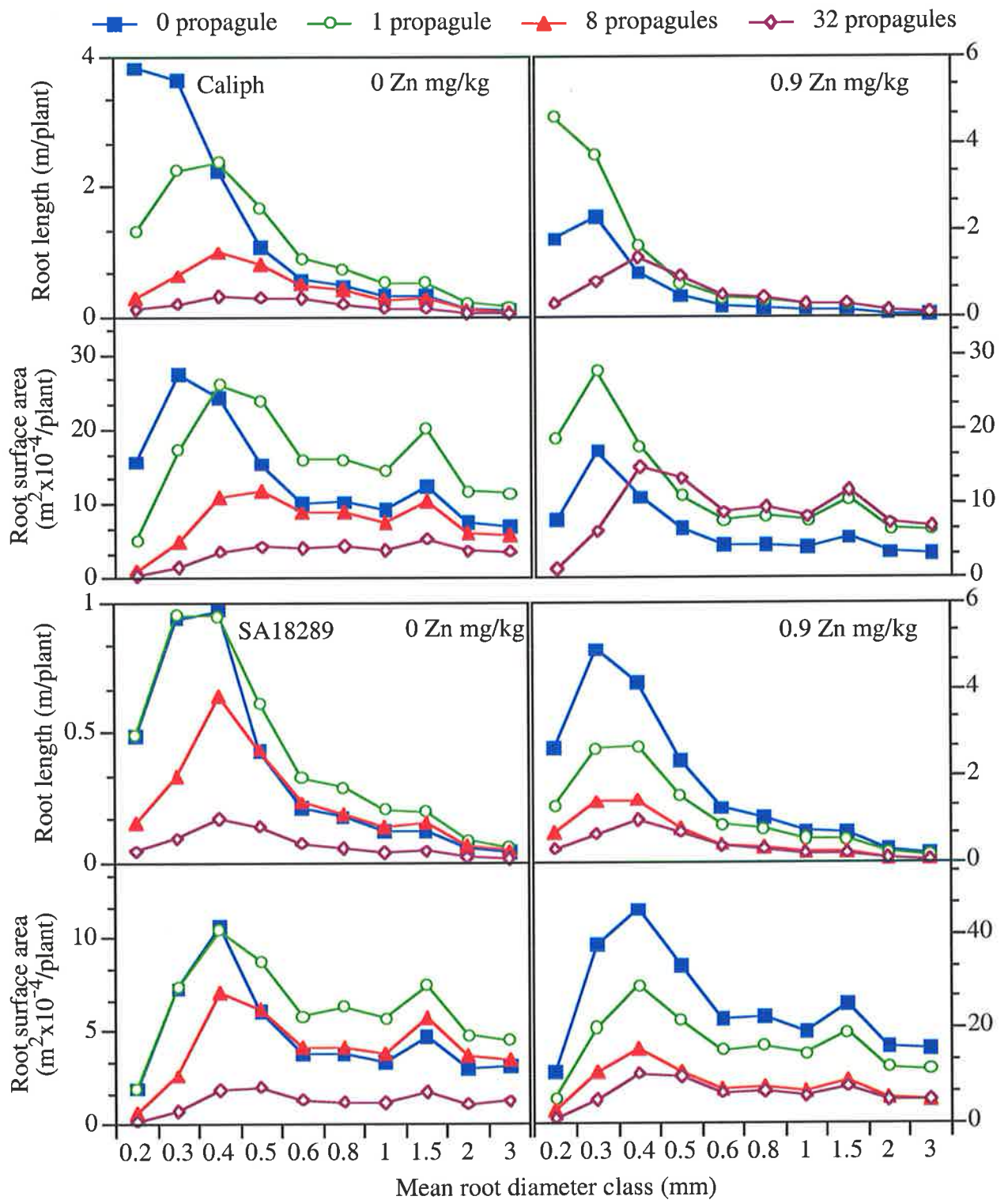


Figure 8.7 (cont)

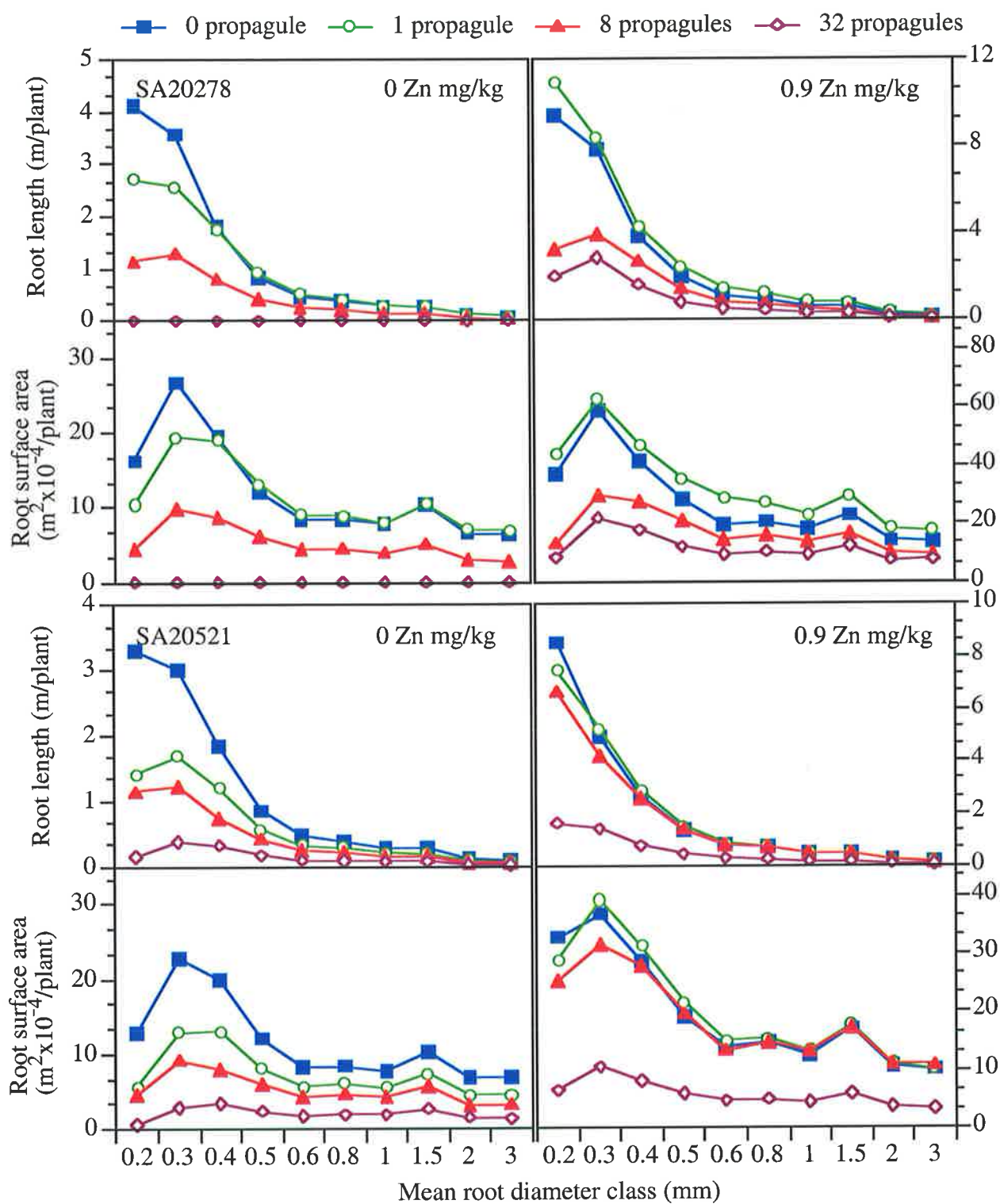
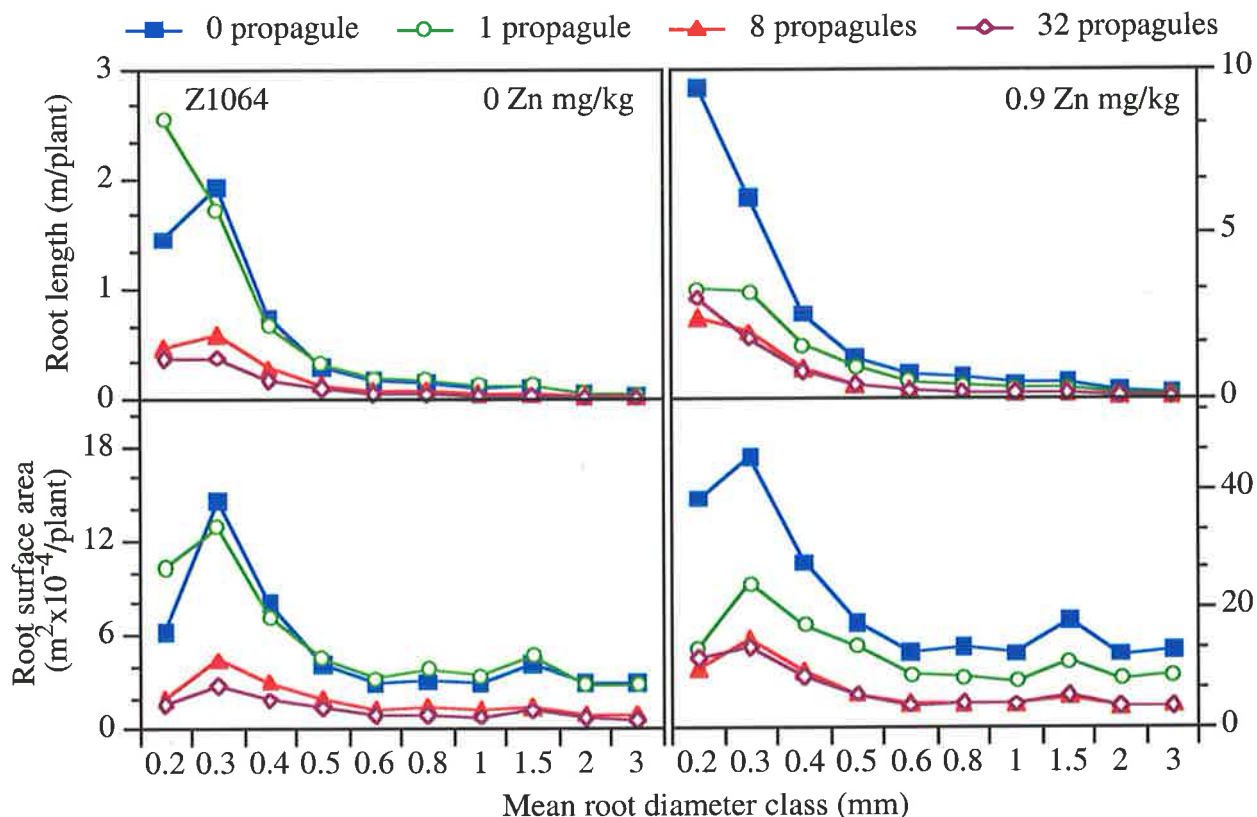


Figure 8.7 (cont)



The addition of Zn resulted in an increase in root length and surface area, while increasing levels of *R. solani* decreased both root length and surface area in all cultivars regardless of Zn treatment (Fig. 8.7). All genotypes had a higher root length and surface area with mean root diameter class 0.2 and 0.3 mm, followed by a decrease in larger root diameter classes. Caliph was the only genotype to maintain a surface area higher than the control in the presence of *R. solani* at both levels of Zn (Fig. 8.7). SA18289 maintained a higher surface area with 1 propagule compared to the control when deficient in Zn, while SA20278 maintained a higher surface area than the control when Zn was applied to the soil.

8.5 Discussion

All genotypes had lower growth under Zn deficiency in the presence of *R. solani*, with genotypes ranked as Zn-efficient in Chapter 7 (SA20278 and SA20521) maintaining a stable nutrient concentration as inoculum level increased from 0 to 8 propagules. This indicates Zn-efficient genotypes have a greater tolerance to the pruning effect of *R. solani* under Zn deficiency than other genotypes. Roots of Zn-efficient genotypes may have a capacity to increase the rate of nutrient uptake per remaining roots as a compensation for root loss. With the highest level of inoculation, the concentration of all nutrients was at their lowest in SA20521 while SA20278 demonstrated no tolerance to that level of pathogen.

The decrease in Zn concentration in all genotypes with increasing inoculum is a result of the pruning effect of *R. solani* exacerbating Zn deficiency. The concentration of P increased in SA20521 and SA20278 only, with 1 and 8 propagules of *R. solani*. This is most probably due to the increase in membrane permeability caused by the combined effects of Zn deficiency and infection by *R. solani*. Due to the greater root length of SA20521 and SA20278 infection by *R. solani* did not effect P uptake enough to cause a reduction in concentration similar to that observed in SA18289.

Although Z1064 was ranked as the most Zn-efficient medic genotype in this experiment, other factors suggest otherwise. When Zn was applied to the soil, Z1064 was the only genotype to be deficient in most elements in the absence of *R. solani*. This indicates the basal fertiliser was not adequate to sustain growth of Z1064. However, when Zn was applied to the soil in Chapter 7, Z1064 was not deficient in any nutrient. In Chapter 7 plants were derived from seed with a higher nutritional status, implying maximum growth potential was not obtained in this experiment for Z1064 due to a lower seed nutritional quality. Since maximum yield potential was not reached, the Zn efficiency ranking obtained for Z1064 in this present study is therefore unreliable. When seed nutritional status was poor, the concentrations of all nutrients in Z1064 supplied with Zn increased with higher levels of *R. solani* to concentrations required

for normal growth. Since shoot dry weight was reduced, the higher concentration is most likely due to the nutrients present in a more concentrated form and not due to a higher rate of uptake compared with uninfected Z1064. For Z1064, not only was Zn-efficiency ranking irrelevant, but nutrient analysis was also inadequate in determining the cause for reduced growth when plants were derived from seed with low nutritional levels.

This experiment has also demonstrated the addition of Zn increases tolerance to disease providing the severity of disease is below a "critical" level. In this experiment a "critical" level of disease was reached between 8 and 32 propagules per kg of soil, 32 propagules resulting in reduced shoot growth. At this high level of disease, the addition of adequate nutrients is of no benefit to the plant.

A reduction in shoot growth caused by *R. solani* under conditions of adequate levels of nutrients has been observed in wheat by Kirkegaard *et al.* (1998). They observed a reduction in leaf elongation with infection of *R. solani* only when the disease score exceeded 6 on a 0-10 scale where 0 represents no infection. This range is equivalent to a disease score greater than 3 on the 0-5 scale used in this experiment with medic genotypes. Values greater than 3 on the disease scale correlate with the reduction in growth of all medic genotypes; in this study, a mean disease score of 4.5 under Zn deficiency and a score of 3.5 when Zn was applied was found. It has also been observed that 40% of the root system can be removed due to infection by *R. solani* from wheat seedlings before leaf growth is reduced (James *et al.*, 1997). In all medic genotypes, root length was reduced by more than 60% at the highest level of infection, at both levels of Zn.

As a consequence of disease score exceeding 3 on a 0-5 scale and with more than 60% of roots pruned by *R. solani*, most nutrients were deficient in the shoots when treated with the highest level of inoculum. Pruning of the root system by *R. solani* will therefore affect the nutrient status of the plant if the level of infection exceeds the "critical" point, even if adequate nutrients are present in the soil.

Previously, it was assumed that the pruning effect of the fungus results in nutrient deficiencies in the infected plant that in turn cause a reduction in shoot growth. However, since Kirkegaard *et al.* (1998) observed a reduction in leaf elongation despite adequate water and nutrient supply they hypothesised that a hormonal message is involved in reducing leaf growth at the higher levels of infection. The authors then suggested the direct hormonal signals were responsible for the reduction in shoot growth in plants infected with *R. solani*, challenging the conventional "supply" explanation for reduced growth. However, if hormonal control was solely responsible for directing shoot growth, it would be expected that all nutrients would be in adequate supply, despite reduced shoot growth. Although Kirkegaard *et al.* (1998) did not measure the concentration of any nutrient in the wheat plants used in their study, reduced nutrient concentration has previously been demonstrated in wheat infected by *R. solani* (Wall *et al.*, 1994). It may therefore be a combination of both reduced supply of water and nutrients to the shoot as roots are pruned by *R. solani* and a hormonal message that is responsible for the reduced shoot growth at higher levels of disease under nutrient-sufficient conditions.

When nutrients are limiting, eg. Zn deficiency in this experiment, the pruning effect of *R. solani* is exaggerated, resulting in greater levels of stress for the plant. Not only will this cause a more severe level of Zn deficiency for the plant, but a hormonal release may occur at lower levels of infection that may at least partly be responsible for reduction in shoot growth. The "critical" level of infection is therefore affected by the level of available nutrients, with the overall result being that plants deficient in Zn are more sensitive to infection by the fungus.

It should also be noted that although Kirkegaard *et al.* (1998) state the existence of direct signals, they suggest that the initial growth reduction caused by *R. solani* can not be overcome by agronomic treatments such as increased water or nutrient supply. However, the addition of Zn in a field situation was beneficial to wheat infected by *R. solani* (Thongbai *et al.*, 1993a). The addition of Zn has also been beneficial to medic infected with low levels of inoculum, resulting in improved tolerance to *R. solani* (see Chapter 5), and hence should not be ignored as a potential agronomic solution.

A "critical" level of infection, above which plants demonstrate visible shoot foliar symptoms, has implications for the ecology of *R. solani*. Infection by *R. solani* is visible as patches of stunted plants in the field, yet infection may also be observed on plants outside of patches that show no visible above-ground symptoms. Since patch expansion occurs during the growing season (MacNish *et al.*, 1993) those plants within patches are believed to have been infected early in the season as seedlings, which are more susceptible to disease. Plants showing symptoms on their roots but occurring outside of the patch are thought to have been infected later in the season when plants are less susceptible. The abruptness of the patch outline has been thought to be due to the positioning of the fungus in the soil early in the season when plants are most susceptible. However, it may be that patchiness is caused by higher concentration of the fungus present at levels exceeding the "critical" point for the plant at the beginning of the season or the presence of micronutrient deficiencies reducing the plants tolerance to infection. Many chemical properties of the soil have been compared inside and outside a patch including: CaCO₃, pH, total nitrogen, total organic carbon, total phosphorus, bicarbonate-extractable phosphorus, chloride, soil conductivity, water penetration, NH₄-nitrogen, NO₃-nitrogen, and soil bulk density (Rovira and Simon, 1985; MacNish, 1996), with no differences detected. However, Zn availability of the soil has not been tested, and it still remains to be seen if plants are more sensitive to disease in the patch due to higher inoculum level or a reduction in tolerance with the additional stress of Zn deficiency. Plants outside of patches may still be infected early in the season but if the level of infection is below this "critical" level, above ground symptoms will not be observed.

In conclusion, the greater root length of Zn-efficient cultivars improves the tolerance of medic to *R. solani* by maintaining adequate nutrition under Zn-deficient conditions. This effect may be more pronounced if the seed nutritional status was not a limiting factor in the growth of the plant. As discussed in Chapter 5, plants derived from seed with lower nutritional status demonstrate reduced tolerance to *R. solani*.

The data presented in this thesis provides the basis for understanding the relationship between Zn nutrition and *R. solani*, and for more effective control measures of *R. solani* through the use of a combination of Zn fertiliser and Zn-efficient medic cultivars. Some of these points are discussed in this chapter.

9.1 Improving Tolerance of *Medicago* species to *Rhizoctonia solani*

Resistance to *R. solani* has been observed in a number of species including rice, rapeseed, mustard, bean, and sugar beet (Lafitte *et al.*, 1984; Stockwell and Hanchey, 1985; Massaquoi and Rush, 1987; Yang *et al.*, 1992; Bugbee, 1993) where mechanical barriers and enzymatic inhibitors are involved. In this thesis, no such resistance was observed in medics to infection by *R. solani* on the root system, although it must be remembered that only five genotypes were screened.

The five genotypes screened in Chapter 8 all sustained a similar level of damage on their root systems, implying a similar amount of *R. solani* was present on each genotype. Resistance refers to the ability of the host to “limit the penetration, development, and/or reproduction of invading pathogens” (Graham and Webb, 1991). If a medic genotype was resistant to *R. solani*, the amount of visible damage would be lower than that for less resistant genotypes. However, tolerance which is measured by the ability of the host to “maintain its own growth or yield in spite of the infection” (Graham and Webb, 1991) was observed. Tolerance to *R. solani* was demonstrated in Chapters 5 and 8 and varied with the level of soil applied Zn, the genotype, and the nutritional quality of the seed sown.

9.1.1 Zn Application

Medic genotypes supplied with sufficient levels of Zn had a higher tolerance to disease than Zn-deficient genotypes. When medics were deficient in Zn, a reduction in dry matter production, root length and root surface area occurred, along with an increase in the concentration of most cations. The metabolic changes in Zn-deficient plants are quite complex owing to the large number of enzymes activated by Zn (Vallee and Auld, 1990). These include changes to the metabolism of carbohydrates, proteins, and auxins, impaired membrane integrity and changes in gene expression (see Chapter 2). The increase in membrane permeability with severe Zn deficiency resulted in an increase in the concentration of B, Cu, Fe, Mg, S, and P in the shoots of medics. Increased cell membrane permeability is also an initial host response to infection by *R. solani* (Weinhold and Sinclair, 1996), along with pruning of the root system, resulting in reduced surface area. Zinc-deficient plants may be less tolerant to infection by *R. solani* due to both Zn deficiency and *R. solani* affecting membrane permeability, root length and root surface area. Changes in the root geometry with Zn deficiency may be more important in tolerance to *R. solani* as a lower surface area is available for uptake of the limiting nutrient, in this case soil-immobile Zn.

9.1.2 Genotypic Variation

Genotypic variation was observed with two Zn-efficient genotypes, as determined in Chapter 7 (SA20248 and SA20521), maintaining a stable nutrient concentration in the shoots under Zn deficiency when exposed to *R. solani* (excluding severe levels of infection). Greater tolerance to infection by *R. solani* was also observed in SA20521 with adequate levels of soil Zn. Zinc-inefficient genotypes were more sensitive to infection by *R. solani* when deficient in Zn, with a parallel reduction in concentration of most nutrients in the shoot. This indicates root pruning by *R. solani* is affecting nutrient uptake to a point where plant growth is affected more severely in Zn-inefficient genotypes.

9.1.3 Seed Quality

Rhizoctonia solani infects plants early in the seedling stage when the developing plant is reliant, at least partly, on seed reserves. Plants derived from seed with a high nutritional value may therefore be more tolerant to early infection as they are more vigorous and have a higher growth than seed with a lower nutritional content. This was demonstrated in Chapter 8 when the nutritional quality of the seed sown was shown to have an effect not only on the tolerance to *R. solani* under Zn-deficient conditions, but also on tolerance under non-limiting conditions. Medics derived from seed with lower nutritional value had a lower maximum growth, were more sensitive to Zn deficiency (and therefore being less Zn-efficient) and were less tolerant to infection by *R. solani*. By maintaining adequate levels of nutrients in the growing pasture, in particular Zn since its deficiency is so wide-spread, the quality of seed set for regeneration will assist in maintaining tolerance to *R. solani* at the most sensitive stage of infection in the following generation.

9.2 Mechanisms for Increased Tolerance

Zinc, applied at levels to achieve adequate growth of wheat, does not reduce saprophytic fungal growth (Thongbai, 1993). This, combined with the greater amount of *R. solani* on the roots of Zn-sufficient plants in Chapter 5 excludes the possibility of Zn being at levels toxic to the fungus. The mechanism of Zn related increase in tolerance to *R. solani* is therefore due to the role of Zn in the physiology of the medic plant.

Thongbai *et al.* (Thongbai *et al.*, 1993a; Thongbai *et al.*, 1993b) hypothesised that the effect of Zn deficiency on root membrane leakage is involved in the susceptibility of Zn-deficient wheat to infection by *R. solani*. They suggested that the greater quantity of root exudates from Zn-deficient plants may attract and promote infection by *R. solani* compared with Zn-sufficient

plants. If this were occurring, a lower amount of *R. solani* would be present on plants with adequate levels of Zn. As already mentioned, this did not occur (Chapter 5).

The increase in fine root length that occurred under low levels of infection in Zn-deficient medic plants only, suggests Zn-deficient medics are compensating for the pruning effect of *R. solani*. Medics supplied with Zn generally have more than enough roots to supply the Zn requirements to the plant, and therefore overcome any potential nutrient deficiencies. At severe levels of infection, Zn-deficient plants are unable to compensate by increasing their nutrient-scavenging power and deficiencies in other nutrients can occur. If the plant is stressed to a greater extent, for example by originating from the seed of low nutritional status, the effect of infection by *R. solani* is exacerbated in both Zn-deficient and Zn-sufficient medics.

9.3 The Potential for Future Breeding of Zn Efficiency in Medics

Breeding for micronutrient-efficient crops, including Zn efficiency, will assist in alleviating micronutrient deficiencies not only in the human population but also in other grain-fed animals. To date, applying modern breeding techniques to adapt annual medic plants to soils of low nutritional status has not been attempted. In breeding for improved Zn efficiency for example, not only will the amount of pasture be improved so will the quality of the pasture, alleviating any potential Zn deficiency disorders in foraging ruminants. In order to breed for Zn-efficient medics a number of factors need to be considered. According to Graham (1984), to breed for nutritional characteristics, in this case Zn, it is necessary to demonstrate that:

- the soil is capable of supplying Zn to an improved genotype;
- genetic diversity of Zn efficiency exists; and
- sound agronomic and economic reasons exist for pursuing a breeding solution to Zn deficiency.

Firstly, in considering soil availability of Zn, it has already been demonstrated that Zn deficiency is largely due to availability of Zn and not the absolute amount of Zn present in the

soil (Graham, 1978), with the total amount of soil-Zn adequate for thousands of crops. The remaining two factors are discussed in detail below.

9.3.1 Genetic Diversity for the Zn Efficiency trait in Medics

Genetic diversity for Zn efficiency in medics was demonstrated in the field (Chapter 6) and under controlled conditions (Chapter 7 and 8) with *M. tornata* and *M. littoralis* consistently Zn-efficient. Both species are predominantly found naturally on sandy soils, such as coastal regions (Lesins and Lesins, 1979), with the genotypes tested in the thesis specifically selected from trials on light, sandy soils in South Australia (A. Lake, pers. comm.). This implies that Zn efficiency is an important trait in this environment. Genotypes from *M. tornata* (specifically SA20248, SA20278 and SA20521) appear to be Zn-efficient by virtue of their larger and more extensive root system. These genotypes are also able to extract a high amount of Zn per plant from the soil and yield well on Zn-deficient soils. Their maximum yield potential, however, is only obtained under adequate levels of Zn. Cultivar Rivoli on the other hand was able to reach maximum yield potential under Zn-deficient conditions, but its potential was low. These results imply at least two mechanisms for Zn efficiency can occur in *Medicago* spp. and were observed in only one screening for Zn efficiency. With a broader screening for Zn efficiency a number of other mechanisms may be observed. Crossing these two types (eg. SA20278 and Rivoli) with the aim of isolating progeny with all three factors: a high uptake of Zn, high yield potential and the capacity to reach maximum yield potential under Zn-deficient conditions, is a reasonable expectation.

Theoretically, it is also possible to incorporate the Zn-efficiency genes that have been identified in rye into a medic genotype. Studies on rye chromosomes are currently underway as a potential source of micronutrient efficiency genes for wheat. For example, a wheat-rye chromosome translocation (4B/5R) has increased the Mn and Fe shoot concentration from that of the parent wheat line by almost 100%, and the concentration of Zn by 15% (Cakmak *et al.*, 1997a). Zinc efficiency genes in rye appear to exist predominantly on chromosomes 1R and 7R (Cakmak *et al.*, 1997a), with addition lines of wheat involving the 1R chromosome having

a higher shoot dry weight compared with the parent wheat line under Zn-deficient conditions (Cakmak *et al.*, 1997a). Improved Zn efficiency of wheat, by transfer of rye chromosomes, was related to the total amount of Zn per shoot, suggesting a greater genetic ability to take up Zn from Zn-deficient soils (Cakmak *et al.*, 1997a). The authors also demonstrated that the higher efficiency of rye for Zn uptake was only partly transferred into wheat.

Genetic engineering in perennial medics to improve the nutritive value is already being performed. For example, Tabe *et al.* (Tabé *et al.*, 1995) incorporated chimeric genes encoding a ruminally stable, sulfur amino acid-rich protein from sunflower seeds into lucerne. Although the gene was expressed, the level of sulfur amino acids were not sufficient for the required purposes and more work is required.

Considering the amount of variation observed in this thesis with such a small number of genotypes, further exploration of the large germplasm pool available in medics (annual and perennial), may be the most efficient means of developing Zn-efficient genotypes in the short term.

9.3.2 Agronomic and Economic Reasons for Pursuing a Breeding Solution to Zn Deficiency in Medics

Currently, many medic pastures are deficient in Zn, results from Chapter 4 and 6 demonstrating recommended levels of Zn fertiliser are inadequate for maintaining optimum growth in medics. As discussed in Chapter 1, Zn deficiency in medics is an ongoing problem, and can have quite a severe impact on profitability, both through reductions in pasture and crop yield and through additional fertiliser costs needed to correct deficiencies. Hence, breeding for Zn efficiency in medics will reduce Zn fertiliser costs. Breeding for plants which are able to explore a greater area of the soil will also benefit other diffusion-limited nutrients and therefore reduce the cost of micronutrient fertilisers even further.

Although more work is required, results from Chapter 8 suggest Zn-efficient genotypes are more tolerant to *R. solani* under Zn deficiency. With Zn-efficient genotypes, a higher amount of Zn is also expected in the seed, improving seed quality and therefore increasing seedling vigour and tolerance to disease further in the subsequent generations. These genotypes will be better able to persist and yield well despite infection by *R. solani*, thus leading to greater farming profitability.

Medics are the main source of essential mineral elements required by grazing ruminants, and it therefore follows that improving the Zn content of the forage and seed will assist in overcoming Zn deficiency in animals. Marginal Zn deficiency is typically observed in sheep and cattle on pastures containing less than 20 mg Zn/kg and is characterised by suboptimum growth, reduced fertility and mild skin disorders (White, 1993). As White (1993) points out, it should be of evolutionary significance that the concentration of Zn required for optimum pasture growth is similar to the requirements for sheep growth. It therefore follows that a Zn-deficient medic pasture results in Zn-deficient sheep and/or cattle. Breeding for Zn-efficient medics will prevent this from occurring.

9.4 How Does the Zn-*R. solani* Interaction Relate to the "Medic Decline" Currently Occurring Across the State?

This thesis has found the tolerance to infection by *R. solani* is substantially reduced in Zn-deficient medics and in medics derived from seed with lower nutritional value. Poor performance of medic-based pastures is currently being studied extensively in Australia with field surveys and field trials supporting the work in this thesis with a combination of soil infertility and root diseases involved in the "medic decline" (Gillet *et al.*, 1998).

Setting aside for a moment other factors influencing the growth of medics in the field and considering only Zn deficiency and infection by *R. solani*, one possible scenario contributing to the medic decline is that Zn-deficient medics with a reduced tolerance to *R. solani* will suffer

greater Zn deficiency due to the root pruning effect of the pathogen. Other micronutrients will also be reduced, limiting growth, seed yield and the nutritional status of the seed. Regenerating pastures will therefore grow from seed with a lower nutritional value leading to further reduction in tolerance to *R. solani* and hence an accelerated decrease in quality of medic pasture. In order to break this cycle, re-sowing with nutritionally-sufficient seed of Zn-efficient medic genotypes, along with fertilisation of Zn (assuming all other nutrients are in sufficient supply) will be necessary. This will result in medics with a greater tolerance to *R. solani* leading to improved pasture growth and seed with adequate levels of nutrients. The higher amount of organic matter in the soil produced by improved root systems of pastures may also promote population growth of other organisms which will compete with *R. solani*. After a number of years, not only will seed nutrition be maintained for the regenerating pasture, but the suppressiveness of the general microbial population will take effect and a reduction in inoculum potential of *R. solani* may result.

Micronutrient disorders and root diseases have largely gone unnoticed or ignored in pastures with Gillet *et al.* (1998) highlighting low farmer expectations for their pastures. If farmers are unable to recognise poor pasture performance or are unaware of the maximum yield potential possible this therefore raises the question: do farmers need to be re-educated about maintaining adequate pasture nutrition?

Suggestions for Future Research

Applied Research

- Screening for Zn efficiency in a wider range of closely related medic cultivars, in particular *M. tornata* genotypes;
- Determine the effect of seed nutrition on growth of Zn-efficient medics, their response to Zn (and other micronutrients) and the effect on Zn efficiency in more detail;

- Study the efficacy of new seed Zn coatings in relation to other forms of Zn application (soil, seed content, foliar spray);
- Determine the extent of Zn deficiency in pastures in South Australia (and other micronutrient deficiencies);
- Determine the effect of Zn efficiency on seed Zn content;
- Determine conclusively if Zn-efficient medic genotypes are more tolerant to *R. solani*;

Basic Research

- Determine how Zn is taken up from the soil;
- Quantify the effect of Zn in the seed on tolerance to *R. solani*;
- Determine if root exudates from Zn-efficient and Zn-inefficient genotypes under Zn-deficient and Zn-sufficient conditions influence infection by *R. solani*;
- Understand the genetic control underlying Zn efficiency in medics.

This thesis is the first systematic attempt to investigate the relationship between Zn nutritional status and the degree of infection by *R. solani* in *Medicago* spp. in field and controlled experiments, and genotype effects. From the results of all experiments described from Chapter 4 to 8 it may be concluded that:

- a) A correlation was established in the field, where Zn deficiency and *R. solani* occur concurrently, between Zn concentration in the shoot and the disease score on the roots.
- b) In controlled experiments, the addition of Zn to Zn-deficient soils reduced the above-ground symptoms caused by *R. solani* on medics. Application of Zn above an adequate amount for plant growth did not assist tolerance of medics to *R. solani* any further.
- c) Zinc application does not reduce the amount of *R. solani* on the root system. The amount of infection by *R. solani* is higher on medics supplied with adequate levels of Zn. This indicates medics benefit with an increase in tolerance to *R. solani* with Zn application, and not resistance as was hypothesised initially.
- d) The pruning effect by *R. solani* on the root system, if severe enough, will result in reduced nutrient uptake, and most likely water uptake as well. This effect is enhanced when plants are deficient in Zn. However, at lower levels of infection, medics are able to maintain a stable nutrient composition in the shoots.
- e) Improved tolerance to the disease appears to be related to the increase in root length and surface area that occurs with Zn application. This may allow for improved compensation for nutrient and water uptake in the presence of *R. solani* by uninfected root axes.
- f) If an additional stress is present, eg. by low seed nutrition or reduced quality of light, lower nutrient concentrations occur despite adequate Zn supply.

- g) Genetic variation exists within *Medicago* spp. with *M. tornata* genotypes appearing more Zn-efficient than other species. *Medicago tornata* genotypes tested in this thesis were specifically selected from trials on light, sandy soils in South Australia, implying that Zn efficiency is an important trait in sandy soil environments.
- h) Under Zn deficiency, Zn-efficient genotypes were able to maintain a stable concentration of all nutrients in the shoots in the presence of *R. solani* (excluding severe levels of infection), while nutrient concentration in Zn-inefficient genotypes decreased.
- i) Seed nutritional status is an important factor in maintaining tolerance to *R. solani*, with low seed nutrient status increasing sensitivity of young seedlings to Zn deficiency and infection by *R. solani*.

Appendix 1 Yeast Mannitol Agar - for *Rhizobium meliloti*

Ingredients	1 Litre	Stock Solution
Mannitol	5.0 g	
Sodium Glutamate	0.5 g	
Na ₂ HPO ₄ .12H ₂ O	10 ml from stock	36 g/L distilled H ₂ O
MgSO ₄ .7H ₂ O	10 ml from stock	10 g/L distilled H ₂ O
CaCl ₂ .6H ₂ O	1 ml from stock	79.1 g/L distilled H ₂ O
FeCl ₃ .6H ₂ O	1 ml from stock	6.67 g/L distilled H ₂ O
Agar	20.0 g	
Yeast Extract	0.5 g	
Distilled H ₂ O	960 mL	

Sterilise at 15 psi for 20 minutes

Appendix 2 Acid Washing Procedure

1. Pyroneg wash, scrub with brush;
2. Rinse with tap water three times;
3. Rinse with RO water three times;
4. Rinse with MilliQ water;
5. Place in 10% HNO_3 for at least 24 hours;
6. Rinse with MilliQ water three times;
7. Dry in oven at 40°C.

Note: not suitable for metal

Appendix 3 Media used for growing *Rhizoctonia solani*

Neutral Dox Yeast (NDY) (Warcup, 1955) plus antibiotics

NaNO ₃	2 g/L	
KH ₂ PO ₄	1 g/L	
MgSO ₄	0.5 g/L	
KCl	0.5 g/L	(autoclave 20 minutes at 121°C, 100kPa)
FeSO ₄	0.01 g/L	
Yeast Extract	0.05 g/L	
Sucrose	30 g/L	
Agar (oxoid No.1)	15 g/L	
Water	1 L	

1/6 NDY represents one-sixth of all ingredients above, except for the agar which is full strength.

Mix NaNO₃
 KH₂PO₄
 MgSO₄
 KCl
 FeSO₄

- Melt agar in microwave, or autoclave, with 500 mL water.
- Mix the above nutrients in the remaining 500 mL of water and add to the agar solution
- autoclave for 20 minutes at 121°C, 100kPa

Streptomycin sulphate 100 µg/mL
 Tetracycline hydrochloride 50 µg/mL

- As solution cools, but before it sets, add antibiotics (Streptomycin sulphate and Tetracycline hydrochloride)

Appendix 4 Effect of increasing inoculation with *R. solani* on the concentration of nutrients in the shoots and roots, relative transport of nutrients to the shoot and the total uptake of nutrients per gram of root for Caliph grown in experiment 4.1 with adequate nutrient supply.
ns = not significant, * = significant at 10%, ** = significant at 5%

Inoculum (prop./kg)	MEAN SHOOT NUTRIENT CONCENTRATION (mg/kg)											
	Fe	Mn	B	Cu	Mo	Zn	Ca	Mg	Na	K	P	S
day 14												
0	111.04 a	84.55 a	37.68 a	9.54 a	55.5 a	30.82 a	6713 a	3508 a	360 a	54450 a	10687 a	4268 a
1	123.17 a	77.78 a	41.86 a	11.06 ab	67.5 a	33.10 a	7932 a	3751 a	341 a	57679 a	10573 a	4148 a
8	112.72 a	50.93 a	40.74 a	12.99 b	45.0 a	39.85 a	8402 a	3975 a	463 b	60742 a	10400 a	4004 a
ANOVA	ns	ns	ns	*	ns	ns	ns	ns	**	ns	ns	ns
day 28												
0	77.24 a	240.71 a	49.09 a	16.62 a	68.25 a	42.88 a	24174 a	5550 a	324 a	55204 a	13934 a	5053 a
1	72.69 ab	197.07 a	50.60 a	16.52 a	69.50 a	44.86 a	26183 a	5494 a	335 a	50885 a	15196 a	4809 ab
8	64.96 b	108.54 b	49.05 a	17.99 a	54.75 b	45.26 a	24298 a	5163 a	282 a	51449 a	11062 b	4280 b
ANOVA	*	**	ns	ns	**	ns	ns	ns	ns	ns	**	**
day 42												
0	27.95 a	204.53 a	29.74 a	12.98 a	48.19 a	36.73 a	25787 a	4616 a	285 a	38648 a	5725 a	2724 a
1	35.19 a	103.88 b	29.72 a	11.53 a	49.63 a	37.19 a	34197 b	3877 b	302 ab	36575 a	4767 a	2724 a
8	30.70 a	94.27 b	30.70 a	13.28 a	57.57 b	42.92 a	34013 b	4104 b	321 b	38867 a	5251 a	3048 a
ANOVA	ns	**	ns	ns	**	ns	**	**	*	ns	ns	ns
day 56												
0	37.93 a	108.94 a	12.13 a	9.74 a	50.78 a	30.36 a	28721 a	2971 a	170 a	20917 a	3031 a	2246 a
1	34.20 a	79.55 b	11.69 a	9.26 a	44.85 ab	31.57 a	33687 a	2840 a	181 a	19401 a	2715 a	2082 a
8	28.72 a	69.04 b	11.08 a	7.31 a	36.43 b	31.51 a	31583 a	2564 a	169 a	18977 a	2553 a	2081 a
ANOVA	ns	**	ns	ns	*	ns	ns	ns	ns	ns	ns	ns

Appendix 4 (cont)

Inoculum (prop./kg)	MEAN ROOT NUTRIENT CONCENTRATION (mg/kg)											
	Fe	Mn	B	Cu	Mo	Zn	Ca	Mg	Na	K	P	S
day 14												
0	346.21 a	500.47 a	19.23 a	36.95 a	196.0 a	74.11 a	4732 a	1108 a	682 a	58987 a	9628 a	5491 a
1	300.12 a	235.07 a	12.55 a	32.38 ab	130.5 b	64.57 a	3411 a	809 b	639 a	40346 b	6190 b	3483 b
8	274.21 a	114.02 a	13.02 a	25.85 b	91.5 b	47.61 a	3168 a	832 b	607 a	38328 b	5463 b	3354 b
ANOVA	ns	ns	ns	*	**	ns	ns	*	ns	**	**	**
day 28												
0	288.71 a	1379.02 a	19.11 a	65.33 a	86.5 a	176.75 ab	8829 a	2186 a	489 a	41861 a	8767 a	5316 a
1	427.05 a	1617.34 a	22.38 a	69.13 a	31.5 a	201.80 a	11123 a	2768 a	241 a	9196 a	7242 a	2975 a
8	287.03 a	653.92 b	20.49 a	63.21 a	61.75 a	140.43 b	8762 a	2478 a	390 a	36832 a	8878 a	4717 a
ANOVA	ns	**	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
day 42												
0	203.93 a	642.78 a	15.04 a	59.51 ab	231.74 a	109.41 a	10431 a	2422 a	1137 a	46515 a	6631 a	6104 a
1	335.57 a	356.14 a	14.10 a	69.49 a	195.97 a	111.98 a	14038 a	3322 b	1424 a	44609 a	6660 a	5581 a
8	238.54 a	450.91 a	15.38 a	52.97 b	132.25 b	154.59 b	10942 a	3275 b	1198 a	48001 a	6535 a	5835 a
ANOVA	ns	ns	ns	*	**	*	ns	*	ns	ns	ns	ns
day 56												
0	261.06 a	205.28 a	9.59 a	47.93 a	112.01 a	43.92 a	19571 a	3060 a	967 a	16006 a	2867 a	7911 a
1	400.37 a	167.30 a	9.36 a	51.31 a	96.9 a	49.48 a	27651 a	3887 a	911 a	16039 a	2960 a	8465 a
8	1250.08 a	268.63 a	11.99 a	59.55 a	89.33 a	85.17 b	23598 a	5255 a	1252 a	20310 a	4042 a	5621 a
ANOVA	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns

Appendix 4 (cont)

[illegible]

Appendix 4 (cont)

Inoculum (prop./kg)	MEAN TOTAL UPTAKE ($\mu\text{g/g}$ root)											
	Fe	Mn	B	Cu	Mo	Zn	Ca	Mg	Na	K	P	S
day 14												
0	557.36 a	661.45 a	90.23 a	55.08 a	300.52 a	132.74 a	17317 a	7718 a	11363 a	161343 a	29856 a	13557 a
1	491.92 a	353.83 a	76.65 a	49.47 a	232.75 ab	115.45 a	15558 a	6568 a	1168 a	128002 a	22462 a	9871 a
8	452.83 a	195.73 a	77.61 a	46.60 a	162.16 b	110.26 a	16475 a	7170 a	1346 a	135189 a	22098 a	9711 a
ANOVA	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns
day 28												
0	387.51 ab	1580.21 ab	130.51 a	98.03 a	310.04 a	260.68 a	68700 a	15675 a	1381 a	212927 a	45405 ab	19541 a
1	651.23 a	2227.06 a	178.67 b	120.17 b	245.53 b	340.35 b	91777 b	19722 b	1275 a	166492 b	54133 a	17827 a
8	389.91 b	647.55 b	153.85 ab	100.47 ab	248.67 b	229.44 a	73377 a	15659 a	1285 a	200370 a	37775 b	17875 a
ANOVA	**	**	*	*	**	**	**	**	ns	**	*	ns
day 42												
0	332.57 a	1593.57 a	154.32 a	119.89 a	445.76 a	280.32 a	129966 a	23862 a	2464 a	226485 a	33436 a	18807 a
1	454.07 a	707.52 b	114.84 a	108.36 a	388.64 a	237.69 a	128785 a	16425 b	2444 a	168150 b	22792 b	14800 b
8	352.23 a	800.11 b	128.59 a	101.88 a	311.34 b	312.51 a	137993 a	18426 a	2385 a	191640 ab	25942 ab	17119 ab
ANOVA	ns	**	ns	ns	**	ns	ns	**	ns	**	*	**
day 56												
0	381.68 a	545.06 a	47.50 a	79.43 a	274.24 a	140.85 ab	111733 a	12410 a	1501 a	81490 a	12445 a	15113 a
1	486.80 a	372.03 a	38.96 a	75.73 a	217.62 a	130.84 a	116307 a	11110 a	1372 a	64843 a	9909 a	13964 a
8	1342.06 a	489.10 a	47.37 a	82.82 a	205.27 a	185.83 b	124314 a	13442 a	1792 a	80871 a	12185 a	12263 a
ANOVA	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns

Appendix 5 Binomial test for the field survey data from Experiment 4.2

The binomial test may be applied to determine whether or not a two-category data distribution is unbiased. It tests whether or not the observed proportion of individuals from one category falls within the confidence limits of a binomial distribution.

In this case, the plants were either infected by *R. solani* or not. This test was used to determine if a randomly selected plant was as equally likely to be infected or not, which would indicate an unbiased sampling procedure. The null hypothesis was therefore that infection by *R. solani* was equally as likely as no infection.

So if p is the probability of infected of a given sample, then

$$H_0: p = 0.5; H_1: p \neq 0.5$$

From the field survey, the total number of plants sampled:

$$N = 1045$$

The number of observations of infected plants:

$$X = pN$$

The confidence limits are used as critical values (Zar, 1984). The lower confidence limit is

$$L_1 = \frac{X}{X + (N - X + 1)F_{\alpha(2), v_1, v_2}},$$

where $v_1 = 2(N - X + 1)$

and $v_2 = 2X$

The upper confidence limit is

$$L_2 = \frac{(X + 1)F_{\alpha(2) v_1' v_2'}}{N - X + (X + 1)F_{\alpha(2) v_1' v_2'}},$$

where $v_1' = 2(X + 1) = v_2 + 2$

and $v_2' = 2(N - X) = v_1 - 2$

Evaluating these gives:

$$X = 522.5$$

$$v_1 = 1043$$

$$v_2 = 1045$$

$$v_1' = 1047$$

$$v_2' = 1041$$

From table B4 (Zar, 1984),

$$F_{0.05(2), 1043, 1045} \approx F_{0.05(2), \infty, \infty} = 1$$

$$F_{0.05(2), 1047, 1041} \approx F_{0.05(2), \infty, \infty} = 1$$

The lower confidence limit is thus

$$L_1 = 0.49952$$

The upper confidence limit is

$$L_2 = 0.50048$$

From the field survey data, the number of plants infected by *R. solani*, $X = 884$

Hence

$$\hat{p} = 884 / 1045 = 0.846$$

As this estimate lies outside the range $0.49952 \leq \hat{p} \leq 0.50048$, we reject the null hypothesis H_0 . This set of data is distributed with bias towards *R. solani* infected plants.

Appendix 6 Correlation Matrix for field survey, Experiment 4.2. Numbers in bold are significant with the critical value at 5% ≥ 0.22

plants/samp.	-															
root length	0.10	-														
disease score	-0.30	-0.46	-													
shoot dry wt	0.03	0.18	0.03	-												
root dry wt	-0.07	0.16	0.12	0.10	-											
shoot [Fe]	-0.06	-0.05	-0.21	-0.16	-0.15	-										
shoot [Mn]	0.28	0.12	-0.49	-0.18	-0.30	0.43	-									
shoot [B]	-0.27	-0.35	0.11	-0.13	-0.06	0.45	0.01	-								
shoot [Cu]	0.03	0.13	-0.16	0.19	0.04	0.06	0.36	-0.04	-							
shoot [Zn]	0.10	0.24	-0.47	-0.12	-0.08	0.16	0.43	-0.22	0.24	-						
shoot [Ca]	-0.10	0.33	-0.07	0.07	0.09	0.11	-0.10	0.13	-0.10	-0.18	-					
shoot [Mg]	-0.09	-0.39	0.32	-0.09	0.00	0.12	-0.02	0.51	0.01	-0.41	0.33	-				
shoot [Na]	0.25	-0.37	0.04	-0.05	-0.08	-0.09	0.05	0.15	0.03	0.04	-0.62	0.00	-			
shoot [K]	-0.08	0.34	-0.16	-0.03	0.15	0.13	-0.06	0.14	-0.08	-0.02	0.59	0.01	-0.51	-		
shoot [P]	0.09	-0.32	0.04	-0.20	-0.12	0.25	0.33	0.41	0.38	0.02	-0.16	0.54	0.24	-0.09	-	
shoot [S]	0.08	-0.22	-0.17	-0.20	-0.20	0.27	0.58	0.06	0.35	0.31	-0.32	0.28	0.40	-0.34	0.51	-
	plants/ sample	root length	disease score	shoot dry wt	root dry wt	shoot [Fe]	shoot [Mn]	shoot [B]	shoot [Cu]	shoot [Zn]	shoot [Ca]	shoot [Mg]	shoot [Na]	shoot [K]	shoot [P]	shoot [S]

Appendix 7 Summary of statistical analysis on the dry weight and Zn concentration on the shoot tissue and seed at days 43, 81, 117 and final harvest. Analysis used a Wald test to compare the effect of large and small seed sown at Lameroo in 1996, with 2 levels of soil applied Zn (0 and 8 kg/ha) for Caliph and Paraggio medics.

ns - not significant; * $p \leq 0.05$

Interaction	day 43		day 81		day 117	seed	
	shoot	[Zn]	shoot	[Zn]	shoot	dry	[Zn]
	DW		DW		DW	weight	
cultivar x seed size x Zn	ns	*	ns	ns	ns	ns	ns
cultivar x seed size	ns	ns	*	ns	ns	*	ns
cultivar x Zn	*	ns	ns	ns	*	*	ns
seed size x Zn	ns	*	ns	ns	ns	ns	ns
cultivar	ns	ns	ns	ns	ns	ns	ns
seed size	*	ns	ns	ns	ns	ns	ns
Zn	ns	*	*	*	ns	ns	*

Appendix 8a - mean shoot concentration of 10 elements for 19 medic cultivars grown in Mt Compass sand with four levels of soil applied Zn (see Chapter 7).

	Zn	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight				g/kg shoot dry weight					
Caliph	0	93	214	59	16	6	33	7	41	25	9
	0.1	61	209	40	16	15	30	6	50	12	5
	0.3	51	183	34	13	24	46	5	51	7	4
	0.9	49	282	30	15	61	31	5	39	8	3
SA12591	0	79	144	45	15	8	34	6	35	10	5
	0.1	50	91	25	13	12	26	5	33	9	2
	0.3	57	85	20	11	15	27	4	26	4	3
	0.9	60	122	21	13	37	26	4	26	6	3
SA18289	0	89	232	42	21	10	34	7	35	26	8
	0.1	56	155	21	18	13	26	6	35	10	4
	0.3	54	145	24	14	17	32	5	28	4	4
	0.9	57	159	20	13	34	30	4	25	4	4
Z942	0	41	129	34	8	8	23	5	33	8	3
	0.1	52	138	36	12	10	29	6	40	12	4
	0.3	62	107	22	14	16	36	5	31	8	4
	0.9	53	141	22	15	36	39	5	29	8	5
Rivoli	0	64	133	37	26	13	37	5	35	9	9
	0.1	59	141	38	26	19	29	5	38	9	9
	0.3	53	155	41	27	33	38	5	37	6	11
	0.9	63	195	43	23	80	41	5	40	6	11
Tornafeld	0	60	148	50	18	11	26	6	35	14	4
	0.1	39	157	41	20	16	36	6	36	7	3
	0.3	43	190	39	20	27	23	5	39	9	4
	0.9	56	227	39	20	65	32	5	36	7	4
SA20278	0	86	220	43	33	13	41	8	27	12	4
	0.1	45	216	35	28	18	40	6	26	6	3
	0.3	47	228	33	31	34	49	6	25	7	4
	0.9	48	234	34	26	72	43	5	26	8	4
SA20248	0	79	231	55	26	13	58	8	31	22	4
	0.1	33	212	38	20	20	33	6	28	9	3
	0.3	34	217	39	23	35	36	6	29	7	4
	0.9	50	353	38	28	92	47	6	29	9	4

Appendix 8a (cont.)

	Zn	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight				g/kg shoot dry weight					
SA20521	0	53	255	48	30	12	39	8	35	9	3
	0.1	52	182	27	26	16	29	6	29	5	2
	0.3	56	208	30	31	29	34	6	34	8	4
	0.9	68	316	31	33	85	41	6	33	8	4
Harbinger	0	65	238	47	20	11	26	5	38	17	6
	0.1	64	164	43	17	13	26	5	41	8	6
	0.3	64	165	36	16	20	21	4	35	5	6
	0.9	64	201	38	14	56	32	4	39	7	7
Herald	0	59	183	56	15	9	26	5	34	19	6
	0.1	58	194	49	17	10	28	5	35	12	6
	0.3	59	224	45	20	22	27	4	44	11	7
	0.9	66	265	43	17	54	27	4	44	10	8
SA15485	0	84	154	47	13	9	16	6	36	10	4
	0.1	64	142	42	11	12	22	5	40	9	4
	0.3	56	134	36	10	20	22	4	39	6	4
	0.9	67	152	38	11	39	27	4	39	6	4
Santiago	0	104	145	71	15	11	20	7	34	19	6
	0.1	60	133	59	15	17	24	5	40	10	5
	0.3	58	146	50	18	29	22	4	39	7	5
	0.9	68	173	55	19	73	25	4	38	8	5
SA4188	0	67	110	47	12	8	16	5	21	14	5
	0.1	95	124	51	15	15	27	5	32	11	5
	0.3	63	134	39	17	26	29	4	29	7	5
	0.9	81	160	37	20	83	26	4	29	8	5
Z1064	0	73	224	57	19	9	23	6	31	29	5
	0.1	77	212	49	19	13	35	5	33	18	5
	0.3	68	174	36	15	24	24	4	35	6	5
	0.9	73	220	37	15	66	30	4	36	6	6

Appendix 8a (cont)

	Zn mg/kg	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight					g/kg shoot dry weight				
Z1065	0	60	198	45	19	10	24	5	35	16	6
	0.1	58	142	37	16	13	31	4	35	13	5
	0.3	67	116	27	13	20	21	3	35	7	4
	0.9	72	181	27	13	57	24	3	36	7	5
Z1068	0	63	230	58	17	8	26	5	30	20	5
	0.1	71	213	49	17	12	34	5	32	11	5
	0.3	78	169	37	15	23	20	4	39	10	5
	0.9	61	251	38	15	61	21	4	38	7	5
Sava	0	84	157	45	14	10	31	5	33	11	5
	0.1	60	101	26	12	14	37	4	35	5	4
	0.3	57	93	24	12	16	42	4	31	4	3
	0.9	66	98	21	13	52	44	4	30	4	2
Paraponto	0	98	299	86	26	12	27	6	41	21	7
	0.1	81	188	53	18	14	27	5	55	11	7
	0.3	60	185	46	16	23	22	4	43	7	6
	0.9	60	230	51	15	60	24	4	42	9	8
Zn		**	**	**	**	**	ns	**	**	**	**
cultivar		**	**	**	**	**	**	**	**	**	**
Zn by cultivar		**	ns	**	**	**	**	**	**	ns	**

ns- not significant, * $p \leq 0.05$, ** $p \leq 0.01$

Appendix 8b - mean root concentration of 10 elements for 19 medic cultivars grown in Mt Compass sand with four levels of soil applied Zn (see Chapter 7).

Cultivar	Zn	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight				g/kg shoot dry weight					
Caliph	0	296	1367	24	84	20	9	3	24	5	4
	0.1	198	2273	18	109	38	9	2	59	6	7
	0.3	213	888	13	59	42	7	2	29	5	4
	0.9	193	1237	17	68	182	11	2	67	8	8
SA12591	0	214	1393	18	17	27	9	3	55	5	6
	0.1	1846	800	12	47	22	10	5	43	5	5
	0.3	467	480	12	40	25	22	7	30	4	10
	0.9	376	612	13	44	63	17	5	34	7	10
SA18289	0	167	1699	19	84	29	8	3	54	9	10
	0.1	254	957	16	65	26	14	4	70	5	11
	0.3	230	527	11	43	27	20	6	41	4	11
	0.9	327	438	13	46	63	28	7	38	4	14
Z942	0	323	1531	20	94	29	8	3	45	7	8
	0.1	432	1344	15	97	29	12	5	59	5	11
	0.3	325	627	11	67	28	21	6	36	7	12
	0.9	620	551	12	50	55	19	6	35	8	14
Rivoli	0	206	1140	16	98	22	6	3	61	6	18
	0.1	2184	1006	16	105	33	7	3	68	8	18
	0.3	173	997	15	91	44	6	3	75	7	20
	0.9	659	863	14	85	113	6	3	75	6	20
Tornafeld	0	208	1099	16	85	22	6	3	54	6	8
	0.1	2518	743	15	79	27	9	3	65	7	8
	0.3	204	765	15	95	36	7	3	64	10	9
	0.9	1694	635	14	80	78	7	4	62	7	9
SA20278	0	309	1155	15	84	23	9	3	59	5	11
	0.1	216	675	12	82	24	12	6	70	5	14
	0.3	2210	941	17	111	53	16	8	92	15	19
	0.9	474	509	12	65	118	7	5	65	7	12
SA20248	0	202	1552	16	126	25	8	2	56	9	9
	0.1	224	1019	13	93	32	9	3	61	8	9
	0.3	1333	670	16	64	43	10	3	63	5	11
	0.9	354	1108	14	80	150	9	4	74	8	10

Appendix 8b (cont.)

Cultivar	Zn	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight					g/kg shoot dry weight				
SA20521	0	437	1615	18	126	33	10	3	54	5	9
	0.1	252	760	13	78	27	19	6	53	4	11
	0.3	398	633	13	76	31	17	5	43	6	12
	0.9	172	936	13	88	137	11	4	54	6	10
Harbinger	0	232	1942	18	97	26	6	3	46	7	10
	0.1	222	809	13	71	21	6	4	57	5	11
	0.3	164	662	11	61	24	9	5	53	5	13
	0.9	158	546	15	78	113	7	4	79	7	14
Herald	0	250	885	19	134	30	7	4	47	9	13
	0.1	199	779	17	80	24	6	4	60	7	12
	0.3	176	670	16	84	34	5	3	64	8	13
	0.9	141	820	18	86	134	6	3	75	8	13
SA15485	0	104	2536	19	122	22	7	2	73	8	15
	0.1	143	1580	16	71	29	7	1	64	6	13
	0.3	168	1028	13	58	36	8	2	59	6	11
	0.9	139	1421	16	70	173	9	2	65	7	11
Santiago	0	188	1754	23	105	28	8	3	44	5	11
	0.1	238	1255	19	86	33	8	3	58	6	11
	0.3	175	1023	15	66	39	8	3	61	6	10
	0.9	203	1410	17	66	145	7	3	68	7	10
SA4188	0	217	1893	27	110	30	8	3	45	6	10
	0.1	198	1285	18	101	32	8	3	66	7	10
	0.3	156	763	14	77	38	9	5	60	9	10
	0.9	172	1193	16	71	133	9	5	77	11	11
Z1064	0	189	2112	29	100	36	9	2	27	8	5
	0.1	233	2016	22	106	42	9	2	41	9	6
	0.3	222	763	13	69	34	9	3	49	5	10
	0.9	2289	781	14	76	104	9	4	59	6	12

Appendix 8b (cont.)

Cultivar	Zn	Fe	Mn	B	Cu	Zn	Ca	Mg	K	P	S
		mg/kg shoot dry weight					g/kg shoot dry weight				
Z1065	0	228	1731	22	110	39	9	3	36	6	11
	0.1	281	871	16	77	29	8	3	33	6	7
	0.3	1177	675	16	62	35	13	7	38	7	10
	0.9	388	917	15	65	96	13	6	40	6	12
Z1068	0	191	2164	32	111	38	11	3	23	7	5
	0.1	262	1302	19	75	45	11	2	25	5	5
	0.3	377	1120	15	89	41	8	3	54	10	9
	0.9	217	1308	15	91	117	8	3	58	7	11
Sava	0	219	1300	18	55	24	6	1	63	7	7
	0.1	212	972	13	50	32	11	4	51	5	7
	0.3	173	686	10	44	29	11	4	41	5	7
	0.9	175	846	11	43	95	14	7	45	5	10
Paraponto	0	342	1445	20	119	18	7	2	42	7	9
	0.1	322	785	14	62	28	6	2	43	5	9
	0.3	435	727	13	53	37	6	2	45	5	9
	0.9	223	1051	16	72	105	6	2	77	10	14
Zn		ns	**	**	**	**	**	**	**	ns	**
cultivar		ns	**	**	**	**	**	**	**	ns	**
Zn by cultivar		ns	ns	ns	ns	**	**	**	**	ns	ns

ns- not significant, * $p \leq 0.05$, ** $p \leq 0.01$

REFERENCES

- Ambler, J.E. and Brown, J.C. (1969) Cause of differential susceptibility to zinc deficiency in two varieties of navy beans (*Phaseolus vulgaris* L.). *Agron. J.* **61**: 41-43.
- Anderson, N.A. (1982) The genetics and pathology of *Rhizoctonia solani*. *Ann. Rev. Phytopath.* **20**: 329-347.
- Armentrout, V.N., Downer, A.J., Grasmick, D.L. and Weinhold, A.R. (1987) Factors affecting infection cushion development by *Rhizoctonia solani* on cotton. *Phytopath.* **77**: 623-630.
- Ayling, S.M. (1989) The growth, yield and nutrient uptake of winter wheat following root-pruning. *Ann. Appl. Biol.* **114**: 567-577.
- Baker, K.F. (1970) Types of *Rhizoctonia* diseases and their occurrence. In: *Rhizoctonia solani*, Biology and Pathology. (Ed.) J.R. Parmeter Jr. Berkeley, University of California Press. pp. 125-148.
- Baugher, T.A. and Miller, S.S. (1991) Growth suppression as a control for nectarine pox. *HortSci.* **26**: 1268-1270.
- Benedict, W.G. and Mountain, W.B. (1956) Studies on the etiology of a root rot of winter wheat in southwest Ontario. *Can. J. Bot.* **34**: 159-174.
- Bertagnolli, B.L., Dal Soglio, F.K. and Sinclair, J.B. (1996) Extracellular enzyme profiles of the fungal pathogen *Rhizoctonia solani* isolate 2B-12 and of two antagonists, *Bacillus megaterium* strain B153-2-2 and *Trichoderma harzianum* isolate Th008. I. Possible correlations with inhibition of growth and biocontrol. *Physiol. Mol. Plant Path.* **48**: 145-160.
- Blevins, D.G., Dropkin, V.H. and Luedders, V.D. (1995) Macronutrient uptake, translocation, and tissue concentration of soybeans infested with the soybean cyst nematode and elemental composition of cysts isolated from roots. *J. Plant Nutr.* **18**: 579-591.
- Bolle-Jones, E.W. and Hilton, R.N. (1956) Zinc-deficiency of *Hevea brasiliensis* as predisposing factor to *Odium* infection. *Nature* **177**: 619-620.
- Bowen, J.E. (1969) Absorption of copper, zinc, and manganese by sugarcane leaf tissue. *Plant Physiol.* **44**: 255-261.
- Brennan, R.F., Armour, J.D. and Reuter, D.J. (1993) Diagnosis of zinc deficiency. In: *Zinc in Soils and Plants*. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 167-181.
- Brennan, R.F. and McGrath, J.F. (1988) The vertical movement of zinc on sandy soils in southern Western Australia. *Aust. J. Agric. Res.* **26**: 211-216.
- Brown, J.C. (1979) Effect of zinc stress factors affecting iron uptake in navy bean. *J. Plant Nutr.* **1**: 171-183.

- Brown, P.H., Cakmak, I. and Zhang, Q. (1993) Form and function of zinc plants. *In: Zinc in Soils and Plants*. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 93-106.
- Buchhorn, S.C. (1988) Evidence of the relationship between manganese and 'take-all' of wheat - a possible correlation between the variable virulence and manganese oxidation of strains of *G. graminis* var. *tritici*. Honours Thesis, The University of Adelaide, South Australia.
- Bugbee, W.M. (1993) A pectin lyase inhibitor protein from cell walls of sugar beet. *Phytopath.* **83**: 63-68.
- Cakmak, I., Derici, R., Torun, B., Tolay, I., Braun, H.J. and Schlegel, R. (1997a) Role of rye chromosomes in improvement of zinc efficiency in wheat and triticale. *In: Plant Nutrition for Sustainable Food Production and Environment*. (Ed.) T. Ando, K. Fujita, T. Mae, H. Matsumoto, S. Mori and Sekiya, Kluwer Academic Publishers. pp. 237-241.
- Cakmak, I., Ekiz, H., Yilmaz, A., Torun, B., Köleli, N., Gültekin, I., Alkan, A. and Eker, S. (1997b) Differential response of rye, triticale, bread and durum wheats to zinc deficiency in calcareous soils. *Plant and Soil* **188**: 1-10.
- Cakmak, I., Gülüt, K.Y., Marschner, H. and Graham, R.D. (1994) Effect of Zn and iron deficiency on phytosiderophore release in wheat genotypes differing in Zn efficiency. *J. Plant Nutr.* **17**: 1-17.
- Cakmak, I. and Marschner, H. (1988a) Enhanced superoxide radical production in roots of zinc-deficient plants. *J. Exp. Bot.* **39**: 1449-1460.
- Cakmak, I. and Marschner, H. (1988b) Increase in membrane permeability and exudation in roots of zinc deficient plants. *J. Plant Physiol.* **132**: 356-361.
- Cakmak, I. and Marschner, H. (1988c) Zinc-dependent changes in ESR signals, NADPH oxidase and plasma membrane permeability in cotton roots. *Physiol. Plant.* **73**: 182-186.
- Cakmak, I. and Marschner, H. (1990) Decrease in nitrate uptake and increase in proton release in zinc deficient cotton, sunflower and buckwheat plants. *Plant and Soil* **129**: 261-268.
- Cakmak, I., Marschner, H. and Bangerth, F. (1989) Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolus vulgaris* L.). *J. Exp. Bot.* **40**: 405-412.
- Cakmak, I., Sari, N., Marschner, H., Ekiz, H., Kalayci, M., Yilmaz, A. and Braun, H.J. (1996b) Phytosiderophore release in bread and durum wheat genotypes differing in zinc efficiency. *Plant and Soil* **180**: 183-189.
- Cakmak, I., Sari, N., Marschner, H., Kalayci, M., Yilmaz, A., Eker, S. and Gülüt, K.Y. (1996a) Dry matter production and distribution of zinc in bread and durum wheat genotypes differing in zinc efficiency. *Plant and Soil* **180**: 173-181.
- Camp, A.F. (1945) Zinc as a nutrient in plant growth. *Soil Sci.* **60**: 157-164.
- Carter, E.D. and Challis, S. (1987) Seed, seedling and species dynamics of grazed annual

- pastures in South Australia. 14th International Grasslands Congress, Kyoto, Japan. pp. 654-656.
- Carter, E.D., Wolfe, E.C. and Francis, C.M. (1982) Problems of maintaining pastures in the cereal-livestock areas of southern Australia. Proc. 2nd Australian Agronomy Conference, Wagga Wagga, New South Wales. pp. 68-82.
- Caten, C.E. (1972) Vegetative incompatibility and cytoplasmic infection in fungi. *J. Gen. Microbiol.* **72**: 221-229.
- Chvapil, M. (1973) New aspects in the biological role of zinc: A stabilizer of macromolecules and biological membranes. *Life Sci.* **13**: 1041-1049.
- Coleman, J.E. (1992) Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. *Ann. Rev. Biochem.* **61**: 897-946.
- Colhoun, J. (1973) Effects of environmental factors on plant disease. *Ann. Rev. Phytopath.* **11**: 343-364.
- Colquorn, H. (1937) Probable application of root amputation to the take-all problem. *J. Aust. Inst. Agric. Sci.* **3**: 169.
- Cotterill, P.J. (1991) Evaluation of in-furrow fungicide treatments to control *Rhizoctonia* root rot of wheat. *Crop Prot.* **10**: 473-478.
- Cotterill, P.J., Ballinger, D.J. and Kollmorgen, J.F. (1990) Evaluation of methods for the chemical control of *Rhizoctonia* root rot of wheat. *Crop Prot.* **9**: 275-280.
- Crawford, E.J., Lake, A.W.H. and Boyce, K.G. (1989) Breeding annual *Medicago* species for semiarid conditions in southern Australia. *Adv. Agron.* **42**: 399-437.
- Davies, W.J., Mansfield, T.A. and Hetherington, A.M. (1990) Sensing of soil water status and the regulation of plant growth and development. *Plant Cell Envir.* **13**: 709-719.
- de Silva, R.L. and Wood, R.K.S. (1964) Infection of plants by *Corticium solani* and *C. praticola* - effect of plant exudates. *Trans. Brit. Mycol. Soc.* **47**: 15-24.
- Dillon-Weston, W.A.R. and Garrett, S.D. (1943) *Rhizoctonia solani* associated with a root rot of cereals in Norfolk. *Ann. App. Biol.* **30**: 79.
- Dodman, R.L. and Flentje, N.T. (1970) The mechanism and physiology of plant penetration by *Rhizoctonia solani*. In: *Rhizoctonia solani*, Biology and Pathology. (Ed.) J.R. Parmeter Jr. Berkeley, University of California Press. pp. 149-160.
- Donald, C.M. (1967) Innovation in agriculture. In: *Agriculture in the Australian Economy*. (Ed.) D.B. Williams. Sydney, Sydney University Press. pp. 57-86.
- Donald, C.M. and Prescott, J.A. (1975) Trace elements in Australian crop and pasture production, 1924-1974. In: *Trace Elements in Soil-Plant-Animal Systems*. (Ed.) D.J.D. Nicholas and A.R. Egan, Academic Press, Inc. pp. 7-37.
- Dong, B., Rengel, Z. and Graham, R.D. (1995) Root morphology of wheat genotypes differing in zinc efficiency. *J. Plant Nutr.* **18**: 2761-2773.
- Dubé, A.J., Eichner, A., Murray, M. and Whittle, P.J.L., Eds. (1987) 1987 *Rhizoctonia* Workshop: Priorities for Research on *Rhizoctonia* Root Rot of Cereals. Report on

- workshop held on 23rd September, 1987 at the Waite Agricultural Research Institute, Glen Osmond, South Australia, Wheat Research Committee for South Australia and the Barley Research Committee for South Australia.
- Erenoglu, B., Cakmak, I., Marschner, H., Römheld, V., Eker, S., Daghan, H., Kalayci, M. and Ekiz, H. (1996) Phytosiderophore release does not relate well with zinc efficiency in different bread wheat genotypes. *J. Plant Nutr.* **19**: 1569-1580.
- Falchuk, K., Hardy, C., Ulpino, L. and Vallee, B. (1977) RNA polymerase, manganese and RNA metabolism of zinc sufficient and deficient *E. gracilis*. *Biochem. Biophys. Res. Commun.* **77**: 314-319.
- Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **133**: 6-13.
- Flentje, N.T., Dodman, R.L. and Kerr, A. (1963) The mechanism of host penetration by *Thanatephorus cucumeris*. *Aust. J. Biol. Sci.* **16**: 784-799.
- Flentje, N.T. and Stretton, H.M. (1964) Mechanisms of variation in *Thanatephorus cucumeris* and *T. praticolus*. *Aust. J. Biol. Sci.* **17**: 686-704.
- Foy, C.D. (1983) Plant adaptation to mineral stress in problem soils. *Iowa State J. Res.* **57**: 355-391.
- French, R.J. (1987) Future productivity of our farmlands. Proceedings of the 4th Australian Agronomy Conference, Melbourne, Victoria. pp. 140-149.
- Fujii, T. (1954) Presence of zinc in nucleoli and its possible role in mitosis. *Nature* **174**: 1108-1109.
- Fukutomi, M. and Takada, H. (1979) Ultrastructure of infection process of *Rhizoctonia solani* Kühn in cucumber hypocotyls. *Ann. Phytopath. Soc. Jap.* **45**: 453-462.
- Gildon, A. and Tinker, P.B. (1983) Interactions of vesicular-arbuscular mycorrhizal infections and heavy metals in plants. I: The effects of heavy metals on the development of vesicular-arbuscular mycorrhizas. *New Phytol.* **95**: 247-261.
- Gillet, D., Neal, J. and Dennis, J. (1998) Medic pasture responses to nutrition and disease treatments. Farming Systems Developments, Adelaide, South Australia. pp. 73-74.
- Giordano, P.M., Noggle, J.C. and Mortvedt, J.J. (1974) Zinc uptake by rice, as affected by metabolic inhibitors and competing cations. *Plant and Soil* **41**: 637-646.
- Goldschmidt, V.M. (1954) Geochemistry, Oxford University Press.
- Goubran, F.H. and Richards, D. (1979) The estimation of root length in samples and subsamples. *Plant and Soil* **52**: 77-83.
- Graham, R.D. (1978) Nutrient efficiency objectives in cereal breeding. Proc. 8th International Colloquium of Plant Analysis and Fertilizer Problems, Auckland. pp. 165-170.
- Graham, R.D. (1981) Absorption of copper by plant roots. In: Copper in Soils and Plants. (Ed.) J.F. Lonergan, A.D. Robson and R.D. Graham. New York, Academic Press. pp. 141-163.

- Graham, R.D. (1983) Effect of nutrient stress on susceptibility of plants to disease with particular reference to the trace elements. *Adv. Bot. Res.* **10**: 221-276.
- Graham, R.D. (1984) Breeding for nutritional characteristics in cereals. *Adv. Plant Nutr.* **1**: 57-102.
- Graham, R.D. (1991) Breeding wheats for tolerance to micronutrient deficient soil: present status and priorities. In: Wheat Production Constraints in Tropical Environments. (Ed.) A.R. Klatt. CIMMYT, Mexico. pp. 305-320.
- Graham, R.D., Ascher, J.S. and Hynes, S.C. (1992) Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant and Soil* **146**: 241-250.
- Graham, R.D., Ascher, J.S., Webb, M.J., Robinson, N.H., Hannam, R.J. and Wilhelm, N.S. (1990) Nutrition survey of cereal crops in the Murray Mallee. Biennial Report 1988-1989. Waite Agricultural Research Institute, Adelaide, South Australia. pp. 59-60.
- Graham, R.D. and Rengel, Z. (1993) Genotypic variation in zinc uptake and utilization by plants. In: Zinc in Soils and Plants. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 107-118.
- Graham, R.D. and Rovira, A.D. (1984) A role for manganese in the resistance of wheat plants to take-all. *Plant and Soil* **78**: 441-444.
- Graham, R.D. and Webb, M.J. (1991) Micronutrients and disease resistance and tolerance in plants. In: Micronutrients in Agriculture, 2nd ed. (Ed.) J.J. Mortvedt, F.R. Cox, L.M. Shuman and R.M. Welch. Madison, Soil Science Society of America. pp. 329-370.
- Graham, R.D. and Welch, R.M. (1996) Breeding for staple food crops with high micronutrient density. Working Papers on Agricultural Strategies for Micronutrients, International Food Policy Research Institute, Washington, D.C.
- Graham, R.D., Welch, R.M., Grunes, D.L., Cary, E.E. and Norvell, W.A. (1987) Effect of zinc deficiency on the accumulation of boron and other mineral nutrients in barley. *Soil Sci. Soc. Am. J.* **51**: 652-657.
- Grewal, H.S., Graham, R.D. and Rengel, Z. (1996) Genotypic variation in zinc efficiency and resistance to crown rot disease (*Fusarium graminearum* Schw Group 1) in wheat. *Plant and Soil* **186**: 219-226.
- Grewal, H.S., Stangoulis, J.C.R., Potter, T.D. and Graham, R.D. (1997) Zinc efficiency of oilseed rape (*Brassica napus* and *B. juncea*) genotypes. *Plant and Soil* **191**: 123-132.
- Hall, M. (1984) Are manganese-deficient wheat plants more susceptible to *Rhizoctonia solani* Kühn than manganese-adequate wheat plants?, Honours Thesis, The University of Adelaide, Adelaide, South Australia.
- Halvorson, A.D. and Lindsay, W.L. (1977) The critical Zn^{2+} concentration for corn and the nonabsorption of chelated zinc. *Soil Sci. Soc. Am. J.* **41**: 531-534.
- Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y. and Wu, C. (1983) *Xenopus* transcription factor A requires zinc for binding to the 5S RNA gene. *J. Biol. Chem.* **258**: 14120-14125.

- Hannam, R.J. (1990) Zinc update. Agronomy Technical Conference 1990, Plant Service Division, Dept. Ag., South Australia. pp. 56-57.
- Hannam, R.J. (1991) Nutrition and Zn update. Agronomy Technical Conference 1991, Plant Service Division, Dept. Ag., South Australia. pp. 90-94.
- Harmsen, K. and Vlek, P.L.G. (1985) The chemistry of micronutrients in soil. *Fert. Res.* **7**: 1-42.
- Harris, G.A. and Campbell, G.S. (1989) Automated quantification of roots using a simple image analyzer. *Agron. J.* **81**: 935-938.
- HiFERT (1995) Fertilizers for Pastures No. 2. HiFERT Educational Leaflet.
- Holloway, R.E. (1996) Zinc as a subsoil nutrient for cereals, Ph.D. Thesis, The University of Adelaide, Adelaide, South Australia.
- Huber, D.M. (1989) Soilborne Plant Pathogens: Management of Disease with Macro- and Microelements. (Ed.) A. Englehard, APS Press, St. Paul, MN. pp. 1-8.
- IRRI (1970) Annual Report. *International Rice Research Institute. Los Banos, Philippines.*
- Jackson, M.B. (1993) Are plant hormones involved in root to shoot communication? *Adv. Bot. Res.* **19**: 103-187.
- James, R.A., Kirkegaard, J.A. and Munns, R. (1997) Contribution of *Rhizoctonia* to reduced seedling growth of direct-drilled wheat: studies with intact cores. *Aust. J. Agric. Res.* **48**: 1231-1239.
- Jeffery, E.H. (1983) The effect of zinc on NADPH oxidation and mono-oxygenase activity in rat hepatic microsomes. *Mol. Pharm.* **23**: 467-473.
- Jolley, V.D. and Brown, J.C. (1991) Factors in iron-stress response mechanism enhanced by Zn-deficiency stress in Sanilac, but not Saginaw navy bean. *J. Plant Nutr.* **14**: 257-265.
- Jyung, W.H., Camp, M.E., Polson, D.E., Adams, M.W. and Wittwer, S.H. (1972) Differential response of two bean varieties to zinc as revealed by electrophoretic protein pattern. *Crop Sci.* **12**: 26-29.
- Kabata-Pendias, A. and Pendias, H. (1984) Trace Elements in Soils and Plants. Florida, CRC Press.
- Kabata-Pendias, A. and Pendias, H. (1992) Trace Elements in Soils and Plants, 2nd ed. Florida, CRC Press.
- Kang, B.T. and Osiname, O.A. (1985) Micronutrient problems in tropical Africa. *Fert. Res.* **7**: 131-150.
- Katyal, J.C. and Vlek, P.L.G. (1985) Micronutrient problems in tropical Asia. *Fert. Res.* **7**: 69-94.
- Kefu, Z., Munns, R. and King, R.W. (1991) Absciscic acid levels in NaCl-treated barley, cotton and saltbush. *Aust. J. Plant Physiol.* **18**: 17-24.
- Keijer, J. (1996) The initial steps of the infection process in *Rhizoctonia solani*. In: *Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control.* (Ed.) B. Sneh, S. Jabaji-Hare, S. Neate and G. Dijst, Kluwer Academic

- Publishers. pp. 149-162.
- Kellogg, E.W. and Fridovich, I. (1975) Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J. Biol. Chem.* **250**: 8812-8817.
- Kerr, A. (1956) Some interactions between plant roots and pathogenic soil fungi. *Aust. J. Biol. Sci.* **9**: 45-52.
- Kerr, A. and Flentje, N.T. (1957) Host infection of *Pellicularia filamentosa* controlled by chemical stimuli. *Nature* **179**: 204-205.
- Khan, H.R., McDonald, G.K. and Rengel, Z. (1998) Chickpea genotypes differ in their sensitivity to Zn deficiency. *Plant and Soil* **198**: 11-18.
- Kirchhof, G. (1992) Measurement of root length and thickness using a hand-held computer scanner. *Field Crops Res.* **29**: 79-88.
- Kirkegaard, J.A., Munns, R., James, R.A. and Neate, S. (1998) Does water and nutrient uptake limit leaf growth of *Rhizoctonia*-infected wheat seedlings? *Plant and Soil* (in press).
- Kitagishi, K., Obata, H. and Kondo, T. (1987) Effect of zinc deficiency on 80S ribosome content of meristematic tissues of rice plant. *Soil Sci. Plant Nutr.* **33**: 423-429.
- Kochian, L.V. (1993) Zinc absorption from hydroponic solutions by plant roots. In: *Zinc in Soils and Plants*. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 45-57.
- Kulik, M.M. and Dery, P.D. (1995) Frequency of plants in populations of annual and perennial medics (*Medicago* spp.) that were resistant to damping-off incited by AG4 isolates of *Rhizoctonia solani*. *Z. PflKrankh. PflSchutz.* **102**: 392-398.
- Kulik, M.M., Dery, P.D. and Douglas, L.W. (1995) Frequency of plants in populations of alfalfa (*Medicago sativa* L.) resistant to pre- and post-emergence damping-off incited by AG 4 isolates of *Rhizoctonia solani*. *J. Plant Dis. Prot.* **102**: 51-57.
- Kuo, S. and Mikkelsen, D.S. (1981) Effect of phosphorus and manganese on growth response and uptake of iron, manganese and phosphorus by sorghum (*Sorghum vulgare*). *Plant and Soil* **62**: 15-22.
- Lafitte, C., Barthe, J.P., Montillet, J.L. and Touze, A. (1984) Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endopolygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. *Physiol. Plant Path.* **25**: 39-53.
- Law, D.M. (1987) Gibberellin enhanced indole-3-acetic acid biosynthesis: D-tryptophan as the precursor of indole-3-acetic acid. *Physiol. Plant.* **70**: 626-632.
- Leece, D.R. (1978) Effects of boron on the physiological activity of zinc in maize. *Aust. J. Agric. Res.* **29**: 739-747.
- Leigh, J.H., Halsall, D.M. and Holgate, M.D. (1995) The role of allelopathy in legume decline in pastures. I. Effects of pasture and crop residues on germination and survival of subterranean clover in the field and nursery. *Aust. J. Agric. Res.* **46**: 179-188.
- Leon, L.A., Lopez, A.S. and Vlek, P.L.G. (1985) Micronutrient problems in tropical Latin

- America. *Fert. Res.* **7**: 95-129.
- Lesins, K.A. and Lesins, I. (1979) Genus *Medicago* (Leguminosae): A Taxogenetic Study., Dr. W. Junk bv Publishers.
- Lindsay, W.L. (1972) Zinc in soils and plant nutrition. *Adv. Ag.* **24**: 147-186.
- Loneragan, J.F., Kirk, G.J. and Webb, M.J. (1987) Translocation and function of zinc in roots. *J. Plant Nutr.* **10**: 1247-1254.
- Loneragan, J.F. and Webb, M.J. (1993) Form and function of zinc plants. *In: Zinc in Soils and Plants.* (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 119-134.
- Longnecker, N.E. and Robson, A.D. (1993) Distribution and transport of zinc in plants. *In: Zinc in Soils and Plants.* (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 79-92.
- Ludbrook, W.V., Brockwell, J. and Riceman, D.S. (1953) Bare-patch disease and associated problems in subterranean clover pastures in South Australia. *Aust. J. Agric. Res.* **4**: 403-414.
- MacNish, G.C. (1983) Rhizoctonia patch in Western Australian grain belt. *Aust. Plant Path.* **12**: 49-50.
- MacNish, G.C. (1985a) Mapping Rhizoctonia patch in consecutive cereal crops in Western Australia. *Plant Pathol.* **33**: 355-359.
- MacNish, G.C. (1985b) Methods of reducing Rhizoctonia patch of cereals in Western Australia. *Plant Pathol.* **34**: 175-181.
- MacNish, G.C. (1988) Changes in take-all (*Gaeumannomyces graminis* var. *tritici*), rhizoctonia root rot (*Rhizoctonia solani*) and soil pH in continuous wheat with annual applications of nitrogenous fertiliser in Western Australia. *Aust. J. Exp. Agric.* **28**: 333-341.
- MacNish, G.C. (1996) Patch dynamics and bare patch. *In: Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control.* (Ed.) B. Sneh, S. Jabaji-Hare, S. Neate and G. Dijst, Kluwer Academic Publishers. pp. 217-226.
- MacNish, G.C. and Fang, C.S. (1987) Effect of short chemical fallow on Rhizoctonia bare patch and root rot of wheat at Esperance, Western Australia. *Aust. J. Exp. Agric.* **27**: 671-677.
- MacNish, G.C., McLernon, C.K. and Wood, D.A. (1993) The use of zymogram and anastomosis techniques to follow the expansion and demise of two coalescing bare patches caused by *Rhizoctonia solani* AG8. *Aust. J. Agric. Res.* **44**: 1161-1173.
- MacNish, G.C. and Neate, S.M. (1996) Rhizoctonia bare patch of cereals: An Australian perspective. *Plant Dis.* **80**: 965-971.
- Mader, M. and Amberg-Fisher, V. (1982) Role of peroxidase in lignification of tobacco cells. I. Oxidation of nicotinamide adenine dinucleotide and formation of hydrogen peroxide by cell wall peroxidase. *Plant Physiol.* **70**: 1128-1131.
- Marschner, H. (1995) Mineral Nutrition of Higher Plants, 2nd ed. London, Academic Press.
- Masle, J. and Passioura, J.B. (1987) The effect of soil strength on the growth of young wheat

- plants. *Aust. J. Plant Physiol.* **14**: 643-656.
- Massaquoi, R.C. and Rush, M.C. (1987) Relationship of quantity of epicuticular wax to resistance of rice to sheath blight. *Phytopath.* **77**: 1723.
- Materon, L.A. and Ryan, J. (1995) *Rhizobial* inoculation and phosphorus and zinc nutrition for annual medics adapted to mediterranean environments. *Agron. J.* **87**: 692-698.
- Matthew, J., Herdina and Whisson, D. (1995) DNA probe specific to *Rhizoctonia solani* anastomosis group 8. *Mycol. Res.* **99**: 745-750.
- McDonald, H.J. and Rovira, A.D. (1985) Development of inoculation technique for *Rhizoctonia solani* and its application to screening cereal cultivars for resistance. In: Ecology and Management of Soil-Borne Plant Pathogens. (Ed.) C.A. Parker, A.D. Rovira, K.J. Moore, P.T.W. Wong and J.F. Kollmorgen, APS Press, St. Paul, USA. .
- McRae, D.G. and Thompson, J.E. (1983) Senescence-dependent changes in superoxide anion production by illuminated chloroplasts from bean leaves. *Planta* **158**: 185-193.
- Millikan, C.R. (1938) A preliminary note on the relation of zinc to disease in cereals. *J. Dept. Agric. Vic.* **36**: 409-416.
- Milthorpe, F.L. and Moorby, J. (1979) An Introduction to Crop Physiology. London, Cambridge University Press.
- Moore, A.W., Isbell, R.F. and Northcote, K.H. (1983) Classification of Australian soils. In: Soils: an Australian Viewpoint. CSIRO Division of Soils. London, Academic Press. pp. 254-276.
- Mortvedt, J.J. and Gilkes, R.J. (1993) Zinc fertilizers. In: Zinc in Soils and Plants. (Ed.) A.R. Robson, Kluwer Academic Publishers. pp. 33-42.
- Nable, R.O. and Webb, M.J. (1993) Further evidence that zinc is required throughout the root zone for optimal plant growth and development. *Plant and Soil* **150**: 247-253.
- Neate, S.M. (1984) Minimum cultivation and root diseases. Ph.D. Thesis, The University of Adelaide, Adelaide, South Australia.
- Neate, S.M. (1987) *Rhizoctonia* in wheat. *Trans. Br. Mycol. Soc.* **88**: 157-162.
- Neate, S.M. (1989) A comparison of controlled environment and field trials for detection of resistance in cereal cultivars to root rot caused by *Rhizoctonia solani*. *Plant Path.* **38**: 494-501.
- Neate, S.M., Cruickshank, R.H. and Rovira, A.D. (1988) Pectic enzyme patterns of *Rhizoctonia solani* isolates from agricultural soils in South Australia. *Trans. Br. Mycol. Soc.* **90**: 37-42.
- Obata, H. and Kitagishi, K. (1980) Investigation on pathway of zinc transport in vegetative node of rice plant by autoradiography. *J. Sci. Soil Manur.* **51**: 297-301.
- Ogoshi, A. (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. *Ann. Rev. Phytopath.* **25**: 125-143.
- Ogoshi, A. (1996) The genus *Rhizoctonia*. In: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. (Ed.) B. Sneh, S. Jabaji-Hare, S.

- Neate and G. Dijst, Kluwer Academic Publishers. pp. 1-10.
- Orphanos, P.I. (1975) Spray application of zinc to young apple trees. *Hort. Res.* **15**: 23-30.
- Ozanne, P.G., Shaw, T.C. and Kirton, D.J. (1965) Pasture responses to traces of zinc in phosphate fertilizers. *Aust. J. Exp. Agric. Anim. Husb.* **5**: 29-33.
- Panavasasivam, V. and Axley, J.H. (1982) Effect of sulphur and potassium on zinc absorption by barley. *Plant and Soil* **64**: 393-401.
- Panella, L. and Ruppel, E.G. (1996) Availability of germplasm for resistance against *Rhizoctonia* spp. In: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. (Ed.) B. Sneh, S. Jabaji-Hare, S. Neate and G. Dijst, Kluwer Academic Publishers. pp. 515-528.
- Papavizas, G.C. (1970) Colonization and growth of *Rhizoctonia solani* in soils. In: *Rhizoctonia solani*, Biology and Pathology. (Ed.) J.R. Parmeter Jr. Berkeley, University of California Press. pp. 108-124.
- Parker, D.R., Aguilera, J.J. and Thomason, D.N. (1992) Zinc-phosphorus interactions in two cultivars of tomato (*Lycopersicon esculentum* L.) grown in chelator-buffered nutrient solutions. *Plant and Soil* **143**: 163-177.
- Parmeter Jr., J.R. and Whitney, H.S. (1970) Taxonomy and nomenclature of the imperfect state. In: *Rhizoctonia solani*, Biology and Pathology. (Ed.) J.R. Parmeter Jr. Berkeley, University of California Press. pp. 7-19.
- Passioura, J.B. and Stirzaker, R.J. (1993) Feedforward responses of plants to physically inhospitable soil. *Int. Crop Sci.* **1**: 715-719.
- Pearson, J.N. and Rengel, Z. (1995a) Uptake and distribution of ^{65}Zn and ^{54}Mn in wheat grown at sufficient and deficient levels of Zn and Mn I. During vegetative growth. *J. Exp. Bot.* **46**: 833-839.
- Pearson, J.N. and Rengel, Z. (1995b) Uptake and distribution of ^{65}Zn and ^{54}Mn in wheat grown at sufficient and deficient levels of Zn and Mn II. During grain development. *J. Exp. Bot.* **46**: 841-845.
- Pederson, R.N., Black, I.D., Dyson, C.B. and Hannam, R.J. (1994) Effects of the herbicide metsulfuron-methyl on root length, nutrient levels, grain protein, and yield of barley. *Aust. J. Exp. Agric.* **34**: 499-504.
- Polar, E. (1976) Variations in zinc content of subcellular fractions from young and old roots, stems and leaves of broad beans (*Vicia faba*). *Physiol. Plant.* **38**: 159-165.
- Ponnamperuma, F.N. (1972) The chemistry of submerged soils. *Adv. Agron.* **24**: 29-96.
- Praske, J.A. and Plocke, D.J. (1971) A role of zinc in the structural integrity of the cytoplasmic ribosomes of *Euglena gracilis*. *Plant Physiol.* **48**: 150-155.
- Pumphrey, F.V., Wilkins, D.E., Hane, D.C. and Smiley, R.W. (1987) Influence of tillage and nitrogen fertilizer on *Rhizoctonia* bare patch of winter wheat. *Plant Dis.* **71**: 125-127.
- Raeder, V. and Broder, P. (1985) Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1**: 17-20.

- Ramani, S. and Kannan, S. (1985) An examination of zinc uptake patterns by cultivars of sorghum and maize: Differences amongst hybrids and their parents. *J. Plant Nutr.* **8**: 1199-1210.
- Rashid, A., Chaudhry, F.M. and Sharif, M. (1976) Micronutrient availability to cereals from calcareous soils. III. Zinc absorption by rice and its inhibition by important ions of submerged soils. *Plant and Soil* **45**: 613-623.
- Reay, P.F. (1987) The distribution of nine elements in shoots of *Lupinus albus* L. and *Lupinus angustifolius* L. compared with that of silicon as a measure of passive transport. *Ann. Bot.* **59**: 219-225.
- Reddy, M.N. (1980) Studies on groundnut hypocotyl exudates and the behaviour of *Rhizoctonia solani* in influencing the disease. *Plant and Soil* **55**: 445-454.
- Reeves, T.G. and Ewing, M.A. (1993) Is ley farming in mediterranean zones just a passing phase? Proc. 17th International Grasslands Congress, Palmerston North, Hamilton and Lincoln, New Zealand, and Rockhampton, Australia. pp. 2169-2177.
- Reid, R.J., Brookes, J.D., Tester, M.A. and Smith, F.A. (1996) The mechanism of zinc uptake in plants: Characterisation of the low-affinity system. *Planta* **198**: 39-45.
- Rengel, Z. and Graham, R.D. (1995a) Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution I. Growth. *Plant and Soil* **176**: 307-316.
- Rengel, Z. and Graham, R.D. (1995b) Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake. *Plant and Soil* **176**: 317-324.
- Rengel, Z. and Graham, R.D. (1995c) Importance of seed Zn content for wheat growth on Zn-deficient soil. I: Vegetative growth. *Plant and Soil* **173**: 259-266.
- Rengel, Z. and Graham, R.D. (1996) Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. *J. Exp. Bot.* **47**: 217-226.
- Rengel, Z., Graham, R.D. and Pedler, J.F. (1993) Manganese nutrition and accumulation of phenolics and lignin as related to differential resistance of wheat genotypes to the take-all fungus. *Plant and Soil* **151**: 255-263.
- Rengel, Z., Graham, R.D. and Pedler, J.F. (1994b) Time-course of biosynthesis of phenolics and lignin in roots of wheat genotypes differing in manganese efficiency and resistance to take-all fungus. *Ann. Bot.* **74**: 471-477.
- Rengel, Z., Pedler, J.F. and Graham, R.D. (1994a) Control of Mn status in plants and rhizosphere: Genetic aspects of host and pathogen effects in the wheat take-all interaction. In: Biochemistry of Metal Micronutrients in the Rhizosphere. (Ed.) J.A. Manthey, D.E. Crowley and D.G. Luster, Lewis Publishers. pp. 125-145.
- Reuter, D.J. and Robinson, J.B., Eds. (1997) Plant analysis: an interpretation manual, 2nd edition, CSIRO Australia.
- Rhodes, D. and Klug, A. (1993) Zinc fingers. *Sci. Am.* **2**: 56-65.
- Robson, A.D. and Pitman, M.G. (1983) Interactions between nutrients in higher plants. In: Encyclopedia of Plant Physiology, New Series: Inorganic plant nutrition. (Ed.) A.

- Läuchli and R.L. Bielecki. Berlin, Springer-Verlag, pp. 147-180.
- Roget, D.K. (1995) Decline in root rot (*Rhizoctonia solani* AG-8) in wheat in a tillage and rotation experiment at Avon, South Australia. *Aust. J. Exp. Agric.* **35**: 1009-1013.
- Roget, D.K., Venn, N.R. and Rovira, A.D. (1987) Reduction of the *Rhizoctonia* root rot of direct-drilled wheat by short-term chemical fallow. *Aust. J. Exp. Agric.* **27**: 425-430.
- Rorison, I.H. (1986) The response of plants to acid soil. *Exper.* **42**: 357-362.
- Rothrock, C.S. and Hargrove, W.L. (1988) Influence of legume cover crops and conservation tillage on soil populations of selected fungal genera. *Can. J. Microbiol.* **34**: 201-206.
- Rovira, A.D. (1986) Influence of crop rotation and tillage on *Rhizoctonia* bare patch of wheat. *Phytopath.* **76**: 669-673.
- Rovira, A.D. and Simon, A. (1985) Growth, nutrition and yield of wheat in calcareous sandy loams of South Australia: Effects of soil fumigation, fungicide, nematicide and nitrogen fertilizers. *Soil Biol. Biochem.* **17**: 279-284.
- Samuel, G. (1928) Two 'stunting' diseases of wheat and oats. *J. Agric. (SA)* **32**: 40-43.
- Samuel, G. and Garrett, S.D. (1932) *Rhizoctonia solani* on cereals in South Australia. *Phytopath.* **22**: 827-836.
- Scott, D.B., Visser, C.P.N. and Rufenacht, E.M.C. (1979) Crater disease of summer wheat in African drylands. *Plant Dis. Repr.* **63**: 836-840.
- Sharma, C.P., Sharma, P.N., Bisht, S.S. and Nautiyal, B.D. (1982) Zinc deficiency induced changes in cabbage. Proc. 9th Int. Plant Nutrition Colloquium, Warwickshire, England, Slough: Commonwealth Agricultural Bureaux. pp. 601-606.
- Sharma, C.P., Sharma, P.N., Bisht, S.S. and Nautiyal, B.D. (1987) Zn deficiency and anther development in maize. *Plant Cell Physiol.* **28**: 11-18.
- Sharma, U.C. and Grewal, J.S. (1990) Potato response to zinc as influenced by genetic variability. *J. Indian Potato Assoc.* **17**: 1-5.
- Shaw, A.G.L. (1967) History and development of Australian agriculture. In: Agriculture in the Australian Economy. (Ed.) D.B. Williams, Sydney University Press. pp. 1-28.
- Sheaffer, C.C. and Lake, A.W.H. (1998) Legumes in cropping systems: approaches in Midwest United States and Southern Australia. 18th International Grasslands Congress, 9-18th June, 1997, Winnipeg Manitoba and Saskatoon Saskatchewan, Canada. (Proceedings in press).
- Sherwood, R.T. (1970) Physiology of *Rhizoctonia solani*. In: *Rhizoctonia solani*, Biology and Pathology. (Ed.) J.R. Parmeter Jr. Berkeley, University of California Press. pp. 69-92.
- Shkolnik, M.Y. (1984) Trace Elements in Plants. Amsterdam, Elsevier.
- Shrotri, C.K., Mohanty, P., Rathore, V.C. and Tewari, M.N. (1983) Zinc deficiency limits the photosynthesis enzyme activation in *Zea mays* L. *Biochem. Physiol. Pflanze* **178**: 213-217.

- Sillanpää, M. (1990) Micronutrient assessment at the country level: An international study. *FAO Soils Bull.* 63. FAO, United Nations, Rome.
- Simon, J.P. and Simon, A. (1965) Relationship in annual species of *Medicago*. I. Number and morphology of chromosomes. *Aust. J. Agric. Res.* **16**: 37-50.
- Singh, M. (1981) Effect of zinc, phosphorus and nitrogen on tryptophan concentration in rice grains grown on limed and unlimed soils. *Plant and Soil* **62**: 305-308.
- Sneh, B., Ichielevich-Auster, M. and Shomer, I. (1989) Comparative anatomy of colonization of cotton hypocotyls and roots by virulent and hypovirulent isolates of *Rhizoctonia solani*. *Can. J. Bot.* **67**: 2141-2149.
- Sommer, A.L. and Lipman, C.B. (1926) Evidence of the indispensable nature of zinc and boron for higher green plants. *Plant Physiol.* **1**: 231-249.
- Sparrow, D.H. and Graham, R.D. (1988) Susceptibility of zinc-deficient wheat plants to colonization by *Fusarium graminearum* Schw. Group 1. *Plant and Soil* **112**: 261-266.
- Stephens, G.G., Herriott, R.I., Downes, R.G., Langford-Smith, T. and Acock, A.M. (1945) Soil, land use and erosion survey. *CSIRO Bulletin No.* 18/8.
- Stockwell, V. and Hanchey, P. (1985) Effect of cuticle treatments on infection of *Phaseolus vulgaris* by *Rhizoctonia solani*. *Phytopathol. Z.* **114**: 6-12.
- Suge, H., Takahashi, H., Arita, S. and Takaki, H. (1986) Gibberellin relationships in zinc-deficient plants. *Plant Cell Physiol.* **27**: 1010-1012.
- Sumner, D.R. (1996) Sclerotia formation by *Rhizoctonia* species and their survival. In: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. (Ed.) B. Sneh, S. Jabaji-Hare, S. Neate and G. Dijst, Kluwer Academic Publishers. pp. 207-216.
- Swaine, D.J. (1955) *Commonw. Bur. Soil. Sci. Tech. Commun. No.* 48.
- Sweetingham, M.W. and MacNish, G.C. (1992) *Rhizoctonia* Isolation, Identification and Pathogenicity: a Laboratory Manual, Western Australia Department of Agriculture, Miscellaneous Publication No. 14/92.
- Tabe, L.M., Wardleyrichardson, T., Ceriotti, A., Aryan, A., McNabb, W., Moore, A. and Higgins, T.J.V. (1995) A biotechnological approach to improving the nutritive value of alfalfa. *J. Anim. Sci.* **73**: 2752-2759.
- Takaki, H. and Kusushizaki, M. (1970) Accumulation of free tryptophan and tryptamine in zinc deficient maize seedlings. *Plant Cell Physiol.* **11**: 793-804.
- Takeshige, K. and Minakami, S. (1979) NADH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochem. J.* **180**: 129-135.
- Takkar, P.N., Bansal, R.L., Singh, S.P. and Nayyar, V.K. (1988) Response of pearl millet cultivars to zinc under field conditions. *Intern. J. Tropical Agric.* **6**: 247-251.
- Takkar, P.N. and Walker, C.D. (1993) The distribution and correction of zinc deficiency. In: *Zinc in Soils and Plants*. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 151-

165.

- Tennant, D. (1975) A test of a modified line intersect method of estimating root length. *J. Ecol.* **63**: 995-1001.
- Thompson, J.P. (1990) Soil sterilisation methods to show VA-mycorrhizae aid P and Zn nutrition of wheat in vertisols. *Soil Biol Biochem.* **22**: 229-240.
- Thongbai, P. (1993) The influence of Zn nutritional status on the severity of *Rhizoctonia* root rot of cereals. Ph.D. Thesis, The University of Adelaide, Adelaide, South Australia.
- Thongbai, P., Graham, R.D., Neate, S.M. and Webb, M.J. (1993b) Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity II. Effect of Zn on disease severity of wheat under controlled conditions. *Plant and Soil* **153**: 215-222.
- Thongbai, P., Hannam, R.J., Graham, R.D. and Webb, M.J. (1993a) Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity I. Field observations. *Plant and Soil* **153**: 207-214.
- Thorne, W. (1957) Zinc deficiency and its control. *Adv. Agron.* **9**: 31-65.
- Tinker, P.B. (1981) Levels, distribution and chemical forms of trace elements in food plants. *Philos. Trans. R. Soc.* **294b**: 41-46.
- Trumble, H.C. (1939) Barrel medic (*Medicago tribuloides*, Desr.) as a pasture legume. *J. Agric. South Aust.* **42**: 953-958.
- Vallee, B.L. and Auld, D.S. (1990) Zinc coordination, function, structure of zinc enzymes and other proteins. *Biochem.* **29**: 5647-5659.
- Vallee, B.L., Coleman, J.E. and Auld, D.S. (1991) Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc. Nat. Acad. Sci. USA* **88**: 999-1003.
- Vallee, B.L. and Falchuk, K.H. (1993) The biochemical basis of zinc physiology. *Physiol. Rev.* **73**: 79-118.
- Vere, D.T. and Muir, A.M. (1986) Pasture improvement adoption in south-eastern New South Wales. *Rev. Market Agric. Econ.* **54**: 19-31.
- Vilgalys, R. and Cubeta, M.A. (1994) Molecular systematics and population biology of *Rhizoctonia*. *Ann. Rev. Phytopath.* **32**: 135-155.
- Wall, P.C., Neate, S.M., Graham, R.D., Reuter, D.J. and Rovira, A.D. (1994) The effect of *Rhizoctonia* root disease and applied nitrogen on growth, nitrogen uptake and nutrient concentrations in spring wheat. *Plant and Soil* **163**: 111-120.
- Walter, A., Römheld, B., Marschner, H. and Mori, S. (1994) Is the release of phytosiderophores in zinc-deficient wheat plants a response to impaired iron utilization? *Physiol. Plant.* **92**: 493-500.
- Warcup, J.H. (1955) Isolation of fungi from hyphae present in soil. *Nature* **175**: 953.
- Webb, M.J. and Loneragan, J.F. (1990) Zinc translocation to wheat roots and its implications for a phosphorus/zinc interaction in wheat plants. *J. Plant Nutr.* **13**: 1499-1512.
- Weinhold, A.R. and Sinclair, J.B. (1996) *Rhizoctonia solani*: Penetration, colonization and

- host response. In: *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. (Ed.) B. Sneh, S. Jabaji-Hare, S. Neate and G. Dijst, Kluwer Academic Publishers. pp. 163-174.
- Weir, R.G. and Cresswell, G.C. (1994) Pastures and Field Crops. Melbourne, Inkata Press.
- Welch, R.M., Allaway, W.H., House, W.A. and Kubota, J. (1991) Geographic distribution of trace element problems. In: *Micronutrients in Agriculture*, 2nd ed. (Ed.) J.J. Mortvedt, F.R. Cox, L.M. Shuman and R.M. Welch. Madison, Soil Science Society of America. pp. 31-58.
- Welch, R.M. and Norvell, W.A. (1993) Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv *Herta*): Studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiol.* **101**: 627-631.
- Welch, R.M., Webb, M.J. and Loneragan, J.F. (1982) Zinc in membrane function and its role in phosphorus toxicity. Proc. 9th Int. Plant Nutrition Colloquium, Warwickshire, England, Slough: Commonwealth Agricultural Bureaux. pp. 710-715.
- Weller, D.M., Cook, R.J., MacNish, G.C., Bassett, E.N., Powelson, R.L. and Peterson, R.R. (1986) Rhizoctonia bare patch of small grains favored by reduced tillage in the Pacific Northwest. *Plant Dis.* **70**: 70-73.
- Wheeler, J.L. (1987) Pastures and pasture research in southern Australia. In: *Temperate pastures : their production, use and management*. (Ed.) J.L. Wheeler, C.J. Pearson and G.E. Robards. Melbourne, CSIRO. pp. 3-31.
- White, C.L. (1993) The zinc requirements of grazing ruminants. In: *Zinc in Soils and Plants*. (Ed.) A.D. Robson, Kluwer Academic Publishers. pp. 197-206.
- White, M.C., Chaney, R.L. and Decker, A.M. (1981) Metal complexation in xylem fluid. I. Chemical composition of tomato and soybean stem exudate. *Plant Physiol.* **67**: 292-300.
- White, M.C., Decker, A.M. and Chaney, R.L. (1979) Differential cultivar tolerance in soybean to phytotoxic levels of soil Zn. I. Range of cultivar response. *Agron. J.* **71**: 121-126.
- Wilhelm, N.S., Graham, R.D. and Rovira, A.D. (1990) Control of Mn status and infection rate by genotype of both host and pathogen in the wheat take-all interaction. *Plant and Soil* **123**: 267-275.
- Wiseman, B.M., Neate, S.M., Ophel-Keller, K. and Smith, S.E. (1996) Suppression of *Rhizoctonia solani* anastomosis group 8 in Australia and its biological nature. *Soil Biol. Biochem.* **28**: 727-732.
- Wong, D.H., Barbetti, M.J. and Sivasithamparam, K. (1985) Pathogenicity of *Rhizoctonia* spp. associated with root rots of subterranean clover. *Trans. Br. Mycol. Soc.* **85**: 156-158.
- Wulff, R.D. (1986a) Seed size variation in *Desmodium paniculatum*. II: Effects on seedling

- growth and physiological performance. *J. Ecol.* **74**: 99-114.
- Wulff, R.D. (1986b) Seed size variation in *Desmodium Paniculatum*. III: Effects on reproductive yield and competitive ability. *J. Ecol.* **74**: 115-121.
- Yang, J., Verma, P.R. and Lees, G.L. (1992) The role of cuticle and epidermal cell wall in resistance of rapeseed and mustard to *Rhizoctonia solani*. *Plant and Soil* **142**: 315-321.
- Zar, J.H. (1984) Biostatistical Analysis, 2nd edition, Prentice-Hall International, Inc.
- Zarcinas, B.A. (1984) Analysis of soil and plant material by inductively coupled plasma-optical emission spectrometry: Comparison of digestion procedures for major and trace constituents in plant material, Australian CSIRO Division of Soils Divisional Report No. 70.