Manganese nutrition status and resistance in barley (*Hordeum vulgare L.*) to take-all (*Gaeumannomyces graminis* var. *tritici*)

by

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July, 2000

Declaration

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Date: 19/7/00

Acknowledgements

Ms T Fowles, Mr N Robinson and Mr L Palmer for the ICPAES analysis, Dr P Harvey (CSIRO) for providing the *Ggt* isolate 17916, Dr P Davies (SARDI) who generated the doubled haploid population, Dr J Ascher (Adelaide University) and Dr M Ryder (CSIRO) for additional advice on *Ggt*, Dr H Wallwork and Mr M Butt (SARDI) for the outdoor take-all screen, and Dr Kath Cooper (Adelaide University) for the use of her triticale *Ggt* screen results. In addition, I would like to thank Ms M Pallotta for providing the seeds, the initial genetic linkage mapping of the 4HS Mn efficiency locus and the identification of the RFLP markers which co-segregate with the Mn efficiency trait, and with Ms A M^{c}Casland-Pexton, for construction of the DH mapping population partial map and help in DNA extraction, digestion, electrophoresis and Southern transfer. Professor P Langridge provided the RFLP markers and checked drafts of chapter 6 (Adelaide University). Advice on statistical analysis was obtained from Ms R Middleberg, (Adelaide University), and Ms M Lorimer and Ms H Oakley (BiometricsSA.). I would also like to thank Dr J Stangoulis for help in the determination of the Mn critical levels. Dr M Wallwork (SARDI) gave excellent help with the light and UV fluorescent microscopy, Dr P Kolesiki for the Confocal microscopy, and Dr M Henderson (CEMMSA) with the SEM microscopy. This research has been kindly funded by the Co-operative Research Centre for Molecular Plant Breeding (CRCMPB).

Finally, I would like to sincerely thank my supervisors, Professor Robin Graham, Dr Chunyuan Huang, and Dr Ken Chalmers. In addition, I offer thanks to Dr Susan Barker (University of Western Australia), Professor Peter Langridge and all in the Department of Plant Science. I have appreciated their friendship and advice, and I am indebted to them. Finally, I give my sincere thanks to my friend and mother, Stella, and my friends Val, Anne, Jen and Hansie, and to my friends in WATAC SA.

Publications

Lloyd, J.M., Barker, S.J., Graham, R.D. and Huang, C. (1999). Manganese nutrition status and take-all *Gaeumannomyces graminis* var. *tritici* (*Ggt*) resistance in barley (*Hordeum vulgare L.*) 11th Australian Plant Breeding Conference Proceedings, Adelaide, 19-23 April 1999 Volume 2, p 188.

Conferences

(Invited speaker)

"Manganese nutrition status and take-all *Gaeumannomyces graminis* var. *tritici* (*Ggt*) resistance in barley (*Hordeum vulgare L.*)". Annual Research Meeting, Co-operative Research Centre for Molecular Plant Breeding, The University of Adelaide, Adelaide, July 15th-16th, 1999.

List of abbreviations

A	Adenine
AFLP	Amplified fragment length polymorphism
ATP	Adenosine 5'-triphosphate
Av.LSL	Average length of stelar lesion
bp	base pair
BSA	Bovine serum albumin
°C	degrees Celsius
µCi	microcurie
C	Cytosine
cDNA	complementary DNA
dATP	2'-deoxy adenosine 5'-triphosphate
dCTP	2'-deoxy cytidine 5'-triphosphate
ddATP	2',3'-dideoxy adenosine 5'-triphosphate
ddCTP	2',3'-dideoxy cytidine 5'-triphosphate
ddGTP	2',3'-dideoxy guanidine 5'-triphosphate
ddTTP	2'.3'-dideoxy thymidine 5'-triphosphate
DAPI	4'5-diamidino-2-phenylindole dihydrochloride hydrate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxy nucleotide 5'-triphosphate
DM	Dry matter
DTT	Dithiothreitol
EASEM	Elemental analysis scanning electron microscopy

EDTA	Ethylenediaminetetraacetic acid
µg	microgram
g	grams
G	Guanine
ICPAES	Inductively coupled plasma atomic emission spectrometry
kb	kilobase
µl	microlitre
LOD	log likelihood
µM	micromolar
M	molar
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
nM	nanomole
NIR	Number of infected roots
NOR	Number of roots
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PIPES	Piperazine-N,N'-bis-(2-ethanesulfonic acid)
PIR	Percent of infected roots
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	revolutions per minute

SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TLSL	Total length of stelar lesion
TNSL	Total number of stelar lesions
Tris	Tris(hydroxymethyl)amino methane
UV	Ultraviolet
w/v	weight for volume ratio
QTL	Quantitative trait loci
YEB	Youngest emerged leaf blade

Thesis summary

Wilhelm *et al.,* (1990) had shown that wheat genotypes with increased Mn efficiency when grown under Mn-deficient soil conditions, showed less *Gaeumannomyces graminis* var. *tritici* (*Ggt*) infection compared to Mn-inefficient wheat genotypes. This result suggested the hypothesis that take-all resistance in wheat may be genetically linked to Mn efficiency. In this thesis I set out to test that hypothesis but chose to use barley, a simpler diploid crop species, in which traits such as Mn efficiency are likely to be more simply inherited than in hexaploid wheat. Using both physiological and genetic studies, no causal connection has been found between enhanced resistance to *Ggt* in barley and the presence of manganese efficiency alleles at two loci.

To begin to test if Mn efficiency is associated with resistance to *Ggt*, infection levels were compared between the Mn-efficient Amagi Nijo and the Mn-inefficient breeder's line, WI 2585, when grown in Mn-deficient soil. Amagi Nijo was found to have a higher shoot Mn concentration and low root infection measured as a short root average length of stelar lesion (Av.LSL) (mm/plant), compared to WI 2585.

Comparison of WI 2585 and Amagi Nijo grown over a range of Mn additions with no *Ggt* inoculum found them to have the same critical shoot Mn concentration. It was therefore reasonable to assume that there is equal Mn stress in WI 2585 and Amagi Nijo when they have the same shoot Mn concentrations. When WI 2585 and Amagi Nijo infected with *Ggt* were grown under soil conditions of equally high Mn stress (similar and low shoot Mn concentration), the Av.LSL in WI 2585 was significantly longer than in Amagi Nijo. But the susceptibility of WI 2585 to *Ggt* was not reduced by the elimination of Mn stress.

A major Mn efficiency locus, *Mel 1*, had been mapped using 4 RFLP markers to chromosome 4HS in an F_2 population of a cross between WI 2585 and Amagi Nijo (Pallotta *et al.*, 1999). Further, a WI 2585 x Amagi Nijo doubled haploid (DH) population had been generated by Dr. P. Davies (SARDI). A field trial was conducted using 62 entries from this DH population to search for more loci linked to Mn efficiency. A new locus controlling shoot Mn concentration showed significant linkage with RFLP marker, *Xwg645* on chromosome 2HL.

An investigation of the genetics of Mn efficiency in Amagi Nijo was used to determine if the take-all resistance was an Mn-independent varietal trait, unlinked to Mn-efficient alleles at both the *Mel 1* and *Xwg645* loci on chromosomes 4HS and 2HL respectively. Neither Mn-

efficient allele at *Mel 1* or *Xwg645* showed linkage with a short Av.LSL in a controlled-environment experiment on 29 DH lines grown in Mn-deficient soil.

Chapter 1 Literature Review

1.1 Introduction

This review covers aspects of current knowledge about manganese (Mn) in crop plants, plant available manganese in the soil, genes for manganese efficiency in barley (*Hordeum vulgare* L.) and the possibility that they enhance resistance to root infection by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*).

1.1.1 Scope of research

Manganese deficiency in wheat occurs when it is grown in soils with low plant available Mn, resulting in significant grain yield loss (Reuter *et al.*, 1973a), and increased susceptibility to *Ggt* (Graham and Rovira, 1984 and Wilhelm *et al.*, 1988). Soils with low plant available Mn are widespread in South Australia and in other winter cereal growing areas of the world. Wheat plants deficient in Mn have been shown to be susceptible to the soil-borne fungus disease, *Gaeumannomyces graminis* var. *tritici* (*Ggt*) (Reis *et al.*, 1982; Graham and Rovira, 1984). The incitant organism, *Ggt*, is commonly known as take-all or hay-die. This is because *Ggt* infection of roots in wheat and other cereals results in stelar lesions on seminal roots. In severe cases of *Ggt* infection, there is premature death of the plant (Asher and Shipton, 1981). Of the winter cereal crops, wheat and barley are the most susceptible to take-all, for which no major resistance genes are known.

A significant decrease in *Ggt* infection has been found, both in the field and in pots, with the experimental addition of Mn fertiliser to Mn deficient soils (Graham and Rovira, 1984; Rovira *et al.*, 1985; Huber and Dorich, 1988; Wilhelm *et al.*, 1988, 1990). However, in the field, the availability of soil Mn to the plants is affected by numerous environmental and soil biotic and abiotic factors (Graham, 1983; Huber and Wilhelm, 1988). As a result, the control of take-all in Mn deficient soils has proven to be very difficult.

1.1.2 Adding Mn fertiliser to the soil

Soil factors can diminish the amount of available plant Mn soon after the addition of Mn fertiliser to the soil (Reuter *et al.*, 1973a; Reuter *et al.*, 1973b; and Marcar and Graham, 1986). Severe *Ggt* infection has been observed in alkaline soils (Cook, 1981). This is because

plant available soluble Mn^{2+} is oxidised in alkaline soil to insoluble MnO_2, which is unavailable to the plant. In addition, the presence of *Ggt* in the soil decreases the availability of soil Mn to the plants (Wilhelm *et al.,* 1990). The problem of low soil Mn is compounded by the finding that the application of Mn as a foliar spray to the crops is ineffective in the control of take-all (Reis *et al.,* 1982; Wilhelm *et al.,* 1988; Huber and Wilhelm, 1988).

1.1.3 Mn status and *Ggt* infection

The critical concentration of Mn in barley below which the plant is Mn deficient is 11.0-11.6 mg/kg dry matter (DM) in the youngest emerged leaf blade (YEB) (Hannam *et al.,* 1987). As previously discussed (section 1.1.1), the experimental application of Mn to Mn limiting soils decreased the severity of *Ggt* infection (Graham and Rovira, 1984; Rovira *et al.,* 1985; Huber and Dorich, 1988; Wilhelm *et al.,* 1988, 1990). Pot studies suggested that the Mn deficiency occurred first in the plants, and predisposed the plants to the *Ggt* infection, rather than the root pruning effects of take-all causing reduced uptake of Mn from the soil causing subsequent development of Mn deficiency as growth occurred (Graham and Rovira, 1984; Rovira *et al.,* 1985; Wilhelm *et al.,* 1988).

Besides Mn levels in soil, the genotype of the plant influences tissue Mn levels. Mn efficiency in plants is defined as the enhanced ability of a genotype to grow and yield well in a soil too deficient in Mn for a standard genotype (Graham, 1984). Mn-efficient genotypes have higher Mn levels under Mn limiting conditions than Mn-inefficient genotypes. In wheat, genotypes that are efficient in Mn uptake from Mn limiting soil have a lower level of *Ggt* infection than Mn-inefficient genotypes (Wilhelm *et al.,* 1990; Pedler, 1994). Wheat has a complex genome because it is hexaploid. Therefore, the genetic basis for Mn nutrition in wheat has not been studied, and it is not known if the connection of plant Mn status and resistance to take-all is due to Mn efficiency or other genetic differences between the wheat varieties tested. This connection has never been investigated in barley, in which the genome is diploid and where Mn efficiency is known to be relatively simply inherited.

1.1.4 Aim of this research

The availability of soil Mn to the plant appears to be the unifying factor in resistance of the plant to infection by *Ggt* (Graham, 1983). Major genes have been proven to control

nutritional characters (Epstein, 1972; M^{c} Carthy *et al.,* 1988). One gene, which is associated with the Mn-efficient trait in barley, *Mne 1*, has been identified (Huang, 1996), and the function is now being investigated. Recent work suggests the presence of another two genetic loci associated with the expression of Mn-efficient or Mn-inefficient traits in barley, (Pallotta *et al.,* 2000), that are unrelated to *Mne 1*. One of these loci, *Mel 1,* has been identified, mapped to 4HS and several Restriction Fragment Length Polymorphism RFLP molecular markers found which allow identification between the Mn-efficient allele and the Mn-inefficient allele (Pallottta, *et* al., 2000). Other loci influencing Mn efficiency have not yet been identified.

In this project, physiological and genetic studies will test the hypothesis that barley genotypes grown in Mn limiting soils with the Mn-efficient allele at *Mel 1* will be genetically linked to resistance to take-all compared to genotypes with the Mn-inefficient allele at *Mel 1*. If this hypothesis is proven, studies using Amplified Fragment Length Polymorphism (AFLP) analysis will be done to identify a molecular marker which is specific to *Mel 1*. The AFLP molecular marker is needed to replace the closely linked RFLP molecular marker *Xabg714*, which is difficult to use for allele identification because it identifies multiple loci. Work will also commence to identify other loci influencing Mn efficiency and checked to determine if the loci are linked with resistance to *Ggt* infection. This work will be a foundation for the pyramiding of all alleles for Mn efficiency into elite barley cultivars, and for the possible transfer of the barley alleles into less Mn-efficient wheat cultivars.

1.1.5 Benefits of this research

The gross value of barley produced in Australia for 1998-99 was AUS$ 937 million (ABARE, 1999a). In South Australia, loss in yield from barley growing in Mn-deficient soils with no Mn fertiliser was found to be between 40-70% (Reuter *et al.*, 1973a). Hence, the sowing of Mn-efficient barley genotypes in the low plant available Mn soils of the southern cereal belt of Australia will increase yields and eliminate much of this loss. Identification of barley genotypes expressing the Mn efficiency trait in Mn limiting soils can be done with the use of specific molecular markers for Mn efficiency.

The gross value of wheat produced in Australia for 1998-99 was over AUS$ 3.5 billion (ABARE, 1999a). Australian wheat growers lose on average AUS$ 66.1 million annually due to yield reduction resulting from *Ggt* infection (ABARE, 1999b). The sowing of Mn-efficient

wheat cultivars in Mn limiting soils could be expected to give additional economic returns if the Mn-efficient trait confers not only greater yield but also greater resistance to take-all (*Ggt*). As with barley, the identification of Mn-efficient wheat genotypes in Mn limiting soils can be done with the use of specific molecular markers for Mn efficiency giving considerable additional economic benefit to the Australian cereal industry.

1.2 Mn in plants

Manganese is an essential micronutrient for growth and development in higher plants (M^{c} Hargue, 1922; Samuel and Piper, 1928). Mn is located in the plant in cell walls and in vacuoles (Garnham *et al.,* 1992). In the periodic table, Mn is a transition group metal and can exist in a range of oxidation states. The outer valence shell contains two unpaired electrons, so Mn can have an oxidation state of +2 as a basic divalent cation, Mn^{2+} (Leeper, 1970). In addition, the d orbit of Mn atoms just below the valence shell has 5 unpaired electrons. Consequently, Mn can have an oxidation state ranging up to +7, as it does in the acidic permanganate ion MnO_4^-. Mn shares some common properties with the alkali earth divalent metals, Ca and Mg, and the transition metals, Zn and Fe. All these cations can activate a number of enzymes and act as co-factors to enzymes in biosynthetic processes. As a result, when the Mn concentration in the tissues of plants is below the critical level, plant growth and development suffer a range of deleterious effects (Burnell, 1988).

1.2.1 Symptoms of Mn deficiency in barley

The critical Mn concentration in barley, below which there is decreasing vegetative yields, is 11.0-11.6 mg/kg DM in the YEBs under both field and controlled environment conditions, and 12-12.4 mg/kg DM in whole shoots under controlled environment conditions (Hannam *et al.,* 1987). No visual symptoms of Mn deficiency appear in the plants until the growth is severely depressed by the Mn deficiency (Rovira *et al.,* 1985). Under controlled environment conditions, interveinal chlorosis appears in the youngest leaf. Then the more slowly developing necrosis appears, but is most severe in the second youngest leaf (Longnecker and Graham, 1990; Webb *et al.,* 1993b). Mn deficiency appears on younger leaves as grey-brown necrotic spots and streaks (Nable and Loneragan, 1984a; Nable and Lonergan, 1984b; Marcar and Graham, 1986). Mild Mn deficiency in barley prolongs

vegetative growth, delays reproductive growth and decreases the number of tillers produced. Therefore mild Mn deficiency in barley can result in severe yield loss. Finally, severe Mn deficiency causes plant death before the onset of the stem elongation stage (Longnecker *et al.*, 1991).

1.2.2 Genotypic differences in Mn efficiency in barley

The level of Mn in plants depends on the efficiency of uptake of Mn from the soil. This process involves the reduction of insoluble soil Mn oxides to plant available Mn, and Mn mobilisation and absorption into the root (Graham, 1988; Marschner, 1988). Differences in Mn uptake efficiency were reported within oat species by Vose and Griffiths (1961). The expression of Mn efficiency is defined as the ability of a genotype to grow and yield well in soil too deficient in plant available Mn for a standard genotype (Graham, 1984). Considerable genotypic variation in Mn efficiency has been observed in barley. Barley genotypes have been classified into three different classes of Mn efficiency according to yield, tissue Mn status and chlorosis at tillering (Graham *et al.*, 1983). These classes were found to be associated with different sources of germplasm. Mn-efficient genotypes originated from the old English line Plumage Archer. This line grew well on the soils of the Mn deficient Jurassic-Cretaceous limestone of south-east England. In contrast, the Mn-inefficient genotypes (such as WI 2585) originated from the line, CI 3576, which grew in highly fertile soils near Alexandria, Egypt (Graham *et al.*, 1983).

The Mn efficiency trait seems to be due to one or at the most a very few dominant genes judging from the data of two crosses, Weeah (Mn-efficient) x WI 2585 (Mn-inefficient) and Weeah x Galleon (Mn-inefficient) (M^{c} Carthy *et al.*, (1988). Cereal species have been categorised in order of Mn efficiency: oat > rye > triticale > barley> bread wheats > durum wheat (Gallagher and Walsh, 1943; Nyborg, 1970; Graham *et al.*, 1983; Marcar, 1986), but variation may be nearly as great among genotypes of the one species.

1.3 The role of Mn in biosynthetic processes

Manganese is involved in a range of biosynthetic processes in plants. As a result, Mn deficiency in plant tissue gives a range of deleterious effects to plant growth and development.

1.3.1 Photosynthesis

Manganese is involved in the photosystem II phase of photosynthesis. During photosynthesis, photosystem II results in the evolution of an O_2 molecule from the splitting of two H_2O molecules (Cheniae, 1976). The manganese center, (MnC), which is a component of photosystem II, is a metalloprotein containing four manganese atoms. MnC binds to two water molecules, extracts four electrons and Mn sequentially passes through four oxidation states as the MnC transfers the electrons to the light harvesting reaction center, chlorophyll P680. The electrons excite the P680 reaction center, which becomes reduced to $P680^+$. The excited $P680^+$ reaction center pumps four protons from the stroma to the thylakoid lumen. As a result, oxygen is released and diffuses from the chloroplast (Cheniae and Martin, 1970; Cheniae and Martin, 1971; Cheniae, 1976; Amesz, 1983; Ghanotakis and Yocum, 1990).

Plants suffering mild Mn deficiency have reduced oxygen evolution (Amesz, 1983; Nable *et al.,* 1984), but chlorophyll concentration and the ultrastructure of the chloroplast remain the same (Nable *et al.,* 1984). But in plants with severe Mn deficiency, the lamellar system in the chloroplasts breaks down (Mercer *et al.,* 1962). As a result, some of the light intercepted by the leaves is not consumed by P680, but is emitted as fluorescence at red wavelengths (Kriedemann *et al.,* 1985). Consequently, Mn deficiency can be diagnosed by estimation of leaf fluorescence (Graham *et al.,* 1985; Hannam *et al.,* 1987; Longnecker and Graham, 1990).

1.3.2 Superoxide dismutase and Mn

Superoxide dismutase (SOD) protects cells from the highly reactive and potentially toxic free radical superoxide ion ($O_2^{\cdot}$). The free radical $O_2^{\cdot}$ is generated during photorespiration (Burnell 1988). Photorespiration is most active in plants that are under environmental stress as a result of high illumination, high temperature, or CO_2 depletion. A metalloenzyme, Mn-superoxide dismutase (Mn-SOD) has been found in the chloroplast (Bannister *et al.,* 1987). The action of Mn-SOD is to oxidise one molecule of the toxic free radical $O_2^{\cdot}$ to H_2O_2 while reducing another $O_2^{\cdot}$ molecule to O_2. Plants deficient in Mn have low Mn-SOD activity. Under environmental stress these plants will be more susceptible to cell damage and less able to survive

1.3.3 Mn and the lignin biosynthetic pathway

Mn deficient plants have a reduced ability to produce lignin. Peroxidases are widely distributed in plants, and contain Cu, Fe and other metals. Peroxidases also exist as Mn-peroxidases in cell walls. One of the functions of Mn-peroxidase is to produce H_2O_2 that oxidises cinnamyl alcohol analogues. The corresponding cinnamyl alcohol free radicals self condense into lignin without enzymatic catalyst (Gross, 1980). Deficient Mn in plants will affect lignin production.

Lignin is a major constituent of woody tissues in plants. Via the shikimic acid pathway, four different monophenolic structure analogues of cinnamyl alcohol undergo self-condensation to form the polyphenolic structure of lignin (Gross, 1980; Graham, 1983; Burnell, 1988). The polymerisation is preceded by Mn-peroxidase activation, and results in random sub-unit sequencing of the monophenolic structures (Gross, 1980). The non-enzymic polymerisation gives lignin a high level of immunity from enzymic attack, and therefore lignin is associated with increasing root resistance to invasion by pathogens (Skou, 1981). The powdery mildew pathogen (*Erysiphe graminis* f. sp. *hordei*), has been found to induce the expression of extracellular peroxidase and lignin production (Scott-Craig *et al.,* 1995). Thus soil-borne plant pathogens may elicit lignin production as a defence response in barley. However, invading pathogens may overcome this plant defence mechanism during invasion of the plant root via disruption of the final polymerisation step with the secretion of Mn^{2+}-oxidising exudate (Wilhelm *et al.,* 1987).

Plants deficient in Mn have reduced phenol and lignin production (Brown *et al.,* 1984), and so are likely to be more vulnerable to invasion by root pathogens (Huber and Wilhelm, 1988), as discussed above.

1.3.4 Mn and auxin biosynthesis

Indole-3-acetic acid (IAA) is an auxin (plant growth hormone) that is synthesised via the shikimic pathway in the apical buds of growing shoots. IAA stimulates growth of the main shoot, and inhibits lateral shoot development. Cytokinin is a plant hormone produced in roots, and promotes differentiation in many plant tissues. Auxins and cytokinins work together, and the ratio of auxin to cytokinin will determine if a plant will grow or differentiate.

Manganese is involved both in the synthesis and in the oxidative degradation of IAA (Graham and Webb, 1991). Mn deficient plants showed an increase in IAA oxidase activity (Morgan *et al.,* 1966; Taylor *et al.,* 1968), and exhibited abnormal growth and development (Webb and Dell, 1990).

1.4 Mn in soils

The level of plant available Mn in soil is a major factor affecting Mn nutritional status and consequently the growth and development of plants.

1.4.1 Forms of Mn in soils

In the soil, Mn^{2+} (MnII), and perhaps the transient Mn^{3+} (MnIII), are soluble and available for uptake by plants. Manganese in soil occurs in a range from 20-6000 mg/kg (Krauskopf, 1972). Most Mn in soil exists as an insoluble form. Mn occurs over a wide range of oxidation states:- from Mn_3O_4 (hausmannite), to MnOOH (manganite), to KMn_8O_{16} (cryptomelane) to the more oxidised forms, pyrolusite (δ–MnO_2), lithiophorite and birnessite ($CaMnO_2$) :- all with an oxidation state of +4 (MnIV) (Leeper, 1970). MnO_2 (MnIV), is reactive in its forms of δ-MnO_2 and β-MnO_2, which can be easily reduced to Mn^{2+} (MnII) (Leeper, 1970).

In calcareous soil, most Mn occurs as insoluble manganocalcite, ($MnCO_3$) with an oxidation state of +2 (MnII). The concentration of soluble Mn^{2+} is very low. This is because in soils with a pH value above 5.0, the oxidation of Mn^{2+} (MnII), to MnIII, and MnIII to MnIV is thermodynamically favoured (Leeper, 1970; Norvell, 1988). MnIV occurs as insoluble oxides that are unavailable for uptake by plants.

1.4.2 Factors affecting Mn availability in soil

Calcareous soils have a high buffering and neutralising capacity due to their high content of free $CaCO_3$. The pH varies from 7.3 to 8.5 (Lindsay, 1979), which thermodynamically favours the oxidation process to bind up the Mn in the form of plant unavailable manganese oxides (Jauregui and Reisenauer, 1982a). Calcareous and alkaline soils predominate on Yorke and Eyre Peninsulas in South Australia, where Mn deficiency in wheat and barley crops is a common problem (Reuter, 1972; Graham *et al.,* 1983).

In calcareous soils, the addition of soluble $MnSO_4$ to the soil as a Mn fertiliser only temporarily increases the plant available Mn^{2+}. This is because the oxidation of Mn^{2+} can be autocatalytic (Leeper, 1970; Nealson *et al.,* 1988). It is thought that the auto-catalysis of soluble Mn^{2+} is due to freshly precipitated and reactive MnIII or MnIV oxides (section 1.4.1) catalysing soluble Mn^{2+} to form sparingly soluble MnIII or MnIV oxides (Uren, 1981; Nealson *et al.,* 1988). Therefore, added Mn as a fertiliser in the form of soluble $MnSO_4$ can be easily oxidised, and over time becomes trapped as only sparingly soluble MnIII or MnIV oxides, which are less readily available for uptake by plants.

1.4.3 Biotic and environmental factors

In addition to soil factors, the availability of manganese to plants in the field is dependent on a variety of seasonal and biotic factors (Samuel and Piper, 1928; Leeper, 1970; Batey, 1971). Soil chemical, physical, and biological factors are all linked (Leeper, 1970), and influence soil pH and the solubility of Mn as shown in the following reaction:

$$MnO_2 + 4H^+ + 2e^- \Longleftrightarrow 2H_2O + Mn^{2+}$$

(insoluble) (soluble)

Organic matter such as green manures help create acidic and reducing conditions in which Mn^{2+} (MnII) becomes available to the plants (Leeper, 1970). The reverse reaction occurs in non-acid calcareous soils when micro-organisms, including bacteria, actinomycetes, fungi and algae are present in the soil (Bromfield, 1978). Under these conditions, insoluble MnIV is formed (Timonin, 1946). Desiccation of the soil over time was found to reduce the soil population of Mn oxidising micro-organisms, while the Mn reducing micro-organism population remained the same. Therefore, the soil Mn^{2+} levels were increased over time by the excess of Mn reducing micro-organisms over Mn oxidising micro-organisms (Leeper, 1970; Webb *et al.,* 1993a). However, because the roots of plants are inactive in dry soils, little Mn^{2+} is absorbed (Leeper, 1970). Some of the seasonal and soil factors affecting Mn availability to plants are summarised in the following table (Table 1.1).

Table 1.1 Soil factors affecting the availability of soil Mn (from Graham and Webb, 1991).

Factor	Mn availability
Higher pH	Decrease
Lime	Decrease
NH_4^+ forms of N	Increase
NO_3^- forms of N	Decrease
Cl^- fertiliser	Increase
Green manures	Increase
Cold wet soils	Decrease
Lupin rotation	Increase

The seasonal situation is complicated with the presence of Mn oxide reducing bacteria in the soil, for the availability of soluble Mn^{2+} to plants is increased (Barber and Lee, 1974). In addition, root induced changes in rhizosphere pH and root exudates that mobilise sparingly soluble micronutrients affect micronutrient availability (Marschner, 1991). Cleared zones of reduction of brown MnO_2 precipitation in nutrient agar around the roots of plants can be used to demonstrate changes in Mn reduction in the rhizosphere (Marschner *et al.,* 1982). Further, plant root exudates of reductants, malic acid and acidic phenolics can also increase Mn^{2+} within the rhizosphere of the plant (Bromfield, 1958a, b; Godo and Reisenauer, 1980; Uren, 1981; Stone and Morgan, 1984).

1.4.4 Fertilisers and Mn availability in soil

Application of ammonium fertilisers, sulphur, green manures and high moisture, which lower soil pH, tend to increase plant available manganese in the soil. This is due mainly to the biological reduction of insoluble MnIII and MnIV oxides to soluble MnII (Mn^{2+}) in low pH conditions (Leeper, 1970). Continued acidification of Mn sufficient soils of moderate pH by the application of fertilisers may over-ride the autocatalytic oxidation of Mn^{2+}, and soil Mn levels may rise resulting in Mn toxicity in sensitive plants (Leeper, 1970).

1.5 Plant micronutrient uptake, absorption and translocation

Mn absorption and translocation is a major factor conferring healthy growth and development to the plant. The mechanisms by which these processes occur are known, but not in sufficient detail. Strategy I plants (both dicots and monocots including cereal species) take up micronutrients from the soil solution using constitutive reductase in root redox processes, (which are highly regulated and under genetic control), and acidify the plant rhizosphere in response to low iron soil conditions (Kochian, 1991; Welch, 1995; Marschner, 1991). However strategy II plants (such as cereal species), which have evolved in alkaline soils use phytometallophore uptake systems to increase iron availability (Rîmheld, 1991; Marschner and Rîmheld, 1994). Thus, the strategy II mechanism of micronutrient absorption involves either: **a)** translocation of micronutrients by phytometallophore (which are produced by plants and bind to divalent cations of Fe, Cu, Ni, Zn, Co, and to least of all to Mn), or microbial metallophore chelates, **b)** absorption of micronutrients by chelate-specific transport proteins located in the root cell plasma membranes into the cytosol, or **c)** absorption of micronutrients via an H^+ or K^+ gradient across the root-cell plasma membrane with an amino acid co-transport system (Welch, 1995; Marschner and Rîmheld, 1994).

1.5.1 Mn efficiency and root system size

The Mn efficiency trait seems not to be associated with the size of the plant root system. Barley is more Mn-efficient than wheat, and has a more extensive root system to allow greater uptake of nutrients (Marcar, 1986). But in another study, no significant difference between barley genotypes differing in Mn efficiency has been found in root morphology to account for differences in Mn efficiency. Harbard (1992), found a similar root-shoot ratio for Mn-efficient and Mn-inefficient genotypes of barley growing in Mn sufficient soil. However, in the rate of root growth, the Mn-inefficient genotype was sensitive to low Mn soil, whereas the rate of root growth of the Mn-efficient genotype was not. Harbard (1992) found that root system size of the Mn-inefficient genotype decreased when grown in Mn limiting soil, but in contrast, the root system size of the Mn-efficient genotype when grown in Mn limiting soil was the same as when grown in Mn sufficient soil. Significantly, a larger root system does not confer efficiency for the uptake of specific nutrients like manganese in Mn limiting soils (Graham *et al.*, 1983).

Further evidence that root system size is not a factor in Mn efficiency is derived from the finding that the heritability of Mn efficiency is by one or a very few genes at the most (Graham *et al.,* 1983; M^{c} Carthy *et al.,* 1988). In contrast the heritability of a large root system is very complex (Zobel, 1975), and many more genes are expected to be involved. Finally, older genotypes of wheat have larger root systems, but have less Mn efficiency when grown in low Mn soils compared to modern genotypes which have a greater Mn efficiency but with a smaller root system (Evans and Dunstone 1970; Siddique *et al.,* 1990). For all these reasons, it seems unlikely that the expression of the Mn efficiency trait is associated with the size of the plant root system.

1.5.2 Mn efficiency and root Mn uptake

The site in the plant relevant to the expression of Mn efficiency must be the root system and the root rhizosphere. Soil-grown plant roots detect signals at the root-soil interface and the mobilisation of nutrients from the rhizosphere. In contrast, solution-grown plant roots may not have the same properties. Solution-grown plants can only be used to study characters of nutrient reduction at the root plasma membranes, absorption rate, translocation and utilisation within the plant (Graham, 1984). Huang *et al.,* (1994) found that the Mn efficiency trait was not detected in solution culture of barley genotypes, but it was in soil culture (as determined by the tissue Mn) (Huang *et al.*, 1994). The results suggested that the differences in Mn efficiency is in the ability of genotypes to uptake Mn from the rhizosphere soil near the root-soil interface, rather than there being differences in the ability of the genotypes to utilise and accumulate Mn throughout the plant (Huang *et al.,* 1994; Huang 1996). Therefore, the root-soil interface appears to be the site of the expression of the Mn efficiency trait.

1.5.3 Mn absorption by roots

It is known that Mn^{2+} is absorbed by roots in two phases. The initial uptake is rapid, reversible and independent of metabolism. The Mn is adsorbed into the root cell wall apoplastic space. During this phase, which lasts for a few minutes, Mn^{2+} is exchangeable with Ca^{2+} (Page and Dainty, 1964; Garnham *et al.,* 1992). The second phase of Mn uptake is slower and proceeds over several hours. During this phase Mn is less readily exchangeable

(Maas *et al.,* 1968). However, Mn absorption during the second phase is dependent on metabolism. Darkness, low temperature and the application of metabolic inhibitors all decrease Mn absorption during the second phase, in which Mn is transported into the symplasm (Maas *et al.,* 1968; Garnham *et al.,* 1992). Mn most likely enters the root cell via carrier or channel proteins (Garnham *et al.,* 1992; Welch, 1995) like permeable Ca channels (Piñeros and Tester 1995).

1.5.4 Vesicular-arbuscular mycorrhiza (VAM)

The role of vesicular-arbuscular mycorrhiza (VAM) infection of barley roots in Mn uptake is not likely to be major, but it is uncertain if VAM infection plays a minor role. VAM infection of roots increases the root surface area for plant uptake of micronutrients, and increases the availability of phosphorus (P) to the plant (Baylis, 1982). VAM infection appears to be regulated by the plant, and can be expected to be greatest in soils low in P content. However, the levels of VAM infection of different barley genotypes with similar root mass growing in soil with no added P was only very low, and ranged from 8.6 - 28.6% (Baon *et al.,* 1993). Further, although VAM infection increased the efficiency of soil P uptake, the plant utilisation of P (as measured by P shoot concentration) was decreased (Baon *et al.,* 1992). VAM infection levels in barley increased when soil temperature was increased from 15°C to 20°C (Baon *et al.,* 1994a), but remained at low levels compared to dicotyledons. Unlike dicotyledons, *Graminaceous* plants such as barley have an extensive fibrous root system, which increases their root surface area for micronutrient uptake. Therefore, barley and other *Graminaceous* plants are likely to be less dependent on mycorrhizal infection to increase the root surface area for enhanced micronutrient uptake, compared to dicotyledons.

VAM infection increased the uptake of Zn in citrus (Tinker and Gilden, 1983). However, there is no evidence that VAM infection increases Mn uptake in barley. Baon *et al.* (1992), found that the levels of VAM infection in Mn sufficient soils was greater in wheat than in barley, while the least VAM infection occurred in rye. However, this is the reverse order of Mn efficiency in these cereals when grown in Mn limiting soil (section 1.2.2). Barley and rye have a more extensive root system than wheat (Baon *et al.,* 1992). This finding would explain why wheat may be more dependent on higher levels of VAM infection to increase its root surface area for micronutrient absorption. But in another study, although the Mn-

efficient trait in Mn limiting soils was greater in barley than in wheat, the levels of VAM infection of the roots was similar (Marcar, 1986). These conflicting results suggest that VAM infection levels may change between sufficient Mn and Mn limiting soils. However, a more likely explanation is that the conflicting results are due to the two studies using different methods to quantify VAM infection levels. In addition, Mn limiting soils are often sufficient in P supply, which has led to decreased VAM infection (Hall, 1978; Baon *et al.,* 1992). To summarise, the current evidence suggests that VAM infection is not likely to be associated with the Mn-efficient trait in low Mn soils in barley.

Conversely, current evidence suggests that VAM infection may not increase but in fact decrease the availability of Mn to plants. The Mn requirement for microbial growth is up to one hundred times less than that in plant tissues (Bertrand and Javillier, 1912). Due to the VAM oxidation of Mn, VAM infection has been found to decrease Mn concentration in roots and shoots, thus perhaps protecting roots from Mn toxicity (Biermann and Linderman, 1983; Pacovsky, 1986; Arines *et al.,* 1989; Kothari *et al.,* 1991; Medeiros *et al.,* 1995). Therefore VAM infection in roots may actually slightly decrease the Mn availability to the plant. In conclusion, VAM infection of barley root is likely to be insignificant to Mn uptake and the Mn-efficient trait because of take-all infection, the sufficient P supply in the soil, and the cool moist conditions that favour the growth of fungi such as take-all, do not favour the growth of mycorrhizas (Baon *et al.,* 1992; 1993; 1994a).

1.5.5 Mn translocation in plants

Plants have not evolved efficient mechanisms of Mn translocation within the plant to ensure survival of plants in Mn limiting soils (Graham, 1983). During vegetative growth, Mn is readily transported in the xylem but not in the phloem of the plant. Mn absorbed by a plant root is transported to the xylem, and then via the transpiration stream to the shoots of the plant. Mn is not re-translocated from the leaves to other parts of the plant (Nable and Loneragan, 1984a; El-Baz *et al.,* 1990). Therefore the application of Mn fertiliser in foliar sprays is likely to overcome Mn deficiency in plants only temporarily until a new leaf emerges. It is only during grain development that Mn in the roots and stems is translocated to the ear (Pearson and Rengel, 1994; 1995b). This is important for the survival of the seed, for

high seed Mn levels have been found to be beneficial to early seedling growth (Marcar and Graham, 1986) and dry weight yield (Longnecker *et al.,* 1988).

There is further evidence that Mn is not transported in the phloem from the results of a split root experiment. Mn was not translocated from Mn sufficient root system to a Mn deficient root system in a split root culture of subterranean clover (Nable and Loneragan, 1984b). Therefore, Mn fertiliser applied to one part of the root network will not supply Mn via phloem translocation to the rest of the Mn deficient root system. Plant available Mn needs to be mixed throughout the soil to supply all the root system with Mn and to ensure adequate Mn nutrition for plant growth and development (Graham and Webb, 1991).

1.6 Molecular studies of Mn efficiency

Mn efficiency in plants when grown in Mn limiting soils is critical to the growth and development of the plant (sections 1.2 and 1.3). Therefore, the study of the Mn-efficient trait has the potential to increase significantly the growth and yields of cereal crops.

1.6.1 Plant Mn status and Mn efficiency

A plant has a status of being Mn sufficient if the shoot Mn level is > 12.0-12.4 mg/kg DM in barley varieties (Hannam *et al.,* 1987). The soil is Mn sufficient for the plant, either because the soil has adequate plant available Mn^{2+}, or because the plant is expressing the Mn-efficient trait in a soil too deficient in plant available Mn for a standard genotype (Graham, 1984). Conversely, a plant is Mn deficient if the soil is too deficient in plant available Mn for a standard genotype and the plant has expressed a putative Mn-inefficient trait.

1.6.2 Screening for Mn efficiency

In a controlled environment, two methods are used to screen barley varieties for the Mn efficiency trait. As discussed (section 1.5.2), a solution culture method is used to detect characters operative at the root surface and inwards to the plant interior. This involves loading of Mn in the cell wall, nutrient reduction at the root plasma membranes, absorption rate, translocation and efficiency of nutrient utilisation within the plant (Graham, 1984). In contrast, a soil bioassay is used to detect characters of the Mn-efficient trait operative at the root-soil interface, such as mobilisation of unavailable nutrients from the rhizosphere (section

1.5.2). The expression of the Mn-efficient or Mn-inefficient traits when barley is grown in Mn limiting soil bioassays has been found to agree reasonably well with results obtained under field conditions (Graham, 1984; Wilhelm *et al.,* 1988; Huang, 1996).

As described in section 1.5.2, the Mn efficiency trait in solution culture was not expressed as either high dry mass yield or tissue Mn concentration (Huang *et al.,* 1994). In solution culture, chelators are uniformly distributed throughout the solution. Consequently, there are no concentration gradients generated down which there can be co-transport of Mn towards the root. Hence, this result showed that the mechanism of the Mn-efficient trait is likely to be due to the ability of the barley plant to mobilise Mn in the plant rhizosphere from the soil solid phase via the soil solution to the plant root at the root-soil interface.

There is further evidence that no Mn-efficient trait in monocotyledons can be detected when solution culture is used as a screening method. Barley grown in solution culture under Fe deficient conditions induced phytosiderophore release, which is likely to be involved in Mn availability, uptake or mobilisation (section 1.5.2). But no such phytosiderophore release response from barley was obtained in low Mn solution culture (Gries, *et al.,* 1995). This result suggests that only soil culture bioassay screening methods are appropriate for studying the expression of the Mn-efficient or Mn-inefficient trait in monocotyledons such as barley.

1.6.3 The identification of genes for plant nutrition

There are several strategies available to isolate genes associated with plant nutrition. If the DNA sequence of the gene encoding for the trait of interest is not known, a DNA based strategy can be used to isolate and identify the gene. When the amino acid sequence of the translation product of the gene of interest is known, protein based strategies can be used to isolate and identify the DNA fragment encoding for the protein using a synthesised oligonucleotide probe derived from a unique region of the protein.

Mne-1 was identified using a DNA based strategy. Huang *et al.,* (1996b) constructed a root cDNA library from a Mn-efficient barley genotype Weeah. Using differential screening, Huang *et al.*, (1996b), found that a clone, later designated *Mne-1* (492 bp), was in greater abundance in an RNA gel blot analysis in Weeah compared to the Mn-inefficient genotype WI 2585, when harvested at both 21 and 28 days from sowing into a Mn limiting soil.

Mne-1 cDNA was sequenced, the amino-acid sequence determined, and shown to be a zinc finger protein, Mne-1 (Huang *et al.*, 1995). Mne-1 shows close homology to a human protein, MPS-1 (GenBank and EMBL), which binds to a cAMP response element (Fernandez-Pol *et al.*, 1994; Xynos *et al.*, 1994). The Mne-1 protein may be a DNA binding regulatory transcriptional factor expressed as an adaptive response to Mn limiting soil conditions to regulate genes responsible for the Mn-efficient trait in barley (Huang, 1996).

To check the possible mechanisms of expression of *Mne-1*, a protein based strategy was used. The protein transcribed from *Mne-1* was expressed in *E. coli,* and polyclonal antibodies were raised against it (Huang *et al.*, 1995). These antibodies were used to show that there was more Mne-1 protein in roots of the Mn-efficient variety Weeah compared to the Mn-inefficient variety WI 2585. This may be consistent with the greater abundance of *Mne-1* mRNA found in Weeah. This result suggested that under Mn limiting conditions in soil, there may be a higher expression of *Mne-1*, both as mRNA and Mne-1 protein, in the Mn-efficient genotype compared to the Mn-inefficient genotype (Huang *et al.*, 1996b).

1.6.4 Molecular markers for nutritional traits

Several tools used in molecular biology are available to investigate the genetic basis of the Mn-efficient or Mn-inefficient traits. Restriction Fragment Length Polymorphisms (RFLPs) are used as genetic markers. RFLPs have been used to identify markers genetically linked to the trait of tolerance to low phosphorus stress in maize plants (Reiter *et al.*, 1991), and RFLPs have been used to locate a salt tolerance gene in rice (Zhang *et al.*, 1995). RFLP maps are available for barley (Kleinhofs and Kilian, 1994). As stated in section 1.6.3, *Mne-1* has been found to be associated with expression of the Mn efficiency trait (Huang *et al.*, 1996b). RFLP analysis has been used to detect a genetic locus *Mel 1* located on 4HS between the Mn-efficient Amagi Nijo, and Mn-inefficient WI 2585 (Pallotta *et al.*, 1999).

1.6.5 Genetic strategies used

A cross of the Mn-efficient Amagi Nijo and the Mn-inefficient WI 2585 had resulted in F_2 lines segregating for either the Mn-efficient or the Mn-inefficient trait when grown in Mn limiting soil (Pallotta *et al.*, 1999). Amagi Nijo is a Japanese line, (see section 1.2.2), and WI 2585 is derived from the Egyptian line, CI 3576. Seventeen F_3 families had been generated

from the selfing of selected F_2 plants. In progeny testing, ten F_3 families showed the lowest Mn uptake efficiency similar to the WI 2585 parent. Bulk RFLP analysis of these F_2 lines to map the Mn uptake efficiency suggested a possible linkage with the Mn inefficiency trait in the WI 2585 parent. Seven F_3 families with the highest Mn uptake efficiency showed a Mn uptake efficiency similar to the Amagi Nijo parent in all except one F_3 family. In this F_3 family, the Mn uptake efficiency levels (shoot Mn concentration) were segregating over a range from 6-12 mg/kg DM. This result suggests that there was more than one loci influencing the Mn-efficient trait in one of the selected F_2 lines.

1.6.6 Summary

The 4HS *Mel 1* alleles for Mn efficiency and Mn inefficiency can be identified using RFLP probes *wg622*, *cdo583*, *abg714* and *mwg77* (Pallotta *et al.,* 1999). These probes can be used to identify alleles for Mn efficiency and Mn inefficiency in plants. When plants with the Mn-efficient allele are grown in soil low in plant available Mn, experiments can be done to determine if these plants have a higher resistance to infection by *Ggt* compared to plants with the Mn-inefficient allele.

1.7 Disease resistance in plants

Micronutrient deficiency has been found to be a critical for plant resistance to certain diseases.

1.7.1 Host disease resistance

Disease resistance in a host is defined as the ability to limit the penetration, development, and/or reproduction of invading pathogens (Graham and Webb, 1991; Wallwork, 1996). In resistant crop varieties, less of the disease inoculum survives to infect crops in the following season (Wallwork, 1996). Resistance can vary with genotype of the host and pathogen, host age and changes in the environment. Specific resistance is controlled by one or more major genes. Non-specific resistance is polygenic, and is often degraded by micronutrient deficiency in the host (Western, 1971; Graham and Webb, 1991). Resistance to pathogens like

take-all, which seems to be Mn dependent, is most likely non-specific resistance (Graham, 1983).

1.7.2 Host disease tolerance

Tolerance to disease in the plant host is defined as the ability to maintain its own growth and yield well in spite of infection (Trudgill, 1986; Wallwork, 1996).

1.7.3 Host disease resistance or tolerance?

There is much debate about the definition of plant resistance and tolerance towards disease. But for the plant, resistance and tolerance are independent of each other. This is because resistant plants may still suffer large yield losses when the disease is present, while tolerant plants showing little effects on yield of disease pathogenicity may leave large amounts of disease inoculum resulting in greater damage to subsequent crops (Wallwork, 1996). Thus, it is ideal to breed plant varieties which have both resistance and tolerance to disease.

The difference between resistance and tolerance becomes ambiguous when the pathogen produces toxic substances which inhibit regrowth of the host plant (Wallace, 1987). The most likely defence mechanism barley has against take-all invasion is thought to be increased lignin production (Skou 1981; Graham and Webb, 1991). Therefore, because the plant produces a physical barrier to take-all penetrating the root, the plant exhibits resistance to the pathogen.

1.8 *Gaeumannomyces graminis* var. *tritici* (*Ggt*)

The fungus, *Gaeumannomyces graminis* var. (Sacc) von Arx & Oliver var *tritici* Walker, (*Ggt*) (cf. Walker, 1981) has a world-wide distribution and is a serious disease of crops (Samuel and Piper, 1928; Cook, 1994). In Australia, the disease occurs across the southern grain belt, extending from Western Australia, through to South Australia, Victoria and most of New South Wales (Mac Nish, 1980; Samuel, 1924; Wong, 1983). *Ggt* has mycelium which consists of black-brown thick walled ectopic runner hyphae which grow along the outside of root systems of crop plants forming a net around the root. From the net-like structure, hyaline thin-walled hyphae invade the root (Garrett, 1934a). *Ggt*, when grown

in both culture medium and on wheat roots, has been found to secrete cell wall degrading enzymes to enhance invasion of the root (Dori *et al.,* 1995). The *Ggt* hyphae effectively amputate plant roots by penetrating the root cortex, then the endodermis, and finally penetrating the phloem and blocking the xylem (Asher, 1981).

1.8.1 *Ggt* disease

Severe *Ggt* infection leads to plant death due to the cutting off of supplies of carbohydrates via the phloem to the root tip, and of nutrients and water via the xylem to the shoots (Graham and Webb, 1991). In less severe infection, stelar lesions of the root are visible by the naked eye as discolouration of the root vascular system (the phloem and the xylem together known as the stele) (Asher, 1981). The plant appears normal until hot dry conditions near harvest cause water stress, reduced head formation, and grain yield loss. Moderate infection may be sub-clinical even up to harvesting. However, yield is significantly reduced due to the plant redirecting resources to infection defence mechanisms such as growing new roots rather than shoots (Rovira and Venn, 1985).

The order of susceptibility of cereals to take-all infection is wheat > barley > triticale > rye > oats (Scott and Hollins, 1985; Scott *et al.,* 1985; Hollins *et al.,* 1986; Dewan and Sivasithamparam, 1990). Significantly, this order of susceptibility to *Ggt* is the reverse of the order of the expression of the Mn efficiency trait in many of these cereals except oats (section 1.2.2). The most sensitive measure of infection levels of *Ggt* isolates in association with the level of plant Mn has been found to be the average length of stelar lesions in the plant roots per plant (Wilhelm, 1991; Pedler, 1994).

1.8.2 The biology of *Ggt*

Ggt is a homothallic ascomycete which is haploid during its vegetative phase (Asher, 1981). *Ggt* also has a diploid zygote phase. In culture the ascospores form conidia which germinate into mainly uninucleate hyphae. In soil the ascospores form asexual phialidic conidia which do not germinate (Asher, 1981). Therefore, the ascospores formed by *Ggt* germinate in culture but not in soil.

1.8.3 Pathogenic variation

There are many isolates of *Ggt* with differing degrees of pathogenicity (as measured by the amount of *Ggt* invasiveness of the plant root). The mycelia are heterokaryotic, having nuclei of several genotypes in the same cell or in a common cytoplasm (Asher, 1981). The genetically heterogeneous mycelia can respond to selection pressure. More vigorous mycelia grow faster towards the root in response to root exudates than less vigorous mycelia. Thus, the more vigorous isolates are selected for survival. The pathogenicity of the isolates has not been found to be associated with the presence or absence of melanin in the runner hyphae, or to the rate of growth of the *Ggt* isolates on agar (Asher, 1981). However, it has been found to be related to the manganese-oxidising ability of the isolates (section 1.8.6) (Pedler *et al,* 1996).

1.8.4 *Ggt* survival and crop rotation

Under Australian field conditions, *Ggt* survives dormant but viable in the soil as a saprophyte within the debris of a previous host. As a result, cultivation of "cleaning" or "break" crops not susceptible to take-all is one strategy used to reduce saprophytic survival and thus infection of the subsequent take-all susceptible cereal crops. Conversely, cultivation of cereal crops of greater susceptibility to take-all increases the severity of take-all infection in subsequent susceptible crops ostensibly because of the higher survival of the saprophyte in the soil (Brassett and Gilligan, 1990).

However, the strategy of rotating take-all susceptible/non susceptible crops, to reduce saprophytic survival of *Ggt* inoculum has given ambiguous results in other studies (Kollmorgen *et al.,* 1985b). The strategy of planting crops less susceptible to *Ggt* in between *Ggt* susceptible crops has been found to both decrease (Moore and Cook, 1984; Rovira and Venn, 1985), and increase (De Boer and Kollmorgen, 1987; Rothrock, 1987) the levels of take-all infection.

Under field conditions, time, warm weather and moisture decrease the viability of *Ggt* inoculum (Garrett, 1938; Kollmorgen, 1985a). The inoculum exists mostly in the upper 10-15 cm of the soil (Kollmorgen *et al.,* 1985a). The presence in the soil of live susceptible roots has been found to elicit hyphal growth trophically from plant to plant to a distance of 1.5 m over 8 months, (Wehrle and Ogilvie, 1955). In plate culture, hyphae have been found to grow

rapidly for a distance of 12 mm, after which the growth rate of the hyphae is slowed because of depleted ion availability in the media (Gilligan, 1980).

1.8.5 Attenuation and restoration of *Ggt* pathogenicity

Subculturing of *Ggt* isolates can lead to attenuation of pathogenicity (Cunningham, 1981). Thus, long term storage of *Ggt* isolates by repeated subculturing on agar can lead to loss of pathogenicity (Pedler *et al,*. 1996). However, pathogenicity of the isolate is restored by passage through the host plant. As discussed in section 1.8.3, the restoration of pathogenicity can occur because of selection pressure for potential pathogenic characteristics within the vegetative mycelium. *Ggt* hyphae can respond with vigorous growth after contact with root exudates, and grow throughout the host plant by invasion of the roots (Kollmorgen *et al.,* 1985b).

1.8.6 Environmental conditions and take-all disease

Take-all disease is severe in cool, moist, alkaline or neutral pH soils (Cook, 1981; Huber 1981). As discussed (section 1.4), available Mn is low in these soil conditions (Leeper 1970). Fertilisers, due to their influence on soil pH, alter the amount of plant available Mn in the soil and consequently the severity of *Ggt* infection (Huber, 1981). The addition of nitrate nitrogen fertiliser to soil increases soil pH which promotes the oxidation of the plant available Mn^{2+} to plant unavailable insoluble Mn oxides (Mn IV). When plant available Mn is low in soil, the severity of *Ggt* infection is increased (Huber, 1981). Conversely, the severity of *Ggt* infection is decreased by ammonium based fertilisers because soil pH is lowered, which promotes the reduction of MnIV to increase the amount of plant available Mn^{2+} (Huber 1981). Soil-borne micro-organisms which are either reducers or oxidisers reverse the above processes, and a variety of linked environmental factors which change soil pH, decrease or eliminate the microbial influence on the soil Mn^{2+} (section 1.4) (Leeper, 1970).

Isolates of *Ggt* need Mn for growth, but the available Mn required is only one hundredth of that needed by higher plants (Bertrand and Javillier, 1912). *Ggt* in plate culture has been found to oxidise Mn^{2+} to brown MnO_2, which was also highly correlated with the pathogenicity of the isolate (Wilhelm *et al.,* 1990; Schulze *et al.,* 1995; Pedler *et al.,* 1996). The Mn oxidising ability of *Ggt* could be a mechanism by which *Ggt* can protect itself from

Mn toxicity (Graham, 1983), and at the same time deprive the root of the host plant of Mn and therefore reduce the efficacy of the plant root defence mechanisms involving Mn (section 1.9). However, Mn levels in calcareous soils have not been found to be toxic to *Ggt* growth (Wilhelm *et al.,* 1988).

1.9 Mn plant defence mechanisms against take-all

The nature of the plant defence mechanism involving Mn which protects different crops (section 1.8.1), and genotypes of barley having different susceptibilities to root invasion by take-all is not clear.

1.9.1 Micronutrient stress, and disease resistance and tolerance

Micronutrient deficiencies can not have been frequent enough before the agrarian revolution to limit the survival of crop plants through evolutionary time (Graham, 1983). In addition, crop plants must have co-existed in a delicate balance with their pathogens, without either the plant host or the pathogen eliminating each other. Changes in soil fertility can shift this balance as appears to have happened with the use of nitrogen fertilisers. The development of nitrogen fertilisers have made possible the annual cultivation of crops in previously infertile soils (Graham, 1983). However, micronutrient deficiencies in these soils were not changed, so crops may have suffered more from micronutrient stress such as Mn deficiency after nitrogen and phosphorus deficiencies were eliminated. Further, the Mn deficiency gave crop plants increased susceptibilities to pathogens such as take-all (Graham, 1983).

1.9.2 Lignin biosynthetic pathway and take-all

One plant defence mechanism against root invasion by take-all may be related to increased lignin production (Skou, 1981). Lignin forms a protective ectopic barrier around the roots of plants. The synthesis of phenols and lignin occurs in normal higher-plant growth, and so is an obligate process. However localised lignin synthesis has been induced by biotic "elicitors" (Barber and Ride, 1988). The fungus *Ggt* has been observed to induce the formation of ligneous structures known as lignitubers (Skou, 1981), which may act as a physical barrier to protect the plant roots from invasion by *Ggt*.

As discussed (section 1.3.3), during biosynthesis of lignin, monophenols are oxidised by Mn-peroxidase. Because many oxidised phenols are toxic, phenol production has also been implicated in plant host defences against invading pathogens (Bell, 1981). In addition, phenolics can reduce Mn oxides (Stone and Morgan, 1984), but probably not enough to release soluble Mn^{2+} to the plant in calcareous soils (pH 5-6) (Godo and Reisenauer, 1980). It is likely that the same effect of pH in calcareous soils applies to malic acid, which is a MnO_2 reducing root exudate of wheat (Jauregui and Reisenauer, 1982b). All of these mechanisms that Mn participates in are likely to inhibit infection of the roots of plants by take-all. However, no connection between take-all resistance and production of phenolics and lignin was found in wheat genotypes with different Mn efficiency (Rengel *et al.,* 1993; 1994a).

1.9.3 Mn and aminopeptidase

The role of aminopeptidase is most likely to provide a supply of amino acids for fungal growth. Aminopeptidase production in plants is activated by pathogens. However, aminopeptidase activity is inhibited by the presence of Mn^{2+} (Huber and Keeler, 1977), and this may be another mechanism by which Mn in the soil and in the plant root rhizosphere inhibits the growth of pathogens such as take-all.

1.9.4 Mn and pectin methylesterase

Pectin methylesterase is an enzyme which fungi produce in order to break down the cell walls of the host plant, facilitating invasion of the host plant root. However, like aminopeptidase, Mn also inhibits the activity of pectin methylesterase (Sadasivan, 1965), and Mn^{2+} in the root rhizosphere can be expected to inhibit take-all invasion and infection of plant roots.

1.9.5 Mn and *Pseudomonas fluorescens*

Soil-borne micro-organisms inhibit *Ggt* infection of plant roots. *Bacillus cereus* has been found to reduce insoluble manganese oxides to Mn^{2+} ions (Wilhelm *et al.,* 1987: Wilhelm, *et al.,* 1990). Thus more Mn can be made available to the plant for use in plant defence mechanisms against pathogen invasion. *Pseudomonas fluorescens* is a micro-organism common in soil which suppresses take-all. *P. fluorescens* produce siderophores, which

scavenge free soil Fe^{3+}. Siderophores transport Fe to the root surface and their function is as a chelator (Nielands and Leong, 1986). Mn uptake in the root is thought to be by a similar mechanism. Therefore siderophores may enhance Mn uptake in roots.

Strain 2-79 of *P. fluorescens* has also been found to increase Mn-peroxidase activity (Albert and Anderson, 1987). The increased Mn-peroxidase activity would result in greater lignin production in the plant roots to act as a more impenetrable barrier to pathogen invasion. *P. fluorescens* also produces an anti-fungal compound, phenazine-1-carboxylate. This compound is more toxic to wheat than *Ggt*, but it also reduces the growth of the take-all fungus (Coghlan, 1996). The action of all of these factors can inhibit growth and infection by take-all.

1.9.6 The role of micronutrients in susceptibility to *Ggt*

Mn seems to be the major plant micronutrient affecting susceptibility of the plant to take-all infection. As discussed (section 1.4.3 and section 1.4.4), the addition of ammonium fertilisers to the soil increases soil acidity and subsequently the amount of plant available Mn (Conner, 1932; Leeper, 1970; Reuter *et al.,* 1973b). Copper, (Cu) and zinc, (Zn) become plant available in Cu and Zn deficient soils in a bi-modal acid and alkaline range (Graham and Nambiar, 1981; Kubota and Allaway, 1972). The pH pattern of soil Fe availability to plants resembles that of Mn, but Fe has no effect on take-all, while addition of Cu and Zn decreased take-all infection (Reis *et al.,* 1982; Wood and Robson, 1984). However, the environmental effects on soil pH and soil plant available Mn deficiency in wheat plants was the only significant factor corresponding with the predisposition of the plants to take-all infection. Therefore Mn seems to be the major micronutrient effecting the susceptibility of wheat plants to take-all infection (Graham, 1983).

1.10 Thesis prologue

The aim of this research is to determine, in barley (*Hordeum vulgare* L.) grown in Mn-deficient soil, if there is a causal connection between manganese uptake efficiency and resistance to root invasion by *Gaeumannomyces graminis* var. *tritici* (*Ggt*). My pilot work found that when the Mn-efficient Amagi Nijo and the Mn-inefficient WI 2584 were grown in

Mn deficient soil, the Mn-efficient Amagi Nijo had less *Ggt* infection compared to the Mn-inefficient WI 2585.

Physiological studies

First Null Hypothesis: There is no physiological connection between the expression of the Mn efficiency trait in barley and enhanced resistance to *Ggt*.

1). The aim of the pilot experiments will be to screen different *Ggt* isolates, select one suitable for my experiments, and to see if there is a difference in infection between Mn-efficient genotype Amagi Nijo and Mn-inefficient genotype WI 2585 when grown in Mn limiting soil (chapter 2 and chapter 3).

2). The next experiment will screen Amagi Nijo and WI 2585 in a pot assay to determine if there is a difference in *Ggt* infection which correlates with the Mn status of the plants when grown in soil with levels of Mn fertiliser added that give plants of deficient Mn status and sufficient Mn status (chapter 4).

3). A further time course experiment can show if the Mn-efficient trait and resistance to *Ggt* could be associated with a plant defence mechanism within the root (chapter 4).

4). Additional factors, other than Mn uptake efficiency, (eg root lignin synthesis, *Ggt* ectopic root growth and *Ggt* penetration of the root) will be investigated using fluorescent and Scanning Electron Microscopy (SEM) (chapter 5).

Genetic studies

If the physiological studies suggest a connection between Mn efficiency and resistance to *Ggt*, a second hypothesis will be investigated. **Second Null Hypothesis:** There is no genetic linkage between the recently identified *Mel 1* locus (4HS) Mn-efficient allele and enhanced resistance to *Ggt* (chapter 6).

1). Map the *Mel 1* locus for *Ggt* resistance/Mn efficiency using RFLP analysis of 439 doubled haploid (DH) lines to a particular locus on chromosome 4HS (chapter 6).

2). Use AFLP analysis to identify AFLP molecular markers specific to *Mel 1* to replace the RFLP marker *Xabg714*, which is difficult to use because it identifies multiple loci (chapter 6).

3). Use lines from the DH population where the RFLP and AFLP allele type at *Mel 1* has been determined to search for linkage between Mn efficiency and *Ggt* infection (chapter 6).

4). Identify loci other than *Mel 1* which influence Mn efficiency and determine if other Mn efficiency loci are genetically linked with resistance to *Ggt* infection (chapter 6).

Chapter 2 Pilot studies

2.1 Introduction

Previous bioassays in Wangary soil had shown that when Amagi Nijo and WI 2585 were grown in Mn deficient soil supplemented with Mn, the shoot Mn concentration of Amagi Nijo was above the critical Mn level for barley (11 mg/kg DM), (Hannam *et al.*, 1987), while the shoot Mn concentration of WI 2585 was below the critical level of barley (see section 1.6.5 and section 1.6.6) (Pallotta, *et. al.*, 1999). This result showed that Amagi Nijo is a Mn-efficient genotype while WI 2585 is a Mn-inefficient one. If the expression of Mn efficiency enhances resistance to *Ggt* (see section 1.8 and section 1.8.1), a pilot study with the *Ggt* fungus added to the pots of both Amagi Nijo and WI 2585 grown in Mn deficient soil would show more infection in the Mn-inefficient WI 2585 than in the Mn-efficient Amagi Nijo.

2.1.1 Pilot studies

Repeated subculturing of *Ggt* isolates has lead to attenuation of pathogenicity (Pedler *et al.*, 1996), but the virulence can be restored via one passage through the host plants. The virulence is maintained by culture of *Ggt* on seed of the host plants (Wilhelm, 1991). The appropriate *Ggt* isolate will be selected for use in the experiments after one passage through the host plants, on the basis of a radial growth on 4% PDA that is not inhibited by the addition of increasing concentrations of Mn, and on the basis of showing an increase in pathogenicity of infection in the roots. Levels of infection by *Ggt* are known to give high variability (Wilhelm, 1991, and Pedler, 1994). Therefore, the infection on each genotype will be checked and the sample size calculated to optimise the possibility of obtaining statistically significant differences in infection between WI 2585 and Amagi Nijo.

2.2 Method

2.2.1 Experiment 1: *Ggt* tolerance to Mn

Mn (as $MnSO_4$) was added at the levels 0, 5, 7.5, 10, 15, 20, 30 and 100 mg Mn/L to 4% PDA, (0.156 g Potato Dextrose Agar (PDA) and 1 g Difco Bacto Agar/100 ml milli-Q

H_2O). After the 4% PDA and added Mn were autoclaved at 120°C and 100 kPa for 15 minutes, three petri dishes of each Mn level were prepared for each *Ggt* isolate treatment.

Seven *Ggt* isolates used (provided by Dr P Harvey) were; *Ggt* 23580, *Ggt* 23125, *Ggt* 17916, *Ggt* 51463, *Ggt* C 3, *Ggt* KM 69, and *Ggt* KS 1. From the edge of an actively growing colony on 40% PDA, mycelial cubes (4 mm x 4 mm x 4 mm) were cut and used to inoculate the centre of the petri dishes. Asepsis was used throughout all inoculation procedures. The inoculated dishes were allowed to grow in the dark at 15-20°C for 5-10 days. The radius of *Ggt* growth (mm) was recorded from the average of measurements taken at right angles to each-other to determine the rate of growth of each isolate over the duration of the experiment.

2.2.2 Experiment 2: Increased *Ggt* pathogenicity

Soil treatment and basal nutrients

The soil used was collected from a severely Mn-deficient site, Wangary, Eyre Peninsula, SA. The topsoil (0-10 cm) and sub soil (10-20 cm) were air dried and sieved through a 2-mm stainless steel sieve, mixed (1:1, w/w), and 20% w/w milli-Q water (resistivity, 18.2 MΩ cm) was added. The mixed soil, a calcareous sand, 67% $CaCO_3$ pH 8.63, was sealed in water tight plastic, protected from the light, and incubated at 15°C day/10°C night for 21 days. Pre-incubation oxidises the plant available Mn which was released during the drying phase (Uren, *et al.*, 1988). Before sowing, the following basal nutrients were added and mixed well into the incubated soil at rates of (mg/kg dry soil): $Ca(NO_3)_2.4H_2O$, 918; KH_2PO_4, 179.5; $MgSO_4.7H_2O$, 140; H_3BO_3, 5.6; $ZnSO_4.7H_2O$, 17.16; NaCl, 4.16; $CuSO_4.5H_2O$, 3.9; $CoSO_4.7H_2O$, 0.585; $H_2MoO_4.H_20$, 0.13; K_2SO_4, 28.4; and $FeSO_4.7H_2O$, 17.2.

Potting the soil, Ggt inoculation and sowing of seeds

Mn, as $MnSO_4$, was added at 6 levels corresponding to 0, 7.5, 10, 15, 30, and 100 mg Mn/kg dry soil. The pots (6.5 cm diameter x 15 cm high) were lined on the outside with aluminium foil to exclude light and lined with a polythene bag on the inside. The Mn supplemented soil (430 g) was added to the pots. Then, an intact agar disk (6 cm in diameter), either completely covered with a mat of the *Ggt* isolate 17916 or with no fungus (control) was placed on top of this soil. To germinate the seeds, the seeds were surface sterilised in sodium

hypochlorite (1%) for 15 minutes, rinsed 5 times in sterile milli-Q H_2O, placed in petri dishes on moist Whatman No. 42 ashless filter paper and stored 4°C in the dark 48 hours, then placed at room temperature for 48 hours in the dark. A further 30 g soil of the appropriate Mn addition was added to the pots, 3 seed of either Amagi Nijo or WI 2585 were sown, and finally 30 g more of the Mn supplemented soil was added. There were 6 Mn levels x 2 genotypes x 2 *Ggt* treatments x 3 replicate pots. In all, there were 72 pots.

Growth and harvesting

Plants were grown in a controlled-environment chamber with a cycle of 14 h day/10 h night and 15°C day/10°C night. The photon flux density was set at 500 µmol/m^2/s 15-cm above the surface of the pots. The labelled pots were placed on the bench in a completely randomised design, generated with Microsoft Excel 4.0, and rotated and watered daily to a water content of 20% (w/w) with milli-Q H_2O. Experiments were harvested at 28 days after sowing. At harvest, the two youngest emerged leaves, the remainder of the shoots (YEBs), and the roots were separated, the fresh mass taken and recorded, the samples placed into labelled envelopes, oven dried 80°C for 48 h and weighed. The Mn concentration was determined by digestion in nitric acid (HNO_3 1%) and analysis of mineral elements by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) (Zarcinas *et al.*, 1987). The shoot Mn content (µg/pot) was calculated from the multiplication of the shoot dry weight mass (DM) (mg/pot) by the shoot Mn concentration (mg Mn/kg DM). The mean and standard error (SE) of all data were calculated. Statistical analysis was for a completely randomised design using a 1-way ANOVA or a 2-way ANOVA (Microsoft Excel 4.0) (α = 0.05).

Determination of Ggt infection

After roots were washed, the primary roots were examined. The number of stelar lesions on each plant (NSL) and the length of each stelar lesion (LSL) (mm) were recorded. From these data, the average length of stelar lesion per plant (Av.LSL) (mm) was calculated. Also recorded were the number of primary roots per plant (NOR), and the number of infected primary roots per plant (NIR). From these data the percent of infected primary roots per plant (PIR) (%) was calculated. Statistical analysis was for a completely randomised design

using a 1-way ANOVA or a 2-way ANOVA (Appendix 2) (Microsoft Excel 4.0) ($\alpha = 0.05$). To satisfy the assumptions of analysis of variance so that ANOVAs could be used for determining significant differences, the highly variable infection data was normalised with a log transformation of the Av.LSL data, and a square root transformation on the PIR data (M. Lorimer, BiometricsSA). The mean and SE of all data were calculated.

2.2.3 Experiment 3: Calculation of sample size

A check was made to determine if, after one passage through the host plants, the *Ggt* isolate 17916 had increased pathogenicity. Compared to the Av.LSL obtained at the first passage through the host plants, the second passage through the host plants resulted in the *Ggt* isolate 17916 giving an increased average length of stelar lesion (Av.LSL), that was significantly greater in WI 2585 (44.6 ± 4.8 mm/3 plants, $n = 3$ pots) than in the Mn-efficient Amagi Nijo (16.1 ± 7.6 mm/3plants $n = 3$ pots) ($p = 0.06$) (2-way ANOVA with replication) (section 2.2.2). Thus, the minimum detectable difference expected (δ) was conservatively estimated to be $\delta = 15$. To enhance the possibility of obtaining statistical significance in the highly variable infection level results obtained between Amagi Nijo and WI 2585, the chosen values were $\alpha = 0.05$ with a power $\phi = 90\%$, ie. 0.90. The sample size equation used for the sample size calculation was 11.26 (Zar, 1984). The number of groups (genotypes) ($k = 2$), and using $s^2 = 142.3$, with a guessed sample size, (n), and $v_2 = k(n-1)$, for $n = 6$, $v_2 = 10$ with $\phi = 0.48$, for $n = 12$, $v_2 = 22$ with $\phi = 0.87$, which is close to the required 0.90, and for $n = 24$, $v_2 = 56$ with $\phi > 1.0$. Therefore, the sample size of $n = 12$, with $\alpha = 0.05$ and $\phi = 0.87$ was chosen as the number of replicate pots needed to optimise the possibility of detecting any statistically significant differences in *Ggt* infection levels.

2.2.4 Experiment 4: Confirmation of an adequate sample size

A pilot study was conducted in a completely randomised design with Mn as $MnSO_4$ added at 15 mg Mn/kg dry soil. There were 3 seed of either WI 2585 or Amagi Nijo grown in each of 12 pots that were inoculated with either *Ggt* 17916 or with nil *Ggt* (control) as in section 2.2.2 (2 genotypes x 12 pots x 2 *Ggt* treatments). In all there were 48 pots.

2.2.5 Seed Mn

Seed Mn concentration affects early seedling growth (Marcar and Graham, 1986) and dry weight yield (Longnecker *et al.,* 1988). To eliminate the confounding effects differences in seed Mn concentration may give in the results, the seed sown of each parent genotype or doubled haploid (DH) line was of similar size, germination stage and Mn concentration (Appendix 1, Table 2.1 and Table 2.2). The Mn concentration of seeds was determined (see section 2.2.2).

2.3 Results

The *Ggt* isolate which met all the selection criteria was *Ggt* isolate 17916. The results are given below.

2.3.1 Experiment 1: *Ggt* tolerance to Mn

The tolerance of *Ggt* isolate 17916 to Mn after one passage through the host plants was checked to determine if the radial growth of the isolate was slowed by increasing levels of Mn added to 4% PDA (Fig. 2.1). The growth radius of *Ggt* 17916 gave significant differences between the increasing concentrations of Mn 0-100 added to the 4% PDA ($p < 1.0x10^{-3}$, 1-way ANOVA, $n = 3$). But this was due to the growth radius of the *Ggt* being consistent at all levels of Mn except for Mn 100, where the growth radius was significantly slowed (Fig. 2.2). However Mn 100 is a Mn addition that will not be used when *Ggt* infection is determined in Mn-supplemented soil bioassays.

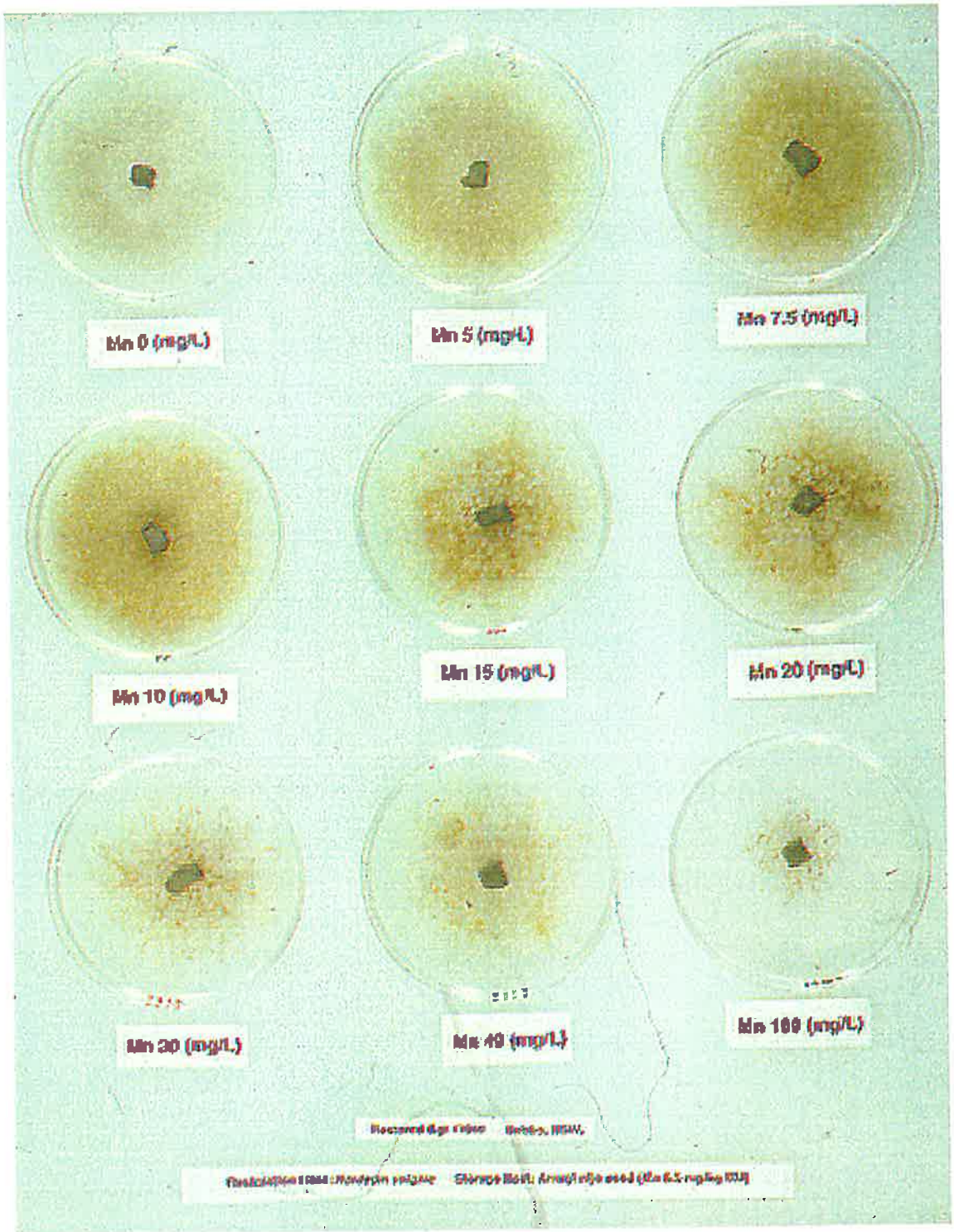


Fig. 2.1 Growth of *Ggt* 17916 on 9 levels of Mn added to 4% PDA over 8 days shows sensitivity to Mn only at Mn 100. Where the Mn as $MnSO_4$ had been added to 4% PDA, the Mn was oxidised by *Ggt* 17916 to brown granules of MnO_2.

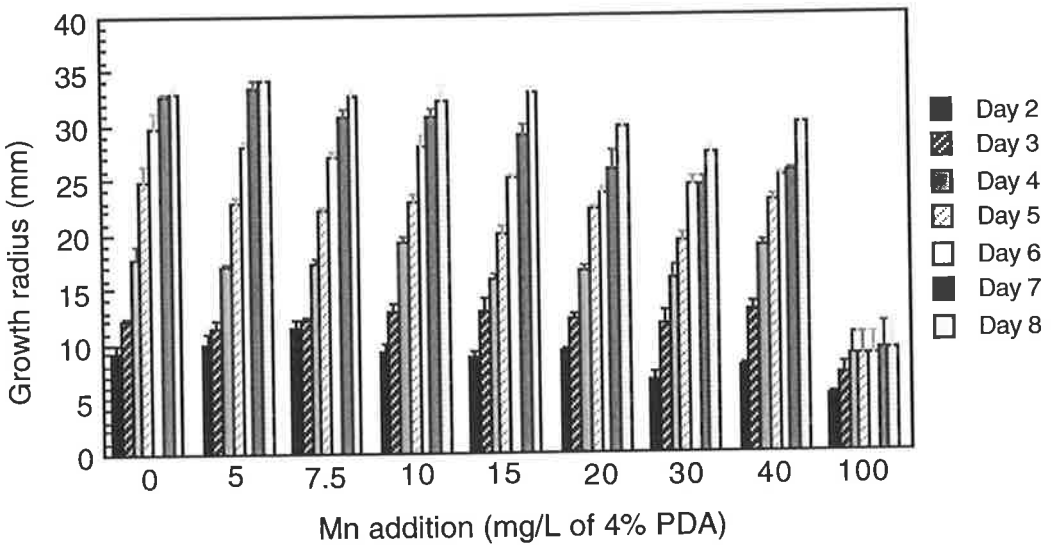


Fig. 2.2 The growth radius (mm) over 8 days of *Ggt* 17916 on 4% PDA with Mn added. Values are the means of 3 replicates x 9 Mn concentrations. Standard errors are shown as vertical bars.

2.3.2 Experiment 2: Increased *Ggt* pathogenicity

The AvLSL (mm) (Fig. 2.3) and the PIR (Fig. 2.4) for all levels of Mn added to the soil was much greater than the small amount of infection obtained upon the first passage of the *Ggt* isolate through the host plants. The interaction between the two genotypes and the soil Mn addition on Av.LSL was not significant ($p = 0.11$, 2-way ANOVA, 3 replications) (Appendix 2, 2.2.3) (Fig. 2.3). In addition, there were no significant differences in Av.LSL between the Mn addition ($p = 0.72$, 2-way ANOVA, $n = 3$). However the longer Av.LSL in WI 2585 than in Amagi Nijo (main effect of genotype) was close to being significant ($p = 0.06$, $\alpha = 0.05$, 2-way ANOVA, $n = 3$). In addition, the Av.LSL in WI 2585 was significantly longer than in Amagi Nijo at soil Mn 15 ($p = 0.043$, 1-way ANOVA, $n = 3$) (Fig. 2.3). There was no significant interaction on PIR between the genotypes and the soil Mn addition ($p = 0.09$, 2-way ANOVA, $n = 3$) (Appendix 2, 2.2.4) (Fig. 2.4). In addition, there was no significant difference in PIR due to the main effect of Mn addition ($p = 0.29$) nor in PIR between WI 2585 and Amagi Nijo ($p = 0.55$) (2-way ANOVA, $n = 3$).

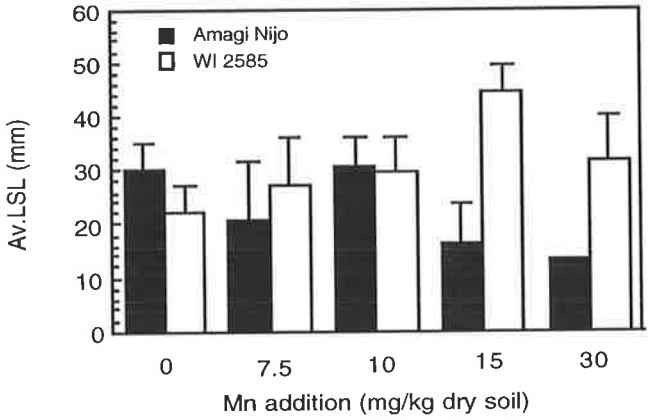


Fig. 2.3 The Av.LSL (mm) on barley grown in a controlled-environment growth chamber under five Mn soil treatments (Mn 0-30), resulting from *Ggt* 17916 infection of both cultivars WI 2585 and Amagi Nijo, after one passage through the host plants. Values are the means of 3 plants/pot and of three replicate pots. Standard errors are shown as vertical bars.

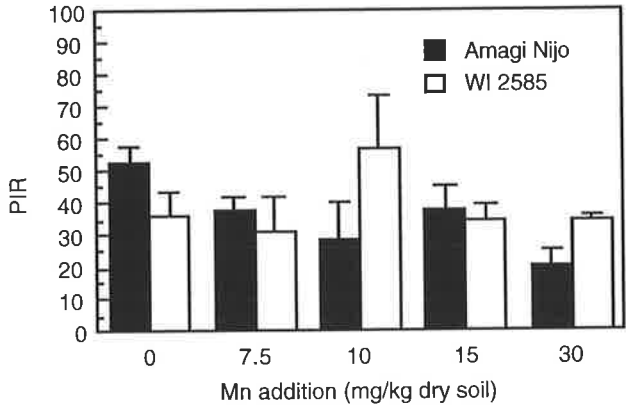


Fig. 2.4 The PIR on barley grown under five Mn soil treatments (Mn 0-30), resulting from *Ggt* 17916 infection of both cultivars WI 2585 and Amagi Nijo after one passage through the host plants. Values are the means of 3 plants/pot and of three replicate pots. Standard errors are shown as vertical bars.

2.3.3 Experiment 4: Confirmation of an adequate sample size

Amagi Nijo and WI 2585 inoculated with nil *Ggt* showed no stelar lesions on the roots, so the soil is essentially free of *Ggt* propagules. There was no significant interaction on shoot Mn concentration between the *Ggt* treatments and genotype ($p = 0.58$, 2-way ANOVA with replication, $n = 12$) (Appendix 2, 2.3.1) (Fig. 2.5). There was no significant difference in shoot Mn concentration between nil *Ggt* and *Ggt* treatments ($p = 0.91$). However, there was a

significant difference in shoot Mn concentration between WI 2585 and Amagi Nijo main effect ($p = 5.1x10^{-17}$, n = 12) (Fig. 2.5).

At soil Mn addition 15 mg/kg with nil *Ggt*, Amagi Nijo expressed Mn efficiency with a shoot Mn concentration of 11.8 ± 0.2 mg/kg DM, n = 12 (Fig. 2.5), which is above the barley critical shoot Mn concentration of 11 mg/kg DM (Hannam *et al.,* 1987). In contrast, at soil Mn 15 mg/kg, WI 2585 expressed Mn inefficiency with a shoot Mn concentration of 8.5 ± 0.2 mg/kg DM, n = 12, which falls below the barley critical shoot Mn level. At the same Mn rate added into the soil and infected with *Ggt* 17916, Amagi Nijo had a shoot Mn concentration of 11.7 ± 0.3 mg/kg DM (n = 12), while WI 2585 had 8.6 ± 0.2 mg Mn/kg DM (n = 12) (Fig. 2.5).

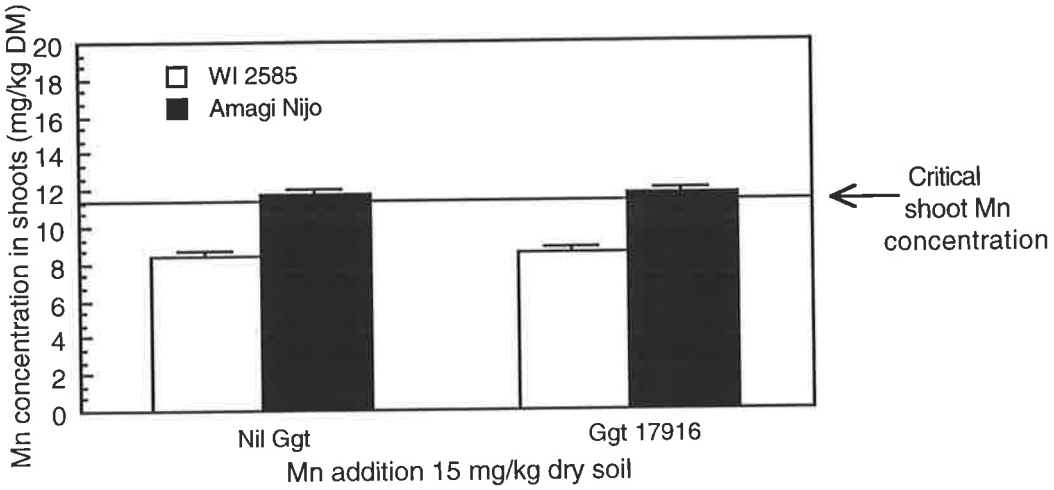


Fig. 2.5 Effect of *Ggt* infection on shoot Mn concentration on WI 2585 and Amagi Nijo grown in a controlled-environment growth chamber in Mn soil treatment Mn 15 with nil *Ggt* and *Ggt* 17916. Values are the means of 12 replicate pots (3 plants/pot). Standard errors are shown as vertical bars.

There was no significant interaction on shoot Mn content (µg/pot) between the *Ggt* treatments and genotype (p = 0.51, 2-way ANOVA, n = 12) (Appendix 2, 2.3.2) (Table 2.3). There was no significant difference in shoot Mn content between nil *Ggt* and *Ggt* treatments. When WI 2585 and Amagi Nijo were infected with *Ggt*, the Mn content of Amagi Nijo was significantly greater than in WI 2585 ($p = 3.0x10^{-17}$, 2-way ANOVA, n = 12) (Appendix 2, 2.3.2). Thus, with or without *Ggt* infection, Mn-efficient Amagi Nijo was absorbing twice as much Mn from the Mn deficient soil as Mn-inefficient WI 2585 (Table 2.3).

Table 2.3 Mn content (μg/pot) of shoots of WI 2585 and Amagi Nijo grown in a controlled-environment growth chamber in Mn soil treatment Mn 15 with nil *Ggt* and with *Ggt*. Values are the means and standard errors of 12 replicate pots (3 plants/pot).

Genotype	Nil *Ggt*	With *Ggt*
WI 2585	2.1 ± 0.1	2.0 ± 0.1
Amagi Nijo	4.6 ± 0.1	4.3 ± 0.3

The Av.LSL in Amagi Nijo, 19.5 ± 2.3 mm/3 plants, was half the Av.LSL in WI 2585, 32.6 ± 0.8 mm/3 plants. The difference was highly significant ($p = 0.006$, 1-way ANOVA, $n = 12$) (Fig. 2.6). In contrast to the significant differences in Av.LSL between genotypes, there were no significant differences between genotypes in PIR. The PIR of Amagi Nijo was 31.4 ± 6.1%, while the PIR of WI 2585 was 36.6 ± 1.8%, ($p = 0.39$, 1-way ANOVA, $n = 12$) (Fig. 2.7).

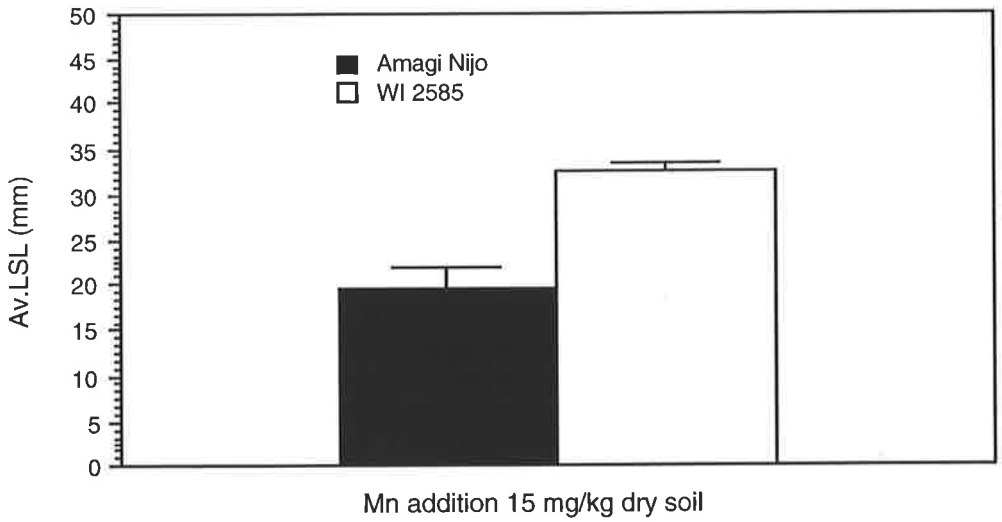


Fig. 2.6 The Av.LSL (mm) for each of WI 2585 and Amagi Nijo grown with *Ggt* under Mn soil treatment Mn 15 in a controlled-environment growth chamber. Values are the means of 12 replicate pots (3 plants/pot). Standard errors are shown as vertical bars.

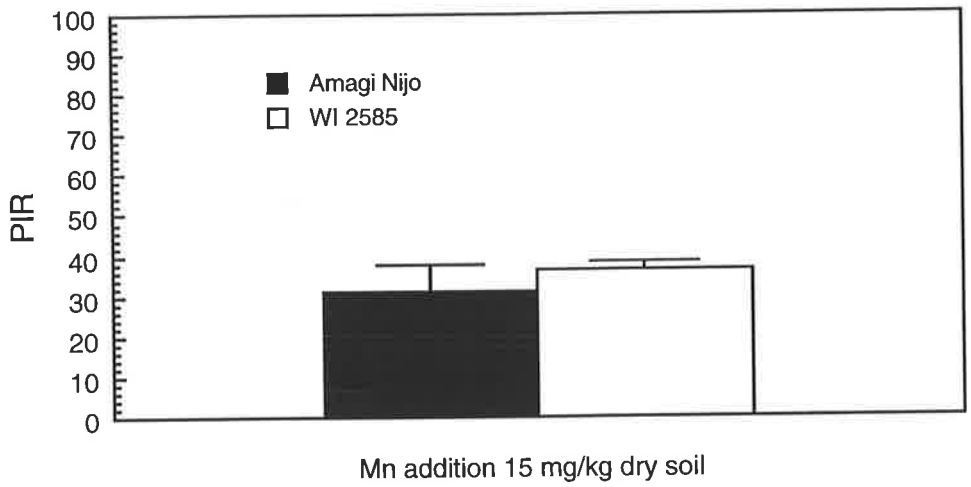


Fig. 2.7 The PIR for WI 2585 and Amagi Nijo grown with *Ggt* under Mn soil treatment Mn 15 in a controlled-environment growth chamber. Values are the means of 12 replicate pots (3 plants/pot). Standard errors are shown as vertical bars.

The analysis of the data taken 28 days after sowing showed that the infection of the plant roots by *Ggt* did not significantly reduce the shoot (Appendix 2, 2.3.3) or root (Appendix 2, 2.3.4) dry mass of the plants of either WI 2585 or Amagi Nijo (Table 2.4).

Table 2.4 Shoot and root DM (g) of WI 2585 and Amagi Nijo grown in a controlled-environment growth chamber in Mn soil treatment Mn 15 with nil *Ggt* and while infected with *Ggt* 17916 (+ *Ggt*). Values are the means of 12 replicate pots (3 plants/pot).

Genotype	Shoots (g)	Roots (g)
WI 2585 (nil *Ggt*)	0.39 ± 0.02	0.18 ± 0.01
Amagi Nijo (nil *Ggt*)	0.51 ± 0.02	0.32 ± 0.04
WI 2585 (+ *Ggt*)	0.37 ± 0.03	0.20 ± 0.004
Amagi Nijo (+ *Ggt*)	0.48 ± 0.05	0.36 ± 0.01

2.4 Discussion

A suitable *Ggt* isolate was obtained through the selection process. The comparison of *Ggt* infection of the roots of the plants is not valid if the growth of the *Ggt* isolate is inhibited by additions of Mn to the soil. For if the Mn addition is toxic to the *Ggt* isolate, the infection of the roots will be low, regardless of the ability of the plants to resist the infection. In the experiment on *Ggt* tolerance to Mn, *Ggt* isolate 17916 gave little or no sensitivity to Mn when grown on 4% PDA, except at Mn 100 (Fig. 2.1 and Fig. 2.2). Mn-efficiency is expressed in Mn deficient soil. Therefore, Mn 100 (mg Mn/kg dry soil) is a high level of Mn addition that may not be needed in a series of experiments to determine if the expression of Mn efficiency in Mn limiting soil enhances resistance to *Ggt*.

Experiment 2 showed that there was more *Ggt* 17916 pathogenicity (infection) after one passage through the host plants WI 2585 and Amagi Nijo than after the small amount of infection obtained upon the first passage through the plants (Fig. 2.3). Further, the *Ggt* infection results suggested that if a greater number of replicates was used, statistically significant differences in Av.LSL between the Mn-inefficient WI 2585 and the Mn-efficient Amagi Nijo may be obtained.

In the experiment to confirm that 12 replicates was an adequate sample size to obtain statistically significant differences in *Ggt* infection between the genotypes, (see section 2.2.3), the Av.LSL in Amagi Nijo, was half the Av.LSL in WI 2585 (Fig. 2.6). This difference was highly significant, confirming the possibility of obtaining statistically significant results with the use of 12 replicates. This result rejects the first Null Hypothesis that there is no physiological connection between the expression of the Mn efficiency trait in barley and enhanced resistance to *Ggt*, (see section 1.10). The PIR between Amagi Nijo and WI 2585 were very similar (Fig. 2.7), again a result consistent with the PIR results obtained previously in *Ggt* infection of Mn-inefficient/Mn-efficient wheat genotypes (Pedler, 1994). The greater Mn efficiency of Amagi Nijo compared to WI 2585 was confirmed by the result that Amagi Nijo had absorbed from the Mn deficient soil (15 mg Mn/kg dry soil) twice as much Mn as WI 2585 (Table 2.3). Further, Amagi Nijo had twice the Mn content of WI 2585, when grown both with or without *Ggt* (Table 2.3).

The *Ggt* infection 28 days after sowing did not significantly reduce the plant shoot or root DM (Table 2.4) when compared with uninfected plants. Therefore, 28 days is a suitable time post inoculation with *Ggt* to harvest the plants for comparison in *Ggt* infection to avoid confounding results due to other effects such as large differences in plant size caused by either the *Ggt* infection or by severe Mn deficiency.

Chapter 3 Critical shoot Mn concentration

3.1 Introduction

The critical nutrient concentration is that concentration of a particular nutrient in a specified plant organ when the plant growth or production is 90% of the maximum growth (Reuter *et al.*, 1986). The critical shoot (YEB) Mn concentration in barley has been found to be between 11.0-11.6 mg/kg DM (Reuter 1986; Hannam *et al.*, 1987). However, the critical concentration of Mn for WI 2585 and Amagi Nijo has not been examined. In addition, the determination of the critical Mn concentration by addition of a range of Mn to soil, will also provide the amounts of soil Mn needed to achieve equal Mn plant status in both WI 2585 and Amagi Nijo.

3.2 Method

3.2.1 The critical shoot Mn concentration experiment

The soil was prepared, incubated, and basal nutrients added as described in section 2.2.2. Mn as $MnSO_4$ was added at 9 levels corresponding to 0, 5, 7.5, 10, 15, 20, 30, 40 and 100 mg/kg dry soil, but the pots were not inoculated with *Ggt*. Three seeds of either Amagi Nijo or WI 2585 were planted/pot (see section 2.2.5) (Appendix 1, Table 2.1). There were 90 pots (2 genotypes x 5 replicate pots x 9 Mn treatments). The pots were placed on the bench in a completely randomised design. Plants were harvested 28 days after sowing as described in section 2.2.2. Statistical analysis of all the results was done (see section 2.2.2). The critical shoot Mn concentrations of Amagi Nijo and WI 2585 were determined from the data.

3.3 Results

3.3.1 Leaf symptoms

When the plants were harvested at 28 days, WI 2585 grown at Mn 0 showed severe symptoms of Mn deficiency (leaf chlorotic streaking, necrosis, and young leaf collapse) (Fig. 3.1). Severe symptoms of Mn deficiency persisted in WI 2585 when the rate of Mn fertiliser addition was increased to Mn 5 and Mn 7.5 (Fig. 3.2 A). When the rate of Mn addition was increased to Mn 10, WI 2585 showed moderate symptoms of Mn deficiency (flecking and necrosis), mild symptoms at Mn 15 (chlorosis), while at Mn 20 WI 2585 showed no

Fig. 3.1 In soil with no added Mn, Mn-inefficient WI 2585 is showing symptoms of Mn deficiency (leaf chlorotic streaking, necrosis and leaf collapse), while Mn-efficient Amagi Nijo shows healthy green growth.

A) WI 2585

B) Amagi Nijo

Fig. 3.2 Mn-inefficient WI 2585 (A), and Mn-efficient Amagi Nijo (B), barley growing in 9 levels of Mn added to the soil, Mn 0, 5, 7.5, 10, 15, 20, 30, 40 and 100 mg Mn/kg dry soil.

symptoms of Mn deficiency. In contrast to WI 2585, Amagi Nijo showed mild symptoms of Mn deficiency (general chlorosis) when grown at Mn 0 (Fig. 3.1), but no symptoms of Mn deficiency when grown at Mn 5 or more (Fig. 3.2 B).

3.3.2 Shoot and root DM

There was a significant interaction between genotype and rate of Mn supply on shoot DM (g/pot) ($p = 3.6x10^{-4}$, 2-way ANOVA with replication, n =5) (Appendix 2, 3.1) (Fig. 3.3 A). The figure shows that the two genotypes differ most in response to Mn at the low rates, and the differences between Amagi Nijo and WI 2585 were significant ($p < 0.05$) at rates 0 to 20 mg/kg Mn by 1-way ANOVAs conducted on the data at each rate.

Similarly, there was a significant interaction on root DM (g/pot) ($p = 0.007$) (Appendix 2, 3.2) (Fig. 3.3 B). Figure 3.3 B shows that the two genotypes differ most in response to Mn at the low and mid rates, and the differences between Amagi Nijo and WI 2585 were significant ($p < 0.05$) at rates 0 to 20 mg/kg Mn by 1-way ANOVAs conducted on the data at each rate.

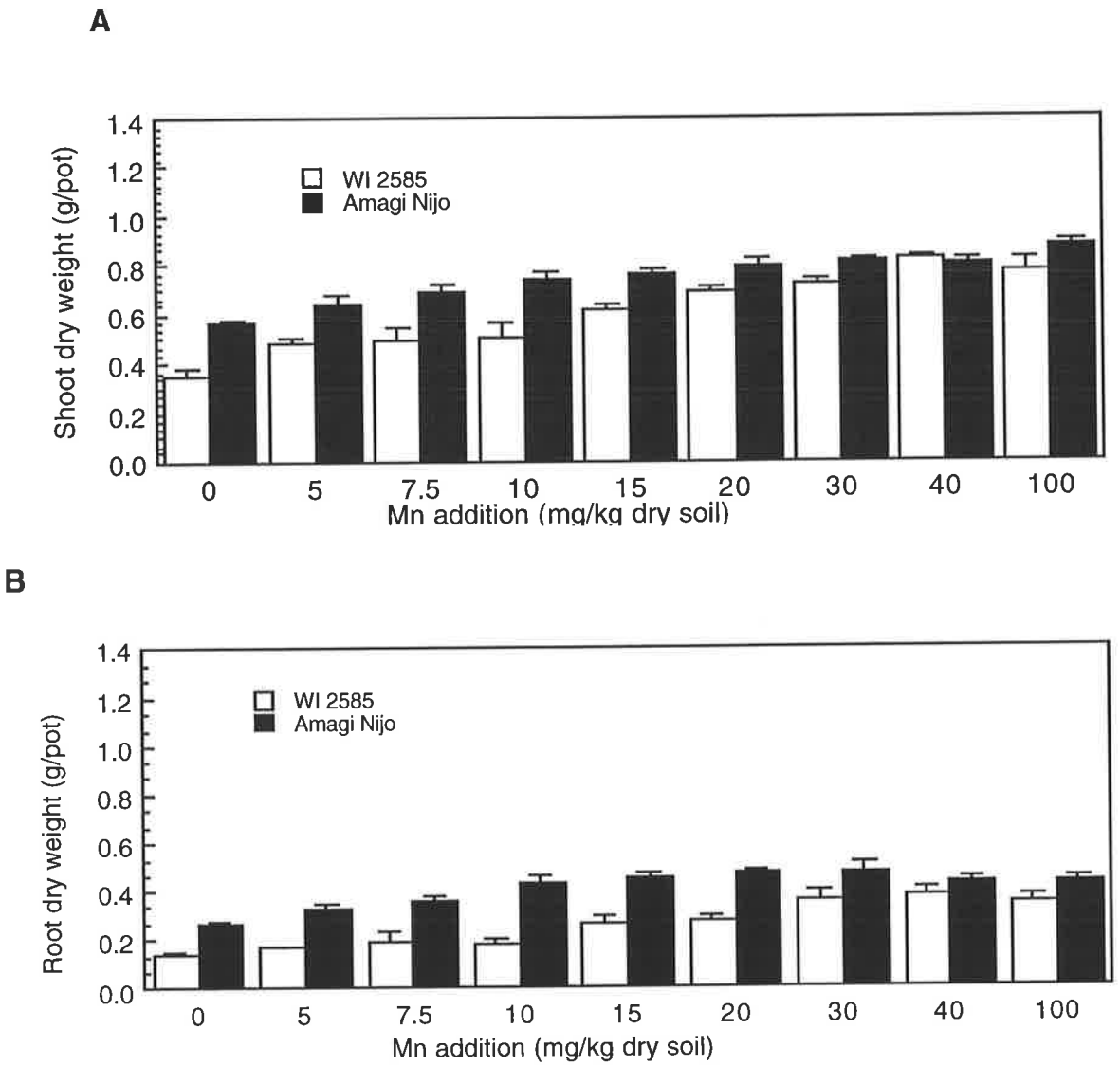


Fig. 3.3 Shoot (DM) (g/pot) (A), and root (DM) (g/pot) (B), of barley plants growing in pots in a controlled-environment growth chamber for 28 days, shows the effect of genotype and rate of Mn fertiliser application to the soil. Values are the means of 5 replicate pots. The standard errors are shown as vertical bars.

3.3.3 Shoot Mn concentration

There was a significant interaction on shoot Mn concentration (mg/kg DM) between genotypes WI 2585 and Amagi Nijo, and soil Mn additions Mn 0 to Mn 100 ($p = 5.3 \times 10^{-5}$, 2-way ANOVA, $n = 5$) (Appendix 2, 3.3) (Fig. 3.4) and both main effects were highly significant ($p < 5.7 \times 10^{-37}$). By 1-way ANOVA on data for one level of Mn separately, the shoot Mn concentration of Amagi Nijo was significantly greater than in WI 2585 ($p < 0.05$) at each level of Mn added to the soil.

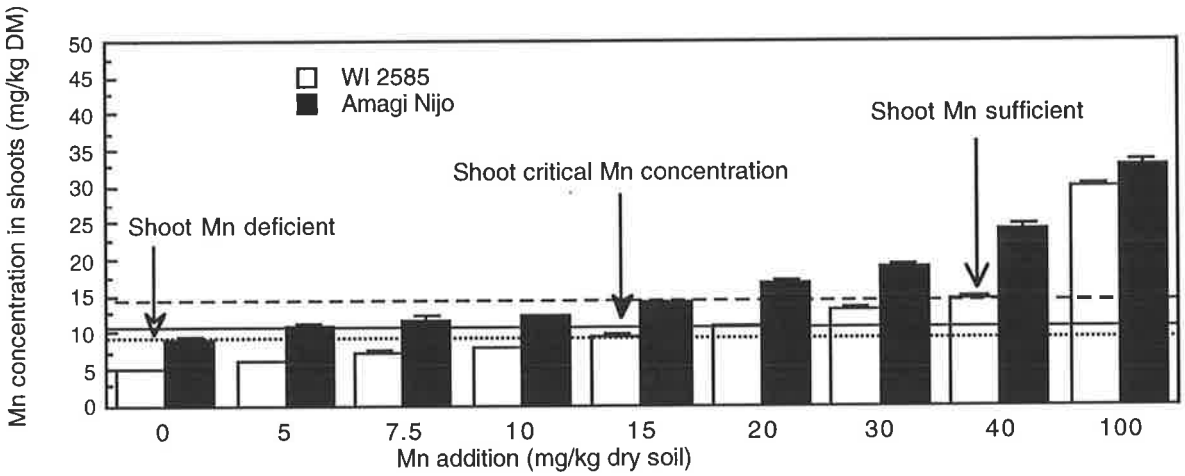


Fig. 3.4 The shoot Mn concentrations (mg/kg DM) of barley plants growing in pots in a controlled-environment growth chamber for 28 days, as a function of genotype and rate of Mn fertiliser application to the soil. Values are the means of 5 replicate pots. The standard errors are shown as vertical bars.

3.3.4 Critical shoot Mn concentration of Amagi Nijo and WI 2585

The relationship between the shoot Mn concentration and the shoot dry weight (DM) of each genotype across 9 levels of Mn added to the soil give nearly identical curves for Amagi Nijo and WI 2585 (Fig. 3.5). Calculation of the critical shoot Mn concentration corresponding to 90% of relative yield gives both Amagi Nijo and WI 2585 similar critical shoot Mn concentrations between 11-12 mg/kg DM. This result is the same as the critical shoot (YEB) Mn concentration for barley determined previously in field work (11.0-11.6 mg Mn/kg DM) (Hannam *et al.*, 1987).

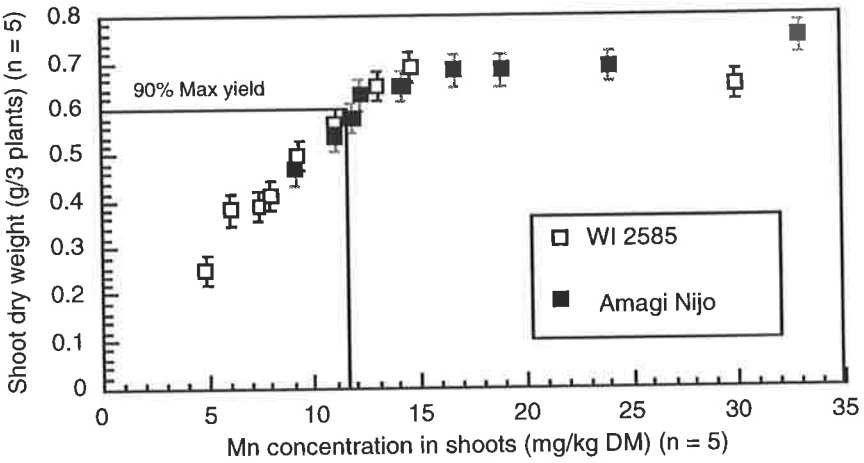


Fig. 3.5 The shoot DM data for Amagi Nijo and WI 2585 plotted against the shoot Mn concentration give nearly identical curves and the same critical shoot Mn concentration 11-12 mg/kg DM. Values are the means of 5 replicate pots. The standard errors are shown as vertical bars.

3.3.5 Determining the rate of soil Mn addition as the basis of comparing the genotypes with equal Mn status

Amagi Nijo grown at soil Mn 15 had sufficient shoot Mn (Fig. 3.4). In contrast, Mn-inefficient WI 2585 grown at soil Mn 15 had a shoot Mn concentration below the Mn critical concentration (section 1.6.1. Fig. 3.4 and Fig. 3.5). Therefore, the plants of WI 2585 grown at soil Mn 15 were Mn-deficient.

When Amagi Nijo was at nil Mn (shoot Mn = 9.1 ± 0.2 mg/kg DM) and WI 2585 was grown at 15 mg/kg added Mn (shoot Mn = 9.3 ± 0.3 mg/kg DM), the genotypes had similar but deficient shoot Mn concentration. On the other hand, Amagi Nijo (shoot Mn = 14.2 ± 0.3 mg/kg DM) and WI 2585 (shoot Mn = 14.6 ± 0.5 mg/kg DM) had similar and sufficient shoot Mn concentration when grown in soil Mn 15 and 40 mg/kg respectively (Fig. 3.4).

3.3.6 Shoot Mn content

At all levels of Mn added to the soil, Amagi Nijo had a significantly greater shoot Mn content (µg/pot) compared to WI 2585 ($p = 7.4 \times 10^{-37}$, 2-way ANOVA, n = 5) (Appendix 2, 3.4) (Fig. 3.6). The statistical analysis showed the same high levels of significance for the main

effects as for the shoot Mn concentration results. The greater shoot Mn content in Amagi Nijo compared to WI 2585 demonstrates that Amagi Nijo is more efficient at absorbing Mn from Mn-deficient soil than WI 2585.

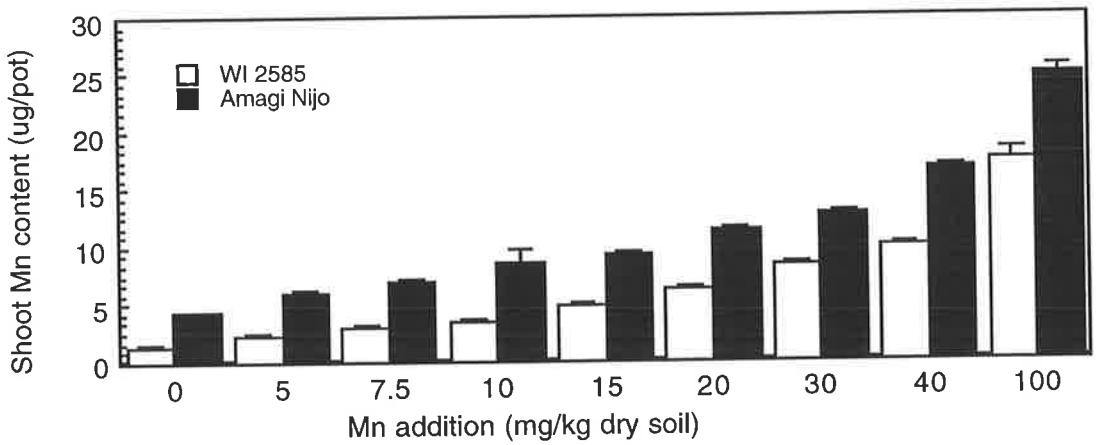


Fig. 3. 6 The shoot Mn content (µg/pot) of barley plants growing in pots in a controlled-environment growth chamber for 28 days, as a function of genotype and rate of Mn fertiliser application to the soil. Values are the means of 5 replicate pots. The standard errors are shown as vertical bars.

3.3.7 Root Mn concentration

There was no significant interaction on root Mn concentration (mg/kg DM) between genotype and Mn soil additions (Mn 0 to Mn 100 mg/kg) ($p = 0.9$, 2-way ANOVA, $n = 5$) (Appendix 2, 3.5) (Fig. 3.7). However the main effect Mn soil additions Mn 0 to Mn 100 was highly significant ($p = 1.0 \times 10^{-35}$, 2-way ANOVA, $n = 5$). By 1-way ANOVA on data for each level of Mn separately, there were no significant differences in root Mn between WI 2585 and Amagi Nijo at each level of Mn added to the soil ($p > 0.05$) (Fig. 3.7).

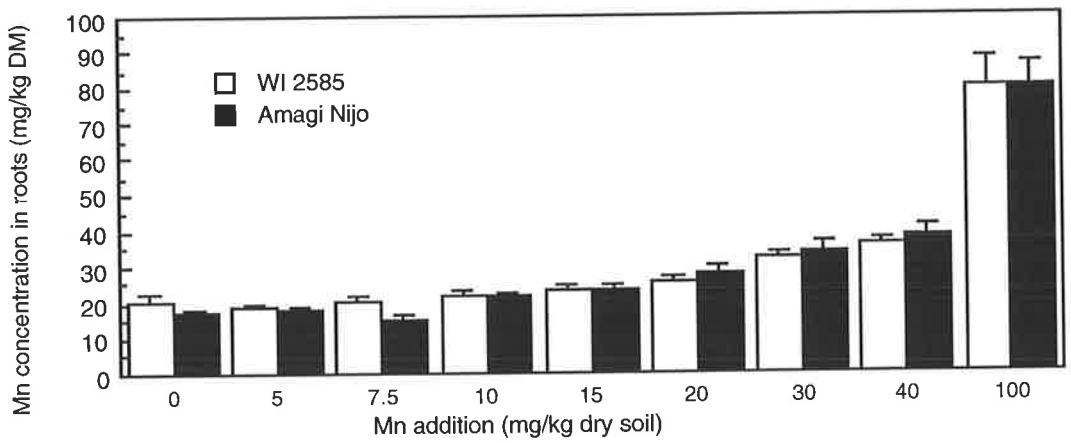


Fig. 3.7 The root Mn concentration (mg/kg DM) of barley plants growing in pots in a controlled-environment growth chamber for 28 days, as a function of genotype and rate of Mn fertiliser application to the soil. Values are the means of 5 replicate pots. The standard errors are shown as vertical bars.

3.4 Discussion

3.4.1 Critical shoot Mn concentration

Many varieties of the one crop have similar critical shoot Mn concentrations, (Reuter *et al.,* 1986 and Hannam *et al.,* 1987). However, differences in critical shoot Mn concentrations have been found in wheat cultivars ranging from 11 mg/kg DM in Bayonet to 17 mg/kg DM in the Canadian cultivar Red Fife (Marcar, 1986). This soil bioassay found that WI 2585 and Amagi Nijo have the same critical shoot Mn concentration (11-12 mg/kg DM) (Fig. 3.5). Therefore, it is reasonable to assume that similar levels of Mn deficiency stress exist in WI 2585 and Amagi Nijo when their shoot Mn concentrations are the same (Fig. 3.4).

However, the greater Mn efficiency in Amagi Nijo compared to WI 2585 was associated with the greater ability of Amagi Nijo to absorb Mn from deficient soil as demonstrated by the greater shoot Mn concentration and content in Amagi Nijo (Fig. 3.4 and Fig. 3.6). The Mn efficiency in Amagi Nijo can be neutralised and made equal in Mn stress to WI 2585 by adding to the soil the amounts of Mn determined in this soil bioassay to achieve equal Mn plant status in both genotypes.

Chapter 4 Physiological studies

4.1 Introduction

Amagi Nijo and WI 2585 have the same critical shoot Mn concentration (11-12 mg/kg DM) (Fig. 3.5). Therefore, when Amagi Nijo and WI 2585 have a similar shoot Mn concentration (Fig. 3.4), it can be assumed that they have equal Mn stress. Therefore, Amagi Nijo should have an Av.LSL as long as in WI 2585 when both have the same deficient shoot Mn concentration, and a short but similar Av.LSL when both have the same sufficient shoot Mn concentration.

Second, the longer Av.LSL in WI 2585 compared to Amagi Nijo when harvested at 28 days (Fig. 2.6) may be because *Ggt* invaded the stele of WI 2585 roots earlier giving the *Ggt* more time to grow within the root. A temporal study of Av.LSL post inoculation with *Ggt* could show if the roots of WI 2585 are invaded earlier than Amagi Nijo to cause the long Av.LSL in WI 2585.

4.2 Method

4.2.1 The *Ggt* experiment

The *Ggt* experiment was performed as in section 2.2.2, except for the following changes. Mn, as $MnSO_4$, was added at the levels 0, 15, and 40 mg/kg dry soil. For both WI 2585 and Amagi Nijo, there was 1 plant/pot and 12 replicate pots for each Mn level where the pots were inoculated with *Ggt* 17916. For the controls with no *Ggt*, there were 6 replicate pots (1 plant/pot) for both WI 2585 and Amagi Nijo for each Mn level. In all, there were 108 pots. The seeds used were as listed in Appendix 1, Table 2.1.

4.2.2 The temporal study

The temporal study was performed as in section 2.2.2 except for these changes. Mn, as $MnSO_4$, was added corresponding to 15 mg/kg dry soil. There were 96 pots inoculated with *Ggt* isolate 17916. There were 2 genotypes (WI 2585 and Amagi Nijo) x 1 plant x12 replicate pots x 4 harvest times 14 days (14D), 21 days (21D), 28 days (28D) and 35 days (35D). The seeds used were as listed in Appendix 1, Table 2.1.

4.3 Results

4.3.1 The *Ggt* experiment

A) Shoot Mn

When WI 2585 and Amagi Nijo were grown with nil *Ggt*, there was no significant interaction on shoot Mn concentration (mg/kg DM) between genotype and rate of Mn supply ($p = 0.27$, 2-way ANOVA). There were significant differences due to both main effects, genotype ($p = 0.01$), and rate of Mn supply ($p = 3.2 \times 10^{-6}$) by 2-way ANOVA (Appendix 2, 4.1.1) (Fig. 4.1).

Fig. 4.1 shows there were no significant differences in shoot Mn concentration when WI 2585 was grown in soil Mn 15 (9.3 ± 0.6 mg Mn/kg DM.), and Amagi Nijo grown in soil Mn 0 (8.1 ± 0.3 mg Mn/kg DM.) ($p = 0.11$, 1-way ANOVA, $n = 6$) (Fig. 4.1). Similarly, the difference in shoot Mn concentration when WI 2585 was grown in soil Mn 40 (15.8 ± 1.2 mg Mn/kg DM.) and Amagi Nijo grown in soil Mn 15 (12.3 ± 1.0 mg Mn/kg DM.) was close to significant ($p = 0.06$, 1-way ANOVA, $n = 6$). At Mn soil addition 15 mg/kg dry soil, Amagi Nijo had a significantly greater shoot Mn concentration than WI 2585 ($p = 0.03$, 1-way ANOVA, $n = 6$) (Fig. 4.1).

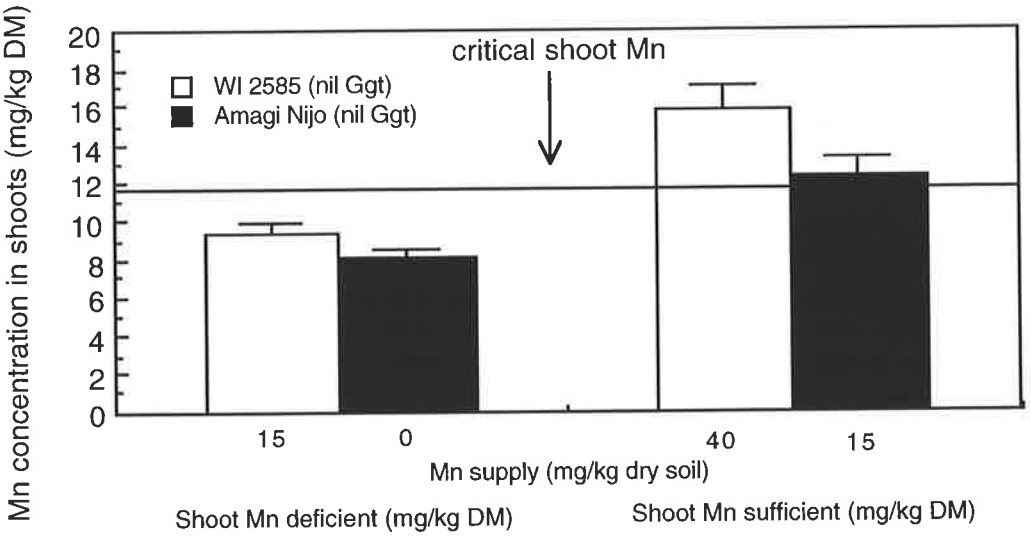


Fig. 4.1 Shoot Mn concentrations (mg/kg DM) of WI 2585 and Amagi Nijo with nil *Ggt* growing in pots in a controlled-environment growth chamber for 28 days show the effect of genotype and rate of Mn supply. Values are the means of 6 replicate pots. The vertical bars show the SE.

When WI 2585 and Amagi Nijo were infected with *Ggt* 17916, there was no significant interaction on shoot Mn concentration (mg/kg DM) between genotype and rate of Mn supply ($p = 0.25$, 2-way ANOVA) (Appendix 2, 4.1.2). There was no significant difference due to main effect of genotype ($p = 0.83$), but the differences between the rate of Mn supply was highly significant ($p = 2.4 \times 10^{-10}$, 2-way ANOVA) (Fig. 4.2).

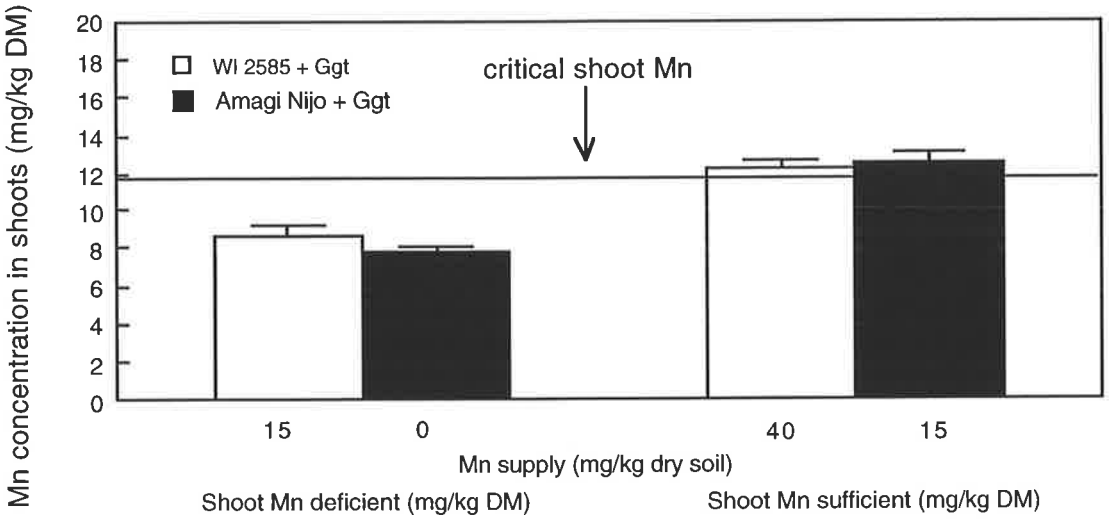


Fig. 4.2 Shoot Mn concentrations (mg/kg DM) of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* in a controlled-environment growth chamber for 28 days show the effect of genotype and rate of Mn supply to the soil. Values are the means of 12 replicate pots. The vertical bars show the SE.

There were no significant differences between genotypes with deficient shoot Mn concentration;- WI 2585 grown in soil Mn 15, (shoot Mn = 8.6 ± 0.6 mg/kg DM.), and Amagi Nijo grown in soil Mn 0, (shoot Mn = 7.8 ± 0.3 mg/kg DM.), ($p = 0.17$, 1-way ANOVA, n =12). In addition, there were no significant differences between genotypes with sufficient shoot Mn concentration;- WI 2585 grown at soil Mn 40 (shoot Mn = 12.2 ± 0.4 mg/kg DM.) and Amagi Nijo grown at soil Mn 15 (shoot Mn = 12.4 ± 0.7 mg/kg DM), ($p = 0.64$, 1-way ANOVA, n = 12) (Fig. 4.2). At Mn soil addition 15 mg/kg dry soil, Amagi Nijo had a significantly greater shoot Mn concentration than WI 2585 ($p = 7.0 \times 10^{-4}$, 1-way ANOVA, n = 12) (Fig. 4.2).

B) Infection (Av.LSL) (mm/plant)

There were no signs of *Ggt* infection on the roots of WI 2585 and Amagi Nijo from uninfected control pots. There was no significant interaction on Av.LSL (mm/12 plants) between genotype and rate of Mn supply interpreted as the level of Mn stress ($p = 0.72$, 2-way ANOVA) (Appendix 2, 4.1.3). There was a significant difference between WI 2585 and Amagi Nijo ($p = 0.02$), but there was no significant difference due to Mn treatment (that is Mn concentration) ($p = 0.28$) by 2-way ANOVA (Fig. 4.3).

Similar to the pilot soil screen with restored *Ggt* 17916 experiment (Fig. 2.6), when the Mn-inefficient WI 2585 and Mn-efficient Amagi Nijo were grown at Mn soil 15, there was a significantly longer Av.LSL in the Mn-inefficient WI 2585 (20.6 ± 5.1 mm/plant) compared to the Mn-efficient Amagi Nijo (6.4 ± 1.7 mm/plant) ($p = 0.01$, 1-way ANOVA, $n = 12$) (Fig. 4.3). But at a deficient shoot Mn concentration, WI 2585 had a high Av.LSL (20.6 ± 5.1 mm/plant), while Amagi Nijo had a shorter Av.LSL (10.9 ± 3.3 mm/plant) ($p = 0.1$, 1-way ANOVA, $n = 12$) (Fig. 4.3). When both genotypes had a sufficient shoot Mn concentration, WI 2585 retained a significantly longer Av.LSL (18.5 ± 4.6 mm/plant) compared to Amagi Nijo (Av.LSL = 6.4 ± 1.7 mm/plant), ($p = 0.02$, 1-way ANOVA, $n = 12$). However, WI 2585 and Amagi Nijo did show a slight but an insignificant decrease in Av.LSL between deficient and sufficient shoot Mn concentration ($p = 0.76$ and $p = 0.23$ respectively, 1-way ANOVA, $n = 12$) (Fig. 4.3).

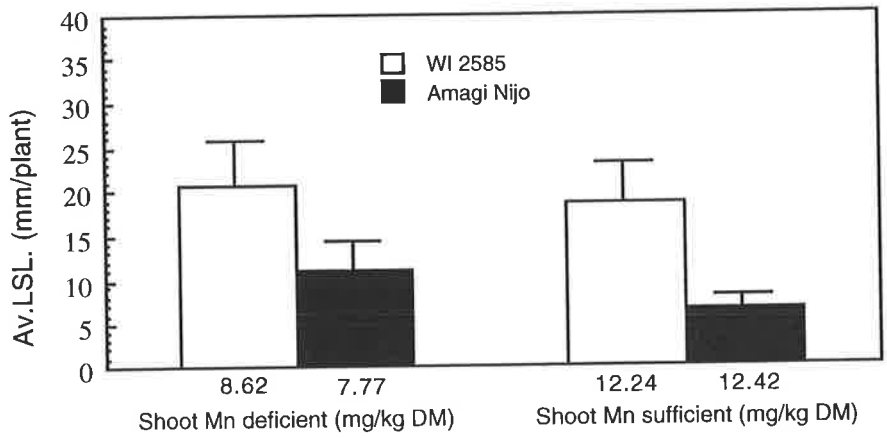


Fig. 4.3 Av.LSL (mm/plant) of WI 2585 and Amagi Nijo under equal Mn stress at a similar shoot Mn concentration growing in pots inoculated with *Ggt* in a controlled-environment growth chamber for 28 days, The figure shows the effect on Av.LSL (mm/plant) of genotype and Mn addition (that is high or low Mn stress). Values are the means of 12 replicate pots. The vertical bars show the SE.

C) Infection (PIR)

There was no significant interaction on PIR between genotype and Mn deficient/Mn sufficient shoot concentrations ($p = 0.14$, 2-way ANOVA, $p = 12$). In addition, there were no significant differences due to either main effect ($p > 0.05$, 2-way ANOVA) (Appendix 2, 4.1.4) (Fig. 4.4).

Similar to the earlier findings in PIR (Fig. 2.7), there were no significant differences in PIR, between WI 2585 ($33.0 \pm 6.7\%$) and Amagi Nijo ($29.8 \pm 4.8\%$), ($p = 0.69$, 1-way ANOVA, $n = 12$) (Fig. 4.4). No significant differences by 1-way ANOVA in PIR between genotypes was found at either level of Mn supply resulting in high or low Mn stress ($p > 0.05$) (Fig. 4.4).

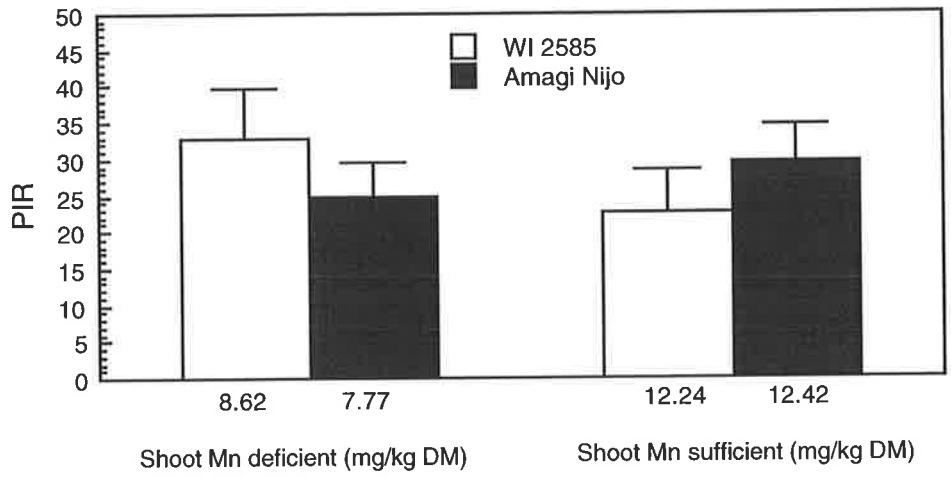


Fig. 4.4 PIR of WI 2585 and Amagi Nijo under equal Mn stress (that is at a similar shoot Mn concentration) growing in pots inoculated with *Ggt* in a controlled-environment growth chamber for 28 days. The figure shows the effect on PIR of genotype and rate of Mn supply. Values are the means of 12 replicate pots. The vertical bars show the SE.

D) Root Mn concentration

When WI 2585 and Amagi Nijo were grown with nil *Ggt*, there was no significant interaction on root Mn concentration (mg/kg DM) between genotype and rate of Mn addition ($p = 0.29$, 2-way ANOVA) (Appendix 2, 4.1.5) (Fig. 4.5). The Mn addition main effect was nearly significant ($p = 0.06$), but there was a significant difference due to genotype main effect with WI 2585 showing a greater root Mn concentration than Amagi Nijo under both rate of Mn addition to the soil ($p = 0.03$, 2-way ANOVA) (Appendix 2, 4.1.5) (Fig. 4.5).

When the pots were inoculated with *Ggt*, there was no significant interaction on root Mn (mg/kg DM) between genotype and rate of Mn addition to the soil ($p = 0.56$, 2-way ANOVA). In addition, there were no significant differences due to the main effect of either genotype or Mn addition ($p = 0.14$, $p = 0.15$ respectively, 2-way ANOVA) (Appendix 2, 4,1,6) (Fig. 4.6).

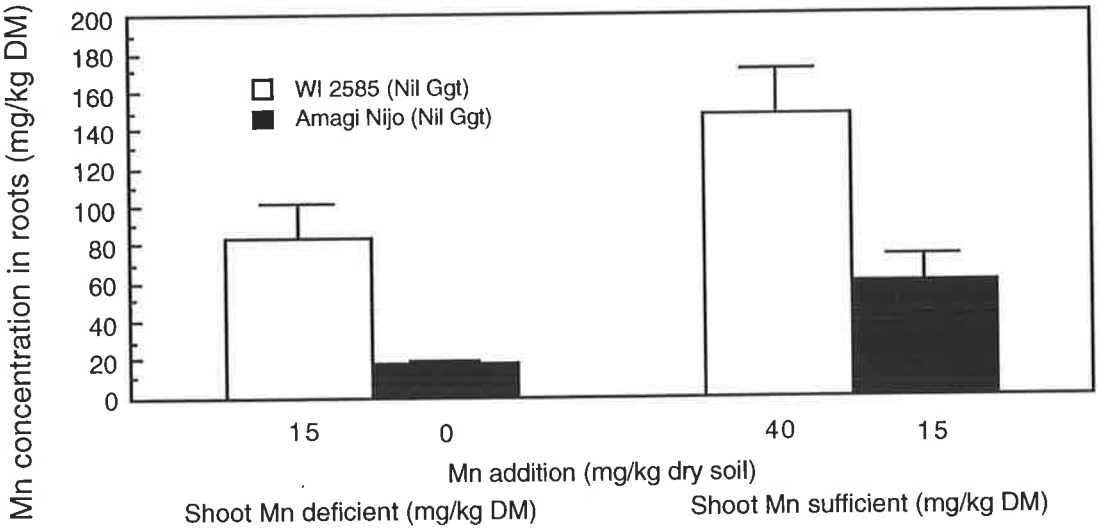


Fig. 4.5 Root Mn concentrations (mg/kg DM) of WI 2585 and Amagi Nijo growing in pots with nil *Ggt* in a controlled-environment growth chamber for 28 days. The figure shows the effect of genotype and rate of Mn addition. Values are the means of 12 replicate pots. The vertical bars show the SE.

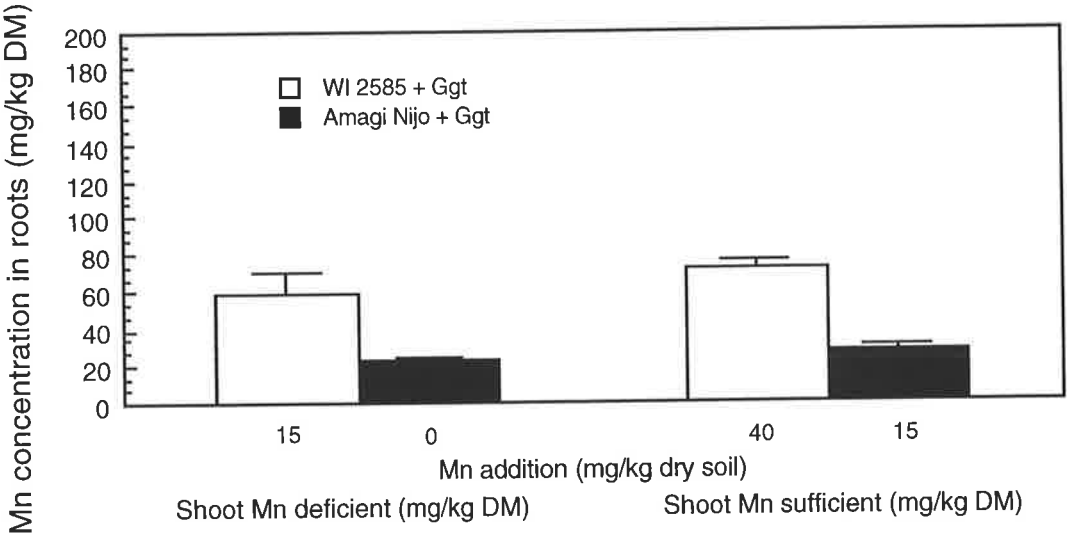


Fig. 4.6 Root Mn concentrations (mg/kg DM) of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* in a controlled-environment growth chamber for 28 days. The figure shows the effect of genotype and Mn addition. Values are the means of 12 replicate pots. The vertical bars show the SE.

E) Dry matter (DM)

When grown with nil *Ggt*, there was no significant interaction on shoot DM (g/pot) between genotype and rate of Mn addition to the soil ($p = 0.82$, 2-way ANOVA) (Appendix 2, 4.1.7), and there were no significant differences due to main effects (Table 4.1). In addition,

there was no significant interaction on root DM (g/pot) between genotype and rate of Mn addition to the soil ($p = 0.40$, 2-way ANOVA) (Appendix 2, 4.1.8) (Table 4.1). There was no significant difference due to genotype or the rate of Mn addition (that is low or high Mn stress) ($p > 0.05$, 2-way ANOVA) (Appendix 2, 4.1.8).

Table 4.1 Shoot and root DM (g/pot) of WI 2585 and Amagi Nijo grown with nil *Ggt* in a controlled-environment growth chamber for 28 days shows the effect on DM of genotype and rate of Mn addition (that is low or high Mn stress). Values are the means and standard errors of 6 replicate pots.

Genotype	Shoots (g/pot) (n = 6)	Roots (g/pot) (n = 6)
Mn deficient		
WI 2585	0.14 ± 0.01	0.06 ± 0.01
Amagi Nijo	0.10 ± 0.004	0.03 ± 0.002
Mn sufficient		
WI 2585	0.15 ± 0.02	0.06 ± 0.02
Amagi Nijo	0.11 ± 0.01	0.05 ± 0.01

When WI 2585 and Amagi Nijo were grown with *Ggt*, there was no significant interaction on shoot DM (g/pot) between genotype and rate of Mn addition the soil ($p = 0.29$, 2-way ANOVA), and there was no significant difference between WI 2585 and Amagi Nijo (Table 4.2). But, there was a significant difference due to soil Mn addition ($p = 0.004$, 2-way ANOVA) (Appendix 2, 4.1.9) (Table 4.2). In addition, there was no significant interaction on root DM (g/pot) between genotype and rate of Mn addition to the soil ($p = 0.50$, 2-way ANOVA) (Appendix 2, 4.1.10) (Table 4.2). There were no significant differences due to main effects (Appendix 2, 4.1.10) (Table 4.2).

Table 4.2 Shoot and root DM (g/pot) of WI 2585 and Amagi Nijo grown in a controlled-environment growth chamber in pots inoculated with *Ggt* for 28 days. The figure shows the effect on DM of genotype and rate of Mn addition (that is low or high Mn stress). Values are the means and standard errors of 12 replicate pots.

Genotype	Shoots (g/pot) (n = 12)	Roots (g/pot) (n = 12)
Mn deficient		
WI 2585 (Ggt)	0.11 ± 0.01	0.03 ± 0.003
Amagi Nijo (Ggt)	0.09 ± 0.004	0.02 ± 0.001
Mn sufficient		
WI 2585 (Ggt)	0.13 ± 0.01	0.03 ± 0.002
Amagi Nijo (Ggt)	0.12 ± 0.01	0.03 ± 0.002

4.3.2 The temporal study

A) Shoot and root Mn concentration

There was no significant interaction on shoot Mn concentration (mg/kg DM) between the genotypes and the harvest time ($p = 0.17$, 2-way ANOVA) (Appendix 2, 4.2.1) (Fig. 4.7). However, both main effects, genotype and the time were highly significant ($p < 4.2 \times 10^{-6}$, 2-way ANOVA). By 1-way ANOVA, Amagi Nijo had a significantly higher shoot Mn concentration than WI 2585 at all harvest times (D14 = $p < 04$), a difference which increased in significance over the course of the experiment (D35 = $p < 2.8 \times 10^{-10}$). The shoot Mn concentration of Amagi Nijo decreased over the course of the experiment, but not significantly so. In contrast, by 1-way ANOVA, the shoot Mn concentration of WI 2585 showed a significant decrease over the course of the experiment (14D-21D $p = 0.01$, 28D-35D, $p = 0.02$, $n = 12$) (Fig. 4.7).

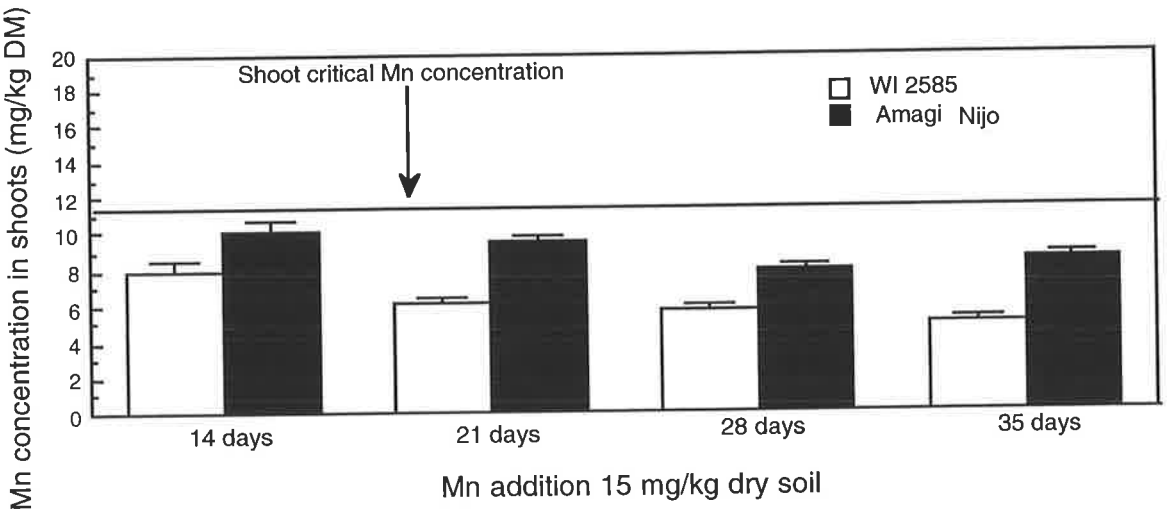


Fig. 4.7 The shoot Mn concentration (mg/kg DM), of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* 17916 in a controlled-environment growth chamber under Mn soil treatment Mn 15. The figure shows the effect on shoot Mn concentration of genotype and harvest time. Values are the means of 12 replicate pots. The vertical bars show the SE.

There was a significant interaction on shoot Mn content (µg/pot) between genotype and the harvest time ($p = 0.003$), and the differences due to both main effects were highly significant ($p < 1.1 \times 10^{-7}$, 2-way ANOVA) (Appendix 2, 4.2.2) (Fig. 4.8). Over the course of the experiment, shoot Mn content in both genotypes increased. By 1-way ANOVA, Amagi Nijo showed significantly greater shoot Mn content than WI 2585, a difference that increased in significance over the course of the experiment (D14 $p = 0.02$, D35 $p = 0.005$) (Fig. 4.8). Amagi Nijo's greater shoot Mn content showed the enhanced ability of this genotype to extract Mn from the soil and transport the Mn into the tissue compared to the Mn-inefficient WI 2585.

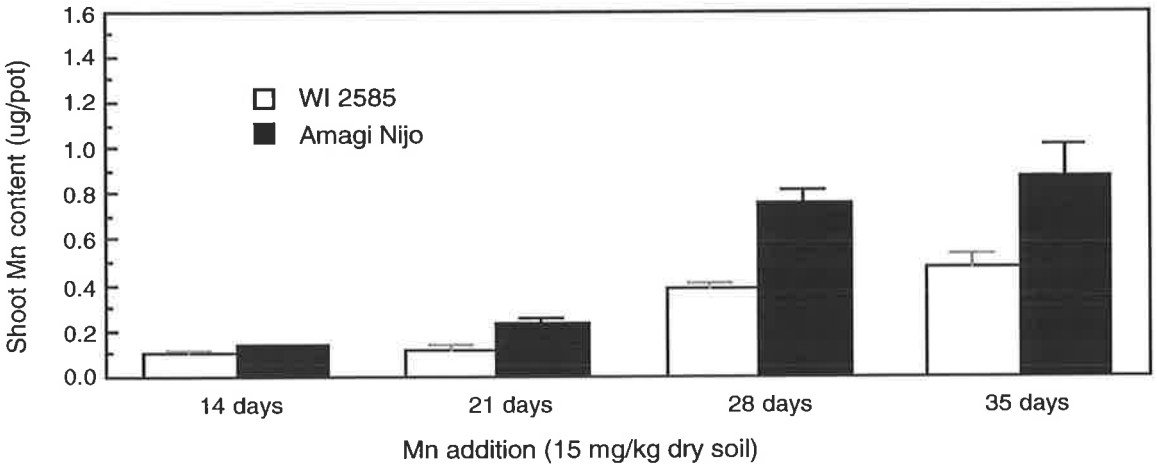


Fig. 4.8 The shoot Mn content (µg/pot), of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* 17916 in a controlled-environment growth chamber under Mn soil treatment Mn 15. The figure shows the effect on Mn content of genotype and harvest time. Values are the means of 12 replicate pots. The vertical bars show the SE.

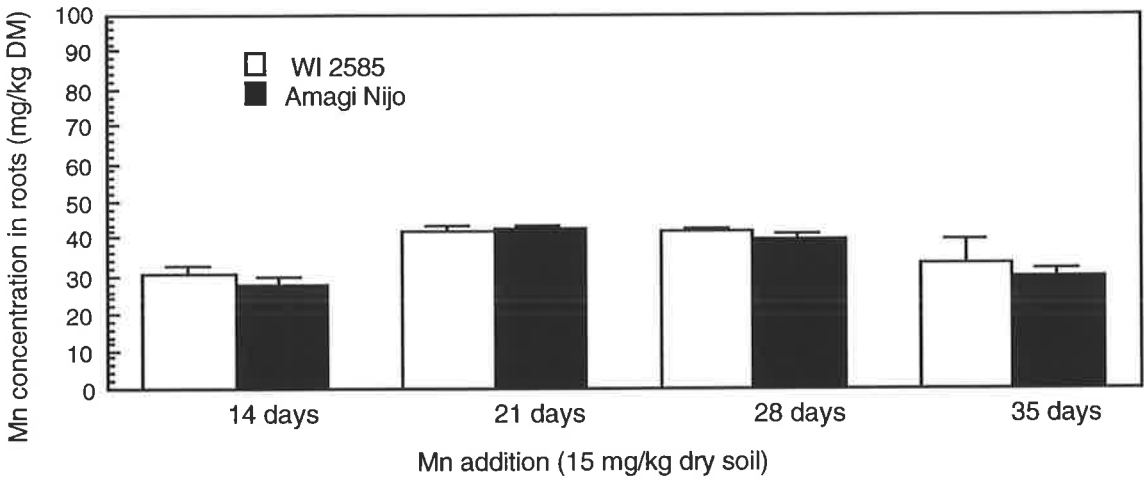


Fig. 4.9 Root Mn concentration (mg/kg DM), of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* 17916 in a controlled-environment growth chamber under Mn soil treatment of Mn 15. The figure shows the effect on root Mn concentration of genotype and harvest time. Values are the means of 12 replicate pots. The vertical bars show the SE.

There was no significant interaction on root Mn (mg/kg DM) between genotype and harvest time ($p = 0.35$, 2-way ANOVA) (Appendix 2, 4.2.3) (Fig. 4.9). However there was a significant difference due to genotype ($p = 0.02$) and due to the harvest time ($p = 3.3 \times 10^{-16}$, 2-way ANOVA) (Appendix 2, 4.2.3). These significant differences are shown by the root Mn

concentrations of WI 2585 and Amagi Nijo increasing from 14 days to 21 days. But over time, as Mn was taken from the soil by the plants, the depletion of available soil Mn is reflected in a decrease in root Mn concentration from 28 days to 35 days (Fig. 4.9). WI 2585 had as much root Mn as Amagi Nijo at 35 days but was dying while Amagi Nijo was not because the shoot Mn in Amagi Nijo was higher still. By 1-way ANOVA, the root Mn concentration shows no significant differences due to genotype at any of the harvest times ($p > 0.05$, $n = 12$) (Fig. 4.9).

B) *Ggt* infection

There was a significant interaction with Av.LSL (mm/plant) between genotype and harvest time ($p = 3.0 \times 10^{-4}$). There was a significant difference due to genotype ($p = 1.0 \times 10^{-4}$), and even more significantly, due to harvest time ($p = 1.24 \times 10^{-7}$) (2-way ANOVA) (Appendix 2, 4.2.4) (Fig. 4.10). By 1-way ANOVA, there were no significant differences in Av.LSL between WI 2585 and Amagi Nijo at either 14D and 21D. But there was a significant increase in Av.LSL in WI 2585 between 21D and 28D ($p = 0.03$); and, more significantly, in WI 2585 between 28D and 35D ($p = 0.02$, 1-way ANOVA, $n = 12$). While the Av.LSL continued to increase significantly with time in WI 2585, there was only a relatively small increase in Amagi Nijo (Fig. 4.10).

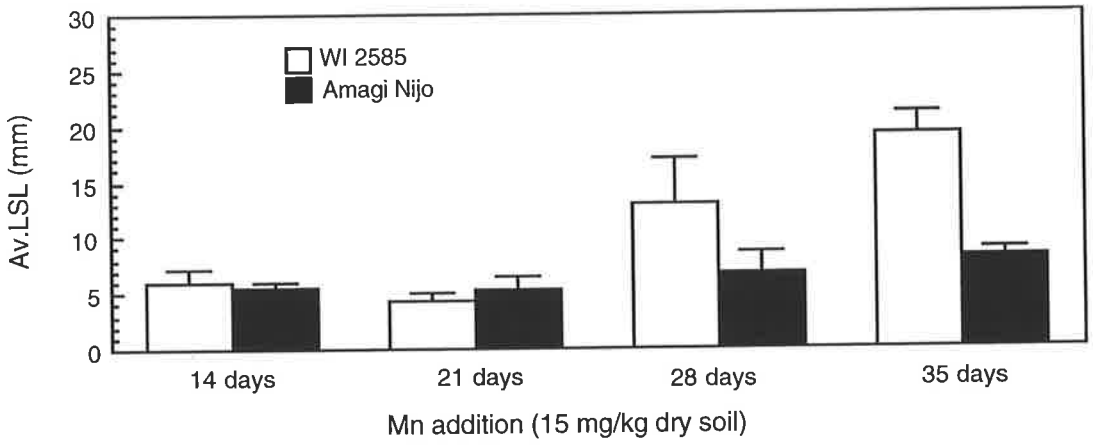


Fig. 4.10 The Av.LSL (mm/12 plants) of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* 17916 in a controlled-environment growth chamber with 15 mg Mn/kg soil treatment. The figure shows the effect on Av.LSL of genotype and harvest time. Values are the means of 12 replicate pots. The vertical bars show the SE.

There was a significant interaction with PIR between genotype and harvest time ($p = 0.02$, 2-way ANOVA) (Appendix 2. 4.2.5) (Fig. 4.11). By 2-way ANOVA, there was also a significant difference in PIR due to harvest time ($p = 0.003$), but none due to the genotype main effect. By 1-way ANOVA, there was no significant difference in PIR between WI 2585 and Amagi Nijo at any of the harvest times ($p > 0.05$, $n = 12$), but there was a significant increase in Amagi Nijo PIR between 28D-35D ($p = 0.02$, 1-way ANOVA) (Fig. 4.11).

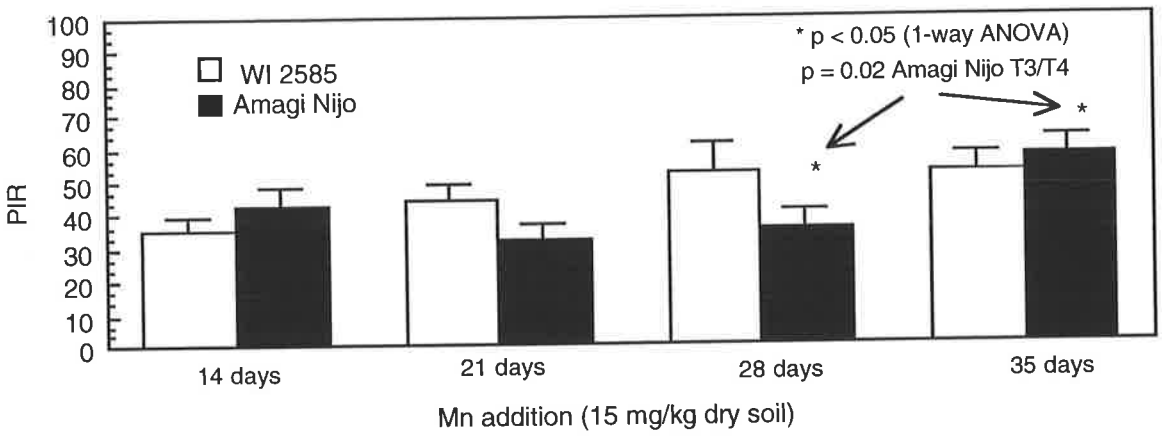


Fig. 4.11 The PIR of WI 2585 and Amagi Nijo growing in pots inoculated with *Ggt* 17916 in a controlled-environment growth chamber with 15 mg Mn/kg soil treatment. The figure shows the effect on PIR of genotype and harvest time. Values are the means of 12 replicate pots. The vertical bars show the SE.

4.4 Discussion

4.4.1 The *Ggt* experiment

The addition of Mn fertiliser and the resulting increase in shoot Mn concentration of WI 2585 to shoot Mn sufficiency concentration did not significantly reduce the Av.LSL in WI 2585 (Fig. 4.3). In addition, the Av.LSL in Amagi Nijo is short with either a shoot Mn concentration below or above the critical shoot Mn concentration (Fig. 4.2 and Fig. 4.3). Further, the Av.LSL in Amagi Nijo was not significantly shortened with an increase in Mn addition to the soil from 0 to 15 mg Mn/kg dry soil. This result suggests that Amagi Nijo may have a Mn-independent resistance to *Ggt*.

The PIR results show no sensitivity to the rate of Mn application to the soil or to shoot Mn concentration of the plants (Fig. 4.4). WI 2585 grown with nil *Ggt* has a significantly higher root Mn concentration compared to Amagi Nijo (Fig. 4.5). When infected with *Ggt*, WI 2585 had a higher root Mn concentration compared to Amagi Nijo, but it was not significantly higher (Fig. 4.6). This result may be due to *Ggt* precipitating Mn when growing within the roots of WI 2585 (with a longer Av.LSL) more than in Amagi Nijo (with a shorter Av.LSL) (see section 1.8).

4.4.2 The temporal study

The Av.LSL in WI 2585 and Amagi Nijo was similar at 14D and 21D (Fig. 4.10). Significantly, the longer Av.LSL in the Mn-inefficient WI 2585 than in the Mn-efficient Amagi Nijo occurs only after 21 days, when the rate in the increase of Av.LSL is slower in Amagi Nijo compared to WI 2585 (Fig. 4.10). Thus, a seedling plant biochemical defence mechanism which is activated after 21 days and by day 28 in the root of Amagi Nijo may be inhibiting the rate of growth of *Ggt* within the root stele resulting in the low Av.LSL in Amagi Nijo compared to WI 2585.

The PIR between WI 2585 and Amagi Nijo are similar at all harvest times (Fig. 4.11); however, the finding that the PIR of Amagi Nijo significantly increases between 28D-35D suggests that this hypothesised pathogen defence mechanism in Amagi Nijo is not sustained after 28D.

Chapter 5 Microscopy investigation

5.1 Introduction

The results of the *Ggt* experiment in chapter 4 (Fig. 4.3) suggest that Amagi Nijo may have a heritable Mn-independent resistance to *Ggt*. The results of the temporal study in chapter 4 (Fig. 4.10) suggest the hypothesis that the *Ggt* resistance in Amagi Nijo may be due to some seedling biochemical or physical mechanism which at 21 days after inoculation with *Ggt* inhibits the rate of growth of *Ggt* within the root. In this chapter, efforts were made to look for an explanation for differences between WI 2585 and Amagi Nijo at the microscopic level.

5.1.1 Lignin and *Ggt*

Mn is involved in lignin production via the shikimic acid pathway (section 1.3.3). Plants deficient in Mn have been found to have low lignin production (Brown *et al.*, 1984). Lignin is thought to serve as a physical barrier to protect the roots from penetration by soil-borne root pathogens such as take-all (Skou, 1981). Therefore, plants deficient in Mn are likely to be more vulnerable to invasion by root pathogens (Huber and Wilhelm, 1988).

In barley, the Mn-efficient Amagi Nijo may be less susceptible to root invasion by *Ggt* compared to the Mn in-efficient WI 2585 because it produces more lignin to have a more efficient ectopic barrier around and within the cells of the roots. No differences in total lignin production has been found between Mn-efficient and Mn-inefficient wheat varieties, (Rengel *et al.*, 1993 and Rengel *et al.*, 1994a). However, the localised lignin production in the root has never been investigated microscopically and compared between Mn-efficient and Mn-inefficient genotypes to account for the reduced *Ggt* infection such as found in Amagi Nijo compared to WI 2585 (Fig. 4.3).

5.1.2 The morphology and pathology of *Ggt* infected roots

In *Ggt* infected wheat roots, ectopic black runner hyphae can be seen (Skou, 1981). Infection hyphae develop from ectopic runner hyphae. The infection hypha appears to penetrate the root by dissolving the cell walls. Within the cell, a lignituber can be seen to form a sheath around the infection hypha. It is thought that as the infection hypha extends and

grows in to the cell, it dissolves the end of the lignituber (Skou, 1981). The morphology of *Ggt* infection in primary roots may be different between the two genotypes. Fluorescent stains for lignin (condensed phenols), DNA and proteins, visualised under a UV microscope may show differences between genotypes in root morphology and *Ggt* pathology within and on the infected roots (O' Brien and Mc Cully, 1981).

5.1.3 SEM and EASEM

The *Ggt* infection can be compared using a temporal investigation of WI 2585 and Amagi Nijo infected roots using Scanning Electron Microscopy (SEM) and Elemental Analysis Electron Scanning Microscopy (EASEM) to detect mineral elements in the samples. The use of these methods may reveal differences between genotypes in the morphology and density of *Ggt* ectopic runner hyphae, root growth morphology, penetration sites of *Ggt* infection hyphae on the surface of the root, and growth within the roots.

5.2 Method

5.2.1 UV fluorescence microscopy

The root samples used were from the *Ggt* experiment (section 4.2.1). The roots selected for UV fluorescence microscopy were the roots of each genotype which were representative of the mean Av.LSL and PIR for each treatment (n = 12). Samples of root 1-2 cm below the crown were cut into 1 cm long sections (O' Brien and Mc Cully, 1981) for analysis as follows:

5.2.2 Fluorescent staining for phenols, DNA and proteins

Auto fluorescence of the root tissue was controlled for by mounting a 1 cm section of the root on the microscope slide in 80% glycerol. At the excitation wavelength, 500 nm, xylem fluoresces green (O' Brien and Mc Cully, 1981).

Acid fuchsin is used to visualise fungal infections of fresh roots. Acid Fuchsin stain in roots infected with fungi shows up the storage proteins of micro-organisms as a fluorescent pink using excitation at 540 nm. The 1 cm long section of root is stained by immersion for 2 minutes in acid fuchsin stain at room temperature (acid fuchsin 0.1 g, lactic acid (lab grade),

875 ml, glycerol 63 ml, milli-Q H_2O to 1 L.). The sample was rinsed in milli-Q H_2O, and mounted onto the slide (O' Brien and Mc Cully, 1981).

Phenolics in lignified cell walls and cell nuclei were visualised by mounting onto a slide in 0.01% ethidium bromide. Ethidium bromide penetrates both fresh whole roots and whole mounts of fungi rapidly. Using excitation at 535 nm, phenolics fluoresce yellow-orange, while round cell nuclei fluoresce red-orange (O' Brien and Mc Cully, 1981).

Cell nuclei in fresh roots were visualised by mounting a 1 cm section of fresh root on a slide in 4',5-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1.5 mg DAPI in 100 ml milli-Q H_2O). Small bright nuclei shine within the root using excitation at 360 nm (O' Brien and Mc Cully, 1981).

To enhance the contrast between phenols in the cell walls and nuclei within the cells, a 1 cm section of fresh root was placed on a slide, stained with DAPI for 10 minutes, and then ethidium bromide (0.01%) was added. Upon using excitation at 535 nm, the yellow-orange background from the phenols is reduced so that the small round nuclei shine orange, while the outline of the lignified cell walls are clearly visible against a black background.

The stained samples were placed on a Sheppard CR 09GP F761 microscope for both light and UV microscopy and the appropriate light filters inserted. With both 20x and 40x magnification, photographs were taken under conditions that most suited features of interest using Kodak RHC 160 ASA colour film.

5.2.3 SEM and EASEM

The samples used for SEM and EASEM were from the temporal study (section 4.2.2). At each harvest and immediately following determination of PIR and Av.LSL, 1 cm long tissue samples of every primary root from each plant of either WI 2585 or Amagi Nijo which showed a mean Av.LSL (mm/12 plants) and PIR (12 plants) were placed into SEM/TEM fixative, and stored 4°C for 4-6 hours. The SEM/TEM fixative (100 ml) was: paraformaldehyde 4 g, NaH_3 0.13 g, and 2.5 ml glutaraldehyde (25% solution, EM grade) in milli-Q H_2O. The samples were fixed and dehydrated in the following procedure.

1). Wash 2 x 10 mins in phosphate buffered saline (PBS) (for 1L mix NaCl 8g, KCl 0.2g, Na_2HPO_4 1.44g, KH_2PO_4 0.24g, pH 7.2), with 4% sucrose.

2). Post-fix in 2% OsO_4 in PBS for 1-2 hour on a rotor.

3). Dehydrate in 70% acetone 3 x 20 mins

90% acetone 3 x 20 mins

95% acetone 3 x 20 mins

100% acetone 2 x 30 mins

100% acetone 1 x 60 mins

4). The samples were then critical-point dried in a critical point drier (Bal-TEC CPD 030).

5). The dried samples were then mounted onto stubs and cut to reveal the interior of the root.

6) Samples to be used for conventional SEM were coated with carbon/gold and stored at room temperature in a dry atmosphere with silica gel in a desiccator.

7). Samples to be used for Elemental Analysis were not washed in OsO_4, and were coated with carbon rather than with carbon/gold.

The samples were examined under a Phillips XL30 Field Emission Scanning Electron Microscope. For the samples used for elemental analysis and with carbon coating only, the Backscattered Electron Detector was put into place. After the image was optimised, labelled photographs and elemental data charts were made and stored on the Centre for Electron Microscopy and Microanalysis of South Australia (CEMMSA) server "ruska".

5.3 Results

5.3.1. Control stain for autofluorescence, 80 % glycerol.

Both the control (nil *Ggt*) and *Ggt*-infected root sections showed green autofluorescence. Since root samples do not contain chlorophyll, no red autofluorescence was found. In the *Ggt* infected root samples, the black runner hyphae of *Ggt* strain 17916 were clearly visible. There were no observable differences between genotypes in the morphology and density of *Ggt* ectopic runner hyphae, root growth morphology and penetration sites of *Ggt* infection hyphae on the surface of the roots.

5.3.2 Acid fuchsin stain for proteins and micro-organisms.

Roots infected with *Ggt* showed pink hyphae within the roots. When placed under confocal microscopy to attempt to reveal the interior root morphology of *Ggt* infection, the hyphae were black. Therefore, the morphology of *Ggt* hyphae within the root was not visible under the confocal microscope.

5.3.3 Ethidium bromide stain for phenols and cell nuclei DNA

In the infected and uninfected roots, ethidium bromide showed yellow-orange fluorescence of the phenolics in the lignified square or oblong cell walls of the root. In addition, orange-red small round nuclei were visible, and clearly distinguishable from the yellow-orange square or oblong sections of the lignified cell walls. *Ggt* hyphae do not take up this stain. *Ggt* infected roots showed dark ectopic runner hyphae and infection hypha root entry points against the fluorescent yellow-orange background.

In both the uninfected and infected *Ggt* roots, there were no visible differences in either location or amount of phenolic lignin synthesis between genotypes at both deficient and sufficient shoot Mn concentrations. However, an increase in the amount of lignin synthesis in both genotypes from deficient shoot Mn to sufficient shoot Mn concentrations was suggested by a more vivid stain in the Mn-sufficient root. The ends of the stelar lesions are visible (Fig. 5.1). However, the detail is not visible at either 20x or 40x magnification to determine any differences between *Ggt* infection hypha within WI 2585 and Amagi Nijo roots.

5.3.4 DAPI staining for cell nuclei DNA

DAPI fluorescent stain showed small round nuclei fluorescing brightly within the root tissue of both genotypes for infected and uninfected roots. While soil grains on the sample were a green colour, black ectopic runner hyphae on the outside of the root, dark thick invasion hypha at the root penetration site, and mycelia growing in the stele and rupturing the root, all showed clearly against the light blue fluorescence of the DAPI stain taken up by the root tissue (Fig. 5.2). A grey mycelial mass was also visible around the outside of the root, as were the brightly shining nuclei in rapidly dividing cells of the growing root hairs. However, no differences in morphology of root penetration site *Ggt* infection hypha between the genotypes and between deficient and sufficient shoot Mn concentrations could be seen.

5.3.5 Double staining: DAPI for DNA and ethidium bromide for phenols and DNA

Similarly, the double staining showed no observable differences between either genotype of deficient and sufficient shoot Mn concentrations. Lignified squared or oblong cell walls and brightly shining DNA in the small round cell nuclei within the cells of the root were visible. In infected roots, black runner hyphae could be visualised on the outside of the root. In addition, the stelar lesions within the root were visible.

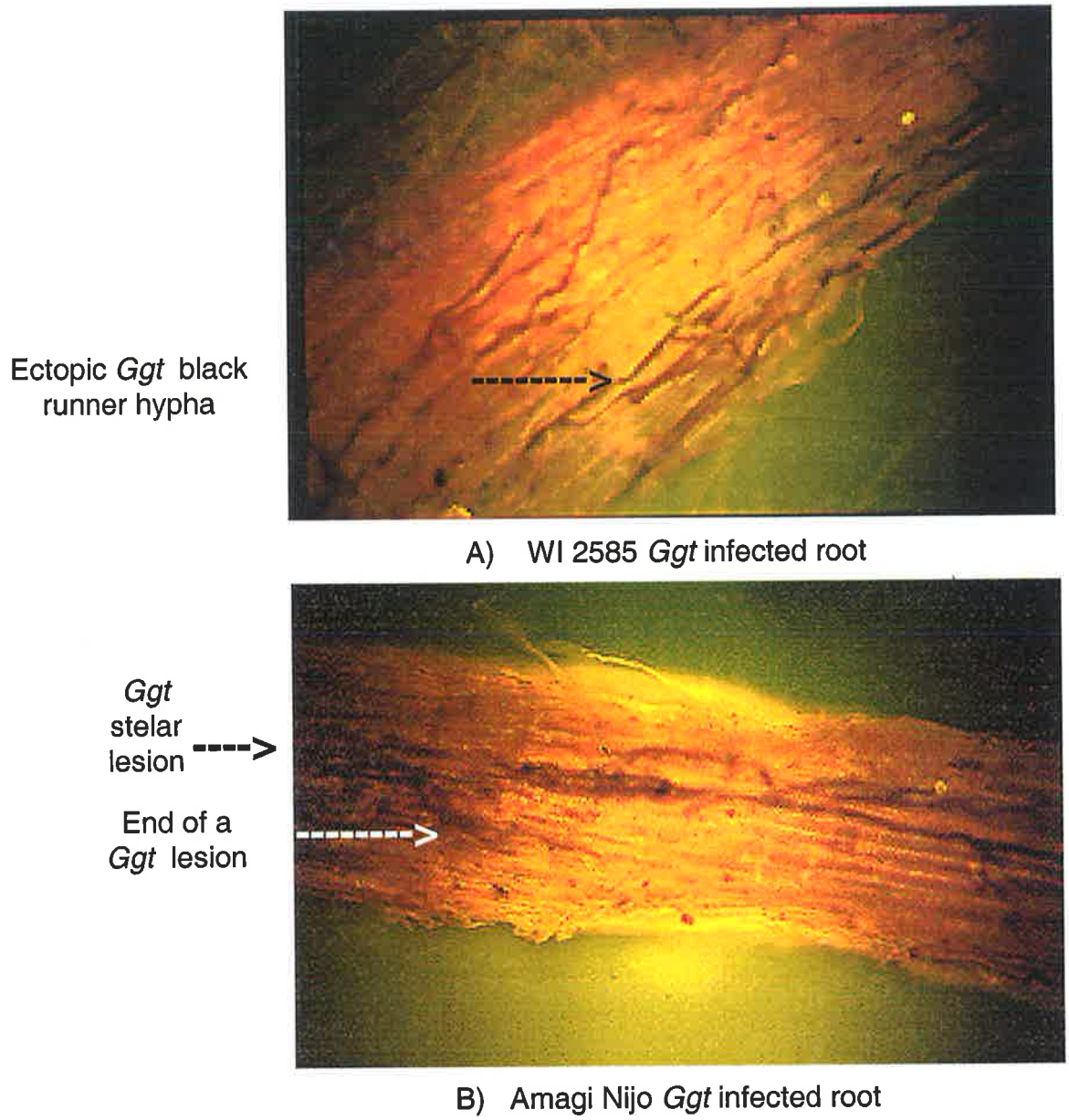


Fig. 5.1 When WI 2585 and Amagi Nijo were grown at 15 mg Mn/kg dry soil, WI 2585 roots were Mn deficient (shoot Mn concentration below the critical shoot Mn concentration, section 3.3.4) (A), while Amagi Nijo roots were Mn sufficient (shoot Mn concentration above the critical shoot Mn concentration, section 3.3.4) (B). Ethidium bromide stain show no differences in the intensity of staining for lignin, or in infection pathology between genotypes (magnification x 20). The lignified cell walls are stained yellow-orange (Exc. λ 535 nm.). The *Ggt* black runner hyphae are visible (arrowed) on the surface of the WI 2585 (A) and Amagi Nijo root (B). The end of a *Ggt* lesion (arrowed) is visible in the Amagi Nijo root (B).

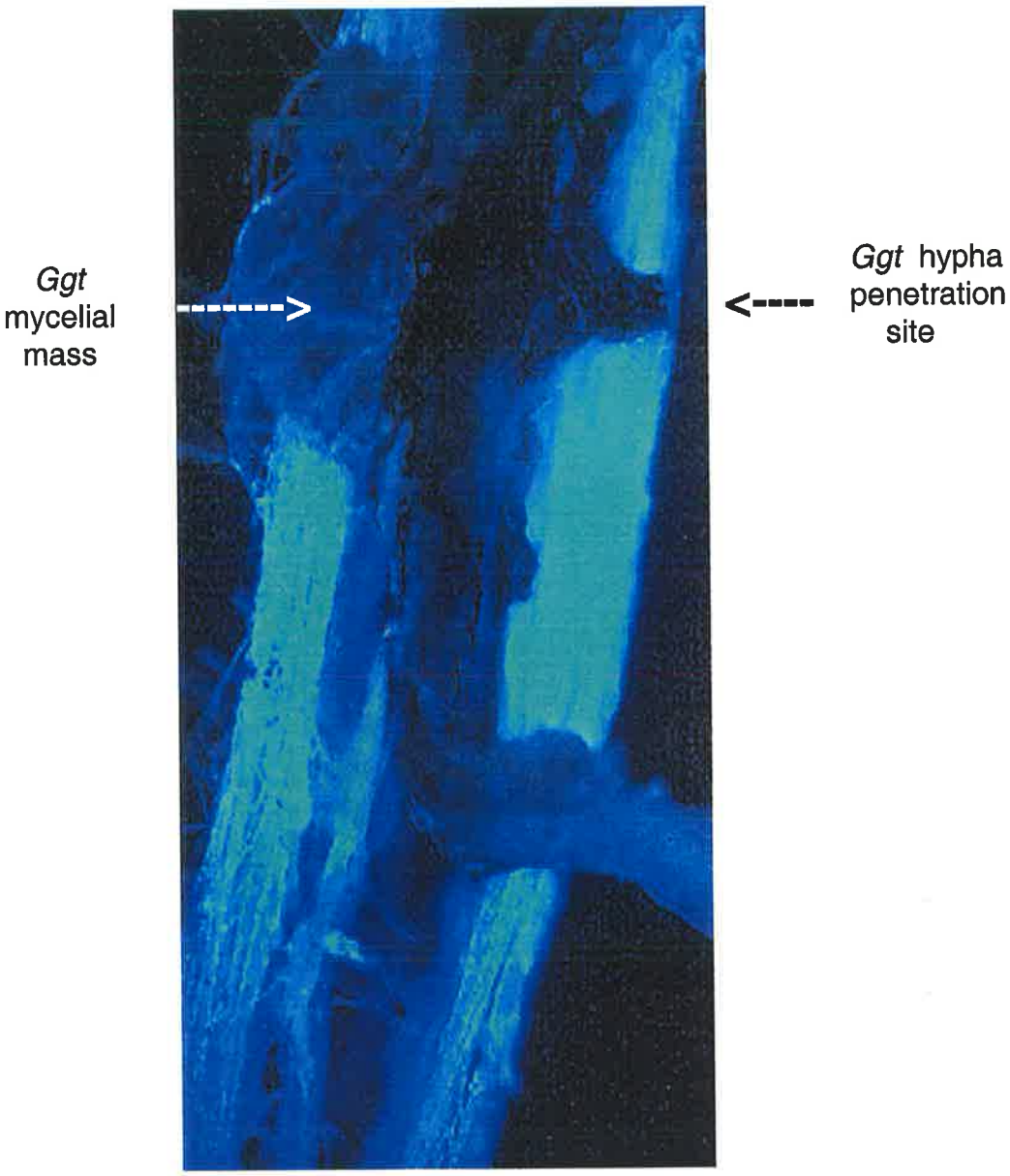
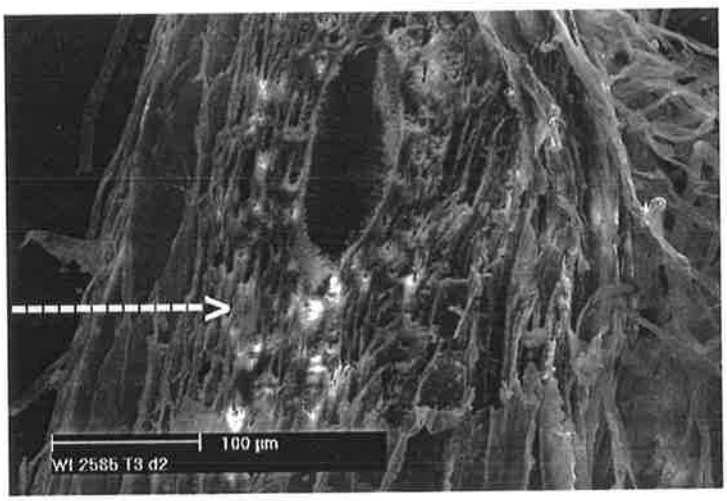
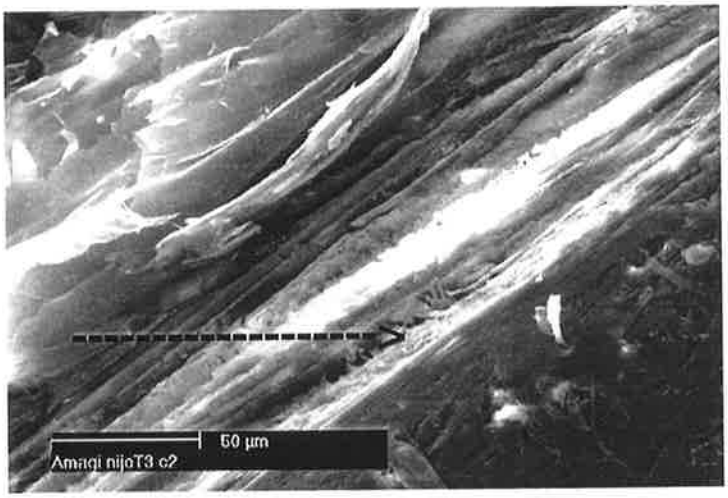


Fig. 5.2 A *Ggt* infected Amagi Nijo root grown in 15 mg Mn/kg dry soil stained with DAPI (magnification x 20, Exc. λ 360 nm.). A root penetration site of a black *Ggt* ectopic runner hypha (arrowed on the right of the root) has ruptured the root revealing the stele clogged with *Ggt* mycelia. An external grey mycelial mass is visible (arrowed on the left of the root).

A) A WI 2585 root infected with *Ggt* shows mycelia clogging the root stele



B) An Amagi Nijo root infected with *Ggt* shows mycelia clogging the root stele



C) AnAmagi Nijo root covered with *Ggt* hyphae at 28 days

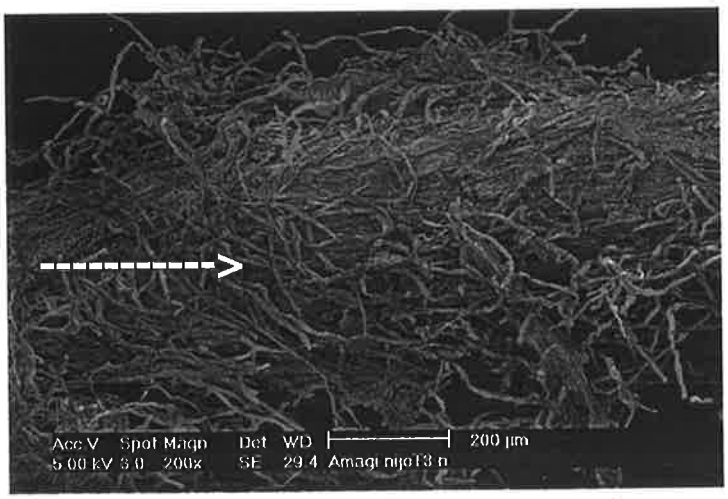


Fig. 5.3 SEM of roots of *Ggt* infected WI 2585 (A) and Amagi Nijo (B) and (C) roots grown for 28 days in soil amended with 15 mg Mn/kg dry soil.

5.3.2 SEM EASEM

At the 14 day harvest, Amagi Nijo appeared to have a less dense cover of ectopic *Ggt* hyphae compared to WI 2585. But no differences in the density of ectopic *Ggt* hyphal cover, internal hyphae or mycelial morphology; or morphology of *Ggt* infection points, were observed at any of the subsequent harvest times. Because all parts of the infected roots of both genotypes at all harvest times were clogged with fine *Ggt* mycelia, the growing tips of infection hypha were totally obscured from view (Fig. 5.3).

5.4 Discussion

5.4.1 UV microscopy

All stains were effective under the staining times and methodology at the given excitation wavelength (Exc. λ), for both control (nil Ggt) and *Ggt* infected root tissue. The acid fuchsin stained roots orange and the *Ggt* hypha pink under light microscopy. However, the *Ggt* hyphae within the root only showed black when placed under the Confocal Microscope. This may be due to the natural colour of the hypha being black and thus absorbing all wavelengths of light.

An increase in the biosynthesis of lignin could be observed in both infected and uninfected roots between deficient shoot Mn and sufficient shoot Mn concentrations. However, there was no reduction in Av.LSL associated with increased lignin synthesis going from deficient shoot Mn to sufficient shoot Mn concentration with either genotype.

5.4.2 SEM and EASEM

Ggt hyphal infection points, and ectopic and internal *Ggt* hyphal and mycelial morphology and density between WI 2585 and Amagi Nijo are indistinguishable at all harvest times. The density of the ectopic *Ggt* infection increased with time in both genotypes. The tips of infection hyphae within the infected roots were totally obscured by the fine mycelia of this particular *Ggt* isolate that completely clogged the stele of the root (Fig. 5.3). Elemental Analysis of root Mn using SEM was not possible due to the root Mn being 100 times below the detectable concentration for this method. Elemental Analysis using Transmission Electron Microscopy (TEM) is 100 times more sensitive, but the magnification of TEM, (x 100,000), is far too great to be of use for investigation of the morphology of *Ggt*. Therefore, it is not

practical to use TEM to detect root Mn within WI 2585 and Amagi Nijo. In conclusion, SEM investigation of the infected roots has not shown any differences between WI 2585 and Amagi Nijo to suggest a mechanism by which the growth of the *Ggt* fungus through the stele of the Amagi Nijo roots is inhibited after 21 days.

Chapter 6 Genetic studies

6.1 Introduction

The studies described in chapter 2 rejected the first Null Hypothesis that there is no physiological connection between the expression of the Mn efficiency trait and enhanced resistance to *Ggt* (section 1.10). In this chapter, genetic studies are used to test the alternative second Null Hypothesis: that there is no genetic linkage between the *Mel 1* (4HS) Mn-efficient allele and enhanced resistance to *Ggt* (section 1.10). For the genetic studies, a doubled haploid (DH) population (439 lines) of a cross between the Mn-efficient Amagi Nijo and the Mn-inefficient WI 2585 were used (Davies and Morton, 1997). DH plants are homozygous at all loci enabling accurate early generation screening (Thomson *et al.,* 1991 and Zivy *et al.,* 1992).

The DH *Ggt* study required the accurate classification of lines into those with the Mn-efficient or Mn-inefficient allele at *Mel 1*. A Restriction Fragment Length Polymorphism (RFLP) marker for *Mel 1* was available, but it was difficult to interpret since it detected multiple loci. Therefore, alternative markers were sought based around the use of Amplified Fragment Length Polymorphism (AFLP). To determine if the presence of the *Mel 1* locus for Mn efficiency on 4HS is linked to resistance to *Ggt*, the DH lines were grown and infected with *Ggt* in soil deficient in plant available Mn under controlled-environment growth conditions.

Second, it was also possible that a clear association between Mn efficiency and resistance to *Ggt* resides at Mn efficiency loci other than *Mel 1*. The bioassays in Mn deficient soil conducted in a controlled-environment suggested that the Mn-efficient allele at *Mel 1* accounts for 70-80% of the variance in Mn efficiency, while in field trials, it accounts for only 50% of the Mn efficiency (Pallotta *et al.*, 1999). This result suggests that genes at other loci than *Mel 1* may contribute to Mn efficiency in the field that do not express in the pot bioassay. A field trial of the DH population at a Mn deficient site at Marion Bay, Southern Yorke Peninsula, South Australia was conducted in an attempt to identify additional loci influencing Mn efficiency.

6.2 Method

6.2.1 RFLP analysis to map *Mel 1* precisely

RFLP analysis of WI 2585 and Amagi Nijo parents, and the 439 doubled haploid lines, including the 29 doubled haploid lines infected with *Ggt*, was performed (Appendix 3, 3.1, 3.2, and 3.2.1 to 3.2.4) using the restriction endonuclease *Dra* 1 and RFLP probes *wg622*, *cdo583*, *abg714*, and *mwg77*. All RFLP results for the DH lines were checked to confirm that the allele type at *Mel 1* was consistent with data for Mn efficiency obtained in both pot bioassays and field Mn efficiency data from two seasons (sections 6.2.4, 6.2.5 and 6.2.6). The mapping programme used to map *Mel 1* precisely on 4HS was Map Manager QT Version b16, $p < 0.01$ (Manly and Cudmore 1997) (Appendix 3, 3.2 and 3.3).

6.2.2 The DH *Ggt* experiment

The soil was prepared, incubated, and basal nutrients added (section 2.2.2). Mn, as $MnSO_4$, was added to correspond to 15 mg Mn/kg dry soil. WI 2585 and Amagi Nijo were the controls for 29 DH lines selected (sections 6.1 and 6.2.1) for infection with *Ggt* isolate 17916 (see section 2.2.2). There were 2 pots x 31 genotype/DH lines x 6 plants/DH line. In all, there were 62 pots. The seeds used were as listed in Appendix 1, Table 2.2. The Mn-efficient allele was present in 15 DH lines, while the Mn-inefficient allele was present in 14 DH lines (see section 6.2.1). Statistical analysis was by 1-way ANOVA for all the results obtained (section 2.2.2) (Appendix 2, 6.1).

6.2.3 Outdoor *Ggt* bioassay

Ggt infection of roots is known to be highly variable. Confirmation of resistance to *Ggt* 17916 in the parents and DH lines was checked by Dr H Wallwork (SARDI) in an outdoor soil bioassay with a mixed *Ggt* inoculum. The DH lines that showed a short Av.LSL in earlier experiments and parents WI 2585 and Amagi Nijo were grown in 5 cm x 15 cm tubes transplanted into sand such that the plant roots grew through the sand. The tubes were placed over a layer of sand that had been fertilised with Osmocote Plus (Total N 16%, P 3.5%, K 10%, S 2.4%, Mg 1.2%, B 0.02%, Cu 0.05%, Fe 0.4%, Mn 0.06%, Mo 0.02%, Zn 0.015%

and organic resin coating 8%) controlled release plant food (0.3 g/tube) (The Scotts Company, Ohio, USA). The soil in the tubes was a modified UC mix pH ~ 6.8. Coarse washed sand (Golden Grove) 350L was pasteurised at 100°C for 30 minutes to kill microbes. Then, Eurotorf peatmoss (250 L) was mixed with the sand for 10 seconds. Ten minutes after the temperature of this mix had dropped to 80°C, the fertilisers $Ca(OH)_2$ 500 g, $CaCO_3$ 500 g, and Nitrophoska (N15 %, P 3.9%, K 12.4%, Mg 1.25%, Ca 3.40% 500g, S 5.30%, Fe 0.30%, Cu 0.0002%, Zn 0.007%, B 0.01% and Mo 0.0003%) were all mixed into it for 20 seconds to ensure that the finished soil had sufficient air porosity. To this was added, at a rate of 0.03% or 0.2 g/L soil, a mixed *Ggt* inoculum consisting of take-all infected ryegrass seeds. Each variety/DH line was tested using 4 blocks of 25 plants. Watering was by overhead irrigation for 20 mins twice a day. The plants were harvested at maturity. *Ggt* infection levels were determined using visual scores from 1 (no infection) to 5 (plant is dead from the infection) of both the severity in blackening of the root stele, and the health and extent of the plant foliage. Statistical analysis was by General Analysis of Variance (Microsoft Statistix Version 2 for Windows), ($\alpha = 0.05$).

6.2.4 Field trial

The field trial was conducted during the winter of 1999 at a severely Mn deficient site at Marion Bay, Southern Yorke Peninsula, South Australia. There were 3 replicates of 62 DH entries in the 1999 field trial. The DH lines were selected against a number of criteria collated in the 1998 field trial to obtain data for analysis that was as free as possible of confounding factors. The seed was taken from the + Mn plots, was similar in size and Mn concentration and had shown mid-maturity in the 1998 field trial.

6.2.5 Field trial design

A split-plot factorial field experiment was conducted, laid out in a randomised complete block design. The main plots were genotype and the split-plots were the Mn treatment. Each main plot was split into two Mn treatments (62 DH lines x +/- Mn treatments x 3 replicate sub-plots). In addition, WI 2585 and Amagi Nijo were used as controls (2 genotypes x +/- Mn treatments x 18 replicate sub-plots). In all, there were 444

sub-plots in the field trial. At sowing, 20 kg Mn/ha as manganese oxysulphate was applied to the + Mn plots. Also applied to the + Mn plots, were two foliar sprays (Mn as $MnSO_4$;- Mangasol 47, Top Australia Ltd.), firstly at maximum tillering to early elongation stage and secondly at early grain filling stage. Each nil Mn (- Mn) and + Mn sub-plot consisted of 6 rows with 15 cm spacing sown at a seeding rate of 170-180 seed/m^2. The plot area harvested was 3.35 m^2.

6.2.6 Analysis of 1999 field trial

Six weeks after sowing, the youngest emerged leaf blade (YEB) shoot samples (16 blades) were taken evenly across each - Mn and + Mn sub-plot for determination of YEB Mn concentration (mg/kg DM) (section 2.2.2). The relative shoot (YEB) Mn concentration was calculated,

relative YEB Mn concentration = 100 x YEB [Mn] from - Mn plot/YEB [Mn] from + Mn plot.

Twelve weeks after sowing, the sampling of YEBs was repeated. The maturity scores (1-5) were recorded (1 all the heads still in boot, 5 all heads fully out). At the same time, the field trial was visually assessed for Mn efficiency (0-100) (%). Comparing the nil Mn plot to the + Mn plot, points were deducted from a maximum of 50 for decreased vegetative growth, from a maximum of 20 for each of increased chlorosis and necrosis, and from a maximum of 10 for increased leaf collapse. These scores were added for each DH line.

At harvest, the relative yield was determined,

relative yield = 100 x yield (g) from - Mn plot/yield (g) from + Mn plot.

Spatial analysis (Gilmour *et al.*, 1997) of the field data was done by H. Oakey, BiometricsSA to remove spatially systematic variation obtained in field trials. The data were used to detect Quantitative Trait Loci (QTLs) (Map Manager QT Version b16, Manly and Cudmore, 1997) influencing Mn efficiency in this DH population (62 DH lines) (log likelihood (LOD) > 3.2, $p < 0.001$) (Appendix 3.2). The significance for a QTL in a minimum interval between markers 20 cM to 40 cM (centimorgan) is given as valid if the LOD score is > 3.2 (Lander and Botstein, 1989). A graphical display of associations between QTLs was displayed using Q-GENE (Nelson 1997) (Appendix 3.2 and 3.3).

6.3 Results

6.3.1 RFLP mapping of *Mel 1*

*Dra*1 was used for the identification of polymorphisms between parents WI 2585 and Amagi Nijo for *Xcdo583* (Fig. 6.1), *Xwg622*, *Xmwg77*, and *Xabg714*. The RFLP analysis of 439 doubled haploid lines suggested that *Mel 1* is located within a region of 1.4 cM between RFLP markers *Xcdo583* and *Xabg714* on chromosome 4HS (Fig. 6.2). The LOD score obtained (LOD = 62.5) is highly significant (Appendix 3, 3.2 and 3.3).

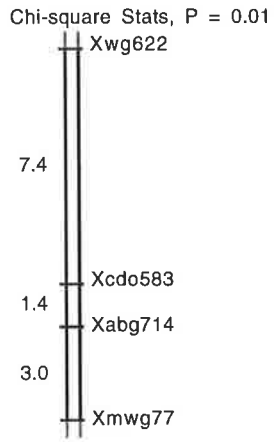


Fig. 6.2 Map of *Mel 1* region of chromosome 4HS.

6.3.2 AFLP mapping of *Mel 1*

The AFLP analysis of the WI 2585 and Amagi Nijo parents and their bulks identified two putative AFLP markers linked to *Mel 1* (Appendix 3.4).

The primer pair *Pst*AA *Mse*CTC identified a clear polymorphism (Fig. 6.3). The polymorphism (arrowed) was identified between WI 2585 (no band) and Amagi Nijo (band), and also between WI 2585-like bulk (no band), and Amagi Nijo-like bulk (band). This AFLP marker showed a segregation pattern of 80% similarity to *Mel 1* with the 20 individual DH lines which made up the WI 2585-like bulk and the Amagi Nijo-like bulk (Fig. 6.3).

The primer pair *Pst*AC *Mse*CAC also identified a polymorphism (Fig. 6.4). This marker co-segregated with *Mel 1* for 70% of the 20 individual bulk DH lines of the WI 2585-like bulk (Fig. 6.4).

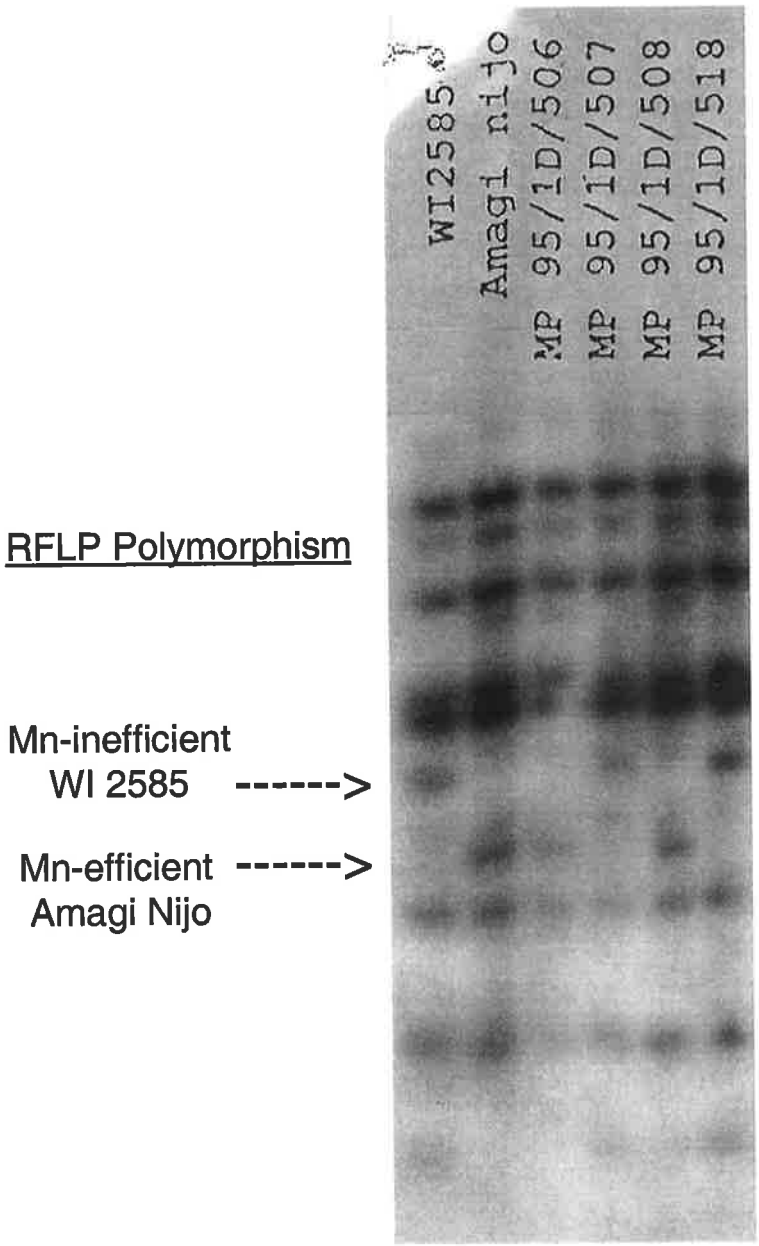


Fig. 6.1 Segregation pattern for RFLP marker *Xcdo583* between the Mn-inefficient WI 2585 and the Mn-efficient Amagi Nijo lines. DNA was digested with *Dra* 1 (the polymorphism is indicated by arrows).

Lane No.	Sample
1	WI 2585
2	Amagi Nijo
3	WI 2585 bulk
4	Amagi Nijo bulk

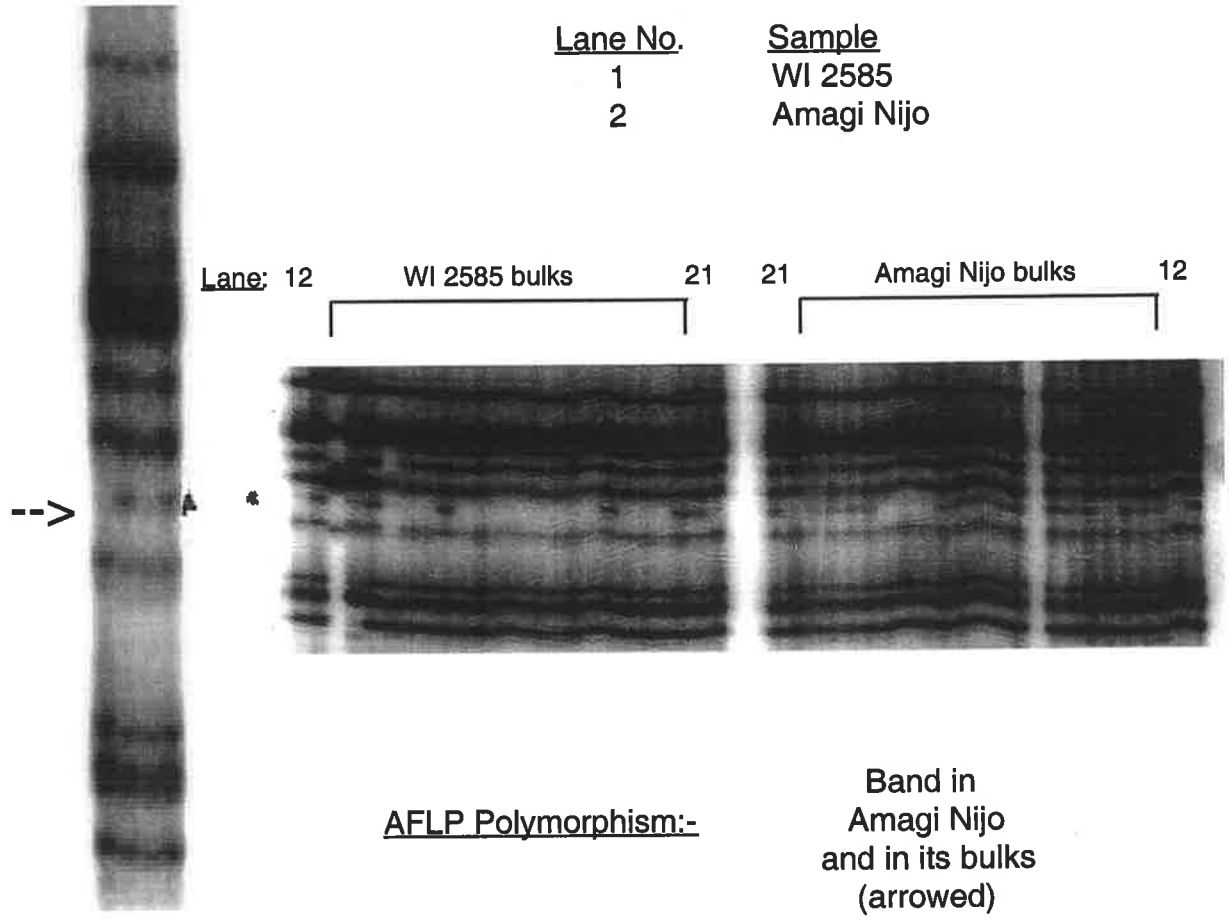


Fig. 6.3 Genomic DNA polymorphism (arrowed) identified between parent WI 2585 (no band) and its bulk (no band), and Amagi Nijo (band) and its bulk (band), using the AFLP selective primers *Pst*AA *Mse*CTC. This AFLP marker co-segregated with *Mel 1* in 80% of the 20 individual DH lines.

Lane No.	Sample
1	WI 2585
2	Amagi Nijo
3	WI 2585 bulk
4	Amagi Nijo bulk

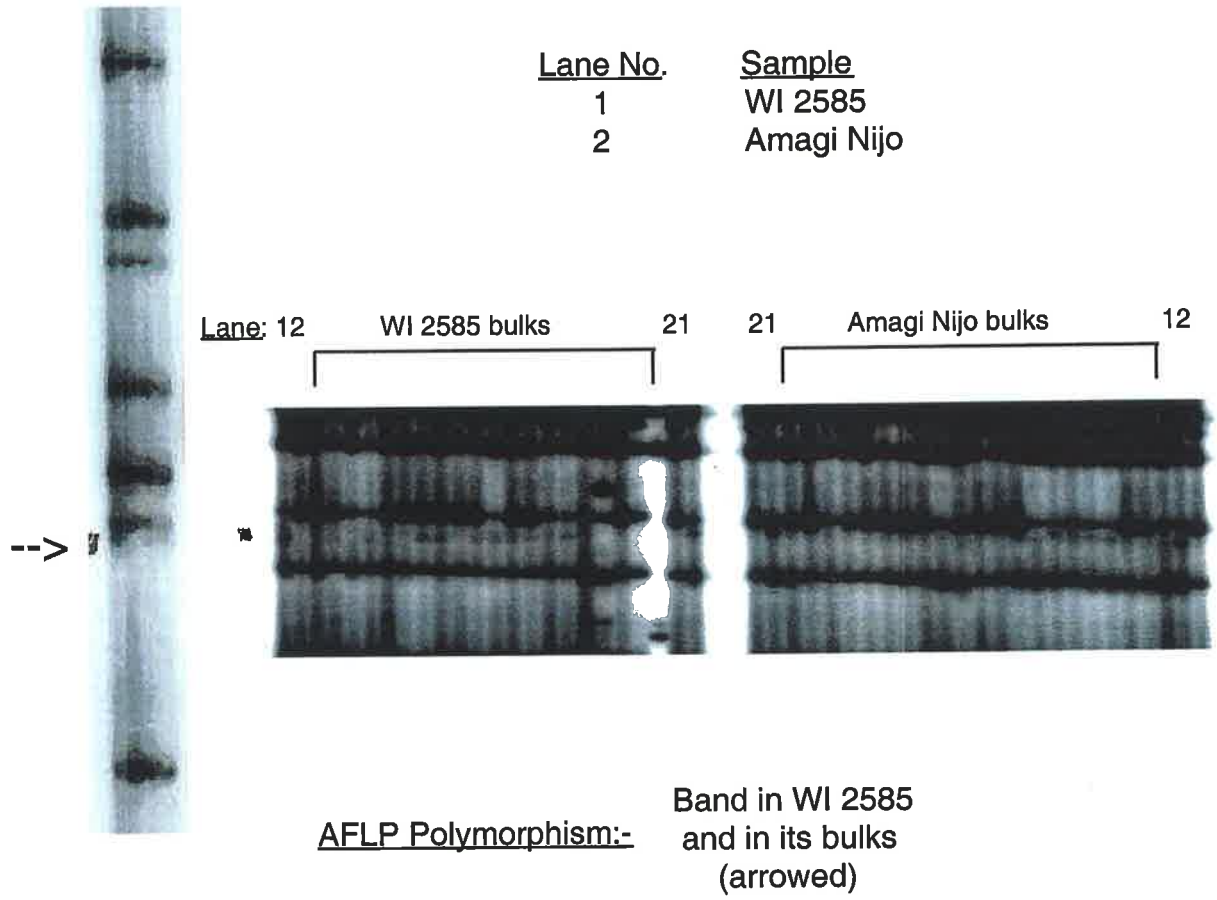


Fig. 6.4 Genomic DNA polymorphism (arrowed) identified between parent WI 2585 (band) and its bulk (band), and Amagi Nijo (no band) and its bulk (no band), using the AFLP primer pair *Pst*AC *Mse*CAC. This AFLP marker co-segregation with *Mel 1* in 70% of the 20 individual DH lines.

Careful checking was done to eliminate scoring errors. Both AFLP markers were confirmed as being linked to *Mel 1* in the 439 DH mapping population (Fig. 6.5). As is often found when markers are added to a genetic map, the map distance between the mapped markers has increased. In addition, because the calculated map distance is a statistical summary of the relationships between mapped markers, *Xabg714* and *Xmwg77* have swapped position (Appels *et al.,* 1998).

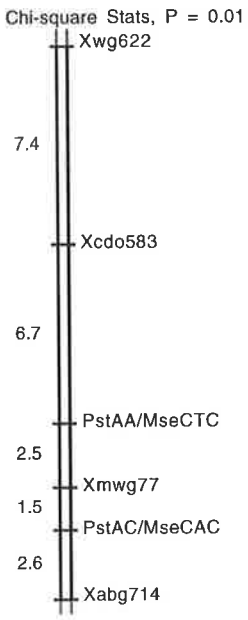


Fig. 6.5 Map of the *Mel 1* region of chromosome 4HS including AFLP markers.

6.3.3 The DH *Ggt* experiment

The DH experiment with *Ggt*, conducted in a controlled-environment, showed that the Mn-efficient parent, Amagi Nijo, and the DH lines with the Mn-efficient allele at *Mel 1* had a significantly greater shoot Mn concentration (mg/kg DM) (Fig. 6.6) and shoot Mn content (µg/pot) (Fig. 6.7) compared to the Mn-inefficient parent WI 2585 and the DH lines with the Mn-inefficient allele at *Mel 1*, ($p = 1.7 \times 10^{-9}$, and $p = 2.16 \times 10^{-5}$, Appendix 2, 6.1.1 and 6.1.2 respectfully). There were no significant differences in mean root Mn concentration between the Mn-efficient Amagi Nijo, the *Mel 1* efficient allele DH lines, Mn-inefficient WI 2585, and the *Mel 1* inefficient allele DH lines ($p = 0.8$). (Fig. 6.8, Appendix 2, 6.1.3).

By 1-way ANOVA, there were no significant differences in Av.LSL ($p = 0.41$) (Fig. 6.9, Appendix 2, 6.1.4) or in PIR ($p = 0.16$) (Fig. 6.10, Appendix 2, 6.1.5) between the Amagi Nijo-like DH lines at *Mel 1* and the WI 2585-like DH lines.

Three WI 2585-like doubled haploids showed a low Av.LSL. (D/5 Av.LSL = 7.9 ± 5.5 mm/plant, n = 6 plants, D/256 Av.LSL = 7.98 ± 2.2 mm/plant, n = 6 plants, and D/701 Av.LSL = 9.04 ± 2.6 mm/plant, n = 6 plants) all of which were of similar length to Amagi Nijo Av.LSL = 8.4 ± 3.7 mm/plant, n = 6 plants. In addition, eleven Amagi Nijo-like doubled haploids showed a longer Av.LSL, (D/2, D/3, D/7, D/25, D/32, D/49, D/678, D/791, D/819, D/843 and D/862), all of which were similar in length to the Av.LSL in WI 2585, 18.2 ± 5.5 mm/plant, n = 6 plants (Fig. 6.9). The shoot Mn concentration higher than 15 mg Mn/kg DM in some Amagi Nijo-like DH lines did not result in a short Av.LSL (Fig 6.6 and Fig. 6.9). These results supports the second Null Hypothesis that there is no genetic linkage between the *Mel 1* (4HS) Mn-efficient allele and enhanced resistance to *Ggt* (see section 1.10 and section 6.1).

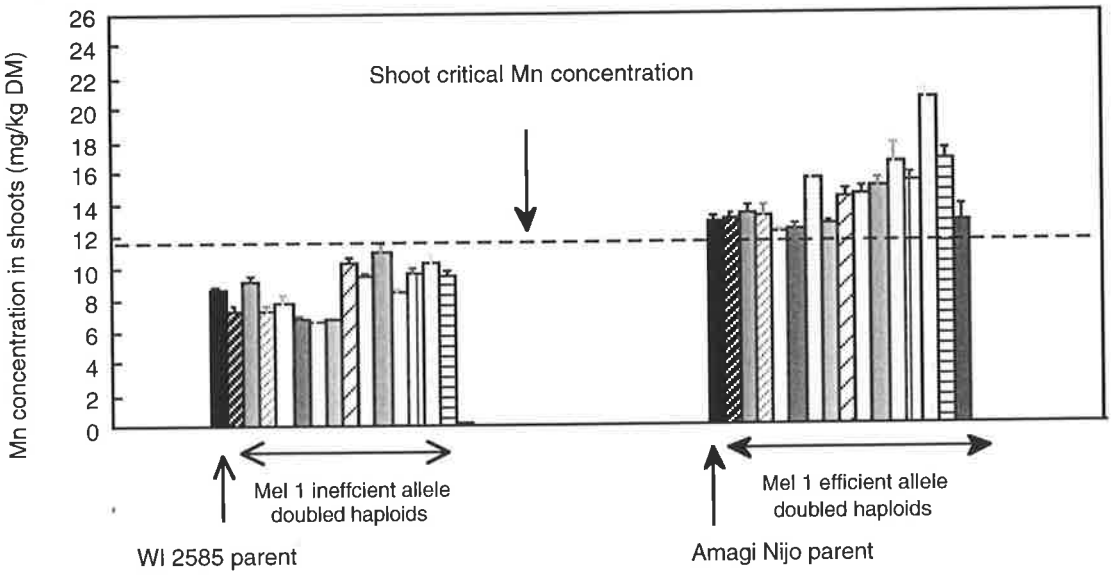


Fig. 6.6 WI 2585 and Mn-inefficient DH lines, and Amagi Nijo and Mn-efficient DH lines grown in pots for 28 days with *Ggt* 17916 in a controlled-environment growth chamber in soil treated with 15 mg Mn/kg. The figure shows the effect of *Mel 1* allele type (1 allele type/DH line) on shoot Mn concentration (mg/kg DM). Values are the means of 6 plants. The vertical bars show the SE.

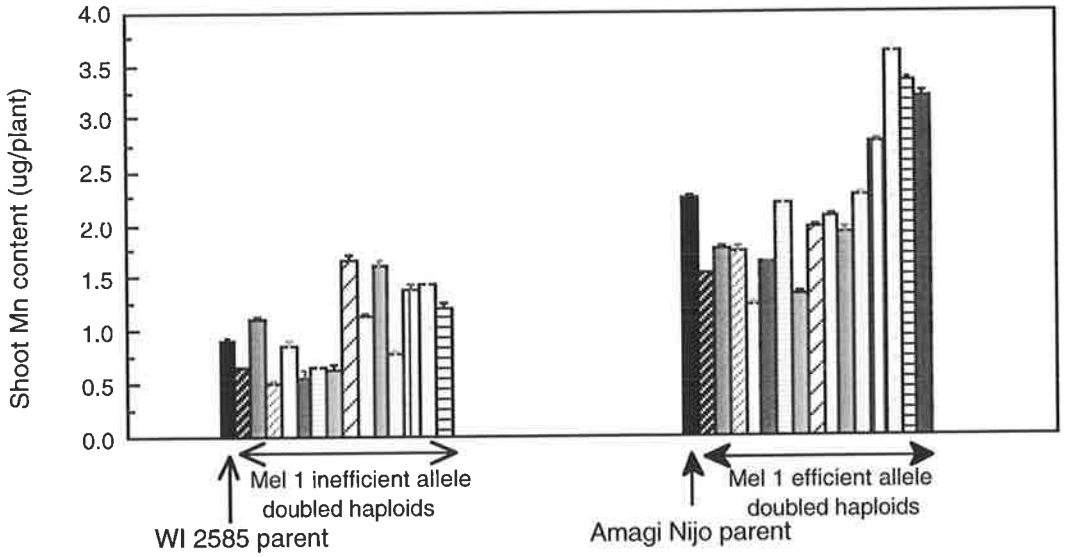


Fig. 6.7 WI 2585 and Mn-inefficient DH lines, and Amagi Nijo and Mn-efficient DH lines grown in pots for 28 days with *Ggt* 17916 in a controlled-environment growth chamber in soil treated with 15 mg Mn/kg. The figure shows the effect of *Mel 1* allele type (1 allele type/DH line) on shoot Mn content (µg/plant). Values are the means of 6 plants. The vertical bars show the SE.

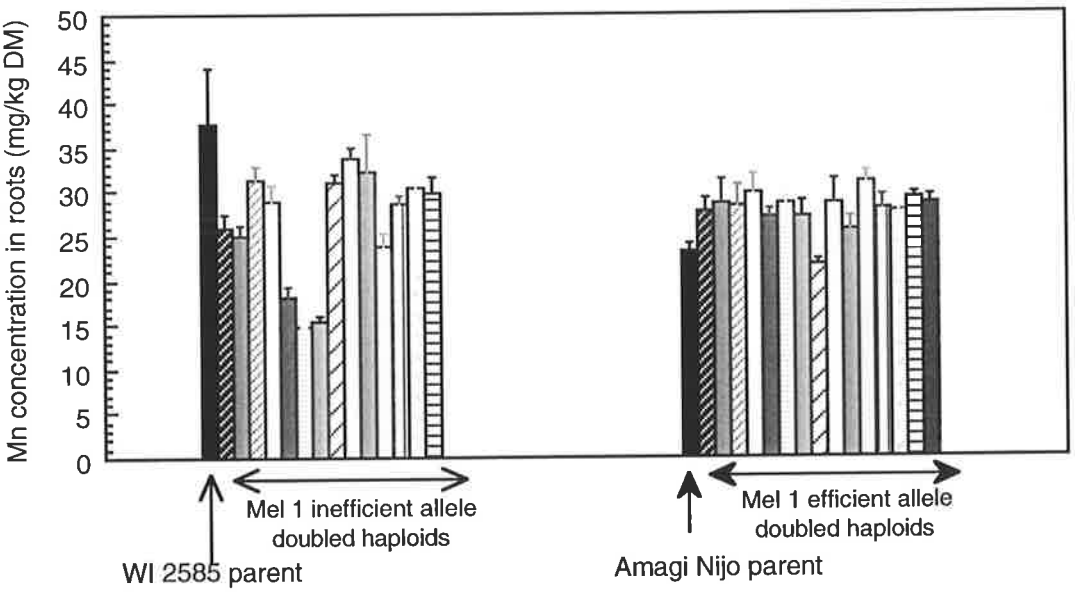


Fig. 6.8 WI 2585 and Mn-inefficient DH lines, and Amagi Nijo and Mn-efficient DH lines grown in pots for 28 days with *Ggt* 17916 in a controlled-environment growth chamber in soil treated with 15 mg Mn/kg. The figure shows the effect of *Mel 1* allele type (1 allele type/DH line) on root Mn concentrations (mg/kg DM). Values are the means of 6 plants. The vertical bars show the SE.

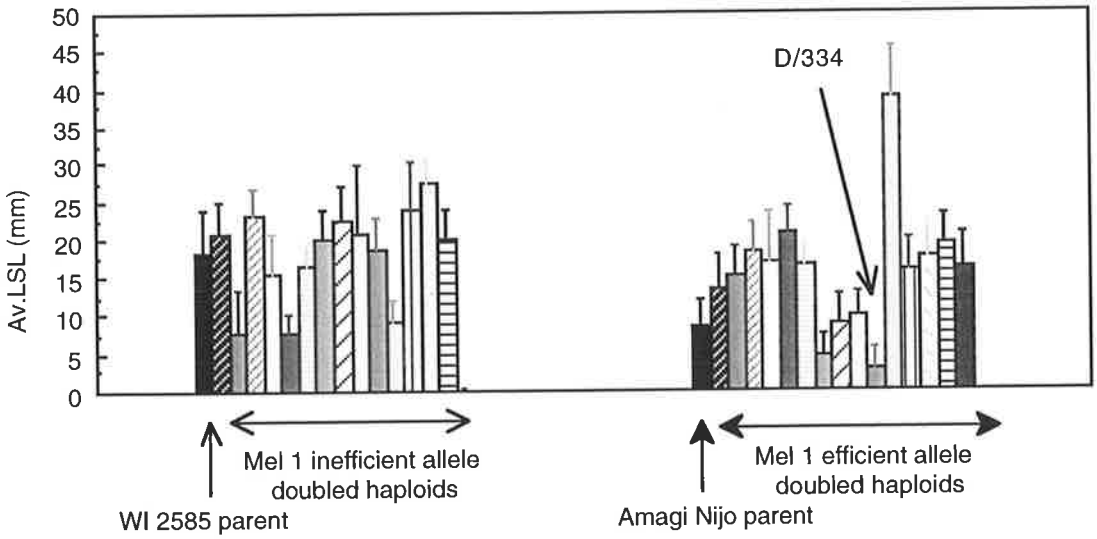


Fig. 6.9 WI 2585 and Mn-inefficient DH lines, and Amagi Nijo and Mn-efficient DH lines grown in pots for 28 days with *Ggt* 17916 in a controlled-environment growth chamber in soil treated with 15 mg Mn/kg. The figure shows the effect of *Mel 1* allele type (1 allele type/line) on Av.LSL (mm). Values are the means of 6 plants. The vertical bars show the SE.

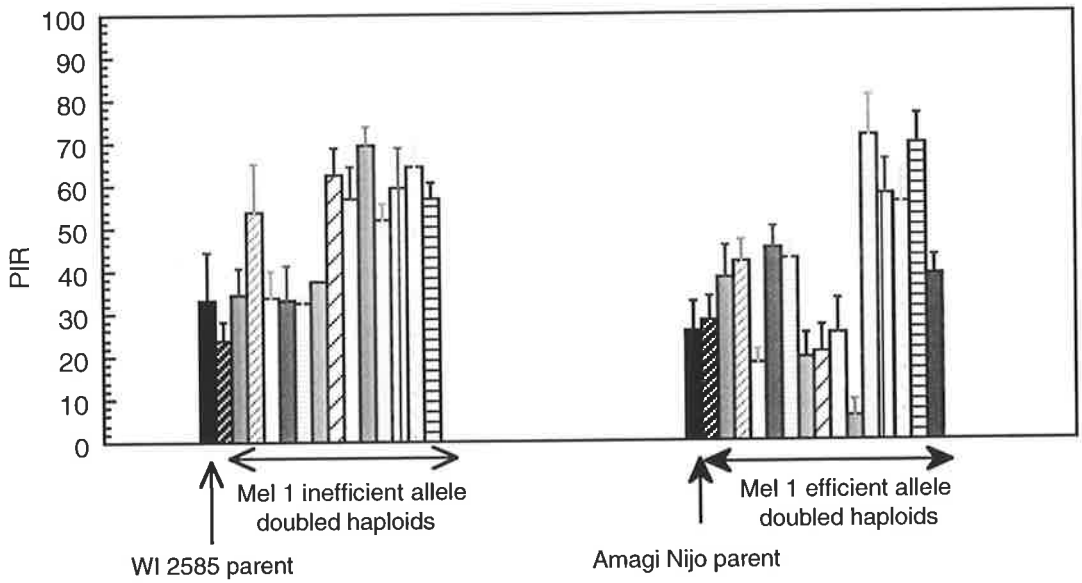


Fig. 6.10 WI 2585 and Mn-inefficient DH lines, and Amagi Nijo and Mn-efficient DH lines grown in pots for 28 days with *Ggt* 17916 in a controlled-environment growth chamber in soil treated with 15 mg Mn/kg. The figure shows the effect of *Mel 1* allele type (1 allele type/DH line) on PIR. Values are the means of 6 plants. The vertical bars show the SE.

6.3.4 Outdoor *Ggt* bioassay

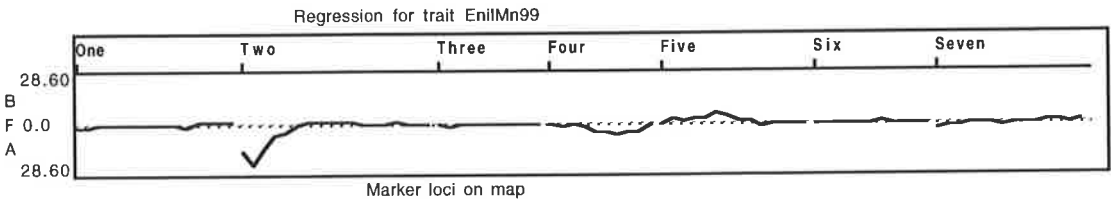
Doubled haploid line D/334 showed putative resistance to *Ggt* 17916 (Av.LSL = 3.0 ± 2.6 mm/plant, n = 6 plants) (Fig. 6.9). However, when D/334 was provided to Dr H Wallwork, this result was not repeated in a outdoor bioassay with a mixed inoculum of *Ggt* (Table 6.1). In this trial, D/334 infection levels were found to be only slightly less than in Amagi Nijo. There were no significant differences in *Ggt* infection between the crop varieties tested ($p > 0.05$), except for the rye variety Bevy, which showed a significant ($p < 0.05$) and useful level of resistance to *Ggt* infection. In addition, Amagi Nijo and doubled haploid line D/334 showed only average resistance to *Ggt* when compared to other crop varieties (Table 6.1).

Table 6.1 Outdoor take-all bioassay *Ggt* infection visual scores from 1 (no infection) to 5 (plant is dead). Only the rye variety "Bevy" showed significantly less *Ggt* infection compared to the other crop varieties ($\alpha = 0.05$) (Wallwork, H. and Butt, M., SARDI). The score of each replicate block is a mean of 25 plants.

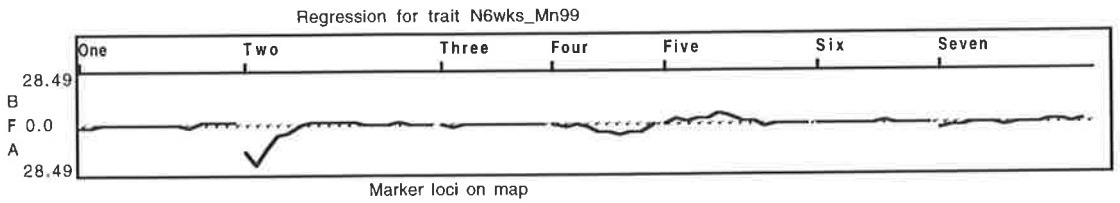
Crop	Variety	Rep 1	Rep 2	Rep 3	Rep 4	Mean	* $p < 0.05$
Barley DH	D/6	3.13	1.94	2.52	2.22	2.45	
Barley DH	D/5	2.90	2.00	2.41	2.25	2.39	
Barley parent	WI 2585	3.43	2.13	2.06	1.91	2.38	
Triticale	Tx93-57-1	2.95	2.50	1.89	1.96	2.33	
Triticale	Abacus	2.73	2.64	2.21	1.73	2.33	
Triticale	Tahara	2.46	2.81	1.65	1.59	2.13	
Triticale	T-type (Abacus sib)	2.17	2.71	2.17	1.43	2.12	
Barley parent	Amagi Nijo	2.65	1.74	1.90	2.00	2.07	
Triticale	Tx93-19-2	2.79	1.91	1.74	1.80	2.06	
Triticale	Treat (Hx86174)	2.37	2.14	1.77	1.87	2.04	
Barley DH	D/334	2.45	1.71	2.00	1.75	1.98	
Triticale	Credit	1.89	2.37	1.59	1.60	1.86	
Triticale	Venus	1.47	1.60	1.17	2.06	1.57	
Rye	Bevy	0.86	0.62	0.47	0.44	0.60	*

6.3.5 QTL analysis of 1999 Marion Bay field trial

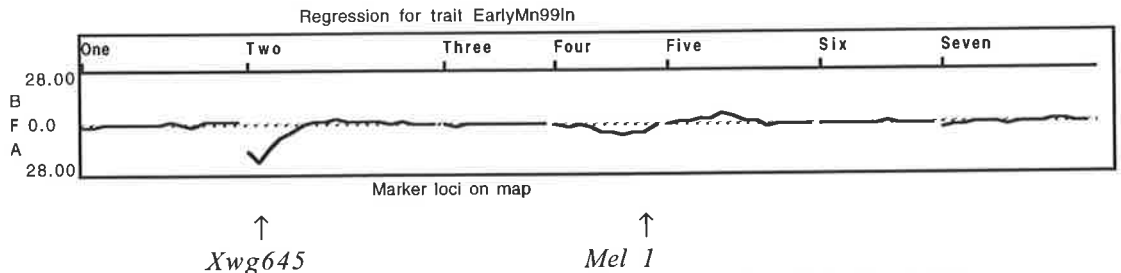
Linkage analysis by Map Manager QT Version b16 of the YEB shoot Mn concentration 6 weeks after sowing from the nil Mn plots, + Mn plots and the relative shoot Mn concentration for the 62 DH lines showed that these traits are significantly linked to RFLP marker loci *Xwg645* (2HL) (LOD = 5.17, LOD = 5.15 and LOD = 5.09 respectively) (Fig. 6.11). RFLP marker loci *Xwg645* (2HL) is not known to be linked with any other morphological or agronomic trait. In contrast, the locus *Xmwg77*, which shows linkage to *Mel 1* in controlled-environment bioassays, showed no significant linkage (LOD < 1.75) to shoot Mn concentration 6 weeks after sowing in the 1999 field trial.



A) Shoot Mn concentration 6 weeks after sowing in nil Mn plots: *Xwg645* (2HL) (LOD = 5.17).



B) Shoot Mn concentration 6 weeks after sowing in + Mn plots: *Xwg645* (2HL) (LOD = 5.15).



C) Relative shoot Mn concentration 6 weeks after sowing : *Xwg645* (2HL) (LOD = 5.09).

Fig. 6.11 Shoot Mn concentration 6 weeks after sowing of 62 DH lines grown at Marion Bay from the data in nil Mn plots (Panel A), + Mn plots (Panel B) and the relative shoot Mn concentration (Panel C). The data for the 62 DH lines showed that all these traits were significantly linked to RFLP marker *Xwg645* (LOD = 5.17, LOD = 5.15 and LOD = 5.09 respectively).

Twelve weeks after sowing (Fig. 6.12), further linkage analysis by Map Manager QT of the YEB shoot Mn concentration on the same day from the nil Mn plots of the 62 DH

+ Mn Nil Mn

WI 2585

+ Mn Nil Mn

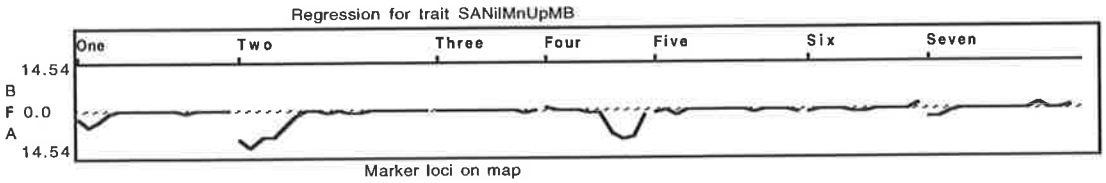
Amagi Nijo

Fig. 6.12 Marion Bay field trial photographed 12 weeks after sowing:- Mn-inefficient WI 2585 showing 21% efficiency, and Mn-efficient Amagi Nijo showing 86% efficiency.

lines showed no significant linkage to the *Mel 1*-linked RFLP *Xabg714(A)* (4HS) (LOD = 2.44), and to RFLP *Xwg645* (2HL) (LOD = 2.91) (Fig. 6.13 A). LOD < 3.2 is less than significant. Given the limited number of DH entries tested, this result suggests the presence of a putative second locus near *Xwg645* on 2HL controlling shoot Mn concentration. The maturity scores mapped to an independent locus near *Xcdo504* on 5HL (LOD = 5.45). Therefore, the shoot Mn concentrations in the DH lines were not influenced by maturity.

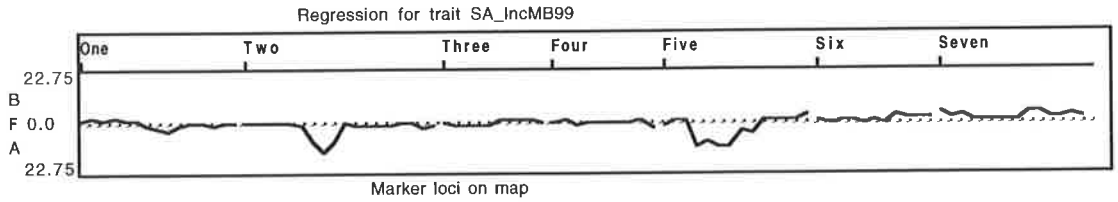
The 62 DH lines (Fig. 6.14) are shown grouped in terms of presence or absence of Mn-efficient allele combinations at *Mel 1* and *Xwg645*. Like the Amagi Nijo parent, the group *Mel 1* and *Xwg645* have the efficient alleles at each of *Mel 1* and *Xwg645*. The *Mel 1* group has the efficient allele at *Mel 1*, and the inefficient allele at *Xwg645*. The *Xwg645* group has the efficient allele at *Xwg645*, but the inefficient allele at *Mel 1*. The "neither" group, like the WI 2585 parent, has the inefficient allele at both *Mel 1* and *Xwg645* (Fig. 6.14).

The presence of both efficient alleles resulted in a significantly greater shoot Mn concentration than in the other groups ($p = 1.7 \times 10^{-9}$) (Fig. 6.14, Appendix 2, 6.2.1). The contribution of each efficient allele at both *Mel 1* and *Xwg645* to YEB shoot Mn concentration in DH lines grown in the nil Mn plots was nearly equal. The shoot Mn concentration in Amagi Nijo was greater than in the DH group with both efficient alleles (Fig. 6.14). This result suggests that more positive or negative loci for shoot Mn concentration may exist in Amagi Nijo or in WI 2585 progeny in the efficient allele groups of the DH lines which were not detected in the DH entries (n = 62) in this field experiment.



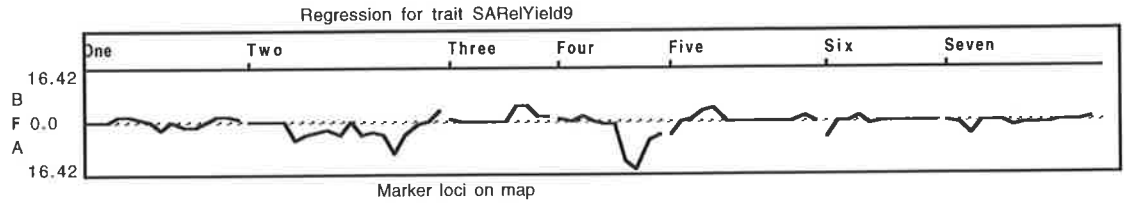
Xwg645 *Mel 1*

A) Shoot Mn concentration (mg/kg DM) after 12 weeks grown in nil Mn plots: *Xwg645* (2HL) (LOD = 2.91), *Xabg714 (A)* (*Mel 1*) (4HS) (LOD = 2.44).



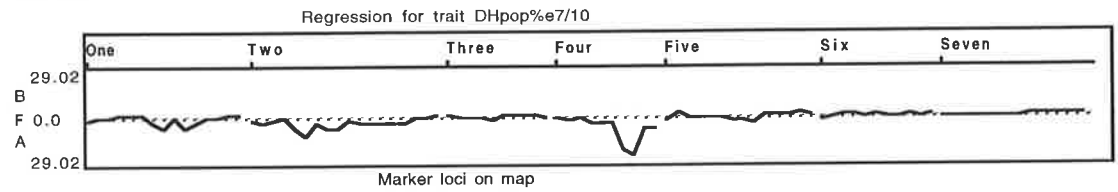
Xwg645 *Xcdo370* *Mel 1* *Xbcd265* *Xmwg514*

B) Relative shoot Mn concentration 12 weeks after sowing: *Xcdo370* (2HS) (LOD = 3.76), *Xbcd265* (5HS) (LOD = 4.15) and *Xmwg514* (5HS) (LOD = 4.09)



Xwg645 *Mel 1*

C) Relative grain yield (g) marker for *Mel 1*, *Xabg714 (A)* (4HS) (LOD = 3.24).



Xwg645 *Mel 1*

D) Visual scores 12 weeks after sowing of Mn efficiency (%) (section 6.2.6):*Mel 1* (4HS) (LOD = 4.76).

Fig. 6.13 Data at 12 weeks after sowing. **A)** Shoot Mn concentration mg/kg DM) of nil Mn plots (n = 3) in 62 DH entries in the 1999 field trial shows no significant linkage to *Mel 1* (4HS) and to RFLP *Xwg645* (2HL) (both LOD < 3.2). **B)** Relative shoot Mn concentration is significantly linked to the loci: *Xcdo370* (2HS), *Xbcd265* (5HS) and *Xmwg514* (5HS). **C)** Relative grain yield (g/plot, n = 3) shows significant linkage with *Mel 1* marker *Xabg714 (A)* (4HS). **D)** Visual assessment of Mn efficiency (%) shows a significant linkage to *Mel 1*.

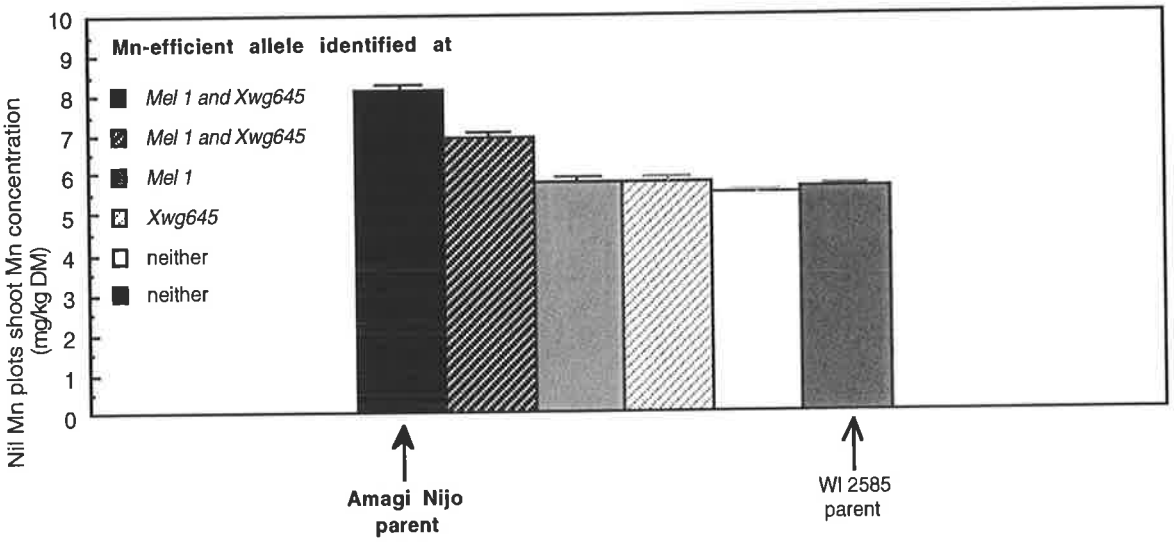


Fig. 6.14 Allele combinations in 62 DH lines for *Mel 1* and *Xwg645*. By 1-way ANOVA, the presence of the efficient allele at both *Mel 1* and *Xwg645* show significant linkage to YEB shoot Mn concentration in the nil Mn plots 12 weeks after sowing at Marion Bay. Values are the means shoot Mn concentration from the nil Mn plots (n = 3) of the allele groups. The vertical bars show the SE.

6.3.6 Allele combinations analysis of Av.LSL in DH *Ggt* experiment

The loci mapped for shoot Mn (mg/kg DM) in the Marion Bay 1999 nil Mn plots on 4HS and 2HL (Fig. 6.11, Fig. 6. 13 A and Fig. 6.14) showed no linkage with the Av.LSL between the four allele group combinations ($p = 0.24$) (Fig. 6.15, Appendix 2, 6.3.1), or between allele groups 1 and 4 ($p = 0.10$) (Fig. 6.15, Appendix 2, 6.3.2).

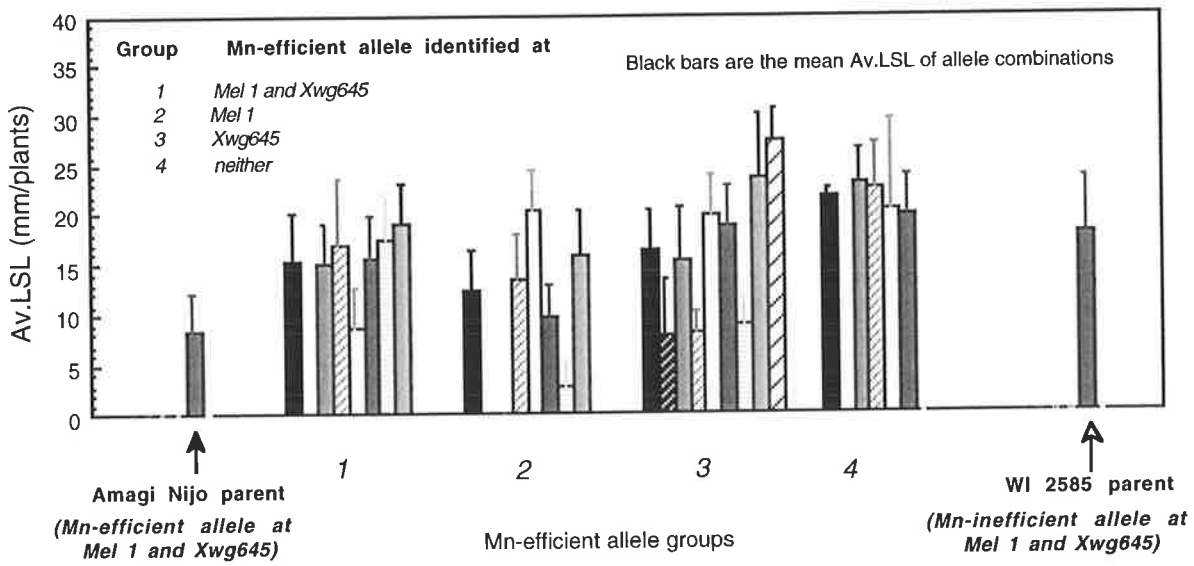


Fig. 6.15 Allele combinations in DH lines for *Mel 1* and *Xwg645* by 1-way ANOVA show no significant association with a low Av.LSL (mm/plant n = 6 plants) when both efficient alleles at *Mel 1* and *Xwg645* are present, only one is present at either locus, or both inefficient alleles are present. Values are the means Av.LSL of the DH lines in the appropriate allele groups. The vertical bars show the SE.

6.4 Discussion

The field trial results provided data suggesting the presence of a second locus linked to *Xwg645* on 2HL associated with Mn efficiency in addition to *Mel 1*. Neither *Mel 1* nor the new locus controlling shoot Mn concentration were associated with resistance to *Ggt*. However, the field trial results provided evidence that there are still further loci influencing shoot Mn concentration in this DH mapping population that would account for the variability in shoot Mn concentration not explained by *Mel 1* and *Xwg645* (Fig. 6.14). These loci need to be investigated and tested for association with resistance to *Ggt*. The use of further DH lines in the field experiments may permit detection and mapping of these additional loci. A more comprehensive partial map of this DH population (Fig. 6.16) would be an important prerequisite for a more detailed genetic study.

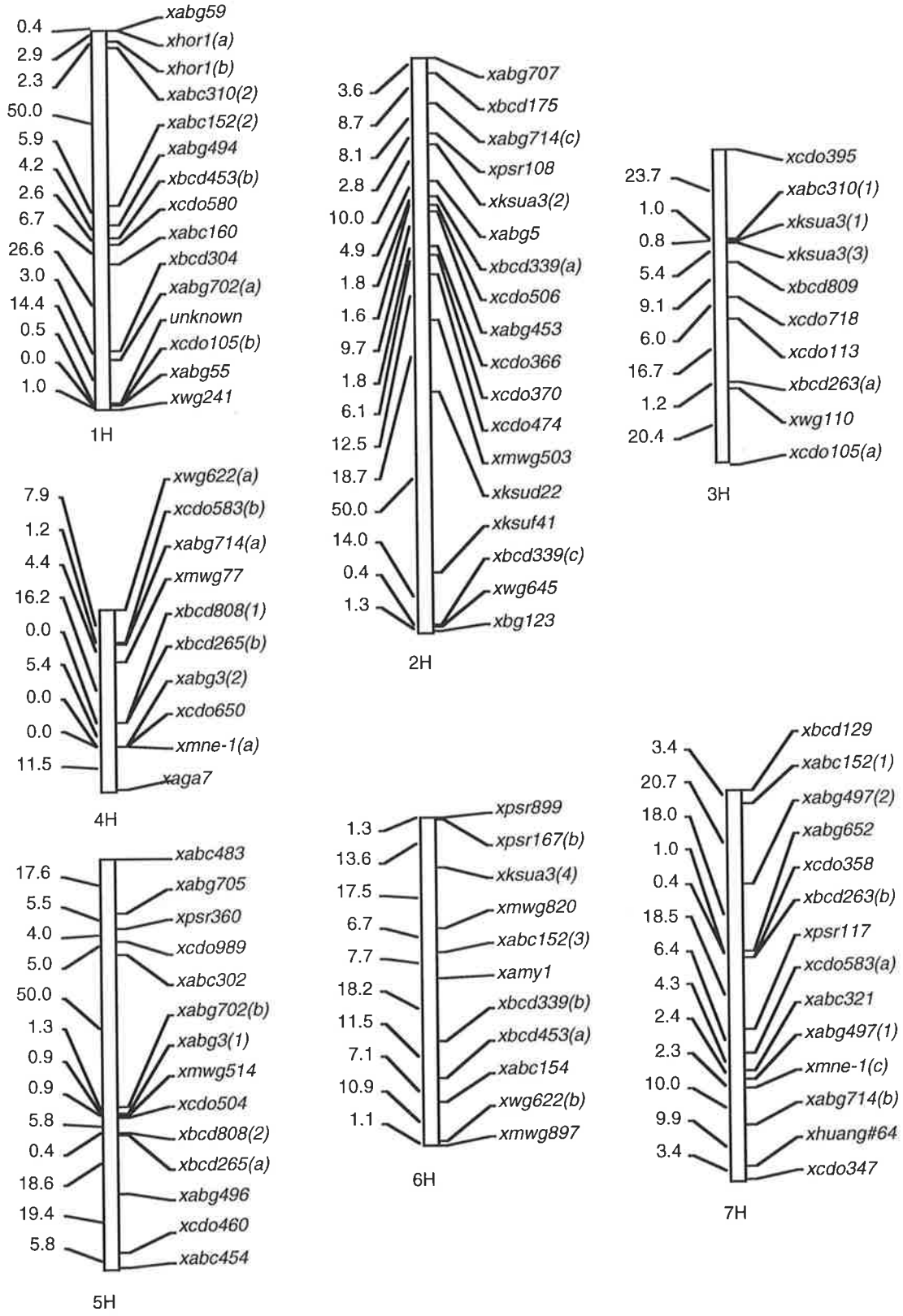


Fig. 6.16 RFLP partial map of WI 2585 x Amagi Nijo DH population using RFLP markers to identify 92 polymorphic loci between WI 2585 and Amagi Nijo on DNA extracted from 140 DH lines of this cross (constructed by McCasland-Pexton, A. and Pallotta, M.).

6.4.1 RFLP partial map of 140 DH lines

The partial map was constructed by McCasland-Pexton, A. and Pallotta, M., using the 140 DH lines screened in the 1999 Marion Bay field trial (Fig. 6.16). The RFLP markers were used to identify 92 polymorphic loci between parents Mn-inefficient WI 2585 and Mn-efficient Amagi Nijo shown photographed on the same day 12 weeks after sowing (Fig. 6.12). The DNA clones were selected to ensure full genome coverage and maximise the probability of detection of linkage to the Mn efficiency trait (Darvasi and Soller, 1994). The aim was to identify markers at a maximum of 30 cM spacing based on a consensus linkage map (Heun *et al.,* 1991, Lui and Tsunewaki, 1991, Gale *et al.,* 1995 and Langridge *et al.,* 1995). Most of these 92 RFLP marker loci (Appendix 3, 3.2) were ordered at an exclusion LOD of > 3.0.

There were few sustained deviations from the expected segregation ratios over adjacent marker loci. However, there were regions of the genome where gaps between markers of more than 50 cM were found (Fig. 6.16). The addition of more markers to this partial map particularly in the regions where these large gaps occur, may allow the identification of further Mn loci in addition to *Mel 1* and *Xwg645*.

6.4.2 Loci contributing to Mn efficiency and linkage with resistance to *Ggt*

If it were possible to identify further loci that significantly influence Mn efficiency, analysis of DH lines infected with *Ggt* may yet reveal that Mn-efficient alleles are predictive of a short Av.LSL and greater resistance to *Ggt*. In addition, increasing the shoot Mn concentrations from those obtained, (7.3-20.6 mg Mn/kg DM), may give results that show linkage of Mn-efficient alleles with resistance to *Ggt*. However, two loci controlling Mn efficiency have now been identified and mapped. Together, these loci account for the major portion of the genetic control of Mn efficiency. For further analysis of plants with a higher shoot Mn concentration infected with *Ggt*, an experimental method needs to be developed that produces data that is as free as possible of the confounding factor of a healthy plant vigour increasing resistance to pathogens rather than the expression of Mn-efficient alleles. If loci linked to resistance to *Ggt* are to be mapped, *Ggt* infection data for at least 60 DH lines with similar shoot critical Mn concentration, and similar shoot and root size and growth habit, would be necessary.

Chapter 7 Discussion

7.1 Introduction

Cook (1994) reported that there are no known wheat genotypes which are resistant to *Ggt*. Wheat genotypes with increased Mn efficiency when grown in soil deficient in plant available Mn were found to have less *Ggt* infection compared to Mn-inefficient genotypes (Wilhelm *et al.,* 1990). This result suggested the hypothesis that Mn efficiency in wheat increases resistance to *Ggt* under Mn-deficient conditions. Barley is a diploid crop species in which traits such as Mn efficiency are more simply inherited than in hexaploid wheat. While *Ggt* is of less significance to the barley crop than to wheat, barley was used in physiological and genetic studies to investigate if Mn efficiency in barley increases resistance to *Ggt*. There was no genetic linkage between enhanced resistance to *Ggt* in barley and the presence of manganese efficiency alleles at two loci on chromosomes 4HS and 2HL.

7.1.1 The physiological and genetic experiments

When grown in Mn deficient soil, the Mn-efficient Amagi Nijo was found to have a reduced Av.LSL compared to the Mn in-efficient WI 2585. But the susceptibility of WI 2585 to *Ggt* was not reduced by the elimination of Mn stress. Further, Amagi Nijo showed a Mn-independent resistance to *Ggt*. An investigation of the genetics of Mn efficiency in a DH population generated from a cross between these two parent genotypes found no linkage between Av.LSL and the known locus for Mn efficiency, *Mel 1* located on chromosome 4HS. Finally, a field experiment using this DH population suggested the presence of another locus controlling shoot Mn concentration on chromosome 2HL linked to RFLP marker *Xwg645*, that when taken together with *Mel 1*, also showed no linkage with Av.LSL and resistance to *Ggt*. Therefore, using both physiological and genetic studies, this research has found no connection between resistance to *Ggt* and the expression of Mn efficiency linked to two loci on 4HS and 2HL when barley is grown within the range of shoot Mn concentrations (7.3-20.6

mg/kg DM) studied. Loci associated with resistance to *Ggt* may or may not be Mn-independent. However, *Ggt* infection data on at least 60 DH lines, all with similar shoot critical Mn concentration, and similar shoot and root size and growth habit, would be necessary for a valid result.

7.2 Future research directions

Wheat, being generally more susceptible to Mn deficiency than barley (Graham *et al.*, 1983; Marcar, 1986), could be used in further pot bioassays and field trials with *Ggt* infection to check both the physiological and genetic results obtained with barley. Wheat genotypes should be chosen in which molecular markers have been identified for the trait of Mn efficiency. The shoot Mn concentrations investigated in the pot bioassays could be increased from between 7 to 50 mg Mn/kg DM. However, experiments such as these involving high levels of Mn added to the soil, need to determine that Mn efficiency is still expressed at these high Mn soil additions. If high shoot Mn concentrations are obtained, an experimental method needs to be developed that eliminates the confounding factor of enhanced plant vigour increasing resistance to *Ggt*, rather than the expression of Mn-efficient alleles. In addition, the experiments with wheat should investigate if high root Mn concentration increases the Av.LSL the same as it may have in barley (see section 4.4.1). If both physiological and genetic studies in *Ggt* infected Mn-inefficient and Mn-efficient wheat genotypes also show no association between Mn efficiency alleles and resistance to *Ggt*, other alternatives are available to minimise the crop losses resulting from *Ggt* infection.

7.2.1 Field solutions

In a field trial at a site not regarded as Mn deficient, the addition of 7.2 kg/ha Mn fertiliser compared to 0 Mn added resulted in the *Ggt* infected wheat having an increased yield over nil Mn, an effect not shown in the absence of *Ggt* (Webb and Graham, 1990). The tissue Mn concentrations for plants in all treatments ranged from 30-37 mg/kg DM, which is supra-optimal in Mn compared to the critical Mn concentration determined previously (Reuter,

1986). The critical Mn concentration in *Ggt* infected wheat plants may be higher in wheat plants not infected with *Ggt*. This result suggests that if supra-optimal levels of Mn fertiliser can be added to crops the loss in yield due to infection by *Ggt* may be minimised.

7.2.2 Plant-pathogen interactions

Investigations of plant-pathogen interactions to date provide no clue as the nature of the inhibition of growth of *Ggt* within the roots of Amagi Nijo observed in the temporal study (chapter 4). Other research has revealed the release of an linoleate 8-dioxygenase protein of unknown biological function from *Gaeumannomyces graminis graminis* (Su and Oliw, 1996), the release of a compound of unknown biological function released by *Gaeumannomyces graminis* var. *tritici* upon exposure to a plant defence phenolic substrate (Thornton *et al.*, 1997), and of the release of another protein similar to a plant chemical defence enzyme lipoxygenase that contains Mn, "Mn-LO", which also has an unknown biological function (Su and Oliw, 1998). The ambiguity of the source and biological function of the compounds released in these plant-pathogen interactions in this research provides no justifiable research direction for investigation into the apparent inhibition of the growth of *Ggt* within the root of Amagi Nijo (chapter 4). Hence, there is no known starting clue to the nature of the hypothesised biochemical mechanism in Amagi Nijo which inhibits the growth of *Ggt* through the stele of the root after 21 days post inoculation with *Ggt*.

7.2.3 Transformation of plant biochemical defence mechanisms

Other avenues of research may have potential for greater success. Avenacin A-1, which is biosynthesised constitutively in the epidermal cells in the roots of oat plants is toxic to *Ggt*. Thus, oats are resistant to *Ggt* (Osbourn *et al.*, 1994). Avenacin is present in the roots of barley and wheat in only minute amounts, so *Ggt* is able to invade the roots (Bowyer *et al.*, 1995). Isolates of *Gaeumannomyces graminis* var. *avenae* (*Gga*) produce an enzyme, avenacinase, which deglucosylates avenacin to a less toxic form, which enables *Gga* to invade

the roots of oats (Osbourn *et al.,* 1991; Bowyer *et al.,* 1995). However, isolates of *Ggt* have a hundred times less avenacinase compared to isolates of *Gga* (Osbourn *et al.*, 1991).

The transformation into barley and wheat of the gene in oats which encodes for the biosynthesis of avenacin A-1 has the potential to result in total resistance to *Ggt*. In addition, plate tests where high concentrations of avenacin A-1 have been included in the agar have inhibited the growth of isolates of *Gga* (Osbourn, *et al.*, 1991). Hence, the biosynthesis in the roots of barley and wheat of avenacin A-1 could give these crops total resistance to *Ggt*, and moderate resistance to *Gga*.

Future work will refine methods for the production of doubled haploids from a cross between diploid oat species. An outdoor screen with *Ggt* could be done on a doubled haploid population derived from a cross between a diploid oat species, *Avena longiglumis*, which lacks avenacin, and so is susceptible to *Ggt* infection, and an avenacin A-1 constitutively synthesising diploid oat species such as *Avena strigosa,* which is resistant to *Ggt*.

The infection data could be used to select DH lines for a bulked segregant analysis on DNA extracted from high *Ggt* infection or little to no *Ggt* infection DH lines. AFLP primer pairs may detect AFLP polymorphic bands segregating for resistance to *Ggt* (and moderate resistance to *Gga*). The AFLP polymorphic band, which is likely to include the DNA sequence for the biosynthesis of avenacin A-1, could be cut from the gel, cloned and sequenced. Then, the sequence containing the gene for biosynthesis of avenacin A-1 may be transformed into wheat and barley, and tested for the biosynthesis of avenacin A-1 giving wheat and barley which is resistant to *Ggt* and has moderate resistance to *Gga*.

The future use of the modern biotechnology strategies, such as transformation of the avenacin A-1 gene into barley and wheat cultivars, has the potential to give more resistance to *Ggt* than the use of molecular makers for the Mn efficiency loci investigated in this thesis. However, the pyramiding of Mn-efficient alleles at *Mel 1* and *Xwg645* on chromosomes 4HS

and 2HL respectively into elite barley and wheat cultivars using breeding strategies now in use may give greater yields and additional economic benefit to the Australian cereal industry.

Appendix 1 Seed Mn concentration

1.1 Seed Mn concentration

The Mn concentration of seeds was determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) (Zarcinas *et al.*, 1987) (see section 2.2.2).

Table 2.1 WI 2585 and Amagi Nijo

Table 2.1 The seed Mn concentration (mg Mn/kg DM) of each of the parent genotypes chosen for use in all experiments.

Experiment	Seed	Harvested	Seed Mn (mg/kg DM)
1) First passage through host plants	WI 2585 B11-2-1-33	November 1995	18.2
	Amagi Nijo B7-3x3	December 1994	18.1
2) Increased *Ggt* pathogenicity	WI 2585 B11-2-1-33	November 1995	18.25
	Amagi Nijo B7-3-2-33	November 1995	19.7
3) Confirmation of sample size	WI 2585 B11-2-1-33	November 1995	18.25
	Amagi Nijo B7-3-3-2-33	November 1995	19.7
4) Critical shoot Mn concentration	WI 2585 B11-2-1-31	November 1995	24.8
	Amagi Nijo B7-3x4	December 1994	20.8
5) *Ggt* experiment	WI 2585 B11-2-1-32	November 1995	17.4
	Amagi Nijo B7-3x3	December 1994	18.1
6) Temporal study	Progeny of WI 2585 B11-2-1-33	January 1997	11.8
	Progeny of Amagi Nijo B7-3x3	January 1997	10.8

Table 2.2 DH lines

Table 2.2 Seed Mn concentration (mg/kg DM) of control parent plants and doubled haploid lines.

Doubled Haploid *Mel 1* **efficient allele**	**Seed Mn (mg/kg DM)**	**Doubled Haploid** *Mel 1* **inefficient allele**	**Seed Mn (mg/kg DM)**
D/2	24.0	D/4	23.7
D/3	24.5	D/5	25.6
D/7	26.3	D/6	26.0
D/25	25.0	D/9	25.7
D/32	26.0	D/256	24.5
D/49	-	D/327	26.7
D/269	27.4	D/460	22.7
D/273	21.1	D/463	27.0
D/320	18.7	D/467	24.0
D/334	26.6	D/653	32.0
D/678	25.0	D/701	25.0
D/791	27.0	D/799	31.0
D/819	24.0	D/888	26.0
D/843	34.0	D/892	26
D/862	35		
Amagi Nijo B7-3x5	25.8	WI 2585 B11-2-1-33	24.8

Appendix 2 Statistical analysis tables for 1-way and 2-way ANOVAs

Chapter 2 Pilot Studies

2.2 Experiment 2: Increased *Ggt* pathogenicity

2.2.3 Fig. 2.3 Av.LSL (mm)

ANOVA Av.LSL

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	605.07243	1	605.07243	4.083504372	0.05689621	4.351250027
Mn addition	309.5484533	4	77.38711333	0.522269071	0.720462925	2.866080706
Interaction	1292.71872	4	323.17968	2.181070514	0.108116942	2.866080706
Within	2963.495933	20	148.1747967			
Total	5170.835537	29				

2.2.4 Fig. 2.4 PIR

ANOVA PIR

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	76.16133333	1	76.16133333	0.377169495	0.546045231	4.351250027
Mn addition	1086.642	4	271.6605	1.345329044	0.287964749	2.866080706
Interaction	1911.702	4	477.9255	2.366803624	0.08739358	2.866080706
Within	4038.573333	20	201.9286667			
Total	7113.078667	29				

2.3 Experiment 4: Confirmation of an adequate sample size

2.3.1 Fig. 2.5 Shoot Mn concentration (mg/kg DM)

ANOVA Shoot Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Nil Ggt/Ggt	0.008008333	1	0.008008333	0.011533537	0.914964379	4.061703862
Genotype	122.88	1	122.88	176.9707854	5.06101E-17	4.061703862
Interaction	0.210675	1	0.210675	0.303412437	0.584537174	4.061703862
Within	30.55148333	44	0.694351894			
Total	153.6501667	47				

2.3.2 Table 2.3 Shoot Mn content (µg/pot)

ANOVA Mn content

Source of Variation	SS	df	MS	F	P-value	F crit
Nil Ggt/Ggt	0.48120075	1	0.48120075	1.25200342	0.26924169	4.06170386
Genotype	70.0495041	1	70.0495041	182.257028	3.0003E-17	4.06170386
Interaction	0.16732408	1	0.16732408	0.43534912	0.51281496	4.06170386
Within	16.9111623	44	0.3843446			
Total	87.6091913	47				

2.3.3 Table 2.4 Shoot DM (g/pot)

ANOVA Shoot DM

Source of Variation	SS	df	MS	F	P-value	F crit
Nil Ggt/Ggt	0.00684019	1	0.00684019	2.00041043	0.16429424	4.06170386
Genotype	0.15743752	1	0.15743752	46.0425475	2.3598E-08	4.06170386
Interaction	0.00024752	1	0.00024752	0.07238738	0.78914959	4.06170386
Within	0.15045325	44	0.00341939			
Total	0.31497848	47				

2.3.4 Table 2.4 Root DM (g/pot)

ANOVA Root DM

Source of Variation	SS	df	MS	F	P-value	F crit
Nil Ggt/Ggt	0.01038408	1	0.01038408	4.2624551	0.05488863	4.06170386
Genotype	0.27210408	1	0.27210408	111.693194	1.181E-13	4.06170386
Interaction	0.00232408	1	0.00232408	0.95398896	0.33404576	4.06170386
Within	0.10719167	44	0.00243617			
Total	0.39200392	47				

Chapter 3 Critical shoot Mn concentration experiment

3.1 Fig. 3.3 A Shoot DM (g/pot)

ANOVA Shoot DM

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	0.39389018	1	0.39389018	89.6221119	2.8389E-14	3.97389499
Mn addition	1.29824482	8	0.1622806	36.9238208	1.602E-22	2.06983231
Interaction	0.14790642	8	0.0184883	4.20665666	0.00035608	2.06983231
Within	0.3164408	72	0.00439501			
Total	2.15648222	89				

3.2 Fig. 3.3 B Root DM (g/pot)

ANOVA Root DM

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	0.4774225	1	0.4774225	150.288559	2.685E-19	3.97389499
Mn addition	0.50506056	8	0.06313257	19.8735981	1.8406E-15	2.06983231
Interaction	0.0749958	8	0.00937448	2.95100532	0.00653705	2.06983231
Within	0.2287228	72	0.00317671			
Total	1.28620166	89				

3.3 Fig. 3.4 Shoot Mn concentration (mg/kg DM)

ANOVA Shoot Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	733.706454	1	733.706454	613.747181	5.7178E-37	3.97389499
Mn addition	3984.56902	8	498.071128	416.637674	4.3763E-57	2.06983231
Interaction	48.3109156	8	6.03886444	5.05152436	5.3287E-05	2.06983231
Within	86.07268	72	1.19545389			
Total	4852.65907	89				

3.4 Fig. 3.6 Shoot Mn content (μg/pot)

ANOVA Shoot Mn content

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	2600.74859	8	325.093574	367.444331	3.6119E-55	2.06983231
Genotype	538.754532	1	538.754532	608.939439	7.369E-37	3.97389499
Interaction	37.2274358	8	4.65342947	5.25964343	3.37E-05	2.06983231
Within	63.7014518	72	0.88474239			
Total	3240.43201	89				

3.5 Fig. 3.7 Root Mn concentration (mg/kg DM)

ANOVA Root Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Genotype	1.9580625	1	1.9580625	0.04989232	0.82388328	3.97389499
Mn addition	31082.8405	8	3885.35506	99.0006052	1.0003E-35	2.06983231
Interaction	151.6615	8	18.9576875	0.48305046	0.86438459	2.06983231
Within	2825.6955	72	39.2457708			
Total	34062.1556	89				

Chapter 4 Physiological Studies

4.1 The *Ggt* experiment

4.1.1 Fig. 4.1 Nil *Ggt* shoot Mn concentration (mg/kg DM)

ANOVA Nil Ggt shoot Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Mn supply	147.460837	1	147.460837	40.6130213	3.2234E-06	4.35125003
Genotype	28.1883375	1	28.1883375	7.76350908	0.01139676	4.35125003
Interaction	4.74370417	1	4.74370417	1.30649033	0.26652957	4.35125003
Within	72.6175167	20	3.63087583			
Total	253.010396	23				

4.1.2 Fig. 4.2 *Ggt* infected shoot Mn concentration (mg/kg DM)

ANOVA Ggt shoot Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Mn supply	194.729633	1	194.729633	66.6051297	2.37E-10	4.06170386
Genotype	0.1323	1	0.1323	0.04525176	0.83252426	4.06170386
Interaction	3.92163333	1	3.92163333	1.34135156	0.25304368	4.06170386
Within	128.6403	44	2.92364318			
Total	327.423867	47				

4.1.3 Fig. 4.3 Av.LSL (mm/plant)

ANOVA Av.LSL. (mm/plant)

Source of Variation	SS	df	MS	F	P-value	F crit
Mn supply	179.800208	1	179.800208	1.19976822	0.27932758	4.06170386
Genotype	946.963333	1	946.963333	6.31888319	0.01567977	4.06170386
Interaction	19.3802083	1	19.3802083	0.12931997	0.72085842	4.06170386
Within	6593.94792	44	149.862453			
Total	7740.09167	47				

4.1.4 Fig. 4.4 PIR

ANOVA PIR

Source of Variation	SS	df	MS	F	P-value	F crit
Mn supply	133.333333	1	133.333333	0.44568809	0.50787636	4.06170386
Genotype	0.08333333	1	0.08333333	0.00027856	0.9867594	4.06170386
Interaction	660.083333	1	660.083333	2.20643462	0.14456609	4.06170386
Within	13163.1667	44	299.162879			
Total	13956.6667	47				

4.1.5 Fig. 4.5 Nil *Ggt* root Mn concentration (mg/kg DM)

ANOVA Nil Ggt root Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	50023.27042	1	50023.2704	4.05327573	0.05774113	4.35125003
Genotype	67172.42042	1	67172.4204	5.44283369	0.03020415	4.35125003
Interaction	14716.35375	1	14716.3538	1.19243382	0.28781944	4.35125003
Within	246828.855	20	12341.4428			
Total	378740.8996	23				

4.1.6 Fig. 4.6 *Ggt* infected root Mn concentration (mg/kg DM)

ANOVA Ggt root Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	5125.333333	1	5125.33333	2.1580577	0.14893781	4.06170386
Genotype	5448.540833	1	5448.54083	2.2941465	0.13701384	4.06170386
Interaction	833.3333333	1	833.333333	0.35088087	0.55664694	4.06170386
Within	104498.905	44	2374.97511			
Total	115906.1125	47				

4.1.7 Table 4.1 Nil *Ggt* shoot DM (g/pot)

ANOVA Nil Ggt shoot DM

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	0.00100881	1	0.00100881	0.74751494	0.39751586	4.35125003
Genotype	0.0061504	1	0.0061504	4.55738178	0.04533778	4.35125003
Interaction	7.0727E-05	1	7.0727E-05	0.0524077	0.8212501	4.35125003
Within	0.02699094	20	0.00134955			
Total	0.03422088	23				

4.1.8 Table 4.1 Nil *Ggt* root DM (g/pot)

ANOVA Nil Ggt root Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	0.00156817	1	0.00156817	2.85996535	0.10633459	4.35125003
Genotype	0.00209067	1	0.00209067	3.81288185	0.06499816	4.35125003
Interaction	0.00040017	1	0.00040017	0.72980942	0.40306212	4.35125003
Within	0.01096633	20	0.00054832			
Total	0.01502533	23				

4.1.9 Table 4.2 *Ggt* infected shoot DM (g/pot)

ANOVA Ggt shoot DM

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	0.00703252	1	0.00703252	9.27989808	0.00390677	4.06170386
Genotype	0.00266114	1	0.00266114	3.51155955	0.06759224	4.06170386
Interaction	0.000867	1	0.000867	1.14406652	0.29062749	4.06170386
Within	0.03334422	44	0.00075782			
Total	0.04390488	47				

4.1.10 Table 4.2 *Ggt* infected root DM (g/pot)

ANOVA Ggt root DM

Source of Variation	SS	df	MS	F	P-value	F crit
Mn addition	0.00020419	1	0.00020419	4.21581355	0.04602309	4.06170386
Genotype	2.8521E-05	1	2.8521E-05	0.58886325	0.44695747	4.06170386
Interaction	2.2687E-05	1	2.2687E-05	0.46842373	0.49730257	4.06170386
Within	0.00213108	44	4.8434E-05			
Total	0.00238648	47				

4.2 The temporal study

4.2.1 Fig. 4.7 Shoot Mn concentration (mg/kg DM)

ANOVA Shoot Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Harvest time	106.8227865	3	35.6075955	10.7812471	4.2046E-06	2.708191
Genotype	215.250651	1	215.250651	65.1734674	3.2877E-12	3.94932442
Interaction	17.13070312	3	5.71023437	1.72894145	0.16687922	2.708191
Within	290.640625	88	3.30273438			
Total	629.8447656	95				

4.2.2 Fig. 4.8 Shoot Mn content (μg/pot)

ANOVA Shoot Mn content

Source of Variation	SS	df	MS	F	P-value	F crit
Harvest time	5.298944615	3	1.76631487	52.2049788	1.7689E-19	2.708191
Genotype	1.13513251	1	1.13513251	33.5498328	1.0573E-07	3.94932442
Interaction	0.507930281	3	0.16931009	5.00410771	0.00299022	2.708191
Within	2.977411583	88	0.03383422			
Total	9.91941899	95				

4.2.3 Fig. 4.9 Root Mn concentration (mg/kg DM)

ANOVA Root Mn

Source of Variation	SS	df	MS	F	P-value	F crit
Harvest time	3155.48115	3	1051.827	39.2724366	3.34437E-16	2.708191
Genotype	144.305104	1	144.3051	5.38797044	0.022587108	3.94932442
Interaction	89.6769792	3	29.892326	1.11610031	0.346989325	2.708191
Within	2356.88917	88	26.782831			
Total	5746.3524	95				

4.2.4 Fig. 4.10 Av.LSL (mm)

ANOVA Av.LSL.

Source of Variation	SS	df	MS	F	P-value	F crit
Harvest time	1092.799936	3	364.266645	14.2161805	1.2389E-07	2.708191
Genotype	412.2202594	1	412.220259	16.0876591	0.00012656	3.94932442
Interaction	540.4207615	3	180.140254	7.03030702	0.00027193	2.708191
Within	2254.857758	88	25.6233836			
Total	4300.298716	95				

4.2.5 Fig. 4.11 PIR

ANOVA PIR

Source of Variation	SS	df	MS	F	P-value	F crit
Harvest time	4669.011979	3	1556.33733	5.00649909	0.00298161	2.708191
Genotype	488.2526042	1	488.252604	1.57063393	0.21343463	3.94932442
Interaction	3143.280313	3	1047.7601	3.37048397	0.02203652	2.708191
Within	27355.97917	88	310.8634			
Total	35656.52406	95				

Chapter 6 Genetic Studies

6.1 The DH *Ggt* experiment

6.1.1 Fig. 6.6 Shoot Mn concentration (mg/kg DM)

ANOVA Shoot Mn concentration

Source of Variation	SS	df	MS	F	P-value	F crit
Mel 1 allele	264.274117	1	264.274117	74.5199434	1.6596E-09	4.18296509
DH lines	102.844273	29	3.54635423			
Total	367.118389	30				

6.1.2 Fig. 6.7 Shoot Mn content (μg/plant)

ANOVA Shoot Mn content

Source of Variation	SS	df	MS	F	P-value	F crit
Mel 1 allele	9.44205238	1	9.44205238	25.5817787	2.1599E-05	4.18296509
DH lines	10.7036935	29	0.36909288			
Total	20.1457459	30				

6.1.3 Fig. 6.8 Root Mn concentration (mg/kg DM)

ANOVA Root [Mn]

Source of Variation	SS	df	MS	F	P-value	F crit
Mel 1 allele	1.64484803	1	1.64484803	0.06658552	0.79819719	4.18296509
DH lines	716.380873	29	24.7027887			
Total	718.025721	30				

6.1.4 Fig. 6.9 Av.LSL (mm/6 plants)

ANOVA Av.LSL

Source of Variation	SS	df	MS	F	P-value	F crit
Mel 1 allele	31.9469759	1	31.9469759	0.69324792	0.41186649	4.18296509
DH lines	1336.40833	29	46.083046			
Total	1368.35531	30				

6.1.5 Fig. 6.10 PIR

ANOVA PIR

Source of Variation	SS	df	MS	F	P-value	F crit
Mel 1 allele	0.506385	1	0.506385	2.05523834	0.16237817	4.18296509
DH lines	7.145237	29	0.24638748			
Total	7.651622	30				

6.2 Marion Bay field trial

6.2.1 Fig. 6.14 Field trial shoot Mn concentration (mg/kg DM)

ANOVA	Shoot [Mn]	concentration	Mel 1 and	Xwg645	allele	combinations
			Ggt			
Source of Variation	SS	df	MS	F	P-value	F crit
combination	19.2319241	3	6.41064138	21.5680607	1.6561E-09	2.76355649
shoot [Mn]	17.2392505	58	0.29722846			
Total	36.4711746	61				

6.3 Linkage of *Mel 1* and *Xwg645* with resistance to *Ggt*

6.3.1 Fig. 6.15 Linkage of Mn-efficient alleles at *Mel 1* and *Xwg645* with Av.LSL (mm/6 plants) between the four allele group combinations

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	140.220244	3	46.7400813	1.51421639	0.24484461	3.15991144
Within Groups	555.615083	18	30.8675046			
Total	695.835327	21				

6.3.2 Fig. 6.15 Linkage of Mn-efficient alleles at *Mel 1* and *Xwg645* with Av.LSL (mm/6 plants) between group 1 and group 4

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	43.9470417	1	43.9470417	3.52446141	0.09730054	5.31764499
Within Groups	99.7532083	8	12.469151			
Total	143.70025	9				

Appendix 3 Materials and methods for molecular biology

3.1 Small scale genomic DNA extraction

The method used to extract DNA from leaves was a mini-prep modified from Rogowsky *et al.,* (1991). Two 10 cm healthy leaf blades were rolled and placed into a sterile 2-ml Eppendorf tube, and frozen in liquid nitrogen. The frozen sample was crushed to a powder in the tube. Then 600 µl DNA extraction buffer (1% sarkosyl 100mM Tris-HCl, 100mM NaCl, 10 mM EDTA, 2% polyvinyl-polypyrrolidone [insoluble] pH 8.5) was added and mixed with the leaf powder to form a slurry. Phenol extraction was done by adding 600 µl phenol:chloroform:isoamyl alcohol (25:24:1) equilibrated with 1 M Tris-HCl pH 8.0 into the slurry and mixing well. After placing at 4°C for 10-30 minutes, the samples were centrifuged at 12K rpm for 15 minutes at 4°C. The supernatant was collected into a fresh tube, and DNA was precipitated after the addition of 60 µl 3 M NaAc (pH to 4.8 with glacial acetic acid) and 600 µl isopropanol. After mixing and leaving at room temperature for 10 minutes, the DNA was pelleted by centrifugation at 12K rpm for 10 minutes at 4°C. The supernatant was gently decanted out of the tube, the remaining pellet of DNA was washed with 1 ml 70% ethanol, centrifugation for 5 minutes at room temperature at 12K rpm, and the supernatant discarded. The DNA pellet was air dried, resuspended and stored at -20°C in 50 µl R40 (40 µg/ml RNAse A (Boehringer Mannheim) in TE buffer [10 mM Tris-HCl, 1 mM EDTA pH 8.0]).

3.2 RFLP analysis and construction of a partial map

A set of RFLPs were found to identify 92 polymorphic loci between parents Mn-inefficient WI 2585 and Mn-efficient Amagi Nijo. The DNA clones were selected to ensure full genome coverage to maximise the probability of detection of linkage to the traits (Darvasi and Soller, 1994) and covered the barley genome at a maximum of 30 cM intervals based on a consensus linkage map for barley (Langridge *et al.,* 1995). Additional markers were selected from genetic maps for barley and wheat (Heun *et al.,* 1991, Lui and Tsunewaki, 1991 and Gale *et al.,* 1995). All DNA clones were obtained through the Australian Triticeae Mapping Initiative.

The RFLP data for 140 DH lines was entered into the mapping programme Map Manager QT Version b16 (Manly and Cudmore 1997). Associations between markers and the

QTL pot bioassay and field data were determined by single-point analysis ($p < 0.001$). A LOD score > 3.2 was taken as the threshold for detecting a QTL for the DH population (Lander and Botstein 1989). The QTL associations with the marker loci was identified using Q-gene (Nelson 1997).

3.2.1 Digestion of genomic DNA for RFLP analysis

Genomic DNA was digested in 1.5 ml Eppendorf tubes containing (3µg) DNA, 4 mM spermidine, 1 µg BSA (acetylated), 100 mM Tris-HCL, 0.5 M potassium acetate, 100 mM magnesium acetate, 10 mM spermidine, 10 mM DTT and 20 units of restriction endonucleases of either *Bam* HI, *Dra* 1, *Eco* RI, *Eco* RV, and *Hind* III (Boehringer Mannheim or Promega) in a total volume 10 µl sterile milli-Q water. Digestion was for 4 hours at 37°C.

3.2.2 Gel electrophoresis and Southern transfer

After digestion, 1 µl 10X Ficoll loading buffer (25% [w/v] ficoll 400, 0.42% [w/v] bromophenol blue, 0.42% [w/v] xylene cyanol FF, pH 8.0) was added to the samples before 11 µl of each sample was loaded onto a 1% agarose gel. Electrophoresis was overnight at 34 V in a tank containing 1X TAE running buffer (400 mM Tris-acetate, 1 mM EDTA pH 8.0 with glacial acetic acid). The DNA bands were visualised and photographed under UV light after staining the gel in ethidium bromide. DNA from the parents WI 2585 and Amagi Nijo were run on each gel as controls for the DNA samples from the DH lines.

Southern transfer was as described by Sambrook *et al.*,(1989), with the following modifications. The ethidium bromide was rinsed from the gel 3x with nanopure water. The gel was rinsed in 0.4M NaOH and placed DNA side up on a sheet of Whatman 3MM filter paper supported by a sponge lying in a reservoir of 0.4 M NaOH. A nylon membrane (Hybond N^+, Amersham), briefly soaked in 0.4 M NaOH was placed on top of the agarose gel. Another sheet of Whatman 3MM filter paper moist with 0.4 M NaOH, was placed on top of the membrane. Finally a 6 cm stack of dry paper towels gently secured in position with a glass plate, the Southern transfer was over 3-4 hours by capillary blotting. The membrane was sealed in a plastic bag for storage at 4°C.

3.2.3 Oligolabelling of RFLP DNA clones

Into a sterile 1.5 ml Eppendorf tube was placed 2 µl DNA (50 ng), 3 µl 9mer random primer mix (0.1 µg/µl), and 3 µl of sterile water. After boiling for 5 minutes and chilling on ice for 5 minutes 12.5 µl oligo buffer (60 µM each of dATP, dTTP, and dGTP (Amersham), and 150 mM Tris-HCl pH 7.6, 150 mM NaCl, 30 mM $MgCl_2$, and 300 µg/ml BSA), and 1 µl of Klenow enzyme (Boehringer Mannheim) and 4 µl [α-^{32}P]dCTP (10 µCi/µl, Amersham) was added. After incubation at 37°C for 1 hour, this mix was passed through a Sephadex G-100 mini-column saturated with TE buffer to separate the labelled probe from the unincorporated [α-^{32}P]dCTP. Then 500 µl of salmon sperm DNA (5 mg/ml) was added.

3.2.4 Hybridisation of DNA probes to membranes

Seven membranes were hybridised simultaneously. For the pre-hybridisation, the membranes were soaked in 5X SSC for 5 minutes, rolled up into a cylinder (DNA side on the inside) and placed into a hybridisation bottle (Hybaid). 7 ml of pre-hybridisation solution was added (0.5 ml water, 3 ml 5x HSB (3 M NaCl, 100 mM PIPES, 25 mM Na_2 EDTA, pH to 6.8 with 4 M NaOH), 3 ml Dextran sulphate (25% w/v), 3 ml Denhardt's III (2% BSA, 2% Ficoll 400, 2% Polyvinyl-pyrrolidone 360, and 10% SDS) and 500 µl denatured salmon sperm DNA (5 mg/ml). The pre-hybridisation step was done over 20 minutes rotation at 65°C in a hybridisation oven. The membranes were unwound by reversing the orientation of the bottle in the oven, and resuming the pre-hybridisation for 2-6 hours at 65°C.

The labelled probe was boiled for 5 minutes, chilled on ice for 5 minutes and 500 µl added to the hybridisation solution in the bottle. The probes were left to hybridise to the DNA on the membranes overnight at 65°C in a rotating hybridisation oven. The membranes were washed for 20 minutes in 2X SSC, 1% SDS in the hybridisation bottle, then removed from the hybridisation bottle, and washed for 20 minutes at 65°C in each of 1X SSC 1% SDS, 0.5X SSC 1% SDS and 0.2X SSC 1% SDS. The membranes were exposed to Fuji RX medical X-ray film for 4-5 days at -80°C.

Following autoradiography, the labelled probe DNA was stripped from the membranes by pouring over the membranes boiling stripping solution (0.1% SDS, 2 mM EDTA), and leaving the membranes immersed in this solution on a rocking platform for 30

minutes. Excess solution was blot dried from the membranes which were then sealed in plastic and stored at 4°C for reuse.

3.3 Linkage analysis for *Mel 1*

The RFLP data of the 439 DH lines for the marker loci *Xwg622*, *Xcdo583*, *Xabg714*, and *Xmwg77* were placed into the mapping programme Microsoft Map Manager QT Version b16 (Manly and Cudmore 1997). The genetic distance between marker loci was calculated using the mapping functions as in Appendix 3.2. The two AFLP primer combinations identified as linked with *Mel 1*, were added to the mapping data set.

3.4 AFLP analysis

The method of bulked segregant analysis (BSA) (Michelmore *et al.,* 1991) was used to identify AFLP markers linked to *Mel 1* (Appendix 3.2 and 2.3) (Vos *et al.*, 1995, and Waugh *et al.*, 1997). To identify putative AFLP markers, genomic DNA from the DH population and the parents was run on gels using 44 primer pairs. DNA was extracted (Appendix 3, 3.1, 3.4.1 and 3.4.2), from WI 2585 and Amagi Nijo. DNA was also extracted from 20 samples of DH lines which by RFLP analysis had the Mn-inefficient allele (WI 2585-like) at all four RFPL markers linked to *Mel 1* (section 6.2.1), and from 20 samples of DH lines which by RFLP analysis had the Mn-efficient allele (Amagi Nijo-like) at all four RFLP markers linked to the *Mel 1* (section 6.2.1). The 20 DNA samples with either the inefficient or the efficient allele at *Mel 1* were made into an inefficient bulk and an efficient bulk DNA samples. The AFLP primer pairs which showed a polymorphic band in WI 2585 and in the inefficient bulk, but no band in Amagi Nijo and in the efficient bulk, or vice versa, were re-tested (Appendix 3, 3.4.1 to 3.4.2) with parents WI 2585 and Amagi Nijo, and with the 20 individual lines which made up each of inefficient bulk and efficient bulk.

Confirmation of the primer pair as identifying a specific AFLP marker for *Mel 1* was validated if the polymorphism present or absent in WI 2585 and in the inefficient bulk, or in Amagi Nijo and in the efficient bulk, or vice versa as seen previously, was also in > 70% of the 20 individual bulks that made up either the inefficient bulk or the efficient bulk. To precisely map *Mel 1*, the confirmed AFLP marker was run with the individual DH lines which

RFLP analysis had found to be recombinant between the RFLP probes *wg622*, *cdo583*, *abg714* and *mwg77* (Appendix 3.2 and 3.3). The AFLP results of the DH lines were entered into the Map Manager QT Version b16 (Manly and Cudmore, 1997) file made for the 439 DH lines as in section 6.2.1 (Appendix 3.2, and 3.3).

3.4.1 Genomic DNA digestion, adaptor ligation and pre-amplification

The AFLP method used was modified from Vos *et al.,* (1995). Genomic DNA (1 µg) was digested with 5 units of *Pst* 1 and *Mse* 1 (Gibco) for 3 hours at 37°C. Double stranded adaptors were ligated to the ends of the restriction fragments. The adaptors were 5 µM *Mse* 1 annealed adaptors, 0.5 µM *Pst* 1 annealed adaptors (Gibco), in 10 mM Tris-HAc, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 1.2 µl of 10 mM ATP, and 1 unit of T4 DNA ligase (Gibco) in a total volume of 73 µl. Pre-amplification was performed using primers specific for the *Pst 1* and *Mse 1* adaptors including one selective nucleotide. The pre-amplification mix contained 75 ng *MseC* primer, 75 ng *PstA* primer (Gibco), 2.5 µl 10X Taq buffer (PCR buffer, Gibco), 4 µl of 1.25 mM of each dNTPs, 50 mM $MgCl_2$, 1 unit of Taq polymerase recombinant (Gibco), and 4 µl of the ligated DNA template mix in a total volume of 25 µl. Pre-amplification PCR consisted of 20 cycles of 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. The pre-amplification template DNA was diluted 1:5 in sterile nanopure water and stored at -20°C.

3.4.2 AFLP selective amplification and electrophoresis

A total of 44 primer pairs were tested to search for putative AFLP markers linked to *Mel 1*. Selective amplification was performed using 11 *Mse* 1 primers with 3 selective bases at the 3' end, and four *Pst* 1 primers with 2 selective bases at the 3' end. The labelling mix was 5 ng *Pst* 1 primer, 1 µl 10X PNK buffer (250 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$ 50 mM DTT, 5 mM spermidine), 2 units of polynucleotide kinase (Gibco), and 1 µl [γ-^{32}P]dATP (10 µCi/µl, Amersham) in a total volume of 10 µl. After incubation for 30-60 minutes at 37°C, 1 µl of the end-labelled 2 selective base primer was added to the selective amplification mix (25 ng of selective primer *Pst* 1, 30 ng of selective primer *Mse* 1 (Gibco), 1 µl 10X Taq buffer (PCR buffer (Gibco), 3.3 µl of 1.25 mM dNTPs, 0.6 µl 50 mM $MgCl_2$, 5 µl of the template

DNA, and 0.5 units of Taq polymerase recombinant (Gibco) in a total volume of 14 µl nanopure water).

The PCR reaction on the selective amplification mix after the addition of the end-labelled primer consisted of one cycle at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by 9 cycles over which the annealing temperature is decreased by 1°C per cycle, with a final run of 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. Loading buffer (20 µl) (98% formamide, 10 mM Na_2EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each sample of selectively amplified PCR sample. The samples were stored at -20°C.

The samples (2 µl) were separated on a 6% denaturing polyacrylamide gel made from Sequegel 6, (48 ml) and Sequegel buffer (12 ml) (National Diagnostics). Before loading on the gel, the samples were denatured for 5 minutes at 95°C and chilled on ice for 5 minutes. The gels were run at 40 W, transferred to Whatman 3MM paper, dried at 80°C for 45 minutes and exposed to Fugi RX medical X-ray film at room temperature 48-120 hours.

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