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BORON TOLERANCE IN GRAIN LEGUMES WITH PARTICULAR REFERENCE TO THE GENETICS OF BORON TOLERANCE IN PEAS

by

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DECLARATION

The work presented in this thesis is my own unless otherwise acknowledged, and has not previously been submitted to any university for the award of any degree or diploma. This thesis may be made available for loan or photocopying provided that an acknowledgment is made in the instance of any reference to this work.

Abdolreza Bagheri Kazemabad

July 1994

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SUMMARY

Boron toxicity is a major problem in dryland farming areas of southern Australia and is a constraint to production of many crop plants, including grain legumes. The present study was undertaken to investigate the extent of genetic variation within grain legumes with particular reference to peas.

The tolerance to boron of nine Australian varieties of pea was investigated to determine the range in genetic variation and also to identify specific responses which may be utilised as selection criteria in genetic studies and in breeding programs. The results showed limited genetic variation in boron tolerance among Australian pea varieties. Symptom expression was the most efficient observation for predicting the response of varieties as determined by dry matter yield and concentrations of boron in shoots. The evaluation of a wide range of pea accessions from the Australian collection revealed considerable genetic variation in tolerance to boron among *Pisum sativum* germplasm. About four per cent of these accessions were rated as more tolerant than the most tolerant Australian varieties and developed only minor symptoms of boron toxicity when grown under high boron conditions. A low degree of symptom expression by tolerant accessions was attributed to low levels of boron in the vegetative tissues.

The inheritance of tolerance to high concentrations of soil boron was studied in five cross combinations including reciprocals. Segregation patterns for boron response in F_2 populations and F_3 derived families were established by visual assessment of leaf damage. The segregation ratios were explained in terms of two major gene loci interacting in an additive manner with incomplete dominance at each locus. Evaluation of selected tolerant and susceptible families indicated that tolerant families contained a significantly lower concentration of boron in shoots than susceptible families.

Linkage analysis with isozymes and DNA markers was used to identify markers linked to a major gene conferring tolerance to boron. The bulk segregant analysis strategy was applied to an F_2 population from a cross between a moderately tolerant Australian variety and one tolerant accession. Random amplified polymorphic DNA (RAPD) and isozyme analyses were conducted upon tissue collected from the F_2 plants and the response to boron was determined for F_3 families. Of 126 random primers screened on the parents, 86 identified polymorphisms. Two primers, OpG02 and OpK07, amplified products that showed polymorphism between the tolerant and sensitive pooled DNA and OpG02 was subsequently shown to be linked to the gene of interest. Amplified RAPD products from the putatively positive primers were cloned and used as probes for Southern analysis. Polymorphisms between the parents were observed but the polymorphic bands were not linked to the tolerance locus. None of the isozyme loci was linked with tolerance to boron.

Further analysis with restriction fragment length polymorphisms (RFLP) in another recombinant inbred population from the John Innes Institute, U.K. revealed that boron tolerance segregating in this cross was controlled by a single major gene which mapped about 10 map units from *dr7-* .This placed the gene about mid-way between the two pairs of markers *i-af* and *d-sym2* on the classical linkage group 1.

The optimisation of polymerase chain reaction for RAPD analysis in peas was investigated and the results were applied to an analysis of the relatedness among five representative Australian varieties and five selected boron tolerant accessions. Genetic similarity among genotypes was estimated on the basis of the percentage of common bands between genotypes and a dendrogram was constructed by the unweighted pair grouping method. The genetic divergence between Australian varieties and the boron tolerant accessions suggests an intensive backcrossing program would be required to transfer boron tolerance to a locally adapted genetic background.

The evaluation of Australian varieties of peas, chickpeas and lentils for their response to high concentrations of boron revealed only a limited genetic variation in boron tolerance exists in Australian breeding materials. On the basis of the most tolerant of the Australian varieties, peas are more tolerant than chickpeas which are more tolerant than lentils. It would appear that there is insufficient genetic variation for boron tolerance in chickpeas and especially lentils in Australia to give adequate levels of adaptation in many soil types in southern Australia.

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CHAPTER 1

INTRODUCTION

Boron toxicity in dryland agriculture in southern Australia was first identified by Cartwright *et al.* (1984). A 17% grain yield reduction in a barley crop was found to be associated with high concentrations of boron in the subsoil and plant tissues (Cartwright *et al.*, 1984). A survey of the concentrations of boron in barley grain from all barley growing areas in South Australia and north west Victoria (1983/1984 and 1989/1990) indicated that large areas produce grain with concentrations equal to or above 2 mg B kg^{-1}(Spouncer *et al.*, 1994; Figure 1.1), a level at which toxicity might occur (Cartwright *et al.*, 1984). High levels of boron have accumulated naturally in the subsoil of alkaline and sodic soils (Cartwright *et al.*, 1986) and therefore removal or amelioration of boron toxicity by management techniques is not economically possible. The only way to ameliorate the problem is through the use of tolerant varieties.

Genetic variation in response to boron has been reported for wheat and barley (Moody *et al.*, 1988; Nable, 1988; Paull *et al.*, 1988; Nable *et al.*, 1990) and medics and peas (Paull *et al.*, 1992). Tolerant lines of all these species maintain relatively low concentrations of boron in roots and shoots (Nable, 1988; Paull *et al.*, 1992). These results imply that there is a great deal of genetic variation in the crop plants in terms of response to high concentrations of boron and breeding for boron tolerance is possible.

Inheritance of tolerance to high concentrations of boron in wheat appears to be under the control of major additive genes (Paull *et al.*, 1991). The deliberate breeding for boron tolerance in field crops, especially wheat, has commenced at the Waite Agricultural Research Institute with the boron tolerant wheat variety BT-Schomburgk recently being produced by the technique of repeated back-crossing and selfing. This variety produces approximately 10% greater grain yield than the recurrent parent, Schomburgk, under high

boron conditions (Moody *et al.*, 1993; Campbell *et al.*, 1993). The selection program which led to this release is the first of several in an overall research and development project aimed at breeding for boron tolerance in crop and pasture species for southern Australia.

Grain legumes are of increasing importance in the Mediterranean-type environment of southern Australia. The areas under cultivation to grain legumes in South Australia and Victoria are 187,500 ha and 227,300 ha, respectively (ABARE, 1990). Grain legumes are grown in rotation with cereals over wide areas in southern Australia where the concentration of boron in the subsoil is often high and cereal crops show symptoms of boron toxicity. The cultivation of grain legumes in these areas would suggest that boron toxicity of legumes may also occur. Initial experiments (Materne, 1989) showed that genetic variation in response to boron exists among Australian pea varieties. However, there is no information available about chickpeas and lentils. Neither is there published evidence on the nature of genetic control of tolerance to boron in peas. Before we can consider the possibility of transferring boron tolerance to local varieties, it is important to understand the genetic basis of boron tolerance in grain legume crops. The objectives of the research reported in this thesis were to:

1. Investigate the range of tolerance to high concentrations of boron of Australian varieties of selected grain legume species (peas, chickpeas and lentils).
2. Screen a collection of *Pisum sativum* germplasm for tolerance to boron and to compare them with Australian varieties with the aim of identifying sources of higher levels of tolerance and to determine if a high frequency of tolerant accessions originates from particular geographical regions.
3. Analyse the concentrations of boron in tissues of Australian varieties with different levels of tolerance to determine whether tolerance to boron may be related to an exclusion mechanism, and to see if the same mechanism applies to the tolerant accessions from the *P. sativum* collection.

4. Determine the number of genes conferring the tolerance of peas to high levels of boron, and their allelic relationships by:

   a. an evaluation of F_1 hybrids and F_2 and F_3 generations in the presence of high concentrations of boron in order to determine the mode of inheritance of boron tolerance in peas, and

   b. developing genetic markers linked to the boron tolerance gene(s). The identification of linked markers would greatly increase the efficiency of selection in a breeding program.

5. Provide the genetic material for field evaluation and contribute toward the development of pea varieties suitable for growing in southern Australia in rotation with cereals.

Fig. 1.1 Boron concentration of barley grain samples collected from South Australia and Victoria in 1984 and 1990. The values greater than 2 mg kg^{-1} indicate a high probability of boron toxicity (Spouncer *et al.*, 1994).

SOUTH AUSTRALIA

VICTORIA

Boron in Barley Grain

indicating the distribution of high-boron soils
in South Australia and North West Victoria

Surveys: 1983/84 and 1989/90

0 - 1 (mg/kg)

1 - 2

2 - 3

> 3

CSIRO Division of Soils - Adelaide, 1991

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 Introduction

The literature review is divided into two sections. Following the introduction is a section considering the species classification within *Pisum* L., *Cicer* L. and *Lens* L. Then follows a section on breeding for nutritional imbalances in crop plants.

The term grain legume is defined by Wijeratne and Nelson (1987) as follows: The term grain-legume is commonly used to denote legumes that produce edible seeds, and to distinguish them from numerous others that produce edible vegetative parts. Pulse is a synonymous term also in common use, particularly in West Asia. Grain legumes are nutritionally important and their seeds are characterised by high contents of protein, up to 40% in dry matter, mainly stored in the cotyledons (Van der Maesen and Somaatmadja, 1989).

Grain legumes were domesticated early in history in the major centres where agriculture originated. It appears from archaeological evidence that peas were one of the earliest crops in the West Asian Neolithic agriculture (Zohary and Hopf, 1988). Barulina (1930) reported the mountainous region between Hind-Kush and the Himalayas as the centre of origin for lentils, but Zohary (1972) believed the occurrence of carbonised lentil seeds in Neolithic settlements in the Middle East to be a sign that lentils were domesticated throughout the fertile crescent. Archaeological finds of chickpeas are not as abundant as those of lentils. The shape of the seed with its prominent beak is conducive to damage, especially in the carbonised state (Van der Maesen, 1984) and therefore is difficult to distinguish from peas. However, archaeological evidence showed that chickpeas belong to the early Neolithic grain crop assemblage of West Asia (Zohary and Hopf, 1988).

A number of the grain legumes that are grown in West Asia are of increasing importance in Australia, including peas, lupins, faba beans, chickpeas, vetches and lentils. Legumes are used as an alternative crop in Australian farming systems in rotation with pasture and cereals. The total area under grain legume crops in the different environmental regions of Australia is about 1,293,000 ha. (ABARE, 1990). Field peas are an important and established grain legume crop in the states of South Australia and Victoria and significant expansion of pea production is also occurring in Western Australia and New South Wales. The production of grain legumes in southern Australia is increasing rapidly as more farmers realise the advantages they offer in cropping rotations. For example, the estimated combined areas under cultivation of peas in South Australia and Victoria in 1982-83 and 1988-89 were 111,600 and 393,000 ha, respectively (ABARE, 1990). Therefore, there is a need to increase the productivity of these crops so that they become more profitable to farmers. One factor known to affect the productivity of crops in the sodic alkaline soils of southern Australia is boron toxicity (Cartwright *et al.*, 1984; 1986). Initial research on boron toxicity in South Australia was conducted on the cereals wheat and barley (e.g. Paull *et al.*, 1988a). As the results for these crops indicated a major yield effect associated with tolerance to boron (Moody *et al.*, 1993) it was considered that boron tolerance would also be a highly desired trait for the rapidly expanding grain legume industry. The effect of high concentrations of boron upon plant growth and genetic variation in response to boron is considered in section 2.3.7.

Sustained research on grain legume breeding in Australia was initiated during the early 1960's. The work was confined to the collection and evaluation of germplasm and the use of this germplasm in breeding programs. At the present time the grain legume research programs are mainly located in New South Wales, Victoria, South Australia and Western Australia. Current chickpea research in Australia is mainly carried out in northern New South Wales and at Horsham, Victoria. Research on peas relative to chickpeas and lentils is relatively advanced and located in South Australia, Victoria and Western Australia. Very little research has been carried out on lentils and there are few commercial cultivars all of which

have been introduced from overseas. The short-term approach in breeding of lentils involves the evaluation of the advanced lines from International Centre for Agricultural Research in the Dry Areas (ICARDA) to select better genotypes for immediate release as cultivars. Some of the selections from ICARDA are under multi-location yield trials (S.M. Ali, pers. comm.).

2.2 Species classification within *Pisum* L., *Cicer* L. and *Lens* L.

In view of the limited breeding history of grain legume crops in Australia and their cultivation in circumstances where only a limited range of genetic variability is available, it was anticipated that the closely related species would be relevant to this project. While preliminary experiments showed that variation in tolerance to boron occurs in *Pisum*, taxonomic considerations could be especially relevant to chickpeas and lentils. Therefore the species classification within these genera were considered.

Among approximately 650 genera within the Leguminosae (or Fabaceae) and about 18,000 species (Polhill *et al.*, 1981) only about 10 or 12 species are economically important (Aykroyd and Doughty, 1964). Leguminosae are the largest family of flowering plants after Compositae and Orchidaceae (Polhill *et al.*, 1981). Most grain legumes belong to one of two tribes, the Vicieae and Phaseoleae. The genera *Lathyrus*, *Lens*, *Pisum* and *Vicia* are placed in the tribe of Vicieae and *Cicer* is now classified in a tribe of its own, Cicereae (Kupicha, 1977).

2.2.1 *Pisum* L.

The genus *Pisum* L. is a small genus belonging to the tribe of Vicieae. Peas have a long history in cultivation and can be dated to Early Neolithic farming villages of West Asia (7000 to 6000 B.C.) (Zohary and Hopf, 1973).

Peas are an important pulse crop as a food source for humans and animals and are cultivated as either field peas for harvesting dry seeds and fodder or as garden peas. The annual world

production of peas is 16.3 Mt with former USSR (7.3 Mt) and France (3.2 Mt) being the largest producers of dry peas (FAO, 1991).

Species and relationship

Classification of the species in the genus *Pisum* has varied considerably in the past and constant revision of the composition of this genus has resulted from new information arising from botanical, morphological, geographical, ecological, evolutionary and genetic studies (Komarov, 1927, 1940; Vavilov, 1931; Zavadskii, 1961, mentioned by Makasheva, 1973).

Since the classification by Davis (1970) to two species *P. sativum* L. and *P. fulvum* Sibth. & Sm. the classification of species has varied considerably, therefore some of these classifications shall be reviewed in this section. Makasheva (1973) listed eight previously described species of *Pisum* , namely:

(1) *P. abyssinicum* A. Br.	The abyssinicum pea
(2) *P. aucheri* Jaub. & Spach	The Aucher pea
(3) *P. arvense* L.	The field pea (maple pea)
(4) *P. elatius* Bieb.	The Mediterranean pea
(5) *P. formosum* (Stev.) Alef.	The ornamental pea
(6) *P. fulvum* Sibth. & Sm.	The red-yellow pea
(7) *P. sativum* L.	The garden pea
(8) *P. syriacum* (Berger) Lehm.	The Syrian pea (*P. humile* Boiss. & Noë)

However, on the basis of comparative taxonomic studies of the N.I. Vavilov Institute of Plant Industry (VIR) pea collection, experiments on crosses and a review of the literature, Makasheva (1973) suggested the following four species:

(1) *P. formosum* (Stev.) Alef.	The ornamental pea (a perennial rock pea)
(2) *P. fulvum* Sibth. & Sm.	The red-yellow pea

(3) *P. syriacum* (Berger) Lehm. The Syrian pea

(4) *P. sativum* L. Sensu amplissimo Govorov The garden pea

Makasheva (1973) also classified the garden pea (*P. sativum* L.) into the following subspecies:

subsp. *transcaucasicum* Govorov.

subsp. *elatius* Schmalh

subsp. *asiaticum* Govorov

subsp. *abyssinicum* Govorov

subsp. *sativum* (or subsp. *commune* Govorov).

Among these subspecies, subsp. *sativum*, subsp. *asiaticum*; subsp. *abyssiniaum* and subsp. *transcaucasium* are cultivated. Gentry (1971) divided *P. sativum* L. into six subspecies:

subsp. *abyssinicum*

subsp. *jomardi*

subsp. *syriacum* Berger

subsp. *elatius* (Stev.) Alef.

subsp. *arvense* Poir.

subsp. *hortense* Asch. & Graeb.

On the combined basis of morphology, ecology and cytogenetics Davis (1970) classified the genus into only two species: *P. sativum L.* and *P. fulvum* Sibth. & Sm. and this classification is generally accepted. He mentioned the following names under the species of *P. sativum* L.:

subsp. *elatius* var. elatius

subsp. *elatius* var. pumilio

subsp. *elatius* var. brevipedunculatum

subsp. *sativum* var. arvense

subsp. *sativum* var. sativum

According to Zohary and Hopf (1973) two types of wild *Pisum* are genetically closely related to the cultivated peas: *P. elatius* Bieb, a tall omni-Mediterranean wild pea; and *P. humile* Boiss. & Noë (synonymous to *P. syriacum* (Berger) Lehm.) a smaller wild pea restricted to the Near East. A cytogenetic study conducted on *P. sativum, P. arvense, P. elatius, P. abyssinicum* and *P. jomardi* found only two crosses (*P. sativum* x *P. arvense* and *P. arvense* x *P. elatius*) to be successful (Fouzdar and Tandon, 1976). Karyotypes of *P. sativum, P. arvense, P. elatius, P. sativum* x *P. arvense* and *P. arvense* x *P. elatius* were basically similar. *P. abyssinicum* and *P. jomardi* also show a close relationship with each other and *P. elatius* has an intermediate position between these two groups of related species.

Ben Zé ev and Zohary (1973) considered the possibility of cross pollination among the three main wild annual species, namely: *P. elatius* Bieb., *P. humile* Boiss. & Noë (= *P. syriacum* (Berger) Lehm.) and *P. fulvum* Sibth. and Sm. and also their relationships with *P. sativum* L.. The combined evidence of morphology and results of F_1 hybrids indicated that hybrids among the four species were fertile or largely interfertile (Table 2.1). They showed that *P. humile, P. elatius* and *P. sativum* are generally closely related and all these peas could be grouped together into a single biological species (*P. sativum*). These biological species all have 2n=14 chromosomes and can be easily crossed, and there is no genetic isolation mechanism which would prevent gene transfer between any of the wild or cultivated forms (Smartt, 1984). On the basis of this evidence, Ben Zé ev and Zohary (1973) suggested that wild *humile* forms should be regarded as the direct ancestors from which the cultivated pea was domesticated.

Table 2.1 The fertility of F_1 hybrids from crosses between *Pisum* species according to Ben Zé ev and Zohary (1973).

Female	Male			
	P. elatius	*P. fulvum*	*P. humile*	*P. sativum*
P. elatius		Semi-fertile to fertile	Semi-sterile	Semi-sterile
P. fulvum	Semi-sterile		Semi-sterile	Semi-sterile
P. humile	Semi-fertile to fertile	Semi-sterile		Fully-fertile to semi-fertile
P. sativum	Semi-fertile	Semi-sterile	Fully-fertile to semi-fertile	

A comparative study of seed proteins by a competitive enzyme-linked immunosorbent assay (ELISA) (Helbing *et al.*, 1987) has also shown that *P. sativum* and *P. elatius* are closely related taxa, while *P. fulvum* is taxonomically quite distinct. F_2 analyses of crosses between *P. elatius*, *P. humile*, *P. sativum*, *P. abyssinicum* and *P. fulvum* revealed that all the ELISA patterns are inherited as single units, as alleles at a single locus (Blixt and Przybylska, 1988).

Based on the taxonomic study, the perennial form *P. formosanum* (Stev.) Alef., was separated from the genus of *Pisum* and is known as the independent genus *Vavilovia* A. Fed. (*V. formosa* Stev.) (Ben Zé ev and Zohary, 1973; Lamprecht, 1974 cited in Gritton, 1980; Kupicha, 1981). Analysis of proteins by electrophoretic methods indicated that *V. formosa* has five specific components absent from the other species of *Pisum* (Tarlakovskaya, 1987).

In summary, all members of the genus *Pisum* are annual, diploid (2n=14) and from the literature it appears that the revised classification of peas in two species (*P. sativum* L. and *P. fulvum* Sibth & Sm.) by Davis (1970) is most acceptable. *P. sativum* contains cultivated

peas and the wild types which can readily cross with each other and F_1 hybrids are fully or almost fully fertile. *P. elatius* Breb. and *P. humile* Boiss. & Noë are two main morphological types of *P. sativum*. *P. fulvum* Sibth & Sm. is considerably divergent from *P. sativum* and their hybrids are semi-sterile (Zohary and Hopf, 1988).

2.2.2 *Cicer* L.

The genus of *Cicer* was classified in the tribe of Vicieae until 1977, when it was placed in the tribe of Cicereae Alfeld by Kupicha (1977, 1981) who believed that Cicereae is closely related to Trifolieae.

Chickpeas are a grain legume that are very important for human consumption in some developing countries. They are cultivated in the Mediterranean basin and West Asia. The total area grown to this crop in the world is about 11 million ha. India produces 74%, Pakistan 10% and Ethiopia 4% of the total crop (Auckland and Van der Maesen, 1980).

Chickpeas are classified into two groups based on seed size, and are referred to as either microcarpa (small seeded) or macrocarpa (large seeded) (Cubero, 1975). Desi are small seeded, angular and coloured while Kabuli are large seeded, ram-shaped and beige coloured. The Desi types, also known as Bengal gram, constitute about 85% of annual world production and are cultivated in the Indian subcontinent, Ethiopia, Mexico and Iran. Kabuli types (or garbanzo) are cultivated in some parts of India, Afghanistan, West Asia, Northern Africa, Southern Europe and the Americas, but not Mexico (Smithson *et al.*, 1985). From a geographical point of view, these two types were separated a long time ago, probably resulting in appreciable genetic divergence between the two types, although hybridisation between the Kabuli and Desi types is possible and can be used to introduce exotic genes into locally adapted cultivars (Hawtin *et al.*, 1980).

Species and genetic relationship

All cultivated varieties are diploid (2n=16 chromosomes) and interfertile (Zohary and Hopf, 1988). The chromosome number of most species in the genus *Cicer* is 2n=16, but 14, 24, 32, and 33 chromosomes have also been reported. This genus consists of about 42 wild relatives of which eight are annual (Van der Maesen, 1989) (Table 2.2).

The species of *Cicer* differ in their range of habitats, and Van der Maesen (1984) described four major types of environments in which different species grow:

(1) The cultivated species, including *Cicer arietinum* L., only occur in cultivation, as an escape from cultivation or as a volunteer and are unable to colonise successfully without intervention by man.

(2) Weedy habitat species such as *C. reticulatum* and *C. bijugum* grow in fallow or disturbed habitats, roadsides, cultivated fields of wheat, and other places completely untouched by man or cattle.

(3) Species such as *C. pungens* and *C. yamashitae* are found on mountain slopes among rubble. Apparently seed dispersal is less hampered by predation that in habitats lacking stones, as the seed are protected between stones.

(4) Species such as *C. montbretii* and *C. floribundum* grow in broad-leaf or pine forests. The humus-rich layer is exploited but a deep taproot is present. These species also prefer some shade.

The genetic relationship between *C. arietinum* and other species of *Cicer* has been investigated to identify wild relatives suitable for gene transfer to the cultivated types. Ladizinsky and Adler (1976b) examined the relationship between *C. arietinum* and six other annual *Cicer* species. *C. reticulatum* was the only species able to hybridise readily with *C. arietinum* and produce a fully fertile hybrid with normal meiosis. Therefore, *C. reticulatum* was placed in the primary gene pool of the *C. arietinum*. *C. echinospermum* differed from *C. arietinum* and *C. reticulatum* by a major reciprocal translocation and their hybrids were highly sterile, so *C. echinospermum* Davis. was placed in the secondary gene pool

(Ladizinsky and Adler, 1976a). The wild species *C. reticulatum* was regarded as a wild race of the cultivated crop and therefore referred to as *C. arietinum* subsp. *reticulatum* (Zohary and Hopf, 1988). Crosses between *C. arietinum* and *C. judaicum* Boiss., *C. pinnatifidum* Jaub & Spach and *C. bijugum* Rech. and *C. cuneatum* produced no viable seed (Ladizinsky and Adler, 1976a; Zohary and Hopf, 1988) and these species were placed into the tertiary gene pool (Ladizinsky and Adler, 1976a).

Further evidence on the relationship between *C. arietinum* and wild relatives is provided by the variation in the patterns of the seed protein profiles in 88 cultivars and six wild species by polyacrylamide gel disc-electrophoresis (Ladizinsky and Adler, 1975). The profile of *C. reticulatum* was similar to the *C. arietinum* and differed for only one band.

The results of the genetic relationships among species of *Cicer* indicate that *C. reticulatum* may be used as a ready source of genetic variation for *C. arietinum*. However, on the basis of results for other species, Smithson *et al.* (1985) suggested that the genetic relationships among *Cicer* species are complex and deserving of further study.

Table 2.2 The geographical distribution of annual and perennial species of the genus *Cicer*.

	Afghanistan	Bulgaria	Egypt	Ethiopia	Greece	India	Iran	Iraq	Italy	Lebanon	Morocco	Pakistan	Spain	Sudan	Syria	Turkey	USSR C Asia	USSR Caucasia
C. acanthophyllum	•											•					•	
C. anatolicum							•	•								•		•
C. arietinum	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
C. atlanticum										•								
C. balcaricum																		•
C. baldshuanicum																	•	
C. bijugum								•							•	•		
C. chorassanicum	•						•											
C. cuneatum			•	•										•				
C. echinospermum																•		
C. fedtschenkoi	•																•	
C. flexuosum																	•	
C. floribundum																•		
C. garanicum																	•	
C. graecum					•													
C. grande																	•	
C. heterophyllum																•		
C. incanum																	•	
C. incisum						•		•								•		•
C. isauricum																•		
C. judaicum										•								
C. kermanense							•											
C. korshinskyi																	•	
C. macracanthum	•											•					•	
C. microphyllum	•					•						•					•	
C. mogoltavicum																	•	
C. montbretii		•		•												•		
C. multijugum	•																•	
C. nuristanicum	•					•						•						
C. oxyodon	•						•	•								•		
C. pauncijugum																	•	
C. pinnatifidum								•							•	•		
C. pungens	•																•	
C. rechingeri	•																	
C. reticulatum																•		
C. songaricum																	•	
C. spiroceras							•											
C. stapfianum							•											
C. subaphyllum							•											
C. tragacanthoides							•										•	
C. yamashitae	•																	

Source: Van der Maesen (1984).

2.2.3 *Lens* L.

The genus of *Lens* Miller. holds an intermediate position between *Vicia* and *Lathyrus*, although closer to *Vicia* Sect. Ervum (Davis and Plitmann, 1970). Lentils are probably one of the most ancient cultivated plants, dating back to prehistorical times, as far as agriculture itself (Barulina, 1930).

Lentils are an important pulse crop for human consumption. The major areas under cultivation of lentils are in central and south-west Asia, Southern Europe, North Africa, Ethiopia and North and South America (Muehlbauer *et al.*, 1985). They are grown on more than 1.8 million ha throughout the world, with an annual seed production of 1.1 Mt. The major producing country is India, with an anual production of 428,000 t from 925,000 ha (Jansen, 1989).

Species and genetic relationship

According to Davis and Plitmann (1970), the genus contains five annual species: *Lens culinaris* Medik., *L. orientalis* (Boiss.) Hand.-Mazz., *L. ervoides* (Brign.). *L. nigricans* (Bieb.) Godr. and *L. montbretii* (Fisch. & Mey.). *L. montbretii* (Fisch. & Mey.) Davis & Plitm. with 2n=12 chromosomes is synonymous with *Vicia montbretii* Fisch. & Mey; *Ervum kotschyana* Boiss.; *Vicia bombycina* stapf ext Post and *Lens kotschyana* (Boiss.) Nab (Ladizinsky and Sakar, 1982). This species has some morphological differences from the other species and it has a strong similarity to the *Vicia*. Davis and Plitmann (1970) questioned its position in the genus of *Lens* and this species now has been transferred to the genus *Vicia montbretii* Fisch. & Mey.. Therefore, four species with 2n=14 chromosomes (Ladizinsky and Sakar, 1982) are in this genus, as follows:

(1) *L. nigricans* (Bieb.) Godr.

Syn: *Ervum nigricans* Bieb. ; *L. culinaris* Medik. subsp. *nigricans* (Bieb.).

Morphologically this species is very similar to *L. culinaris*.

(2) *L. ervoides* (Brign.)

Syn: *Cicer ervoides* Brign.; *Ervum lenticula* Schreb. or *L. lenticula* (Schreb). Alef.

(3) *L. orientalis* (Boiss.) Hand.-Mazz.

Syn: *Ervum orientalis* Boiss. ; *Ervum cyaneum* Boiss. & Hoh. From a morphological point of view this species is very similar to the cultivated lentil (*L. culinaris*) (Zohary, 1972), and it is probably the wild progenitor of the cultivated *Lens* (Barulina, 1930; Ladizinsky, 1979a).

(4) *L. culinaris* Medik.

Syn: *Ervum lens*. L.; *L. esculenta* Moench. *L. culinaris* is the only species in cultivation. This species is divided into two subspecies : *L. culinaris* subsp. macrosperma (large seeded type) and *L. culinaris* subsp. microsperma (small seeded type) (Barulina,1930). The two subspecies hybridise readily and their F_1 hybrids are fully fertile. For this reason Williams *et al*. (1974) believed that lentils should not be subgrouped on the basis of seed size.

Studies have been conducted to determine the possibility of cross pollination between *L. culinaris* and other species. Ladizinsky (1979a) produced hybrids between *L. culinaris, L. nigricans* and *L. orientalis*. There were three chromosome interchanges between *L. culinaris* and *L. nigricans,* but only one between *L. culinaris* and *L. orientalis*. Ladizinsky (1979a) concluded that *L. orientalis* is more likely to be the wild progenitor of lentils, although for breeding purposes, *L. orientalis* and *L. nigricans* could be exploited almost equally well as sources of genes.

The seed-protein profiles of *L. culinaris*, *L. orientalis* and *L. nigricans* were similar to each other (Ladizinsky, 1979b), but different to the wild species *L. ervoides*. Also, hybridisation between *L. culinaris* and *L. ervoides* has been unsuccessful. The behaviour of hybrids between *L. culinaris* and *L. nigricans* has also been studied (Goshen *et al*., 1982). F_1 hybrids were partially fertile and in the F_2 generation about 19% of the plants were fully fertile. Therefore this species also has potential as a source of genetic variation for breeding programs.

2.3 Breeding for nutritional imbalances in crop plants

Agricultural production in the arid and semi-arid regions of the world is limited by poor water resources, limited rainfall and the detrimental effects associated with an excess of soluble salts. Salt affected soils occur extensively in Asia and Australia. Since there is an overlap between different stresses, the characterisation of the world's problem soils is difficult. However, as a rough breakdown, mineral stresses occur in Ferralsols, Acrisols, Nitrosols and Podzols which occupy approximately 2,960,800,000 ha, representing 22.47% of the world's land area (Dudal, 1976).

Mineral stresses considered here are nutritional deficiencies or toxicities which are inherent to the morphology and chemical composition of the soil and often create a serious problem in plant production. Most nutritional stresses such as boron toxicity are difficult to control by cultural practices.

2.3.1 The concept of nutrient efficiency in crop plants

Nutrient efficiency of a genotype is defined as the ability of a genotype or crop to produce a high yield in a soil which is limiting in an element for a standard genotype (Graham, 1984). The functions of plant nutrient efficiency can be considered as internal factors and directly related external factors (Vose, 1963). Internal factors include absorption, translocation, assimilation and detoxication. The most important external factors are root exudates, particularly in relation to the mycorrhiza flora, soil dissolution and element availability. It is relatively easy to establish that varieties of crop plants differ in absorption and translocation but difficult to prove that certain genotypes function effectively with a lesser content of an element under deficiency conditions, or at high level of an element in the case of toxicity (Vose, 1984). The physiological aspects will not receive attention in this section. Instead, emphasis will be placed on aspects which would seem to be of greater interest to plant breeding.

2.3.2 Screening plants for nutrient imbalances

Useful genetic variation apparently exists in all major field crops for tolerance to nutritional imbalances. There are large differences between plants in their capacity to extract and absorb nutrients from relatively unavailable sources and in tolerance to and efficiency of utilisation of nutrient elements.

Screening techniques for determining plant responses to nutrient imbalances are an active area of research and a variety of methods have been developed. The following factors have proved important in increasing the efficiency of screening methods for plant breeding. An assay should:

(1) provide values which are both accurate and precise;

(2) minimise the fluctuation in values due to environmental effects;

(3) characterise the genotype of the zygote rather than the maternal effects;

(4) provide the maximum expression of genetic variation, and

(5) be rapid and labour efficient

In general, it is necessary to confirm that the response measured by any artificial screening system relates to results in the field.

<u>Screening plants in soil</u>

Many workers have screened plants in soil, using fields, field plots or pots. Although field screening and selection might be theoretically ideal, there are numerous reasons that have led workers to choose artificial systems for screening. These include:

(1) the inconvenience of a distant test field;

(2) the need to avoid site and seasonal variability;

(3) the need to screen a large number of acquisitions or selections for which there is too little seed available for field plots;

(4) the desirability of working during a longer season than is possible in the field;

(5) the need to keep costs down;

(6) the need to define more closely the selection environment; and

(7) the need to select for a character impractical in the field e.g. root morphology, rate of root growth, root excretions or reaction (adapted from Vose, 1990).

Despite the above limitations, field screening has been used extensively. At ICARDA in Syria, field screening has been used for evaluation of wheat, barley and triticale germplasm for salt tolerance. The method consisted of planting in two rows, 1.5 m long and 30 cm apart. A standard variety was grown in every twentieth row as the check. Seedling and plant vigour were visually evaluated on a scale of 1 to 9 (Srivastava and Jana, 1984). Field screening also has been used extensively by Brazilian wheat breeders for aluminium and manganese stress (Da Silva, 1976). The use of soil as a screening medium for aluminium and manganese tolerance has also been described by Foy (1976) and Andrew (1976).

At International Rice Research Institute (IRRI) in the Philippines, shallow plastic trays of soil were used for screening a diverse collection of rice for tolerance to salinity and alkalinity. Common salt and sodium carbonate were added to the soil for salinisation and alkalinisation, respectively (Ponnamperuma, 1976; 1984). Plants were scored four weeks after transplanting according to the percentage of dead leaves. This method made possible the screening of 4000 varieties per year using 104 m^2 of glasshouse bench space. Salt-tolerant cultivars identified in the glasshouse were used as parents in the hybridisation program. Progeny from the breeding program were screened on field plots of a nearly neutral clay treated with common salt to give an electrical conductivity (EC) of 8-10 m mhos cm^{-1} at 25°C. Extensive evaluation of the tolerant lines under controlled conditions in the experimental field and in farmers fields showed that salt tolerance in modern rices conferred a comparative yield advantage of 2t ha^{-1}.

Moody *et al.* (1988) screened a large number of wheat genotypes for boron tolerance under controlled conditions in a soil to which a high level of boron had been applied at the Waite Agricultural Research Institute, South Australia. A number of lines selected for tolerance in

an initial glasshouse screening were evaluated at the boot stage in field trials under naturally occurring high levels of boron. Tolerant lines showed significantly lower tissue boron concentrations than Halberd, the most boron tolerant Australian variety of wheat. Moody *et al.* (1988) concluded that the seedling screening technique for boron tolerance was efficient as genotypes identified as tolerant by this procedure showed low tissue boron concentrations under field conditions.

Screening plants in solution/sand culture

Solution/sand culture techniques have been used extensively for screening for genetic variation in response to nutrient imbalances. With this method it is possible to vary the concentration of a single nutrient while maintaining constant levels of others. Furlani and Clark (1981) developed a rapid method of screening genotypes for aluminium tolerance. This method consisted of placing 8-day-old seedlings in 0 and 148 μM aluminium for 10 days. At this time aluminium toxicity symptoms on roots were visually assessed and rated in whole number increments from 0= no aluminium toxicity to 4= severe aluminium toxicity.

Howeler and Cadavid (1976) compared rice cultivars in nutrient solution and under field conditions for aluminium tolerance. For the 240 cultivars studied, a good relationship was found between the laboratory assay as determined by relative root length and grain yield at a low pH in the field.

Other investigators have also found good agreement between results obtained for cultivars screened in nutrient solution and in appropriate soils. However, Randhawa and Takkar (1976) screened crop varieties for their response to micronutrient imbalances in solution/sand culture, pot culture and field experiments. They found that the relative order of susceptibility under controlled conditions (solution/sand culture) differed from those under pot culture and field conditions. They pointed out that this difference could result from interactions between variety/soil and environmental conditions. A number of other reports using solution/sand culture as a screening method for nutrient problems are summarised in Table 2.3.

Table 2.3 Summary of experiments using solution/sand culture to identify tolerance to nutrient imbalances.

Kind of medium	Nature of research	References
Solution/sand culture	Mn tolerance of lucerne	Dessureau (1958)
"	Al and Mn toxicities in plants	Vose & Randall (1962)
Solution culture	Adaptation of crops to salinity	Epstein (1976)
"	Salt tolerance of crop plants	Epstein & Raines (1987)
"	Al tolerance of barley	Reid (1976)
"	Physiology of metal toxicity in plants	Foy *et al.* (1978)
"	Al tolerance of sorghum	Furlani & Clark (1981)
"	Mn toxicity of soybean	Mascarenhas & Camargo (1988)
"	Al tolerance of wheat	Briggs *et al.* (1989)
"	Mn tolerance of wheat	Macfie *et al.* (1989)
"	Genetics of Al tolerance in wheat	Aniol (1990)
"	Fe tolerance of rice	Fageria *et al.* (1990)
"	Al tolerance of wheat	Scott & Fisher (1989)
"	B tolerance of wheat and barley	Nable, 1988; Jenkin (1993)
		Nable *et al.* (1990)

In vitro screening methods

The use of cell suspensions and tissue culture techniques for screening genotypes in terms of nutritional imbalances is a modern technique which has become increasingly attractive. The application of *in vitro* selection in plant breeding was reviewed by Haines (1993). He listed the fundamental attractions of *in vitro* selection, as follows:

(1) the ability to screen large numbers quite rapidly - millions in the case of cell culture;

(2) a greater control over exposure to the agent is often achievable - reducing environmental variation and thus increasing heritability;

(3) the possibility of coupling selection with somatic embryogenesis systems and the generation of variation for somatic tissues.

Cell lines tolerant of elevated levels of salt or metals in the medium have been selected in many studies. The isolation of salt-resistant cell lines from haploid cells of *Nicotiana* spp. has been reported by many workers (Dix and Street, 1975; Nabors *et al.*, 1975; Hasegawa *et al.*, 1980) but Watad *et al.* (1991) showed that plants regenerated from resistant cell lines did not display such tolerance. Rarely has the resistance observed in culture been associated with resistance at the whole plant level (Orton, 1980; Warren and Gould, 1982). In contrast, in cases such as alfalfa (Winicov, 1990; 1991), *Coteus blumei* (Ibrahim *et al.*, 1992), *Brassica juncea* (Jain *et al.*, 1991) and *Citrus sinesis* (Spiegel-Roy and Thorpe, 1986) plants regenerated from the cell lines displayed increased tolerance to salinity in glasshouse and field trails.

The lack of success in developing new salt-tolerant genotypes by *in vitro* screening can be attributed to many factors. These include equivocal salt tolerance of regenerated plants from selected cells, multi-genic inheritance of tolerance, inadequate knowledge of salt tolerance mechanisms (which depend on the structural and physiological integrity of the whole plant), and differences in mechanisms of salt tolerance between cells in culture and cells in whole plants (Dracup, 1993).

Genotypic differences in response to boron toxicity have been reported in wheat genotypes using tissue culture in media containing high concentrations of boron. Excised root tips of boron susceptible genotypes produced less callus than tolerant genotypes and only tolerant genotypes were able to initiate callus in the presence of 25 mM boron in the agar medium (Huang and Graham, 1990). The response of genotypes in tissue culture concurred with response at the whole plant level.

2.3.3 Inheritance of response to nutritional stresses

Before any nutritional character can be used in a crop improvement program, there must be an adequate range of variation in the character, particularly in the direction in which improvement will be sought. Given this, it is necessary to confirm to what degree the character is heritable and the mode of gene action, whether for example it is dominant or recessive, simple or multi genic, additive or non additive (Vose, 1984).

One of the first investigators to combine nutritional and genetic studies was Weiss (1943). Soybean (*Glycine max*) was grown in a calcareous soil and in a synthetic nutrient medium low in available Fe. Some strains developed chlorosis typical of severe iron deficiency, other strains were without symptoms. Inheritance studies demonstrated a single pair of alleles to be responsible for susceptibility to Fe deficiency. Efficiency was dominant, with only *fefe* plants becoming chlorotic. Cianzio *et al.*, (1980) re-examined inheritance of the character using field evaluations on calcareous soils. The difficulty with a single gene model in explaining the control of iron utilisation in soybeans was that discrete classes were not observed when a large number of soybean lines were grown on calcareous soils in the field. This research concluded that Fe efficiency is controlled by a major gene, but that additional genes with quantitative inheritance also contribute to iron efficiency on calcareous soils. The inheritance of Fe efficiency in tomato (*Lycopersicum esculentum*) is also under simple genetic control (Brown and Wann, 1982). Seedlings from an F_2 population of iron efficient x iron-inefficient parents, when subjected to Fe-deficiency, segregated for the single gene (*fer*) in the expected 3:1 phenotypic ratio with efficiency dominant.

Pope and Munger (1953a,b) demonstrated that single gene differences controlled susceptibility to magnesium and boron deficiency in celery (*Apium graveoleus*). Chemical analysis showed that a magnesium susceptible cultivar Utah 10B contained significantly less Mg than tolerant cultivars. Wall and Andrus (1962) described a mutant of tomato (*Lycopersicon esculentum*), T3238, which developed the stem and petiole brittleness characteristic of boron deficiency in a nutrient medium in which the cultivar Rutgers grew without developing deficiency symptoms. Brittle stem (*btl*) susceptibility was controlled by a single recessive gene.

Considerable genetic variation exists for most, if not all, essential plant nutrients and selection for improved nutritional characteristics is therefore possible.

2.3.4 Aluminium and manganese toxicity stress

Aluminium and manganese toxicities are important growth limiting factors in many acid soils. There has probably been more research on the genetic control of tolerance to these two toxicities than for any other nutritional problems. This research is reviewed briefly below to provide examples of the processes involved in breeding for tolerance to nutritional toxicities.

2.3.5 Aluminium toxicity stress

Aluminium toxicity is an important growth limiting factor for plants in many acid soils below pH 5.0, but it can occur at pH levels as high as 5.5 (Foy *et al.*, 1978). Root growth in acid soils is usually greatly inhibited (Foy, 1974). Aluminium also interferes with the uptake and transport of phosphorous, calcium, magnesium and other essential elements (Foy *et al.*, 1978). Genetic variation for tolerance of aluminium toxicity has been reported in crops representing a diverse flora (Table 2.4).

Table 2.4 Genetic variation in response to aluminium toxicity among various plant taxonomic groups. (RGR, relative growth rate; RE, root elongation; RTI, root tolerance index; RGS, root growth score).

Plant species	Source of variation	No. of lines	Basis of assessment	Experimental condition	References
White clover (*T. repens*)	Cultivars	14	Shoot dry weight	Pot	Mackay *et al.* (1990)
Alfalfa (*M. sativa)*	Cultivars	6	Visual, top & RGS	"	Devine *et al.* (1976)
Wheat (*T. aestivum*)	Cultivars	30	Root weight	Solution & field	Briggs *et al.* (1989)
White clover (*T. repens)*	Populations	15	RGR& RE	Solution	Caradus (1987)
Spring wheat (*T. aestivum*)	Cultivars	20	RTI	Pot	Taylor & Foy (1985)
P. aquatica	Accessions & cultivars	39	Root length	Solution	Culvenor *et al.* (1986)
P. aquatica	Accessions	23	Dry weight	Pot	"
Soybean (*Glycine max*)	Cultivars	48	Top & root yield	Pot	Armiger *et al.* (1968)
Sorghum (*Sorghum bicolor*)	S1 progeny & inbred lines	294	Visual on root	Solution	Furlani and Bastos (1990)

Inheritance of tolerance of aluminium toxicity

The existence of major genes for tolerance to aluminium has been reported in a number of species. For example, the wheat cultivar Druchamp has a major dominant gene compared to the susceptible cultivar Brevor (Kerridge and Kronstad, 1968). While the existence of these major genes has been noted, many authors also indicate that the major genes account for only part of the observed variation. Campbell and Lafever (1978) found that inheritance of aluminium tolerance in wheat was more complex than a single gene with incomplete dominance as additive effects also appear to be involved (Aniol, 1983). Slootmaker (1974) investigated tolerance to soil acidity in the wheat-related species, rye (*Secale cereale*) and Triticale. He demonstrated that the A-genome of the *Triticum* species contributes to tolerance. Also the D-genome carries one or more genes which contribute to tolerance to high soil acidity in hexaploid wheat, differentiating hexaploid wheat from barley, wild and cultivated einkorn and emmer wheats.

An attempt was made to locate genes for tolerance to available aluminium in wheat (*Triticum aestivum* L.) using nullisomic-tetrasomic and ditelosomic lines of the moderately tolerant wheat cultivar "Chinese Spring" (Aniol and Gustafson, 1984). Genes for aluminium tolerance were found to be localised in chromosome arms 6AL, 7AS, 2DL, 3DL, 4DL and 4BL and on chromosome 7D. Rye addition and substitution lines in different wheat varieties were also included and results indicated that major genes for aluminium tolerance in rye seem to be located on 3R and 6RS, with other genes on 4R.

In similar studies, an evaluation of the aluminium tolerance of three sets of wheat/rye addition lines indicated that the tolerance in rye is controlled by different chromosomes (mainly 3R, 4R and 5R in the CS/King II rye addition lines, 5R and 6R in the CS/Imperial rye addition lines and 2R and 5R in CS/*S. montanum* rye addition lines) although the effects were predominantly on chromosome 5R (Manyowa *et al.*, 1988).

There are a number of reports on the genetic control of aluminium tolerance in other species of the tribe Triticeae, namely: maize, barley, *Phalaris aquatica*, sorghum and phalaris. The difference in aluminium tolerance between Dayton and Smooth Awn 86, two winter barleys, was controlled by a single dominant gene (Reid, 1970). Furlani *et al.* (1982) indicated that control of aluminium tolerance in F_1 hybrids of sorghum appeared to be expressed by dominant genes. In maize a single major gene for aluminium tolerance, with multiple alleles, was reported by Rhue *et al.* (1978). The influence of aluminium tolerance in *P. aquatica* was investigated and explained by a two gene model in which tolerance required at least one dominant allele at each locus (Culvenor *et al.*, 1986).

Research on aluminium toxicity has not been as extensive in legumes as in other crop species and the inheritance appears to be complex (Helyar, 1978). Aluminium tolerance in alfalfa is a heritable trait and recurrent selection has been used effectively to develop strains having a greater level of tolerance to aluminium toxic soils (Devine *et al.*, 1976). In soybean, significant variation for tolerance to aluminium toxicity was reported by Armiger *et al.* (1968) and Devine (1976). Differential tolerance of individual white clover (*Trifolium repens* L.) cultivars to aluminium has been demonstrated by Caradus (1987) and Mackay *et al.* (1990). They suggested that superior aluminium tolerance in white clover was related to germplasm with wide agronomic adaptation. The aluminium tolerance in white clover appeared to be inherited predominantly as a recessive character since narrow sense heritability was 0.4 (Caradus *et al.*, 1991).

2.3.6 Manganese toxicity stress

Manganese is considered to be one of the main toxic factors in some strongly acid soils (below pH 5.5) in which the original materials were high in total manganese. Manganese toxicity may also occur at higher pH levels under reducing conditions created by flooding, compaction or organic matter accumulation (Foy *et al.*, 1978). Species in which differential tolerances to manganese have been reported include wheat, apple, triticale, soybean, cotton,

flax (Foy, 1983), subclover, bean, rice, tobacco, orchard grass, bulbous canary grass, amaranth, Japanese persimmon, red fescue and cowpea (Foy *et al.*, 1988).

Inheritance of tolerance of manganese toxicity

Camargo (1988) investigated the tolerance of wheat cultivars to different levels of manganese in nutrient solution and pointed out that the variability in wheat is under genetic control and selection for tolerance to manganese toxicity would be effective in early generations. Screening of wheat cultivars has shown that a wide range of response to manganese toxicity exists (Macfie *et al.*, 1989; Moroni *et al.*, 1991a). Inheritance of tolerance to manganese in wheat appears to be under the control of many genes. For example, the P_1, P_2, F_1, F_2, BC_1 and BC_2 generations derived from crosses among tolerant, intermediate and sensitive genotypes of wheat were studied for tolerance of manganese (Moroni *et al.*, 1991b). Total chlorophyll content was used to determine manganese tolerance, and it was concluded that manganese tolerance in wheat is a quantitatively inherited character. A preponderance of additive effects coupled with high heritability and small dominance (potence ratio) estimates indicate that selection for this character should be highly effective in early generations. The analysis of specific Chinese Spring wheat and Imperial rye chromosomes suggested that the tolerance genes are located on more than one chromosome (Manyowa, 1989). The study of Chinese Spring *Th. bessarabicum* addition lines showed lines possessing chromosome $5E^b$ or a $5E^b$/ $6E^bL$ translocation chromosome exhibited a level of manganese stress tolerance similar to that of the Chinese Spring /*Th. bessarabicum* amphiploid, suggesting that these chromosomes each carry a dominant gene(s) for manganese tolerance (Manyowa, 1989).

The inheritance of the response of lettuce to excess manganese was studied by Eenink and Garretsen (1977). Five lettuce genotypes *Lactuca sativa* cvs. Neptune, Plenos, Troppo and Celtuce (all sensitive) and an accession of *L. serriola* (insensitive) were intercrossed. Various population analyses showed that different numbers of genes for insensitivity were present in the parents, varying from one to four genes. Three of the loci were linked in the repulsion phase.

2.3.7 Boron toxicity stress

Response of plants to boron

Boron is a micronutrient essential to the normal growth of plants, but plants differ widely in their ability to absorb boron from soils and water. The metabolic requirement for boron appears to be in the range of 5 to 100 $\mu g\ g^{-1}$ dry weight, with monocotyledonous plants tending to have a lower requirement than dicotyledonous plants (Tanaka, 1967; Gupta, 1983; Pilbeam and Kirkby, 1983). Boron is transported in the transpiration stream and accumulates in the tips and margins of the leaves with very little remobilisation to other parts of the plant (Jones, 1970). The biochemical role of boron in plant metabolism is still perhaps the least well understood of all the plant nutrients (Pilbeam and Kirkby, 1983). The functions of boron are related to some basic processes including metabolism of carbohydrate and transport of sugars through membranes (Berger, 1949; Sisler *et al.*, 1956), root extension and synthesis of components of the cell wall (Jackson and Chapman, 1975; Cohen and Lepper, 1977) and ATPase activity (Pollard *et al.*, 1977).

Boron in the soil

The total amount of boron in the surface soil varies widely from 1 to 467 mg kg^{-1} and its average content ranges from 9 to 85 mg kg^{-1} (Kabata-Pendias and Pendias, 1992). Sandy and loamy soils of Poland and New Zealand were found to contain a low level of boron and soils considered to contain the highest concentrations are lateritic soils of India, solonchaks of the U.S.S.R. and calcareous soils of Israel (Kabata-Pendias and Pendias, 1992).

Boron is present in all the rocks of the earth's crust, but the amount of boron varies relative to the nature of the rock. Sedimentary rocks of marine origin have a high amount of total boron which can attain 500 mg kg^{-1} or more (Aubert and Pinta, 1977). Boron occurs in a number of minerals including hydrous borates, anhydrous borates and complex borosilicates, which include tourmaline, the original source of boron in most soils (Norrish, 1975).

Tourmaline is very resistant to weathering and boron in this form is therefore unavailable to plants.

The soils of arid and semi-arid regions generally have average to high total boron concentrations (Aubert and Pinta, 1977; Norrish, 1975). In Israel, brown isohumic soils contain from 25 to 40 mg kg^{-1} and Indian vertisols contain from 25 to 50 mg kg^{-1} of total boron (Aubert and Pinta, 1977). The boron concentrations in saline soils are often higher than average and sometimes very high (Singh and Singh, 1972; Aubert and Pinta, 1977; Keren and Bingham, 1985). Examples of saline soils with high concentrations of boron include Yugoslav solonetses (40-65 mg kg^{-1}), saline alkaline soils of Uzbekistan (100 mg kg^{-1}) and saline alluvial soils of Israel (150-170 mg kg^{-1}). Mediterranean red soils of Israel, which are formed on limestone, are also very rich in total boron (190 mg kg^{-1}) (Aubert and Pinta, 1977).

The concentrations of plant available boron are generally much less than in the parent rock material. Most of the plant available boron comes from the decomposition of soil organic matter and from boron adsorbed and precipitated onto the surface of soil particles (Bingham, 1973). It is known that boron is the most mobile element in the soil relative to other micronutrients, although its water soluble fraction is low and varies from 3.2 to 5.3% of the total content (Cumakov, 1988). Boric acid is the most common form of boron in soil solution and is the form that plant roots absorb most efficiently (Oertli and Grgurevic, 1975). An equilibrium exists between the solution boron and adsorbed boron and experimental evidence suggests that plants only respond to the portion of boron in solution (Hatcher *et al.*, 1959; Keren *et al.*, 1985). Stinson (1953) and Wear and Patterson (1962) also found the concentration of boron in alfalfa shoots to be directly correlated with the soluble boron content of the soil. Thus these experiments demonstrate that plant response to boron provides a better basis for assessing the boron status of the soil rather than total or adsorbed soil boron.

Although boron in soil is often the most significant source of boron, many other factors may also influence the concentrations of this element available to plants. Reports from the Punjab (Singh and Kanwar, 1963); Rajasthan (Mathur *et al.*, 1964), western parts of Uttar Pradesh (Mehrotra *et al.*, 1980), other parts of India (Chauhan and Powar, 1978; Chauhan and Asthana, 1981), Spain (Salinas *et al.*, 1986) and the United States (Francois and Clark, 1979, Francois, 1984) indicated high concentrations of boron in well water being used for irrigation. High concentrations of boron have also been reported in river water in Spain (Salinas *et al.*, 1981) and in agricultural sub surface drain water from the San Joaquin Valley of California (Saiki *et al.*, 1992). Over fertilisation with fertilisers which are high in boron may also lead to boron accumulation in the soil (Gupta *et al.*, 1976; Francois and Clark, 1979). Another possible source of boron, which is of increasing concern, is the reuse of sewage water (Francois, 1984).

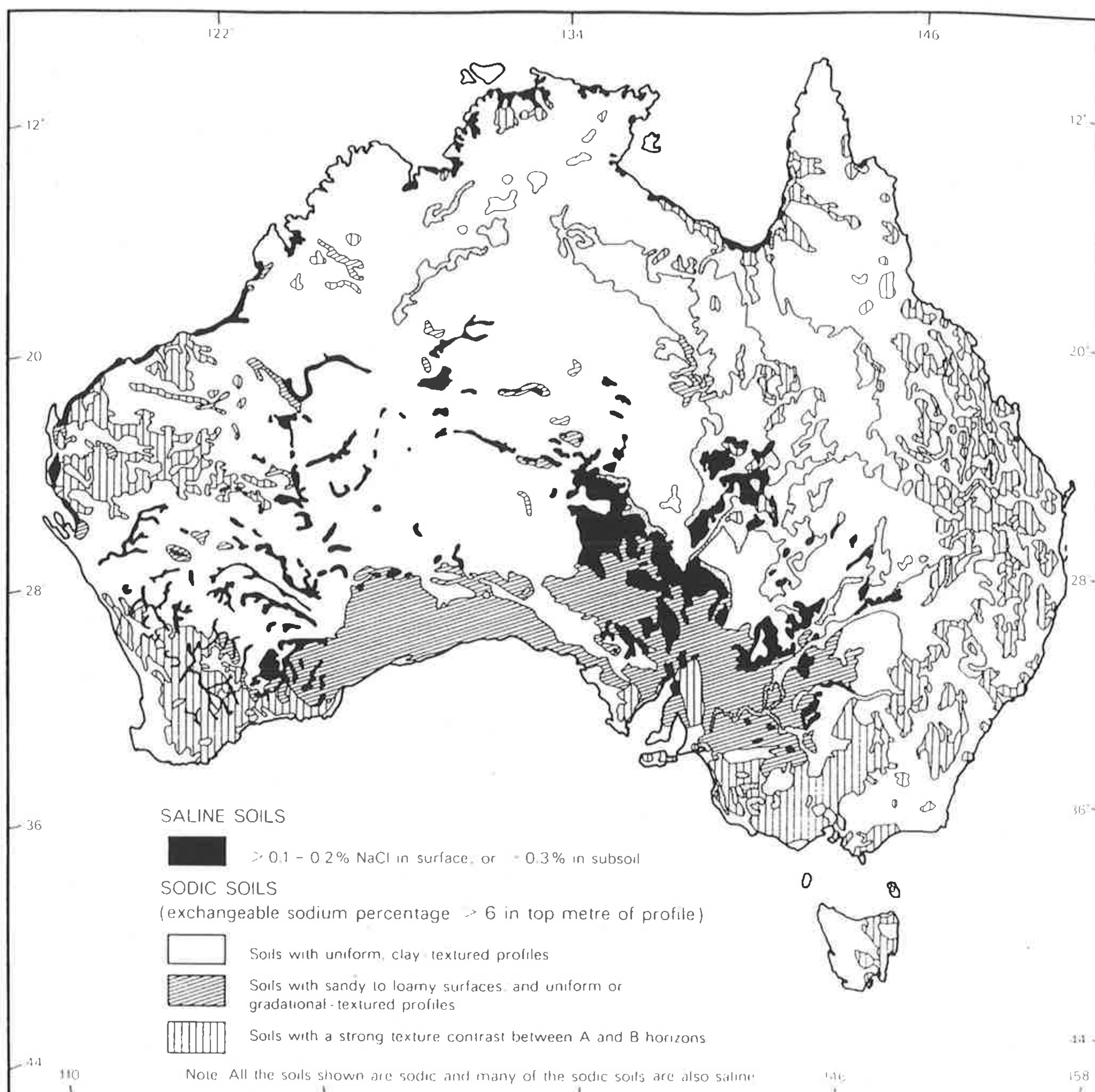
Boron toxicity problems in Australia

Many of the arable soils in Australia are affected by salinity or sodicity. Northcote and Skene (1972) reported that sodic soils occupy 27.6% of the total land surface in Australia, while saline soils occupy 5.3%. Salt-affected soils occur extensively in Australia as shown in Fig. 2.1. Sodic soils are dominated by a high level of exchangeable sodium, relative to other cations (K, Ca, Mg) and sodicity is expressed as exchangeable sodium percentage (ESP). An ESP value below 6 is associated with normal soils, but values of 6 or more indicate that sodium salts are important and the soils are described as sodic while those with an ESP of 15 or greater are strongly sodic (Northcote and Skene, 1972). More recently, Rengasamy and Olsson (1991) proposed a scheme for classifying sodic soils with respect to three important soil properties, namely, the sodium adsorption ratio (SAR), electrical conductivity (EC) and pH. Based on this classification, saline-sodic soils are soils with an SAR > 3 and EC greater than the threshold concentration (TC), which is generally > 0.4 dSm^{-1}. When SAR exceeds 3 but EC is below TC, the soils are defined as sodic. Acidic sodic soils have pH < 6; neutral sodic soils have pH between 6 and 8, and alkaline sodic soils have pH > 8. Most sodic soils in Australia have a dense subsoil with an alkaline pH (Northcote and Skene, 1972).

South Australian soils are generally alkaline throughout or have an alkaline reaction trend with depth (Cartwright *et al.*, 1987; Rathjen *et al.*, 1987). The soils of this region have high concentrations of boron and usually soluble boron concentrations are highest in the subsoil (Cartwright *et al.*, 1984). Soils with high levels of boron include red brown earths (calcic natrixeralfs), calcareous earths (calciorthids) and calcareous sands (xerochrepts) with free carbonates and high pH (Cartwright *et al.*, 1986). The concentration of extractable boron is often above 20 mg kg^{-1} and may occasionally exceed 100 mg kg^{-1} where sodic or saline-sodic conditions are dominant (Rathjen *et al.*, 1987). Boron toxicity of crop plants in South Australia was first reported by Cartwright *et al.* (1984). They found that a 17% grain yield reduction in a barley crop could be attributed to a high concentration of boron in the subsoil (Cartwright *et al.*, 1984). Cartwright *et al.* (1986) found a significant correlation between extractable boron and ESP, cation exchange capacity (CEC) and clay content of soils of southern Australia. Holloway and Alston (1992) examined the interaction between boron and sodium cloride toxicities in solonised brown soils (alkaline sodic soils) of South Australia and found that wheat plants were affected more by boron than salt.

Symptoms of boron toxicity in barley have been reported in Western Australia (Riley, 1987). The occurrence of boron toxicity symptoms in citrus trees (Penman and McAlpin, 1949) and sultana vines (Sauer, 1958) in the Mildura area of north-western Victoria has also been reported. In recent years, considerable effort has been directed towards the identification of plants that may tolerate high concentrations of boron and to understand the heritability and genetics of boron tolerance.

Fig. 2.1 The distribution of saline and sodic soils in Australia (Northcote and Skene, 1972).


122°
134
146
12°
12°
20
20°
28
28
36
36°
44
44
110
146
158
SALINE SOILS
> 0.1 – 0.2% NaCl in surface, or > 0.3% in subsoil
SODIC SOILS
(exchangeable sodium percentage > 6 in top metre of profile)
Soils with uniform, clay-textured profiles
Soils with sandy to loamy surfaces, and uniform or gradational-textured profiles
Soils with a strong texture contrast between A and B horizons
Note All the soils shown are sodic and many of the sodic soils are also saline

Boron deficiency and toxicity symptoms

Boron deficiency is common on a world-wide scale in several plant species and is of agronomic concern (Tehrani *et al.*, 1971; Blamey *et al.*, 1984; Bell *et al.*, 1990). Limits between deficiency and toxicity are very narrow and applications of boron can be extremely toxic to some plants at concentrations only slightly above the optimum for others (Eaton, 1944; Gupta, 1983). Symptoms of boron deficiency and boron toxicity have been reported for numerous plant species (Eaton, 1944; Gandhi and Mehta, 1959; Bradford, 1966; Gupta, 1979; Gupta, 1983; Gupta *et al.*, 1985; Keren and Bingham, 1985). Symptoms of boron deficiency in crop plants first appear as abnormal development of growing points and blue-green colour of young leaves. As deficiency progresses, the terminal growing points die, growth of the whole plant is reduced and flower and fruit formation is markedly restricted (Sauchelli, 1969). In contrast to boron deficiency, where symptoms develop from the young leaves, the symptoms of boron toxicity develop from the old leaves. They consist of marginal and tip chlorosis which is quickly followed by necrosis (Brenchley, 1914; Eaton, 1944) and usually boron has a tendency to accumulate in the margins of leaves (Oertli and Kohl, 1961).

Genetic variation, mechanism and inheritance of boron tolerance

Differential response to boron stress has been reported for many species, and useful genetic variation apparently exists in all major field crops for both boron deficiency (Blatt, 1976; Blamey *et al.*, 1984; Prasad and Singh, 1988; Prasad *et al.*, 1988; Mandal and Singharoy, 1989; Sakal *et al.*, 1991) and toxicity (Blatt, 1976; Mehrotra *et al.*, 1980; Cayton, 1985; Mandal and Das, 1988; Moody *et al.*, 1988; Paull *et al.*, 1988a; Picchioni and Miyamoto, 1991; Paull *et al.*, 1992).

Several investigators have found the concentrations of boron in roots and shoots are lower for tolerant than sensitive genotypes of wheat and barley (Nable, 1988; Paull *et al.*, 1988a; Nable *et al.*, 1990) and a similar mechanism appears to control the tolerance of peas and medics to boron (Paull *et al.*, 1992). The mechanisms by which roots are able to limit the

uptake of boron by tolerant plants are not yet clearly understood. There are three mechanisms that enable plants to tolerate toxic concentrations of mineral elements in soils, namely avoidence, exclusion and internal tolerance (Rathjen *et al.*, 1987; Nable and Paull, 1991).

There are several reports of major gene control of response to low boron conditions. For example, susceptibility of celery and tomato to boron deficiency are controlled by single recessive genes (Pope and Munger, 1953b; Wall and Andrus, 1962; respectively) while susceptibility of red beet to boron deficiency was reported to be controlled by a single dominant gene (Tehrani *et al.*, 1971). In a field study of the response of six sunflower inbreds and their 15 hybrids to boron deficiency, general combining ability was highly significant. This, together with a significant positive correlation between the means and general combining ability effect of inbred lines, suggests that susceptibility of a hybrid to boron deficiency can be predicted from the performance of its parents (Blamey *et al.*, 1984).

Inheritance of boron tolerance in wheat (*Triticum aestivum*) was determined from crosses among highly sensitive (Kenya Farmer), sensitive (W1*MMC), moderately sensitive (Warigal), moderately tolerant (Halberd) and tolerant (G61450) genotypes (Paull, *et al.*, 1988b: Paull *et al.*, 1991). The F_1 hybrid appeared to be intermediate between the two parents, indicating that boron tolerance in wheat is controlled by an incompletely dominant gene (s). Based on the segregation of F_2 and F_3 generations, it was shown that the inheritance of tolerance to high concentrations of boron appears to be under additive genetic control (Paull *et al.*, 1991). Three genes, *Bo1*, *Bo2* and *Bo3* were identified among the Australian material. Analysis of the Chinese Spring (Kenya Farmer) substitution lines suggested that wheat chromosome 4A has a major effect in controlling the response to a high concentration of boron (Paull *et al.*, 1988b; Paull *et al.*, 1992). Further studies using monosomic lines revealed that the *Bo1* gene is located on chromosome 7B (Chantachume *et al.*, 1993). Jenkin (1993) studied the response of barley varieties and progeny to boron and observed that F_1 hybrids among Sahara 3771 (tolerant), CM 72 (moderately tolerant) and Stirling (sensitive) were intermediate to the parents indicating boron tolerance in barley is also

expressed as a partially dominant trait. F_2 and F_3 populations among these barley genotypes were tested for segregation in response to boron. Tolerance was found to be controlled by allelic loci in the two tolerant genotypes. A continuous frequency distribution of segregating generations indicated that boron tolerance in barley is a quantitatively inherited trait and it was proposed that at least three major genes determine boron tolerance in Sahara 3771 and two in CM 72.

Inheritance of response to boron in other crops also has been investigated. For instance, Gorsline *et al.* (1964) investigated the mode of inheritance of response to boron and 10 other elements in corn by diallel analysis. The results showed additive gene action for ear leaf concentrations of all elements. A diallel analysis for different characters using wheat plants grown in boron-deficient and boron-supplemented soil showed that values for general combining ability (GCA) and specific combining ability (SCA) were variable in direction and magnitude at different levels of boron (Mandal, 1988). Another study of yield components, in eight genotypes of wheat and their 28 hybrids, in low boron soils, revealed the existence of high GCA effects in some of the genotypes (Mandal and Singharoy, 1989). These statistical analyses, while indecisive, are generally compatible with more precise genetic investigations of Paull *et al.* (1988b, 1991, 1992).

Genetic variation in response to high concentrations of boron has been reported among pea genotypes (Paull *et al.*, 1992). However, there is no published evidence of differences in boron tolerance among genotypes or varieties of other grain legumes. Since differences in boron tolerance have been found in many crops, it is reasonable to assume that differences might also be found within grain legume crops. The objectives of the present investigation were to identify genetic variation of grain legumes to high concentrations of boron with particular reference to peas, to study the mode of inheritance and to identify chromosomal location of gene (s) conferring tolerance to boron using isozyme and molecular markers.

CHAPTER 3

EFFECT OF EXCESS BORON ON AUSTRALIAN COMMERCIAL PEA VARIETIES

3.1 Introduction

Initial experiments (Materne, 1989) showed that genetic variation for response to boron exists in Australian pea varieties which were classified into three categories: tolerant: Alma, Early Dun, Maitland, Derrimut and Dundale; moderately tolerant: Collegian; intolerant (sensitive): Pennant, Buckley and Dinkum. The classification of response by Materne (1989) relied to a large extent on expression of symptoms of toxicity supported by the response in total dry matter and the rate of development for six varieties grown at three levels of applied boron.

The present investigation was undertaken to confirm the genetic variation of Australian pea varieties, to study the mechanism of tolerance and to identify selection parameters which could be used for breeding varieties tolerant to boron.

3.2 Materials and methods

Soil

The soil used was a bulk sample of silty clay loam texture from the surface (0-10 cm) of a red brown earth (Typic Haploxeralf) collected from the Glenthorne Research Farm, O'Halloran Hill, South Australia (under the name Glenthorne III soil) (Paull *et al.*, 1988a) which was kindly provided by Dr J.G. Paull, Waite Agricultural Research Institute. Chemical and physical properties of the soil are presented in Table 3.1, while the boron adsorption capacity of the soil determined by the method of Elrashidi and O'Connor (1982) is presented in Table 3.2.

To ensure a uniform soil texture, the field soil sample was sifted through a one cm screen. The boron was mixed through the soil in a modified stock feed mixer (capacity 500 kg). Boron (as H_3BO_3) was dissolved in warm water and applied in solution at the rates of 0, 10, 20, 30 and 40 mg kg^{-1} soil while the soil was being mixed. These treatments are designated as B0, B10, B20, B30 and B40.

Table 3.1 Physical and chemical properties of soil collected from the CSIRO Glenthorne Research Farm, O'Halloran Hill, South Australia.

pH	7.4	Na mmol kg^{-1}	4.7
E.C. dS m^{-1}	0.25	K mmol kg^{-1}	28.6
Cl mg kg^{-1}	67.0	Total ex Cat.	257.0
C (total)%	4.50	Texture (%)	
N (total)%	0.27	Clay	34.0
P ($NaHCO_3$ extr) mg kg^{-1}	144.0	Silt	24.0
Exchangeable cations pH 8.5			
Ca mmol kg^{-1}	199.0	Sand - fine	37.0
Mg mmol/kg	25.1	Sand - coarse	5.0

Adapted from Paull (1990).

To ensure that there were no nutrient deficiencies and also to improve the water holding capacity and aeration of the soil, blood meal and peat were added at the rates of 1.2g kg^{-1} and 20g kg^{-1}, respectively. Four kilograms of treated soil were added to each pot (200 mm diameter) lined with a water-tight polythene bag (38 micron x 305 x 455 mm). Prior to seeding, sufficient water was added to germinate weeds.

Table 3.2 Boron adsorption capacity of the soil collected from the CSIRO Glenthorne Research Farm, O'Halloran Hill, South Australia.

Conc. of B in $CaCl_2$ solution (mg kg^{-1})	Available B in filtrate (mg kg^{-1})	Adsorbed %
0	0.2	0.0
30	10.2	65.9
60	25.7	57.1
100	51.3	48.7
150	84.5	43.6
200	124.7	37.6

Adapted from Paull (1985)

Genotypes and seed treatment

The pea varieties Alma, Pennant, Dinkum, Buckley, Maitland, Derrimut, Early Dun, Dundale and Collegian were used. The pedigrees, breeding institutions and years of release of these varieties are listed in Table 3.3. All seeds were kindly provided by Dr S. M. Ali, South Australian Research and Development Institute. To ensure uniform germination, seeds were placed in plastic petri dishes containing moist filter paper, stored at 2-4° C for two days and then at room temperature for one day

Experimental design

Before seeding, the pots were arranged in a randomised complete block as a split-plot design with three replicates. Four seeds were sown two cm below the soil surface in each pot. The pots were watered by adding distilled water as required.

Table 3.3 Pedigree, breeding institution and year of release of Australian pea varieties grown in this experiment.

Variety	Breeding Institution	Year of release	Pedigree
Early Dun	-	pre-1900	Introduction, probably from U.K.
Collegian	RAC (SA)[a]	1939	White Brunswick x Early Dun
Derrimut	D.A. (Vic)[b]	1964	Collegian x MU 244 C (Italian-East Africa)[c]
Buckley	D.A. (Vic)	1970	White Brunswick x MU33
Dundale	D.A. (SA)[d]	1970	Selection from Early Dun
Pennant	WARI (SA)[e]	1977	White Brunswick x CPI 15247 (Mediterranean line)
Alma	D.A. (SA)	1986	White Brunswick x PI 173052 (Turkish line)
Maitland	D.A. (SA)	1986	Early Dun x JI 143 (John Innes line, U.K.)
Dinkum	D.A. (Vic)	1988	Complex cross involving Victorian Dippes Gelbe, Early Dun, Buckley and other introductions [f]

[a] Roseworthy Agricultural College, South Australia

[b] Department of Agriculture, Victoria

[c] MU = Melbourne University

[d] Department of Agriculture, South Australia

[e] Waite Agricultural Research Institute, South Australia

[f] Daratech Pty. Ltd. (1988)

<u>Growth measurements</u>

Emergence was measured one and two weeks after sowing. After establishment (21 days after sowing) seedlings were thinned to two plants per pot. At this time, the plant characteristics such as number of shoots, number of nodes, height of plants and visual symptoms of boron toxicity were recorded. Thinned plants from the three replicates of each treatment were combined, oven dried, ground and analysed for concentration of boron in the plant tissue. As the values for concentrations of boron represented composite samples from all three replicates, statistical analysis was not possible for this stage. Date of flowering for varieties in each treatment was recorded during the experiment.

<u>Harvesting and tissue analysis</u>

Plants were harvested at the time when most varieties had commenced flowering at the B0 treatment (45 days after sowing), and plant characteristics (number of shoots, height of plants, number of nodes and visual symptoms of boron toxicity) were measured for each pot. Visual symptoms of boron toxicity were recorded on a scale of 0-8 (Table 3.4), adapted from Materne (1989). In order to gain an understanding of the effect of boron toxicity on leaf area, leaf dry matter and stem dry matter, the stems and leaves of two varieties (Alma and Pennant) were separated and leaf areas for all treatments were measured using a Paton Electronic Planimeter. All samples were then dried at 80°C for 48 hours and ground. The plant samples were digested in 70% nitric acid at 140°C and the concentrations of boron determined by inductively coupled plasma spectrometry (ICP) (Zarcinas *et al.*, 1987).

<u>Statistical analysis</u>

Analysis of variance was based on a split-plot design (replicates, boron treatments, varieties). Separations of means were always based on the significance level of factors using Duncan's multiple range test and LSD test (Cochran and Cox, 1966). A paired t-test (Steel and Torrie, 1960) was performed to determine the significance of differences between the two varieties Alma and Pennant for leaf and stem dry matter.

Table 3.4 Visual scoring system[a] based on the severity and appearance of foliar symptoms of boron toxicity in peas.

Score	Symptoms
0.0	No apparent symptoms
0.5	Necrotic spotting, slight chlorosis, no marginal necrosis
1.0	Marginal necrosis on the oldest set of leaves
1.5	As 1, plus necrotic spotting of second set of leaves
2.0	Marginal necrosis on less than or equal to 25% of total leaf area
2.5	As 2, plus necrotic spotting of third set of leaves
3.0	Marginal necrosis on 26% to 50% of total leaf area
3.5	As 3, plus necrotic spotting of fourth set of leaves
4.0	Marginal necrosis on 51% to 75% of total leaf area plus complete necrosis of bottom leaves
4.5	As 4, plus necrotic spotting of fifth set of leaves
5.0	Marginal necrosis on greater than 75% of total leaf area plus complete necrosis of second bottom leaves
5.5	All leaves with marginal necrosis except youngest leaves
6.0	Plants wilted
7.0	Only stem green
8.0	Dead

[a] Modified from the visual scoring method described by Materne (1989) .

3.3 Results

Emergence

The percentage emergence was measured one and two weeks after sowing. Boron treatments had no significant effect on emergence (Table 3.5) and approximately 70% and 90% of plants had emerged after one and two weeks, respectively. The emergence percentages of Pennant and Buckley were significantly lower than for all other varieties (Table 3.6). However, as this effect was observed at all treatments, it could not be attributed to the effect of boron.

Visual symptoms

Varieties were rated for the severity of symptoms of boron toxicity 21 and 45 days after sowing. The initial symptoms were characterised by light brown specks near the margin of the distal half of the leaf and developed first on the bottom set of leaves. The development of boron toxicity symptoms followed a similar progression to that described by Brenchley (1914) and Salinas *et al.* (1981) for pea seedlings. As toxicity progressed, the light brown specks turned necrotic and symptoms developed progressively from the leaf margin to the centre of the leaf and, in severe cases, resulted in death of the leaf (Plate 3.1). Symptoms were first observed for the highest boron rate (40 mg kg^{-1}) 10 days after sowing.

The severity of symptoms of boron toxicity increased with successively higher boron treatments and symptoms were more severe at the second stage of scoring (Table 3.5). The rapidity at which necrosis appeared and the severity of the symptoms varied among genotypes (Table 3.6, Plate 3.2 and 3.3) and the variety x treatment interaction was highly significant (Table 3.7). Early Dun, Dundale, Derrimut and Alma developed the least severe symptoms of toxicity, while symptoms for Dinkum were most severe.

Table 3.5 Percentage emergence[a] and visual symptoms of toxicity for nine pea varieties when grown at five levels of boron.

Treatment ($mg\ B\ kg^{-1}$)	Percentage emergence		Visual score	
	7 days after sowing	14 days after sowing	(21)[b]	(45)[b]
B0	73.1	92.6	0.00 d	0.00 e
B10	78.7	92.6	2.43 c	2.85 d
B20	62.0	91.7	3.40 b	3.70 c
B30	66.6	90.7	3.88 b	4.20 b
B40	63.0	89.8	4.45 a	5.04 a
Significance levels	n.s	n.s	*P<0.01*	*P<0.01*

[a] Values within a column followed by a common letter are not significantly different according to Duncan´s multiple range test.

[b] (21) and (45) indicate days of first and second scoring.

Table 3.6 Percentage emergence[a] and visual symptoms of toxicity for nine pea varieties when grown at five levels of boron.

Variety	Percentage emergence		Visual score	
	7 days after sowing	14 days after sowing	(21)[b]	(45)[b]
Alma	85.0 ab	100.0 a	2.47 e	2.67 d
Dundale	74.9 b	95.0 a	2.54 e	2.73 d
Early Dun	75.0 b	98.3 a	2.50 e	2.77 d
Maitland	76.7 b	95.0 a	2.70 cde	3.13 c
Collegian	96.7 a	98.4 a	3.00 bc	3.57 b
Buckley	33.3 c	61.7 c	2.90 bc	3.10 c
Derrimut	81.7 ab	100.0 a	2.67 de	2.87 d
Pennant	48.3 c	78.3 b	3.20 ab	3.50 b
Dinkum	46.7 c	96.7 a	3.40 a	4.10 a
Significance levels	*P<0.01*	*P<0.05*	*P<0.01*	*P<0.01*

[a] Values within a column followed by a common letter are not significantly different according to Duncan´s multiple range test.

[b] (21) and (45) indicate days of first and second scoring.

Table 3.7 Symptoms of toxicity for nine pea varieties when grown at five levels of boron, at 21 and 45 days after sowing.

Variety	Symptoms of boron toxicity									
	B0		B10		B20		B30		B40	
	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)
Alma	0.0	0.0	1.8	2.2	3.2	3.3	3.5	3.5	3.8	4.3
Dundale	0.0	0.0	2.5	2.5	3.0	3.3	3.3	3.5	3.8	4.3
Early Dun	0.0	0.0	2.2	2.7	3.2	3.3	3.5	3.7	3.7	4.2
Maitland	0.0	0.0	2.5	3.2	2.8	3.7	4.0	4.0	4.2	4.8
Collegian	0.0	0.0	2.3	3.3	3.7	4.0	4.5	5.0	4.7	5.5
Buckley	0.0	0.0	2.2	2.7	3.3	3.3	4.0	4.7	5.0	5.3
Derrimut	0.0	0.0	2.5	2.7	3.2	3.0	3.5	3.7	4.2	5.0
Pennant	0.0	0.0	2.8	2.8	3.7	4.3	4.7	4.8	5.3	5.5
Dinkum	0.0	0.0	3.0	3.7	4.3	5.0	4.3	5.5	5.3	6.3

Interactions for 21 days and 45 days were significant ($P < 0.01$) and ($P < 0.05$), respectively. To compare values of first stage of scoring: in a column LSD 0.01 = 0.18; in a row, LSD 0.01 = 0.37 and to compare values for second stage of scoring: in a column LSD 0.01 = 0.10; in a row, LSD 0.01 = 0.35.

Plate 3.1 Range of boron toxicity symptoms on the leaves of peas showing the marginal necrosis and brown spotting.

Boron toxicity symptoms

(Fieldpea)

Plate 3.2 Response of Australian pea varieties to high concentrations of soil boron.

(a) From left to right: Collegian, Maitland, Pennant, Early Dun, Derrimut, Dundale, Dinkum, Buckley and Alma. Plants were sown in soil with 10 mg B kg^{-1}.

(b) From left to right: Collegian, Maitland, Pennant, Early Dun, Derrimut, Dinkum, Dundale Buckley and Alma. Plants were sown in soil with 40 mg B kg^{-1}.

(a)

(b)

Plate 3.3 Effect of five boron treatments on two pea varieties.

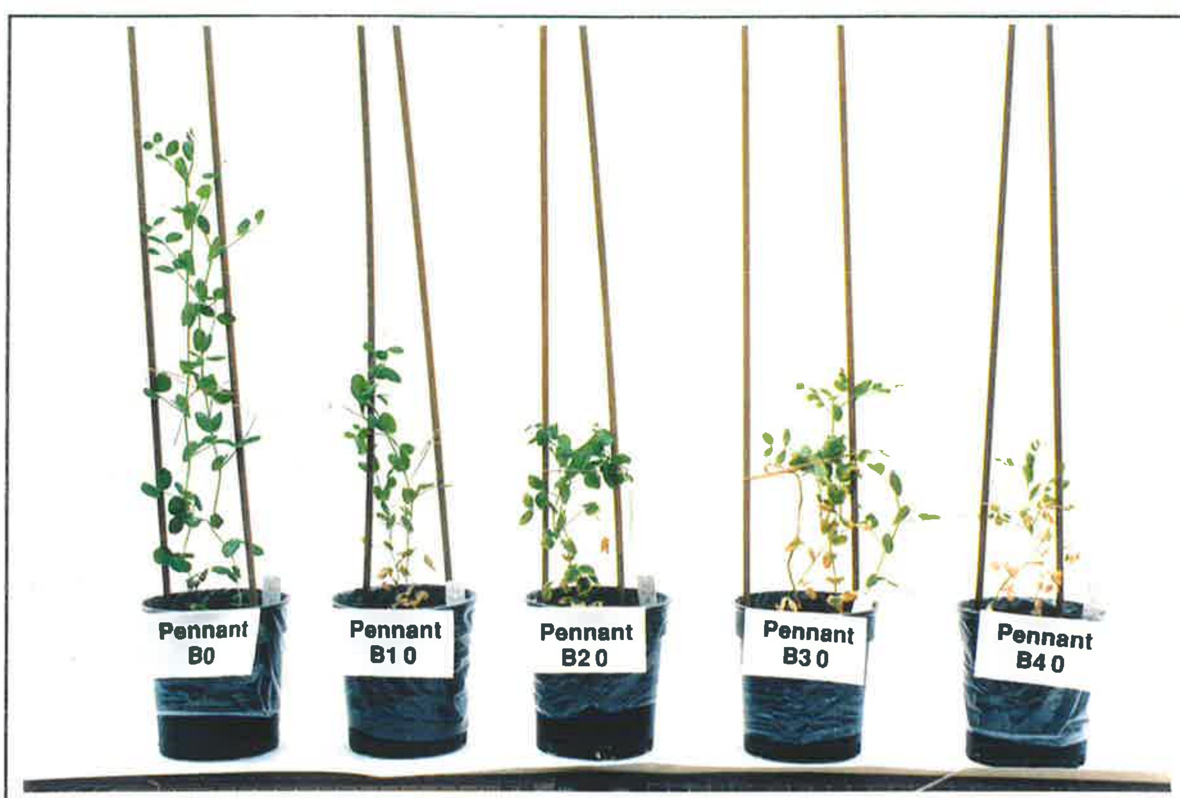
(a) Alma (moderately tolerant)

(b) Pennant (sensitive)

Treatments are from left to right are: 0, 10, 20, 30 and 40 mg B kg^{-1}.

(a)

(b)



Rate of plant development

The effect of boron treatments upon the development of secondary branches could not be assessed as plants generally did not develop these even at the control treatment, although Derrimut and Buckley produced a limited number of shoots at B10, B20 and B30. The experiment was undertaken in summer, so the low degree of branching was probably related to the growing season.

Varieties differed in length of time to the commencement of flowering. All varieties had started flowering at the B0 treatment prior to harvest, while flowering was delayed by 3-4 days at B10 and B20 and by 5-7 days at B30 and B40 relative to B0. At the time the experiment was harvested, Buckley, Pennant and Dinkum had not commenced flowering at B40.

Number of nodes

The number of nodes for the five boron treatments at 21 and 45 days are given in Table 3.8. The number of nodes decreased with increasing levels of boron, and particularly at B40 where plants developed 8% fewer nodes than in the control treatment for the first stage of scoring. Number of nodes at B0 was higher than for other treatments, although there was no significant difference between B0, B10, B20 and B30 at the first stage. However, there were significant differences between B0 and other treatments at the second stage. Differences were observed among varieties for the number of nodes (Table 3.9). The varieties with the highest number of nodes included Collegian, Alma, Early Dun and Maitland, while the semi-dwarf variety Dinkum produced significantly fewer nodes than all other varieties. The variety x treatment interaction at the first stage was also significant ($P < 0.05$, Table 3.10).

The height of plants

Table 3.8 shows the influence of boron treatments on the height of plants. Boron treatments did not appreciably affect the height of plants, with the exception of B40, at

which treatment heights were reduced 14% and 24% relative to B0 at 21 and 45 days after sowing, respectively.

Varieties differed in their height and had a similar pattern at both stages of measurement (Table 3.9). There was no significant difference among Alma, Collegian, Maitland, Early Dun and Dundale, and these varieties were taller than Derrimut, Pennant and Buckley, which were not significantly different. Dinkum was significantly shorter than all other varieties. The variety x treatment interaction at both the first and second stages was also highly significant ($P < 0.01$, Table 3.11), reflecting the differing responses of the varieties to increasing levels of soil boron.

Table 3. 8 Number of nodes[a] and height for nine pea varieties when grown at five levels of boron, 21 and 45 days after sowing.

Treatment (mg B kg^{-1})	Number of nodes		Height (mm)	
	(21)	(45)	(21)	(45)
B0	10.9 a	19.0 a	502 a	983 a
B10	10.5 ab	18.2 b	515 a	979 a
B20	10.4 ab	17.8 b	489 a	950 a
B30	10.4 ab	17.7 b	495 a	945 a
B40	10.0 c	16.3 c	434 b	748 b
Significance levels	$P<0.05$	$P<0.01$	$P<0.01$	$P<0.01$

[a] Values within a column followed by a common letter are not significantly different according to Duncan´s multiple range test.

Table 3.9 Number of nodes[a] and height for nine pea varieties when grown at five levels of boron, 21 and 45 days after sowing.

Variety	Number of nodes		Height (mm)	
	(21)	(45)	(21)	(45)
Alma	11.2 b	20.3 a	619 a	1177 a
Dundale	10.6 bc	18.0 b	532 bc	1032 b
Early Dun	11.0 b	19.2 ab	578 ab	1086 ab
Maitland	11.2 b	19.4 ab	602 ab	1140 ab
Collegian	12.3 a	20.0 a	619 a	1079 ab
Buckley	9.0 d	16.3 d	403 d	853 c
Derrimut	10.7 bc	17.9 bc	461 cd	856 c
Pennant	10.0 c	16.4 cd	407 d	781 c
Dinkum	7.9 e	12.7 e	170 e	288 d
Significance levels	*P<0.01*	*P<0.01*	*P<0.01*	*P<0.01*

[a] Values within a column followed by a common letter are not significantly different according to Duncan´s multiple range test.

Table 3. 10 Number of nodes 21 days after sowing for nine pea varieties when grown at five levels of boron.

Variety	Number of nodes				
	B0	B10	B20	B30	B40
Alma	11.0	11.3	11.6	10.7	11.2
Dundale	10.3	11.3	10.5	10.3	10.5
Early Dun	10.5	11.5	11.7	11.2	10.2
Maitland	10.3	11.5	10.7	11.5	12.0
Collegian	12.5	12.2	12.2	12.5	12.2
Buckley	10.7	10.5	7.2	9.0	7.8
Derrimut	10.5	10.7	10.5	11.2	10.6
Pennant	9.7	10.3	10.5	10.5	9.0
Dinkum	9.2	11.3	10.5	10.3	10.5

Interaction was significant ($p < 0.05$). To compare values in a column LSD 0.05 = 0.36; in a row, LSD 0.01 = 1.07.

Table 3.11 Height (mm) for nine pea varieties when grown at five levels of boron, at 21 and 45 days after sowing.

Variety	Plant height (mm)									
	B0		B10		B20		B30		B40	
	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)
Alma	640	1170	627	1083	672	1281	578	1278	578	1075
Dundale	520	1022	662	1194	545	1116	423	930	510	898
Early Dun	646	998	506	1168	638	1258	598	1101	601	907
Maitland	633	1227	622	1168	530	1115	655	1192	570	998
Collegian	643	1123	615	1200	570	986	737	1150	530	935
Buckley	462	1013	567	1067	343	768	397	896	247	521
Derrimut	437	944	445	823	472	933	449	845	505	738
Pennant	422	867	427	857	463	738	470	923	253	521
Dinkum	243	452	170	292	170	315	153	242	117	143

Interactions were significant (*$P < 0.01$*) for both times of measurement. To compare values of first measurement in a column LSD 0.01 = 31.1; in a row, LSD 0.01 = 54.4 and to compare values for second measurement in a column LSD 0.01 = 44.0; in a row, LSD 0.01 = 79.7.

Dry matter

The data in Tables 3.12 and 3.13 indicate significant effects of both treatments and varieties upon dry matter. The lowest dry matter yield was obtained with the application of 40 mg B kg^{-1} of soil. Treatments B10, B20, B30 and B40 resulted in mean yield decreases of 18, 31, 42 and 64 per cent relative to the control, respectively.

Considerable variation was observed among varieties for dry weight (Table 3.13). Alma and Early Dun produced more than other varieties. However, they were not significantly different from Dundale and Collegian. Derrimut, Pennant and Maitland did not differ in dry matter but they were significantly higher than Buckley while Dinkum produced the lowest yield.

The varieties x treatment interaction was not significant (Table 3.14).

Concentration of boron in shoots

The results of plant analysis at two harvest times are given in Table 3.12. The concentration of boron in shoots increased significantly with an increase in the boron concentration in the soil (Table 3.12) and there was significant variation among the varieties in tissue boron concentrations (Table 3.13). The interaction between varieties and treatments for concentration of boron in shoots was statistically significant ($P < 0.01$, Table 3.15). Boron concentrations in tissues were lowest for Alma, Dundale, Maitland and Early Dun. In general, concentrations of boron in shoots were highest for Dinkum, while Collegian was intermediate. The ranking of varieties was similar between treatments, although there were differences between treatments in distinguishing between varieties. For example, at B20 the boron concentration of Dinkum was much higher than in Buckley, Derrimut and Pennant, but there was little difference between these four varieties at B40.

Table 3.12 Means[a] for dry matter yield and boron concentrations in shoots for nine pea varieties when grown at five levels of boron, at 21 and 45 days after sowing.

Treatment (mg B kg^{-1})	Dry matter (g)[b]	Shoot boron (mg kg^{-1})	
		(21)[c]	(45)
B0	5.15 a	33	39 e
B10	4.24 b	183	192 d
B20	3.57 c	371	373 c
B30	3.00 d	551	654 b
B40	1.85 e	971	1045 a
Significance levels	*P<0.01*		*P<0.01*

[a] Values within a column followed by a common letter are not significantly different, according to Duncan´s multiple range test.

[b] Dry matter yield at 45 days after sowing.

[c] Not statistically analysed.

Table 3.13 Means[a] for dry matter yield and concentrations of boron in shoots for nine pea varieties when grown at five levels of boron, at 21 and 45 days after sowing.

Variety	Dry matter (g)[b]	shoots boron (mg kg^{-1})	
		(21)[c]	(45)
Alma	4.77 a	336	373 e
Dundale	4.25 ab	358	343 e
Early Dun	4.59 a	352	396 de
Maitland	3.74b	299	379 e
Collegian	4.20 ab	440	447 d
Buckley	2.33 c	427	480 bc
Derrimut	3.53 b	435	492 bc
Pennant	3.45 b	509	511 b
Dinkum	1.30 d	602	725 a
Significance levels	*P<0.01*		*P<0.01*

[a] Values within a column followed by a common letter are not significantly different according to Duncan´s multiple range test.

[b] Shoot dry matter at 45 days after sowing.

[c] Not statistically analysed.

Table 3.14 Dry matter yield (g) for nine pea varieties 45 days after sowing when grown at five levels of boron.

Variety	Dry matter yield (g)				
	B0	B10	B20	B30	B40
Alma	6.20	5.59	5.59	4.02	2.47
Dundale	5.11	5.09	4.78	3.57	2.70
Early Dun	5.59	5.53	5.11	3.89	2.82
Maitland	5.10	3.95	3.56	3.20	2.44
Collegian	6.34	5.37	3.45	3.52	1.98
Buckley	4.34	3.47	1.81	1.44	0.58
Derrimut	5.07	3.81	3.37	3.48	1.87
Pennant	4.99	4.19	3.53	3.23	1.33
Dinkum	3.21	1.21	0.93	0.64	0.47

The variety x treatment interaction was not significant.

Table 3.15 Concentration of boron (mg kg^{-1}) in shoots for nine pea varieties when grown at five levels of boron, at 21 and 45 days after sowing.

Variety	Boron concentration in shoots (mg kg^{-1})									
	B0		B10		B20		B30		B40	
	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)	(21)	(45)
Alma	31	35	60	172	312	275	446	514	731	869
Dundale	23	36	32	163	275	297	387	503	700	717
Early Dun	24	41	27	156	270	291	544	524	787	966
Maitland	27	34	52	178	333	350	411	521	572	812
Collegian	34	35	78	174	471	384	564	676	911	965
Buckley	46	37	222	181	362	379	644	607	851	1193
Derrimut	44	39	225	196	414	394	628	613	862	1219
Pennant	33	41	211	235	431	345	645	683	1230	1242
Dinkum	37	53	242	275	526	629	754	1244	1410	1422

Interaction was significant (*P< 0.01*). To compare values of the second shoot analysis (45 days), in a column LSD 0.01 = 28; in a row, LSD 0.01 = 70.6.

<u>The effect of boron treatments on leaf area of Alma and Pennant</u>

The leaf area of both Alma and Pennant decreased at increasing levels of boron. Alma developed a greater leaf area than Pennant at B0 and all other treatments. At B40, the percentage reduction in leaf area relative to B0 was 80% for Pennant and 39% for Alma (Table 3.16). The difference in response of leaf area to boron treatments between Alma and Pennant was statistically significant ($t = 8.09$, $P < 0.05$).

Table 3.16 Leaf area (cm^2) of two pea varieties when grown at five levels of boron.

Variety	Leaf area (cm^2)				
	B0	B10	B20	B30	B40
Alma	914	840	832	739	556
Pennant	695	600	463	456	139

<u>The effect of boron treatments on leaf, stem and tissue dry matter of Alma and Pennant</u>

The boron treatments affected the dry matter of leaves and stems of both Alma and Pennant (Table 3.17). There is a significant difference between varieties for stem dry matter and the stems of Pennant were affected by boron treatments to a much greater extent than those of Alma.. There was no significant difference between the two varieties for the reduction in yield of leaves at B40 relative to the control.

Table 3.17 Leaf and stem dry matter (g) for two pea varieties when grown at five levels of boron. Relative dry matter (% of B0) are presented in brackets.

Variety	Dry matter (g)				
	B0	B10	B20	B30	B40
Leaf dry matter[a]					
Alma	2.83	2.66 (94)	2.67 (94)	1.98 (70)	1.06 (37)
Pennant	2.31	1.87 (81)	1.57 (68)	1.48 (64)	0.82 (36)
Stem dry matter					
Alma	3.36	2.92 (87)	2.04 (87)	2.04 (61)	1.38 (41)
Pennant	2.67	2.31 (86)	1.75 (73)	1.74 (62)	0.50 (19)

[a] t values comparing leaf and stem dry matter between varieties are 2.38 (n.s.) and 5.93 ($P < 0.05$), respectively.

3.4 Discussion

Substantial genetic differences were found among Australian pea varieties in growth response and concentrations of boron in tissues when grown under high boron conditions. The concentrations of boron in the shoots of varieties which produced high dry matter yields at high boron treatments were significantly lower than those of the other varieties. Alma, Early Dun, Maitland and Dundale had lower concentrations of boron in tissues, while this value was very high for Dinkum (Table 3.15).

The symptoms of boron toxicity were more severe at higher boron treatments and the maximum differences between varieties occurred at the second time of scoring at the B40 treatment, although significant differences between varieties also resulted at the lower boron treatments and at the first stage of scoring. Alma developed the least severe symptoms of toxicity, while symptoms for Dinkum were very severe (Table 3.7). The significant genotype x treatment interaction ($P < 0.01$) for visual score could be attributed to a greater increase in the severity of symptoms for the sensitive varieties at the higher boron treatments. High concentrations of boron in tissues were associated with boron toxicity symptoms which were more severe in intolerant varieties than moderately tolerant varieties.

The joint distribution of values of relative tissue dry matter, tissue boron concentration and visual symptoms of boron toxicity for the nine varieties at the different levels of boron demonstrate the genetic variation for boron response among varieties (Fig. 3.1). This variation among varieties is consistent over treatments but is most pronounced at higher boron treatments.

The response of varieties in terms of concentration of boron in tissues and visual score of toxicity symptoms was consistent with results for dry weight. The correlation coefficients that resulted between the three parameters shoot dry weight, concentration of boron in shoots and visual score of boron toxicity were as follows:

(1) relative dry matter and concentration of boron in shoots ($r = -0.78$, $P< 0.01$, Fig. 3.2 a)

(2) relative dry matter and visual score (r = -0.74, $P < 0.01$, Fig. 3.2 b) and,

(3) concentration of boron in shoots and visual score (r = 0.81, $P < 0.01$, Fig. 3.2 c).

Based on the results for these three parameters, the varieties can be grouped into four categories:

- Moderately tolerant: Alma, Early Dun, Dundale and Maitland,
- Moderately sensitive: Collegian and Derrimut,
- Sensitive: Buckley and Pennant,
- Very sensitive: Dinkum.

Fig 3.1 Distribution of relative tissue dry matter, shoot boron concentration and visual symptoms of boron toxicity for nine Australian pea varieties at four levels of boron

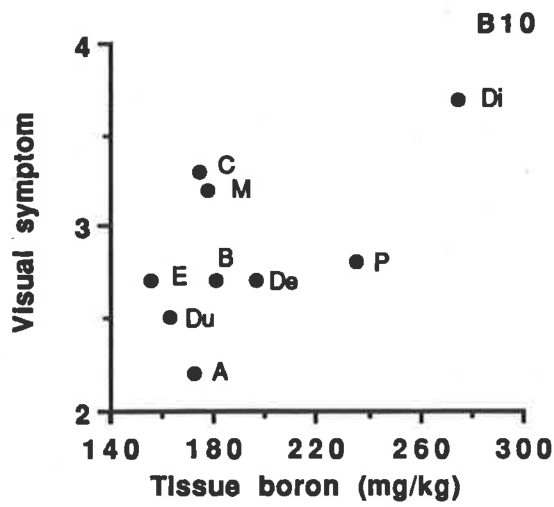
B10 and B20 treatment:

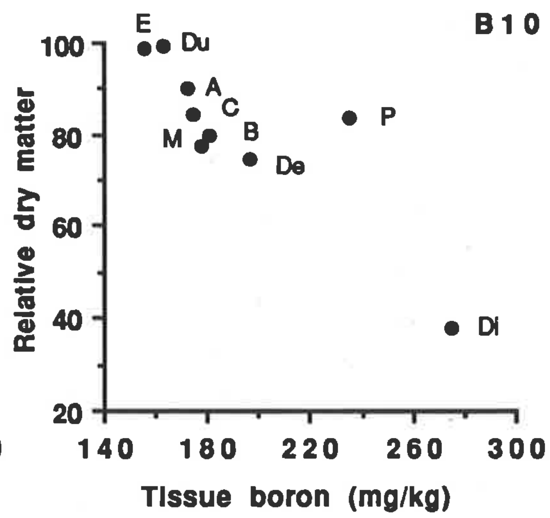
Visual symptoms of boron toxicity V tissue boron concentration

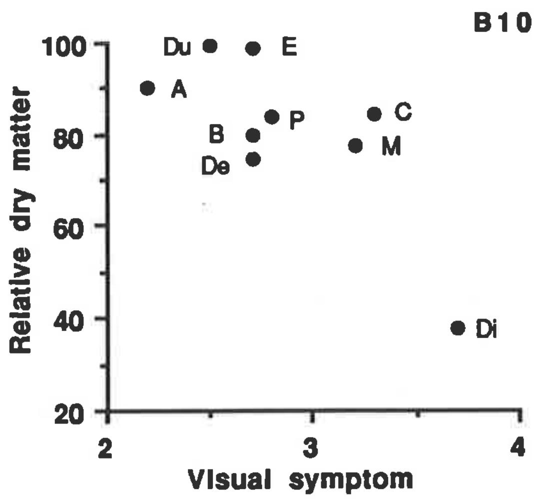
Relative dry matter V tissue boron concentration

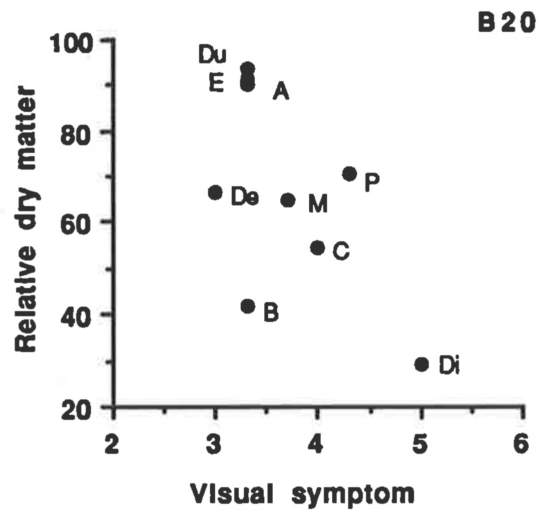
Relative dry matter V visual symptoms of boron toxicity

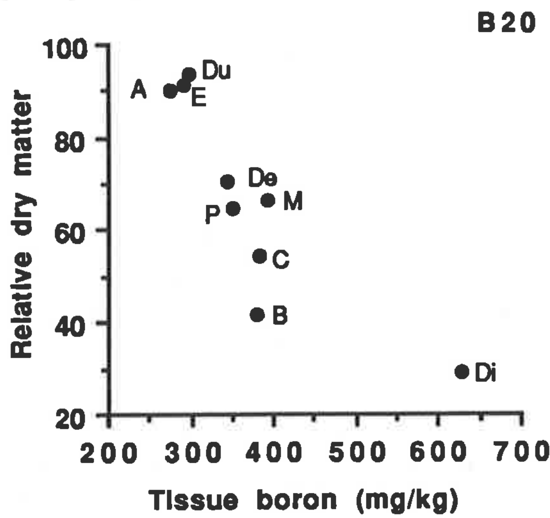
A, B, C, De, Di, Du, E, M and P refer to Alma, Buckley, Collegian, Derrimut, Dinkum, Dundale, Early Dun, Maitland and Pennant.


B10
Visual symptom
Tissue boron (mg/kg)
4
3
2
140
180
220
260
300
Di
C
M
B
E
De
P
Du
A


B10
Relative dry matter
Tissue boron (mg/kg)
100
80
60
40
20
140
180
220
260
300
E
Du
A
C
P
M
B
De
Di


B10
Relative dry matter
Visual symptom
100
80
60
40
20
2
3
4
Du
E
A
C
P
B
M
De
Di


B20
Relative dry matter
Visual symptom
100
80
60
40
20
2
3
4
5
6
Du
E
A
P
De
M
C
B
Di


B20
Relative dry matter
Tissue boron (mg/kg)
100
80
60
40
20
200
300
400
500
600
700
Du
A
E
De
M
P
C
B
Di

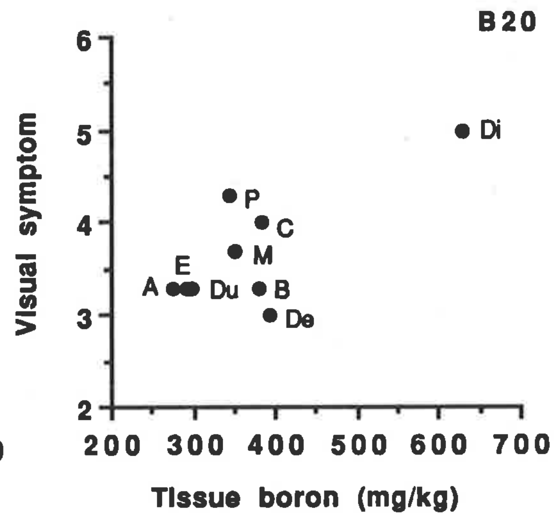

B20
Visual symptom
Tissue boron (mg/kg)
6
5
4
3
2
200
300
400
500
600
700
Di
P
C
M
E
A
Du
B
De

Fig 3.1 (continued)

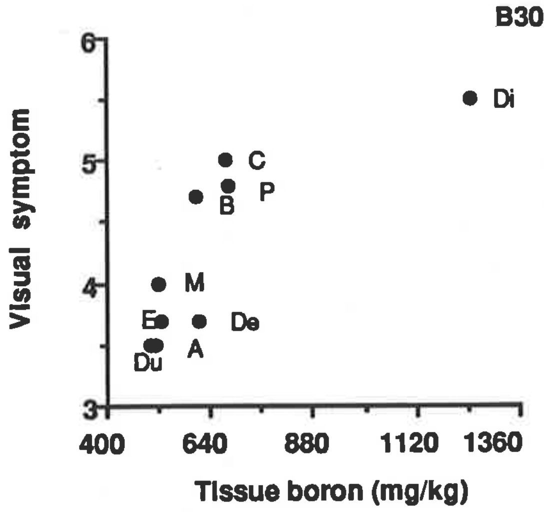
B30 and B40 treatment:

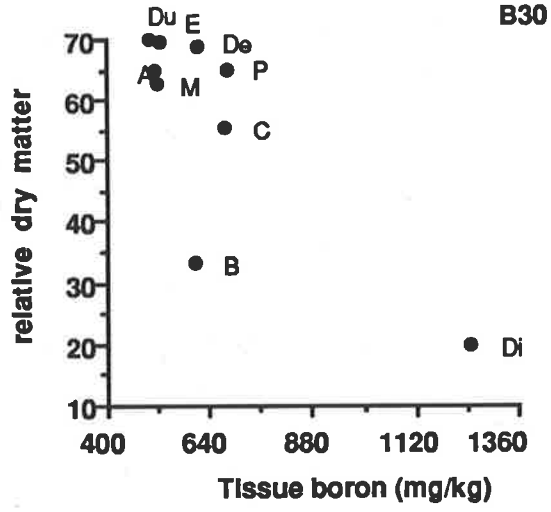
Visual symptoms of boron toxicity V tissue boron concentration

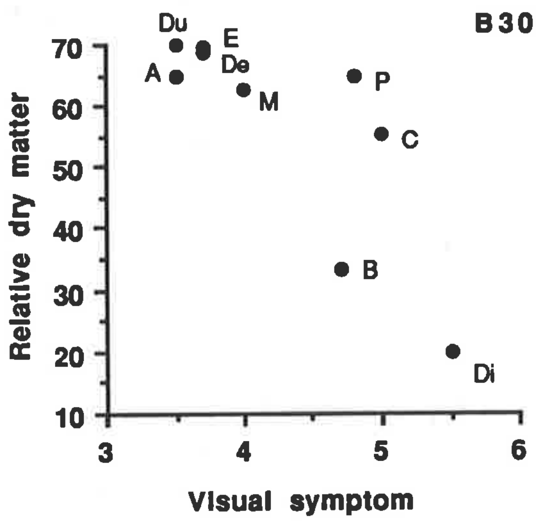
Relative dry matter V tissue boron concentration

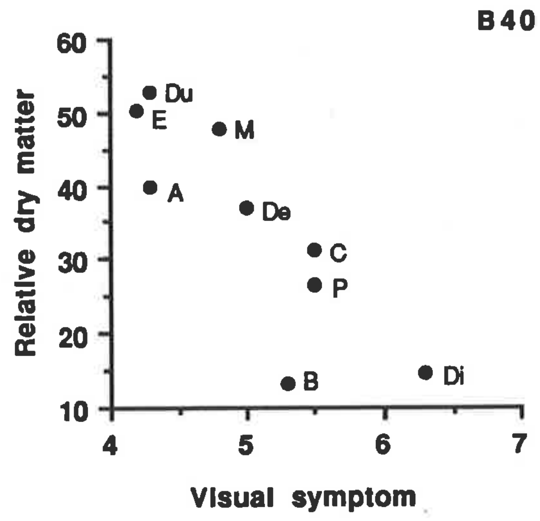
Relative dry matter V visual symptoms of boron toxicity

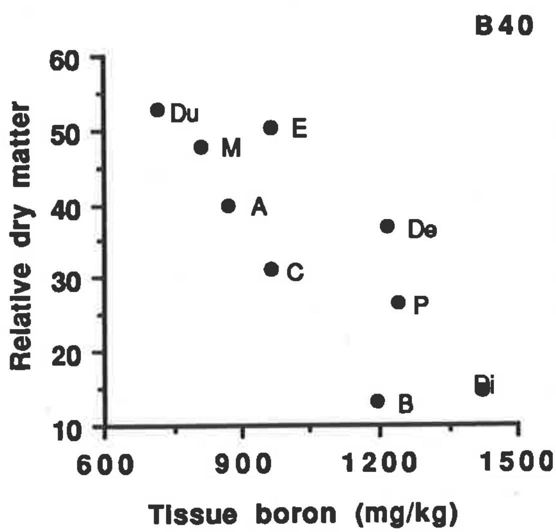
A, B, C, De, Di, Du, E, M and P refer to Alma, Buckley, Collegian, Derrimut, Dinkum, Dundale, Early Dun, Maitland and Pennant.


B30
Visual symptom
Tissue boron (mg/kg)
3
4
5
6
400
640
880
1120
1360
Di
C
P
B
M
E
De
Du
A


B30
relative dry matter
Tissue boron (mg/kg)
10
20
30
40
50
60
70
400
640
880
1120
1360
Du
E
De
P
A
M
C
B
Di


B30
Relative dry matter
Visual symptom
10
20
30
40
50
60
70
3
4
5
6
Du
E
De
A
M
P
C
B
Di


B40
Relative dry matter
Visual symptom
10
20
30
40
50
60
4
5
6
7
Du
E
M
A
De
C
P
B
Di


B40
Relative dry matter
Tissue boron (mg/kg)
10
20
30
40
50
60
600
900
1200
1500
Du
E
M
A
De
C
P
Di
B

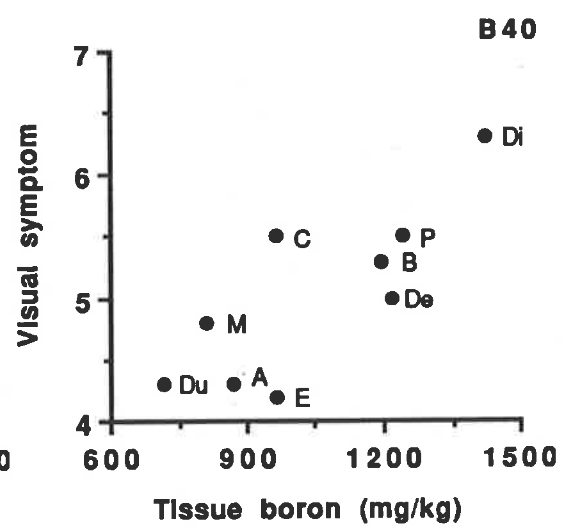
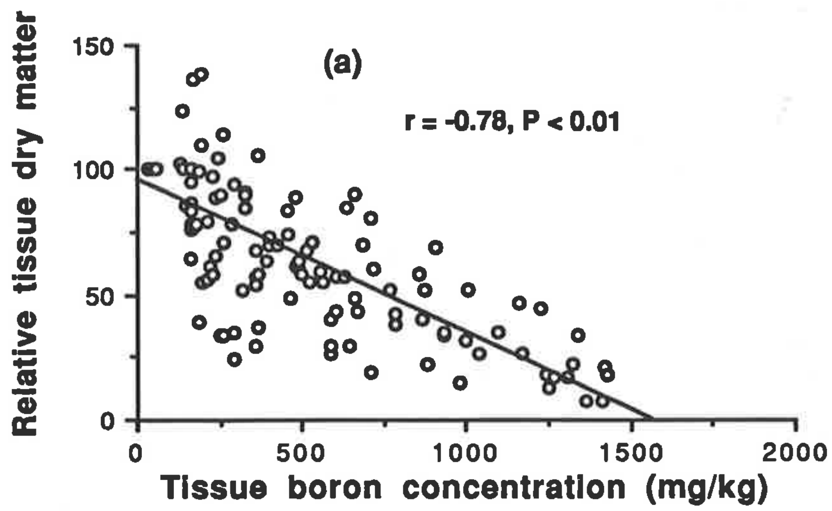

B40
Visual symptom
Tissue boron (mg/kg)
4
5
6
7
600
900
1200
1500
Di
C
P
B
De
M
Du
A
E

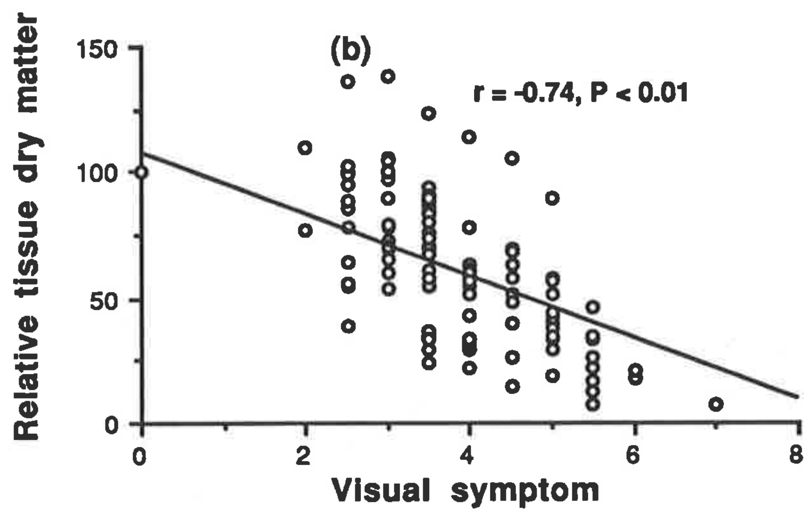
Fig. 3.2 Correlations between growth response parameters of peas to high levels of soil boron.

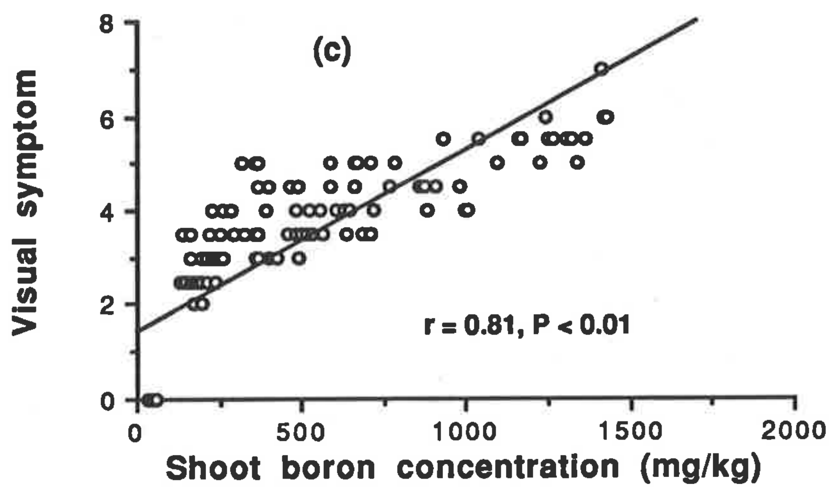
(a) relative tissue dry matter V tissue boron concentration

(b) relative tissue dry matter V visual score of boron toxicity

(c) tissue boron concentration V visual score of boron toxicity


(a)
r = -0.78, P < 0.01
Relative tissue dry matter
150
100
50
0
0
500
1000
1500
2000
Tissue boron concentration (mg/kg)


(b)
r = -0.74, P < 0.01
Relative tissue dry matter
150
100
50
0
0
2
4
6
8
Visual symptom


(c)
r = 0.81, P < 0.01
Visual symptom
8
6
4
2
0
0
500
1000
1500
2000
Shoot boron concentration (mg/kg)

The pedigree map of Australian varieties which are used in this experiment is presented in Fig. 3.3. The evaluation of, JI 143, PI 173052, L58 and Viktoria Dippes Gelbe for response to high concentrations of boron was undertaken by Materne (1989) and the response of White Brunswick was recorded in Chapter 4. There has been a limited use of exotic germplasm in Australian pea breeding and Early Dun and White Brunswick are two important parental genotypes of Australian pea varieties. Early Dun could be a direct introduction from the United Kingdom, possibly during the early 1900, and White Brunswick is an English garden pea (Hedrick *et al.*, 1928). The varieties (e.g. Buckley) that have White Brunswick as one parent are sensitive except Alma which is moderately tolerant and the tolerant response of Alma appears to have been derived from PI 173052 (Materne, 1989). On the other hand, varieties that have Early Dun as a parent include both moderately tolerant (e.g. Maitland) and moderately sensitive (e.g. Collegian) types.

This experiment was conducted to identify the most reliable and efficient parameters for distinguishing between pea varieties for response to high concentrations of boron in the soil. Emergence, plant height and number of nodes were less suitable than symptom expression, boron concentrations in the shoot and dry weight production for predicting the response of the varieties to boron. While there was a highly significant interaction between varieties and boron treatments for plant height at both times of scoring, with the relative heights of the sensitive varieties Dinkum, Buckley and Pennant being affected to a much greater extent by high boron treatments than the more tolerant varieties, there was significant variation among the heights of varieties at the control treatment. For this reason the height of plants would not be suitable as a selection criterion in a screening program where lines are grown at only a single applied boron treatment. The number of nodes also differed significantly between varieties at the control treatment, with the tallest varieties having the greatest number of nodes. The response of varieties to boron, with respect to number of nodes, was inconsistent between the two times of scoring and the interaction was significant ($P< 0.05$) at only the first stage. Emergence of varieties was not affected by boron treatments.

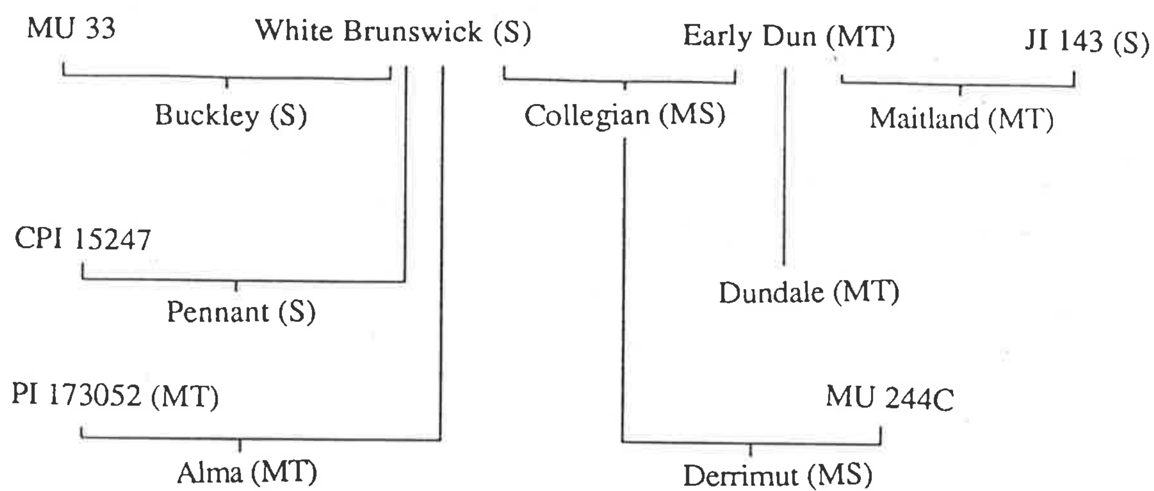
A significant reduction in shoot dry weight occurred at each increase in the level of boron and yields were lowest at the B40 treatment (Table 3.12) but the interaction among genotypes and treatments was non significant, an unexpected finding in view of the other results. Among the genotypes studied Alma, Dundale, Early Dun and Collegian produced the highest and Dinkum the least dry weight (Table 3.14). The high mean dry weight yield of Collegian could be attributed to high yield at the control treatment rather than at high boron treatments. On the other hand, the semi-leafless variety Maitland, which produced a low overall mean yield and a low yield at the control treatment was among the highest yielding varieties at B40 and on this basis would be considered tolerant to boron.

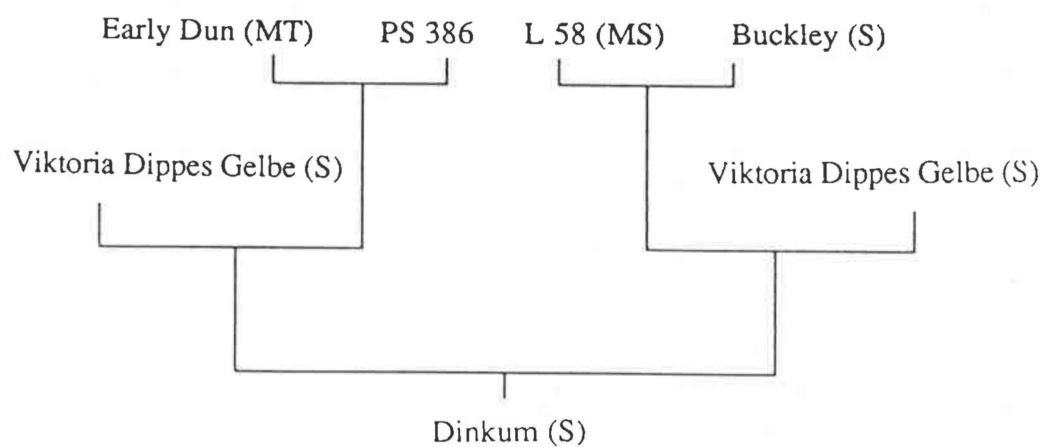
The data in Table 3.14 indicate that the lowest boron treatment (B10) was toxic for pea and a 17.6% reduction in dry weight of shoots occurred in this treatment. The concentration of boron in shoots at this level over all varieties was 192 mg kg^{-1}, although the value differed significantly among varieties (Table 3.15). For example, Alma had the lowest concentration of boron in shoots (172 mg kg^{-1}), while the concentration was highest for Dinkum (275 mg kg^{-1}). Salinas *et al.* (1981) suggested that a leaf boron concentration in the range of 50-300 mg kg^{-1} at harvest time might be considered as sufficient for normal pea growth. They also reported that a leaf boron concentration of 350 mg kg^{-1}, when associated with 2 mg B l^{-1} for solution culture, is within the toxicity range (Salinas *et al.*, 1986). Toxicity of peas has also been reported when 4 mg kg^{-1} boron was supplied in irrigation water, leading to 2 mg kg^{-1} soil solution boron and 213 mg kg^{-1} boron in plant tissues (Chauhan and Powar, 1978). Gupta and MacLeod (1981) reported the critical level for foliar toxicity symptoms as > 61 mg B kg^{-1}. This low critical value for toxicity may reflect the use of symptom development as the criterion for toxicity, whereas other workers have measured dry weight reduction. Although there is considerable variation in toxic concentrations of boron reported in the literature, the concentrations of boron in plants associated with boron toxicity described in this chapter are within the range of those reported by previous researchers.

There are several mechanisms that would enable plants to tolerate high concentrations of mineral elements in soils, namely avoidance (e.g. a shallow root system to avoid elements, such as boron, which accumulate in the subsoil), exclusion from the root system, and internal tolerance (Rathjen *et al.*, 1987). Several investigators have reported the concentration of boron in shoots is lower for tolerant than sensitive genotypes of wheat and barley (Nable, 1988; Paull *et al.*, 1988a; Nable *et al.*, 1990) and a similar mechanism appears to control the tolerance of peas to boron. Nable (1988) also measured low concentrations of boron in the roots of tolerant wheat and barley varieties and suggested that the tolerance was governed by the ability of varieties to exclude boron, but the mechanisms limiting uptake of boron are not yet understood.

This experiment has demonstrated that variation in boron tolerance exists among Australian commercial pea varieties. A wide range of pea accessions should now be examined to determine the extent of genetic variation within the species *P. sativum* and to identify lines more tolerant than the Australian varieties which may be used to introduce tolerance to the commercial pea varieties by breeding.

Fig. 3.3 Pedigrees of Australian pea varieties grown in this experiment. The evaluation of, JI 143, PI 173052, L58 and Viktoria Dippes Gelbe for response to high concentrations of boron was undertaken by Materne (1989).


MU 33
White Brunswick (S)
Early Dun (MT)
JI 143 (S)
Buckley (S)
Collegian (MS)
Maitland (MT)
CPI 15247
Pennant (S)
Dundale (MT)
PI 173052 (MT)
MU 244C
Alma (MT)
Derrimut (MS)


Early Dun (MT)
PS 386
L 58 (MS)
Buckley (S)
Viktoria Dippes Gelbe (S)
Viktoria Dippes Gelbe (S)
Dinkum (S)

CHAPTER 4
EVALUATION OF THE RESPONSE OF EXOTIC GERMPLASM OF PEAS TO HIGH CONCENTRATIONS OF SOIL BORON

4.1 Introduction

The experiment described in Chapter 3 demonstrated that a limited range of genetic variation in boron tolerance exists among Australian pea varieties. Also, preliminary investigations indicated that the variation in response to boron among the Australian international pea collection was greater than that in Australian commercial varieties (Materne, 1989; Paull *et al.*, 1992).

In general, highly variable soil conditions in the field mitigate against evaluation, especially during the screening of early generations when replication is difficult or impossible (Lewis and Christansen, 1981). This is especially the case with boron in soil which is heterogenous both laterally and vertically (Cartwright *et al.*, 1984; 1986), so field screening of a large number of genotypes or breeding lines is impractical (Paull *et al.*, 1988a). As a consequence, the procedure adopted for screening for tolerance to boron at the Waite Agricultural Research Institute consists of growing plants under controlled conditions in a soil to which a high level of boron has been applied (Moody *et al.*, 1988).

The first major objective of this work was to gain a comprehensive summary of the extent and geographical distribution of boron sensitivity and tolerance in *P. sativum* and to identify sources of tolerance for the breeding program. A second major objective was to confirm selection criteria identified in Chapter 3 for screening for boron tolerance in pea breeding programs.

Following the identification of genotypes more tolerant to boron than that currently available in Australia, more detailed investigations were undertaken at a range of boron treatments to

confirm the performance of the putative boron tolerant accessions compared to Australian varieties. In this experiment, the concentrations of boron in tissues were measured as a critical evaluation of the relative level of tolerance.

As already mentioned (Chapter 2), South Australian soils are generally alkaline throughout or have an alkaline reaction trend with depth (Cartwright *et al.*, 1987; Rathjen *et al.*, 1987). High concentrations of boron occur in the subsoil and are associated with sodicity (Cartwright *et al.*, 1986). As chemical analysis of plant tissue by ICP-spectrometry enables the concentrations of many elements to be monitored simultaneously, the concentrations of sodium in the tissue of selected tolerant lines were also examined.

4.2 Materials and methods

4.2.1 Glasshouse screening

The response to high concentrations of boron was measured for 617 accessions representative of the geographical distribution of *P. sativum* and including Australian varieties and advanced breeding lines. All seeds were kindly provided by Dr. S.M. Ali, South Australian Research and Development Institute.

The screening was conducted in boxes (2m x 1m x 0.25 m) containing soil (Chapter 3) to which boron had been applied at the rate of 100 mg kg^{-1} soil. The concentration of boron extractable in hot $CaCl_2$ (Spouncer *et al.*, 1992) was 58 mg kg^{-1}.

To ensure uniform germination, seeds were allowed to imbibe on moist filter paper in plastic petri dishes, stored at 2-4°C for two days and placed at room temperature (approximately 20-25°C) for one day. The accessions were randomised for planting. Six seeds from each accession were sown 2 cm deep in each of two randomly allotted rows. Four replicates of Alma (the most tolerant Australian cultivar) were sown systematically throughout each box to act as a check for comparisons among genotypes. The boxes were located in an evaporatively cooled glasshouse and watered as required.

Four weeks after planting, genotypes were scored for severity of symptoms on the basis of leaf damage on a scale of 0-8, described in Chapter 3. The mean was calculated for each accession and accessions were then assigned to one of four categories, namely: tolerant, moderately tolerant, moderately sensitive and sensitive. The check variety Alma was classified as moderately tolerant. The scale used was as follows:

Score	Classification
≤ 3.0	Tolerant
$3.0 < X < 4.0$	Moderately tolerant
$4.0 \leq X < 5.0$	Moderately sensitive
≥ 5.0	Sensitive

4.2.2 Glasshouse experiment

This study was conducted to confirm the observations of the initial screening, in which a number of accessions of peas more tolerant than Alma to high concentrations of boron were identified. As the initial classification was based on the expression of symptoms of boron toxicity under a single boron treatment, and therefore relied upon the correlation between symptom expression and the boron tolerance as defined in the experiment reported in Chapter 3, a more precise confirmation of the boron tolerance was warranted.

Genotypes and seed treatment

Nine putative tolerant pea accessions, selected to represent geographically widespread origins, were compared to three Australian pea varieties and two South Australian advanced lines (Table 4.1). Seeds were germinated as described previously (Section 4.2.1) and five seeds were sown 2 cm below the soil surface in each pot. The plants were watered with deionised water as required. The seedlings were thinned to three evenly spaced plants per pot, three weeks after sowing.

Table 4.1 Collection number, pedigree and country of origin of pea genotypes grown in a pot experiment. (PIG, Plant Industry Genetics (CSIRO, Canberra)); CPI, Commonwealth Plant Introduction; SA, South Australian Research and Development Institute; NGB, Nordic Gene Bank, Sweden.

Genotype and collection number	Pedigree	Country of origin
PIG 16	-	India
PIG 36	-	Colombia
CPI 65352	-	Unknown
SA 132 (NGB 1430)	-	Afghanistan
SA 395	-	India
SA 213 (NGB 1779)	-	Netherlands
SA 448	-	India
SA 310 (NGB 2126)	-	Afghanistan
NGB 1574	-	Ethiopia
SA 1512	-	S. A. advanced line
M93	Early Dun x JI 143	S. A. advanced line
Alma	White Brunswick x PI 173052	Australian variety
Early Dun	Introduction from UK	Australian variety
Pennant	White Brunswick x CPI 15247	Australian variety

Soil

The soil and the procedure for its preparation were the same as described in Chapter 3. Treatments comprised four levels of applied boron, 0, 20, 40 and 60 mg kg^{-1} soil (B0, B20, B40 and B60, respectively). The concentration of boron extractable in 20 ml 0.01 M $CaCl_2$ (Spouncer *et al.*, 1992) was determined (Table 4.2). Pots (200 mm diameter) were lined with water-tight polythene bags, filled with 4 kg of treated soil and arranged in a glasshouse as a randomised complete block with three replicates.

Table 4.2 Boron extractable from the soil used for the pot experiment. Values are the means of two samples.

Boron applied (mg kg-1)	0	20	40	60
Extractable boron (mg kg^{-1})	2.3	16.1	31.1	47.2

Harvesting and tissue analysis

The plants were harvested one centimetre above ground level seven weeks after sowing and morphological characteristics including visual score of toxicity symptoms, height of plants, number of shoots and number of leaves were recorded. The plants were dried at 80°C for 48 hours and weighed. Dried shoots were ground by hand, digested in nitric acid and analysed for the concentration of boron and other elements by ICP spectrometery (Zarcinas *et al.*, 1987).

Statistical analysis

Data for dry matter production and tissue boron concentrations were subjected to square root and logarithmic (log_e) transformations, respectively, to ensure homogeneity of variance. All data were analysed by factorial analysis and the significance between means was calculated by the LSD test (Gomez and Gomez, 1984).

4.3 Results

4.3.1 Glasshouse screening

The 617 accessions of *P. sativum*, including Australian commercial varieties and advanced breeding lines, exhibited a wide range of symptoms (Plate 4.1). A high proportion of accessions (52%) were classified as being sensitive whereas only 3.5% were classified as tolerant (Table 4.3). Tolerant accessions were identified from all geographical regions. Although the number of lines tested was low and therefore may not be fully representative, there appear to be differences in the proportion of tolerant to sensitive lines between regions. A high proportion of tolerant lines occurred in collections from Asia and South America but relatively few European accessions were tolerant. Tolerant genotypes were identified from 12 individual countries (Table 4.4).

Table 4.3 Geographical origin of *P. sativum* germplasm and response to a high concentration of soil boron. Frequency (%) of each class is indicated in brackets.

Origin	Visual response			
	S[a]	MS	MT	T
Asia	31 (48)	19 (30)	9 (14)	6 (9.0)
Africa	22 (50)	13 (29)	7 (16)	2 (4.5)
North & Central America	15 (55)	8 (30)	3 (11)	1 (4.0)
South America	2 (18)	5 (45)	2 (18)	2 (18)
Europe	85 (53)	55 (34)	16 (10)	4 (2.5)
USSR	34 (58)	18 (30)	4 (7)	3 (5.0)
Oceania	27 (40)	24 (36)	15 (22)	1 (1.5)
Unknown	107 (58)	51 (28)	23 (12)	3 (2.0)
Total	323	193	79	22
Frequency (%)	52	31	13	3.5

[a] S = sensitive, MS = moderately sensitive, MT = moderately tolerant, T = tolerant

Plate 4.1 (a and b) Boxes containing a high concentration of boron (100 mg kg^{-1}) for screening pea accessions for response to boron. The concentration of boron extractable in hot $CaCl_2$ was 58 mg kg^{-1}. Plants are four weeks old.

(a)

(b)

Table 4.4 The response[a] of accessions of *P. sativum* from individual countries to a high concentration of soil boron.

Origin	Visual response S	MS	MT	T	Total accessions
Asia					
Afghanistan	16	13	7	3	39
China	2	1	-	1	4
India	7	3	-	2	12
Nepal	1	-	-	-	1
Palestine	1	-	1	-	2
Thailand	1	-	-	-	1
Turkey	3	2	1	-	6
Africa					
Algeria	-	-	1	-	1
Ethiopia	20	9	5	2	36
Morocco	2	2	-	-	4
Sudan	-	1	1	-	2
Zambia	-	1	-	-	1
North and Central America					
Canada	6	3	-	-	9
Mexico	1	-	-	1	2
USA	8	5	3	-	16
South America					
Chile	2	2	1	1	6
Colombia	-	3	1	1	5
Europe					
Albania	2	-	-	-	2
Bulgaria	1	-	-	-	1
Czechoslovakia	4	5	1	-	10
France	1	-	-	-	1
Germany	1	1	-	-	2
Greece	24	13	1	-	38
Hungary	10	3	-	-	13
Italy	-	-	1	-	1
Netherlands	1	-	1	2	4
Portugal	1	-	-	-	1
Spain	-	6	1	-	7
Sweden	36	23	9	1	69
U.K.	3	2	1	1	7
Yugoslavia	1	2	1	-	4
USSR	34	18	4	3	59
Oceania					
Australia	19	20	14	1	54
New Zealand	8	4	1	-	13
Unknown	107	51	23	3	184
Total	323	193	79	22	617

[a] S = sensitive, MS = moderately sensitive, MT = moderately tolerant, T = tolerant

4.3.2 Glasshouse experiment

Emergence

Boron treatments did not significantly influence percentage emergence at any time of measurement (Table 4.5), but there were significant differences between genotypes; in particular, the percentage emergence of CPI 65352 and SA 213 was low. These differences between genotypes may be attributed to environmental or genetic factors other than response to boron. The interaction between varieties and treatments was not statistically significant (Table 4.5).

Number of nodes and branches

A number of genotypes (SA 395, SA 310, PIG 16, SA 448 and SA 132) produced numerous branches at the B0 treatment while the majority of plants of all other genotypes did not produce branches (Table 4.6). Boron treatments significantly reduced the number of branches of the former group of genotypes and the contrast in plant habit resulted in a significant genotype x treatment interaction ($P < 0.05$; Table 4.6).

As a consequence of reduction in branches there was also a significant reduction ($P < 0.01$) in nodes per plant (Table 4.6). Application of 60 mg B kg^{-1} resulted in a 25% decrease in the number of nodes. Differences among the genotypes were also significant ($P < 0.01$). SA 448, SA 395 and PIG 16 produced the most nodes; these genotypes produced the most branches as well. CPI 65352 produced the least nodes even at the control treatment.

The high branching plants produced more nodes and were more affected by the application of boron compared with the other genotypes, and this could be related to reduction in number of branches of these genotypes at high levels of boron.

Table 4.5 Percentage emergence for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron.

Genotype	Percentage emergence							
	One week				Two weeks			
	B0	B20	B40	B60	B0	B20	B40	B60
PIG 16	100	100	100	93	100	100	100	100
PIG 36	100	87	100	87	100	93	100	100
CPI 65352	60	73	80	73	67	87	80	100
SA 132	100	100	100	100	100	100	100	100
SA 395	93	100	100	80	100	100	100	100
SA 213	60	60	53	80	60	87	87	93
SA 448	93	93	100	100	100	100	100	100
SA 310	93	93	100	87	100	100	100	100
NGB 1574	80	100	80	93	87	100	93	100
SA 1512	87	73	87	93	100	100	100	100
M93	80	93	87	87	100	100	100	100
Alma	100	67	80	87	100	100	100	100
Early Dun	93	87	80	87	93	93	100	100
Pennant	67	73	100	87	73	93	100	100

Table 4.6 The number of nodes and branches for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron.

Genotype	Nodes				Branches			
	B0	B20	B40	B60	B0	B20	B40	B60
PIG 16	39	36	31	31	5.2	4.1	4.1	3.7
PIG 36	16	15	13	12	1.2	1.0	1.1	1.0
CPI 65352	12	11	9	9	1.3	1.2	1.0	1.0
SA 132	22	19	17	16	2.0	2.0	1.8	1.6
SA 395	52	39	33	30	7.8	6.1	4.1	4.2
SA 213	16	16	13	13	1.0	1.0	1.0	1.0
SA 448	48	45	36	33	8.1	6.8	5.2	4.5
SA 310	22	20	20	16	3.1	2.9	2.7	1.9
NGB 1574	15	13	11	11	1.3	1.0	1.0	1.0
SA 1512	14	14	12	12	1.0	1.2	1.0	1.0
M93	14	14	13	13	1.0	1.0	1.0	1.0
Alma	15	14	14	13	1.0	1.0	1.0	1.0
Early Dun	16	15	13	13	1.0	1.0	1.0	1.0
Pennant	13	13	13	13	1.0	1.0	1.0	1.0

To compare values of nodes in a column LSD 0.01 = 2.54; in a row, LSD 0.01 = 1.36 and to compare values for branches in a column LSD 0.01 = 0.57; in a row, LSD 0.01 = 0.3.

The height of plants

The heights of plants were significantly ($P < 0.01$) influenced by the application of boron. There was also significant variation among the genotypes and CPI 65352, PIG 16, SA 395 and SA 448 were the shortest genotypes. The range in per cent reduction of height of genotypes at B60 in comparison with the control was 6 to 34 per cent with the smallest reduction being for M93. The genotype x treatment interaction was also significant ($P < 0.05$; Table 4.7).

Visual symptoms

Plants were scored for severity of foliar symptoms of boron toxicity by the scale described in Chapter 3. Symptoms typical of boron toxicity, initially consisting of light brown specks near the leaf margin but later turning necrotic, were observed on most genotypes, although CPI 65352, SA 132, M93 and SA 310 developed only necrotic areas on the leaves. SA 395 and SA 448 showed boron toxicity symptoms on the leaves of the main stem and the main stem remained small. However, the secondary shoots were affected to only a minor extent.

Symptoms increased significantly ($P < 0.01$) on all genotypes as a result of the application of boron, but the magnitude varied among genotypes resulting in a significant genotype x boron treatment interaction ($P < 0.01$) at both four and seven weeks after sowing (Table 4.8 and Plate 4.2). Symptoms were more severe seven weeks after sowing than at four weeks. The symptoms of boron toxicity were least severe for PIG 16, SA 132 and SA 310 and in general less severe for the exotic accessions than for the Australian varieties and were therefore consistent with the response in the initial screening.

Dry matter

There was a significant ($P < 0.01$) decrease in yield of dry matter with each increase in the level of boron.

Large differences were recorded among genotypes for dry matter production at the control treatment and in particular the landraces were of low vigour. The mean tissue dry matter

over all genotypes in the control was 5.32 g pot^{-1} and this was decreased by 32, 58 and 68 per cent in the B20, B40 and B60 treatments, respectively.

The trends differed among genotypes resulting in a significant genotype x treatment interaction ($P < 0.01$). M93 showed the lowest percentage yield reduction while the sensitive variety Pennant was the most severely affected (Table 4.9).

Table 4.7 Height for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron.

Genotype	Plant height (mm)			
	B0	B20	B40	B60
PIG 16	511	451	375	349
PIG 36	825	800	580	545
CPI 65352	420	410	355	316
SA 132	815	713	705	640
SA 395	339	339	323	291
SA 213	1046	957	828	794
SA 448	393	306	301	256
SA 310	719	634	441	524
NGB 1574	909	851	701	615
SA 1512	963	911	759	688
M93	767	841	773	723
Alma	914	807	766	704
Early Dun	865	943	783	784
Pennant	867	708	653	574

To compare values in a column LSD 0.05 = 74; in a row, LSD 0.05 = 29.

Table 4.8 Expression of symptoms of boron toxicity by nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron, four and seven weeks after sowing.

Genotype	Visual symptoms							
	Four weeks				Seven weeks			
	B0	B20	B40	B60	B0	B20	B40	B60
PIG 16	0.0	0.0	1.7	1.7	0.0	0.3	1.7	2.2
PIG 36	0.0	0.7	2.5	3.7	0.0	1.0	2.7	4.3
CPI 65352	0.0	0.0	1.5	2.3	0.0	0.2	1.8	3.2
SA 132	0.0	0.0	0.5	1.0	0.0	0.0	0.5	1.7
SA 395	0.0	1.7	2.6	3.2	0.0	1.7	2.7	3.3
SA 213	0.0	1.8	2.2	2.7	0.0	2.3	3.3	4.0
SA 448	0.0	1.2	2.5	3.8	0.0	1.7	2.8	3.8
SA 310	0.0	0.0	0.5	1.6	0.0	0.0	1.0	1.7
SA 1512	0.0	1.7	2.7	3.7	0.0	2.0	3.4	4.5
NGB 1574	0.0	2.0	2.8	3.8	0.0	2.7	4.0	4.8
M93	0.0	0.0	0.5	1.8	0.0	0.2	2.2	3.5
Alma	0.0	2.2	3.3	3.8	0.0	2.3	4.0	4.6
Early Dun	0.0	2.3	3.2	4.0	0.0	2.3	4.2	4.7
Pennant	0.0	2.5	3.2	5.2	0.0	2.8	4.5	5.7

To compare values of first scoring in a column LSD 0.01 = 0.31; in a row, LSD 0.01 = 0.16 and to compare values for second scoring in a column LSD 0.01 = 0.27; in a row, LSD 0.01 = 0.14.

Plate 4.2 Comparison of response of the moderately tolerant variety Alma with tolerant lines to high concentrations of soil boron. From left to right; PIG 16, SA 132 (PIG 390), SA 310 (PIG 483), M93 and Alma. Plants were sown in soil with (a) 40 mg B $kg^{-1}$and (b) 60 mg B kg^{-1}.

(a)

(b)

Table 4.9 Dry matter (g) (B0) and relative dry matter yield (B20, B40 and B60) for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron. Statistical analyses were performed upon the transformed (square root) data presented in brackets. Significance levels refer to the transformed data.

Genotype	Dry matter (g)	Relative dry matter (% of B0)		
	B0	B20	B40	B60
PIG 16	4.35 (2.09)	74.4 (1.77)	48.8 (1.56)	41.8 (1.36)
PIG 36	5.76 (2.40)	68.9 (2.00)	37.9 (1.48)	25.9 (1.23)
CPI 65352	4.03 (1.99)	75.0 (1.74)	45.0 (1.34)	40.0 (1.24)
SA 132	4.45 (2.10)	75.0 (1.82)	54.4 (1.54)	43.2 (1.40)
SA 395	3.98 (1.99)	65.0 (1.60)	47.5 (1.40)	37.5 (1.23)
SA 213	6.97 (2.64)	53.6 (1.90)	34.8 (1.55)	29.0 (1.39)
SA 448	3.51 (1.87)	68.6 (1.54)	51.4 (1.34)	37.1 (1.15)
SA 310	3.43 (1.85)	76.5 (1.61)	47.0 (1.27)	38.2 (1.16)
NGB 1574	5.24 (2.29)	65.3 (1.85)	42.3 (1.47)	23.0 (1.09)
SA 1512	6.82 (2.61)	58.8 (2.00)	35.3 (1.54)	25.0 (1.31)
M93	5.39 (2.32)	92.6 (2.40)	68.5 (1.93)	51.9 (1.68)
Alma	6.82 (2.61)	66.2 (2.13)	35.3 (1.54)	25.0 (1.32)
Early Dun	6.88 (2.62)	75.4 (2.28)	34.8 (1.54)	31.8 (1.46)
Pennant	6.84 (2.61)	48.5 (1.82)	32.3 (1.46)	19.1 (1.14)

To compare values in a column LSD 0.01 = 0.14; in a row, LSD 0.01 = 0.07 .

Concentrations of boron in shoots

Increasing applications of boron produced an increase in the concentration of boron in tissues while the genotype x treatment interaction was highly significant ($P < 0.01$; Table 4.10). The ranking of genotypes for concentration of boron in tissues followed a similar pattern to that of relative tissue dry matter with SA 132 and SA 310 having the lowest concentrations of boron. M93 was an exception and contained a higher level of boron at B60 than would have been expected on the basis of dry matter response.

Most accessions selected as tolerant in the initial screening had lower boron concentrations than those of Australian varieties. PIG 36 appears to have been misclassified by the initial screening as it had a high level of boron in tissues, and reacted in a progressively more sensitive manner, relative to the other genotypes, as the soil boron increased.

Concentrations of sodium in shoots

There were significant ($P < 0.01$) differences among genotypes for concentrations of sodium, even in the control treatment (Table 4.11). The concentration of sodium in the tissues of CPI 65352 was considerably lower than in the others (Fig. 4.1). A significant interaction between genotypes and treatments ($P < 0.01$) also occurred. An excess of soil boron significantly ($P < 0.01$) depressed the concentration of sodium in shoots of all genotypes, except Pennant at the B40 and B60 treatments, resulting in a mean decrease of 31% in B60 relative to the control.

Table 4.10 Concentrations of boron in whole shoots for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron. Statistical analyses were performed upon transformed data ($\log_e$) presented in brackets. Significance levels refer to the transformed data.

Genotype	Boron concentration (mg kg^{-1})			
	B0	B20	B40	B60
PIG 16	24 (3.19)	259 (5.56)	583 (6.37)	1079 (6.98)
PIG 36	23 (3.13)	416 (6.02)	1170 (7.06)	1855 (7.52)
CPI 65352	19 (2.96)	231 (5.43)	668 (6.50)	1253 (7.13)
SA 132	19 (2.94)	167 (5.11)	413 (6.01)	611 (6.41)
SA 395	26 (3.27)	244 (5.49)	661 (6.49)	1019 (6.92)
SA 213	23 (3.10)	300 (5.69)	563 (6.33)	861 (6.76)
SA 448	40 (3.67)	255 (5.54)	673 (6.50)	948 (6.85)
SA 310	21 (3.02)	148 (5.00)	440 (6.08)	638 (6.45)
NGB 1574	21 (3.02)	359 (5.87)	1172 (7.07)	1538 (7.34)
SA 1512	21 (3.02)	285 (5.64)	869 (6.76)	1583 (7.37)
M93	19 (2.94)	276 (5.62)	750 (6.61)	1373 (7.22)
Alma	23 (3.12)	289 (5.66)	869 (6.77)	1215 (7.09)
Early Dun	21 (3.02)	249 (5.50)	777 (6.65)	1166 (7.05)
Pennant	22 (3.10)	340 (5.82)	918 (6.82)	1538 (7.33)

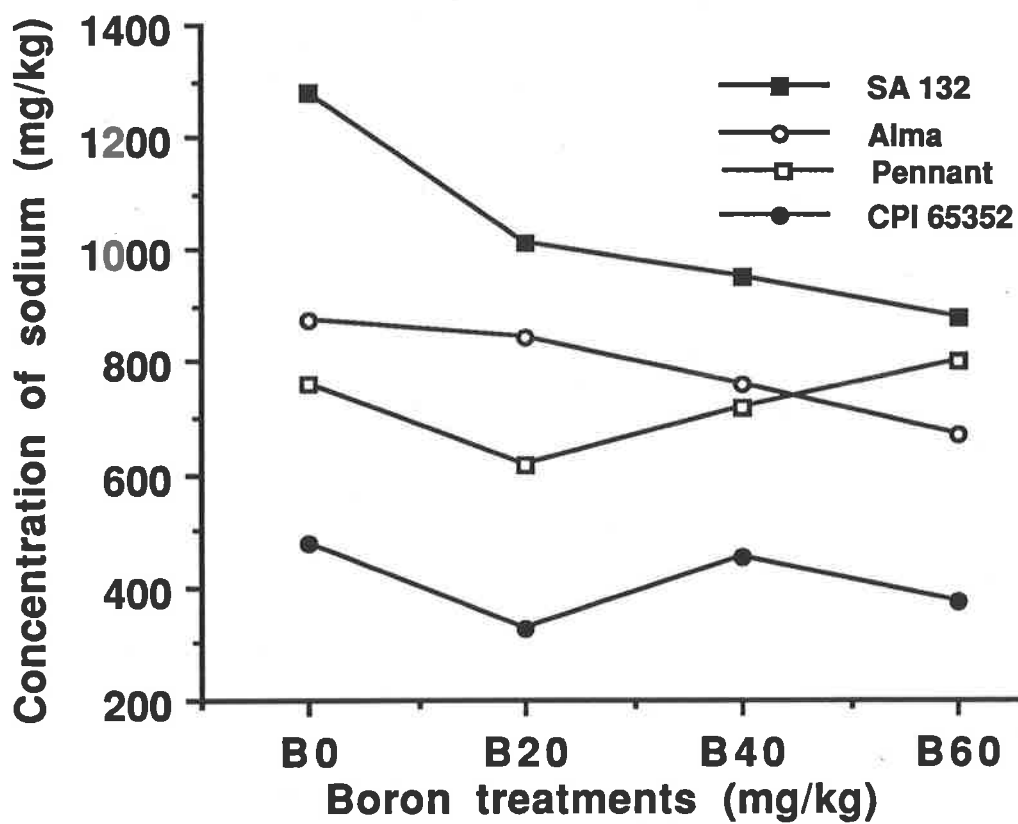
To compare values of tissue boron in a column LSD 0.01 = 0.105; in a row, LSD 0.01 = 0.56 .

Table 4.11 Concentrations of sodium in whole shoots for nine exotic pea accessions and five Australian varieties and advanced lines when grown at four levels of boron.

Genotype	Sodium concentration (mg kg^{-1})			
	B0	B20	B40	B60
PIG 16	1378	1085	1076	1042
PIG 36	755	654	575	669
CPI 65352	475	325	456	377
SA 132	1282	1014	951	880
SA 395	834	714	702	696
SA 213	1058	1090	904	765
SA 448	721	525	550	473
SA 310	1484	904	1003	858
NGB 1574	1254	1320	1125	1006
SA 1512	870	769	788	667
M93	1210	827	712	654
Alma	874	845	762	670
Early Dun	911	827	721	691
Pennant	758	615	721	803

To compare values of tissue sodium in a column LSD 0.01 = 89; in a row, LSD 0.01 = 47.

Fig. 4.1 Changes in concentrations of sodium in tissues with the application of boron for four genotypes.


1400
1200
1000
800
600
400
200
Concentration of sodium (mg/kg)
SA 132
Alma
Pennant
CPI 65352
B0
B20
B40
B60
Boron treatments (mg/kg)

4.4 Discussion

Considerable genetic variation in response to boron was demonstrated among exotic accessions of *P. sativum* with a number being more tolerant than Alma and Early Dun, two moderately tolerant Australian pea varieties. Genetic variation in response to high concentrations of boron for several crop species has been investigated and there are similarities in the origin of tolerant lines between peas and these other crops. Moody *et al.* (1988) evaluated 1579 wheat genotypes from the Australian Winter Cereals Collection, and found that wheat varieties vary widely in their tolerance to boron. About 6% of these genotypes were more tolerant than the most tolerant Australian wheat varieties.

Many tolerant lines of wheat (Moody *et al.*, 1988) and barley (R.C.M. Lance, pers. comm.) originated along the major continental fault lines and volcanic zones of West, Central and East Asia and from the Andean region of South America (Morgan, 1980). Other tolerant accessions, such those from the Indian subcontinent, originate from areas where high concentrations of boron are associated with sodic and saline soils. Tolerant accessions of medics also originate from Western Asia and North Africa (Paull *et al.*, 1992). Far more data is needed for Asian regions, and also on the response of other species to soil boron, before we can be certain about the centres of genetic diversity in response to boron. However, in general it is reasonable to accept that Asia is one of the main centres of genetic tolerance to boron for wheat, barley, medics and peas. There are many reports in the literature of an excess of boron in the salt-affected area of Delhi (Paliwal and Anjaneynlu, 1967; Singh and Randhawa, 1980), the saline alkaline soils of the Punjab and Rajastan (Aubert and Pinta, 1979) and irrigation water from Agra (Chauhan and Powar, 1978; Chauhan and Asthana, 1981) in India. Therefore, the tolerance of accessions from such areas as India to high concentrations of boron is likely to be related to natural selection at the site of selection.

The current Australian pea varieties have been classified as moderately tolerant (e.g. Alma, Early Dun), moderately sensitive (e.g. Collegian), sensitive (e.g. Pennant) and very

sensitive (e.g. Dinkum) to high concentrations of boron (Paull *et al.*, 1992; Chapter 3). In this experiment Pennant developed more severe symptoms of boron toxicity (Table 4.8), produced a lower dry matter yield at B60 (Table 4.9) and contained higher concentrations of boron in shoots at all levels of applied boron (Table 4.10) than Alma and Early Dun. These two tolerant varieties are very widely grown in Australia, especially in areas with sodic soils. Their agricultural distribution reflects the pattern of Halberd, a moderately tolerant wheat variety (Rathjen and Pederson, 1988) and they consistently outyield sensitive varieties at all breeding sites in South Australia (M.S. Ali, pers. comm.).

The response of M93 was somewhat anomalous and despite developing the least symptoms of boron toxicity and producing the greatest yield at the high boron treatments, the concentrations of boron in its tissues were higher than those of the other tolerant accessions. This may be a reflection of the semi-leafless habit which may change the transpirational pattern and therefore alter the toxic effects of boron on vegetative tissues. M93 is a breeding line, developed from a cross between Early Dun and JI 143. Early Dun was introduced into South Australia from the United Kingdom, possibly during the early 1900s. JI 143 is semi-leafless line and originated in the John Innes Institute, UK. M93 has been bred for spring sowing in the South East of South Australia. Its main attribute is mildew resistance (S.M. Ali, pers comm.) and was recently released under the name of Glenroy (Ali and Hawthorne, 1993).

A low degree of symptom expression by tolerant accessions could be attributed to the exclusion of boron from the shoots as they maintained lower concentrations of boron in tissues than did other lines. A similar response was reported for barley and wheat (Nable, 1988) and peas and medics (Paull *et al.*, 1992; Chapter 3). In all species low concentrations of boron in the shoots of tolerant genotypes could be attributed to an exclusion of boron from the root system rather than a restricted translocation of boron from roots to shoots.

The results showed that the ranking of the genotypes was the same in terms of

(a) visual assessment of boron toxicity;

(b) relative dry matter production; and,

(c) concentrations of boron in shoots (Fig 4.2).

The correlation coefficients that resulted among these three parameters were as follows: visual score and shoot dry matter: r = - 0.68 ($P < 0.01$); visual score and concentration of boron in shoots: r = 0.88 ($P < 0.01$) and shoot dry matter and concentrations of boron in shoots: r = - 0.73 ($P < 0.01$). As the three parameters are significantly correlated and effective at identifying tolerant genotypes, selection based upon visual assessment of symptom expression would be the most appropriate for a breeding program as such selection is non-destructive and may be conducted during seedling growth.

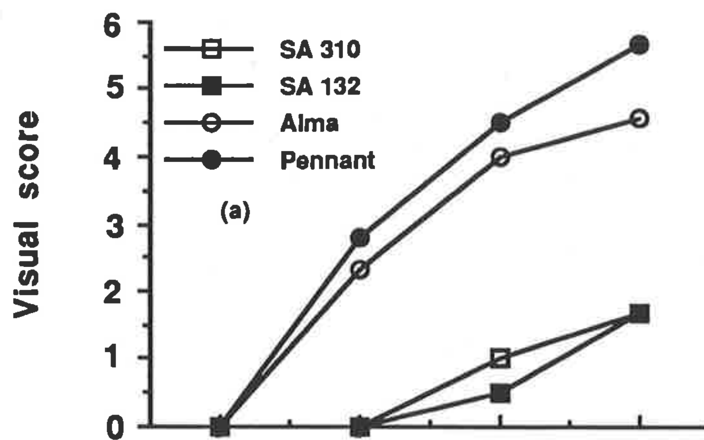
The findings have confirmed that genotypes more tolerant than current Australian pea varieties are available for pea breeding programs. The concentration of boron in tissues and expression of toxicity symptoms were least for SA 132 and SA 310, so these two accessions are particularly promising as progenitors in breeding programs for improving the adaptation of peas to high boron soils. It should be noted that most tolerant accessions are slow growing, bushy in growth habit, late flowering and have poor seed quality (Plates 4.3 and 4.4); hence it would be necessary to incorporate the tolerant allele (s) through backcrossing to recover the adapted genetic background of the recurrent parent. The significance of genetic distance between the tolerant accessions and Australian varieties is examined further in Chapter 7.

The boron-tolerant accessions come from diverse origins and may therefore include different genes conferring tolerance to boron. Intercrossing of tolerant lines and subsequent selection could result in the development of varieties with increased levels of tolerance to boron as was shown for wheat where transgressive segregation was observed among the progeny of moderately tolerant and tolerant genotypes (Paull *et al.*, 1991).

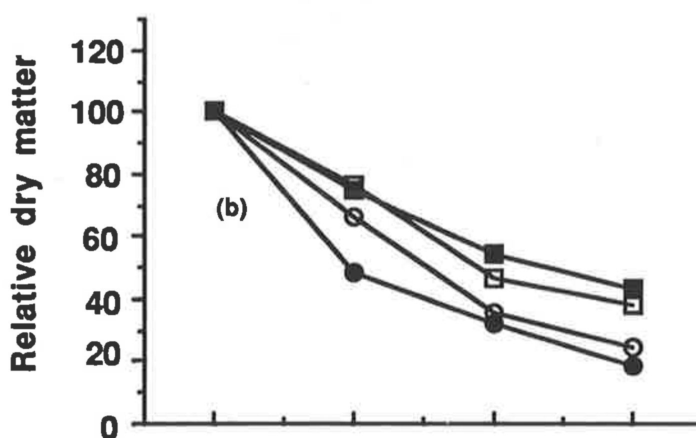
In South Australia, soils with high concentrations of boron are generally sodic (Cartwright *et al*., 1984; 1987). In this experiment, increasing levels of applied boron markedly depressed sodium concentration in tissues of all lines except the sensitive variety Pennant (Table 4.11). Sodium concentration in tissue was not the same for all varieties and it is interesting to note the very low concentrations of sodium in shoots of CPI 65352. Tolerance to sodicity has been reported to be associated with low concentrations of sodium in shoots of rice and wheat (Sharma, 1986; Gupta and Sharma, 1990). If the same mechanism operates in *Pisum*, CPI 65352 may be of interest for the development of pea varieties tolerant to sodicity.

The results of the pot experiment are in substantial agreement with those of the initial screening and indicate that considerable genetic variation exists among exotic accessions of *P. sativum*. The genetic relationships, with respect to boron tolerance, between selected tolerant accessions and Australian varieties and also within Australian varieties are examined in the next Chapter to determine the possibility of and strategies for transferring tolerance to a locally adapted genetic background.

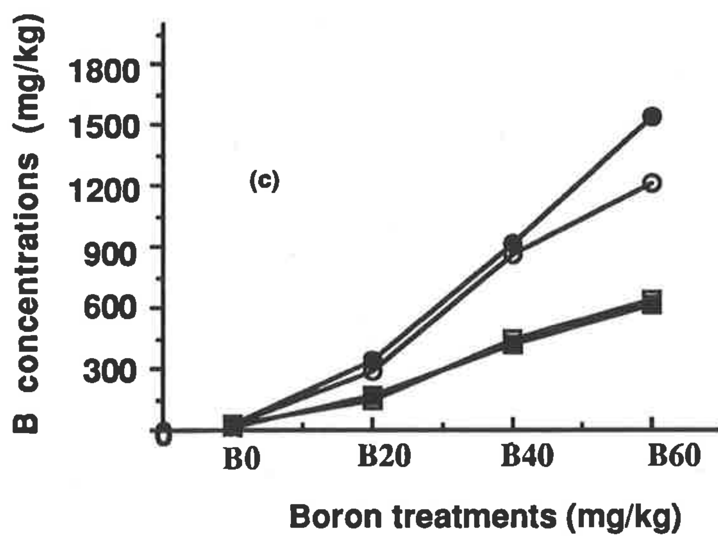
Fig. 4.2 (a) Visual score of boron toxicity, (b) relative dry matter yield and (c) concentrations of boron in shoots of the four pea genotypes SA 310, SA 132, Alma and Pennant grown at four levels of soil boron.



6
5
4
3
2
1
0
Visual score
SA 310
SA 132
Alma
Pennant
(a)



120
100
80
60
40
20
0
Relative dry matter
(b)



1800
1500
1200
900
600
300
0
B concentrations (mg/kg)
(c)
B0
B20
B40
B60
Boron treatments (mg/kg)

Plate 4.3 Comparison of growth habits between the moderately tolerant Australian variety Alma and exotic tolerant accessions. From left to right Alma, SA 448,PIG 16, SA 132 and SA 310.

1
2
3
4
5

Plate 4.4 (a) Comparison of seed size and seed colour between the moderately tolerant Australian commercial variety Alma and exotic tolerant accessions. From left to right Alma, SA 448, PIG 16, SA 132 and SA 310.
(b) Comparison of pod size between the sensitive Australian commercial variety Pennant and two exotic tolerant accessions SA 395 and SA 310.

(a)

(b)

CHAPTER 5

GENETICS OF TOLERANCE TO HIGH CONCENTRATIONS OF SOIL BORON IN *P. SATIVUM*

5.1 Introduction

A sequential survey in the Australian international pea collections (Chapter 4) revealed that boron tolerant genotypes exist which, however, are poor in terms of growth habit and yield (Chapter 4). Knowledge of the inheritance of boron tolerance of pea germplasm is necessary before considering its transfer to other genotypes. Although information on the genetic control of boron tolerance in other crop plants is limited, in wheat and barley the inheritance has been shown to be under the control of a series of major genes (Paull *et al.*, 1991; Jenkin, 1993). The present study was undertaken to investigate the mode of inheritance of boron tolerance in *P. sativum* and to assess the possibility of transferring this tolerance into locally adapted varieties.

5.2 Material and methods

Three boron tolerant accessions and two Australian varieties were used as parents (Table 5.1). The Australian variety Alma was chosen because of its good adaptation and desirable agronomic traits such as good seedling establishment, early vigour, good cooking quality and tolerance to black-spot infection (S.M. Ali, pers. comm.) as well as moderate tolerance to boron toxicity (Paull *et al.*, 1992; Chapter 3). The other Australian variety Pennant was chosen because of its sensitivity to boron. Although early maturing and of good quality it has never been widely grown. The tolerant parents PIG 16, SA 132 and SA 310 were selected on the basis of low symptom expression and concentration of boron in tissue (Chapter 4) and to represent geographically different origins, namely India and Afghanistan.

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A topcrossing scheme was undertaken with Alma being crossed to all the other parental lines and Pennant being crossed to PIG 16.

Hybridisations were conducted in a glasshouse and F_1 hybrids were also grown in a glasshouse to produce F_2 seeds. About 100 F_2 derived families were produced by self pollinating F_2 plants of these crosses. The parents were grown along with each generation and used as controls in all boron screening experiments.

Treatments comprised two levels of boron, 60 and 100 mg kg^{-1} (B60 and B100) added to the soil as described in Chapter 3. B100 was used for screening populations derived from crosses between tolerant accessions with Alma, and the B60 treatment for screening populations derived from the cross between Alma and Pennant. Large boxes (as described in Chapter 4) and plastic trays (400 mm x 285 mm x 120 mm) were used for B100 and B60 treatments, respectively. The experiments were conducted in an evaporatively cooled glasshouse, normally ranging from 15-25^oC.

To ensure uniform germination, seeds were placed in petri-dishes containing moist filter paper, stored at 2-4^oC for two days and then at room temperature of about 20-25° C for one day. Seeds of F_2 populations were sown in rows 2 cm deep and at a spacing of 5 cm x 5 cm. F_3 seeds were also sown with the same spacing with 10 to 12 seeds sown for each F_3 family. Four replicates of each parent, each consisting of 10 plants, were sown throughout each box to act as checks.

Boron toxicity symptoms generally began to appear ten days after seedling emergence and final visual assessments of individual plants were recorded four weeks after sowing on the basis of leaf damage on a scale of 0-8 (Chapter 3) where eight indicated necrosis of all leaf tissue.

Samples of plant tissue from six tolerant and six sensitive F_3 families of the Alma x SA 132 cross were taken at the time of scoring to determine concentrations of boron in shoots. Five

plants per family were cut one centimetre above ground level, dried at 80°C, ground and digested in nitric acid at 140°C. The boron concentrations were determined by ICP spectrometry (Zarcinas *et al.*, 1987).

Segregation ratios observed in the F_2 and F_3 were tested for goodness of fit to genetic models by chi-square analysis.

Table 5.1 Genotypes used for the crosses and their origin. (PIG, Plant Industry Genetics, Division of Plant Industries, CSIRO, Canberra; SA, South Australian Department of Primary Industries).

Genotype	Response to boron	Origin
Alma	Moderately tolerant	Australia
Pennant	Sensitive	Australia
PIG 16	Tolerant	India
SA 310	Tolerant	Afghanistan
SA 132	Tolerant	Afghanistan

5.3 Results

The responses of the F_1 hybrids were intermediate to the parents, indicating that tolerance is expressed as a partially dominant trait. There was no difference in any cross in the severity of symptoms expressed by reciprocal F_1 hybrids, so F_2 seeds derived from reciprocal crosses were pooled (Table 5.2). The reaction of the parental lines, tested at the same time as their F_1, F_2 and F_3 progeny, were consistent with previous results (Chapter 3 and 4).

Table 5.2 Response of parents and F_1 hybrid plants of reciprocal crosses to high concentrations of boron, rated on the basis of visual symptoms of boron toxicity. Data are the average of seven to ten plants.

Parents and crosses	Treatments	Visual symptoms
Alma	B60	3.10
Alma x Pennant	B60	4.10
Pennant x Alma	B60	4.25
Pennant	B60	5.00
Alma	B100	3.25
Alma x PIG 16	B100	2.30
PIG 16 x Alma	B100	2.45
PIG 16	B100	0.00
Pennant	B100	5.20
Pennant x PIG 16	B100	3.80
PIG 16 x Pennant	B100	3.70
PIG 16	B100	0.00
Alma	B100	3.25
Alma x SA 310	B100	1.88
SA 310 x Alma	B100	1.80
SA 310	B100	0.00
Alma	B100	3.25
Alma x SA 132	B100	2.57
SA 132 x Alma	B100	2.65
SA 132	B100	0.00

5.3.1 F_2 generation

The segregation in response to boron in the F_2 plants confirmed the hybrid status of the F_1 plants. Ratings of individual plants in the F_2 population of Alma x PIG 16 were between 0 and 4.5, while for the population from Alma x Pennant were between 3 and 5 on a scale from 0 to 8 (Fig. 5.1). Individual F_2 plants were assigned to categories delineated by the reaction of the parents (e.g. F_2 plants were classified as sensitive if symptoms of boron toxicity were within the range of the sensitive parent, and similarly for the tolerant category). Segregation ratios in the F_2 populations Alma x Pennant and Alma x PIG 16 fitted a monogenic ratio of 1:2:1 (tolerant: intermediate: sensitive) when scored four weeks after sowing (Table 5.3). This suggests that boron tolerance in both crosses is controlled by an incompletely dominant gene. Alma x PIG 16 fitted a 3: 1 ratio better than 1: 2: 1 which may be explained by misclassification of some heterozygous plants into the tolerant class with the cut-off score between tolerant and intermediate set between 2 and 2.5.

Ratings of individual F_2 plants of the cross Pennant x PIG 16 at the B100 treatment were between 0 and 5 (Fig. 5.1). Out of 87 plants, 45 plants appeared to be as tolerant as PIG 16, seven plants showed severe damage, similar to Pennant, and the others were intermediate. Therefore, the plants were divided into two groups:

(1) tolerant and intermediate - similar to the tolerant parent with no symptoms of boron toxicity and expression of boron toxicity with a score of 2 to 4, respectively and,

(2) sensitive - similar to the sensitive parent with extensive necrosis of the bottom leaves.

Segregation of the F_2 generation, on the basis of symptom expression after four weeks, corresponded to a 15:1 ratio but not a 3:1 (Table 5.3). In particular, the low frequency of F_2s as sensitive as Pennant indicates that more than a single gene is segregating in this cross. The results suggest that the sensitive variety Pennant and the tolerant accession PIG 16 differed at two genes with respect to tolerance to boron.

5.3.2 F_3 generation

Table 5.4 shows the F_3 segregation pattern of F_2-derived populations. The F_3 families of crosses among Alma and other lines showed a segregation ratio of 1 (homogenous tolerant): 2 (segregating): 1 (homogenous sensitive). All plants within the homogenous tolerant and homogenous sensitive families expressed symptoms similar to the tolerant and sensitive parents, respectively (Plate 5.1). However, the F_3 of the cross Pennant x PIG 16 showed low frequencies of homogenous tolerant and homogenous sensitive families and the other families were classified into three segregating or intermediate classes based on the expression of symptoms which ranged from severely damaged to almost symptom free. These families were also independently rated by Dr J.G. Paull and his results were in agreement with those presented here. On the basis of the hypothetical mode of inheritance for genetic control of two independent, partially dominant loci, the genotypic constitutions of the classes would be as follows:

		Genotype	Phenotype
1	1	1 AABB	All tolerant
:	: 4	2 AABb 2 AaBB	Tolerant - intermediate
14	: 6	1 AAbb 1 aaBB 4 AaBb	Predominantly intermediate but some families have tolerant or sensitive plants
:	: 4	2 Aabb 2 aaBb	Sensitive - intermediate
1	: 1	1 aabb	All sensitive

The observed frequencies (Table 5.4) of these groups fitted a 1: 14: 1 ratio. Thus, the F_3 data support the results of F_2 analyses indicating that boron tolerance of these genotypes is controlled by two incompletely dominant unlinked genes.

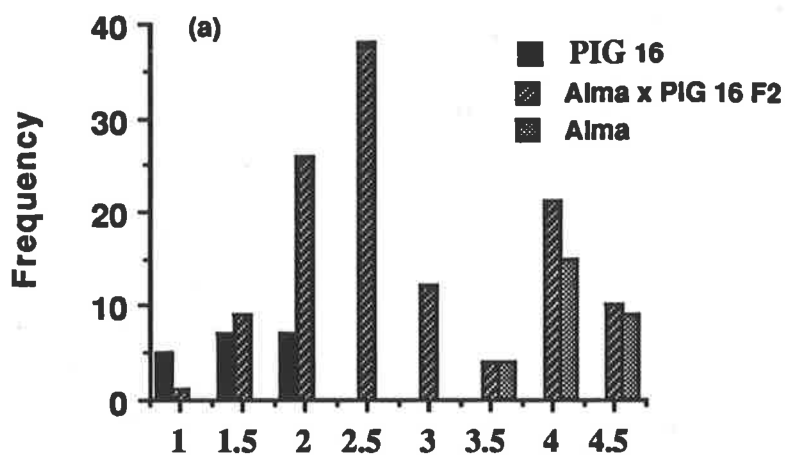
The results of tissue analysis of six tolerant and six sensitive F_3 families from the cross Alma x SA 132, selected on the basis of symptom expression, are shown in Table 5.5. The tolerant and sensitive selections were significantly different ($P < 0.01$) from each other. Mean tissue boron concentrations of all of the tolerant families were similar to SA 132 and the sensitive families were similar to Alma.

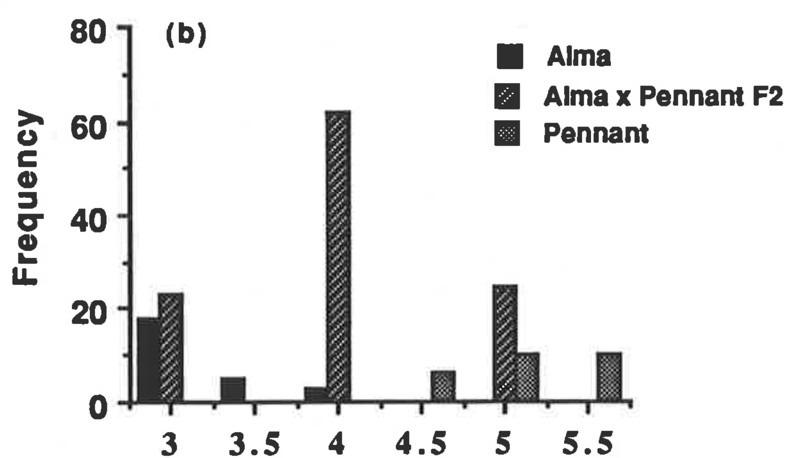
Fig 5.1 Frequency distributions of F_2 individuals from crosses among genotypes with different levels of boron tolerance screened on the basis of visual symptoms at 100 or 60 mg B kg^{-1}:

(a) Alma x PIG 16 (Alma and PIG 16 had average scores of 4.0 and 1.9, respectively)

(b) Alma x Pennant (Alma and Pennant had average scores of 3.2 and 5.3, respectively)

(c) Pennnat x PIG 16 (Pennant x PIG 16 had average scores of 4.9 and 0.0, respectively).


(a)
Frequency
40
30
20
10
0
1
1.5
2
2.5
3
3.5
4
4.5
PIG 16
Alma x PIG 16 F2
Alma


(b)
Frequency
80
60
40
20
0
3
3.5
4
4.5
5
5.5
Alma
Alma x Pennant F2
Pennant

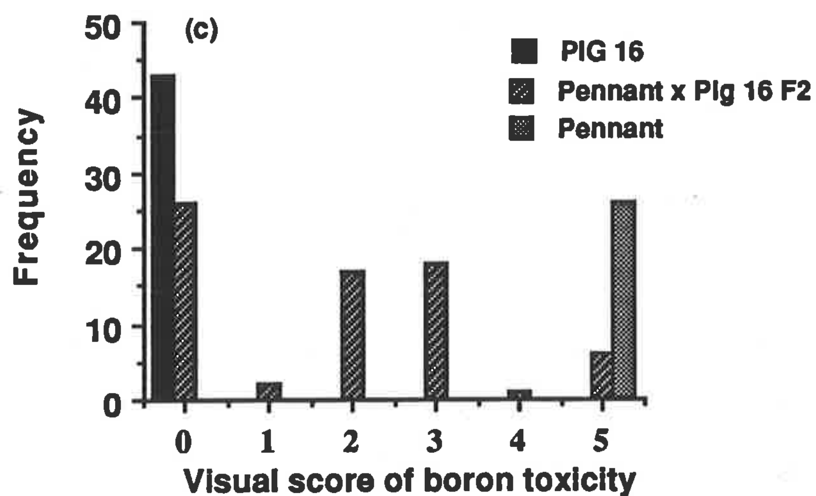

(c)
Frequency
50
40
30
20
10
0
0
1
2
3
4
5
Visual score of boron toxicity
PIG 16
Pennant x Pig 16 F2
Pennant

Table 5.3 The response to high concentrations of boron of three F_2 populations screened on the basis of visual symptoms at 60 or 100 mg kg^{-1}.

Cross combination	Treatments	Model		Observed and expected frequencies			
				Tolerant[a]	Intermediate	Sensitive	χ^2_2
Alma x PIG 16	B100		Obs.	36.0	50.0	35.0	
		1 : 2 : 1	Exp.	30.2	60.5	30.2	3.65
				Tolerant + intermediate		Sensitive	χ^2_1
Alma x PIG 16	B100		Obs.	86.0		35.0	
		3 : 1	Exp.	90.7		30.25	0.90
				Tolerant[a]	Intermediate	Sensitive	χ^2_2
Alma x Pennant	B60		Obs.	23.0	62.0	25.0	
		1: 2 : 1	Exp.	27.5	55.0	27.5	1.75
				Tolerant + intermediate		Sensitive	χ^2_1
Pennant x PIG 16	B100		Obs.	80.0		7.0	
		15 : 1	Exp.	81.56		5.43	0.48
		3 : 1	Exp.	65.25		21.75	13.33

[a] The terms tolerant and sensitive indicate responses similar to the tolerant and sensitive parents , respectively.

P	0.50	0.20	0.05	0.01
χ^2_1	0.45	1.64	3.84	6.63
χ^2_2	1.93	3.22	5.99	9.21

Table 5.4 The response to high concentrations of boron of five F_3 populations screened on the basis of visual symptoms of boron toxicity at 60 or 100 mg B kg^{-1}.

Cross combination	Treatments	Model		Observed and expected frequencies			
				Homogenous tolerant	Segregating	Homogenous sensitive	χ^2_2
Alma x Pennant	B60		Obs.	18.0	49.0	18.0	
		1 : 2 : 1	Exp.	21.2	42.5	21.2	1.98
Alma x PIG 16	B100		Obs.	24.0	36.0	20.0	
		1 : 2 : 1	Exp.	20.0	40.0	20.0	1.00
Alma x SA 310	B100		Obs.	23.0	36.0	22.0	
		1 : 2 : 1	Exp.	20.2	40.5	20.2	1.02
Alma x SA 132	B100		Obs.	21.0	41.0	23.0	
		1 : 2 : 1	Exp.	21.2	42.5	21.2	0.20
Pennant x PIG 16	B100		Obs.	8.0	69.0	6.0	
		1 : 14 : 1	Exp.	5.19	72.6	5.19	1.83

P	0.50	0.20	0.05	0.01
χ_2^2	1.39	3.22	5.99	9.21

Plate 5.1 Comparison of the response of the parents and plants from F_2 derived families to high concentrations of soil boron.

(a) From left to right: Alma, Alma x Pennant segregating family, Pennant.

(b) From left to right Alma, Alma x PIG 16 segregating family and PIG 16

(a)

(b)

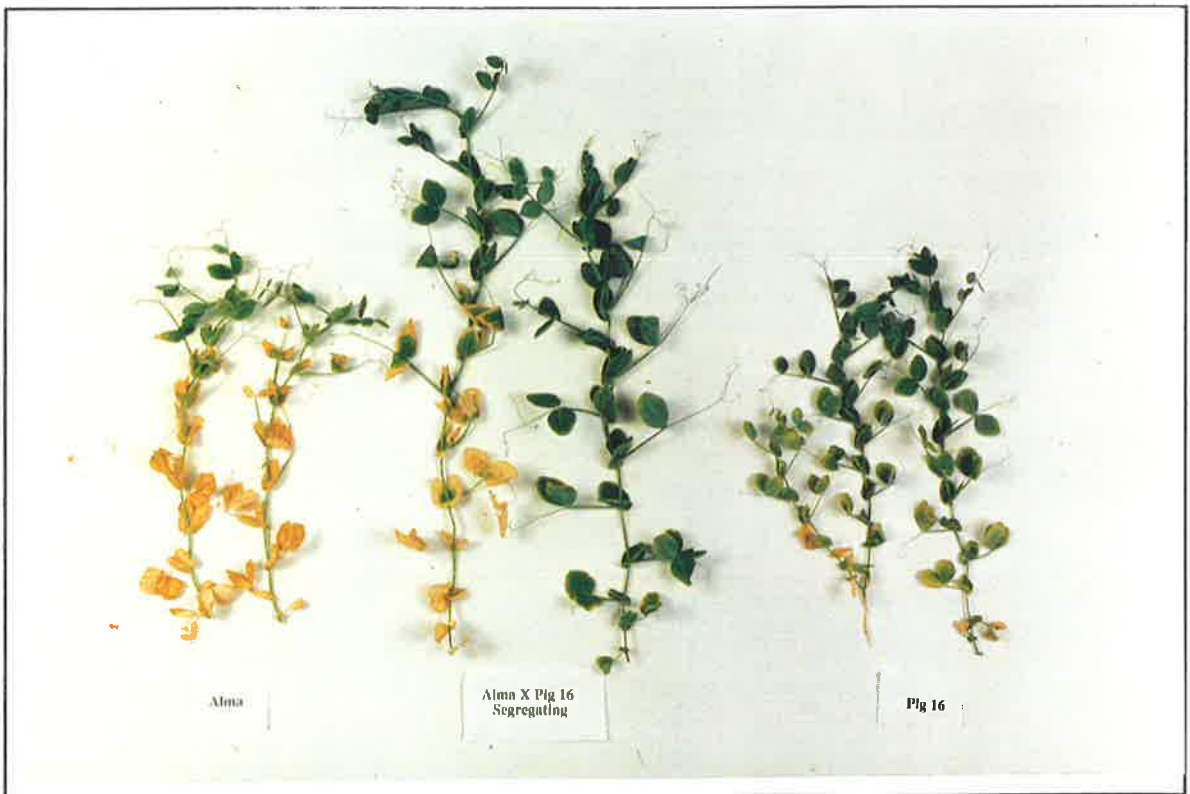


Table 5.5 Concentration of boron[a] in shoots of twelve selected F_3 families derived from tolerant and sensitive F_2 plants of Alma x SA 132, and their parents, grown at the B100 treatment.

Tolerant family	B (mg kg^{-1})	Sensitive family	B (mg kg^{-1})
1	526	1	743
2	533	2	667
3	372	3	795
4	505	4	791
5	496	5	850
6	407	6	787
SA 132	340	Alma	915

[a] Average of three replicates. $t = 6.20$ ($P < 0.01$).

5.4 Discussion

The objective of this study was to determine the mode of inheritance of boron tolerance in Australian varieties of peas and exotic lines previously shown to be tolerant to high concentrations of soil boron.

As boron tolerance is controlled by incompletely dominant gene (s) (Table 5.3) and response is based on a quantitative observation of expression of symptoms, analysis of F_3 families is a more accurate method of determining genetic control than the response of F_2 segregants. The frequency of segregating families can be compared to the frequency of non-segregating families of both parental types. Furthermore the genotype of an F_2 plant from which an F_3 family was derived can be inferred by the response of plants within the family. A family expressing a large variation in response, or segregation, would imply the F_2 plant was heterozygous while an F_3 family in which all plants were similar to either of the parents would imply the F_2 plant was homozygous. Unfortunately, it was not possible to screen successively the F_2 and the F_3 here as sensitive F_2 plants produce little or no seed after testing for response to boron. In this experiment, the F_3 families were derived from unscreened plants.

The segregation ratios observed in the F_2 and F_3 generations suggest that genetic variation in response to boron can be attributed to relatively few genes of major effect. The simplest hypothesis to explain the genetic variation among these five lines is two major unlinked genetic loci interacting in an additive manner with a single allele difference between Alma and the three tolerant lines and variation at a second gene between Pennant and the other genotypes including Alma.

A similar inheritance pattern to boron was reported in wheat (Paull *et al.*, 1991) and barley (Jenkin, 1993). Inheritance of boron tolerance in wheat (*Triticum aestivum*) was determined from crosses among genotypes ranging from highly sensitive to tolerant (Paull *et al.*, 1991).

The F_1 hybrids were intermediate to the two parents with respect to both growth response and concentration of boron in tissues, indicating that boron tolerance in wheat is controlled by an incompletely dominant gene(s). Based on segregation of F_2 and F_3 generations, it was shown that the inheritance of tolerance to high concentrations of boron was under additive genetic control (Paull *et al.*, 1991). Three genes, *Bo1*, *Bo2* and *Bo3* were identified. Jenkin (1993) studied the tolerance of barley varieties to boron and observed that F_1 hybrids among Sahara 3771 (tolerant), California Mariout 72 (moderately tolerant) and Stirling (sensitive) barley genotypes were intermediate to the parents indicating partial dominance. The F_2 and F_3 generations were tested for segregation and tolerance to boron was found to be controlled by three major genes.

F_3 progeny, from the cross Alma x SA 132 selected as tolerant and sensitive on the basis of symptom expression, contained low and high concentrations of boron in tissues, respectively (Table 5.5). This conforms with previous results on the mechanism of tolerance to boron (Nable and Paull, 1991) and indicates that tolerant lines can be selected directly using F_2 populations or F_3 families assessed on the basis of symptom expression of plants grown in soil with a high concentration of boron. In general, the box screening technique should be considered as a valuable selection technique in a pea breeding program because it is quick and the results correlate well with concentrations of boron in tissues (Chapter 3).

Based on the evidence presented, the proposed genotypes of Pennant, Alma and PIG 16 are given in Table 5.6. Since this is the first report on the inheritance of tolerance to boron toxicity in peas, the allele symbols *Bo* and *bo*, for boron tolerance and susceptibility, respectively, have been tentatively assigned, corresponding to those in wheat (Paull *et al*, 1991). The designation of gene symbols in the other tolerant lines must wait until further test crosses are made.

Further studies need to be undertaken to determine whether genetic control of boron tolerance varies among PIG 16, SA 310 and SA 132 (tolerant accessions). This could be achieved by intercrossing the three lines in all combinations and examining the response of the F_2 generations under high boron conditions. If the genetic control of tolerance was the same in all three lines no segregation would be expected in the F_2, but if the tolerance of these lines was controlled by different genes, transgressive segregation would be expected as was observed in wheat (Paull *et al.*, 1991).

This knowledge of the genetics of boron tolerance in peas will increase the efficiency in breeding boron tolerant varieties. As tolerance is under major gene control it should be relatively simple to transfer boron tolerance from the tolerant accessions into current commercial varieties by the well-known technique of repeated back-crossing and selfing. This procedure has already been shown to effect yield improvement in wheat (Moody *et al.*, 1993; Campbell *et al.*, 1993). As tolerant accessions identified here are poor in terms of agronomic characters (Chapter 4), further studies using molecular techniques were conducted (Chapter 7) to clarify the genetic relatedness among the geographically distant collections and Australian adapted varieties of *P. sativum* before using them in breeding programs.

Table 5.6 Genotypes of three pea lines for genetic control of response to high concentrations of boron.

Line	Response to boron	Genotype
Pennant	Sensitive	*bo1bo1 bo2bo2*
Alma	Moderately tolerant	*Bo1Bo1 bo2bo2*
PIG 16	Tolerant	*Bo1Bo1 Bo2Bo2*

CHAPTER 6

CHROMOSOMAL LOCALISATION OF GENES MEDIATING TOLERANCE TO BORON IN PEAS

6.1 Introduction

A limited range in genetic variation in tolerance to boron occurs among Australian pea varieties (Chapter 3) and lines more tolerant than Australian varieties were identified among a collection of *P. sativum* (Chapter 4). The segregation ratios observed in the F_2 and F_3 generations for crosses between genotypes contrasting in response to boron indicate that genetic variation is controlled by several partially dominant, additive genes (Chapter 5). These genes were classified as *Bo1* and *Bo2* where allelic variation at the *Bo1* locus accounts for the variation between the Australian varieties Alma (moderately tolerant) and Pennant (sensitive). The variation between Alma and the tolerant accession PIG 16 was attributed to the *Bo2* locus (Chapter 5). The objective of the present study was to discern linkage relationships between the gene(s) conferring tolerance to boron and markers which would provide convenient selection criteria in breeding programs.

Morphological, isozyme and molecular markers have been used in genetic studies. In the early twentieth century morphological traits were employed as markers in the development of methods for mapping gene position on chromosomes (Morgan, 1911). By the late 1950s, isozyme assays also became available as biochemical markers for determination of genetic relationships (Markert and Moller, 1959). Since, isozyme studies have been used in many areas of plant biology including plant breeding, plant population genetics, systematics, evolutionary genetics and somatic cell genetics. An important application of isozymic variation has been the construction of gene linkage maps for plant chromosomes. Rick and Fobes (1974) discovered a strong linkage between the gene for nematode resistance in tomato (*Mi*) and the allozyme variant Aps^1. This marker has now been used in commercial tomato breeding for selection of nematode resistant plants. In peas, the enzyme locus *Pgm-p*

has been reported as a marker for *Mo*, a gene which controls resistance to bean yellow mosaic virus (Weeden *et al.*, 1984). Locally, Wallwork (pers. comm.) has used the linkage between *Yr10* and brown glumes, located on chromosome 1BS (Macer, 1975 and Unrau, 1950, respectively) in the breeding for stripe rust resistance of the wheat variety Angas. This also is linked to the one of the better alleles, '*j*', for low molecular weight glutenin at the *GluB 3* locus (G. Cornish, pers. comm.).

Although isozymes are a valuable tool in linkage analyses, recent developments in DNA-based technology are providing tools suitable for rapid and detailed genetic analysis of higher organisms including plant species. These molecular marker techniques include restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980) and random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990) and other polymerase chain reaction (PCR) based methods. These systems generate a unique range of DNA fragments whose size can be readily determined by agarose gel electrophoresis resulting in a characteristic pattern of bands in the gel. Molecular markers are potentially powerful tools in linking genes of interest (Beckmann and Soller, 1983; Tanksley, 1983; Tanksley *et al.*, 1989) and the much higher level of polymorphism generated by these methods may overcome the major limitation of isozyme markers. Genetic linkage maps based upon DNA markers have been produced for many crop species (e.g. *Zea mays*, Coe *et al.*, 1990; *Brassica oleracea*, Slocum *et al.*, 1990) while linkage has been identified between a number of DNA markers and agronomic traits. For instance, RFLP markers have been identified that are tightly linked to genes for resistance to tobacco mosaic virus (Young *et al.*, 1988), downy mildew in lettuce (Landry *et al.*, 1987) and *Fusarium oxysporum* in tomato (Sarfatti *et al.*, 1989).

RAPDs are a more recent development than RFLPs and overcome some of the technical limitations (e.g. length of time for the test) of RFLP analysis (Williams *et al.*, 1990; Rafalski *et al.*, 1991, Rafalski and Tingey, 1993). Polymorphisms generated by RAPDs can be

detected between different individuals after gel electrophoresis. RAPDs are almost always dominant markers whereas RFLPs are co-dominant.

RAPDs provide potential for the rapid construction of linkage maps (Williams *et al.*, 1991) and, in recent years, several reports have appeared using this technique as an efficient tool to identify molecular markers which are linked to traits of interest (Paran *et al.*, 1991; Martin *et al.*, 1991; Haley *et al.*, 1993). Using the RAPD approach, Michelmore *et al.* (1991) showed that linkage between the trait of interest and RAPD markers can be identified using two pooled DNA samples from the contrasting homozygous individuals of an F_2 population. Within each bulk, the individuals are identical for the trait or gene of interest but are random genotypes at loci unlinked to the selected region. Thus, the probability that a polymorphism between the two pools generated by RAPD amplification is genetically linked to the locus determining the trait is high. Linkage between polymorphic markers and the target locus can be confirmed by testing the segregating population.

The genetic map of *P. sativum* has been extensively developed. Seven linkage groups involving morphological and physiological trait loci (Blixt, 1974), isozyme (Weeden and Marx, 1987) and isozyme and other protein variants, RFLPs and structural genes from DNA clones (Weeden and Wolko, 1990) have been reported (Fig. 6.1). More recently, Ellis *et al.* (1992) employed RFLP markers to construct linkage maps of several recombinant inbred populations (Fig. 6.2). Based on the data presented, the maps are not universally consistent between different crosses and previously mapped linkage groups but indicate a number of translocations.

Research presented in this Chapter involved attempts to determine linkage between a gene conferring tolerance to boron and three types of markers, namely isozymes, RAPDs and RFLPs. As there was no indication of the location of the gene for boron tolerance, and specific probes for studying RFLPs in peas were not available in Australia, this study first

focused on isozyme and RAPD techniques. The RFLP analysis was conducted in collaboration with Dr T.H.N. Ellis, John Innes Institute, Norwich, UK.

Fig. 6.1 Linkage map of *P. sativum* involving the combination of morphological, isozyme and DNA markers (Weeden and Wolko, 1990).

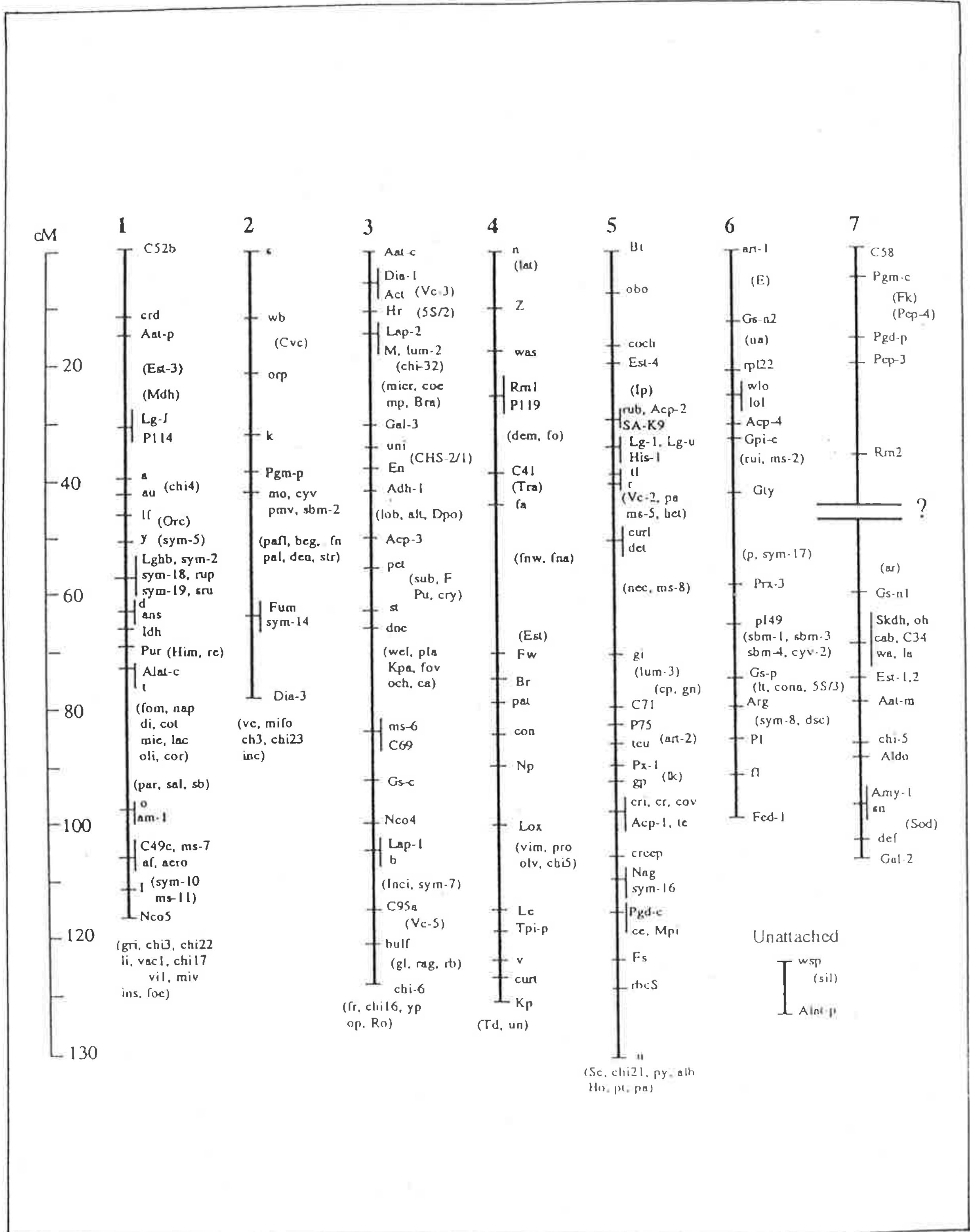
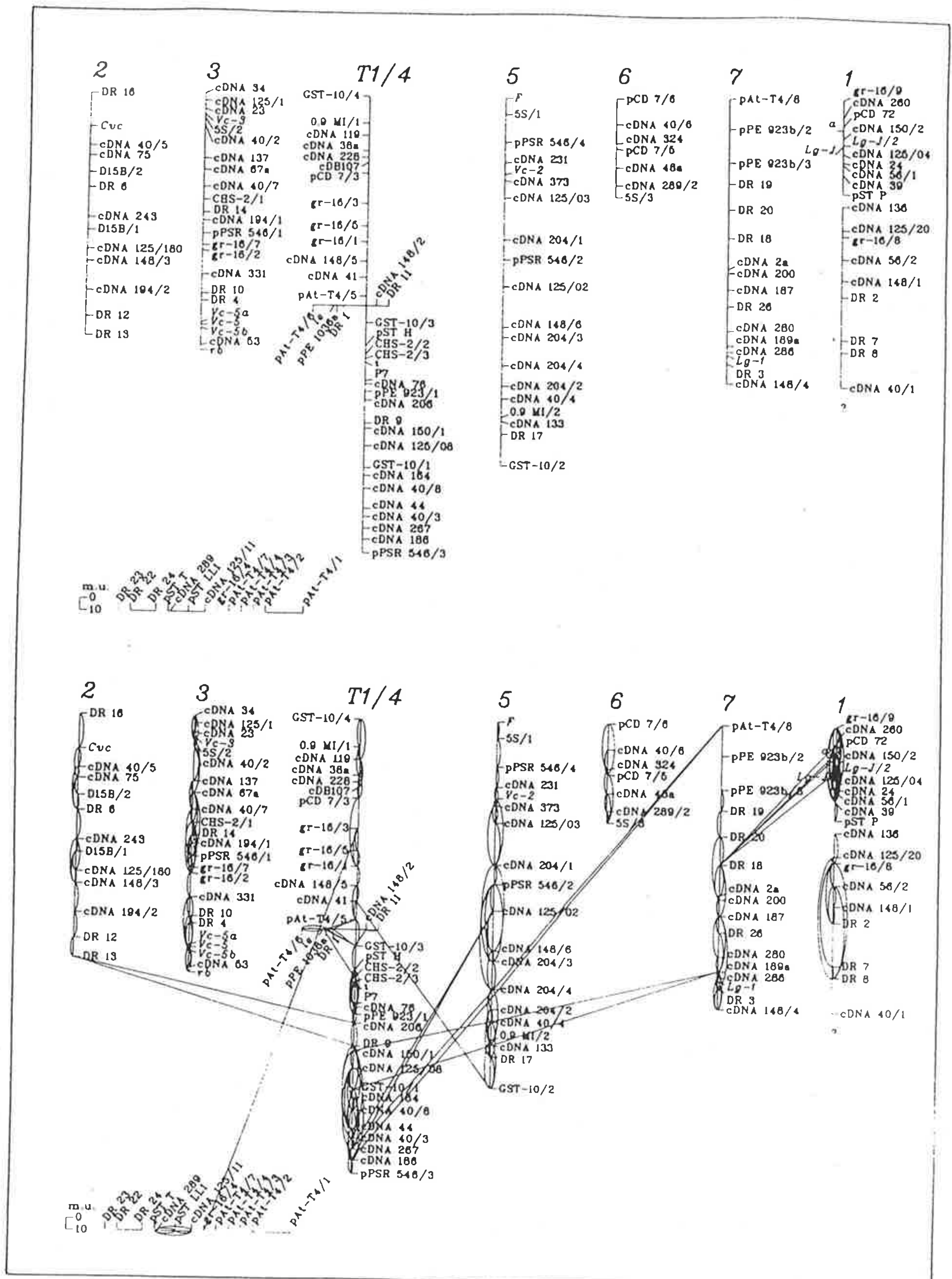

cM
20
40
60
80
100
120
130
1
C52b
crd
Aat-p
(Est-3)
(Mdh)
Lg-J
P114
a
au (chi4)
lf (Orc)
y (sym-5)
Lghb, sym-2
sym-18, rup
sym-19, sru
d
ans
ldh
Pur (Him, re)
Alat-c
t
(fom, nap
di, cot
mie, lac
oli, cor)
(par, sal, sb)
o
am-1
C49c, ms-7
af, aero
l (sym-10
ms-11)
Nco5
(gri, chi3, chi22
li, vac1, chi17
vil, miv
ins, foc)
2
s
wb
(Cvc)
orp
k
Pgm-p
mo, cyv
pmv, sbm-2
(pafl, beg, fn
pal, dea, str)
Fum
sym-14
Dia-3
(vc, mifo
ch3, chi23
inc)
3
Aat-c
Dia-1
Act (Vc-3)
Hr (5S/2)
Lap-2
M, lum-2
(chi-32)
(mier, coe
mp, Bra)
Gal-3
uni
En (CHS-2/1)
Adh-1
(lob, alt, Dpo)
Acp-3
pet
(sub, F
Pu, cry)
st
doc
(wel, pla
Kpa, fov
och, ca)
ms-6
C69
Gs-c
Nco4
Lap-1
b
(Inci, sym-7)
C95a
(Vc-5)
bulf
(gl, rag, rb)
chi-6
(fr, chi16, yp
op, Ro)
4
n
(lat)
Z
was
Rm1
P119
(dem, fo)
C41
(Tra)
fa
(fnw, fna)
(Est)
Fw
Br
pat
con
Np
Lox
(vim, pro
olv, chi5)
Le
Tpi-p
v
curt
Kp
(Td, un)
5
Bt
obo
coch
Est-4
(lp)
rub, Acp-2
SA-K9
Lg-1, Lg-u
His-1
tl
r
(Vc-2, pa
ms-5, bet)
curl
det
(nec, ms-8)
gi
(lum-3)
(cp, gn)
C71
P75
tcu (art-2)
Px-1
gp (lk)
cri, cr, cov
Acp-1, te
creep
Nag
sym-16
Pgd-c
ce, Mpi
Fs
rbcS
u
(Sc, chi21, py, alb
Ho, pt, pa)
6
art-1
(E)
Gs-n2
(ua)
rpl22
wlo
lol
Acp-4
Gpi-c
(rui, ms-2)
Gty
(p, sym-17)
Prx-3
pl49
(sbm-1, sbm-3
sbm-4, cyv-2)
Gs-p
(lt, cona, 5S/3)
Arg
(sym-8, dsc)
Pl
fl
Fed-1
7
C58
Pgm-c
(Fk)
(Pcp-4)
Pgd-p
Pcp-3
Rm2
?
(ar)
Gs-n1
Skdh, oh
cab, C34
wa, la
Est-1,2
Aat-m
chi-5
Aldo
Amy-1
sn
(Sod)
def
Gal-2
Unattached
wsp
(sil)
Alnt-p

Fig. 6.2 Linkage map of *P. sativum* based on DNA markers (Ellis *et al.*, 1992).


2
DR 16
Cvc
cDNA 40/5
cDNA 75
D15B/2
DR 6
cDNA 243
D15B/1
cDNA 125/180
cDNA 148/3
cDNA 194/2
DR 12
DR 13
3
cDNA 34
cDNA 125/1
cDNA 23
Vc-3
5S/2
cDNA 40/2
cDNA 137
cDNA 67a
cDNA 40/7
CHS-2/1
DR 14
cDNA 194/1
pPSR 546/1
gr-16/7
gr-16/2
cDNA 331
DR 10
DR 4
Vc-5a
Vc-5
Vc-5b
cDNA 63
T1/4
GST-10/4
0.9 MI/1
cDNA 119
cDNA 36a
cDNA 228
cDB107
pCD 7/3
gr-16/3
gr-16/5
gr-16/1
cDNA 148/5
cDNA 41
pAt-T4/5
cDNA 148/2
DR 11
pAt-T4/6
pPE 1036a
DR 1
GST-10/3
pST H
CHS-2/2
CHS-2/3
P7
cDNA 76
pPE 923/1
cDNA 206
DR 9
cDNA 150/1
cDNA 125/08
GST-10/1
cDNA 164
cDNA 40/8
cDNA 44
cDNA 40/3
cDNA 267
cDNA 186
pPSR 546/3
5
F
5S/1
pPSR 546/4
cDNA 231
Vc-2
cDNA 373
cDNA 125/03
cDNA 204/1
pPSR 546/2
cDNA 125/02
cDNA 148/6
cDNA 204/3
cDNA 204/4
cDNA 204/2
cDNA 40/4
0.9 MI/2
cDNA 133
DR 17
GST-10/2
6
pCD 7/6
cDNA 40/6
cDNA 324
pCD 7/5
cDNA 46a
cDNA 289/2
5S/3
7
pAt-T4/8
pPE 923b/2
pPE 923b/3
DR 19
DR 20
DR 18
cDNA 2a
cDNA 200
cDNA 187
DR 26
cDNA 280
cDNA 189a
cDNA 286
Lg-1
DR 3
cDNA 148/4
1
gr-16/9
cDNA 260
pCD 72
a
cDNA 150/2
Lg-J/2
Lg-J
cDNA 125/04
cDNA 24
cDNA 56/1
cDNA 39
pST P
cDNA 136
cDNA 125/20
gr-16/8
cDNA 56/2
cDNA 148/1
DR 2
DR 7
DR 8
cDNA 40/1
m.u.
0
10
DR 23
DR 22
DR 24
pST T
cDNA 289
pST LL1
cDNA 125/11
gr-16/4
pAt-T4/7
pAt-T4/3
pAt-T4/2
pAt-T4/1

6.2 Materials and methods

6.2.1 Plant materials

The plant materials used in this study were the varieties and accessions of *P. sativum* listed in Table 6.1. A population consisting of 100 F_2 plants and F_2 derived families was produced from the cross between Alma and SA 310 for testing for linkage between marker loci and the gene controlling tolerance to boron. Alma and SA 310 are moderately tolerant and tolerant to boron, respectively (Chapter 4, Plate 6.1a) and this difference is conferred by allelic variation a single locus (Chapter 5).

Plant tissue samples were collected from the F_2 plants for isozyme, RAPD and RFLP studies and response to boron was determined for the F_3 families (Chapter 5).

Table 6.1 List of *P. sativum* varieties and accessions used for isozyme assays.

Genotype	Origin	Response to boron
Alma	Australia	Moderately tolerant
Collegian	Australia	Moderately sensitive
Pennant	Australia	Sensitive
PIG 16	India	Tolerant
SA 448	India	Tolerant
SA 310	Afghanistan	Tolerant

6.2.2 Isozyme analyses

Both starch and cellulose acetate (cellogel) gel electrophoresis techniques were used in this study.

Starch gel electrophoresis

Young actively growing leaves or seeds were used for isozyme analyses. Leaves were collected and placed in petri dishes on moist filter paper. Alternatively, seeds were soaked overnight in distilled water. One half of each leaf, or two or three 1 mm diameter pieces of seeds were transferred to a 1.5 ml centrifuge tube (Eppendorf® brand). After adding extraction buffer to each tube, a sharpened plastic knitting needle was used to break up the sample. The tubes were centrifuged for 2 min at 10,000 rpm. Homogenised samples were kept in an ice box to prevent degradation. Clear supernatants were used for electrophoresis.

Sample extraction buffer

Two extraction buffers were used in order to optimise allozyme resolution and enzyme stability (Table 6.2). Buffer I consisted of 0.07 M Tris-maleate, pH 7.4, containing 20% glycerol (v/v), 10% soluble polyvinylpyrolidone (PVP-40), 0.5% Triton X-100 and 14 mM 2-mercaptoethanol. The last two constituents were added immediately before use. The second extraction buffer (Buffer II) was 80 mM potassium phosphate, pH 7.0, containing 20% sucrose (w/v), 5% PVP-40, 0.5% Triton X-100 and 14 mM 2-mercaptoethanol. Again, the last two components were added just before use (Weeden and Marx, 1984).

Electrode buffer and starch gel preparation

The isozyme loci, extraction buffers and buffer systems used for isozyme analyses are given in Table 6.2. Three kinds of buffer systems were used, depending on the enzymes studied. Tris-citrate/lithium borate buffer, pH 8.1 (Weeden and Marx, 1984) was used for asparatate amino transferase (AAT or GOT), NADP-specific isocitrate dehydrogenase (DIA III), glucose-phosphate isomerase (PGI) or (GPI), alcohol dehydrogenase (ADH), leucine aminopeptidase (LAP), shikimate dehydrogenase (SKDH), peroxidase (PRX) and phosphoglucomutase (PGM). The gel buffer consisted of Tris-citrate and lithium borate buffer (9:1). The lithium borate buffer was used as the electrode buffer.

For the enzymes 6-phosphogluconate dehydrogenase (6-PGD) and NADH diaphorases (DIA I) the histidine buffer system, pH 6.5, was used. Histidine buffer (pH 6.5) was used to prepare the gels and the electrode buffer was tri-sodium citrate (pH 8.0) (Weeden and Marx, 1984).

The buffer system citrate/N-(3-amino propyl) morpholine (Weeden and Marx, 1984) was used for NADP-specific isocitrate dehydrogenase (IDH), acid phosphatase (ACP), methyl umbelliferyl esterase (EST), and peptidase (PEP). A 2 mM citrate/N-(3-amino propyl) morpholine buffer (pH 6.1) was used to prepare the gel and 40 mM citrate/N-(3-amino propyl) morpholine buffer (pH 6.1) used as the electrode buffer.

Table 6.2 Isozyme loci, extraction buffers and buffer systems used for isozyme analyses (starch gel).

Isozyme locus	Extraction buffer	System	Reference
PGI	Tris maleate (I)	Tris citrate/lithium borate (pH 8.1)	5
PGM	"	"	1
DIAIII	"	"	5
AAT	"	"	1
ADH	"	"	2
LAP	"	"	1
SKDH	"	"	1
PRX-3	"	"	3
6-PGD	Phosphate (II)	Histidine (pH 6.5)	1
DIA I	"	"	4
IDH	"	Citrate/N-(3-amino propyl) morpholine (pH 6.1)	1
ACP	"	"	1
EST	"	"	1
PEP	"	"	2

References: 1. Weeden and Marx (1984); 2. Weeden and Marx (1987); 3. Weeden and Provvidati (1991); 4. Wolko and Weeden (1988); 5. Wolko and Weeden (1990).

Ten percent potato starch gels (Sigma brand) were prepared and a slot was cut in the gel in the direction of electrophoresis, 4 cm from one end. With clean forceps, paper wicks (6 x 4 mm Whatman #3) were dipped in the supernatant of homogenised samples and loaded in the slots. After 30 min, the sample wicks were removed and the gel was run as shown in Table 6.3.

Table 6.3 Buffer systems and running conditions used in assays of pea isozymes.

System	Voltage (V)	Current (mA)	Running conditions (Hours)
Tris-borate (pH 8.1)	200	75	3.00
Histidine (pH 6.5)	150	25	5.30
Citrate/N-(amino propyl) morpholine (pH 6.1)	300	75	5.30

Staining

Staining assays for the different enzymes are presented in Table 6.4. Staining solutions were prepared immediately before the end of the gel run. Slices of the gel were immersed in the staining solution with the cut side up and incubated at 37°C in the dark until the isozyme bands were stained. After staining, gels were fixed in 10% acetic acid.

Table 6.4 Staining solutions for detection of isozymes by starch gel electrophoresis.

Enzyme	Stain composition	References
AAT	45 ml H_2O, 5 ml 1M Phosphate buffer[a] (pH 7.0), 0.125 g L-Asparatic acid, 0.1 g α–Ketoglutaric acid, 0.1 g Fast Blue BB, 0.006 g Pyridoxal-5-Phosphate	Brown *et al.*(1978)
ACP	50 ml 0.5 M acetate[b] (pH 5.0), 50 mg Black K salt, 50 mg Na-α-naphthyl acid phosphate	Shaw and Prasad (1970)
ADH	60 ml 0.2 M Tris HCl (pH 8.0), 2.6 ml NBT, 0.6 ml PMS, 3 mg β NAD, 2.6 ml ethanol (absolute)	Brown (1983)
DIA	50 ml 0.1 M Tris HCl (pH 8.5), 20 mg NADH, 20 mg MTT, 0.5 mg 2, 6 dichlorophenol	Wolko and Weeden (1988)
EST	0.05 g Fast blue RR, 5 ml 0.5 M Tris-HCl (pH 7.1), 0.015 g α naphthyl acetate, 0.015 g β naphthyl acetate, 43.5 ml H_2O	Shaw and Prasad (1970)
GPI	50 ml 0.1 M Tris HCl (pH 8.0), 2 mM $MgCl_2$, 0.5 mM NADP, 0.1 mg ml^{-1} PMS, 0.4 mM MTT, 1 unit Glucose 6 Phosphate dehydrogenase, 10 mM Fructose 6-Phosphate	Wolko and Weeden (1990)
IDH	50 ml Tris HCl (pH 8.0), 50 mg $MgCl_2$, 75 mg Isocitric acid, 2 ml NBT, 5 mg NADP, 0.5 ml PMS	Brown and Munday (1982)
PEP	50 ml 0.2 M Tris HCl (pH 8.0), 10 mg $MnCl_2$, 25 mg $MgCl_2$, 50 mg O-dianisidine dihydrochloride, 10 mg L-amino acid oxidase (Snake venom), 20 mg peroxidase, 50 mg l-Leucyl-L alanine	Brown *et al.*(1978)
PRX-3	25 ml 0.1 M acetate (pH 5.0), Two drops 3% H_2O_2, 25 mg 3-amino-9-ethylcarbazole (dissolve in 2 ml N,N-dimethylformamide)	Weeden and Provvidati (1991)
SKDH	50 ml Tris HCl (pH 8.5), 1.5 mM shikimic acid, 0.3 mM MTT, 100 μM PMS, 0.001 g NADP	Weeden and Gottlieb (1980)
6-PGD	100 ml 0.2 M Tris HCl (pH 8.0), 0.4 ml $MgCl_2$ (10% solution), 0.02 g trisodium 6-phosphogluconic 0.01 g NADP, 4 ml Nitro-Blue-tetrazolium (0.005 g ml^{-1}), acid 1 ml phenazine methosulphate (PMS)(0.005 g ml^{-1})	Brown *et al.*(1978)

[a] Phosphate buffer (1M, pH 7.0): A, 20.4 g KH_2PO_4 in 150 ml de-ionized water; B, 52.25 g K_2HPO_4 in 300 ml de-ionized water, mix B into A to pH 7.0

[b] 0.5 M acetate pH 5.0: 6.8 g Na acetate, 3 H_2O; 14.8 ml HCl (1N); 1000ml H_2O and adjust pH with 0.1 N HCl.

Cellulose acetate (cellogel) gel electrophoresis

Cellogel (Chemtron, Italy) electrophoresis was used to screen the F_2 population for enzymes which showed polymorphism between parents in the starch gel electrophoresis. Fresh leaf material from the parents was extracted and subjected to isozyme analysis to check that the same isozyme pattern was present as determined in the starch gel. Leaf material (0.4 g) was ground with 0.15 g poly-vinylpolypyrolidone and 2.0 ml of extraction buffer containing 0.05 M Tris, 0.15% citric acid, 0.12% cystein HCl, 0.1% ascorbic acid, pH 8.0. Grinding was carried out in a mortar. The mixture was then centrifuged at 3,000 rpm for 30 min and the supernatant was used for gel electrophoresis. The running buffer for electrophoresis was 0.05 M Tris-maleate (pH 7.8) for LAP, AAT and SKDH and 0.025 M Tris-glycine (pH 8.5) for PGM.

Cellogel electrophoresis was carried out as described by Richardson *et al.* (1986). Gel slices (20x15 cm) were soaked in 300 ml of electrophoresis buffer for 3 hours at 4°C before being placed in gel trays containing 700 ml cold electrode buffer. The gel trays were designed such that the gel made direct contact with the electrode buffer. Approximately 1 µl of sample was loaded on the gel using a drawing pen and electrophoresis was carried out at 200 V and 4°C for approximately 2 hours.

Following the run, staining for AAT, LAP and PGM was carried out as described by Richardson *et al.* (1986). For SKDH, staining solution was made up immediately prior to use by adding 6 mg shikimic acid, 0.1 ml 25 mM NADP, 0.1 ml 14.5 mM MTT and 0.1 ml 6.5 mM PMS to 2.0 ml 0.1M Tris (pH 8.5). Stained gels were incubated in an oven at 37°C to allow the isozyme pattern to develop and then scored.

6.2.3 RAPD analyses

Mini-preparation of template DNA

The F_2 population of 82 plants from the cross between Alma and SA 310 was used to identify RAPD markers linked to the boron tolerance gene using the strategy described by Michelmore *et al.* (1991). 0.5 g leaf samples were collected from each F_2 plant and parents, placed in 2.0 ml Eppendorf tubes and kept at -80°C . The leaves were ground to a powder under liquid nitrogen and mixed with 750 µl DNA extraction buffer (Appendix 1a), then with 750 µl phenol/chloroform/iso-amyl-alcohol (25: 24: 1). The mixture was shaken for 20-30 s, extracted on an orbital rotor for 30 min and the aqueous phase recovered after centrifugation. The phenol/chloroform/iso-amyl-alcohol extraction was repeated. The aqueous phase was transferred to a fresh tube, an equal volume of chloroform added and extracted again on an orbital rotor for 30 min. After centrifugation, the aqueous phase was transferred to a fresh tube. DNA was precipitated by adding 1/10 volume of 3M Na-acetate pH 4.8 and an equal volume of isopropanol. To recover the DNA, samples were centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the pellet washed twice in 70% ethanol. The sample was then dried under vacuum for 5 min before being resuspended in 50 µl R40 (Appendix 1a).

Polymerase chain reaction materials

The 10-mer oligonucleotides used as random primers in the PCR were purchased from Operon Technologies Inc. (Alameda Calif., USA) and used as single primers for the amplification of RAPD sequences. *Taq* DNA polymerase, together with 10x concentrated PCR buffer was supplied by Promega (Madison, USA). The PCR was performed in a PTC-100 (Programmable Thermal Controller, MJ Research, Inc, USA).

Amplification reaction conditions

The amplification conditions were tested rigorously in order to optimise the generation of RAPDs from pea DNA. The polymerase chain reactions were carried out in a 25 µl volume containing 0.5 units *Taq* polymerase (Promega), genomic DNA template (ranging from 5 to

200 ng), 15 ng of primer, 0.2 mM each of dATP, dCTP, dGTP and dTTP; $MgCl_2$ (ranging from 0.5 to 3 mM) and 3 µl of 10 x *Taq* reaction buffer (Promega, Appendix 1a).

The reaction mixture was overlayed with one drop of paraffin oil and subjected to PCR. Amplification was for 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C followed by one cycle of 5 min at 72°C after which samples were cooled to 25°C for 5 min. Samples of 10 µl PCR products were analysed on 1.5% agarose gels (Promega) in 1x TBE buffer system (Appendix 1a) running at 50 mA for two hours and visualised under UV light following staining with ethidium bromide (Appendix 1a). The size marker used for the gels was *Spp*-1 phage DNA restricted with *Eco*RI (Bresatec Ltd, Adelaide).

6.2.4 Molecular cloning of RAPD products linked to boron tolerant gene(s)

Amplification reaction conditions

Two 10-mer primers, OpG02 and OpK07, that amplified regions which appeared linked to the boron tolerance gene on the basis of the bulk segregation analysis, were used for the amplification. Reaction mixtures (25 µl) contained 2.5 µl of 10x Vent® reaction buffer (New England Biolabs, Inc.; Appendix 1b), 0.4 mM of each dNTP, 15 ng primer, 1 µl template DNA (1 µl of 1/10 dilution) and 1 unit of Vent® DNA polymerase (New England Biolabs, Inc.) overlayed with paraffin oil.

DNA was amplified using the program described earlier. Amplified DNA fragments were separated by electrophoresis on 1.5% agarose gels in 1x TBE buffer. The gel was stained with ethidium bromide and the bands corresponding to the DNA of the desired length, as determined by comparison with a DNA size standard, were excised. The DNA was recovered from the gel by using the Geneclean®II kit (Bio 101, La Jolla, CA). The excised bands were dissolved by soaking them in 4.5 volumes of 6M NaI and 1/2 volume of TBE modifier for 5 min at 50°C. The DNA was bound with 5 µl glassmilk (suspension of silica matrix in water) incubated at room temperature for 5 min and centrifuged for a few seconds. The upper solution was removed and the pellet was washed three times with New Wash®

solution (Appendix 1b). The pellet was resuspended in 10 µl TE buffer (Appendix 1b) and incubated at 50°C for five minutes. The aqueous supernatant phase was collected and used as insert DNA in the ligation mixture.

Preparation of the cloning vector

The pBluescript SK (+) plasmid was chosen as the cloning vector. This vector enables α-complementation of the β–galactosidase gene (Sambrook *et al.*, 1989) allowing blue/white colour selection of recombinant colonies on agar plates when supplemented with IPTG and X-gal.

One µg of pBluescript SK plasmid was digested with 5 units *Sma* I restriction enzyme in the presence of the appropriate restriction buffer (provided by the manufacturer, Boehringer Mannheim) at room temperature for two hours. The digested vector was resolved by gel electrophoresis on 1.5% agarose with 1 x TBE buffer, excised from the gel, and purified using the Geneclean® II kit.

Ligation of RAPD product DNA to a linearized plasmid vector

The vector was ligated to the insert in a 10 µl solution containing 2 µl 5x ligation buffer (Appendix 1b), 0.5 mM ATP, 3 units T4 DNA ligase (Bresatec Ltd, Adelaide) and 40 ng of the vector. The RAPD product DNA was added to maintain vector to insert ratios of approximately 2:1, 1:1 and 1:5. This was achieved by estimation of the amount of insert DNA in comparison with a standard size marker (*Spp*-1 phage DNA/*EcoR*I) by gel electrophoresis. The ligation mixture was incubated at room temperature (25°C) overnight.

Transformation of electrocompetent DH5α cells with recombinant plasmids by high-voltage electroporation electroshock transformation

Preparation of electrocompetent DH5α cells

A single colony of *E. coli* DH5α was removed from a streak plate and grown overnight in 15 ml of 2YT medium (Appendix 1b). One litre of 2YT medium was inoculated with 10 ml of the overnight culture. The cells were grown for about four hours in a shaken non-baffled 2 litre flask at 37°C until the optical density (OD) at λ=600 nm was 0.9 on a spectrophotometer. The culture was placed on ice for 15 minutes. The cells were repelleted in a cooled GSA rotor at 3,000 rpm and 4°C for 15 min and carefully resuspended in one litre of cold sterile nanopure water. The cells were again pelleted by centrifugation as described above. The supernatant was carefully decanted and the pellet was resuspended in 0.5 L 10% glycerol. The cells were again centrifuged, the supernatant decanted and the pellet resuspended in 20 ml 10% glycerol, and pelleted by centrifugation. After decanting the supernatant, the pellets were resuspended in 2 ml 10% glycerol. Finally, the suspension was dispensed as 40 µl aliquots of cells into chilled 1.5 ml Eppendorf tubes and snap frozen in liquid nitrogen. The aliquots were stored at - 80°C.

Precipitation purification of DNA (ligation mixture) for electroshock transformation

DNA of the ligation mixture was precipitated by adding 90 µl TE buffer to the ligation reaction and 1µl 10 mg ml^{-1} tRNA and adjusting the solution to 0.3 M sodium acetate. 2.5 volumes of cold ethanol were added and the mixture stored on ice for 20 min. The DNA was pelleted by centrifuging at 14,000 rpm for 20 min in an Eppendorf centrifuge and the supernatant decanted. The pellet was rinsed with 70% ethanol and centrifuged for 10 min. The supernatant was carefully removed and pellets dried under vacuum for 5 min. The pellets were resuspended in 10 µl of nanopure water.

Transformation of competent cells

2 μl of the ligated plasmid DNA were transferred to a chilled electroporation cuvette and 40 μl of the competent cells were added and mixed gently. Electric field pulses were applied by the electric capacitor discharge method using a BioRad Gene Pulser® unit. The cells were electroshocked at 1.8 kV. About 1.5 ml of 2YT medium was added to each cuvette and then the mixture was transferred to Eppendorf tubes and incubated at 37°C for one hour. Cells were plated out as 300 μl aliquots onto agar plates with LB medium containing 50 μg ml^{-1} ampicillin, X-Gal and IPTG and incubated at 37°C overnight to allow growth of colonies. Recombinant colonies containing insert appeared as white colonies on this medium and the others were blue.

Mini-preparation of recombinant plasmids from bacteria cells

Recombinant plasmids were isolated from the bacterial cells by a minipreparation alkaline lysis method. White clonies were picked from the plate and used to inoculate 3 ml 2YT medium containing 100 μg ml^{-1} of ampicillin. The cultures were incubated overnight at 37°C with vigorous shaking. A 1.5 ml aliquot of each culture was pelleted by centrifugation at 14,000 rpm for three minutes. The supernatant was removed by aspiration and the cells were resuspended in 100 μl GET solution (Appendix 1b) by pipetting up and down. Cells were lysed by 200 μl of a freshly prepared mixture of 0.2 N NaOH and 1% SDS and placed on ice for about 5 min. The solution was neutralised by adding 150 μl of KAcF (Appendix 1b), mixed by inversion and then incubated on ice for 5 min. The debris was removed by centrifugation at 14,000 rpm for 8 min and the supernatant containing the plasmid was transferred to a new tube. Plasmid DNA was precipitated with 400 μl isopropanol and pelleted by centrifugation at 14,000 rpm for 15 min. The pellets were washed in 70% ethanol, dried under vacuum for 5 min, resuspended in 40 μl TE buffer and stored at -20°C.

Analysis of the sizes of inserts in recombinant plasmids by restriction enzyme digestion

The recombinant plasmids were analysed for the presence of insert DNA of the correct size by digestion with *Pvu*II according to the manufacturer´s (Promega) directions. Digestion

was performed overnight at 37°C. 200 units RNase A (DNase-free) were added to each tube and incubated at room temperature for 20 minutes. The reaction mixture was then electrophoresed in a 1.5% agarose gel in 1x TBE buffer using *Spp*-1 phage DNA/*EcoR*I as the DNA size standard.

6.2.5 RFLP analyses

RFLP analysis with clones of RAPD products linked to the boron tolerance gene.

Two cloned RAPD products, which were suspected as being linked to the gene conferring tolerance to boron on the basis of the initial bulk segregation analysis, were used as probes for RFLP analyses in an attempt to develop alternative and more robust markers.

Isolation of insert DNA from the plasmid

Recombinant plasmid DNA was amplified by PCR using M13 forward and reverse primers. This amplification provided an efficient method of isolating the DNA insert from within the pBluescript plasmid as the M13 forward and reverse primers anneal to sites at either end of the polycloning site. The PCR reactions were prepared in a volume of 50 µl containing 1.0 unit *Taq* polymerase, 50 ng per reaction plasmid DNA template, 150 ng per reaction of each of M13 forward and reverse primers, 0.4 mM each of dATP, dCTP, dGTP and dTTP; 3 mM $MgCl_2$ and 5 µl of 10 x *Taq* reaction buffer. The reaction tubes were overlaid with one drop of paraffin oil and placed in a Thermal cycler. The PCR conditions consisted of an initial denaturing treatment at 94°C for 5 min followed by 35 cycles of annealing at 55°C for 2 min and denaturing at 94°C for 1 min. The reaction was completed by 72°C for 10 min and 25°C for 5 min.

An aliquot of the PCR sample was fractionated by electrophoresis on a 1% agarose gel in 1x TAE buffer (Appendix 1c), stained with ethidium bromide (10 µg ml^{-1}) and viewed under UV light. DNA of the desired length, as determined by comparison with the DNA size standard, was excised. The DNA was recovered from the gel by using the Geneclean® II kit. Concentration of the DNA was approximated by comparison with the size marker of known concentration.

Digestion of genomic DNA with restriction enzymes, electrophoresis and transfer of DNA to nylon membranes

Genomic DNA was digested by four 6-base pairs recognition restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I) (Promega). DNA was digested in a reaction containing 6 µl DNA solution (concentration approximately 0.5 µg μl^{-1}), 14 units of restriction enzyme, 1 µl spermidine, 1 µl BSA and 1.2 µl of 10x restriction enzyme buffer (Appendix 1c). The reaction was made up to a total volume of 12 µl by the addition of sterile water. All reactions were incubated at 37°C for 6-12 hours. Digested DNA was fractionated in 1% agarose gels in 1x TAE buffer running at 20-25 mA overnight. After electrophoresis, gels were stained in 10 µg ml^{-1} ethidum bromide for 20 min and viewed under UV light. Gels were soaked for 30 min in 200 ml denaturing solution (Appendix 1c) and rinsed with 5x SSC for 1 min. The DNA was transferred to a nylon membrane (Hybond-N^+, Amersham) by capillary blotting (Southern, 1975) using 20x SSC for 4-6 hours. Upon completion of the transfer, the membranes were rinsed in 5x SSC for 10-15 sec and dried. DNA was fixed by soaking the membranes, DNA facing upwards, for 20 min on Whatman filter paper (3MM Chr) saturated in 0.4 M NaOH. The membranes were placed in neutralising solution (Appendix 1c) for 5 min and then rinsed with 2x SSC for 2 min, dried and sealed in a plastic bag.

Labelling of probes

Probes were labelled by the random priming method (Feinberg and Vogelstein, 1983). Approximately 50 ng samples of probe DNA were mixed with 3 µl random primer (9mer), denatured by boiling in water for 5 min and then chilled on ice to prevent reannealing of the separate strands. Then 12.5 µl specific oligolabelling buffer, 3 µl [α-^{32}P]dCTP and 1.5 units Klenow enzyme (Promega) were added, mixed and incubated at 37°C for 60 min. Labelled DNA was separated from unincorporated nucleotides on a Sephadex G-100 column, mixed with 250 µl of salmon sperm DNA (5 µg ml^{-1}), boiled in water for 10 min and chilled on ice.

Prehybridization, hybridisation of DNA probe to membrane and autoradiography

The membranes were prehybridized in a buffer containing 1.75 ml sterile water, 3.0 ml 5x HSB, 2.0 ml 10x Denhardts III solution, 3 ml 25% dextran sulphate and 250 µl salmon sperm DNA (5 µg ml^{-1})(Appendix 1c). Salmon sperm DNA was boiled in water for 5 min and chilled on ice before being added to the buffer. The membrane was placed in a hybridisation bottle and the solution, which was pre-warmed to 65°C, was added. Membranes were prehybrized at 65°C for 6-12 hours in a Hybaid® oven.

The labelled probes were added to the hybridisation mixture in the bottle and hybridisation was performed at 65°C overnight. Unbound DNA probe was removed by washing the membranes in 2x SSC, 0.1% SDS for 30 min, 1x SSC, 0.1% SDS and 0.5x SSC, 0.1% SDS at 65°C. The membranes were sealed in plastic and X ray film (Fuji Medical-X ray Film) exposed inside light proof autoradiograph cassettes at -20°C for 1 hour and -80°C for one to three days, depending on signal strength, and then scored.

<u>RFLP analysis of recombinant inbred populations</u>

As stated previously, specific RFLP probes for pea DNA are unavailable in Australia. However, the possibility of locating genes conferring tolerance of pea to boron using existing RFLP maps was investigated in collaboration with Dr T.H.N. Ellis, John Innes Institute, UK. Dr Ellis has produced linkage maps of pea, incorporating RFLP and morphological markers, for several recombinant inbred and F_2 populations (Ellis *et al.*, 1992). The parents of these populations were screened for tolerance to boron in a quarantine glasshouse by the method described in Chapter 4 (Table 6.5). Two replicates of Alma and Pennant as moderately tolerant and sensitive Australian varieties, respectively (Chapter 3), were sown as checks for comparisons among genotypes. Based on the screening results (Table 6.5), the mapping population derived from the cross JI 15 x JI 399 (Plate 6.1b) was chosen for further testing. Two to ten seeds of each of 27 recombinant inbred lines (F_{11} progeny), depending on availability of seeds, were screened in the quarantine glasshouse

and visual symptoms of boron toxicity were recorded four weeks after sowing. The parents were grown along with the recombinant inbred lines and used as controls. The data were compared by Dr T.H.N. Ellis with morphological and RFLP markers already mapped in this population, to find a linkage to the boron response.

Table 6.5 Response of the parents of John Innes inbred populations to high concentrations of soil boron together with the Australian varieties Alma and Pennant.

Line	Characteristics of the lines	Boron response
JI 15	=WBH 1458, a classical marker stock	Sensitive
JI 61	=WBH 761, carrier of a 3/5 translocation	Moderately sensitive
JI 281	Landrace from Ethiopia	Moderately sensitive
JI 399	cv Cennia	Moderately tolerant
JI 813	From cv Vinco, carrier of *yp*	Moderately tolerant
JI 1194	MISOG-1 conventional	Moderately sensitive
JI 1201	MISOG-1 *af*, *st*, *tl*	Moderately sensitive
Alma	Australian variety	Moderately tolerant
Pennant	Australian variety	Sensitive

RFLP analyses of the F_2 population derived from the cross between Alma x SA 310 by probes selected from linkage group 1

Further RFLP analyses were performed with the following five DNA markers: cDNA 44, cDNA 150, cDNA 206, SHMT and pIT26-74. These probes which map to chromosome 1 (Ellis *et al.*, 1992; Turner *et al.*, 1993) in the region of the boron gene in the JI 15 x JI 399 population were kindly provided by Drs T.H.N. Ellis and C. Domney of John Innes Institute, Norwich, UK. DNA from the parents was digested with the enzymes *Eco*RI, *Eco*RV, *Bam*HI, *Dra*I and *Hin*dIII and Southern analyses were performed as described

previously. The F_2 population of Alma x SA 310 was then tested with enzyme/probe concentrations that revealed RFLPs between the parents.

6.2.6 Association of the boron tolerance gene with allozyme, RAPD and RFLP markers

The mapmaker program (Mapmaker Macintosh V1.0 Du Pont) was used to assess the association of the response to boron with allozyme, RAPD and RFLP markers. LOD scores (Edward, 1992) were also calculated. A LOD (log odds) score is calculated by the logarithm of the ratio of two probability densities or hypotheses. In the case of linkage mapping the alternative hypotheses are the possible orders of genes.

Plate 6.1 Response of the parents of mapping populations to high concentrations of soil boron.

(a) Alma and SA 310 at 100 mg kg^{-1}

(b) JI 399 and JI 15 at 60 mg kg^{-1}

(a)

(b)

6.3 Results

6.3.1 Isozyme analyses

Biochemical markers, which are encoded by genes previously mapped to the seven chromosomes of pea, were investigated. Nineteen enzyme systems were studied and, of these, four enzymes (AAT, SKDH, LAP and PGM) showed clear bands, reproducible banding patterns and polymorphisms between parents (Table 6.6, Fig 6.3). These four marker loci occurred on three chromosomes. Other enzymes including IDH and G6PD produced reliable banding patterns, but there was no polymorphism observed between the parent lines. The remaining enzymes showed insufficient or no activity (e.g. SOD), complex and hard to reproduce zymograms (e.g. EST) or inconsistent patterns (e.g. PEP).

Details for all enzymes studied are presented in Table 6.6. In general, Australian varieties tended to show similar banding patterns for the enzymes AAT, LAP, PGM and SKDH. The tolerant accessions were similar to each other, but different to the Australian varieties, for these enzymes. Details of the band staining patterns of the four enzymes tested on the segregating population were:

a) AAT

Samples showed five zones of band mobility. The fastest of these bands showed the weakest staining activity (Fig. 6.4). The lines PIG 16, SA 310 and SA 448 showed similar banding patterns (bands 3 and 5) while the other lines tested showed different banding patterns (Alma, bands 1, 4 and 5; Pennant, 1, 2, 4 and 5; Collegian, bands 4 and 5). Bands 3 and 4 appeared to be allelic and homozygous individuals showed only one band. However, heterozygotes of the mapping population showed a band of intermediate mobility between bands 3 and 4. This polymorphism (bands 3 and 4) was used to assay the degree of linkage with the boron tolerance character in the mapping population. This locus is located in chromosome 7, and was named *Aat-m* by Weeden and Marx (1984).

b) LAP

LAP isozyme gels showed two zones of similar staining intensity (Fig 6.3). Lines tested showed either the fast migrating band (Alma, Pennant and Collegian) or the slower band (PIG 16, SA 310 and SA 448). The heterozygous types within the F_2 population were characterised by three bands corresponding to the fast and slow bands of parental types and a third band of intermediate mobility. This enzyme locus is located in chromosome 3 (Weeden and Marx, 1984).

c) SKDH

These gels showed two zones of similar staining intensity (Fig. 6.3). Lines showed either the fast migrating band (Alma, Pennant and Collegian) or the slower band (PIG 16, SA 310 and SA 448). Heterozygotes of the mapping population were distinguishable by the presence of fast and slow bands (parental) and a band of intermediate mobility. SKDH is located in chromosome 7 (Weeden and Marx, 1984).

d) PGM

This system also showed two zones of similar staining intensity (Fig 6.3). Lines showed either the fast migrating band (PIG 16, SA 310 and SA 448) or a slow band (Alma, Pennant and Collegian). Heterozygotes of the mapping population were distinguishable by the presence of fast and slow bands (parental) and an intermediate band. This enzyme locus is located in chromosomes 2 and 7 (Weeden and Marx, 1984; 1987).

<u>Association of the boron tolerance gene with allozymes</u>

The Mapmaker program was used to assess the association of the response to boron with allozymes. There was no association between any of the allozymes and tolerance to boron. Two-way contingency tables are presented in Table 6.7.

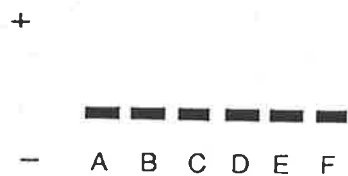
Table 6.6 List of enzymes, types of gel used for electrophoresis, presence or absence of polymorphisms and comments on results for each enzyme.

Isozyme	Starch gel	Cellogel (buffer)[a]	Polymorphism	Comment
ACP	+[b]	+ (C)	?	Poor reproducibility
ADH	-	+ (B)	?	Hard to score
DIA I	-	+ (A)	?	Low activity
DIA III	-	+ (A)	?	Low activity
EST	+	+ (A)	+	Complex and hard to score
AAT	+	+ (C)	+	High reproducibility
GPI	+	+ (C)	?	Low activity
IDH	+	+ (C)	-	High reproduciblity
LAP	+	+ (C)	+	High reproduciblity
LOX	n.t.	+ (A)	-	Low activity
MDH	n.t.	+ (A & B)	?	Low activity
MPI	n.t.	+ (A)	?	Low activity
PEP	+	+ (C)	+	Poor reproducibility
PGM	+	+ (A)	+	High reproducibility
PRX-3	+	n.t.	-	Low activity
SKDH	-	+ (C)	+	High reproduciblity
SOD	n.t.	+ (A)	?	Low activity
6-PGD	+	n.t.	+	Poor reproducibility
G6PD	n.t.	+ (B)	-	High reproducibility

[a] Cellogel buffers: A, 0.05 M Tris-maleate (pH 7.8); B, 0.02 M phosphate, pH 7.0 and C, 0.025 M Tris-glycine (pH 8.5).

[b] +, enzyme activity detected; -, no enzyme activity; n.t. not tested.

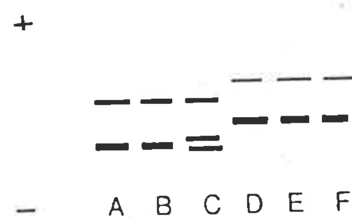
Fig 6.3 Zymogram representation of electrophoretic banding patterns for IDH, G6PD, LAP, PGM, GPI, 6PGD and SKDH. A, B, C, D, E and F refer to Alma, Pennant, Collegian, PIG 16, SA 310 and SA 448, respectively. The movement of bands was from - to +.



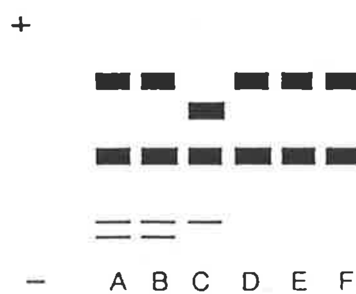
(IDH & G6PD)

(LAP)

(PGM)



(GPI)

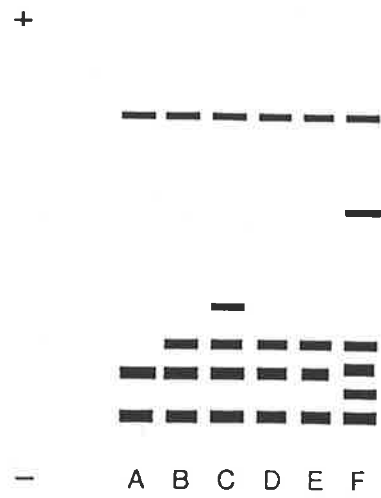


(6PGD)

(SKDH)

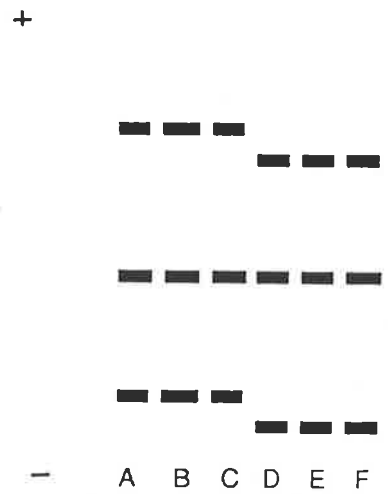
Fig 6.3 (continued) Zymogram representation of electrophoretic banding patterns for AAT, EST, ACP and PEP. A, B, C, D, E and F refer to Alma, Pennant, Collegian, PIG 16, SA 310 and SA 448, respectively. The movement of bands were from - to +.

(AAT)



(EST)

(ACP)



(PEP)

Table 6.7 Two-way contingency tables between genotypes with respect to boron tolerance and Isozyme markers.

Enzyme system		Boron			
		BoBo	*Bobo*	*bobo*	Total
GOT					
SA 310 type		5	13	5	23
Heterozygote		8	21	8	37
Alma type		6	15	5	26
	Total	19	49	18	86
LAP					
SA 310 type		2	11	5	18
Heterozygote		10	27	8	45
Alma type		7	9	6	22
	Total	19	47	19	85
SKDH					
SA 310 type		6	7	2	15
Heterozygote		10	26	13	49
Alma type		7	9	6	22
	Total	23	42	21	86
PGM					
SA 310 type		1	4	3	8
Heterozygote		14	26	10	50
Alma type		4	16	7	27
	Total	19	47	19	85

6.3.2 RAPD analyses

Preliminary investigations were conducted to optimise conditions for PCR. One factor known to affect the stringency of PCR amplification is the magnesium concentration. The concentration of magnesium, as $MgCl_2$, was varied between 0.5 and 3 mM. A concentration of 3 mM resulted in the most reliable results (Plate 6.2). A constant banding pattern was obtained with the concentration of template DNA ranging from 5 to 200 ng per reaction, but the concentration of 50 ng per reaction produced the most intense amplification bands (Plate 6.2).

The F_2 individuals used for isozyme analyses (derived from a cross between Alma and SA 310) were used for RAPD analyses. 126 primers of arbitrary nucleotide sequence were tested against template DNA from the parent genotypes Alma and SA 310. A high frequency of the primers produced amplified products and a large number of easily scored polymorphisms were found. Overall, the frequency of polymorphic primers between parents was 86 out of the 108 successful primers. Band profiles comprised of from one to five major products plus a varying number of minor products. Examples of polymorphic bands detected in DNA from parental lines are shown in Plate 6.3.

Following the identification of polymorphisms between parents, the segregating population was tested by bulked segregant analysis. Ten individuals from either homozygous tolerant or homozygous sensitive families were pooled and screened along with the parents. Two primers amplified products where the tolerant and sensitive pooled DNAs corresponded to the tolerant and sensitive parents, respectively, indicating a high probability of linkage to the gene for boron tolerance. These primers were $OpG02_{800}$ and $OpK07_{400}$. ´Op´ for the source of the primers (Operon Technologies), G02 and K07 for the specific kits of (G), primer number (2) and (K), primer number (7), respectively, and 800 and 400 for the sizes (bp) of the polymorphic fragments. Both primers are decamers and their sequences are OpG02: 5´-GGCACTGAGG-3´ and OpK07: 5´-AGCGAGCAAG-3´. These two primers were then analysed against the DNA of individual plants from the F_2 population (Plate 6.4) and results compared with boron response of the F_3 families. The F_3 families segregated for

boron response in the monogenic ratio of 1 (homogeneous tolerant): 2 (segregating): 1 (homogeneous sensitive) (Chapter 5).

The RAPD reactions were scored for the presence or absence of a single band. Segregation for presence : absence of the bands fitted the ratio of the 1: 3 for both primers (Table 6.8). These ratios are consistent with monogenic segregation where the presence of the band was expressed as a recessive trait. For both primers, the critical band was derived from the sensitive parent Alma. The F_2 population was therefore scored as either homozygous "SA 310-type" or heterozygous (i.e. band absent) or as "Alma-type" (i.e. band present). The program Mapmaker (V1.0 Du Pont) was employed to calculate the percent recombination between these markers and the boron tolerance gene. OpG02 showed 25.9% recombination with the boron tolerance gene with a LOD score of 2.12, but the polymorphic band generated by OpK07 was not linked to response to boron.

Table 6.8 Two-way contingency tables between genotypes with respect to boron tolerance and two RAPD markers, OpG02 and OpK07.

RAPD marker	Boron			
	BoBo	*Bobo*	*bobo*	Total
OpG02				
SA 310 type or Heterozygote type	17	23	8	48
Alma type	0	1	9	10
Total	17	24	17	58
OpK07				
SA 310 type or Heterozygote type	14	17	6	37
Alma type	2	5	9	16
Total	16	22	15	53

Plate 6.2 Ethidum bromide stained 1.5% agarose gels of RAPD products from a reaction using primer J 11 (Operon Technologies, Inc.) on the pea variety Alma.

(a) PCR products with the following template concentrations:

Lane 1. DNA size marker

Lane 2. 5 ng

Lane 3. 10 ng

Lane 4. 20 ng

Lane 5. 50 ng

Lane 6. 100 ng

Lane 7. 200 ng

(b) PCR products with the following $MgCl_2$ concentrations:

Lane 1. DNA size marker

Lane 2. 0.5 mM

Lane 3. 1.0 mM

Lane 4. 1.5 mM

Lane 5. 2.0 mM

Lane 6. 2.5 mM

Lane 7. 3.0 mM

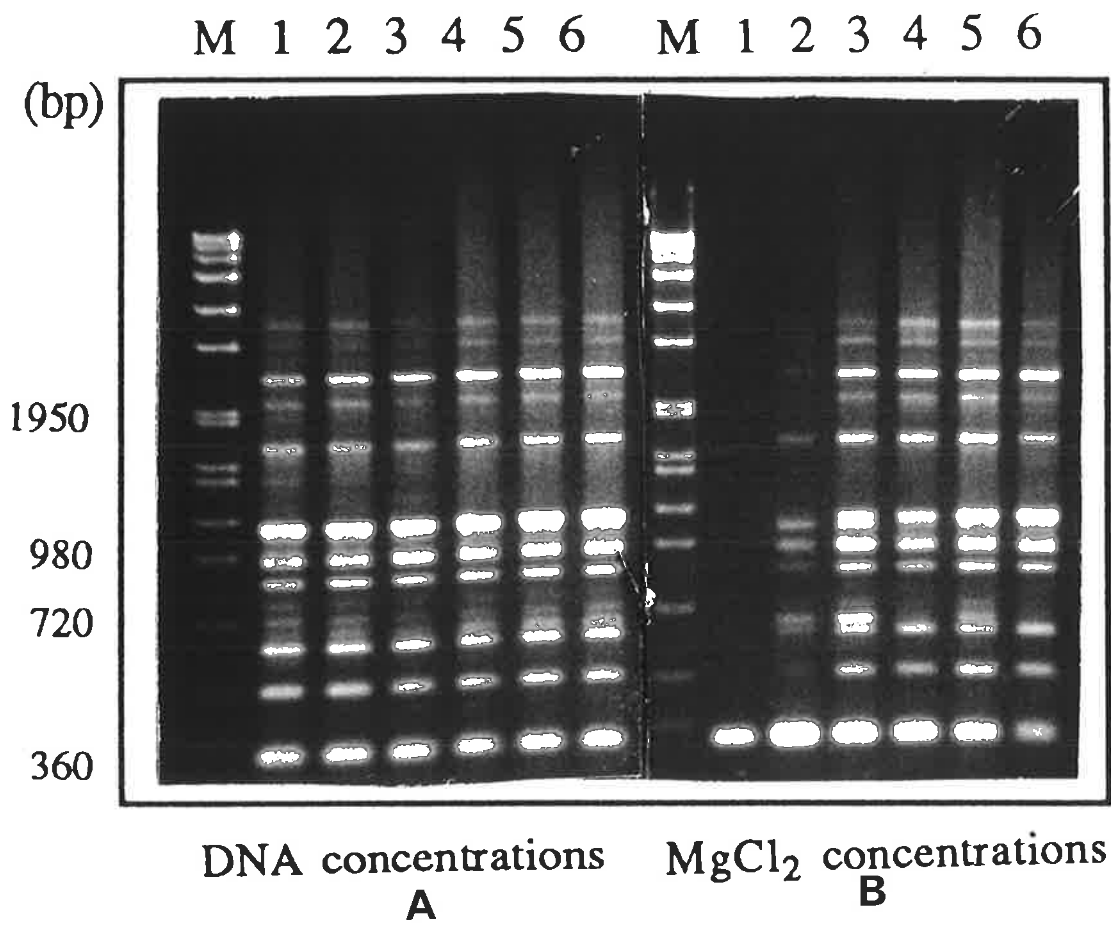

M 1 2 3 4 5 6 M 1 2 3 4 5 6
(bp)
1950
980
720
360
DNA concentrations
A
MgCl2 concentrations
B

Plate 6.3 DNA polymorphisms generated by 10 mer primers OpG07, OpG10, OpG16, OpI13 and OpI14 with pea varieties Alma and SA 310.

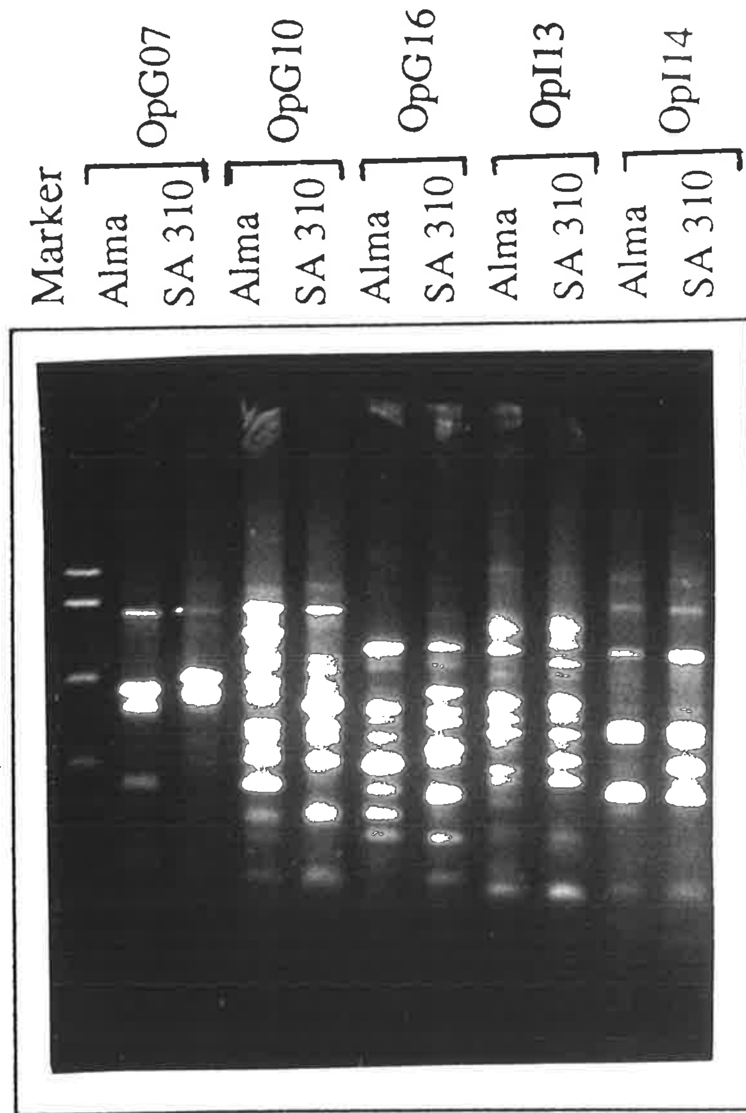
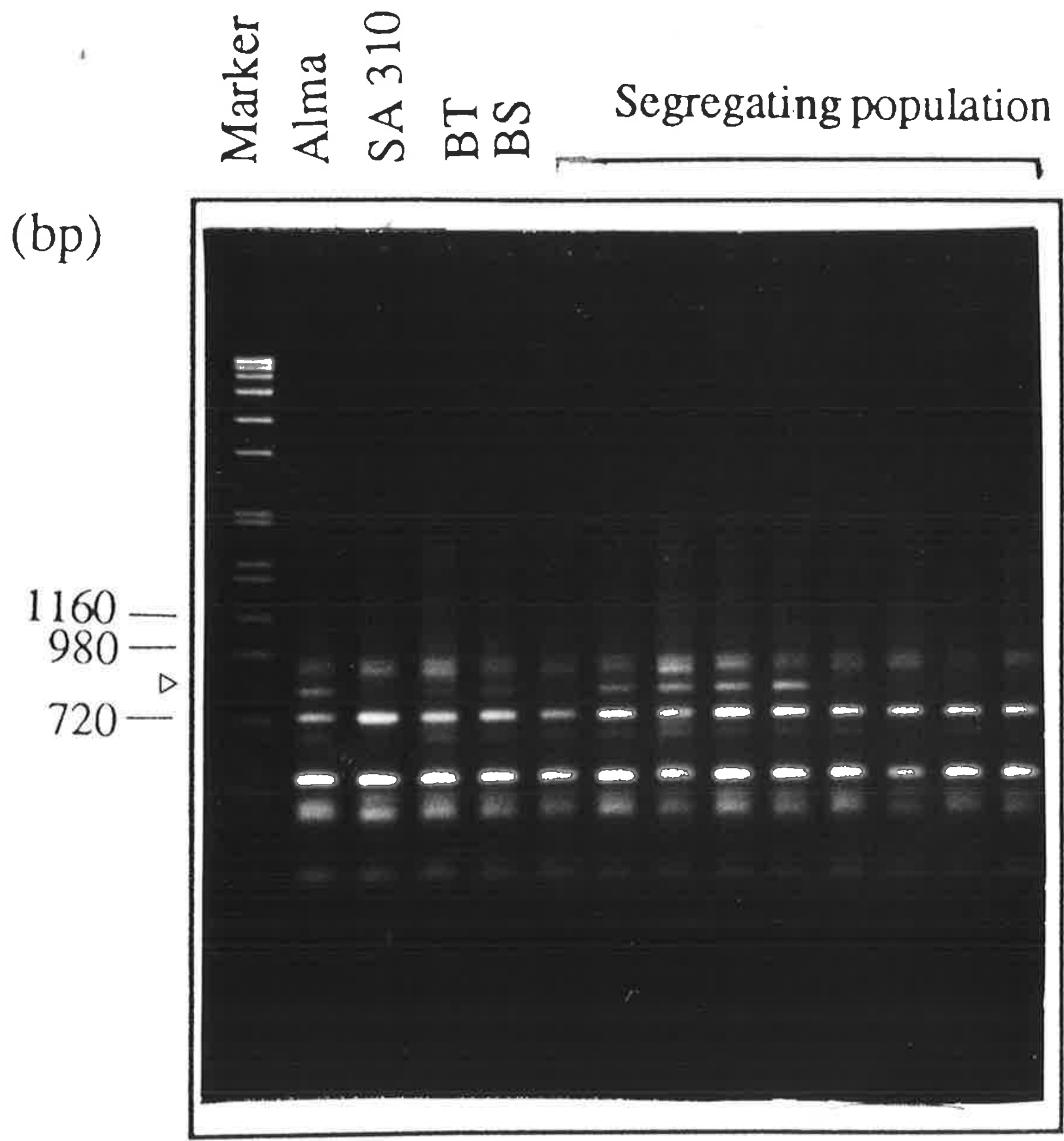

Marker
Alma
SA 310
OpG07
Alma
SA 310
OpG10
Alma
SA 310
OpG16
Alma
SA 310
OpI13
Alma
SA 310
OpI14
(bp)
2149
1763
1097
692

Plate 6.4 Segregation of a RAPD band generated by the primer OpGo2 linked to response to boron in the cross between Alma and SA 310.



Marker
Alma
SA 310
BT
BS
Segregating population
(bp)
1160
980
720

6.3.3 RFLP analyses

RFLP analysis with clones of RAPD products linked to the boron tolerance gene

The recombinant cells containing the RAPD products were selected on the basis of colour. Recombinant colonies were white and non-recombinant clonies were blue. Mini-preparations of the recombinant plasmids were analysed for the size of inserts by digestion with restriction enzymes and gel electrophoresis. Four clones had inserts which corresponded in size to the cloned RAPD products. Two amplified inserts were used as RFLP probes (pOpG02-800 and pOpK07-400). Both clones hybridised to parental DNA but only pOpK07-400 revealed RFLPs between the two parents (Plate 6.5). Bands generated from the pOpG02-800 probe were not scorable because of high background signal strength. The pOpK07-400 probe was tested against the F_2 population of Alma x SA 310 digested with two restriction enzymes (Table 6.9). These two enzymes identified RFLPs which segregated independently of each other and with boron response and therefore were not linked to response to boron.

Plate 6.5 Polymorphisms between Alma and SA 310 detected with the probe pOpK07-400. Genomic DNA was digested by *Eco*RI, *Hind* III, *Eco*R V and *Bam*HI restriction enzymes. The arrows shows the polymorphic bands.

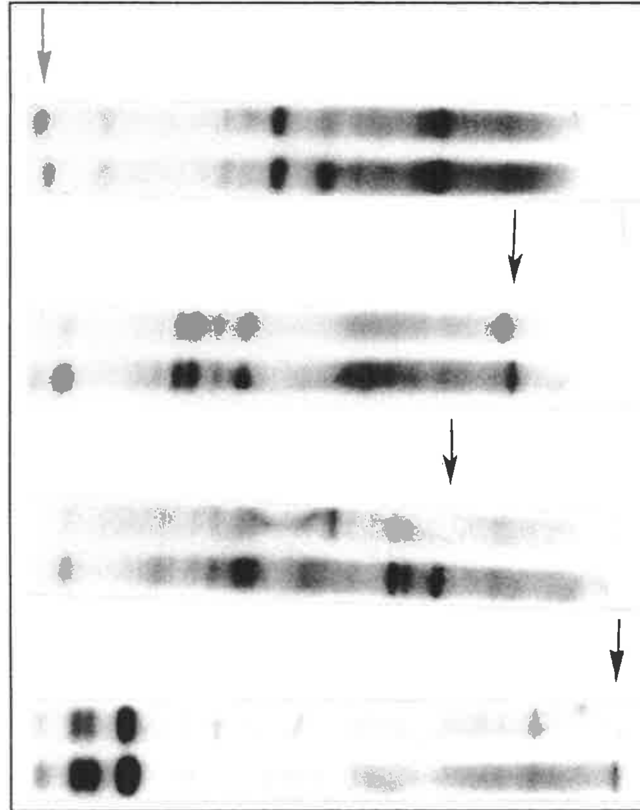

Alma
SA 310
Alma
SA 310
Alma
SA310
Alma
SA 310

Table 6.9 Two-way contingency tables between genotypes with respect to boron tolerance and RFLP markers detected by the probe pOpK07-400.

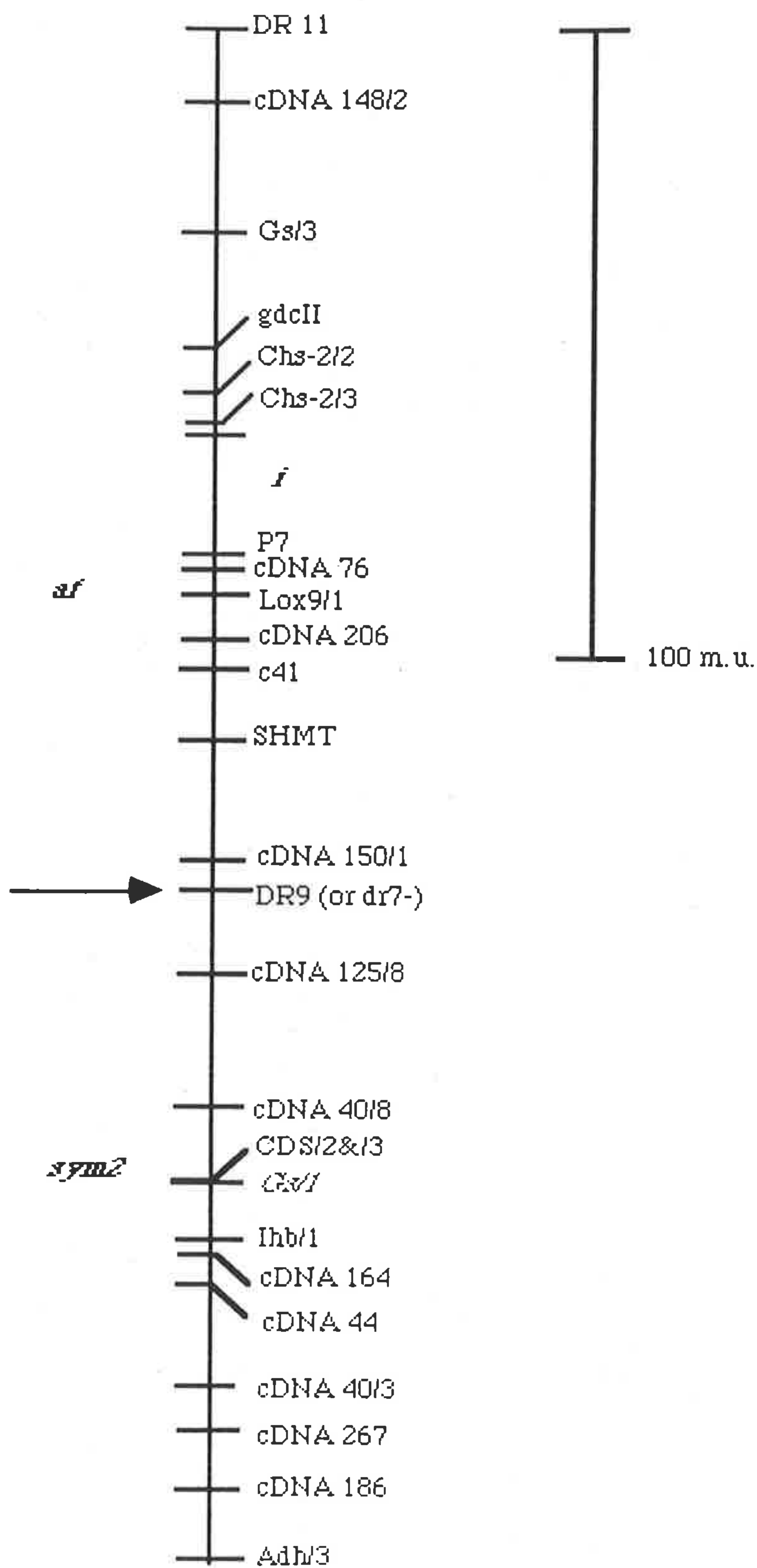
Probe and enzyme		Boron			
		BoBo	*Bobo*	*bobo*	Total
pOpK07-400/*Eco*RV					
SA 310 type		7	15	6	28
Heterozygote		7	15	7	29
Alma type		3	11	8	22
	Total	17	41	21	79
pOpK07-400/*Dra*I					
SA 310 type		2	9	5	16
Heterozygote		9	15	8	32
Alma type		5	9	1	15
	Total	16	33	14	63

RFLP analysis of the recombinant inbred population

The boron response of the recombinant inbred population (JI 15x JI 399) is controlled by a single major gene which maps about 10 cM from the dispersed repeat marker *dr7-* with a LOD score of 2.3. The band to which response to boron is linked is from JI 399 and this band also segregates in the cross (JI 281 x JI 399). However, the marker is called DR9 in this cross (T.H.N. Ellis, pers. comm.). From the combined linkage maps for JI 15 x JI 399 and JI 281 x JI 399, the gene for boron tolerance and *dr7-* are placed about midway between the two pairs of markers *i* - *af* and *d* - *sym2* on the classical linkage group 1. A tentative map of the gene loci in this population is given in Fig. 6.4.

Fig. 6.4 An RFLP map of chromosome 1 of *P. sativum*, showing markers linked to a gene conferring tolerance to boron (*Bo*) in the recombinant inbred population (JI 15 x JI 399). The arrow shows the possible region of the *Bo* gene.

Linkage group 1



RFLP analysis of the F_2 population derived from the cross between Alma x SA 310 by probes selected from linkage group 1

The F_2 population of Alma x SA 310 was tested by RFLP analysis using probes from linkage group 1 to determine whether the boron tolerance gene in this population is located in the same position as in the recombinant inbred population (JI 15 x JI 399). Five probes were tested and four (cDNA 44, cDNA 150, cDNA 206 and pIT26-74) revealed RFLPs between Alma and SA 310. A fifth probe (SHMT) did not hybridise to the digested DNA. The data from all the hybridisations are summarised in Table 6.10. The segregation of the four polymorphic probes was independent of boron tolerance in the F_2 generation (Table 6.11). Therefore, it is possible that the gene conferring boron tolerance in the population Alma x SA 310 is different from in JI 15 x JI 399.

Table 6.10 RFLPs detected between Alma and SA 310 with five selected probes from linkage group 1.

Probe	Enzymes				
	*Bam*HI	*Eco*RI	*Eco*RV	*Dra*I	*Hin*dIII
cDNA 150	+[a]	+	+	+	+
cDNA 206	+	–	+	+	–
pIT 26-74	+	+	+	+	+
cDNA 44	n.t.	n.t.	+	n.t.	+
SHMT[b]					

[a] +, polymorphic; -, non-polymorphic and n.t., not tested.

[b] This probe did not hybridise to the digested DNAs.

Table 6.11 Two-way contingency tables between genotypes with respect to boron tolerance and RFLP markers generated by four selected probes from linkage group 1 digested with different restriction enzymes.

DNA probe and enzyme		Boron			
		BoBo	*Bobo*	*bobo*	Total
cDNA 150/*Dra*I					
SA 310 type		10	5	1	16
Heterozygote		1	21	8	30
Alma type		5	6	7	18
	Total	16	32	16	64
cDNA 150/Eco*R V*					
SA 310 type		2	2	1	5
Heterozygote		5	3	2	10
Alma type		5	3	4	12
	Total	12	8	7	27
cDNA 150/*Bam*HI					
SA 310 type		1	2	5	8
Heterozygote		4	5	5	14
Alma type		2	0	2	4
	Total	7	7	12	26
cDNA 150/*Hind*III					
SA 310 type		1	0	3	4
Heterozygote		0	6	3	9
Alma type		0	2	1	3
	Total	1	8	7	16
cDNA 206/*Dra*I					
SA 310 type		3	0	2	5
Heterozygote		6	0	4	10
Alma type		2	0	1	3
	Total	11	0	7	17
cDNA 206/*EcoR*V					
SA 310 type		4	4	3	11
Heterozygote		9	14	7	30
Alma type		1	2	5	8
	Total	14	20	15	49

Table 6.11 (Continued).

DNA probe and enzyme		Boron			
		BoBo	*Bobo*	*bobo*	Total
pIT26-74 /*Eco*RI (a)					
SA 310 type		3	4	2	9
Heterozygote		9	9	4	22
Alma type		5	6	8	19
	Total	17	19	14	50
pIT26-74/*Eco*RI (b)					
SA 310 or Heterozygote type		7	6	5	18
Alma type		10	12	10	32
	Total	17	18	15	50
pIT26-74/*Eco*RI (c)					
SA 310 or Heterozygote type		6	5	3	14
Alma type		11	13	12	36
	Total	17	18	15	50
pIT26-74/*Eco*RI (d)					
SA 310 or Heterozygote type		5	6	2	13
Alma type		12	13	12	37
	Total	17	19	14	50
pIT26-74/*Eco*RI (e)					
SA 310 or Heterozygote type		4	4	2	10
Alma type		13	14	12	39
	Total	17	18	14	49
cDNA 44/*Eco*RI (a)					
SA 310 type		2	3	1	6
Heterozygote		3	4	2	9
Alma type		1	5	2	8
	Total	6	12	5	23
cDNA 44/*Eco*RI (b)					
SA 310 or Heterozygote type		21	28	17	66
Alma type		6	5	5	16
	Total	27	33	22	82

6.4 Discussion

Segregating allozyme and DNA markers were used to try to establish linkage to boron tolerance in two populations. In the present investigations, difficulties were encountered in identifying isozyme separation systems capable of resolving polymorphism between tolerant and sensitive pea lines. The principle approach used was to locate published literature on previous isozyme studies with peas and then attempt to reproduce banding patterns. However, problems encountered included, (a) lack of any observed enzyme activity e.g. SOD, (b) observed bands of low activity e.g. EST and (c) clear banding patterns but showing no polymorphism e.g. IDH.

The isozyme patterns of Weeden and Marx (1984; 1987) were confirmed for four enzyme loci (*Aat-3, Lap, Skdh, and Pgm*) which map to the three linkage groups 2, 3 and 7. According to the results generated by the Mapmaker program all of these isozyme markers segregated independently to the response to boron. This suggests that the boron tolerance gene distinguishing SA 310 from Alma may be located on a linkage group other than groups 2, 3 and 7.

Sarawat *et al.* (1994) investigated isozyme systems in selected Australian pea varieties and other pea accessions to estimate the genetic distance and its association with heterosis in peas. Fifteen enzyme systems were used in their study, some of which (e.g. ADH) showed successful band resolution which was not achieved in the present study. Unfortunately, the results of Sarawat *et al.* (1994) were not available at the time the present experimental procedures were underway and direct adoption of her methods could not be made.

The major limitations of the isozyme investigations were the lack of enzyme markers on several chromosomes and insufficient detectable variation at some of the previously mapped enzyme loci. Although these isozyme markers have been mapped, it would appear that the methods used to resolve them need to be improved before they can be used routinely as genetic markers. The low level of polymorphism revealed by isozymes indicates that it is highly unlikely that isozymes would provide sufficient marker loci for the seven

chromosomes of peas to enable a comprehensive mapping project. Therefore, further studies focused on RAPD and RFLP markers.

The establishment of standard conditions for RAPD analysis in peas allowed this technique to be used for linkage analysis. A high level of genetic variation was easily detected among the parental lines. Using this approach, with bulked segregant analysis of pooled DNA samples of homozygous individuals, one RAPD marker putatively linked to the boron gene was identified, demonstrating the possible use of RAPD markers for linkage studies in peas. However, the recessive nature of this marker resulted in a low LOD score. It would appear that a greater number of F_2 plants would need to be screened to improve the confidence of the use of RAPDs for linkage analysis.

In RFLP studies, clones were isolated from the RAPD products which, on the basis of bulk segregation analysis, appeared to be linked to the boron gene. These clones were used as hybridisation probes and one provided very clear polymorphisms between parents. However, these RFLPs were shown to be independent of response to boron when tested against the segregating F_2 population. This is perhaps not surprising as OpK07400, the only clone to produce a clear hybridisation pattern, was isolated from a band which proved not to be linked to boron tolerance when the F_2 population was tested by RAPD analysis. The clone derived from the band generated by OpG02 which was linked to tolerance to boron produced an unscorable pattern due to excessive background hybridisation.

RFLP mapping of the recombinant inbred population (JI 15 x JI 399) indicated that the gene conferring boron tolerance in this population is located on linkage group 1. On the other hand, response to boron among the F_2 population of Alma x SA 310 segregated independently of markers from linkage group 1. This suggests that the two populations segregate at different loci with respect to boron tolerance.

The F_2 derived families of the cross of Alma x SA 310 segregated in the ratio of 23 (homogenous tolerant): 36 (segregating): 22 (homogenous sensitive) with respect to boron (Chapter 5) thus fitting well to a 1: 2: 1 ratio. Segregation ratios of Alma with other genotypes indicated at least two genes for boron tolerance that act in an additive manner. One dominant gene is present in Alma (*Bo1Bo1bo2bo2*) while the genotype of Pennant is proposed as *bo1bo1bo2bo2* (Chapter 5). Although the necessary crosses were not tested to determine the genotype of SA 310 relative to Pennant, Alma and PIG 16, both SA 310 and PIG 16 were phenotypically similar for response to boron and both segregated at a single gene relative to Alma. SA 310 is therefore tentatively assigned the genotype *Bo1Bo1Bo2Bo2*.

The responses of JI 15 and JI 399 are comparable to those of Alma and Pennant, respectively, (Chapter 5) and the segregation ratio in this population indicated monogenic control with respect to boron. Based on the assumptions presented above it is probable that different genes control boron tolerance in these two mapping populations. It is therefore possible that the locus conferring boron tolerance in the population of JI 15 x JI 399 corresponds to *Bo1*. To confirm this hypothesis it would be necessary to test the F_2 populations of JI 15 x Pennant and JI 399 x Alma for response to boron to establish that the genotypes of JI 15 and Pennant and of JI 399 and Alma are the same with respect to boron tolerance.

The *Bo1* gene has been of great value for adaptation to high boron soil in Alma and the introduction of a second allele could further assist in breeding boron tolerant varieties. From the plant breeding point of view it is necessary to find tight linkage between markers and the gene of interest before they can used as selection criteria. When linkage is identified, the RAPD technology is relatively rapid and may be applied to improvement of varieties by marker assisted selection during screening of segregating populations.

CHAPTER 7
GENETIC DISTANCE DETECTED WITH RAPD MARKERS AMONG SELECTED AUSTRALIAN COMMERCIAL VARIETIES AND BORON TOLERANT EXOTIC GERMPLASM OF PEAS

7.1 Introduction

Genotypes more tolerant than current Australian varieties have been identified among a collection of *P. sativum* (Chapter 4). However, tolerant accessions are poor in terms of agronomic characteristics and a knowledge of genetic relationships between the tolerant accessions and local varieties would assist in devising the strategy to transfer boron tolerance to the local breeding program.

Recent developments in DNA-based technology, including random amplified polymorphic DNAs (RAPDs) are providing tools suitable for rapid and detailed genetic analysis of higher organisms including plant species (Williams *et al.*, 1990; Rafalski *et al.*, 1991). The RAPD assay is based on the use of primers that are nine or ten nucleotides long in a DNA-amplification reaction and do not require any specific sequence information about the target genome. Polymorphisms generated by these primers from genetically different individuals can be detected between the amplification products by agarose gel electrophoresis resulting in characteristic patterns of bands in the gel. This technique has been used extensively for varietal identification and phylogenic studies in a wide range of crop plants (e.g. Weining and Langridge, 1992; Yang and Quires, 1993; Mailer *et al.*, 1994; Yu and Nguyen, 1994).

The aim of the present study was to determine the genetic relationships among selected Australian commercial varieties of peas and exotic boron tolerant accessions.

7.2 Materials and methods

Plant materials and genomic DNA extraction

Five Australian commercial pea varieties and five tolerant accessions from different geographical regions were chosen for this study (Table 7.1). They were grown in a glasshouse and leaves were collected when plants were four weeks old and frozen in liquid nitrogen. The DNA was extracted using the small-scale DNA isolation method (Chapter 6).

Table 1 Varieties and accessions of *P. sativum* used for RAPD analyses.

Genotype	Boron response	Origin	Pedigree
Alma	Moderately tolerant	Australian variety	White Brunswick x PI 173052
Buckley	Sensitive	Australian variety	White Brunswick x MU 33
Collegian	Moderately sensitive	Australian variety	White Brunswick x Early Dun
Dundale	Moderately tolerant	Australian variety	Selection from Early Dun
Pennant	Sensitive	Australian variety	White Brunswick x CPI 15247
PIG 16	Tolerant	India	–
SA 132	Tolerant	Afghanistan	–
SA 310	Tolerant	Afghanistan	–
SA 395	Tolerant	India	–
SA 448	Tolerant	India	–

Polymerase chain reaction materials and amplification reaction conditions

Thirty-four 10-mer oligonucleotides from sets G, H, I and J, Operon Technologies Inc. (Alameda Calif., USA), were used as single primers for the amplification of sequences (Table 7.2). *Taq* DNA polymerase, together with 10x concentrated PCR buffer, was supplied by Advanced Biotechnologies Ltd (UK). The PCR was performed in a PTC-100 (Chapter 6).

The amplification conditions were the same as described in Chapter 6. Samples of 10 μl PCR products were analysed on 1.5% agarose (Promega) gels in 1x TBE buffer running at 50 mA for two hours and visualised under UV light following staining with ethidium bromide. The size markers used for the gels were *Spp*-1 phage DNA restricted with *Eco*RI (Bresatec Ltd, Adelaide) and pTZ 18U digested with *Dra* I and *Rsa* I.

Data analysis

Gels were scored on the basis of the presence (1) or absence (0) of each band for all genotypes. Pairwise comparisons were made among genotypes and the values used to generate Jaccard's similarity coefficients (Jaccard, 1908). Cluster analysis, using the unweighted pair-group method with arithmetical average (UPGMA) (Sneath and Sokal, 1973) was performed with the GENSTAT 5 statistical package (Genestat 5 Committee, 1987). This statistical analysis was kindly performed by Ms Lynne Giles of the Department of Plant Science, Waite Agricultural Research Institute. These results were used to generate a dendrogram displaying the hierarchical associations among all genotypes.

7.3 Results

Five representative Australian varieties and five selected tolerant accessions derived from different geographical regions were compared using 34 primers which generated a total of 180 polymorphic bands. Band profiles for individual primers comprised from one to 12 bands. Examples of polymorphic bands detected in DNA from different genotypes are shown in Plate 7.1.

In order to compare the overall similarity among genotypes the data from individual primers were combined in one set. Genetic relationships among the Australian varieties and tolerant exotic accessions were measured using Jaccard's similarity coefficient (Jaccard, 1908). Pairwise similarity of genotypes is given in Table 7.3.

The data show that there is a major distance between the Australian varieties and the tolerant accessions. In general, four clusters can be distinguished among these genotypes (Table

7.3). One cluster contains Alma and Dundale which are very close to each other with only 5 % distance. The second cluster contains Collegian, Buckley and Pennant. PIG 16, SA 132 and SA 310 were placed in a loose third cluster. The two Indian accessions of SA 395 and SA 448 are very close (5% distance) as a separate cluster (Fig. 7.1).

Table 7.2 Nucleotide sequence of the random primers (Operon Technologies Inc) and number of polymorphisms generated among 10 *P. sativum* genotypes.

Primer	Sequence	Polymorphic bands
G-03	5´-GAGCCCTCCA-3´	1
G-08	5´-TCACGTCCAC-3´	5
G-09	5´-CTGACGTCAC-3´	3
G-13	5´-CTCTCCGCCA-3´	3
G-16	5´-AGCGTCCTCC-3´	3
H-03	5´-AGACGTCCAC-3´	2
H-06	5´-ACGCATCGCA-3´	11
H-07	5´-CTGCATCGTG-3´	6
H-12	5´-ACGCGCATGT-3´	5
H-14	5´-ACCAGGTTGG-3´	8
H-15	5´-AATGGCGCAG-3´	3
H-16	5´-TCTCAGCTGG-3´	7
H-18	5´-GAATCGGCCA-3´	3
H-19	5´-CTGACCAGCC-3´	5
I-01	5´-ACCTGGACAC-3´	12
I-06	5´-AAGGCGGCAG-3´	1
I-07	5´-CAGCGACAAG-3´	5
I-11	5´-ACATGCCGTG-3´	4
I-14	5´-TGACGGCGGT-3´	2
I-16	5´-TCTCCGCCCT-3´	8
I-18	5´-TGCCCAGCCT-3´	4
I-19	5´-AATGCGGGAG-3´	7
J-01	5´-CCCGGCATAA-3´	10
J-03	5´-TCTCCGCTTG-3´	7
J-04	5´-CCGAACACGG-3´	3
J-10	5´-AAGCCCGAGG-3´	3
J-11	5´-ACTCCTGCGA-3´	6
J-12	5´-GTCCCGTGGT-3´	11
J-13	5´-CCACACTACC-3´	5
J-14	5´-CACCCGGATG-3´	8
J-15	5´-TGTAGCAGGG-3´	7
J-16	5´-CTGCTTAGGG-3´	2
J-18	5´-TGGTCGCAGA-3´	9
J-20	5´-AAGCGGCCTC-3´	1

Plate 7.1 DNA polymorphism generated by 10-mer primer J 11 (Operon Technologies, Inc.) in 10 *P. sativum* genotypes.

Lane 1 M. DNA size Marker (*Spp*-1/ *Eco*RI)

Lane 2. Alma

Lane 3. Dundale

Lane 4. Collegian

Lane 5. Buckley

Lane 6. Pennant

Lane 7. PIG 16

Lane 8. SA 132

Lane 9. SA 310

Lane 10. SA 395

Lane 11. SA 448.

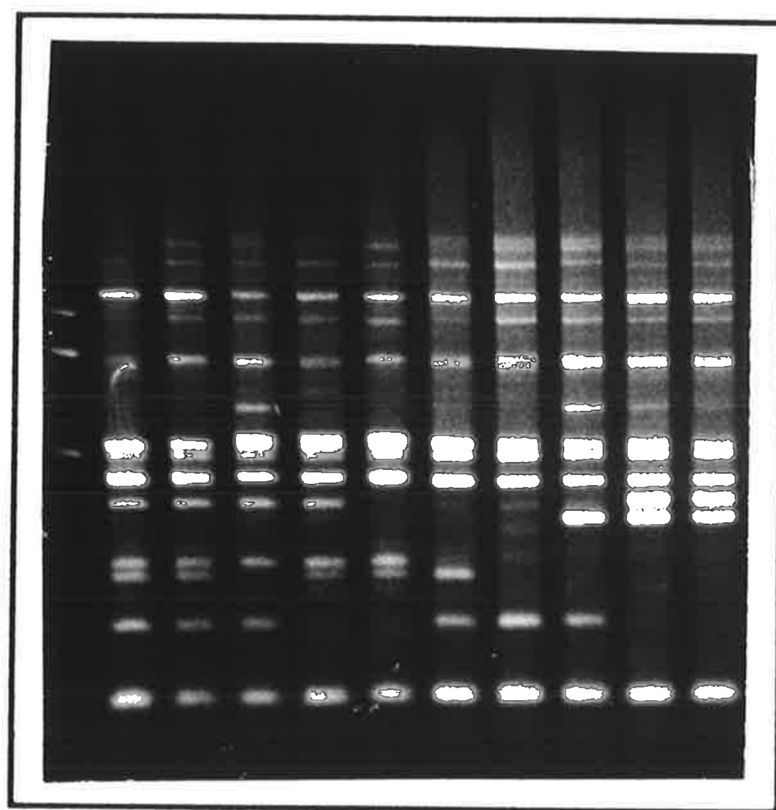
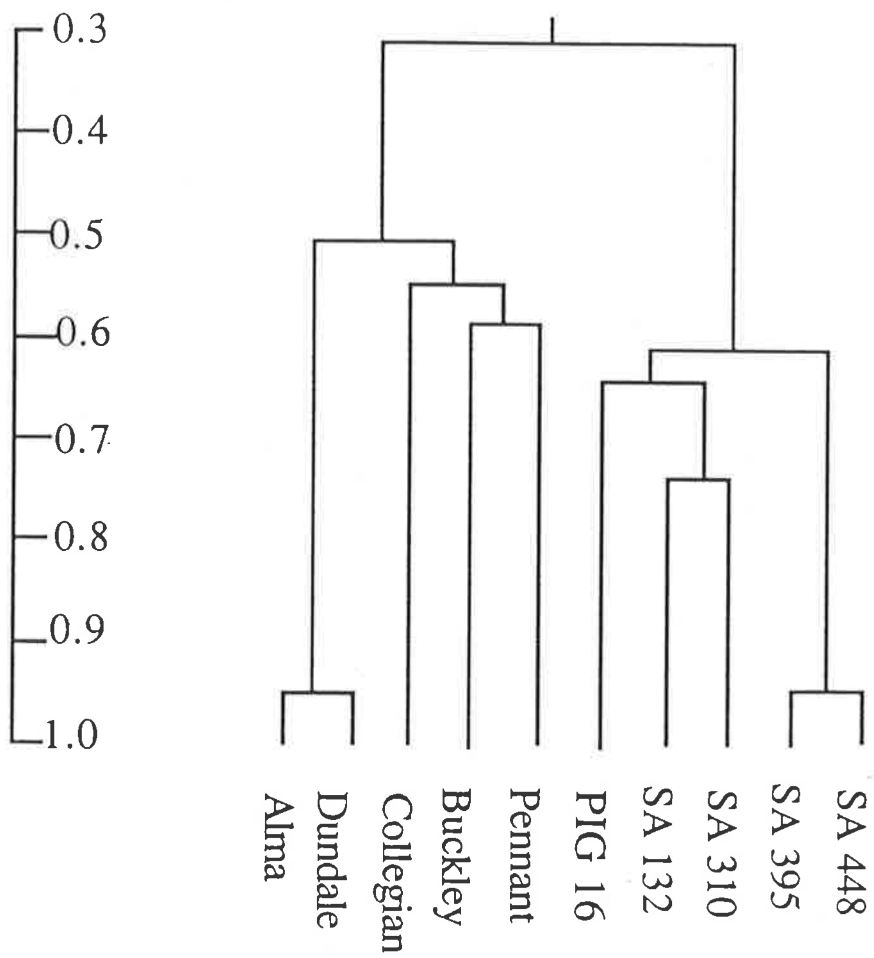

M 1 2 3 4 5 6 7 8 9 10
(bp)
2149
1097
692

Table 7.3 Similarity matrix among selected Australian varieties and boron tolerant exotic accessions of *P. sativum*.

Genotype	Australian varieties					Boron tolerant accessions				
	Alma	Dundale	Collegian	Buckley	Pennant	PIG 16	SA 132	SA 310	SA 395	SA 448
Alma	100									
Dundale	95.2	100								
Collegian	55.0	55.7	100							
Buckley	51.2	49.6	56.6	100						
Pennant	47.0	47.7	54.4	59.6	100					
PIG 16	25.0	26.5	28.7	25.4	23.2	100				
SA 132	29.7	30.4	32.6	27.7	26.4	68.0	100			
SA 310	28.0	28.7	34.5	26.8	26.4	60.2	74.8	100		
SA 395	32.9	33.5	43.4	37.6	32.4	62.6	63.0	60.3	100	
SA 448	33.8	34.4	42.2	37.5	32.5	60.5	63.6	61.0	94.5	100

Fig. 7.1 Dendrogram of genetic distances obtained from RAPD data constructed by the unweighted pair-group method with arithmetical average. The scale indicates similarity among genotypes.


0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Alma
Dundale
Collegian
Buckley
Pennant
PIG 16
SA 132
SA 310
SA 395
SA 448

7.4 Discussion

Measures of genotypic similarity among genotypes of different geographical region provides information regarding the management of breeding programs. The establishment of standard conditions for RAPD analysis in peas (Chapter 6) allowed this technique to be used to determine the genetic similarity among five Australian commercial varieties and five boron tolerant accessions derived from different geographical regions. Genetic similarity was estimated on the basis of the percentage of common bands between genotypes and a dendrogram was constructed. The dendrogram showed clear differences between Australian varieties and exotic boron tolerant accessions with the level of similarity between Australian varieties and tolerant accessions being 31% (Fig. 7.1).

Among the cultivated varieties, Alma and Dundale are very similar. This result is somewhat surprising. Although the two varieties have similar response to boron, they are recorded as being unrelated, with Dundale being a selection from Early Dun and Alma selected from a cross between White Brunswick x PI 173052. Sarawat *et al.* (1994) also observed a relatively high degree of similarity between Alma and Dundale, when comparing genetic distance among Australian varieties and exotic accessions with isozyme and morphological markers. These results raise doubts on the accuracy of the putative ancestry of Alma. Buckley, Collegian and Pennant are relatively similar on the dendrogram. They have one parent in common and it is reasonable to expect that similar fingerprinting patterns would be obtained from these genotypes. The accessions of SA 310 and SA 132, which originate from Afghanistan, are genetically similar. Among the Indian accessions, SA 395 and SA 448 are in one cluster and PIG 16 is placed in another cluster which shows 62% similarity with the other Indian genotypes. Previous results have shown that tolerant accessions mainly originated from Asia (Chapter 4), so the similarity between tolerant accessions from Afghanistan and those tolerant accessions from India might result from common ancestry.

There has been a limited use of exotic germplasm in Australian pea breeding and the utilisation of tolerant accessions as sources of boron tolerance could be useful for pea improvement. The gene(s) conferring tolerance to boron can be transferred to commercial

varieties using the backcrossing method, as for the breeding of the wheat variety BT-Schomburgk which produces a 10% yield advantage over its recurrent parent when grown under high boron concentrations (Moody *et al.*, 1993). The genetic divergence between Australian varieties and the boron tolerant accessions suggests that a comprehensive, intensive backcrossing program would be required to transfer boron tolerance into a locally adapted genetic background. Ignoring linkage drag associated with selection for tolerance to boron, the genetic diversity will halve with each cross, so the initial 69% will be reduced to 17.25% following one backcross. While this is within the range of the diversity in the Australian varieties, it is well above that between Alma and Dundale which might be an appropriate target to give a reasonable probability of being able to select a boron tolerant variety. To get to this level of similarity would require the generation of a BC_3F_2 population with screening for boron tolerance after each backcrossing. The preliminary results of Burton (1993) would support this hypothesis. Crosses between Australian varieties and exotic materials were very poor in terms of agronomic characters when grown at Palmer, South Australia.

These results show RAPD to be useful for clarifying the phylogenic relationships within a species and also to provide useful genetic markers for varietal identification in peas. RAPD fingerprinting is a simple procedure compared with other methods such as RFLP and isozyme analysis and requires the smallest amount of DNA. Furthermore, RAPD analysis permits the analysis of many individuals and has enabled the study of the genetic relationship among the boron tolerant lines from the collection of *P. sativum* and could help in the exploitation and management of these tolerant accessions in a breeding program.

CHAPTER 8
RESPONSE OF AUSTRALIAN VARIETIES OF *PISUM SATIVUM*, *CICER ARIETINUM* AND *LENS CULINARIS* TO HIGH CONCENTRATIONS OF SOIL BORON

8.1 Introduction

Among the food grain legumes, peas are the most important and established crop in South Australia with an estimated area in 1989-90 of 116,000 ha. Chickpeas and lentils are grown over much smaller areas than peas with an estimated 8,000 ha for chickpeas and less than 100 ha for lentils (ABARE, 1990).

Although only a limited range in genetic variation for boron tolerance exists among Australian pea varieties (Chapter 3), considerable genetic variation was demonstrated among exotic accessions of peas, including a number of accessions more tolerant than the most tolerant Australian varieties (Chapter 4). The response of Australian chickpea and lentil varieties to high concentrations of boron has not been evaluated. The objective of this study was to assess the genetic variation for boron tolerance in chickpeas and lentils relative to peas and to study the mechanisms of tolerance.

8.2 Materials and methods

8.2.1 Glasshouse screening of chickpeas and lentils

Australian chickpea and lentil varieties and South Australian advanced lines of lentils (Table 8.1) were kindly provided by Dr S.M. Ali and Mr W. Hawthorne of the South Australian Research and Development Institute.

Table 8.1 Australian varieties of chickpeas and lentils and breeding lines of lentils (crossbred lines from ICARDA) tested for response to boron. (ILL, ICARDA lentil line).

Variety or line	Breeding site	Variety or line	Breeding site
Lentils		**Lentils**	
Laird	Introduction from Canada	ILL5799	ICARDA
ILL5746	ICARDA	ILL4400	ICARDA
Callisto	NSW	ILL20	ICARDA
ILL5731	ICARDA	ILL5828	ICARDA
ILL5728	ICARDA	ILL4606	ICARDA
ILL 4605	ICARDA	ILL5750[a]	ICARDA
ILL5719	ICARDA	**Chickpeas**	
Kye	Introduction from Ethiopia	Garnet	NSW
ILL5698	ICARDA	Kaniva	Victoria
ILL5732	ICARDA	Opal	Unknown
ILL4401	ICARDA	Narayen	Queensland
ILL5770	ICARDA	Semsen	NSW
ILL2194	ICARDA	Tyson (C 245)	Indian variety
ILL5562	ICARDA	Macarena	Spanish variety
ILL975	ICARDA	Barwon	NSW
ILL5729	ICARDA	Dooen	Victoria
ILL5740	ICARDA	Amethyst	NSW
ILL5588	ICARDA	Desavic	South Australia
ILL5774	ICARDA		& Victoria

[a] ILL5750 released as Aldinga by Dr S.M. Ali, South Australian Research and Development Institute in 1994.

The soil and its preparation were as described in Chapter 3. Plastic trays (Chapter 5) containing 15 kg soil to which 30 mg kg^{-1} boron had been applied were used for screening (Plate 8.1). Six seeds from each genotype were sown in each of two randomly allotted rows at the depths of one and three cm below the soil surface for lentils and chickpeas, respectively. Four weeks after planting, genotypes were rated for expression of symptoms of boron toxicity, as described in Table 8.2.

8.2.2 Glasshouse experiment

Genotypes

Three Australian varieties of peas, chickpeas and lentils were grown in four levels of boron in a glasshouse experiment. These varieties were chosen primarily according to their response to boron in preliminary screening experiments to represent the most tolerant, intermediate and sensitive (relatively tolerant: Alma, Garnet and Laird; intermediate: Collegian, Semsen and Callisto; relatively sensitive: Pennant, Macarena and Kye) Australian varieties of each crop.

Soil and treatments

Soil from the same source as described for the initial screening experiment was used. Boron was applied at the rates of 0, 10, 20 and 30 mg kg^{-1} of soil, designated as B0, B10, B20, and B30. Four kilograms of treated soil were added to each pot (200 mm diameter) lined with a water-tight polythene bag. Because of the small production of dry matter by individual plants of lentils and chickpeas relative to peas, different numbers of seeds were sown for each genus. Hence, six seeds of peas, eight of chickpeas and 14 of lentils were sown in each pot at the depths of three, two and one cm below the soil surface, respectively. Three weeks after sowing, pots were thinned to three, six and 12 plants for peas, chickpeas and lentils, respectively. The pots were arranged in a glasshouse in a randomised complete block design as a factorial with three replicates.

Plate 8.1 Plastic trays for growing (**a**) lentils and (**b**) chickpeas in soil with high concentrations of boron (30 mg kg^{-1}) to screen for tolerance to boron toxicity. Plants are four weeks old.

(a)

(b)

Table 8.2 The scale for visually rating the severity of symptom expression in response to high concentration of boron for peas, chickpeas and lentils.

Very Tolerant	Symptoms visible on 0 to 15 per cent of leaves
Tolerant	Symptoms visible on 16 to 25 per cent of leaves
Moderately Tolerant	Symptoms visible on 26 to 35 per cent of leaves and death of the lowest leaves
Moderately Sensitive	Symptoms visible on 36 to 60 per cent of leaves and death of the second to lowest leaves
Sensitive	Symptoms visible on 61 to 80 per cent of leaves and death of 50 per cent of leaves
Very Sensitive	Symptoms visible on 81 to 100 per cent of leaves to complete death of plant

Growth measurement and data analyses

Emergence was measured one and two weeks after sowing. Plants were harvested after seven weeks. At the time of harvesting, visual symptoms of boron toxicity, height of plants and number of branches were recorded. Plants were cut one centimetre above the soil, dried at 70°C for 48 hours and then ground. The plant tissues were digested in nitric acid at 140°C and the concentrations of boron were determined by ICP-spectrometry (Chapter 3).

All data were analysed by factorial analysis. The data for dry matter production and tissue boron concentrations were subjected to square root and logarithmic ($\log_e$) transformations, respectively, to ensure homogeneity of variance before being analysed. As there were different numbers of plants per pot, the data for dry matter production were analysed on an individual plant basis.

8.2.3 Filter paper experiments

In the pot experiment, the chickpea and lentil plants dropped many leaves and this effect was most pronounced for the oldest leaves at the high boron treatments. This raised doubts regarding interpretation of the results of the pot experiment, particularly for data on dry matter production and concentration of boron in tissues. Varieties of the three genera were therefore also compared at a range of boron treatments in filter papers by the method described by Chantachume *et al.* (1993) for wheat. In this assay, response to boron is assessed on the basis of root growth of seedlings under high boron conditions.

Filter paper experiment 1

Two varieties of peas and one variety each of chickpeas and lentils were examined for their response to boron. To ensure uniform germination, seeds were placed in plastic petri dishes on moist filter paper and stored at 2-4°C for two days and then at room temperature for one day.

Filter papers (Ekwip® 32 x 46 cm grade R6) were immersed in solutions containing either 0, 25, 50, 75 or 100 mg B l^{-1} (designated as B0, B25, B50, B75 and B100, respectively) and 0.1 M Ca $(NO_3)_2$. 4 H_2O and 5 mM ZnSO4. 7 H_2O and allowed to drain for one to two minutes. Ten germinated seeds, with the radicle downwards, were placed across the middle of each filter paper. The filter papers were folded and rolled up carefully to avoid damage to the radicle. The rolled filter papers were covered with aluminium foil to prevent evaporation and then stored upright at 15°C. After 12 days the lengths of radicles were measured.

The experimental design was a randomised complete block in a 4 x 6 factorial arrangement with three replications. Data were analysed by factorial analysis.

Filter paper experiment 2

Two Australian varieties and one tolerant accession of peas, three Australian varieties of chickpeas and two Australian varieties of lentils were further examined at four levels of boron, B0, B50, B75 and B100. The methods, experimental design and data analysis were as described for filter paper experiment 1 with the exception that the experiment comprised only two replications.

8.3 Results

8.3.1 Glasshouse screening of chickpeas and lentils

In contrast to peas where symptoms developed around the margin of the leaf, the symptoms for lentils and chickpeas develop from the tip of the leaf and progress to the base, eventually resulting in death of the leaf and senescence (Plate 8.2). However, as for peas, the symptoms of boron toxicity on lentils and chickpeas develop first and are most severe on the oldest leaves.

Genetic variation in expression of symptoms of boron toxicity was observed among both chickpea and lentil varieties (Table 8.3). Of the chickpeas, Garnet, Kaniva, Opal, Narayen and Semsen were rated as moderately sensitive and the remaining varieties were sensitive to boron. Laird was the most tolerant of the lentil varieties (moderately sensitive), Callisto was sensitive and Kye was very sensitive. With the exception of ILL 5746, ILL5731, ILL5728, ILL4605 and ILL5719 the majority of the breeding lines of lentils, introduced from ICARDA, were very sensitive to boron.

Plate 8.2 Boron toxicity symptoms on leaves of peas, chickpeas and lentils.

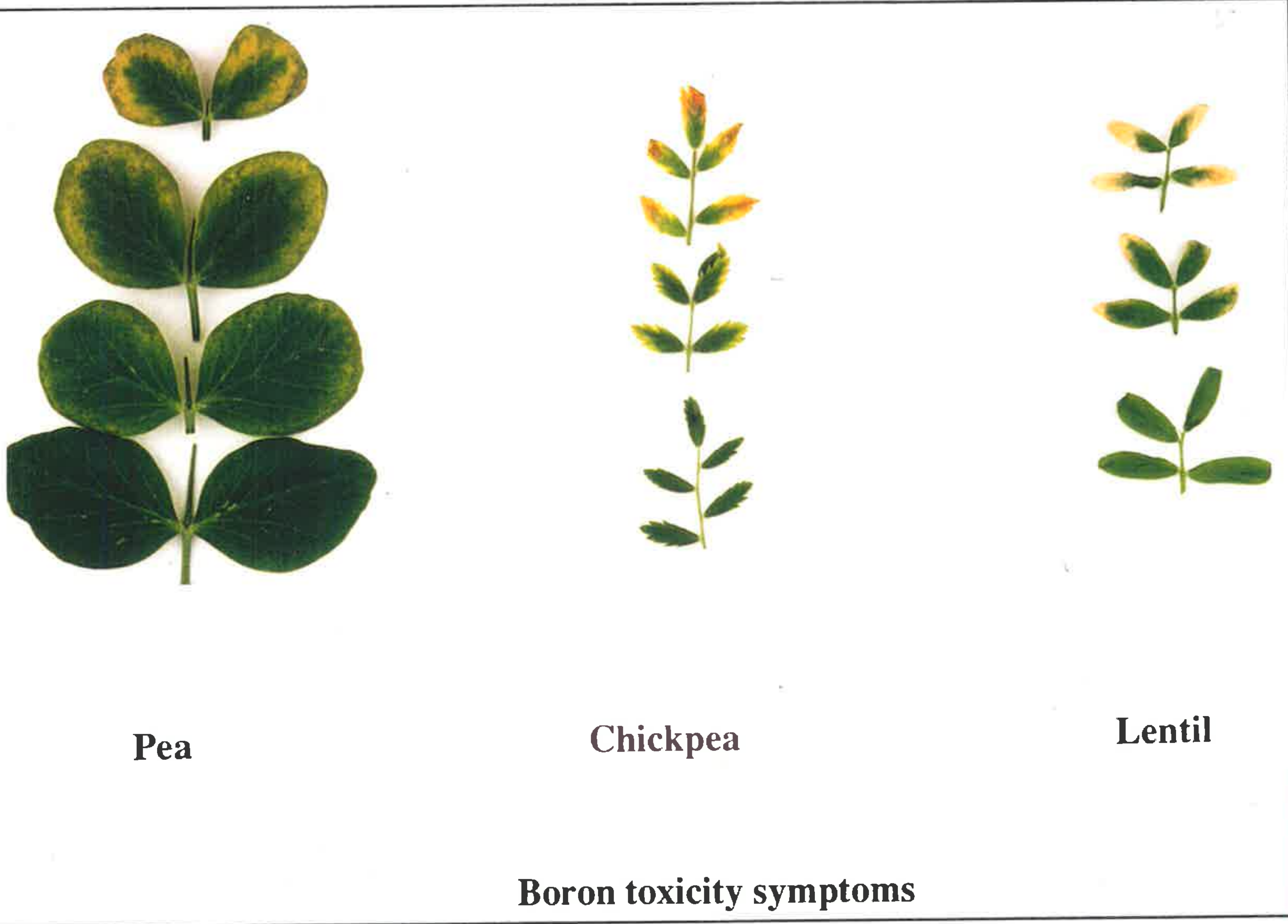

Pea
Chickpea
Lentil
Boron toxicity symptoms

Table 8.3 The response of Australian varieties of chickpeas and lentils and 22 breeding lines of lentils to an excess of soil boron.

Variety or line	Score[a] (%)	Response[b]	Variety or line	Score (%)	Response
Lentils			**Lentils**		
Laird	49	MS	ILL5799	85	VS
ILL5746	50	MS	ILL4400	92	VS
Callisto	70	S	ILL20	98	VS
ILL 5731	77	S	ILL5828	89	VS
ILL5728	76	S	ILL4606	92	VS
ILL4605	80	S	ILL5750	91	VS
ILL5719	69	S	**Chickpeas**		
Kye	100	VS	Garnet	40	MS
ILL5698	84	VS	Kaniva	37	MS
ILL5732	88	VS	Opal	51	MS
ILL4401	88	VS	Narayen	53	MS
ILL5770	92	VS	Semsen	53	MS
ILL2194	82	VS	Tyson	69	S
ILL5562	82	VS	Macarena	68	S
ILL975	88	VS	Barwon	61	S
ILL5729	100	VS	Dooen	67	S
ILL5740	91	VS	Amethyst	69	S
ILL5588	96	VS	Desavic	62	S
ILL5774	92	VS			

[a] Percentage damage.
[b]MS = moderately sensitive, S = sensitive, VS = very sensitive

8.3.2 Glasshouse experiment

Emergence

Percentage emergence was not strongly influenced by boron treatments in absolute terms, although the effect was statistically significant with a trend towards a high rate of germination at the high boron treatment. Differences between varieties were significant ($P < 0.01$) both one and two weeks after sowing but the interaction between

treatments and varieties was only significant at week one (Table 8.4). Whereas Garnet and Kye show a substantial increase in the germination percentage at high compared to low boron treatments, the other varieties do not show any consistent trend.

Symptoms of boron toxicity

Symptoms of boron toxicity were observed on all plants treated with boron and were most severe at the B30 treatment. Symptom expression varied between genera and in particular *Pisum* developed less severe symptoms than *Cicer* and *Lens* (Table 8.5 and 8.9) with no significant difference between *Cicer* and *Lens* except at the B20 treatment. The significant interaction ($P < 0.01$) between varieties and boron treatments reflected the difference in tolerance to boron. Within *Cicer*, Garnet developed the least severe symptoms of boron toxicity and within *Lens*, Callisto was most affected and died five weeks after sowing at the B30 treatment.

Branches and height

The effect of boron treatments upon number of branches varied among genera and also among varieties of lentils. Pea varieties did not produce any branches at any of the treatments, while the numbers of branches developed by chickpeas were not affected by treatments. Among the lentil varieties, Kye produced the most and Laird the fewest branches (Table 8.6).

There were significant differences ($P < 0.01$) among varieties for plant height. Differences between boron treatments were non-significant, although the application of boron resulted in shorter plants in several cases and especially for lentils (Table 8.6).

Dry matter

Dry matter showed a highly significant response to the level of boron supply ($P < 0.01$) (Table 8.7) and the variation among varieties was highly significant ($P < 0.01$). Although the interaction was non-significant, within chickpeas, lentils and peas, the

yield of Garnet, Laird and Alma were least affected by boron treatments as would be expected from the previous results.

Significant differences between genera ($P < 0.01$) for dry matter production could be attributed to differences in the yield potential of individual plants of the three genera (Table 8.9). The dry matter of each genus decreased at increasing levels of boron application and although the magnitude of the effect varied among genera, the interaction was non-significant. At the B30 treatment the mean percentage reductions of dry matter, relative to the control, were 15, 6, 32 per cent for *Pisum*, *Cicer* and *Lens*, respectively.

Tissue boron concentration

Tissue boron concentrations increased significantly ($P < 0.01$) with the application of boron (Table 8.8). At the B30 treatment, concentrations for individual varieties ranged from 280 to 718 mg kg^{-1}. Significant variation for boron concentrations was observed among genera ($P < 0.01$) and among individual varieties. The tissue boron concentration was lowest for *Pisum* whereas the difference between *Cicer* and *Lens* was not significant (Table 8.9). Boron concentrations in shoots of Alma and Collegian were consistently lower than in Pennant. Within *Cicer*, Macarena was lower than the others varieties and within *Lens*, Laird with the lowest tissue boron concentration was not significantly different to Callisto but significantly less than Kye.

Table 8.4 Percentage emergence for Australian varieties of peas, chickpeas and lentils when grown at four levels of boron, one and two weeks after sowing.

Variety	Percentage emergence							
	One week				Two weeks			
	B0	B10	B20	B30	B0	B10	B20	B30
Pisum								
Alma	78	61	67	83	83	72	78	83
Collegian	83	67	72	78	83	83	78	72
Pennant	33	28	28	28	67	61	56	72
Mean	65	52	56	63	78	72	71	76
Cicer								
Garnet	21	29	46	71	58	63	79	92
Semsen	87	79	96	17	79	96	100	100
Macarena	17	4	8	17	58	33	71	54
Mean	42	37	50	35	65	64	83	82
Lens								
Laird	75	75	78	75	92	81	89	81
Callisto	100	67	92	77	100	95	95	95
Kye	56	67	92	95	94	83	95	95
Mean	77	70	87	82	95	86	93	90

To compare values for one week after sowing; in a column LSD 0.01 = 14.2; and in a row, LSD 0.01 = 3.59. Interaction was not significant two weeks after sowing.

Table 8.5 Symptoms of boron toxicity (percentage leaf area affected) for Australian varieties of peas, chickpeas and lentils when grown at four levels of boron.

Variety	Symptom expression (% leaf area)				
	B0	B10	B20	B30	*Mean*
Pisum					
Alma	0.0	1.6	16	35	
Collegian	0.0	12	25	43	
Pennant	0.0	10	26	45	
Mean	0.0	8	22.1	41.1	17.9
Cicer					
Garnet	0.0	2	13	47	
Semsen	0.0	8	55	73	
Macarena	0.0	10	35	65	
Mean	0.0	6.9	34.4	61.7	25.8
Lens					
Laird	0.0	4	18	48	
Callisto	0.0	15	40	77	
Kye	0.0	5	17	53	
Mean	0.0	8.0	25.0	59.7	23.1

To compare values of varieties in a column LSD 0.01 = 7; and in a row, LSD 0.01 = 4.

Table 8.6 Number of branches and height for Australian varieties of peas, chickpeas and lentils when grown at four levels of boron.

Variety	Branches				Height (cm)			
	B0	B10	B20	B30	B0	B10	B20	B30
Pisum								
Alma	1	1	1	1	69	63	70	70
Collegian	1	1	1	1	80	78	79	78
Pennant	1	1	1	1	59	53	57	62
Mean	1	1	1	1	69	65	69	70
Cicer								
Garnet	3.6	3.3	3.3	3.4	20	20	21	20
Semsen	3.8	3.8	3.9	4.0	23	20	21	21
Macarena	4.2	3.8	4.2	4.3	21	21	21	21
Mean	3.9	3.6	3.8	3.9	22	21	21	21
Lens								
Laird	2.2	2.1	1.9	2.1	23	22	20	20
Callisto	3.7	3.2	2.8	1.9	18	15	12	8
Kye	5.6	4.7	4.4	3.7	12	10	10	7
Mean	3.8	3.3	3.0	2.6	17	16	14	12

To compare values for branches in a column LSD 0.01 = 0.5; and in a row, LSD 0.01 = 0.34. Interaction was not significant for height data.

Table 8.7 Dry matter (g plant^{-1}) of Australian varieties of peas, chickpeas and lentils when grown at four levels of boron. Statistical analyses were performed upon the transformed (square root) data and untransformed data are presented in brackets.

Variety	Dry matter (g plant $^{-1}$)				
	B0	B10	B20	B30	*Mean*
Pisum					
Alma	1.00 (1.00)	0.93 (0.87)	1.00 (1.00)	0.95 (0.90)	
Collegian	1.13 (1.29)	1.00 (1.02)	0.96 (0.92)	0.94 (0.89)	
Pennant	0.95 (0.90)	0.81 (0.67)	0.85 (0.74)	0.79 (0.65)	
Mean	1.06 (1.13)	0.92 (0.85)	0.94 (0.90)	0.89 (0.80)	0.95
Cicer					
Garnet	0.63 (0.40)	0.60 (0.37)	0.57 (0.33)	0.58 (0.41)	
Semsen	0.58 (0.34)	0.59 (0.35)	0.63 (0.40)	0.50 (0.30)	
Macarena	0.64 (0.40)	0.52 (0.30)	0.50 (0.26)	0.54 (0.30)	
Mean	0.62 (0.38)	0.57 (0.33)	0.57 (0.33)	0.58 (0.34)	0.58
Lens					
Laird	0.44 (0.20)	0.45 (0.20)	0.40 (0.16)	0.37 (0.14)	
Callisto	0.39 (0.15)	0.33 (0.10)	0.30 (0.09)	0.20 (0.04)	
Kye	0.34 (0.11)	0.30 (0.09)	0.28 (0.08)	0.20 (0.05)	
Mean	0.39 (0.15)	0.36 (0.14)	0.33 (0.11)	0.26 (0.07)	0.34

Interaction was not significant.

Table 8.8 Concentrations of boron in shoots of Australian varieties of peas, chickpeas and lentils when grown at four levels of boron. Statistical analyses were performed upon the transformed ($\log_e$) data and untransformed data are presented in brackets. Significance levels refer to the transformed data.

Variety	Boron concentrations (mg kg^{-1})				
	B0	B10	B20	B30	*Mean*
Pisum					
Alma	2.74 (16)	4.07 (59)	4.89 (133)	5.72 (305)	
Collegian	2.97 (20)	4.26 (71)	5.06 (159)	5.63 (280)	
Pennant	2.81 (17)	4.40 (82)	5.33 (208)	5.81 (348)	
Mean	2.84 (17)	4.25 (71)	5.09 (166)	5.73 (311)	4.48
Cicer					
Garnet	3.23 (25)	5.05 (157)	5.92 (378)	6.60 (718)	
Semsen	3.42 (31)	5.20 (176)	5.94 (394)	6.48 (658)	
Macarena	3.34 (28)	4.87 (133)	5.98 (392)	6.38 (592)	
Mean	3.33 (28)	5.03 (155)	5.94 (388)	6.48 (656)	5.19
Lens					
Laird	3.30 (27)	4.90 (135)	5.76 (321)	6.39 (596)	
Callisto	3.37 (29)	5.00 (149)	5.82 (356)	6.50 (665)	
Kye	3.20 (25)	5.14 (172)	6.00 (409)	6.56 (712)	
Mean	3.29 (27)	5.01 (152)	5.86 (362)	6.48 (658)	5.16

To compare values in a column LSD 0.01 = 0.16; and in a row, LSD 0.01 = 0.10.

Table 8.9 Symptom expression, dry matter yield and tissue boron concentrations of *P. sativum*, *C. arietinum* and *L. culinaris* when grown at four levels of boron. Statistical analyses were performed upon the means of the three varieties and four boron treatments using square root and $\log_e$ transformed data for dry matter and tissue boron, respectively.

Genus	Symptom expression (%)				Dry matter (square root)				Tissue boron ($\log_e$)			
	B0	B10	B20	B30	B0	B10	B20	B30	B0	B10	B20	B30
P. sativum	0.0	7.8	22.8	41.1	1.06	0.91	0.94	0.89	2.84	4.24	5.09	5.73
C. arietinum	0.0	6.9	34.4	61.7	0.61	0.57	0.57	0.58	3.33	5.02	5.94	6.48
L. culinaris	0.0	8.0	25.0	59.6	0.39	0.36	0.33	0.26	3.29	5.01	5.86	6.48

To compare values:

Symptom expression in a column LSD 0.01 = 7.27; in a row, LSD 0.01 = 6.30,

Dry matter in a column LSD 0.01 = 0.06; in a row, LSD 0.01 = 0.05 and,

Tissue boron concentration in a column LSD 0.01 = 0.12 and in a row, LSD 0.01 = 0.10.

8.3.3 Filter paper experiments

Filter paper experiment 1

There was a significant reduction in radicle length when plants were grown under increasing concentrations of boron and, on average, radicle lengths at B25, B50, B75 and B100 were 73%, 53%, 30% and 19% of the control treatment (Fig 8.1a; Plate 8.3).

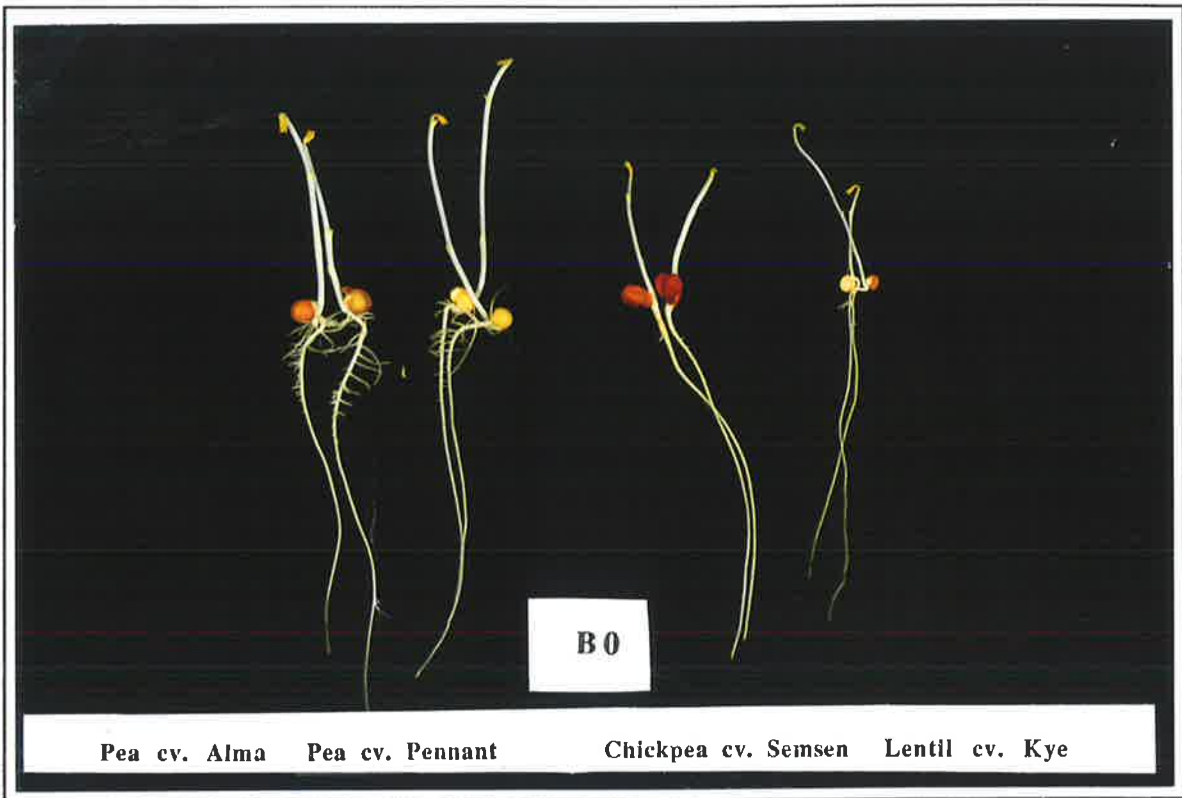
A significant interaction ($P < 0.01$) between varieties and boron treatments for radicle length demonstrates that genetic variation in response to boron among individual varieties of grain legumes is identified by this assay. Although the differences in tolerance to boron were evident at B25 and B50, at the B75 treatment the effects of boron became more severe and the variation among varieties was more evident. At this treatment, radicle lengths were 54%, 30%, 25% and 11% of the control for Alma, Pennant, Semsen and Kye, respectively (Fig. 8.1a; Plate 8.4).

Filter paper experiment 2

In this experiment the effect of boron on eight varieties was studied. Radicle length decreased as boron concentration increased from B0 to B100 with a large variation detected among varieties (Fig. 8.1b; Table 8.10) which resulted in a significant interaction ($P < 0.01$) between boron treatments and varieties. The relative length of radicles showed differences among varieties at individual treatments; for instance the relative lengths of SA 310 and Callisto at B50 were 74 and 42%, respectively. Within genera, *P. sativum* cv. SA 310 and *C. arietinum* cv. Kaniva were less affected by a high concentration of boron than the other varieties while there was little difference between the two varieties of *L. culinaris*..

Plate 8.3 Comparison of radicle length of grain legumes when grown on filter papers treated with **(a)** B0 and **(b)** B75. From left to right *P. sativum* cv. Alma, *P. sativum* cv. Pennant, *C. arietinum* cv. Semsen and *L. culinaris* cv. Kye.

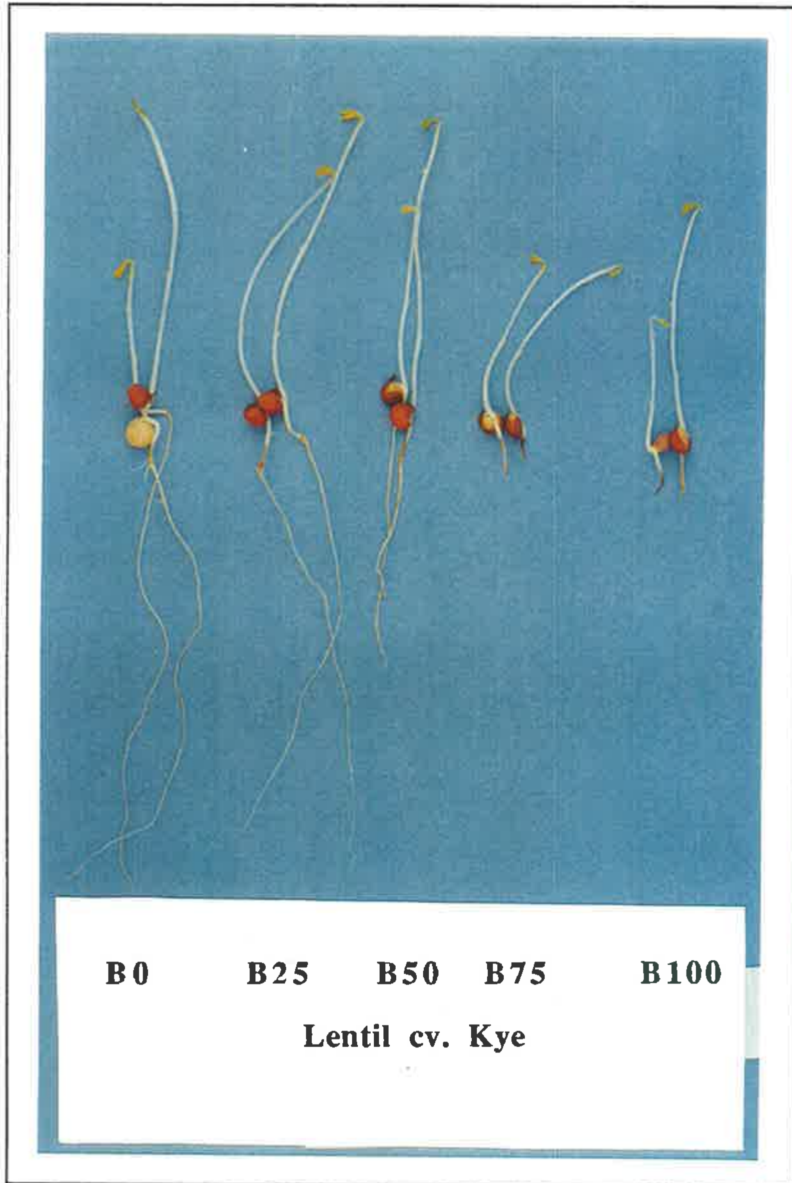
(a)



(b)

Plate 8.4 Effect of boron treatments on radicle length of (**a**) *L. culinaris* cv. Kye and (**b**) *C. arietinum* cv. Semsen .

(a)



(b)

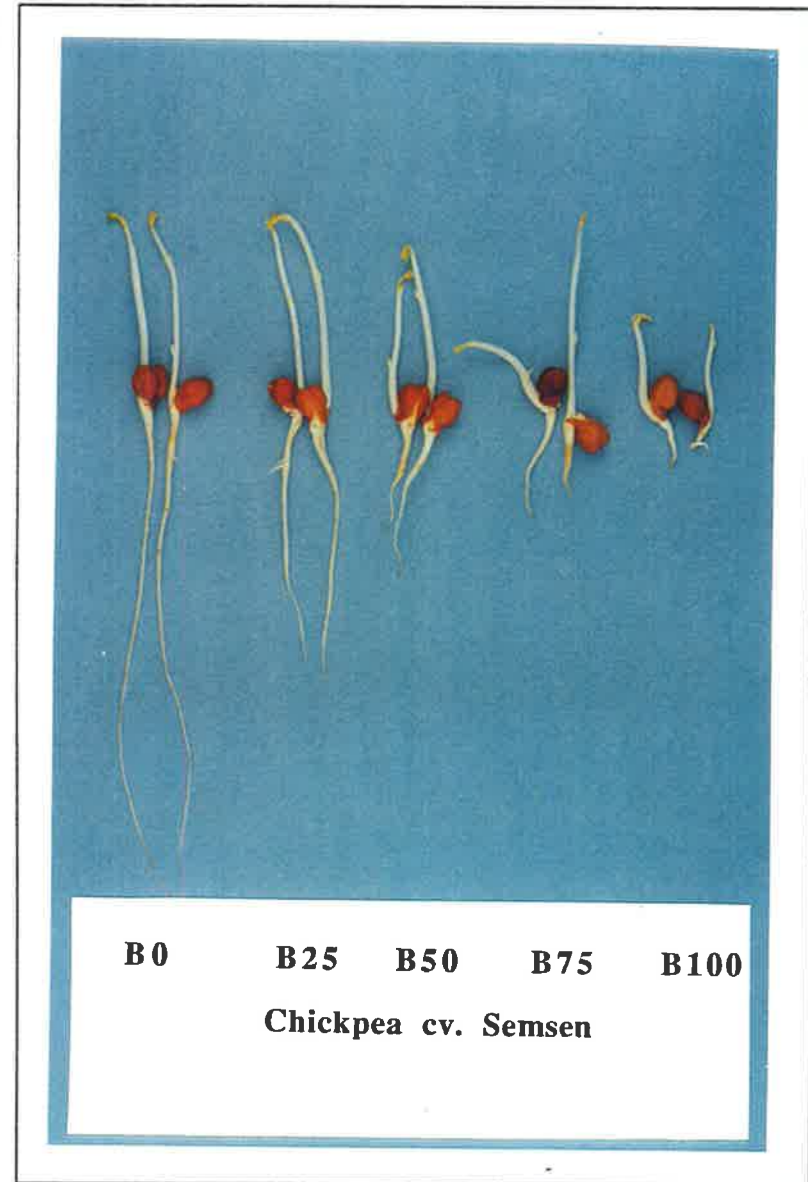
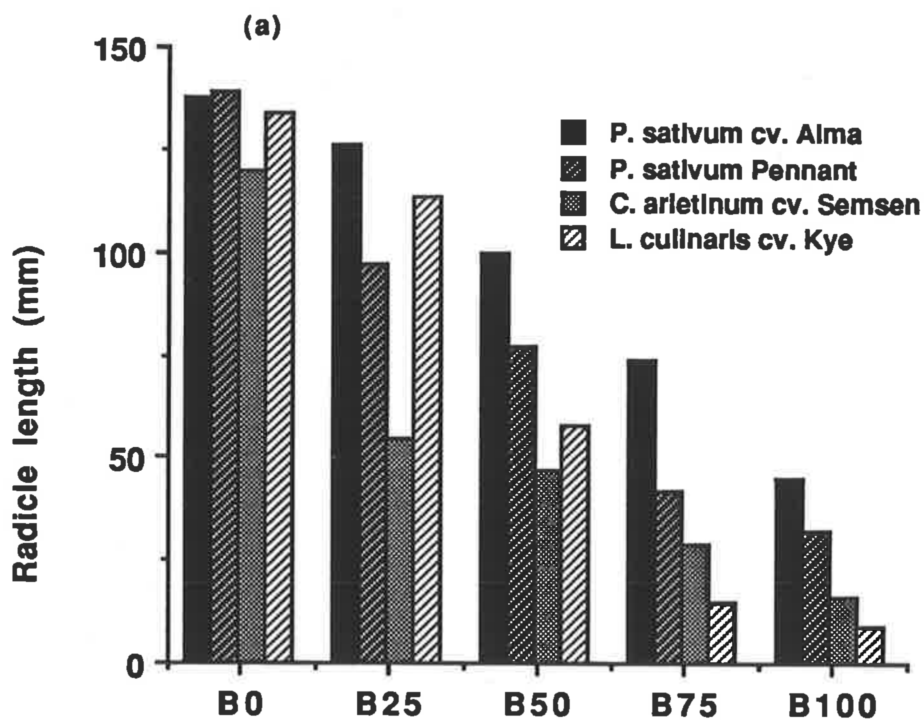


Fig 8.1a Effect of increasing concentrations of boron (mg l^{-1}) on the radicle length of four grain legume varieties Alma, Pennant, Kye and Semsen. The plants were grown for 12 days in a filter paper treated with B0, B25, B50, B75 and B100.

Fig 8.1b Effect of increasing concentrations of boron (mg l^{-1}) on radicle length of eight grain legume varieties consisting of three varieties of *P. sativum* and *C. arietinum* and two of *L. culinaris*. The plants were grown for 12 days in filter papers treated with B0, B50, B75 and B100.


(a)
150
100
50
0
Radicle length (mm)
P. sativum cv. Alma
P. sativum Pennant
C. arietinum cv. Semsen
L. culinaris cv. Kye
B0
B25
B50
B75
B100

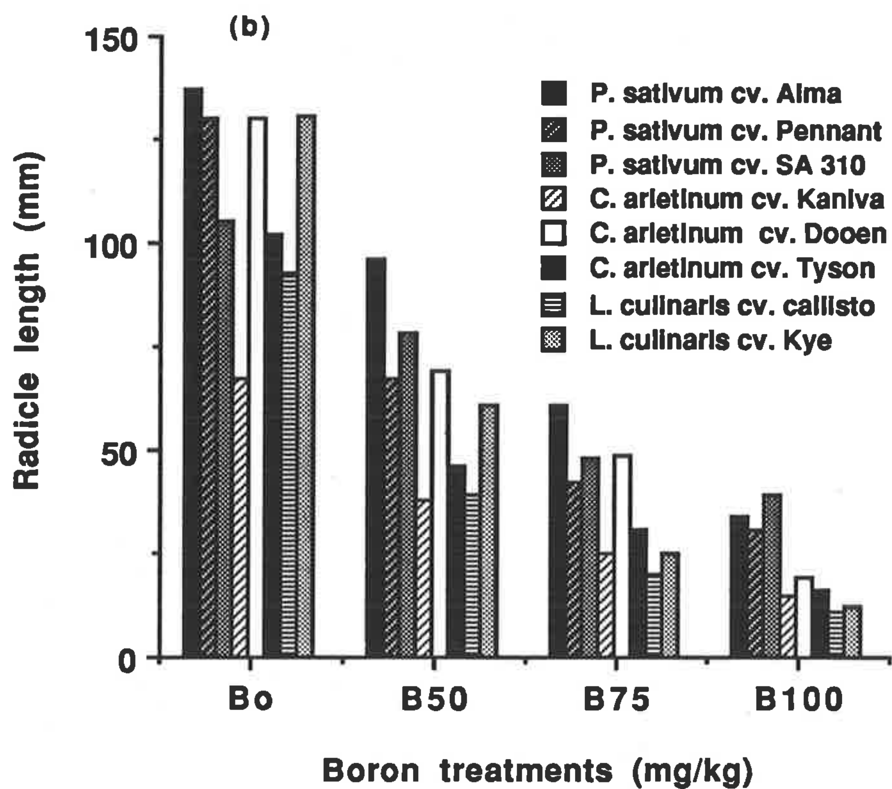

(b)
150
100
50
0
Radicle length (mm)
P. sativum cv. Alma
P. sativum cv. Pennant
P. sativum cv. SA 310
C. arietinum cv. Kaniva
C. arietinum cv. Dooen
C. arietinum cv. Tyson
L. culinaris cv. callisto
L. culinaris cv. Kye
Bo
B50
B75
B100
Boron treatments (mg/kg)

Table 8.10 Radicle length of Australian varieties of peas, chickpeas and lentils grown for 12 days in filter papers treated with four levels of boron (mg l^{-1}). Relative radicle length (% of B0) values are presented in brackets

Variety	Radicle length (mm)				
	B0	B50	B75	B100	*Mean*
Pisum					
Alma	137	96 (70)	61 (45)	34 (25)	
Pennant	130	67 (52)	42 (33)	31 (24)	
SA 310	105	78 (74)	48 (45)	39 (37)	
Mean	124	81 (65)	50 (41)	35 (29)	72
Cicer					
Kaniva	67	38 (57)	25 (38)	15 (23)	
Dooen	130	69 (53)	49 (38)	19 (15)	
Tyson	102	46 (45)	31 (31)	16 (16)	
Mean	100	51 (52)	35 (36)	17 (18)	51
Lens					
Callisto	92	39 (42)	20 (22)	11 (12)	
Kye	131	61 (47)	25 (19)	12 (9)	
Mean	112	50 (45)	23 (21)	11 (11)	49

To compare values in a column LSD 0.01 = 3.17; and in a row, LSD 0.01 = 2.24.

8.4 Discussion

In these experiments the response of varieties of peas, chickpeas and lentils to high concentrations of boron were studied. On the basis of an initial visual assessment of symptom expression, Australian chickpea and lentil varieties were classified as moderately sensitive, sensitive and very sensitive. In order to rank genera and varieties for tolerance to boron various parameters including symptom expression, dry matter yield, tissue boron concentration and radicle length were used.

The response of selected varieties of peas, chickpeas and lentils was examined in a pot experiment with different levels of boron. The results indicated variation among the three genera of grain legumes and among varieties within individual genera in response to high concentrations of boron. The tissue boron concentration in the control plants varied within a range of 16 to 31 mg kg^{-1} between crops (Table 8.8). Significant differences were detected among genera at high boron treatments with tissue boron concentrations of *Pisum* being significantly lower than the others. Difficulties were encountered in the measurement of boron concentrations in shoots of chickpeas and lentils. The lower leaves tended to be dropped when symptoms of boron toxicity were severe. As boron is unevenly distributed throughout plants, with highest concentrations occurring in oldest leaves (Oertli and Kehl, 1961), it is highly probable that the concentrations of boron measured were an underestimate of the total boron accumulation.

On the basis of dry matter production, chickpeas appeared to be more tolerant than peas while lentils were the most sensitive. However, chickpeas would be considered more sensitive than peas on the basis of symptom expression and concentrations of boron in tissues. The morphology of chickpeas with a higher proportion of dry matter in stems than occurs for peas might account for the apparent anomaly in the results and this appears to be analogous the performance of the pea M93 (Chapter 4). Although the variety x treatment interaction for dry matter production was not significant over all varieties, the observation that the lentil variety Callisto died five weeks after sowing at the

B30 treatment and that the number of branches produced by lentil varieties were significantly reduced by boron treatments, but chickpea varieties were not affected, indicates that, in general, the lentil varieties were more sensitive than the chickpeas. The reduction in number of branches could be considered analogous to the reduction and delay in tillering by sensitive wheat varieties in response to boron (Paull *et al.*, 1990)

The ranking of the genotypes for boron tolerance by the filter paper experiments was in close agreement with the ranking in the initial screening according to symptom expression Previous investigations in peas (Chapter 3 and 4) have shown that symptom expression, shoot dry matter and concentrations of boron in shoots were significantly correlated and effective at identifying tolerant genotypes. However, it appears that measurement of shoot dry matter or tissue boron concentrations can not used as a reliable index of boron tolerance across all the genera studied because under high concentrations of boron, plants of *Cicer* and *Lens* senesce their affected leaves. For instance, Semsen with a high percentage of leaf area damage at B30 (Table 8.5) showed a low concentration of boron in its tissues (Table 8.8). Therefore, the results of these three experiments demonstrate that the most effective procedure for selecting boron tolerant genotypes of chickpeas and lentils would be screening in a soil with a high concentration of boron and visual assessment of symptom expression after four weeks. Alternatively, filter paper screening and measurement of radicle length after 12 days could be used as an index of tolerance to boron. One confounding effect associated with this method would be variation in radicle length in the absence of boron (e.g. radicle length of Kaniva was only 67 mm at B0 compared with 130 mm for Dooen). While this would not be a problem when screening fixed lines where a control and a high boron treatment could be used, it would reduce the reliability of selecting single plants of a segregating generation at a single boron treatment. There was variation in radicle length at the control treatment and in seed size for all genera. However, there was not a consistent relationship between radicle length and seed size. For example, among *P. sativum*, the small seeded line SA 310 produced shorter radicles than others, whereas the small seeded lentil variety Kye produced the longest

radicles and among Cicer varieties, the Kabuli type (large seeded and ram-shaped) Kaniva produced shorter radicles at the control treatment than the Desi type (small seeded and angular) varieties.

The majority of ICARDA lines of lentil were more sensitive than current Australian varieties and it is possible that these differences could reflect the selection of the ICARDA lines under lower boron conditions than the Australian varieties. Comparison of the peas, chickpeas and lentils supports the conclusion that lentils have a higher sensitivity to boron toxicity than peas or chickpeas and that sensitivity could be attributed to the accumulation of high concentrations of boron in the tissue. Based on the results of screening germplasm of *P. sativum* (Chapter 4) it might be expected that a wide range of genetic variation could also exist within *Cicer* and *Lens* with genotypes more tolerant than Australian varieties. A wide range of tolerance to boron has been reported for wheat (Moody *et al.*, 1988), barley (R.C.M. Lance, pers. comm.) and peas (Paull *et al.*, 1992; Chapter 4). These reports suggest that boron tolerance of crop plants has originated mostly from West, Central and East Asia and from the Andean region of South America. As the centres of origin and domestication of chickpeas and lentils occur with these regions, it is likely that boron tolerance could be identified among landraces of these crops.

It appears that there is insufficient genetic variation for boron tolerance in chickpeas and especially lentils in Australia to give adequate levels of adaptation in many soil types in southern Australia. In the short term, Kaniva chickpeas and Laird lentils might be expected to be at an advantage for growing in areas where high levels of boron occur in the soil. In the long term there is a need to evaluate a larger number of more diverse genotypes for response to boron. Genetic variation in boron tolerance appears to exist in many natural plant populations, for instance wheat, barley, peas and medics, and it could be expected that variation also exists in the cultivated genera or related wild types of other crops.

CHAPTER 9
GENERAL DISCUSSION

Breeding of field crops for tolerance to high concentrations of soil boron is a major objective under dryland areas of southern Australia where high levels of boron have accumulated in the subsoil of alkaline and sodic soils (Cartwright *et al.*, 1984; 1986). Before starting a breeding program, the breeder needs to know the extent of the genetic variation that is available, the mode of inheritance of the tolerance to boron and preferably the chromosomal location of the genes.

Breeding of peas

In the studies conducted during this project the above points were considered. It was established that there is a wide range of genetic variation for tolerance to high concentrations of soil boron in grain legumes, and in particular in peas, and that only a limited range has been exploited in Australia. This genetic variation was inherited simply and conferred by at least two major loci. Lines were tested for response to boron using several methods, depending upon the objectives of each experiment and the genetic material being tested. The ranking of lines was generally consistent over screening methods and selection criteria. With the exception of M93, the symptom expression was highly correlated with shoot dry matter and the concentration of boron in tissues under high boron conditions and so could be used as a non-destructive selection criterion.

Genetic diversity among Australian varieties and exotic germplasm of *P. sativum* which differed in boron tolerance was identified using the RAPD method. There was a large genetic distance between Australian varieties and exotic tolerant accessions and while it should be possible to increase the range of tolerance in the Australian pea varieties, an intensive backcrossing program accompanied by visual selection for a low level of symptoms in plants grown in high boron conditions would be required to reconstitute an

adapted genetic background. The genetic differences between Australian varieties and tolerant accessions measured by this technique provided some basis for estimating the extent of backcrossing required to achieve a reasonable probability of selecting adapted varieties. This study also indicated that the use of RAPD techniques to detect genetic variation among *P. sativum* germplasm is possible. This will be useful in the future for varietal identification and management of breeding programs.

Before adopting breeding for boron tolerance as an objective, there is need to have some idea of the economic benefit. The backcrossing method was used to transfer the *Bo1* allele from the moderately tolerant wheat variety Halberd to the moderately sensitive Schomburgk. The resulting variety BT-Schomburgk yields approximately 10% more than Schomburgk when grown on high boron soils in southern Australia (Moody *et al.*, 1993; Campbell *et al.*, 1993). The results presented in this thesis, including the development of a screening procedure and the identification of genetic variation under major gene control would enable the Australian pea breeding programs to adopt breeding and selection for tolerance to boron routinely. The yield results obtained for wheat, together with the fact that the mostly widely adapted varieties of peas in southern Australia (Early Dun and more recently Alma) are moderately tolerant to boron suggest that significant yield improvements could be achieved.

From an evaluation of 617 germplasm accessions and breeding lines of *P. sativum*, 3.5% were identified as being more tolerant than the most tolerant Australian varieties. A relatively high proportion of tolerant and moderately tolerant accessions originated from Asia and South America, as would be expected from the intensive investigations with wheat (Moody *et al.*, 1987). There is no need to search for further genetic diversity at present but rather to include the tolerant accessions already identified as parents in Australian pea breeding programs.

Screening of *P. sativum* germplasm in a box containing high boron soil with visual assessment of symptom expression after four weeks was both quick and efficient (Chapter

4) and correlated well with dry weight yield, with the exception of M93, and tissue boron concentrations. The growth of radicles in filter papers treated with a high boron concentration was also a quick test and its results correlated well to symptom expression of plants grown in soil. Two major problems with the filter paper procedure were the level of variation among plants within genetically homogenous lines and variation in root length in the absence of boron. These would reduce the accuracy of adopting this procedure for single plant selection, such as in an F_2 generation. Such problems have not been encountered with bread and durum wheats (Y. Chantachume and S. Jamjod, pers. comm.) and the difference between the species might be related to variation in hard seededness among grain legumes.

The Australian varieties Alma and Pennant and one of the tolerant accessions can be used as standard genotypes for moderately tolerant, sensitive and tolerant response to boron, respectively, in the same way as Warigal, Halberd and the Greek line G61450 are being used in wheat (Paull *et al.*, 1991).

Genetics of boron tolerance in peas

Although information on the genetic control of boron tolerance in crop plants is limited, the inheritance of tolerance of wheat and barley to boron has been shown to be under the control of a series of major genes (Paull *et al.*, 1991; Jenkin, 1993). The expectation that the genetic control of boron tolerance in peas would be relatively simple was confirmed by an analysis of populations segregating for this character. Data from the survey of five segregating generations suggests that two major genes with incomplete dominance were the major factors governing the inheritance of boron tolerance in peas. Selected tolerant families had lower concentrations of boron in tissues and therefore this finding again confirms the use of symptom expression as a selection criterion in breeding programs. The gene symbol *Bo* and *bo* for tolerance and susceptibility to boron respectively, have been tentatively assigned, corresponding to those in wheat (Paull *et al.*, 1991).

Further studies are required to determine whether genetic differences exist among tolerant accessions for reaction to high concentrations of boron. This could be achieved by intercrossing the tolerant lines in all combinations and examining the response of the F_2 generations under high boron conditions. If the genetic control of tolerance was the same in all three lines, no segregation would be expected in the F_2, but if the tolerance of these lines were controlled by different genes, transgressive segregation would be expected, as was observed in wheat (Paull *et al.*, 1991).

Involvement of major gene (s) for boron tolerance allowed the possibility of attempting to identify linked markers that could be used for marker-assisted selection in peas. Segregating DNA markers and allozymes were used to establish the linkage to boron tolerance in two populations (Alma x SA 310 and JI 15 x JI 399). Evaluation of the F_2 of Alma x SA 310 with RAPD markers identified one RAPD marker putatively linked to the boron gene, but the recessive nature of this marker resulted in a low LOD score and it would be necessary to screen a greater number of F_2 plants to improve the confidence of this marker. As visual selection is reliable and quick, linked markers are not necessary for selection for this character. This work illustrates that the technology for selecting peas with linked markers is available to breeders.

RFLP analysis of a recombinant inbred population (JI 15 x JI 399) showed that boron tolerance in this population is located about 10 cM from *dr7*- which occurs on linkage group 1. Evaluation of the F_2 population of Alma x SA 310 with selected probes from linkage group 1 showed independent segregation of RFLP and boron tolerance, suggesting different genes confer boron tolerance in the two populations. The challenge now is to evaluate Alma x SA 310 with more probes from linkage group 1 to confirm the different position of the gene in this population. The parents of the John Innes population should be intercrossed with Alma and Pennant (i.e. the combination that defines the *Bo1* locus), to determine if the lines that are similar phenotypically (Alma and JI 399-moderately tolerant and Pennant and JI 15-moderately sensitive) are the same genetically. If this were the case, it could be assumed

that the *Bo1* locus is located on linkage group 1 and markers identified in the JI 15 x JI 399 population could be tested against Australian genotypes.

Linkage-assisted selection has been an ideal long sought by breeders and molecular markers will meet such a requirement. DNA markers are an especially valuable tool for introgression of desirable traits from exotic germplasm into locally adapted varieties.

The development of a reproducible system for transformation and regeneration of peas (Schroeder *et al.*, 1993) allows the genetic engineering of this crop. The challenge in this area would be the isolation of more genes, particularly those of relevance to particular components of agricultural productivity and also the development of methods for transforming other grain legumes.

Breeding of chickpeas and lentils

The evaluation of Australian varieties of chickpeas and lentils for their response to high concentrations of soil boron revealed a limited range in genetic variation in boron tolerance. The measurements of dry matter or tissue boron concentrations were not reliable indices of boron tolerance for *C. arietinum* and *L. culinaris*. It seems that the most effective procedures for selecting boron tolerant genotypes during backcrossing of these genera would be screening in a soil with a high concentration of boron and visual assessment of symptom expression after four weeks or alternatively, screening in filter papers treated with boric acid and measurement of radicle length after 12 days as an index of tolerance to boron.

Of the most tolerant of the Australian varieties, peas are more tolerant than chickpeas which are more tolerant than lentils. The lack of sufficient genetic variation among Australian varieties is probably due to selection under the favourable environments of Tamworth for chickpeas and ICARDA (Tel Hayda, Aleppo, Syria) for lentil lines. As none of the varieties was grown under high boron conditions in field trials during line selection, genes for boron tolerance may have been lost or are at very low frequencies. The soil at Tel Hadya is low in

boron as boron toxicity symptoms are not expressed in barley compared with other sites in Syria, such as Breda and Bouider (A.J. Rathjen, pers. comm.). Therefore, Tel Hayda is not suitable as the primary site for selection of boron tolerant lines. The soil at Tamworth is also relatively undifferentiated, compared to the alkaline duplex soils of much of southern Australia which not only show boron toxicity, but are also sodic and likely to be deficient in available Zn, Mn, Cu and Fe.

Another important fact is that the use of germplasm materials in Australian chickpea and lentil breeding programs has been limited and therefore it would appear that there is insufficient genetic variation for boron tolerance in chickpeas and especially lentils in Australia to give adequate levels of adaptation in many soil types in southern Australia.

The presence of variation for boron tolerance in peas and other species would suggest that the limited variation for tolerance in chickpeas and lentils can be overcome by systematic screening of a large number of lines from collections or from the closely related species. A wide range of tolerance to boron has been reported for wheat (Moody *et al.*, 1988), barley (R.C.M. Lance, pers. comm.), medics (Paull *et al.*, 1992) and peas (Paull *et al.*, 1992; Chapter 4). These reports suggest that boron tolerance of crop plants has mostly originated from West, Central and East Asia and from the Andean region of South America. *Cicer* and *Lens* spp. are widely distributed in West Asia (Ladizinsky and Alder, 1976 and Zohary, 1972) and therefore this region would be the best place to choose lines for screening for boron tolerance.

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APPENDIX 1

COMPOSITION OF SOLUTIONS AND MEDIA

(a) RAPD analysis

10x *Taq* reaction buffer: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 0.1% (w/v) gelatin

10x TBE buffer: 1M Tris, 10mM Na_2EDTA, 860 mM boric acid, pH 8.3

DNA extraction buffer: 1% sarkosyl, 100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5

Ethidium bromide staining solution: 10 $\mu g\ ml^{-1}$ ethidium bromide in H_20

Phenol/chloroform/isoamylalcohol (25:24:1): redistilled phenol was saturated with 0.5 M Tris-HCl (pH 8.0) and mixed with chloroform and iso-amyl alchol as indicated

R40: 40 $\mu g\ ml^{-1}$ RNase A in TE buffer

(b) Molecular cloning

2YT medium: (per litre distilled water) 16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl adjust pH to 7.0 with 10 N NaOH

5x ligation buffer: 250 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 5 mM DTT, 25% (w/v) polyethylene glycol 8000

10x Vent® reaction buffer: 100 mM KCl, 200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM $(NH4)_2\ SO_4$, 20 mM $MgSO_4$, 1% Triton X-100

GET solution: 50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris-HCl pH 7.5

KAcF solution: 3M potassium acetate pH 4.8, 1.8 M formic acid

LB medium (Luria-Bertaini): (per litre distilled water) 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, pH 7.5

TE buffer: 10 mM Tris-HCl, 1mM EDTA, pH 8.0

(c) RFLP analysis

1x TAE buffer: 40 mM Tris-acetate, 1 mM EDTA pH 7.6

5x HSB: 3M NaCl, 100 mM PIPES, 25 mM Na_2EDTA, pH 6.8 with 4 M NaOH

10x restriction endonuclease buffer B: 6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.5

10x restriction endonuclease buffer D: 6 mM Tris-HCl, 6 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, pH 7.9

10x restriction endonuclease buffer E: 6 mM Tris-HCl, 6 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.5

10x restriction endonuclease buffer H: 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5

Denaturing solution: 1.5 M NaCl, 500 mM NaOH

Denhardts III: 2% gelatin, 2% ficoll, 2% Polyvinylpyrollidone (PVP), 10% SDS, 5% tetrasodium pyrophosphate, filter at 65°C

Neutralising solution: 1.5 M NaCl, 500 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.0

Salmon sperm DNA: add 0.5 g salmon sperm DNA to 100 ml nanopure H_2O, autoclave

Sephadex G-100: To 300 ml TE buffer add 10 g sephadex G-100, incubate with gentle shaking for 2 h at 65°C