The University of Adelaide
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Biochemical studies of tissue glucosinolates for improvement of canola (*Brassica napus*) as a disease break within the southern Australian cereal rotation.

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SUMMARY

The inclusion of canola (*Brassica napus*) in the southern Australian cereal cropping rotation has led to much discussion regarding the qualities of the crop as a 'disease break'. Canola has been reported to 'clean up' the soil, reducing populations of pests and pathogens and therefore providing a healthier soil environment for subsequent crops. However, while many growers and agronomists agree that the crop can be used as a disease control tool, the effect is not reliable, often exacerbating soil disease problems, due primarily to the apparent susceptibility of canola to a range of pests and pathogens.

Field studies reported within this thesis showed that canola (*Brassica napus*) led to at least comparable reductions in soil levels of the root lesion nematode (*Pratylenchus* spp.) as other rotation crops grown in parallel. *Brassica* crops were observed to be particularly effective when green manured (incorporated into the soil), suggesting the release of nematicidal agents as the tissues broke down in the soil. However, the disease break effect of the crop was limited by its susceptibility to infestation by the nematode. Efforts to improve the disease break effectiveness of the crop against *Pratylenchus* spp. must therefore consider both the susceptibility of the plants and the nematicidal potency of the tissues as they degrade in the soil.

Degrading leaf tissues were observed to be more potent against *P. neglectus* than root tissues. However, HPLC studies revealed no relationship between the nematicidal potency of *Brassica* leaves and the glucosinolates within them, suggesting that an alternative allelopathic system was active within these tissues. As leaf tissues are not incorporated into the soil within the cropping system under study, further scrutiny of these tissues were considered beyond the scope of this thesis.

A close association was observed between the levels of glucosinolates within the roots and the nematicidal potency of these tissues. Further study revealed that a single glucosinolate, 2-phenylethyl (2-PE), was entirely responsible for the nematicidal qualities of the root tissues, despite the presence of comparable levels of other glucosinolates, such as 2-propenyl, within some tissues. *In vitro* studies of purified 2-PE isothiocyanate confirmed the nematicidal properties of the molecule. Studies also revealed that root levels of 2-PE glucosinolate were closely associated with the susceptibility of *Brassica* roots to *P. neglectus*. Plants containing

above a critical level of 2-PE glucosinolate were both more resistant and more nematicidal to *P. neglectus*.

The mean 2-PE glucosinolate levels in the most commonly grown canola variety of the southern Australian cereal region, Dunkeld, were below the observed critical level, perhaps explaining the unpredictable nature of the crop as a disease break. However, considerable variation was observed in the levels of 2-PE glucosinolate within its roots, suggesting the potential to increase the mean levels beyond the threshold. Self pollination studies of Dunkeld plants revealed that the variation in 2-PE glucosinolate levels was heritable, with S_1 and S_2 populations maintaining the parental phenotype. The S_2 progeny of a segregating selection exhibited a 'high':'low' 2-PE glucosinolate phenotype in approximately a 3:1 ratio, suggesting single gene inheritance following Mendelian principles. 'High' 2-PE glucosinolate S_2 sub-populations were significantly less susceptible to *P. neglectus* than 'low' 2-PE glucosinolate counterparts.

These same self pollination studies revealed a varied distribution pattern for total glucosinolate levels, suggesting a second heritable character which may have been linked to total seed glucosinolate levels. However, these findings were not further pursued within this thesis, and served only to stress the need to select for % 2-PE glucosinolate levels to minimise the risk of impacting on seed glucosinolate levels and thus quality.

It is likely that 'high' 2-PE glucosinolate sub-populations could be developed from many of the current canola varieties. Such sub-populations should maintain the agronomic characters of the parent line, occupying the same rotational niche as the original varieties. However, being less susceptible and more nematicidal, these lines should perform more reliably as disease breaks against *P. neglectus* within the cereal rotation.

STATEMENT OF ORIGINALITY

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

I give consent for this thesis being made available for photocopy and loan.

17TH SEPT 1998

Mark J. Potter

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PUBLICATIONS ARISING FROM THIS THESIS

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Potter, M., Davies K., and Rathjen A. (1997). *Brassica* tissue suppression of the root lesion nematode (*Pratylenchus neglectus*). Proceedings of the 11th Australian Research Assembly on *Brassicas*, Perth, Western Australia, pp. 161-164.

Potter, M., Davies K., and Rathjen A. (1997). *Brassica* tissue suppression of the root lesion nematode (*Pratylenchus neglectus*). Proceedings of the 11th Biennial Conference of the Australasian Plant Pathology Society, Perth, Western Australia, pp. 259.

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Potter, M., Davies K., Kirkegaard, J. and Rathjen A. (in preparation). Improved resistance to the root lesion nematode (*Pratylenchus neglectus*) in canola (*Brassica napus*) containing elevated root levels of 2-phenylethyl glucosinolate. *Journal of Nematology*.

Potter, M., Davies K., and Rathjen A. (in preparation). Breeding to increase the disease break strength of canola (*Brassica napus*). The inheritance of 2-phenylethyl glucosinolate in the root. *Plant Breeding*.

CHAPTER 1

GENERAL INTRODUCTION

Over 16 million hectares are sown to cereal in Australia each year, producing more than 28 million tonnes of grain with a value of approximately $AUS 4 billion (AWB, 1998). Efforts to maintain the profitability and sustainability of cereal production within southern Australia have emphasised the use of alternative crops, such as legumes and oilseeds, in rotation with cereal production (Mead, 1992). Such rotations provide a 'disease break', a situation where the growth of a non-host crop 'breaks' the reproductive cycle of pest and disease organisms. These practices have the potential to reduce populations of harmful organisms without cultivation of the soil (leading to erosion of soil structure) or costly and contaminating chemical applications for control (Purvis *et al.*, 1990).

Winter sown canola (*Brassica napus* L.) has been integrated into the rotation of many cereal producing areas in southern Australia. The recent release of varieties better adapted to Australian conditions and the development of strong domestic and international markets for the seed oil and meal has increased the security and economic viability of the crop. However, a further incentive for farmers to produce canola has been its effect in improving the early vigour and yield of subsequent wheat crops. This increase has been attributed to many factors, including root microporation of soils, variation in nutrient cycling and water relations (Angus *et al.*, 1991). However, the primary reason for its success is considered to be its role as a disease break, reducing harmful populations of nematodes, fungi, bacteria, viruses and even weed seeds (Brown and Morra, 1997). The disease break effect may be due to more than the effect of a period free of a host for the disease causing organism. Reduced pathogen levels have been reported after canola crops susceptible to the organism (Kollmorgen *et al.*, 1983), implying that the canola reduced pathogen levels by some other means than by failing to nurture the organism. *Brassica* tissues have been reported to actively suppress harmful organisms as the tissues degrade in the soil (Rice, 1984; Bennett and Wallsgrove, 1994; Brown and Morra, 1997).

The tissues of *Brassica* plants produce and store a range of stable, non-toxic molecules called glucosinolates, thought to be involved in plant defence. Upon tissue damage or natural senescence, the glucosinolates are cleaved enzymatically, releasing a range of allelopathic molecules with toxic activity against a broad range of organisms (Underhill, 1980; Poulton and Moller, 1993; Stoewsand, 1995). The disease break associated with canola is thought to be enhanced by the active release of these biotoxic molecules as the tissues break down in the soil (Angus *et al.*, 1991).

Canola is, however, not a reliable disease break crop in the field. Suppression of harmful organisms is not always observed (Johnson *et al.*, 1992), with populations often increased rather than decreased following a canola crop (MacLeod and Warren, 1993; Kirkegaard *et al.*, 1994). Canola is susceptible to a broad range of soil-borne wheat pathogens, potentially undermining the suppressive impact of the degrading tissues. While ample research discusses the biocidal potential of *Brassica* tissues, and the biotoxic impact of purified glucosinolate derivatives, studies linking the two concepts for application to the field condition are rare. In order to maximise the disease break associated with canola, the dynamics of the system must be more thoroughly examined. It is the aim of this project to determine the relationship between the glucosinolates within canola tissues and the susceptibility and nematicidal impact associated with the crop, in an effort to determine the potential to improve the disease break qualities of the crop through manipulation of glucosinolate levels.

CHAPTER 2

LITERATURE REVIEW

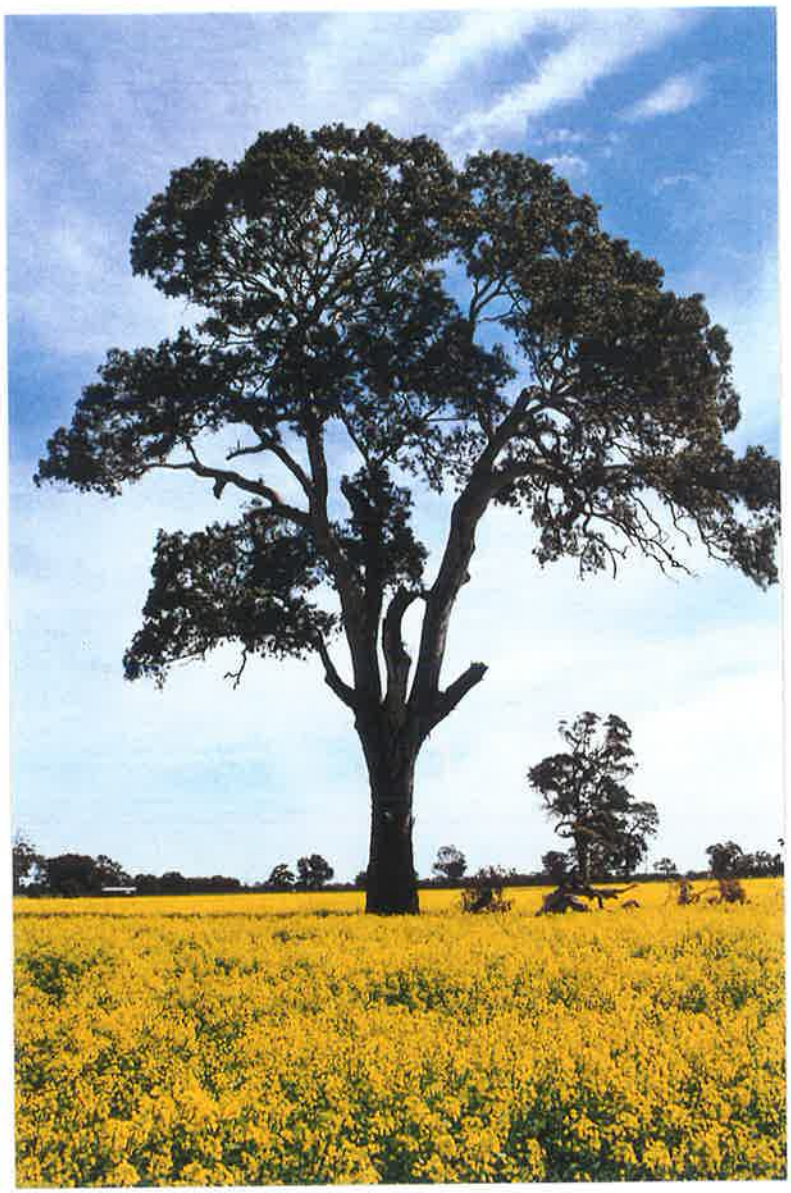
2.1 CANOLA PRODUCTION

Expansion of the world market for vegetable oils for human consumption and industrial use has encouraged development of a number of oilseed crops for inclusion within broadacre agricultural systems. Of these oilseed crops, canola (*Brassica napus* L.) is one of the best adapted to the southern Australian cereal growing area (Plate 2.1).

World production of canola (1997/98) was forecast at 33.2 million tonnes (mt.), produced primarily by China (9.54 mt.), Germany (2.8 mt.), France (3.5 mt.), UK (1.5 mt.), India (6.96 mt.), and Canada (6.2 mt.) (FAO, 1998). The Australian industry, hampered by the susceptibility of early lines to blackleg fungus (*Leptospharia infestans*) and late maturity under Australian conditions, is small in world terms (Buzza, 1995). However, since the development and release of blackleg resistant and earlier maturing lines in the mid 1980's, there has been a rapid expansion in production, increasing from 17,194 tonnes in 1983/84 to over 769,000 tonnes in 1996/97 (FAO, 1998). This expansion is set to continue, due to the high value of the seed, increasing markets for canola oils, the development and release of new, more highly adapted varieties, and the better integration of the canola within Australian agricultural rotations (Buzza, 1995).

The value and stability of the canola industry today reflects the diversity of uses for the crop product, and thus diversity of markets. Canola seed is crushed, yielding both oil and a high protein meal. Until recently, use of the meal was limited due to high levels of toxic oxazolidinethiones, produced as degrading hydroxylated glucosinolates rearranged within the tissue, and causing goitre in livestock (Wallsgrove *et al.*, 1995). Consequently, a major aim of canola breeding programs was the reduction of total seed glucosinolates below the critical level of approximately 10mmol/g tissue (~10% of original levels), allowing the development of markets for the defatted meal, a high protein source (~40% by weight), used as a stock feed.

Plate 2.1: A canola crop (*Brassica napus* cv Dunkeld) in the field at Keith, South Australia, September 1995.



The oil, extracted from the seed by pressing, has a multitude of uses, depending upon the properties of the fatty constituents (Murphy, 1995). As production for human consumption was originally limited by high levels of erucic acid within the oil, a second major aim of breeding programs has been to reduce this fatty acid to acceptable levels (<2%). Varieties have also been developed which produce oil suitable for industrial purposes. High erucic acid rapeseed (HEAR) oils have many industrial applications and the advantages over petrochemical oils of being renewable, biodegradable, non-toxic and non-corrosive. Variation in the saturation of the fatty acid content of the oil alters properties such as viscosity, pressure and temperature sensitivity and biodegradability. The selection for alternative fatty acid profiles within the seed oil has allowed the development of lines producing oils for use as replacements for mineral oils, as lubricants, surfactants, cosmetic oils and polymer bases (Carruthers, 1995). Rapeseed oil has also been used as a substitute for diesel fuels, and, in direct injection engines, pure unprocessed HEAR oil has been used as a fuel hydrocarbon. As plant breeders gain an understanding of the inheritance and means of manipulation of the fatty acid profile within the *B. napus* seed, a range of new lines may become available with fatty acid profiles appropriate for specific industrial requirements (Carruthers *et al.*, 1995; Murphy, 1995). Such lines will further broaden the marketability of canola seed, increasing the attractiveness of the crop for integration as a disease break within the southern Australian cereal rotation.

2.2 TEST ORGANISM

2.2.1 *Choice of test organism*

To study the relationship between tissue glucosinolates and the disease break associated with canola, it was important to select a pathogen for use as a test subject. The suitability of such a pathogen was based upon the following parameters.

- significance to the southern Australian cereal industry,
- disease break as primary means of control,
- rapid lifecycle,
- ease of access to uniform populations,
- ease of population maintenance and inoculation,
- ease of sampling and population assessment,
- apparent variation in the susceptibility of canola varieties to the organism.

The root lesion nematode, *Pratylenchus neglectus* Filipjev, 1936 (syn. *P. minyus*; Order Tylenchida), a significant pathogen of cereals in southern Australia (Vanstone *et al.*, 1993), was adopted as an appropriate test subject (Plate 2.2). At present, crop rotation is the only effective and economic means of control of this nematode within the cereal cropping system and, consequently, the use of poor host rotational crops is paramount in its control. The nematodes can be efficiently extracted from soil by misting (Southey, 1986), and can be cultured under aseptic conditions to produce inoculum for laboratory and glasshouse trials (Vanstone, 1991; Nicol and Vanstone, 1991). The lifecycle of the nematode is relatively quick (one generation in 6 weeks at 25°C; Vanstone, 1991), reproducing parthenogenetically and thus minimising genetic variation within cultured populations. Finally, variation in the susceptibility of *Brassica* crops to this nematode has been observed (Nicol, 1996; Webb, 1996), suggesting potential for improvement of the disease break through plant breeding.

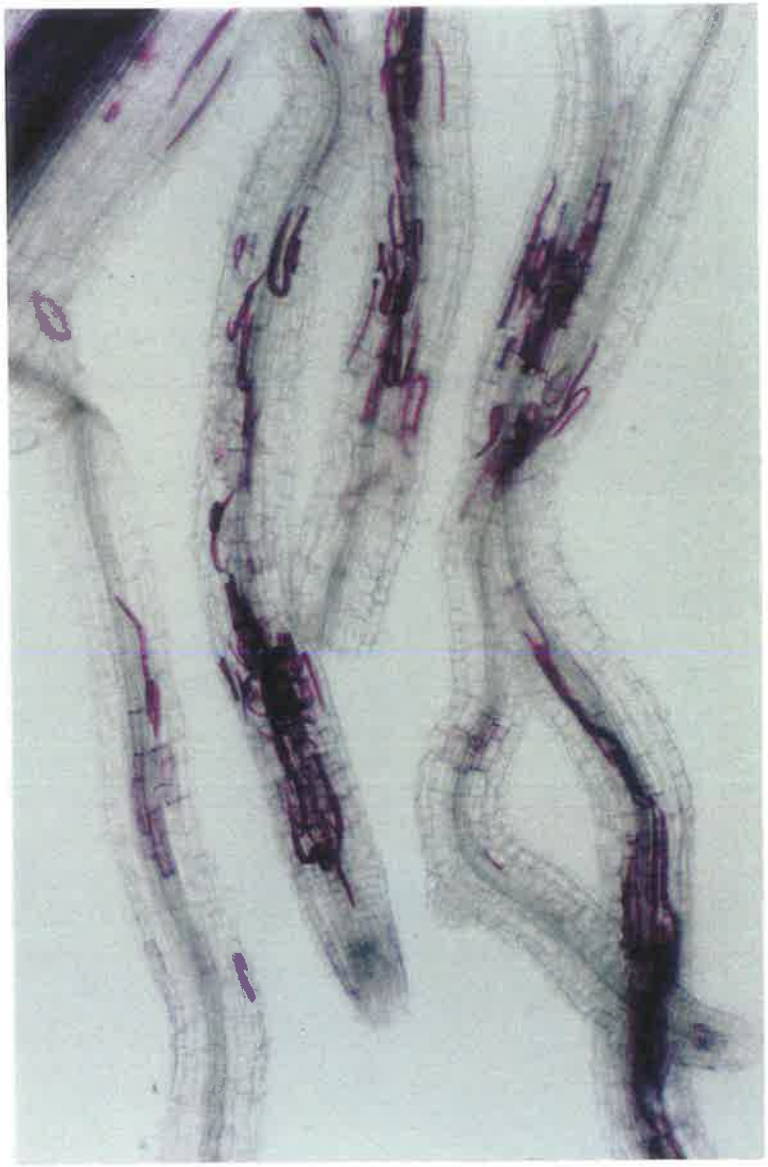
2.2.2 *Agricultural significance*

The impact of plant parasitic nematodes on world agriculture has only lately become appreciated, as the development of chemical nematicides has enabled pathologists to evaluate crop yield with and without nematodes (Stirling, 1992). Actual losses due to nematodes are difficult to assess, as they are often involved in synergistic relationships with other plant parasites. *Pratylenchus* invasion predisposes the plant to infection by other organisms (particularly fungi), creating the potential for development of disease complexes involving two or more pathogens (Taheri, 1996). The impact of nematodes is therefore dependent upon many factors including:

- crop species and variety,
- nematode species,
- abundance of other soil pests and diseases,
- diversity of plant species within the crop rotation,
- environmental factors such as soil type, fertility, temperature and water availability.

Disease incidence and severity is exacerbated by continuous cropping with susceptible crops, allowing nematode numbers to increase. In the Australian agricultural industry, plant parasitic nematodes are estimated to cause losses in crop production of about $400 million per year, of which as much as 25% is in losses in the productivity and yield of wheat (Stirling *et al.*, 1992).

Plate 2.2: *Pratylenchus neglectus* nematodes stained within wheat roots (*Triticum aestivum*). Photograph courtesy of Abdol Taheri.



Within the southern Australian cereal growing region, the most economically significant nematode parasites are the cereal cyst nematode, *Heterodera avenae*, and the root lesion nematodes, *Pratylenchus neglectus* and *P. thornei* (Stirling *et al.*, 1992). While the problems associated with heavy infestations of *H. avenae* have long been recorded, losses have been reduced by the development of resistance in cereals, and improved management practices such as crop rotation (Stirling *et al.*, 1992). In contrast, the root lesion nematode, although identified as a potential problem in the 1960's (Colbran and McCulloch, 1965), has only recently been recognised as an economically significant parasite within the cereal rotation (Thompson *et al.*, 1978; Vanstone, 1991). Although efforts are underway to develop new lines of wheat resistant to this nematode, presently the only economic means of control within the southern Australian cereal belt is the use of rotations with poor host crops (Vanstone *et al.*, 1993; Taylor *et al.*, 1995).

2.2.3 *Root lesion nematodes* (Pratylenchus *spp.*)

Root lesion nematode is found in most regions of the world including Europe, Africa, North and South America, and Australasia (Wiese, 1987). They are polyphagous, infesting plants from at least eighteen botanical families, including cereals (Thompson, 1990), oilseeds (Webb, 1990; Bunte and Friedt, 1995) and legumes including vetch, chickpea, soybean (Mountain, 1954), clover (Townshend and Potter, 1976), and horticultural crops such as peach, potatoes, strawberries and crucifers (Goodey *et al.*, 1965).

At least seven *Pratylenchus* species develop on wheat, including *P. thornei, P. neglectus, P. zeae, P. crenatus* and *P. mediterraneus* (Wiese, 1987). *P. neglectus* has been recorded as an economically important pathogen of wheat in Canada (Benedict and Mountain, 1956), and the United States (Mojtahedi and Santo, 1992), and has recently been identified as economically significant in the southern Australian wheat industry (Vanstone *et al.*, 1998).

Taxonomy and morphology

Over 40 species of *Pratylenchus* have been described (Loof, 1991). There is considerable overlap in terms of morphological parameters and reliable species identification depends upon microscopic assessment of characters such as body length/width ratio, vulval position and number of lip annules. Recent developments in the use of molecular probes and markers offers potential to distinguish species more easily (Orui, 1996).

Adult *P. neglectus* females range from 300 to 800µm in length, with a long, slender vermiform body approximately 20µm wide. The nematodes have a sharp stylet approximately 50µm long used to pierce plant cells for penetration and feeding. The oesophageal region takes up the first 20% of the length of the nematode, tapering to a characteristic diagonal at the proximal end of the alimentary canal, produced by the oesophageal glands overlapping the oesophagus. The position of the vulval region varies between species, but is between 65% and 85% of the length of the nematode, at the rounded tail end (Mai and Mullin, 1996).

Lifecycle

P. neglectus reproduces by mitotic parthenogenesis (Roman and Triantaphyllou, 1969; Townshend and Anderson, 1976). Small numbers of males may be present in populations, but are not known to contribute to the reproductive cycle. Eggs are covered in a thick layer of polypeptide and are very resilient to desiccation, known to survive in an anhydrobiotic state for more than 2 years without significant loss (Mountain, 1954; Baxter and Blake, 1968). Juvenile nematodes reach maturity in about 30 days under favourable conditions (Mountain, 1954), with adults depositing eggs at a rate of one per day as they move through the soil or within the root (Zunke, 1990a). There are multiple generations per year, leading to exponential population growth throughout a season.

Tissue attack

Pratylenchus spp. are attracted along a gradient of exudate from host plant roots (Townshend and Anderson, 1976) and penetrate adjacent to the root cap, at the junction of existing and new roots, or at the zone of elongation (Zunke, 1990a). The nematode enters the root by piercing the epidermis with the stylet, and forces its way into the cortical tissues. Increased root exudates from entry sites attract other individuals, leading to multiple penetrations in one area. The nematodes feed by puncturing and entering cortical cells and consuming cell cytoplasm. This damage induces liberation of phenolics and tannins from the tissues, causing cellular necrosis which can extend beyond the immediate area of infestation, producing the characteristic root lesions (Baxter and Blake, 1967). *Pratylenchus* spp. may also feed as ectoparasites, grazing on root hairs and meristems (Zunke, 1990b) which can limit the development of lateral roots and restrict root elongation (Thorne, 1961). The nematodes are mobile, migrating within and between roots and soil, producing new entry sites and lesions throughout their life (Dropkin, 1980).

Root damage by *Pratylenchus* spp. reduces the potential for the plant to take up water and nutrients from the soil. The impact of the nematode is often more obvious in dry years as infected plants are less tolerant to conditions of stress (Baxter and Blake, 1968). Exposure of seedlings to high root lesion nematode numbers may have a great impact on crop yield, as infestation at this critical period depresses early vigour. Productivity of many crops may be limited by heavy nematode infestations and the farmer's choice of economically desirable crops may actually be prohibited by heavy soil infestations of these nematodes.

Wheat plants grown in soil heavily infested with *P. neglectus* show symptoms characteristic of plants with damaged or restricted root systems (Vanstone, 1991). The plants may be stunted and may wilt readily under dry conditions. Yellowing of lower leaves, characteristic of nutrient deficiency, may also occur (Farsi, 1995). Roots often have brown lesions within the cortex, and the number of lateral roots may be reduced in length and number (Vanstone, 1991). Heavy infestation can lead to reduction in early vigour and an overall reduction in tillering. Significantly, nematode infestation allows invasion of the root system by other pathogens via entry wounds. Synergism between *Pratylenchus* and other potential pathogens may be the primary cause of the reduction in crop yield (Taheri, 1996).

Susceptibility of canola

Many parasitic nematodes may invade and multiply within *B. napus* canola roots, including *Heterodera schachtii* (Evans and Russel, 1993; Theirfelder and Friedt, 1995), *Helicotylenchus pseudorobustus*, *Meloidogyne hapla* and *M. incognita* (Bernard and Montgomery-Dee, 1993), and *Pratylenchus* spp. (Acedo and Rohde, 1971; Webb, 1990). Vigour and final yield of canola crops in the field has been increased by nematicide application, suggesting significant damage due to nematodes (Evans and Webb, 1989). Inoculation of canola tissues with *P. fallax* caused visible root necrosis after sixteen days, although damage and yield loss was dependent on the variety of *B. napus* examined and the species of nematode (Webb, 1996). Zunke (1990b) reported that *Pratylenchus* spp. fed within the root cortex of oilseed rape as endoparasites and on root hairs as ectoparasites, reducing the length of main and lateral roots. Other *Brassica* species, including many horticultural crops such as *B. oleracea,* also support high populations of *Pratylenchus* and show the associated lesioning (Acedo and Rhode, 1971).

The reproductive potential of *Pratylenchus* spp. on *B. napus* is unclear. Bernard and Montgomery-Dee (1993) reported that five *B. napus* cultivars were very poor hosts for *P. scribneri,* and Nicol (1996) found resistance to *P. thornei* within *B. napus* lines. Webb (1993) found that canola varieties were poor hosts for *Pratylenchus,* observing that susceptibility was independent of the potential for nematode damage to the crop. This suggested variation in both tolerance and resistance to the nematode within the species. However, Vanstone *et al.* (1993) found that nine *Brassica* varieties allowed reproduction of *P. neglectus* to levels as high as susceptible wheat lines. The dynamics of the relationship between *Pratylenchus* spp. and *B. napus* lines seems to be dependent upon the particular combination of nematode species and canola variety.

Controlling Pratylenchus *spp. in the field*

The use of nematicides in broad-acre cereal farming in southern Australia is not practical due to the prohibitive cost and the health risks associated with the handling of such chemicals (Clewett and Thompson, 1985). Biological control (Jatala, 1986) offers limited potential for control of *Pratylenchus* spp. at present, due to difficulties in identifying and inoculating field soils with suitable control agents. Until more cereal crops with improved resistance and tolerance to the nematode are released, the industry is reliant upon cultural practices to keep root lesion nematode numbers in check (Farsi *et al.*, 1995). While fertiliser applications have been shown to reduce nematode numbers (Kimpinski *et al.*, 1976; Vanstone, 1991), the primary means of control within the cereal farming system is through cultivation (mechanically damaging nematodes in the soil; Taylor and Vanstone, 1993) and crop rotation (Taylor *et al.*, 1995). However, environmentally sustainable management practices seek to reduce soil cultivation (minimum tillage) to conserve soil structure and moisture and reduce erosion. Reduced cultivation practices limit the control of nematode numbers through physical abrasion and consequently, crop rotation has become the primary means for control.

By rotating susceptible crops with poor host plants which grow well in heavily infested soils (tolerance) without allowing the nematode to multiply (resistance), economic return can be maintained whilst reducing soil populations of nematodes. A number of studies comparing yields of wheat grown after wheat with yields after alternative crops support the use of rotations for nematode control (McEwen *et al.*, 1989; Purvis and Jones, 1990; Mead, 1992).

The roots of the rotation crop are likely to occupy different regions of the soil, tapping resources previously unused in the monoculture, contributing to the cycling of soil nutrients throughout the profile, and increasing soil microporation. Wheat roots can then follow the old root channels, gaining access to alternative areas of soil water and nutrients (Angus *et al.*, 1991). However, perhaps the greatest advantage is the potential for a 'break' in the disease cycle, leading to significant decreases in pathogen numbers in the soil, thus improving the early vigour of the following crop (Mead, 1992).

The poor adaptation of the majority of the alternative agricultural crop species to the southern Australian cropping regions restricts the number of crops that can be economically produced. Without a variety of appropriate resistant crops, the usefulness of rotations to control soil pests and diseases is limited. Comprehensive control of the root lesion nematode within the southern Australian cereal production industry is therefore reliant on the development and integration of new or improved disease break crops, to disrupt the cycle of pests and pathogens within the rotation.

2.3 BRASSICAS AS DISEASE BREAK CROPS

2.3.1 *Disease break*

*Brassica*s have been successful as break crops, leading to greater increases in wheat yield than crops such as linseed or oats (McEwen *et al.*, 1989; Christen *et al.*, 1992). Angus *et al.* (1991) observed that the rotational affect of both canola (*B. napus*) and mustard (*B. juncea*) led to greater yield improvements in following wheat crops than linseed, oats or a second crop of wheat. Kirkegaard *et al.* (1994) also observed a strong effect of *Brassica* within the wheat rotation, leading to 12-48% increases in wheat biomass after a canola crop. The mode of action for this improvement was unknown, but a range of factors may have been involved. Field studies have shown that improvements in wheat following *Brassica* crops were not due to enhanced nutrient or water status, suggesting instead that the advantage was associated with a reduction in soil-borne disease (Angus *et al.*, 1991). The most significant contribution of the *Brassica* crop to the cereal rotation is likely to be its effectiveness as a disease break.

2.3.2 *Active suppression by* Brassica

The disease break associated with a *Brassica* crop is augmented by the release of chemicals which suppress organisms as the vegetative tissues break down in the soil (Brown and Morra, 1997). This allelopathic phenomenon is due to the presence of glucosinolates[1], which are produced and stored within *Brassica* tissues, breaking down to release biocidal isothiocyanates during tissue damage or senescence. The biocidal potential of the tissues has been used in agriculture by incorporation of crops into the soil (green manuring), reducing populations of a range of organisms including nematodes, fungi, bacteria, insects and weed seeds (Brown and Morra, 1997). Different plant tissues have variable toxicity, depending on the organism and the environment of exposure. The seed meal of many species is considered particularly effective, and has been used to control a wide variety of nematodes (Akhtar and Alam, 1991; Rathore, 1994; Tiyagi and Alam, 1995; Shukla and Haseeb, 1996), *Aphanomyces* fungi (Smolinska *et al.*, 1997), weeds (Brown and Morra, 1995) and insect larvae (Elberson *et al.*, 1996). However, vegetative tissues have also been reported to have strong nematicidal effects (Akhtar and Alam, 1991; Owino *et al.*, 1993; Jing and Halbrendt, 1994; Kanwar and Bhatti, 1994; Walker, 1997).

Due to the non-specificity of the molecules released from degrading *Brassica* tissues (Kroll *et al.*, 1994), beneficial soil organisms are also likely to be influenced by degrading *Brassica* crops. Symbiotic organisms, such as mycorrhizal fungi, nitrogen fixing bacteria, and potential biological control agents, such as predacious nematodes and nematode-trapping fungi (Jatala, 1986), are likely to be adversely affected. Schreiner and Koide (1993) observed that *B. nigra* and *B. kaber* roots inhibited germination of the spores of vesicular-arbuscular mycorrhizal fungi. While this review of the literature revealed no studies examining the effect of *Brassica* tissues or isothiocyanates upon nitrogen fixing bacteria such as *Rhizobia,* isothiocyanates have been observed to be inhibitory to many bacteria (Brabban and Edwards, 1995). *Brassica* chemicals have also been observed to inhibit seed germination (Bialy *et al.*, 1990; Kirkegaard *et al.*, 1994; Brown and Morra, 1995; Cheam, 1996), and it has been observed that the emergence of cereal crops has been reduced by the preceding *Brassica* crop in the rotation. Vera *et al.* (1987) and Purvis (1990) discussed the potential for canola stubbles to reduce cereal germination in the field. However, Kirkegaard *et al.* (1994) found that reductions in plant emergence following *Brassica* spp. were more than offset by the subsequent stimulation

[1] The biochemistry of the glucosinolates will be discussed in detail later in this review.

of vegetative growth, leading to biomass production and yield comparable to other rotation crops tested. While the preceding *Brassica* crop may have initially reduced the germination of the cereal, the emerging crop more than made up for such a loss due to the associated reduction in soil disease.

2.4 ALLELOPATHY

2.4.1 *Allelopathy*

Allelopathy can be defined as the harmful effect of one organism on another owing to the release of secondary metabolic products (allelochemicals) into the environment (Halbrendt, 1996). Detrimental effects on many organisms have been observed due to the release of allelochemicals from plant tissues (Bennett and Wallsgrove, 1994). Plant allelochemicals may be leached directly from growing crops, liberated during tissue decomposition or synthesised by microorganisms utilising dead plant material (Purvis, 1990). Within the cereal rotation, retained *Brassica* stubbles have been shown to have allelopathic effects upon populations of soil pests and pathogens, and upon following crops (Angus *et al.*, 1991; Purvis and Jones, 1990, respectively).

2.4.2 *Secondary metabolism*

Plant growth and development involves the two distinct processes of:

- cellular growth (division and enlargement), and
- development (differentiation and specialisation) (Herms and Mattson, 1992).

The physiological processes involved in both may be divided into primary metabolism, supporting morphological growth and differentiation, and secondary metabolism, producing a wide variety of compounds with distinct purposes other than primary growth and development (Herms and Mattson, 1992). Secondary metabolites are often key components of plant defence mechanisms. Vascular plants produce an array of defence-related secondary chemicals, including complex organic acids, simple phenols, phenolics, phenylacetic acids, coumarins, quinones, flavanoids, tannins, terpenoids, alkaloids, steroids, cyanogenic glycosides and the aforementioned glucosinolates (Purvis and Jones, 1990). The presence of these compounds within plant tissues may decrease the ability of pests and pathogens to identify, feed or reproduce on the plant, thus providing an advantage for the plant as it grows and develops (Bennett and Wallsgrove, 1994).

The production of defensive secondary metabolites is often physiologically expensive, and may compete with primary metabolism for resources (Siemens and Mitchellolds, 1998). Kakes (1989) observed a negative relationship between tissue levels of cyanogenic glycosides and the number of flowers produced by individuals within a population of *Trifolium repens*. Cyanogenic individuals were protected against feeding by snails, offsetting the disadvantages associated with the decreased number of flowers in environments where snail damage was severe. Because plants have limited access to resources, a balance must be struck between primary processes (growth and development/photosynthetic potential) and secondary processes such as defence. In periods of high vegetative growth, secondary metabolism is likely to suffer, limited by the drain of resources and energy required for vegetative production (Herms and Mattson, 1992). This was illustrated by Clossais-Besnard and Larher (1991), who found that as the rate of vegetative growth increased, glucosinolate concentrations within *Brassica* tissues declined due to dilution within the expanding tissues. A balance must be struck to maximise the vegetative growth and development of the plant whilst maintaining the production of secondary metabolites for defence.

2.4.3 *Allelopathy in the* Brassica

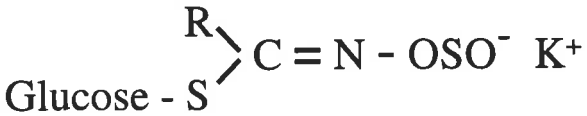
Glucosinolates are considered to be the primary allelochemicals responsible for the biocidal nature of *Brassica* tissues (Brown and Morra, 1997). However, *Brassica* tissues are known to produce other defence-related secondary metabolites, including toxic amino acid derivatives such as s-methyl cysteine sulphoxide (Wynne Griffiths *et al.*, 1994), fatty acid derivatives such as alkanones, alkenals and alkanoic acids (Bradow, 1991) and derivatives of glucosinolates, including carbinols (Beier, 1996). These molecules may supplement the impact of the glucosinolate within the *Brassica*, and must not be discounted when examining the allelopathic nature of the tissues.

2.5 GLUCOSINOLATES

2.5.1 *Introduction*

Glucosinolates are found in dicotylendonous plants, most particularly within order Capparales (Fenwick *et al.*, 1994). They are abundant within family Cruciferae, and are found in every member of the genus *Brassica.* Glucosinolates, stored in the cell vacuoles of most plant tissues, first gained recognition due to the products of their degradation, the mustard oils, which are responsible for the characteristic taste of many of the kohl and mustard crops. Although mustard oils were extracted and purified by distillation in the early 1600s, the first of the glucosinolates, sinalbin, was not purified until 1830 (Fenwick *et al.*, 1994). The general structure of the glucosinolates (Figure 2.1) was formulated in 1897 after study of its degradation products, and confirmed by X-ray crystallography in 1963 (Waser and Watson, 1963).

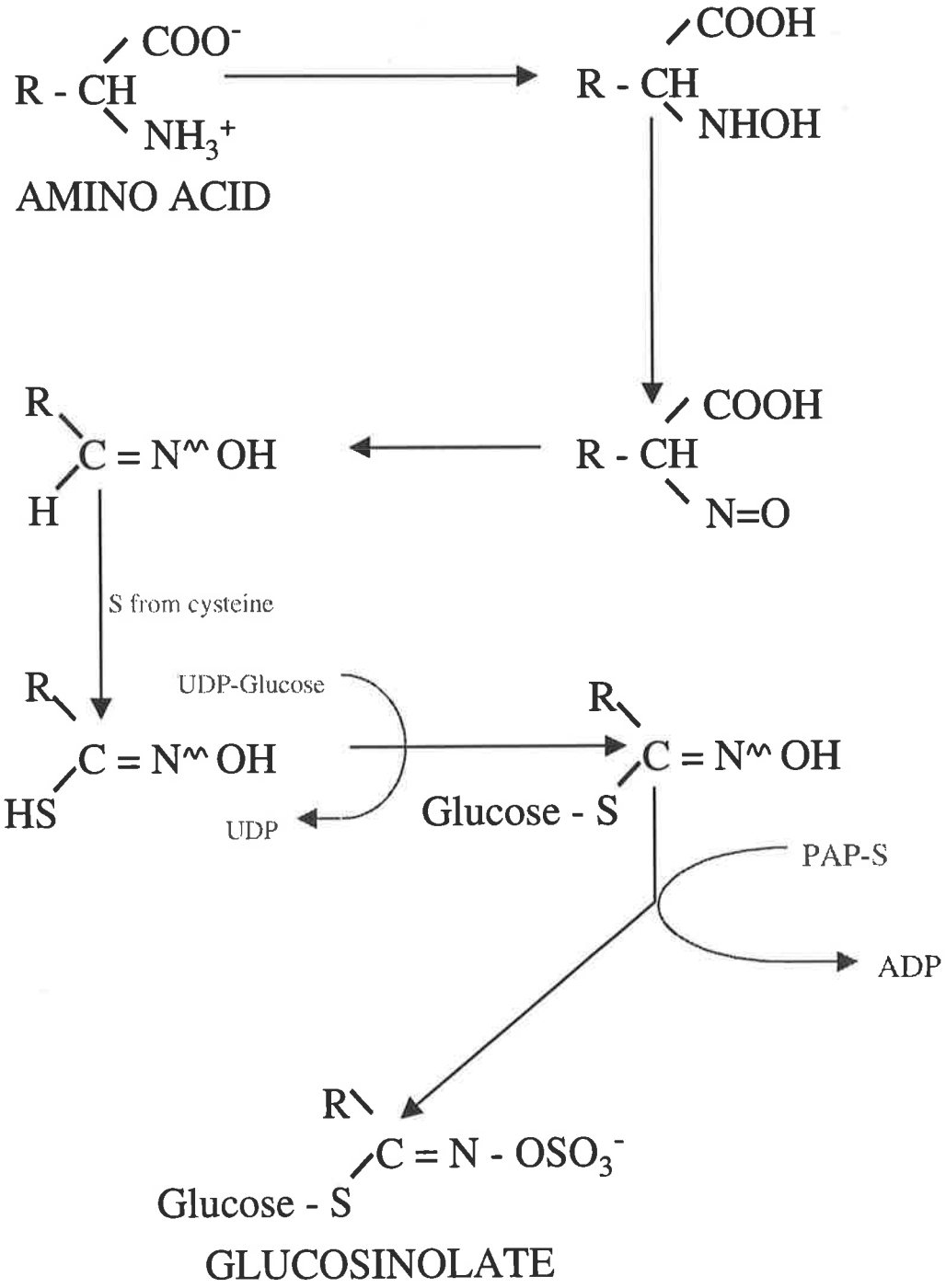
Figure 2.1: Generalised structure of a glucosinolate, invariably isolated as a potassium salt (K^+). The R~group is variable, characterising the different glucosinolates. (Source: Underhill, 1980).



Extensive research into the biosynthesis of glucosinolates has revealed that they are derived from amino acids, using the central carbon, the nitrogen atom and the side chain (R~group) of the original molecule (Underhill *et al.*, 1962). A generalised mechanism for the biosynthesis of glucosinolates appears in Figure 2.2, showing the complexity of biosynthesis. Three major classes of glucosinolates have been characterised, each originating from different amino acids; the aliphatic (synthesised from methionine), the indolyl (from tryptophan) and the aralkyl (from phenylalanine). Manipulation and elongation of the side chain results in a large array of glucosinolates (Magrath *et al.*, 1994), with over 90 different glucosinolates characterised within the three classes (Daxenbichler *et al.*, 1991). Glucosinolates within classes share similar properties due to the chemical nature of their amino-derived side chain.

A single plant species will produce only a few glucosinolates, the relative amounts varying between the different plant tissues (Fenwick *et al.*, 1994). *Brassica napus* (canola) tissues have been reported to contain fifteen glucosinolates (Sang *et al.*, 1984), predominantly aliphatic glucosinolates in the seed, indolyl in the leaves, and aralkyl in the roots (Josefsson, 1967).

Figure 2.2: Generalised mechanism for the biosynthesis of glucosinolates from an amino acid (after Larsen, 1981). Three different amino acids (methionine, tryptophan and phenylalanine) are used to produce aliphatic, indolyl and aralkyl glucosinolates, respectively.



2.5.2 *Glucosinolates and plant development*

Both total and individual glucosinolate levels vary in the tissues as the plant grows and develops (Clossais-Besnard and Larher, 1991; Fieldsend and Milford, 1994). High levels are normally found in the seed and cotyledon, providing early protection for seedlings against grazing pressure. As the seedling develops, total glucosinolate concentrations within the leaf tissues drop, as glucosinolates become diluted throughout the increasing tissue bulk (Clossais-Besnard and Larher, 1991; Iwasa *et al.*, 1996). However, as the plant begins to mature and vegetative growth slows, leaf glucosinolate levels often rise, peaking just prior to flowering (Giamoustaris *et al.*, 1994). This implies a constant production of glucosinolate during the vegetative cycle, with tissue concentrations diluted in periods of high growth. Once the plant begins to flower, leaf glucosinolate levels drop, being re-mobilised from vegetative tissues to accumulate in the silique for packaging into the seed (Qinzheng *et al.*, 1991). While little information is available regarding the production of glucosinolates within the roots, Clossais-Besnard and Larher (1991) observed that root levels were low relative to other vegetative tissues, increasing only during the later period of vegetative growth.

Glucosinolates (both totals and individuals making up the profile) may fluctuate as tissue grow and develop (Poulton *et al.*, 1993; Fieldsend and Milford, 1994) and can also vary within a 24 hour period (Rosa, 1997). As their levels are also responsive to environmental variation, such as temperature, water availability and soil nutrition (MacLeod and Pikk, 1979), great care must be taken to standardise experimental conditions when studying glucosinolates *in vivo*.

2.5.3 *Glucosinolates and plant defence*

It has been suggested that glucosinolates act as a glucose and sulphate reservoir for the plant, and that the indolyl glucosinolates serve as intermediaries in the biosynthesis of plant auxins, including indole acetic acid (Fenwick *et al.*, 1994). However, the major function of the glucosinolates is related to defence against grazing pests, with parallels made to the cyanogenic glycosides (defence-related secondary metabolites) (Bennett and Wallsgrove, 1994). Variation in glucosinolate levels within genetically similar plants can cause significant alterations in plant relations to both specialist and generalist pests (Blight *et al.*, 1995; Giamoustaris and Mithen, 1995). The relationship between tissue glucosinolates and plant defence against a broad range of pests and diseases has been reviewed by Brown and Morra (1997).

2.5.4 *Enzymatic degradation of glucosinolates*

Glucosinolates are generally stable and relatively non-toxic molecules despite their relationship to plant resistance (Lazzeri *et al.*, 1993; Poulton *et al.*, 1993). Upon tissue damage, the glucosinolate comes into contact with an enzyme, myrosinase (Section 2.5.5) which cleaves the glucose unit from the molecule producing an unstable aglucone which then spontaneously re-arranges to produce a wide range of biologically active products (Figure 2.3). The products of glucosinolate degradation depend upon the chemical nature of the R~group and the environment under which the reaction takes place (Underhill, 1980). The primary product under normal cellular conditions is the isothiocyanate (Brown and Morra, 1997), although other biologically active molecules include thiocyanates (predominating at high pH) and nitriles (predominating at lower pH and in the presence of ferrous ions) can result (Larsen, 1981). Aliphatic glucosinolates containing R~groups with a hydroxyl functional group produce isothiocyanates that are unstable, and rearrange spontaneously to form oxazolidinethiones which limited the use of seed mean as an stock feed due to their anti-nutritional qualities (Fenwick *et al.*, 1994).

2.5.5 *Myrosinase*

Myrosinase, a thioglucosidase, is responsible for the cleavage of glucosinolates within the *Brassica* tissues (Poulton *et al.*, 1993). Activated by ascorbate, it cleaves the glucose moiety from the glucosinolate at the sulphur linkage, destabilising the structure of the molecule, which then spontaneously rearranges to produce molecules such as the isothiocyanate. The enzyme has several isoforms, differing in electrophoretic mobility and response to ascorbate (Xue *et al.*, 1992). Immunosorbency studies have confirmed that myrosinase is compartmentalised within membrane bound "myrosin grains" found within discrete cells (myrosin cells) scattered diffusely throughout the tissues of the plant (Thangstad *et al.*, 1991; Bones and Rossiter, 1996; Plate 2.3). However, myrosinase activity has also been associated with cells lacking these myrosin grains (Iverson *et al.*, 1979). Myrosinase may also be present within glucosinolate containing cells, bound to the plasma membrane, and separated from the glucosinolates and ascorbate (which activates the enzyme), which are located in the vacuole (Luthy and Matile; 1984; Bones and Rossiter, 1996; Figure 2.4).

Plate 2.3: The distribution of myrosinase with *Brassica* root tissues. After Bones *et al.* (1991).

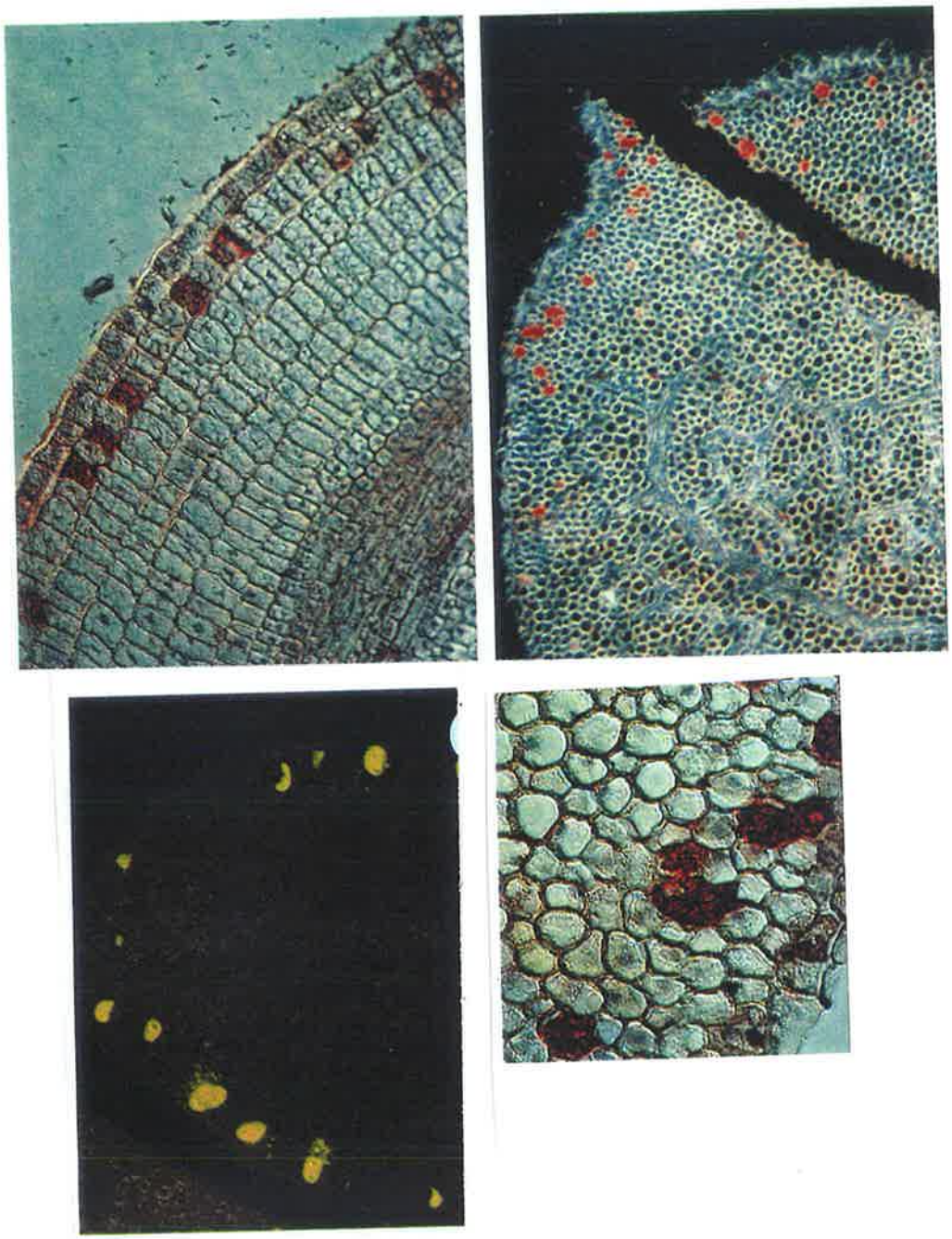


Figure 2.3 Enzyme induced hydrolysis of glucosinolates by myrosinase. The unstable aglucone spontaneously degrades, liberating a sulfate molecule and producing nitrile, thiocyanate and isothiocyanate, depending on the chemical environment (after Larsen, 1981).

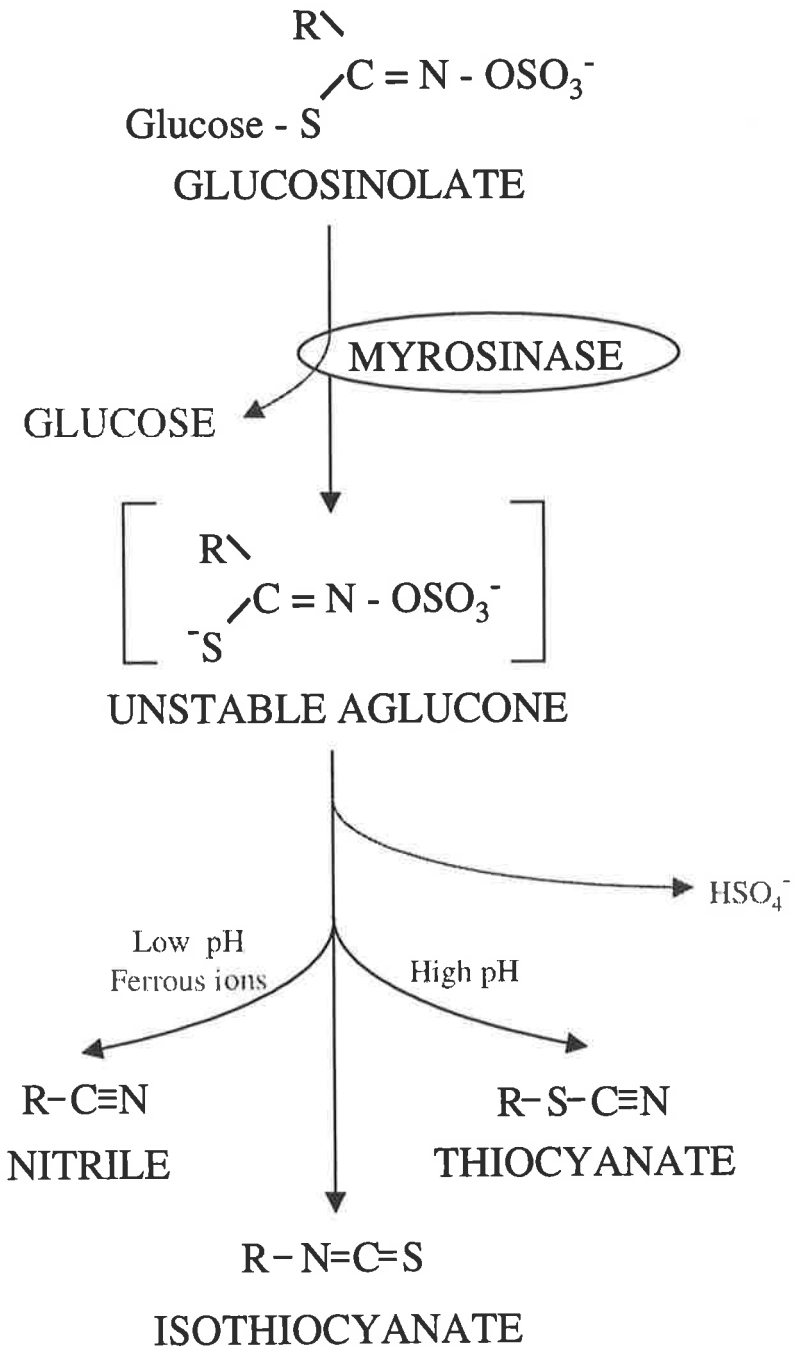
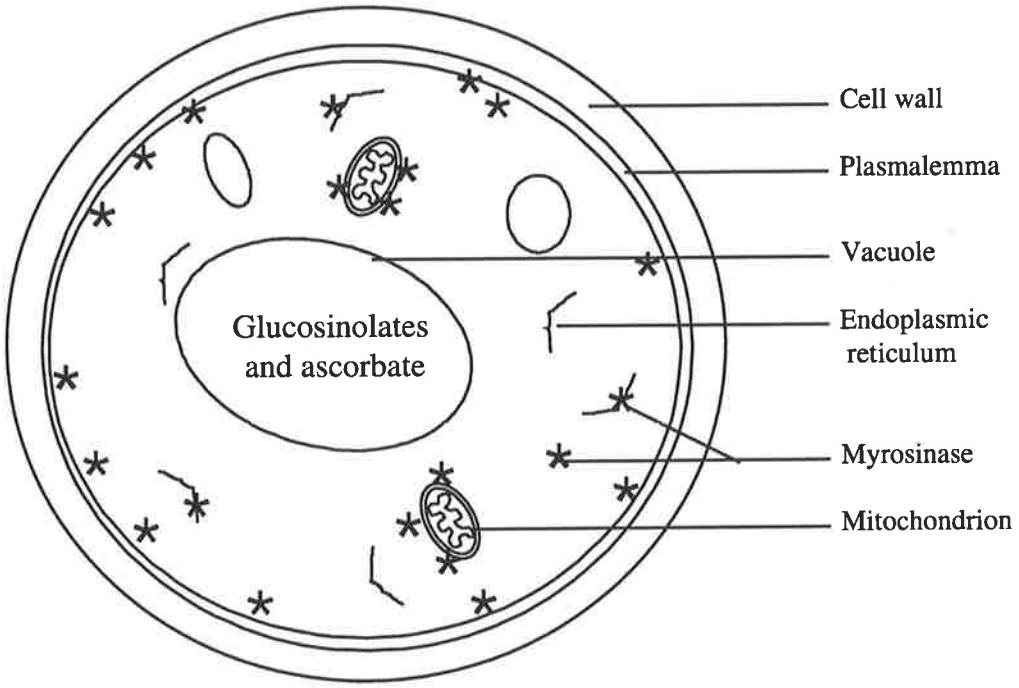


Figure 2.4: The 'myrosinase bomb'. Distribution of the glucosinolate, myrosinase and myrosinase activator, ascorbate within a plant cell (after Luthy and Matile; 1984) .



The location of myrosinase within the tissue is instrumental to the control of the glucosinolate defence system (Poulton *et al.*, 1993). As the products of glucosinolate cleavage have been shown to be active herbicides (Brown and Morra, 1995), premature exposure of glucosinolates to the enzyme is likely to release products which can damage the surrounding tissues of the plant. The location of the enzyme and its substrate, whether in separate cells (Bones and Rossiter, 1996), or within subcellular compartments (Luthy and Matile, 1984), ensures that the glucosinolate and the enzyme do not come into contact unless the tissue undergoes extensive physical damage. The mode of action of the glucosinolate defence system therefore seems to be based upon the release of toxic molecules after grazing damage, acting as a deterrent to further feeding, rather in defence against microscopic organisms. However, as the tissues are thought to liberate toxic molecules into the soil as they degrade, the system may have a role in soil fumigation, reducing pests and diseases in the soil in readiness for the next plant generation (Kirkegaard *et al.*, 1993; Brown and Morra, 1997). This is further supported by observations of myrosinase activity in a number of soil bacteria, which may use the thioglucosidase to liberate the glucose moiety from glucosinolates in the soil (Brabban and Edwards, 1994; Palop *et al.*, 1995).

2.5.6 *Toxicity of isothiocyanates*

Isothiocyanates are toxic to a broad range of organisms (Mithen *et al.*, 1986; Brabban and Donkin *et al.*, 1995; Edwards, 1995; McCaffrey *et al.*, 1995; Brown and Morra, 1996; Mojtahedi and Santo, 1996). The role of the isothiocyanate as a biocide was discussed by Bennett and Wallsgrove (1994). Isothiocyanates act to denature protein by interacting with thiol linkages on amino acids within the polypeptide chain, disrupting the protein structure (Kawakishi *et al.*, 1983; Kawakishi and Kaneko, 1985; Kroll *et al.*, 1994). An example of such an interaction is presented in Figure 2.5.

Figure 2.5: The reaction between protein and isothiocyanate leading to interference with the structure of the protein. (after Kroll *et al.*, 1994)

The toxic qualities of the isothiocyanates are due to the activity of the cyanide and thiol linkage making up the 'thiocyanate' functional group. However, parameters of the R~group, such as chain length and the presence of different chemical groups, alter the chemical properties of the molecule, changing properties such as solubility and vapour pressure. To illustrate, 2-propenyl isothiocyanate (molecular weight of 99.16) is hydrophilic and a gas at room temperature, whereas 2-phenylethyl isothiocyanate (molecular weight of 163.2) is hydrophobic and a liquid. It is likely that these molecules will impact differently in the soil environment due to their differences in chemistry. Shaaya and Desmarchelier (1995) examined the sorption of a series of isothiocyanates on wheat residues, finding that isothiocyanates with small R~groups were bound and inactivated by the tissue. Matthiesson *et al.* (1996) found that a large percentage of purified methyl isothiocyanate, added to soil as a fumigant, was adsorbed and inactivated in the soil environment. The degree of sorption was related to the soil type and structure, with loamy soils adsorbing far more isothiocyanate than sandy soils.

Due to variation in the structure of the side chain (R~group), different isothiocyanates show varying toxicity to various pathogens (Brown and Morra, 1997), demonstrated *in vitro* against *Meloidogyne* and *Pratylenchus* nematodes (Mojtahedi and Santo, 1996), *Leptosphaeria maculans* fungus (Mithen *et al.*, 1986), germinating cereal seeds (Bialy *et al.*, 1990), and the

eggs of the black vine weevil (Borek *et al.*, 1995). Mithen *et al.* (1986) stated that isothiocyanate produced from sinigrin (2-propenyl or allyl glucosinolate) was the most toxic isothiocyanate to various organisms *in vitro*. Industry has capitalised on the biocidal nature of isothiocyanates, producing nematicides based upon methyl isothiocyanate such as Vapam and Metham Sodium (Goldwasser *et al.*, 1995; Saeed *et al.*, 1996; Taylor *et al.*, 1996), and a weapon (mustard gas) based on 2-propenyl (allyl) isothiocyanate.

Thus the characteristics of the R~group influence not only the impact of the molecule on a particular organism, but also the efficacy of the isothiocyanate under different environments. Consequently, the profiles of glucosinolates within *Brassica* tissues are likely to be critical to the biocidal potential of the tissues. The tissue glucosinolate profile varies between *Brassica* species and the accessions within them (Sang *et al.*, 1984), perhaps contributing to differences in their disease break strength under field conditions.

2.5.7 *Isothiocyanate release from* Brassica *tissues*

The effectiveness of isothiocyanates against organisms is often dose dependent (Drobnica *et al.,* 1967; Mithen *et al.*, 1986; Bialy *et al.*, 1990), and thus the level of isothiocyanate released from degrading tissues is critical in determining the potency of the tissue. The biocidal impact of *Brassica* will therefore be a function of the total glucosinolates within the tissues, the levels of tissue present in the soil, the rate of tissue breakdown and the longevity of the isothiocyanate within the soil. Consequently, the state of the tissue (whole or crushed) and soil conditions such as moisture, temperature and microbial content, which affect the speed of tissue degradation, will alter the effectiveness of the tissue (Brown and Morra, 1997). Chan and Close (1987) reported that the effect of *Brassica* tissue upon *Aphanomyces* oocytes in the field was greater after six weeks than after three weeks, suggesting that the longer period allowed a greater release of isothiocyanate into the soil. However, isothiocyanates released into soil do not persist for a long period, dissipating or being bound, inactivated or broken down after only a few days (Brown *et al.*, 1991). Maximisation of the speed of tissue degradation through residue management has the potential to improve the biocidal impact of *Brassica* residues in the soil.

2.5.8 *Increasing glucosinolate levels*

The relationship between tissue glucosinolates and plant defence suggests that the disease break associated with canola crops could be further improved by increasing the levels of glucosinolates within the tissues. The following sections discuss the potential to maximise glucosinolate levels within current varieties, and to increase the potential of current and new varieties through plant breeding.

Cultural practices

MacLeod and Pikk (1979) studied the impact of a variety of cultural practices on the total glucosinolate levels within *B. oleracea* vegetative tissues, observing that factors such as plant spacing, water availability, temperature and nutrition affected glucosinolate production. Light intensity and wavelength also influenced the levels of glucosinolates produced by *Brassica* plants (Antonious *et al.*, 1996). As glucosinolates are a secondary plant product, and are metabolically expensive (Section 2.4.2; Herms and Mattson, 1992), the health and productivity of the plant will affect glucosinolate production. Plants grown under optimal conditions are likely to contain maximum levels of glucosinolates.

Glucosinolate levels are responsive to nutrient availability, in particular to sulphur and nitrogen (Poulton *et al.*, 1993). As glucosinolates are synthesised from amino acids, any factor which limits the ability of the plant to produce amino acids will limit glucosinolate production (Underhill, 1962). Biosynthesis is reported to rely on an oversupply of precursor amino acids, which, if not required for protein synthesis, become available for glucosinolate production (Chisholm and Wetter, 1966). A slight reduction in resources for amino acid production may therefore reduce glucosinolate levels without leading to notable reduction in protein synthesis and crop yield (Josefsson, 1970). As glucosinolates contain two sulphur atoms, one from cysteine, the other from PAP-S (Figure 2.2), sulphur availability is likely to affect their synthesis. Aliphatic glucosinolates, which also require methionine for synthesis (also containing sulphur), are particularly dependent on sulphur availability. Deficiency in available sulphur, or reductions in ability of the plant to utilise available sulphur, can lead to marked decreases in total glucosinolate levels in the seed (Zhao *et al.*, 1994) and in vegetative tissues (Eaton, 1942; MacLeod and Nussbaum, 1977; Booth *et al.*, 1991). Nitrogen nutrition also influences glucosinolate levels within *Brassica* tissues, although a wide variation in response to nitrogen has been observed (Zhao *et al.*, 1994). Josefsson (1970) showed a

reduction in glucosinolate levels in response to an increase in nitrogen, supporting the theory that levels may be diluted as nitrogen availability allows increases in plant biomass (Bilsborrow and Evans, 1995). However, Bilsborrow *et al.* (1993) observed increases in glucosinolate levels with increased nitrogen availability, perhaps due to an increase in plant health and amino acid production. It has been suggested that a significant interaction exists between sulphur and nitrogen, and that the balance plays an important role in the regulation of total glucosinolate biosynthesis (Zhao *et al.*, 1993). However, as side chain manipulation is controlled by a different system to total glucosinolate production (Magrath *et al.*, 1993), alteration in total glucosinolate levels is unlikely to influence levels of individual glucosinolates relative to each other.

Insect attack and mechanical damage simulating insect attack can lead to systemic changes in glucosinolate levels within the plant. Tissue damage normally increases total glucosinolate levels, primarily due to an increase in the levels of indolyl glucosinolates (Birch *et al.*, 1992; Wynne-Griffiths *et al.*, 1994). While chemical changes following herbivorous damage are often associated with the development of induced resistance (Karban, 1987), Birch *et al.* (1992) and Bodnaryk (1992) observed that increases in glucosinolates were not correlated with improvements in resistance to further attack. Little information is available regarding the function of the increased indolyl levels, but as they can act as intermediaries for auxin synthesis (Poulton *et al.*, 1993) their increase may be indicative of increased auxin production within the damaged tissue. The systemic nature of damage-induced increases in glucosinolates suggests the release of a diffusable element which stimulates glucosinolate production (Bodnaryk, 1992). Two such elements, considered to be involved in the development of systemic acquired resistance, methyl jasmonate and salicylic acid, have been observed to increase glucosinolate levels within vegetative tissues. Kiddle *et al.* (1994) observed that foliar applications of salicylic acid increased production of 2-phenylethyl (2-PE) glucosinolate (aralkyl) within leaf tissues while Doughty *et al.* (1995) found that leaves exposed to methyl jasmonate accumulated indolyl glucosinolates. Consequently, foliar application of methyl jasmonate or salicylic acid may provide a means to increase tissue glucosinolate concentrations before the tissue is incorporated into soil as a green manure.

Genetic manipulation

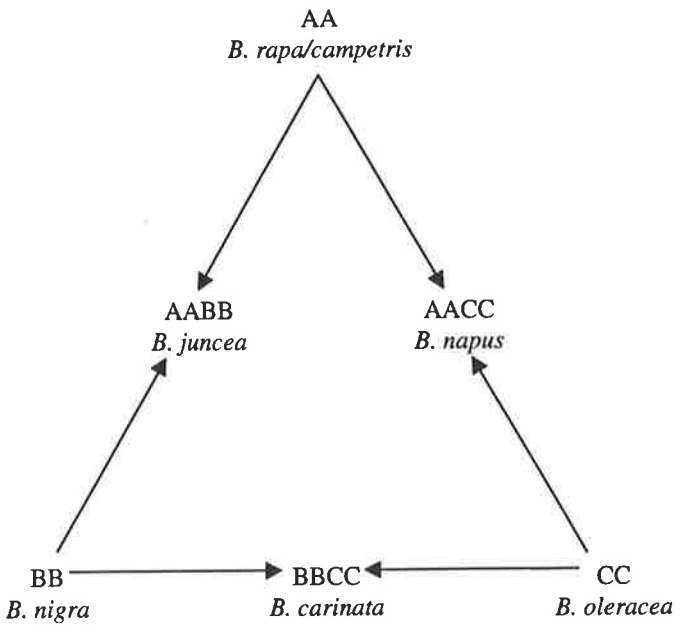
While glucosinolate levels in existing lines can be maximised by providing the most appropriate environment for production, genetic limitations will eventually restrict the levels attainable. The potential to manipulate glucosinolate levels genetically must therefore be explored. Most efforts to manipulate the levels of glucosinolates within *Brassica* crops have sought to reduce total seed levels (Poulton and Moller, 1993). However, recent efforts have aimed to manipulate levels of individual glucosinolates within vegetative tissues in an effort to explore the relationship between the glucosinolates and the pest and disease relations of the plant (Mithen, 1992; Giamoustaris *et al.*, 1994; Mithen *et al.*, 1995).

The biosynthetic pathway for glucosinolate production is controlled by many enzymatic steps (Figure 2.2), and thus the genetic regulation of total glucosinolate production is complex (Rucker and Robbelen, 1994; Wallsgrove *et al.*, 1995). Variation in the efficiency of these enzymes is likely to lead to variation in total glucosinolate levels between different genotypes (Rucker and Robbelen, 1994). However, as mentioned in Section 2.5.2, individual glucosinolates are under different genetic control from total glucosinolate production, being produced through side chain manipulation of already synthesised glucosinolate precursors (Magrath *et al.*, 1993; Rucker and Robbelen, 1994). Should one glucosinolate be found to be of significance to the *Brassica* disease break, it may be possible to increase its levels without altering the total amount of glucosinolates in the tissues.

Glucosinolate production within the different tissues of the plant also appear to be under separate genetic control, as reductions in seed levels have not led to significant alterations in the levels in roots or leaves (Porter *et al.*, 1991; Mithen, 1992; Fieldsend and Milford, 1994). Lines bred to contain low seed glucosinolate levels ('double zero' canola lines) appear no more susceptible to pests and diseases than previously grown high glucosinolate ('single zero') lines (Rawlinson *et al.*, 1989; Inglis *et al.*, 1992). Also, many types of glucosinolates found within vegetative tissues are not present in seed tissues (Sang *et al.*, 1984), suggesting tissue specific genetic control of the different glucosinolates produced by the plant. In the Australian cereal cropping system, only the root tissues of a canola crop release glucosinolates within the soil, and therefore efforts to improve the disease break of the crop must target the root tissues. As distinct tissue-based genetic regulation is apparent, it should be possible to manipulate the root glucosinolate levels without altering the amounts in the seed, thus maintaining its quality.

Brassica napus is thought to have developed by natural hybridisation between *B. rapa* and *B. oleracea* (Figure 2.6; U, 1935). More recent cytogenetic examinations have suggested that the species may have originated from a small number of crosses, implying a limited genetic base for the improvement of the species (Prakash and Hinata, 1980; Thormann *et al.*, 1994). The introduction of desirable traits into *B. napus* varieties has therefore been performed through hybridisation with closely related species (Somda, 1995), or through the re-synthesis of *B. napus* through hybridisation of *B. rapa* and *B. oleracea* genotypes exhibiting the desired trait (Chen and Heneen, 1989).

Figure 2.6: Cytogenetic relationship between the major *Brassica* species (after U, 1935).



The malleability of the glucosinolate production system presents a good opportunity to breeders, allowing the potential to manipulate glucosinolate profiles of a particular tissue without adversely influencing the other tissues. Great potential to manipulate the levels of different glucosinolates within *Brassica* tissues via molecular avenues exists. Many of the crucial enzymes of the glucosinolate system are currently being sequenced and cloned (Bones and Rossiter, 1996), and transgenic *Brassica* plants are routinely produced through use of *Agrobacterium* and tissue culture (Knutzon *et al.*, 1992). Transgenic *Brassica* plants with reduced seed glucosinolate levels have already been produced (Lefebvre, 1993). However, limited scope exists for the application of molecular technologies to improve the crop in the short term.

Historically, however, Australian canola breeding programs have sought to improve the crop through pedigree selection whilst maintaining the hybrid vigour of the population (Buzza, 1995). As no selection regime targeting the levels of glucosinolates within canola roots has been employed, the variation inherent to the species should still be present within the varieties available today. It may therefore be possible to capitalise on variation already present within *B. napus* or closely related species, providing a means to manipulate root glucosinolates within a population through traditional intercrossing and selection techniques. Such avenues should be explored before committing resources to direct molecular intervention.

CHAPTER 3

GENERAL METHODS

3.1 SOURCE OF NEMATODES

Cultures of *Pratylenchus neglectus* were required for the glasshouse and laboratory studies described in Sections 3.3 and 3.4. These nematodes were supplied by Dr. Vivien Vanstone, Department of Plant Science, University of Adelaide. Nematodes were grown aseptically on carrot root pieces using the method of Nicol (1996), and extracted using the mister technique (Southey, 1986). A detailed description of the culturing and extraction technique is presented in Nicol and Vanstone (1993).

3.2 SOIL

The soil used for tissue toxicity (Section 3.3) and susceptibility (Section 3.4) studies was the Waite version of standard University of California (UC) soil mix, consisting of 400L of coarse washed sand which was steam treated at 100°C for 30 minutes, before being mixed with eight cubic feet of Euroturf® peatmoss. Once cooled to 80°C, 700g calcium hydroxide, 480g calcium carbonate and 600g Nitrophoska® (N-P-K, 15-4-12) was mixed in, resulting in a soil pH of approximately 6.8. The soil was passed through a 3mm sieve to remove stones and agglomerations of peat.

3.3 TISSUE TOXICITY TRIALS

Trials examining the toxicity of *Brassica* tissues to *P. neglectus* were a modification of techniques used to assess chemical nematicides (McBeth and Bergeson, 1953; Taylor *et al.*, 1957), with dehydrated *Brassica* tissues substituted for the chemical.

3.3.1 *Tissue growth, preparation and storage*

Plants were grown in standard UC soil (Section 3.2) in the glasshouse under ambient light and temperature conditions. The tissues were harvested just prior to flowering, as glucosinolate levels within vegetative tissues peak during this period (Giamoustaris *et al.*, 1993). Harvest techniques were developed to avoid loss of glucosinolates due to tissue damage (Section 2.5.4) and consequently were performed with care and haste. Plants were removed from pots, and roots were washed with running tap water over a 3mm sieve to remove as much of the

soil from the root system as possible. The roots were then cut from the tops using a sharp razor blade, placed whole in perforated crispy wrap bags (Cryovac Division®, W R Grace, Australia), which was then submerged in liquid nitrogen. The tissue was chemically stable at this temperature as no enzyme activity (and thus no glucosinolate hydrolysis) could occur. Plants harvested in the field were placed immediately on ice, washed upon return to the laboratory, bagged and immersed in liquid nitrogen.

Frozen tissues were placed into a freeze drier drum at -40°C, lyophilised and then ground into a powder using a domestic coffee grinder. While tissues remained desiccated, glucosinolate levels remain stable (Williams *et al.*, 1993). Tissues were therefore stored over anhydrous silica gel until required for toxicity trials.

3.3.2 *Microcosms*

The susceptibility trials were conducted in 30ml glass screw-top scintillation vials (Pharmaglass®), which provided a closed environment. A stock (5kg) of sterilised and sieved UC soil (Section 3.2) was collected and dried thoroughly in a forced-air dehydrator (80°C for 48 hours). Sufficient amended soil was prepared for the appropriate number of replicates (10g per vial) by physically mixing the ground tissue at the required rate into the prepared UC soil. Because the soil was dry, the tissue remained anhydrous, and thus no glucosinolate breakdown and isothiocyanate release was expected before hydration.

3.3.3 *Soil hydration and nematode inoculation*

The soil was hydrated in the vials by adding 2ml distilled water, and mixing thoroughly with a stainless steel spatula. As the mixing process could damage the nematode population (Section 2.2.2; Taylor and Vanstone, 1993), 1ml of nematode suspension (500 nematodes/ml) was added after hydration. Once infested, the containers were sealed as quickly as possible to prevent the loss of volatile isothiocyanates into the environment. The microcosms were stored in the dark at 20°C for 80 hours before the surviving nematodes were retrieved.

3.3.4 *Nematode retrieval*

Nematodes were extracted from the soil by a modified mister extraction technique (Southey, 1986). The mister extraction apparatus is shown in Figure 3.1. The soil from each vial was washed into a wire basket (50mm tall, 80mm diameter) lined with three layers of coffee filter paper (Altra Filters Inc, USA) with distilled water. The basket was placed into a funnel attached to a plastic tube which was firmly clamped to ensure that no liquid could escape. The funnel was then placed into a rack in the mister apparatus which sprayed the baskets with warmed, filtered water for ten seconds every ten minutes. The nematodes moved out of the soil due to the flooded conditions, though the filter paper and were washed down the funnel to collect in the hose above the clamp. After five days, the funnels were removed from the mister, and the nematodes collected into 50ml graduated centrifuge tubes by unclamping the hose and allowing 15ml of water to run through. Nematodes were stored at 4°C until counting.

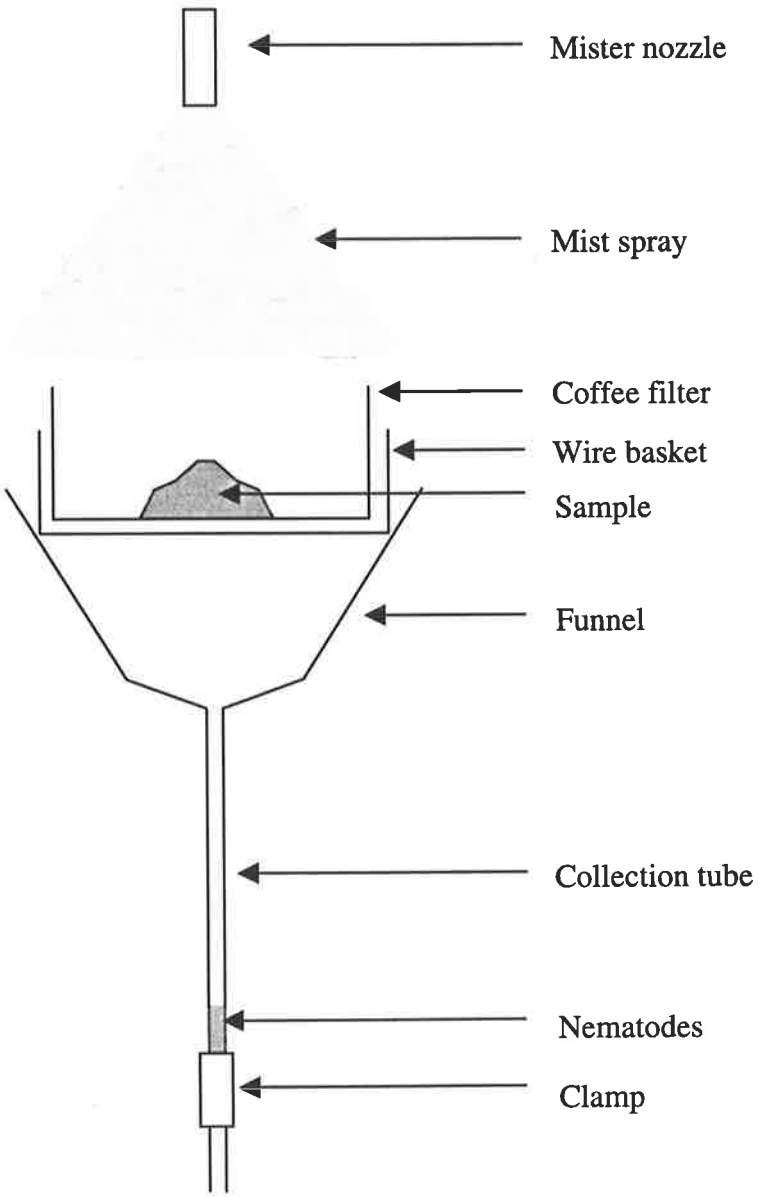
In preliminary studies, soil was returned to the mister for a further 5 days to ensure that all the living nematodes had been collected. As no significant numbers were collected, we concluded that nematodes had not been merely anaesthetised by the toxic effect of the tissues.

3.3.5 *Counting*

The nematodes were counted using a modification of the method of Doncaster (1962). The volume of nematode suspension was recorded and the solution vortexed to distribute the nematodes uniformly in the water. One millilitre of the suspension was pipetted into a counting dish, and nematode numbers counted under a dissecting microscope.

The nematicidal impact of the *Brassica* tissue was calculated relative to the nematode numbers retrieved after exposure to a wheat tissue control, to distinguish between normal loss due to the experimental environment, general tissue toxicity, and glucosinolate related toxicity.

Figure 3.1: Mister apparatus for the extraction of *Pratylenchus* nematodes from root or soil samples.



3.4 SCREENING FOR SUSCEPTIBILTY

The susceptibility of various *Brassica* accessions and related species was assessed in pot experiments in the glasshouse. Because both the nematode (Zunke, 1990a) and glucosinolate levels within *Brassica* tissues (Underhill, 1980) were responsive to environmental changes, it was vital that uniform conditions, particularly with regard to soil temperature and fertility, were maintained throughout these studies.

3.4.1 *Pot trials*

Plants were sown into soil in Hygienic Lily® 300ml (105mm tall by 60mm diameter) plastic cups with no drainage holes. Moistened soil was pressed firmly into the pots until 10mm from the lip (~350g moist, sieved UC soil, Section 3.2), and watered with 100ml of distilled water.

3.4.2 *Growing conditions*

Susceptibility trials were carried out in a glasshouse under ambient conditions. However, soil temperature was maintained at $19^{+}/-2^{\circ}C$ by placing the pots in a controlled temperature water-bath. Plants were watered with distilled water as necessary, and were fertilised after four weeks growth using 25ml of a 0.5g per litre solution of the pot plant fertiliser, Phostrogen® (Phostrogen Ltd., UK).

3.4.3 *Seed*

Seed was surface-sterilised by immersing in 70% ethanol for ten seconds, then washing in excess distilled water. Two seeds were sown directly into each pot at a depth of approximately 5mm. In this environment, the seedlings emerged after 3-5 days. After 7 days, one of the seedlings was removed at random from each pot such that only one plant per pot remained. If no germination occurred in a pot, a seedling was transplanted from another pot. The seedlings were left for another week (fourteen days after germination) to allow any root damage from transplanting to be repaired before inoculation with the nematodes.

3.4.4 *Inoculation*

Nematodes were harvested from carrot cultures (Section 3.1) by misting (Section 3.3.4). As this method of extraction was based upon the movement of live nematodes, the extract obtained contained very few eggs or dead nematodes. After extraction, nematodes were

collected on a 20μm filter, and washed with distilled water to remove bacterial contaminants. The final volume of the nematode suspension was adjusted using distilled water to produce a nematode density of 400 nematodes/ml. Two week old plants were inoculated with 3ml of the nematode suspension (1200 nematodes per pot). In order to distribute nematodes more evenly throughout the soil, three tunnels were made using a 2mm skewer to allow vertical distribution of the nematode, each tunnel receiving 1ml of the solution. The pots were watered with distilled water, to collapse the tunnels.

3.4.5 *Plant sampling*

A six week period of plant growth was the longest period possible, as many of the *Brassica* lines examined began to elongate towards flowering at this time, potentially changing the levels of glucosinolates within their vegetative tissues (Giamoustaris *et al.*, 1994). Plants were removed from the pot, and the roots washed thoroughly over a 3mm sieve. Roots were separated from the plant tops using a razor blade, chopped into 30mm sections and nematodes extracted by misting.

3.4.6 *Nematode extraction from roots*

Nematodes were extracted from the roots using a modification of the method outlined in Section 3.3.4 (Southey, 1986). The root sections were placed into a wire basket (50mm tall, 80mm diameter), which was lined with a single layer of coffee filter paper (Altra Filters Inc, USA). The mister apparatus was set up in an identical fashion as employed in Section 3.3.4 (Figure 3.1), and the period of misting and volume of elutant was also the same. Once nematodes had been collected, the root tissues were removed from the baskets and placed in a forced-air dehydrator at 80°C for at least 48 hours before being weighed to allow evaluation of nematode numbers per gram dry root.

3.4.7 *Nematode counting*

The nematodes were counted using a modification of the method of Doncaster (1962), as described in Section 3.3.5.

3.4.8 *Susceptibility assessment using stained roots*

Soil was gently washed from the roots before they were cut into 20mm long pieces with a razor blade. A known weight of roots were placed into 1.5% NaOCl for four minutes, washed

under running water and suspended in fresh distilled water for twenty minutes. After this, the roots were drained and placed in 30ml distilled water to which 1ml of fuchsin stain (3.5g acid fuchsin, 250ml acetic acid, 750ml distilled water) had been added. The suspension was placed on a heating block and allowed to boil for 60 seconds. After cooling to room temperature, roots were placed into 30ml of glycerol, acidified with five drops of 5N HCl. If required, over-stained roots were brought to the boil in the glycerol, and cooled to room temperature. Slides were made by placing roots in a drop of acidified glycerol and squashing between two glass microscope slides. Nematode numbers within the roots were counted using a light microscope, and calculated as number of nematodes per gram dry root tissue.

3.5 HPLC ANALYSIS OF GLUCOSINOLATES

Glucosinolates in the *Brassica* vegetative tissues were assessed by HPLC in a desulphated form, following the protocol of Heany *et al.* (1986).

3.5.1 *Tissue harvest, preparation and storage*

The tissues used for HPLC were harvested, lyophilised, ground and stored under identical conditions as outlined in Section 3.3.1.

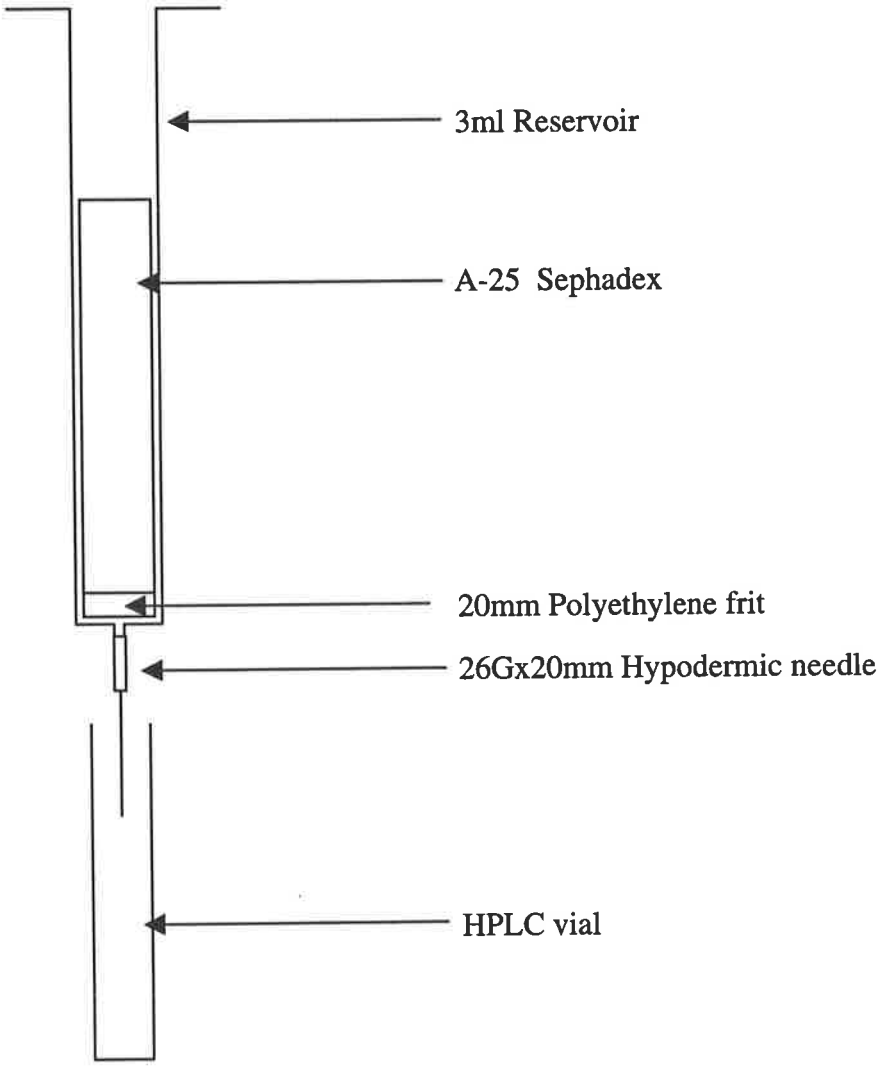
3.5.2 *Glucosinolate extraction*

Powdered tissue (0.3g) was weighed into a 50ml centrifuge tube and 10ml of hot (70°C) 70% ethanol solution added. To this, 100µl of a 0.01M solution of a glucosinolate standard, not present within the tissues, was added, the tube capped and the solution mixed by vortex. The tubes were placed in a 70°C waterbath for twenty minutes, vortexing every ten minutes. After this period, the tubes were removed from the waterbath, allowed to return to room temperature, and centrifuged at 3000g for five minutes. The supernatant was then run through a desulphation column, as prepared in Section 3.5.3.

3.5.3 *Column preparation*

The desulphation columns consisted of a plastic syringe and stainless steel needle, with a porous polyethylene frit inserted to filter the extract (Table 3.1; Figure 3.2). They were prepared by adding 0.5ml of a suspension of A-25 Sephadex® in 0.02M sodium acetate

Figure 3.2: Desulphoglucosinolate extraction apparatus.



(Section 3.4.8), and allowing the buffer to drip through beyond the frit to be discarded. The Sephadex® column was washed with two 0.5ml aliquots of distilled water.

Table 3.1: HPLC extraction apparatus.

Column apparatus	Source
Reservoir (3ml)	Analytichem, U.K.
Hypodermic needle, size 26Gx20mm	Philip Harris, U.K.
Polyethylene frit, 20µm (for 3ml reservoir), part no. 120-1033-B	Isolute Accessories, U.K

3.5.4 *Column loading*

After centrifugation, a 3ml aliquot of the supernatant from the ethanol extraction was added to the prepared column, allowing the glucosinolates to bind to the sephadex exchange resin as supernatant dripped through. The resin was washed with two 0.5ml aliquots of distilled water to remove water soluble components and two 0.5ml aliquots of 0.02M sodium acetate were allowed to run through in preparation for the addition of the sulphatase enzyme (Section 3.5.5).

3.5.5 *Glucosinolate desulphation*

A 75µl aliquot of a saturated sulphatase solution was gently layered over the resin of each loaded column, and left at room temperature for at least 8 hours. After this period, the desulphated glucosinolates were eluted from the column with two 0.5ml aliquots of distilled water, and collected in 1ml HPLC vials (Figure 3.3). Vials were stored at -20°C until required for HPLC analysis.

3.5.6 *HPLC analysis of desulphated glucosinolates*

Desulphated glucosinolates were assessed using a Waters Lambda Max LC Spectrophotometer (Model 481) and a Waters Spherisorb S5 ODS2 column (4.6mm x 250mm). The solvents employed by the two HPLC pumps, and the pump schedule are displayed in Table 3.2 and Table 3.3, respectively. Glucosinolates were identified relative to the absorbance of known standards (Table 3.4) at 229nm. and quantified with reference to an internal standard of 2-propenyl (sinigrin), benzyl glucosinolate (glucotropaelin) or para nitrophenyl sulphate (Table 3.5) depending the presence of glucosinolates within the tissues being assessed.

Figure 3.3: Water elution of desulphoglucosinolates, cleaved by sulphatase, from the A-25 Sephadex column.

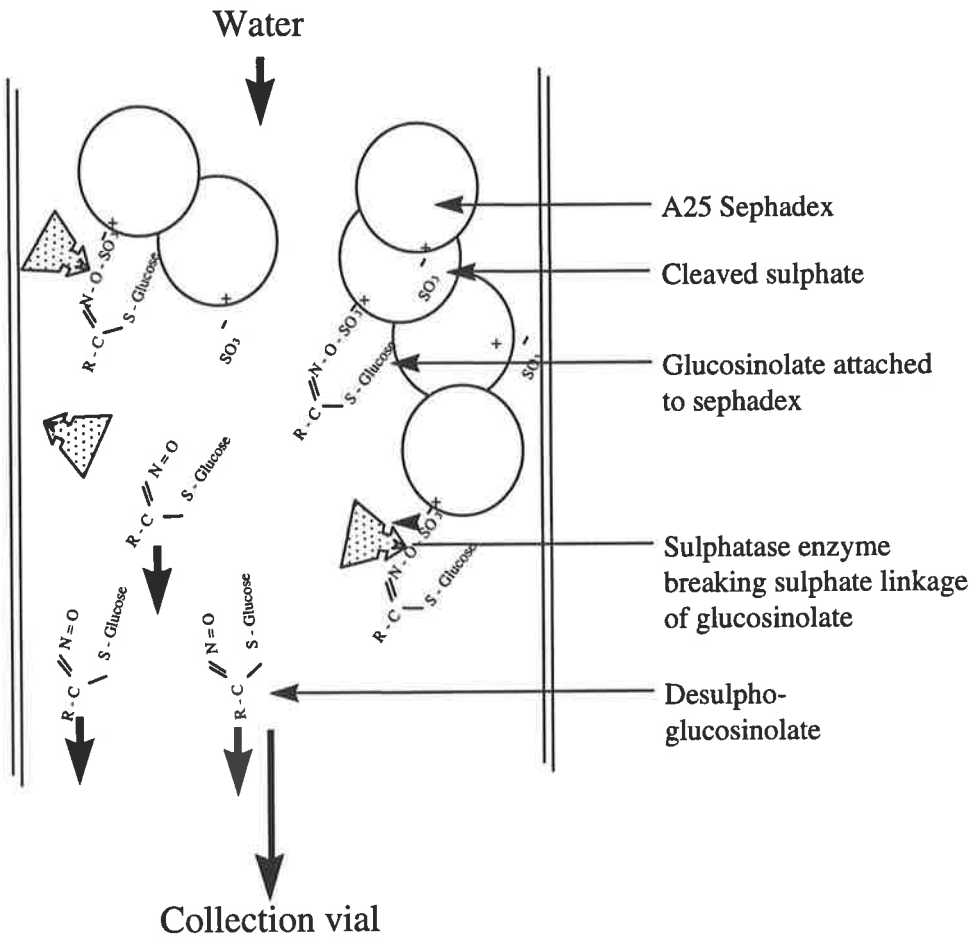


Table 3.2: HPLC solvents

A-pump solvent	100% water
B-pump solvent	60% acetonitrile

Table 3.3: HPLC pump schedule

Time (min)	Flow (ml/min)	A-pump (%)	B-pump (%)
0	1	100	0
3	1	88	12
20	1	1	99
23	1	99	1
35	1	100	0

Table 3.4 Retention time and correction factors (relative to sinigrin) for desulphoglucosinolates.

Glucosinolate	Common name	Retention time (min)	Correction factor
Artifact	-	2.3	-
pNPS (standard)	-	5.4	1.82 ($^1/_{0.549}$)
2-hydroxy-3-butenyl	Progoitren	10.9	1.09
2-propenyl (standard)	Sinigrin	11.6	1.00
4-methyl-sulphinyl-butyl	Glucoraphenin	13.4	1.07
2-hydroxy-4-pentenyl	Gluconapoleiferin	14.3	1.00
benzyl (standard)	Glucotropaelin	14.9	0.95
4-methyl-thio-butyl	Glucoerucin	15.8	1.07
3-indolyl-methyl	Glucobrassicin	16.3	0.29
4-methoxy-3-indolyl-methyl	4-methoxyglucobrassicin	16.8	0.25
2-phenylethyl	Gluconasturtiin	17.2	0.95
1-methoxy-3-indolyl-methyl	Neoglucobrassicin	18.4	0.2

Table 3.5 HPLC standards

Standard	Source	per 100ml water
Sinigrin	Sigma: S1647	0.415g (0.01M)*
Benzyl glucosinolate	Dr Mithen, John Innes Centre, Norwich, U.K	0.720g (0.01M)
p Nitrophenyl sulphate (pNPS)	Sigma: N3877	0.257g (0.01M)

* The standards employed within the studies reported in Chapters 5 and 6 were sampled from a different batch to those used in the studies reported in Chapters 7 and 8. Unfortunately, due to arithmetical inconsistencies, the concentration of sinigrin did differ between the standard solutions. Consequently, while the relative proportions of the individual glucosinolates remain the same, the glucosinolate quantification presented in Chapters 5 and 6 will not be directly comparable to those presented in Chapters 6 and 7. The expression of data as % 2-phenylethyl glucosinolate (amount of 2-phenylethyl glucosinolate relative to the total amount) was performed to accommodate these differences in quantification.

3.5.7 *Data analysis*

Glucosinolate levels were calculated from the area under the peak as μmol of glucosinolate per gram tissue relative to the area of a known amount of glucosinolate standard, using the following equation:

$$\text{Glucosinolate (}\mu\text{mol/g tissue)} = \left(\frac{\text{Peak area}}{\text{Peak Area of standard}}\right)\left(\frac{(\mu\text{l standard added}) \times (\text{correction factor})}{\text{Tisse dry weight (g)}}\right)$$

p-Nitrophenyl sulphate was used as a standard when samples contained sinigrin, as it would be impossible to distinguish between added and native sinigrin within such samples. In this case, an 'expected' area due to sinigrin was calculated based upon the linear regression between sinigrin and pNPS levels (above; Figure 5.3). The 'expected' sinigrin figure was then used to calculate glucosinolate levels within the sample.

3.5.8 *HPLC solutions*

0.02M Sodium Acetate, pH 5.0

1.2ml of acetic acid was added to 990ml distilled water and the pH adjusted to 5.0 by the addition sodium hydroxide. The total volume was made up to 1000ml with distilled water.

A-25 Sephadex®

13g of DEAE Sephadex (Sigma: A25-120) was added to 100ml of 0.02M sodium acetate and left to expand for 30 minutes. The solution was then filtered, washed with water, resuspended in excess sodium acetate, and stored at 4°C until required.

C-25 Sephadex®

13g of CM Sephadex (Sigma: C25-120) was added to 100ml of 0.02M sodium acetate and left to expand for 30 minutes. The solution was then filtered, washed with water, resuspended in excess sodium acetate, and store at 4°C until required.

Purification of sulphatase:

Sulphatase enzyme (type H1 from *Helix pomatia*) was obtained from Sigma (S-9626) and 300mg dissolve into 12ml ice cold water, to which 12ml of cold ethanol was added. After stirring to dissolve, the solution was centrifuged at 3000g for six minutes. The supernatant was retained and combined with 1.5 volumes of cold ethanol, mixed by vortexing, and returned to the centrifuge for a further six minutes at 3000g. The precipitate was dissolved in 8ml of cold distilled water, which was then dripped through four A-25 prepared columns, then four C25 columns. The elutant was collected and stored at -20°C until required for glucosinolate desulphation.

3.6 NON-DESTRUCTIVE SAMPLING OF *BRASSICA* ROOTS

The major aim of the breeding program developed within this project was to determine the potential to manipulate root levels of 2-PE glucosinolates. It was therefore necessary to harvest root tissues in a non-destructive fashion, enabling the root glucosinolate profile to be determined, while plants continued to grown on and produce seed for further study.

Plants were grown singly under ambient glasshouse conditions in 200mm diameter pots containing sterilised UC soil and watered with tapwater as required. Root tissues were harvested as the plants began to elongate prior to flowering, washed over a 3mm sieve and placed in liquid nitrogen for HPLC analysis of the glucosinolate content (Section 3.5).

The stems of plants were cut into 100mm pieces, removing all of the leaves except for the uppermost two fully expanded leaves and associated buds of each stem piece. The lower region of the stem was scored with a razor blade and dipped into Plant Cutting Powder® (Arthur Yates and Co. Pty Ltd.). Cuttings were placed in 150mm x 50mm pots with drainage holes containing a moist mix of vermiculite and perlite (1:1). The pots were placed in a basin containing 50mm water and covered with a clear plastic box to maintain plant turgidity. Roots began to appear through the drainage holes of the pots after 3-5 weeks at which time the cuttings were removed from the pots and replanted into UC soil. Plants produced new leaves and eventually elongated to flower, producing pods and seeds after a further 4-6 weeks. The development of adventitious cuttings is displayed in Plate 3.1.

Plate 3.1: The development of adventitious roots on *B. napus* cv Dunkeld stem cuttings.

CHAPTER 4

BRASSICA SPP. AS DISEASE BREAKS IN CROPPING ROTATIONS

4.1 INTRODUCTION

The disease break associated with *Brassica* crops has been a matter of much discussion at grower field days throughout the period of this project. It is an interesting subject, with as many reports supporting the canola (*B. napus*) as a disease break crop as suggesting that it exacerbates rather than reduces some disease problems. Usually, the effectiveness of the break is gauged by the performance of the following crop, ignoring factors such as seasonal and nutritional variation from one year to the next. As the disease break effect will depend on many factors including the pathogen, the variety of *Brassica* employed, the intensity of cultivation, and environmental factors such as soil nutrition, soil type, rainfall and temperature, it is not surprising that apparent results are somewhat variable.

As observed in Section 2.4.3, *Brassica* crops have been associated with reductions in soil populations of harmful organisms within established rotational practices (Angus *et al.*, 1991). However, as *Brassica* plants have also been shown to host *Pratylenchus* spp. (Webb, 1990), other factors must have played a part in the suppressive effect of the *Brassica* on *Pratylenchus* populations in the field (Parker, 1994). The release of toxic glucosinolate hydrolysis products from the tissues as they degrade in the soil is likely to contribute (Brown and Morra, 1997). As tissue glucosinolate levels are very sensitive to environmental fluctuations, particularly with reference to soil nutrition (Zhao *et al.*, 1994), it is likely that field experiments aiming to test the disease break impact of *Brassica* crops will rarely produce repeatable results. While the studies of Angus *et al.* (1991) and Kirkegaard *et al.* (1994) provided evidence that *Brassica* crops can play a positive role in the control of disease in the field, it was important that the research conducted for this project be performed with respect to first hand observations the impact of *Brassica* on *Pratylenchus* spp. To this end, a series of field trials were undertaken (1995/1997), aiming to examine the disease break against *Pratylenchus* spp. associated with *B. napus* crops under field conditions.

4.2 MATERIALS AND METHODS

4.2.1 *Trials*

Three field trials were conducted under different environments and cropping practices within South Australia. Similar techniques of sampling, nematode extraction and population assessment were used for each. The three trials examined the impact of *Brassica* crops on final soil populations of *Pratylenchus* spp. in relation to other crops grown concurrently.

4.2.2 *General methods*

Soil sampling

Soil samples were taken using a flat trowel or hollow PVC tube (10cm wide x 15cm deep). It was important to minimise soil disturbance during sampling, as nematode populations can be reduced by mechanical damage such as abrasion by soil particles (Taylor and Vanstone, 1993). A multiple number (10-20) of soil cores were sampled across each plot (varying with plot size) at a depth of 0-15cm. Sampled soils were bulked into a plastic bag, which was placed on ice. After return to the laboratory, nematodes were extracted from four 50g sub-samples of the bulked soil from each plot.

Nematode extraction and evaluation

Nematodes were extracted from the soil using the mister method described in Section 3.3.4. Field soils with high levels of clay required a greater number of coffee filters than sandy soils to avoid clouding the samples with clay particles which made subsequent counting difficult. Replicated studies had shown that no significant alteration in nematode retrieval was observed when one, two three or four coffee filters had been used (Vanstone, unpublished). In all trials, the numbers of *Pratylenchus* spp. nematodes were counted using the standard method described in Section 3.3.5. At site 2 (Lameroo), *total* nematode numbers were also determined.

4.2.3 *Field sites*

The effect of *B. napus* crops on soil levels of *P. neglectus* was examined at three field sites, chosen to represent a range of environments and cropping practices. The details of the three sites are presented in Table 4.1.

Table 4.1 Details of field sites examined.

Field site	Site 1	Site 2	Site 3
Location:	Condada, Eyre Peninsula, S.A.	Lameroo, Murray Mallee, S.A.	Woodside, Mt. Lofty Ranges, S.A.
Primary crop:	Wheat	Wheat	Potato
B. napus crop employed:	Canola	Canola	Fodder
Soil Type:	Sandy loam	Loamy clay	Sandy loam
Av. Yearly Rainfall:	296mm	360mm	520mm
Predominant *Pratylenchus*	*P. neglectus*	*P. neglectus*	*P. crenatus*
Trial type:	Green manuring	Conventional cropping	Green manuring
No. treatments:	5	3	2
No. replicates:	4	3	4

Site 1: Condada. Green manuring trial within the cereal rotation

This trial aimed to examine the effects of growing and incorporating (green manuring) a series of different crops (Table 4.2) on the final soil levels of *Pratylenchus* spp. under a subsequent wheat crop. The various treatments were sown as part of a research project managed by Mr Matthew Cook, Department of Plant Science, University of Adelaide, South Australia. The soil under the following wheat crop was sub-sampled at anthesis for the purposes of this study.

Table 4.2: Crop treatments sown at Condada (site 1), 1994.

Crop treatment	Variety	Sowing density	Susceptibility to *P. neglectus*
Wheat (green manured)	Janz	65kg/ha	Susceptible
Vetch (green manured)	Languedoc	70kg/ha	Moderately resistant
Canola (green manured)	Rainbow	6kg/ha	Unknown
Canola (harvested)	Rainbow	6kg/ha	Unknown
Fallow (unplanted plot*)	-	-	-

Four replicates of each of the five treatments were sown into plots (17m long by 1.6m wide) on the 16th June, 1994. The plants were allowed to grow and develop using management practices typical of local farming either before being incorporated into the soil by cultivation on 19th September, 1994, or being harvested in late October, 1994. The following crop of wheat (cv Janz) was sown at 65kg/ha on 18th May, 1995, and five soil samples were taken for nematode assessment at anthesis (18th September, 1995). Bulked soil was sub-sampled and assessed for total *Pratylenchus* numbers using the methods described in Section 4.2.2. The predominant *Pratylenchus* species was identified as *P. neglectus* by Dr V. Vanstone, Department of Plant Science, University of Adelaide, South Australia.

* Unplanted plots were kept free of vegetation to ensure that no host plants were available to support the nematode.

Site 2: Lameroo. Conventional cropping within the cereal rotation

This trial aimed to examine the effects of different crops (Table 4.3) on final soil levels of *Pratylenchus* spp. within a conventional cereal cropping system. The trial was established by Mr Trent Potter, South Australian Research and Development Institute, Struan, South Australia.

Table 4.3: Crop treatments sown at Lameroo (site 2), 1995

Crop	Variety	Sowing density	Susceptibility to *P. neglectus*
Wheat	Janz	65kg/ha	Susceptible
Pea	Dundale	60kg/ha	Resistant
Canola	Hyola 42	5kg/ha	Unknown

Three replicates of each of the three treatments were examined. Plots were 8m long by 2m wide, and were sown on 12th June, 1995. The plants were allowed to grow and develop until anthesis (~16weeks), before nematode populations were assessed. Twenty soil samples were taken per plot and sub-sampled for assessment of total *Pratylenchus* numbers using the methods described in Section 4.2.2. The predominant *Pratylenchus* species was identified as *P. neglectus* by Dr V. Vanstone, Department of Plant Science, University of Adelaide, South Australia.

Site 3: Woodside. Green manuring trial within a potato rotation

This trial aimed to examine the effects of green manuring two different fodder crops (Table 4.4) on soil levels of *Pratylenchus* spp. nematodes within a potato cropping system.

Table 4.4: Crop treatments sown at Woodside (site 3), 1995.

Crop	Variety	Sowing density	Susceptibility to *P. crenatus*
Oat	Coolabah	75kg/ha	Host, but degree of susceptibility unknown
Canola	Rangi Rape	23kg/ha	Unknown

Four replicates of each of the two treatments were examined. Plots were 5m wide by 10m long with a 1m buffer between treatments. Plants were sown on the 10th May, 1995, and were cultivated into the soil at floral initiation of the *Brassica* (16th October, 1995). Numbers of nematodes in the soil under the plants were assessed two days prior to green manuring, and then 10, 17, 24 and 32 days after the cultivation. Twenty five soil samples were taken per plot and sub-sampled for assessment of total *Pratylenchus* spp. numbers using the methods

described in Section 4.2.2. The predominant *Pratylenchus* was identified as *P. crenatus* by Mrs Frances Reay, South Australia Research and Development Institute, Waite Campus, South Australia.

4.3 RESULTS

Site 1: Condada

Significant variation (P<0.05) in the final numbers of *Pratylenchus* spp. in the soil was observed after the five different treatments (Figure 4.1; Table 4.5*a*). Populations under wheat after green manured wheat, vetch or harvested canola treatments were not significantly different (Table 4.5*f*), although the canola treatment led to only 66% of the population as the wheat or the vetch.. The green manured canola treatment led to significantly fewer nematodes than did the harvested canola treatment (Figure 4.1; Table 4.5*c*), resulting in nematode numbers not significantly different to those in soils under wheat following the fallow treatment (Table 4.5*g*).

Table 4.5: Statistical comparison of *Pratylenchus* spp. numbers under wheat cv Janz after crops at Condada (site 1), 1995.

Crop comparison	Fprob.
(a) All five treatments	0.013*
(b) All crop treatments	0.039*
(c) Canola (GM[1]) versus Canola (HVST[2])	0.024*
(d) Wheat versus Canola (GM)	0.020*
(e) Wheat versus Fallow	0.034*
(f) Wheat versus Vetch versus Canola (HVST)	0.456
(g) Canola (GM) versus Fallow	0.327

*Significantly different at 5% level
[1] GM ~ green manured
[2] HVST ~ harvested

Site 2: Lameroo

Significant variation (P<0.05) was observed in the final numbers of *Pratylenchus* spp. in the soil under the different crop treatments examined (Figure 4.2A; Table 4.6*a*). The numbers of *Pratylenchus* spp. were significantly higher under the wheat crop than under the pea or canola crops (Table 4.6*b*,*c*). Numbers under the canola and pea crops were not significantly different (Table 4.6*d*). No significant difference in *total* nematode numbers was observed after the three crops (Figure 4.2B; Table 4.7).

Figure 4.1: Populations of *Pratylenchus* nematodes per gram dry soil sampled under wheat at anthesis following the green manuring (GM) of three different rotational crops, the harvest (HVST) of another and a period of fallow. Means of four replicates and standard deviations shown. Columns with the same letter ('a' or 'b') are not significantly different at the 5% level.

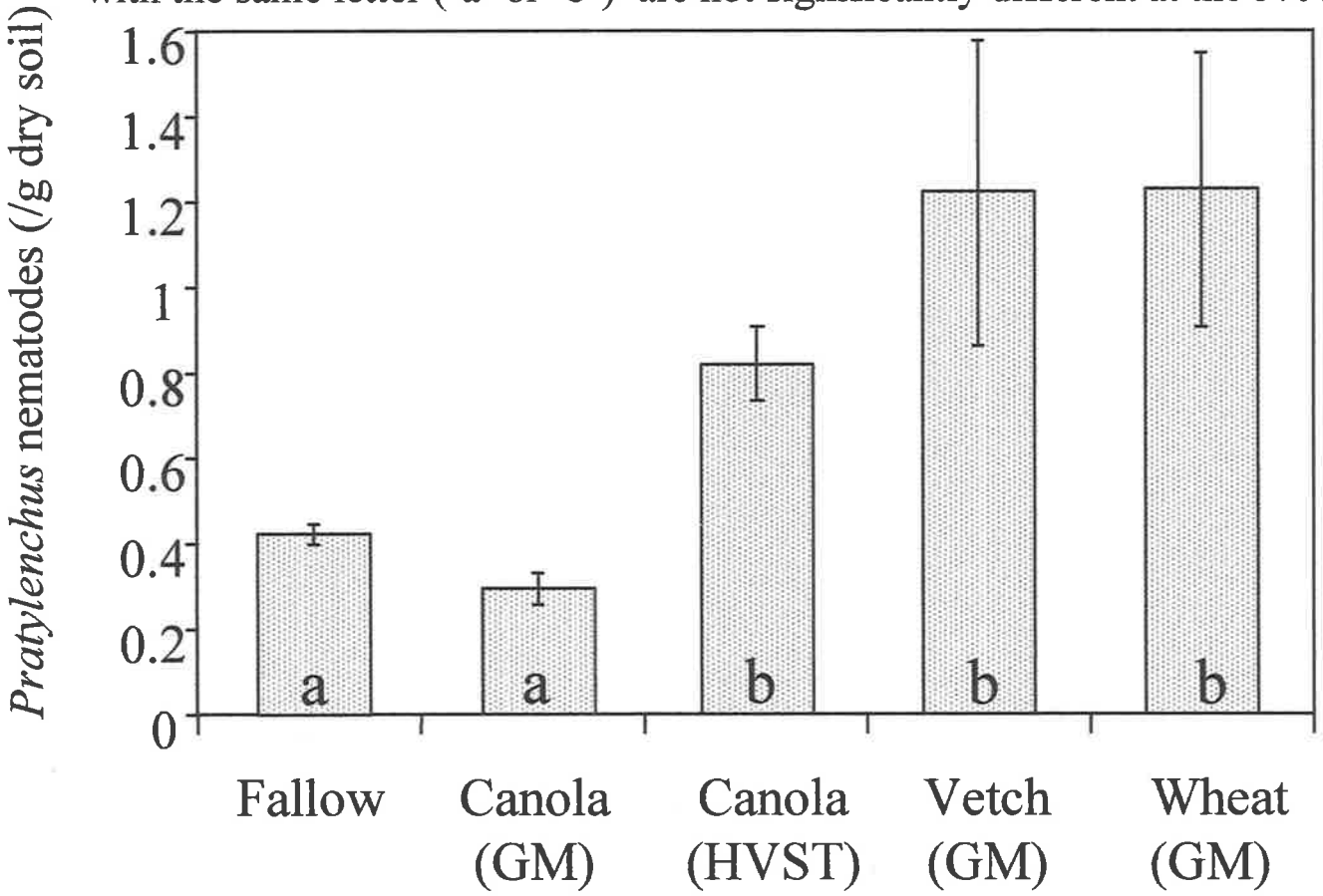


Figure 4.2: Numbers of *Pratylenchus* (A) and total (B) nematodes per gram dry soil sampled from three different rotational crops. Means of three replicates and standard deviations shown. Columns with the same letter ('a', 'b' or 'c') are not significantly different at the 5% level

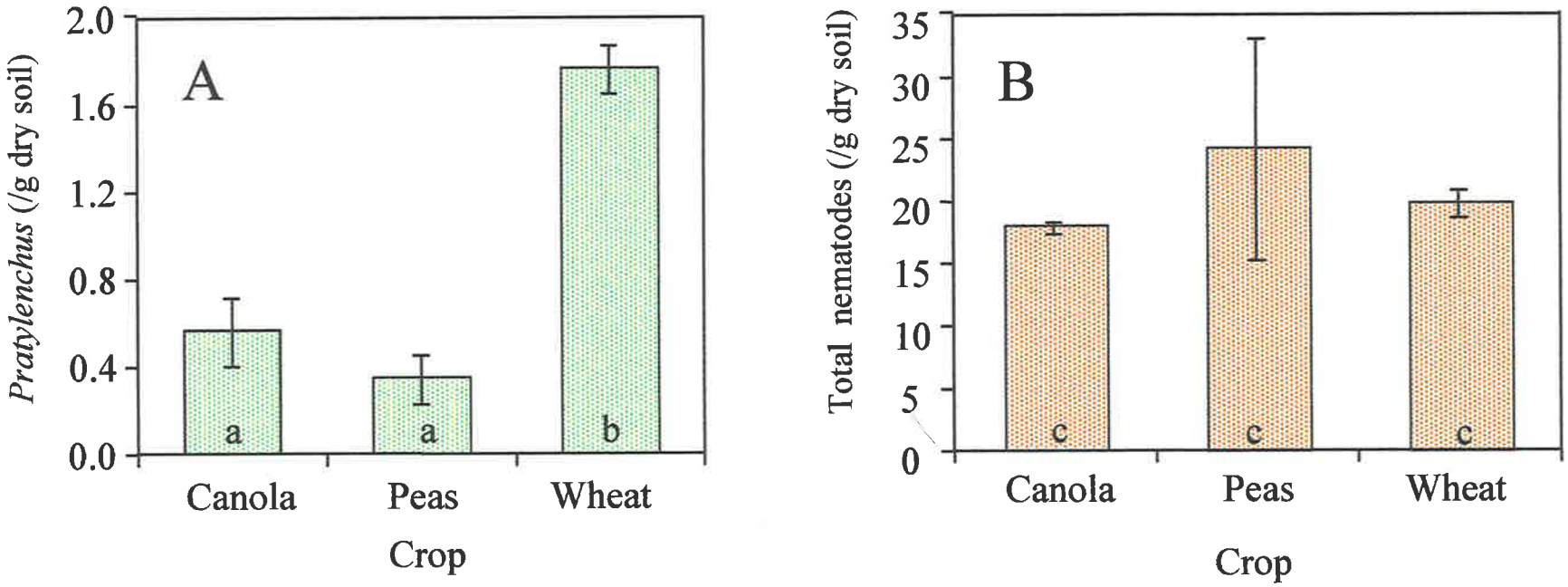


Table 4.6: Statistical comparison of *Pratylenchus* spp. numbers after crops at Lameroo (site 2), 1995.

Crop comparison	Fprob.
(a) All treatments	<0.001*
(b) Wheat/Canola	<0.001*
(c) Wheat/Pea	<0.001*
(d) Canola/Pea	0.357

*Significantly different at 5% level

Table 4.7 Total nematode numbers present in soils after crops at Lameroo (site 2). Means of three replicates displayed.

Treatment	Mean number of total nematodes per gram dry soil
Canola	17.79
Peas	24.11
Wheat	19.67

Fprob (0.05) = 0.765 (not significantly different)

Site 3: Woodside

A significant difference between the impact of the two fodder crops on the numbers of *Pratylenchus* spp. was observed (Table 4.8*a*; Figure 4.3), with a greater number of nematodes found under the rape (*B. napus*) than under the oat (Table 4.8*f*). The numbers of nematodes under the oat did not change significantly after incorporation (Table 4.8*c*). However, a significant decline in nematode numbers followed the green manuring of the rape crop (Table 4.8*h*). The reduction in nematode numbers associated with the *Brassica* was greatest in the period immediately after green manuring, dropping to levels not significantly different to the oat in the first ten days (Table 4.8*g*). The decline in numbers was then slower, but a significant reduction was observed during the following 25 days (Figure 4.3; Table 4.8*d*).

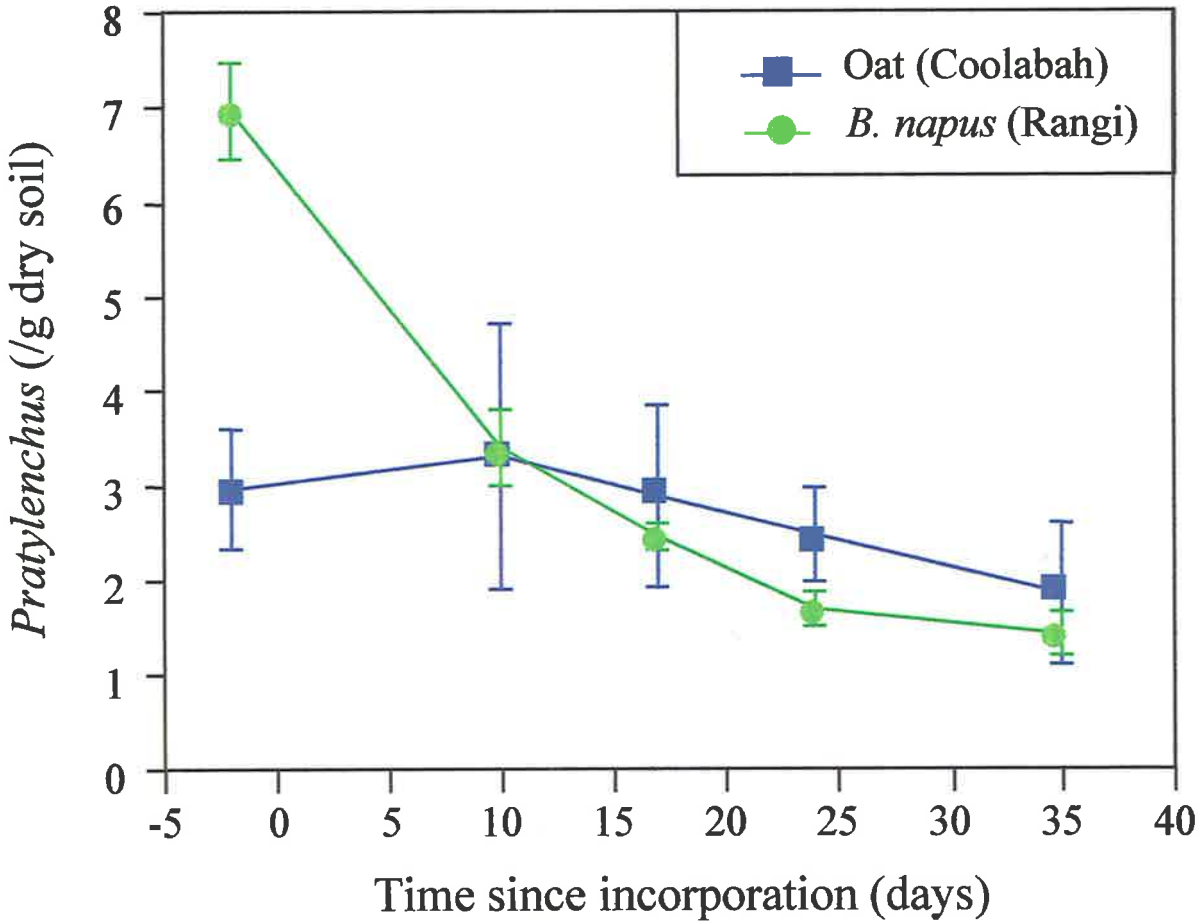
Table 4.8: Statistical comparison of *Pratylenchus* spp. numbers under fodder crops at Woodside (site 3), 1995.

Crop comparison	Fprob.
(a) Rape versus Oat (entire sampling period)	<0.001*
(b) Rape (entire sampling period)	<0.001*
(c) Oat (entire sampling period)	0.091
(d) Rape (sampling period after GM[1])	<0.001*
(e) Oat (sampling period after GM)	0.091
(f) Rape versus Oat (Two days prior to GM)	<0.001*
(g) Rape (10 days after GM) versus Oat (10 days after GM)	0.904
(h) Rape (Two days prior GM) versus Rape (10 days after GM)	<0.001*

* Significantly different at 5% level

[1] GM ~ green manured

Figure 4.3: Number of *Pratylenchus* nematodes per gram dry soil sampled under two different fodder crops before and after incorporation. Means of four replicates and standard deviations shown.



4.4 DISCUSSION

The green manuring trial performed at Condada suggested a nematicidal effect against *Pratylenchus* of canola tissues as they degraded in the soil. Comparisons of the green manured and the harvested canola treatments showed that the incorporation of *Brassica* tissues into the soil reduced nematode populations to a greater degree than did the same *Brassica* line harvested for seed. While an argument could be presented that the differences were due to the impact on *Pratylenchus* populations of the extra cultivation associated with the green manuring, no such impact was observed when wheat and vetch plants were incorporated. The reduction of nematode numbers was specific to the cultivation of the *Brassica*, fitting the hypothesis that the tissues release nematicidal molecules upon damage or during senescence.

In this study, the treatments providing best nematode control were the green manured *Brassica* (potentially nematicidal) and the period of fallow (where no host was present to allow nematode multiplication). However, it is important to realise that both the green manuring treatment and the fallow are not economic in the southern Australian cereal cropping system (as discussed in Section 2.2.2). Thus these results, while suggesting that canola tissues can lead to nematode control, are not appropriate. The trial did, however, suggest that the *Brassica* treatment grown to harvest, as is the practice within this farming system, led to lower nematode numbers than the green manured wheat and vetch treatments. While this difference was not statistically significant at the 5% level, it was large enough to suggest that the wheat crop following the canola would have been exposed to initially fewer nematodes, and thus had a greater potential for economic return.

Without information on the initial nematode numbers into which the succeeding wheat crop was sown, it is difficult to draw any firm conclusions from this trial. The observed differences in nematode numbers under the succeeding wheat crop may have been due to differences in the susceptibility of the various preceding crops, and thus how many nematodes developed throughout the first season. However, it can be said that green manuring increased the impact of the canola plots and that even the harvested canola treatment led to lower nematode populations than green manured wheat or vetch. The canola treatment provided as good or better control of the nematode in the soil as any of the other crops examined.

The Lameroo trial was more comparable to the conventional Australian cereal cropping system as the crops examined were grown through to harvest, not green manured. The degree of nematode control associated with these crops was therefore a function of the susceptibility of the plants to the nematode during the season and any allelopathic impact as the root tissues degraded in the soil (as the plant tops were never incorporated as in the green manuring studies).

The canola crop led to *Pratylenchus* populations which were not significantly different to those after peas, known for their resistance to *P. neglectus* and *P. thornei* (Vanstone *et al.*, 1993). Both of these treatments led to fewer nematodes in the soil (less than 50%) than the wheat treatment. This is a significant finding, considering that the *Brassica* is reputed to be susceptible to the nematode, allowing multiplication at rates comparable to the wheat (Vanstone et al., 1993; Webb, 1996). Once again, conclusions are limited as no estimate of the initial nematode densities was undertaken, and thus we have no measurement of the susceptibilities of the crops under these field conditions. It appears that the *Brassica* led to low nematode numbers despite its potential as a host to the nematode. Once again, this implies that the crop has some system distinct from resistance to control nematode populations, although this assertion is based only on circumstantial evidence. It may only be concluded, once again, that the *Brassica* provided as good or better control of the nematode in the soil as any of the other crops examined within the trial.

The Lameroo trial also examined the impact of the crops on the total number of nematodes in the soil samples, including the free living, non-plant parasitic nematodes. This second assessment was conducted to provide information on the effect of *Brassica* tissues on the general soil ecology, exploring the concept that the crop has the potential to destroy beneficial as well as harmful organisms as the tissues broke down in the soil. The assessment revealed that there were no significant differences in the impact of any of the three crops on the total numbers of nematodes (including free living and plant parasitic nematodes). The effect of the canola root tissues against *P. neglectus* (Figure 4.2A) seems therefore to have been localised. As any allelopathic chemical (such as isothiocyanate) released from senescing tissues would have been concentrated near the root tissues, a decrease in toxicity would have been expected as distance increased from the root surface. The allelochemical released from the *B. napus* tissues may therefore have been sufficient to kill only nematodes intimately associated with

the root system, predominantly the plant parasitic nematodes such as *P. neglectus*, which contribute only a small proportion to the total nematode numbers in the soil.

While the trials discussed above were limited by insufficient sampling in each case, the canola treatments were observed to be as effective in reducing soil *Pratylenchus* spp. populations as any crop examined. This suggests that either the canola was relatively resistant, or that it was actively reducing numbers by some other means, such as the allelopathy discussed in Section 2.4.3, or both. The trial at Woodside was better designed to explore the allelopathy associated with *B. napus*. In this case, assessments of initial nematode populations were undertaken, and the effect of the degrading tissues on the nematodes was monitored by successive sampling after the incorporation of the crops.

The oat and rape crops clearly displayed differences in susceptibility to the dominant *Pratylenchus* spp. (*P. crenatus*), as initial sampling showed significantly greater nematode populations under the *Brassica* than under the oat. This supports the suggestion that the *Brassica* are susceptible to the root lesion nematode during vegetative growth (Vanstone *et al.*, 1993; Webb, 1996). However, the incorporation of the *B. napus* crop resulted in a significant reduction in *Pratylenchus* populations, without a comparable reduction after the cultivation of the oat. This suggests that the nematode reduction associated with the green manure *Brassica* was due to some quality specific to the tissues, rather than due to nematode death due to the mechanical damage of the cultivation. The cultivation of the *Brassica* crop could have led to a large release of isothiocyanate in the short term, actively reducing nematode numbers within the initial ten days. After this period, tissues not immediately macerated by cultivation presumably began to degrade, releasing isothiocyanate and killing some of the remaining nematodes in the soil as observed in Figure 4.3. The *Brassica* treatment was sufficient to reduce the number of nematodes in the soil to a level comparable to those under the oat, despite its greater susceptibility.

The *Brassica* treatments at all three sites resulted in nematode numbers as low or lower than any of the other crops examined, despite the apparent susceptibility of canola to the nematode. The hypothesis that *Brassica* tissues contribute to the disease break strength of the crop through the release of allelopathic chemicals, resulting in mortality of nematodes close to

degrading tissues in the soil has not been disproved by these studies. The trials did suggest that the green manuring of the entire plant was more effective than allowing the plant to flower and seed, which limited the allelopathic influence to the root system. However, the trials also suggested that *B. napus* grown to seed could provide a reasonable disease break. While these studies gave only limited information due to basic flaws in their design, they do highlight the contribution of both resistance and allelopathy to the disease break against *Pratylenchus* spp associated with the *Brassica*. Studies evaluating the disease break effect at the biochemical level should therefore consider both the *susceptibility* of the crop during the season, and its *allelopathic potential* as it breaks down in the soil.

CHAPTER 5

GLUCOSINOLATES IN *BRASSICA* TISSUES

5.1 INTRODUCTION

As discussed in Section 2.5.1, glucosinolates are molecules found within the tissues of all *Brassica* species, including the cultivated species such as *B. napus* canola. While these molecules are stable and relatively non-toxic, upon tissue damage they are hydrolysed by the enzyme myrosinase, producing a range of biologically active molecules, including isothiocyanates. These isothiocyanates play a role in the pest and pathogen relations of the plant, reducing the palatability of the tissues to a wide variety of organisms (Waligora and Krzymanska, 1995). Their presence in the seed meal of oilseed rape has limited its use as a high protein meal as a cattle feed (Underhill, 1980). However, international breeding programs have responded to this problem, leading to the release of varieties containing total seed glucosinolates at levels below detectable toxicity (from >100mmol/g tissue to <20mmol/g tissue) (Buzza, 1995). More recently, the impact of glucosinolates within *B. napus* canola vegetative tissues has come under scrutiny, as efforts are made to understand the pest and pathogen relations of the crop (Brown and Morra, 1997). It has been suggested that glucosinolates contribute to the disease break impact of canola, as isothiocyanates released from the tissues act to suppress populations of harmful organisms in the soil (Chapter 4; Angus *et al.*, 1991; Kirkegaard *et al.*, 1994).

As this project examines *B. napus* canola in the southern Australian wheat cropping rotation, where plants are grown to seed, the root tissues are of prime importance as only these tissues will release isothiocyanates into the soil environment. *B. napus* root tissues contain high levels of the aralkyl glucosinolate, 2-phenylethyl (2-PE), with trace levels of indolyl and aliphatic glucosinolates (Sang *et al.*, 1994). The presence of 2-PE glucosinolate in *B. napus* root tissues, almost to the exclusion of all others, suggests a role for this glucosinolate in the pest and pathogen relations of the root system. Certainly, 2-PE isothiocyanate is known to have biocidal effects against a broad range of organisms (Drobnica *et al.*, 1967; Ahman, 1986; Borek *et al.*, 1995; Adesida *et al.*, 1996; Potter *et al.*, 1998).

Glucosinolates are notoriously difficult to work with, as the levels within plant tissues are influenced strongly by the external environment, and, while stable in undamaged tissues, they are quickly degraded after tissue maceration (Underhill, 1980). This chapter addresses the environmental and genetic factors influencing glucosinolate levels in *Brassica* tissues in an effort to standardise techniques and develop reliable protocols for the major thrust of the research in this thesis, as described in Chapters 6, 7 and 8.

5.2 GLUCOSINOLATE ASSESSMENT IN *BRASSICA* TISSUES

5.2.1 *Introduction*

Many methods for the assessment of glucosinolates in *B. napus* have been developed to assist breeding efforts to reduce the high seed levels which have limit the use of oilseed rape seed meal as cattle feed. However, to maximise the simplicity and efficiency of breeding methods, quality standards for seed meal have been based upon total glucosinolate levels (Joseffson, 1968; Van Etten *et al.*, 1974; Wilkinson *et al.*, 1984) and are therefore not suitable for the studies within this thesis. Qualitative assessment, suitable for the studies reported here, has been performed predominantly by High Performance Liquid Chromatography (HPLC) (Heany *et al.*, 1986) or Gas Chromatography (GC) (Brown and Morra, 1996). Other potential methods include Micellular Electrokinetic Capillary Chromatography (MECC) (Michaelson *et al.*, 1992) and Enzyme Linked Immunosorbent Assay (ELISA) (Hassan *et al.*, 1988).

The HPLC method of Heany *et al.* (1986), targeting desulphated glucosinolates, has limitations, as this does not measure levels of isothiocyanate liberated from degrading plant tissues, thought to be the major contributors to the impact of the crop against the nematodes. However, HPLC assessment of the more stable glucosinolates has been used in many studies (Mithen *et al.*, 1986; Lazzeri *et al.*, 1993; Sarwar and Kirkegaard, 1998), with researchers inferring isothiocyanate release from the glucosinolates detected within the tissues. Glucosinolate assessment therefore acts merely as a gauge of potential isothiocyanate release. As the isothiocyanate is not necessarily the only product of glucosinolate breakdown by myrosinase (Section 2.5.4), these studies have been forced to assume that, providing uniform conditions of tissue degradation were maintained, the proportion of glucosinolates breaking down to isothiocyanate will have been constant. Despite this drawback, the studies undertaken within this project employed the HPLC method of Heany *et al.* (1986) to assess desulphated glucosinolates within *Brassica* vegetative tissues.

5.2.2 *HPLC assessment of glucosinolates*

The methods for root tissue extraction and assessment of glucosinolates were described in Section 3.5. A typical HPLC trace for root and leaf tissue extracts is presented in Figure 5.1.

5.2.3 *Identification of peaks and validation of standards*

Peaks were identified with reference to *B. oleracea* samples containing predominantly single glucosinolates verified by Gas Chromatography/Mass Spectrometry (GC/MS) (kindly supplied by Dr Richard Mithen, Department of Brassicas and Oilseeds, John Innes Centre, Norwich, UK). The individual glucosinolates within an extract, separated by HPLC, were identified with reference to the retention times of glucosinolate peaks as identified by GC/MS studies of Dr Richard Mithen, John Innes Centre, Norwich, U.K. Peaks were quantified relative to peak area produced by a known amount of standard added to the tissue before extraction. Sinigrin (allyl glucosinolate) was used as a standard for most studies of canola tissues, as this glucosinolate is not produced by *B. napus*. However, the presence of sinigrin in the tissues of many *Brassica* species (particularly *B. nigra*, *B. carinata* and *B. juncea*) would have confounded quantification, and consequently, para-nitrophenyl sulphate (pNPS) was adopted as an alternative standard when studying these species. The sources and concentration of these standards are presented in Table 3.4.

Standard curves were developed, ensuring that a linear increase in added chemical led to a linear increase in HPLC peak area at their respective retention time. Aliquots of 20, 50, 100, 200, 350 or 500µl standard solutions (0.01M; Table 3.5) were added to 0.3g samples of lyophilised *B. napus* cv Dunkeld root tissue, and extracted and assessed by HPLC as described in Section 3.5. Both standards produced absorbance peaks at retention times which did not encroach on other relevant glucosinolates within the *Brassica* root or leaf samples (Figure 5.1), and thus would not confound area calculations for peak quantification. A linear relationship was observed between added standard and peak area in both cases (Figure 5.2), suggesting that the standards did not interact with the root tissues and were therefore suitable for use in the HPLC assays.

Figure 5.1 HPLC generated traces of desulphated glucosinolates within extracts from *B. napus* cv Dunkeld root (A) and leaf (B) tissues.

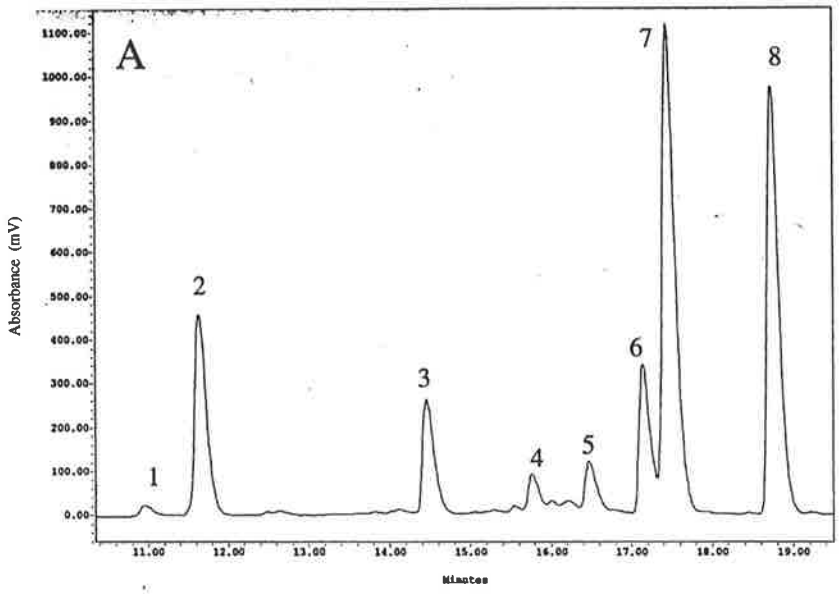


Figure 5.1A: HPLC trace of desulphated glucosinolates in extract from *B. napus* cv Dunkeld root tissue.
1 = Hydroxy-3-butenyl; 2 = 2-Propenyl (standard); 3 = 3-Butenyl;
4 = 4-Methyl-sulphinylbutenyl; 5 = 3-Indolylmethyl; 6 = 1-Methoxy-3-indolyethyl;
7 = 2-Phenylethyl; 8 = 4-Methoxy-3-indolylmethyl.

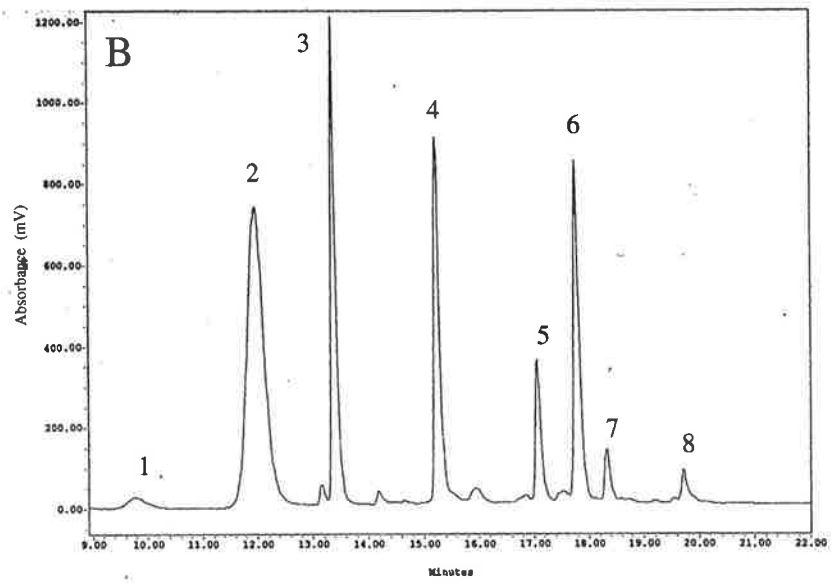
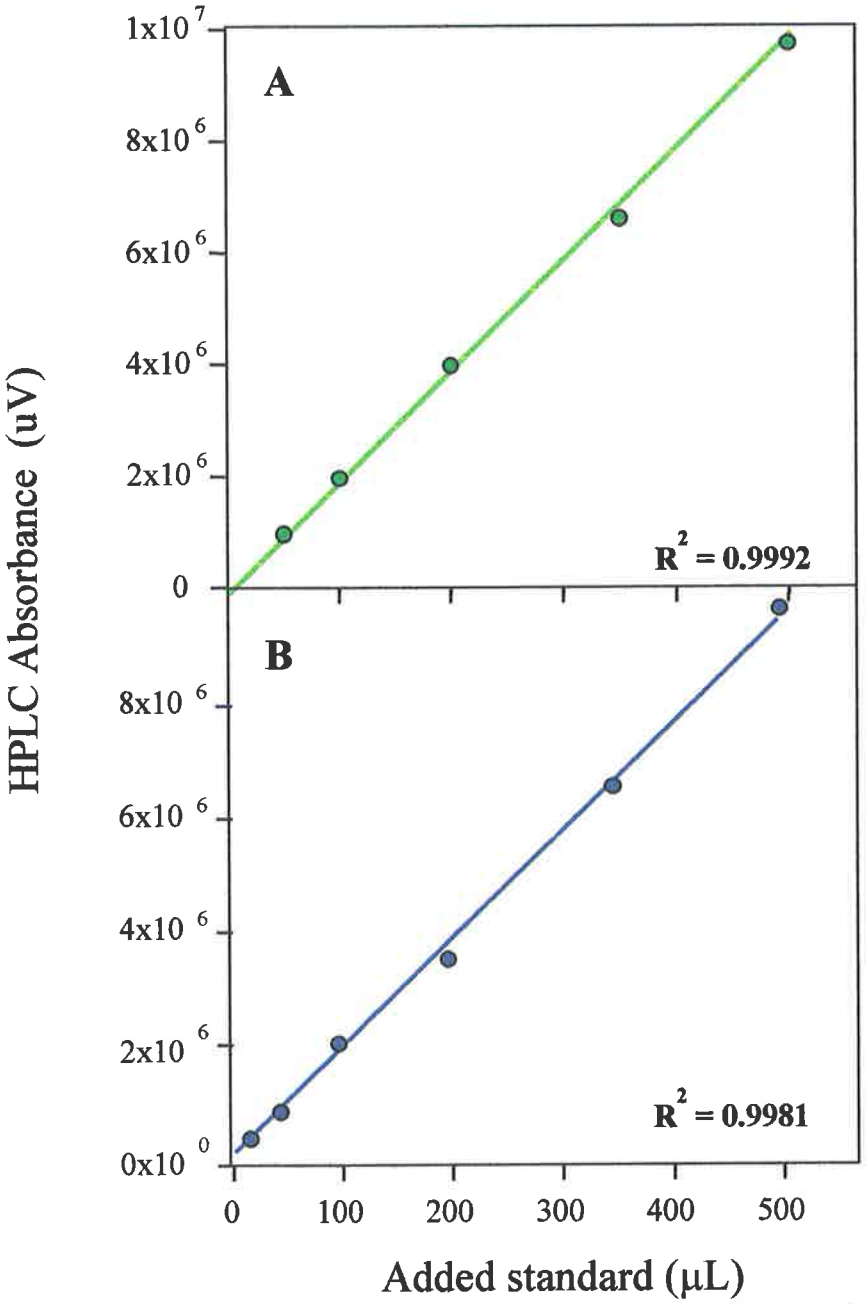


Figure 5.1B: HPLC trace of desulphated glucosinolates in extract from *B. napus* cv Dunkeld leaf tissue.
1 = Hydroxy-3-butenyl; 2 = 2-Propenyl (standard); 3 = 3-Butenyl;
4 = 4-Pentenyl 5 = 3-Indolylmethyl 6 = 1-Methoxy-3-indolymethyl;
7 = 2-Phenylethyl; 8 = 4-Methoxy-3-indolylmethyl.

Figure 5.2: Increases in absorbance (at 226nm) in HPLC analyses due to the addition of sinigrin (A) and para-nitrophenyl sulphate (B) standards to *B. napus* cv Dunkeld tissue extracts.



The quantification of glucosinolates based on the HPLC peak area was calculated with reference to the peak area produced by a known amount of standard. As different desulphoglucosinolates have different intensities of uV absorbency in the HPLC detector, calculations include a correction factor (Table 3.3), based on the absorbency of the desulphoglucosinolate relative to the standard employed. The correction factors displayed in Table 3.3 are developed with reference to sinigrin, and are therefore not directly applicable to calculations using pNPS as a standard. The linear regression (below) defining the relationship between the absorbency of sinigrin and pNPS (Figure 5.3) allowed the calculation of a 'predicted' sinigrin absorbance from the 'observed' pNPS absorbance. Estimating sinigrin absorbance from observed pNPS absorbance avoided having to calculate new correction factors for each desulphoglucosinolate when using pNPS as a standard.

$$\text{Sinigrin} = 0.549 \times p\text{NPS}$$

Using sinigrin and pNPS as standards, preliminary extractions of *B. napus* tissue performed at the Waite Campus were sent for verification of peak identity and sample quantification to an external laboratory (Dr M. Sarwar, CSIRO Plant Industry, Canberra, A.C.T., Australia). Although retention times varied due to variation in HPLC programs and hardware, peak identification and quantification of the studies undertaken in Chapter 5 were comparable (Table 5.1).

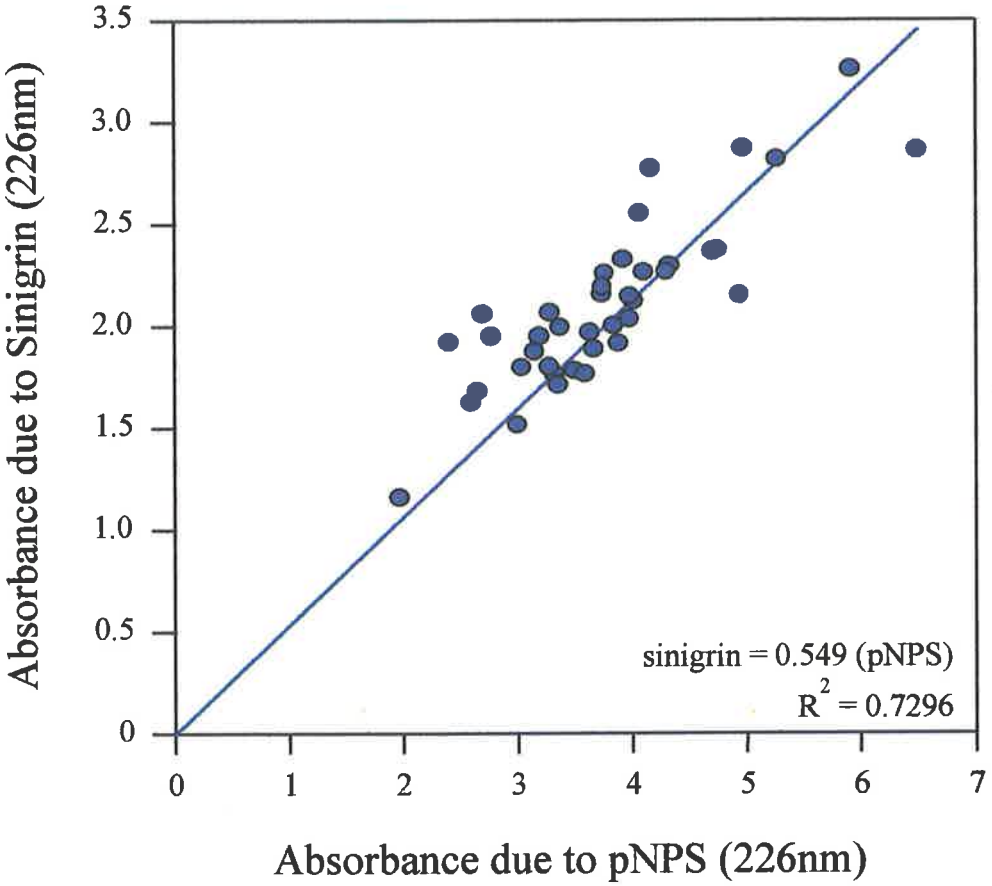
Table 5.1: Comparisons of HPLC determinations of glucosinolates within a *B. napus* cv Dunkeld root tissue extract, determined by the author, and by Dr M. Sarwar (CSIRO Plant Industry, Canberra, A.C.T. Australia).

	Waite	CSIRO
Glucosinolate	(µmol/g tissue)	
3-Indolylmethyl	0.19	0.17
4-Methoxy-3-indolylmethyl	0.21	0.19
2-Phenylethyl	8.25	8.68
1-Methoxy-3-indolylmethyl	1.22	1.01
Total	9.87	10.05

5.2.4 *Repeatability of HPLC analysis*

The repeatability of the HPLC analysis method was evaluated by assessing three sub-samples of bulked root tissue sampled from three separate developmental stages of glasshouse grown *B. napus* (cv Dunkeld) plants. Twenty-one *B. napus* cv Dunkeld plants were grown under

Figure 5.3 Relationship between the absorbance (at 226nm) due to HPLC separated additions of sinigrin and para-nitrophenyl sulphate standards to *B. napus* cv Dunkeld tissue extracts. The simple linear equation (sinigrin = 0.549 pNPS) allows the prediction of sinigrin absorbance for quantification calculations in samples containing native sinigrin, using pNPS as a standard.



standard glasshouse conditions (Section 3.4). Roots from seven plants were harvested, lyophilised and bulked at days 21, 35 and 49 after germination. Bulking the roots of seven plants at each sampling date was undertaken in an effort to minimise the confounding effect of any genetic variation between individual *B. napus* cv Dunkeld plants. Three separate 0.3g sub-samples were assessed on each of the three sampling dates using methods described in Section 3.5. Variation between sub-samples within each sampling date was assessed by analysis of variance.

Repeated HPLC quantification of glucosinolates sub-sampled from the same pool of ground tissue revealed no significant differences in any glucosinolate, at any of the sampling dates (Table 5.2), confirming the repeatability and reliability of the extraction and HPLC procedures. Significant variation in root glucosinolate profiles between the sampling dates was not unexpected, and will be discussed in Section 5.5.7. As "between sample" variation was significant, while "within sample" variation was not, the sensitivity of the method was judged appropriate for the analyses carried out in this project.

Table 5.2: Levels of the four detectable glucosinolates in four samples of bulked *B. napus* cv Dunkeld root tissue, sampled at three different stages of plant growth and development.

	P value (0.05)			
		Within		Between
Glucosinolate	Day 21	Day 35	Day 49	sampling dates
3-Indolylmethyl	0.259	0.986	0.177	>0.001*
4-Methoxy-3-indolylmethyl	0.936	0.971	0.952	>0.001*
2-Phenylethyl	0.905	0.979	0.953	>0.001*
1-Methoxy-3-indolylmethyl	0.914	0.983	0.973	>0.001*
Total	0.881	0.981	0.961	>0.001*

*Significantly different at 5% level

5.3 DEVELOPMENT OF RELIABLE PROTOCOLS MATERIALS AND METHODS

5.3.1 *Glucosinolate assessment of* Brassica *vegetative tissues*

Unless otherwise stated, plant tissues used in the following glucosinolate studies were grown in UC soil under the standard glasshouse conditions described in Section 3.4. Tissues were harvested directly into liquid nitrogen at floral initiation, lyophilised and ground into a powder, and stored as described in Section 3.5.1. Glucosinolate extraction and analysis was

performed as described in Section 3.5. Variation in glucosinolate levels between lines was assessed by analysis of variance.

5.3.2 *Inter-specific variation*

Six accessions were chosen to represent each of the major species of the *Brassica* (Table 5.3). Appropriate material of the *B. oleracea* (CC) species was unavailable and therefore an accession from the *B. oxyrrhina* species was selected. While the *B. oxyrrhina* (a common European weed) lies within its own cytodeme, it is considered comparable with the *B. oleracea* (CC) genome (Harberd, 1976). Leaf and root tissues from seven plants from each accession were bulked, and a sub-sample was taken from each for glucosinolate analysis.

Table 5.3: *Brassica* accessions selected to represent the major *Brassica* species (U, 1935) for examination of inter-specific variation in leaf and root glucosinolate levels.

Species	Genome	Accession no.
B. napus	AACC	Dunkeld
B. oxyrrhina	CC*	95060**
B. nigra	BB	95067**
B. carinata	BBCC	94044**
B. juncea	AABB	99Y11***
B. rapa	AA	90139**

*see above text
** Germplasm Collection, VIDA, Horsham, Australia.
***CSIRO Plant Industry, Canberra, Australia.

5.3.3 *Inter-varietal variation*

Four *B. napus* varieties from diverse genetic backgrounds were chosen for study (Table 5.4). Root tissues from seven plants from each variety were bulked and a sub-sample taken for glucosinolate analysis.

Table 5.4: *B. napus* varieties selected from a broad genetic base to examine inter-varietal variation in root glucosinolate levels.

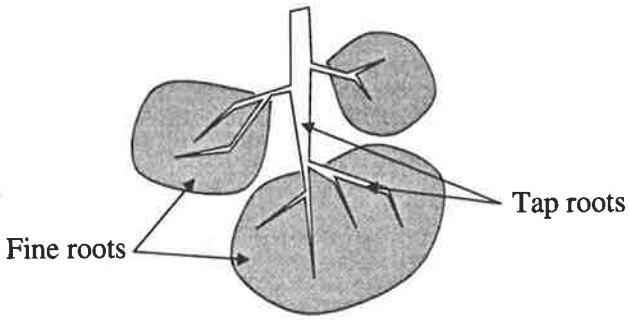
Variety	Source
Narendra	Australia
Dunkeld	Australia
Westar	Canada
Topas	Europe

5.3.4 *Intra-varietal variation*

The glucosinolate profile was determined in tissue extracts from the individual root systems of nine single plants from *B. napus* cv Dunkeld.

5.3.5 *Glucosinolate variation in tap and fine roots*

The root systems of seven individual *B. napus* (cv Dunkeld) plants were divided into tap and fine root sections (as below) using a razor blade. Comparisons of glucosinolate profiles were made between each section from each plant.



5.3.6 *Glucosinolates in adventitious roots*

The root systems of ten *B. napus* (cv Dunkeld) plants were harvested, lyophilised, ground and stored at -80°C. Plant tops were treated to initiate adventitious roots (Section 3.6) and successful cuttings were returned to UC soil for three weeks to recover. The first root harvest suspended floral development, allowing the development of the adventitious roots which were harvested as the cuttings began floral elongation. Glucosinolate levels within original and adventitious root tissues were compared.

5.3.7 *Developmental variation*

One hundred *B. napus* (cv Dunkeld) plants were sown into UC soil in individual 15cm pots. Roots from five plants were harvested on days 14, 23, 29, 36, 42, 51, 71 and day 95 after seeding and stored at -80°C. Glucosinolate variation during development was assessed by comparisons of means and standard deviations.

5.3.8 *Effect of nematode extraction on root glucosinolates*

The roots of forty *B. napus* (cv Dunkeld) plants were harvested at floral initiation. Roots were sectioned laterally with a razor blade and bulked into two samples, each containing equal amounts of both fine and tap root material. One half of the tissue was placed in liquid

nitrogen and stored at -80°C, while the other half was placed on the mister apparatus (Section 3.4.6) for 120 hours. Root tissues were removed from the mister and placed into liquid nitrogen, before HPLC extraction and assessment.

5.3.9 *Impact of* P. neglectus *on root glucosinolates*

Twenty-one *B. napus* (cv Dunkeld) plants were grown under standard glasshouse conditions. Fourteen days after germination, fourteen plants were infested with *P. neglectus* nematodes (3ml of a 350 nematode/ml suspension added to soil), while the remaining seven plants were untreated as controls, receiving 3ml distilled water only. After four weeks, roots were harvested from all plants. Glucosinolates were assessed in the roots of the seven control and seven infested plants. Roots from the remaining seven plants were placed on the mister apparatus for 120 hours to determine the extent of nematode infestation (Section 3.4.6).

5.4 DEVELOPMENT OF RELIABLE PROTOCOLS RESULTS

5.4.1 *Variation in glucosinolates in vegetative tissues*

Glucosinolate profiles differed between leaf and root tissues in all six accessions examined (Tables 5.5 and 5.6). Leaf tissues generally contained higher glucosinolate levels than root tissues, with high proportions of aliphatic glucosinolates, including 2-propenyl and 3-butenyl. Root tissues generally contained high levels of 2-PE glucosinolate, which was observed either at trace levels or not at all in the leaf tissues. 2-Propenyl glucosinolate was also observed in the roots of some species. Indolyl glucosinolates were present in both leaf and root tissues at low and variable levels.

5.4.2 *Inter-specific variation*

The different *Brassica* species displayed variation in the glucosinolate profiles of their leaf and their root tissues (Tables 5.5 and 5.6). 2-Propenyl glucosinolate dominated *B. nigra*, *B. juncea* and *B. carinata* leaf profiles. The weed species (*B. oxyrrhina*) had the highest leaf glucosinolate levels of those studied, but was peculiar as it contained high levels of 3-butenyl and 4-pentenyl glucosinolate rather than 2-propenyl glucosinolate. The *B. rapa* and *B. napus* accessions contained low leaf levels of glucosinolates, lacking the high levels of aliphatic glucosinolates observed in the other lines. Trace levels of indolyl and aralkyl glucosinolates were present in the leaf tissues of all species. However, small peaks were often difficult to

distinguish from the baseline due to large aliphatic and aralkyl peaks produced by some extracts and therefore they were regarded as being present at insignificant levels.

Table 5.5: The levels of nine detectable glucosinolates as determined by HPLC of desulphated extracts from the leaf tissue of six *Brassica* accessions, each representing a single *Brassica* species.

GLUCOSINOLATE	CLASS	SPECIES (μmol/g tissue)					
		B. napus	*B. oxyrrhina*	*B. rapa*	*B. nigra*	*B. juncea*	*B. carinata*
2-Hydroxy-3-butenyl	aliphatic	0.38	nil	0.52	nil	nil	nil
2-Propenyl	aliphatic	Nil	nil	nil	25.18	23.68	40.52
4-Methyl sulphonyl butyl	aliphatic	Nil	0.43	0.15	nil	nil	nil
3-Butenyl	aliphatic	0.10	41.69	0.10	nil	nil	nil
4-Pentenyl	aliphatic	0.19	8.48	1.59	nil	nil	nil
3-Indolylmethyl	indolyl	0.99	0.65	0.31	nil	nil	0.37
4 –Methoxy-3-indolylmethyl	indolyl	0.14	0.40	0.40	nil	nil	nil
1 –Methoxy-3-indolylmethyl	indolyl	0.27	0.18	0.05	nil	nil	nil
2-Phenylethyl	aralkyl	0.12	0.64	0.21	nil	nil	nil
Total aliphatic	-	0.67	50.60	2.36	25.18	23.68	40.52
Total indolyl	-	1.40	1.23	0.76	nil	nil	0.37
Total aralkyl	-	0.12	0.64	0.21	nil	nil	nil
Total glucosinolate	-	2.19	52.47	3.33	25.18	23.68	40.89

nil: nil or trace levels observed

Table 5.6: The levels of seven detectable glucosinolates as determined by HPLC of desulphated extracts from the root tissue of six *Brassica* accessions, each representing a single *Brassica* species.

GLUCOSINOLATE	CLASS	SPECIES (μmol/g tissue)					
		B. napus	*B. oxyrrhina*	*B. rapa*	*B. nigra*	*B. juncea*	*B. carinata*
2-Propenyl	aliphatic	nil	nil	nil	3.08	10.42	1.64
3-Butenyl	aliphatic	nil	0.63	nil	nil	nil	nil
4-Pentenyl	aliphatic	nil	0.63	0.14	nil	nil	nil
3-Indolylmethyl	indolyl	0.15	nil	nil	nil	nil	nil
4-Methoxy-3-indolylmethyl	indolyl	0.17	nil	nil	nil	nil	nil
1- Methoxy-3-indolylmethyl	indolyl	0.22	nil	nil	nil	nil	nil
2-Phenylethyl	aralkyl	12.73	14.41	3.00	4.45	6.68	0.87
Total aliphatic	-	nil	1.26	0.14	3.08	10.42	1.64
Total indolyl	-	0.54	nil	nil	nil	nil	nil
Total aralkyl	-	12.73	14.41	3.00	4.45	6.68	0.87
Total glucosinolate	-	13.27	15.67	3.14	7.53	17.10	2.51

Nil: nil or trace levels observed

Root tissues from all lines contained a high proportion of 2-PE glucosinolate, although *B. juncea*, *B. nigra* and *B. carinata* accessions also contained relatively high levels of 2-propenyl glucosinolate (Table 5.6). Low and inconsistent levels of indolyl glucosinolates were observed in the roots of all lines. As with leaf tissues, the highest glucosinolate levels were observed in *B. oxyrrhina* roots, due primarily to high levels of 2-PE glucosinolate.

5.4.3 *Inter-varietal variation*

The varieties examined contained the same complement of root glucosinolates (Table 5.7). No significant difference was observed between the varieties in terms of total amount or 2-PE glucosinolate levels at the 5% significance level, despite a moderately wide range in total glucosinolate (8.15 to 11.35µmol/g tissue) and 2-PE glucosinolate (6.80 to 9.08µmol/g tissue) levels. Significant variation between the varieties was observed only in the levels of 3-indolylmethyl and 1-methoxy-3-indolylmethyl glucosinolates.

Table 5.7: The levels of four detectable glucosinolates and totals as determined by HPLC of desulphated extracts from the root tissue of four *B. napus* varieties from a wide genetic base.

		Variety (µmol/g tissue)				P value
Glucosinolate	Class	Dunkeld	Narendra	Topas	Westar	(0.05)
3-Indolylmethyl	Indolyl	0.22	0.4	0.15	0.22	0.002*
4-Methoxy-3-indolylmethyl	Indolyl	0.22	0.2	0.25	0.22	0.954
1-Methoxy-3-indolylmethyl	Indolyl	1.83	1.75	0.95	2.29	0.046*
2-Phenylethyl	Aralkyl	9.08	8.27	6.80	7.15	0.640
Total		11.35	10.62	8.15	9.88	0.595

*Significantly different at 5% level

5.4.4 *Intra-varietal variation*

The roots of individual *B. napus* cv Dunkeld plants had significantly different levels of each detectable glucosinolate (Table 5.8). Total levels ranged from 3.82 to 12.10µmol/g tissue, based primarily on variation in levels of 2-PE (3.18 to 8.66µmol/g) and 1-methoxy-3-indolylmethyl (3.18 to 8.66µmol/g) glucosinolates.

Table 5.8: The levels of detectable glucosinolates as determined by HPLC of desulphated extracts from the root tissue of nine individual *B. napus* cv Dunkeld plants.

		Dunkeld selection (µmol/g tissue)									P value
Glucosinolate	Class	1	2	3	4	5	6	7	8	9	(0.05)
3-Indolylmethyl	Indolyl	0.05	0.03	0.03	0.28	0.64	0.59	0.06	0.03	0.03	>0.001*
1-Methoxy-3-indolylmethyl	Indolyl	0.65	0.45	0.51	1.88	1.99	2.40	0.75	0.48	0.45	>0.001*
4-Methoxy-3-indolylmethyl	Indolyl	0.27	0.16	0.18	0.37	0.81	0.81	0.21	0.13	0.14	>0.001*
2-Phenylethyl	Aralkyl	5.31	4.57	4.85	4.34	8.66	6.01	3.83	3.18	4.11	>0.001*
Total	Total	6.28	5.21	5.57	6.87	12.10	9.81	4.85	3.82	4.73	>0.001*

*Significant at the 5% level.

5.4.5 *Glucosinolate variation in tap and fine roots*

Fine roots had significantly greater levels of the two indolyl glucosinolates, 4-methoxy-3-indolylmethyl and 1-methoxy-3-indolylmethyl, than tap root tissues (Table 5.9). Levels of 2-PE, 3-indolylmethyl and total glucosinolates were not significantly different (at the 5% level).

Table 5.9: The levels of detectable glucosinolates as determined by HPLC of desulphated extracts sampled from the fine and tap root tissues from seven individual *B. napus* cv Dunkeld plants.

	Mean (µmol/g tissue)		P value
Glucosinolate	Tap	Fine	(0.05)
3-Indolylmethyl	0.32	0.50	0.130
1-Methoxy-3-indolylmethyl	0.44	1.74	0.002*
4-Methoxy-3-indolylmethyl	0.44	1.84	>0.001*
2-Phenylethyl	5.28	3.78	0.086
Total	6.48	7.86	0.426

* Significant at the 5% level

5.4.6 *Glucosinolate variation in adventitious roots*

No significant variation was observed between the total glucosinolates or levels of 3-indolyl and 2-PE glucosinolates within the original and adventitious roots (Table 5.10). However, significant variation was observed in the levels of 3-indolylmethyl and 1-methoxy-3-indolylmethyl glucosinolates. Differences between totals (original and adventitious, 8.16 and 9.96µmol/g tissue, respectively) were due primarily to greater levels of 1-methoxy-3-indolylmethyl in the adventitious root tissues.

Table 5.10 The levels of detectable glucosinolates as determined by HPLC of desulphated extracts sampled from original and adventitious root tissues from ten individual *B. napus* cv Dunkeld plants.

Glucosinolate	Original (µmol/g tissue)	Adventitious (µmol/g tissue)	P value (0.05)
3-Indolylmethyl	0.14	0.18	0.096
1-Methoxy-3-indolylmethyl	1.42	2.72	0.016*
4-Methoxy-3-indolylmethyl	0.09	0.15	0.013*
2-Phenylethyl	6.51	6.91	0.290
Total	8.16	9.96	0.076

*Significant at 5% level

5.4.7 *Developmental variation*

Plant growth and development was categorised into the three growth stages (Table 5.11).

Table 5.11 Timing of growth stages of *B. napus* cv Dunkeld plants grown in UC soil under standard glasshouse conditions (Section 3.3.1).

Stage	Growth Habit	Days
Stage 1	Vegetative	0-39
Stage 2	Floral initiation, elongation and podding	40-69
Stage 3	Plant senescence	69-100

Root levels of 2-PE glucosinolate and total glucosinolate levels are displayed as µmol of glucosinolates per total root system and per gram tissue in Figure 5.4. Trends in glucosinolate levels were consistent between total and 2-PE glucosinolate levels throughout the life of the plants. Levels rose within the total root system (µmol/root system) during the initial stages of development, peaking just prior to flowering (day 36), then falling as the plants began to elongate (day 42). Once flowering was completed, levels slowly increased (days 42 to 71), then fell gradually as the plant tissues began to senesce. Concentration (µmol/g tissue) peaked earlier (day 23) than total root levels (µmol/g plant), and fell as the root system continued vegetative growth, and through flowering, podding and plant senescence.

5.4.8 *Impact of nematode extraction on root glucosinolates*

A significant reduction in all detectable root tissue glucosinolates was observed after a period of 120 hours in the mister (Table 5.12). The greatest reduction was of 2-PE glucosinolate (8.0 to 5.2µmol/g tissue), contributing to a large reduction in total glucosinolate levels in the tissues that had been misted.

Figure 5.4: Trends in the levels of total and 2-phenylethyl glucosinolates (A, per plant; B, per gram root) within developing *B. napus* cv Dunkeld plants grown in the glasshouse under conditions described in Section 3.3.1.

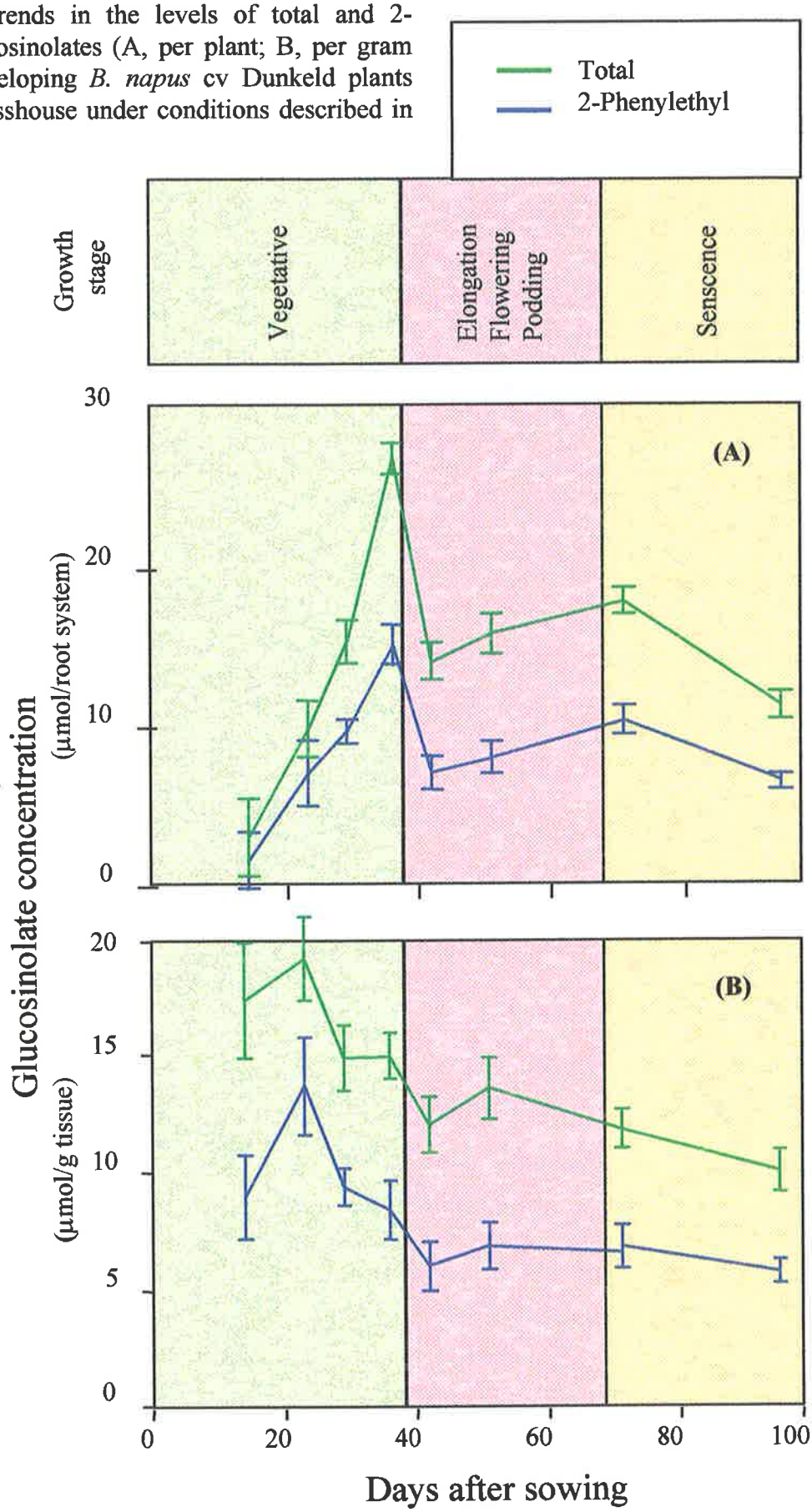


Table 5.12: The levels of detectable glucosinolates as determined by HPLC of desulphated extracts fresh sampled from *B. napus* cv Dunkeld plants root tissues, and those exposed to the mister conditions for five days at room temperature (Section 3.2.7).

Glucosinolate	Before misting (µmol/g tissue)	After misting (µmol/g tissue)	P value (0.05)
3-Indolylmethyl	1.10	0.56	0.038*
1-Methoxy-3-indolylmethyl	1.73	1.14	0.027*
4-Methoxy-3-indolylmethyl	1.73	1.14	>0.001*
2-Phenylethyl	8.01	5.20	0.006*
Total	12.57	8.04	0.021*

* Significance at 5% level

5.4.9 *Impact of* P. neglectus *on root glucosinolate levels*

Inoculation of pots with 1000 *P. neglectus* led to a population of 600 nematodes per gram dry root after six weeks. Control plants (not inoculated) contained no *P. neglectus*. Infestation at this level did not significantly affect the levels of any detectable glucosinolate in the roots of *B. napus* cv Dunkeld (Table 5.13).

Table 5.13: The levels of detectable glucosinolates as determined by HPLC of desulphated extracts sampled from un-inoculated *B. napus* cv Dunkeld root tissues, and those containing 600 nematodes (*P. neglectus)* per gram root.

Glucosinolate	Un-inoculated (µmol/g tissue)	Inoculated (µmol/g tissue)	P value (0.05)
3-Indolylmethyl	0.08	0.05	0.570
1-Methoxy-3-indolylmethyl	0.55	0.51	0.624
4-Methoxy-3-indolylmethyl	0.04	0.03	0.464
2-Phenylethyl	8.82	9.56	0.750
Total	9.49	10.15	0.872

5.5 DEVELOPMENT OF RELIABLE PROTOCOLS DISCUSSION

5.5.1 *Variation in glucosinolates in vegetative tissues*

The observed differences in total glucosinolate levels between leaf and root tissues and the predominance of different classes in each tissue type (aliphatic in leaf tissues and aralkyl in root tissues; Tables 5.5 and 5.6) support the findings of Sang *et al.* (1984). The selective advantage governing the distribution of glucosinolates within the different plant tissues is likely to relate to the properties of the isothiocyanates released after tissue damage. Mithen *et al.* (1995) suggested that different grazing pressures could lead to a gradual alteration of

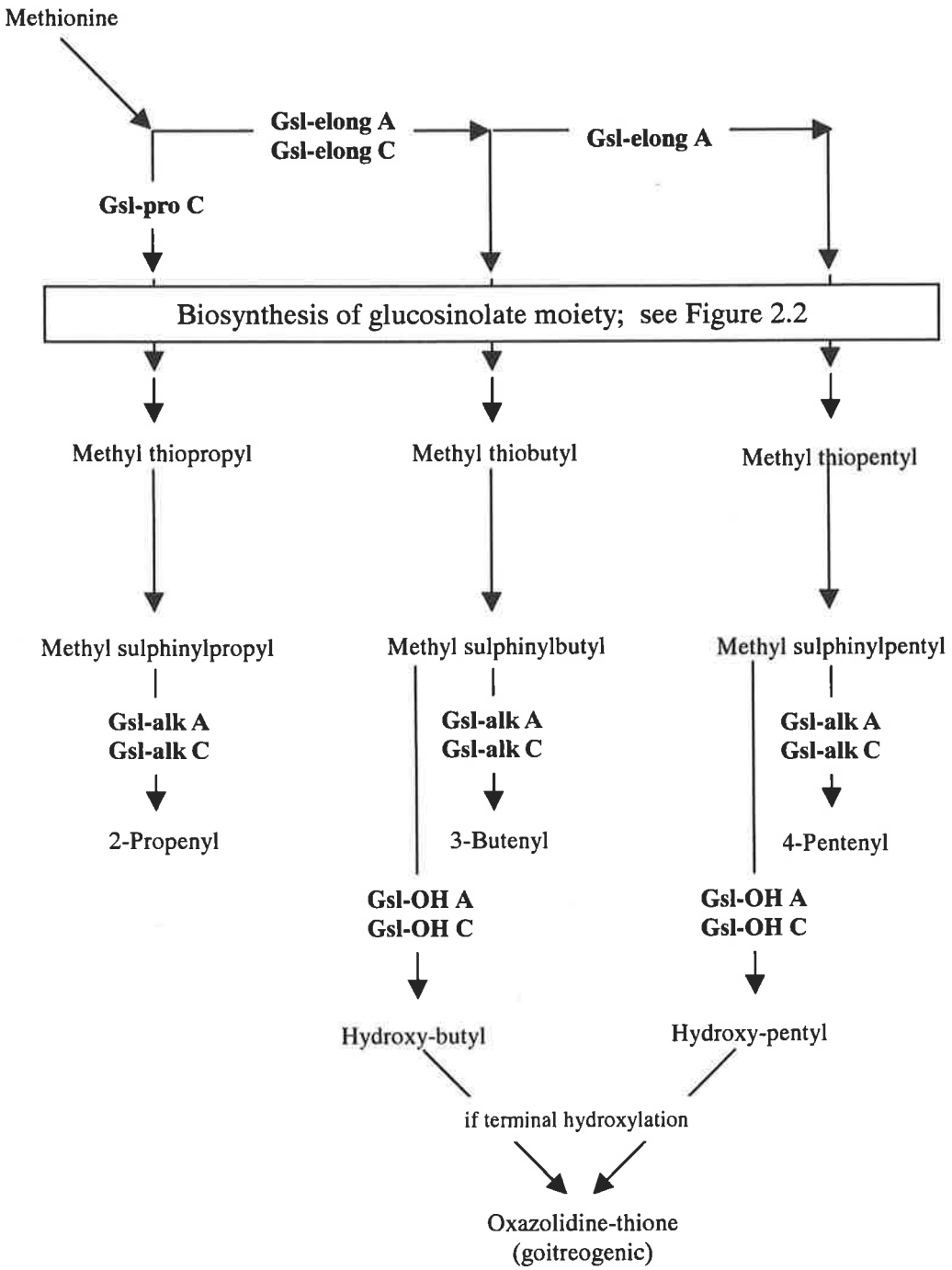
glucosinolate profiles in the leaf tissues in wild plant populations such that the most appropriate glucosinolates to deter grazing were present in the tissues under threat. The high metabolic cost involved in the production and maintenance of secondary plant products (Bell and Charwood, 1980) is likely to provide a selective advantage to plants containing the most efficient glucosinolates in the appropriate tissues. The prevalence of the aliphatic glucosinolates in the above ground parts suggests that their resulting isothiocyanates, which are reasonably volatile, may be more effective in the aerial environment. The aralkyl glucosinolates, which dominate the profile of the below ground parts of the plant, may liberate isothiocyanates more suited for activity in the soil environment.

5.5.2 *Inter-specific variation*

The qualitative and quantitative glucosinolate variation observed between the accessions from the six *Brassica* species examined reflect the wide genetic base available to the breeder. While the tissues of the different species contained common glucosinolates (Section 5.5.1), variation was observed in the actual levels and distribution of the glucosinolates within the tissues.

The absence of 2-propenyl glucosinolate in the leaves of the *B. napus* accession supports the absence of a functional Gls-pro allele (proposed by Magrath *et al.*, 1994; Figure 5.5), responsible for the production of 2-propenyl glucosinolates from a common aliphatic precursor. The glucosinolate profile in the *B. oxyrrhina* leaves also supports the same model, with high levels of 3-butenyl and 4-pentenyl glucosinolates being produced by functional Gls-elong alleles from an aliphatic precursor not being converted to 2-propenyl glucosinolate due to a null Gls-pro allele. The *B. nigra*, *B. juncea* and *B. carinata* accessions all contained high leaf levels of 2-propenyl glucosinolate, implying the presence of an active Gls-pro allele. It is noteworthy that the functional Gls-pro allele appeared to be present in each line containing a B genome (*B. nigra* (BB), *B. juncea* (AABB) and *B. carinata* (BBCC)), suggesting that the functional allele may reside in this genome. No information was available on the inheritance of aliphatic glucosinolates in the roots of the *Brassica*, although lines exhibiting 2-propenyl in the leaves also contained this glucosinolate in the roots, confirming the activity of the Gls-pro allele in root tissues.

Figure 5.5: The inheritance of aliphatic glucosinolates in *Brassica napus*. The genetic model of aliphatic glucosinolate biosynthesis, as proposed by Magrath *et al.* (1993). The authors suggest that the R~group of the glucosinolate is developed through side chain elongation from a common resource of the amino acid, methionine. The enzymes coded by these alleles utilise the methionine substrate in a competitive fashion. Should a 'null' allele be present at any of the glucosinolate elongation (Gsl-elong) loci (as observed at the GSL-pro C locus in *B. napus*), the resultant increase in the availability of methionine is considered to lead to a corresponding increase in the production of the other glucosinolates across the biochemical pathway.



The indolyl glucosinolates were present at trace levels in both the root and leaf tissues of the accessions examined. The function of the indolyl glucosinolates is unclear in *Brassica* spp. tissues, but they may act as precursors to plant hormones and intermediaries in the biosynthesis of other plant products (Fenwick *et al.*, 1994). Variation in the levels observed in the root and leaf tissues may be due to differential production of plant hormones at the particular physiological stage at which the plants were harvested. As levels were low and generally unpredictable, little relevant information could be determined from these studies.

2-PE glucosinolate was found only at trace levels in the leaf tissue but dominated the glucosinolate profile of the root. While no information could be found in the literature regarding the inheritance of aralkyl glucosinolates in the *Brassica*, the variation observed here suggests that manipulation of root levels of 2-PE glucosinolate may be possible through inter-specific crossing, for instance through hybridisation of *B. napus* with *B. oxyrrhina.*

5.5.3 *Inter-varietal variation*

No significant variation was observed in total or 2-PE glucosinolate concentrations between the four different varieties examined, despite a range of mean levels observed (total: 8.15 to 11.35μmol/g tissue; 2-PE: 6.80 to 9.08μmol/g tissue). It is possible that the statistical analysis of the inter-varietal variation was confounded by the large and significant intra-varietal variation as observed when examining individual plants from *B. napus* Dunkeld (Table 5.8).

The limited variation observed between the varieties could be explained by the narrow genetic base associated with canola (Thormann *et al.*, 1994). It has been suggested that cultivated *B. napus* (AACC) was developed from a limited number of natural crosses between wild *B. rapa* (AA) and *B. oleracea* (CC) plants, leading to a very narrow base of germplasm (Song and Osborne, 1992; Thormann *et al.*, 1994). Genetic diversity in *B. napus* has been further limited by selective breeding from low seed glucosinolates, required for the use of meal as a stock feed (Section 2.5.1), obtained through hybridisation with the variety, Bronowski. Fundamental differences in root glucosinolate profiles, which have never undergone deliberate selection in breeding programs, are therefore unlikely. The Dunkeld and Narendra varieties are closely related, having been released from the same breeding program within two years of each other, sharing many common progenitors.

Thus, the lack of significant variation in root glucosinolate levels observed between the four varieties may be explained by their genetic similarity. However, the high degree of intra-varietal variation observed (Section 5.4.4) suggests that the lack of variation between the varieties was based more on similarities of the means in the varietal populations, than on absence of variation in the germplasm.

5.5.4 *Intra-varietal variation*

Significant variation was observed in the levels of all detectable glucosinolates within the roots of nine single plants of the variety Dunkeld. Dunkeld is the result of a complicated crossing program, incorporating the genetic material from more than ten different *B. napus* sources. Selection has been based upon a modified pedigree method, and varieties have been released containing reasonable genetic diversity. As the plants within these populations are likely to outcross at approximately 10%, it is not surprising to find variation in traits which have not been directly selected for in breeding programs, such as root glucosinolate profiles. Despite the fact that the variation in this species is considered restricted, it seems that significant levels of variation can be present within plant populations grown under identical conditions. This is an important finding, identifying a source of genetic variation for breeding programs aiming to improve the disease break potential of the crop by alteration of root glucosinolate profiles (Chapter 8).

5.5.5 *Glucosinolate variation in tap and fine roots*

The fine and tap roots of *B. napus* cv Dunkeld differed in their levels of 4-methoxy-3-indolylmethyl and 1-methoxy-3-indolylmethyl glucosinolates, but contained similar total levels and levels of 3-indolylmethyl and 2-PE glucosinolate. However, the limited number of replicates within this trial may have limited statistical sensitivity, as a reasonable level of variation was observed despite the lack of significance. Parker (1994) observed significant variation in the toxicity of *B. napus* fine and tap roots against *P. neglectus*, suggesting that tap roots contained greater levels of glucosinolates releasing toxic isothiocyanates than fine roots. Further studies performed to relate the glucosinolate levels within fine and tap root tissues to the toxicity of the tissues will be presented in Chapter 6.

5.5.6 *Glucosinolates in adventitious roots*

A method for non-destructive root sampling was required in the breeding program so that glucosinolate profiles within single plants could be characterised, while still allowing the plants to produce seed for further study. In order to maintain the plants after root harvest, adventitious roots were induced on stem material (Section 3.6). These adventitious roots, produced from stem tissue, contained total levels and concentrations of 3-indolylmethyl and 2-PE glucosinolate similar to those of original roots, harvested earlier. Significant differences were observed in levels of the two methoxy-indolylmethyl glucosinolates, but the glucosinolate profile of the adventitious roots otherwise resembled that of the original root tissues. This is not surprising, considering that both root systems were harvested at a similar developmental stage (Section 5.5.7). As the indolyl glucosinolates have been reported to act as precursors of plant hormones (Fenwick *et al.*, 1994), an auxin-related response to plant wounding at first harvest, or the biochemical processes of adventitious root initiation, may have been responsible for the observed variation in the levels of 4-methoxy-indolylmethyl and 1-methoxy-indolylmethyl glucosinolates.

5.5.7 *Developmental variation*

Glucosinolate profiles change during the development of the plant, particularly as the plant undergoes floral elongation (Giamoustaris *et al.*, 1994). Qinzheng *et al.* (1991) described the remobilisation of glucosinolates from the vegetative tissues to the seed pods during the floral period of growth, for translocation to the developing seed. To develop a reliable comparison between trials in this project, it was essential to determine the phase of plant development where root glucosinolate levels were maximised. The results of this study support the findings of Fieldsend and Milford (1994), Clossais-Besnard and Larher (1991) and Qinzheng *et al.* (1991), demonstrating a gradual increase in total and 2-PE glucosinolates in the whole root system during vegetative growth, before falling during the period of elongation, flowering and podding. The glucosinolate concentration per gram root declined earlier than total levels within the whole root system (by day 23), suggesting that the increase in root biomass was greater than the rate of glucosinolate biosynthesis in these tissues. These results confirm that the most appropriate timing for tissue harvest for glucosinolate analysis is just prior to the period of floral development. Plants in glasshouse trials were therefore harvested at the first sign of elongation. These results also support the timing of incorporation (green manuring) of the crops in the field studies discussed in Chapter 4.

5.5.8 *Impact of nematode extraction on root glucosinolates*

The nematode extraction procedure (Section 3.4.6) involved exposure of harvested roots to repeated saturation with water at room temperature for 120 hours. While no obvious root degradation was observed during this period, it was likely that some glucosinolate degradation could occur, potentially liberating isothiocyanates into the mister baskets. The results presented in Table 5.12 confirm that there was a significant reduction in all detectable glucosinolates after 120 hours on the mister apparatus. This has implications for the susceptibility studies discussed in Chapter 7, as isothiocyanates released from degrading tissues might kill or weaken nematodes leaving the roots, reducing the numbers detected by this extraction method. The impact of this glucosinolate loss on nematode retrieval is further discussed in Section 7.2.1.

5.5.9 *Impact of* P. neglectus *nematodes on root glucosinolates*

A number of studies have discussed the impact of tissue damage due to herbivores on the concentrations of glucosinolates in *Brassica* vegetative tissues (Nicholas *et al.*, 1990; Bodnaryk, 1992; Wynne-Griffiths, 1994). In most cases, it was the indolyl glucosinolates that were influenced, often observed at higher levels after tissue damage. However, salicylic acid (considered to play a role in systemic plant defense) has been observed to induce higher levels of 2-PE glucosinolate in *Brassica* spp. leaf tissues (Doughty *et al.*, 1995). As experiments described in Chapter 7 aimed at relating root glucosinolate levels to the susceptibility of the tissues to nematode infestation, it was important to determine if infestation by nematodes significantly affected root glucosinolate levels. The results (Table 5.13) suggest that nematode infestation at rates comparable to those used in later work (Chapter 7) did not change the levels of any detectable glucosinolate in the roots of *B. napus* cv Dunkeld. Any correlation between root glucosinolates and susceptibility to *P. neglectus* is therefore unlikely to be confounded by the effect of the nematode on the root glucosinolate profile.

CHAPTER 6

ALLELOPATHIC POTENTIAL OF *BRASSICA* SPP. TISSUES AGAINST *P. NEGLECTUS*

6.1 INTRODUCTION

The biocidal qualities of *Brassica* tissues against soil-borne organisms, including nematodes, was reviewed by Brown and Morra (1997). The presence of glucosinolates within tissues has been closely associated with this trait (Jing and Halbrendt, 1994; Waligora and Krzymanska, 1995), although *in vitro* studies suggest that it is not the glucosinolates themselves, but the products of their hydrolysis which are responsible for this effect (Brown and Morra, 1997). In particular, the isothiocyanates are considered highly nematicidal, displaying variable potency depending on the chemical nature of their defining R~group (Mojtahedi and Santo, 1996). In Australian dryland cereal rotations, canola residues, particularly root tissues, have been observed to reduce the level of inoculum of soil borne pathogens as these residues degrade in the soil, leading to increased vigour and eventual grain yield of following cereal crops (Angus *et al.*, 1994; Kirkegaard *et al.*, 1994). The reduction in pest and disease levels is thought to be due to the release of isothiocyanates from the degrading tissues (Kirkegaard *et al.*, 1994), although the literature fails to report an association of any single glucosinolate contained in canola roots with this phenomenon.

This chapter presents studies aimed to establish the relationship between the range of individual glucosinolates within *B. napus* canola leaf and root tissues and the impact of these tissues on the survival of *P. neglectus* in the soil environment. Identification of the key components contributing to the biocidal effect of the tissues would aid breeding programs aiming to improve the disease break effect of canola within the rotation.

6.2 MATERIALS AND METHODS

6.2.1 *General methods*

The trials discussed in this chapter aimed to determine the relationship between glucosinolates and the biocidal qualities of *Brassica* leaf and root tissues against *P. neglectus*. To this end, an assay was developed, based on the nematicide screening assays of McBeth and Bergeson (1953), in which nematodes were exposed to soil amended with various *Brassica* tissues (Section 3.3).

Unless otherwise described, *Brassica* spp. were grown to floral initiation under glasshouse conditions and leaf and root tissues harvested, dried and stored as described in Section 3.3.1. *Triticum aestivum* cv Machete leaf and root tissues, used as controls, were grown and prepared under identical conditions. Amended soils were prepared by mixing a predetermined amount of dehydrated tissue into sterilised and sieved (3mm) UC soil. Microcosms were prepared as described in Section 3.3.2 and soils hydrated and inoculated with *P. neglectus* as described in Section 3.3.3. Surviving nematodes were recovered from soils by misting (Section 3.3.4), and counted as described in Section 3.3.5.

6.2.2 *Nematode survival in un-amended soil*

Sixty microcosms were prepared, each containing 10g of unamended soil hydrated with 2 ml of sterile, distilled water. One millilitre of a 500/ml suspension of nematodes was added to the hydrated vials and the number of retrievable nematodes determined (five replicates) after incubation for twelve different periods at 20°C (0 hours, 36 hours, 84 hours, 120 hours then at 12 noon on days 8, 12, 15, 19, 22, 25, 40 and 52). Numbers retrieved at each sampling time were expressed as a percentage of the number originally inoculated.

6.2.3 *Nematicidal effect of* B. napus *cv Dunkeld leaf and root tissues*

The impact of increasing levels of *B. napus* cv Dunkeld leaf and root tissue amendments on *P. neglectus* was assessed in microcosms. Seven replicates of microcosms, each containing 10g soil amended with the appropriate tissue, were prepared for each level (leaf: 0, 0.5, 1.0, 2.0g/100g soil; root: 0, 0.4, 0.8, 1.2, 2.0, 3.0, 4.0 g/100g soil). The nematicidal impact of a 4g/100g amendment of wheat cv Machete tissues was also assessed. The numbers of nematodes retrieved were used to calculate nematode loss due to tissue amendment as a percentage of the original inoculum added.

$$\text{Nematode loss (\%)} = \frac{\text{no. nematodes retrieved}}{\text{no. nematodes inoculated}} \times \frac{1}{\text{no. nematodes retrieved from Machete wheat}}$$

6.2.4 *Nematicidal impact of six* Brassica *spp.*

Dried and powdered root and leaf tissues from accessions of six different *Brassica* species were gathered from the same source as harvested for glucosinolate analysis in Chapter 5 (Section 5.4.2), which had been stored under silica gel as described in Section 3.3.1. Wheat cv Machete (leaf and root) tissues were used as controls. Two grams of tissue were mixed thoroughly into 100g of prepared UC soil (Section 3.3.2). Ten grams of amended soil from each of the fourteen tissue treatments ((6 *Brassica* + 1 control) x 2 tissue types) was then added to seven replicate microcosms. Microcosms were hydrated and inoculated with 500 *P. neglectus* as described in Section 3.3.3. Mean numbers of retrievable nematodes were determined after 80 hours (Section 3.3.4) and expressed as a percentage of the initial population relative to the nematode loss associated with the appropriate wheat tissue amendment (as below).

The levels of various glucosinolates within the tissues (Section 5.4.2) were expressed as nmol/g soil and related to the observed nematode loss associated with the tissue through simple linear regression. Nematode loss associated with leaf tissue was related to the total, total aliphatic, total indolyl, 2-propenyl, 3-indolylmethyl, and 2-PE glucosinolates within the tissue. Loss due to root tissue amendments was related to the total, total aliphatic, 2-propenyl, and 2-PE glucosinolates within the root tissues.

6.2.5 *Nematicidal variation in root components*

Dried and powdered tap and fine root tissues from *B. napus* cv Dunkeld plants were gathered from the same source as harvested for glucosinolate analysis in Chapter 5 (Section 5.4.5), which had been stored under silica gel as described in Section 3.3.1. Wheat cv Machete root tissues were used as a control. Two grams of tissue was mixed thoroughly into 100g of prepared UC soil (Section 3.3.2). Ten grams of amended soil from each of the three tissue treatments (2 *B. napus* root tissue types + 1 control) was added to seven replicate microcosms. Microcosms were hydrated and inoculated with 500 *P. neglectus* as described in Section 3.3.3. Mean numbers of retrievable nematodes were determined after 80 hours (Section 3.3.4) and expressed as a percentage of the initial population relative to the nematode loss associated with the wheat tissue amendment (as above).

The levels of total and 2-PE glucosinolates within the tissues (Section 5.4.5) were expressed as nmol/g soil and then related to the observed nematode loss associated with the tissue through simple linear regression.

6.2.6 In vitro *studies with 2-PE isothiocyanate*

In vitro studies using purified 2-PE isothiocyanate (Sigma Chemicals; P-2179) were based on the microcosm studies (Section 3.3), with replacement of the ground tissue amendments by aliquots of pure isothiocyanate. As 2-PE isothiocyanate is insoluble in water, a stock solution of 5µmol/ml 2-PE isothiocyanate was made up in 100% ethanol. Aliquots containing 0.1ml, 0.2ml, 0.3ml, 0.4ml or 0.5ml of the isothiocyanate solution, made up to 0.5ml with ethanol, were added to microcosms containing 10g sieved soil (Section 3.2) previously hydrated with 1.5ml sterile distilled water. Controls received 0.5ml ethanol only. After mixing, 1ml of a 500 nematode/ml suspension was added, and the vials were sealed and incubated in the dark at 20°C for 80 hours. Surviving nematodes were extracted (Section 3.3.4) and numbers lost expressed as a percentage of the initial population relative to the loss associated with the ethanol control (as above).

6.3 RESULTS

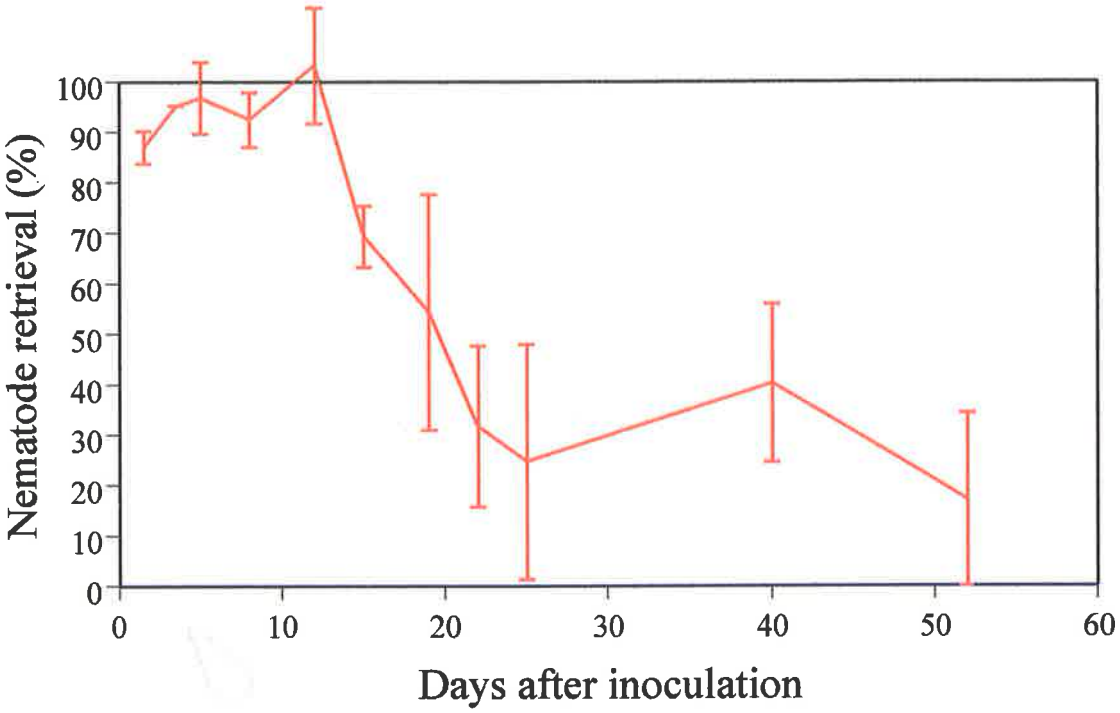
6.3.1 *Nematode survival in un-amended soil*

Almost all nematodes (>90%) in the microcosms could be recovered during the first twelve days (Figure 6.1). After this time, numbers declined slowly to approximately 30% of initial levels by day 22. After twelve days in the soil, many nematodes showed banding symptoms associated with starvation (Mai and Mullin, 1996). Nematode numbers recovered from replicate microcosms after day 22 were inconsistent, with some vials yielding no nematodes while others still yielded >90% of the original population (Table 6.1).

Table 6.1: Variation in the numbers of nematodes retrieved from individual microcosm vials containing UC soil after 25, 40 and 52 days with no host plant.

	Days since inoculation		
Replicate	25	40	52
1	0	52	429
2	588	88	0
3	0	490	0
4	0	143	0
5	27	234	0

Figure 6.1: Effect of time without a host on the retrieval of *P. neglectus* from sterile UC soil by misting. Means of five replicates and standard error of the mean displayed.



6.3.2 *Nematicidal effect of* B. napus *cv Dunkeld leaf and root tissues*

Approximately 95% of nematodes were retrieved from the unamended UC soil (Figure 6.2), confirming the efficiency of the mister technique and the ability of the nematode to survive within the microcosm environments for short periods (<12 days; Figure 6.1). Amendment of microcosms with 4g wheat tissues/100g soil led to a decline of approximately 30% from the initial nematode population regardless of the tissue type (leaf or root).

Brassica napus leaf tissue was associated with a decrease in nematode retrieval even at the lowest level of amendment (0.4% soil by weight; Figure 6.2). As the level of leaf amendment increased, *P. neglectus* populations were further depressed. Over 90% of the population was not retrievable above the 2g/100g level. Root tissue amendments did not significantly impact on the nematode populations at levels below 1.6g/100g soil (Figure 6.2). Once the 1.6g/100g soil level was passed, a rapid reduction in nematode retrievability was observed, resulting in the loss of approximately 90% of the population at the 2g/100g level.

6.3.3 *Nematicidal impact of six* Brassica *spp.*

Leaf tissues

The leaf tissue amendments led to the loss of more nematodes than did the root tissues for all the accessions examined (Figure 6.3). The leaf tissues from the accessions studied reduced nematode populations by a minimum of 55%. No significant difference was observed between the reduction in numbers associated with the *B. napus*, *B. nigra*, *B. rapa*, *B. carinata* or *B. juncea* leaf amendments. However, the *B. oxyrrhina* leaf tissues were associated with the loss of more nematodes than the leaves from the other accessions (>90% reduction).

No significant relationship was observed between any of the glucosinolates present in the leaves and the reduction in nematode numbers associated with the leaf tissue amendments (Figure 6.4 A-F). A base level of nematode loss (~55%) was associated with all the leaf amendments despite some containing negligible levels of glucosinolates (~8.8nmol/g soil and ~12.1nmol/g soil; *B. napus* and *B. rapa*, respectively). When this base level was subtracted from the observed data (leaving only the effect due to glucosinolates), a positive trend was observed between the reduction in nematode retrievability and tissue glucosinolates only after levels had exceeded 200nmol/g soil (*B. oxyrrhina* accession) (Figure 6.5).

Figure 6.2: Percentage reduction of *P. neglectus* populations with increasing levels of soil amendments of *B. napus* cv Dunkeld and wheat cv Machete leaf and root tissue (4g/100g soil). Means of seven replicates and standard errors of the means shown.

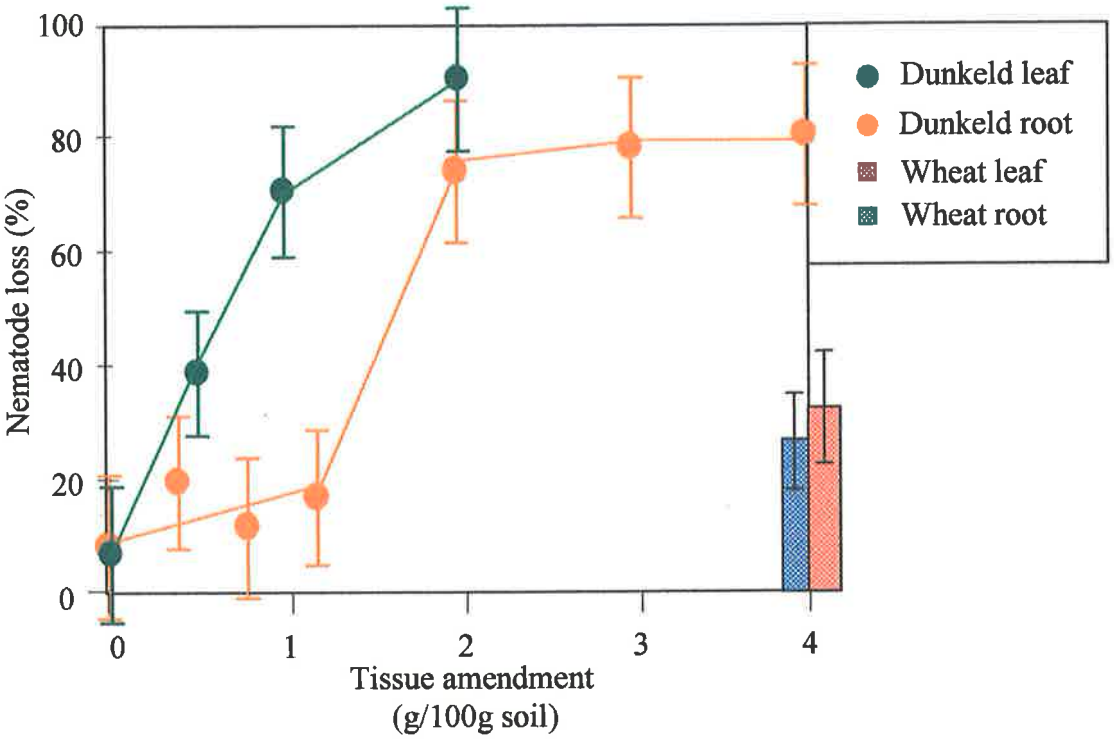


Figure 6.3: The reduction in retrievability of *P. neglectus* associated with 2g/100g soil amendments of *Brassica* spp. leaf and root tissues. Means of seven replicates and standard errors of the means are shown.

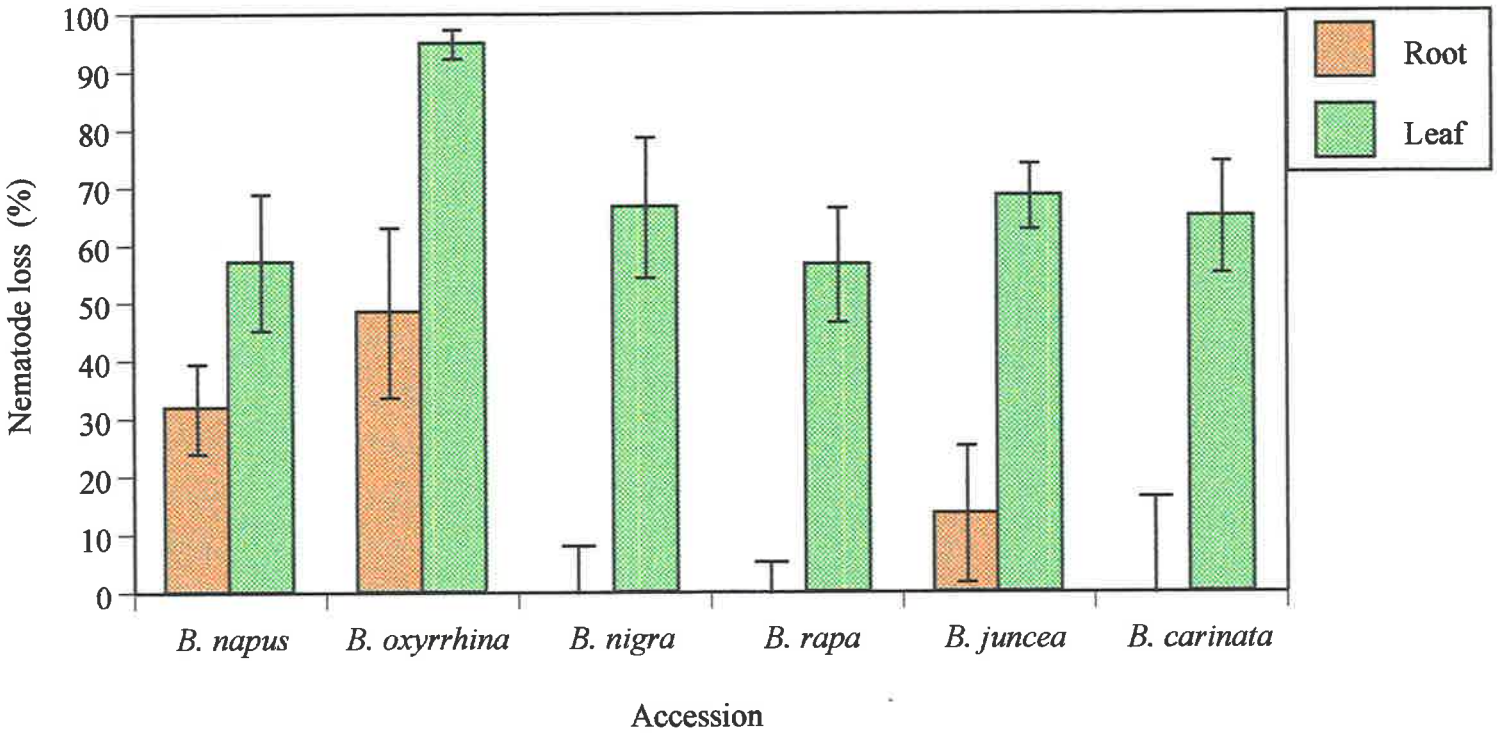


Figure 6.4: Correlation between reduction in *P. neglectus* populations associated with amendment of leaf tissue from six *Brassica* species and glucosinolate levels in the soil due to the amendments.

A = total; B = total aliphatic; C = total indolyl; D = 2-propenyl; E = 3-indolylmethyl; F = 2-phenylethyl glucosinolate.

1 = *B. napus*; 2 = *B. oxyrrhina*; 3 = *B. rapa*; 4 = *B. carinata*; 5 = *B. nigra*; 6 = *B. juncea* (as per Table 5.3).

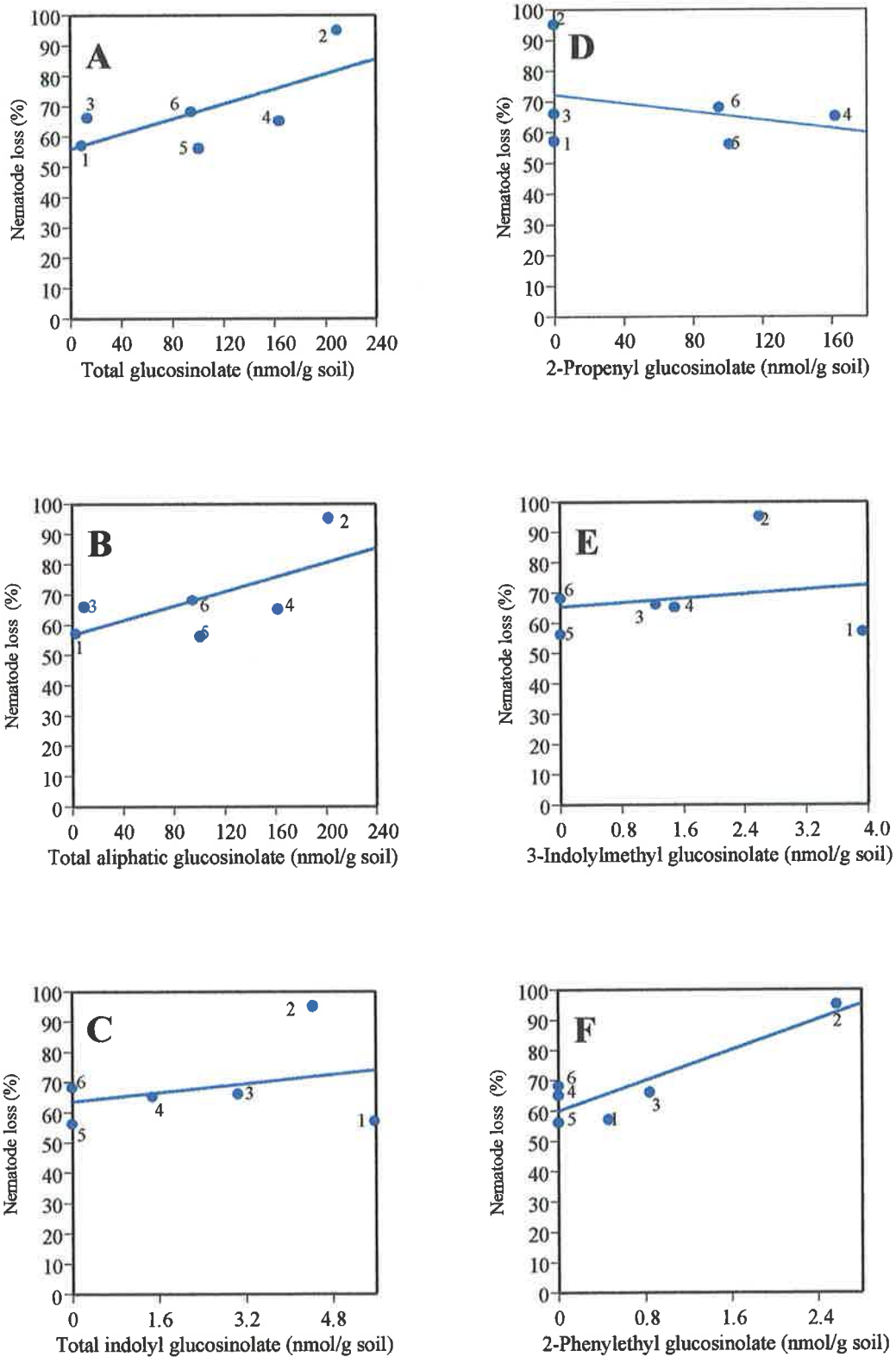
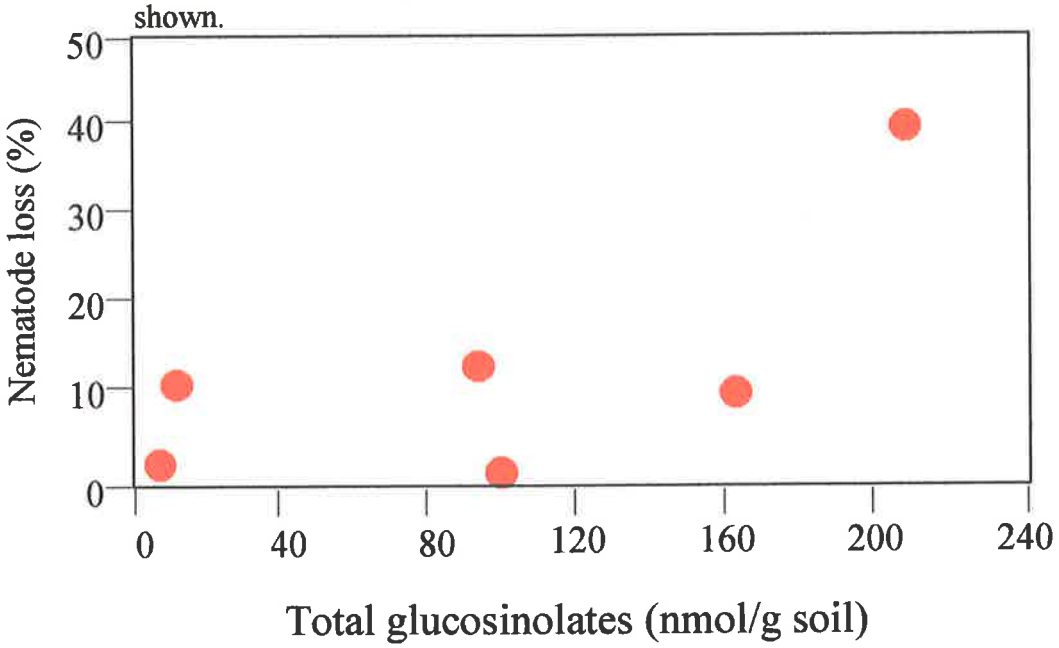


Figure 6.5: Loss of *P. neglectus* associated with total glucosinolates within six *Brassica* spp. leaf tissue amendments relative to the base level of nematode loss associated with *Brassica* leaf tissue containing no glucosinolates. Means of seven replicates shown.



Root tissues

The root tissues from the *B. nigra*, *B. carinata* and *B. rapa* accessions did not reduce nematode recovery relative to the wheat root tissues (Figure 6.3). However, the *B. juncea*, *B. napus* and *B. oxyrrhina* amendments were associated with a reduction in nematode recovery (13%, 38% and 42% relative to wheat root tissues respectively).

A positive relationship was observed between total glucosinolate levels and nematode loss due to the root amendments (Figure 6.6A). No relationship was observed between total aliphatic glucosinolates, or 2-propenyl glucosinolate and nematode loss (Figure 6.6B, C), despite amendments contributing more than 32nmoles glucosinolate/gram soil. However, a strong and significant positive relationship (R^2 = 0.98) was observed between nematode loss and soil levels of the aralkyl glucosinolate, 2-PE (Figure 6.6D). After a critical level was passed (~45nmol/g soil; *B. juncea* accession), increases in soil levels of 2-PE glucosinolate led to a linear increase in nematode loss (Figure 6.6E).

6.3.4 *Nematicidal variation in root components*

The amendment of soil with 2g/100g tap root tissue was associated with a greater loss of nematodes than the amendment of fine root tissues (~80% and ~60% relative to wheat, respectively; Figure 6.7). A strong and positive relationship (R^2=0.84) was observed between the levels of 2-PE glucosinolate and nematode loss associated with the tap root amendments (Figure 6.8). However, despite the fact that glucosinolate levels were comparable between the two root tissue types (Table 5.9), there was a poor relationship (R^2=0.21) between nematode loss and levels of 2-PE glucosinolate in fine root tissue amendments.

6.3.5 In vitro *studies with purified isothiocyanate*

The ethanol used as the control reduced nematode populations by approximately 40%. Allowing for this, increasing levels of 2-PE isothiocyanate were associated with a linear decrease in nematode retrievability (Figure 6.9).

Figure 6.6: Correlation between reduction in *P. neglectus* populations associated with amendments of root tissue from six *Brassica* species and glucosinolate levels in the soil due to the amendments.

A = total; B = total aliphatic; C = 2-propenyl; D = 2-phenylethyl; E = linear section of 2-phenylethyl glucosinolate.

1 = *B. napus*; 2 = *B. oxyrrhina*; 3 = *B. rapa*; 4 = *B. carinata*; 5 = *B. nigra*; 6 = *B. juncea* (as per Table 5.3).

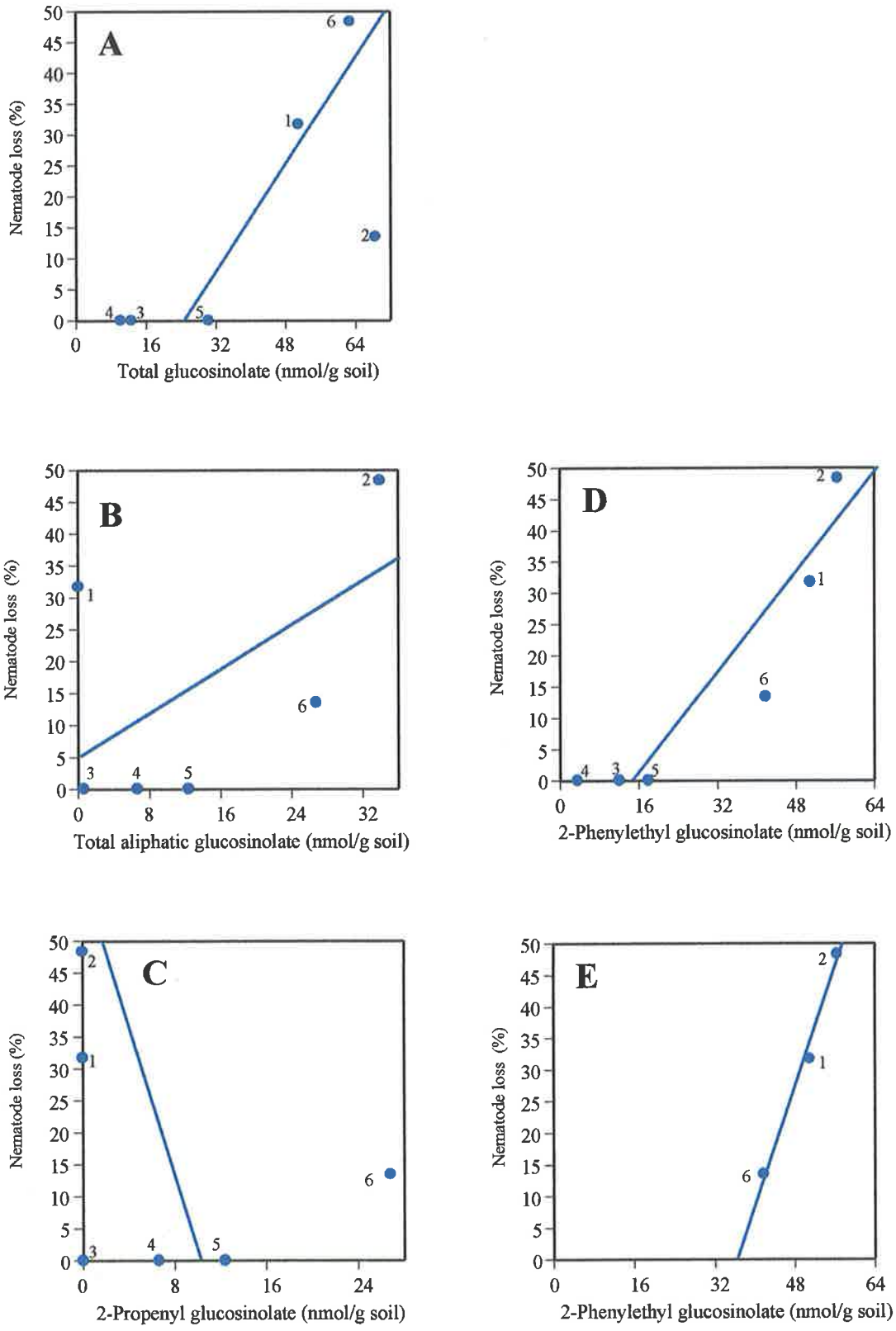


Figure 6.7: Nematicidal effect of *B. napus* cv Dunkeld fine and tap root amendments on *P. neglectus*. Means of seven replicates relative to wheat cv Machete root tissues and standard errors of the means shown.

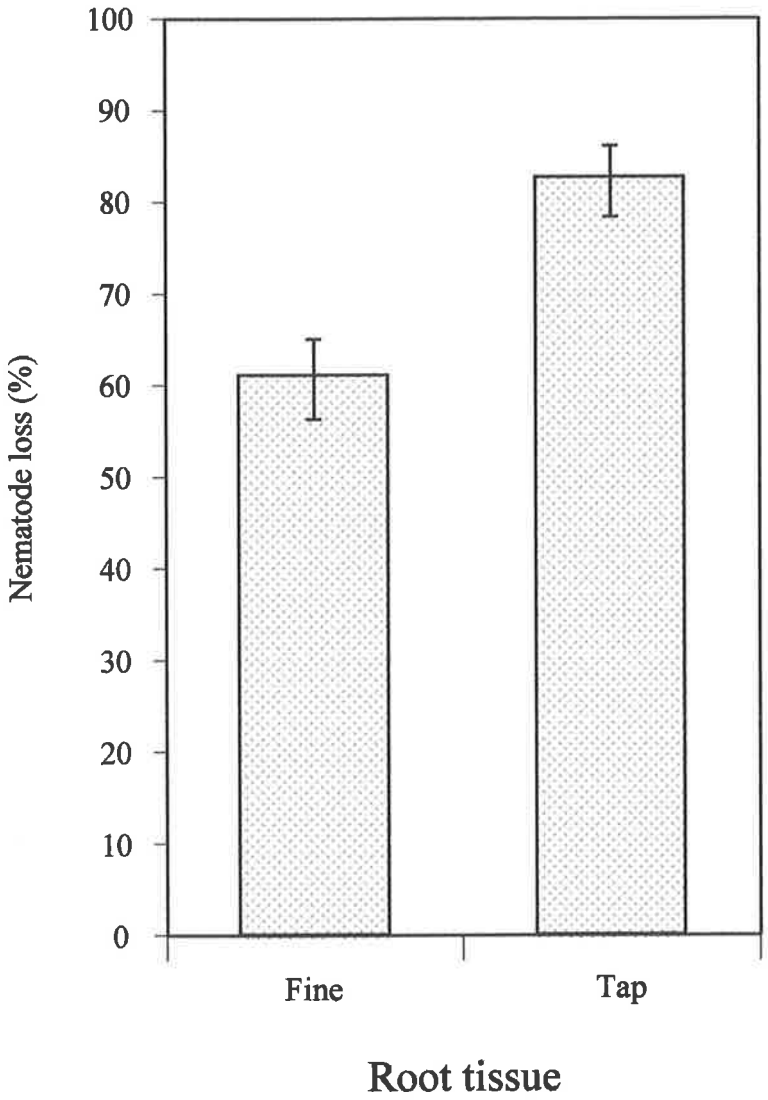


Figure 6.8: Relationship between suppression of *P. neglectus* by *B. napus* cv Dunkeld fine and tap root tissue amendments and soil levels of 2-phenylethyl glucosinolate due to tissue amendment.

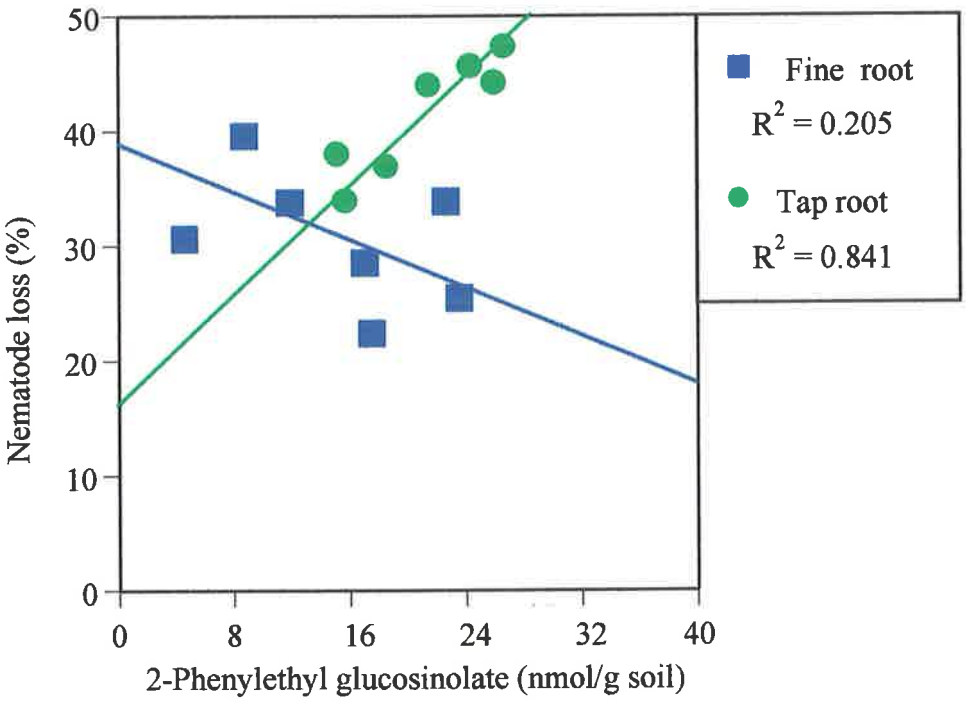
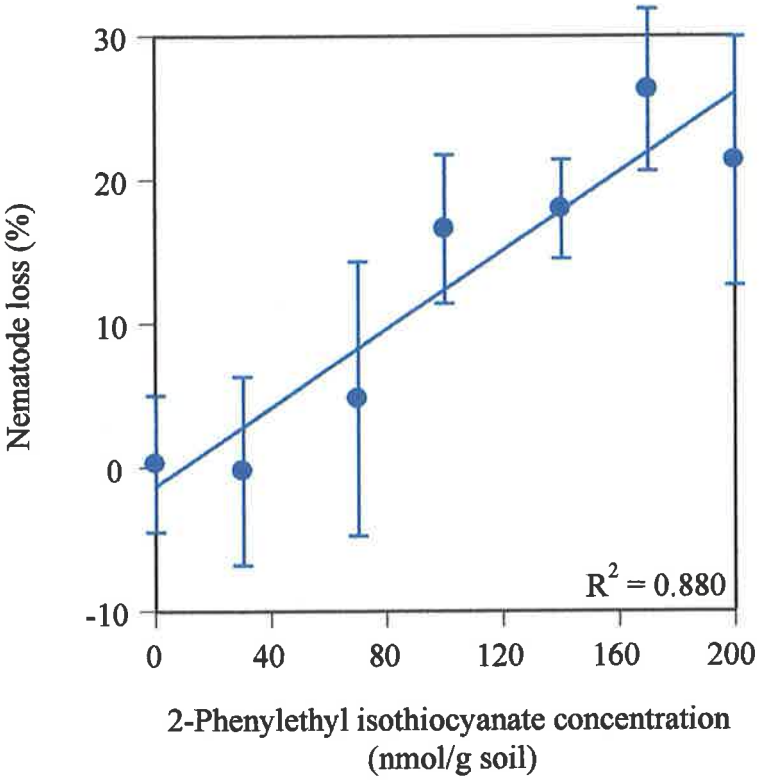


Figure 6.9: Impact of purified 2-phenylethyl isothiocyanate amendments upon the retrievability of *P. neglectus* in the soil. Means of seven replicates and standard errors of the means are shown.



6.4 DISCUSSION

6.4.1 *Nematode survival in microcosms*

Nematode survival within the microcosm is dependent on many factors, including temperature, soil moisture and micro-organism attack. However, as long as aseptic conditions are maintained, it should be expected that the predominant cause of nematode death over time within the microcosm would be starvation. In this study, large numbers of nematodes could be retrieved for the first twelve days, after which numbers declined slowly. After twelve days of incubation, many nematodes displayed banding characteristic of starvation (Mai and Mullin, 1996), suggesting that the lack of food was at least partially responsible for the declining numbers. However, some nematodes were retrieved from microcosms with no symptoms of starvation even after more than 50 days without food. As some vials in the latter periods of sampling yielded high numbers of nematodes, while others yielded very few, some factor other than starvation may have been involved in the decline of the nematode populations. These nematodes may have become inactive or quiescent, minimising use of energy reserves, which allowed them to avoid starvation during the period of the trial. Further studies are required to resolve this matter.

The major aim of this trial was to ensure that the microcosm environment did not adversely influence the recovery of nematodes during the incubation period in subsequent studies. As no significant nematode loss was observed within the first twelve days (280 hours) in the microcosm, it was concluded that the 80 hour period employed in subsequent trials would not significantly impact on the nematode populations.

6.4.2 *Nematicidal effect of* B. napus *cv Dunkeld leaf and root tissues*

Many of the natural degradation products of plant tissues are considered to have biocidal effects on soil organisms as the tissues break down in the soil (Vera *et al.*, 1987; McEwen *et al.*, 1989; Christen *et al.*, 1992). As the studies in this chapter aimed to investigate the relationship between glucosinolates within *Brassica* tissues and the nematicidal impact of the tissues, it was important to discriminate between the impact of general vegetative tissue (as discussed by Vera *et al.*, 1987) and glucosinolate related loss. As *Brassica* leaf tissues containing negligible glucosinolate levels were still observed to exhibit strong nematicidal qualities (Figure 6.4), a means to approximate the non-glucosinolate nematicidal qualities of plant tissues was required. Hence, wheat tissues, containing no glucosinolates, were

employed as a control in these studies, providing a gauge of the nematode loss associated within vegetative tissues alone.

The nematicidal impact of the leaf and root tissues differed in the studies undertaken. At the lower rates, leaf tissue amendments appeared more toxic to the nematodes than the root tissues, being associated with significant loss at 0.4g/100g soil while roots had no effect at levels less than 2g/100g soil. This was not unexpected as leaf tissues have been generally considered to be more potent than root tissues throughout the literature, possibly as they generally contain a higher glucosinolate content (Brown and Morra, 1997). Increases in the level of leaf tissues were associated with a gradual reduction in nematode retrieval (Figure 6.2), suggesting that the nematicidal component(s) of the leaves were highly potent in the soil environment. In contrast, a similar increase in root tissue amendments did not lead to decreases in nematode retrieval until root amendments exceeded 1.2g/100g soil. However, once a critical level had been passed (>1.2g/100g soil), nematode control was maximised (~90% population lost) and further decreases in retrievability were not observed. This suggests a basic difference in the nematicidal component(s) of the leaf and root tissues.

This study aimed to determine a minimum level of tissue amendment which could be used in subsequent nematode control studies. The studies described here revealed that the same level of tissue was required to reach maximum nematode loss (~90% of population) by both leaf and root tissues (2g/100g soil), despite the greater impact of leaf tissues at lower rates compared to roots. The 2g/100g soil amendment level was therefore adopted for all further studies.

6.4.3 *Nematicidal impact of six* Brassica *spp*

Leaf tissues

Leaf tissues from all accessions examined were more potent than root tissues. Most biocidal studies of *Brassica* spp. have examined the leaf tissues (Brown and Morra, 1997), with few studies of root tissues (Glenn *et al.*, 1988; Schreiner and Koide, 1993; Angus *et al.*, 1994; Potter *et al.*, 1998). The observations of much of the literature have suggested that the greater nematicidal potency of the leaf tissues may be due to the fact that they have higher levels of glucosinolates (compare Tables 5.5 and 5.6; Sang *et al.*, 1984). However, as will be discussed below, this does not seem to be the case.

The lack of variation in impact of the leaf tissues of the various accessions examined, excluding *B. oxyrrhina*, was unexpected given the variation in the glucosinolate profiles between the species, particularly in 2-propenyl glucosinolate levels (Table 5.5; Sang *et al.*, 1984). The glucosinolate profile of the tissue did not seem to directly influence the nematicidal impact of the leaf tissues. A significant loss in nematodes (>55%) was associated with both *B. napus* and *B. rapa* leaf tissues, despite the fact that they imparted negligible levels of glucosinolates to the soil (<20nmol/g soil; Figure 6.4A). It therefore seems likely that other factors, independent of the glucosinolate allelopathic system, contributed to the nematicidal impact of the leaf tissues. Many biologically active organic chemicals, including alkenals and alkanols, are released from senescent tissues (Bradow, 1991; Jiang *et al.*, 1996) and may contribute to the nematicidal impact of the *Brassica* leaf tissues.

Subtracting the base level of loss (where negligible glucosinolates were present), showed that only the levels of glucosinolates within *B. oxyrrhina* leaf amendments were associated with a significant decrease in nematode retrieval (Figure 6.5). It was possible that the particularly high total levels of glucosinolate within the *B. oxyrrhina* tissue passed a critical level (>160nmol/g soil) after which sufficient isothiocyanate was liberated to impact on the nematode population. As only the glucosinolates of this one accession were related to any effect, no correlation between individual glucosinolates and the nematicidal potency of the tissue could be made. However, the presence of very high levels of 3-butenyl and 4-pentenyl glucosinolates within the *B. oxyrrhina* amendment (leading to soil levels of >160 and 30nmol per gram soil, respectively) may have been responsible for the observed loss above the base level. This is supported by Mojtahedi and Santo (1996), who observed significant loss in *P. penetrans* numbers in soils amended with purified 3-butenyl isothiocyanate.

Amendments with *B. nigra*, *B. juncea* and *B. carinata* leaf tissues did not significantly alter nematode retrieval beyond the base level, despite imparting high levels of 2-propenyl glucosinolate (>160nmol/gram soil). This was in contrast with findings of Mojtahedi and Santo (1996) which suggest that soils amended with 2-propenyl isothiocyanate were more nematicidal than those amended with 3-butenyl isothiocyanate. Mayton *et al.* (1996) and Williams *et al.* (1993) also reported high biocidal activity of 2-propenyl isothiocyanate. Liberation of this isothiocyanate from the glucosinolate may have been inhibited in some way

in the work reported here, or the structure or small molecular weight of the isothiocyanate may have limited its effectiveness within the soil environment.

The results reported here suggest some scope to improve the potential of *B. napus* leaf tissues through inter-specific crossing with other species, such as *B. oxyrrhina*. However, as breeding efforts to increase the nematicidal impact of the leaf tissue are not relevant to the Australian broadacre cereal cropping in which green manuring is not practiced, this issue was not pursued further. Horticultural industries, in which incorporation of entire immature crops into the soil as a green manure can be economic, could, however benefit from such breeding work.

Root tissues

While no reduction in nematode retrievability was associated with root tissues from the *B. rapa, B. nigra* or *B. carinata* accessions examined, significant reductions were associated with the *B. oxyrrhina, B. napus* and *B. juncea* root amendments. As *B. oxyrrhina* leaves were the most potent observed, increases in the potential of *B. napus* roots may be possible through inter-specific crossing with this line. However, the extensive differences in phenotype between the two species makes it unlikely that the cross would be successful commercially. Many undesirable traits from the *B. oxyrrhina* line, such as poor seed quality and yield, would be introduced into *B. napus* canola. However, this study does have implications for the improvement of *B. juncea* roots, as the more potent alleles could be transferred from *B. napus* into *B. juncea* using hybridisation techniques similar to those employed by Somda *et al.* (1995).

Glucosinolate levels were closely associated with nematode loss in the root studies, in contrast with the leaf studies. The strong, positive relationship between total levels and tissue potency (Figure 6.6A) suggests that one or more of the glucosinolates within the root profile contributed to the impact of the amendments. As in the leaf studies, no relationship was observed between nematode loss and any of the aliphatic glucosinolates, including 2-propenyl, despite significant levels (Figure 6.6B, C), further suggesting that 2-propenyl isothiocyanate release or activity was not effective within the soil environment of the microcosm. However, the strong relationship between nematode loss and the levels of 2-PE glucosinolate in the soil (Figure 6.6D) suggested that this glucosinolate was involved in the

nematicidal potential of the tissues. The nematode suppression occurred after a critical level of 2-PE glucosinolate had been imparted via the tissues amendments (~45nmol/g soil). This implies that the nematode response to the isothiocyanate may be dose dependent, or that perhaps the soil environment requires saturation with isothiocyanate before enough is free to impact on the nematodes. As 2-PE glucosinolate contributed between 40% and 90% of the total glucosinolates within the tissues examined (Table 5.6), the positive relationship observed between total glucosinolates and tissue potency (Figure 6.6A) is likely to be due to the strong association with 2-PE glucosinolate.

6.4.4 *Nematicidal variation in root components*

The observed difference in nematicidal potency of the fine and tap roots is significant given that both tissues contained similar total of total glucosinolate levels (Table 5.9). This observation suggested that the level of glucosinolates within the root tissues was not directly related to the nematicidal potency of the tissue, and that the reduction in nematode populations observed may have been due to another factor.

However, Figure 6.8 suggested that 2-PE glucosinolate levels were integrally associated with the potency of the tap root tissues. The differences between the impact of the fine and tap roots may therefore be related to a difference in the myrosinase-mediated release of isothiocyanate from the glucosinolates within the two tissues. As the nematicidal impact of these tissues differed (Figure 6.7), despite the fact that levels of 2-PE glucosinolate were not significantly different (Table 5.9), there may have been differences in the levels of myrosinase or numbers of 'myrosin' cells in the tissues. Bones and Rossiter (1996) found that myrosinase, responsible for the glucosinolate hydrolysis releasing isothiocyanate, was located in distinct cells, discretely distributed throughout the cortex of the tap root tissues (Plate 2.3). If the distribution of this enzyme was not uniform throughout the tissues of the plant and/or its level differed between the fine and tap roots, less enzyme may have been present to cleave glucosinolates in the fine root amendments, despite the fact that the two tissues contained similar levels of glucosinolates. To explain the differences between the nematicidal potential of these tissues, further microcosm studies, which include the incorporation of active myrosinase with the two tissue types, are required.

6.4.5 In vitro *studies with purified isothiocyanate*

As mentioned in Section 2.5.6, the association between glucosinolates and the nematicidal potential of the tissue is likely to be due to the release of isothiocyanates after damage, rather than to any toxic qualities of the glucosinolates themselves. Quantification of glucosinolate levels therefore provide only an indication of the potential isothiocyanate release. The biocidal properties of 2-PE isothiocyanate have been reported from *in vitro* studies using insect eggs (Borek *et al.*, 1995), fungi (Drobnica *et al.*, 1967) and cultured human lymphoma cells (Horakova, 1966). Mojtahedi and Santo (1996), examining a range of isothiocyanates, omitted 2-PE isothiocyanate, but found that benzyl isothiocyanate (also aralkyl) exhibited strong nematicidal qualities. The studies above (Figure 6.9) demonstrated the nematicidal qualities of 2-PE isothiocyanate within the soil environment, supporting the assertion of a relationship between tissue 2-PE glucosinolate levels and amendment related nematode loss (Section 6.4.3).

The literature has embraced the biocidal qualities of aliphatic isothiocyanates (particularly 2-propenyl), leading to the development of aliphatic-based commercial fumigants such as Vapam and Metham Sodium (using methyl isothiocyanate; Saeed *et al.*, 1996). However, the conclusions of the literature have been based primarily upon *in vitro* studies of the purified chemical, or upon correlation between the biocidal qualities of the leaf tissues containing high 2-propenyl glucosinolate levels. If aliphatic isothiocyanates do not act efficiently in *in vivo* studies, as supported by Matthiesson *et al.* (1996), and a second system of allelopathy does exist within the leaf tissues (as postulated after examination of Figures 6.4 and 6.5), the association between aliphatic isothiocyanates and *in vivo* tissue activity in the soil might be overstated by the literature. Aliphatic glucosinolates are, after all, found in only limited amounts within the roots of many *Brassica* species. While the literature does not consider the nematicidal qualities of 2-PE isothiocyanate, the results of this chapter suggest that it does have a significant and negative impact on *P. neglectus* in the soil. Further *in vivo* studies are now required to compare the nematicidal potential of 2-PE and aliphatic isothiocyanates, such as 2-propenyl, in an effort to better determine the nematicidal potency of plants containing differences in the levels of these chemicals within their tissues.

CHAPTER 7

SUSCEPTIBILITY OF *BRASSICA* TISSUES TO *P. NEGLECTUS*

7.1 INTRODUCTION

The number of *P. neglectus* in the soil following a canola crop will be a function of the crop's qualities as a host for the nematode during the season (susceptibility), and the nematicidal impact of the tissues as they degrade in the soil at the end of the season. Consequently, efforts to enhance the ability of canola crops to control *P. neglectus* within the cereal rotation must both consider the nematicidal potency of the tissues (as discussed in Chapter 6) and the susceptibility of the crop to the nematode.

The *Brassica* are generally considered to be susceptible to root lesion nematodes (Acedo and Rohde, 1971; Evans and Webb, 1989; Webb, 1996), although Vanstone *et al.* (1993) found that the degree of susceptibility to different *Pratylenchus* species varied. The nematodes damage the roots by feeding on root hairs as ectoparasites, and also by invading the cortex and penetrating individual cells (Webb, 1990; Zunke, 1990a; Zunke, 1990b). Given that damaged *Brassica* tissues are known to be nematicidal (Chapter 6; Brown and Morra, 1997), it is surprising that the nematodes can invade at all. However, as discussed in Chapter 6, the discrete distribution of the enzyme myrosinase may allow nematodes to infect the roots without releasing the nematicidal compounds. The nematodes may instinctively avoid the myrosin cells or may cause insufficient damage to release enough isothiocyanate to act as a deterrent to feeding or multiplication.

This chapter describes investigations to determine the degree of variation in susceptibility to *P. neglectus* within common *Brassica* species, in an effort to determine the potential to increase the resistance of common *B. napus* canola lines through breeding. Further, the relationship between susceptibility and root glucosinolates is explored, to evaluate the potential to improve plant resistance by increasing root glucosinolate levels.

7.2 MATERIALS AND METHODS

7.2.1 *Impact on nematode recovery of glucosinolates lost during the mister treatment*

This study was undertaken to determine the impact of glucosinolates lost from root tissues during the mister procedure (as reported in Table 5.12) on nematode recovery. The susceptibility of eight plants from *B. napus* cv Dunkeld, and wheat cv Machete, respectively, was tested as outlined in Section 3.4. However, once plants had been removed from the soil and roots washed, the root systems were weighed and divided into two parts with a razor blade, each containing approximately equal amounts of tap, secondary lateral and fine roots. Half of the root material was placed in the mister apparatus to determine the degree of nematode infestation (Section 3.4), while the other half was stained (Section 3.4.8) to enable nematodes to be counted in root squashes.

7.2.2 *Variation in susceptibility within the* Brassica

Thirty one accessions were selected for study (Table 7.1), representing the species *B. napus*, *B. oxyrrhina*, *B. juncea*, *B. carinata*, *B. nigra* and *B. rapa*. *Triticum aestivum* cv Machete plants were used as the control in this study. Ten replicates of each accession were each sown into single 300ml pots, and grown under the controlled conditions outlined in Section 3.4.2. After two weeks, plants were inoculated with 1000 *P. neglectus* (J2-adult) as discussed in Section 3.4.4. After six weeks root tissues were harvested (Section 3.4.5) and nematodes extracted (Section 3.4.6) and counted to determine susceptibility relative to wheat roots, as described in Section 3.4.7.

Table 7.1: Accessions selected to assess variation in the susceptibility of *Brassica* spp. to *P. neglectus*.

Species	Accession	Species	Accession
B. napus	Lirawell	*B. napus*	AG32
B. napus	Cresus	*B. oxyrrhina*	Oxy 1
B. napus	Lirakotta	*B. oxyrrhina*	Oxy 2
B. napus	Bievenu	*B. rapa*	90119
B. napus	Oscar	*B. rapa*	90139
B. napus	Ridana	*B. rapa*	Turnip 1
B. napus	Korina	*B. nigra*	95067
B. napus	Start	*B. nigra*	Black Mustard
B. napus	Dunkeld	*B. nigra*	91072
B. napus	Midas	*B. juncea*	Mustard 1
B. napus	Norin 16	*B. juncea*	Mustard 2
B. napus	Tamara	*B. juncea*	94200
B. napus	Eureka	*B. juncea*	Cutlass
B. napus	Libravo	*B. carinata*	94044
B. napus	Rangi	*B. carinata*	94036
B. napus	Narendra		

7.2.3 *Root glucosinolates and susceptibility within the* Brassica

These studies were undertaken to relate the susceptibility of *Brassica* spp. to *P. neglectus* to the levels of glucosinolates within their roots.

Trial 1 *Preliminary correlation between field and glasshouse tested plants*

The accessions selected for study are presented in Table 7.2. Glucosinolate analyses were performed by Dr J. Kirkegaard and Dr M. Sarwar (CSIRO Plant Industry, Canberra), following a similar protocol to that outlined in Section 3.4. Twenty plants were sown into each of three replicate plots at Ginninderra, Canberra, on the 20th of May, 1996, and the root tissues were harvested as each accession came to flower (between 107 and 126 days, depending on the accession). At harvest, the whole root system was removed from the soil with a shovel, then washed before lyophilisation. Glucosinolates were extracted from 0.3g sub-samples from a bulk of tissue from >10 plants from each plot, and presented as a mean level for each accession.

The susceptibility to nematodes of ten glasshouse grown plants from each accession was individually assessed at the University of Adelaide, Waite Agricultural Research Institute, following the protocol outlined in Section 7.3.4. The mean nematode numbers from all ten replicates of each accession were calculated for comparison with the glucosinolate levels determined at CSIRO, Canberra.

Table 7.2: Accessions selected to assess the relationship between the levels of 2-phenylethyl glucosinolates in the field and the susceptibility of the plants to *P. neglectus* in the glasshouse (Trial 1).

Accession	Species	Accession	Species
Lirawell	*B. napus*	Syn	*B. napus*
Dunkeld	*B. napus*	90119	*B. rapa*
Cresus	*B. napus*	90139	*B. rapa*
Lirakotta	*B. napus*	94044	*B. carinata*
Bievenu	*B. napus*	94036	*B. carinata*
Oscar	*B. napus*	91072	*B. nigra*
Ridana	*B. napus*	95067	*B. nigra*
Korina	*B. napus*	94200	*B. juncea*
Midas	*B. napus*	90300	*B. juncea*
Norin 16	*B. napus*	Cutlass	*B. juncea*
Tamara	*B. napus*		

This study was performed to gain an indication of the relationship between the susceptibility of plants to *P. neglectus* and the levels of glucosinolates within the roots. However, as the tissues were produced under different environments, it was likely that the levels of glucosinolates within the glasshouse and field grown plants were not comparable (Josefsson, 1970; Zhao *et al.*, 1994). Consequently, the results presented in this section were used as a guide only, leading to two further trials which were designed to minimise the impact of such variation.

Trial 2 Brassica *spp. studies*

In order to maximise the chances of detecting variation in root glucosinolates, accessions were selected from a broad genetic base, representing the 6 major *Brassica* genomes. The same six accessions used to examine inter-specific variation in glucosinolates (Chapter 5) and nematicidal potential (Chapter 6) were selected for this study. The details of these accessions are presented in Table 5.3. Ten plants from each accession were sown into single 300ml pots, and grown under the controlled conditions outlined in Section 3.4.2. However, due to loss of plants during the trial, only 10 *B. napus*, 8 *B. carinata*, 2 *B. oxyrrhina*, 4 *B. nigra*, 8 *B. rapa* and 6 *B. juncea* replicates were assessed, making a total of 38 individual plants.

The susceptibility of these plants was assessed as discussed in Section 3.4. However, the method was modified in that the root systems were weighed after washing and divided in half with a razor blade, each containing approximately equal amounts of tap, lateral and fine roots. Half the root material was placed in the mister apparatus to extract nematodes (as described in Section 3.4.6). The other half was placed directly into liquid nitrogen, lyophilised, bulked for each accession, ground and total, 2-propenyl and 2-PE glucosinolates were assessed as in Section 3.5.

Trial 3 B. napus *studies*

Variation in root glucosinolate levels was observed between the individual plants from the variety *B. napus* cv Dunkeld (Table 5.8). Based upon this variation, this study aimed to compare the susceptibility of current canola varieties with their total and 2-PE glucosinolate levels. The accessions chosen for study were Oscar (10 replicates), Dunkeld (7), Barossa (10), Yickadee (7), Narendra (6), Karoo (6), Hyola (12), Monty (9), LL96-2 (9) and AGA95-01 (9), making a total of 85 plants. Plants were grown, harvested and assessed as in Trial 2.

7.3 RESULTS

7.3.1 *Impact on nematode recovery of glucosinolates lost during the mister treatment*

Marginally more nematodes were found using the staining procedure than with the misting technique, for wheat and canola (Table 7.3). No significance differences in detected nematodes could be attributed to two different methods.

Table 7.3 Mean numbers of *P. neglectus* within wheat cv Machete and *B. napus* cv Dunkeld roots as detected by two different assessment techniques (staining and misting). Means of eight replicates for each crop are shown.

	Stain (nematodes/ g dry root)	Mister	Ratio (sem)
Wheat	1615	1396	1.22 (0.51)
Canola	1727	1677	1.13 (0.23)

7.3.2 *Variation in susceptibility within the* Brassica

A high degree of variability in susceptibility was observed between the different plants within the accessions studied (Figure 7.1). Plants from *B. napus* accessions yielded from 20% to almost 200% as many nematodes as the Machete control. However, while this 'within accession' variation was sufficient to confound statistical studies between the accessions studied, trends were observed in the susceptibility of the different species (Figure 7.2). The *B. napus* populations as a whole (based upon the mean of the population) hosted fewer nematodes than the Machete wheat control which is considered very susceptible (Vanstone *et al.*, 1993). The two *B. rapa* accessions showed similar susceptibility to the wheat. The roots of those accessions containing the B genome (*B. juncea*, *B. carinata* and *B. nigra*) all yielded greater numbers of nematodes than Machete. In contrast, both *B. oxyrrhina* accessions showed very low levels of susceptibility relative to all other accessions examined.

7.3.3 *Root glucosinolates and susceptibility within the* Brassica

Trial 1 *Preliminary correlation between field and glasshouse tested plants*

At lower glucosinolate levels (<10µmol/g tissue; Figure 7.3), no relationship could be observed between root levels of 2-PE glucosinolate and the susceptibility of those roots to *P. neglectus*. However, tissues with greater than 10µmol/g tissue did show significantly reduced susceptibility to the nematode (Table 7.4).

Figure 7.1: Variation in susceptibility to *P. neglectus* of single plants from *B. napus* cv Hyola (A) and Dunkeld (B) relative to the susceptibility of wheat cv Machete. Nematode number = (total nematodes recovered/mean number of nematodes recovered from Machete control).

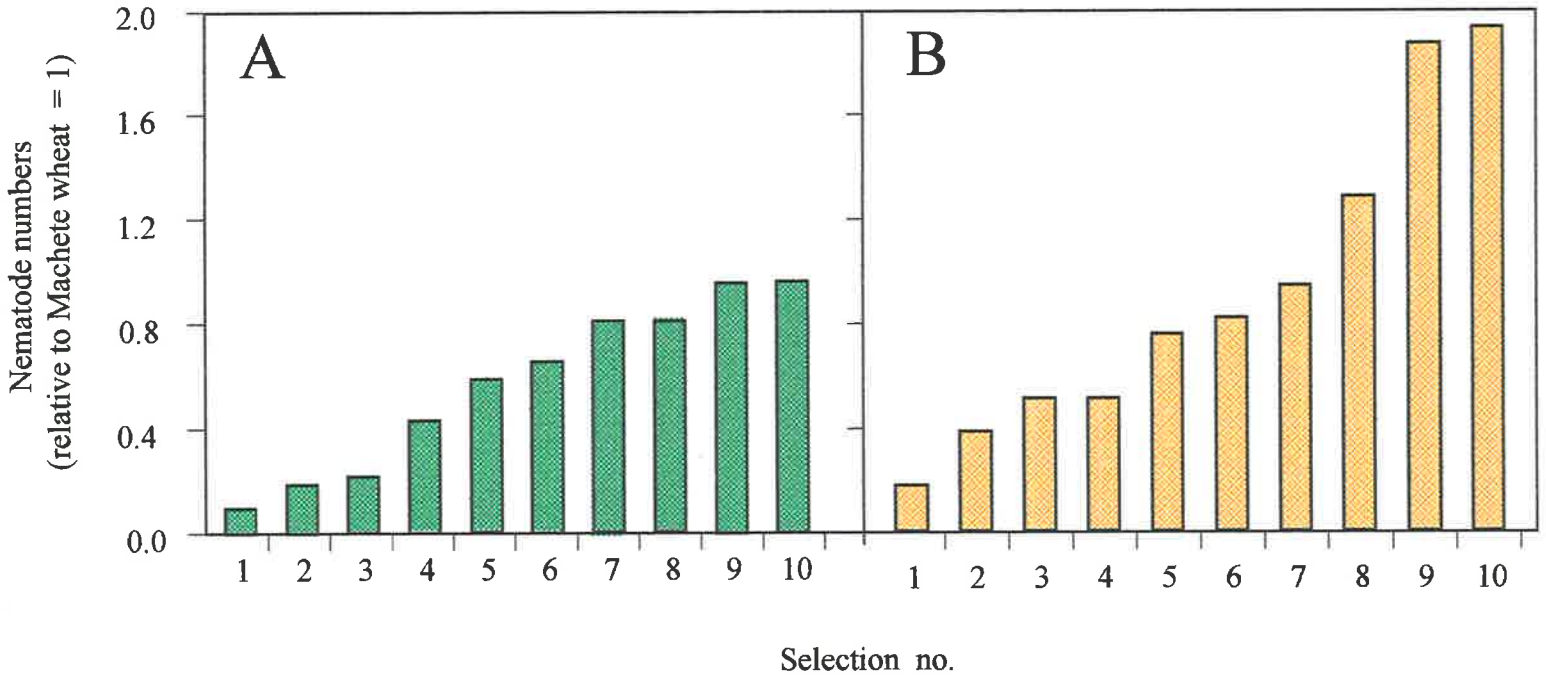


Figure 7.2: Variation in susceptibility to *P. neglectus* within 32 accessions from different *Brassica* spp. relative to the susceptibility of wheat cv Machete (=1). Means of ten individual plants shown. High levels of variation within the plants of the accessions led to no significant difference between the lines. However, the individual species were significantly different from one another (see graph inset below).

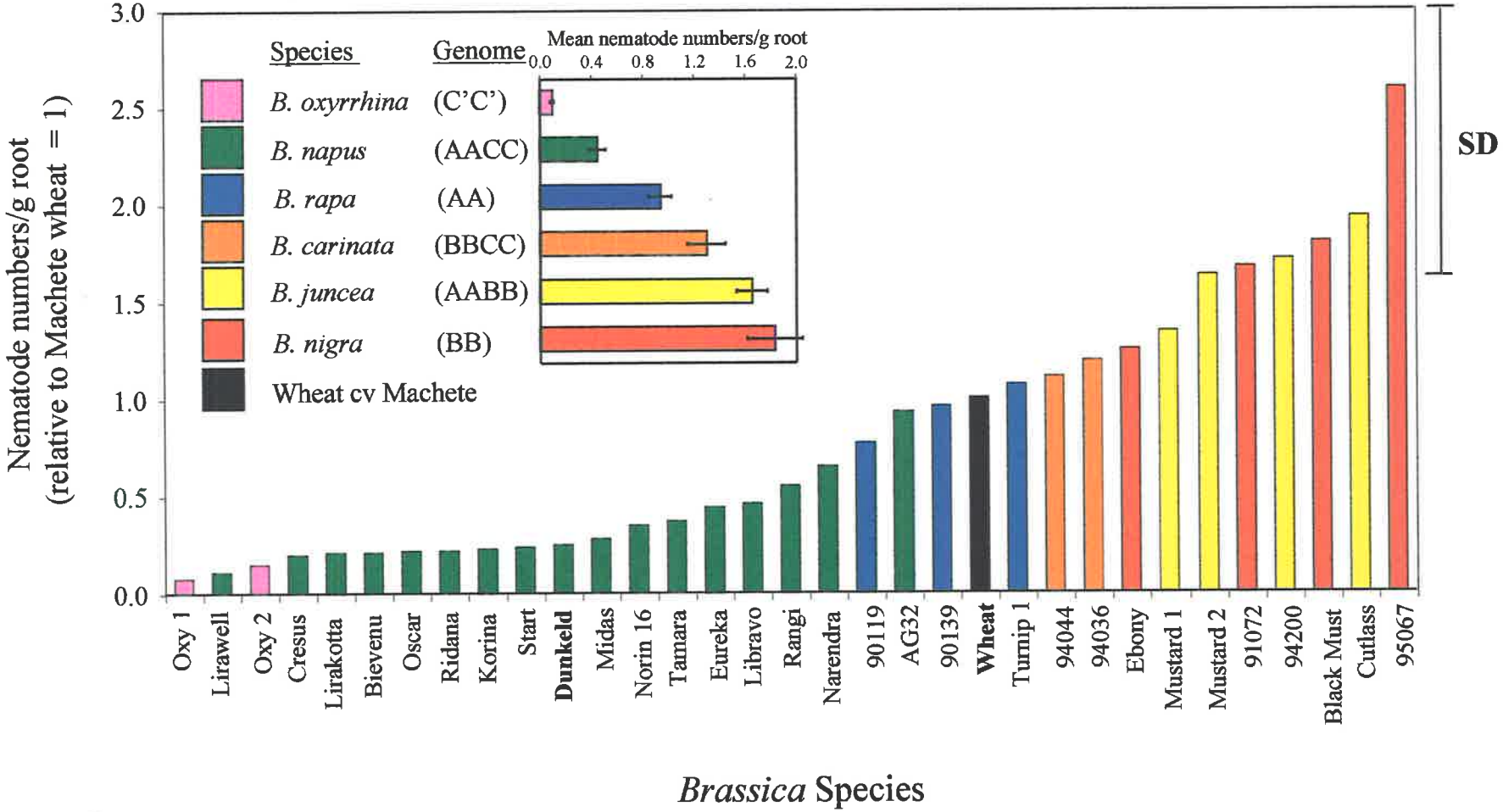


Figure 7.3: The relationship between the susceptibility of *Brassica* spp. to *P. neglectus* of 21 accessions grown in the glasshouse and root levels of 2-phenylethyl glucosinolates in the same accessions grown in the field. Each point represents mean susceptibility (ten replicates) and 2-phenylethyl glucosinolate levels in a bulked sample of > ten plants from each *Brassica* spp. accession. Susceptibility is expressed relative to the susceptibility of wheat cv Machete (=1).

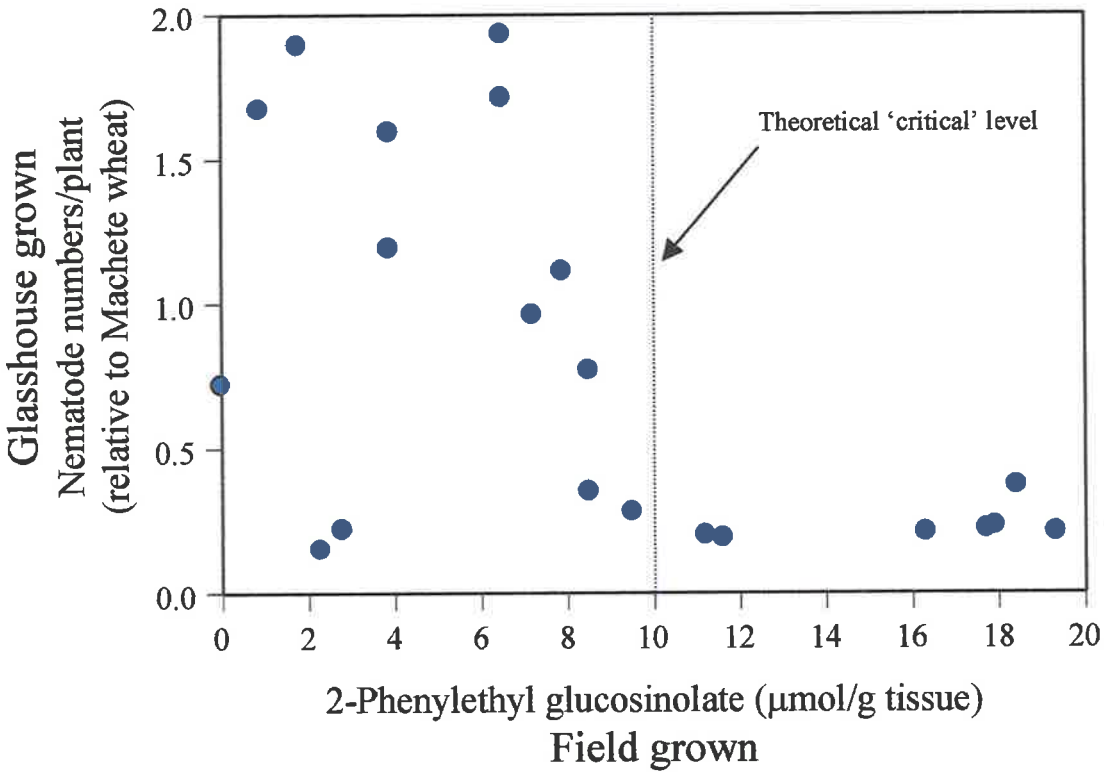


Table 7.4: Average nematode recovery from the roots of individual plants from accessions from six different *Brassica* spp. with levels of 2-phenylethyl glucosinolate below or above a theoretical critical level.

Range of 2-phenylethyl (µmol/g tissue)	No. plants Assessed	Average nematode recovery (relative to Machete wheat)	Standard error
0-10	14	1.04	0.17
10-19	7	0.23	0.02

Trial 2 Brassica *spp. studies*

No relationship was found between total and 2-propenyl glucosinolates and the susceptibility to nematodes of the individual plants from the six *Brassica* species examined (Figure 7.4). However, when 2-PE glucosinolate levels were compared with susceptibility, a pattern developed, such that accessions with glucosinolate levels above a critical level (~8µmol/g tissue) had significantly fewer nematodes in the roots (Table 7.5).

Table 7.5: Average nematode recovery from the roots of individual plants from accessions from six different *Brassica* spp. with levels of 2-phenylethyl glucosinolate below or above a theoretical critical level.

Range of 2-phenylethyl (µmol/g tissue)	No. plants assessed	Average nematode recovery (relative to Machete wheat)	Standard error
0-8	31	0.54	0.07
8-14	7	0.27	0.06

Trial 3 B. napus *studies*

Examination of the relationship between the susceptibility of *B. napus* to *P. neglectus* and total glucosinolates suggested that, as total levels rose beyond 20µmol glucosinolate/g tissue, the numbers of nematodes recovered from the roots declined (Figure 7.5). This pattern was accentuated in the comparison between susceptibility and the concentrations of 2-PE glucosinolate (Figure 7.5), where nematode recovery from the roots actually began to decline after 2-PE glucosinolate levels exceeded 12µmol glucosinolate/g tissue (Table 7.6).

Table 7.6: Average nematode recovery from the roots of individual plants from accessions from six different *Brassica* spp. with levels of 2-phenylethyl glucosinolate below or above a theoretical critical level.

Range of 2-phenylethyl (µmol/g tissue)	No. assessed	Average nematode recovery (relative to Machete wheat)	Standard error
0-12	71	0.68	0.08
12-20	13	0.46	0.06

Figure 7.4: The relationship between susceptibility to *P. neglectus* and root levels of 2-propenyl, 2-phenylethyl and total glucosinolates within single plants from six different *Brassica* spp. grown in the glasshouse. Each point represents the susceptibility and glucosinolate levels of a single plant. Susceptibility is expressed relative to that of wheat cv Machete (=1).

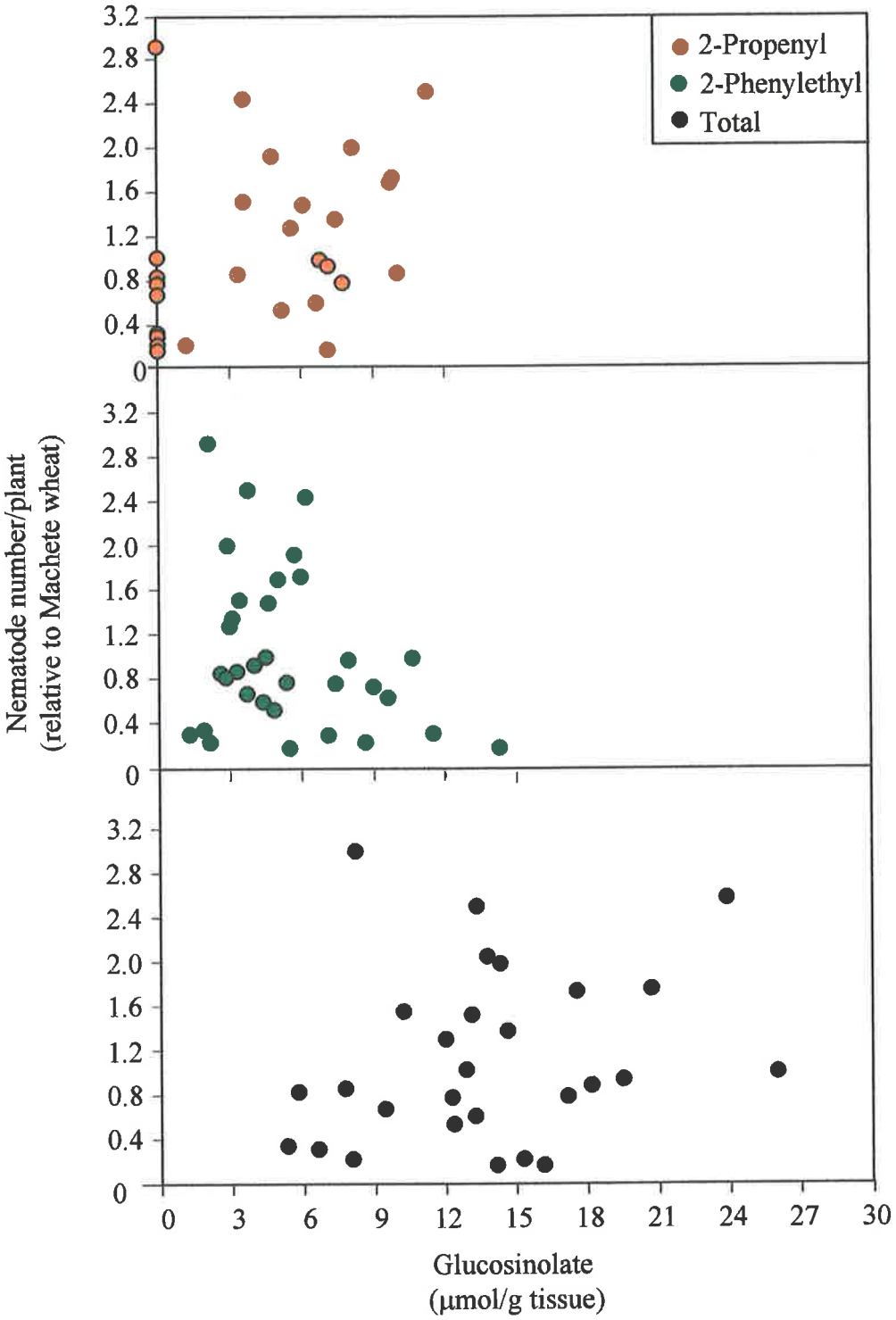
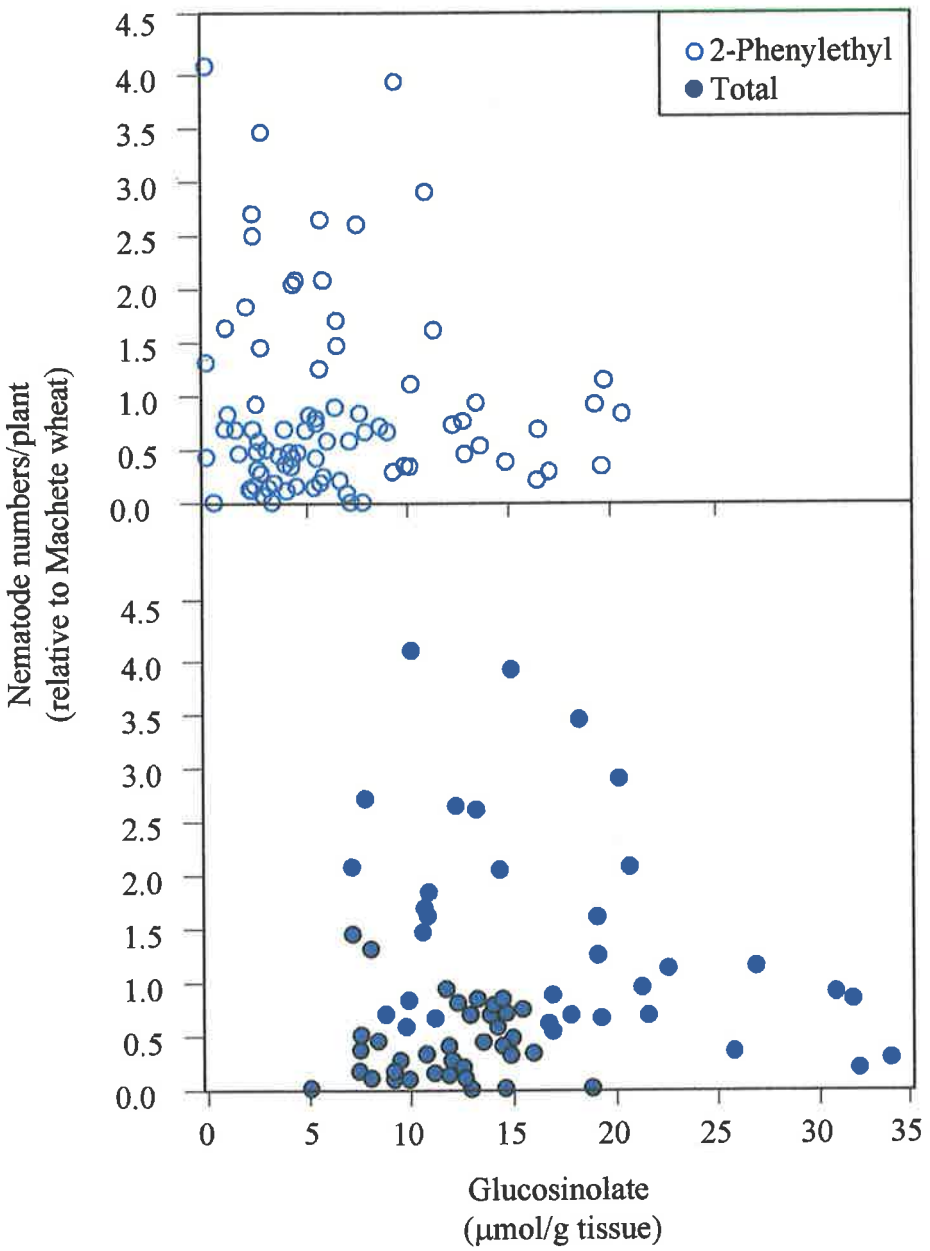


Figure 7.5: The relationship between susceptibility to *P. neglectus* and root levels of 2-phenylethyl and total glucosinolates within single plants from ten common *B. napus* accessions grown in the glasshouse. Each point represents the susceptibility and glucosinolate levels of a single plant. Susceptibility is expressed relative to that of wheat cv Machete (=1).



7.4 DISCUSSION

7.4.1 *Impact on nematode recovery of glucosinolates lost during the mister treatment*

As root sampling is likely to result in the loss of glucosinolates (Underhill, 1980), the *in vivo* study of glucosinolates within *Brassica* tissues requires that tissue damage is minimised, and that tissues are frozen or dried as soon as possible after sampling. Unfortunately, the recovery of nematodes from *Brassica* roots (when testing for plant susceptibility) using a mister apparatus dictated that the fresh tissue remained at room temperature for an extended period (>100 hours). As reported in Section 5.4.8, a significant loss in glucosinolates from *B. napus* cv Dunkeld root tissues was observed during this period (particularly in 2-phenylethy; (2-PE) glucosinolate, which decreased by approximately 35%). It is not known if the loss in glucosinolate is due to myrosinase activity, but if so, the products of myrosinase hydrolysis, particularly isothiocyanate, may influence the recovery of nematodes during the period on the mister apparatus.

Although fewer nematodes were detected using the mister method than by staining, the degree of the decline did not significantly differ between the Dunkeld and Machete root tissues (Table 7.3). If the glucosinolates lost from the *Brassica* tissues were affecting the potential to recover the nematodes by misting, significant differences associated with the impact of the mister between the *Brassica* and the wheat tissues (containing no glucosinolates) would be expected. As this was not the case (Table 7.3), the loss of glucosinolates from the *Brassica* tissues (as observed in Section 5.4.8) does not appear to influence the recovery of the nematodes extracted using the mister technique. Thus, a reasonable level of confidence can be placed on the use of this method for use in the following studies.

7.4.2 *Variation in susceptibility within the* Brassica

Given the outcrossing nature of *B. napus*, and that canola breeding programs have not yet targeted resistance to *P. neglectus*, it was not surprising to find a substantial degree of variation in the susceptibility of individual plants within the accessions examined. If glucosinolates affect the susceptibility of the plant, the significant levels of glucosinolate variation within the tissues of individual plants (as reported in Section 5.4.4) might also contribute to the variation in susceptibility observed (Figure 7.1). Variation was greater within plants from the Dunkeld accession (ranging from 20% to 190% of Machete) than within plants from Hyola (ranging from 10% to 90% of Machete). Hyola plants are F1

hybrids, and therefore it is not surprising that they exhibit less phenotypic variation than conventionally produced crops.

Despite the high levels of variation in susceptibility *within* the accessions, Figure 7.2 shows a strong trend in susceptibility *between* the different species examined. More nematodes were recovered from the plants of all *B. napus* accessions than were inoculated, suggesting that the species is susceptible to *P. neglectus*, and supporting the findings of Vanstone *et al.* (1993), Bernard and Montgomery-Dee (1993) and Webb (1996). However, of all the commercial *Brassica* species examined, *B. napus* canola lines had the greatest resistance to *P. neglectus.* Consequently, limited potential exists to improve current *B. napus* canola lines through inter-specific hybridisation with already agronomically adapted accessions. While the two *B. oxyrrhina* lines did appear less susceptible to the nematode than most *B. napus* lines studied, their use in the improvement of *B. napus* through hybridisation is limited due to the potential for introduction of undesirable traits to the *B. napus* accessions, as discussed in Section 6.4.3.

The high susceptibility of the *B. nigra*, *B. carinata* and *B. juncea* accessions suggests a link between susceptibility and the B genome, present in all three species (U, 1935). The mean nematodes numbers recovery from these lines was in the order of 150% to 200% of Machete wheat, itself considered to be a susceptible variety (Vanstone *et al.*, 1993). These results have implications for the use of B genome crops in districts with established *P. neglectus* problems, particularly in the application of 'canola quality' mustards (*B. juncea*) being developed for use within the southern Australian cereal rotation. The widespread use of such crops could lead to increased numbers of *P. neglectus* in the field, particularly as the root residues do not appear to have a significant nematicidal effect as they break down in the soil (Figure 6.3). However, this method of assessment considers susceptibility in isolation, and ignores the possibility of subsequent nematicidal impact of the degrading *Brassica* root and leaf tissues (as discussed in Chapter 6). Consequently, the observed susceptibility of the crops may be offset by a reduction in nematode populations due to degrading plant materials in the soil.

7.4.3 *Root glucosinolates and susceptibility within the* Brassica

The three trials examining the relationship between susceptibility and root glucosinolate levels were carried out as a logical progression, each trial reducing potentially confounding external factors. Trial 1 was a preliminary study only, with plants grown in radically different environments, which could have caused variation in the levels of glucosinolates within the

compared tissues (Josefsson, 1970; Zhao *et al.*, 1994). The comparison considered the mean glucosinolate and nematode levels of plant populations, and thus did not take into account the variability within the individual plants, as observed in Section 5.4.4 (glucosinolates) and in Section 7.4.2 (susceptibility). Trial 2 and Trial 3 were performed to address these problems. The relationship between susceptibility and glucosinolate levels was assessed in individual plants by dividing the root systems in half, each section assessed for a separate character. This method overcame the problems of variability due to environment and the genetic diversity between the plants. Trial 2, in an effort to examine as much potential variation as possible, employed individual plants from a broad range of *Brassica* spp. However, the results from this trial were likely to be confounded by genetic variation between the different species examined. Consequently, Trial 3 was designed to limit this genetic variability by examining plants from a single species (*B. napus*). A fourth trial, examining plants from a single *B. napus* accession, Dunkeld, (observed to contain variation in both glucosinolates (Table 5.8) and susceptibility (Figure 7.1B)) was not undertaken due to the time constraints of the project.

All three trials showed a negative relationship between the levels of 2-PE glucosinolate in the roots and the susceptibility of the accession. If no relationship existed between this glucosinolate and the susceptibility of the tissues, data points would have been arrayed in a random fashion. Instead, accessions containing 2-PE glucosinolates beyond a critical level of contained fewer nematodes in the roots (Figures 7.3, 7.4 and 7.5). In Trial 2, no such relationship was observed between susceptibility and total glucosinolates (Figure 7.5), supporting the hypothesis that the individual glucosinolates have different roles within the tissues of the *Brassica* (Giamoustaris *et al.*, 1994). In Trial 3, higher total glucosinolate levels were associated with nematode susceptibility, as totals passed 25µmol/g tissue (Figure 7.5). However, as a stronger relationship was observed when comparing susceptibility to 2-PE glucosinolate levels (Figure 7.5), and due to the fact that this glucosinolate contributes approximately 75% of the total (Table 5.7), it is likely that the observed relationship between susceptibility and total glucosinolates was due to the high levels of 2-PE glucosinolate. No relationship was observed between susceptibility and 2-propenyl glucosinolate, despite being present at levels comparable to 2-PE, suggesting that the levels of this glucosinolate did not affect the ability of the nematodes to penetrate, feed and multiply within the root systems. The hypothesis that 2-propenyl glucosinolate does not play a significant role in the nematode relations of the roots was supported by the findings of Section 6.3.3, where no relationship

could be discerned between this glucosinolate and the nematicidal potential of the tissue, despite being present at levels comparable to 2-PE glucosinolate.

Many other factors may be involved in determining *Brassica* susceptibility to *P. neglectus*. In all three trials, plants containing negligible levels (<3µmol/g tissue) of 2-PE glucosinolate were observed to have very few nematodes in the root systems. While beyond the scope of this study, opportunity obviously exists to decrease the susceptibility of *B. napus* crops by identifying and selecting for these alternative resistance mechanisms. However, high levels of 2-PE glucosinolate within the roots appear to aid in the plant's defence against the nematode. Efforts to increase the mean levels of 2-PE glucosinolate within *Brassica* roots should lead to decreases in susceptibility to the root lesion nematode. Increased root levels of 2-PE glucosinolate may also reduce the susceptibility of the plants to other *Pratylenchus* spp., and to other plant parasitic nematodes which infest the roots in a similar fashion to *Pratylenchus*.

CHAPTER 8

GENETIC VARIATION IN 2-PE GLUCOSINOLATE IN *BRASSICA* ROOT TISSUES

8.1 INTRODUCTION

The levels of 2-PE glucosinolate within *Brassica* roots are closely linked to the susceptibility of the plants to the root lesion nematode (*P. neglectus*) and to the nematicidal qualities of the tissues as they degrade in the soil (Chapters 6 and 7). Consequently, canola varieties with increased levels of 2-PE glucosinolate in the roots are likely to be more efficient as a rotational break against *P. neglectus*, allowing greater control of the pest within the cereal rotation.

The biosynthesis of the different glucosinolates relies upon a complex biochemical pathway, using protein amino acids as precursors (Figure 2.2). While high total levels of glucosinolates in the seed are heritable (Rucker and Robbelen, 1994; Lethenborg, 1995), efforts to manipulate these levels have been limited by the number of genes involved. The low seed glucosinolate phenotype, first observed in the line Bronowski, is controlled by at least four recessive genes (Rucker and Robbelen, 1994).

Studies of the inheritance of aliphatic glucosinolates within vegetative tissues suggest that the partitioning of substrates for individual glucosinolate synthesis is under genetic control independent of total glucosinolate production (Magrath *et al.*, 1993; Rucker and Robbelen, 1994; Stringam and Thiagarajah, 1995). The model proposed by Magrath *et al.* (1993) suggests that once the glucosinolate precursors have been synthesised, individual aliphatic glucosinolates are produced by the manipulation and elongation of the side chain, controlled by simple Mendelian genetics. While no information could be found describing the inheritance of 2-PE glucosinolates, Dawson *et al.* (1993) suggested that the biosynthesis of the aralkyl (from phenylalanine) and aliphatic (from methionine) glucosinolate precursors occurred in a similar fashion. If the side chain manipulation of the aralkyl glucosinolate precursor is controlled in a similar manner to the aliphatic system (Magrath *et al.*, 1993), the inheritance of 2-PE glucosinolate should be controlled by relatively simple genetics. The inheritance within *B. napus* root tissues is likely to be particularly simple, as 2-PE glucosinolate is the only aralkyl glucosinolate found within these tissues (Table 5.6; Sang *et al.*, 1984).

As mentioned in Section 2.5.8, the genetic variation within *B. napus* is thought to be limited due to the small number of original crosses from which the crop developed (Thormann *et al.*, 1994). Despite this limitation, significant variation in the levels of 2-PE glucosinolate in the roots has been observed within *B. napus* cv Dunkeld (Table 5.8). Should there be a genetic basis to this variation, it would be possible to increase the mean level of 2-PE glucosinolate within the roots of *B. napus* canola varieties, and thus improved disease break qualities, simply by applying a selection pressure favouring plants with high root levels of 2-PE glucosinolate.

8.2 MATERIALS AND METHODS

8.2.1 *Selection for variation in levels of 2-PE glucosinolate in* B. napus *cv Dunkeld*

Inter-varietal variation and selection of parental material

Sixty-five single *B. napus* plants from four varieties (11 Westar, 7 Topas, 17 Narendra and 30 Dunkeld) were grown individually in 200mm pots containing UC soil under standard glasshouse conditions (Section 3.5.1). Root tissues were harvested at floral initiation, washed and placed into liquid nitrogen. Glucosinolates were assessed as described in Section 3.4. The plant tops were retained and treated to initiate adventitious root formation (Section 3.3.6) and rooted plants were returned to UC soil in 200mm pots. As the plants began to flower, floral heads were bagged to ensure self pollination. S_1 seed was collected as the siliques matured.

Nine single plants (designated D1-D9) from the parental population of *B. napus* cv Dunkeld were selected for further study, representing intermediates and extremes of the variation in root levels of 2-PE glucosinolate, as observed by HPLC analysis.

Assessment of the S_1 material and generation of S_2 populations

S_1 seed collected from each of the nine selections (described above) was sown singly into 200mm pots containing UC soil. The numbers of S_1 seed sown from each selection were D1 (19 plants), D2 (12), D3 (13), D4 (18), D5 (13), D6 (15), D7 (16), D8 (16) and D9 (15). Fifteen *B. napus* cv Dunkeld plants (parental population) were also grown, to monitor environmental influences upon root 2-PE glucosinolate levels. S_1 and parental plants were grown under standard glasshouse conditions and roots were harvested for glucosinolate assessment at floral initiation (Section 3.5). The plant tops were grown on by vegetative

propagation and flowering heads bagged to ensure self pollination. S_2 seed was harvested as the siliques matured. S_1 seed collected from D1, D5 and D9 was sent to Mr Wayne Burton, Victorian Institute for Dryland Agriculture, Horsham, Victoria, Australia for assessment of glucosinolate levels by NIR (Near Infra-Red) spectroscopy.

Four of the nine S_1 populations were selected for further study, representing the extremes of the variation in root levels of 2-PE glucosinolate. D2 and D3 represented the 'high' extremes of the S_1 populations and D8 represented the 'low' extreme. D6 was selected, as the D6-S_1 progeny displayed heterogeneity in terms of root 2-PE glucosinolate levels and therefore may have undergone segregation.

Assessment of the S_2 material

The S_2 seeds from a single S_1 selection were sown individually into 200mm pots containing UC soil. As the D6-S_1 population appeared to contain two different phenotypes in terms of 2-PE glucosinolate levels (high and low), two selections were made, representing the extremes of each sub-population. The number of S_2 seed sown from each S_1 selection was D2 (29 plants), D3 (20), D6 'high' phenotype (39), D6 'low' phenotype (41), and D8 (28). Twenty five *B. napus* cv Dunkeld plants (parental population) were also grown and assessed as a control, to determine the effects of the selection program on the levels of 2-PE glucosinolate within the populations. Plants were grown under standard glasshouse conditions and the roots harvested for glucosinolate assessment at floral initiation (Section 3.5). Plant tops once again were retained, and treated to form adventitious roots (Section 3.6), and grown to flowering. Floral heads were bagged to ensure self pollination. However, due to a heavy infestation of white fly (*Trialeurodes vaporariorum*), no S_3 seed was recovered from these lines.

8.2.2 *Susceptibility to* P. neglectus *of S_2 selections from* B. napus *cv Dunkeld*

The susceptibility of a parent population of *B. napus* cv Dunkeld plants to *P. neglectus* was compared to that of the D6-S_2 'high' and D6-S_2 'low' *B. napus* cv Dunkeld selections. Seven plants from each line were sown individually into 300ml pots, and the susceptibility of these plants to the nematode was assessed (Section 3.4).

8.3 RESULTS

8.3.1 *Selection for variation in 2-PE glucosinolate in* B. napus *cv Dunkeld*

Total glucosinolate levels are readily influenced by environmental variation (Poulton et al., 1993) while the individual glucosinolates contributing to that total are generally considered to be influenced only by genetic variation (Magrath et al., 1993). Consequently, the variation in 2-PE glucosinolate levels within Dunkeld was displayed as a percentage of the total levels within the tissue. While efforts were made to limit environmental variation throughout these studies, selection for percentage (%) 2-PE glucosinolate levels limited the potential for environmental variation to impact on our selection decisions.

Inter-varietal variation

A high degree of variability was observed in the root levels of 2-PE glucosinolate within the individual plants from both the *B. napus* cv Dunkeld and *B. napus* cv Westar populations. *B. napus* cv Narendra and *B. napus* cv Topas exhibited less intra-varietal variation (Figure 8.1). While differences existed in the degree of variation observed between the varieties, similar mean levels of total and 2-PE glucosinolate were observed (Table 8.1), supporting findings presented in Table 5.8.

Table 8.1: Levels of total and 2-phenylethyl glucosinolate within the roots of four *B. napus* canola varieties. Means and standard deviations are shown.

Variety	Number of plants	Total glucosinolate (µmol/g tissue) Mean	SD	2-Phenylethyl glucosinolate (µmol/g tissue) Mean	SD
Dunkeld	30	12.39	6.59	9.55	5.96
Narendra	17	11.84	3.34	8.23	2.42
Topas	7	9.00	1.55	6.30	1.59
Westar	11	10.98	4.33	6.85	4.27

Parental generation

B. napus cv Dunkeld was selected for further study as it contained the greatest variation in root 2-PE glucosinolate levels (Tables 5.8 and 8.1; Figure 8.2). Nine selections (D1-D9) were chosen to represent the intermediates and extremes of % 2-PE glucosinolate levels within the parental population (Figure 8.2).

Figure 8.1: Variation in 2-phenylethyl levels relative to total glucosinolates within populations of plants from four *B. napus* canola accessions (Dunkeld, Narendra, Topas and Westar).

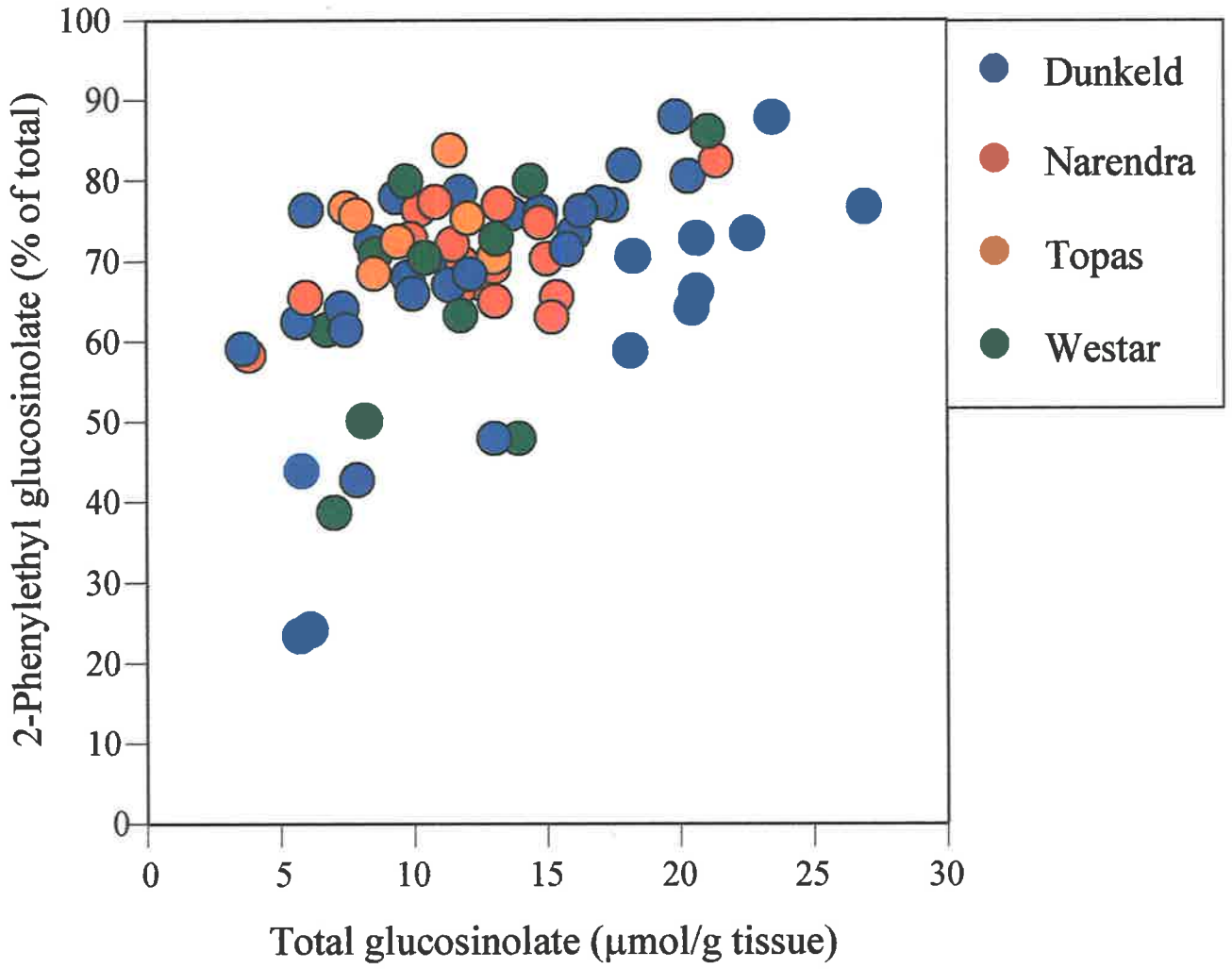
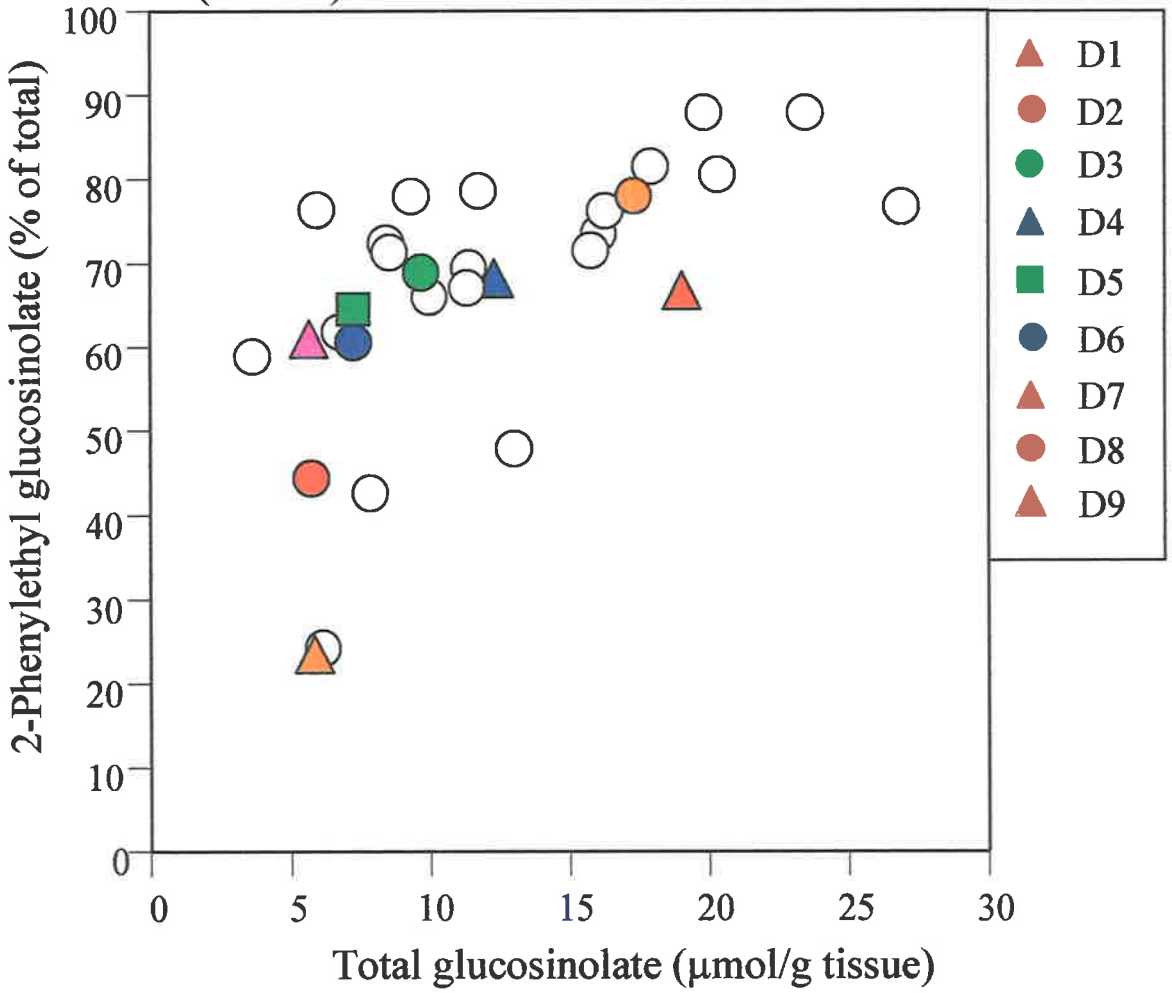


Figure 8.2: Variation in 2-phenylethyl levels relative to total glucosinolates within parental population of *B. napus* cv Dunkeld. Individual plants selected for further study are shown in colour (D1-D9).



S_1 generation

Considerable variation was present within the S_1 selections in traits such as flowering time, vernalisation requirement and nutrient efficiency (Plate 8.1). In terms of root glucosinolates, the S_1 population separated into two distinct groups (Figure 8.3), distinguished by levels of 2-phenyethyl glucosinolate as 'high' (>45% of total) and 'low' (<45%) phenotypes. S_1 plants selected from parents at the extremes (D1, D2 'high', D8, D9 'low') of the population (Figure 8.2) maintained the root phenotypes of their parental lines. The parent lines with phenotypes closer to the mean of the original population (D3, D4, D5, D7) produced S_1 progeny exhibiting a single phenotype at one of the extremes of the original population (Table 8.2). The S_1 population derived from the D6 parent line showed both phenotypes, with the population divided between the 'high' and the 'low' phenotypes in a 6 'high' to 8 'low' ratio. Four of the S_1 populations (two 'high', D2, D3; one 'low', D8; and one possible segregating population, D6) were retained for further study (Figure 8.4).

A second distribution was apparent within the S_1 populations. Examination of Figure 8.3 revealed both a 'high' and 'low' *total* glucosinolate phenotype, truncated at 10 µmol/glucosinolate per gram root. The D1, D4, D5, D7, and D8 parental plants and S_1 populations contain 'low' total glucosinolates, distinct from the D9 parent and S_1 population which contained 'high' total levels. The D3-S_1 population, produced from an intermediate parent plant, appeared to be splitting between the two phenotypes.

Table 8.2: Variation in the percentage 2-phenylethyl glucosinolate of the total glucosinolates of the root tissue of *B. napus* cv Dunkeld parental and S_1 selections.

Parental line	Parental phenotype	S_1 Phenotype
D1	High	High
D2	High	High
D3	Medium	High
D4	Medium	High
D5	Medium	Low
D6	Medium	Heterogeneous
D7	Medium	High
D8	Low	Low
D9	Low	Low

Plate 8.1: Morphological variation within S1 populations selected from *B. napus* cv Dunkeld. Lines differed in such traits as flowering time, nutrient efficiency and vernalisation requirement

Figure 8.3: Variation in 2-phenylethyl levels relative to total glucosinolates within S_1 progeny arising from self fertilisation of nine *B. napus* cv Dunkeld parent selections (as outlined in Figure 8.2).

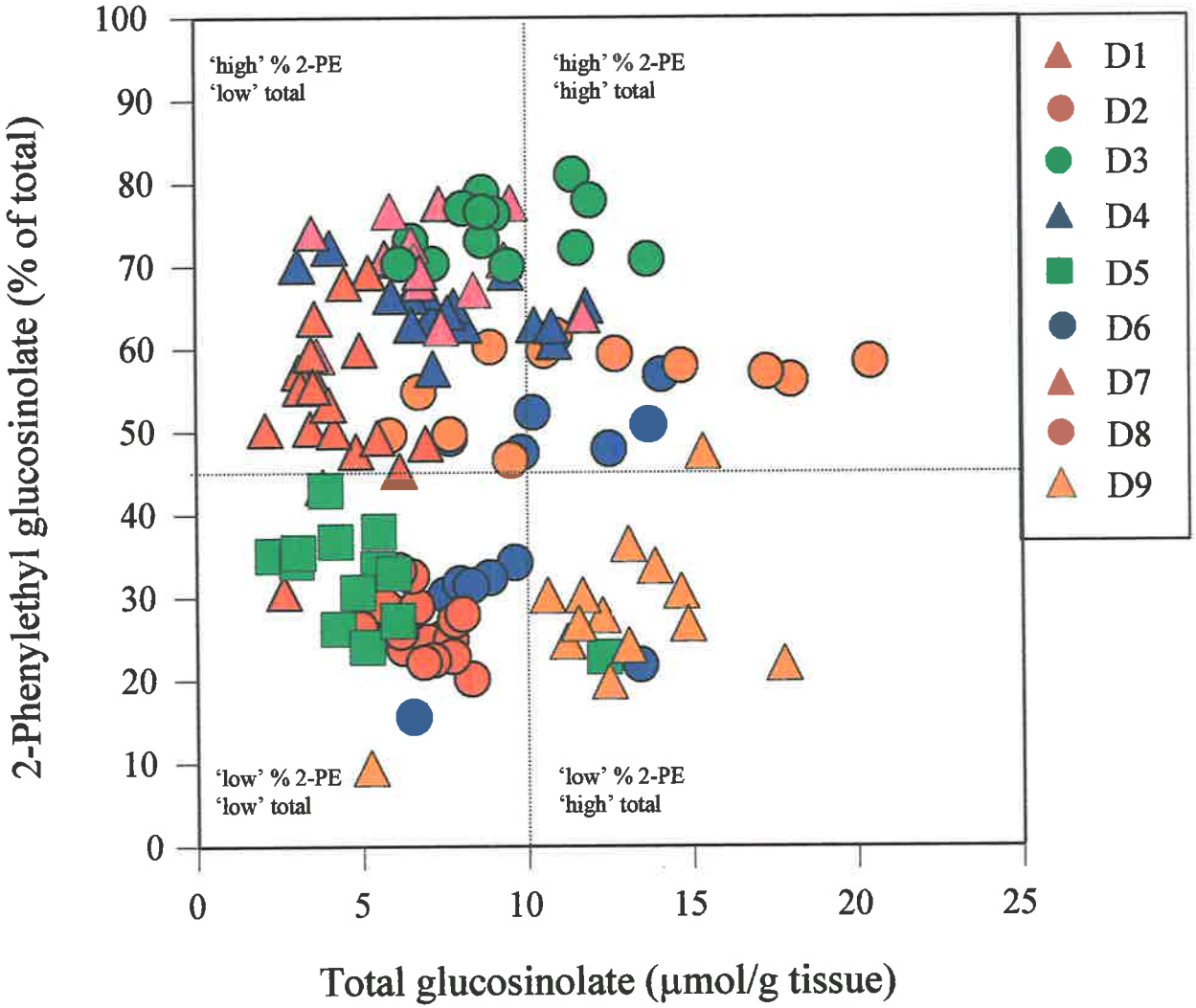
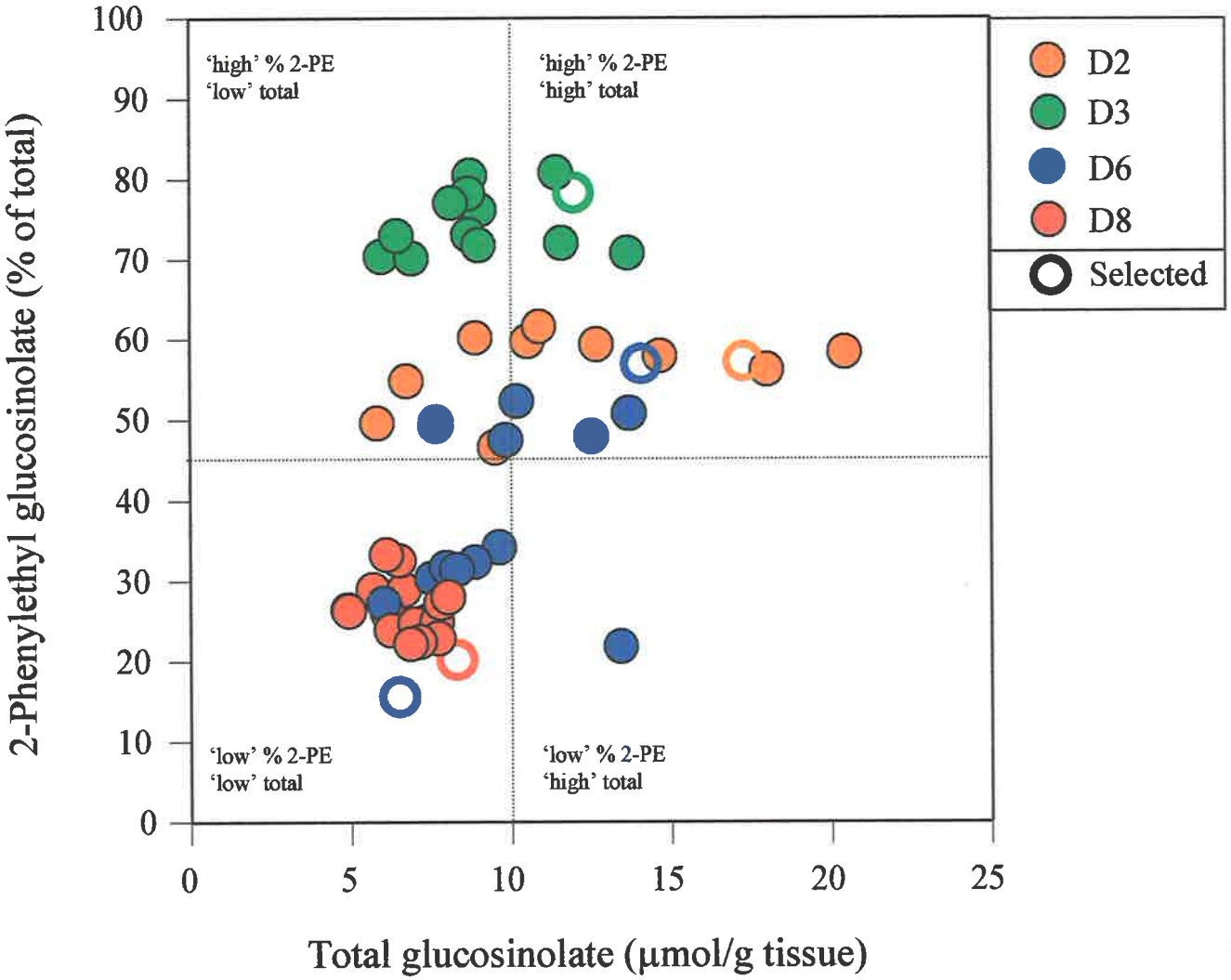


Figure 8.4: Variation in 2-phenylethyl levels relative to total glucosinolates within S_1 *B. napus* cv Dunkeld populations selected for further study. The open circles represent the plants selected for selfing to produce the S_2 generation.



Glucosinolate levels in S_1 seed analysed at the Victorian Institute for Dryland Agriculture, Horsham, Victoria were observed to vary from the parental population, although this variation did not appear to be related to %2-PE glucosinolate levels within the roots (Table 8.3). However, the D9-S_1 population, displayed 'high' total glucosinolate levels in both root and seed tissues, while the D1-S_1 and D5-S_1 populations displayed low total glucosinolate levels in both tissue (Table 8.3), suggesting a link between total glucosinolate levels in the root and seed.

Table 8.3: Comparison of total glucosinolate levels within the root and seed tissues of *B. napus* cv Dunkeld S_1 selections. Root data represent the mean of N individual plants, whilst the seed data represent a single analysis of 50 S_1 seeds from the same plants 'bulked' together.

		Total glucosinolate (µmol/g tissue)	
Selection	N	S_1 root	S_1 seed
Dunkeld*	15	12.39*	8.49
D1-S_1	19	3.92	9.47
D5-S_1	13	4.36	8.77
D9-S_1	15	13.08	13.77

Parental population*

S_2 generation

The S_2 progeny of the D2-S_1, D3-S_1 and D8-S_1 selections maintained the phenotype of the parental lines, with populations distributed on either side of the 45% 2-PE glucosinolate level (Figure 8.5). The two selections from the segregating line, D6-S_1, produced distinct populations, following the phenotype of the S_1 parent (Figure 8.6). The S_2 progeny of the 'low' phenotype D6-S_1 selection produced progeny almost exclusively exhibiting the 'low' phenotype (37 out of 39). The S_2 progeny of the D6-S_1 'high' selection contained individuals exhibiting both phenotypes, distributed in a 31 'high' to 10 'low' ratio. This distribution led to the formulation of the hypothesis that the inheritance of the high 2-PE glucosinolate trait within *B. napus* cv Dunkeld root tissues was controlled by a single gene.

Hypothesis: That the 'high' 2-PE (%) trait is coded by a dominant allele at a single locus and that the individual representing the 'high' phenotype in the D6-S_1 population was heterozygous (*Pe pe*), resulting in S_2 progeny segregating for 'high' and 'low' phenotypes in a 3:1 ratio (Table 8.4).

Figure 8.5: Variation in the levels of 2-phenylethyl glucosinolate (relative to the total glucosinolates) within the roots of S_2 populations from four single plant S_1 selections of *B. napus* cv Dunkeld.

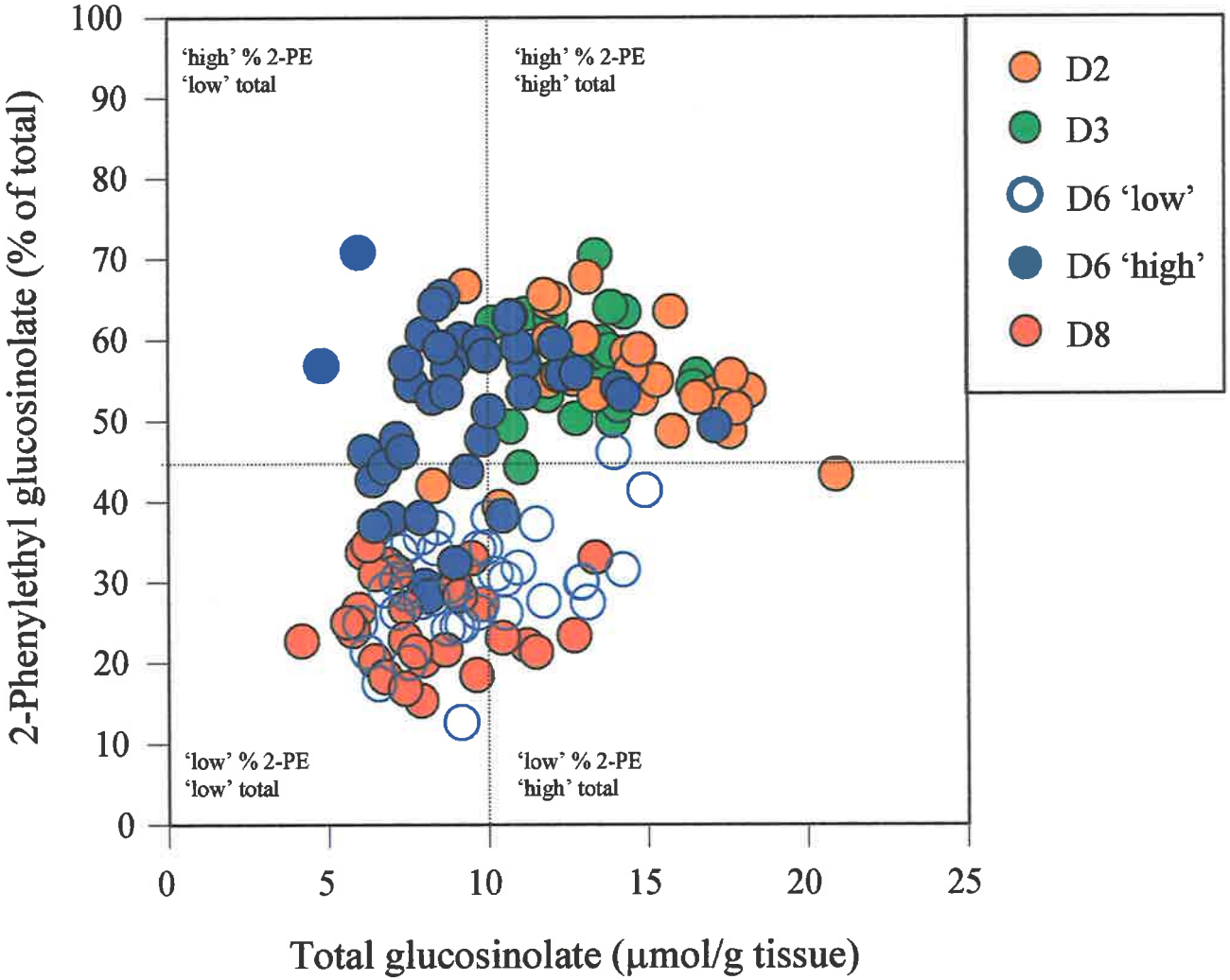


Figure 8.6: Frequency distribution of 2-phenylethyl glucosinolate levels as a percentage of the total glucosinolates in the roots of the S_2 populations produced from selfing a 'high' and a 'low' plant selected from the D6-S_1 *B. napus* cv Dunkeld population.

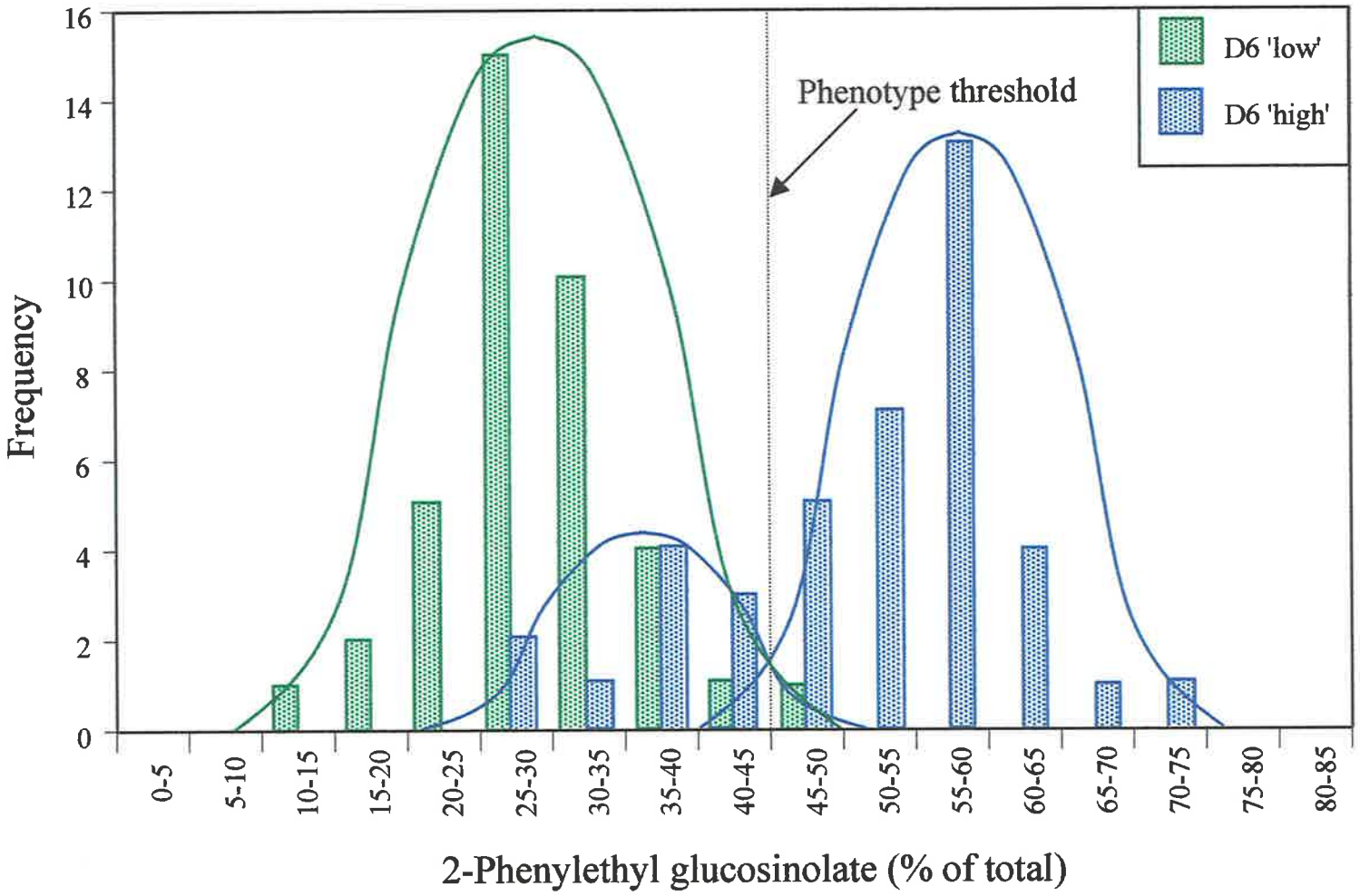


Table 8.4: Observed and expected population distributions appropriate to hypothesis that 'high' root % 2-PE glucosinolate levels are coded as a dominant allele at a single locus following Mendelian principles.

	Observed	Expected
'Low' phenotype (<45% total)	10	10.25
'High' phenotype (>45% total)	31	30.75
Total	41	41

$$\chi^2_P = (10-10.25)^2/10.25 + (31-30.75)^2/30.75 = 0.0041$$

As χ^2_P < 3.84 at the 5% level, we do not reject the hypothesis, suggesting that the 'high' 2-PE glucosinolate phenotype is encoded by a single dominant gene, and that the selected S_1 parent was heterozygous for this trait (*Pe pe*).

A comparison of % 2-PE glucosinolate levels within the roots of a parental *B. napus* cv Dunkeld population with the S_2 selections and those within a parental population shows the impact of the program of selection (Figure 8.7). A frequency distribution is also presented, showing the effect of the selection regime on the S_2 sub-populations (Figure 8.8). While the % 2-PE glucosinolates did alter within the roots of the S_2 populations (Figure 8.8A), no great impact on total glucosinolate levels within the tissue was observed (Figure 8.8B).

Once again, a 'high' and 'low' *total* glucosinolate phenotype was apparent within the populations, truncated above and below 10µmol/glucosinolate per gram tissue. The D2-S_2 and D3-S_2 populations exhibited 'high' total glucosinolate levels as did the parent (S_1) selections, while the 'low' % 2-PE D6, and the D8 populations exhibited 'low' total glucosinolate levels as did the parent (S_1) selections. It should be noted that no true 'low' % 2-PE glucosinolate/'high' total glucosinolate population was present, as no selection was made from S_1 plants with this phenotype (such as from the D9 S_1 population).

Figure 8.7: Comparison between the levels of 2-phenylethyl glucosinolate (relative to total glucosinolate levels) within parental and S_2 populations of *B. napus* cv Dunkeld. D6-S_2 data omitted due to segregation.

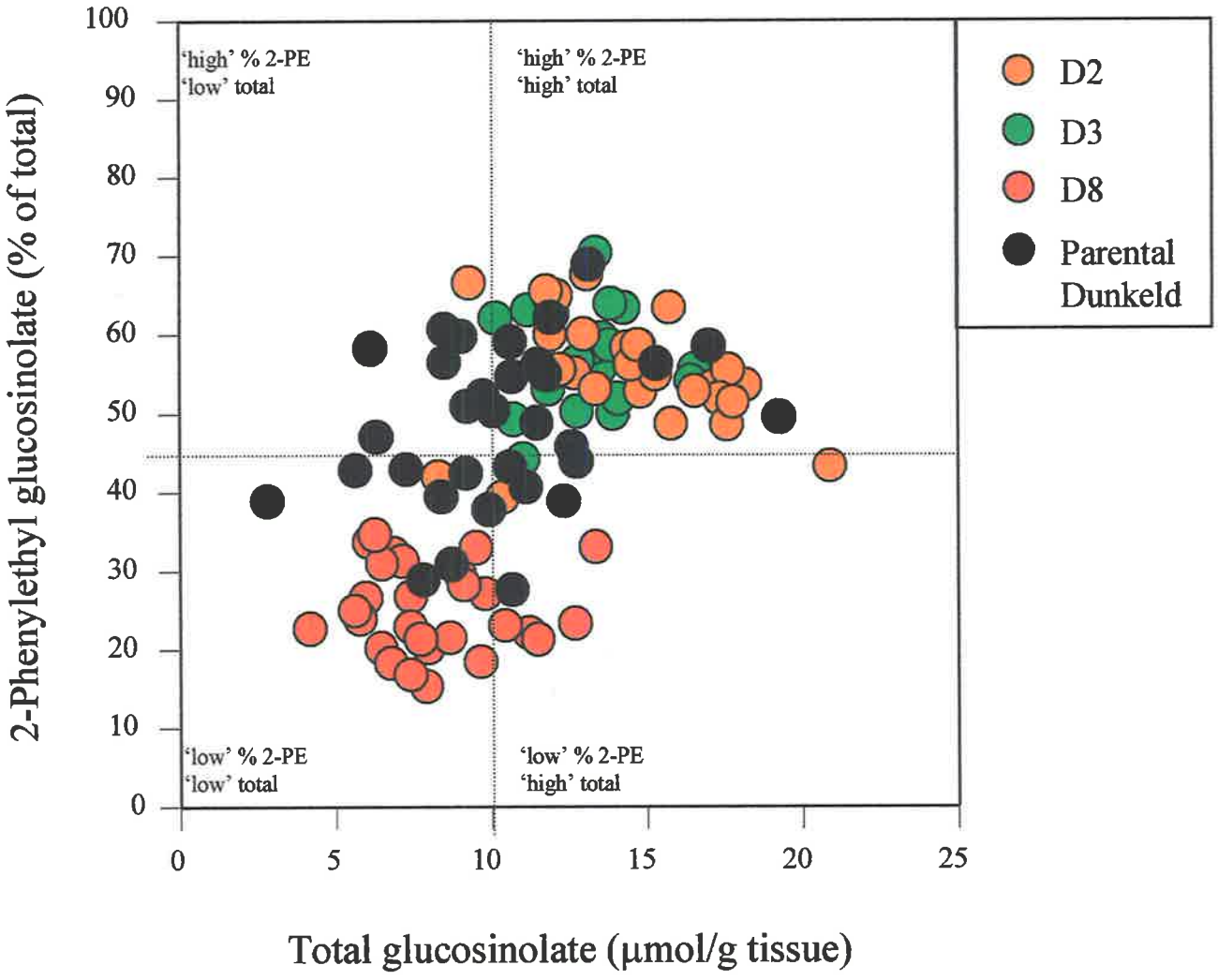
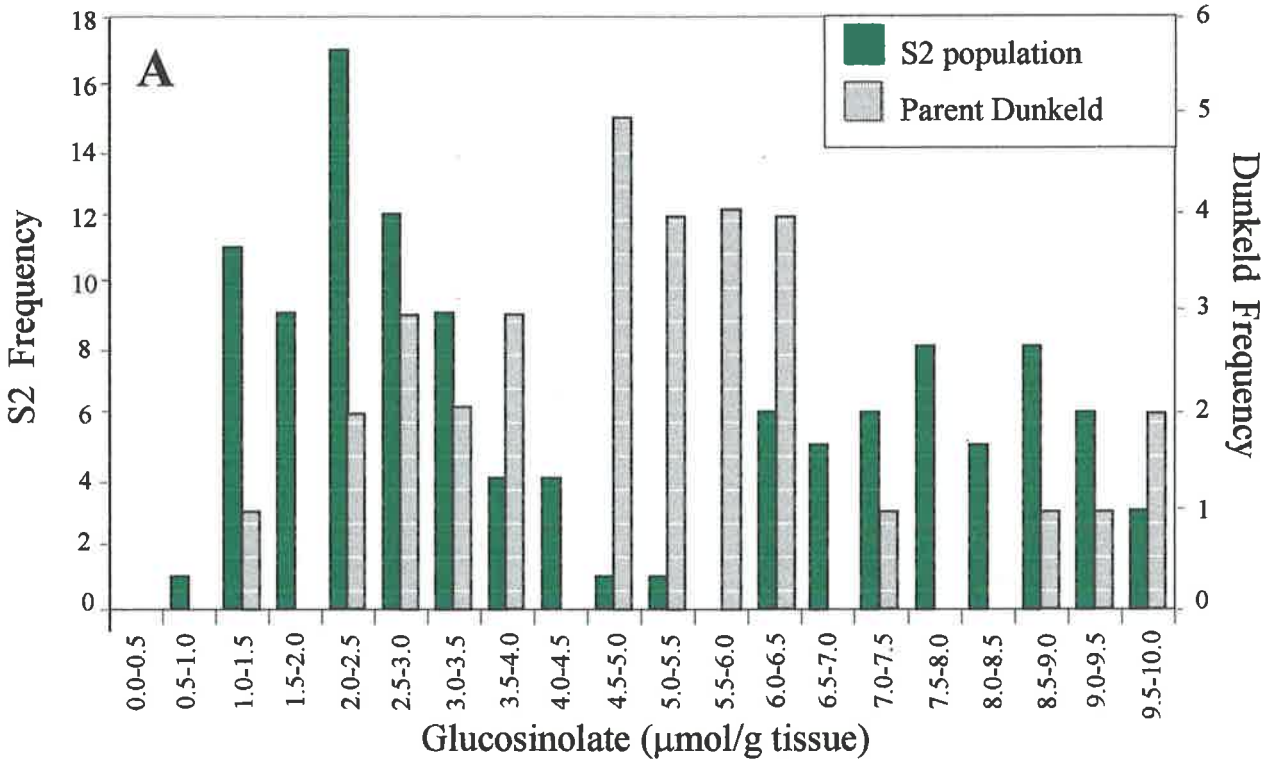
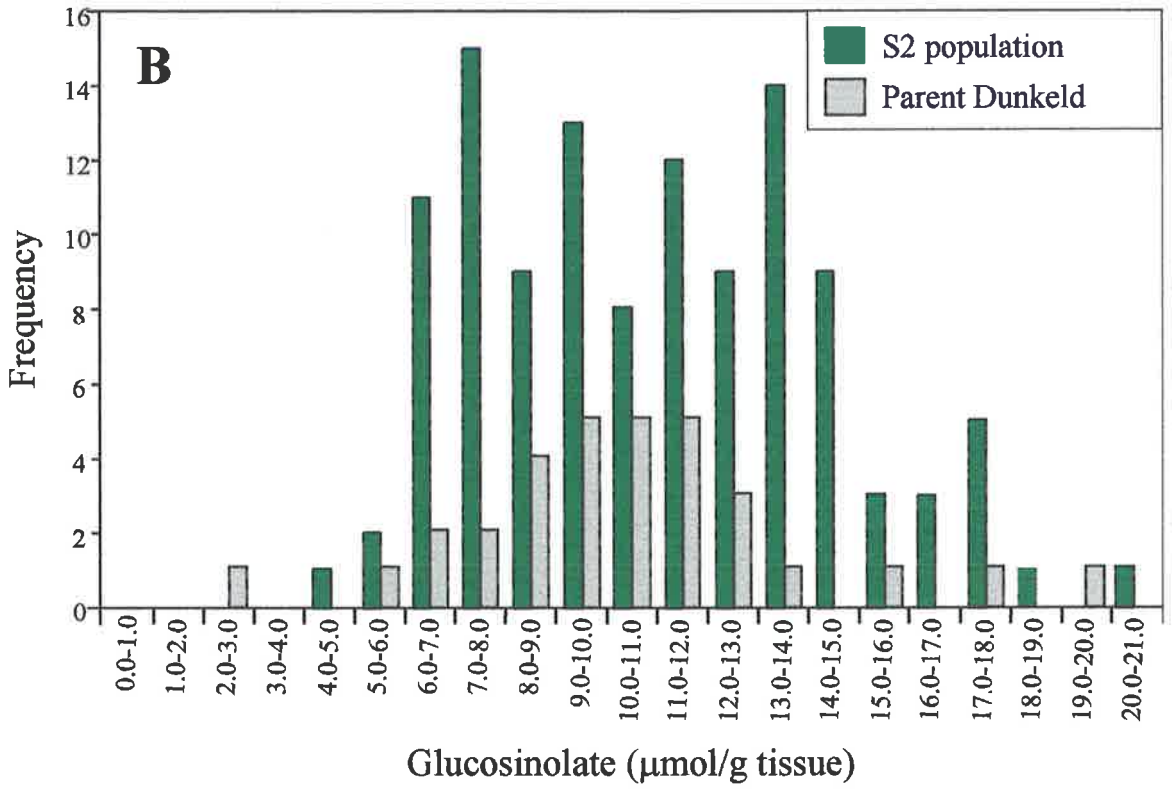


Figure 8.8: Frequency distributions of the levels of 2-phenylethyl (A) and total (B) glucosinolates within individual plants from parental and S_2 *B. napus* cv Dunkeld populations. D6- S_2 populations omitted due to segregation.





8.3.2 *Susceptibility to* P. neglectus *of S_2 selections from* B. napus *cv Dunkeld*

The 'low' % 2-PE glucosinolate population was more susceptible to *P. neglectus* than both the 'high' population and the parental population of *B. napus* cv Dunkeld (Figure 8.9). While the susceptibility of the D6-S_2 'high' plants was not statistically different (at the 5% level) from the parent *B. napus* cv Dunkeld population, the differences in the means (229 nematodes/g root, 355 nematodes/g root; D6-S_2 'high', Dunkeld, respectively) suggest that with more replication a significant difference could have been established.

Table 8.5: Comparisons of the susceptibility to *P. neglectus* of parental *B. napus* cv Dunkeld and 'high' and 'low' S_2 selections from *B. napus* cv Dunkeld. (n = 7).

Susceptibility comparison	P (0.05) value
'high' Vs Dunkeld	0.152
'low' Vs Dunkeld	0.037*
'high' Vs 'low'	0.005*

* Significantly different at the 5% level

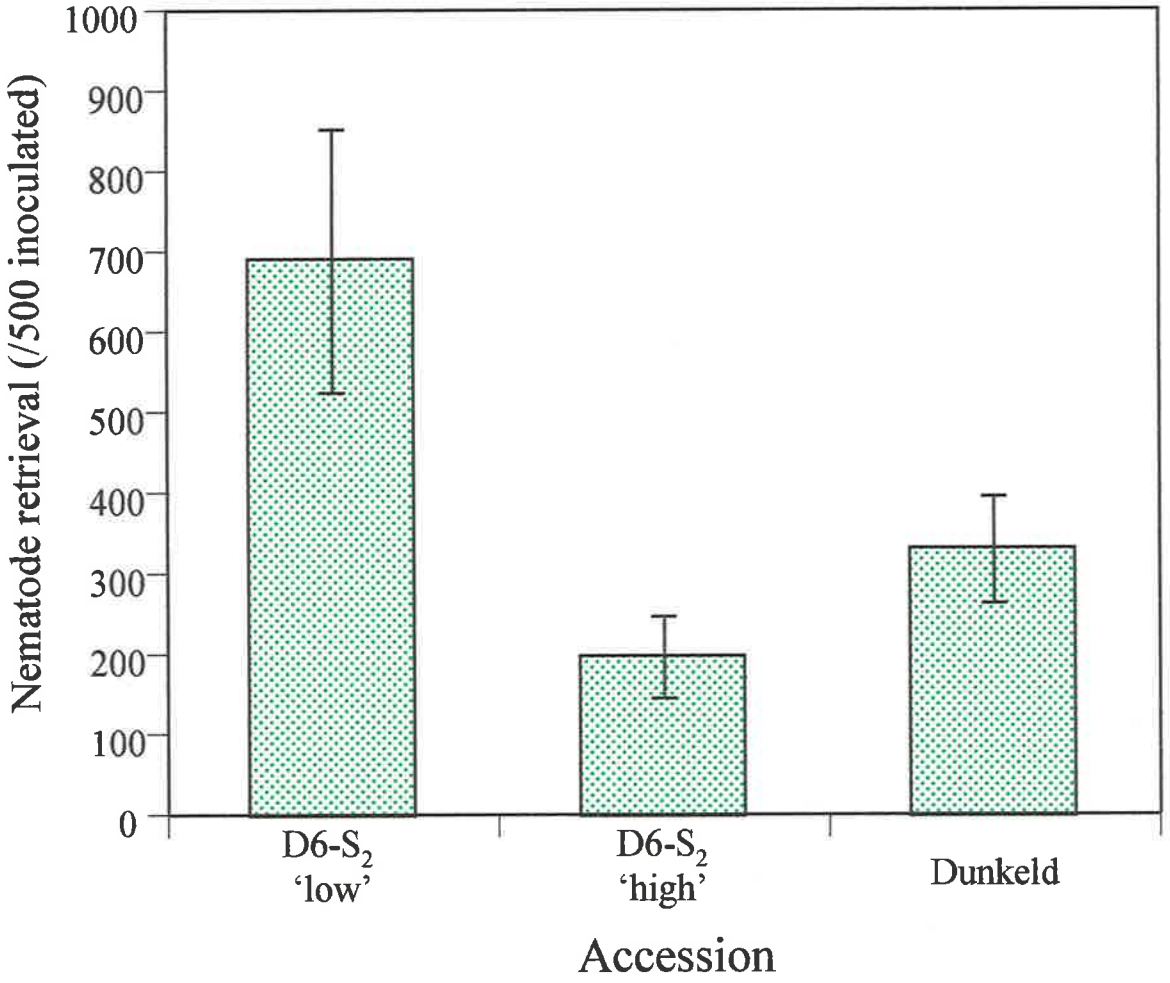
8.4 DISCUSSION

8.4.1 *Selection of variation in 2-PE glucosinolate within* B. napus *roots*

While variation in total and 2-PE glucosinolate levels was observed between the individual plants from each of the four *B. napus* varieties (Figure 8.1), the varieties did not differ greatly in their mean total and 2-PE glucosinolate levels (Table 8.1), supporting earlier studies (Table 5.7, Table 5.8). Because 2-PE glucosinolate is the only aralkyl glucosinolate present in the root of *B. napus* cv Dunkeld, it would not be surprising to find little competition for the resources of its biosynthesis within the tissue, as is known to occur during the synthesis of aliphatic glucosinolates (Magrath *et al.*, 1993)..

The diversity observed within the S_1 populations of *B. napus* cv Dunkeld in terms of agronomic traits (flowering time, nutrient efficiency etc; Plate 8.1) suggests the potential for variation in traits not directly selected for during the breeding of the crop, such as root levels of 2-PE glucosinolate. Indeed, the degree of variation in root 2-PE glucosinolate levels within *B. napus* cv Dunkeld populations appeared to be sufficient to allow an increase in the mean levels within the population (Figure 8.2). Provided that the variation within the population had a genetic basis, it appeared likely that increases could be made without the introduction of exotic germplasm into the Dunkeld genetic background. It was therefore necessary to gather information about the heritability of 2-PE levels within the roots of *B. napus* cv Dunkeld.

Figure 8.9: Susceptibility to *P. neglectus* of the progeny of *B. napus* cv Dunkeld selections (S_2), with 'high' and 'low' 2-phenylethyl glucosinolate levels in their roots to *P. neglectus*. Mean and standard deviation shown (n = 7).



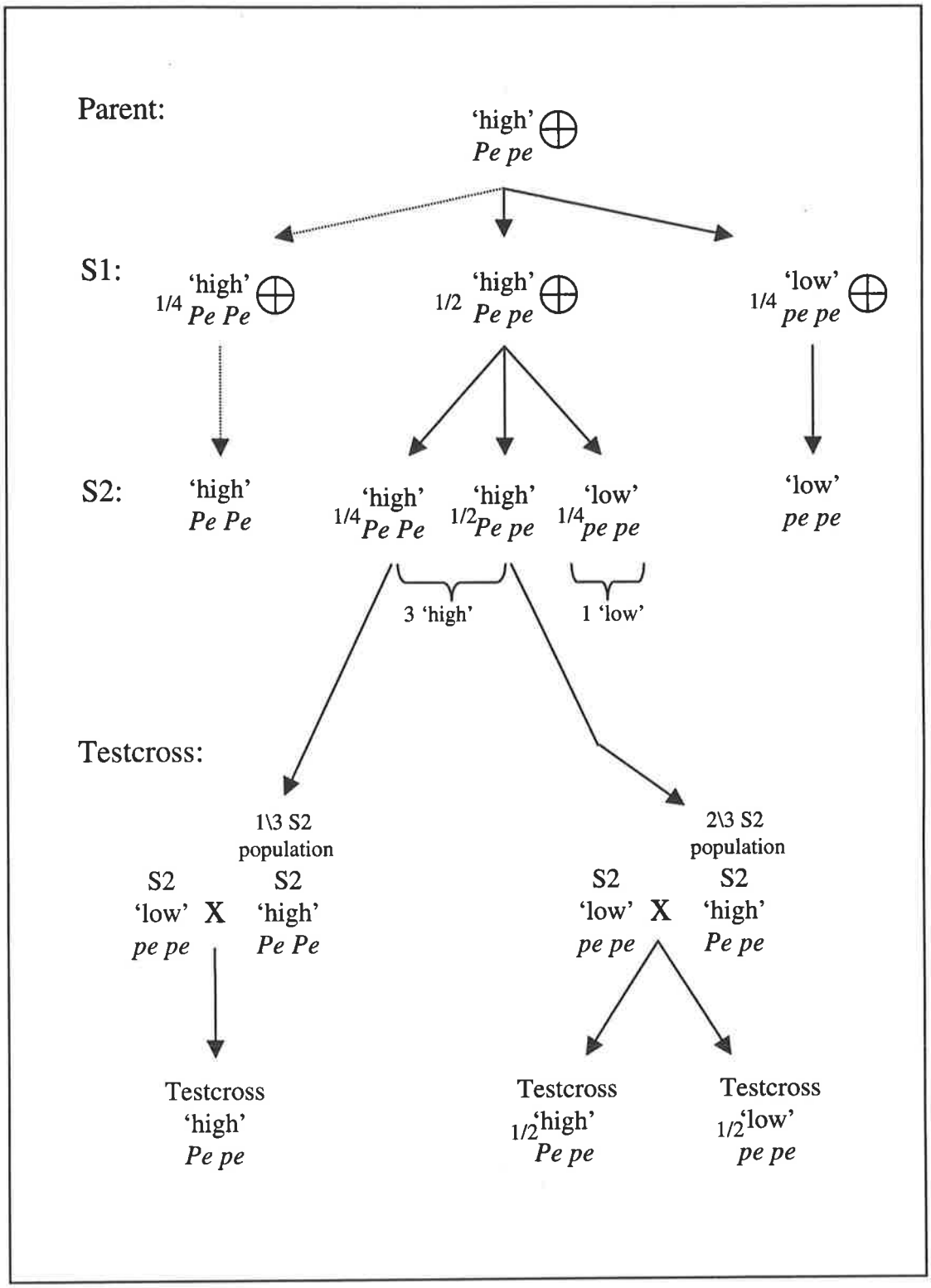
As discussed previously, glucosinolate levels are readily influenced by the environment (Underhill *et al.*, 1962; MacLeod and Nussbaum, 1997), and despite efforts to maintain experimental uniformity, it had to be assumed that at least some of the observed variation was due to minor differences in the growing conditions of the plants. However, by assessing the levels within plants grown under identical environments over a number of generations, it was possible to elucidate the inheritance of the high 2-PE glucosinolate character.

A selection program was developed based on the percentage of 2-PE glucosinolate in the profile such that genes involved in 2-PE glucosinolate production from established glucosinolate precursors were targeted, rather than the total level of precursor available for glucosinolate biosynthesis. In order to study % 2-PE glucosinolate inheritance, a level of 45% 2-PE glucosinolate was chosen, based upon the bimodal distribution of non-segregating populations within the S_1 and S_2 generations (Figures 8.3 and 8.5, respectively).

Studies following the phenotypes of the D2, D3 and D8 lines through the three generations (parental, S_1 and S_2) found that each generation exhibited the same phenotype as the parent, (ie. the root % 2-PE glucosinolate levels were heritable, and the parents were homozygous for the trait). In the D6 line, the parental and S_1 individuals segregated into the two phenotypes. The parent line contained 'high' % 2-PE glucosinolate levels (~60% 2-PE glucosinolate), and gave rise to S_1 progeny in two distinct populations, either side of the 45% mark. Hence, the D6 parent must have been heterozygous, exhibiting a 'high' phenotype, and producing both 'high and 'low' phenotypes in the S_1 generation (Figure 8.4).

In the D6-S_2 generation, the progeny of the 'low' phenotype D6-S_1 selection maintained the 'low' phenotype, suggesting that the S_1 parent plant was homozygous for the trait as only one plant out of thirty nine exhibited the 'high' phenotype, and this marginally so (45.8%; likely to be due to environmental variation within the trial). The progeny of the 'high' phenotype D6-S_1 selection again showed bimodal distribution, exhibiting sub-populations lying either side of the 45% cut-off. The selected 'high' D6-S_1 individual was heterozygous with a 'high' phenotype whilst producing a progeny population (S_2) segregating in a 3:1, 'high':'low' ratio. As the chi-square test supported the single gene model (Table 8.4), a pedigree for the plants examined in the selection program was developed (Figure 8.10A).

Figure 8.10: Genetic flow of single gene model for the inheritance of 2-phenylethyl glucosinolate within the root of *B. napus* cv Dunkeld though three generations. The two phenotypes are based upon the percentage that 2-phenylethyl glucosinolate contributes to the total glucosinolates levels of the root ('high' >45%, 'low' <45%).



Using the model (Figure 8.10A), the D2, D3 and D8 parental lines would have been homozygous for the % 2-PE glucosinolate trait (D2, D3 being *Pe Pe*, and D8 *pe pe*), explaining the consistency of the phenotypes throughout the S_1 and S_2 generations. The D6 parent line must have been heterozygous (*Pe pe*) to produce two sub-populations of D6-S_1 plants. While D6-S_1 progeny were distributed in a 6:8, 'high':'low' ratio, this study involved too few individuals to accurately determine the inheritance of the root % 2-PE glucosinolate trait. From the model, all plants in the 'low' phenotype D6-S_1 sub-population should have been homozygous recessive (*pe pe*), producing only 'low' (*pe pe*) D6-S_2 progeny. The 'high' phenotype D6-S_1 sub-population would have contained a mixture of homozygous dominant (*Pe Pe*) and heterozygous (*Pe pe*) individuals in a 1:2 ratio. Self-fertilisation of the selected 'high' D6-S_1 plant produced a segregating S_2 population which implied that the D6-S_1 plant selected was heterozygous for the 2-PE glucosinolate trait (*Pe pe*). The S_2 population would therefore contain a mixture of genotypes (1 *Pe Pe* : 2 *Pe pe* : 1 *pe pe*), and would exhibit both phenotypes in a 3:1, 'high':'low' ratio (as confirmed by chi-square analysis).

Further study is required to confirm the single gene model for inheritance of % 2-PE glucosinolates within *B. napus* cv Dunkeld. A test-cross should be undertaken (Figure 8.10B), hybridising one of the 'low' D6-S_2 lines (*pe pe*) with individuals from the 'high' D6-S_2 population (2 *Pe pe* : 1 *Pe Pe*). If the genetic model is correct, crosses between the 'low' S_2 plant and a homozygous dominant 'high' S_2 (*Pe Pe*) would produce all 'high' progeny, whilst crosses with a heterozygous 'high' S_2 (*Pe pe*) would produce populations segregating into 'high' and 'low' phenotypes in a 1:1 ratio.

8.4.2 *Variation in* total *glucosinolate levels within* B. napus *roots*

Heritable variation in total levels of root glucosinolates was apparent in the S_1 and S_2 generations. Insufficient data was obtained to determine the nature of the inheritance, or any potential linkage to the inheritance of % 2-PE glucosinolate within that total. However, the relationship highlights the importance of selection for % 2-PE glucosinolates, such that the disease break qualities of the crop may be maximised without increasing total glucosinolate levels. Lines derived from populations such as D9-S_1 are likely to be undesirable, as, while they may contain higher actual levels of 2-PE glucosinolate than lines developed from D4-S_1 and D7-S_1 populations, they may also lead to higher seed glucosinolate levels.

The findings of Section 8.3.1 suggest an avenue to increase the percentage of 2-PE glucosinolate within the total through simple selection. If the advances made through this mode of selection become limiting, further increases in 2-PE glucosinolate levels may be feasible through selection for increased total glucosinolate levels. However, such efforts will have to be considerate of their potential to also increase total seed glucosinolate levels, which may undermine seed quality.

8.4.3 *Susceptibility to* P. neglectus *of S_2 selections from* B. napus *cv Dunkeld*

The significant variation in susceptibility between plants from the 'high' and the 'low' D6-S_2 populations is likely to be due to variation in root levels of 2-PE glucosinolate. Although the actual root levels of 2-PE glucosinolate within these two populations were not measured at the same time as the susceptibility, glucosinolate levels assessed in plants from the same populations revealed significant differences in the % 2-PE glucosinolate levels between the populations, although they contained similar total glucosinolate levels (Figure 8.7). A strong relationship was observed between the tissue levels of 2-PE glucosinolate and the susceptibility of the plants to the nematode, supporting the claim made in Section 7.4.3. The results are particularly significant as the 'high' and 'low' D6-S_2 populations were otherwise highly genetically similar and thus these results were not confounded by unaccounted for genetic variation. Further, the 'high' and 'low' D6-S_2 populations contained comparable total levels of glucosinolates, highlighting the significance of the single glucosinolate, 2-PE, to the ability of *B. napus* cv Dunkeld to resist the invasion and multiplication of *P. neglectus*.

CHAPTER 9

GENERAL DISCUSSION

A great deal of conjecture surrounds the benefits of canola as a rotational crop to control pests and diseases in the soil of southern Australian cereal cropping systems. Grower discussion during the course of this project has revealed mixed reaction, with many growers suggesting that canola exacerbates soil disease problems rather than providing disease break benefits to the rotation. Within the southern Australian cereal cropping system, the disease break impact of canola is, at best, unpredictable, leading to uncertainty as to the suitability of canola for inclusion within the rotation.

Replicated field studies do suggest that the crop can lead to improved early vigour of following wheat crops at least comparable with other rotational crops such as the nitrogen fixing pulses (Kollmorgen *et al.*, 1983; Purvis, 1990; Christen *et al.*, 1992). Further field studies have suggested that the advantage is based on the negative impact of degrading *Brassica* tissues on populations of harmful organisms in the soil (Angus, 1991; Kirkegaard *et al.*, 1993). Laboratory studies clearly show that degrading canola tissues are biocidal to a wide range of organisms (Akhtar and Alam, 1991; Owino *et al.*, 1993; Jing and Halbrendt, 1994; Smolinska *et al.*, 1997), due to the liberation of toxic molecules as the tissues degrade in proximity to the organism (Brown and Morra, 1997). Clearly, canola tissues have potential to reduce soil populations of harmful organisms.

The field studies reported in Chapter 4 did not refute the postulate that *Brassica* crops can lead to a reduction in harmful soil organisms. At the three sites examined, the *Brassica* rotation led to fewer *Pratylenchus* in the soil than the other crops examined. Trials performed at Condada and Woodside showed that the incorporation of the whole plants into the ground (green manuring) reduced the numbers of nematodes in the soil (Figures 4.1 and Figure 4.3, respectively). The decrease in nematode numbers associated with green manuring was particularly effective in the *Brassica* rotations, suggesting that it was not due to cultivation alone. Some factor, responsive to tissue damage and unique to the *Brassica* crops used in the trials, was involved in the observed effect, suggesting the glucosinolate system of plant defence.

Green manuring is likely to maximise the biocidal benefits of the *Brassica* crop, as it not only incorporates both leaf and root tissues, but also macerates the tissues, maximising glucosinolate breakdown. However, such activities are not generally appropriate to the southern Australian cereal cropping system, being limited by economic feasibility and the requirement for an extra cultivation. The roots are the only tissues to break down in the soil when canola is allowed to develop for seed harvest, and therefore attention must be directed to the allelopathic nature of the root systems. The studies at Condada and Lameroo demonstrated that canola crops allowed to develop to seed led to lower nematode numbers relative to the other crops examined, but as initial nematode numbers were not determined, it is unclear in what manner the crop impacted upon nematode numbers.

The field studies at Woodside also demonstrated the susceptibility of the *Brassica* to *Pratylenchus* spp. (Figure 4.3), supporting the findings of Vanstone *et al.* (1993) and Webb (1996). In this case, the nematode numbers following the *Brassica* were comparable to the more resistant oat only after the green manuring treatment. As the number of nematodes present in the soil following a *Brassica* crop will be a function of both nematode multiplication during the season (susceptibility), and the subsequent nematicidal impact of the tissues as they break down in the soil (nematicidal potency), both characteristics require attention when considering improvement of the disease break associated with canola.

Investigation of varieties from a broad range of *Brassica* species revealed a great deal of variation in both these characters. However, the *B. napus* canola accessions examined were generally the least susceptible and the most nematicidal of all the cultivated lines examined (Figures 7.2 and 6.3, respectively). The only line examined with greater resistance combined with greater nematicidal potential than *B. napus* was a wild type (uncultivated) *B. oxyrrhina* line, which also contained many undesirable genetic traits unsuitable for introduction to cultivated canola. Thus, these findings suggested a limited potential to improve the disease break associated with crop through inter-specific crossing. Consequently, further studies were directed towards understanding the physiology of the *Brassica* allelopathy such that a selection program could be developed based on biochemical measurements.

The fact that no relationship was observed between the high nematicidal potency of the leaf tissues and the levels of total or any individual glucosinolates within those tissues, suggested that other allelopathic factors were present within the *Brassica* leaves. Investigations into the non-glucosinolate allelopathic factors would be very important to efforts seeking to improve the green manuring potential of *Brassica*. However, the leaf tissues of field grown canola plants wither and die on the stem as the seed matures, and therefore their glucosinolates are lost before the tissues are incorporated into the soil. As the impact of the leaves on soil pests and diseases within the cereal rotation would be negligible, further biochemical studies of the leaves were considered to be beyond the scope of this project.

The studies reported here show that root 2-PE glucosinolate levels were closely related to the nematicidal potential of the root tissues (Figure 6.6). As root tissues containing low levels of 2-PE had no significant nematicidal impact, the levels of this glucosinolate alone may have been responsible for the nematicidal effect of the root tissues. The significance of 2-PE glucosinolate to the nematicidal potential of the root tissues was supported by *in vitro* studies demonstrating the nematicidal qualities of purified 2-PE isothiocyanate (Figure 6.9).

The close relationship between root tissue levels of 2-PE glucosinolate and the nematicidal potential of the tissue raises the question of the susceptibility of the crop to the nematodes, as discussed by Acedo and Rhode (1971) and Webb (1996). Given that glucosinolates release nematicidal isothiocyanates as a result of tissue damage (Brown and Morra, 1997), and that *Pratylenchus* infection of roots can cause tissue necrosis leading to large lesions, it is unclear how the nematodes can develop in *Brassica* roots at all. The nematode must move and feed within the plant root in such a way as to avoid significant release of nematicidal isothiocyanates. Tap roots were observed to be more nematicidal than fine roots (Figure 6.7), suggesting that nematodes may be able to develop in some root tissues, which are perhaps not so well protected by the glucosinolate system. Alternatively, as *Pratylenchus* spp. invade individual cells one at a time to feed on the cytoplasmic contents (Zunke, 1990a), it is possible that insufficient isothiocyanate is released by the damage associated with nematode feeding. The nematodes, drawing cytoplasm (likely to include the glucosinolate and the myrosinase activator, ascorbate) from the cells through the stylet, may not allow mixing of the glucosinolate with the membrane-bound hydrolytic enzyme (myrosinase), and thus may avoid isothiocyanate liberation altogether. Further, as much of the myrosinase present may be

sequestered into special 'myrosin' cells scattered diffusely throughout the plant tissues (Plate 2.3; Iverson *et al.*, 1979; Bones *et al.*, 1991), nematode feeding would not liberate isothiocyanate unless the nematode directly invaded a 'myrosin' cell. While the liberation of isothiocyanate due to nematode feeding requires further study, it seems that the glucosinolate system responds most effectively to gross tissue damage, such as from the grazing of larger herbivores, rather than against tissue invasion by smaller parasites.

As if in support of this, *Brassica* spp. have been shown to be susceptible to the root lesion nematode throughout the vegetative life of the crop (Chapter 7; Webb, 1996). However, further studies revealed that plants containing higher root levels of 2-PE glucosinolate contained significantly fewer nematodes than their lower 2-PE glucosinolate counterparts (Figures 7.3, 7.4C and 7.5B). Clearly, the ability of the nematode to invade and multiply within the roots is reduced in roots with higher levels of 2-PE glucosinolate. The mechanism behind this observation is unclear. As even healthy vegetative tissues may continuously release small amounts of isothiocyanate (Tang, 1971), it is possible that roots containing higher 2-PE glucosinolate levels release sufficient isothiocyanate, perhaps in the root exudate which nematodes rely upon to locate the host root (Section 2.2.2), to deter some nematode species from invasion. Variation in the ability of different *Pratylenchus* spp. to invade and multiply within *Brassica* roots may be due to subtle variation in the feeding patterns of the nematodes, and hence variation in their exposure to the glucosinolate system of defence. Further studies to determine the relationship between nematode invasion, root glucosinolate content and myrosinase location are required to better understand the susceptibility of the *Brassica* to *Pratylenchus* spp.

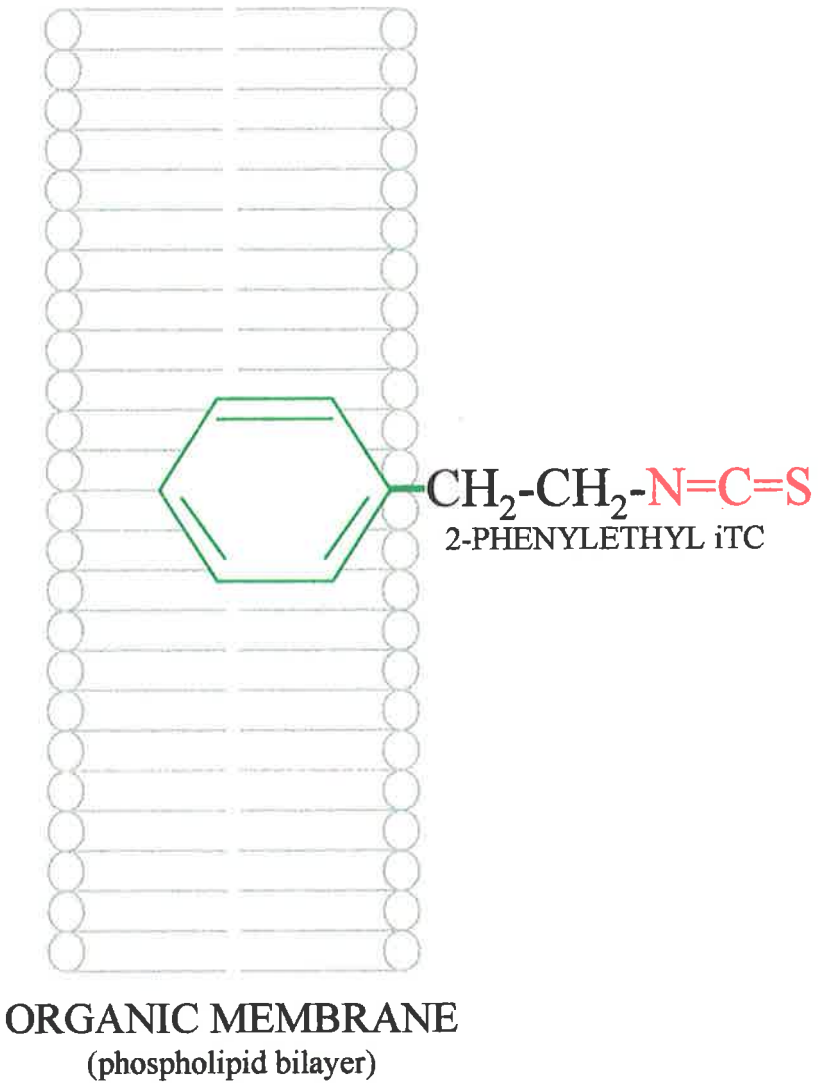
The assertion that 2-propenyl isothiocyanate is a highly effective nematicide (Smedley, 1939; Lazzeri *et al.*, 1993; Donkin *et al.*, 1995;; Mojtahedi and Santo, 1996) was not supported by this study. Tissue amendments imparting over 160nmol 2-propenyl glucosinolate/gram soil had no significant effect on the nematode (Figure 6.4), despite the fact that amendments providing less than one third of this amount (~54nmol/g soil) of 2-PE glucosinolate reduced nematode retrieval by almost 50% (Figure 6.6). Root levels of 2-propenyl glucosinolate were also found to be unrelated to the susceptibility of the tissues (Figure 7.4B), despite this chemical being present at comparable levels to 2-PE glucosinolate in some of the plants examined.

The chemical structure of 2-PE isothiocyanate may be particularly well adapted for action in the soil environment (Figure 9.1). The molecule is less volatile than the smaller aliphatic type of isothiocyanates (including 2-propenyl) and may lodge within the membranes of soil organic matter due to the hydrophobic nature of the phenyl group. The ethyl bridge, linking the phenyl group with the functional isothiocyanate, may act to hold the isothiocyanate free from the soil particles allowing contact with soil organisms. The smaller isothiocyanates, such as methyl and 2-propenyl, may not be efficient in the soil environment, being bound, inactivated or lost from the soil profile, as suggested by Matthiesson *et al.* (1996) and Williams *et al.* (1993). Mithen *et al.* (1995) suggested that plants producing isothiocyanates with physiologically varied activities may be responsive to selection pressures, leading to the development of a tissue profile best suited to the environment in which the isothiocyanate is likely to be released. As 2-PE glucosinolate is produced almost to the exclusion of all others within the root tissues of *Brassica* spp. (Table 5.6; Sang *et al.*, 1984), it would not be surprising to find that 2-PE isothiocyanate is particularly effective in the soil environment.

The apparent efficiency of 2-PE isothiocyanate as a nematicide within the soil environment was sufficient to inspire the GRDC (Grains Research and Development Corporation), the Department of Plant Science (University of Adelaide) and Luminis Pty Ltd (University of Adelaide) to develop a provisional patent for the development of the molecule as a soil fumigant within Australia. The details of this patent are presented in Appendix 1 of this thesis. The patent was held for a provisional period of twelve months, before it was allowed to lapse due to the need for publication (Potter *et al.*, 1998) of the relevant information.

Once a critical level was surpassed, 2-PE glucosinolate levels in the roots of *B. napus* were related to both the susceptibility to *P. neglectus* and the nematicidal potential of the tissues. Unfortunately, in *B. napus* cv Dunkeld, the average level of 2-PE glucosinolate within the population was below this critical level. A population of *B. napus* cv Dunkeld is therefore unlikely to exhibit the disease break advantages associated with the glucosinolate system unless environmental conditions are particularly conducive to glucosinolate production. The unreliability of canola within the cereal rotation may be due to the fact that tissues only produce more than the critical level of glucosinolates when management practices and seasonal conditions provide such an optimal environment. Canola lines routinely producing higher 2-PE glucosinolate levels irrespective of the environment would therefore be more likely to provide a reliable disease break effect.

Figure 9.1: Hypothesised interaction of 2-phenylethyl isothiocyanate (iTC) with organic matter in the soil environment.



Significant variation in root levels of 2-PE glucosinolate was detected within the commercially available canola variety, Dunkeld (Tables 5.8 and 8.1; Figure 8.1). Breeding studies (Chapter 8) revealed that root levels of 2-PE glucosinolate were inherited following Mendelian principles of segregation, implying control by a single gene. By applying selection pressures based upon 2-PE glucosinolate levels in the roots, *B. napus* cv Dunkeld sub-populations were developed displaying the extremes of the original population (Figure 8.6). S_2 plants with the 'high' 2-PE phenotype were significantly less susceptible to the nematode than otherwise identical lines exhibiting the 'low' 2-PE phenotype, confirming the validity of the original observations regarding 2-PE glucosinolates and susceptibility to *P. neglectus* (Figures 7.3, 7.4C and 7.5B). Unfortunately, parallel comparisons of the nematicidal potential of the root tissues from these S_2 plants could not be performed due to limitations in the amount of available root tissue. Based upon the observations in Chapter 6, it is hypothesised that such a trial would have shown the 'high' 2-PE roots to be more nematicidal than their 'low' 2-PE counterparts.

These same breeding studies revealed a second apparently heritable distribution within the progeny, this time for the total glucosinolate character. Two distinct total glucosinolate phenotypes were observed, truncated at 10µmol glucosinolate/g tissue. While these findings were not further pursed within the project, a link was suggested between total glucosinolate levels within the root and the seed tissues. Of the lines compared, those containing 'high' total glucosinolates levels in their roots tended to also contain relatively high seed levels. These findings reinforce the importance of selection to manipulate only the relative proportions of glucosinolates within the root, until the relationship between total levels within the root and the seed can be better elucidated.

The degree of heritability of root % 2-PE glucosinolate levels suggests that a sub-population could be selected from *B. napus* cv Dunkeld plants which exhibit the 'high' % 2-PE phenotype, and therefore have decreased susceptibility combined with increased nematicidal potential against *P. neglectus*. As the selection program was specific, targeting only root levels of % 2-PE glucosinolate, such a sub-population is likely to maintain the positive agronomic traits associated with the original variety, whilst breeding true for the 'high' root % 2-PE glucosinolate phenotype. Although questions still exist regarding the relationship between root and seed glucosinolates (Table 8.3), parallel analysis of seed quality could be

undertaken to ensure that seed glucosinolate levels do not rise significantly. While self-pollination (required to develop pure breeding (homozygous) 'high' % 2-PE glucosinolate lines) risks inbreeding depression within the sub-populations, a number of homozygous 'high' plants, selected from different parents, could be intercrossed, thus maximising the hybrid vigour of the original variety while maintaining the 'high' % 2-PE glucosinolate trait. The selection program developed for the purposes of this project yielded five (out of nine) parental varieties which produced pure breeding 'high' S_1 offspring (D1, D2, D3, D4 and D7), all of which could be intercrossed to maintain the hybrid vigour of the original variety. After testing in the glasshouse and the field to establish the improvement of disease break benefits and the maintenance of the agronomic qualities relative to the original *B. napus* cv Dunkeld parental population, it is likely that the sub-population could be re-released as an 'enhanced' variety in its own right. Such a variety would fit into the same rotational niche as the original parent variety, but would perform more reliably to break disease cycles within the southern Australian cereal cropping system. Sub-populations with enhanced root disease break qualities could be developed from any established *B. napus* canola variety displaying comparable variation in root 2-PE glucosinolate levels.

While this study has used *Pratylenchus* spp. as a model to investigate the biochemistry of the *Brassica* disease break, isothiocyanates act to denature protein (Kroll *et al.*, 1994) and thus will be effective against a wide range of organisms. The effect observed against the root lesion nematode in this study is likely to be seen against a broad range of not just pathogenic nematodes, but fungi, bacteria and other harmful soil organisms. Enhancing the levels of 2-PE glucosinolate in canola roots has positive implications for general disease control within the cropping system, but may also have a negative impact upon beneficial soil organisms such as natural biological control agents, nitrogen fixing bacteria and mycorrhizal fungi. While the nematicidal effect of degrading *Brassica* did seem to be localised (not affecting free living nematode populations; Figure 4.2A), increases in the potential of canola allelopathy may alter the soil ecology in an unfavourable manner. Further, *Brassica* residues liberating increased levels of isothiocyanate may affect the emergence of following wheat crops, as isothiocyanates have been observed to reduce germination (Brown and Morra, 1995). Consultation with farmers has already revealed occasional difficulties in the emergence of cereals following conventional canola crops, particularly when associated with seed sown into the canola wind-row position. If the liberation of isothiocyanate from canola tissues is

responsible for reduced emergence, 'enhanced' canola lines may act to exacerbate this problem. Clearly, the integration of 'enhanced' canola lines into the cereal cropping system will require careful management. While comparable to the original lines in all other agronomic qualities, 'enhanced' lines may only be appropriate when disease control is a high priority. Once stable 'high' 2-PE glucosinolate sub-populations have been developed in the glasshouse, a great deal of further field work will be required to properly integrate these 'enhanced' lines into the southern Australian cereal cropping system.

APPENDIX 1: Patent document

Regarding the development of 2-phenylethyl (2-PE) isothiocyanate as a soil fumigant.

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention Title: SOIL FUMIGANT

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The invention is described in the following statement :

This invention relates to a method of sterilising soil and a soil fumigant formulation for use in soil.

It will be understood that the term sterilising or sterilant or similar terms in this specification relates to the significant reduction in populations of soil microorganisms and does not necessarily mean killing of all organisms within an area of soil that is treated. A significant reduction will minimise the risk of an undesired pathogen having an adverse affect on a plant or crop.

Intense agriculture leads to the build up of plant pathogens especially where cultivation of the same or similar plants occurs repeatedly in the same soil. Such plant pathogens have a strongly adverse effect on crops and can make certain agricultural pursuits practically unworkable unless addressed.

In certain fields of agriculture such as in the cultivation of vegetables, it has become practice to sterilise soil before planting a crop. Some of the soil pathogens are particularly resilient to treatment perhaps as a result of their association with various particular constituents of the soil that confer additional resistance to treatment or where such pathogens are in an inactive

state for example as spores. Some plant pathogens such as nematodes are very resilient to killing by agricultural sterilants. As a result particularly severe sterilants are required and thus highly toxic sterilants such as the halogen fumigant methyl bromide are commonly used. These sterilants are toxic for a broad spectrum of life including plants and after fumigation, the soil is left untouched for a short period, such as two weeks before commencement of planting out.

A difficulty with the use of methyl bromide is a result of its halogen compound. Halogen is released into the atmosphere and is thought to play a role in depletion of that layer of the atmosphere known as the ozone layer and over which great concern has been expressed in recent times. As a consequence there is a desire to minimise the use of such compounds, and indeed at least some countries have, or are about to have, implemented a ban on the use of such compounds as soil fumigants. Furthermore there is some concern about residues of halogenated compounds that result from soil sterilisation being maintained in the environment for an unacceptably long period of time.

There is therefore a desire to find replacement compounds which can be effective as a soil sterilant. Amongst those that have been screened are some naturally occurring isothiocyanate compounds that are found in cruciferous plants such as *Brassica*s. One such compound being methyl isothiocyanate is presently being used as a fumigant under the trade mark METHAM and VAPAM. The difficulty with this compound is, however, that the cost of using the compound is quite high. The compound is applied at a rate of about 500L/ha for potatoes, at present, representing a cost in Australia of approximately $700 per hectare.

Whilst the compound is more toxic than methyl bromide it has been found that the effectiveness of methyl isothiocyanate in soil is significantly lower than might be predicted by *in vitro* toxicity tests, in fact the predicted level is somewhere in the order of five times less. An explanation of this phenomenon is not entirely clear, however, it would presumably arise as a result of the fact that the agricultural soil is very complex in its make up, and perhaps some of the methyl isothiocyanate is complexed to take on an inactive form, alternatively perhaps methyl isothiocyanate is simply too volatile and a majority of the compound escapes the soil without effecting sterilisation.

It is known that the isothiocyanates as a class are somewhat toxic, and the present invention is a result of the hope that other members of this class of compound may be more effective than the methyl isothiocyanate in its capacity to act as a soil fumigant.

Isothiocyanates are a catabolic product released from cruciferous plants. Cruciferous plants accumulate glucosinolates in various parts, including leaves, and roots. The glucosinolates stored in these tissues are not particularly toxic to microorganisms or other plants. On tissue damage, however, the glucosinolates are converted under the influence of the enzyme myrosinase to products, the most predominant of which are the isothiocyanates. Generally three different classes of naturally occurring glucosinolates exist in the cruciferous plant, the indolyl glucosinolates which are broken down to compounds that are predominantly compounds other than isothiocyanates, the aliphatic glucosinolates whereby an aliphatic group is present and the aralkyl glucosinolates whereby a phenyl, or substituted phenyl group is present. Catabolism of compounds of the latter two classes generally results predominantly in the production of isothiocyanates.

There is very large range of isothiocyanates produced naturally (Fenwick *et al* 1994) and different cultivars of the various cruciferous plants are found to have different types of glucosinolates present, and the levels of these will vary depending on the genetic background of the plants and to some extent the level of the glucosinolates will be dependent upon environmental factors. Additionally there is some variation in distribution of the glucosinolates depending upon the plant part that is examined, thus the roots of cruciferous plants generally have a different glucosinolate profile than do the leaves.

It has been shown that green manures of various plant parts of the cruciferous plants have a nematicidal activity which reflects the level of isothiocyanate released into the soil. Isothiocyanates released into the soil are difficult to measure and thus the level of aralkyl and alkyl glucosinolates present in a part of a plant applied to the soil is used as an initial estimate of the level of isothiocyanate released into the soil under the influence of myrosinase when the plant part is injured or damaged.

There has been considerable work on the toxicity of the more commonly occurring isothiocyanates. As many of these tests have been conducted *in vitro* however, the results are not uniformly applicable. It is found that the toxicity of isothiocyanate is dependent upon the micro-organism against which it has been tested. Furthermore as alluded to above a compound that is effective *in vitro* is not necessarily effective in soil.

Various of the isothiocyanates have also been tested in the soil and for example US 3113908 discloses the superiority of methyl isothiocyanate relative to other isothiocyanates in the soil. Phenyl isothiocyanate was amongst the compounds tested and is shown to be inferior to methyl isothiocyanate.

A number of *in vitro* tests using a variety of compounds including 2-PE isothiocyanate have been conducted including ones reviewed in Fenwick *et al.* (1994) pp123 (see pp176 to 177) where 2-PE isothiocyanate is shown to generally be one of the more effective *in vitro* against a variety of pests including certain moulds and insects, however, as far as the inventor is able to determine, 2-PE isothiocyanate has never been tested for its effectiveness as a soil sterilant so that it can be determined whether it will be an effective sterilant, or whether it will suffer the shortcomings of methyl isothiocyanate.

OBJECT OF THE INVENTION

It is an object of the present invention to provide a soil sterilant that obviates or minimises any one of the above problems or at least provides the public with a useful choice.

SUMMARY OF THE INVENTION

It has been found by the inventor that parts of plants producing a higher level of 2-PE glucosinolates are significantly more nematicidal in soil, than parts of plants whereby 2-propenyl glucosinolates are produced.

As a result of the present findings it is thought that 2-PE isothiocyanate is considerably more effective than 2-propenyl isothiocyanate or methyl isothiocyanate by reason of its structure.

It is thought that the aromatic ring (a phenyl group) assists in the retention of the structure within hydrophobic constituents of the soil such as membranes of various plants or micro-organisms or debris of the same. It is thought that the aralkyl group need not be a phenyl but may also be a benzyl group and still be effective. The short ethyl chain may provide a means of then holding the lethal isothiocyanate group away from the supporting substrates as to expose it to passing micro-organisms, or where the compound is lodged within the membrane of a target micro-organism the short chain may give the isothiocyanate group sufficient mobility to provide an effective contact with the micro-organism.

In one form the invention could be said to reside in a method of sterilising soil comprising contacting the soil with an effective amount of a sterilising compound said sterilising compound comprising

$$X\text{-}(CH_2)_n\text{-}N{=}C{=}S$$

wherein X is a phenyl group and wherein n is at least 1. In a preferable form n is 2. Alternatively n is 1.

The upper limit of n is likely to be the result of the degree to which the length of the bridge impacts on the stability of the molecule or the extent to which the molecule is water miscible.

It will be understood that the invention includes substitutions within the aromatic ring or phenyl group. It is thought that a variety of substitutions may be possible, for example with short aliphatic groups CH_3-$(CH_2)_{n2}$ where n2 equals 0 to 3, Cl, I, CH_3-O-,NO_2-, groups. Any such substitutions will need to maintain the hydrophobic nature of the aromatic ring.

Whilst the $(CH_2)_n$- group is very much preferred the invention may encompass another generally neutral link between the isothiocyanate group and X.

In an alternative form X is an aromatic ring.

In a further alternative form the sterilising compound is an aralkyl isothiocyanate, and most preferable is 2-PE isothiocyanate.

It will be understood that the sterilising compound may be suspended in a carrier which stabilises the isothiocyanate compound, and may also include a wetting agent to enhance the capacity of the sterilising compound to enter the soil, comprising a soil sterilant composition.

The procedures used will reflect current sterilising techniques presently used, thus present fumigant procedures, with the usual precautions to avoid contact with the sterilant will be employed. Thus the sterilant composition may be applied by a blade or tyne soil injector, by a sprinkler or trickle tape under mulch or a rotary hoe incorporating a following boom spray applicator. This will be applied in an approved manner. There will also be a period typically 2 to 3 weeks, and perhaps longer in heavier soils or soils with a larger organic content before the sterilised soil is to be worked, because many of the isothiocyanate including 2-PE isothiocyanate are phytotoxic.

The levels at which the sterilant may be applied will vary, and can be determined empirically. It is anticipated that the levels will either be in the order of 2 to 4 fold less than those used with methyl isothiocyanate preparations.

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