THE EFFECT OF *Plasmodiophora brassicae* INFECTION, PHOSPHONATE AND BION TREATMENT ON GLUCOSINOLATE LEVELS IN BROCCOLI

A thesis submitted in fulfilment of the requirements for the degree of Master of Science in Agriculture

Faculty of Agriculture, Food and Natural Resources
The University of Sydney
New South Wales
Australia
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Thesis submitted by
Md Nazrul Islam
SID No. 307050351

Thesis Research Supervisor
Professor David Guest

Faculty of Agriculture, Food and Natural Resources
The University of Sydney
New South Wales
Australia

2010

DECLARATION OF AUTHORSHIP

This thesis contains no material which has been submitted for a degree or diploma in any university or any other institution and no matter was previously published or written by other people, except acknowledgement is made in the text of this thesis.

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Abstract

Broccoli (*Brassica olerecea* L. ssp. *italica* cv. Marathon) seedlings are a rich source of secondary metabolites including glucosinolates such as 4-methylsulfinylbutyl glucosinolate (glucoraphanin), the precursor of the chemo-protective isothiocyanate, sulforaphane. The aim of this thesis is to investigate the levels of glucosinolate in root and aerial tissues of broccoli following *Plasmodiophora brassicae* infection and potassium phosphonate and Bion (acibenzolar) treatment under glasshouse condition.

Three inoculation techniques of *P. brassicae* in broccoli seedling were evaluated under glasshouse condition. Combination of spore extraction injected into the root zone and infected gall slurry amended in potting mix exhibited faster and effective disease development than any of the single inoculation method.

Field infections of clubroot changed glucosinolate profiles in *Brassica rapa ssp.chinensis* cv. pak choy. Aliphatic glucosinolate levels were significantly lower in leaf and stem tissues of diseased plants, while indole glucosinolate levels were nearly three times higher in infected root tissues.

In the glasshouse trial, the clubroot pathogen *Plasmodiophora brassicae* affected glucosinolate levels in both root and aerial tissues during primary, secondary and mature gall formation. Total aliphatic glucosinolate levels (glucoiberin, progoitrin, glucoraphanin, gluconapin) remained unchanged in aerial tissues but significantly increased (1.5 times) in root tissues during symptom development (28 days post inoculation). Among aliphatic glucosinolates, glucoraphanin significantly ($P<0.05$) increased after 28 days in root tissues and 14 days in aerial tissues. Progoitrin

production also increased both in root and aerial tissues after 28 and 14 days respectively, compared to healthy plants.

Total indole glucosinolates (4-hydroxy glucobrassicin, glucobrassicin and neoglucobrassicin) in root tissues increased 2.5 fold during symptom development to mature gall formation stage (21 to 42 days) and also significantly increased ($P<0.05$) in aerial tissues (1.25 to 2 fold) between primary infection and gall formation. Among indole glucosinolates, glucobrassicin in root tissues increased 8 times during symptom development.

Glucosinolate levels and clubroot disease severity were affected by foliar application of potassium phosphonate or Bion. Aliphatic glucosinolate levels in root tissues remained unchanged until 42 days following chemical treatments in both inoculated and uninoculated plants. Combined chemical treatment with phosphonate plus Bion significantly suppressed ($P<0.01$) aliphatic glucosinolate levels in uninoculated plants, however it significantly increased levels in inoculated plants. Indole glucosinolate levels in inoculated root tissues were lower in phosphonate plus Bion treated plants. Bion, or combinations of phosphonate plus Bion, did not affect indole glucosinolate levels, but phosphonate alone significantly ($P<0.001$) increased indole glucosinolate levels in root tissues in uninoculated plants. Aliphatic and indole glucosinolate levels in aerial tissues was lower following chemical treatment in both inoculated or uninoculated plants. Only neoglucobrassicin significantly ($P<0.001$) increased in inoculated plants.

The effects of clubroot on glucosinolate levels in both field and glasshouse grown brassica showed similar patterns.

Phosphonate significantly suppressed gall formation (46%) and fresh gall weight (70%) followed by Bion compared to inoculated control.

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Chapter 1

Review of Literature

1.1 Introduction

The Brassicaceae family includes vegetables such as broccoli, cabbage, cauliflower, collards, kale, turnip greens and leaf rape. They are commonly grown and consumed worldwide. Glucosinolates are the major class of secondary metabolites found in brassica crops.

The first observations on the unique properties of glucosinolates were recorded at the beginning of the 17th Century as a result of the study on the chemical origin of the sharp taste of mustard seeds (Fahey *et al.* 2001). Glucosinolates known as Sinigrin (2-propenyl) and Sinalbin (4-hydroxybenzyl) were isolated early in the 1830s from black (*Brassica nigra*) and white (*Sinapis alba*) mustard seeds, respectively. In 1956, Ettlinger and Lunden (1956) proposed the correct structure of glucosinolates and they described the first chemical glucosinolate synthesis (Fahey *et al.* 2001).

Different epidemiological studies have indicated that diet and cancers are closely interlinked and natural phytochemicals such as glucosinolates have anticarcinogenic properties (Talalay and Fahey 2001; Anilakumar *et al.* 2006). Results have consistently shown that the chemo-protective agents derived from brassicas have an influence on carcinogenesis during the initiation and promotion phases of cancer development. Reports from clinical trials support this notion (Smith *et al.* 2005; Farnham *et al.* 2004; Rosa *et al.* 1997). Epidemiological data show that a diet rich in brassica vegetables can reduce the risk of a number of cancers and the risk can be significantly reduced by an intake of as little as 10 g per day (Kohlmeier and Su 1997;

Wattenberg 1993; Graham 1983). Isothiocyanates are able to inhibit the growth of cancer (Munday and Munday 2004).

Recent studies have shown broccoli sprouts to be the best source of glucosinolates, particularly glucoraphanin (4-methylsulfinylbutyl) the precursor of sulforaphane, and one of the most potent naturally occurring inducers of detoxification enzymes, Phase II enzymes (Zang and Talalay 1994; Hecht 2000; Fahey *et al.* 2002a; Anilakumar *et al.* 2006).

Glucosinolates are phytochemicals that break down upon tissue disruption and react with an enzyme called myrosinase to produce isothiocyanates. Tissue disruption can be caused by environmental factors, insect invasion or disease.

Glucosinolate levels in brassica are affected by many factors. Factors that may contribute to the quality and quantity of glucosinolates include soil type, fertilizer application, plant spacing, and date of harvest (Rosa *et al.* 1996; Griffiths *et al.* 1991; Josefsson, 1970). The level of glucosinolates and their corresponding isothiocyanates vary within plants due to external environment, including light intensity, temperature, water stress and micro-organism interference such as disease (Bouchereau *et al.* 1996; Rosa and Rodrigues 1999a). Levels and types of glucosinolates also vary between species and cultivars and between plant tissues. Seeds have the highest levels of glucosinolates in nearly all plants observed, followed by leaves and shoots (Sexton *et al.* 1999). Glucosinolates and their breakdown products are thought to play a role in disease resistance against insect and fungal pathogens and in host pathogen recognition (Glen *et al.* 1990).

The following review aims to introduce the important role of glucosinolates during disease development, focusing on clubroot of broccoli and Chinese cabbage. Different management options for clubroot disease are discussed. This review also includes the different HPLC methods of measuring and extraction procedure of glucosinolates. I also review the significance of glucosinolates as bioactive compounds for human nutrition and health and examine the influence of environmental conditions, including biotic stress imposed by the clubroot pathogen *Plasmodiophora brassicae* on glucosinolate levels in broccoli seedlings. This review also focuses on glucosinolate biosynthesis and highlights recent advances made in understanding glucosinolate pathways.

1.2 The Origin, Distribution and Classification of Cultivated Broccoli

Broccoli has gained increasing importance for its potential health benefits. Although it does not constitute a significant portion of most people's diets worldwide, broccoli consumption increased by nearly 34 percent between 1990 and 1998, and the production of crucifers is in the top three vegetables after potato and tomato (FAO 2006).

The botanical family of broccoli is the Brassicaceae, also known as the Mustard family. The Brassicaceae is a large family comprised of approximately 3,000 described species apportioned among 350-380 genera. The precise number of genera varies depending on the authority (Keil & Walters 1988; Heywood 1978). For example, the scientific name for broccoli, *Brassica oleracea* (L.), is also shared by cabbage, Chinese cabbage, cauliflower, collards, Brussels sprouts, kale, kohlrabi, and tronchuda kale. Despite the fact that all of the aforementioned varieties are similar to one another and to broccoli, and are therefore referred to as *B. oleracea*, they are

nevertheless separate entities. Most authorities today consider there are two major varieties of broccoli, *B. oleracea* (L.) var. *botrytis* or cauliflower broccoli and *B. oleracea* (L) var. *italica* or sprouting broccoli (Keil & Walters 1988; Heywood 1978; Terrell 1977; Harlan 1975) (Figure 1.1).

Crucifer vegetables include broccoli, cabbage, Brussels sprouts, cauliflower and kale, which evolved from the wild ancestral cabbage nearly 3000 years ago (Phillips and Rix 1993). Broccoli was introduced into England in the early 16th Century where it was known as "Italian asparagus". Members of this family are found in most parts of the world but are mainly concentrated in the north temperate region, in the countries surrounding the Mediterranean basin and in south-western and central Asia.

As far as the two broccoli varieties are concerned, the cauliflower broccoli (var. *botrytis*) is more widely grown in Europe than else where. It is a hardier variety than sprouting broccoli and is grown to be harvested in the winter and spring. In the U.S.A, and some of Asian countries sprouting broccoli (var. *italica*) is more common and is planted as a summer annual (Schery 1972). Siberian broccoli, a small, hardy, purple member of this family was first planted at Norfolk Island, Australia in 1788. Throughout the 19th century, purple and green varieties were available in Australia but broccoli was not as popular as ordinary cabbage but its demand is increasing day by day (Anonymous 2009b). Currently, brocolli is being cultivated all over Australian territories and major growing areas are shown in Figure 1.2. The primary use of broccoli, both modern and ancient, is as a source of food. The part of the broccoli plant consumed is the fleshy-stemmed flowering head (Schery 1972).

Cultivation of a cabbage may have occurred as early as 8,000 years ago along the Northern European coast. The wild ancestral cabbage was later introduced into the Mediterranean, Eastern Europe and even into the Near East and Orient (Heywood 1978; Schery 1972). Theophrastus (370-285 B.C.) indicated that several different coles were already used in Greece, (Snogerup 1980). The Romans Plinius (23-79 A.D.) and Cato (234-149 B.C.) also mentioned the cultivation of a number of different forms of coles, primarily cabbages and kales (Snogerup 1980). Indeed, it is more than likely that "The first selection of sprouting broccoli was probably made in Greece and Italy in the pre-Christian era" (Heywood 1978).

The frequency of hybridization and outcrossing is greatly enhanced by a high degree of self-incompatibility among the brassicas. In light of these considerations, it is not surprising to find so many different varieties among the brassicas and the *B. oleracea* group in particular. In fact, the close proximity of various cultivars of *B. oleracea* with their wild relatives will more than likely result in further hybridization (Snogerup 1980). The taxonomy of common cruciferous/brassica vegetables are shown in Table 1.1.

Figure 1.1: Two heads of common broccoli variety (*Brassica oleracea* var. *italica* group CV Marathon)

Figure 1.2 Major broccoli growing areas in Australia

Table 1.1: Worldwide cultivated *Brassica* varieties and cultivar groups

Common name	**Genus**	**Specific Epithet**	**Cultivar Group**
kale	*Brassica*	*oleracea*	Acephala Group
collards	*Brassica*	*oleracea*	Acephala Group
chinese broccoli (gai laan)	*Brassica*	*oleracea*	Alboglabra Group
cabbage	*Brassica*	*oleracea*	Capitata Group
brussets sprout	*Brassica*	*oleracea*	Gemmifera Group
kohlrabi	*Brassica*	*oleracea*	Gongylodes Group
broccoli	***Brassica***	***oleracea***	**Italica Group**
broccoflower nn	*Brassica*	*oleracea*	Italica Group × Botrytis Group
broccoli romanesco	*Brassica*	*oleracea*	Botrytis Group / Italica Group
cauliflower	*Brassica*	*oleracea*	Botrytis Group
wild broccoli	*Brassica*	*oleracea*	Oleracea Group
Bok choy	*Brassica*	*rapa*	*cinensis*
mizuna	*Brassica*	*rapa*	*nipposinica*
broccoli rabe	*Brassica*	*rapa*	*parachinensis*
flowering cabbage	*Brassica*	*rapa*	*parachinensis*
chinese cabbage, napa cabbage	*Brassica*	*rapa*	*pekinensis*
turnip root; greens	*Brassica*	*rapa*	*rapifera*
rutabaga	*Brassica*	*napus*	*napobrassica*
siberian kale	*Brassica*	*napus*	*pabularia*
canola/rape seeds; greens	*Brassica*	*napus*	*oleifera*
wrapped heart mustard cabbage	*Brassica*	*juncea*	*rugosa*
mustard seeds, brown; greens	*Brassica*	*juncea*	
mustard seeds, white	*Brassica*	*hirta*	
mustard seeds, black	*Brassica*	*nigra*	
tatsoi	*Brassica*	*rosularis*	
ethiopian mustard	*Brassica*	*carinata*	
radish	*Raphanus*	*sativus*	
daikon	*Raphanus*	*sativus*	*longipinnatus*
horseradish	*Armoracia*	*rusticana*	
Real wasabi (not horseradish)	*Wasabia*	*japonica*	
rocket (arugula)	*Eruca*	*vesicaria*	
watercress	*Nasturtium*	*officinale*	

Citation: Wood R, 1999; Zohary and Hopf, 2000; Dixon GR (2007) and web site visited on 12-12-08 http://encyclopedia.thefreedictionary.com/Cruciferous+vegetables.

1.3. Economic Importance of Broccoli in Australia

Horticulture in Australia is a $7 billion industry (Anonymous 2008a). It is extremely diverse and comprises fruit, vegetables, nuts, nursery, extractive crops, cut flowers and turf. Total horticultural exports in 2006/07 were $763 million. Total horticulture exports (fresh fruit, vegetable, nuts and plants including flowers) were $751 million (12 months to May 2008). It is the fastest growing industry in agriculture with 17,273 individual enterprises. Horticulture employs over 100,000 people; this translates to about 20 per cent of total employment in agriculture (Anonymous 2008b).

In Australia, vegetable crops account for 36% of horticultural production of which 13% are exported. Broccoli and cauliflower are among the 9 highest annual producing crops. Broccoli was the seventh most valuable crop in 2006-07, with 40,032 tonnes produced valued at $87.5 million. Victoria is the largest producer (42%) followed by Queensland (25%) and New South Wales accounts for only 9%. Severe competition from China is reducing Australia's share of exports amongst Asian economies. Partially offsetting this development is the fact that new markets in the Middle East are being developed (Anonymous 2008c).

1.4 Glucosinolates

The glucosinolates are a group of over 130 nitrogen and sulphur-containing natural products derived from glucose and an amino acid. They are found almost exclusively in plants of the Brassicaceae and other related families of the order Capparales (Brown and Morra, 1997; Fahey *et al.* 2001). Every glucosinolate contains a central carbon atom which is bound via a sulphur atom to the thioglucose group (making a sulfated ketoxime) and via a nitrogen atom to a sulphate group (Figure 1.3). In addition, the central carbon is bound to a side group, different glucosinolates have

different side groups, and it is variation in the side group that is responsible for the variation in the biological activities of these plant compounds (Fenwick *et al.* 1983a; Chew 1988a; Rask *et al.* 2000; Lambrix *et al.* 2001).

About 130 different glucosinolates are known to occur naturally in plants. Glucosinolates can be classified by their precursor amino acid and the types of modification to the R group. So called aliphatic glucosinolates derived from mainly methionine, but also alanine and leucine. Most glucosinolates are actually derived from chain elongated homologues of these amino acids, e.g. glucoraphanin is derived from dihomomethionine, which is methionine chain elongated twice (Brown *et al.* 2003). Variations of glucosinolate levels among the *Brassica* plants observed in previous investigations (Kiddle *et al.* 2001; Moreno *et al.* 2006) are listed in Table 1.2.

The Brassicas contain the enzyme myrosinase which, in the presence of water, cleaves off the glucose group from a glucosinolate molecule. The remaining molecule then quickly converts to an isothiocyanate, a nitrile, or a thiocyanate. These are the active substances that serve as defence for the plant (Glen *et al.* 1990; Doughty *et al.* 1991). The standard product of the reaction is the isothiocyanate (mustard oil), the other two products mainly occur in the presence of specialised plant proteins that alter the outcome of the reaction (Burow *et al.* 2007). To prevent damage to the plant itself, the myrosinase and glucosinolates are stored in separate compartments of the cell and come together following physical injury or cellular disruption.

Because the use of glucosinolate containing crops as a primary food source for animals was shown to have toxic effects, food crops have been developed that contain

2very low amounts of glucosinolates (e.g. canola). On the other hand, plants producing large amounts of glucosinolates are also desirable, because substances derived from glucosinolates can serve as biological pesticides (Nicholls *et al.* 1999) (effective in soil fumigation) and are under investigation in the prevention of cancer with sulphoraphane in broccoli being the best known example (Hecht 2000).

Glucosinolates are well known for their toxic effects at high doses (mainly as goitrogens) in both humans and animals. Their hydrolytic and metabolic products act as chemo-protective agents against chemically-induced carcinogens and have been shown to block the initiation of tumors in a variety of rodent tissues eg. liver, colon, mammary gland, pancreas, etc (Verhoeven *et al.* 1997; Talaly and Fahey 2001; Fahey 2002a; Conaway *et al.* 2002).

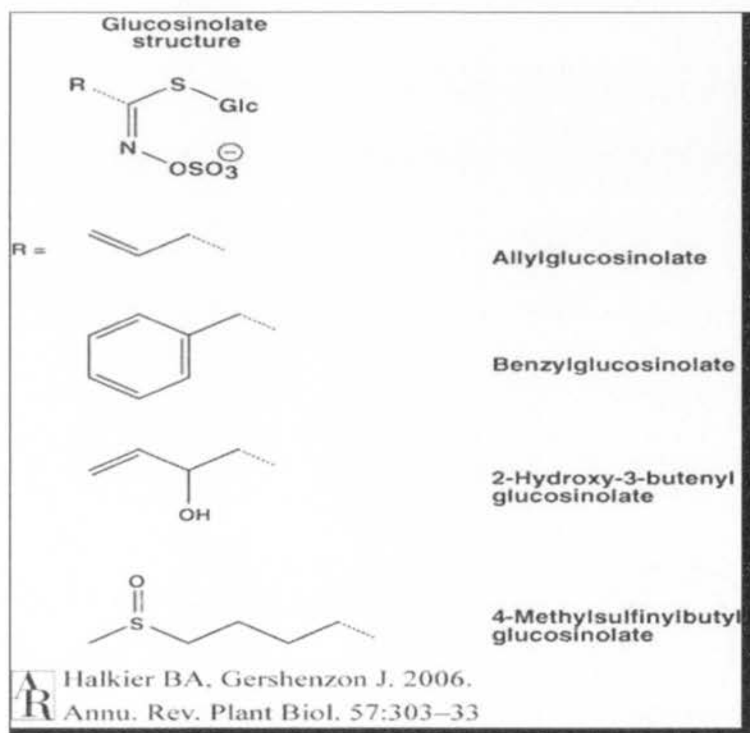


Figure 1.3: The common structure of glucosinolate and some specific glucosinolates showing typical variation in the structure of the side chain.

Table 1.2. Some of the brassica plants and tissues source of specific glucosinolate group

Brassica species	Plant Organ	Major Glucosinolate Class
Arabis hirsuta	Hairy rock cress, young leaves	Aromatic
Barbarea praecox	Land cress, young leaves	Aromatic
Barbarea vulgaris	Bitter winter cress, young leaves	Aromatic
Brassica campestris	Chinese cabbage, seeds	Indolyl
B. juncea	Brown mustard young leaves	Alkenyl
B. napus	Oilseed rape young leaves	Mixed
B. nigra	Black mustard young leaves	Alkenyl
B. oleracea var. *botrytis* subvar. *Cymosa*	Calabrese broccoli florets	Mixed
B. oleracea var. *Italica*	Broccoli head	Mixed
Conringia orientalis	Hares ear cress young leaves	Alkyl
Isatis tinctoria	Woad young leaves	Indolyl
Lepidium sativum	Garden cress young leaves and roots	Aromatic
Nasturtium officinalis	Watercress young leaves	Mixed
Reseda luteola	Dyers rocket young leaves	Mixed
Reseda alba	White mignonette young leaves	Alkyl
Sibara virginica	Young leaves	Mixed
Tropaeolum majus	Nasturtium young leaves	Aromatic

Extracted and modified from Fahey *et al.* 2001; Kiddle *et al.* 2001; Fahey 2002b; and Moreno *et al.* 2006.

1.4.1 Biosynthesis of Glucosinolate

The formation of glucosinolates can be divided into three separate phases. First, certain aliphatic and aromatic amino acids are elongated by inserting methylene groups into their side chains (Figure 1.4). The three principal steps are: (1) condensation with acetyl-CoA, (2) isomerisation, and (3) oxidation-decarboxylation (Halkier and Gershenzon 2006).

Second, the amino acid moiety itself, whether elongated or not, is metabolically reconfigured to give the core structure of glucosinolates (Figure 1.5). CYP79 enzymes

catalysing the conversion of amino acids to aldoximes are the only side-chain-specific step in the pathway (Halkier and Gershenzon 2006). The products from the CYP83s are too reactive to be isolated, but are proposed to be either *aci*-nitro compounds or their dehydrated analogs, nitrile oxides. The sulphur-donating enzyme is the only enzyme that remains to be identified, and is proposed to be a glutathione-*S*-transferase-like enzyme that uses cysteine as a substrate (Halkier and Gershenzon 2006).

Third, the initially formed glucosinolates are modified by various secondary transformations (Figure 1.6). AOP2 and AOP3 indicate the 2-oxoacid-dependent dioxygenases catalysing these reaction types in *Arabidopsis*. For each category of glucosinolate, a different range of chain lengths is known to occur naturally (Halkier and Gershenzon 2006).

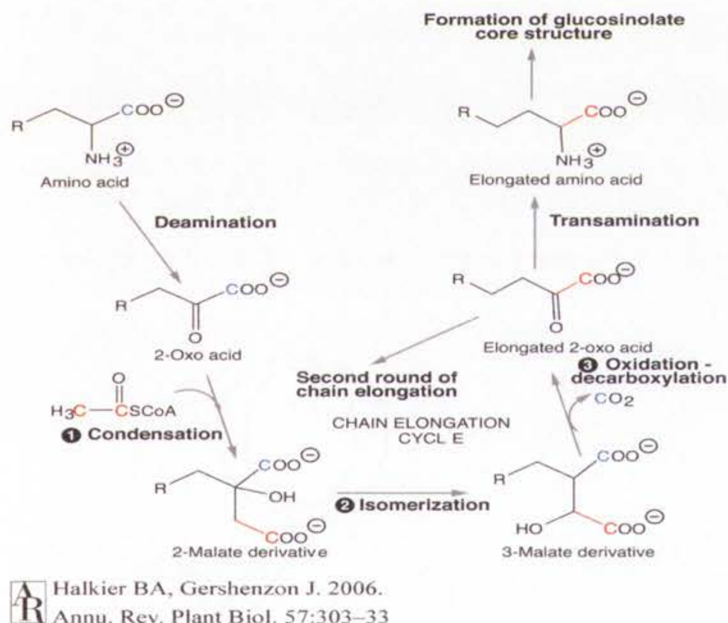


Figure 1.4: Glucosinolate biosynthesis and amino acid chain-elongation cycle. Abbreviations: R, variable side chain.

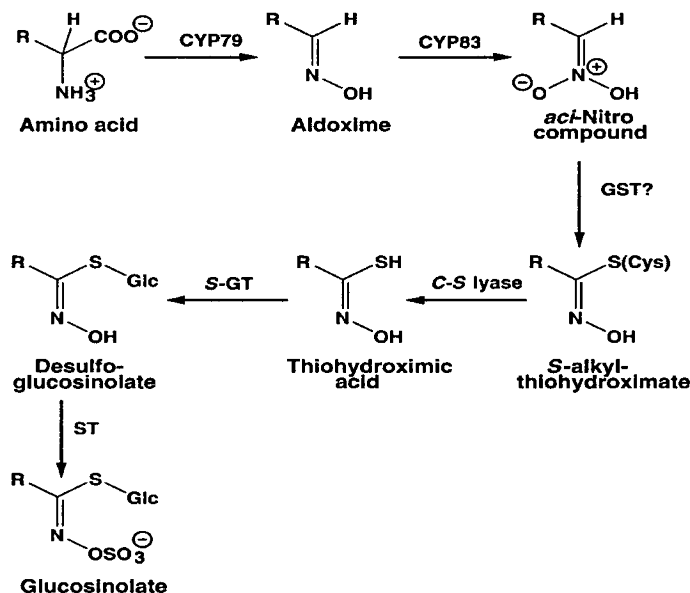


Figure 1.5: Biosynthesis of the glucosinolate core structure. Abbreviations; R, variable side chain; GST, glutathione-*S*-transferase; *S*-GT, *S*-glucosyltransferase; ST, sulphotransferase and C-S lyase.

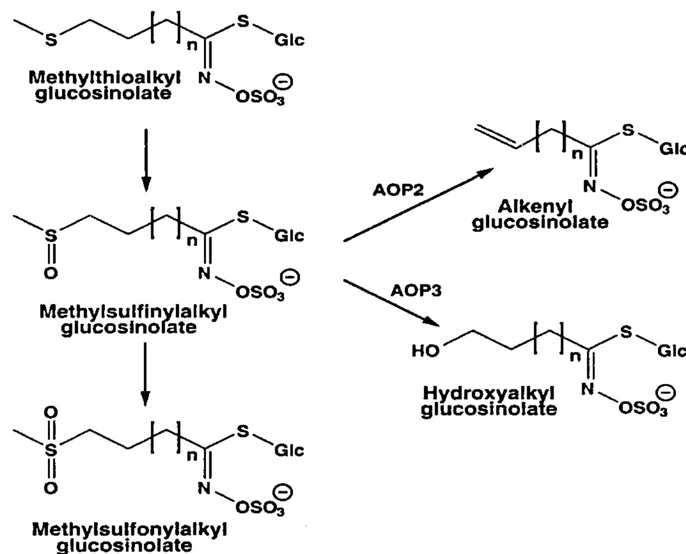


Figure 1.6: Some common oxidative secondary transformations of methionine-derived glucosinolates. For methylthioalkyl and methylsulphonylalkyl, n = 1–8; for methylsulphinylalkyl, n = 1–9; for alkenyl, n = 1–5; for hydroxyalkyl, n = 0–2. Abbreviations-AOP2 and AOP3: 2 & 3-oxoacid-dependent dioxygenases catalyzing agent.

1.4.2 Hydrolysis of Glucosinolate

Glucosinolates are degraded upon plant damage to a variety of hydrolysed products responsible for most of the biological activity. The process begins with myrosinase-catalysed hydrolysis of the thioglucoside linkage, leading to the formation of glucose and an unstable aglycone (Bones and Rossiter 1996; Rask *et al.* 2000). Depending on the structure of the side chain and the presence of additional proteins and co-factors, the aglycone then rearranges to form different products, including isothiocyanates, oxazolidine-2-thiones, nitriles, epithionitriles, and thiocyanates (Figure 1.7).

The most common glucosinolate hydrolysis products in many plant species are isothiocyanates, which are formed from the aglycone by a Lossen rearrangement involving the migration of the side chain from the oxime carbon to the adjacent nitrogen (Halkier and Gershenzon 2006). When the glucosinolate side chain bears a hydroxyl group at C-2, the isothiocyanates formed are unstable and cyclize to oxazolidine-2-thiones, a class of substances known to cause goitre.

In other plants, a major percentage of glucosinolate hydrolysis products are nitriles (Cole 1976; Lambrix *et al.* 2001). The formation of nitriles *in vitro* is favoured at a pH of less than 3 or in the presence of the Fe^{2+} ion (Gil and Macleod 1980; Galletti *et al.* 2001). However, protein factors may be involved in nitrile formation *in vivo*, such as the epithiospecifier protein (ESP) (Tookey 1973; MacLeod and Rossiter 1985; Bernardi *et al.* 2000; Foo *et al.* 2000). When the glucosinolate side chain has a terminal double bond, ESP promotes the reaction of the sulphur atom of the thioglucoside linkage with the double bond to form a thirane ring. The process occurs only in the presence of myrosinase. ESP is not known to have any catalytic abilities

by itself. The recent isolation of an *Arabidopsis* gene encoding an ESP showed that this protein not only promotes the formation of epithionitriles, but also the formation of simple nitriles from a large variety of glucosinolates (Lambrix *et al.* 2001).

Other hydrolysis products include thiocyanates, which are formed from only three glucosinolates: benzyl-, allyl-, and 4-methylsulphinylbutyl-glucosinolate (Figure 1.3), all of which form stable side-chain cations. Like nitrile formation, thiocyanate production is also associated with specific protein factors (Hasapis & Macleod 1982), but these have not yet been identified. The hydrolysis of indole glucosinolates is somewhat different from that of the other glucosinolate types, because the initially formed isothiocyanates are unstable at neutral or slightly acidic pH, and are converted to further metabolites, including indole-methanols, ascorbic acid conjugates, and oligomeric mixtures (Latxague *et al.* 1991; Agerbirk *et al.* 1998; Buskov *et al.* 2000).

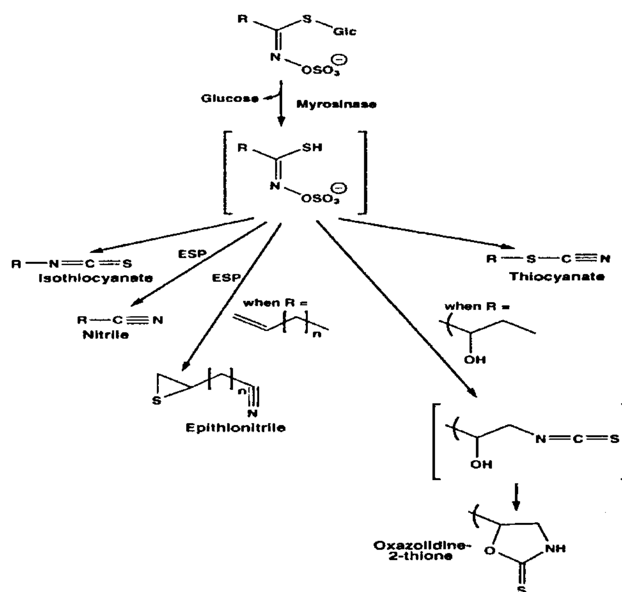


Figure 1.7: Glucosinolate hydrolysed product and unstable intermediates (within brackets). Abbreviations: ESP, epithiospecifier protein; R, variable side chain.

1.4.3 Glucosinolate Content Variations among Plant Tissues

Glucosinolate content in plants is about 1% of dry weight in some tissues of *Brassica* vegetables (Rosa *et al.* 1997), although the content is highly variable (Farnham *et al.* 2004), and can approach 10% in the seeds of some plants, where glucosinolates may represent one-half of the sulphur content of the seeds (Josefsson 1970).

Distribution of the glucosinolates that have been examined varies among plant organs, with both content and diversity differences between roots, leaves, stems and seeds. For example, seeds or young sprouts of broccoli (*Brassica oleracea* var. *italica*) can contain 70–100 □mol total glucosinolates / g FW, with <1% contributed by indole glucosinolates and the balance consisting almost entirely of the aliphatic glucosinolates, glucoraphanin, glucoerucin and glucoiberin (Fahey *et al.* 1997). Late vegetative to reproductive stage plants of the same cultivar typically may contain only about 1–4 □mol of total glucosinolates/g FW, with aliphatic and indole glucosinolates present at roughly equivalent levels (Fahey *et al.* 1997; Fahey and Stephenson 1999).

Plant age is a major determinant of the qualitative and quantitative glucosinolate composition of plants. Environmental factors such as soil fertility (Booth and Walker 1992; Fahey and Stephenson 1999), pathogen challenge (Butcher *et al.* 1974), wounding (Bodnaryk 1992) or plant growth regulators (Bodnaryk 1994; Bodnaryk and Yoshihara 1995) also have significant effects on levels of specific glucosinolates in the growing plants and may affect distribution among plant organs. Variation of glucosinolates in different plant tissues has been summarised below (Table 1.4 & 1.5).

Table 1.3. Glucosinolate variation within different plant species and tissues

Species	Glucosinolate concentration in μmol per gram dry weight									
	Organ	Seed	Sprout	Stem	Root	leaves	Leaves	Infloresence	Siliques	References
	Age of plant (days)		matured	matured	matured	10 days	50-70 days	70 days		
	Common name									
Brassica oleracea botrytis	Var. Cauliflower							3.22*		Tian *et al.* 2005
Brassica oleracea	Var, Brussel sprouts, Gemmifera						9.40*			Tian *et al.* 2005
Brassica oleracea italica	Broccoli							2.08*		Tian *et al.* 2005
Brassica oleracea italica	Broccoli		4.02*							Tian *et al.* 2005
Brassica oleracea acephala	Ornamental kale, chidori white						9.07			Kushad *et al.* 1999
Brassica oleracea acephala	Ornamental kale, Nagoya white						10.6			Kushad *et al.* 1999
Brassica oleracea acephala	Ornamental cabbage, Tokyo white						11.53			Kushad *et al.* 1999
Brassica oleracea acephala	Ornamental cabbage, Tokyo Red						14.18			Kushad *et al.* 1999
Brassica napus	Ssp. Rape, Pabularia						19.85			Font *et al.* 2005
Brassica campestris pekinensis var. Granat	ssp. Chinese cabbage	14.72			10.0					Ludwig-Müller *et al.* 1997
Brassica campestris pekinensis var.	ssp. Chinese cabbage, Granat	8.28			5.0					Ludwig-Müller *et al.* 1997
Wasabi japonica	Wasabi	>150		9.1						Sexton *et al.* 1999
Brassica juncea	Indian mustard	85		1.61						Sexton *et al.* 1999
Brassica napus	Canola	22		0.56						Sexton *et al.* 1999
Arabidopsis thaliana Heyn	Thale Cress	63.4			18.6	17.9	7.4	30.2	15.4	Brown *et al.* 2003
Brassica oleracea	Broccoli, italica							12.8 (edible portion)		Kushad *et al.* 1999

* μmol per g fresh weight

Table 1.4. Mean individual glucosinolates (expressed in μmol per gram dry mass) among edible tissues of *Brassica oleracea*

Glucosinolates		*B. oleracea* varieties				
Group	Form	Broccoli (Marathon)	Brussels sprouts	Cabbage	Cauliflo wer	Kale
Aliphatic	Sinigrin	0.1	8.9	7.8	9.3	10.4
	Gluconapin	1.0	6.9	0.7	0.3	1.0
	Progoitrin	1.0	2.4	0.2	0.3	0.6
	Glucoiberin	0.1	0.0	0.0	0.0	0.0
	Glucoraphanin	7.1	1.0	0.1	0.5	1.0
Indole	Glucobrassicin	1.1	3.2	0.9	1.3	1.2
	4OH glucobrassicin	0.2	0.6	0.3	1.6	0.1
	4 CH_3OH glucobrassicin	0.4	0.4	0.3	1.0	0.2
	Neoglucobrassicin	0.2	0.2	0.2	0.2	0.1

Extracted from Faulkner *et al.* 1998; Kushad *et al.* 1999.

1.4.4 Factors Affecting the Level of Glucosinolates in Plants

Variation in the amount of glucosinolates has been attributed to genetic and environmental (biotic and abiotic) factors. Glucosinolates contain a significant proportion of sulphur and nitrogen. Therefore it might be expected that fertilisers will influence the concentrations of glucosinolates in *Brassica* crops. It has been suggested that under conditions of sulphur deficiency, sulphur bound in glucosinolates of *Brassica* species can be remobilised by enzymatic cleavage with myrosinase (Schnug *et al.* 1993). The mechanism for this is thought to involve the control of myrosinase activity by the ascorbate/glutathione cycle. Studies have shown a close relationship between sulphur status, glucosinolate concentrations and glutathione (Schnug *et al.* 1995), although inter-dependencies with ascorbate are less apparent.

Glucosinolate profiles of four oilseed rape cultivars (Bienvenue, Ariana, Cobra and Capricorn) have been determined at vegetative growth stages and showed substantial differences in their profiles (Fieldsend and Milford 1994). Changes in the profiles of glucosinolates throughout the plant's development are thought to have implications for pests and diseases. A decreasing sulphur supply to the plants results in a decrease

in free sulphate and glucosinolate concentrations and an increase in myrosinase activity (Underhill, 1980; Schnug 1990). This implies that the increase in myrosinase activity during sulphur stress could have the function of a remobilisation of sulphate sulphur from glucosinolates, because sulphate and isothiocyanates can be utilised as sulphur sources in the primary metabolism of the plants (Machev and Schraudolf 1978).

Aires *et al.* (2006) investigated the effect of fertilisation on glucosinolate levels in broccoli (*Brassica oleracea* L. var. *italica* cv. Marathon) roots and leaves. Nitrogen was tested at 0, 45.5, 91.0 mg L^{-1} and sulphur at 0, 14.6 and 29.2 mg L^{-1}. The results showed that total glucosinolates in the aerial part were significantly higher ($P < 0.001$) than in the roots. The major glucosinolates in the aerial part were 4-methylsulfinylbutyl (glucoraphanin) and 3-methylsulphinylpropyl (Glucoiberin) whereas in the roots they were 2-phenylethyl (gluconasturtiin) and 4-methylthiobutyl (glucoerucin). Fertilisation of broccoli sprouts had a significant ($P < 0.001$) detrimental effect on the levels of aliphatic glucosinolates whereas the opposite was noted for indole and aromatic glucosinolates.

Jones *et al.* (2007) showed that nitrogen applications over 30 kg/ha caused a decrease in the content of glucoraphanin (18-34%), progoitrin content was not affected while glucobrassicin increased by up to 44% with nitrogen application >30 kg/ha. Sulphur applications of 50 or 100kg/ha had no significant effect on any tested glucosinolates.

Numerous comparative studies of glucosinolate profiles indicate significant differences among cruciferous crops (VanEtten *et al.* 1976; Carlson *et al.* 1987a; Kushad *et al.* 1999 Ciska *et al.* 2000). Apart from glucosinolates profile, large

differences in the levels of both aliphatic and indole glucosinolate have been observed in brassica plants, presumably due to the use of different varieties, analytical methods and growing conditions (Cartea *et al.* 2008).

It is clear that the glucosinolates/myrosinase system is dynamic, responding to environmental changes and to plant damages. Pretreatment with elicitor compounds, which stimulate glucosinolate accumulation (Kiddle *et al.* 1994, Doughty *et al.* 1995), can enhance resistance of the plant to subsequent infection by pathogens (Doughty *et al.* 1991). The few reports of glucosinolates accumulation patterns in *Brassica* crops following *Plasmodiophora brassicae* infection are summarised below (Table 1.5).

Table 1.5: Glucosinolate content (μmol /g DW) in *Brassicas* following *Plasmodiophora brassicae* infection

Plant varieties	Benzyl glucosinolates						
	Root (control)	Root (infected)	Old leaves (control)	Old leaves (Infected)	Young leaves (Control)	Young leaves (infected)	References
Tropaeolum majus	6.85	9.76 nmol	6.33	0.959	5.36	13.41	Ludwig-Müller *et al.* 1999b
Carica papaya	7.36	11.84	1.935	2.074	5.63	4.00	Ludwig-Müller *et al.* 1999b
Reseda alba	6.59 (progoitrin) 553 (glucobrassicin) 3.199 (neoglucobrassicin)	4.01 (progoitrin) 0.398 (glucobrassicin) 3.02 (neoglucobrassicin)	-	-	-	-	Ludwig-Müller *et al.* 1999b
Brassica campestris ssp pekinensis (Yuki, Parkin – resistant & Granat, Osiris susceptible var.)	Total Glucosinolates- 9.31 (Var.Yuki)	Total Glucosinolates- 14.72 (Var.Granat)	-	-	-	-	Ludwig-Müller *et al.* 1997
	8.28 (Var.Parkin)	30.31 (Var.Osiris)	-	-	-	-	

1.4.5 Microbial Interaction and Glucosinolates and Isothiocyanates

The antibacterial activities of isothiocyanates (Kjaer and Conti 1954; Virtanen 1962; Wagner *et al.* 1965; Johns *et al.* 1982; Uda *et al.* 1993; Brabban and Edwards 1995; Detaquis and Mazza 1995; Hashem and Saleh 1999; Lin *et al.* 2000) and their

antifungal activity (Drobinca *et al.* 1967; Kojima and Ogawa 1971; Mari *et al.* 1993; Detaquis and Mazza 1995; Mayton *et al.* 1996; Manici *et al.* 1997; Hashem and Saleh 1999) have been recognised for many decades. Activity against a range of soil-borne fungal and bacterial plant pathogens is profound, and has been extensively characterised (Brown and Morra 1997; Rosa and Rodrigues 1999b; Chen *et al.* 2000).

The activity of isothiocyanates such as sulphoraphane against numerous human pathogens (e.g. *Escherichia coli, Salmonella typhimurium, Candida* sp.) could even contribute to the medicinal properties ascribed to cruciferous vegetables, such as cabbage, and mustard, which have been used as wound poultices and antitumor agents for centuries (Hartwell 1982; Gordon and Newman 2005).

Glucosinolates are widely recognized as defensive compounds against generalist herbivores and are likely to be involved in host plant recognition by specialist predators, thus acting both as an insecticide and as an insect feeding attractant (Louda and Mole 1991; Rask *et al.* 2000).

1.4.6 Broccoli, Glucosinolate and Cancer Chemo-protection

Broccoli is considered a good source of nutrients because it is rich in vitamin C, carotenoids, fibre, calcium, and folate. It is also the source of many phyto-chemicals that may have anti-cancer properties (Verhoeven *et al.* 1997; Shapiro *et al.* 1998; Conaway *et al.* 2002). A glucosinolate derivative identified in broccoli appears to be more effective than modern antibiotics against the bacteria which cause peptic ulcers (Traka and Mithen 2009). Moreover, tests in mice show that the compound offers tremendous protection against stomach cancer - the second most common form of cancer in the world (Anonymous 2002). For example, broccoli contains several isothiocyanates, including sulphoraphane and indole-3-carbinol (I3C), which have

been considered as possible anti-cancer agents in recent years (Basten *et al.* 2000). Early studies have shown these substances may act as antioxidants and may boost detoxifying enzymes in the body. Some studies have also suggested they may alter body oestrogen levels, which might affect breast cancer risk (Traka and Mithen 2009).

Studies demonstrating that broccoli consumption or supplementation reduces the risk of cancer are frequent in the scientific literature, with multiple references to different types of cancers, and the complexity affecting the study of gene–diet interactions and cancer risk in humans (Finley *et al.* 2000; Lampe and Peterson 2002; Ambrosone *et al.* 2004; Jansen, 2004; Walters 2004). Examples of the wide range of studied effects of dietary anticancer bioactives from broccoli include the antiproliferative effects of sulphoraphane in human breast cancer (Jackson and Singletary 2004; Brandt *et al.* 2006; Rose *et al.* 2005) reduced risk of cancer via decreased damage to DNA (Gill *et al.* 2004; Wiseman *et al.* 2005), effects on the regulation of intestinal cell growth and death in colon cancer (Parnaud *et al.* 2004), the cancer-protective effect of high-selenium broccoli (Finley *et al.*2000) and the protective effect against prostatic tumours (Zhang et *al.* 1992; Giovannucci et *al.* 2003; Canene-Adams *et al.*2005a and 2005b).

Bioactive isothiocyanates from broccoli suppress bladder carcinoma cells (Munday and Munday 2004 and Tang and Zang 2004), protect against cellular oxidative stress (Eberhardt et *al.* 2005) and lower serum cholesterol effects (Suido *et al.* 2003). Glucosinolates protect against cardiovascular disease (Sesso *et al.* 2003) and *Helicobacter pylori* infections (Galan *et al.* 2004). These studies demonstrate that incorporation of brassica glucosinolate through diet provides levels of glucosinolates

that the lower incidence of different types of cancer and diseases in individuals who regularly consume such vegetables.

Little is known about the direct effect of broccoli sprouts on human health, even though *in vitro* and *in vivo* data provide evidence that supports the belief that young cruciferous sprouts with their high concentrations of phytochemicals may be a potent source of protective chemicals against cancer (Gill *et al.* 2004). Broccoli sprouts are a rich source of glucosinolates and isothicyanate that induce Phase II detoxication enzymes, boost antioxidant status, and protect animals against chemically induced cancer (Munday and Munday 2004; Tang and Zang 2004).

There have been a large number of studies that have used animal models to show that glucosinolate breakdown products such as isothiocyanates inhibit chemically-induced carcinogenesis by up to 100%. The anticarcinogenic properties of broccoli depends both on the concentration of glucoraphanin and on the conversion of this compound into isothiocyanate on autolysis (Zang *et al.* 1992).

1.4.7 Toxic Effect of Glucosinolate

Glucosinolates have previously caused controversy because of their goitrogenic and growth retardation activities (Mawson *et al.* 2006). The glucosinolate breakdown product oxazolidine-2-thiones derived from progoitrin (Rosa *et al.* 1997) and generally found in several oil meals may induce morphological and histological abnormalities of internal organs in animals (Schöne *et al.* 1994). Increased thyroid weight in pigs and poultry, as well as depressed growth, goitres, poor egg production, and liver damage have also been identified (Tawfiq *et al.* 1994). Glucosinolates are

well known for their toxic effects (mainly as goitrogens) in both humans and animals at high doses.

The potential negative effects of glucosinolates require a further examination, as this topic has been scarcely addressed in recent years. The anti nutritional nature of these compounds are reviewed by Fenwick *et al.* (1983a), Rosa *et al.* (1997), Griffiths *et al.* (1998) and Anilakumar *et al.* (2006).

1.5 Glucosinolate Extraction and Analytical Procedures

The abundance and structural variety of the glucosinolates and their breakdown products makes analysis very complicated (Mithen *et al.* 2000). Early identifications relied on paper or thin-layer chromatography of the glucosinolates or of their presumptive hydrolysis products (e.g. an investigation of the glucosinolates from the seeds of 151 different crucifers by Danielak and Borkowski 1969).

The analytical methods available have been extensively reviewed by McGregor *et al.* (1983) and Verkerk *et al.* (1998). The most extensively studied glucosinolates are the aliphatic, □-methylthioalkyl, aromatic and heterocyclic (e.g. indole) glucosinolates, found in the *Brassica* vegetables (Moreno *et al.* 2006). The largest single group (one-third of all glucosinolates) contain a sulphur atom in various states of oxidation. Another small group of benzyl glucosinolates have an additional sugar moiety, rhamnose or arabinose, in glycosidic linkage to the aromatic ring (Fahey *et al.* 2001; Fahey *et al.* 2002b and Rangkadilok *et al.* 2002).

Because glucosinolates coexist with myrosinase in the plant, fresh plant material processing in the presence of water (grinding, cutting) initiates a rapid hydrolysis of

the parent compounds. Inhibition of myrosinase activity is essential for analysis of intact glucosinolates. Before disruption of the material, samples should be completely dried by freeze-drying or frozen in liquid nitrogen. The use of aqueous methanol for extraction in combination with high temperatures also inhibits myrosinase (Heaney and Fenwick 1993; Mithen *et al.* 2000).

West *et al.* (2002) reported a single column approach with reversed-phase liquid chromatograph separations using hydrophilic endcapped C_{18}-bonded silica and a 50 mM ammonium acetate-methanol gradient mobile phase to resolve both non-polar and polar glucosinolates present in isolates obtained, using boiling water extraction. This procedure is extremely useful and valuable to other researchers studying brassica glucosinolates as well.

To date, many plant glucosinolates have not been rigorously identified by modern analytical and spectroscopic methods such as HPLC, NMR, mass spectroscopy, near-infrared spectroscopy or supercritical fluid chromatography with light scattering detection (Fenwick *et al.* 1982; West *et al.* 2004; Font *et al.* 2005). There was, and still is, an extreme paucity of high purity chromatographic standard glucosinolates available to researchers.

One of the major problems in the analysis of glucosinolates has been the lack of suitable standards. The only commercially available pure (more than 99%) glucosinolates are benzylglucosinolate (glucotropaeolin) and 2-propenyl-glucosinolate (sinigrin). Sinigrin is not as ideal internal standard because of the presence of this compound in most *Brassicaceae* plants, but glucotropaeolin is not normally present in *Brassica* and has been used as internal standard (Brown *et al.* 2003). Inconsistent measurement of glucosinolate among the investigations depends

on numerous scientific analytical instruments used and different extraction procedures. The advantages and disadvantages of available methods were briefly summarised in Table 1.6.

Table 1.6: Advantages and disadvantages of some commonly used methods for the analysis of Glucosinolates and their breakdown products

Compound	Method	Advantage	Disadvantage	References
Total glucosinolates	Palladium chloride and thymol assays	Provide only quantitative data for total glucosinolates	Characterization of glucosinolates is not possible	Heaney *et al.* 1988; Heaney and Fenwick 1993; Kiddle *et al.* 2001; Galan *et al.* 2004
	Glucose- and sulphate-release enzyme assays	This is a rapid and simple method, efficient for measuring total glucosinolate contents. High skilled for operation is not required.	Method was not designed for measuring intact/individual glucosinolates, Myrosinase activity may interrupt the isolation of glucosinolates	Ettlinger and Lundeen 1956b; Ettlinger and Lundeen 1957; Thies 1976; Heaney *et al.* 1988; Daxenbichler *et al.* 1991; Mithen *et al.* 2000; Kiddle *et al.* 2001
	ETISA	Cost and time involvement are lower than HPLC system	The method tends to overestimate glucosinolates	van Doorn *et al.* 1998; van Doorn 1999; Mithen *et al.* 2000
	Near infra-red reflectance (NIR) spectroscopy; alkaline degradation and thioglucose detection	Cost and time are lower than HPLC and minimize repeated measurement cost	Not designed for characterization of glucosinolates	Ettlinger and Lundeen 1956b; Ettlinger and Lundeen 1957; Underhill and Kirkland 1971; Thies 1976; Mithen *et al.* 2000
	High resolution nuclear magnetic resonance (NMR) spectroscopy	Low time consuming and mostly accuracy in measurement	Costly and not possible to detect individual glucosinolates	Fenwick *et al.* 1982; Prestera *et al.* 1996; Zhang *et al.*1996; Font *et al.* 2005; Kiddle *et al.* 2001; West *et al.* 2004
	Microchip Capillary Electrophorosis /CE (u-CE)	Single step procedures	Not widely used method, needs more experimentation for more accuracy	Fouad *et al.* 2008.

Table Continued…

Individual intact glucosinol ates	Reverse phase HPLC	Potential for measuring intact glucosinolates; more accurate for determining individual glucosinolates, known as robust, powerful and selective method	Not cost effective, time consuming, needs comparing to standards of glucosinolates; results are variable within different HPLC set up	Underhill and Kirkland 1971; Thies 1976; Heaney and Fenwick 1993; Karcher and Rassi 1999; Mithen *et al.* 2000
	Thermospray LC with tandem MS; high performance capillary electrophoresis; capillary GC–MS, GC–MS, GC–MS–MS	LC-MS, LC-MS-MS or capillary electrophoresis systems offer either intact or individual glucosinolates, the most accurate forms of identification and quantification possible	This methods are high cost and multi-step procedures and high skill require	Karcher and Rassi 1999; Kiddle *et al.* 2001; Tian 2005
	Capillary zone electrophoresis (CZE) coupled to electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS)	Provide identification of a broad series of glucosinolates, easy and rapid method for analysis of intact, non-desulfated glucosinolates	Very selective, time consuming and high cost involvement, not suitable for measuring total glucosinolates	Bringmann *et al.* 2005.
Desulpho-glucosinol ates	Reverse phase HPLC	Efficient for measuring individual glucosinolates	No suitable for measuring total glucosinolates, time consuming, multi-step procedures, subject to difficulties for effect of pH, and enzyme activity of desulphation products	Olsen and Sorensen 1979; Helboe *et al.*1980; Truscott *et al.* 1983; van Doorn1999 ; Mithen *et al.* 2000; Kiddle *et al.* 2001; West *et al.* 2002; Vallejo *et al.* 2004
Degradatio n products	X-ray fluorescence spectroscopy (XRF); GC or GC–MS; HPLC	Efficient for measuring hydrolysis product of Glucosinolates than intact glucosinolates, GC-MS may have more accurate identification & quantification of glucosinolates than GC alone.	GC based technique generally unsuitable for accurate identification of any form of glucosinolates	Daxenbichler *et al.* 1991; Zhang 1996; Chiang 1998; Mithen *et al.* 2000; Kiddle *et al.* 2001; Jackson and Singletary 2004

Extracted and modified from Moreno *et al.* 2006

1.6 Life Cycle and Host Pathogen Interaction of *Plasmodiophora brassicae* Causing Clubroot Disease in *Brassica* Plants

Plasmodiophora brassicae is the casual agent of club root disease of crucifers. Records of club root back to the 13th Century in Europe. In the late 19th Century, a severe epidemic of club root destroyed large proportions of the cabbage crop in St. Petersburg, Russia. (Grabowski 2005). Woronin, a Russian scientist successfully identified the cause of club root as a "Plasmodiophorous organism" in 1875, and gave it the name *Plasmodiophora brassicae* (Karling 1968).

The life cycle of *P. brassicae* consists of two phases (Figure 1.7) the primary phase is restricted to root hairs and epidermal cells of the host, and the secondary phase which occurs in the cortex and stele of roots and hypocotyl and leads to abnormal development (Ingram and Tommerup 1972).

Not much is known about histological events during the primary infection phase, although the mechanism of penetration has been well described (Buczacki 1983). Resting spores germinate and release zoospores which penetrate the root hair by an apparatus consisting of a 'Stachel' and 'Rohr' (Braselton 1995), thereby releasing the complete protoplast of the pathogen into the root hair. Then, a multinucleate primary plasmodium grows until zoospores are formed which are again released into the soil (Ludwig-Müller and Schuller 2008). These can again infect root hairs or penetrate the root cortex by an unknown mechanism. Whether plasmodia have to fuse prior to penetration has not been clearly established, however, the observation of dikaryotic myxamoeba during this phase of infection would point to plasmogamy of two zoospores (Kobelt *et al.* 2000).

Since it is also possible to generate root galls from a single zoospore, it is assumed that different mating types are not necessary (Klewer *et al.* 2001). After the secondary infection has occurred the dikaryotic amoeba migrate, through the root cortex in direction of the central stele (Kobelt *et al.* 2000; Mühlenberg *et al.* 2002) and in the early stages of the second infection cycle, young secondary plasmodia are formed (Figure 1.8). Finally, multinucleate secondary plasmodia are formed which is accompanied by strong hypertrophy of infected cells (Ludwig-Müller *et al.* 1999a & 1999b; Siemens *et al.* 2006). In this stage karyogamy has been observed followed by meiosis of the diploid nuclei (Ingram and Tommerup 1972). These processes lead to the cleavage of the plasmodium to yield numerous resting spores (Figure 1.8).

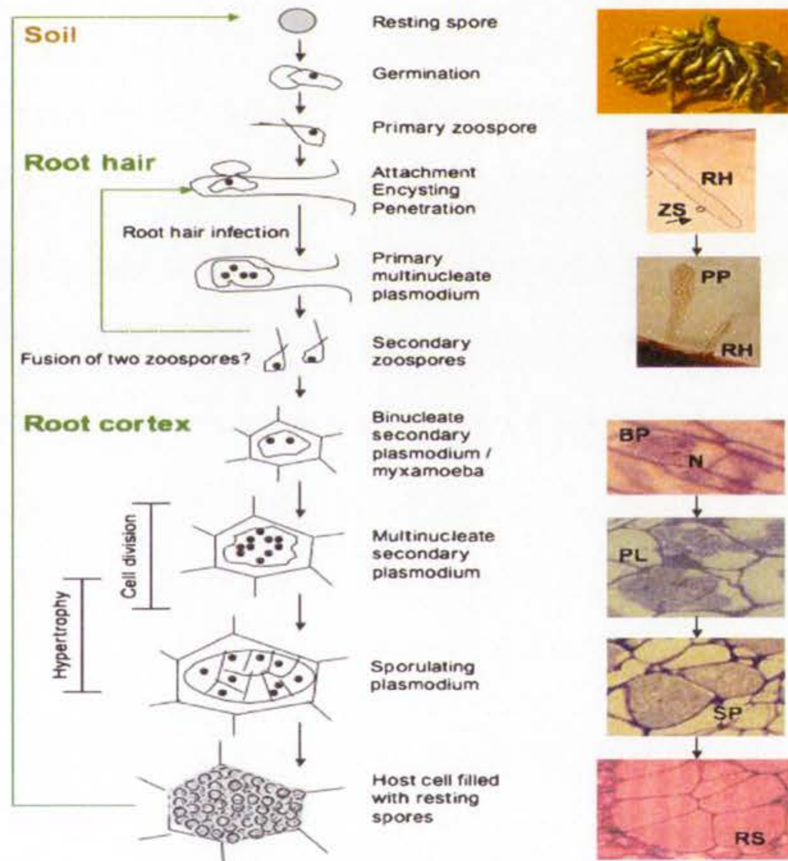


Figure 1.8: Microscopic pictures and characteristic developmental stages of *Plasmodiophora brassicae* were illustrated by Ludwig-Müller (1990). Abbreviations as from the top of right side: ZS, zoospore (arrow);RH, root hair; PP, multinucleate primary plasmodium; BP binucleate secondary plasmodium (myxamoeba); N, host nucleus;, PL, multinucleate secondary plasmodium; SP, sporulating plasmodium; RS, resting spores.

Obligate biotrophic plant pathogens have evolved together with their host and therefore exploit the host metabolism for their own needs. The symptoms caused by *P. brassicae* are root galls. The final stage of disease development leads to changes in water and nutrient supply due to destruction of vasculature and thus causing wilting in the green parts of the plant (Rausch *et al.* 1983). In earlier stages of the infection, the pathogen uses plant signalling molecules such as cytokinins to re-distribute assimilates from the shoot to the root to guarantee its own nutrition (Evans and Scholes 1995; Siemens *et al.* 2006). Mono, disaccharides and starch increase in roots colonised by *P. brassicae* (Evans and Scholes 1995; Brodmann *et al.* 2002). In addition, the production of auxin is stimulated which in turn causes the root cells to enlarge (Grsic-Rausch *et al.* 2000; Ludwig-Müller and Schuller 2008). This intricate relationship is difficult to investigate because the pathogen can not be cultivated without its host.

1.6.1 Resistance Mechanism to Clubroot Disease

The understanding of the mechanisms of disease development is required because the disease is difficult to control under field conditions. Breeding programs have yielded resistant cultivars, but on the other hand the pathogen itself has evolved more virulent pathotypes on these plants (Voorrips 1995).

QTL analysis of brassicas has identified specific genes involved in resistance mechanisms (Rocherieux *et al.* 2004; Piao *et al.* 2004; Hirai 2006; Saito *et al.* 2006). Recent transcriptome and proteome analysis on the interaction of *P. brassicae* with *A. thaliana* (Devos *et al.* 2006; Siemens *et al.* 2006) and Brassica (Cao *et al.* 2007) could reveal novel targets for resistance breeding.

1.6.2 Role of Aliphatic and Aromatic Glucosinolate during Clubroot

The role of glucosinolates in plant defence is proposed to involve the release of toxic thiocyanates and isothiocyanates (Fenwick *et al.* 1983b; Chew 1988a). The conversion of glucosinolate to nitriles would yield less toxic products (Bones and Rossiter 1996). Little information on the role of aliphatic and aromatic glucosinolates during the development of the clubroot disease is available, because most research has focussed on indole glucosinolates (Agerbirk *et al.* 2008).

Devos *et al.* (2006) showed myrosinase was upregulated at a very early time point of infection, whereas Siemens *et al.* (2006) found evidence of downregulation of myrosinase transcripts in *A. thaliana* clubroots at later time points of gall development. It has not yet been shown by functional analysis that myrosinase is a limiting factor in gall formation. Additional factors such as binding protein (MBP) myrosinase associated protein (MyAP), epithiospecific protein (ESP) and ESM1 (Epithiospecifier modifier, a protein belonging to a known class of myrosinase-associated proteins, which inhibits ESP-mediated nitrile formation) would determine the outcome of metabolism and toxicity (Lambrix *et al.* 2001; Eriksson *et al.* 2002; Halkier and Gershenzon 2006; Burow *et al.* 2008).

In resistant and susceptible Chinese cabbage (*Brassica rapa*) cultivars, aliphatic and aromatic glucosinolates level was analysed (Ludwig-Müller *et al.* 1997). The total glucosinolate content in roots of the two susceptible varieties was higher throughout the experimental period than in roots of the two resistant cultivars (Ludwig-Müller *et al.* 1997). While the aliphatic glucosinolate were induced in the two susceptible cultivars compared to the resistant ones, the two resistant cultivars showed an increase

in aromatic GSL, indicating there may be a dual role for these compounds (Ludwig-Müller *et al.* 1999b). In addition, in one of the resistant cultivars inductions of aliphatic and aromatic glucosinolates were observed after jasmonate treatment (Ludwig-Müller *et al.* 1997). Overall, the observations did not entirety fit with the hypothesis that the group of aliphatic glucosinolate are induced as defence response.

In an investigation on the host range of *P. brassicae* in various non-brassica species, a dual role of aromatic glucosinolates during club formation was presented (Ludwig-Müller *et al.* 1999b). Symptom development was correlated with specific glucosinolates in one species, while the increase in other glucosinolates might be regarded as a defence response. In glucosinolate containing non-brassicas, *Tropaeolum majus* and *Carica papaya*, the concentrations of benzyl-glucosinolate were increased markedly in roots following *P. brassicae* infection compared with the controls (Ludwig-Müller *et al.* 1999b). Slight gall formation was observed in *T. majus* (Ludwig-Müller *et al.* 1999b) and it was hypothesised that benzyl-Glucosinolates could act as precursor for phenylacetic acid (PAA-Fig-1.8), which has auxin activity in *T. majus* (Ludwig-Müller and Cohen 2002).

Two alkenyl-glucosinolates were only detectable in *P. brassicae* infected roots of mutants of *A. thaliana*. Total aliphatic glucosinolates were slightly increased in clubroots (Haughn *et al.* 1991). Consequently, a mutant in a gene encoding an enzyme involved in the biosynthesis of aliphatic glucosinolates (Kroymann *et al.* 2001) did not show any tolerance to clubroot (Ludwig-Müller *et al.* 1999a) (Figure 1.8).

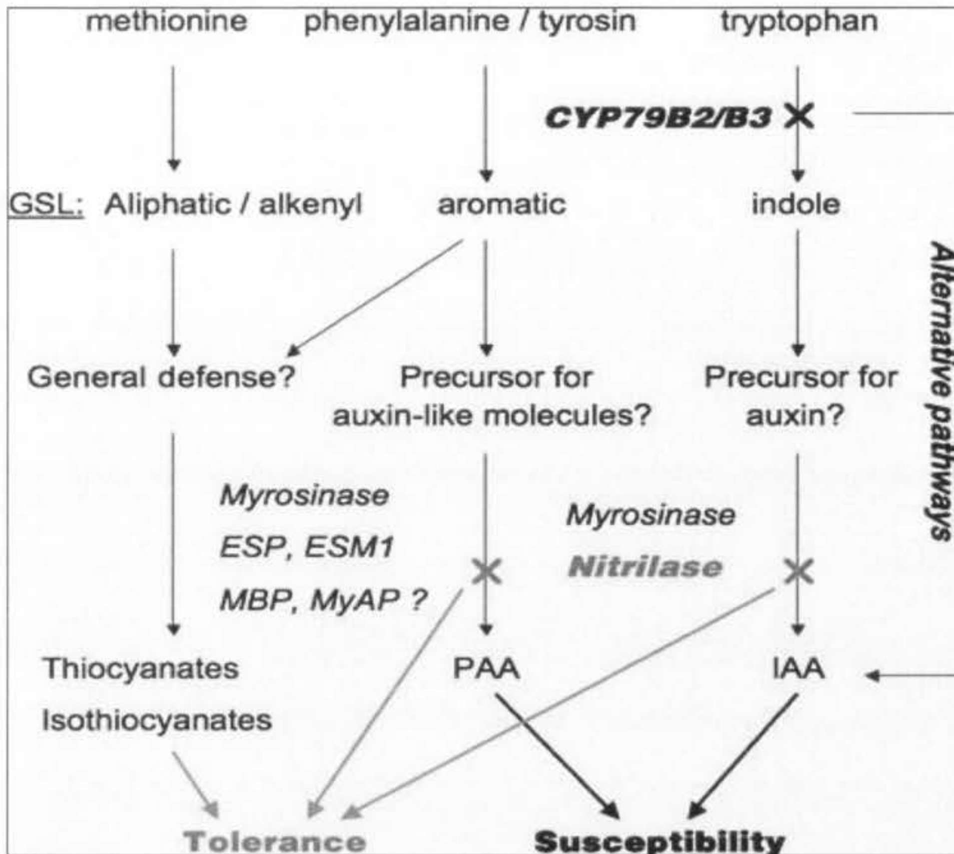


Figure 1.9: Model proposed by Ludwig-Muller *et al.* (1999a; 1999b) suggesting the probable pathway of glucosinolates during clubroot formation. Alipathic glucosinolates derived from methionine might play a role as defence compounds leading to a level of tolerance. The aromatic glucosinolates could also play defensive role but they might serve as precursors for auxin like molecules such as phenylacetic acid (PAA). Indole glucosinolates derived from tryptophan likely serve as auxin precursors and are intermediate for Indole 3- acetic acid (IAA) increasing IAA production which pathway would lead to susceptibility. Inhibition of the enzymes (nitrilase) in this pathway would help the plant more immune in nature. Blocking early steps in the pathway of CYP79B2/B3 would not lead to tolerance because alternative pathways might operate. The role of Myrosinase binding protein (MBP), Myrosinase associated protein (MyAP) have not yet been demonstrated. Epithiospecific protein (ESP) and Epithiospecifier modifier 1 (EMS1) have involvement in regulating glucosinolates hydrolysis and influence the out come of the reaction leading to determine the synthesis of specific defense compounds.

1.6.3 Indole Glucosinolate and Clubroot Disease Development

Indole glucosinolates play a vital role as precursors for Indole Acetic Acid (IAA) production during clubroot formation (Butcher *et al.* 1974). It was emphasised that if plants have large amounts of indole glucosinolate, then after tissue disruption also large amounts of IAA would be produced, thereby increasing susceptibility. Another assumption is that plants with low indole glucosinolate levels and therefore a lower

capacity for IAA synthesis by turnover of indole glucosinolates, should be more tolerant to clubroot formation (Figure 1.8).

However, conflicting results have been published on this topic. For example Butcher *et al.* (1974) and Ockendon and Buczacki (1979) found clear correlations between resistance and low indole glucosinolate content in Brassicaceae. Chong *et al.* (1981 & 1984). Mullin *et al.* (1980) were not able to correlate indole glucosinolate content with resistance to clubroot. Two resistant and two susceptible *B. rapa* cultivars showed differences in the indole glucosinolate patterns (Ludwig-Müller *et al.* 1997). Two susceptible cultivars reacted with an increase in indole glucosinolates after infection with *P. brassicae* 14 and 20 day post inoculation in roots, whereas there was no difference between infected and control roots of the two resistant cultivars. All cultivars tested showed increased glucosinolate levels after treatment with salicylic acid (SA) and jasmonic acid. Jasmonic acid induced mainly indole glucosinolates in the leaves, whereas SA induced the indole glucosinolates both in leaves and roots of both cultivars.

In *A. thaliana* Columbia, indole glucosinolate levels were increased during clubroot development (Ludwig-Müller *et al.* 1999a). Two mutant lines (lines tu3 and tu8) with altered glucosinolate content (Haughn *et al.* 1991) showed reduced symptom development compared to the wild type. In tu8 indolic glucosinolates decreased while in tu3 were unchanged compared to the wild type (Ludwig-Müller *et al.* 1999a).

1.7 Chemical Management of Clubroot Disease

Although some soil fungicides are available for the control of clubroot, these have limited efficacy where there is a high density of resting spores and highly virulent populations of *P. brassicae* (Tanaka *et al.* 1999, Porter *et al.* 1998; Donald *et al.*

2001). Fungicides such as cluazinam, flusulfamide, cyazofamid and mancozeb have also been found to be effective (Humpherson-Jones 1993; Dixon *et al.* 1994; Mitani *et al.* 2003) against clubroot of Brassicas.

Mitani *et al.* (2003) demonstrated that cyazofamid at 0.3 mg/L inhibited resting spore germination of *P. brassicae* by 80%, cyazofamid at 3-10 mg/L exhibited fungicidal activity to resting spores of *P. brassicae* 1-10 days after treatment. Cyazofamid was applied to infested soil, both root-hair infections and club formation caused by *P. brassicae* were strongly inhibited at 1-3 mg kg^{-1} dry soil, suggesting that cyazofamid directly inhibits resting spore germination, thereby leading to the inhibition of root-hair infection and club formation.

In 1996, fluazinam formulated as 50% suspension concentrate, was registered in Australia for use as a soil drench to control clubroot disease of brassica crops. Commercially suitable methods for application of fluazinam were evaluated in field trials conducted in Victoria and Western Australia by Donald *et al.* (2001) and they demonstrated that incorporation of fluazinam into the soil in bands 23 cm wide along the transplant row (to a depth of about 15–20 cm) immediately before transplanting was the most effective method of application. The banded soil incorporation treatment consistently increased the marketable yield of broccoli and cauliflower.

Knowledge of the mode of action allows more effective application of fungicides. However, the determination of the mode of action of fungicides against *P. brassicae* is made more difficult because it is an obligate pathogen and cannot be cultured axenically. Nevertheless, biological modes of action of some fungicides against *P. brassicae* have been postulated e.g. trichlamide and fluazinam may inhibit resting-

spore germination, and primary or secondary infections (Naiki and Dixon 1987; Suzuki *et al.* 1995). In contrast, quintozene possesses relatively low suppressive activity against resting-spore germination (Naiki and Dixon, 1987; Suzuki *et al.* 1995) but does act against *P. brassicae* established within host cortical tissues in a manner similar to Benomyl (Dixon *et al.* 1972; Naiki 1985).

1.8 Alternate Management Strategies

P. brassicae survives in soil for a long time as resting spores (Wallenhammar 1996). Liming has been employed as a traditional and conventional technique and disease-suppressive effects of the neutralisation of soil acidity are well documented (Colhoun, 1953; Dobson *et al.* 1983; Campbell *et al.* 1985; Murakami *et al.* 2002; Tremblay *et al.* 2005).

Webster & Dixon (1991) employed the sand-solution culture system to investigate the effects of pH on primary (root-hair) infection and clubroot development in the absence or presence of calcium. They observed independent and synergistic effects of pH and calcium on the suppression of zoospore invasion, zoosporangial maturation and clubroot development. It was demonstrated that an increase in soil pH as a result of the accumulation of calcium was the primary cause of the suppression of root-hair infection and that the degree of effectiveness was dependant upon the level of Ca applied.

Although the suppression mechanism of soil neutralisation and that of calcium enrichment need to be elucidated comparatively, all these observations confirm that the occurrence of clubroot disease is suppressed effectively under neutral soil pH in the presence of calcium. It is evident that either spore germination or root hair

infection, each a part of the primary phase of the life cycle of the pathogen, is inhibited under such conditions (Niwa *et al.* 2008).

Niwa *et al.* (2008) provided the first direct evidence that the spore germination of *P. brassicae* in the rhizosphere is inhibited under neutral conditions created by the application of calcium-rich organic matter or $CaCO_3$. Myers & Campbell (1985) demonstrated that neutralization by calcium application did not reduce the viability of the resting spores, suggesting that the effects were not fungicidal, but fungistatic to the pathogen. Webster & Dixon (1991) suggested that an increase in soil pH by calcium application might not eradicate the pathogen directly, but create unfavourable conditions affecting the infection processes, e.g. enhancing host resistance to infection. The result clarifies a part of the mechanism underlying the inhibition of the disease under neutral soil pH and raises new questions of how spore germination is inhibited under these conditions.

It has been suggested that root exudates stimulate spore germination (Macfarlane 1970; Suzuki *et al.* 1992; Kowalski and Bochow 1996). It is hypothesised that soil pH may influence the response of *P. brassicae* spores to root exudates or that qualitative and/or quantitative changes in the exudates may occur under neutral conditions. Identification of the germination-stimulating factors in exudates would be an important step in understanding this mechanism (Macfarlane 1970)

The application of food factory sludge compost (FSC) to soil increases soil pH, calcium, carbon, nitrogen and phosphorus levels, which may lead to additional effects of these elements on disease suppression (Niwa *et al.* 2007). However, this is unlikely because the suppressive effect in FSC-treated soils was cancelled out by acidification with sulphuric acid (Niwa *et al.* 2007).

In Australia, an integrated program to control and prevent the spread of clubroot has been developed, based on detection and quantification of *P. brassicae*, improved farm and nursery hygiene and strategic application of in-field controls including lime (calcium oxide), calcium, boron and the fungicide fluazinam (Donald *et al.* 2006).

1.9 Bion and Phosphonate Application against *Plasmodiophora brassicae*

The plant activator acibenzolar (Bion) is a non fungicidal compound used to combat plant pathogens by inducing the host plant's natural defence mechanisms (Tally *et al.* 1999). This is a functional analogue of salicylic acid shown to accumulate in plants challenged by a pathogen (Friedrich *et al.* 1996). Following accumulation of salicylic acid, plant defence gene(s) are induced (Lawton *et al.* 1996) triggering the production of defence compounds.

Phosphorous acid has been shown to have a number of modes of action including being directly fungisatic (Grant *et al.* 1990) as a plant activator (Afek and Sztejnberg 1989), and as an inhibitor of pathogen defence suppressor (Grant *et al.* 1990). The simple anion of phosphonic acid, phosphonate has been demonstrated to be a remarkably cost effective agent for the control of a number of plant diseases (Pegg *et al.* 1985; Guest *et al.*1988; de Boer *et al.* 1990). Phosphonate application leads to a more rapid accumulation of phytoalexin when compared to untreated control (Dercks and Creaser 1989; Guest 1984; Guest *et al.* 1988; Nemestothy and Guest 1990; Saindrenan *et al.* 1988). Cheah *et al.* (2000) demonstrated that phosphorous acid (Foli-R-FOS 400) as a plant drench (2 mL/L) significantly reduced clubroot formation in Chinese cabbage cv. Wong bok (*Brassica chinensis* L.) in glasshouse condition.

A number of field trials were carried out by Abbasi and Lazarovits (2006) to test the effect of phosphonate fungicide on the severity of clubroot of bok choy/Pak choy (*B. rapa* var. *chinensis*) and cabbage (*B. rapa* var. *perkinensis* and *B. oleracea* var. *capitata)* in clubroot infested field. Phosphonate concentrations of 0.07 and 0.14% a.i. applied as soil drench before or after planting consistently reduced clubroot severity. Fresh weight of bok choy was increased or not affected by phosphonate treatments.

1.10 RATIONALE AND SIGNIFICANCE

There is evidence that glucosinolates reduce the risk of cancer in human and enhance the response to disease infection in some plant groups. It has been demonstrated that certain glucosinolate levels rise upon pathogen invasion as part of a resistance mechanism (Mithen 1992). However, the effects of glucosinolates accumulation in different plant tissues following infection is not well understood. There is minimal information available on the level of glucosinolates during different crop management practices such as fertilisation, irrigation, planting date, etc. There is also lack of information on the content of aerial and root glucosinolates during clubroot disease development at early stages of plant growth.

Changes of glucosinolates have long been associated with clubroot disease symptoms. Results show that several glucosinolates are induced in root galls, while aliphatic glucosinolates are regarded as defence compounds. Analysis of cabbage cultivars as well as *A. thaliana* mutants provided correlative evidence between disease severity and root indole glucosinolates content. The potential to use chemicals like potassium phosphonate and Bion to increase glucosinolate levels, and thus enhance the defence response has not been explored. The effect of clubroot disease on accumulation of

particular group of glucosinolates in aerial parts of *Brassica oleraecea italica* is yet to be investigated.

The interaction between leaves-shoot and root induction of glucosinolates in *Brassica oleracea* and *B. nigra* were studied applying the signalling hormones jasmonic acid and salicylic acid (van Dam *et al.* 2003). Interestingly, there is no information on the changes of individual glucosinolates throughout the aerial and root parts of plants in response to interaction with clubroot disease and fungicide action over time.

Based on the previous findings, this investigation will measure the level of individual glucosinolates both in aerial and root tissues during the early stages of the development of *P. brassicae.* The findings of this work will aid in designing future experimentation to elucidate the role of individual glucosinolates in plant defence and the maintenance of higher levels of beneficial glucosinolates in *Brassicas* that potentially reduces the risk of human diseases.

Chapter II. General Materials and Methods

2.1 Plant material

Hybrid Broccoli (*Brassica oleracea* L. var. *Italica*), cultivar Marathon F1 (Fairbanks, Lot no. 156490, 542 Footscray Road, West Melbourne 3003, Australia) was used as a susceptible host of *P. brassicae* in this experiment.

2.2 Pathogen material

A field isolate of *Plasmodiophora brassicae* Woronin, was collected by Dr Caroline Donald (DPI Victoria) from a broccoli farm in Werribee, Victoria, Australia in 2006. Also fresh clubroot (galls) were collected at the beginning of this study from newly harvested bok choy (*Brassia rapa* L. var. *chinensis* cv. Hanelt) at a commercial vegetable farm in Camden, New South Wales, Australia in March, 2008. The galls were washed and stored in a freezer at -20°C until use.

2.3 Inoculation techniques

Three different methods of inoculation were tested to find out one suitable technique for effective and rapid disease development. The viability of *Plasmodiophora brassicae* spores in frozen clubroots was tested before inoculation into the seedlings by staining with 0.01% (w/v) hypertonic neutral red in 10 mM phosphate buffer (pH 7.5) (O'Connell *et al.* 1985).

Inoculation method-1 (IM-1) consisted of injecting spore suspension into the root zones of 12 day old seedlings (Donald and Porter 2004). Root galls (clubroots) containing resting spores of *P. brassicae* were homogenised 1:3 with distilled water (w/v) in a mechanical blender. The extract was filtered through nylon stocking. The

filtered spore suspensions were cleaned by repeated centrifugation in 20 mL Falcon tubes at 2000 g for five minutes and resuspended in sterilise distilled water (Donald and Porter 2004). The final concentration of resting spores in each suspension was estimated using a haemocytometer. The spore suspension was diluted to 10^8 spores/mL. The 12 day old transplanted seedlings were inoculated by pipetting 200 μL of resting spore suspension (10^8 spores/mL) at the base of each seedling.

The second inoculation technique (IM-2) was modified from the method of Toxopeus and Janssen (1975). Inoculum slurry was prepared by 3 min high speed blending of washed club roots with distilled water (1:5 w/v). About 50 mL of infected root slurry (10^7 resting spores per mL) per kilogram potting mix was thoroughly mixed up 3 days prior to 12 day old seedling transplantation.

The third inoculation technique (IM-3) was a combination of IM-1 and IM-2. Twelve day old seedlings were transplanted in to the potting mix containing 10^7 spores/mL of slurry mixed 3 days prior to seedling transplantation (IM-2). After immediate transplanting seedlings, 200 μL of cleaned resting spore suspension containing 10^8 resting spores per mL was injected around the rhizosphere of seedlings (12 day old).

In pilot tests the third inoculation method (IM3) showed the highest infection rate and was used in further experiments. Glucosinolates were measured following pathogen infection of 12 day old seedlings. Chemical treatments were applied to 10 day old seedlings (experiments described in Chapter 4).

2.4 Plant culture

Plastic pots (15 cm diameter, 20 cm high) were filled with modified UC (University of California) potting mix consisting of coco peat and washed sand (1:1-w/v). Osmocote slow release fertilizer (Scotts Australia Pty. Ltd) was added at 10 g kg^{-1} potting mix 3 days before transplanting or seed sowing. Seedlings were maintained for the duration of the experiment in Darlington Glasshouse, at The University of Sydney from November 2008 to July 2009. Glasshouse room temperature was maintained between 22 to 25°C during day and 18 to 20°C at night. Relative humidity was maintained at 80 to 90%. Seedlings were irrigated three times daily for 3 minutes. Pots were thoroughly watered immediately prior to inoculation and seed sowing.

2.5 Microscopic observation of diseases development process

Stages of pathogen development within the root hair and cortical cell infection towards gall formation were observed using a light microscope. A solution of aniline-blue was used to stain primary and secondary infection structures of *P. brassicae* infecting root hairs and the cortical cell region. Four days after inoculation, potting mix was rinsed from seedling roots under water. One hundred root hairs on the tap root (0-7 cm from the basal part of the hairy root) were identified and the numbers of root hair infections were counted (Asano *et al.* 2000). Each seedling was placed with its roots in 125 ppm aniline-blue solution in 50% (v/v) acetic acid for 1 min at room temperature and then rinsed with tap water for 1 minute (Voorrips 1992).

The roots of 8 plants for each inoculation method (2 plants from one pot) were examined under the light microscope using Nomarski optics (Olympus-DP70-BX51,

Olympus Australia, North Ryde, NSW, Australia). This process was repeated every 4 days up to 36 days post inoculation (DPI). Root hair infection, including differentiated stages of primary phases of plasmodial developmental (primary plasmodia, mature zoosporangia, partially evacuated zoosporangia and fully evacuated zoosporangia), cortical cell infection by dikaryotic and multinucleate secondary plasmodium and specific time point of gall formation were recorded.

Invasion of secondary plasmodium in the cortex region was observed using aniline blue staining of 0.5 cm segments from the midsection of the root. The frequency (%) of infection was calculated from the ratio of the number of infected cells to the total number of cells observed in each area of the cortical cells (n=?).

Disease severity (fresh gall weight in g/plant) among the inoculation methods were assessed at 12, 16, 20 24, 28, 32 and 36 DPI.

2.6 Statistical Analysis

All the analysis (data from three inoculation methods) was performed with the statistical package GraphPad Prisam 5.2, 2009 (GraphPad Software Inc, Sandeigo California, USA). A two-way ANOVA (inoculation method x days post inoculation) was to determine the effect of each inoculation method on the frequency of root hair infection, cortical cell infection and fresh gall weight per plant.

2.7 Sample collection for glucosinolate measurement

For glucosinolate analysis, inoculated and uninoculated roots and leaves-shoots were sampled every 7 days post inoculation to 42 days (total of 6 intervals). Eight plants were collected each time with roots and leaves-shoots washed and dried prior to snap freezing in liquid nitrogen. Samples were stored at -20^0C in polyethylene Ziploc bags until freeze drying.

2.8 Freeze drying

Frozen samples were removed and immediately placed into the freeze drying chamber (DYNAVAC-FD300 freeze drier, Rowville, Victoria, Australia). Drying cycle continued until all water was removed from the plant tissue. Freeze dried samples were ground with a mechanical coffee blender to a fine powder. Powdered material was stored in air tight containers at room temperature until HPLC analysis. Glucosinolate content was assumed to remain constant in freeze- dried samples held at relative temperature (Jones *et al.* 2007).

2.9 Glucosinolate extraction

Powdered broccoli (0.4 g) was preheated for 10 minutes in near boiling water (90^0C) in 10 mL Oakridge centrifuge tubes. Ten mL of boiling micro filtered deionised water was added and the tubes heated for a further 10 minutes. Samples were centrifuged at 4,000 rpm for 10 minutes. The supernatant was collected and transferred to a 25 mL volumetric flask. The pellet was resuspended using a vortex mixer with 10 mL ultra pure boiling water and re-centrifuged at same speed. The supernatant was combined and the volume adjusted to 25 mL. The extracted supernatants were filtered (PHENEX NY, 0.45 μm regenerated cellulose) into auto-sampler vials (2 mL AMB

RAM vials, 9 MM THD) for High Performance Liquid Chromatography (HPLC) analysis (Jones *et al.* 2007).

2.10 HPLC – analyses and UV Detection

Glucosinolates were separated on a C-18 column as described by Jones *et al.* (2007), modified from West *et al.* (2002). A binary gradient of 50 mmol/L ammonium acetate in pure water at pH 6.74 (mobile phase A) and 20% methanol in 50 mmol/L ammonium acetate (v/v) (mobile phase B) at a flow rate of 1 mL/min. Gradient was maintained at 0% (mobile phase B), for 10 minutes, then increased to 40% (mobile phase B) over 5 min, maintained at 100% (mobile phase B) for 10 minutes before re-equilibrating the column with the 100% (mobile phase A) for 15 min. Column temperature was maintained to 30^{0}C and column pressure maintained at 11.5 MPa. The column was protected with an appropriate guard column. The injection volume was 20 µL. Wavelength for detecting glucosinolates (UV detector) in all instances was 230 nm. Glucoiberin (3-methylsulphinyl-propyl), progoitrin (2OH-3-butenyl) glucoraphanin (4-methylsulphinyl butyl), gluconapin (-3butenyl), 4-hydroxyglucobrassicin (4OH-3-indolylmethyl) glucobrassicin (Indol 3 ylmethyl), neoglucobrassicin (1-methoxyindol-3-ylmethyl) were measured throughout the experiments. The standards were purchased from C_2 Bioengineering (www.glucosinolates.com), Hovedgaden 12, 2690 Karlslunde, Denmark, verified 25 September 2007. All other chemicals used were analytical grade and mostly purchased from Sigma-Aldrich.

CHAPTER III

3.1 Introduction

Brassica rapa L. ssp. *cinensis* (L.) Hanelt cv. Pak Choi is now produced mainly by Chinese and Vietnamese Australians and partly by traditional brassica growers who wish to diversify. This vegetable is generally known as Chinese cabbage and also by local names bok choy, baak choi or tsing pak choi. This leafy vegetable is grown commercially in all states of Australia (Lee 1995; Moore *et al.* 1998). A number of diseases, including clubroot, cause at least 10% yield loss every year (Anonymous 2009a). Clubroot occurs in most of the major vegetable brassica growing regions of Australia and is probably the most serious disease of these crops world-wide. Infected plants wilt, become stunted and, in severe cases, die. Infection occurs on roots at any stage of growth. Symptoms are not obvious until the final stages of disease development, when knotted swellings on the roots cause plants to be stunted and wilt, particularly in hot-dry weather.

In this later stage of gall development the pathogen causes abnormal tissue proliferation which is the result of an altered metabolism of auxins (Butcher *et al.* 1974 &1976; Kavanagh & Williams 1981). The biosynthesis of auxin in Brassicaceae is believed to be produced via the indole glucosinolate pathway (Mahadevan & Stowe 1972; Ludwig-Müller *et al.* 1993). Glucosinolates are a group of secondary plant metabolites found in all species of Brassicaceae. It is a widely studied class of plant chemical compounds with a large structural diversity. Over 120 glucosinolates have been identified, mainly in species belonging to the Brassicaceae (Fahey *et al.* 2001). Glucosinolates influence important quality and flavour characteristics of the produce,

as well as resistance against non-adapted pathogen and insect pests (Chew, 1988b; Mithen 2001).

Glucosinolate levels in brassicas are also affected by the growing location and conditions (Shelp and Maclellan 1993). The clubroot causing pathogen may alter the levels of different glucosinolate among above ground and belowground organs of brassica crops (Rostas *et al.* 2003; Mithen 1992). Previous studies on glucosinolates in brassica vegetables have indicated environmental, including biotic and abiotic stresses, and cultural factors affecting glucosinolate levels. Therefore, the main objective of this study was to compare the levels of major glucosinolates in field and glasshouse grown brassica crop following symptom development.

The major glucosinolate profiles of root and shoot tissue of *Brassica oleracea* (broccoli) following *P. brassicae* infection in glasshouse condition (see Chapter 4) were monitored. Although the original aim was to compare similar cultivars of Broccoli grown under field and glasshouse conditions. Instead of broccoli a large field of severely clubroot infested Chinese cabbage (*Brassica rapa* ssp. *chinensis* cv. pak choi) was used. Both diseased and healthy plants were collected and root and aerial tissues analysed to establish a comparison between clubroot infested glasshouse and field grown brassica. It is hypothesised that there will be a similar pattern of glucosinolate concentration between brassica vegetables following infection by the clubroot causing obligate pathogen, *Plasmodiophora brassicae* under glasshouse and field conditions.

3.2 Materials and Methods

3.2.1 Plant materials and sample collection:

Five week old *B. rapa* ssp. *chinensis* cv. Pak Choy, commonly named as Chinese cabbage (bok choy or pak choy) were collected from Camden, NSW, Australia in March of 2008. The field was known to be heavily infected with *Plasmodiophora brassicae* for several years. The following groups of plant specimens were collected for this experiment:

1. Diseased (clubroot) roots
2. Healthy roots
3. Leaves and shoot of diseased plant
4. Leaves and shoot of healthy plant

Whole plant samples (root + leaf + shoot) were kept in airtight polyethylene bags with moist field soil. After 3 hours plant samples were washed thoroughly dried between filter papers then immediately immersed into the liquid nitrogen and stored at -20^{0}C until freeze drying.

3.2.2 HPLC condition and UV detection

All of the collected pak choy field samples were analysed at the Postharvest Laboratory, Department of Primary Industries, Knoxfield, Victoria. Sample storage, freeze drying and extraction were described in Chapter 2. Field sample analysis was performed on HPLC (GBC, Australia) equipped with an LC5100 UV-VIS diode array detector. Glucosinolates were separated on a C-18 column (250 x 4.6 mm, 5 um;

Alltech Associates) as modified by Jones *et al.* (2007) from West *et al.* (2002). The standards of glucosinolates used were glucoiberin (3 methylsulphinyl- propyl), progoitrin (2 OH butenyl), glucoraphanin (4-methylsulphinyl butyl), gluconapin (3 butenyl), 4 OH glucobrassicin (4 OH 3 indolylmethyl), glucobrassicin (Indol 3 ylmethyl) and neoglucobrassicin (1 methoxyindol 3 ylmethyl). The standards were purchased from C_2 Bioengineering (www.glucosinolates.com), Hovedgaden 12, 2690 Karlslunde, Denmark, verified 25 September 2007. All other chemicals used were analytical grade and mostly purchased from Sigma-Aldrich.

3.2.3 Data analysis

Diseased and healthy plant samples were randomly collected (5 plants for each category of treatment). HPLC results of individual glucosinolates were subjected to an analysis of variance. The limit of significance between differences of treatments was set at $P < 0.05$ by a two sample t-test using GenStat v 11.2 edition developed by Lawes Agricultural Trust (Rothamsted Experimental Station), UK.

Figure 3.1. Five week old field grown Chinese cabbage (*B. rapa* ssp. *chinensis* cv. Pak Choi) A. Clubroot B. Healthy roots.

3.3: Effect of clubroot on glucosinolate contents in field grown Chinese cabbage (Pak-Choy)

3.3.1 Aliphatic glucosinolate levels in aerial tissues

Five week old cabbage plant exhibited young galls (Figure 3.1). The major aliphatic glucosinolates in the aerial parts of 5 week old field grown clubroot diseased and healthy Chinese cabbage were progoitrin, glucoraphanin and with minor amounts of gluconapin.

Progoitrin levels were 18% lower (P=0.013) in diseased plants compared to healthy plants (Figure 3.2). There were no significant differences in glucoraphanin content between aerial tissues of diseased and healthy. Gluconapin levels were significantly (P=0.03) higher in diseased aerial tissues compared with healthy aerial tissues.

The total concentrations of three aliphatic glucosinolates in cabbage leaves and shoot were significantly (P=0.03) lower in *P. brassicae* infected plants, and infection caused 13% lower aliphatic glucosinolate accumulation compared with healthy plant leaves-shoot parts (Figure 3.2).

3.3.2. Indole glucosinolate levels in aerial tissues

Clubroot disease of field grown Chinese cabbage significantly affected indole glucosinolate contents during young gall forming stage of *Plasmodiophora. brassicae* infection.

Glucobrassicin could not be detected in healthy plant leaves, whereas infected plant leaves produced 0.44 µmole/g DW. The changes in neoglucobrassicin in leaves were increased 5 fold following *P. brassicae* infections.

The total indole glucosinolate contents of clubroot infected aerial tissues were significantly higher (0.74 µmole/g DW). This was 11 times higher than in healthy plant tissues (Figure 3.3).

3.3.3. Aliphatic glucosinolate levels in roots

The levels of progoitrin in Chinese cabbage root significantly increased during young gall formation. The progoitrin level in diseased root was 6.37 µmole/g DW, almost 4 times higher than healthy root (1.46 µmole/g DW).

Glucoraphanin production in roots was very low and infected plant roots contained significantly ($P<0.001$) higher amount of glucoraphanin (0.86 µmole/g DW) compared with healthy roots (0.26 µmole/g DW). Diseased roots contained half the level of gluconapin ($P<0.001$) of healthy roots.

The total aliphatic glucosinolate content of field grown Chinese cabbage was markedly increased in infected roots ($P<0.001$), 3 times higher than in healthy roots (Figure 3.4).

3.3.4. Indole glucosinolate levels in root

A remarkable increase in indole glucosinolate content was observed following clubroot disease infestation in roots of field grown pak-choy. *P. brassicae* infection strongly enhanced glucobrassicin accumulation rate in diseased root compared with healthy roots. Approximately 8-fold higher levels of glucobrassicin were detected during young gall formation. Healthy roots accumulated only 0.15 μmole/g DW. Neoglucobrassicin levels were 0.67 μmole/g DW in healthy root while diseased root produced significantly ($P<0.01$) higher amounts of neoglucobrassicin (1.39 μmole/g DW) (Figure 3.5).

The resulting mean of individual indole, total indole glucosinolate showed significantly higher levels ($P<0.001$) in infested roots (2.81 μmole/g DW) than in healthy roots (0.83 μmole/g DW) (Figure 3.5).

3.3.5. Total aliphatic and indole glucosinolate levels distributed throughout the aerial tissues and root organs

Levels of total glucosinolate (aliphatic and indole) in aerial tissues of healthy plants were significantly ($P=0.002$) higher than those of diseased aerial tissues. Diseased roots accumulated significantly ($P=0.002$) higher total glucosinolate levels (aliphatic and indole) (Figure 3.6). This is a clear indication that *P. brassicae* infection at the time of young gall formation alters production of individual and total glucosinolates.

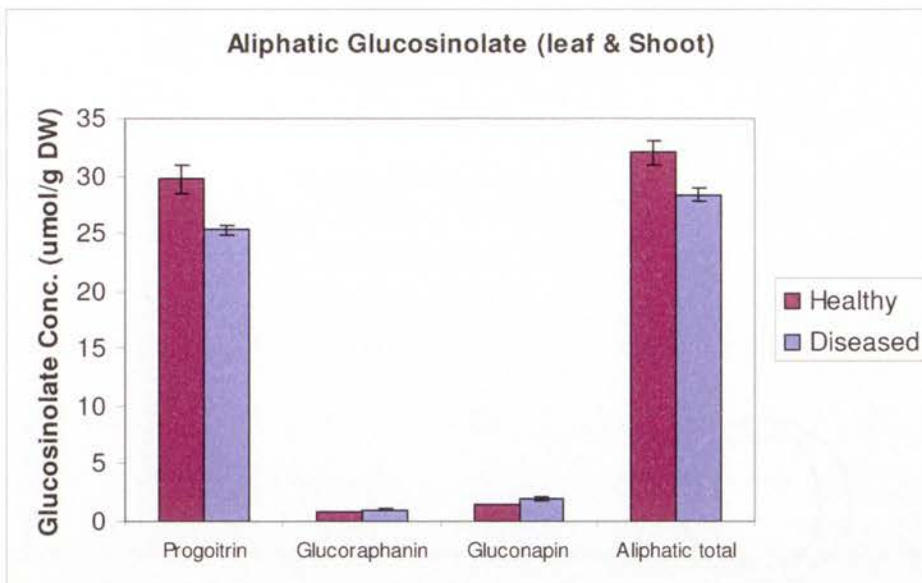


Figure 3.2. Progoitrin, glucoraphanin, gluconapin and total aliphatic glucosinolate in leaves and shoots of 5-week old field grown healthy and diseased (clubroot) Chinese cabbage (pak-choy). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

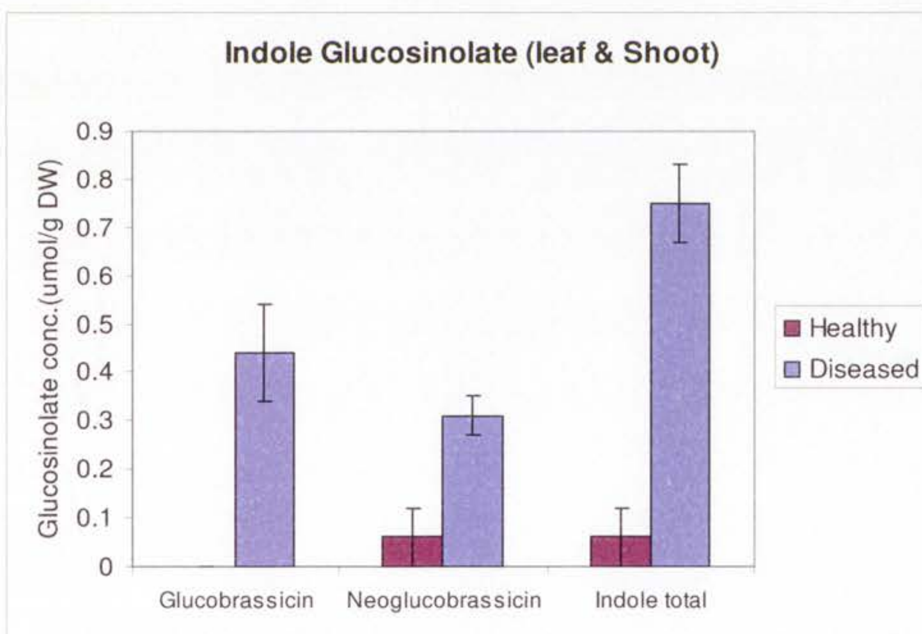


Figure 3.3. Glucobrassicin, neoglucobrassicin and total indole glucosinolate in leaves and shoots of 5-week old field grown healthy and diseased (clubroot) Chinese cabbage (pak-choy). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

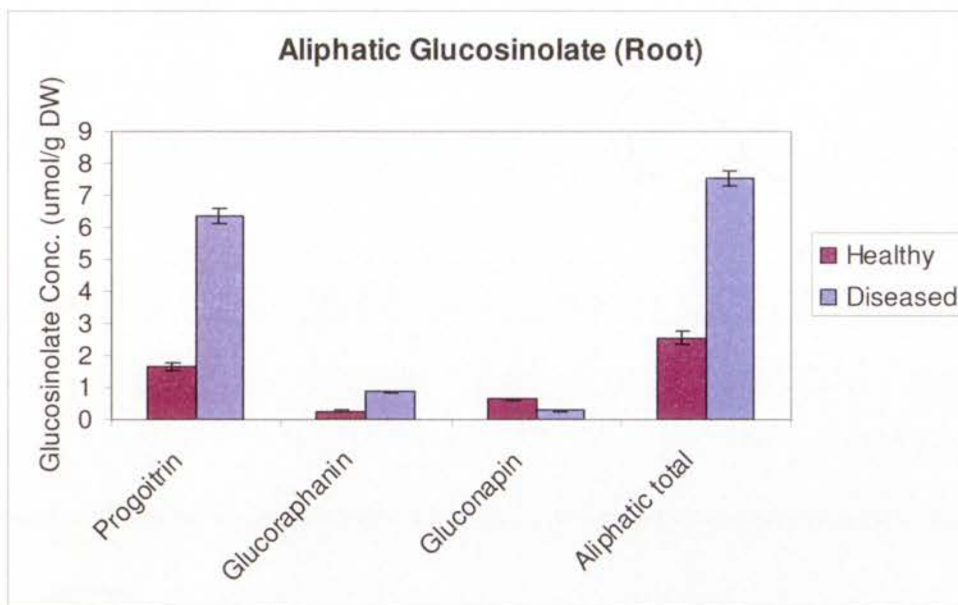


Figure 3.4. Progoitrin, Glucoraphanin, Gluconapin and total aliphatic glucosinolate in root tissues of 5-week old field grown healthy and diseased (clubroot) Chinese cabbage (pak-choy). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

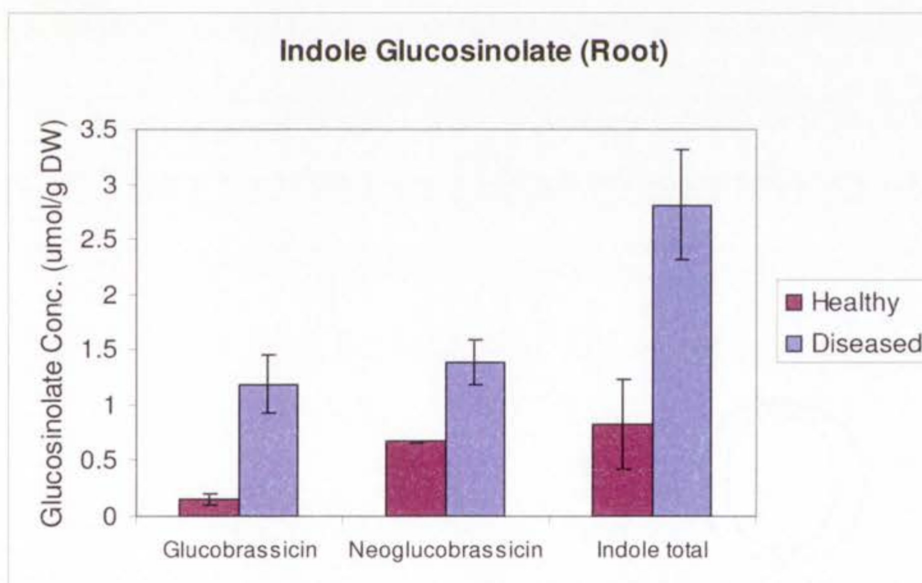


Figure 3.5. Glucobrassicin, neoglucobrassicin and total indole glucosinolate in root tissues of 5-week old field grown healthy and diseased (clubroot) Chinese cabbage (pak-choy). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

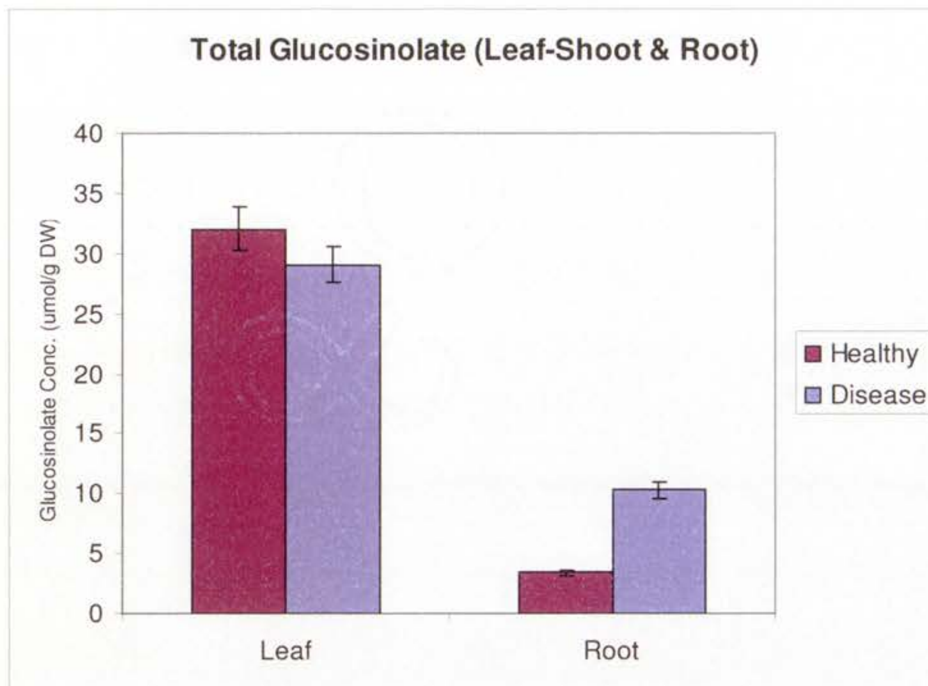


Figure 3.6. Total glucosinolates (aliphatic & indole) in leaves and root of 5-week old field grown healthy and diseased (clubroot) Chinese cabbage (pak-choy). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

3.4: Effect of clubroot on glucosinolate contents in field grown Chinese cabbage (Pak-Choy)

Individual and total aliphatic glucosinolate levels in aerial and root tissues of 35 day old field grown pak-choy were evaluated using HPLC. Aliphatic (progoitrin, glucoraphanin and gluconapin) and indole glucosinolates (glucobrassicin and neoglucobrassicin) were detected both in healthy and diseased aerial tissues. Total aliphatic glucosinolate level was significantly higher in healthy aerial tissues (28.33 μmole/g DW) than in diseased aerial tissues. Glucobrassicin was not identified in healthy aerial tissues but was abundant in aerial tissues of diseased plants, contributing to total indole glucosinolate levels that were 12.5 times higher in aerial tissues from diseased compared to healthy plants. Total glucosinolate levels were however dominated by aliphatic glucosinolates, which were 3 – 30 times more abundant in leaf and shoot tissue.

These results indicate that during the characteristic gall formation caused by *P. brassicae* the synthesis of aliphatic glucosinolates, particularly progoitrin, is suppressed (Figure 3.2) in the edible parts of this pak-choy cultivar. However, infection caused enhanced glucobrassicin accumulation in aerial tissues. Glucosinolate accumulation can also be induced by insect herbivory (Birch *et al.* 1990) and fungal infection (Doughty *et al.* 1991). This induction does not lead to similar increases in the content of all glucosinolates in the tissues, but rather to increase of certain compounds during the development of disease (Ludwig-Müller *et al.* 1997, 1999a & 1999b).

There are no available published data on glucosinolate levels in aerial plant parts following *P. brassicae* infection. Previous investigations relating to clubroot and glucosinolate concentrated on indole glucosinolate and gall development factors. Information has been published on the occurrence of glucosinolates in nonbrassicas, including *Tropaeolum majus* and *Carica papaya, Reseda alba* and *Beta vulgaris,* following *P. brassicae* infection by Ludwig-Müller *et al.* (1999b). However, they did not investigate the concentration of either aliphatic or indole glucosinolate levels, rather benzylglucosinolates such as glucotropaeolin, gluconasturtiin and glucomalcomiin. They reported that young leaves of *P. brassicae* infected plants of *T. majus* contained more than 150% benzylglucosinolate levels than leaves from uninoculated plants. Benzylglucosinolate levels of *C. papaya* young leaves following infection were decreased by 29% and old leaves increased by 7% than health leaves. This information demonstrates that *P. brassicae* infection can interfere directly or indirectly in the biosynthesis pathway of glucosinolate accumulation in aerial tissues.

Aliphatic (progoitrin, glucoraphanin and gluconapin) and indole glucosinolate were detected in the root tissues of field grown pak-choy. Diseased root showed significantly higher levels of aliphatic glucosinolate. Progoitrin production (6.37 μmole/g DW) is nearly 4 times higher than in healthy root tissues (1.66 μmole/g DW). Total aliphatic glucosinolate accumulations in diseased root tissues were 3 times higher than in healthy root tissue (Figure 3.4). A similar pattern was observed in indole glucosinolate production following infection. Glucobrassicin increased nearly 8 times and neoglucobrassicin production was more than doubled following *P. brassicae* infection at the time of characteristic gall formation. This result suggests the biosynthetic pathway of both aliphatic and indole glucosinolates might be interrupted

due to complicated interactions during clubroot development. Ludwig-Müller *et al.* (1997) demonstrated that roots of the Chinese cabbage variety, Osiris, total indole glucosinolate levels strongly increased during the symptom development stage (28 DAI) compared with the resistant cultivar, Parkin. They also reported that the infected roots of the variety Osiris showed a higher peak of indole glucosinolate levels compared to Parkin due to increasing level of neoglucobrassicin which is similar to our results. Bennett *et al.* (1996) also showed that young leaves of 35 day old Chinese cabbage produced higher neoglucobrassicin (type of indole glucosinolate) following infection.

Ludwig-Müller *et al.* (1997) confirmed that *P. brassicae* infection in susceptible Chinese variety showed increased levels of aliphatic glucosinolate during root hair infection stage and higher indole glucosinolate at the symptom development stage. Aromatic glucosinolate remained unchanged in susceptible varieties (Granat and Osiris) of Chinese cabbage. Aromatic glucosinolate levels were not analysed in our study.

Total glucosinolates (aliphatic and indole) in aerial tissue were significantly higher in healthy plants (32.04 μmole/g DW) than in diseased plants (29.08 μmole/g DW). This happened due to production of a higher concentration of aliphatic glucosinolates (range 25.00 to 30.00 μmole/g DW) compared to indole glucosinolate (range 0.1 to 0.7 μmole/g DW) in aerial tissues of pak-choy. This could indicate that *P. brassicae* infection is capable of suppressing aliphatic glucosinolate in aerial tissues, however further research should be conducted to confirm this indications. Total glucosinolates

(aliphatic and aromatic) in root tissues were significantly higher in diseased plant (10.32 μmole/g DW) than in healthy plants (3.38 μmole/g DW).

The most likely explanation for the observed differences in root and shoot patterns is that both organs have a different regulation of glucosinolate biosynthesis and turnover. Transportation of glucosinolates via the phloem over long distance (Chen *et al.* 2001) is not likely to be the main cause. This is supported by the observation that induction of specific indole glucosinolates by aphid feeding occurs in detached leaves as well, precluding a role for transport from the roots (Kim and Jander 2007). Moreover glucosinolate metabolism is highly integrated with plant growth. For example, there is a direct link between indole glucosinolate biosynthesis and the auxin, indole 3 acetic acid (IAA) metabolism, as both derive from tryptophan and share the first dedicated step in their biosynthesis (Grubb and Abel 2006). IAA is a key regulator in plant development in tissue differentiation (De Smel and Jürgens 2007). This also implies that environmental factors, both biotic and abiotic, affect IAA-regulated changes in growth rate or shoot/root ratios and may affect the levels of indole glucosinolates.

Additionally glucosinolate biosynthesis may also be interfering with defence signalling pathways. Hence, the question of what makes the glucosinolate profile different between roots and shoots may be intimately associated with the physiological differences between root and shoot metabolisms in general., Some general patterns have been identified with the current results of glucosinolate levels from field grown samples that may help us to better understand the role of these compounds in natural and controlled growth condition.

CHAPTER IV

4.1: Introduction

The glucosinolate content of Brassica tissues is influenced by genetics and the environment. This can include the external and cell environment (Jones *et al.* 2006). Besides the glucosinolate type varying between Brassica varieties, tissue type and the age of the plant affect glucosinolate levels. The accumulation of glucosinolates can be induced after wounding and pathogen attack (Koritsas *et al.* 1991; Bodnaryk 1992). Glucosinolates and their breakdown products are thought to play a role in disease resistance against insects and fungal pathogens (Glen *et al.* 1990; Mithen 1992).

Plasmodiophora brassicae causes clubroot and is one of the most damaging pathogens of the Brassicaceae family worldwide (Woronin 1878; Ludwig-Müller 1999a & 1999b). The effect of plant pathogens, particularly *P. brassicae,* on glucosinolate concentrations in broccoli is relatively unknown, as is information on the relationship of individual glucosinolates between aerial and root tissues of broccoli following *P. brassicae* infection process. There is some evidence that indole glucosinolate levels increase upon the development of clubroot in *B. campestris (renamed: Brassica rapa* L. subsp. *campestris* (L.) A. R. Clapham) *and B. oleracea* along with an increased synthesis of auxin precursors (Anonymous 2009c; Ludwig-Müller 1999b).

The plant defence activators phosphorous acid and acibenzolar (Bion) have been evaluated for the controlling numbers of plant pathogens. The plant activator acibenzolar (Bion) is a non fungicidal compound used to combat plant pathogens by inducing the host plant's natural defence mechanisms (Tally *et al.* 1999). This

chemical is a functional analogue of salicylic acid (SA), a plant signal shown to accumulate in plants challenged by a pathogen (Friedrich *et al.* 1996).
Phosphorous acid has been shown to have a number of modes of action including being directly fungistatic (Grant *et al.* 1990), and as a plant activator and enhance the of plant defence responses (Guest *et al.* 1988; Afek and Sztejnberg 1989; Grant *et al.* 1990; de Boer *et al.* 1990).

There is no available published information on the effect of potassium phosphonate and Bion against *P. brassicae* invasion or the levels of individual glucosinolates of their distribution among the above and below ground organs either in broccoli or any of the Brassica crops.

In this study, susceptible *Brassica oleracea* ssp. *italica* cv. Marathon (broccoli) was studied in a glasshouse to evaluate the complex interactions between the pathogen attack,, application of defence activators and levels of aliphatic and indole glucosinolate compounds distributed in above and below ground tissues.

Based on previous studies in cultivated species, it is hypothesised that phosphonate and Bion application will elicit a glucosinolate response and that these responses are systemic (Guest *et al.* 1988; Ludwig-Müller *et al.* 1997; Bartler *et al.* 1999). Individual classes of glucosinolates respond differently to chemical treatment and to the root damage following pathogen infection.

The aim of this chapter is to study whether phosphonate and Bion applications trigger differential induction of specific classes of glucosinolate and systemic responses throughout the plant. Changes in glucosinolate levels were also compared to the

effects of activators on gall formation, which may indicate the future efficacy of these chemicals to manage clubroot.

4.2: Materials and Methods

4.2.1 Chemicals

Treatments with phosphonate (Agrifos Supa 600, Agrichem Manufacturing Industries, Loganholme, Queensland, Australia) 1 g a.i / L, Bion (500 g/kg a.i. acibenzolar-S-methyl;Syngenta) 25 mg a.i. /L of water and equal combination of phosphonate and Bion were sprayed once 10 days after seed germination. Sprays were applied until runoff (approx. 250 mL solution per tray containing 100 seedlings). Tap water was sprayed at the same time on untreated control plants. Potting mix and glasshouse condition were described in Chapter 2.

4.2.2 Seedling Inoculation Technique

Inoculation Method-3 (IM-3) is the combination of IM-1 (direct spore injection at the base of seedlings) and IM-2 (resting spores of *Plasmodiophora brassicae* amended potting mix) was applied for rapid disease development in this experiment. According to the outcome from preliminary trial of three inoculation techniques, double inoculation method (IM-3) showed significantly faster symptom development (Figure- 4.1, 4.2 and 4.3). So inoculation Method-3 (IM3) was used for these experiments (Figure 4.9 to 4.35), where variation of glucosinolates accumulation following pathogen infection and chemicals treatments was determined. Twelve day old seedlings were transplanted into the potting mix containing 10^7 spores/mL of slurry mixed 3 days prior to seedling transplantation (IM-2). After transplanting

seedlings, 200 μL of cleaned resting spore suspension containing 10^8 resting spores per mL was injected around the rhizosphere of seedlings (IM1).

4.2.3 Treatment Combination

The following eight (8) treatments were used to collect the samples for glucosinolate measurements:

1. Potassium Phosphonate (PP) treated seedlings + *Plasmodiophora brassicae* (Pb)
2. Bion (Bn) treated seedlings + Pb
3. PP + Bn treated seedlings + Pb
4. PP treated seedlings + no pathogen
5. Bn treated seedlings + no pathogen
6. Bn + PP treated seedlings + no pathogen
7. Pathogen inoculation (Pb only)
8. No chemical treatment and no pathogen inoculation/Healthy plant (HLY)

The design was Randomized Complete Block Design (RCBD) with four replications. Samples were collected at 0, 21 and 42 days after inoculation. To observe sequential disease development and determine glucosinolate variations, samples were also collected at 7, 14, 28 and 35 days intervals from both inoculated and uninoculated plants. Plants in treatment 7 (inoculated) and treatment 8 (uninoculated) are referred as diseased control and healthy control throughout the text.

Sample collection, storage, freeze drying, glucosinolate extraction procedures were described in Chapter 2.

4.2.4 HPLC and UV Detection

Samples were analysed in the Laboratory of Plant Pathology, Faculty of Agriculture, Food and Natural Resources at The University of Sydney during June 2008 to July 2009. Glucosinolate extraction protocols, HPLC condition such as mobile phase, flow rate and temperature are described details in Chapter 2. For this experiment, the HPLC (Dionex Pty Lt, Australia) equipped with PDA100 photo diode array detector. Glucosinolates were separated on a Agilent ZORBAX Eclipse plus C18 column (4.6x250mm; 5µm) Agilent technologies, USA. In the beginning, duplicate samples (those were used in Postharvest Lab, Knoxfield DPI, Victoria- see Chapter 2) ran with the standard- sinigrin purchased from Sigma Aldrich, Australia. The differences of retention time of sinigrin between the Knoxfield Lab and Sydney Uni (Plant Pathology Lab) HPLC set up was estimated. The retention times of glucoiberin, progoitrin, glucoraphanin, gluconapin, 4-hydroxy-glucobrassicin, glucobrassicin and neoglucobrassicin were matched with the relative retention time of sinigrin. Due to unavailability of pure standards, the amount of above individual glucosinolate was determined as µmoles/L sinigrin equivalents.

Sinigrin was used as an external standard to calibrate the concentration and peak area to measure the individual glucosinolate concentration equivalent to sinigrin concentration. Four different concentrations (□ mole) were used e.g. 12.5, 25, 50 and 100 □mole. The relationship between concentration of standard (sinigrin) and the peak area was calculated based on the formula $Y= ax + b$ where, 'x' is the concentration of glucosinolate (□mole/g dry weight), 'Y' is the peak area and 'r^2' is the correlation of the equation and 'a' (slope) = 0.1821 and 'b' intercept = 0.087. The

correlation coefficients confirm excellent linear relationships between the concentration of the glucosinolates and their response peak area (Figure 4.1).

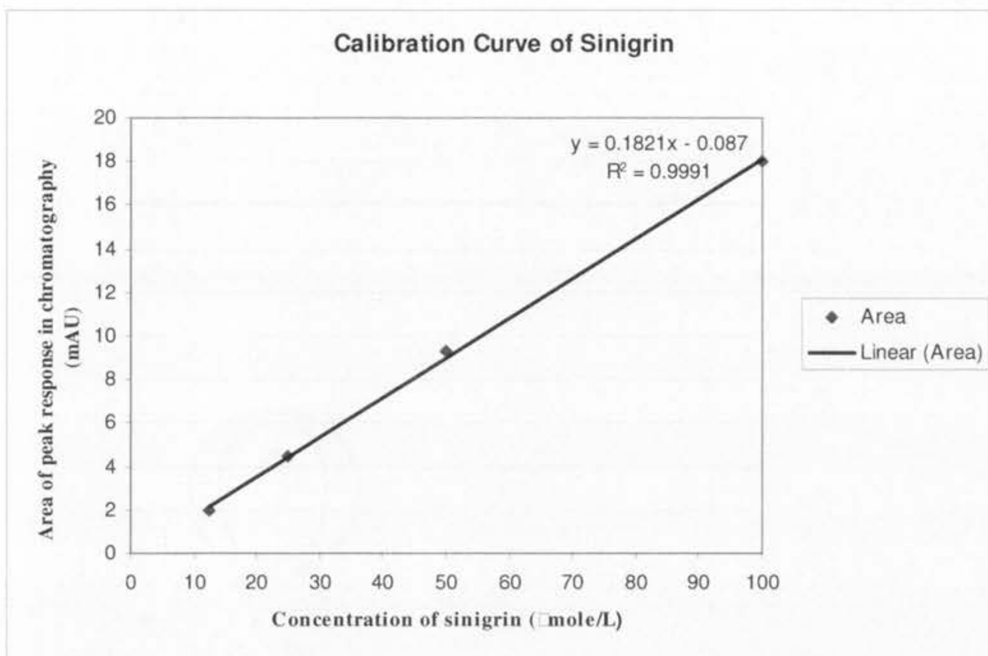


Figure 4.1 Calibration curve of sinigrin in chromatography used as external standard

4.2.5 The effect of Bion and Phosphonate on clubroot severity

The following five (5) treatments were used to evaluate gall suppression in broccoli

1. Potassium Phosphonate (PP) treated plant + *Plasmodiophora brassicae* (Pb) inoculation
2. Bion (Bn) treated plant + *Plasmodiophora brassicae* (Pb) inoculation
3. Combination of PP and Bn treated plant + Pb inoculation
4. Fresh plant (no chemical treatment) + Pb inoculation
5. Fresh plant (no chemical and no pathogen)

A total of 10 x 5 x 2 = 100 seedlings from 5 different treatments were assessed for disease severity as well as different growth parameters. Plants were harvested 42 days after inoculation. Pots were arranged in RCBD with 10 replications.

4.2.6 *Disease assessment*

The effect of chemical applications on disease severity was assessed at the end of the trial (42 days post inoculation). Plants at 42 days post inoculation were assessed visually by washing root galls using a scale of six disease classes (Friberg *et al.* 2006) modified from Buczacki *et al.* (1975) and Wallenhammar *et al.* (2000). The modified disease classes are:

0 no symptoms;

1 small club formations on lateral roots;

2 small club formations on the main root;

3 large club formations on the main roots, lateral roots with small or few clubs only:

4 large club formations on the main roots and numerous clubs on lateral roots; and

5 severe club formations on the main roots leading to partial degradation, lateral roots completely destroyed, plant growth markedly affected.

4.2.7 *Statistical Analysis*

Data analysis of chemically treated, diseased and healthy control plants were performed with the statistical package GraphPad Prisam 5.2, 2009 (GraphPad Software Inc, San Deigo California, USA). Two way analysis of variance (ANOVA) was used to determine different treatment factors over time factor. Mean comparisons

were also estimated at level 5% of confidence interval (*P=0.05*) and standard error of mean. Mean separation was also determined for the effect of chemical treatment and pathogen inoculation on specific individual glucosinolates in broccoli according to Bonferroni post test.

Ordinal regression analysis was performed for different chemical application data in suppressing club root disease severity (0 to 5 scales). Growth parameters such as fresh and dry weight of plant leaf-shoot, fresh gall weight were analysed by analysis of variance and using GenStat 11.2 Edition package program, 2009, VSN International Ltd.

4.3 Results: Optimisation of Inoculation Method

Three different inoculation techniques of *Plasmodiophora. brassicae* were tested against susceptible broccoli seedlings under glasshouse condition. Inoculation methods were evaluated to find a fast and effective technique based on the frequency of root hair infection, cortical cell infection and fresh gall weight at different time point after inoculation.

The percent root hair infection of three inoculation techniques was estimated at 4, 8, 12 and 16 days after inoculation. There was no significant difference between inoculation method 1 (spore injection at the base of seedlings) and inoculation method 2 (inoculum slurry amended with potting mix) on the number of root hair infections by primary zoospore of *P. brassicae* at any of the sampling times. Method 3 (combination of IM-1 and IM-2) gave significantly higher ($P<0.001$) percent of root hair infection compared with method-1 and 2 at any time, post inoculation. Method-3 exhibited 97% root hair infection compared to 77% and 75% of root hair infection by

method-2 and method-1, respectively at 16 days after inoculation. Similar ratios of percent root hair infection were recorded at 4, 8 and 12 days post inoculation (Figure 4.2).

Inoculation methods were evaluated by counting the percent of root cortical cells occupied by multinucleate secondary plasmodia from 12 to 36 days post inoculation. Methods-1 and 2 did not show any significant differences in cortical cell infection between 12 to 24 days post inoculation but Method 1 showed significantly lower ($P<0.001$) infected cortical cell at 24 and 28 days post inoculation compared to Method 2. Method-3 showed significantly higher ($P<0.001$) percent of cortical cell infection 12 to 24 days after inoculation compared with both Methods 1 and 2, however Method-3 was significantly different after 28 days compared with Method-2. Method-1 had significantly lower ($P<0.001$) percent of cortical cell infection at 24 and 28 days compared with Method-3. Method 3 caused 80% cortical cell infection while Method-1 and 2 gave only 3.75% and 8.25% cortical cell infection respectively, however, nearly one hundred percent cortical cells were infected with the multinucleate plasmodia of *P. brassicae* by each inoculation method 36 days after inoculation (Figure 4.3).

Fresh galls were weighed every 4 days between 12 to 36 days following infection. Initially (12 dpi), Method-3 produced gall like symptoms (hypertrophy of hypocotyl root) that weighed only 0.75 g/plant. No galls developed using Methods 1 and 2 until 20 days after inoculation. An average of 1.25 g/plant fresh gall was collected from Method -2 while Method-3 produced 9.00 g/plant fresh gall, which was significantly higher ($P<0.001$) than Method-2. Fresh gall weight from method 1 and 2 did not show

any significant differences until 32 days post inoculation, but a significantly higher ($P<0.01$) weight of fresh galls was observed in Method 2 at 36 DPI compared to Method 1. Method 3 exhibited higher fresh gall weight at every time point of sampling. At 36 DPI, galls from method 3 were 1.8 and 3.2 times larger than those from Method 2 and Method 1, respectively (Figure 4.4).

Sequential development of primary and secondary infection stages were observed in the plant roots where inoculation method 3 (spore injection and inoculum slurry) was used. Primary plasmodia, differentiated plasmodia (early zoosporangia) and also fully differentiating plasmodia (early zoosporangia) in root hairs were seen under microscope 0 to 7 day after inoculation (Figure 4.5). Secondary plasmodia were first identified at 7 DPI in cortical cell region. A small and big cluster of secondary plasmodia with cell proliferation in cortical cells were observed 10 DPI. Multinucleate secondary plasmodia and destroyed cortical cell walls were also observed 10 DPI (Figure 4.6). At the same time, initial gall initiation was seen under microscope. Visible galls were harvested at 14 DPI. Resting spore formation inside young galls were identified during 21 to 28 dpi and matured resting spores were successfully extracted from 42 day old galls of broccoli, those were used further experiments (Figure 4.6).

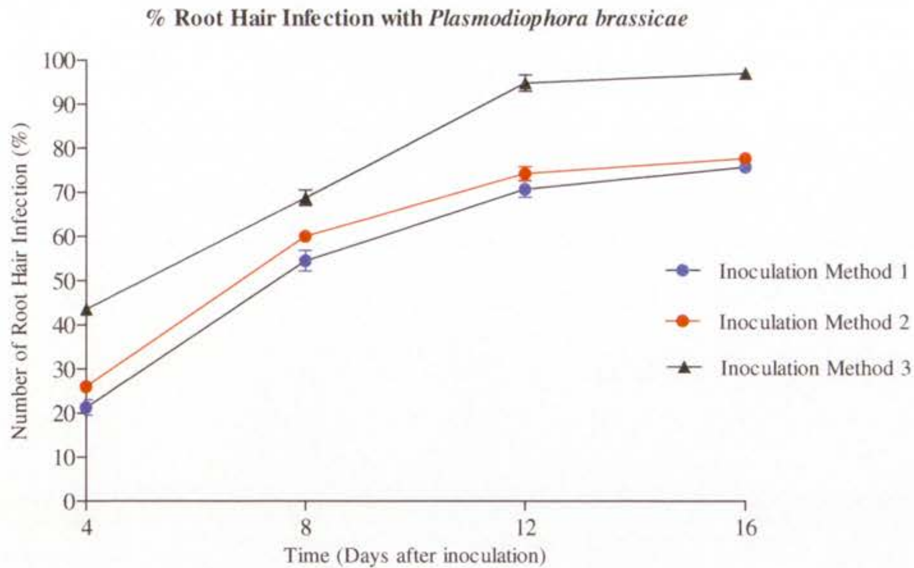


Figure 4.2. Effect of inoculation methods on root hair infection by *Plasmodiophora brassicae* of 4 to 16 days after inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

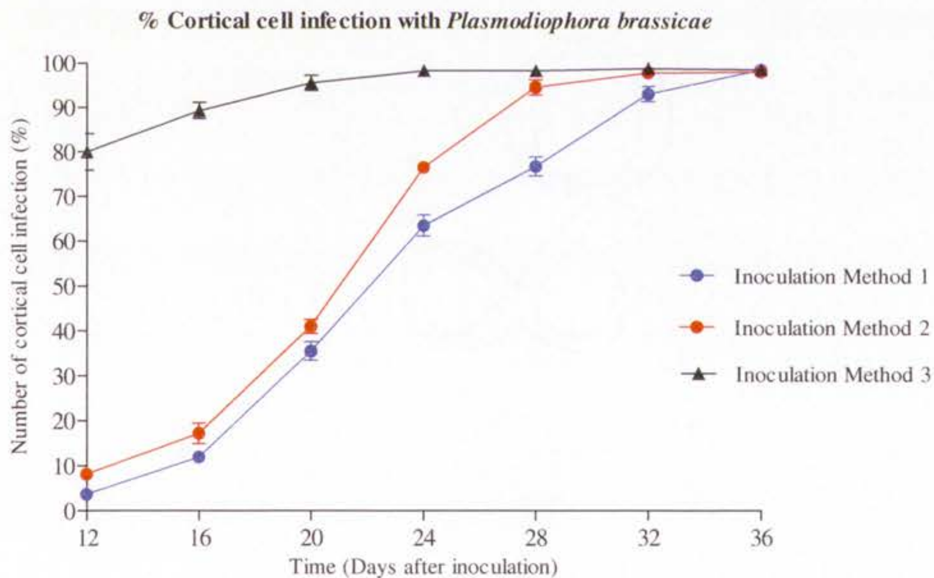


Figure 4.3. Effect of inoculation methods on cortical cell infection by *Plasmodiophora brassicae* of 4 to 16 days after inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

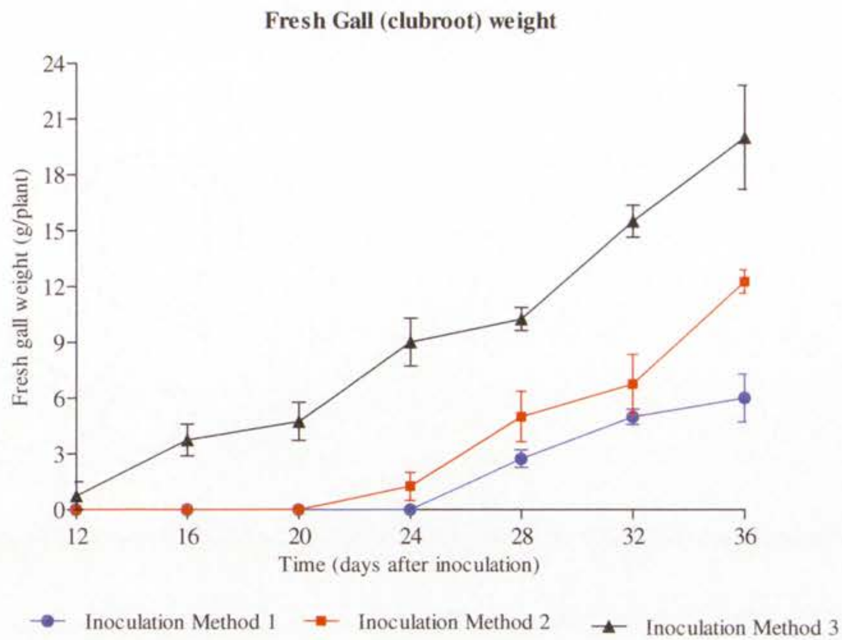
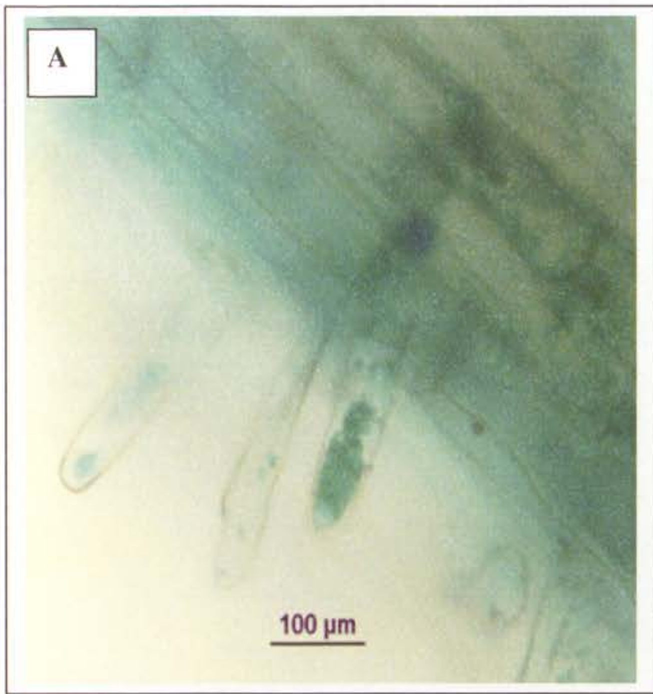
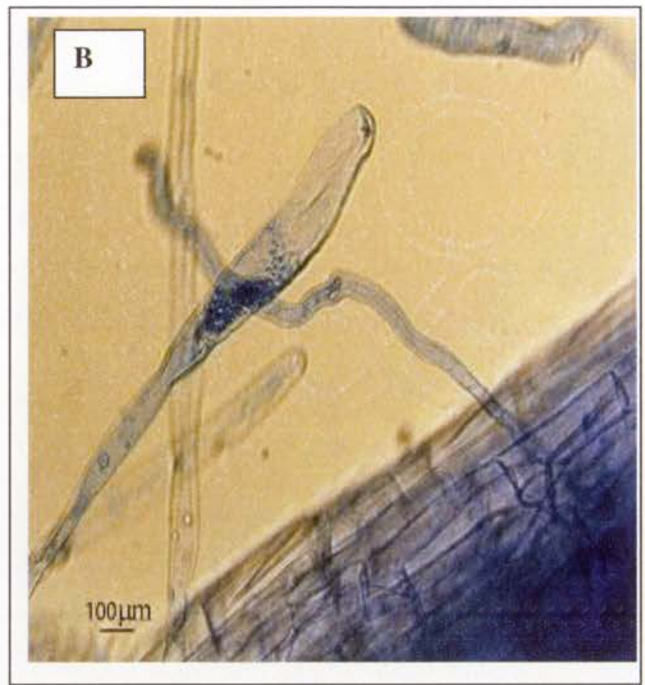
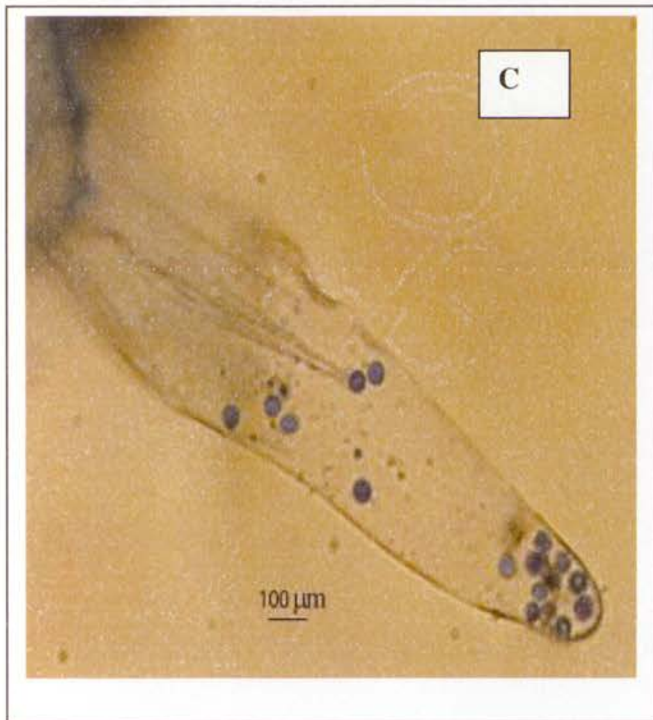


Figure 4.4. Effect of inoculation methods on fresh gall weight by *Plasmodiophora brassicae* of 4 to 16 days after inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).







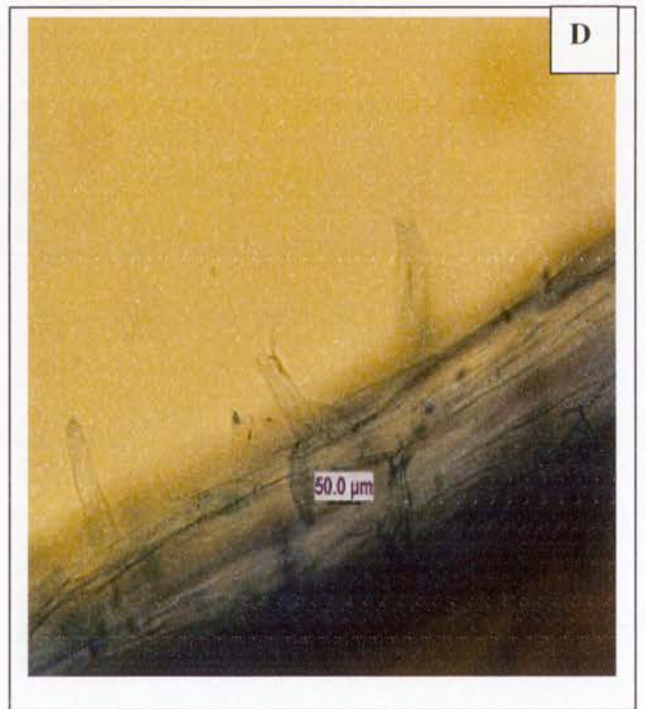


Figure 4.5. Sequential development of primary stage of infection in broccoli root hair occurred 0 to 7 days after inoculation A. Primary plasmodium in root hairs B. Differentiating plasmodium (early zoosporangia) in root hairs C. Root hair with secondary plasmodia, fully differentiated zoosporangium D. Empty root hair - Secondary plasmodia (sp) released from the tip of hairs.

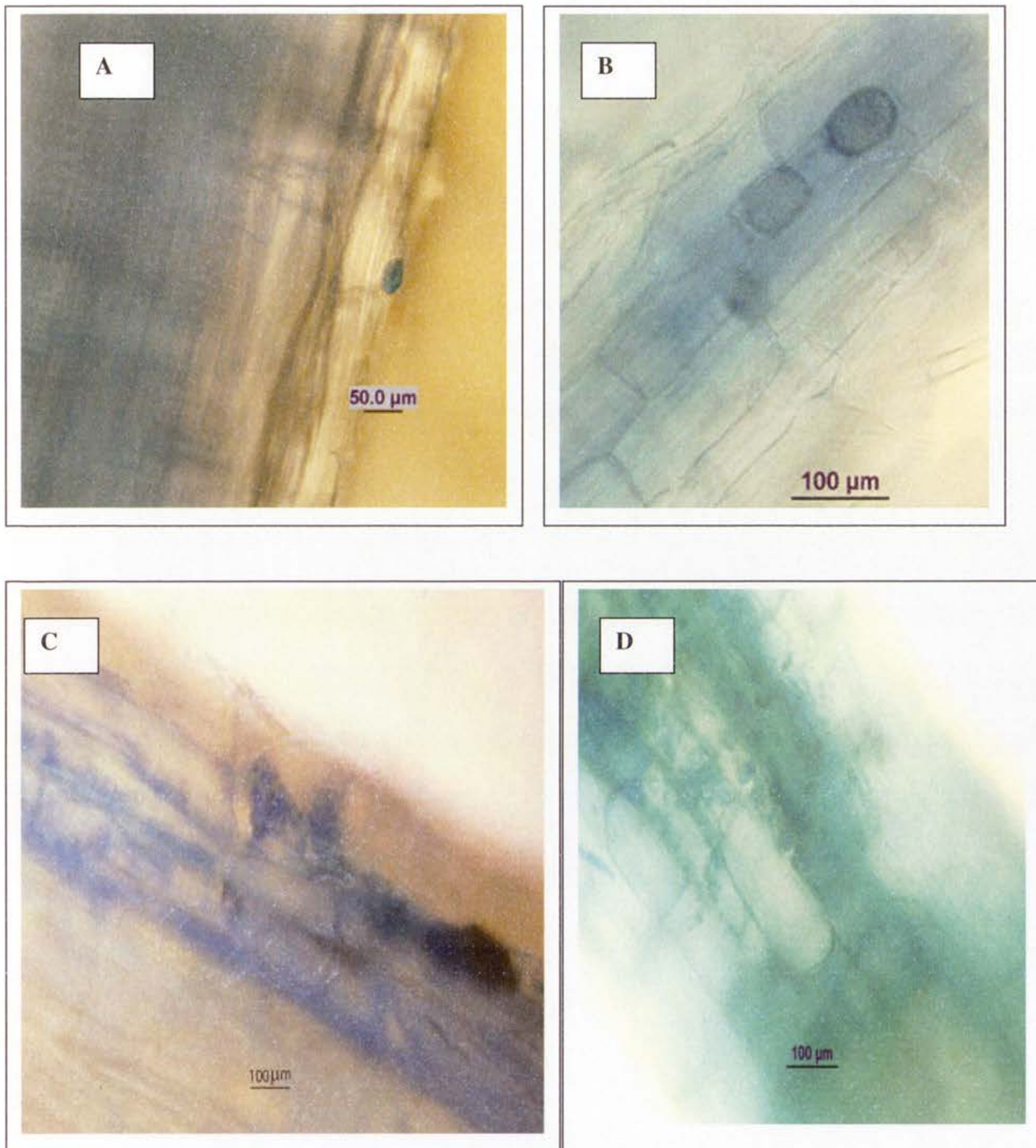
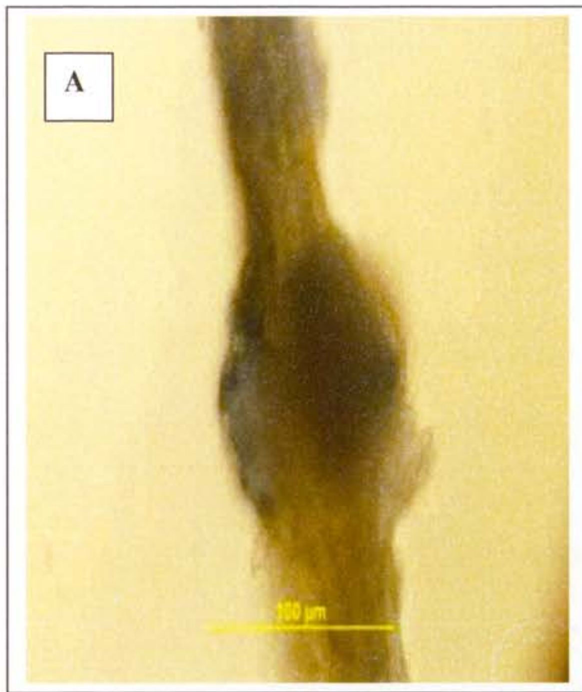
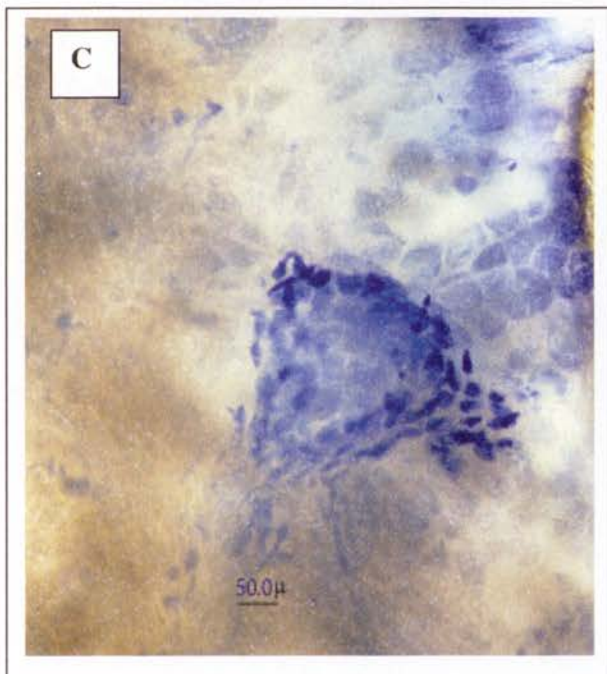


Fig 4.6. Sequential development of secondary infection stage in root cortical cell region of broccoli A. Secondary plasmodia (observed at 7 dpi) B. Small cluster of secondary plasmodia C. large cluster of secondary plasmodia occupied cortical cells and cell proliferation starts D. Multinucleate secondary plasmodia and destroyed cortical cell walls.





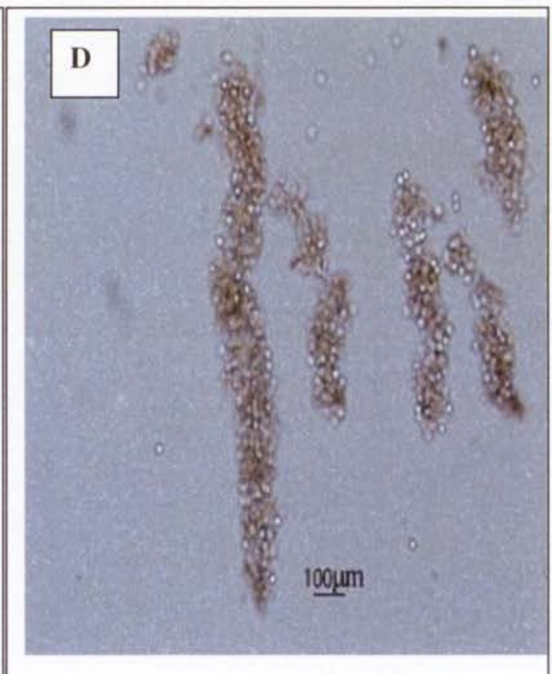
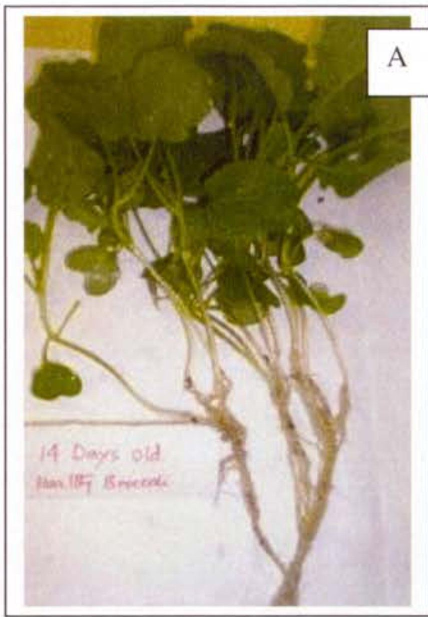


Figure 4.7 A. Gall initiation at 10 DP seen under light microscope B. Visible gall at 14 DPI C. Resting spore formation from dissecting gall (21 DPI) D. Extracted resting spores from characteristic gall at 42 day after inoculation



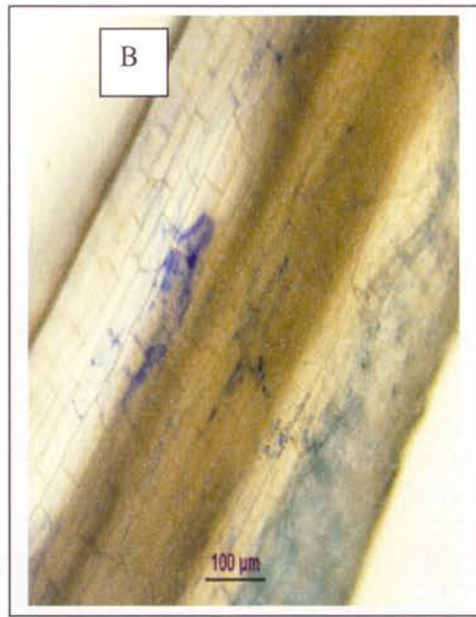


Figure 4.8 A. Root of uninoculated control plant at 14 day old plant B. Cortical cell of uninoculated seedling root

4.4 Discussion

In this study, inoculation method 1 injected *P. brassicae* spore suspension into the root zone of 12 days old seedling of broccoli. Inoculation method 2 mixed inoculum slurry with potting mix. Method 1 and 2 did not show any differences in root hair infection. Microscopic observation of infected cortical cells showed method 2 exhibited significantly higher number of cell infection at 24 and 28 days post inoculation than method 1. Method 2 (inoculum slurry) also caused a significant increase in fresh gall weight at 36 DPI. However, Methods 1 and 2 did not show consistent differences in relation to cortical cell infection and fresh gall weight.

Johnston (1968) regarded incorporating diseased tissue into the growth medium as an imprecise method for inoculating cabbage plants with *Plasmodiophora brassicae.* He reported that a spore suspension technique was more precise and for experiments where inoculum must be precisely quantified, similar procedures may be preferable to inoculum consisting of diseases root tissue. The most aggressive method of producing disease is not always preferred i.e. disease control studies, bio-control studies where subtle differences may be present. The presence of mixed pathogens in populations, the inoculum used, the more likely that mixed pathogens will be present. This is important in many studies eg. resistance. However, in this study the soil inoculation with infected root slurry resulted in a better infection rate in cortical tissue and produced clubs weighing significantly more than spore injecting inoculation method. For work intended to maintain or increase stocks of *P. brassicae*, there would seem to be no compelling reason to use a more complicated and more time consuming procedure. Considering, time and labour, infected root slurry is much easier, less time consuming preparing inoculum than extracted spore suspension injection technique.

Inoculation method 3 (IM3) was the combination of Method 1 and 2 applied together into broccoli seedlings. This combined application (double inoculation) of spore suspension and inoculum slurry into the broccoli seedlings resulted strong infection at every sampling observation of root hair, cortical cell infection and fresh gall size. The likely reason for a rapid infection by IM-3 might be due to higher inoculum pressure causing gradual increase of primary and secondary infection rate during 0 to 36 days post inoculation compared with any of single inoculation technique (Method 1 and 2). Method 3 caused consistent infections to root hairs and cortical region and produced clubs weighing significantly more than other two inoculation methods.

The slurry mix ensured that large numbers of resting spores are in close proximity to plant roots. Possibly, combining Method 1 and 2 increased the inoculum pressure in the seedling roots and resulted in more than 80% root hair and 40% cortical cells infected 4 and 12 days post inoculation, respectively, while separately, spore suspension and inoculum slurry gave 10% and 20% root hair and cortical cell infection, respectively. The first young gall appeared following the combined inoculation method (IM3) at 12 dpi whereas first young galls were seen on spore suspension (IM1) and (inoculum slurry (IM2) inoculated plants at 24 and 28 dpi, respectively. Dekhuijzen (1979) reported visible galls of susceptible variety of *B. campestris* var. *rapa* infected with sterile resting spores of *P. brassicae* from senescent callus 14 dpi. He also reported the presence of amoeboid structures within cortical cells of the susceptible variety 10 days after inoculation. The double inoculation technique (IM 3) induced clubroot symptoms earlier than reported by Dehhuijzen (1979). The presence of decaying root tissue used as slurry may enhance

the germination of resting spores (Williams and McNabola 1967; Macfarlane, 1970). Inoculating seedlings with infected root slurry prior to injection of extracted spore was rapid and effective method likely to be useful to researchers maintaining population of the organism in the glasshouse.

Previous studies (Samuel and Garrett 1945; Naiki *et al.* 1978) reported that the age of root hairs might affect root hair infection by *P. brassicae*. It is interesting to note roots hairs on 5 – 6 day old roots were more susceptible to infection by *P. brassicae* than younger or older root hairs (Asano *et al.* 2000). In this study, inoculum slurry was added to potting mix 3 days prior to seedling transplantation thus the early stage of root hairs become exposed to germinated resting spores resulted rapid infection. In the case of spore suspension techniques, spores injected to root zone of 12 days old transplanted seedlings means root hairs were older than used in slurry inoculation method. This explanation is in good agreement with the findings of Samuel and Garrett (1945) and Naiki *et al.* (1978). So far, there is no published report of a double inoculation technique of *P. brassicae*. This technique gives rapid and consistent formation of galls 14 DPI.

4.5: Results: Glasshouse trial on the effect of *Plasmodiophora brassicae* inoculation on glucosinolate levels in aerial and root tissues of broccoli.

4.5.1 Aliphatic glucosinolate production in broccoli root

Inoculated and uninoculated plant roots were assayed using High Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) to observe changes of different individual glucosinolate levels during primary (0-7 DPI), secondary (7-14 DPI), symptom development (14-28 DPI) and characteristic gall formation phases (28-42 DPI) of clubroot disease. Aliphatic glucosinolate levels such as glucoiberin, progoitrin, glucoraphanin and gluconapin concentration were determined following infection from 0 to 42 days compared with uninoculated plants. Sequential developmental stages of infection are marked as

- primary stage of infection: 0 to 7 DPI,
- secondary stage of infection: 7 to 42 DPI, of which
    - symptom development stage or initial gall formation stage: 7 to 21 DPI and
    - characteristic gall formation stage: 21 to 42 DPI.

There were no significant differences in glucoiberin and gluconapin production between inoculated and uninoculated roots during 0 to 21DPI. However, infected roots showed significantly ($P<0.001$) higher levels of glucoiberin and gluconapin accumulation during the symptom development stage (28 to 35) of clubroot (Fig. 4.9 & 4.12). Glucoraphanin production was similar in infected and healthy roots at any stage of the *P. brassicae* life cycle (Fig.4.11). Progoitrin accumulation in inoculated

root showed significantly higher at 14, 28 and 35 dpi compared with uninoculated, healthy plants (Fig. 4.10).

Glucoiberin, progoitrin and gluconapin production significantly increased in inoculated plant roots, which resulted in higher levels of total aliphatic accumulation at 21 to 42 DPI compared with uninoculated plant (Figure 4.13).

4.5.2 Indole glucosinolate production in broccoli root

Changes in individual indole glucosinolate accumulations were observed during symptom development stage of infection to characteristic gall formation (7 to 42 DPI). 4–hydroxy glucobrassicin contents in inoculated plants increased at the secondary infection stage then decreased at symptom development stage and increased again to 42 days post inoculation (Fig. 4.14). Glucobrassicin accumulation rates were continuously higher ($P<0.001$), except during primary phases of infection (0 to 7 DPI) when it was higher in uninoculated plants (Fig.4.15). During primary infection, neoglucobrassicin in inoculated root significantly ($P<0.001$) dropped, and then increased significantly ($P<0.01$) until gall formation, compared with uninoculated roots (Fig. 4.16).

Significantly lower levels of total indoles accumulated following root hair infection, however, with the progression of infection indole accumulation in root organs significantly increased until characteristic gall producing stage (42 DPI). Infected roots showed 2.5 times higher levels of total indole accumulation than uninoculated roots 28 DPI (symptom development stage) of *P. brassicae* infection (Figure 4.17).

4.5.3. Aliphatic glucosinolate production in aerial tissues

P. brassicae inoculation did not cause significant differences in the accumulation of glucoiberin, progoitrin and gluconapin in aerial tissues except at the time of symptom development (21 DPI). During the symptom development phase of clubroot, infection caused a 50% decrease ($P<0.05$) in glucoiberin accumulation. A similar pattern was observed in progoitrin and gluconapin accumulation in aerial organs compared with healthy plants. Glucoraphanin accumulation was significantly enhanced ($P<0.05$) following infection and it was nearly 1.5 times higher than in uninoculated plants at the secondary infection time (14 DPI) (Figure 4.18, 4.19 and 4.20 and 4.21).

The resulting total aliphatic content of leaves and shoots of inoculated and uninoculated plants was not significantly different at any time point of clubroot infection (0 to 42 DPI) (Figure 4.22).

4.5.4 Indole glucosinolate levels in aerial tissues

There was a significantly ($P<0.001$) higher 4-OH glucobrassicin accumulation in shoots from inoculated compared to uninoculated plant, except at the secondary infection phase (14DPI). 4-OH glucobrassicin levels were nearly 80 times higher than in uninoculated tissues 35 DPI. Glucobrassicin production levels were significantly ($P<0.001$) higher in inoculated plants 7, 14 and 28 DPI. *P. brassicae* infection did not cause significant variations in neoglucobrassicin levels except at the time of symptom development when neoglucobrassicin was not detected in healthy

aerial tissue and was 1.69 µmole/g DW in infected tissues (Figures 4.23, 4.24 and 4.25).

A sharp increase in total indole glucosinolate levels was observed following infection and significantly higher (P>0.001) levels of total indole glucosinolate were recorded at primary and symptom development phase and gall forming stage (P<0.05) of clubroot (Figure 4.26).

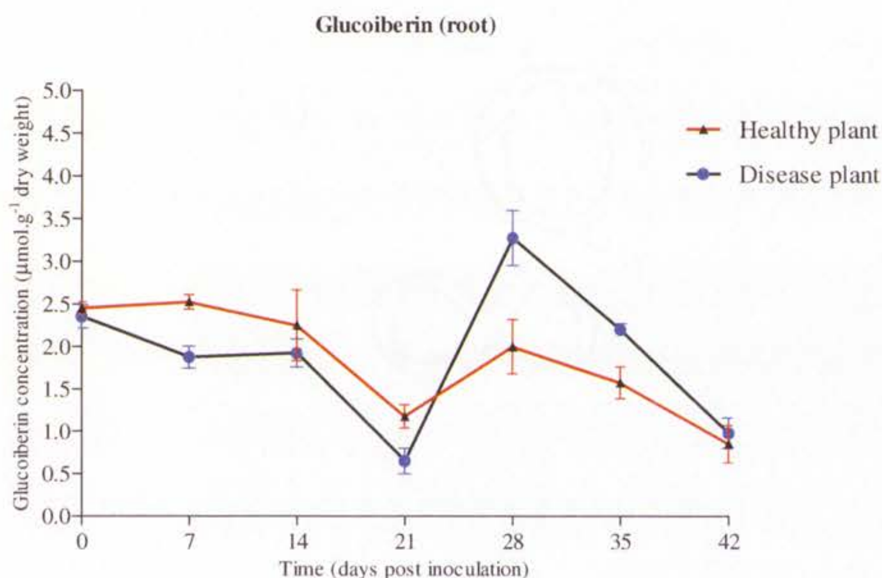


Figure 4.9. Glucoiberin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

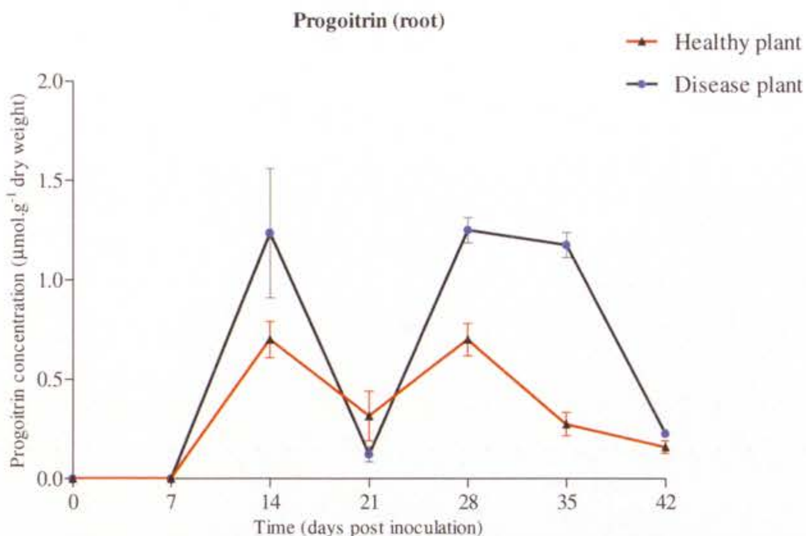


Figure 4.10. Progoitrin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

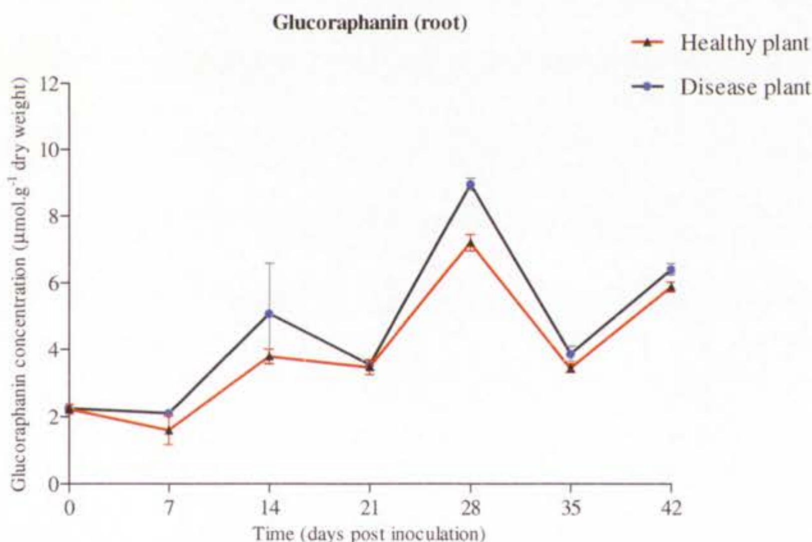


Figure 4.11. Glucoraphanin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

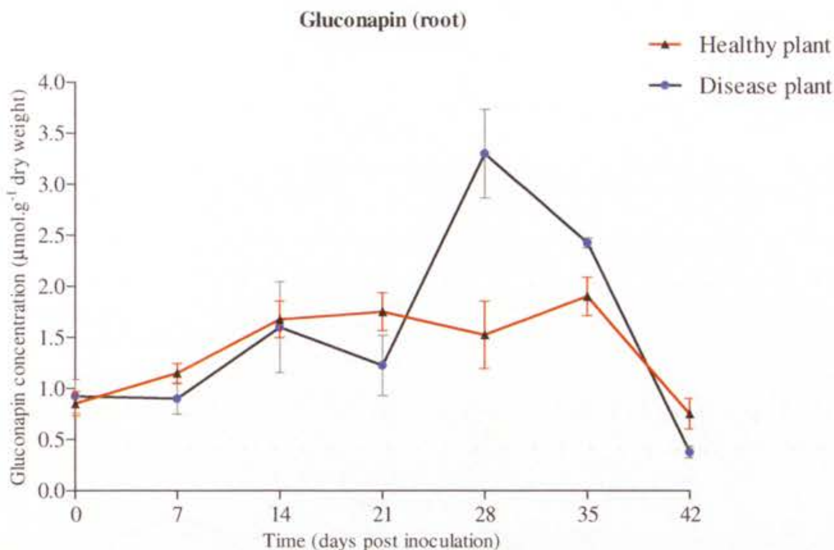


Figure 4.12. Gluconapin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

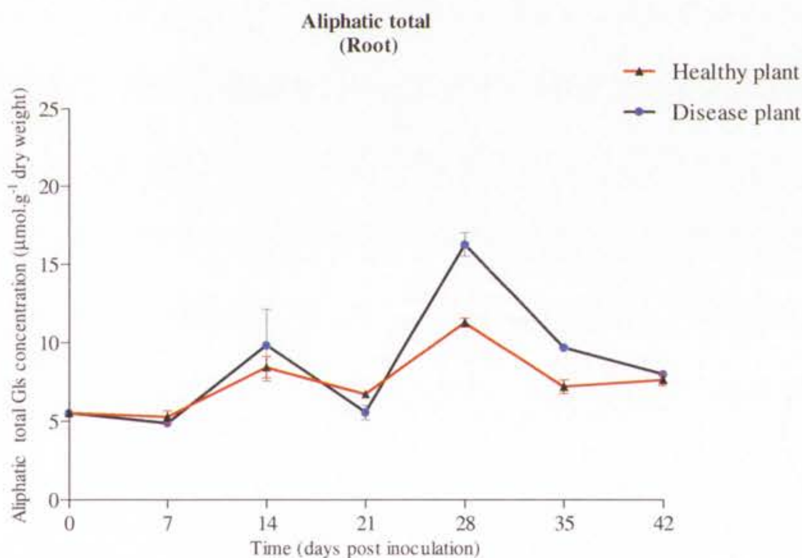


Figure 4.13. Total aliphatic glucosinolate content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

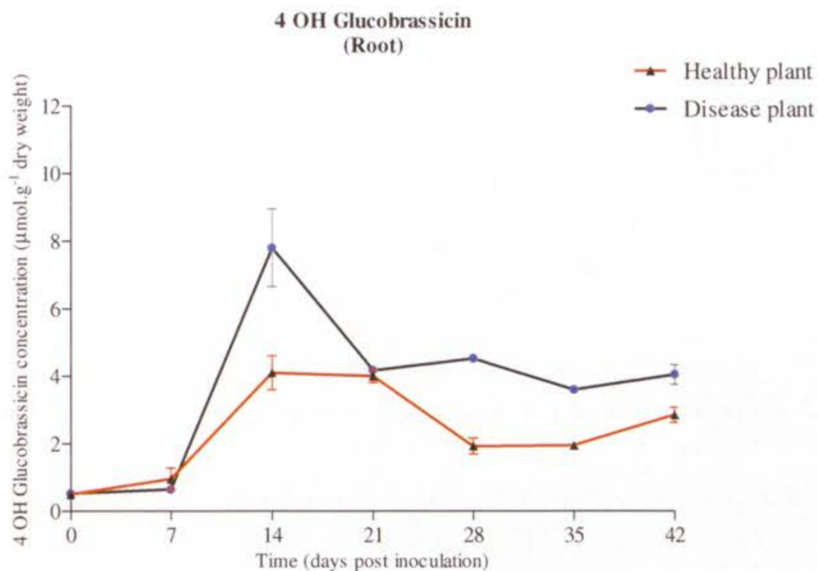


Figure 4.14. 4 OH glucobrassicin content in *Brassica oleracea*(broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

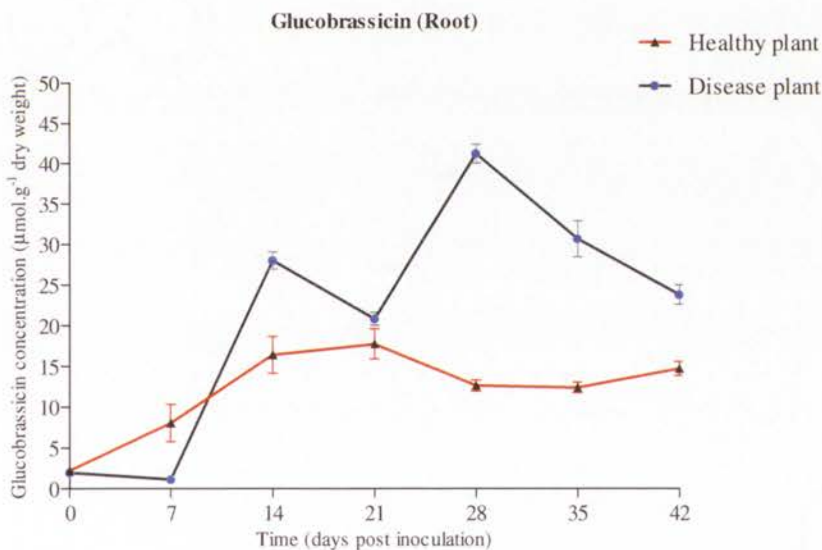


Figure 4.15. Glucobrassicin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculatio Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

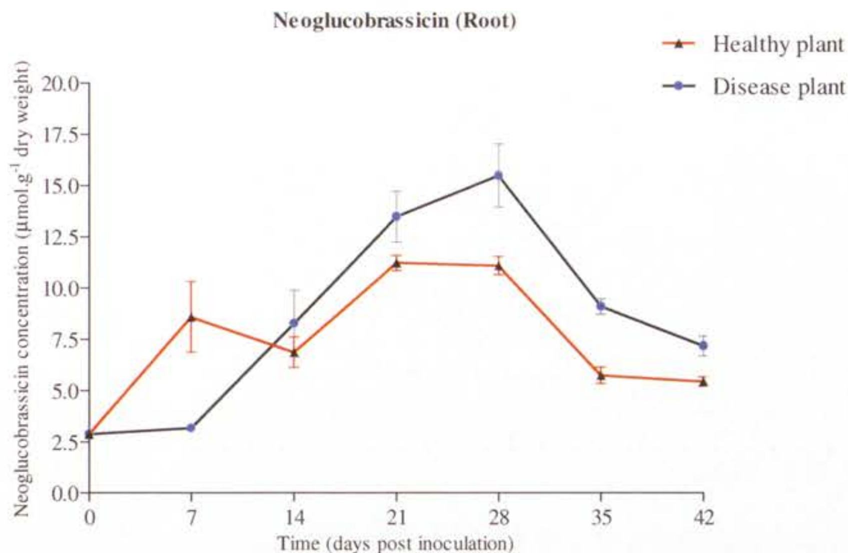


Figure 4.16. Neoglucobrassicin content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

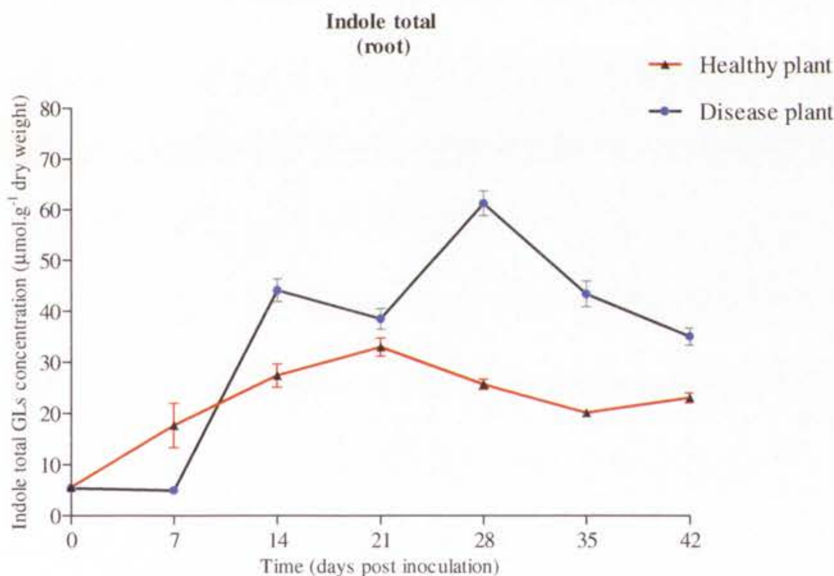


Figure 4.17. Total indole glucosinolate content in *Brassica oleracea* (broccoli) root tissues of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation. Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

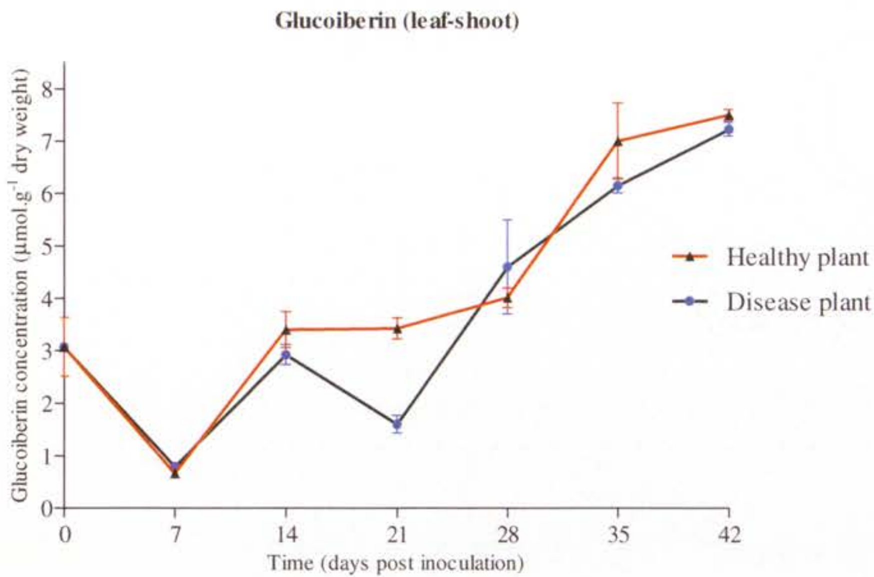


Figure 4.18. Glucoiberin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

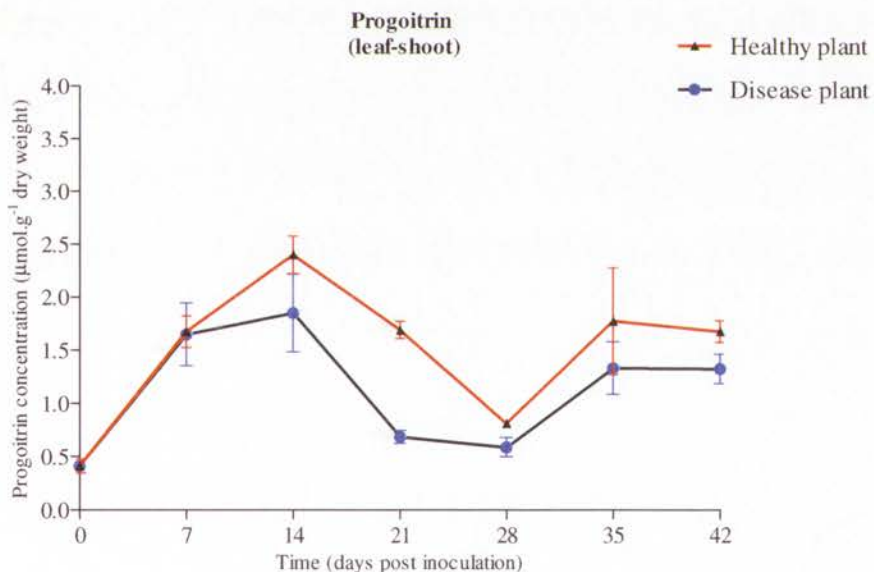


Figure 4.19. Progoitrin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation) Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

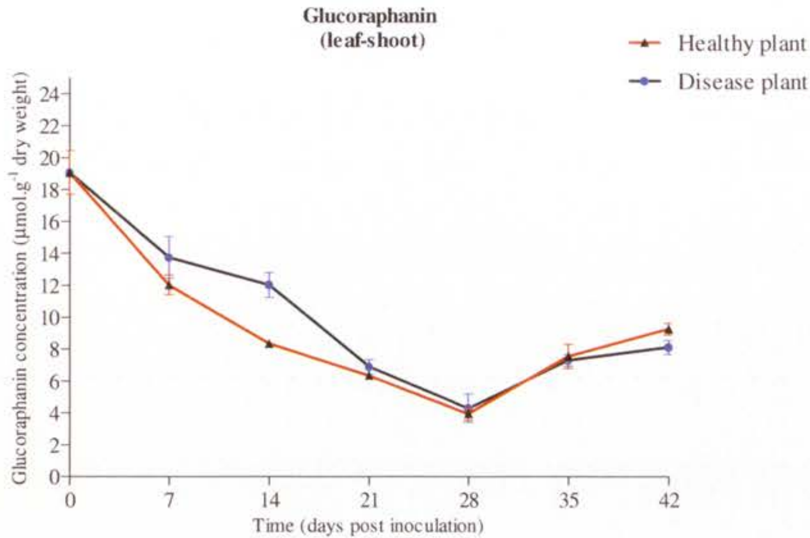


Figure 4.20. Glucoraphanin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

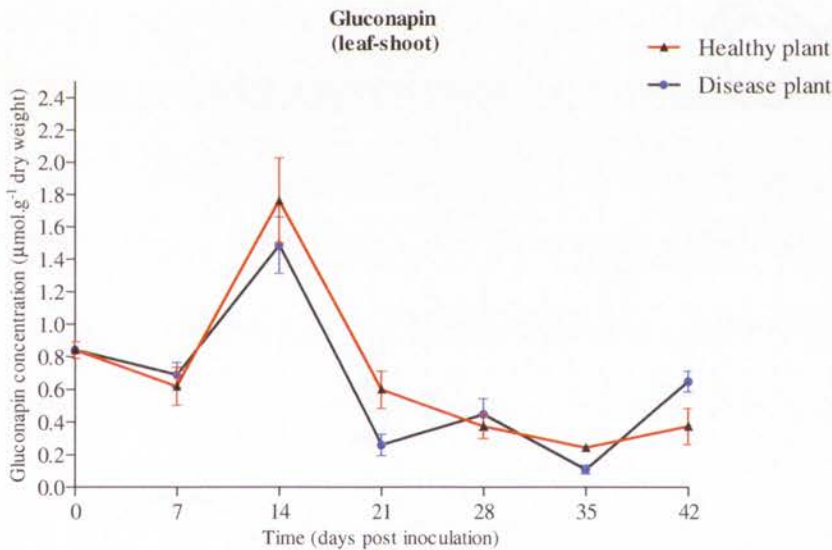


Figure 4.21. Gluconapin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation) Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

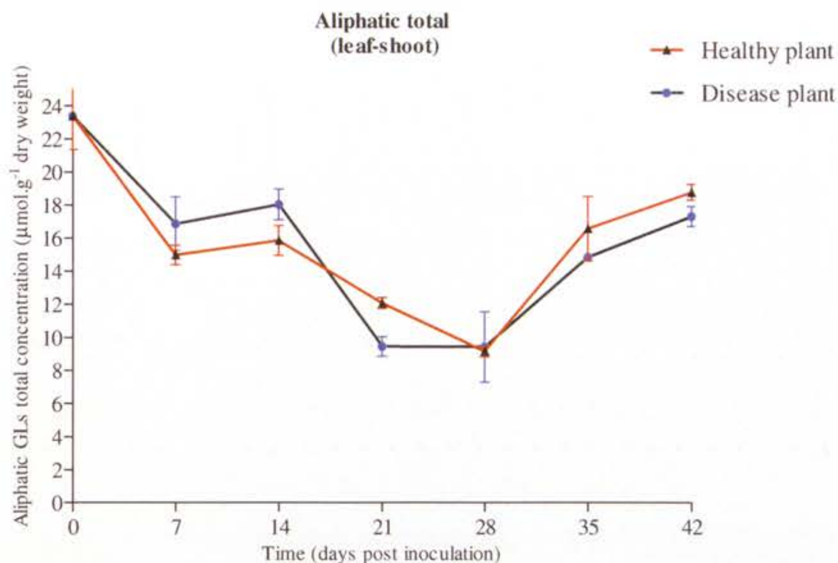


Figure 4.22. Total aliphatic glucosinolate content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

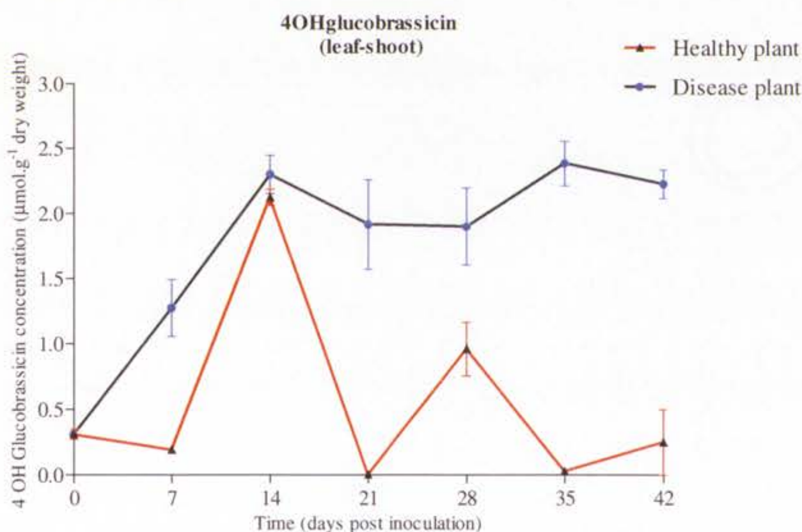


Figure 4.23. 4-hydroxyGlucobrassicicn content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation) Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

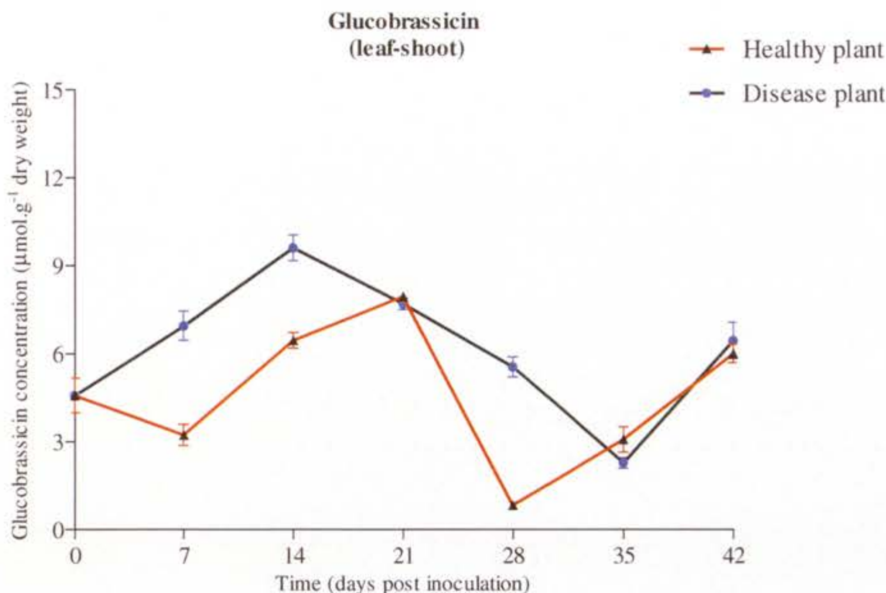


Figure 4.24. Glucobrassicin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation) Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

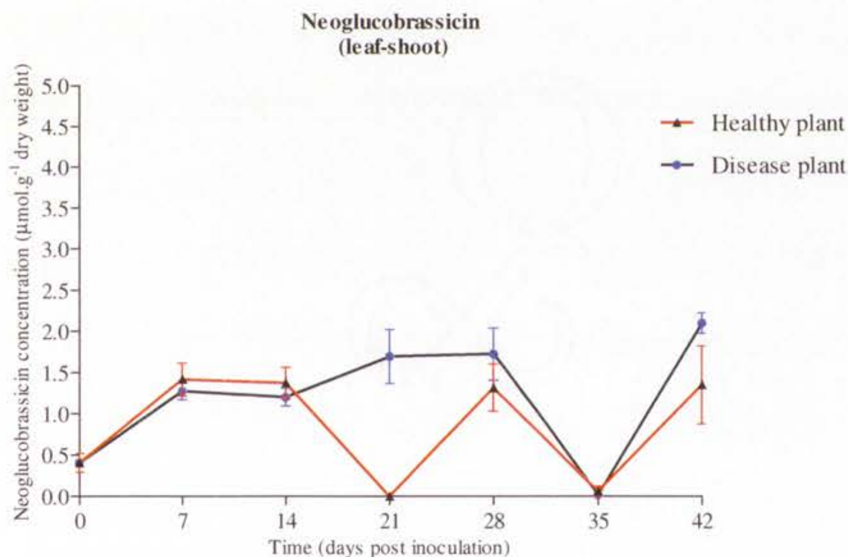


Figure 4.25. Neoglucobrassicin content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation) Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

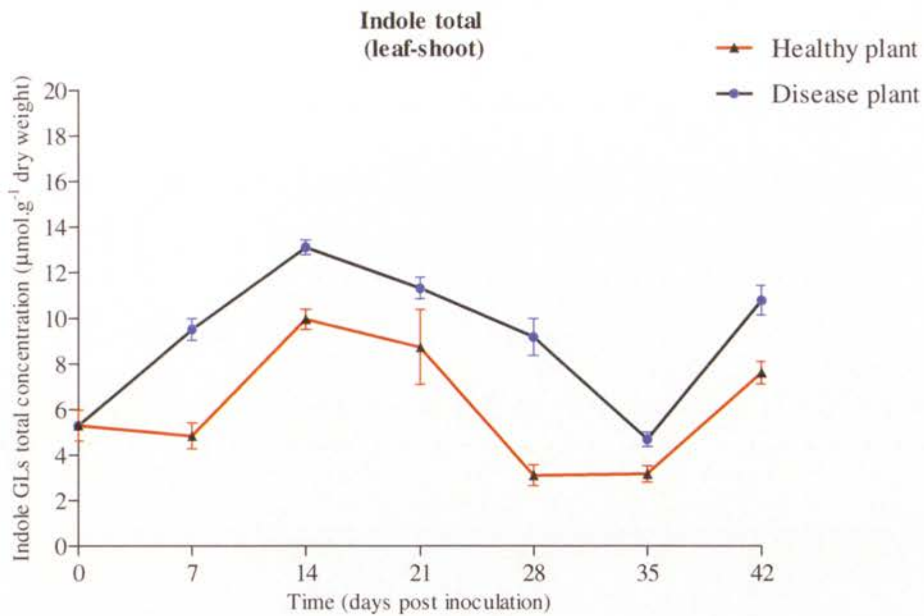


Figure 4.26. Total indole glucosinolate content in *Brassica oleracea* (broccoli) aerial tissues (leaves & shoots) of *P. brassicae* inoculated (diseased) and uninoculated (healthy) plant (0 to 42 days post inoculation Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

4.6 Discussion: Levels of individual and total glucosinolates in roots and aerial tissues of *Plasmodiophora brassicae* inoculated and uninoculated plants

Glucosinolates are believed to play a role in the host pathogen interaction. There is evidence for the association of glucosinolates and their breakdown products in resistance to fungal pathogens and insects (Mithen 1992; Ludwig-Müller 1999a & 1999b; Rangkaddilok *et al.* 2002). In this study, the main objective was to monitor changes in particular glucosinolates and their total levels in a susceptible variety of broccoli (*B. oleracea* ssp. *italica* cv. Marathon) following *Plasmodiophora brassicae* infection.

Glucosinolate levels in both the roots and aerial tissues were strongly influenced, although in different ways, by *P. brassicae* infection. Aliphatic glucosinolates including glucoiberin, progoitrin, glucoraphanin and gluconapin were present in both root and aerial tissues. Individual glucosinolate levels showed different responses to symptom development.

Results from root tissue analysis clearly indicate that during symptom development of clubroot disease, glucoiberin, gluconapin levels increase more than progoitrin. Glucoraphanin is the dominant aliphatic glucosinolate produced by broccoli seedlings. During any of the infection phases there is a limited association with infection and glucoraphanin accumulation. The highest level of glucoraphanin at 28 DPI was 8.95 µmole/g DW in diseased plants, not significantly different to healthy plant roots. A similar concentration of glucoraphanin in healthy roots was reported in broccoli cv. Marathon by Kushad *et al.* (1999).

Our results showed that *Plasmodiophora brassicae* infection induced nearly 4 times higher total indole glucosinolate (61.22 μmole/g DW at 28DPI) accumulation than total aliphatic glucosinolate (16.27 μmole/g DW at 28 DPI). This suggests that during gall formation, indole glucosinolate respond more dramatically than aliphatic glucosinolates.

This study monitored indole glucosinolates including 4-hydroxy glucobrassicin, glucobrassicin and neoglucobrassicin. Glucobrassicin levels have a strong correlation with following the early secondary infection (14 DPI) to characteristic gall forming phase (matured galls) (42 DPI). At every time point glucobrassicin levels were higher in inoculated plants, and the highest levels (41.22 μmole/g DW) were identified during symptom development phase of clubroot disease.

Butcher *et al.* (1974) proposed that indole glucosinolates are converted by the fungus to indole acidic acid (IAA) during clubroot formation. The outcome of this study is supported by those findings however, that during primary infection glucobrassicin accumulation significantly decreased compared with healthy root. There were also some conflicting results published in this aspect. Mullin *et al.* (1980) were not able to correlate changes in indole content with clubroot formation in susceptible cabbage seedlings.

Neoglucobrassicin levels increased during primary infection, however, diseased plants produced significantly lower concentrations during gall formation. 4-hydroxy

glucosinolates also showed similar patterns during secondary infection. Initially (0- 7 DPI), it increased and then decreased during symptom development.

Ultimately, total indole production was significantly higher in inoculated plants throughout the disease development process, except at the primary stage of infection when accumulation was lower in diseased root tissues. These findings are similar to the results shown by Ludwig-Müller (1999a). It has been shown that indole glucosinolate content of cabbage seedlings susceptible to *P. brassicae* increased compared to resistant varieties. The work by Ludwig-Müller (1999a) did not clarify the particular indole glucosinolate levels during the infection process, however the concentration of indole glucosinolates was estimated by analysing whole plants rather than root tissues only.

Infection caused a significant reduction of glucoiberin, gluconapin and progoitrin production during symptom development. However, glucoraphanin was significantly enhanced during the later stage of secondary infection compared with uninoculated plants. Interestingly, total aliphatic accumulation in aerial tissues did not differ significantly at any stage of the infection process. There is insufficient information on the role of aliphatic glucosinolates during development of clubroot, as previous studies focused on indole glucosinolates (Agerbirk *et al.* 2008). However, the aliphatic glucosinolates were enhanced in infected roots of Chinese cabbage (Granat), and Osiris showed a very high content of aliphatic glucosinolates in root tissue during the whole infection period of clubroot (Ludwig-Müller *et al.* 1997). Devos *et al.* (2006) showed myrosinase was upregulated at very early time point of infection

whereas Siemens *et al.* (2006) found evidence of down regulation of myrosinase in *Arabidopsis thaliana* clubroot at later time point of gall formation.

This information indicates that the different types of glucosinolates were affected differently during infection. Our results also showed the unequal distribution of aliphatic and indole glucosinolates between aerial and root tissues following infection. Haughn *et al.* (1991) observed that total aliphatic glucosinolate accumulation in *A. thaliana* during gall formation increased slightly. This information is not directly comparable with current findings, because Haughn *et al.* (1991) used different plant species and analytical methods.

Diseased leaves showed significantly higher levels of particular individual indole glucosinolates, such as 4–hydroxy glucobrassicin, glucobrassicin and also neoglucobrassicin, during most of the infection process. As a result, total indole glucosinolates accumulation significantly increased throughout clubroot development. It is not yet known how leaf indole glucosinolates interact with root infection with *P. brassicae.* The only available information is from the investigation by Rostas *et al.* (2003) who monitored cabbage plants infected with *Alternaria brassicae.* A distinct rise of indole glucosinolates, glucobrassicin, neoglucobrassicin and 4-methoxy–glucobrassicin, was observed.

These results support our current understanding that fungal leaf infection may alter glucosinolate levels and more likely increased individual and total indole glucosinolate in aerial parts. However this investigation shows increased indole glucosinolate levels following infection of root tissues. More investigation is required to gain a better understanding the influence of infection of either leaf or root tissue on glucosinolate accumulation in infected or distant leaf and root tissues.

4.7: Results: The effect of phosphonate and Bion on glucosinolate levels in *P. brassicae* inoculated and uninoculated plants

4.7.1 Aliphatic Glucosinolate Production in Broccoli Root Tissue

Glucoiberin production

Combined application of phosphonate and Bion in inoculated plants significantly ($P < 0.01$) increased levels of glucoiberin in broccoli root organs during gall maturation stage (Figure 4.27).

There was no difference in glucoiberin production between *P. brassicae* infected and healthy control plants at transplantation and mature gall formation phase. During symptom development stage, *P. brassicae* infection caused significantly ($P<0.05$) higher levels of glucoiberin production than uninoculated plants. Concentrations were 45% higher in uninoculated plants (1.17 □mole/gDW) compared to inoculated plants (0 .65 □mole/gDW) (Figure 4.27).

There was no significant difference in glucoiberin production among the chemical treatments at the time of transplanting. Glucoiberin production in chemically treated inoculated and uninoculated plant gradually decreased over time, varying from 3.5 to 2.75, 2.0 to 2.5 and 0.6 to 1.8 µmole/gDW at 0, 21 and 42 dpi, respectively.

Progoitrin Production

Combined application of phosphonate and Bion to infected plants resulted in a significantly ($P < 0.05$) higher production of progoitrin at gall maturation stage, which was double that of phosphonate treated inoculated plant (Figure 4.28). There was no significant difference between the levels of progoitrin in *P. brassicae* inoculated and uninoculated plants any stage of the pathogen life cycle.

At transplanting both inoculated and uninoculated plants had not produced progoitrin, whereas phosphonate and Bion treated plants produced progoitrin, and there were no significant differences among the treatments. No progoitrin could be detected in phosphonate treated and untreated plants during the symptom development phase. Phosphonate application to inoculated plants resulted in progoitrin production (0.26 mole/g DW) however, no progoitrin could be detected in phosphonate treated uninoculated plant at the stage of gall maturation (Figure 4.28).

Glucoraphanin production

There was no significant difference in glucoraphanin production in root tissues among the chemical treatments in either inoculated or uninoculated plants at early, mid and later disease development phase, however chemically treated plants contained significantly ($P<0.001$) a higher (4 times) amount of glucoraphanin than inoculated and non-inoculated plants at seedling transplantation.

P. brassicae infection had no effect on levels of glucoraphanin in roots at any stage of the infection process compared with uninoculated healthy plants.

Glucoraphanin production in chemical treated inoculated and uninoculated plant was enhanced 1.5 fold compared to non-chemical treated plants during symptom development phase.

When phosphonate was applied separately or in combination with Bion to uninoculated plants, glucoraphanin production was significantly reduced ($P<0.001$) compared with both infected and healthy plants only at 42 DPI (Figure 4.29).

Gluconapin

At any phase of the life cycle, infection with *P. brassicae* did not result in any significant changes on the content of gluconapin in broccoli roots compared with healthy plants. Phosphonate and the combination of phosphonate and Bion treatments resulted in greater gluconapin production during early stages of seedling growth (0 to 14 DPI) both in inoculated and uninoculated plants compared with inoculated and uninoculated control plants. There is no significant difference in gluconapin production among chemical treatments in inoculated and uninoculated however Bion treated uninoculated plant had significantly lower ($P<0.01$) gluconapin concentrations than both inoculated and uninoculated control plants (Figure 4.30).

Total aliphatic glucosinolate production in root tissues

Combined application of phosphonate and Bion in uninoculated plant significantly ($P<0.01$) reduced total aliphatic glucosinolate levels than uninoculated control plant (Figure 4.31). There was no difference in total aliphatic glucosinolate levels in the roots of inoculated and uninoculated broccoli plants at any stages of disease cycle. During early stages of seedling growth/transplanting time (0 DPI), phosphonate, Bion and their combination significantly increased production of aliphatic glucosinolates, which was more than 3 fold higher than chemical untreated control plants. There was no significant difference in total aliphatic production in broccoli root among chemical application or diseased or healthy plants during clubroot symptom and characteristic gall formation stages (Figure 4.31).

4.7.2 Indole glucosinolate levels in broccoli root

4-hydroxy glucobrassicin production

There was no significant difference in 4-hydroxy glucobrassicin production in broccoli root between *P. brassicae* infected and healthy plant during transplanting time and symptom development stage of clubroot. There was a significant difference ($P<0.01$) between the 4-hydroxyglucobrassicin content of uninoculated and inoculated control plants by 42 DPI, in which, concentrations were 42% higher in inoculated control plants (4.05 □mol/g DW) compared with healthy control plants (2.85 □mol/g DW) (Figure 4.32).

Significantly lower concentrations of 4-hydroxy glucobrassicin accumulated when phosphonate, Bion and their combination were applied to uninoculated plants Phosphonate, Bion and their combined applications in inoculated plants significantly ($p<0.001$) increased 4-hydroxy glucobrassicin production (Figure 4.32).

Glucobrassicin Production

At the time of transplanting there were no significant differences identified in root glucobrassicin content among the treatments. Glucobrassicin content had increased in all treatments during symptom development stages however, between 21 and 42 days, the content of glucobrassicin in some treatments increased while in others it decreased (Figure 4.33).

Bion and phosphonate application in uninoculated plants significantly ($p<0.001$) reduced glucobrassicin production, compared to uninoculated control plants during characteristic gall formation stage. Bion and combined application of phosphonate and Bion in inoculated plants significantly reduced glucobrassicin levels ($P<0.001$)

compared with inoculated and uninoculated controls at 42 DPI. Phosphonate application in uninoculated plant significantly reduced ($P<0.01$) glucobrassicin levels, while Bion and phosphonate-Bion combined application had no effect and remained unchanged compared to uninoculated control plants (Figure 4.33).

Neoglucobrassicin Production

Neoglucobrassicin content in roots at the time of transplanting was not equal in all treatments. The concentration of neoglucobrassicin due to combined treatment of phosphonate and Bion both in inoculated and uninoculated plants was absent at transplanting time (Figure 4.34).

Phosphonate, Bion or their combined application in inoculated plants suppressed the production of neoglucobrassicin in plant roots during symptom development. However, phosphonate application in uninoculated plants significantly ($P<0.05$) enhanced neoglucobrassicin accumulation compared to healthy control plants. (Figure 4.34). Phosphonate application to inoculated plants significantly reduced neoglucobrassicin production while Bion and their combined application in inoculated plants had no effect and remained unchanged compared to inoculated control plant during matured gall formation stage. However phosphonate ($P<0.001$) Bion ($P<0.01$) and their combined ($P<0.05$) application in uninoculated plant significantly increased neoglucobrassicin production compared to uninoculated control plant during gall formation stage (Figure 4.34).

Total indole glucosinolate production in roots

Total indole glucosinolate production in roots at the time of inoculation was very low in all treatments and there were no significant differences among chemical treated and inoculated control (Figure 4.35).

Level increased by at least 5 times at symptom development and mature gall formation phases of clubroot disease, however, phosphonate and Bion application in inoculated plant significantly ($P<0.001$) suppressed the level of total indole glucosinolate but their combined treatment did not change total indole glucosinolate content compared to inoculated but untreated control plants.

Phosphonate application in uninoculated plants significantly ($P<0.001$) increased the accumulation of total indole glucosinolates. Bion or combined application had no effect compared to uninoculated control plant during gall forming stage (Figure 4.35).

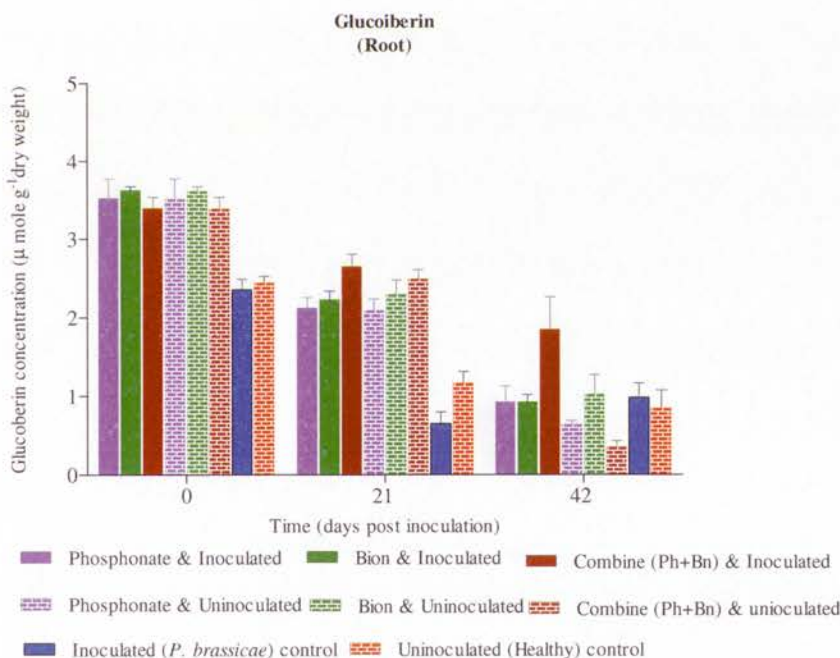


Figure 4.27. The effect of phosphonate and Bion on glucoiberin content of *Brassica oleracea* (Broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

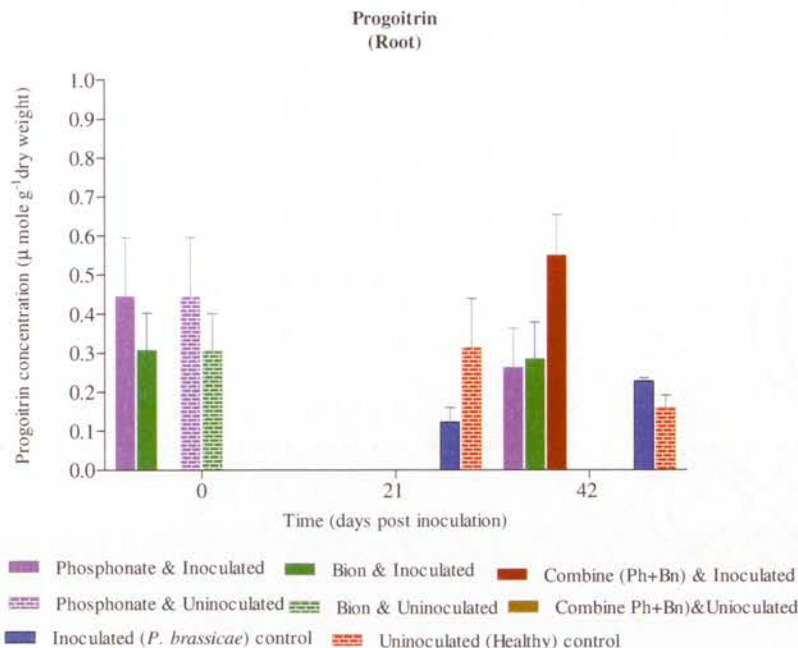


Figure 4.28. The effect of phosphonate and Bion on progoitrin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

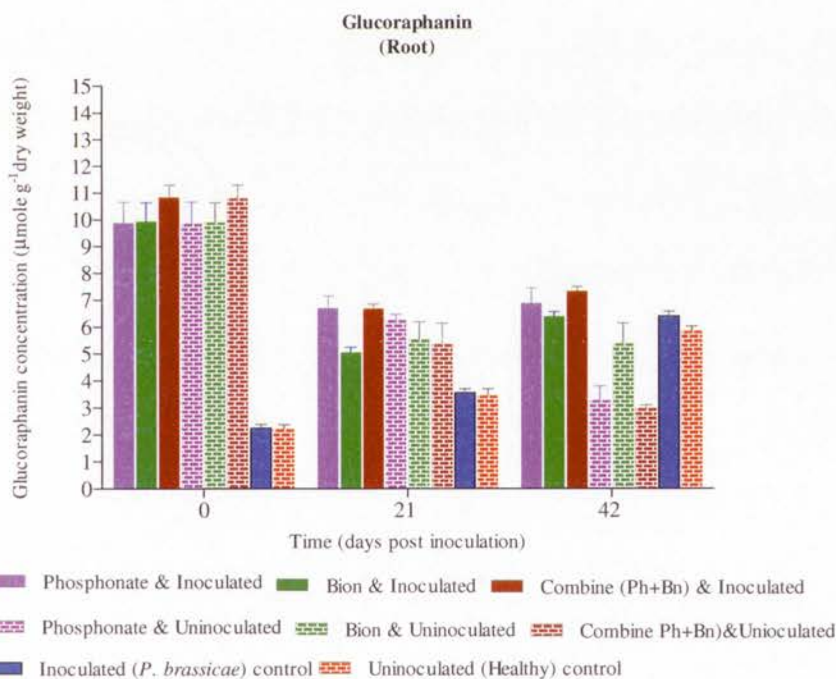


Figure 4.29. The effect of phosphonate and Bion on glucoraphanin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

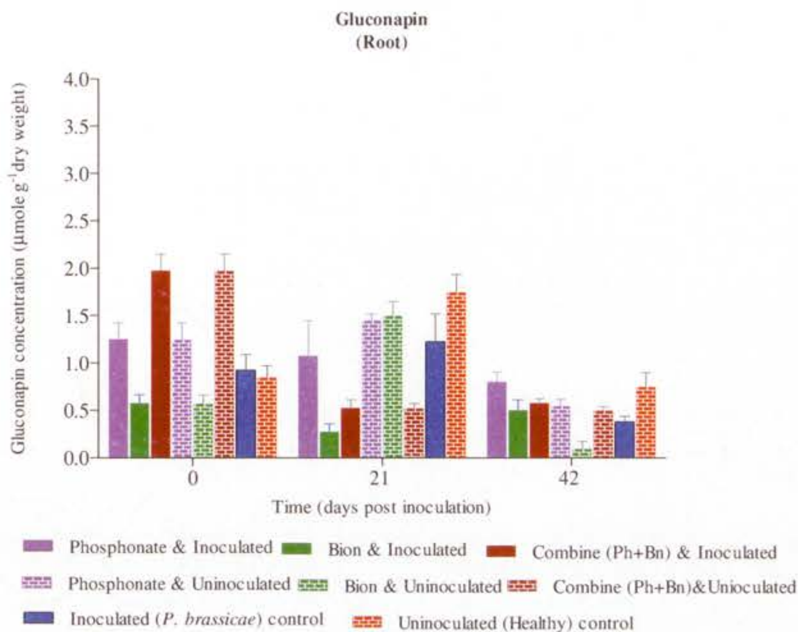


Figure 4.30. The effect of phosphonate and Bion on gluconapin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

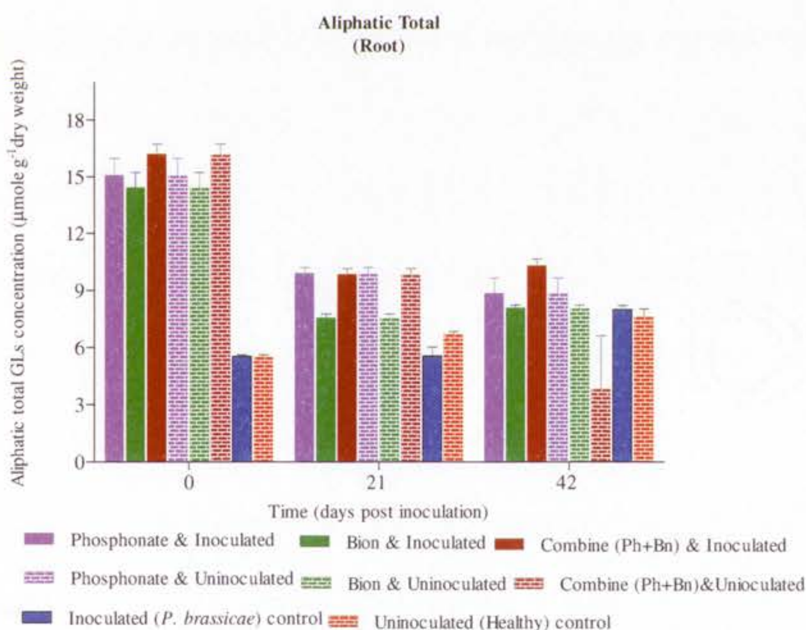


Figure 4.31. The effect of phosphonate and Bion on total aliphatic glucosinolates content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

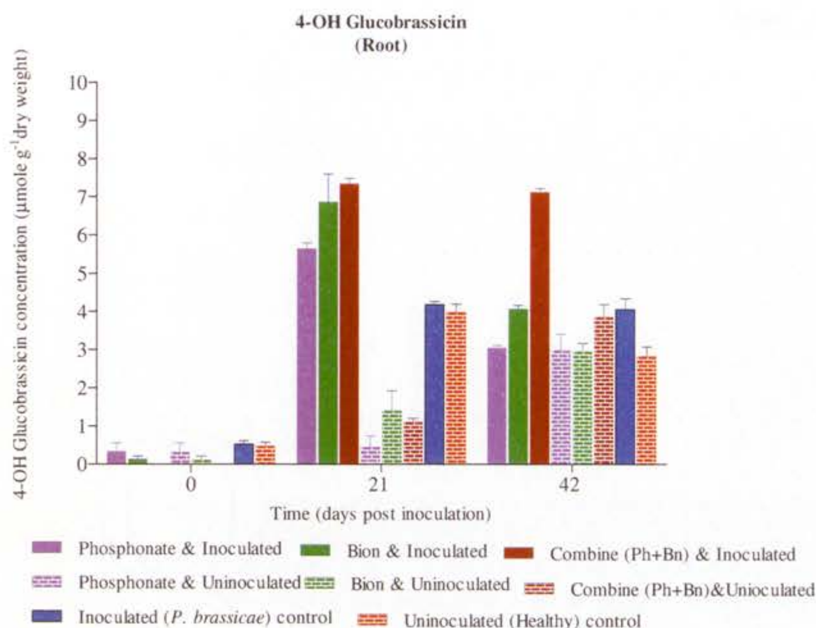


Figure 4.32. The effect of phosphonate and Bion on 4-hydroxy glucobrassicin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

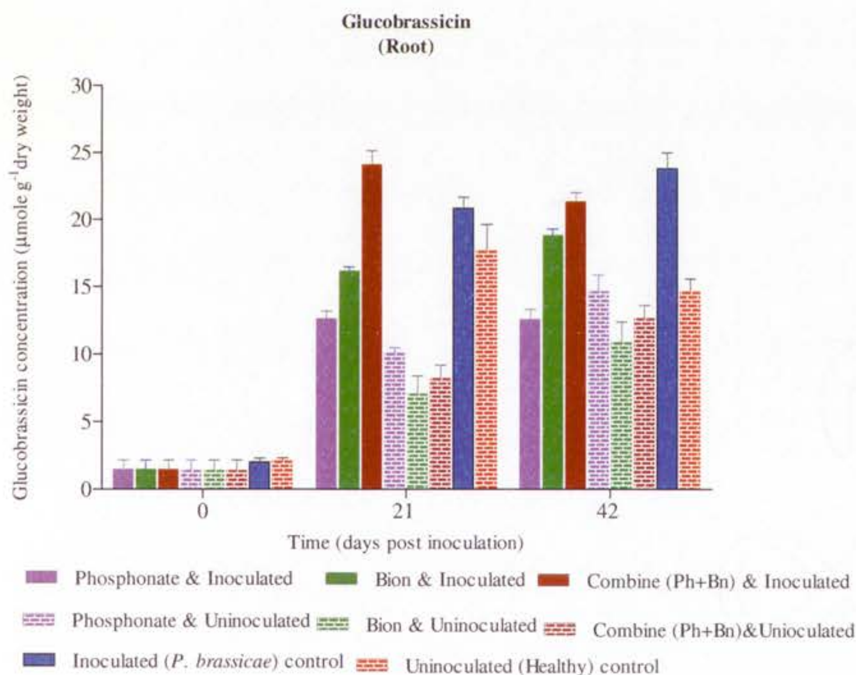


Figure 4.33. The effect of phosphonate and Bion on glucobrassicin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

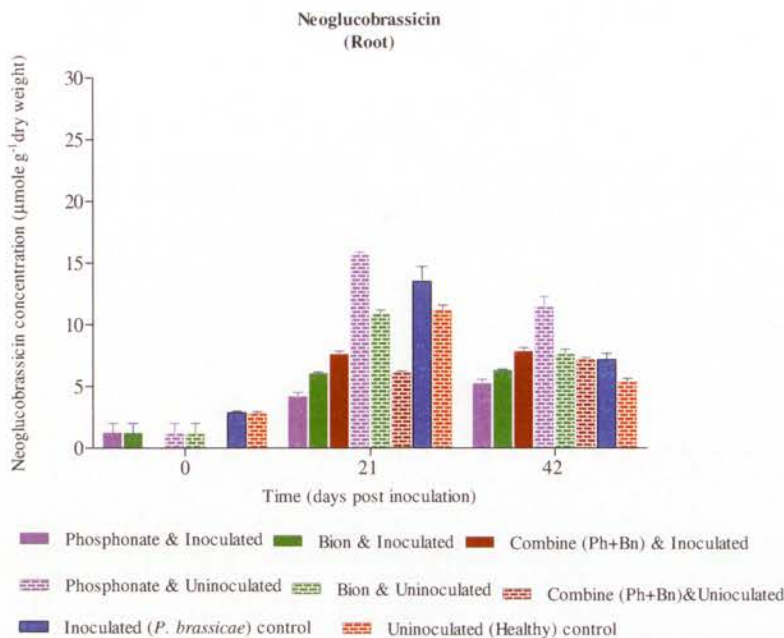


Figure 4.34. The effect of phosphonate and Bion on neoglucobrassicin content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

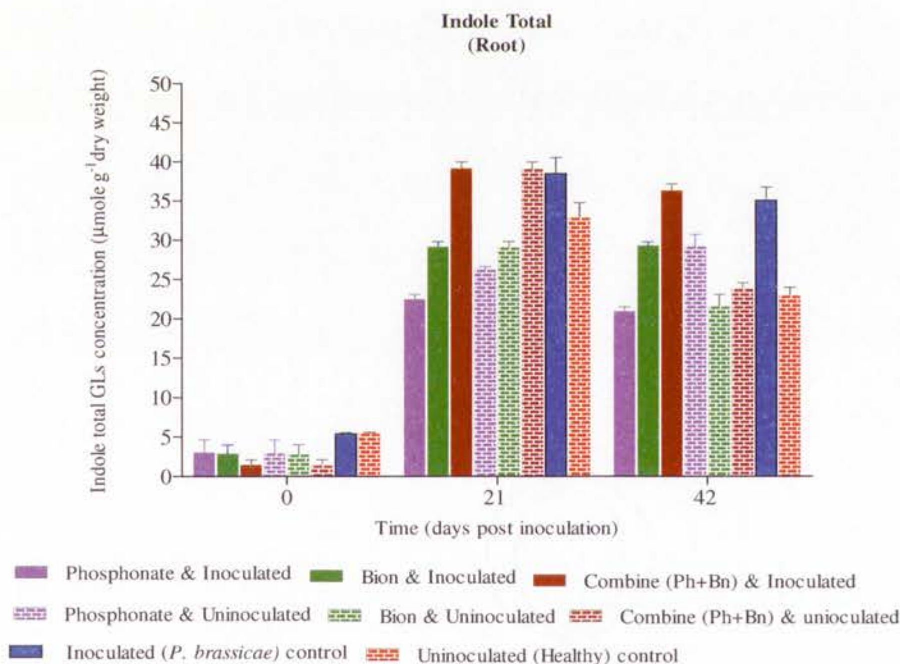


Figure 4.35. The effect of phosphonate and Bion on total indole glucosinolate content of *Brassica oleracea* (broccoli) root tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

4.7.3 Aliphatic Glucosinolate Production in Aerial Tissue of Broccoli

Glucoiberin production

Plasmodiophora brassicae infection in broccoli seedlings had no significant effect on glucoiberin production in aerial tissue of broccoli during gall formation stage, but infection caused a significant reduction ($P<0.001$) of glucoiberin production in broccoli aerial tissue compared to healthy control plants during symptom development stage.

Phosphonate, Bion and their combined application in both inoculated and uninoculated plant suppressed glucoiberin production at all stages of disease development except 21 DPI, however chemical application in inoculated plants gave significantly ($P<0.001$) higher levels of glucoiberin compared to chemical treated uninoculated plants during mature gall formation (Figure 4.36).

Progoitrin production

Plasmodiophora brassicae infection caused a significant reduction ($P<0.001$) of progoitrin during symptom development stage (21 DPI), which was a two-fold decrease compared to healthy controls. There was no difference in glucoiberin content between infected and healthy plant at later stages of disease development (42 DPI).

Phosphonate, Bion and their combined application in both inoculated and inoculated plants suppressed levels of progoitrin in aerial tissues at early and later stages of disease development however chemical treated inoculated and uninoculated plants gave lower amounts of progoitrin than untreated uninoculated control plants (Figure 4.37).

Glucoraphanin production

The concentration of glucoraphanin did not change in response to infection with *P. brassicae* at any stage of disease compared to uninoculated healthy plants. Glucoraphanin production in broccoli leaves and shoots were three times higher in inoculated and uninoculated plants at transplanting time compared to symptom development and gall formation stages.

Throughout disease development, combined application of phosphonate and Bion in both inoculated and uninoculated plants gave lower amounts of glucoraphanin compared to single application of phosphonate and Bion (Figure 4.38).

Gluconapin production

P. brassicae infection had no significant effect on gluconapin production in broccoli aerial parts at the gall formation stage (42 DPI). At symptom development, gluconapin production decreased two-fold in infected plants compared to healthy plants. The amount of gluconapin was higher at the transplant stage than both symptom development and gall formation stages.

Single applications of phosphonate or Bion either in inoculated or uninoculated significantly increased the levels of gluconapin. However their combined application did not enhance the gluconapin accumulation and concentrations of gluconapin remained the same in inoculated and healthy control plant at transplanting time (Figure 4.39).

Total aliphatic glucosinolate

Considering all treatments, contents of total aliphatic glucosinolate were significantly higher ($P<0.001$) at the time of seedling transplantation compared to later symptom (21 DPI) and gall formation (42 DPI) stages of disease development. There were no significant differences in total aliphatic glucosinolate accumulation in aerial tissues from inoculated plants compared with healthy plants at any stage of the disease cycle. Phosphonate and Bion application in either inoculated or uninoculated plants reduced aliphatic glucosinolates accumulation 1.5 to 2 fold at the symptom and gall producing stage of clubroot disease in broccoli (Figure 4.40). However, combined application of Phosphonate and Bion significantly reduced the total aliphatic glucosinolate content compared with application of phosphonate ($P<0.001$) and Bion ($P<0.01$) alone at the time of seedling transplanting (Figure 4.40).

4.7.4 Indole glucosinolate production in broccoli aerial tissues

4-hydroxy glucobrassicin production

Chemical treatment (phosphonate, Bion or their combination) in both inoculated and uninoculated plant completely suppressed the accumulation of 4-hydroxy glucobrassicin during mature gall forming stage of disease development. At the symptom development stage, phosphonate application in uninoculated plants significantly increased ($P<0.001$) 4-hydroxy glucobrassicin production (1.77 μmole/g DW) compared to phosphonate treated inoculated plant (0.45 μmole/gDW). At early stages of disease development, no significant differences were observed among chemical treatments and concentrations varied between 0.25 to 0.45μmole/g DW among all treatments including control plants (Figure 4.41).

There was a significant difference ($P<0.001$) between the content of 4-hydroxy glucobrassicin in *P. brassicae* infected and healthy plants at the mature gall formation stage. Concentrations were nine fold higher in infected plants (2.22 μmole/gDW) compared with healthy plants (0.25 μmole/g DW). During the symptom development stage, infection caused seven times higher 4-hydroxyglucosinolate production compared to healthy plants.

Glucobrassicin production

The glucobrassicin content in infected and healthy control plants was not different at both symptom development and gall formation stage. At every stage of the pathogen life cycle, chemical treatment of both inoculated and uninoculated root reduced the levels of glucobrassicin. No significant differences in glucobrassicin concentration were detected among the chemical treatments either in inoculated or uninoculated seedlings during early stage of the pathogen life cycle however, large variations of glucobrassicin production were observed among the chemical treated plants during symptom development and gall forming stages of the pathogen life cycle. Phosphonate and Bion in inoculated plants and Bion in uninoculated plants had no significant differences on levels of glucobrassicin at both symptom and severe gall forming stages, whereas combined application of phosphonate and Bion significantly ($P<0.01$) reduced glucobrassicin accumulation in leaves and shoots of broccoli seedling at same or later stages of pathogen infection (Figure 4.42).

Neoglucobrassicin production

Neoglucobrassicin production in above ground organs was not detected in chemical treated inoculated and uninoculated plants at 0 DPI and also in Bion treated

inoculated plant at 21 DPI, whereas *P. brassicae* infection significantly (P<0.001) enhanced neoglucobrassicin accumulation (1.65 μmole/g DW) at symptom development stage (21 DPI). Neoglucobrassicin content was low during initial infection stages (0.3 μmole/g DW) but increased four-fold in infected plant (2.0 μmole/g DW) at later stages of infection (42 DPI) which was significantly (P<0.05) lower compared to healthy plants (1.3 μmole/g DW).

Phosphonate and phosphonate-Bion combined application to inoculated plant significantly (P<0.01) increased neoglucobrassicin levels compared with untreated inoculated plants during matured gall forming stage (42 DPI). Chemically treated uninoculated plant had significantly (P<0.05) less neoglucobrassicin production in above ground organ compared with both non-chemically treated plants during gall formation stages (Figure 4.43).

Total Indole glucosinolate production

Total indole glucosinolate levels in leaves and shoots were significantly higher in untreated *P. brassicae* infected control plants than in healthy plants at both symptom (P<0.05) and mature gall forming stage (P<0.01).

Initially, indole levels were low and gradually increased during the infection process. Chemical activator application in both inoculated and uninoculated plant suppressed the accumulation of total indole in above ground organs compared to non treated diseased and healthy control plants during symptom and gall formation stages of clubroot in broccoli (Figure 4.44).

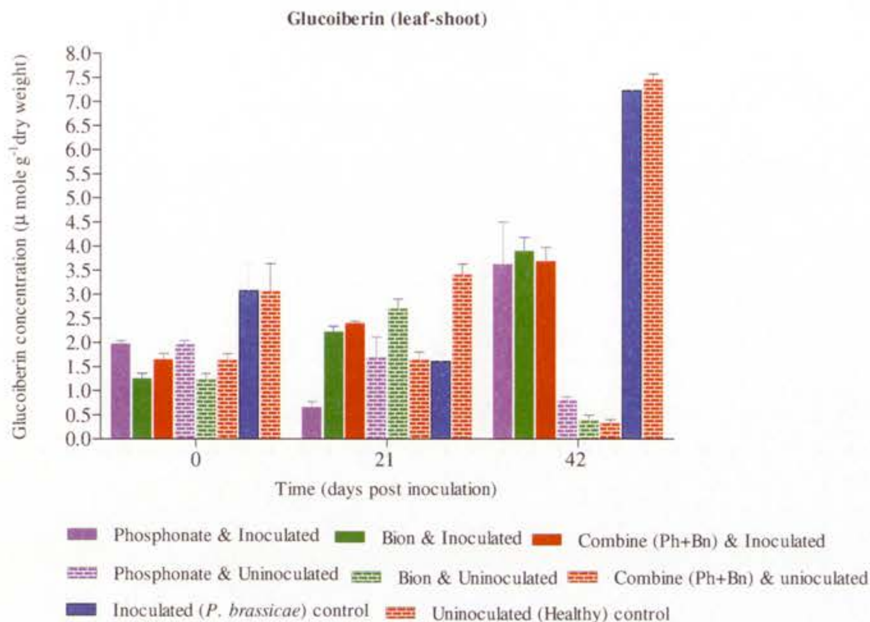


Figure 4.36. The effect of phosphonate and Bion on glucoiberin content of *Brassica oleracea* aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

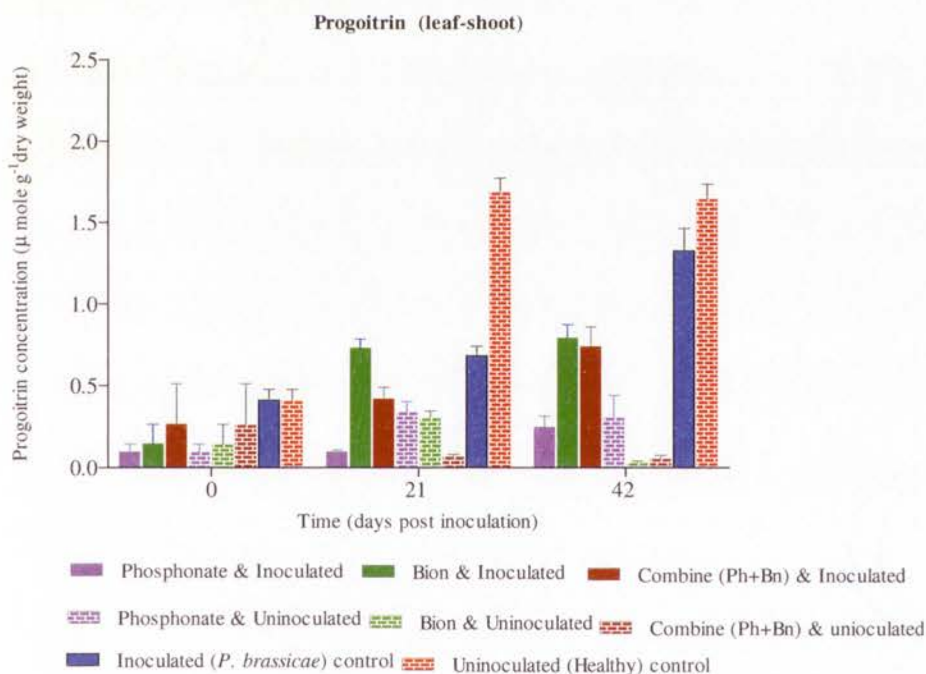


Figure 4.37. The effect of phosphonate and Bion on progoitrin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

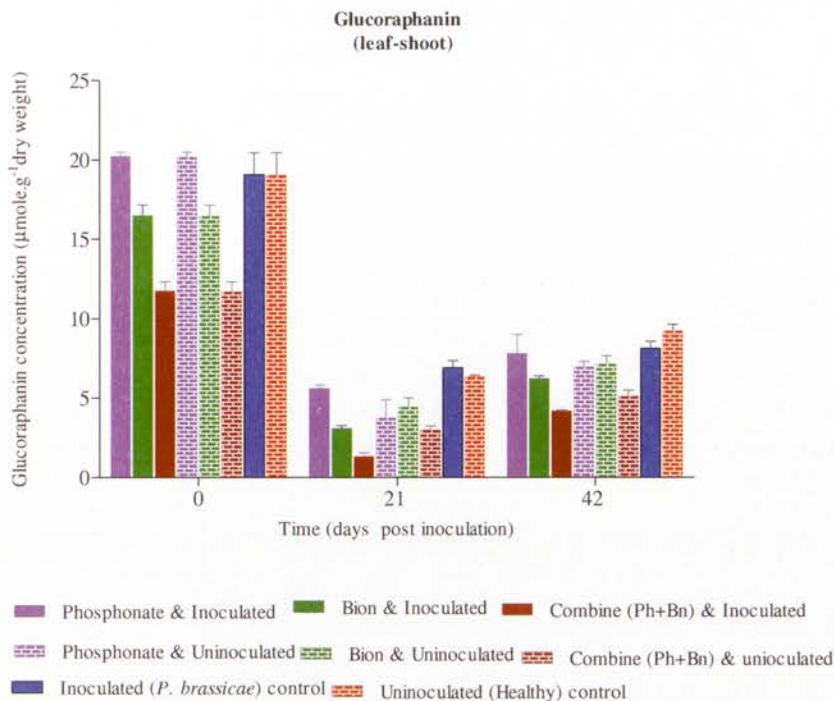


Figure 4.38. The effect of phosphonate and Bion on glucoraphanin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

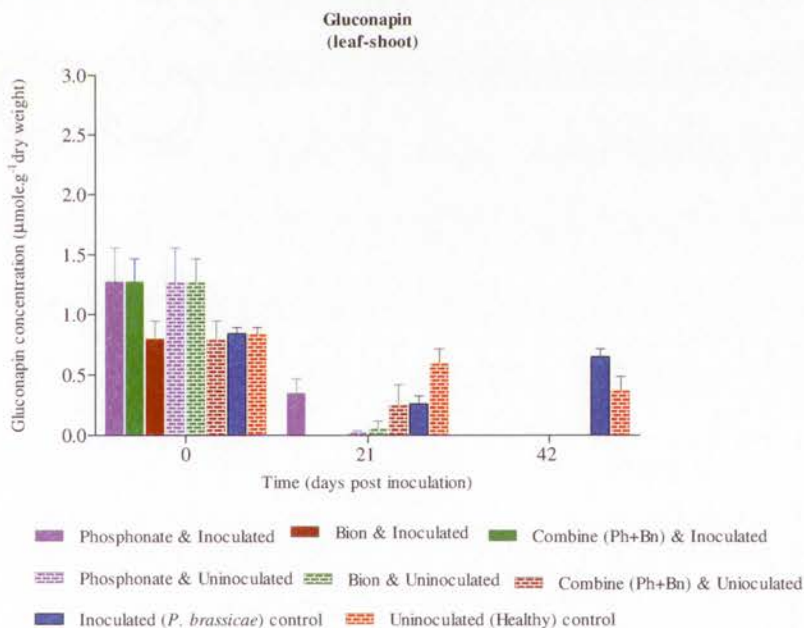


Figure 4.39. The effect of phosphonate and Bion on gluconapin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

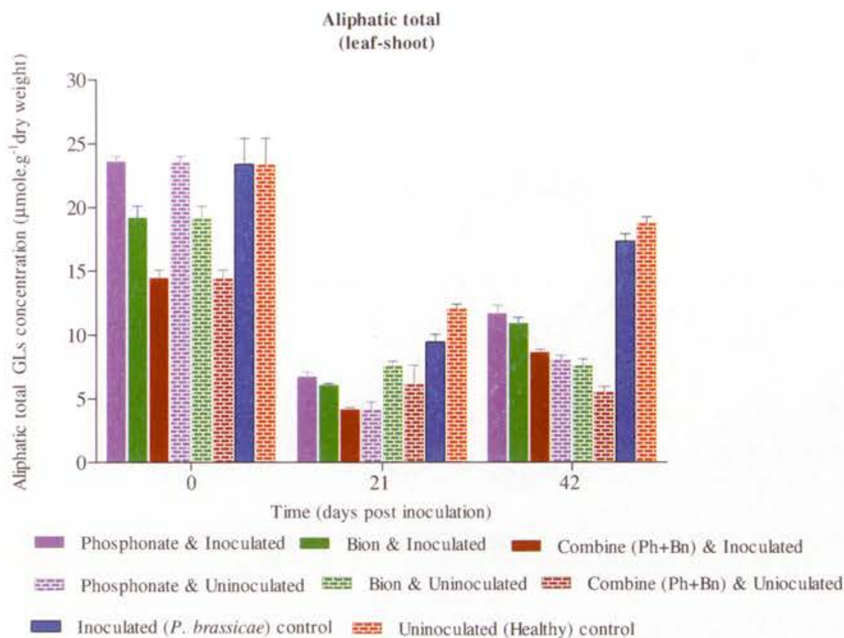


Figure 4.40. The effect of phosphonate and Bion on total aliphatic glucosinolate content of *Brassica oleracea* (broccoli) in aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

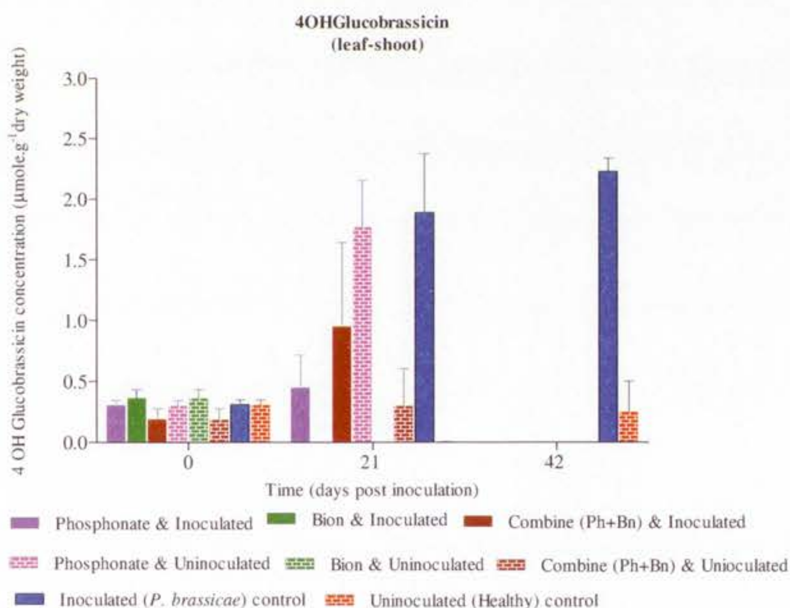


Figure 4.41. The effect of phosphonate and Bion on 4 OH glucobrassicin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

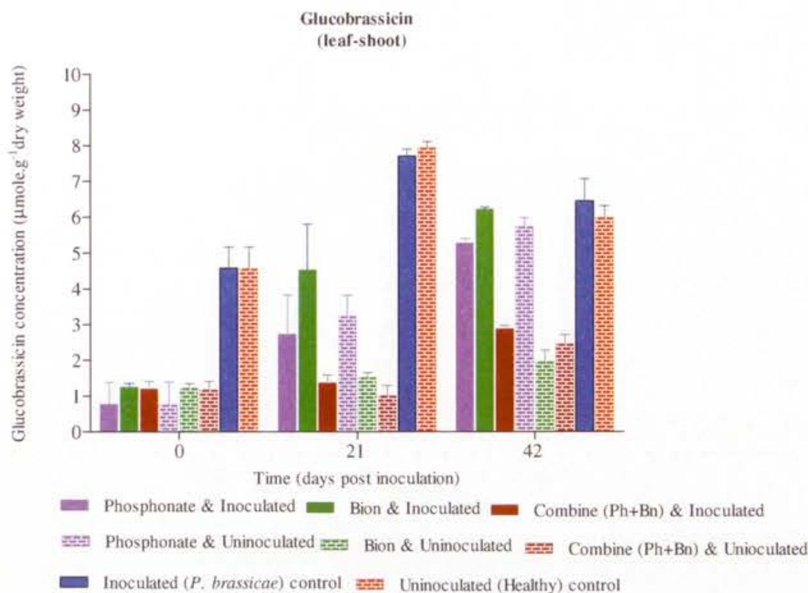


Figure 4.42. The effect of phosphonate and Bion on glucobrassicin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

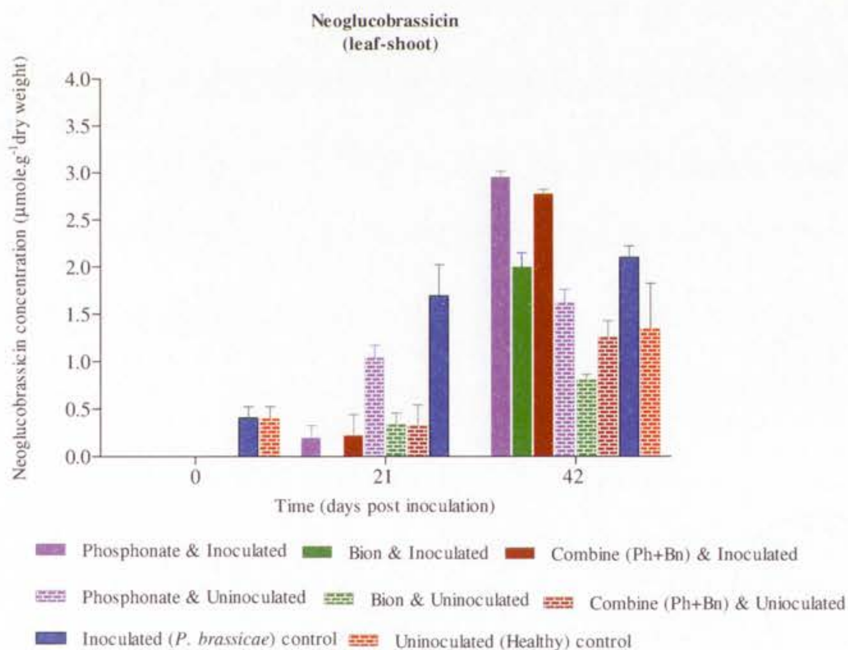


Figure 4.43. The effect of phosphonate and Bion on neoglucobrassicin content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

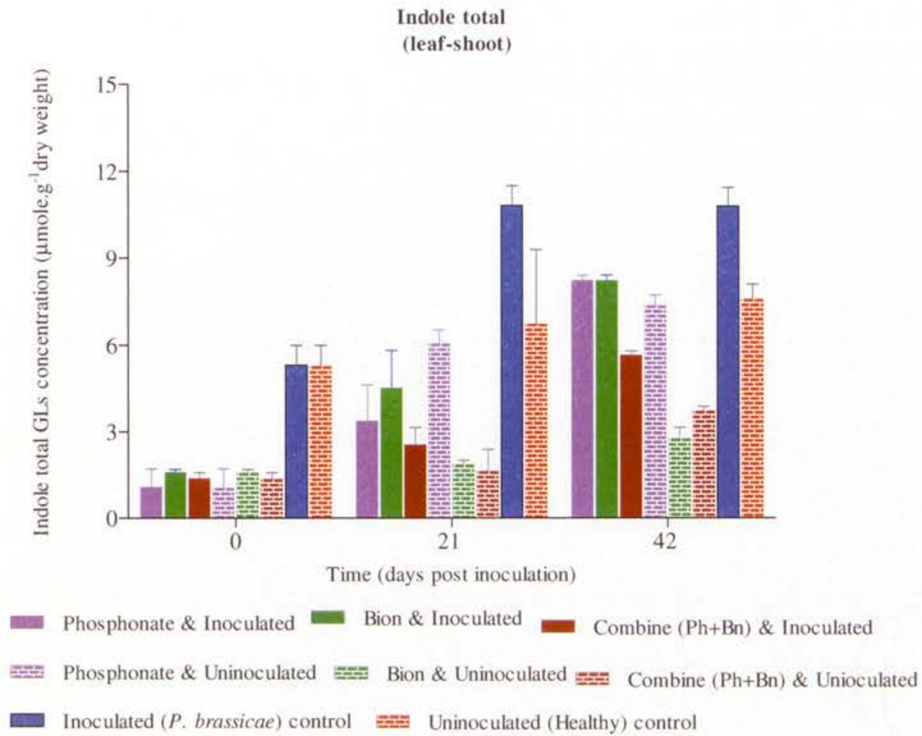


Figure 4.44. The effect of phosphonate and Bion on total indole glucosinolate content of *Brassica oleracea* (broccoli) aerial tissues following *Plasmodiophora brassicae* infection (0 to 42 days post inoculation). Data points represent the mean of four experimental replicates and error bars are the standard errors (±SE).

4.8 Discussion: The effect of phosphonate and Bion on glucosinolate levels following *P. brassicae* inoculated and uninoculated plant

This is the first report analysing glucosinolate levels in Brassica inoculation with *Plasmodiophora brassicae* prior to foliar sprays of phosphonate or Bion. This study provides the first direct evidence that these plant defence chemicals can strongly affect aliphatic and indole glucosinolate levels both in aerial and root tissues during root infection by the obligate clubroot pathogen, *Plasmodiophora brassicae*. This study focused on the analysis of aerial and root tissues at the initial infection (0-7 DPI), clubroot symptom development stage (7-28 DPI) and finally the later stage of this infection process (42 DPI, time of characteristic gall formation).

Results at the final stage of clubroot gall maturation (42 dpi) on variations of glucosinolate levels following the application of phosphonate and Bion are summarized (Table 4.1). Results have been displayed to show the changes glucosinolate levels in treated, uninoculated plants and treated in inoculated plants, compared with non treated uninoculated and inoculated control plants respectively. Significant changes in glucosinolate levels occurred at the stage of characteristic gall formation.

Phosphonate or Bion application has a significant systemic impact on total indole root glucosinolate levels in *B. oleracea* cv. Marathon. No significant changes in root aliphatic glucosinolate levels following single applications of phosphonate or Bion in either pathogen inoculated or uninoculated plants.

Table 4.1. Summary of the variations of individual aliphatic and indole and total aliphatic and indole glucosinolate following *P. brassicae* inoculation and chemical treatment in aerial and root tissue of *B. oleracea* spp italica cv. Marathon (Broccoli) during matured gall formation (42 DPI)

Treatments	Individual and total aliphatic and indole glucosinolate profile								
	GB	PN	GR	GN	Aliphatic Total	4OH GBS	GBS	NGBS	Indole Total
Root tissue	Compared with uninoculated, untreated(healthy) plant								
Unino & Ph	ns	nd	*** □	ns	ns	ns	ns	***□	***□
Unino & Bion	ns	nd	ns	**□	ns	ns	**□	**□	ns
Unino & Ph+Bn	ns	nd	*** □	ns	**□	*□	ns	*□	ns
Inoculated	Compared with inoculated, untreated (diseased) plant								
Ino & Ph	ns	ns	ns	ns	ns	*□	*** □	*□	***□
Ino & Bn	ns	ns	ns	ns	ns	ns	*** □	ns	***□
Ino & Ph+Bn	**□	**□	ns	ns	**□	*** □	ns	ns	ns
Aerial tissue	Compared with uninoculated, untreated(healthy) plant								
Unino & Ph	*** □	*** □	*□	***□ nd	***□	ns/nd	ns	ns	ns
Unino & Bion	*** □	*** □	*□	***□ nd	***□	ns/nd	*** □	ns	***□
Unino & Ph+Bn	*** □	*** □	*** □	***□nd	***□	ns/nd	*** □	ns	***□
Inoculated	Compared with inoculated, untreated (diseased) plant								
Ino & Ph	*** □	*** □	ns	***□ nd	***□	*** □nd	ns	***□	ns
Ino & Bn	*** □	*** □	ns	***□ nd	***□	*** □nd	ns	ns	ns
Ino & Ph+Bn	*** □	*** □	*** □	***□nd	***□	*** □nd	*** □	**□	***□

GB; Glucoiberin, PN; Progoitrin, GR; Glucoraphanin, GN; Gluconapin; 4OHGBS; 4 OH Glucobrassicin, GBS; Glucobrassicin, NGB; Neoglucobrassicin, Ph; potassium phosphonate, Bn; Bion, Nd; Not detected, Ns; not significant at P<0.05, □; Decreased, □; Increased, *; P<0.05, **; P<0.01 and ***; P<0.001.

A single application of phosphonate or Phosphonate + Bion in uninoculated plant significantly decreased the production of glucoraphanin in root tissue while no changes were observed in inoculated plants. A single application of phosphonate or Bion has almost no effect on root total aliphatic glucosinolate levels either in inoculated or uninoculated plants. Interestingly, total aliphatic glucosinolate production was significantly reduced in uninoculated, but then increased in inoculated plants following the combined application of phosphonate and Bion signifying that the aliphatic glucosinolate synthesis pathway shifted following infection. Furthermore, combinations of phosphonate and Bion might regulate accumulation of aliphatic glucosinolate levels in root tissues of broccoli. This indicates that the root pathogen triggers signalling pathways, and may significantly alter food quality by stimulated biosynthesis pathway of particular compounds (van Dam *et al.* 2003).

Phosphonate or Bion application to shoot in inoculated plant significantly ($P<0.001$) reduced indole levels in root tissues compared to inoculated control plants, whereas phosphonate application in uninoculated plants stimulated accumulation rate of indole levels. The application of Bion to uninoculated plants did not change total indole levels however, glucobrassicin and neoglucobrassicin significantly increased and decreased, respectively. This result indicates that *P. brassicae* infection interacting with single application of phosphonate or Bion suppressed the production of indole glucosinolates in root tissue.

The total indole reduction might be correlated to gall formation. There is evidence that indole glucosinolates are implicated in gall formation. Plants with lower indole production have a lower capacity for indole acidic acid synthesis and turnover of

indole glucosinolates (Chong *et al.* 1984; Ludwig-Müller *et al.* 1997). Disease severity score (Figure 4.47) clearly indicates that single applications of phosphonate or Bion significantly reduce gall severity and gall size compared with non-treated inoculated control plants. These results from our observation certainly establish a significant correlation between glucobrassicin reduction and suppression of symptom development. Metabolites of glucobrassicin may be associated with the development of galls (Ockendon and Buczacki 1979; Ludwig-Müller and Cohen 2002).

Ludwig-Müller (1997) showed that after inoculation with *P. brassicae*, symptoms of clubroot in the Chinese cabbage varieties, Granat and Osiris showed higher glucosinolate levels, especially indole glucosinolates. Ludwig Muller also confirmed through scanning electron microscopy that plasmodia of *P. brassicae* were confined in the resistant variety (Parkin), demonstrating that indole glucosinolate levels affect the auxin content and may influence symptom development of clubroot disease. The absence of these compounds does not confer resistance to the plant, but rather prevents club development. Evidence from other literature concerning this point is conflicting. Butcher *et al.* (1976) and Chong *et al.* (1981 & 1984) found correlations between low indole glucosinolates content of the plants and resistance to clubroot, however Mullin *et al.* (1980) did not confirm these results. Previous demonstration by Ludwig-Müller *et al.* (1993) indicated that indole glucosinolates only increased when club formation was observed. Invasion of roots of the susceptible variety Granat in liquid culture did not lead to clubroot symptoms, and the indole glucosinolates content did not increase compared with controls.

The data summarised in Table 4.1 indicate that either individual or total aliphatic glucosinolate production in aerial tissues was significantly ($P<0.001$) lower following both single and combined applications of phosphonate and Bion in inoculated and uninoculated plants. These results suggest that phosphonate and Bion induce a systematic response resulting in a reduction of aliphatic glucosinolate levels. This also indicates that *P. brassicae* infection did not affect aliphatic glucosinolate accumulation in aerial tissues. Information published by Kiddle *et al.* (1994) and Ludwig-Müller (1997) showed that Bion application in did not increase overall glucosinolate levels in shoots of *B. nigra* and *B. campestris.*

The levels of indole glucosinolates in aerial tissue remained unchanged following phosphonate application in uninoculated and inoculated plants. Phosphonate and Bion combined induced significant ($P<0.001$) reduction of total indole levels, but a single application of Bion in uninoculated plant significantly reduced total indole levels. Total indole glucosinolates of inoculated plant roots remained unaffected. This suggests both Bion application and pathogen infection influenced the signalling pathway in *B. oleracea*. This also indicates that a decrease or no change in root glucosinolate synthesis would not necessarily decrease levels in shoots as well, because the above ground and below ground aliphatic and indole profiles are regulated independently (Sang *et al.* 1984; Potter *et al.* 1999).

The significant overall correlation due to phosphonate and Bion application particularly in root tissues in *P. brassicae* infected and uninfected plants throughout the experiment (0 to 42 DPI) indicated that metabolites of glucosinolates may be associated with the progression of gall formation. Phosphonate has been shown to

enhance the plant defence responses, including lignification, phytoalexin accumulation and hypersensitive cell death (Guest 1984; Saindrenan *et al.* 1988). In several plant species, jasmonic acid and salicylic acid (plant signalling hormone) have systemic activity (van Dam *et al.* 2001; Rostas *et al.* 2003). Based on the previous knowledge, it was hypothesised that phosphonate and Bion application against clubroot pathogen could play an important role altering defence compound like glucosinolates and may also varying roots and shoot induced responses. Recent demonstration by Van Dam *et al.* (2003) suggested that SA applications reduced root glucosinolates in root treated plants whereas shoot levels remained similar. This indicates that the physiochemical regulation, clarification of signalling pathways of major glucosinolate profiles in root-shoot tissues of brassicas requires further investigation, including the effects of phosphonate and Bion on both root and shoot organs.

Thus decreased (□) levels of indole glucosinolates reflect suppression of symptom development by phosphonate or Bion.

The full significance of these findings reported here and the effect of phosphonate and Bion on other pathogens in field and glasshouse condition has yet to be assessed. The results also suggested that current ideas regarding the symptom development and relative importance of indole glucosinolate synthesis pattern (MacDanell *et al.* 1988; Ludwig-Müller *et al.* 1999a & 1999b) needs to be revaluated.

4.9 Results: The effect of phosphonate and Bion on clubroot disease severity

In the glasshouse trial, potassium phosphonate, Bion or their combination were sprayed to evaluate their suitability in suppressing clubroot severity. Plants were harvested 42 days post inoculation. Gall size was scored (0 to 5 scale) visually and fresh weight of gall (g) was also compared to determine a suitable management option for clubroot disease of broccoli.

There were significant differences in disease scores of broccoli plants among the chemical treatments 6 weeks post inoculation. Phosphonate application showed significantly lower ($P<0.001$) disease score (2.5) followed by Bion (3.25), combined treatment (3.50) compared with inoculated control treatment (4.75). There was no significant difference between Bion and phosphonate-Bion combined application in suppressing gall formation however, Bion and combined treatment significantly ($P<0.05$) reduced gall weight compared with the inoculated control treatment (Figure 4.45 and 4. 46).

The same trend was seen in fresh gall weights among chemical and non-chemical treated control plant. Fresh weights of galls were affected by the chemical treatments. Highest fresh gall weight (56.8 g) was harvested from the non treated plant which had significantly higher ($P<0.001$) fresh gall weights compared with the rest of the treatments (Figure 4.46). Phosphonate treated plants produced significantly lower fresh gall weight followed by Bion (32.5 g) and the combined treatment (45.8 g) plants. Phosphonate treated plant contained galls at least 3 times smaller than control plants (Figure 4.47).

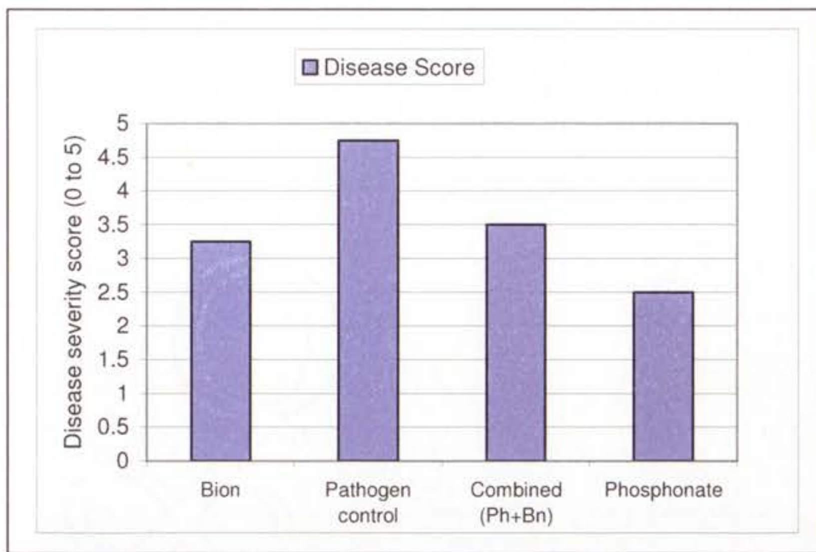


Figure 4.45. The effect of phosphonate and Bion on clubroot disease severity (gall score) 42 day after inoculation. Least significant differences (LSD): 0.832.

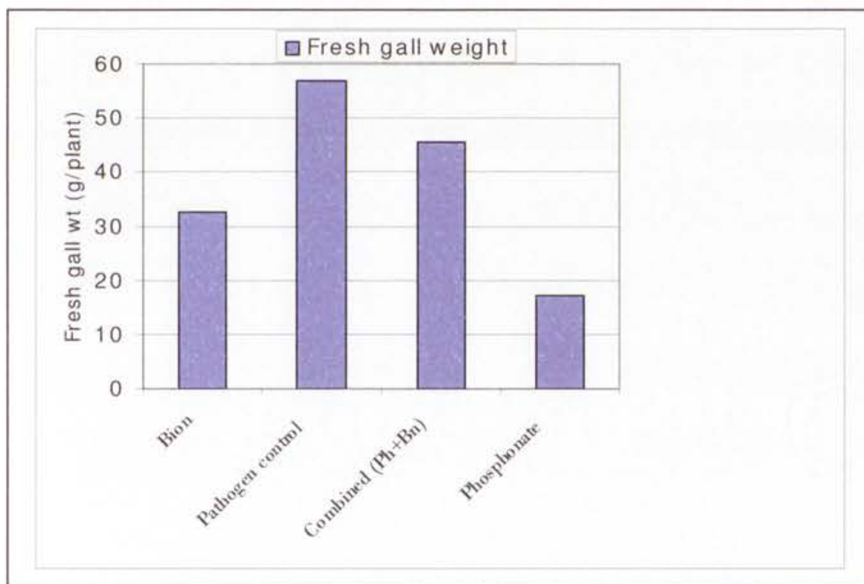


Figure 4.46. The effect of phosphonate and Bion on fresh gall weight following *Plasmodiophora brassicae* harvested at 42 day after inoculation. Least significant differences (LSD): 6.074.

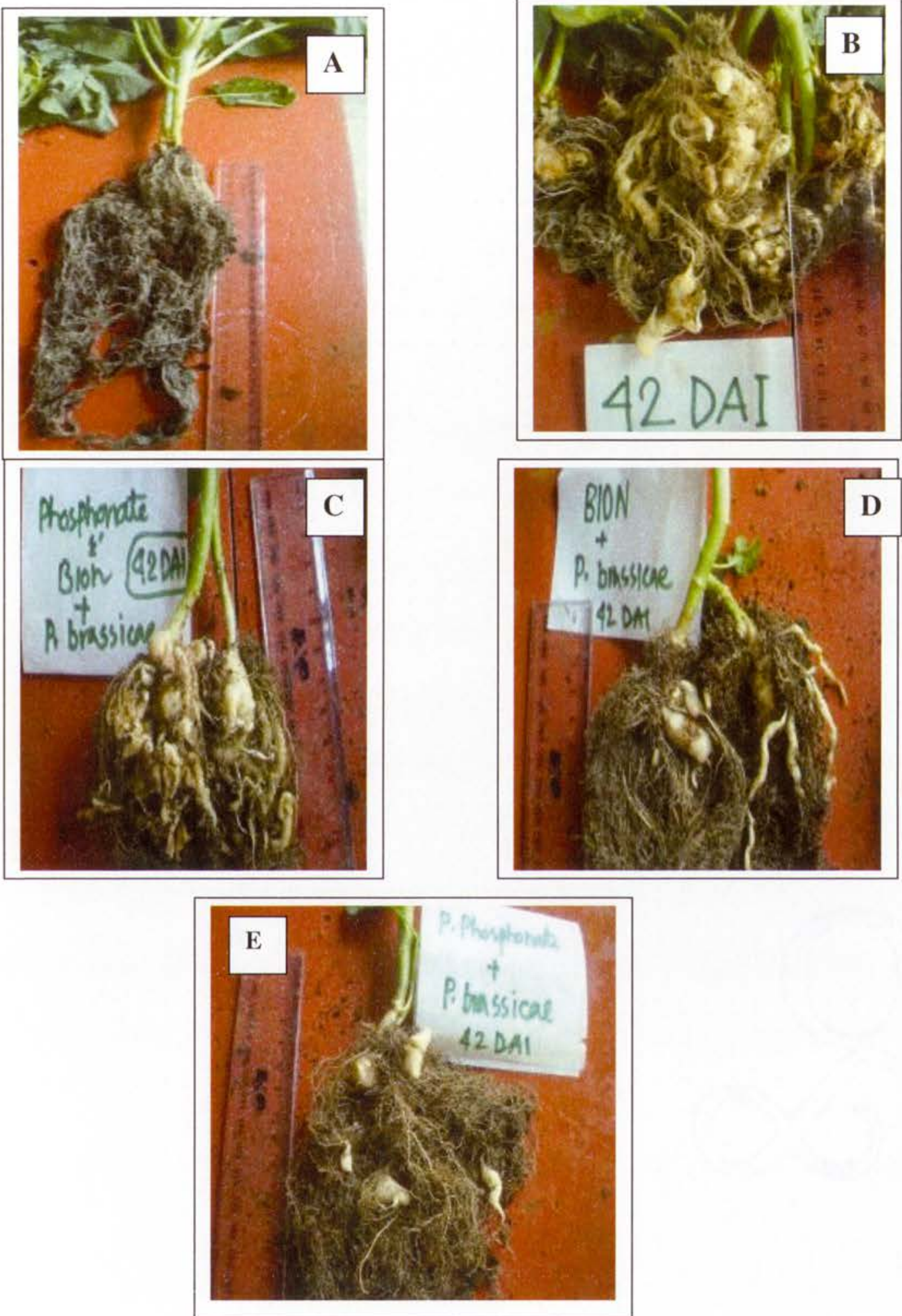


Figure 4.47 The effect of chemicals on clubroot disease severity, A. Untreated uninoculated healthy root B. Untreated inoculated (*P. brassicae*) control root C. Phosphonate & Bion treated root D. Bion treated root E. Phosphonate treated root.

4.10 Discussion

Plants absorb phosphonate through leaves and stems, then show increased defence responses following inoculation (Guest and Bompeix 1990; Grant *et al.* 1990). This may inhibit the formation of infective and reproductive structures. Bion (acibenzolar, 50% a.i. wettable granule formulation) is a non fungicidal compound used to combat plant pathogens by inducing the plant's natural defence mechanisms (Tally *et al.* 1999). This chemical is a functional analogue of salicylic acid shown to accumulate in plants challenged with a pathogen (Friedrich *et al.* 1996). Following accumulation of salicylic acid, plant defence gene(s) are induced (Lawton *et al.* 1996) triggering the production of defence compounds.

In this study chemicals were sprayed on 10 day old seedling leaves 4 days prior to seedling transplanting into the inoculated potting mix. Phosphonate (Agrifos 600) significantly reduced the severity of clubroot of broccoli in glasshouse trials more than Bion or a combination of phosphonate and Bion. Similar results were observed when gall size was measured, indicating that phosphonate is most effective in reducing fresh gall weight compared to other treatments.

Phosphonate is directly toxic to target pathogens but also may protect plants by enhancing the defence response of the treated plant against the invading pathogen (Guest *et al.* 1988; Grant *et al.* 1990; Guest and Bompeix 1990).

A low dose of phosphonate (1g a.i./L of water) was applied because of possible side effects such as leaf burning, and stunting have been documented under phosphate limiting conditions (Carswell *et al.* 1996). Also excessive concentrations can result in

severe phytotoxicity (Pilbeam *et al.* 2000; Hardey *et al.* 2001). Phosphonate treatment reduced gall formation 6 weeks after inoculation, and the average gall score was 2.5 out of 0 to 5 scale, whereas non treated, inoculated control plant had an average gall score of 4.75.

A higher rate of phosphonate application may provide excellent clubroot control however they also may not be economically feasible and may cause phytotoxicity. In this instance, further studies need to be undertaken to optimise the doses and minimise phytotoxicity, both in glasshouse and field trials.

The first report on the efficacy of phosphonate to control clubroot of *B. rapa* var. cinensis, *B. rapa* var. *perkinensis* and *B. oleracea* var. *capitata* in naturally infested muck soils was reported by Abbasi and Lazarovits *at al.* (2006). Their published results support the findings of our glasshouse trial, however they applied phosphonate as a number of soil drenches before and after transplanting. Phosphonate concentration of 0.07 and 0.14% a. i /L. as soil drenching before and after planting significantly reduced clubroot severity under glasshouse condition.

Chapter V. General Discussion

Optimised inoculation procedures for *Plasmodiophora brassicae* on broccoli under greenhouse conditions were developed. Changes in glucosinolate levels in root and shoot organs were demonstrated following natural and artificial infection with *P. brassicae*. Both phosphonate and Bion were shown to alter glucosinolate levels following pathogen infection, and phosphonate was shown to reduce clubroot severity.

Root and aerial tissues from naturally and artificially infected plants were analysed for four aliphatic glucosinolates (glucoiberin, progoitrin, glucoraphanin and gluconapin) and three indole glucosinolates (4-hydroxy glucobrassicin, glucobrassicin and neoglucobrassicin). In aerial tissues, aliphatic glucosinolate levels were unchanged following *P. brassicae* infection. Indole glucosinolate levels were significantly higher in inoculated plants at each sampling time point (7, 14, 21, 28, 35 and 42 DPI) compared to uninoculated plants. Particular glucosinolate levels increased or decreased mostly at symptom development and mature gall forming stages.

In root tissue, aliphatic glucosinolate levels in diseased plants were significantly higher at symptom development phase (28 DPI) while no significant changes were observed between diseased and healthy roots at primary, early secondary infection stage and later mature gall forming stages. Total indole glucosinolate levels declined at the primary stage of infection, but significantly increased during symptom development phase to gall forming stages.

Data from these experiments suggest that aliphatic glucosinolates, both in root and in aerial tissues, are less affected than indole glucosinolates by clubroot infection. Changes

in indole glucosinolate levels following clubroot in relation to clubroot disease development have been reported in previous studies. The concentration and synthesis of auxin and cytokinins are increased in *P. brassicae* infected tissues. While it was demonstrated that the vegetative secondary plasmodia of the pathogen produce cytokinins (Muller and Hilgenberg 1986), the increased amount of IAA might be due to the increased synthesis and turnover of auxin precursors in infected roots (Searle e*t al.* 1982; Rausch *et al.* 1983). The break down of indole glucosinolates, which could lead to the release of relatively large amounts of auxin, is responsible for inducing clubroot symptoms (Butcher *et al.* 1974). This implies that levels of indole glucosinolates do not increase in resistant host. This hypothesis is consistent with the evidence shown by Ludwig-Müller *et al.* (1997) that indole glucosinolate increased during development of the clubroot disease in two susceptible varieties, but did not change in two *Plasmodiophora*-resistant Chinese cabbage varieties.

This study aimed to observe the changes of glucosinolate concentration following *P. brassicae* infection and foliar spray of potassium phosphonate and Bion. Glucosinolate profiles and suppression of gall formation were monitored. The levels of aliphatic glucosinolate in root tissues increased 2.5 times in treated plants compared to untreated plants 4 days after chemical treatment. Glucosinolate accumulation gradually decreased both in inoculated and uninoculated plants over time. During gall formation chemical treatments of either inoculated or uninoculated plants induced no changes in glucosinolate levels compared to untreated inoculated or inoculated plant. This result indicates aliphatic glucosinolate levels are almost unaffected due to *P. brassicae* infection or chemical treatment.

At final stage of gall formation, the accumulation of indole glucosinolates in Bion and phosphonate treated inoculated plant root were suppressed significantly compared to inoculated plants but treated uninoculated plants showed almost similar levels of indole glucosinolates to uninoculated plants.

There is good evidence that pathogen infection stimulated the accumulation of indole glucosinolates but that chemical application inhibited the growth of the pathogen and ultimately resulted in a suppression of indole production. This finding might be correlated with the results from the clubroot gall score and fresh gall size significantly reduced in phosphonate and Bion treated plants. Previous studies show that gall formation is directly or indirectly correlated with the production of indole glucosinolates. In particular, glucobrassicin may play vital role in gall formation. This was also investigated by Butcher *et al.* (1974) who found that plants having little or no indole glucosinolates were resistant to clubroot and developed fewer symptoms. In this study, glucobrassicin levels in phosphonate and Bion treated inoculated plant at 42 DPI dropped nearly 200% and 20% compared to untreated inoculated plants respectively. Similar data was recorded for neoglucobrassicin and 4-hydroxy glucobrassicin. Gall development was suppressed by phosphonate and Bion indicating that these chemicals suppress the pathogen growth and development, simultaneously reducing indole glucosinolate accumulation or as in the case of phosphonate as directly inhibitory to the pathogen.

There might be another possibility that the chemical directly interrupted the biosynthesis of indole glucosinolate in plant root. Because phosphonate application in uninoculated plants slightly increases neoglucobrassicin accumulation resulted significant increase of total indole glucosinolate levels (42 DPI) than untreated uninoculated plants. Bion and

combined application of Bion and phosphonate had a dual effect as glucobrassicin level decreased significantly in uninoculated plant and neoglucobrassicin significantly increased compared to untreated uninoculated plant (42 DPI). A similar pattern was observed in combined applications of phosphonate and Bion treated plant. For this reason, the second hypothesis needs to be further investigated as there is no available evidence published elsewhere with such a interaction between Bion or phosphonate, *P. brassicae* infection and glucosinolate profiles in brassica. Abbasi and Lazarovits *et al.* (2006) tested the effect of phosphonate on clubroot formation and found similar effectiveness to this study, but they did not monitor glucosinolate levels following infection.

Chemical application in aerial tissues of inoculated and uninoculated plants, significantly suppressed total aliphatic glucosinolate levels. Phosphonate application in uninoculated plants did not cause any changes in total indole glucosinolates levels, but Bion and combined applications significantly reduced indole production levels compared to untreated uninoculated plants. Phosphonate and Bion application did not cause any change in indole glucosinolate levels in inoculated plants but combined applications significantly reduced levels of total indole compared to untreated inoculated plants. These results show a complex pattern of indole glucosinolate accumulation in aerial plant tissues following pathogen infection of roots.

The most plausible physiological explanation for the observed differences in root and shoot patterns is that both organs have a different regulation of glucosinolate biosynthesis and turnover. Transportation of glucosinolates via the phloem over long distances (Chen *et al.* 2001) is not likely to be the main cause. This is supported by the observation that

the induction of specific indole glucosinolates by aphids occurs in leaves as well, producing a role for transport from the roots (Kim and Jander 2007). Progress has been made in identifying transcription factors of glucosinolate biosynthesis (Yan and Chen, 2007). Several of these glucosinolate transcription factors showed organ specific expression patterns (Gigolashvili *et al.* 2007a & 2007b & 2008) However, a more detailed experimentation and analysis of tissue specific regulation of glucosinolates synthesis and turn-over, as well as integration into the general metabolism is needed to elucidate the mechanism of specific gene involvement in specific glucosinolates accumulation rate throughout the biosynthetic and signalling pathway of particular glucosinolate.

This study has demonstrated the effect of clubroot on aliphatic and indole glucosinolates in root and aerial tissues of 35 days field grown brassica (*B. rapa* cv. *cinensis*). Our attempt was to compare glucosinolate profiles of managed and naturally grown clubroot infected brassica crop. Interesting results from both natural and controlled systems were found. This glasshouse trial was good model of field grown samples. In both cases, clubroot disease strongly enhanced indole glucosinolate accumulation both in root and aerial tissues. Only a small dissimilarity was observed in aliphatic glucosinolates. In the glasshouse trial, clubroot infection did not affect total aliphatic glucosinolates in the aerial parts while field grown sample analysis showed 12% reduction of total aliphatic accumulation in clubroot infected aerial tissues. In roots, total aliphatic glucosinolates increased 3 times and similarly, in glasshouse trial, total aliphatic glucosinolates increased 1.5 times during gall development (28 DPI). The evidence of this comparative

study suggests that the changes in glucosinolate profiles following *P. brassicae* infection followed a similar trend in field and glasshouse-grown plants of different species.

This study focused on changes in glucosinolate profiles following clubroot infection, rather than defensive roles of particular glucosinolates. Individual glucosinolate levels strongly responded to pathogen infection, particularly the indole glucosinolates. A possible reason for this is the direct or indirect involvement of Indole Acidic Acid (IAA) in gall formation. Myrosinase may preferentially convert indole glucosinolates to their corresponding nitriles and then to IAA (Fenwick *et al.* 1983a) hence, indole glucosinolates can be an alternative pathway of auxin production in brassica. The induction of auxin via indole glucosinolates may be responsible for many of the symptoms in brassica following pathogen attack (Mithen 1992). There are several factors that still need to be investigated before a precise role can be attributed, including changes to TrpOXE (Tryptophan Oxidase Enzyme) activity after infection, the activity of nitrilase and the pH of infected cells, and how this influences the products from myrosinase-catalysed degradation (Ludwig-Müller *et al.* 1999a & 1999b).

One aliphatic glucosinolate, progoitrin (2 hydroxy-3 butenyl) significantly increased both in root and aerial tissues following infection of *P. brassicae*, both in field and glasshouse trial study. It is necessary to investigate if there is any involvement of progoitrin in disease resistance. The toxic activity of hydrolysis products of indole glucosinolates to some pests and pathogens have been reported (Mithen *et al.* 1986). The hydrolysis product of hydroxyl aliphatic glucosinolates like progoitrin had no toxic activity towards *Leptosphaeria maculans* (Mithen *et al.* 1986).

The evidence from suppression of gall formation and fresh gall size after chemical treatment shows that phosphonate is strongly effective and Bion moderately effective against *P. brassicae* development but combined application was not effective in suppressing gall development. Conflicting patterns of glucosinolate profiles both root and shoot tissues due to combined applications of phosphonate and Bion application were found. This suggests a need to further investigate glucosinolate profiles and clubroot management following combined applications of phosphonate and Bion, both in foliar and root treatment using different doses. Effectiveness of phosphonate might be due to its ability to be absorbed rapidly and translocate within the plant in both the xylem and phloem (Cohen and Coffey 1986). It can persist in plant tissues for extensive periods (Carswell *et al.* 1996) have protective and curative properties (Wicks *et al.* 1991; Marks and Smith 1992) and displays a complex mode of action, ranging from direct effects such as activation of host defence responses against a number of plant pathogens (Grant *et al.* 1990; Guest and Bompeix 1990).

In vitro studies have demonstrated that a range of isothiocyanates, such as sulforaphane derived from glucoraphanin, the most abundant glucosinolate in broccoli, inhibited Phase I enzymes responsible for activation of carcinogens and induces Phase II detoxification enzyme systems, the body's cancer defence mechanisms (Zang *et al.* 1992; Johnson 2000; Talalay and Fahey 2001; Munday and Munday 2004). In our study, glucoraphanin significantly increased in aerial tissues following infection during symptom development time (14 DPI) and also significantly increased in root tissues during gall maturation (28 DPI). This information might be good sign for breeders who are working on breeding for beneficial glucosinolate rich brassica varieties. Considering overall increase of

glucosinolates that occurred in root tissue following infection, researchers may concentrate further on developing glucosinolate rich varieties of root brassica crops such as radish.

In this study of glucosinolate levels in roots and shoots some general patterns have been identified that may help us to understand better the role of different glucosinolates compounds in natural and controlled conditions. The levels and distribution of glucosinolates have been much better defined in relation to clubroot. The relationship between clubroot and aerial tissue glucosinolate profiles and fungicide interaction to root-shoot glucosinolates distribution is mostly unknown. More effort should go into analysing glucosinolate profiles in above ground tissues following root infection and chemical spray both as foliar and root treatments (soil drenching). This would provide insights into possible mechanisms causing the observed diversity in glucosinolate profiles. In addition, it would greatly benefit plant breeders wishing to manipulate glucosinolate composition in respect of health benefits and pest and pathogen specific defence responses.

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