Chapter 1 General introduction

1.1 Background

Sturt's desert pea (*Swainsona formosa*) is an ornamental leguminous plant, native to Australia (Williams and Taji, 1992), from the subfamily Faboideae within the family Fabaceae (Williams, 1996). High flower production (up to 150 flowers) per plant and the availability of white, pink and red lines makes *S. formosa* an ideal ornamental species (Williams and Taji, 1991). This plant has been used as a container plant and for cut flowers in some flower markets (Williams and Taji, 1991; Kirby, 1996). *Swainsona formosa,* with a prominent bird-eye spot, called a boss, in the centre of each flower, is a popular flower. A complex design of three *S. formosa* lines planted among other ornamental plants in parks and within roundabouts can be spectacular. The potential utility of *S. formosa* as an ornamental plant is, however, affected by the susceptibility of *S. formosa* to soilborne pathogens (Williams, 1996). Observations from the *S. formosa* breeding program at the University of New England suggested that white- or pink-flowered varieties are more susceptible to root diseases, particularly under greenhouse conditions (unpublished information). Such susceptibility creates a challenge for growers who supply markets where a preference for white or pink flowers exists.

To produce white and pink flowers, it is necessary to control root diseases which are a constraint to production. Chemical control of soilborne pathogens, aside from negative environmental consequences, requires specialised apparatus and expertise (Cook et al., 1996). In addition, the pathogen often becomes resistant or tolerant to chemicals (Staub, 1991). Moreover, biotic pathogens are a natural component of all ecosystems, and are therefore unlikely to be eradicated (Mussell, 1980, cited in Glover and Scott, 1998). Microbial biocontrol of damaging soil microorganisms can be too expensive (Cook et al., 1996) to use in the production of ornamental plants. Therefore, white- or pink-flowered lines of *S. formosa* with high levels of resistance to root diseases would be highly desirable. Before attempting to breed or select such varieties, it is necessary to know whether appreciable variation in resistance to soilborne diseases occurs in this plant, and the extent to which this resistance is linked to flower colour.

Phytophthora cinnamomi is a typical soilborne pathogen, has a wide-host-range, and has been reported as a limiting factor in the production of many horticultural, ornamental, agricultural, pastural and plantation forestry industries throughout the Australian continent (Irwin et al., 1995). This research assesses the reaction of greenhouse grown *S. formosa* lines to *P. cinnamomi*. Chemical control of *P. cinnamomi* is difficult because of its wide host range and the ability of the pathogen to survive saprophytically as chlamydospores in symptomless plants and in the soil (Robinson and Cahill, 2003). Although the production of resistant cultivars is difficult and time-consuming, and resistance may be overcome by the appearance of new pathogenic races (Bletsos, 2005), the use of lines and cultivars resistant to *Phytophthora* diseases is the best approach to control (Schmitthenner, 1985; Denman and Sadie, 2001).

1.2 Thesis outline

A review of the genetics and physiology of the resistance to fungal pathogens in plants is followed by a critical review of general biological resistance to *Phytophthora* species in plants, then specifically in leguminous plants (Chapter 2, Literature review). Other subjects discussed in Chapter 2 include the relationship between flower colour and disease resistance, from which the project originated, and the biochemistry of flower pigment synthesis and other phytocompounds that have defensive functions in incompatible interactions of non-host plants and pathogens.

Variation in resistance to *P. cinnamomi* among the three *S. formosa* lines, and the inheritance of disease resistance in F_2 generations from resulting crosses between the red (resistant) and white (susceptible) lines is investigated in Chapter 3. Soilborne fungi were simultaneously isolated from roots of naturally infected greenhouse-grown *S. formosa* white line, identified, and their pathogenicity to white, pink and red lines tested (Chapter 4).

A comprehensive investigation of the physiological mechanisms of resistance to *P. cinnamomi* in *S. formosa* is given in Chapter 5. As in most cases resistance to diseases involves more than one phytocompound (Ebel and Grisebach, 1988), the phytoalexin activity of different phytocompounds including enzymes (β-1,3-glucanase), plant pigments (anthocyanins), and total phenolic compounds was investigated. Subsequently, the possible involvement of end products of the

phenylpropanoid pathway, such as leucoanthocyanidins and proanthocyanidins, extracted from three *S. formosa* lines were assessed. The growth inhibitory effect on *P. cinnamomi* of polymeric and non-polymeric phenolic compounds extracted with acetone from *S. formosa* lines was also investigated.

It was hypothesised that the linkage between flower colour and disease resistance was due to the synthesis of condensed tannins from anthocyanidins. The anthocyanidin reductase (ANR) pathway has been reported in other plants (Xie et al., 2003; Bogs et al., 2005; Fujita et al., 2005; Takos et al., 2006) but not in *S. formosa*. The presence or absence of ANR in *S. formosa* lines was investigated using DNA molecular methods, and is presented in Chapter 6. The results of the experiments are summarised and discussed in Chapter 7.

1.3 Research objectives

The objectives of this project were the following:

1. To establish whether a link exists between flower colour and resistance to *P. cinnamomi*
2. To identify the biochemical and physiological mechanisms of resistance
3. To determine whether it is possible to obtain white- or pink-flowered varieties of *S. formosa* with high levels of resistance to soilborne diseases

Chapter 2 Literature review

2.1 Introduction

Sturt's desert pea (*Swainsona formosa*) is a legume from the subfamily Faboideae within the family Fabaceae (Williams, 1996). Breeding disease resistant plant cultivars and lines is one approach to overcome soilborne fungal pathogens. Identification of pathogens, recognition of plant cultivars resistant to the pathogen, and understanding the mechanisms of resistance in resistant plant cultivars are necessary to breed disease resistant plant cultivars. There is little published information on diseases of *S. formosa*, or on breeding or selecting for disease resistance in this species. This review describes the general biology of *Phytophthora* species, and the physiological and genetic defence mechanisms of plants to fungal pathogens. Furthermore, the review specifically deals with the resistance mechanisms of leguminous and other plants to *Phytophthora* species in incompatible interactions between pathogen and non-host plants. In addition, the relationship between disease resistance and flower colour and other phytocompounds is reviewed, including a description of the major enzymes and enzymatic activities in the phenylpropanoid or flavonoid pathway (Winkel-Shirley, 2001; Xie et al., 2004a) involved in the biosynthesis of plant pigments and colourless phytocompounds reported to be effective in plant defence mechanisms.

2.2 *Phytophthora* diseases of legumes

2.2.1 Major diseases caused by *Phytophthora* species in legumes

Phytophthora is a cosmopolitan genus comprising 67 described species and varieties, many of which are economically important plant pathogens (Ribeiro, 1978; Erwin and Riberio, 1996). The most typical diseases produced by *Phytophthora* species are root rot, stem rot, damping-off and blight (Erwin et al., 1983). *Phytophthora* species are mostly soilborne, including both necrotrophs and hemibiotrophs, and species with broad host ranges and those with narrow host ranges (Irwin, 1997). *Phytophthora cinnamomi* has been recorded from over 900 plant species from 67 countries (Zentmayer, 1980). Plant reactions to *P. cinnamomi* range from fully susceptible to various levels of resistance (Cahill et al., 1989). Host

recognition by this pathogen occurs through recognition of chemical, electrical, and physical features of plant roots (Irwin, 1997). Both host-specific factors, such as isoflavones, and host-nonspecific factors such as, amino acids, calcium, and electrical fields, influence zoospore taxis, encystment, cyst germination, and hyphal chemotropism in guiding the pathogen to potential infection sites (Tyler, 2002).

In a study performed in south-west Australia to determine which plant species in eucalyptus forests were susceptible to *P. cinnamomi*, 25 species from 10 genera of Faboideae were identified (Shearer and Dillon, 1995). In addition, this research identified 16 plant species from two genera in Mimosoideae as susceptible to *P. cinnamomi* (Shearer and Dillon, 1995). In similar research carried out in Perth, Western Australia, 21 species from 12 Faboideae genera were also identified as susceptible to *P. cinnamomi* (Shearer and Dillon, 1996). Members of the Mimosoideae and Faboideae are monophyletic leguminous plants (Herendeen and Bruneau, 2000), and have similar susceptibility or resistance to diseases.

The most prevalent *Phytophthora* species that attack leguminous plants worldwide are *P. sojae* (which attacks soybean and lupin), *P. medicaginis* (which attacks lucerne and chickpea), and *P. vignae* (which attacks cowpea and adzuki) (Irwin, 1997). After *P. cinnamomi*, *P. sojae* is the most destructive fungal disease and is an ongoing problem in soybean-growing areas around the world, particularly in the United States (Glover and Scott, 1998; Tyler, 2002). *Phytophthora* species that attack leguminous plants in Australia include *P. cinnamomi* on *Lupinus digitatus* and ornamental plants, *P. clandestina* on subterranean clover (*Trifolium subterraneum*), *P. cryptogea* on cluster bean (*Cyamopsis tetragonoloba*), *P. dreschsleri* on *Stizolobium deeringianum* and pigeon pea (*Cajanus cajan*), *P. macrochlamydospora* and *P. sojae* on soybean, *P. medicaginis* on lucerne and chickpea, *P. megasperma* on white clover (*Trifolium repens*), and *P. vignae* on cowpea (*Vigna unguiculata*) (Irwin et al., 1995; Shearer and Dillon, 1995; Irwin et al., 1996; Shearer and Dillon, 1996).

Susceptible plants exhibit different symptoms depending on the *Phytophthora* species, the plant species and prevailing environmental conditions (Zentmayer, 1980). In susceptible species, progressive symptom development can include water soaking of tissues, lesions that extend through the root to the hypocotyl, and root death accompanied by wilting, leaf chlorosis, shoot die-back and plant death (Cahill et al., 1989). Where the hypocotyl is the first organ infected, the lesion often progresses upward and the infected areas become necrotic. This is a primary characteristic of

Phytophthora stem rot in, for example, cowpea, soybean and pigeon pea (Irwin et al., 1995).

Legumes resistant to some *Phytophthora* species have been identified. These include soybean resistant to *Phytophthora sojae* (Shan et al., 2004), lucerne resistant to *Phytophthora medicaginis* (Vandemark and Barker, 2003), and cowpea resistant to *Phytophthora vignae* (Ralton et al., 1988).

Australian plant species from the subfamily Faboideae resistant to *P. cinnamomi* include *Gompholobium tomentosum*, *G. capitatum*, *Hardenbergia comptoniana*, *Hovea trisperma, Kennedia prostrata, K. coccinea* and *Mirbelia dilatata* (Shearer and Dillon, 1995; Shearer and Dillon, 1996).

2.2.2 Biology of *Phytophthora*

2.2.2.1 Asexual reproduction

Phytophthora species typically produce sporangia, zoospores and chlamydospores through asexual reproduction. Sporangia in some species such as *P. infestans* are freely released from aerial hyphae and serve as dispersal agents. Sporangia can directly germinate to produce hyphae or can differentiate to produce 10-30 zoospores. As zoospores lack a cell wall and have two flagella for swimming, they move quickly. Zoospore production is typically triggered by flooding. They are generally short-lived (hours) and quickly differentiate to form adhesive cysts, which in turn germinate to produce hyphae. Zoospores are the most important route to root infection, especially when the soil is flooded. To infect plants, the zoospores encyst on the root surface from where the hyphae produced from the cyst directly penetrate the root. Zoospores, as well as sporangia, can also be spread to the upper parts of the plant by splashing (Erwin et al., 1983). *Phytophthora* species are able to survive saprophytically as chlamydospores in symptomless plants, and in the soil (Robinson and Cahill, 2003).

2.2.2.2 Sexual reproduction

Phytophthora species may either be homothallic (selfing) or heterothallic (exhibiting two mating types). Homothallic species and heterothallic species, where both compatible mating types (A_1 and A_2) exist, produce oospores (Ribeiro, 1978).

The oospores are formed from the fusion of two morphologically different gametangia called the oogonium and antheridium. Meiosis occurs in the gametangia shortly before gamete fusion and oospore formation. Oospores can survive in the soil for many years (Drenth et al., 1995) and germinate by producing one or more germ tubes, which can form either diploid hyphae or terminate in a sporangium that can germinate to produce diploid hyphae or produce zoospores (Shaw, 1988).

2.3 Mechanisms of resistance to *Phytophthora*

2.3.1 Mechanisms of disease resistance in plants

Plants are in frequent contact with potential pathogens and have consequently evolved effective mechanisms to resist infection (Swain et al., 1977). These include passive or mechanical barriers to infection, preformed chemical defences, and resistance mechanisms that are activated by exposure to pathogens (Hammond-Kosack and Jones, 1996).

However, in many cases disease resistance results from the activation of more than one biochemical defence mechanism (Ebel and Grisebach, 1988). Disease resistance in plants involves two distinct forms of chemical communication with the pathogen, recognition and defence, which begins with non-self recognition through the perception of pathogen-derived signal molecules, or elicitors (Hahlbrock et al., 2003). A defence response against pathogens requires sensitive and specific recognition mechanisms for pathogen-derived signals in plants (Nurnberger et al., 1997). Hadwiger (1999) stated that an adapted compatible pathogen generates a weak response and thus can more easily infect the plant tissue, whereas plant pathogens known to be incompatible with a given plant species can elicit strong disease resistance responses. The elicitors derived from pathogens include oligosaccharides gained from chitin, glucan, and pectin; and fungal cell wall derivatives, extracellular glycoproteins, polysaccharides, and harpins produced by pathogenic and non-pathogenic bacteria and fungi (Punja, 2004). The production of characteristic flavonoids by plants, particularly those exuded from the roots by leguminous plants, appear to be used as signals for various pathogens (Ndakidemi and Dakora, 2003). Some evidence suggests that the activation of the phenylpropanoid pathway and the accumulation of its products is an important early event in incompatible host-pathogen combinations (Hahlbrock and Scheel, 1989). The final products derived

from the phenylpropanoid pathway which are effective in disease resistance, are isoflavonoids, flavones, leucoanthocyanidins, anthocyanidins, condensed tannins (proanthocyanidins) and phlobaphenes (Winkel-Shirley, 2001).

The different defence mechanisms used by plants against pathogens are described below.

2.3.1.1 Preformed compounds

Some plants resist microbial attack by using preformed compounds. The preformed antimicrobial compounds or phytoanticipins are constitutive and present at an inhibitory level to the pathogens in intact plant tissues before infection, without a *de novo* synthesis after microbial attack (Schlosser, 1994; Slusarenko et al., 2000). The activation of the pre-existing constituents often involves plant enzymes that are released as a result of cell breakdown (Osbourn, 1996). Some of these compounds exist in healthy plants in their biologically active forms and others, such as cyanogenic glycosides and glucosinolates, exist in healthy plants as inactive precursors that are activated in response to pathogen attack (Osbourn, 1996). The distribution of phytoanticipins within plants is often tissue specific (D'Mello et al., 1991). Although preformed antifungal compounds are commonly sequestrated in vacuoles or organelles in healthy plants, there is a tendency for these compounds to be concentrated in the outer cell layers of plant organs (Osbourn, 1996). The nature and level of preformed inhibitors to which a potential pathogen is exposed varies, depending on factors such as host genotype, age and environmental conditions (D'Mello et al., 1991). Despite the fact that preformed inhibitors may be effective against a broad spectrum of potential pathogens, successful pathogens are likely to be able to circumvent their effectiveness, either by avoiding them completely or by tolerating or detoxifying them (Bennett and Wallsgrove, 1994).

A large number of constitutive plant compounds with antimicrobial activity have been reported. Well-known examples consist of phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, cyanogenic glycosides, glucosinolates (including aliphatics, indolyls and aralkyl α-amino acids), and saponins which are glycosylated compounds and are divided into three major groups including triterpenoids, steroids and steroidal glycoalkaloids (D'Mello et al., 1991; Bennett and Wallsgrove, 1994; Schlosser, 1994; Osbourn, 1996).

Antimicrobial phenols have been found in all plants investigated to date. Although many phenolic compounds are formed in attempted defence responses, some of them occur constitutively and are thought to function as preformed inhibitors associated with non-host resistance (Nicholson and Hammerschmidt, 1992; Schlosser, 1994). The antifungal activities of phenolic compounds is enabled by the accumulation of soluble phenolic compounds, their deposition on cell walls, melanisation and lignification (Prats et al., 2003). Condensed tannins, a group of the phenolic compounds, in sufficient concentrations protected plants against pathogenic microorganisms (Schlosser, 1994). Condensed tannins are rather unspecific enzyme inhibitors, and have been effective in binding to the extracellular hydrolases secreted by pathogens (Schlosser, 1994). In addition, tannins have been reported to prevent basidiospore germination and germtube elongation of *Crinipellis perniciosa*, the causal agent of witches broom of cocoa (Brownlee et al., 1990; Brownlee et al., 1992). The resistance of the carnation cultivar Novada to *Fusarium oxysporum* f. sp. *dianthi*, the causal agent of *Fusarium* wilt, is related to the presence of a constituent fungitoxic flavonol triglycoside called kaempferide 3-O- beta –rutinoside (Curir et al., 2005). This phytoanticipin could cooperate with the unconstitutive and post-infectional phytoalexins, for the plant defensive response against *Fusarium* attacks (Curir et al., 2005). A sunflower line resistant to *Sclerotinia sclerotiorum* was found to have a greater amount of soluble phenolic compounds than the susceptible line. In addition, the induced phenolic content as well as phenylalanine ammonia-lyase (PAL) activity was greater in the resistant line (Prats et al., 2003). These observations show that both phytoalexins and phytoanticipins are responsible for resistance to *Sclerotinia sclerotiorum* in the resistant sunflower line.

2.3.1.2 Physical defence barriers

Most plants normally withstand attack by potential pathogens via defence barriers, such as the cuticle and the cell wall, which render them resistant to such attacks (Hammond-Kosack and Jones, 1996). Strengthening makes cell walls more difficult to penetrate and reduces the intracellular spread of pathogens (Slusarenko et al., 2000). Ficke et al. (2004) reported that a preformed physical or biochemical barrier near the cuticle surface of the plant prevents infestation by pathogens. Lignin and lignin-like materials are often deposited in a zone surrounding the site of tissue penetration during defence reactions to pathogenic attack (Slusarenko et al., 2000).

Callose deposition in the roots of some resistant plant species may also limit pathogen growth (Cahill and Weste, 1983; Kpemoua et al., 1996). In the same way, lignosuberised tissues of jack pine (*Pinus banksiana*) resistant to the fungus *Gremmeniella abietina*, the causal agent of Scleroderris canker and necrophylactic periderms on Douglas-fir roots in response to *Armillaria ostoyae,* confined the pathogens within the necrotic areas (Robinson and Morrison, 2001; Simard et al., 2001).

2.3.1.3 Induced defence response

Induced defence response is a common form of resistance. Induced defence responses resulting from interaction between elicitors (primary or secondary products of avirulence genes) and host receptors, according to their distinct temporal and spatial expression patterns, can be assigned to four major categories (Lauge and de Wit, 1998; Punja, 2004).

2.3.1.3.1 Hypersensitive reaction

Hypersensitive reactions (HR) occur when immediate and rapid defence responses are initiated in the cells adjacent to those invaded by a pathogen, as a result of signals being transferred to those neighboring cells, frequently leading to rapid collapse and death of a few challenged host cells. Hypersensitive reactions occur in incompatible interactions between plant and pathogen and are often related to the establishment of systemic acquired resistance. Hypersensitive reaction can be recognised within a few hours after contact between plant and pathogen. The invaded cells or tissues prevent the spread of the pathogen to the rest of the plant (Ebel and Grisebach, 1988; Ryals et al., 1996). Salicylic acid has a key role in the activation of disease resistance, and is frequently associated with a special type of cell death (Dangl et al., 1996).

2.3.1.3.2 Gene activation

Subsequent to recognition, abundant biochemical and metabolic plant modifications take place in close vicinity to infection sites, as a result of local gene activation. Gene activation leads to the synthesis of proteins involved in the formation

of antimicrobial compounds called phytoalexins, the reinforcement of the plant cell wall through the synthesis of lignin-like materials, the deposition of callose (β-1,3 glucan), or the accumulation of hydroxyproline-rich glycoproteins (Ebel and Grisebach, 1988; Punja, 2004).

2.3.1.3.3 Pathogenesis-related proteins

The production of pathogenesis-related (PR) proteins including β-1,3-glucanases and chitinases is a result of the systemic activation of genes. Pathogenesis-related proteins occur in plant tissues at a distance from the initial infection site, resulting in the establishment of an immunity to secondary infections, and are termed systemic acquired resistance (Sticher, 1997). Pathogen-induced plant proteins, according to their functional characteristics, are involved in plant cell signalling, inhibition of enzymes excreted by pathogens, plant cell wall stabilisation, triggering of apoptosis, enzymatic activity that lyses the cell walls of pathogenic fungi and bacteria, enzymatic activity in the metabolic pathways of phenylpropanoid and terpenoid phytoalexins, and affecting pathogens directly via disturbing cell membrane function or by ribosome deactivation (Tarchevsky, 2001).

2.3.1.3.4 Secondary metabolites

Some plant species defend themselves against pathogens through the accumulation of plant secondary metabolites. For example, anthocyanin accumulation in a resistant sugarcane cultivar in response to red rot pathogen (*Colletotrichum falcatum*) inoculation was greater at the inoculation site and at 2.5 cm away from the inoculation site than in susceptible cultivars (Viswanathan, 2002). Cyanidin 3-dimalonyl glucoside is another plant pigment that accumulates in uninfected, healthy epidermal cells surrounding restricted lesions on the leaves of maize resistant to *Bipolaris maydis* (Hipskind et al., 1996). These cells, which were affected but not infected by the pathogen, stopped pathogen growth in the resistant cultivar 18 hours post-infection, well before pigment accumulation (Hipskind et al., 1996).

2.3.2 Mechanisms of resistance to *Phytophthora* in legumes

Leguminous plants such as bean and soybean defend themselves against *Phytophthora* species by rapid activation of a multitude of defence reactions. These include reinforcement of the cell wall by the rapid insolubilisation of pre-existing proline-rich structural proteins in the cell wall (Bradley et al., 1992), callose deposition (Ebel and Grisebach, 1988), hypersensitive reactions (Klarman and Corbett, 1974), accumulation of polycyclic compounds, phytoalexin and phytoanticipins before, during and after attack (Dakora and Phillips, 1996), and the production of pathogenesis-related proteins (Dann et al., 1996; Yi and Hwang, 1996).

Some phytoalexins, such as kievitone, phaseollin isoflavon and phaseollin, have been isolated from bean seedling roots grown in soil infected with *Pythium* spp. (Liu et al., 1995), which are from the same family as *Phytophthora*. Glyceollin-I, which is an isoflavonoid, is the major phytoalexin that accumulates at the site of infection in the incompatible interaction between soybean and *P. sojae* (Ebel and Grisebach, 1988). Some other phytoalexins with high production levels have been isolated from soybean plants resistant to *P. sojae*. For example, when the resistant soybean cultivar Harosoy 63 and the susceptible cultivar Harosoy were infected *in vitro* by *P. sojae* to investigate the level of phytoalexin production, identified as 6-α-hydroxyphaseollin (Keen, 1971), the phytoalexin accumulated in the resistant cultivar 10 to 100 times faster than in the susceptible cultivar (Keen, 1971). Histopathological studies of the hypocotyl tissues of susceptible and resistant soybean cultivars to *P. sojae* revealed that pathogen hyphae spread intercellularly and intracellularly into all tissues of the susceptible hypocotyls, but in the resistant cultivar only those cells directly surrounding the inoculation wound were colonised by the pathogen, and organelles of adjacent uninfected host cells were normal (Klarman and Corbett, 1974). In the resistant cultivar, some host cells in the vicinity of the infected site were filled with granular, dark-staining cytoplasm, which appeared to form a barrier to prevent further pathogen movement (Klarman and Corbett, 1974).

Production of pathogenesis-related proteins is one of the approaches that legumes employ to overcome *Phytophthora* species. Soybean seedlings exposed to *P. sojae* increased greatly the synthesis and accumulation of β-1, 3-glucanases and chitinases in their hypocotyls and leaves (Yi and Hwang, 1996). These enzymes catalyse the hydrolysis of glucan and chitin respectively in the fungal cell walls (Yi

and Hwang, 1996). Isoforms of β-1, 3-glucanase and chitinase accumulated in diseased soybean tissue during symptom development in both compatible and incompatible interactions, but the accumulation of these enzymes in soybean hypocotyls was more pronounced in the incompatible interaction of soybean and *P. sojae* than in the compatible interaction (Yi and Hwang, 1996). Similarly, β-1, 3-glucanases and chitinases accumulated in leaves of green bean (*Phaseolus vulgaris*) in response to *P. sojae* (Dann et al., 1996).

Leguminous plants defend themselves against *Phytophthora* species by other defence mechanisms such as a reduction in abscisic acid concentration (Cahill and Ward, 1989; McDonald and Cahill, 1999). Abscisic acid, by influencing the biochemistry of resistance, has been shown to be effective not only in incompatible interactions between legumes and *Phytophthora*, but also in incompatible interactions between legumes and other pathogens (Dunn et al., 1990; Ryerson et al., 1993). It is known that abscisic acid regulates the expression of at least 150 genes (Anderson et al., 1994). Cahill and Ward (1989) reported that abscisic acid was a potential regulator of the outcome of an interaction between host and pathogen. They demonstrated that in incompatible interaction of *P. sojae* and soybean, the concentration of abscisic acid decreased rapidly between two and four hours after inoculation at lesion sites and in tissues adjacent to the lesioned area.

2.3.3 Mechanisms of resistance to *Phytophthora* in other plants

Other plants, like legumes, use defence mechanisms against *Phytophthora* species. These include hypersensitive cell death (Zhang et al., 2003), cell wall reinforcement (Schmidt et al., 1998; Schmelzer, 2002), phytoalexin production (Afek and Sztejnberg, 1988; Sulistyowati et al., 1990; Mucharromah et al., 1995), expression of various pathogenesis-related proteins (Christ and Mosinger, 1989) and the generation of reactive oxygen species (Hawksworth, 2005).

In a challenge between a non-host plant and *Phytophthora*, various defence mechanisms initiate. For example, the response of non-host tobacco variety to *P. palmivora* 9-12 hours after challenge included hypersensitive cell death and phytoalexin capsidiol accumulation (Perrone et al., 2003). Often, an increase in PAL activity occurs during the challenges between *Phytophthora* species and non-host plants, resulting in phytoalexin production (Ralton et al., 1988; Nemestothy and Guest,

1990; Cahill and McComb, 1992; Cahill et al., 1993). In a tobacco cultivar, NC 2326, which is resistant to race 0 of the black shank pathogen *P. nicotianae* var. *nicotianae,* in response to stem inoculation of the pathogen, PAL activity at the infection site increased rapidly and sesquiterpenoid phytoalexins accumulated quickly (Nemestothy and Guest, 1990). Similarly, in a cultivar of *Eucalyptus calophylla* resistant to *P. cinnamomi*, an increase in PAL activity was observed at the root tip of inoculated roots within 24 hours of inoculation. The activity of PAL declined to below control levels over the following 1-2 days. That process of increasing PAL activity followed by a decline in activity was continued for four of the five sequential 1 cm root segments away from the root tip (Cahill and McComb, 1992). Apparently, changes in PAL activity in the successive segments resulted in cell wall reinforcement and phytoalexin accumulation in the infected sites and areas where the disease had progressed. After progress of the pathogen to 3-4 cm from the inoculation site, the disease ceased, presumably because reinforcing of the adjacent cell walls and phytoalexin production was sufficient to protect the tissue. Cahill et al. (1992) described that in *E. calophylla* inoculated with *P. cinnamomi*, root lignin increased substantially, and total soluble phenolic compound level in the third root segment from the root tip was at its maximum at 97% above the control (Cahill and McComb, 1992). Therefore, lignin and soluble phenolic compounds were responsible for the resistance of *E. calophylla* against *P. cinnamomi* (Cahill and McComb, 1992). The same changes in PAL activity, lignin concentration and soluble phenolic compounds were observed when resistant *Eucalyptus marginata* clonal lines bred, through tissue culture, were inoculated with *P. cinnamomi* (Cahill et al., 1993).

The presence of constitutive compounds in healthy plants prevents plant infection by *Phytophthora* species. Although Cahill et al. (1993) reported that an increase in soluble phenolic compounds in the resistant *Eucalyptus marginata* clonal lines inoculated with *P. cinnamomi* was a resistance response, as the increase was trivial, it seems that high levels of soluble phenolics (94%) as constitutive compounds in the healthy clonal lines anticipate their infestation by the fungus. In an experiment conducted by Mozzetti et al. (1995) to measure the activity of a pool of enzymes in the leaves and cell suspensions of two near-isogenic pepper lines, which were susceptible and resistant respectively to *Phytophthora capsici* and *Phytophthora nicitianae* var. *parasitica*, higher constitutive chitinase, β-1, 3-glucanase and

peroxidase activities were detected in the non-inoculated leaves and cell cultures of the resistant line.

Hawksworth (2005) stated that the production of peroxide and other reactive oxygen species, which is a ubiquitous defensive reaction of cells presented with toxins and potential pathogens, may play an important role in signal transduction, serving to induce other reactions against fungal invaders and to promote pigment biosynthesis. In an incompatible interaction between *P. parasitica* and tobacco, a form of vacuolar cell death in the plant required protein synthesis and was associated with the production of intracellular reactive oxygen species (Galiana et al., 2005).

There are numerous examples of constitutive natural antibiotics that protect plants against pathogenic *Phytophthora* species. Avocado (*Persea*) species inhibited *P. cinnamomi* growth *in vitro* by a preformed antifungal substance called borbonol. This phytoanticipin was extracted from all parts of *Persea borbonia* plants and from the twigs of *P. caerulea*, *P. skutchii*, and *P. pachypoda* (Zaki et al., 1980). Taxol and related compounds such as cephalomannine, which are preformed antifungal defence compounds and are present in *Taxus* species, prevent, *in vitro*, the growth of several fungi, particularly *Phytophthora* species (Wagner and Flores, 1994). The volatile phytoanticipin allicin extracted from garlic (*Allium sativum* L.) has antimicrobial activity against a wide range of plant pathogens including *Phytophthora* (Curtis et al., 2004).

Resistance of plant roots to *P. cinnamomi* depends not only on biochemical mechanisms but also on the morphological barriers of cells (Cahill and McComb, 1992). In *Themeda australis* resistant to *P. cinnamomi,* rapid cell wall lignification separates invaded tissue from healthy tissue, and the pathogen is restricted to the lesion zone, whereas, in susceptible individuals the pathogen invariably penetrates and colonises the phloem and xylem (Cahill et al., 1989). In potato plants attacked by *P. infestans*, cytoskeleton rearrangement resulted in massive cell-wall reinforcement (Schmelzer, 2002). With this response, the cytoplasm and cell nucleus are translocated to the fungal penetration site, and local apposition of barrier material occurs around the penetration site (Schmelzer, 2002). Cuticle thickness of non-wounded mature green and red pepper fruit can be a factor in resistance to *P. capcici* (Biles et al., 1993).

In conclusion, in incompatible interactions between non-host plants and pathogens, different changes can occur in plants to prevent infestation by the

pathogens. These include changes in the morphology and biology of plants, and vary depending on the plant and pathogen species.

2.4 The genetics of resistance to *Phytophthora*

2.4.1 Measurement of resistance

2.4.1.1 Current methods and their limitations

Current methods for measuring resistance to *Phytophthora* species include the use of field-based disease nurseries (Thies and Barnes, 1991; Irwin et al., 2003), infestation of soil surrounding 6 to 8 week-old plants in the greenhouse (Irwin, 1974), and sowing seed into infested potting mix for assessing levels of post-emergence damping-off (Irwin et al., 1979). The disadvantage of these assays for mature plants is that considerable greenhouse space or field areas are required for prolonged time periods ranging from 6-8 weeks to 3 months. In seedling assays, strict control of inoculum levels is required. In addition, the use of mycelial mats as inoculum presents difficulties in accurately quantifying associated infective propagules due to differences in the maturity of isolates. Moreover, all these methods are costly (Irwin et al., 2003).

2.4.1.2 Newly identified methods and their advantages

Recently, detached stem (Huberli et al., 2002), hypocotyl (Burnham et al., 2003a) and detached leaf (Irwin et al., 2003; Moragrega et al., 2003; Baral et al., 2004) assays have been used to assess plant susceptibility to pathogens. In the new methods for assessing lucerne root reactions to *Phytophthora medicaginis,* detached leaf and cotyledon of lucerne (cv. D, susceptible to, × cv. W116, resistant to, *P. medicaginis* UQ5614) cultivars and the mature root of original seed-grown parent lucerne were used to measure their resistance to isolate UQ5614 of *P. medicaginis*. Plant materials were inoculated with different concentrations of zoospore suspensions, and after being maintained in special conditions for a determined time, the reactions of the plant materials to the pathogen were plotted and their resistances measured. In this study, the cotyledon reaction showed a higher level of agreement with the mature root reaction of individuals than the detached leaf reaction (Irwin et al., 2003).

Major potential advantages of the cotyledon and leaf assays over root inoculation include the rapidity of obtaining a result, savings in greenhouse costs, and the flexibility that the leaf assays would provide to a breeding program (Irwin et al., 2003).

2.4.2 Genetics of resistance to *Phytophthora* in legumes

Genes activated in plant disease resistance can be divided into three classes (Takemoto et al., 2000): genes encoding transcriptional regulators, genes encoding enzymes that are involved in secondary metabolic pathways, and genes encoding for PR-proteins. Expression of resistance genes in resistant plants, or treating pathogens with elicitors such as chitosan inhibits plant infestation. Both activities can result in the rapid activation of a subset of genes called pathogenesis-related genes, generally regarded as the genes that functionally develop disease resistance (Hadwiger, 1999).

2.4.2.1 Single dominant host resistance genes

Host resistance has been an effective trait for controlling *Phytophthora* damping-off and stem rot in soybean (Schmitthenner, 1985). Single dominant host resistance genes (Rps genes) in soybean, which interact with *P. sojae* in a gene for gene system, have been used extensively (Schmitthenner, 1985; Burnham et al., 2003a). Resistance of some other leguminous plants to *Phytophthora* species is reportedly managed by a single gene. For example, cowpea resistant to *P. vignae* has been identified as vertically (Purss, 1972). In lucerne complete forms of resistance to *P. megasperma* have been identified (Irwin et al., 1981a; Irwin et al., 1981b).

Unfortunately, a single dominant host resistance gene termed complete or vertically resistant, is not durable. The most widely used Rps genes have remained useful for single-gene *Phytophthora* root rot protection for about 8-10 years (Schmitthenner et al., 1994). For example, in soybean numerous physiological races of *P. sojae* have developed in response to the deploying of a single resistance gene (Dorrance and Schmitthenner, 2000).

2.4.2.2 Tolerance

Horizontal resistance or tolerance is controlled by several genes (Glover and Scott, 1998). Tolerance is also termed partial resistance, field resistance, general resistance, and rate-reducing resistance in soybean literature (Tooley and Grau, 1984). Tolerance is described as the relative ability of plants to survive infection, either natural or artificial, without showing severe symptoms such as death, stunting, or yield loss (Young et al., 1994). The influence of parental number on heritability of tolerant multiple-parent populations remains unknown (Glover and Scott, 1998). Tolerance heritability to *Phytophthora* root rot is reported to be quite high in the soybean germplasm (McBlain et al., 1991). Tolerance does not have a negative effect on yield in the absence of disease (St. Martin et al., 1994).

Soybean tolerance to *Phytophthora* root rot is a form of protection that may prevent the build-up of races capable of defeating resistance genes (McBlain et al., 1991). Soybean partial resistance is inherited as a quantitative trait (Walker and Schmitthenner, 1984; Glover and Scott, 1998) and is governed by a few genes with relatively large effects (Walker and Schmitthenner, 1984) to several genes (McBlain et al., 1991). Tolerance of soybean to *P. sojae* can be partitioned into root resistance, slow rotting or rate-reducing resistance, and endurance (McBlain et al., 1991). Tolerance is effective in soybean against all races of *P. sojae* (Tooley and Grau, 1984). Partial resistance in legumes against *Phytophthora* may not be effective in young seeds and seedlings (Dorrance and McClure, 2001). Unfortunately, environmental conditions and various levels of disease pressure affect partial resistance (Dorrance et al., 2003).

2.5 Relationship between flower colour and resistance

Coloured plant tissues are usually resistant to pathogen attack (Prasad and Weigle, 1969; Statler, 1970). Phenolic compounds, such as anthocyanins, flavonols and flavones, are responsible for different colours in flowers (Zhao et al., 2004), seed coats (Nozzolillo et al., 1989), fruits and vegetables (Rababah et al., 2005). In wild type plants it is impossible to predict the content of any colourless phenolic based on the extent of presence of anthocyanins in the same or other tissues (Waterhouse, 2002). Many white-flowered ornamental species produce flavonols in abundance, and may produce anthocyanins for a brief period during flower development (Davies et al.,

2002). However, it has been observed that plants containing greater levels of phenolic compounds are more resistant to plant diseases (Prasad and Weigle, 1969; Statler, 1970; Cahill and McComb, 1992; Cahill et al., 1993) and pests (Boughdad et al., 1986).

Synthesis of the pigment complex in plants constitutes a defence response to pathogens (Nicholson et al., 1987). Some current white-flowered cultivars of *Vicia faba* (faba bean) suffer from various post-emergence plant diseases (Kantar et al., 1996). Pascual Villalobos and Jellis (1990) observed that coloured-flowered faba bean were resistant to soilborne fungal pathogens such as *Fusarium culmorum* and *F. solani*, while tannin-free, white-flowered faba beans were susceptible to these pathogens. Kantar et al. (1996) achieved similar results. They noticed that white-flowered faba bean lines and cultivars were more susceptible to soilborne fungal pathogens than coloured-flowered lines and cultivars during seed germination, but all lines and cultivars were equally resistant during seedling growth. The negative effect of faba bean tannin content on the growth of pathogenic soilborne fungi of *Vicia faba* reported earlier by Pascual Villalobos and Jellis (1990), led Kantar et al. (1996) to examine the effect of tannin extracted from coloured-flowered faba bean seeds and its influence on faba bean pathogenic fungi *in vitro*. The tannin inhibited the mycelial growth of all the faba bean pathogenic fungi tested (Kantar et al., 1996), confirming that the tannin of coloured-flowered faba beans, which exists as a constitutive compound in faba bean testa, has an antifungal activity against soilborne diseases during seed germination. The observation that Kantar et al. (1996) made that all lines of and cultivars of faba bean were equally resistant to fungal pathogens during seedling growth may indicate that the tannins were located in the testa and did not accumulate in the growing seedlings.

The earlier investigations regarding the association of flower and other tissue colour and disease resistance comes back to Link et al. (1929), who reported that coloured onion varieties with greater amounts of phenolic compounds were more resistant to onion diseases than white varieties. Later, Prasad and Weigle (1969) reported that snap bean varieties with purple hypocotyls and black testae were resistant to *Rhizoctonia solani*, while varieties with green hypocotyls and white testae were susceptible to this soilborne fungal pathogen. The resistant snap bean cultivars had high amounts of polyphenols and flavonoid pigments, while these secondary phytocompounds were not found in white-seeded snap bean cultivars (Prasad and

Weigle, 1969). The amounts of the secondary metabolites in the inoculated susceptible snap bean seedlings was not significantly different from non-inoculated control seedlings, indicating that polyphenols and flavonoid pigments prevented disease development in resistant seedlings. Similarly, bean varieties with purple hypocotyls and black testae were more resistant to *Fusarium solani* f.sp. *phaseoli*, the causal agent of *Fusarium* root rot, than varieties with green hypocotyls and white or pinto seeds (Statler, 1970). The difference in the hypocotyl and testa colours of the two bean groups originated from their soluble phenolic compound content. Bean varieties with purple hypocotyls and black testae contained greater amounts of soluble phenols than varieties with green hypocotyls and white or pinto testae. The soluble phenolic compounds present in the varieties with purple hypocotyls and black testae inhibited their infection by the fungus (Statler, 1970). To confirm this, the effects of certain phenolic compounds on fungal growth were tested *in vitro,* and the growth inhibitory impact was observed (Statler, 1970). In recent research by Punyasiri et al. (2005) to identify tea cultivars resistant to *Exobasidium vexans*, the causal agent of blister blight, the TRI 2043 cultivar with a purple green leaf, containing high levels of anthocyanins compared to other cultivars, was resistant to the fungal pathogen. The researchers reported that the high resistance of the TRI 2043 cultivar was related to high catechin and epicatechin content, both of which are precursors of proanthocyanidins and are synthesised from anthocyanidins.

The association between testa colour and disease resistance to pests and diseases has been extensively studied. In *in vitro* research performed by Guevara et al. (1986) to determine the resistance/susceptibility of French bean (*Phaseolus vulgaris*) varieties with various seed testa colours to *Rhizoctonia solani*, it was observed that white-seeded varieties were more susceptible than red- and black-seeded varieties. The plants most resistant to *R. solani* were the ones which grew from mottled seeds. The result of another study revealed that Desi chickpea (*Cicer arietinum* L.) with dark-coloured seeds was resistant to *Fusarium oxysporum* f. sp. *ciceris*, the causal agent of *Fusarium* wilt, while the traditional Kabuli-type cultivars with cream-coloured seeds were susceptible to the disease (Cobos et al., 2005). In 1987, Higuera and Murty performed crosses among three white-seeded and five black-seeded-soybean varieties to investigate resistance/susceptibility of the progenies in the F_2 generation to a fungal (*Macrophomina*) and a bacterial (*Xanthomonas*) pathogen. The results showed that all white-seeded progeny from the three white-seeded varieties

were highly susceptible to both pathogens. In contrast to the above work, van Loon et al. (1989) reported white-flowered cultivars of *Vicia faba* have equal or higher levels of resistance to attack by soilborne pathogens compared to cultivars with anthocyanin pigmented flowers. However, the resistance of pigmented tissues to plant diseases has been reported to be related to high phenolic content in the plant. The higher resistance of white-flowered *Vicia faba* cultivars compared to plants with pigmented flowers against soilborne pathogens may be related to the presence of other phytocompounds in the white-flowered plants and the lack of those compounds in plants with pigmented flowers.

Linkage between tissue colour and resistance to diseases has not only been observed in herbaceous plants but also in trees and products derived from trees. Dark-coloured redwood boards (*Sequoia sempervirens*) were reported to be more resistant to decay than light-coloured boards (Wilcox and Piirto, 1976). Dark-coloured boards had a greater concentration of ethanol-soluble extractives (phenolic compounds) than the light-coloured boards (Wilcox and Piirto, 1976), so the resistance of the dark-coloured boards was associated with their higher level of phenolic compounds.

There is a strong relationship between genes controlling tannins and pigment compounds (Kristiansen, 1984). Although most of the earlier studies on tannin genetics were concerned with quantitative (presence or absence) genetics, indicating that the anthocyanin pigments, leucoanthocyanidins and proanthocyanidins are absent in white-flowered plants (Dickinson et al., 1957; Bond, 1976; Crofts et al., 1980; Nozzolillo et al., 1989; van Loon et al., 1989), Cabrera and Martin (1989) reported that homozygous dominant plants, with genes that control the diffuse distribution of pigment in the flowers in the F_2 generations, had significantly higher tannin contents in the testa than recessive genotypes. Similarly, homozygous recessive plants in the F_2 generation, with genes controlling the diffuse distribution of pigment in the flowers, had higher tannin content than plants with spotted flowers. In contrast, when the spotted flowers were governed by a dominant allele, the effect on tannin content was the opposite. However, a study by Cabrera and Martin (1989) revealed a relationship between pigmentation in flowers or testa colour and tannin content. The white-flowered lines carry genes that influence tannin content in quite a different way. The two recessive genes in *Vicia faba* that control the white flower colour, also control the absence of tannin in the tannin-free lines (Crofton et al., 2000).

2.6 Biosynthesis of flower pigments and defence compounds

Plants attacked by pathogenic microbes respond with a number of protective biochemical changes (Tarchevsky, 2001). Flavonoids, which are phenolic compounds, are one of the major defensive compounds. Flavonoids, including plant pigments (such as phlobaphenes, anthocyanidins and anthocyanins) and colourless compounds (such as flavones, flavonols, flavanones, dihydroflavonols, leucoanthocyanidins and proanthocyanidins), are synthesised in the phenylpropanoid or flavonoid pathway (Winkel-Shirley, 2001; Davies et al., 2002). Different enzymes regulate metabolite synthesis and its levels in various branches of the flavonoid pathway. The crucial enzymes involved in this pathway consist of chalcone synthase (CHS), PAL, isoflavone reductase (IFR), flavonoid 3'-hydroxylase (F3`H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) (Figure 2.1).

Flavonoids are responsible for a series of metabolic changes in plants, from phytoalexin biosynthesis, to lignification and suberisation to prevent pathogen ingress (Regnault-Roger et al., 2005). The key enzyme for the synthesis of phenolics in plants is PAL, constituting the defence system (Dayaram et al., 2003). By challenging lucerne seedlings with the fungal pathogen *Colletotrichum trifolli,* Saunders and O'Neill (2004) reported three enzymes to be involved in flavonoid biosynthesis (Figure 2.1): PAL, CA4H and IFR. The PAL and CA4H enzymes lead to many different metabolic pools including flavonoids, coumarins and lignin-derived compounds (Saunders and O'Neill, 2004). In legumes, IFR rearranges the flavonoid carbon skeleton, leading to the accumulation of a wide range of simple isoflavonoids, coumestans, pterocarpans and isoflavans (Dixon and Paiva, 1995; Jung et al., 2000).

A key reaction in the biosynthesis of the phytoalexin medicarpin in legumes is 4'-O-methylation of an isoflavonoid intermediate (He and Dixon, 2000). The production of isoflavone O-methyltransferase (IOMT) from lucerne *in vitro,* as well as *in vivo* in unchallenged leaves of transgenic lucerne, converts the isoflavone daidzein to 7-O-methyl daidzein (isoformononetin). When lucerne plants were infected with *Phoma medicaginis* fungus, overexpression of IOMT resulted in increased induction of the phenylpropanoid/isoflavonoid pathway, and further, resulted in a greater accumulation of formononetin (4'-O-methyl daidzein) and medicarpin in the leaves of infected compared to control plants, in which no

isoformononetin was produced (He and Dixon, 2000). Plants with IOMT-overexpressed were resistant to *P. medicaginis* (He and Dixon, 2000). The enzyme F3`H is involved in the formation of dihydroflavonols from flavanones (narangenin) (Winkel-Shirley, 2001).

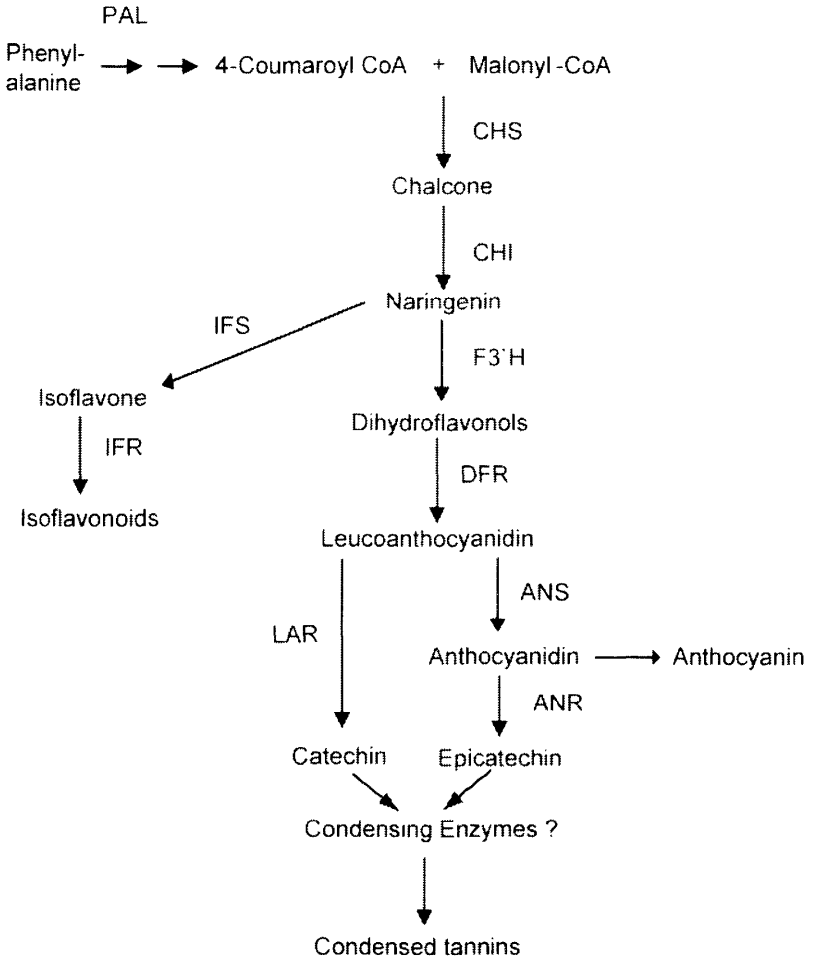

Figure 2.1. Schematic of the major branches of the phenylpropanoid pathway. Phenylalanine is the first substrate used. This substrate is converted to 4-coumaroyl coenzyme A (CoA) under catalysis of phenylalanine ammonia-lyase (PAL) and two subsequent enzymes. The incorporation of 4-coumaroyl CoA and malonyl CoA is catalysed by chalcone synthase (CHS) in the main branch to produce chalcone. This substrate is catalysed by chalcone isomerase (CHI) and produces flavanones (naringenin) which is used as a substrate for three branches. Two of the branches are catalysed by the flavonoid 3` hydroxylase (F3`H) lead to the production of dihydroflavonols (only one of these two branches is shown). Dihydroflavonol 4-reductase (DFR) catalyses the conversion of dihydroflavonols to leucoanthocyanidins. This substrate is converted to anthocyanidin by anthocyanidin synthase (ANS). Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) catalyse conversion of anthocyanidin and leucoanthocyanidin respectively to epicatechin and catechin which are proanthocyanidin precursors. In the other branch chalcone is catalysed by isoflavone synthase (IFS) to produce isoflavone. Isoflavone is catalysed by five enzymes including isoflavones reductase (IFR) and finally produces the end products in this branch called isoflavonoids.

Boddu et al. (2004) reported that in sorghum, ingress of *Cochliobolus heterostrophus* in the first 24 hours after infection induced 3-deoxyanthocyanidin phytoalexins, apigeninidin and luteolinidin synthesis. Characterisation and comparison of the cDNA sequence of two phytoalexins showed that their cDNA sequences correspond to a

putative F3'H. The transcription of F3'H was regulated with that of CHS and DFR, and expression of these genes was induced within the first 24 hours of fungal challenge. Although, Boddu et al. (2004) reported that the synthesis of apigeninidin and luteolinidin followed the induced expression of the F3'H gene, Halbwirth et al. (2002) reported that the F3'H activity seemed to be blocked. Consequently, the enzymatic reduction of flavanones to flavan-4-ols resulted in the accumulation of the 3-deoxyflavonoids.

A later key enzyme in the phenylpropanoid pathway, DFR, (Figure 2.1) conducts the flux into biosynthetic branches resulting in anthocyanidin and condensed tannin production (Xie et al., 2004a). It catalyses the formation of leucoanthocyanidins, which are common precursors for anthocyanidin and proanthocyanidin synthesis from three types of dihydroflavonols: dihydrokaempferol, dihydroquercetin and dihydromyicetin (Xie et al., 2004a). Induction of DFR activity in plants leads to accumulation of proanthocyanidin (Xie et al., 2004a) and anthocyanins (Liu et al., 2005; Shimada et al., 2005). In contrast, inactivation of the DFR gene in yellow onions (*Allium cepa*) results in blockage of anthocyanidin production (Kim et al., 2004; Kim et al., 2005).

The crucial branch of the phenylpropanoid pathway is the conversion of leucoanthocyanidins to anthocyanidins and proanthocyanidins (Figure 2.1). Anthocyanidins never accumulate in the free states. They are extremely unstable and only exist as transient intermediary compounds, probably bound to the enzyme complexes that synthesise them (Waterhouse, 2002). Of course, 3-deoxy anthocyanidins that act as phytoalexins in sorghum (Nicholson, et al., 1987) and possibly in sugarcane tissues (Viswanathan, 2002) are exceptions. 3-O-glucosylation of anthocyanidins results in formation of anthocyanins (Xie et al., 2004a). Anthocyanins, which are colour compounds, are responsible for different flower colours, consisting of red, pink, orange, scarlet, purple, blue, blue-black and some yellow (Davies et al., 2002). No known normal (not 3-deoxy-) anthocyanins have any proven direct role in disease resistance (Hammerschmidt and Nicholson, 1977), although they can accumulate after infection (Hipskind and Nicholson, 1996). It is now well understood that anthocyanins tend to accumulate in damaged cells (Steyn, et al., 2002).

The expression of flavonoid biosynthetic genes is under developmental control during anthocyanidin production in flower petals or seedling cotyledons (Davies et al.,

2002). Anthocyanidin synthase catalyses the conversion of leucoanthocyanidin to anthocyanidin (Holton and Cornish, 1995; Tanner et al., 2003; Xie et al., 2004b; Bogs et al., 2005). Then, ANR catalyses the NADPH-dependent reduction of anthocyanidins to 2,3 cis flavan-3-ols such as epicatechins (Holton and Cornish, 1995; Marles et al., 2003; Tanner et al., 2003; Xie et al., 2003; Xie et al., 2004b; Bogs et al., 2005), whereas, LAR, another enzyme in this cascade, converts leucoanthocyanidins to 2,3 trans flavan-3-ols such as catechins (Devic et al., 1999; Tanner et al., 2003). The factors controlling the condensation of monomeric 2,3 cis flavan-3-ols and 2,3 trans flavan-3-ols to form condensed tannin polymers are still unknown (Xie et al., 2004a; Xie et al., 2004b). However, a new development in this area has revealed that ANR is encoded by a single gene, while LAR is encoded by two highly related genes (Bogs et al., 2005).

Production of white flowers in *Vicia faba* is controlled by two different recessive genes (Crofton et al., 2000). These genes are linked to the absence of tannin (Crofton et al., 2000). However, it has been reported (Davies et al., 1993; Nakatsuka et al., 2005) that production of white-flowered plants is the result of two mutations in the phenylpropanoid pathway. Two mutations occurred in gentian which has blue flowers in nature. The mutations resulted in the production of two white-flowered cultivars, Homoi and Polano. Tests were performed to determine mutation loci (Nakatsuka et al., 2005). Protein (enzyme) measurements using the Western blot technique revealed that the Homoi cultivar lacked ANS. In addition, Southern blot analysis confirmed the deficiency of one or two ANS loci in the Homoi cultivar. Transient expression of ANS in flower petals also strongly suggested that the Homoi cultivar was derived from the ANS mutation (Nakatsuka et al., 2005). However, the mutation that occurred in the blue-flowered cultivar to create the white-flowered cultivar Polano, is completely different from the Homoi cultivar. Northern blot analysis of Polano RNA showed decreased expression of the ANS gene as well as 10 flavonoid biosynthetic structural genes, including CHS, F3`H, DFR (Figure 2.1), which were previously reported to be temporally regulated in the naturally blue-flowered cultivar. Moreover, analysis of stress-induced flower pigmentation suggested that a defect in one or more regulatory factors controlling the later steps of flavonoid biosynthesis is responsible for white colouration in the Polano cultivar (Nakatsuka et al., 2005).

2.7 Conclusion

Breeding disease resistant plant cultivars and lines is the best control approach to overcome soilborne fungal pathogens. Identification of the pathogen, recognition of resistant plant cultivars to the pathogen, and knowing the mechanisms of resistance in resistant plant cultivars are necessary to breed disease resistant plant cultivars. There wwer observations from the *S. formosa* breeding program at the University of New England that white- or pink-flowered varieties were more susceptible to root disease, particularly in greenhouse conditions (unpublished information). As there is little published information on diseases of *S. formosa*, or on breeding or selection for resistance in this plant, the review has focused on leguminous plants.

In many cases, resistance to disease results from the activation of more than one biochemical defence mechanism (Ebel and Grisebach, 1988). However, resistant plant cultivars resist pathogens through passive or mechanical barriers to infection, preformed chemical defences, and resistance mechanisms that are activated by exposure to pathogens (Hammond-Kosack and Jones, 1996).

A relationship has been observed between disease resistance and coloured flowers and other coloured plant tissues, such as coloured seeds and coloured hypocotyls. Synthesis of the pigment complex in plants constitutes a defence response to pathogens (Nicholson et al., 1987). This phenomenon has been observed in many genera in Fabaceae (Guevara et al., 1986; Higuera and Murty, 1987; Cobos et al., 2005).

Generally, resistant plants defend themselves against microbial attack through either preformed secondary metabolites called phytoanticipins or induced phytocompounds synthesised in challenges between pathogens and non-host plants in incompatible interactions. Both compounds can be either tissue specific or nonspecific. Phenolic compounds synthesised through the phenylpropanoid pathway include the main defensive compounds in leguminous plants. The concentration of these compounds in coloured plant tissues is higher than in colourless or white tissues.

Preformed antimicrobial compounds or phytoanticipins are constitutive and present at an inhibitory level to pathogens in intact plant tissues before infection without a *de novo* synthesis after microbial attack (Schlosser, 1994; Slusarenko et al., 2000). Some phenolic compounds occur constitutively and are thought to function as

preformed inhibitors associated with non-host resistance (Nicholson and Hammerschmidt, 1992; Schlosser, 1994). Tannins, which are phenolic compounds, inhibit fungal growth in high-tannin-containing cultivars (Bond and Smith, 1989; Kantar et al., 1996) and also bind to proteins (enzymes) and block their activities (Schlosser, 1994).

The experimental chapters following (Chapters 3-6) examine the correlation between flower colour and resistance to *P. cinnamomi* and some other fungi, and the variety and form of phytocompounds responsible for resistance, and the genetic basis of resistance.

Chapter 3 Variation in, and inheritance of, resistance to *Phytophthora cinnamomi* among lines of Sturt's desert pea

3.1 Introduction

Plant tissues with different colours exhibit different reactions to fungal disease (Statler, 1970; Kantar et al., 1996; Punyasiri et al., 2005), bacterial disease (Park et al., 1999), insect attack (Boughdad et al., 1986; Lattanzio et al., 2005) and mechanical damage (Ochodzki and Piotrowska, 2002).

White-flowered lines of *Vicia faba* are more susceptible than coloured lines to infection with the fungal soilborne pathogens *Fusarium culmorum* and *Pythium debaryanum* (which is from the same family as *P. cinnamomi*) during seed germination, but are equally resistant during seedling growth (Kantar et al., 1996). Similarly, bean varieties with green hypocotyls and white testae were reported to be more susceptible to *Fusarium solani*, the causal agent of *Fusarium* root rot, and *Rhizoctonia solani* than varieties with purple hypocotyls and black testae (Prasad and Weigle, 1969; Statler, 1970). Observations, from the breeding program for *Swainsona formosa* at the University of New England, that white- or pink-flowered lines were more susceptible to root diseases, particularly under greenhouse conditions (unpublished information).

Understanding the inheritance of resistance to disease is essential for effective breeding of resistant cultivars because breeding strategies vary depending on whether resistance is based on a limited number of genes or multiple genes (Jansky et al., 2004; Lithourgidis et al., 2005). It is also important to know whether resistance is governed by dominant or recessive alleles. If the resistance is dominant, it can be simply transferred to desirable germ plasm (Jansky et al., 2004).

There is little published information regarding *S. formosa* diseases (Williams, 1996; Summerell et al., 1997). The main objectives of this work were to determine (1) the resistance or susceptibility of three *S. formosa* lines with different flower colours to the typical soilborne pathogen *Phytophthora cinnamomi*; (2) the most useful assay for a breeding program, and (3) the mode of the inheritance of resistance to *P. cinnamomi*.

3.2 Materials and methods

In all experiments *S. formosa* white, pink and red lines were used. Seeds were supplied from the *S. formosa* breeding program conducted at the University of New England. *Phytophthora cinnamomi* isolate DU041 was supplied by Dr David Cahill, Deakin University, Victoria, Australia.

3.2.1 Seed germination

Seeds from greenhouse-raised plants were surface-sterilised in 1.0% (w/v) sodium hypochlorite for 10 minutes, rinsed in sterile distilled water, and rinsed again in just-boiled water for 10 sec. Sterilised seeds were soaked overnight in cool, sterile distilled water. Within 24 hours, swollen seeds were plated onto moistened double-layer sterile filter paper in Petri dishes. The Petri dishes were placed on a shelf in a culture room at a temperature of 23 ± 2 °C under a 16-hour photoperiod. Seeds germinated after 48 hours. To obtain uniform seedlings, testae were removed soon after radicle emergence. Seedlings were transplanted into Jiffy™ compressed peat pots (GIGS-Company, 2005) two days after removing testae. Jiffy pots were prepared by soaking in water for 10 hours. Seedlings were kept in a shaded greenhouse, with a light intensity ranging from 100 to 170 μmol m^{-2} s^{-1}, a day/night temperature of 25/18 °C and a relative humidity of approximately 80%. Three weeks after germination, seedlings were transplanted into 20 cm diameter plastic pots (5 litre capacity), containing a 3: 1 (v/v) sand: peat mixture at a density of one plant per pot. Before use, the sand and peat mixture was wetted down and pasteurised at 80 °C for 30 minutes to eliminate pathogenic microorganisms and weed seeds. Seedlings were kept in a greenhouse with an average temperature range of 25 to 32 °C, and light intensity ranging from 650 to 1200 μmol m^{-2} s^{-1}. Seedlings were watered once a day using an automatic drip-watering system. Approximately 200 mL per plant of supplementary liquid fertiliser, Hortico Aquasol™ (23% N, 4% P, 8% K, 0.05% Zn, 0.06% Cu, 0.0013 Mo, 0.15% Mn, 0.06% Fe, and 0.11% B) was applied weekly at a concentration of 1 g L^{-1} (Zulkarnain, 2003).

3.2.2 Zoospore production

Zoospores of *P. cinnamomi* were produced using the technique of Byrt and Grant (1979). Circles of cheesecloth were rinsed with glass-distilled water and were

autoclaved prior to placement on V8 agar for inoculation. One litre of V8 agar consisted of 200 mL V8 juice (Campbell's Soups, Sydney, Australia), 2 g calcium carbonate, 20 g agar and 800 mL distilled water, adjusted to pH 6.0-6.5 prior to autoclaving. Each cheesecloth plate was inoculated with five to seven pieces of colony (2 mm^2) taken from a seven-day-old culture of *P. cinnamomi* grown on V8. The cheesecloth plate was then incubated at 24 °C for four to six days in darkness.

The cheesecloth pad was lifted gently from the agar surface and transferred to a 250 mL Erlenmeyer flask containing 100 mL cleared V8 juice broth (100 mL V8 juice, 4 g calcium carbonate and 100 mL of glass-distilled water, centrifuged at 800 *g* for 20 minutes, the supernatant filtered through Whatman number 1 filter paper, diluted 10-fold with glass-distilled water, and adjusted to pH 6.0-6.5 prior to autoclaving). Flasks were shaken at 160 rpm under fluorescent light with a light intensity of 40 µmol m^{-2} s^{-1} for 19 hours at 24 °C. The mat was washed twice in a mineral salt solution (calcium nitrate 0.01 M, potassium nitrate 0.005 M, magnesium sulphate 0.004 M, plus chelated iron) for 45 minutes each time (Chen and Zentmyer, 1970). The chelated iron solution was added at 1 mL per 1 litre of mineral salt solution after autoclaving. This chelated iron solution contained 13.05 g EDTA, 7.5 g potassium hydroxide, 24.9 g ferrous sulphate heptahydrate and distilled water to 1 litre and was sterilised by filtration through a Millipore filter of 0.22 µm pore size. Finally, the mats were suspended in 40 mL of mineral salt solution and shaken by orbital shaker for a further 24 h at 160 rpm under fluorescent light at 24 °C, during which time the sporangia were formed.

Zoospores were released by using cold shock treatment with three 5-10 minutes washes with 4 °C sterile glass-distilled water. Following the addition of 40 mL of sterile glass-distilled water, each Erlenmeyer flask containing a mat was cooled on ice for 30 minutes, then transferred to a dark incubator for 1 hour at 24 °C. Zoospores were released from the sporangia by washing with sterile glass-distilled water, pre-cooled to 18 °C, and then incubating in 40 mL of glass-distilled water per mat at 18 °C for a further 50-90 minutes.

Zoospore suspensions were filtered using Whatmann number 541 filter paper to remove mycelia and chlamydospores, and zoospores were encysted by vigorous shaking in plastic bottles for two minutes on a wrist action shaker to facilitate counting. Zoospore concentration was determined using a light microscope, in the lens magnification of 10×, and a haemocytometer. When required, the suspension was

diluted using a basic salt solution containing 1.75 mM calcium chloride, 1 mM potassium chloride and 1 mM magnesium sulphate (Mitchell and Yang, 1966; Kong et al., 2003).

3.2.3 Root dip assay

The root assay was performed using a completely randomised design with five replicates plus a control for each line of Sturt's desert pea. This experiment was performed once. Each experimental unit consisted of two, three-week-old *S. formosa* seedlings. After germination, each seedling was transferred to a small pot containing sand and perlite (v:v; 1:1). The potting mixture had been moistened and autoclaved at 121 °C for 1 hour for two consecutive days prior to potting up (Babai-Ahary et al., 2004). From this mixture, the seedlings could be removed easily for inoculation and lesion measurement. The seedlings were inoculated with *Phytophthora cinnamomi* zoospores at 1000 zoospores per mL. Inoculation was performed after removing the seedlings from the pots, rinsing with distilled water and drying with a paper towel.

To prevent contamination of the aerial portions of the seedlings, roots of each seedling were placed into a 5 mL pipette tip which was lowered into a culture tube containing the zoospore suspension (Figure 3.1). Approximately 0.5 cm of the root tip was submerged into the inoculum (Wagner and Wilkinson, 1992).

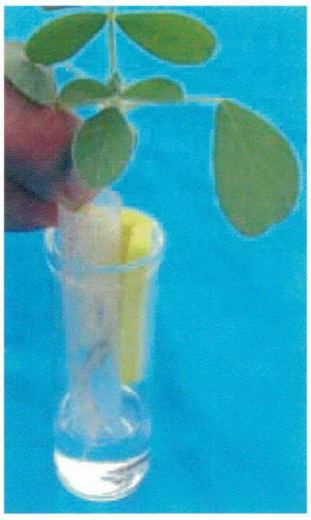

Figure 3.1. Inoculation of *Swainsona formosa* with *Phytophthora cinnamomi* zoospores by placing roots in a pipetted tip and immersing 0.5 cm of the roots into a zoospore suspension.

After 20 minutes exposure to the inoculum, the seedlings were re-potted and returned to the greenhouse as described above. After three weeks the seedlings were removed from the pots, and the root systems rated for disease symptoms as follows:

0 = no symptoms

1 = browning extends less than 2 cm from tip of main root; no symptoms observed on lateral roots

2 = browning present on first 2-3 cm of main root; less than 20% of lateral roots are browned

3 = browning present on first 3-4 cm of main root; browning occurs on more than 50% of length of lateral roots; seedling wilted

4 = browning is present on more than 4 cm of main root; lateral roots are completely browned, seedling dead

Diseased roots were harvested. The fungus was reisolated from diseased roots, and cultured on V8 agar. The fungus was compared with the initial *P. cinnamomi* used for root inoculation.

3.2.4 Hypocotyl assay

A completely randomised design with five replicates plus control for each of the three lines was used to determine the susceptibility of the *S. formosa* hypocotyl to *Phytophthora* infection. Each replicate (experimental unit) consisted of two, three-week-old seedlings grown in separate Jiffy™ pots. Two inoculated seedlings of each replicate were placed on an inverted Petri dish in a small colourless plastic container containing water to a depth of 1 cm (Figure 3.2). The plastic containers were covered to increase humidity. This experiment was conducted twice.

Inoculum for infection of the hypocotyl was prepared by growing *P. cinnamomi* for 1 week on V8 agar. Seedlings were inoculated with a hypodermic syringe after the method of Burnham et al. (2003a). Colonised agar from a plate of *P. cinnamomi* was forced through the syringe to create a slurry. The slurry was drawn back into the syringe. Seedlings were inoculated by scratching the hypocotyls with the needle of the syringe and placing a drop of agar-mycelium mixture onto the wound. Seedling hypocotyls were about 6 cm long, and the distance between apex and the

inoculation site was approximately 3 cm. As a control for each line, the stems of control plants were scratched and a drop of sterile distilled water was applied to the wound. Following inoculation, seedlings were replaced into plastic containers. The containers were incubated at 24 °C with a 12 hour light period of 30 μmol m^{-2} s^{-1}. Recording was done five, eight and 10 days after inoculation according to the scale below:

0 = no symptoms

1 = browning beyond inoculation site up to 5 mm long

2 = lesion length more than 5mm, but had not reached the cotyledon

3 = lesion at cotyledon, cotyledon wilted

4 = bending/breaking of stem

5 = plant death

The score for each replicate was the average score of the two seedlings.

The fungus was reisolated from diseased hypocotyls, and cultured on V8 agar. The fungus was compared with the initial *P. cinnamomi* used for hypocotyl inoculation.

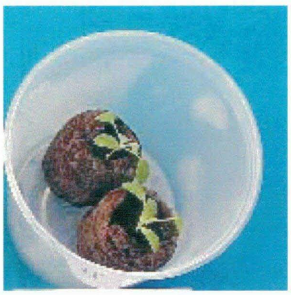

Figure 3.2. Inoculated *Swainsona formosa* seedlings. The seedling hypocotyl was inoculated with *Phytophthora cinnamomi* mycelium. Two seedlings of each replicate were placed on an inverted Petri dish in a small colourless plastic container. The container contained water to a depth of 1 cm.

3.2.5 Detached stem assay

The detached stem assay was conducted as a completely randomised design with seven replicates for each line, plus a control (non-infected detached stem). Each replicate consisted of two 11 cm detached stems from eight-week-old plants. Stems were cut from the ends of branches and trimmed of leaves after cutting. Prior to inoculation, the stems were surface-sterilised with 70% ethanol for 5 seconds, rinsed

in sterile distilled water and blotted dry. The cut ends of both stems were placed in one-week-old *P. cinnamomi* cultures growing in 1 cm of V8 agar contained within 12 cm tall glass jars. The basal 1 cm of each stem segment was placed in the medium. The length of lesion of the stems, above the surface of the medium, was measured two, three, and four days after inoculation. The fungus that covered the surfaces of the infected stems was reisolated and cultured on V8 agar. The fungus was compared with the initial *P. cinnamomi* used for stem inoculation. This experiment was performed twice.

3.2.6 Detached leaf assay

This assay was conducted based on the method of Irwin et al. (2003). A completely randomised experimental design with five replicates for each *S. formosa* line, plus a control (non-infected detached leaflets) was used. Three inoculum concentrations: 2000, 5000 and 10000 *P. cinnamomi* zoospores per mL of water were used. Each replicate consisted of three young leaflets from an individual plant with the abaxial side placed on the surface of a Whatman number 1 filter paper in the centre of a Petri dish. Filter papers were moistened with 1.5 mL sterile distilled water. Each leaflet was inoculated with 10 µL of the zoospore solution. Control leaflets were inoculated with 10 µL sterile distilled water. Plates were incubated at 24 °C with a 12 h light period of 30 µmol m^{-2} s^{-1}. Recording was performed 48, 72, 96 and 120 h after inoculation under a stereomicroscope according to a 1-5 disease scale, where:

1= no symptoms (necrosis, chlorosis, or cell collapse)

2= necrosis and chlorosis only at inoculation site and no cell collapse present

3= necrosis, chlorosis and cell collapse beyond the inoculation site, but confined to <25% of the leaf area

4= necrosis, chlorosis and cell collapse confined to >25% but ≤50% of the leaf area

5= no necrosis or chlorosis present at the inoculation site, and >50% of the leaf showing cell collapse.

3.2.7 Stigma receptivity

Swainsona formosa flowers are suspended in groups of three to seven, and mostly in groups of six, on a long peduncle. A second inflorescence sometimes forms above the main inflorescence (Williams, 1996). In the subfamily Faboideae, the

corolla is comprised of a standard petal, two wing petals, and a keel made up of two petals fused at the margin (Nair et al., 2004). The bases of nine of the ten stamens are fused together around the pistil to form a staminal column (Nair et al., 2004). *Swainsona formosa* is hermaphroditic plant in which both male and female floral organs are fully functional and the flowers produce a large amount of pollen (Barth, 1990; Williams, 1996). The *S. formosa* stigma is covered by a stigmatic cuticle that, unless ruptured, prevents self-pollination (Jusaitis, 1994). A cross was performed on the flowers of one inflorescence after determining the stigmatic receptivity of one flower from each inflorescence. Stigma receptivity was determined using the Galen and Plowright (1987) test as follows. One stigma from each inflorescence was placed into a Petri dish. A drop of 6% hydrogen peroxide was trickled onto the stigmatic surface and its reaction observed under a stereomicroscope or unaided. Bubbles formed on receptive stigmas immediately after the addition of the hydrogen peroxide.

3.2.8 Production of F_1 and F_2 plants

In most investigations of inheritance of disease resistance, resistant cultivars have been selected as male (Alzate-Marin et al., 1999; de Arruda et al., 2000; Burnham et al., 2003a). In this research in the cross between the resistant (red) line and susceptible (white) line, the resistant line chosen was also male. In addition, other reciprocal crosses were made in the greenhouse between the red line and the pink line and also between the pink line and the white line of *S. formosa*. Crosses were made after determining the stigma receptivity of each inflorescence. By bending back the keel of the receptive white flower to expose the stamens, flowers were emasculated and their stigmas were ruptured, using a fine paint brush. Pollen grains from the newly opened flowers of the male parent in each cross were applied to stigmas of the female parent. The pollinated inflorescences were enclosed in nylon organza bags to exclude insect pollinators. The F_1 plants grown in the greenhouse were allowed to self-pollinate (without access by insects), by rupturing their stigmas, to produce F_2 seeds.

3.2.9 Assessment of inheritance of disease resistance

Parental and F_2 plants were grown in the greenhouse. At the flowering stage, a detached stem assay of the F_2 progeny of *S. formosa,* with the stems of the parental

plants as the control, was performed to investigate the resistance or susceptibility of the F_2 progeny to *P. cinnamomi*. Five individual stems of each parental line, and individual stems from 96 plants of the segregated F_2 population were inoculated, after recording their flower colour, by dipping into a *P. cinnamomi* culture as described in Section 3.2.5. Four days after inoculation, uninfected stems were recorded as resistant and completely infected stems were recorded as susceptible. There were no intermediate symptoms. To assess the relationship between the observed and the expected data, a Chi-square (χ^2) value was calculated using the formula designed by Jansky et al. (2004) as follows:

$\chi^2 = [(R - Er)^2/Er] + [(S - Es)^2/Es]$

where: R = Number of resistant progenies

Er = Expected number of resistant progenies

S = Number of susceptible progenies

Es = Expected number of susceptible progenies.

3.2.10 Data analysis

As the data for hypocotyl and root assays for disease susceptibility were categorical, the Kruskal-Wallis test was used to compare treatments. ANOVA was used for data analysis in the other experiments. All analyses were conducted using the SPSS™ statistical package. The criterion for significance of treatment effects was $P < 0.05$ unless otherwise indicated.

3.3 Results

3.3.1 Root assay

There was a significant ($P < 0.01$) difference between lines in the severity of symptoms. No lesions were seen on the roots of the red line, which remained similar to the controls. Lesions extended over most of the roots in the white line (Figure 3.3) and most of these plants died. The reaction of the pink line was intermediate between the other two. The pathogen was successfully reisolated from the infected roots.

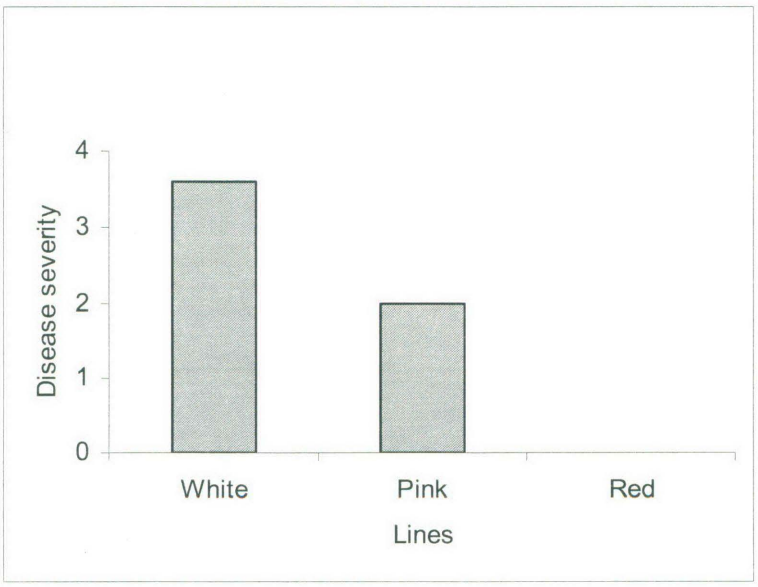

Figure 3.3. Root reaction to *Phytophthora cinnamomi* in three lines of *Swainsona formosa* inoculated by dipping roots in a zoospore suspension. There was five replicates plus a control for each line. Each experimental unit consisted of two, three-week-old *S. formosa* seedlings. Disease was rated three weeks after inoculation on a 0-4 scale where 0 = no symptoms and 4 = plant death.

3.3.2 Hypocotyl assay

In the hypocotyl assay the effect of variety was highly significant ($P < 0.01$) in all three lines, 5, 8 and 10 days after inoculation. Table 3.1 and Figure 3.4 illustrate that disease severity in the white line was high at each of the three post-inoculation scoring times. This line was susceptible to *P. cinnamomi*. The red line showed no symptoms. Therefore, the red line can be confirmed as resistant. The pink line showed an intermediate response to the disease. Ten days after inoculation, the white line was dead; the hypocotyl of the pink line was brown, and the hypocotyl of the red line showed no symptoms. The pink-flowering plants were relatively higher resistant than the white-flowering plants. Only an issue of slower development of the disease was observed, thus the inoculated seedlings eventually died. The pathogen was successfully reisolated from the infected seedlings. This experiment was conducted twice with similar results in each repetition.

Table 3.1 Severity of symptoms on hypocotyls of three-week-old seedlings of 3 lines of *Swainsona formosa* after inoculation with *Phytophthora cinnamomi*. Disease severity was measured 5, 8 and 10 days after inoculation, using a scale of 0-5 where 0 = no symptoms, and 5 = plant death

Lines	Mean Severity Score (± SE)		
	5th day	8th day	10th day
White	2.8 ± 0.2	4 ± 0.31	5 ± 0
Pink	1 ± 0	1.4 ± 0.24	2 ± 0
Red	0	0	0

Figure 3.4. Hypocotyl reaction to *Phytophthora cinnamomi* in three lines of *Swainsona formosa* 10 days after inoculation.

3.3.3 Detached stem assay

Symptoms appeared on the fourth day after inoculation, and there was a significant ($P < 0.01$) difference between lines in the severity of symptoms. No lesions were observed on the red line, which remained similar to the controls. Lesions extended over most of the length of detached stems taken from the white line (Table 3.2), while the pink line showed symptoms intermediate between the other two. The

pathogen was successfully reisolated from the infected stems. This experiment was conducted twice with similar results in each repetition.

Table 3.2. Mean lesion length in the detached stem assay of *Swainsona formosa*. This experiment was conducted with 7 replicates. Each replicate consisted of two, 11 cm trimmed detached stems from eight-week-old plants placed in the culture of *Phytophthora cinnamomi*. Lesion length was measured four days after inoculation.

Lines	Lesion length (mm)	Standard Error
Red	0	0
Pink	31.7	3.36
White	92.1	1.62

3.3.4 Detached leaf assay

No symptoms of disease were observed on inoculated detached leaves of any of the three lines.

3.3.5 Stigma receptivity and crossing

Stigmas were receptive when the flower-bud lengths exceeded 32 mm. They remained receptive until flower-bud length reached the maximum size (65-70 mm), and even after the stigmas protruded from the keels. The pollen grains appeared synchronously with the standard petal opening, when the flower-bud lengths were about 47 mm. Crosses carried out on 32-38 mm long flowers failed to produce pods. All 78 crosses performed on flowers within this size range produced no pods. The crossed flowers abscised before or shortly after pod production. The most successful crosses were the ones carried out on flowers 39-45 mm in length. Almost 55% of these crosses produced mature pods. No cross was performed on flowers greater than 45 mm because of pollen grain release.

3.3.6 The inheritance of flower colour in the F_1 and F_2 generation

From the crosses made between the *S. formosa* lines, only the red × white cross produced pods with viable seeds. The other crosses either failed to produce pods or produced pods with non-viable seeds. All F_1 plants grown from red × white crosses

produced red flowers. In the F_2 generation, 23 plants produced white flowers and 73 plants produced red flowers. The Chi-square analysis of the data confirmed that the observed ratio (3.17:1) was not significantly different from 3:1 (χ^2), suggesting that segregation was consistent with a single locus, with red colour being dominant over white colour.

3.3.7 The inheritance of disease resistance

All detached stems of individuals of the parental red line (resistant control) inoculated with *P. cinnamomi* were asymptomatic at four days after inoculation. In contrast, all inoculated stems of individuals of the parental white line (susceptible control) were completely infected four days post-inoculation. Inheritance of disease resistance in the F_2 progeny segregated in a Mendelian fashion. All red-flowered F_2 progeny remained healthy while all white-flowered F_2 progeny were completely infected. Chi-square analysis of the data ($\chi^2 = 0.056$, $P<0.05$) confirmed that the resistant to susceptible ratio in the F_2 generation was 3:1. This ratio suggests that segregation was consistent with a single structural gene in *S. formosa* red line being responsible for its resistance to *P. cinnamomi*.

3.4 Discussion

The detached stem, hypocotyl and root assays revealed a wide range of reactions of the three *S. formosa* lines to *P. cinnamomi*, from the red line which was completely resistant to the white line which was highly susceptible. As would be expected if there was a close link between flower colour and disease resistance, the reaction of the pink line was intermediate in all experiments. The three lines revealed no difference to damage caused by manipulation, and the pathogen was successfully reisolated from the infected plants in all experiments. By themselves these experiments do not prove that differences in flower colour were responsible for the differences in resistance, but they do lend support to the observations of growers that at least some white- and pink-flowered lines are more susceptible to root diseases than red lines. Because white lines of this ornamental plant are preferred in some markets, it is important to determine whether it is possible to obtain white-flowered lines with high disease resistance. Some large-scale crossing experiments were conducted to determine the strength of any genetic linkage between flower colour and disease resistance.

The F_1 seeds obtained from the cross between the white- and red-flowered lines in the F_1 generation produced only red-flowered plants. In addition, the red to white flower colour ratio in the F_2 population was 3:1, indicating that the red flower colour is dominant over the white flower colour. This is in concert with Mendelian inheritance and has been reported in similar studies (Khan, 1990; Ren et al., 2000; Huh et al., 2001).

All the red-flowered plants in the F_2 population obtained from the red × white crosses were resistant to *P. cinnamomi*, while all the white-flowered plants were susceptible. The phenotypic ratios (resistant:susceptible) of 3:1 in the F_2 progeny, which is based on Mendelian rule and has been reported by others (Alzate-Marin et al., 1999; Alzate-Marin et al., 2000; de Arruda et al., 2000; Burnham et al., 2003a), indicates that the inheritance of the resistance in the *S. formosa* red line to *P. cinnamomi* is controlled by a single dominant gene.

The high level of resistance of the red line to infection by *P. cinnamomi* indicates that this fungus is not likely to be a significant contributor to the root rot syndrome in wild-type plants. However, it enabled very good discrimination in resistance between the three lines and may therefore be useful as an indicator for general pathogen resistance in *S. formosa*. Conversely, the presence of high, low and intermediate levels of resistance in the one plant species may make this a useful model for mechanisms of resistance to *Phytophthora* diseases in legumes.

The relative disease resistance of the three *S. formosa* lines was the same in both the root system and aerial parts of the plants. Although root rots are the major problem in cultivation, conducting disease assays on roots is difficult and time-consuming. Furthermore, considerable greenhouse space is required for prolonged time periods ranging from 4-6 weeks. In contrast, the major potential advantage of using aerial portions such as the hypocotyl assay is the rapidity of obtaining a result, (4 days after inoculation) and saving in greenhouse space. One disadvantage of using the hypocotyl assay is that, a few days after seedling infection and recording disease symptoms, the seedlings destroy. Thus, it is not possible to determine both disease resistance and flower colour on the same plant, which is desirable when screening to segregate populations. Detached leaf and stem assays can be conducted on plants at the flowering stage. Detached leaf assays are very efficient in terms of space and quantity of plant tissue required, and have been used to assay *Phytophthora* resistance in other legumes (Irwin et al., 2003). However, the detached leaf assay did not work

in this study, presumably because of the difficulty of inoculating a plant with very hairy leaves. The detached stem assay gave a sensitive, quantitative and reproducible discrimination in resistance level between lines and appears to be the best method to use in a breeding program.

The resistance or susceptibility to *P. cinnamomi* in *S. formosa* parental lines and in the F_2 population of the only successful cross was observed to be closely related to flower colour. It appears that in *S. formosa* red line, the genes responsible for flower colour and resistance to *P. cinnamomi,* and presumably other soilborne fungal pathogens, are closely linked, and it seems very likely that they are the same gene. The resistance to pathogen attack in many plants is achieved by plant secondary metabolites with antifungal activities. These substances produced by structural genes exist in healthy plants in their biologically active forms (Osbourn, 1996). The lack of a structural gene which is responsible for the production of a phytocompound with antifungal activity to *P. cinnamomi* in the white line and its presence in the red line may be a possible explanation for the susceptibility and resistance, respectively, of these lines to *P. cinnamomi*.

To my knowledge, no data are available in the literature regarding the genetic control of resistance to *P. cinnamomi* and other pathogens in *S. formosa*. The resistance of other leguminous plants to fungal pathogens, particularly *Phytophthora* species, is commonly controlled by a single dominant gene. For instance, the resistance of soybean to *P. sojae* (Moots and Nickell, 1987; Burnham et al., 2003a), cowpea to *P. vignae* (Ralton et al., 1988), faba beans to *Sclerotinia* stem rot (Lithourgidis et al., 2005), common bean (*Phaseolus vulgaris*) to rust (*Uromyces appendiculatus*) (Liebenberg and Pretorius, 2004) and white mold disease (*Sclerotinia sclerotiorum*) under field conditions (Genchev and Kiryakov, 2002) are controlled by a single dominant gene in each case, and in each case a 3:1, resistant to susceptible, ratio has been observed in the F_2 progeny. Because of the linkage between flower colour and resistance or susceptibility of *S. formosa* lines to *P. cinnamomi*, it was expected that from the cross of resistant × susceptible *S. formosa* lines, in the F_2 generation the phenotypic traits of resistance related to a single gene would segregate. The results showed that the resistance of *S. formosa* red line to *P. cinnamomi* was related to a single dominant gene.

The linkage between flower colour and resistance also formed the basis for the hypothesis that anthocyanidin or possibly some precursor(s) of the anthocyanidin

and/or some other colourless phenolic compounds in the phenylpropanoid pathway (Dixon and Paiva, 1995; Winkel-Shirley, 2001) were responsible for the resistance of *S. formosa* red line to *P. cinnamomi*. In other words, the resistance of the red line of *S. formosa* to *P. cinnamomi* may be related to the presence of a high level of anthocyanin in the red line (Zhao et al., 2004), or an increase in the precursors of anthocyanidin, such as leucoanthocyanidins, in the phenylpropanoid pathway. Proanthocyanidins (condensed tannins), which are derivatives of anthocyanidin (Xie et al., 2003; Xie et al., 2004b) or other phytocompounds related to other final groups, such as isoflavonoids (Lozovaya et al., 2004) and lignin (Cahill and McComb, 1992; Slusarenko et al., 2000), may be involved in *S. formosa* red line resistance to *P. cinnamomi.* The resistance of this line would result from either the presence of antimicrobial preformed substances, phytoanticipins, or the production of phytoalexins produced as a result of challenge between *P. cinnamomi* and *S. formosa* red line.

A link between flower colour and resistance to *P. cinnamomi* was observed in the three *S. formosa* lines. Chapter 4 considers the response of these three lines to some other fungi.

Chapter 4 Isolation, purification, identification and testing of pathogenicity of soilborne fungi which interact with the roots of greenhouse grown *Swainsona formosa*

4.1 Introduction

Many different fungal species exist in the soil (Singleton et al., 1992), but the factors that determine which species are able to colonise living plants are complex and poorly understood (Carter et al., 1999). In general, soilborne fungi may be saprophytes, parasites or fungi that have lost their ability to cause disease (Toussoun and Nelson, 1968). Some soilborne pathogens are spread worldwide and many are economically important plant pathogens (van der Plaats-Niterink, 1981; Erwin and Riberio, 1996; Nicholson et al., 2003; Mule et al., 2004).

Plant pathogens have the considerable ability to manipulate biochemical, physiological and morphological processes in their host plants through a diverse array of extracellular activities that can either promote infection or trigger defence responses (Staskawicz et al., 2001). However, interactions between the plant and the microbial community, which develop in and around the plant root, affect plant health and development (Carter et al., 1999). Many pathogenic soilborne fungi produce root rot, crown rot, wilt or damping-off in leguminous plants (Hancock, 1983; Tu, 1991).

Isolation of microorganisms and subsequent rapid, accurate and sensitive identification of them, where they are problematic, can be crucial for effective and long-term management strategies (Moorman et al., 2002; Kong et al., 2003). In addition to physiological changes, the morphology of soilborne fungi changes when confronted with unfavourable environmental conditions (Appel and Gordon, 1994), and this change needs to be considered during identification.

Swainsona formosa white line showed high mortality in the greenhouse. In this chapter the isolation and identification of any soilborne fungi present in the roots of *S. formosa* white line is reported. The pathogenicity of these fungi to *S. formosa* white, pink and red lines are tested. In addition, the reaction of the three *S. formosa* lines to *Botrytis cinerea* was investigated. *Botrytis cinerea* has been demonstrated to be a pathogen of *S. formosa* (Summerell et al., 1997).

4.2 Materials and methods

4.2.1 Isolation

Roots of *S. formosa* white line plants showing dieback symptoms were collected from greenhouse grown plants and immediately transferred to the laboratory. The roots were placed into a 500 mL beaker with a wire-mesh net over the beaker mouth and washed under running tap water for 10 minutes, then surface sterilised in 70% ethanol for 30 seconds. They were transferred immediately from the ethanol to sterile distilled water to remove the ethanol. Small root pieces (about 5 mm) from the areas between the diseased and healthy parts were cut using a sterile scalpel, and cultured to several media. There were three media used:

1. **Acidified water agar (AWA)**, consisting of 20 g agar per litre, and 6 drops of 88% lactic acid per litre to acidify the medium and suppress bacterial development. Since this culture lacks nutrients, it is suitable for the isolation of fast growing fungi including *Pythium* species (Watanabe, 2002).

2. **One quarter strength acidified potato dextrose agar (¼ APDA)**, consisting of 10 g potato dextrose agar, 11 g extra agar and 6 drops of 88% lactic acid per litre.

3. **Peptone PCNB agar**, modified from the recipe of Singleton et al. (1992) with different antibiotics being used. One litre of medium consisted of 20 g agar, 15 g Difco peptone, 1 g potassium dihydrogen phosphate, 500 mg hydrated magnesium sulphate, 100 mg streptomycin, 30 mg penicillin, 1 g pentachloronitrobenzene (PCNB) or terraclor and 1 litre distilled water. Antibiotics (streptomycin 0.1 g and penicillin 0.03 g) were dissolved in 10 mL sterile distilled water in a sterile McCartney bottle and this solution was added to the medium, after the medium was autoclaved at 121 °C for 20 minutes and then cooled to about 45 °C.

4.2.2 Purification of the fungi and production of reproductive organs for identification

When mycelia appeared, hyphal tips from the advancing edges of the colonies were subcultured onto selective media to produce the sexual and asexual reproductive

organs required for morphological identification. Spezieller nahrstoffarmer agar (SNA) medium (Singleton et al., 1992) was used for the *Fusarium* species. One litre of the medium consisted of 1 g potassium dihydrogen phosphate, 500 mg hydrated magnesium sulphate, 1 g potassium nitrate, 500 mg potassium chloride, 200 mg glucose, 200 mg sucrose, 20 g agar and 1 litre distilled water. The medium was autoclaved at 121 °C for 20 minutes and, when cooled to about 45 °C, was dispensed into 9 cm diameter sterile Petri plates. To enhance sporulation during medium solidification, two pieces of sterile filter paper (1 cm^2 each) were placed onto the medium surface in each plate.

Pythium mycelia growing on ¼ APDA were transferred to grass leaf culture (Singleton et al., 1992) to produce sporangia and sex organs. Rice leaves were cut to about 2 cm in length. The cut leaves were placed into a 500 mL beaker containing 100 mL distilled water. The mouth of the beaker was covered with a piece of aluminium foil, and the contents boiled for 10 minutes. Three mats of fungal colony (each about 1 cm^2) were taken from the advancing edge of a colony, growing on PDA, and were placed into a 9 cm diameter sterile Petri plate at a distance of about 2.5 cm from each other. The mats were submerged in sterile distilled water, and three pieces of boiled rice leaf were placed into each plate. The plates were incubated at 25 °C.

4.2.3 Identification of the isolated fungi

4.2.3.1 Pythium

Morphological characteristics were used for identification. Within 2-6 days, when abundant sporangia and sex organs had developed on the grass culture, a small piece of colony (about 25 mm^2) containing these reproductive organs was cut off, from the distance between mats, and mounted on a slide, as described by Zeng et al. (2005). Using a light microscope, the morphological characteristics of the sporangia, oogonia, and antheridia, and whether the antheridia were amphigynous or paraginous were recorded. At least 20 measurements were taken to determine each organ size. The identification keys of van der Plaats-Niterink (1981) and Watanabe (2002) were used for species identification.

4.2.3.2 Fusarium

Fusarium species were identified, based on the morphology of the macroconidia, microconidia, chlamydospores, conidiophores, general colony morphology and taxonomic descriptions of *Fusarium* species by Singleton et al. (1992), Burgess et al. (1994) and Rodrigues and Menezes (2005).

4.2.4 Pathogenicity test

The pathogenicity of the isolated fungi to the white, pink and red lines of *S. formosa* was tested using the detached stem and the root dip assays described below. In addition, the reactions of the three *S. formosa* lines to *Botrytis cinerea,* reported to be the causal agent of *S. formosa* stem rot (Summerell et al., 1997), was tested using the detached stem assay. A fresh and virulent isolate of *B. cinerea* was obtained from the University of New England teaching culture collection.

4.2.4.1 Detached stem assay

A completely randomised design with five replicates plus five controls for each of the three *S. formosa* lines was used to examine the pathogenicity of each identified fungus and *Botrytis cinerea* on each *S. formosa* line. Each replicate consisted of two 11 cm trimmed detached stems from seven-week-old plants grown in the greenhouse. Inoculum preparation, stem preparation, surface sterilisation of trimmed stems, and the inoculation method were performed as described in Chapter 3 (Section 3.2.5). The length of stem lesions above the medium surface was measured four days post-inoculation when the white line stems were severely infected. A ruler was used to measure the length of the lesions.

4.2.4.2 Root dip assay

1. Experimental design and preparation of plant medium

The experimental design and the replicate numbers of inoculated and non-inoculated control plants for the pathogenicity testing of each identified fungus on each *S. formosa* line was the same as for the detached stem assay mentioned above. Each replicate consisted of two three-week-old seedlings grown separately in 10 cm diameter plastic pots in a mixture of sand and perlite (v:v, 1:1). The potting mixture

had been moistened and autoclaved at 121 °C for 1 hour for two consecutive days prior to potting up (Babai-Ahary et al., 2004).

2. *Inoculum production*

The prepared inoculum of *Pythium irregulare* was based on the method of Deng et al. (2005). The fungus was grown in the dark on ¼ APDA at 31 °C overnight. Five small agar plugs (25 mm^2) from the advancing edge of the colony were transferred into each sterile Petri plate. The plugs were covered to the top with autoclaved V8 broth. The broth was made by mixing 350 mL V8 juice with 5 g calcium carbonate. The mixture was filtered through a coffee filter, diluted four times and autoclaved. Mycelial mats were produced by incubating the plates at 31 °C for two days in darkness. The mats were washed three times with sterile distilled water to remove the broth. They were then flooded in 100 mL sterile water in a 200 mL bottle, and incubated at room temperature for 48 hours under cool-white fluorescent light. The mats were blended for 2 minutes at high speed, using a clean blender. The slurry was filtered through a four-ply layer of sterile cheesecloth to remove agar pieces. The colony-forming-unit (CFU) concentration in the suspension was determined using a haemocytometer. The concentration of 1×10^4 CFU per mL was obtained by diluting the suspension with sterile distilled water.

The prepared inoculum of the *Fusarium* species was based on the method of Ykema and Stutz (1991). *Fusarium oxysporum* and *F. solani* isolated from infested roots were grown on V8 juice agar plates under fluorescent light of 30 $\mu molm^{-2}s^{-1}$at 25 °C for 1 week. The V8 juice agar medium consisted of 20% V8 juice, 2% agar and 0.2% calcium carbonate. A suspension of fungal conidia (microconidia and macroconidia) in water was prepared. The concentration of the conidia was determined using a haemocytometer. The inoculum concentration of 2×10^5 spores per mL was prepared by diluting the suspension with sterile distilled water.

3. *Inoculation and disease development*

The root dip assay described in Chapter 3 (Section 3.2.3) was used to test the pathogenicity of *P. irregulare, F. solani and F. oxysporum* on the three *S. formosa* lines. The seedling roots were submerged in the spore suspension for 20 minutes. The

control seedlings were submerged in sterile distilled water for 20 minutes. Seedlings were inoculated late in the afternoon to avoid the highest daily temperatures. After inoculation, the seedlings were returned to the pots and kept in the greenhouse with temperatures favourable for infection and root rot development. The best temperature for fungal development is reported to be 30-37 °C during the day and 26-28 °C at night (Deng et al., 2005). The seedling media were moistened by watering 2-3 times daily.

4. Pathogenicity and resistance evaluation

The seedling response of the three *S. formosa* lines to *P. irregulare, F. solani* and *F. oxysporum* were recorded three weeks post-inoculation using a modified scale of 0 to 4 after the method of Deng et al. (2005) where:

0 = all root tissues were healthy without any rotting symptoms
0.5 = 1-4% of root tissue on the root ball surface rotted
1 = 5-14% of root tissue on the root ball surface rotted
1.5 = 15-34% of root tissue on the root ball surface rotted
2 = 35-54% of root tissue on the root ball surface rotted
2.5 = 55-84% of root tissue on the root ball surface rotted
3 = 85-94% of root tissue on the root ball surface rotted
3.5 = 95-99% of root tissue on the root ball surface rotted
4 = 100% root infestation

The level of root rot resistance from the above scale was rated on the basis of the following scale:

≤ 0.5 = resistant
$> 0.5 \leq 1.5$ = moderately resistant
$> 1 \leq 2$ = susceptible
> 2 = very susceptible

4.2.4.3 Reisolation and identification of pathogens from infected seedlings

Diseased roots were harvested, and the fungi reisolated using the same techniques and media employed for the initial isolation of each fungus from the infected plants.

4.2.4.4 Data analysis

A one-way ANOVA was used to analyse data from the pathogenicity tests in both the detached stem and root dip assays. All analyses were conducted using the SPSS™ statistical package. The criterion for significance of treatment effects was $P < 0.05$, unless otherwise indicated.

4.3 Results

Two *Fusarium* species and one *Pythium* species were isolated from the naturally infected roots of *S. formosa* white line grown in unautoclaved soil in the greenhouse.

4.3.1 Identification of *Fusarium* species

The *Fusarium* species were identified using available keys and taxonomic descriptions by Singleton et al. (1992), Burgess et al. (1994), and Rodrigues and Menezes (2005).

4.3.1.1 Fusarium oxysporum Schlecht

Colonies on APDA medium were initially white, but subsequently (after 3 days) changed to purple. Colonies on SNA medium were colourless. Large numbers of macroconidia, each with an attenuated apical cell and a pedicellate basal cell (Figure 4.1-a), were formed on short branched or unbranched conidiophores (monophialides) (Figure 4.1-b), or on sporodochia (Figure 4.2). The macroconidia were three septate. Microconidia were produced on short and unicellular conidiophores as a false head and were mostly unicellular (Figure 4.1-c). Their shapes varied from oval-ellipsoid to cylindrical, and from straight to curved. The fungus formed terminal or intercalary chlamydospores (Figure 4.1-d) with smooth walls. The chlamydospores were mostly single.

Figure 4.1. The reproductive organs of *Fusarium oxysporum*. a: macroconidia, b: monophialide, c: microconidia, d: chlamydospore.

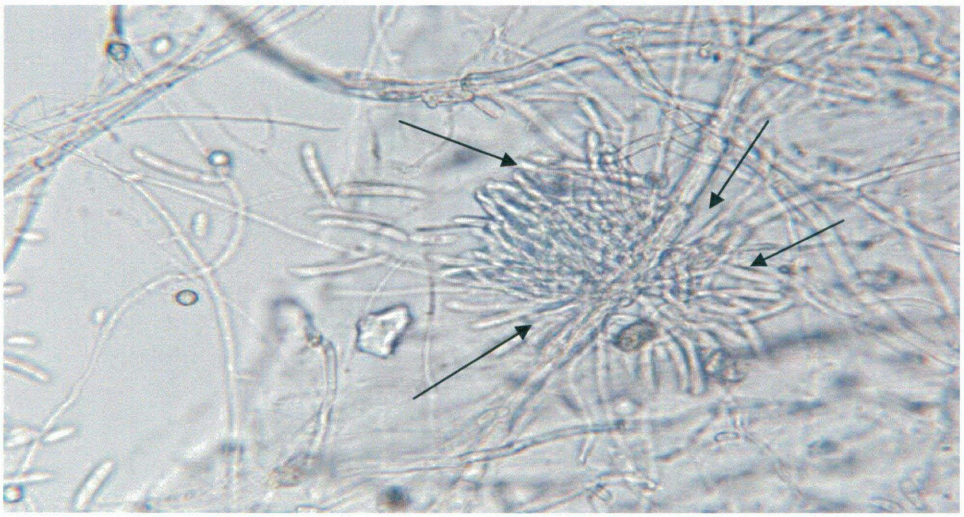

Figure 4.2. Sporodochium of *Fusarium oxysporum*. Arrows point to sporodochium.

4.3.1.2 Fusarium solani (Mart.) Sacc.

Colonies on APDA were slow-growing, and cream in colour. The typical morphological characteristic of *F. solani* used for identification is the formation of long lateral monophialides (Singleton et al., 1992; Summerell et al., 2001; Rodrigues and Menezes, 2005) (Figure 4.3) which produce microconidia. Microconidia, as are shown in (Figure 4.4-a), were unicellular, narrowing at the apex, and were oval or

kidney-shaped Macroconidia were 3-5 septate and generally cylindrical for almost their entire length (Figure 4.4-b). Chlamydospores were globular or oval with smooth or wrinkled walls, formed singly or in pairs (Figure 4.4-c).

Figure 4.3. Monophialides or conidiophores of *Fusarium solani*. Monophialides produce microconidia. Arrows point to monophialides.

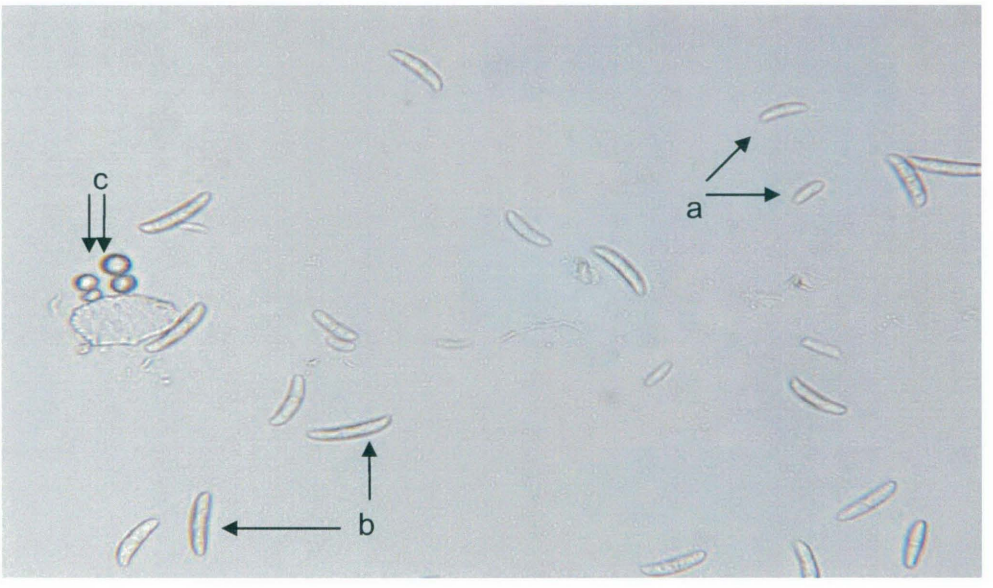

Figure 4.4. Asexual reproductive spores of *Fusarium solani*: a: microconidia, b: macroconidia, c: chlamydospores.

4.3.2 Identification of *Pythium irregulare* Buisman

When the agar discs from the purified *Pythium irregulare* culture were grown on grass culture, the fungus produced an abundance of mycelia and sexual and asexual reproductive organs. The hyphae were coenocytic. The widths of the main hyphae were 2-6 μm. The oogonia were predominantly intercalary and sometimes terminal. They were globose to irregular, and their diameters were between 16 to 22.5 μm, but mostly 17.5 μm. The surfaces of the oogonia were smooth or had 1-5 blunt cylindrical projections (Figure 4.5) between 2-5 μm long. About 50% of the oogonia were irregular with projections. Each oogonium was conjugated paragynously with one or two monoclinous antheridia. Antheridia originated mostly from the same hyphae as the oogonia (homothallic), and were 10-15 × 4-5 μm. The antheridia made apical contact with the oogonia (Figure 4.6 and Figure 4.7). The oospores were aplerotic. The oospore wall thickness varied between 1 to 5 μm.

Sporangia were both intercalary and terminal. They were globose and their diameter varied from 10 to 20 μm. They produced zoospores of approximately 8 μm in diameter. The characteristics and the sizes of different organs were in concert with those described by van der Plaats-Niterink (1981).

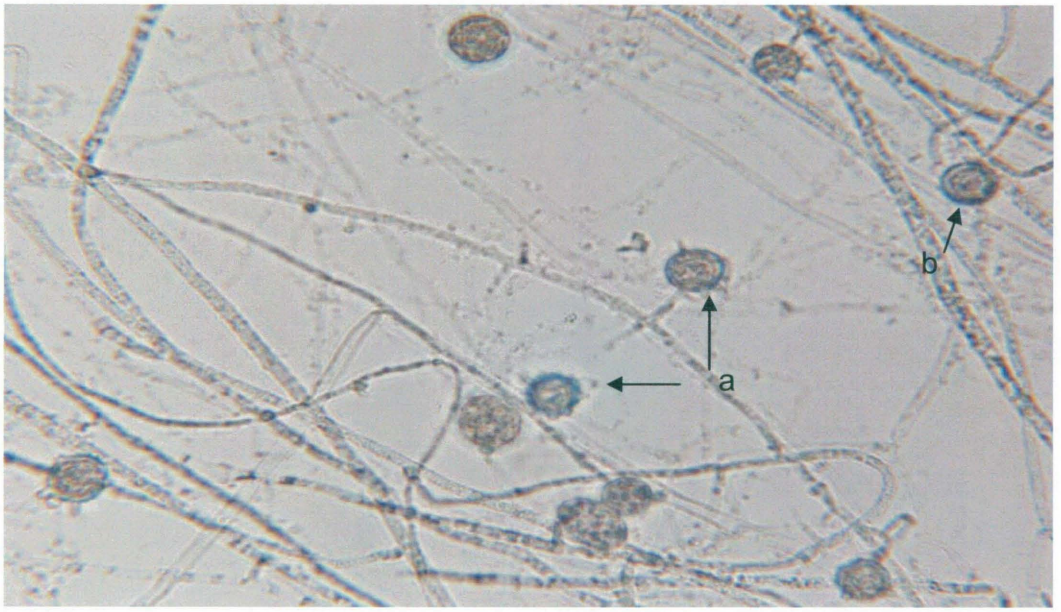

Figure 4.5. Oogonia of *Pythium irregulare*: a: irregular oogonium with projections, b: smooth and globose oogonium.

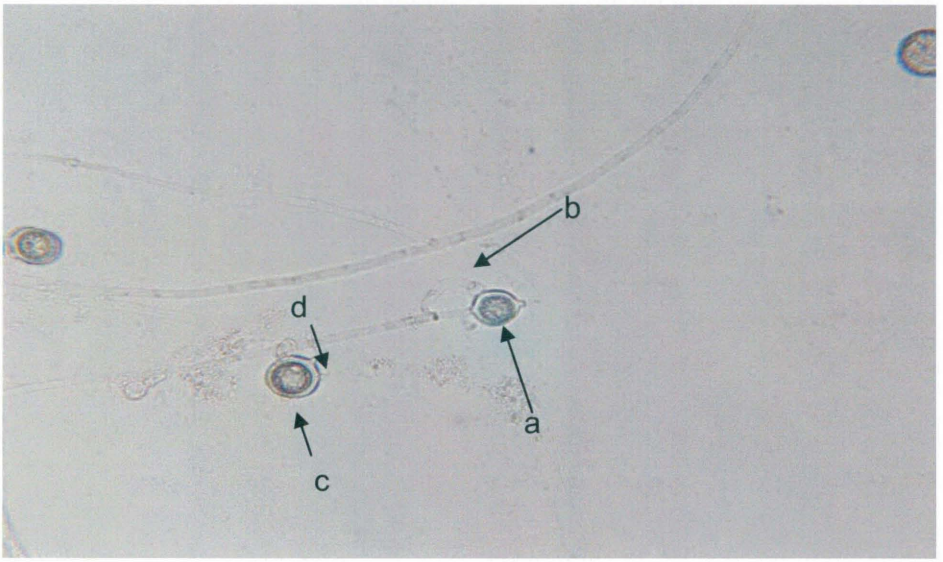

Figure 4.6. Conjugation of antheridium and oogonium of *Pythium irregulare*: a: oogonium, b: antheridium, c: globose oogonium with projections, d: projection.

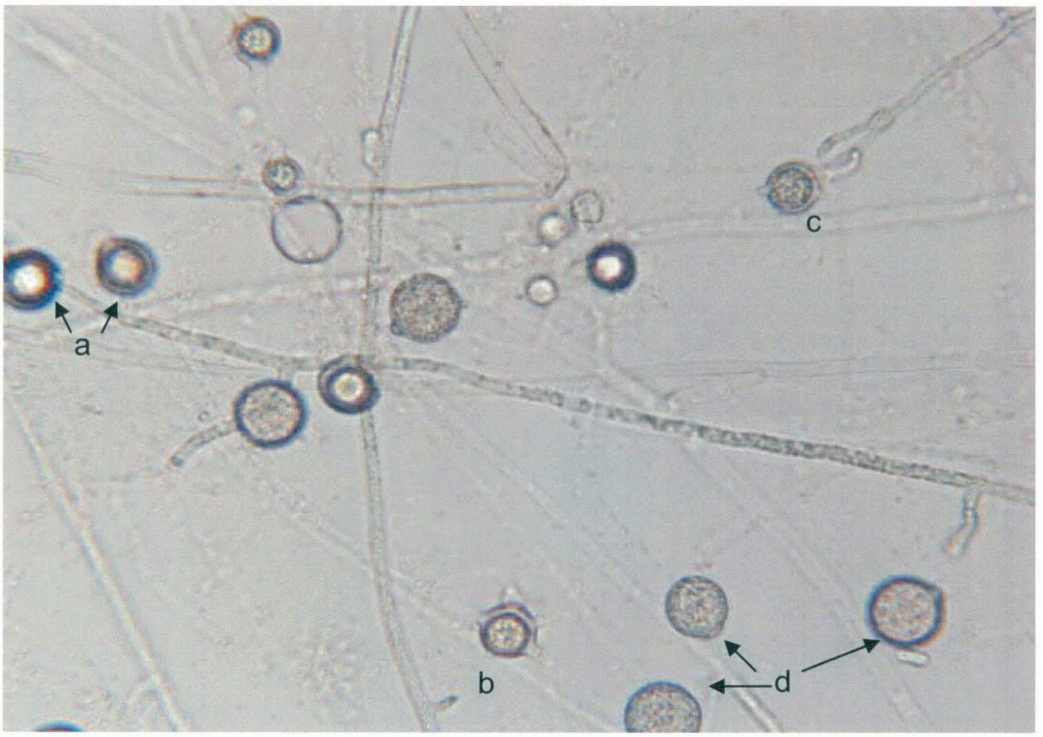

Figure 4.7. Sexual and asexual organs of *Pythium irregulare*: a: smooth and globose oogonium, b: irregular oogonium with projections, c: conjugation of oogonium and antheridium (paragynous), d: sporangia

4.3.3 Pathogenicity test

The response of the three *S. formosa* lines to *F. solani* isolated from the roots of diseased *S. formosa* white line plants was significantly (0.01) different in both assays (Table 4.1 and Figure 4.8). *Swainsona formosa* white line was very susceptible to *F. solani* in both assays, while the red line was resistant, and the pink line moderately resistant. The reaction of the three *S. formosa* lines to *P. irregulare* was similar to their reactions to *F. solani* in both assays (Table 4.1 and Figure 4.9). The inoculated white line plants revealed similar symptoms to naturally infected plants and the same pathogen was reisolated from artificially inoculated infected plants.

There was no significant difference in the response of the three *S. formosa* lines to *F. oxysporum* in either assay. Inoculated plants were asymptomatic and remained as healthy as the control plants (data not shown).

No significant difference was apparent between lines in the severity of symptoms to *B. cinerea* in the detached stem assay. The stems of the three *S. formosa* lines were highly infected post-inoculation (data not shown).

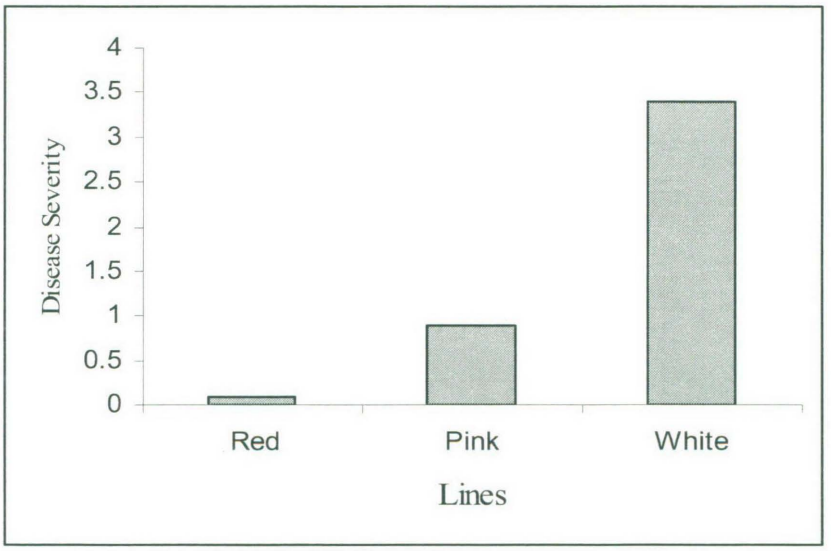

Figure 4.8. Root reaction to *Fusarium solani* in three lines of *Swainsona formosa* inoculated by dipping roots into a spore suspension. Disease was rated three weeks after inoculation on a 0-4 scale, where 0 = no symptoms and 4 = 100% root infection.

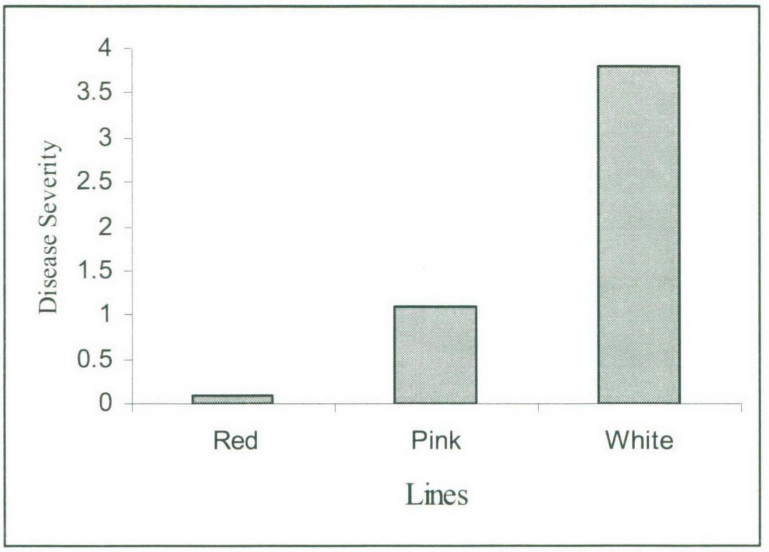

Figure 4.9. Root reaction to *Pythium irregulare* in three lines of *Swainsona formosa* inoculated by dipping roots in a spore suspension. Disease was rated three weeks after inoculation on a 0-4 scale, where 0 = no symptoms and 4 = 100% root infection.

Table 4.1. Mean lesion length in the detached stem assay of *Swainsona formosa* inoculated with *Fusarium solani* and *Pythium irregulare*. These experiments were conducted with five replicates. Each replicate consisted of two, 11 cm trimmed detached stems from seven-week-old plants placed separately in cultures of *F. solani* and *Pythium irregulare*. Lesion length was measured four days after inoculation.

Variety	Lesion length (mm) ± S.E.	
	Fusarium solani	*Pythium irregulare*
Red	0	0
Pink	49.4 ± 1.21	48.6 ± 1.5
White	94.8 ± 1.46	94.2 ± 1.39

4.4 Discussion

Two *Fusarium* and one *Pythium* species were consistently isolated from rotted roots of naturally infected *S. formosa* white line grown in unautoclaved soil in the greenhouse. The *Fusarium* species, based on the morphology of the colony, microconidia, macroconidia, conidiophores, chlamydospores and taxonomic descriptions of species by Singleton et al. (1992), Burgess et al. (1994) and Rodrigues and Menezes (2005), were identified as *F. solani* (Mart.) Sacc. and *F. oxysporum* Schlecht. The *Pythium* species, based on the morphology of the oospores, oogonia, antheridia, sporangia and taxonomic descriptions by van der Plaats-Niterink (1981) and Watanabe (2002), was identified as *P. irregulare* Buisman. The pathogenicity of each species on the white line was determined separately using the detached stem and the root dip assays. *Pythium irregulare* and *F. solani* severely infected the inoculated *S. formosa* white line and produced similar symptoms to those of the naturally infected plants. In each case, the same fungus was reisolated from the infected plants. Therefore, it can be concluded that these fungi are pathogenic to *S. formosa* white line.

The *S. formosa* white line plants inoculated with *F. oxysporum* were asymptomatic, indicating that this fungus is not pathogenic to *S. formosa*. Reisolation of *F. oxysporum* was not attempted. *Fusarium oxysporum* comprises both pathogenic and non-pathogenic forms (Lemanceau et al., 1993). Reports indicate that non-

pathogenic forms of this species can colonise the root cortex without causing disease symptoms (Kaur et al., 2003). Some non-pathogenic *F. oxysporum* forms have been isolated from other leguminous plants including common bean (Coelho Netto and Dhingra, 1999), pigeon pea (Chakraborty and SenGupta, 2000) and chickpea (Kaur et al., 2003). Although some non-pathogenic forms of *F. oxysporum* are antagonistic toward plant pathogens (Kaur et al., 2003), the function of non-pathogenic *F. oxysporum* forms on *S. formosa* has not been studied.

Pythium irregulare has been reported to be pathogenic to a broad range of plant species (van der Plaats-Niterink, 1981; Denman et al., 1995; Pankhurst et al., 1995; Moorman et al., 2002). Some legumes susceptible to *P. irregulare* include soybean (Southern et al., 1976; Forbes and Davet, 1990; Rizvi and Yang, 1996), pea (Persson et al., 1997), lentil (Paulitz et al., 2004), alfalfa or lucerne (Hancock, 1991; Denman et al., 1995), chickpea (Trapero et al., 1990), *Cicer milkvetch* (Hou et al., 1997), arrow-leaf clover (*Trifolium vesiculosum*) (Pemberton et al., 1998), and the annual legume *Kummerowia* (Mihail et al., 2002).

Fusarium solani is generally organised into *formae speciales* (f. sp.) and infects the roots of a number of different plant species including legumes (Hartman et al., 2004). It causes root rot and vascular wilt diseases (Hartman et al., 2004). Phytotoxins produced by some strains of *F. solani* inhibit root growth and are translocated to leaves causing interveinal chlorosis and necrosis (Baker et al., 1981; Hartman et al., 2004). *Fusarium solani* has been isolated from diseased leguminous plants, such as bean (Miller et al., 1980; Furuya et al., 1999), broad bean (Beshir and Degago, 1999; Ren et al., 2003), soybean (Roy, 1997; Cho et al., 2001), pea (Panka and Sadowski, 1999), and chickpea (Patel and Anahosur, 2001).

In addition to testing the pathogenicity of the isolated fungi on *S. formosa* white line, the responses of the red and pink lines to the isolated fungi were assessed in both assays. The results were: (1) the red and pink lines showed no symptoms when inoculated with *F. oxysporum*; (2) the red line remained asymptomatic when it was inoculated with either *P. irregulare* or *F. solani*; and (3) the pink line showed moderate resistance when inoculated with either *P. irregulare* or *F. solani*.

The three *S. formosa* lines were highly susceptible to *B. cinerea,* and showed similar symptomatic intensity to the pathogen. Susceptibility of *S. formosa*, presumably the red line, to *B. cinerea* has been reported by Summerell et al. (1997). The three *S. formosa* lines were equally susceptible to *B. cinerea,* and no correlation

was observed between susceptibility/resistance to this aerial pathogen and flower colour. Kantar et al. (1996) reported that the incidence of aerial diseases, such as *Botrytis fabae*, and foliar diseases, such as *Uromyces viciae*, on *Vicia faba* was not related to flower colour.

The reaction of the three *S. formosa* lines to *P. irregulare* and *F. solani* was similar to the reaction of these lines to *Phytophthora cinnamomi* (Chapter 3). A close relationship between flower colour and susceptibility and resistance to these pathogens was observed. The white line with white-coloured flowers was very susceptible, while the red line with red-coloured flowers was resistant. The reaction of the pink line was intermediate between the other two.

In this chapter, the relationship between flower colour and resistance to some soilborne pathogens was described. In chapter 5 the possible involvement of different derivatives from the phenylpropanoid pathway, from which flower colour originates, and some other compounds in resistance to *P. cinnamomi* are investigated.

Chapter 5 Investigation into the inhibitory effects of some of *Swainsona formosa* phytocompounds on the growth of *Phytophthora cinnamomi*

5.1 Introduction

The chemical basis of plant disease resistance is related to the presence of preformed and/or induced antimicrobial substances (Slusarenko et al., 2000). A large number of phytocompounds, including phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates, have been reported to have antifungal activities (Bennett and Wallsgrove, 1994; Osbourn, 1996). A relationship has been observed between disease resistance and coloured flowers and other coloured plant tissues, such as coloured seeds and coloured hypocotyls (Dixon et al., 2005). The experiments in Chapter 3 showed a link between flower colour and resistance/susceptibility of *S. formosa* lines to *P. cinnamomi*. It appears that flavonoids or phenolic compounds involved in flower colour may have a role in the resistance of *S. formosa* red line to *P. cinnamomi*.

Synthesis of the pigment complex in plants constitutes a defence response to pathogens (Nicholson et al., 1987). This phenomenon has been observed in many genera in Fabaceae (Guevara et al., 1986; Higuera and Murty, 1987; Cobos et al., 2005). Most plants, particularly leguminous plants, resist pathogen attack via various classes of phenylpropanoids, either in the form of phytoalexin or phytoanticipin (Bednareka et al., 2001; Dixon and Ferreira, 2002). Earlier studies suggest that anthocyanins may be involved in plant disease resistance (Hammerschmidt and Nicholson, 1977). Antifungal effects of anthocyanins to *Helminthosporium* species in maize has been reported (Heim et al., 1983), and anthocyanins are known to inhibit spore germination (Kraft, 1977) and aflatoxin B_1 production by *Aspergillus parasiticus* and *A. flavus* in some food crops (Norton, 1999). These pigments also accumulate in close vicinity to the inoculation site in sugarcane resistant to *Colletotrichum falcatum* pathotype Cf671, the causal agent of red rot (Viswanathan, 2002).

Other phenolic compounds such as proanthocyanidins (condensed tannins) (Statler, 1970; Snyder et al., 1991) and leucoanthocyanidins (Snyder et al., 1991) are involved in the preinfection and postinfection defence responses of plants against a

number of pathogens. Some white-flowered *Vicia faba* (faba bean) cultivars suffer from various post-emergence plant diseases (Kantar et al., 1996). In contrast, coloured-flowered faba beans were observed to be resistant to soilborne fungal pathogens, such as *Fusarium culmorum* and *F. solani* (Pascual Villalobos and Jellis, 1990). The tannin present in faba bean with coloured flowers inhibits the mycelial growth of all faba bean pathogenic fungi (Kantar et al., 1996). Similarly, white-seeded varieties of French bean (*Phaseolus vulgaris*) are reported to be more susceptible to *Rhizoctonia solani* than red- and black-seeded varieties (Guevara et al., 1986). Furthermore, the high resistance of the TRI 2043 tea cultivar with purple-green leaves to *Exobasidium vexans* was related to a high content of catechin and epicatechin, precursors of condensed tannins (Punyasiri et al., 2005). Tannins extracted from *Liquidambar formosana* leaves had fungicidal properties against *Alternaria alternata,* the causal agent of pepper black spot (Zheng et al., 2005), and some condensed tannins extracted from peanut testae and cotyledons inhibited *Aspergillus parasiticus* growth *in vitro* and reduced the levels of aflatoxin production (Azaizeh et al., 1990). The objective of this chapter is to investigate whether coloured compounds (pigments) of the phenylpropanoid pathway and/or colourless compounds of this pathway, or other phytocompounds in the *S. formosa* red line have growth inhibitory effects against *P. cinnamomi.*

5.2 Materials and methods

Different techniques were used to determine which phytocompounds in the red line, and in which form, were responsible for resistance to *P. cinnamomi.* The phytocompound concentrations, in most cases, were measured spectrophotometrically. Before measurement of a phytocompound concentration, the appropriate wavelength for its measurement was determined. As there is a direct correlation between optical density (OD) of a compound and its concentration, and in all issues the compound levels were compared among the three *S. formosa* lines, the OD values were directly used for comparison.

5.2.1 Measurement of some phytocompounds in inoculated and non-inoculated *S. formosa* lines

A link between flower colour and resistance to *P. cinnamomi* in *S. formosa* lines was observed in the vegetative parts of the plants (Chapter 3, Sections 3.3.1-3.3.3). To determine which phytocompounds in the red line were responsible for resistance to *P. cinnamomi*, and the form in which they occur, the concentrations of the following compounds were measured in inoculated and non-inoculated plants.

5.2.1.1 Free phenolic compounds

The free phenolic content of inoculated and non-inoculated hypocotyls and stems were measured. The tissues were inoculated as was performed in Chapter 3 (Sections 3.2.4. and 3.2.5) to measure their responses to infection. The hypocotyl assay had a completely randomised design with five replicates for each line, and the detached stem assay a completely randomised design with seven replicates for each line. The number of replicates of non-inoculated controls was the same as for the inoculated plants of each line (five and seven respectively). Four days after hypocotyl inoculation, and three days after stem inoculation, a 500 mg sample was taken from each replicate. Samples were removed from the hypocotyls 0.5 cm above the inoculation site, and from the detached stems 2 cm above the inoculation site. Control samples were obtained from the same regions as on the inoculated samples.

Immediately after weighing, each sample was placed into a 2 mL microcentrifuge tube. One millilitre of 95% ethanol containing 0.1% HCl (extraction solution) was added to each microcentrifuge tube and the contents were homogenised using a drill bit turned by hand. A further 0.5 mL of extraction solution was added to each microcentrifuge tube, which was then incubated at 4 ^{o}C overnight. Samples were centrifuged for 5 minutes at 15000 *g* (Huang, 2004).

To measure soluble phenolic compounds, 0.5 mL of extract was put into a test tube, followed by 0.5 mL 95% ethanol, 2.5 mL distilled water, and 250 µL of 2N Folin-Ciocalteu reagent (Sigma Chemical Company). The mixture was vortexed, 0.5 mL of 5% sodium carbonate was added and the solution mixed completely. The mixture reacted for 60 minutes in darkness at room temperature. Samples were again homogenised with a vortex mixer and 2 mL from each sample were transferred into a

spectrophotometer cuvet where the absorbance for each sample was measured at 725 nm using a spectrophotometer (Huang, 2004). The blank contained all materials except the extract.

5.2.1.2 Anthocyanins

The anthocyanin content of inoculated and non-inoculated hypocotyls and stems were measured. The tissues were inoculated as was performed in Chapter 3 (Sections 3.2.4. and 3.2.5) to measure their responses to infection. Plant extracts were obtained from 250 mg of inoculated hypocotyls, detached stems and their controls in 1.5 mL of acidified 95% ethanol, as was described in the free phenolic assay (Section 5.2.1.1). One half millilitre of extract was diluted with 1.5 mL of extraction solution and the absorbance was measured at 525 nm.

5.2.1.3 Activity of β-1, 3-glucanase

The activity of β-1, 3-glucanase was measured as an indicator of general defence responses of the plant tissues. Tissue segments were obtained from inoculated hypocotyls, detached stems and their controls, as described in Sections 3.2.4 and 3.2.5, respectively. Enzymes were extracted with an acetate-polyvinylpyrrolidone-EDTA buffer as described by Dann et al. (2000). For each replicate, 0.4 mL potassium acetate buffer (10 mM, pH 5.0) and 0.1 mL plant extract were added to a microcentrifuge tube and allowed to equilibrate at 30 °C for 3 minutes. The reaction was initiated by the addition of 0.1 mL azurine-crosslinked pachyman (AZCL-pachyman Megazyme, Ireland, 15 mg mL^{-1} in distilled water) and was terminated after 10 minutes by the addition of 0.7 mL 20% w:v Tris. Tubes were vortexed, maintained at room temperature (≈ 23 °C) for 5 minutes, vortexed again and centrifuged at approximately 9000 *g* for 2 minutes. The supernatant from each microcentrifuge tube was transferred to the spectrophotometer cuvet; colour development from each sample was measured at 610 nm using the spectrophotometer.

5.2.2 The inhibitory effect of flower pigments on growth of *P. cinnamomi*

5.2.2.1 Extraction of flower pigments

To determine the growth inhibitory property of the flower pigments, 2 g (fresh weight) of flower petals from 5 individuals of each *S. formosa* line were separately weighed (total n = 15). Samples were extracted using 20 mL of a solution containing 95% ethanol and 0.1% HCl. Flowers were macerated in 15 mL of the solution using a homogeniser or omni mixer (Sorvall Inc., USA) at 8000 revolutions per minute (rpm) for 2 minutes. The homogeniser was rinsed with 5 mL of the solution, which was mixed with the original homogenate. The homogenates were completely extracted by being placed in the refrigerator at 4 °C overnight and then being centrifuged (Heim et al., 1983) at 2500 *g* for 10 minutes. The ethanol and hydrochloric acid were removed from the extract by means of vacuum distillation at 45-50 °C using a rotary evaporator (Pretorius et al., 2003). The remaining substance (2 mL), termed the crude extract, was transferred into a sterile vial in a sterile air flow cabinet. The crude extracts of the red, pink and white lines were intense red, light reddish to moderately pink, and yellowish, respectively.

5.2.2.2 The inhibitory effect tests

The reaction of *P. cinnamomi* to flower-colour pigments was determined by the application of three different techniques:

Poison food technique

This experiment was based on the method of Schmourlo et al. (2005). A completely randomised design with five replicates plus five controls of each line was used. Each experimental unit included a Petri dish containing a mixture of 20 mL of 1/5 strength V8 culture medium (Chapter 3) and 600 µL of the crude extract equivalent to 600 mg of fresh petals from an individual plant. Crude extract was added to the V8 medium after autoclaving at 121 °C for 20 minutes and cooling to about 45 °C. After solidification of the medium, a 2-5 mm diameter plug from the advancing edge of a four-day-old *P. cinnamomi* culture was placed in the centre of each plate. The control plates lacked the crude extract. The plates were incubated at

24 °C in darkness. The radial growth of the fungus was measured daily for four days using a ruler.

Paper disc diffusion technique

This experiment, with five replicates plus five controls of each *S. formosa* line, was based after the method of Bandopadhyay et al. (2003) with a major modification as outlined below. Twenty millilitres of 1/5 strength V8 medium, after autoclaving at 121 °C for 20 minutes and cooling to about 45 °C, was incorporated with 0.5 mL of the homogenised *P. cinnamomi* mycelia in sterile distilled water. The fungal suspension was prepared from an approximate 5 cm^2 piece of fungus culture from a four-day-old colony that covered the surface of the V8 medium. The piece was mixed with 10 mL sterile distilled water and homogenised into a 10 mL sterile syringe. Three 1 cm diameter round pieces of sterilised paper discs cut from Whatman number 4 filter paper were placed on the surface of the solidified inoculated medium in each Petri plate. One hundred microlitres of the crude extract equivalent to 100 mg of fresh petals from an individual plant was placed onto each disc. The control plates were treated with sterile distilled water. Plates were incubated at 24 °C in darkness. At twenty-four hours post-inoculation, the length of the colony growth from the edge of each disc was measured using a ruler.

The agar well diffusion technique

This experiment was carried out with five replicates of each *S. formosa* line after the method of Aqil et al. (2005). The medium preparation and inoculation procedure were the same as for the paper disc diffusion technique. After inoculation and solidification of the medium, three wells 8 mm in diameter were punched into each medium plate using a cork borer. Each well was filled with 100 µL of the crude extract equivalent to 100 mg fresh petals from an individual plant. The control plates were filled with sterile distilled water. The incubation conditions and time, and the recording time for measuring the length of growth from edge of each well, were the same as in the paper disc diffusion technique.

5.2.3 Toxicity of soluble phenolics and measurement of concentration of some phenolic compounds

Soluble phenolic concentration in non-inoculated stems and hypocotyls of the three *S. formosa* lines changed in the same direction as resistance to *P. cinnamomi* (Tables 5.2 and 5.3), so it was likely that soluble phenolics present in the red line were responsible for the resistance. To examine this, the toxicity of extracts containing soluble phenolics from the three lines were investigated. The optical density of leucoanthocyanidins and proanthocyanidins in the soluble phenolics were then measured to determine the relationship between these compounds and resistance to *P. cinnamomi*. As these compound levels were compared among the three *S. formosa* lines, the optical density values were directly used for comparison.

To have tissues of similar age, and can easily macerate the tissues, soluble phenolic compounds were extracted from stem buds. Immediately after collection from the greenhouse plants, the samples were cut into small pieces and dried using a freeze-drying machine. Dried samples were pulverised using a mixer mill at 30 hertz for 20 minutes, and were extracted using an acetone solvent as described below. Otherwise, the freeze-dried samples were stored at -4 ^{o}C.

Plant materials were extracted with acetone using the method of Dalzell and Kerven (1998), Kennedy and Jones (2001), and Rakhmani (2005). Six grams of dry ground stem buds from five individuals of each *S. formosa* line were extracted separately with 60 mL of 70% aqueous acetone in a covered centrifuge tube at room temperature for 24 hours. To decrease proanthocyanidin oxidation, solutions were sparged with nitrogen (N_2), and the extraction was performed in darkness. Samples were centrifuged at 2500 *g* for 10 minutes and the supernatant collected. Acetone was removed from the extract by vacuum distillation at 35 ^{o}C using a rotary evaporator, and the extract was lyophilised to dryness using a freeze-drying machine.

5.2.3.1 Toxicity of acetone extracted soluble phenolics on the growth of *P. cinnamomi*

The poison food technique was used to investigate the inhibitory activity of the extracts on *P. cinnamomi*. Five replicates from each *S. formosa* line were used. Each replicate consisted of a Petri plate containing a mixture of 20 mL of 1/5 strength

V8 medium (Chapter 3) and 20 mg of lyophilised extract redissolved in 2 mL of absolute methanol. Each replicate contained media with the extract from an individual plant added to the medium when the medium had cooled to about 45 ^{o}C. The control plates contained a mixture of 1/5 strength V 8 medium and 2 mL absolute methanol added to the medium when it had cooled to about 45 ^{o}C. The inoculation technique, incubation conditions and recording times were the same as for the poison food technique used for the flower colour pigments.

5.2.3.2 Free phenolic, leucoanthocyanidin and proanthocyanidin concentrations of acetone-extracted compounds

Leucoanthocyanidins, which are monomeric colourless molecules, convert to coloured anthocyanidins in acidic solutions such as HCl-butanol as a result of a dehydration reaction (Haskins and Gorz, 1986), whereas proanthocyanidins, which are polymeric colourless molecules, convert to anthocyanidins in acidic solutions as a result of a depolymerisation reaction (Haskins and Gorz, 1986). In acidic solutions, leucoanthocyanidins convert easily to anthocyanidins without heating, while proanthocyanidins convert to anthocyanidins under heating conditions (Watterson and Butler, 1983).

To measure the concentration of these compounds, the lyophilised extract of stem buds from five individuals of each *S. formosa* line was dissolved separately in methanol at a concentration of 1 mg mL^{-1}. The free phenolic concentration was measured using Folin-Ciocalteu reagent, as described earlier in this chapter (Section 5.2.1.1). The leucoanthocyanidins and proanthocyanidins of the extract were converted to anthocyanidins using a slightly modified butanol-HCl technique (Watterson and Butler, 1983). For each sample, 1 mL of the extract solution (1 mg mL^{-1}) was added to 500 mg of treated polyvinylpolypyrrolidone (PVPP) in a screw-top centrifuge tube. The PVPP was treated by boiling for 10 minutes in 10% HCl, and the residue filtered through a Whatman number 1 filter paper and dried (Watterson and Butler, 1983). Seven millilitres of butanol:HCl (70:30; v:v) was added to each tube. A blank was prepared in an identical manner, but it contained 1 mL of methanol instead of 1 mL extract solution. The tubes were agitated on an agitator at ambient temperature for 1 hour to convert the leucoanthocyanidins to anthocyanidins, and were then centrifuged at 3500 *g* for 5 minutes. The supernatant was separated and its

absorbance was measured at 525 nm. The supernatants were returned to the respective tubes and the tubes were vigorously shaken and heated in a boiling water bath for 1.5 hours to destroy the unstable anthocyanidins produced from the leucoanthocyanidins in the acidic solution, and to convert the proanthocyanidins of the extract to anthocyanidins. The optical density of the supernatant, after centrifugation for 5 minutes at 3500 g, was measured at 525 nm using a spectrophotometer (Watterson and Butler, 1983).

5.2.4 Proanthocyanidin precipitation by Bovine serum albumin, and assessment of supernatant and precipitate

The proanthocyanidin fraction of the soluble phenolics appeared to have different concentrations among the three *S. formosa* lines. The toxicity of the proanthocyanidins to *P. cinnamomi* was evaluated indirectly by precipitating proanthocyanidins from the extract, and testing the toxicity of the supernatant.

5.2.4.1 Proanthocyanidin precipitation

Proanthocyanidins are functionally defined by their capacity to bind proteins and produce insoluble protein-proanthocyanidin complexes (Hagerman and Carlson, 1998a; Hagerman et al., 1998b). They bind to proteins because they are rich in hydrophobic aromatic rings and hydroxyl groups that can interact with biological molecules with hydrogen bonds and hydrophobic interactions (Abrahams et al., 2002). Bovine serum albumin (BSA), casein and ribulose bisphosphate carboxylase (rubisco) are common proteins which have been used for proanthocyanidin precipitation (Rakhmani et al., 2005). In this experiment, proanthocyanidins were precipitated from the extracts using BSA, and the toxicity of the remaining fractions of the extracts were examined, as described below. This experiment was performed to see whether the substances remaining in the extracts after proanthocyanidin removal were toxic to the fungus.

A completely randomised design with five replicates was used to measure the BSA precipitated proanthocyanidins of the three *S. formosa* lines. Each replicate consisted of a 10 mL mixture of BSA and lyophilised extract at a concentration of 1:1 (v:v) after the method of Rakhmani et al. (2005). Bovine serum albumin was

dissolved at a concentration of 2 mg mL^{-1} in a 0.1 M sodium acetate buffer (pH 4.5) containing 17 mM sodium chloride. The lyophilised extract (containing proanthocyanidins) of each individual plant was dissolved at a concentration of 1 mg mL^{-1} in 50% methanol. The BSA:extract mixture was incubated at 4 °C for 1 hour, and was then centrifuged at 10000 *g* for 15 minutes at 4 °C. The supernatant and precipitate were separated and kept refrigerated for further analysis.

To confirm an excess of protein in the precipitation medium, the protein (BSA) concentration in the supernatant of the three *S. formosa* lines was measured using the Bradford (1976) dye-binding assay with BSA as standard.

5.2.4.2 Supernatant toxicity test

A completely randomised design with five replicates from each line plus five controls for each line was used to determine the supernatant toxicity of each line. Each treatment replicate consisted of a Petri plate containing 20 mL of autoclaved 1/5 strength V8 medium. When the medium had cooled to about 45 °C, it was mixed with 1.7 mL of supernatant from an individual plant. The supernatant of each plant was prepared by mixing 1 mL of 20 mg mL^{-1} lyophilised extract in 50% methanol with 1 mL of 40 mg mL^{-1} BSA in sodium acetate buffer. The proanthocyanidins reacted with BSA, and were separated from the supernatant after centrifugation. For the controls of each line, 20 mL of autoclaved 1/5 strength V8 medium was incorporated with 20 mg of lyophilised extract from an individual plant of that specific line. The extract was dissolved in a mixture of 1 mL of 50% methanol plus 1 mL of 0.1 M sodium acetate buffer (pH 4.5) containing 17 mM sodium chloride, and was added to the medium, when the medium had cooled to about 45 °C. All the Petri plates were inoculated with a piece of colony of approximately 25 mm^2 from an advancing edge of a four-day-old *P. cinnamomi* culture placed in the centre of each. Hyphal growth was measured daily for four days using a ruler.

5.2.4.3 Concentrations of free phenolics, leucoanthocyanidins and proanthocyanidins of the supernatant

The free phenolic, leucoanthocyanidin and proanthocyanidin contents of the supernatant were measured using the techniques described earlier in this chapter (Sections 5.2.1.1 and 5.2.3.2).

5.2.4.4 Proanthocyanidin concentration of the precipitate

Proanthocyanidins were separated from the precipitate after the technique of Rakhmani et al. (2005). The precipitate was dissolved in 1 mL of a mixture consisting of 1% sodium dodecyl sulphate and 5% of 0.1 M tris, 10 mM EDTA, and 50 mM sodium acetate adjusted to pH 7.2. Two millilitres of absolute methanol were added to the solution. A 0.5 mL aliquot from each sample was diluted with 0.5 mL of absolute methanol. The proanthocyanidins were converted to anthocyanidins using the butanol-HCl technique described in Section 5.2.3.2, and absorbance of each sample was measured at 525 nm using a spectrophotometer.

5.2.5 Separation of phenolic compounds by high performance liquid chromatography

The HPLC analysis of the extracts of the three *S. formosa* lines was performed to determine whether there were obvious differences in the phenolic profiles of the three lines, and in particular, in catechin and epicatechin content, two compounds from which proanthocyanidins are biosynthesised.

5.2.5.1 Equipment

An Automated Gradient Controller system (Model 680 Waters/Millipore, USA) with two high pressure pumps (Model 510 Waters/Millipore, USA), and a Lambda-Max UV detector (Model 481 Waters/Millipore, USA) coupled to a Data Module 746 chromatograph (Waters/Millipore, USA) were used for the solvent delivery system and detection of compounds. For the stationary phase, a μ-Bondapak^{TM} C18 column, 3.9 × 300 mm, with 10 μm particle sizes from Waters/Millipore, was used with a flow

rate of 0.5 mL minute^{-1}. A manual sample injector with a 25 µL sampling loop (SGE, Australia) was used for sample injection into the system.

5.2.5.2 Standards and plant samples

Gallic acid, catechin, epicatechin, quercetin and naringenin were used as standards for the detection of these compounds in the samples. Epicatechin was purchased from Sigma (Sigma Co., Australia), and the other phenolic compounds were from chemicals from the School of Environmental Sciences and Natural Resources Management, University of New England, purchased from Sigma (Sigma Co., Australia). Pure standards were dissolved in methanol at a concentration of 200 mg L^{-1} (Snyder et al., 1988), filtered through 0.45 µm Metricel membrane filters (Ann Arbor, Michigan, USA), and stored at 4 °C in darkness for a maximum period of 1 week.

To prepare plant samples, 500 mg of dry bud sample from an individual plant from each *S. formosa* line was extracted in 70% acetone and lyophilised as described earlier, Section 5.2.3. The lyophilised extract was dissolved in 1 mL absolute methanol and filtered through a 0.45 µm Metricel membrane filter (Ann Arbor, USA).

5.2.5.3 Injection

To obtain a chromatogram of visible phenolic compounds at 280 nm from each line and to identify some of the peaks using standards, 10 µL from a plant sample or a standard were injected into the system each time. In addition, for further confirmation of the presence or absence of catechin and epicatechin in the plant samples, 0.2 mg of both catechin and epicatechin were spiked into 1 mL of each plant sample and 10 µL of the mixture were injected into the system. The resulting chromatograms were compared with the main chromatograms.

5.2.5.4 Solvents and separation conditions

To separate phenolic compounds that are strongly different in polarities with a single run of high performance liquid chromatography (HPLC), a binary gradient elution with increasing concentration of acetonitrile after the method of Lamuela-

Raventós and Waterhouse (1994) was used. Solvent A contained 50 mM dihydrogen ammonium orthophosphate adjusted to pH 2.6 using phosphoric acid. The water used was HPLC grade, deionised and purified in a USF-ELGA water purification system (Permutit, Sydney, Australia). Solvent B was a mixture of 20% solvent A and 80% absolute acetonitrile. The solvents were filtered separately through a 0.45 μm filter paper (Millipore, USA), and simultaneously degassed under vacuum on a stirrer. The column was washed for 15 minutes with 100% solvent A before the next injection.

The solvent gradient employed to separate the phenolic compounds was based on the gradient of Lamuela- Raventós and Waterhouse (1994), with a considerable modification as is shown in Table 5.1. The last solvent combination, as is shown in Table 5.1, was 75% solvent A and 25% solvent B where the last peaks were fully eluted. The concentration of phenolic compounds was measured at 280 nm on the instrument detector.

Table 5.1. Solvent gradient conditions with linear gradient.

Final time	Flow rate (mL/min)	A%	B%	Curve
0	0.5	100	0	6
5	0.5	100	0	6
15	0.5	96	4	6
25	0.5	93	7	6
26	0.5	92	8	6
35	0.5	85	15	6
40	0.5	80	20	6
45	0.5	75	25	6
50	0.5	100	0	6

5.2.6 Data analysis

A one-way ANOVA was used for data analysis. All analyses were conducted using the SPSS™ statistical package. The criterion for significance of treatment effects was $P < 0.05$ unless otherwise indicated.

5.3 Results

5.3.1 Concentration of some phytocompounds in inoculated and non-inoculated plants

5.3.1.1 Soluble phenolic compounds

Soluble phenolic content was significantly different ($P < 0.01$) in both detached stems and hypocotyls of the three *S. formosa* lines. The red line had the highest level of soluble phenolics, while the white line had the lowest level and the pink line was intermediate (Table 5.2 and Table 5.3). Soluble phenolic content was not significantly different between inoculated tissues and their controls. Therefore, no response to infection was observed. This is consistent with some types of phenolics being involved in resistance as phytoanticipins, rather than an induced response.

5.3.1.2 Anthocyanin levels

The anthocyanin level did not increase in the inoculated tissues compared with their controls (Table 5.2 and Table 5.3); therefore, no response to infection was observed. Although the anthocyanin content in non-inoculated detached stems of the three lines was significantly different ($P < 0.01$) and varied directly with resistance (red line> pink line> white line (Table 5.3)), there was no difference in anthocyanin content between the pink and red line hypocotyls (Table 5.2), suggesting that the coloured compounds are not directly responsible for differences in resistance.

Table 5.2. Hypocotyl defence responses to inoculation with *Phytophthora cinnamomi* in the three *Swainsona formosa* lines. Hypocotyls of three-week-old seedlings were inoculated with *P. cinnamomi* mycelia and measurements were taken four days later.

Line	Treatment	Anthocyanins ($\Delta OD_{525\ nm}$)	β-glucanase activity ($\Delta OD_{610\ nm}$)	Soluble phenolics (ΔOD725 nm)
White	Control	0.043 ± 0.0059A	0.030 ± 0.0024	0.201 ± 0.010
	Infected	0.038 ± 0.0099	0.030 ± 0.0011	0.20 ± 0.011
Pink	Control	0.064 ± 0.0048	0.030 ± 0.0008	0.410 ± 0.003
	Infected	0.071 ± 0.0036	0.030 ± 0.0010	0.404 ± 0.004
Red	Control	0.063 ± 0.0023	0.030 ± 0.0003	0.620 ± 0.007
	Infected	0.066 ± 0.0023	0.030 ± 0.0006	0.615 ± 0.006

[A]Values are means ± standard error (n = 5)

Table 5.3. Detached stem defence responses to inoculation with *Phytophthora cinnamomi* in the three *Swainsona formosa* lines. Sections of trimmed stems 11 cm long were placed into cultures of *P. cinnamomi*. Assays were conducted three days later using tissue from 2 cm above the surface of the culture.

Line	Treatment	Anthocyanins ($\Delta OD_{525\ nm}$)	β-glucanase activity ($\Delta OD_{610\ nm}$)	Soluble phenolics (ΔOD725 nm)
White	Control	0.013 ± 0.0006A	0.247 ± 0.0069	0.339 ± 0.011
	Infected	0.014 ± 0.0006	0.244 ± 0.0077	0.342 ± 0.008
Pink	Control	0.027 ± 0.0008	0.239 ± 0.0050	0.608 ± 0.005
	Infected	0.028 ± 0.0005	0.242 ± 0.0051	0.610 ± 0.008
Red	Control	0.056 ± 0.0011	0.242 ± 0.0062	0.893 ± 0.009
	Infected	0.058 ± 0.0015	0.243 ± 0.0047	0.864 ± 0.011

[A]Values are means ± standard error (n = 7)

5.3.1.3 Activity of β-1, 3-glucanase

No significant difference was detected in β-1, 3-glucanase activity of either the hypocotyls or detached stems between the three *S. formosa* lines (Table 5.2 and Table 5.3). There was also no significant difference in β-1, 3-glucanase activity between the inoculated tissue and controls (Table 5.2 and Table 5.3).

5.3.2 The inhibitory effect of flower pigments on growth of *P. cinnamomi*

The effect of the flower pigments of each *S. formosa* line on growth of *P. cinnamomi* did not differ significantly ($P<0.01$) in the three techniques tested. The extent of *P. cinnamomi* growth in the presence of different flower pigments was also not significantly different ($P<0.05$) from the controls (Figure 5.1).

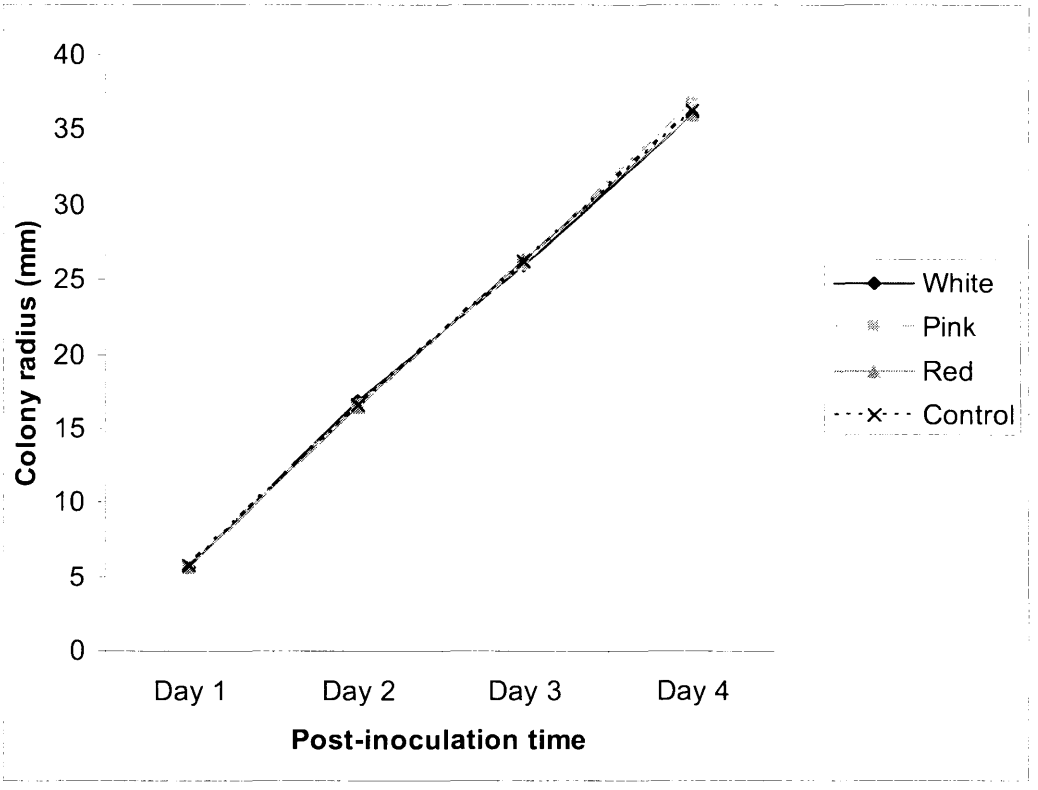

Figure 5.1. The reaction of *Phytophthora cinnamomi* hyphae to anthocyanins extracted from the three *Swainsona formosa* lines. An aliquot of 600 µL concentrated extract from each *S. formosa* individual was mixed with 20 mL of 1/5 strength V8 medium, the medium autoclaved and then cooled to about 45 °C. Each incorporated culture was inoculated with a small piece of *P. cinnamomi* placed in the centre of the plate. The radial growth of the fungus was measured daily for 4 days, using a ruler.

5.3.3 The inhibitory effect of acetone-extracted phenolics on the growth of *P. cinnamomi*

The influence of phenolic compounds on the growth of *P. cinnamomi* in each *S. formosa* line was highly ($P<0.01$) significant. The phenolic compounds present in the red line had an absolute inhibitory impact on *P. cinnamomi* growth, and no growth inhibitory effect was observed with the white line; the influence of the white line was the same as the control. The growth inhibitory effect of the phenolic compounds from the pink line on *P. cinnamomi* was intermediate between those of the red and white lines (Figure 5.2).

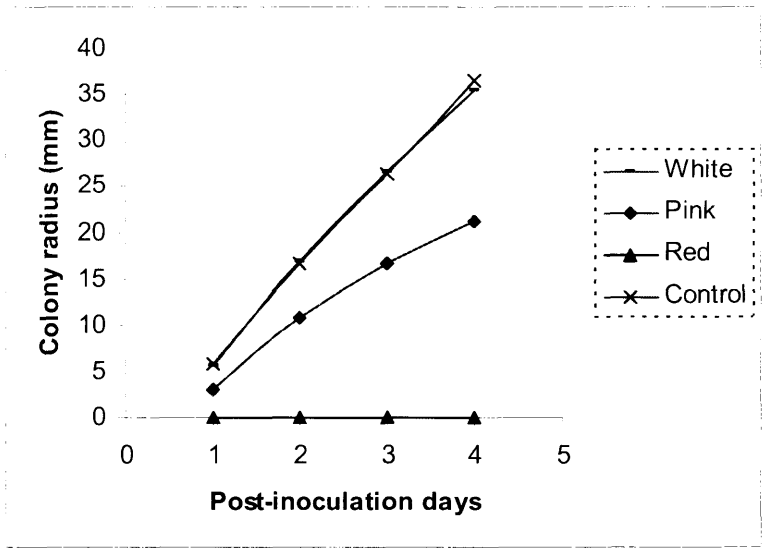

Figure 5.2. The reaction of *Phytophthora cinnamomi* hyphae to phenolic compounds extracted from the three *Swainsona formosa* lines. Twenty milligrams of acetone-extracted lyophilised extract from each individual of *S. formosa* was dissolved in 1 mL methanol and incorporated with 20 mL of 1/5 strength autoclaved V8 medium at about 45° C. Each infused culture was inoculated with a small piece of *P. cinnamomi* placed in the centre of the plate. The radial growth of the fungus was measured daily for 4 days.

5.3.4 The bovine serum albumin concentration in the supernatant of the three *S. formosa* lines after precipitating proanthocyanidins

The mixture of BSA and the red line extract had the highest precipitate. Therefore, if the BSA of this mixture is sufficient to precipitate all condensed tannins, the BSA of the other mixtures should also be sufficient. After proanthocyanidin precipitation, 0.2 and 0.13 mg mL^{-1} BSA remained in the supernatant of the pink and

red lines, respectively. It indicates that all condensed tannins from the extracts of these lines were precipitated by the protein, and some excess protein remained in the precipitation media. The extract from the white line produced no precipitate (Figure 5.3), and the concentration of BSA in its supernatant was higher (0.7 mg mL^{-1}) than for the red and pink lines.

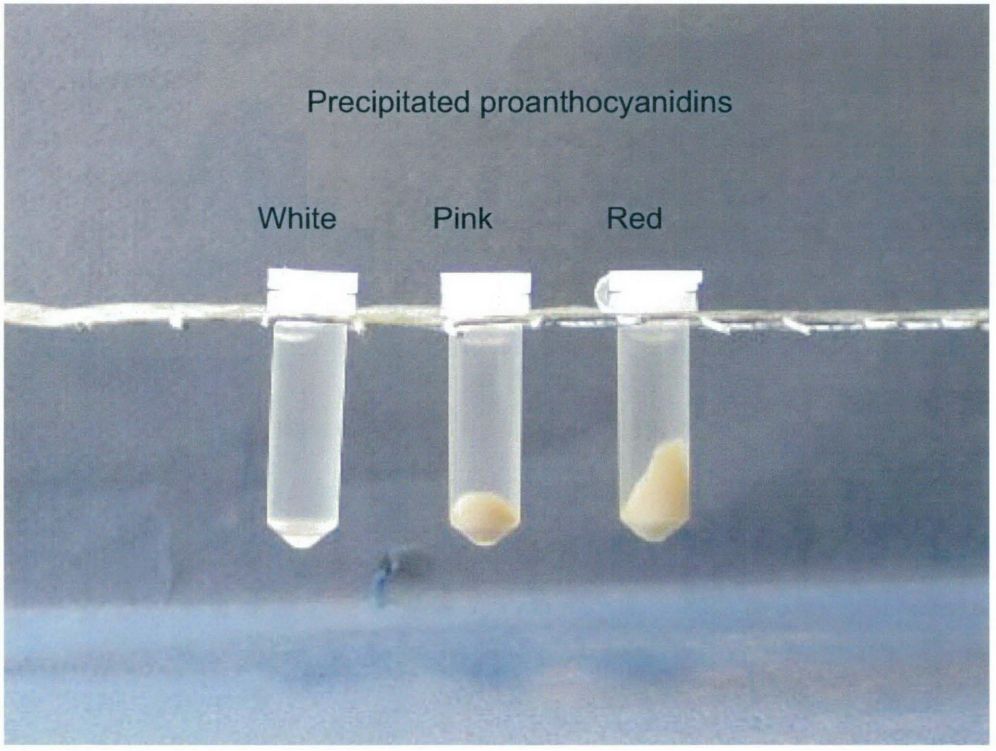

Figure 5.3. Supernatant, and precipitated condensed tannins of the three *S. formosa* lines. The lyophilised extracts of individual plants from each line and bovine serum albumin (BSA) were dissolved in appropriate solvents at concentrations of 1 mg mL^{-1} and 2 mg mL^{-2}, respectively, and mixed. The condensed tannins reacted with BSA and were precipitated by centrifugation.

5.3.5 The inhibitory effects of supernatant on the growth of *P. cinnamomi*

No significant differences ($P<0.01$) in the growth rate of *P. cinnamomi* were observed when the supernatants from the three *S. formosa* lines were incorporated into the fungal media. The supernatants from the three lines, which lacked proanthocyanidins, had no inhibitory effect on the growth of *P. cinnamomi* (Table 5.4). In contrast, a significant difference ($P<0.01$) was detected on the fungal growth rate of the control plates where the mixtures of buffer and original lyophilised extracts were incorporated into the fungal media. The extracts from the red line inhibited

fungal growth absolutely, while the extract from the white line had no effect on hyphal growth. The hyphal growth on the incorporated media with the extract from the pink line was intermediate between those of the red and white lines (Table 5.4).

Table 5.4. Growth rate of *Phytophthora cinnamomi* on media incorporating supernatant. The experiment was conducted with five replicates plus five controls for each line. Each replicate was a Petri plate containing 20 mL of 1/5 strength V 8 medium incorporated with about 2 mL supernatant from an individual plant. The controls were 1/5 strength V 8 medium incorporated with about 2 mL solution of sodium acetate buffer and 20 mg mL^{-1} of lyophilised extract from an individual plant (1:1; v:v).

Lines	Colony diameter (mm) ± S.E.	
	Protein treated extract	Untreated extract
White	39 ± 0.40	39 ± 0.32
Pink	39 ± 0.32	24.4 ± 0.40
Red	38 ± 0.45	0

5.3.6 Soluble phenolic and leucoanthocyanidin contents of the acetone-extracted original extract and supernatant

There was a significant difference in the total or soluble phenolic concentration of the original extracts from the three *S. formosa* lines ($P<0.01$). The red line recorded the highest amount of phenolic compounds, the white line the lowest amount and the pink line was intermediate between those of the red and white lines (Figure 5.4).

Free phenolic levels were significantly different ($P<0.01$) in the supernatant of each *S. formosa* line. The pink line had a lower concentration of free phenolics than the red and white lines (Figure 5.4).

In both the original extract and the supernatant, leucoanthocyanidin concentration of the three *S. formosa* lines was very low. However, the leucoanthocyanidin concentration of the red line was significantly higher ($P < 0.01$) than that of the pink and the white lines, which did not differ significantly from each other (Figure 5.4).

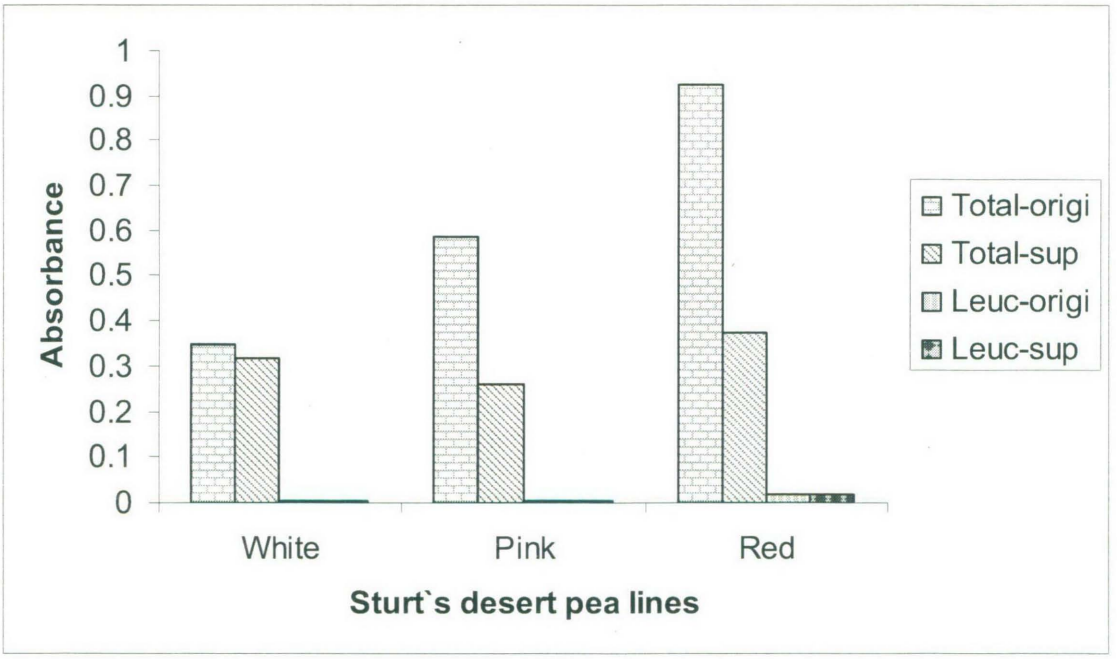

Figure 5.4. Total phenolic and leucoanthocyanidin concentration of the original extract and supernatant. Total-origi: total phenolics of original extract, Total-sup: total phenolics of supernatant, Leuc-origi: leucoanthocyanidin of original extract, and Leuc-sup: leucoanthocyanidin of supernatant. The lyophilised extract (original) was dissolved in methanol at a concentration of 1 mg mL^{-1} and was chemically converted to anthocyanidin by adding butanol-HCl, and its concentration was measured at 525 nm using a spectrophotometer. The leucoanthocyanidin concentration of supernatant was measured in the same approach.

5.3.7 Concentration of proanthocyanidins of the original extract, supernatant and precipitate

The concentration of proanthocyanidins in the original extract and precipitate was significantly different ($P<0.01$) among the three *S. formosa* lines. In both the original extract and the precipitate, the proanthocyanidin concentration was higher in the red than in the pink line. The proanthocyanidin concentration of the white line in the original extract, precipitate and supernatant was approximately zero (Figure 5.5). No significant difference ($P<0.01$) between supernatant proanthocyanidins was observed in the red and pink lines.

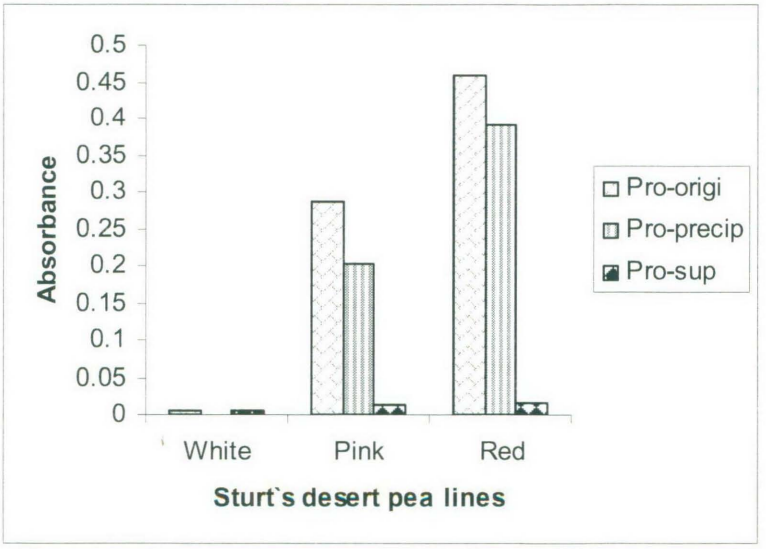

Figure 5.5. Proanthocyanidin concentration of original extract, precipitate and supernatant. Pro-origi: proanthocyanidin of original extract, Pro-precip: proanthocyanidin of precipitate, and Pro-sup: proanthocyanidin of supernatant. In all cases, the proanthocyanidin was chemically converted to anthocyanidin by adding butanol-HCl, and was measured at 525 nm using a spectrophotometer.

5.3.8 Comparison of phenolic compounds of *S. formosa* lines on HPLC chromatogram

Phenolic compounds were analysed using HPLC. Many phenolic compounds with similar retention times were observed in the three *S. formosa* lines, although the relative proportion of some peaks differed between the lines. Some peaks were identified in comparison with standards, and were distinguished as gallic acid, catechin, naringenin, quercetin and epicatechin with retention times of 6, 8, 47, 55 and 56 minutes, respectively. Except for epicatechin, which was not found in the white line, all the other compounds were detected in the three *S. formosa* lines, although the relative proportion of each peak differed between the lines (Figure 5.6).

Catechin and epicatechin were separated from the column at different times. The identity of catechin and epicatechin was confirmed by spiking the extracts from the three lines with catechin and epicatechin and obtaining an increase in absorbance of catechin in the three lines, and epicatechin in the red and pink lines. This also confirmed the presence of catechin (the second peak in Figure 5.6) in the three *S. formosa* lines, and the absence of epicatechin in the white line. All stages of this experiment were conducted twice with similar results in each repetition.

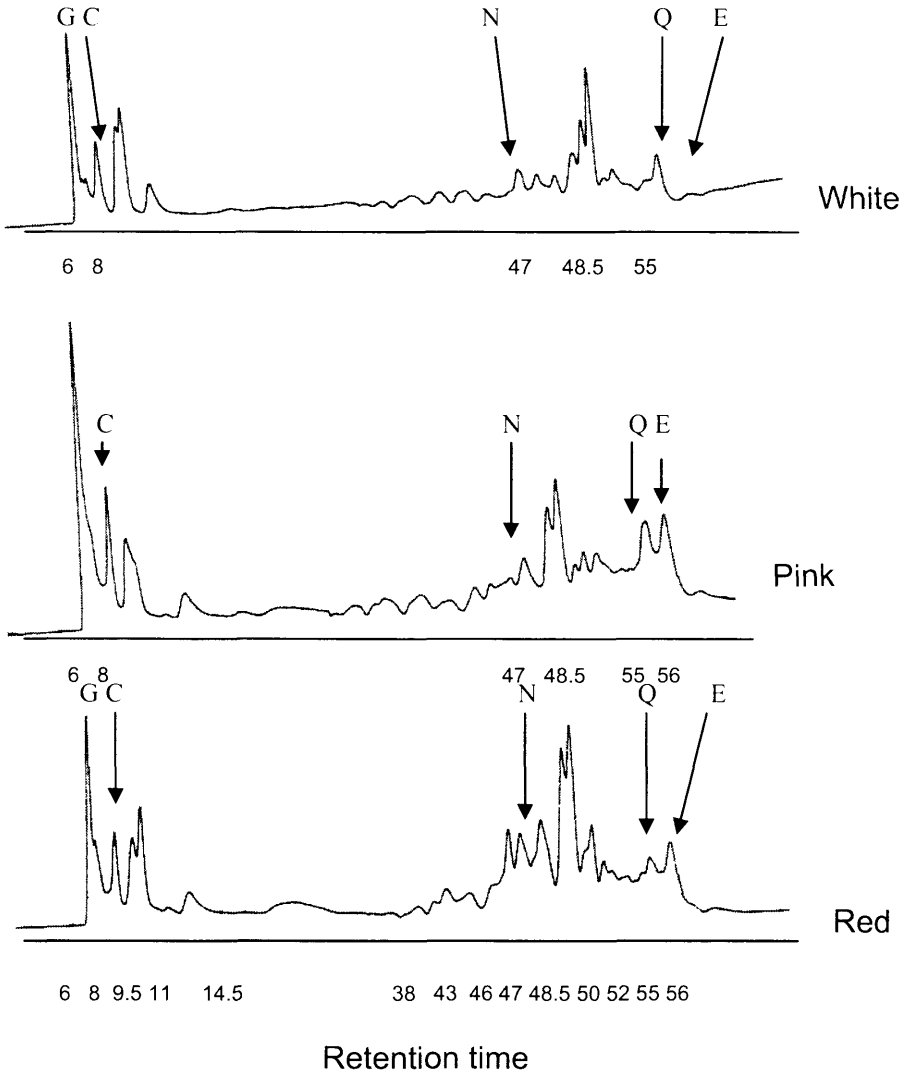

Figure 5.6. High performance liquid chromatography chromatogram of phenolic compounds in the three *Swainsona formosa* lines. G: gallic acid, C: catechin, N: naringenin, Q: quercetin and E: epicatechin. The acetone extracted phenolic compounds were analysed by HPLC and measured at 280 nm. Attenuation was 128 and sheet speed was 0.25 cm minute^{-1}. Gallic acid, catechin, naringenin, quercetin and epicatechin were run as standards. Chromatograms are overlapped for comparison. Retention time for gallic acid, catechin, naringenin, quercetin and epicatechin were 6, 8, 47, 55 and 56 minutes, respectively.

5.4 Discussion

The effects of some polyphenolics such as anthocyanins, and free phenolics, some of which are related to flower colour, were initially tested because of the link between flower colour and resistance to *P. cinnamomi*. In addition, β-1, 3-glucanase

activity was tested to investigate the possible involvement of pathogenesis-related proteins. Total phenolic levels varied among non-inoculated lines in direct relation to resistance to *P. cinnamomi*. The red line contained the highest concentration of soluble phenolics, while the white line contained the lowest and the pink line was intermediate between the other two lines. The levels of β-1, 3-glucanase were similar among the three lines, suggesting that resistance to *P. cinnamomi* was not due to β-1, 3-glucanase. When the involvement of free phenolics, anthocyanins, and β-1, 3-glucanase from inoculated and non-inoculated plants, as induced and/or preformed defence responses were evaluated, there was no evidence for the involvement of active defence mechanisms in the differing resistance of the three lines. This suggested that preformed defences, probably chemical, were responsible. Phenolics and phenolic glycosides have been reported as phytoanticipin forms preventing pathogen infestation (Bennett and Wallsgrove, 1994; Osbourn, 1996). Preformed chemicals are commonly secreted in the vascular system or the organelles of healthy plants at levels anticipated to have antimicrobial properties (Grayer and Harborne, 1994; Morrissey and Osbourn, 1999), and to prevent plant infection by necrotrophic pathogens (Osbourn, 1996). However, it was concluded that some phenolic compounds present in the red line completely inhibited plant infestation by *P. cinnamomi*. In the same way, the partial resistance of the pink line could be related to a lower concentration of phenolic compounds.

The link between flower colour and disease resistance led to the hypothesis that the differences in disease resistance among the three lines was related to the anthocyanin contents of the tissues. Anthocyanin content of the detached stems varied in accordance with resistance to *P. cinnamomi*. However, there was no difference in anthocyanin content between the pink and red line hypocotyls, suggesting that the coloured compounds are not directly responsible for differences in resistance.

To confirm that anthocyanins were not responsible for the resistance of the red line to *P. cinnamomi,* and also to confirm the involvement of soluble phenolics as phytoanticipins the toxicity of these phytocompounds to the fungus was tested. Results showed that the anthocyanins present in the three lines were not toxic to *P. cinnamomi*, while the hyphal growth rate was significantly different when the fungal media were incorporated with soluble phenolics extracted from the three lines. The free phenolic compounds from the red line, as expected, inhibited fungal growth,

while the extract from the white line had no growth inhibitory effect on the fungus. An intermediate response was observed with extract from the pink line.

To identify which phenolic compound(s) from the red line were associated with resistance to *P. cinnamomi*, the concentration of flower-colour dependent phenolic compounds (leucoanthocyanidins and proanthocyanidins) was measured in the three lines. The pink and white lines, with different reactions to *P. cinnamomi*, contained the same amount of leucoanthocyanidins; therefore partial resistance in the pink line was not related to leucoanthocyanidin content.

The proanthocyanidin component of the soluble phenolics from the three lines was found to have a higher concentration in the red line than the pink line, and was absent in the white line. As the soluble phenolics were toxic to the fungus, it was assumed that the proanthocyanidins would also be toxic to the fungus. The toxicity of proanthocyanidins to *P. cinnamomi* was indirectly examined. Proanthocyanidins were precipitated from the extracts of the red and pink lines by BSA, and toxicity to the fungus of the remaining substances in the supernatant was tested using the poison food technique. The supernatants of the red and the pink lines lacking proanthocyanidins were not toxic to the fungus; therefore the complete and partial resistances of the red and pink lines, respectively, were correlated with proanthocyanidin content.

The antimicrobial activity of proanthocyanidins has been reported (Scalbert, 1991; Snyder et al., 1991; Zheng et al., 2005). Kantar et al. (1996) reported that white-flowered *Vicia faba* lines were more susceptible to fungal infection during seed germination than lines with coloured flowers. This, however, appeared to be due to differences in seed coat tannin content. Dixon et al. (2005) suggested that the major function of the proanthocyanidins and monomeric flavan-3-ols and their derivatives present in the fruits, bark, leaves and seeds of many plants, is to protect them against microbial pathogens, insect pests and large herbivores. Susceptibility to diseases and pests in some plants has been associated with the lack or low concentration of proanthocyanidins. For example, susceptibility of coffee varieties to the fungal pathogen *Hemileia vastatrix* was reported to be associated with low proanthocyanidin levels (Gonzalez de Colmenares et al., 1998).

The absence of proanthocyanidins in the white line could result from a blockage or inactivation in biosynthesis or activity of ANS, ANR, regulatory genes for ANS and ANR, or enzymes upstream of ANS and ANR in the phenylpropanoid

pathway. In each case epicatechin, a precursor of proanthocyanidin, is not biosynthesised. Anthocyanidin synthase activity is followed by ANR. Anthocyanidin synthase catalyses the conversion of leucoanthocyanidin to anthocyanidin (Holton and Cornish, 1995; Tanner et al., 2003; Xie et al., 2004b; Bogs et al., 2005). Anthocyanidin reductase subsequently catalyses the reduction of anthocyanidins to 2,3 cis flavan-3-ols such as epicatechins (Holton and Cornish, 1995; Marles et al., 2003; Tanner et al., 2003; Xie et al., 2003; Xie et al., 2004b; Bogs et al., 2005). The absence of epicatechin in the white line was confirmed by HPLC. However, the presence of catechin in the white line plants suggests that they are able at least to biosynthesise leucoanthocyanidins, the precursor of both catechin and anthocyanidins; it may be reasonable to assume that it is loss of function of ANS that leads to loss of colour. The white line plants may still have functional ANR, but they lack epicatechin and consequently proanthocyanidins because they lack anthocyanidins, the substrate for ANR.

The genetic basis of *P. cinnamomi* resistance is considered in the next chapter.

Chapter 6 Identifying a gene for anthocyanidin reductase in *Swainsona formosa*

6.1 Introduction

In *Swainsona formosa*, a strong link was observed between flower colour and susceptibility to *P. cinnamomi* and some other soilborne fungi (Chapters 3 and 4). It was shown that susceptibility was due to the lack of proanthocyanidins (Chapter 5). The classical models of the flavonoid biosynthetic pathways (Dixon and Paiva, 1995; Winkel-Shirley, 2001) cannot explain why reduction in flower pigments should be linked to reduced proanthocyanidin content. Xie et al. (2003) discovered genes for a novel enzyme, anthocyanidin reductase (ANR), in *Arabidopsis thaliana* and *Medicago truncatula* that leads to the biosynthesis of epicatechin from anthocyanidins. Anthocyanidins are therefore the precursors of the major building blocks of proanthocyanidins in these plants (Xie et al., 2003; Xie et al., 2004b; Xie et al., 2006). In a recent study on apple fruit skin, the same function for ANR has been reported (Takos et al., 2006). Epicatechin, and catechin, which is converted from leucoanthocyanidins by leucoanthocyanidin reductase (LAR) (Tanner et al., 2002; Tanner et al., 2003), are condensed by unknown enzymes (Xie et al., 2003) to produce proanthocyanidins. The existence of this pathway through ANR has not been widely demonstrated in other plants. The occurrence of a gene for ANR in *S. formosa* would indicate that this species also synthesises epicatechin, and hence proanthocyanidins, from anthocyanidins. The objective of this chapter was to investigate whether *S. formosa* contains a gene homologous to *M. truncatula* ANR gene.

6.2 Materials and methods

6.2.1 Plant materials and genomic DNA extraction

DNA was extracted from fresh, newly opened leaflets of the three *S. formosa* lines (red, pink and white) after the method of Doyle and Doyle (1987), and Fatemi (2005). Briefly, 100 mg of fresh tissue from each line were placed separately in 1.5 mL microfuge tubes. Samples were macerated and homogenised at room temperature using tungsten carbide beads in a Qiagen MM 300 mixer mill. Genomic substances were extracted by adding 1 mL of lysis buffer to each tube. The lysis buffer for DNA

extraction for 15 samples consisted of 15 mL cetyl trimethyl ammonium bromide (CTAB) buffer (1 litre buffer contains 100 mL of 1 M tris with pH 8.0, 280 mL of 5 M sodium chloride, 40 mL of 0.5 M EDTA and 20 g CTAB), 600 mg polyvinylpyrrolidone (PVP), 75 µL β-mercaptoethanol, 15 mg diethyldithio-carbamate (DIECA) and 15 mg ascorbic acid. The tubes were stirred and mixed thoroughly. Samples were incubated at 60 °C in a block digester for at least 1 hour to completely lyse the cells. Proteins were removed by adding 500 µL of chloroform:isoamyl alcohol (v:v; 24:1) to each tube. The tubes were inverted and vigorously shaken for 1 minute to form an emulsion, and then centrifuged at 15000 *g* for 5 minutes. After centrifugation, three layers formed in each tube: an aqueous phase containing genomic DNA on the top, a solid phase formed from debris and proteins in the middle and a chloroform phase at the bottom of the tube. The upper supernatant from each tube was carefully transferred into a labelled, 1.5 mL microfuge tube using a plastic pipette. Five hundred microlitres of chloroform:isoamyl alcohol (24:1) was added to each tube, and each tube inverted and vigorously shaken for 1 minute to form an emulsion. The samples were again centrifuged at 15000 *g* for 5 minutes, and the upper phase was transferred into a fresh labelled tube. To precipitate the DNA, 600 µL of cold isopropanol were added to each tube and the tubes were gently inverted to mix their content. Samples were stored at -20 °C for at least 1 hour, and then centrifuged at 9000 *g* for 15 minutes. The supernatant was carefully discarded and a DNA pellet was left at the bottom of each tube. The pellets were washed with cold (-20 °C) 70% ethanol, as follows. One millilitre of cold 70% ethanol was added to each tube and the tube gently vortexed. The tubes were centrifuged at 9000 *g* for 5 minutes and the supernatant discarded. Each pellet was washed as above with 1 mL of cold (-20 °C) 90% ethanol. The tubes containing the DNA pellets were placed in the fumehood for 40 minutes to dry. Each DNA pellet was resuspended in 100 µL of 10 mM tris, which is alkaline, and stored at -20 °C.

6.2.2 Primer design

Before designing primers, highly conserved regions of putative ANR genes were identified. Primers were designed from these regions based on the *Medicago truncatula* sequence, other putative ANR sequences from legumes, and matching

expressed sequence tags (ESTs) from legumes. The sequence for the *M. truncatula* ANR gene (accession number AY184243) determined by Xie et al. (2003) was obtained from the GenBank database through the 'National Centre for Biotechnology Information (NCBI, 2006:online). The 'Basic Local Alignment Search Tool' (BLASTn) (NCBI/BLAST, 2005:online) was used to find matching sequences. Regions on the *M. truncatula* gene that were identified by BLAST as being similar to those on other plant ANR or putative ANR genes were identified. These other sequences came from *Phaseolus*, apple, grape, cotton and tea. Primer3 software (Primer3, 2004:online) was used to design primers from the *M. truncatula* sequence that were located in conserved regions. Primers were compared with the *Phaseolus coccineus* sequence and matching ESTs from other legumes, to select those that were likely to amplify all known legume sequences. Table 6.1 shows the gene sequences that were used for primer design. Primers were synthesised by Geneworks, Adelaide, Australia. These were used in all possible combinations of left and right primer (Table 6.2).

Table 6.1. Sequences of anthocyanidin reductase (ANR) and highly similar expressed sequence tags (ESTs) from legumes used in design of primers for ANR in Sturt's desert pea.

Accession number	Species	Type of sequence
AY184243	*Medicago truncatula*	ANR, complete mRNA
BN000164	*Phaseolus coccineus*	Putative ANR, complete mRNA
AV771486	*Lotus corniculatus*	EST
BI1642387	*Robinia pseudoacacia*	EST
CO985210	*Glycine max*	EST
BM092813	*Glycine max*	EST
AV774784	*Lotus japonicus*	EST

Table 6.2. Forward and reverse primers for anthocyanidin reductase. The primer name indicates the position in the *Medicago truncatula* sequence (AY184243) of the first base pair in the primer.

Primer name	Primer length	Forward/Reverse	Primer sequence: 5` to 3`
L522	20	Forward	CCC ACT TGG GGT TAT CCT GT
R855	20	Reverse	GCT CGG GAA CAC TGG TAT TG
L286	21	Forward	TCC AAC TTG CTA CAC CTG TGA
R781	20	Reverse	TCC AAC TTG CTA CAC CTG GA
L317	23	Forward	TCA AGA TCC TGA GAA TGA CAT GA
R877	20	Reverse	CGT TTG CTG AGA AAC TTT GC

6.2.3 Polymerase chain reaction conditions

The DNA was amplified in 200 µL tubes, using a Corbett gradient thermocycler model CG1-96. After preparation, the amplification mixture (master mix) was dispensed into thermocycler tubes. Each amplification reaction was performed in a total volume of 50 µL containing 5 µL 10× PCR buffer, 200 nM of each primer (forward and reverse), 1 µL of the dNTP mixture consisting of 200 µM of each dNTP, 2.5 units (=0.25 µL) *Taq* DNA polymerase (Qiagen), 5 µL template DNA, 2.5 mM magnesium chloride which was 2 µL of 25 mM magnesium chloride and 35.75 µL sterile deionised water. Other concentrations of magnesium chloride including 1.5, 2.5 and 3 mM were also tested.

The thermocycler was programmed for 3 minutes at 95 °C to initiate DNA denaturation, followed by 30 cycles of the denaturation step at 95 °C for 30 seconds, an annealing temperature step at 55 °C for 30 seconds and an extension step at 72 °C for 1 minute. After the last cycle, the reaction was incubated at 72 °C for 10 minutes to allow completion of uncompleted strands. The cooling temperature chosen was 4 °C to keep the amplified products cool until they were transferred to the refrigerator. The experiment was performed four times, each time with a newly extracted DNA from each line.

The amplified products were separated on a 1.4% agarose gel in 1× tris-borate buffer (89 mM tris, 89 mM boric acid, 2 mM EDTA). The gel was stained for at least 20 minutes in a solution of 0.5 μg mL^{-1} ethidium bromide in water. The gel was then washed with tap and distilled water respectively and was photographed under ultra-violet light.

6.2.4 DNA sequencing and sequence similarity searching of EST-PCR products

Amplicons were purified using the Qiaquick PCR purification kit (Qiagen, 2002). They were mixed with primers according to recommendations of the sequencing company (Sydney University Prince Alfred Macromolecular Analysis Centre, SUPAMAC), and were sent to SUPAMAC for sequencing. Ten picomoles (pmol) of forward primer was mixed with 100 nanograms (ng) of purified amplicon and diluted to 16 μL with distilled water. Similarly, 10 pmol of reverse primer was mixed with 100 ng of purified amplicon and diluted to 16 μL with distilled water. There, the purified amplicons were completely sequenced from 5' and 3' ends, using an automated laser sequencer. Sequences were compared with those in GenBank, using BLASTn algorithm (NCBI/BLAST, 2005:online) to determine if the sequences of the PCR products were homologous to the known sequences for anthocyanidin reductase genes.

6.3 Results

Of the nine primer pair combinations used to amplify the ANR gene if present in the genome of each *S. formosa* line, only one fragment of 1278 bp (Figure 6.1) was amplified in the red and pink lines using forward L286 and reverse R877 primers. No amplicon was produced from the amplification of white line DNA extracts. The best magnesium chloride concentration was 3 mM.

The PCR product amplified from the red and pink lines was sequenced, and subjected to a BLAST search against the DNA database at NCBI. The most similar sequences identified were known putative ANRs. Comparison of the *S. formosa* sequence with those in the database revealed the presence of three introns. The introns were edited out following comparison of the *S. formosa* sequence with the cDNA sequences of *M. truncatula* (AY184243) and *Phaseolus coccineus* (BN000164) and a genomic sequence from *Vitis vinifera* (AB199315). This resulted in a sequence of 492

bases, or 164 amino acids, which is approximately half of the length of the known ANR sequences. The *S. formosa* sequence showed 84% nucleic acid identity and 83% amino acid identity with the known ANR sequence from *M. truncatula*, and a similar level of similarity to putative ANR genes or proteins from other plants (Table 6.3). The next most similar set of sequences were for dihydroflavanol-4-reductases, but these showed less than 50% amino acid identity to the *S. formosa* sequence. It was concluded that the amplicon was part of a gene for anthocyanidin reductase.

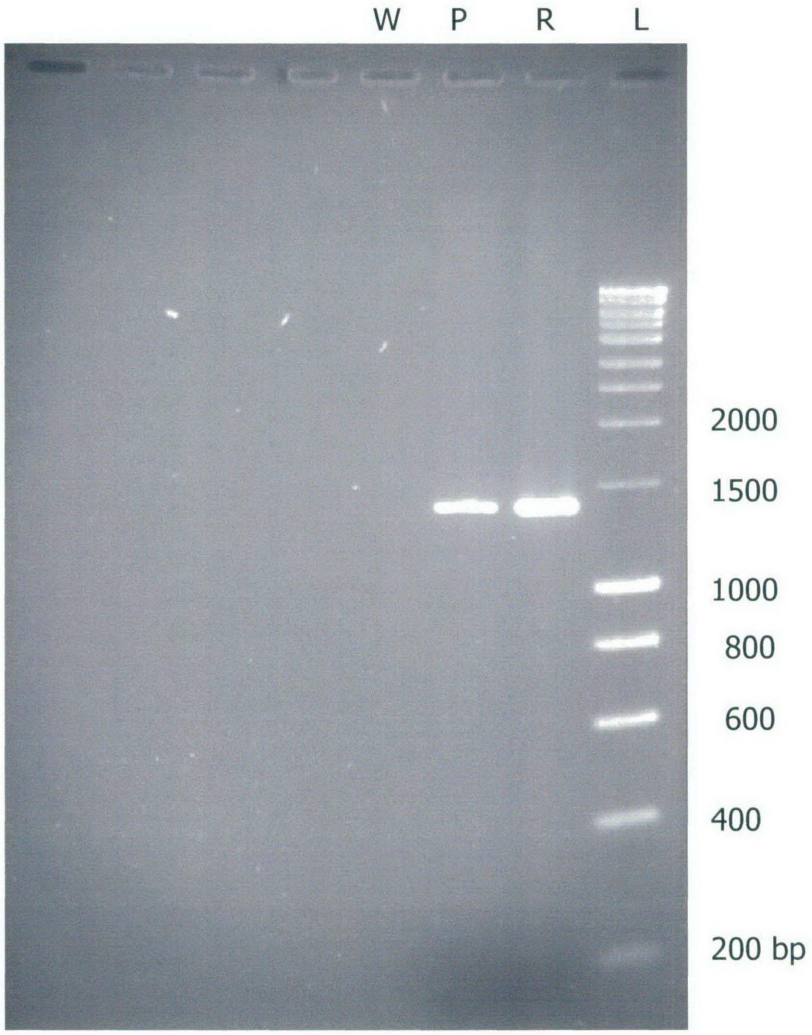

Figure 6.1. An expressed sequence tag marker generated by the primer set designed in this study. Two primers, L286 (5`- TCC AAC TTG CTA CAC CTG TGA-3`) and R877 (5`- CGT TTG CTG AGA AAC TTT GC-3`), were used for PCR amplification. L: Ladder/DNA molecular marker (Hyper ladder 1 from Bioline company, Australia), R: *Swainsona formosa* red line, P: *S. formosa* pink line and W: *S. formosa* white line.

Table 6.3. Sequence similarity of protein translation of *Swainsona formosa* amplicon to known or putative anthocyanidin reductases from other species. ID: identification. Comparison based on 164 amino acids: known ANR proteins are 336-340 amino acids long.

Accession	Organism	ID/function[1]	Amino acid identity (%)
CAD91909	*Phaseolus coccineus*	Putative ANR	90
AAN77735	*Medicago truncatula*	ANR	83
CAD91910	*Gossypium arboreum*	Putative ANR	81
AAT68773	*Camellia sinensis*	Putative ANR	81
BAD89742	*Vitis vinifera*	ANR	79
AAX12184	*Malus domestica*	Putative ANR	78
AAR27014	*Medicago truncatula*	DFR	45

[1]ANR anthocyanidin reductase; DFR dihydroflavanol-4-reductase.

6.4 Discussion

A part of a putative ANR gene with high DNA and amino acid sequence similarity to the ANR gene of *M. truncatula* and other plants was amplified from *S. formosa* red and pink line genomes. Adams et al. (1991) demonstrated that the possible function of an anonymous gene can be inferred solely on the basis of partial nucleotide homology to the genes of known function or deduced amino acid sequence homology to the products of known function genes. This enzyme converts anthocyanidins to epicatechin, one of the proanthocyanidin precursors, in other plants (Xie et al., 2003; Xie et al., 2004b; Takos et al., 2006; Xie et al., 2006). The presence of epicatechin in *S. formosa* red and pink lines was observed by HPLC (Chapter 5). The presence of a homologue of the gene for ANR in *S. formosa* indicates that epicatechin is also synthesised from anthocyanidins in this plant. This could explain why reduction in anthocyanin pigments is linked to reduction in proanthocyanidin content.

The failure to amplify an ANR gene from the white line genome may mean that it lacks this gene. The lack of this gene in the white line, or a mutation making the gene non-functional, would result in a lack of epicatechin. The absence of epicatechin in the white line was shown earlier (Chapter 5). As this compound is a precursor of proanthocyanidin, the lack of it leads to the lack of proanthocyanidins in the white line. However, the lack of proanthocyanidins in the white line plants may be the result of the lack of anthocyanidins, which are the substrate for epicatechin biosynthesis (Xie et al., 2003; Xie et al., 2004b), or mutations in regulatory genes in the proanthocyanidin pathway. Therefore, even if ANR is present and fully functional in the white line, it still could not produce proanthocyanidins. Loss of ANR function would not explain the lack of pigments in the white line. The reason why no ANR gene fragment was amplified in the white line is uncertain.

This chapter and the preceding experimental chapters examined the relationship between flower colour and resistance to soilborne fungal pathogens in the three *S. formosa* lines. The experiments sought to determine which phytocompounds were responsible for resistance or susceptibility, and whether a gene homologous to ANR, present in other legumes, particularly *M. truncatula*, existed in *S. formosa*. The general discussion in Chapter 7 draws together the findings and proposes future areas of research.

Chapter 7 General discussion and future directions

Observations from the *S. formosa* breeding program at the University of New England suggested that white or pink-flowered lines are more susceptible to root diseases, particularly under greenhouse conditions (unpublished information). Such susceptibility creates a challenge for growers who supply markets where a preference for white or pink flowers exists. To produce white and pink flowers, it is necessary to control root diseases which are a constraint to production.

As a first step in this project, the resistance of three *S. formosa* lines with different flower colours to the soilborne pathogen *Phytophthora cinnamomi* were compared. A number of different assay methods were tested to evaluate the resistance of different plant tissues and also to determine which ones would be most useful in a breeding program. The test results showed that the red line was resistant, while the white line was susceptible and the pink line showed intermediate resistance between the other two lines. The reaction of the three *S. formosa* lines to *Pythium irregulare* and *Fusarium solani* was similar to their reactions to *P. cinnamomi*. In the resistant line, all above-ground (stems and hypocotyls) and under-ground (root) parts were resistant; in the susceptible line all above- and under-ground parts were susceptible. The detached stem assay proved to be the best assay for the breeding program. When some stems from individual F_2 plants are excised and used for resistance/susceptibility assessment, the remaining plant parts can be used for DNA extraction or for determining flower colour.

After identifying the resistant and susceptible lines, a cross was made between the resistant and susceptible lines to determine the mode of inheritance for resistance to *P. cinnamomi* in the F_2 population. Segregation in the F_2 population suggested that a single dominant gene controls the resistance to *P. cinnamomi*, and that this was completely linked to the gene controlling flower colour.

It has been reported that some physiological changes occur in resistant plants exposed to pathogen attack (Ebel and Grisebach, 1988; Yi and Hwang, 1996; Saunders and O'Neill, 2004). Trials were conducted to identify physiological mechanisms of resistance to *P. cinnamomi* in *S. formosa* red line. However, because of the putative link between flower colour and disease resistance in this species, it was anticipated that one or more coloured or colourless phenolic compounds in the phenylpropanoid pathway were possibly responsible for the resistance of the red line

to *P. cinnamomi*. It has been reported that in incompatible interactions, resistant plants defend themselves against different pathogens by means of preformed (phytoanticipins) or inducible (phytoalexins) defense mechanisms (Slusarenko et al., 2000), and a resistant plant initially prevents the penetration or entrance of pathogens into its tissues (Ebel and Grisebach, 1988; Yi and Hwang, 1996). The experiments in Chapter 5 measured the phenolic content before and after inoculation.

Soluble phenolics, which include a wide range of coloured and colourless phenolics in the phenylpropanoid pathways, were hypothesised to be related to disease resistance. In the experiments in Chapter 5 total phenolic content did not change in inoculated tissues of the three *S. formosa* lines compared to non-inoculated plants, so no induced response to disease was observed with soluble phenolic compounds. The free phenolic content of non-inoculated hypocotyls and stems of the three lines revealed a relationship between flower colour and *P. cinnamomi* resistance. The free phenolic concentration was greatest in the red line and least in the white line, and that was in conformity with low relative levels of resistance. Therefore it could be concluded that soluble phenolics in a preformed or phytoanticipin form inhibited the infestation of *S. formosa* red line by the fungus.

Other examples showing anticipation of plant diseases by the presence of soluble phenolics in the plants have been reported. Bean varieties with purple hypocotyls and black testae, which contained a great amount of soluble phenols, were more resistant to *Fusarium solani* f. sp. *phaseoli*, the causal agent of *Fusarium* root rot, than varieties with green hypocotyls and white or pinto seeds, which contained a low amount of soluble phenols (Statler, 1970). The inhibitory effect of certain phenolic compounds *in vitro* on the growth of *F. solani* f. sp. *phaseoli* has also been reported (Statler, 1970). Similarly, Prasad and Weigle (1969) reported that snap bean varieties with purple hypocotyls and black testae were resistant to *Rhizoctonia solani*, while varieties with green hypocotyls and white testae were susceptible to this soilborne fungal pathogen. Resistant snap bean cultivars had high amounts of polyphenols and flavonoid pigments, while these phytocompounds were absent in white-seeded snap bean cultivars (Prasad and Weigle, 1969). Link et al. (1929) reported that coloured onion varieties with greater amounts of phenolic compounds were more resistant to onion diseases than white varieties.

Anthocyanins, which are coloured phenolic compounds responsible for flower colour, were next hypothesised to be related to the red line resistance to *P. cinnamomi*.

The relationship between anthocyanin content and resistance of detached stem and hypocotyl assays was inconsistent. The result of the hypocotyl assay revealed no relationship between *P. cinnamomi* resistance and flower colour, although this was not the outcome of the detached stem assay. Nevertheless, the results of the poison food assay performed with anthocyanins from the three lines showed no relationship between *P. cinnamomi* resistance and plant pigments, and confirmed the results of the hypocotyl assay.

To identify which component of the total phenolic compounds inhibited growth of *P. cinnamomi*, the concentrations of some phenolic compounds in the three lines were measured, and the growth inhibitory effects of the one(s) that had different concentration in the three lines were tested. Leucoanthocyanidins with very low concentrations in the three lines did not display any difference between the pink and white lines, so differences in resistance to *P. cinnamomi* were not associated with leucoanthocyanidin content.

Proanthocyanidins were the only phenolic compound group identified to have different concentrations in the three lines. The red line had a higher proanthocyanidin concentration than the pink line, and the pink line a higher concentration than the white line, which contained a very low (approximately zero) level. Proanthocyanidin toxicity to *P. cinnamomi* was examined indirectly by precipitating proanthocyanidins of extracts by Bovine serum albumin and testing the toxicity of the supernatant of *S. formosa* red and pink lines to the fungus. This was done to see whether by removing proanthocyanidins from the soluble phenolics, soluble phenolic toxicity was removed. The supernatant of either the red or pink lines lacking proanthocyanidins was not toxic to the fungus, therefore complete and partial resistance of the red and pink lines respectively were associated with proanthocyanidin content.

The antimicrobial activity of proanthocyanidins has been reported (Scalbert, 1991; Snyder et al., 1991; Zheng et al., 2005). In recent research, by Punyasiri et al. (2005), to identify cultivars of tea resistant to *Exobasidium vexans*, the causal agent of blister blight, the TRI 2043 cultivar with a purple-green leaf containing high levels of anthocyanins compared to other cultivars was found to be resistant. The researchers reported that the high resistance of the TRI 2043 cultivar was related to high catechin and epicatechin content; both are proanthocyanidin precursors and are synthesised from anthocyanidins. Kantar et al. (1996) found that white-flowered lines of *Vicia faba* were more susceptible to fungal infection during seed germination than lines

with coloured flowers. This appeared to be due to differences in the tannin content of the seed coats. Tannins extracted from *Liquidambar formosana* leaves had fungicidal properties against *Alternaria alternata,* the causal agent of pepper black spot (Zheng et al., 2005). Some condensed and water-soluble tannins extracted from peanut testae and cotyledons inhibited *Aspergillus parasiticus* growth *in vitro* and reduced aflatoxin production (Azaizeh et al., 1990). Dixon et al. (2005) identified that the major function of proanthocyanidins and monomeric flavan-3-ols and their derivatives, which are present in the fruits, bark, leaves and seeds of many plants is to protect plants against microbial pathogens, insect pests and large herbivores. On the other hand, disease and pest susceptibility in some plants has been reported to correlate with the absence (or low concentration) of proanthocyanidins. For example, coffee varieties susceptible to the fungal pathogen *Hemileia vastatrix* contain low proanthocyanidin levels (Gonzalez de Colmenares et al., 1998). Proanthocyanidins have antimicrobial activities not only against plant diseases and pests but also against clinical microbes. For instance, rumen microbes *in vitro* were sensitive to all proanthocyanidin fractions extracted from the forage legume *Dorycnium rectum* (Sivakumaran et al., 2004).

As the production of various phytocompounds from their precursors is catalysed by different enzymes encoded by different genes, it was assumed that the anthocyanidin reductase (ANR) enzyme involved in the production of proanthocyanidins from anthocyanidins may be absent or inactive in *S. formosa* white line. In addition, the absence of proanthocyanidins in the white line could be the result of the absence of or a mutation in anthocyanidin synthase (ANS), which converts leucoanthocyanidins to anthocyanidins (Holton and Cornish, 1995; Tanner et al., 2003; Xie et al., 2004b; Bogs et al., 2005), regulatory genes, or even enzymes upstream to ANS and ANR in the phenylpropanoid pathway. In each case, epicatechin which is a proanthocyanidin precursor is not biosynthesised. The absence of epicatechin in *S. formosa* white line was confirmed in HPLC chromatograms.

The presence of ANR in *S. formosa* was investigated using DNA molecular techniques. Primers were designed from parts of the ANR gene of the model legume *Medicago truncatula* which has high sequence similarities with ANR from other plants. The amplified product from the genomic DNA of *S. formosa* had a high level of sequence similarity to other ANR genes. This indicated that the pathway via ANR

was present in this plant and explains why changes in flower colour affect proanthocyanidin levels.

A link between flower colour and colours of other tissues and resistance to diseases has been observed not only in leguminous plants such as Sturt's desert pea (Chapters 3 and 4), *Vicia faba* (Cabrera et al., 1989; Kantar et al., 1996), bean (Statler, 1970), snap bean (Prasad and Weigle, 1969) and chickpea (Cobos et al., 2005), but also in non-legluminous plants such as onion (Link et al., 1929), redwood (*Sequoia sempervirens*) (Wilcox and Piirto, 1976) and tea (Punyasiri et al., 2005). Anthocyanins are synthesised in sepals and vegetative organs (e.g. stems and senescent leaves) (Rosati et al., 2003). The lack of expression of anthocyanidin synthase (ANS) might constitute the major block in anthocyanin synthesis in petals (Rosati et al., 1999). Inhibition of flavonol synthase (FLS) production through introduction of an FLS antisense RNA construct in petunia led to anthocyanin production and a pink-flowered phenotype (Davies et al., 2002). However, there are a number of white-flowered ornamental species in which the flowers may show a blush of anthocyanin or produce anthocyanins for a brief period during flower development, and which also produce flavonol in abundance (Davies et al., 2002).

Proanthocyanidins act as phytoanticipins in incompatible interactions between non-host plant cultivars and pathogens, to prevent plant infestation by pathogens. The susceptibility to diseases of white-flowered plants is due to the lack of proanthocyanidins, which are a component of soluble phenolics (Cabrera et al., 1989). Loss of proanthocyanidins in plants increases susceptibility to pathogens. The link between flower colour and proanthocyanidins is due to anthocyanidins, which are a substrate for ANR, one of the key enzymes in proanthocyanidin synthesis. The observation associating flower colour, proanthocyanidin content and resistance to soilborne diseases in leguminous plants can now be understood in the light of new knowledge about biosynthetic pathways.

Future work to breed *S. formosa* pink and white lines resistant to *P. cinnamomi* could use transformation of these lines. Transformation of the model legume *Medicago truncatula* has been successfully performed using an *Agrobacterium* mediated technique (Chabaud et al., 2003; YongLin et al., 2003; Araujo et al., 2004), vacuum infiltration and floral dip techniques (Ye et al., 1999; YoungWoo and DaeHae, 2004). As *S. formosa* is readily reproduced through biotechnological techniques such as micropropagation (Taji and Williams, 1989),

somatic embryogenesis (Tapingkae, 1998; Sudhersan and AboEl-Nil, 2002), and organogenesis (Tapingkae, 1998), it is reasonably feasible to transform *S. formosa* pink and white lines using *Agrobacterium*-mediated, vacuum infiltration and floral dip techniques. Although there do not seem to be any technical problems for the transformation of these lines, the challenge would be to identify genes that reduce flower pigmentation without reducing proanthocyanidin content.

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Appendix 1

Consensus sequence for *S. formosa* homolog of anthocyanidin reductase gene. Constructed from alignment of the pink line left and right, and the red line right sequences, obtained using L286 and R877 primers.

AGGCATTGAATTAGTACTTGTATTCTAAATTTAACAAGAAAATTGAATGCTCTTACGCATACATGTGTG
TGTTGAAGAAAAACACTAATGAGGAAATTTTTGCAACTACTATTCAGAATGACATGATCAAGCCTGCAA
TCTCAGGCGTATTGAATGTGTTGAAAGCATGTGCGCGGGCAAAAGATGTCAAACGAGTCATCTTAACAT
CTTCTGCAGCTGCAATAACCATAAGCGATGTCAAGGGGACTGGTCTGGTTATGGATGAAAGCAACTGGA
CTGATGTTGAGTTCTTGAGCACTGCAAAGCCACCCACTTGGGTAAAATTCAAACCTTATTCAGTGCGTG
TTTGAGTCAGCGCTTGCAAAATTGATTTTGATTAAAACTGATGTGGAAGTTAAGTGATTATGAATGCAT
GATTTTACTTCAAAAACAACTTGGTAACTAAACTCAATATAGAAGTTTTTTCGCAAGATAGAAGTTACT
TAAATTCAAAATTAATTTTAGACCCAGAATCAATTCCTCAACTGAGACTAGGTAAATGTTTGGTTGTCT
AGCCAAGATCCACGTTTAGCTTTGAATCATGTCAAAAATGATGCAAAAGCAACTATCTCTTGTTTCCAT
GATCCATGTCCAACTTGTACAAACAGGAATCTAAATAGGCCATAAAATATGAAGATTTACTTAAAATCA
CTATTTCTCAACATGATTTTGGATGATGCAACATAAATACAAACACGCCTTTAGAACTTGGGATGGGGA
TGAGGAATGGCTCTTTGCACTGATTTGCCTTACTGTCATTCCTGGTTGCAATGGATAACTACTCATTGG
GTAGATTCTGAAGTAGAATGTTCACCCAAACATGCAGGGGTACCCTGTCTCCAAAACACTAGCTGAGAA
GACAGCATGGAAATTTGCTGAAGAAAATCACATTGATCTCATCACTGTGATTCCTTCTCTCACAACTGG
TCCTTCTATCACTCCAGACATCCCTTCAAGTGTTGGCCTCGCCACGTCCCTTATAACAGGTTCAAAGCT
TCCAAATTTAATGTTTTGAGGACTTCTAGAAATGTTAAATAATTATGTTTTTTAAGGACCGTGGTGAAT
GGCCAAAATGTTTTTCAATGGAATTTTTCTTTGTACCTTGCAGGCAATGATTTCCTCATAAACGCTTTG
AAAGGCATGCAGATGCTTTCTGGTTCAATATCCATTACTCATGTGGAGGATATTTGCCGAGCACATGTA
TTCGTGGCAGAGAAAGAATCCGCTTCTGGTAGATAC

Coding region obtained by comparison with *Phaseolus coccineus* and *Medicago truncatula* mRNA, and *Vitis vinifera* genome sequences.

AATGACATGATCAAGCCTGCAATCTCAGGCGTATTGAATGTGTTGAAAGCATGTGCGCGGGCAAAAGAT
GTCAAACGAGTCATCTTAACATCTTCTGCAGCTGCAATAACCATAAGCGATGTCAAGGGGACTGGTCTG
GTTATGGATGAAAGCAACTGGACTGATGTTGAGTTCTTGAGCACTGCAAAGCCACCCACTTGGGGGTAC
CCTGTCTCCAAAACACTAGCTGAGAAGACAGCATGGAAATTTGCTGAAGAAAATCACATTGATCTCATC
ACTGTGATTCCTTCTCTCACAACTGGTCCTTCTATCACTCCAGACATCCCTTCAAGTGTTGGCCTCGCC
ACGTCCCTTATAACAGGCAATGATTTCCTCATAAACGCTTTGAAAGGCATGCAGATGCTTTCTGGTTCA
ATATCCATTACTCATGTGGAGGATATTTGCCGAGCACATGTATTCGTGGCAGAGAAAGAATCCGCTTCT
GGTAGATAC

Amino acid translation of coding region.

NDMIKPAISGVLNVLKACARAKDVKRVILTSSAAAITISDVKGTGLVMDESNWTDVEFLSTAKPPTWGY
PVSKTLAEKTAWKFAEENHIDLITVIPSLTTGPSITPDIPSSVGLATSLITGNDFLINALKGMQMLSGS
ISITHVEDICRAHVFVAEKESASGRY