Confocal and light microscopy of infection by, and resistance to, *Ciborinia camelliae* in *Camellia* species and the potential for biocontrol

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To my wife, Malliha and children, Neelujah and Keerthanan:
With my deepest gratitude and love, forever.

Abstract

In this thesis, factors affecting *C. camelliae* ascospore germination, germ tube penetration, infection and subsequent disease development and resistance in C*amellia* species were studied in detached camellia petals by microscopy. Potential biocontrol agents were also isolated and evaluated against *C. camelliae* in the laboratory and in the field.

Trypan blue in lactophenol was used to stain for fungal structure in bright field microscopic investigations of factors affecting ascospore germination, germ tube penetration and infection of *C. camelliae*. Relative humidity affected ascospore germination and germ tube penetration, but temperature had little or no effect although low temperature restricted the growth of penetration hyphae in the tissue and disease development. Absence of free water affected ascospore germination and germ tube penetration when ascospores were inoculated as suspensions using a pipette or atomizer. Ascospores released from apothecia and allowed to settle on petals by gravity germinated and the germ tube penetrated petal tissue in both the absence and presence of free water. A possible reason for high spore germination and germ tube penetration in the absence of free water could be the association of liquid drops adhering to freshly released ascospores that could have acted as a reservoir of moisture. Multiple germ tubes and swollen hyphae were common and many germ tubes grew across the petal surface without penetration in brush inoculated (ascospores directly transferred from apothecia using a paint brush) petals under free water conditions. Production of antifungal materials over the petal surface due to petal damage by brush bristles during inoculation may be the reason for the malfunction of some germ tubes.

A glutaraldehyde technique was developed to increase image contrast by reduction of background fluorescence from plant tissue in confocal microcopy. Ascospores were found to germinate and produce a short germ tube that directly penetrated the petal cuticle within 6 h. During next 12 h, the penetrated germ tube transformed into a subcuticular swelling underneath the cuticle. From this, a narrow tube enlarged into a

subcuticular hypha that grew underneath the cuticle to the junction between two cells. The mycelium continued to develop intercellularly for the next 60 hours but without causing extensive destruction of the cell walls. The fungus appears to follow the infection process strategy of subcuticular intramural pathogen such as *Colletotrichum capsici*.

Resistance mechanisms of *Camellia* species against *C. camelliae* were investigated by light and confocal microscopy. *C. cuspidata* expresses resistance to *C. camelliae* by formation of papillae and a hypersensitive reaction. *C. lutchuensis* and *C. transnokoensis* express their resistance by papillae and production of antifungal metabolites such as PR proteins respectively. Excess microorganisms were observed in *C. polyodonta* with distorted ascospores and in *C. tricocarpa* with abnormal swollen hyphae which suggests biocontrol activities of the organisms.

A total of 13 bacterial isolates and 6 yeast biocontrol agent (BCA) candidates were obtained from an attachment assay. Bacterial isolates, 07L1B and 04S2B, gave maximum disease control when tested *in vivo* on intact petals and in the field. Direct contact of these bacterial isolates with ascospores totally inhibited germination. Biocontrol was not effective if germ tube penetration occurred before BCA arrival. Biocontrol efficacy was observed for at least 72 h both in the laboratory and in the field condition although the bacterial populations declined. Antibiotic production was observed from these two isolates and was the prime mode of action against *C. camelliae*.

The main goal of this thesis was achieved by understanding some basic biology of *C. camelliae* and these principles were used in the biocontrol strategy and study of resistance mechanism of *Camellia* species.

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Chapter One

General Introduction

1.1. The genus *Camellia*

1.1.1. History of camellia

A large number of plants that now grace the gardens of different parts of the world came from eastern Asia. Camellias too, have come from the same area and different camellia species have grown as native plants on the main land of Eastern Asia from Indo-China to Korea and on the islands that lie off shore (Hume 1951; Clere 1991). Camellias are not only a garden ornamental but are also used as food, cosmetic and culinary oil, fuel as high-grade charcoal, and most importantly as a beverage i.e. tea (Durrant 1982). Plants for the early culture of camellia were raised from seeds and when they came into bloom some specimens bore flowers of unusual form and attractiveness. They were appreciated for their beauty, and because of their aesthetic values were grown in pots and in gardens (Hume 1951). Because of their economic and aesthetic value they were introduced in Europe and wherever the European colonised in the world. Camellias were first introduced to Europe in late 1700s and to USA, Australia and New Zealand in early 1800s (Hume 1951). Alabama State in the USA adopted the camellia flower as their State flower in 1959 and emphasized its potential for earning millions of dollars for camellia growers, and for helping the State economy by attracting tourists (Anonymous 1999). Camellias are now widely cultivated in most countries (Clere 1991) and a number of societies are keen to promote their cultivation, breeding and propagation and distribution around the world.

1.1.2. Classification of camellia

Camellia is the largest genus (Chang & Bartholomew 1984) among the thirty genera of the family Theaceae (Kobuski 1978). Members of the genus are slow-growing evergreen shrubs or tree (Feathers 1978; Sealy 1958) and more than 267 species of the genus *Camellia* were registered by 1991 (Savige 1993).

The name camellia was given by Linnaeus in 1735 in memory of George Joseph Kamel (or Camellus), a Jesuit missonery in the Philippines (Macoboy 1998). He named this plant, which he had never seen, based on a description given by others (Hume 1951; Brown

1978). Later in 1753 he gave it the binominal name, *Camellia japonica* and he also named the tea plant, *Thea sinensis*, later changed to *Camellia sinensis* by O. Kuntze (Hume 1951; Brown 1978).

The botanical hierarchy of the genus *Camellia* has developed considerably from the original species, *C. japonica* and *C. sinensis*, listed by Linnaeus as the genera *Camellia* and *Thea*, up to the present day 267 species with numerous varieties (Savige 1993).

The detail classification scheme is that of Chang and Bartholomew (1984)

1. Subgenus Protocamellia.

- 1. Section Archecamellia.
- 2. Section Stereocarpus.
- 3. Section Piquetia.

2. Subgenus Camellia.

- 4. Section Oleifera.
- 5. Section Furfuracea.
- 6. Section Paracamellia.
- 7. Section Psuedocamellia.
- 8. Section Tuberculata.
- 9. Section Luteoflora.
- 10. Section Camellia

3. Subgenus Thea.

- 11. Section Corallina.
- 12. Section Brachyandra.
- 13. Section Longpedicellata.
- 14. Section Chrysantha.
- 15. Section Calpandria.
- 16. Section Thea.
- 17. Section Longissima.
- 18. Section Glaberrima.

4. Subgenus Metacamellia.

- 19. Section Theopsis.
- 20. Section Eriandria

At present, the economically most important species is *Camellia sinensis* (L.) O. Kuntze for black and green tea production, and horticulturally, *Camellia japonica* Linnaeus, *Camellia reticulata* Lindley and *Camellia sasanqua* Thunberg for evergreen ornamental shrubs and

trees. These horticultural species offer a wide range of flower forms (single, semi-double, anemone, formal Double, Rose form Double, Loose Peony form, Full Peony from), colours White, green, pink, rose pink, red, black red, purple and blue), and size (~ 1cm –23 cm diameter) (Feathers 1978).

1.1.2.1. *Camellia japonica* Linnaeus.

Camellia japonica is native to Japan (Macoboy 1998), but Simpson (1978) pointed out that it may have been introduced into Japan by Chinese Seafarers, and thus he claimed that the *C. japonica* could be a native of China. *Camellia japonica* is a shapely shrub, slowly becoming a tree (Macoboy 1998) and shows an almost infinite variation in size, form, colour and arrangement of petals and shape and size of leaves, when grown from seeds (Durrant 1982). It grows in quite a wide range of climate from subtropical (Taiwan) to bitterly cold area (Hill of Northern China) (Macoboy 1998). The plant starts to flower during early winter and continues up to early summer (Macoboy 1992 and 1998).

1.1.2.2. *Camellia reticulata* Lindley.

Camellia reticulata produces dull-green leaves which last only for a year, falling in late spring as the new growth develops. Flowers are pink to deepest red in colour, the petals are swirled and fluted, so the flowers rarely open quite as flat as many japonicas do. The pollen passes from gold to grey as the flowers age. The flowers tend to appear late in the winter season, after the japonicas have reached their peak during spring (Macoboy 1992 and 1998).

1.1.2.3. *Camellia sasanqua* Thunberg.

Camellia sasanqua produces medium sized, mostly single or semi-double flowers, with golden stamens and fluted, ruffled petals. Their colours mostly range from white through to deep pink with a very few described as red. Their flowering time is during autumn to early winter (Macoboy 1998) and thus most cultivars are classified as “early season” (Macoboy 1992 and 1998).

1.1.3. Diseases of camellia

Camellias are relatively untroubled by pest and diseases (Bieleski 1991; Bond 1994), but are susceptible to a few diseases including flower blight caused by *Ciborinia camelliae*; *Botrytis* flower spot (or grey mold) caused by *Botrytis cinerea*; *Pestalotia* leaf spot caused by *Pestalotia* spp; Die back incite by *Glomerella cingulata* causing stem cankers and the death of branches; *Phytophthora* root rot caused by *Phytophthora cinnamomi*; *Armillaria* root rot casued by *Armillaria mellea*; Damping off caused by *Rhizoctonia solani, Pythium aphanidermatum* and *Thielaviopsis basicola*; Virus diseases (Raabe et al. 1978). Flower blight caused by *Ciborinia camelliae* is considered the most serious disease of the genus (Raabe et al. 1978).

1.1.3.1. *Botrytis* flower blight (Grey mould disease)

Botrytis flower blight or grey mould is a common disease of camellia but is not usually serious in New Zealand (Peter Long Personal communication). This disease, caused by *Botrytis cinerea,* is often confused with the spotting caused by the flower blight fungus, *Ciborinia camelliae* (Raabe et al. 1978). The difference in appearance is that *Botrytis* forms a grey mould of mycelium and spores amongst the petals, whereas *Ciborinia* forms a collar or a partial collar of hyphae at the base of an infected flower under the calyx (Blumenthal and St. Ives. 1995). Botrytis develops woolly grey conidiophores and conidia on infected petals in high humidity conditions and this also differentiates it from flower blight. There is no collar of fungus tissue as in camellia flower blight. Cool humid conditions as well as dead or dying tissues usually promote the disease and frequently the fungus invades the flower through wounds or mechanical injuries.

1.1.3.1.1. Botrytis cinerea Pers: Fr

Contrary to *Ciborinia camelliae,* the *Botrytis cinerea* is a wide host range pathogen. It attack numerous fruits, vegetables, flowers and ornamental plants (Maude 1980). *Botrytis cinerea* is classified in the family Moniliaceae, and the perfect stage is *Botryotinia fuckeliana* (de Bary) Whetzel (Agrios 1988). This sexual stage (an apothecium) is seldom found in nature (Polach and Abawi 1975). *Botrytis cinerea* produces abundant grey mycelium and long branched conidiophores with rounded apical cells bearing clusters of colourless or grey, one-celled, ovoid conidia which contain 4-18 nuclei (Jarvis 1980; Agros

1988). The sclerotium is an overwintering structure under natural conditions. It produces hyphae, conidia, or (rarely) an apothecium the following spring and summer (Coley-Smith 1980).

1.1.3.2. *Pestalotia* leaf spot

Pestalotia leaf and flower spot disease (caused by *Pestalotia* spp.) is also a minor disease in camellia plants. This fungus is often associated with injuries resulting from sunscald, insects, wounds and infection from other fungi. The spots resulting from such inversions are usually silver gray on the upper surface, light brown on the lower surface, and frequently dotted with small dark fruiting bodies (acervuli) of the organisms (Mordue & Holliday 1971).

1.1.3.3. Flower blight

Flower blight destroys the beauty and renders flowers worthless for decorative purposes. This disease, caused by *Ciborinia camelliae* Khon (formally *Sclerotinia camelliae* Hara), was first recorded in Japan in 1919 (Durrant 1982). It was reported in California, USA in a nursery near Hyward in 1938 (Hansen and Thomas 1940 and 1994) and later in a second nursery (Thomas and Hansen 1946; Hansen and Thomas 1946 and 1994). Raabe et al (1958) stated that infected flowers had been seen in scattered areas in Northern California by nurserymen prior to 1938. However, since the first identification it has spread throughout of the USA. Both nurseries had imported plants from Japan and it is probably these imported plants that introduced the disease (Hansen and Thomas 1940, 1946 and 1994; Thomas and Hansen 1946). In New Zealand, Stewart and Neilson (1993) first identified it in the Wellington region in 1993 and they stated that it is likely to have existed in this area for at least 2 previous seasons. Neall et al. (1998) reported that the disease had been found in Waikanae, Wanganui and in a farm at Kauangaroa, 22 km east of Wanganui in 1996. Now the disease is widespread in the lower, central and westen North Island, within Christchurch and in the northern region of the South Island of New Zealand (Taylor 1999). It has also been confirmed in Italy (Garibaldi et al. 2001) and reported elsewhere in Europe (Taylor and Long 2000) and in China (Christine Taylor – Personal communication).

1.1.3.3.1. Symptoms of flower blight

The disease attacks only flowers. The symptoms first appear as small, irregular brown spots on the petals during the peak of the flowering season. Within a few days these small spots gradually enlarge to cover greater part of the petal and eventually whole flower (Louis 1959; Stewart and Neilson 1993). The diseased blossoms completely turn into dull brown colour. The blighted petals are dry or leathery but do not crumble when handled. Once the blooms are killed, they will drop from the plant. A white or grey ring of mycelium of the fungus can be seen at the base of the flower, underneath of the calyx to confirm the disease. This feature represents a positive identification of the disease (Plate 1.1).

Plate 1.1. Symptoms of camellia flower blight. A) Uninfected flower. B) Small brown lesions (arrows) which spread until the flower completely brown. C) Base of the flower before petals completely brown. D) The characteristic ring of white or grey mycelium around the base of the flower (arrow head) where the calyx is removed.

Flower blight symptoms can be confused with cold injury. Cold injury, however, can be distinguished from flower blights by noting the following: the browning almost always starts from the outside margin of the flower petals and no signs of the fungus occur as in the flower blights.

1.2. *Ciborinia camelliae* Kohn

Ciborinia camelliae is an inoperculate Discomycete belongs to the family Sclerotiniaceae where the members produce apothecia from stroma or sclerotia (Agrios 1988; Alexopoulos and Mims 1979). This pathogen was first named by Hara as *Sclerotinia camelliae* Hara in 1919 in Japan (Hara 1919 – cited by Kohn and Nagasawa 1984) and in 1938 by Hansen and Thomas as *Sclerotinia camelliae* Hansen and Thomas in California, USA (Hansen and Thomas 1940) without knowing Hara's work. Later Hansen and Thomas acknowledged Hara's work and recognised *Sclerotinia camelliae* Hara, but they pointed out that dimension of asci and ascospores were smaller in their sample than in Hara's one (Hansen and Thomsan 1946). Kohn (1979) studied the holotype of both Hara's and fresh Californian samples and concluded that the two species were not the same and described the Californian specimens as *Ciborinia camelliae* Kohn. Later, Kohn and Nagasawa (1984) studied the holotypes of *Ciborinia camelliae* Kohn and *Sclerotinia camelliae* Hara and suggested that both are synonymous. Now, the name *Ciborinia camelliae* Kohn remains as the correct name of the flower blight pathogen of camellia.

The genus *Ciborinia* erected by Whetzel (1945) to contain species in which either wholly or partially digested host tissue is incorporated into the sclerotial medulla, but genus *Sclerotinia* do not contain host tissue in its sclerotial body. Genus *Ciborinia* contain twenty known species and are all host specific pathogens (Batra and Korf 1959; Batra 1960). Batra and Korf (1959), and Batra (1960) reported that most of these species including *Ciborinia camelliae* (Hansen and Thomas 1940) produce microconidia, but contrary to these reports, Kohn (1979) reported that no *Ciborinia* species produced microconidia. However, *Ciborinia camelliae* produces microconidia in New Zealand (Taylor and Long 1998).

Isolation of *C. camelliae* is rather difficult from diseased petals (Winstead et al. 1954; Taylor 1999), but can be easily obtained from sclerotia by aseptically cutting out hyphae from the medulla and plating (P G Long personal communication) on Difco PDA (Taylor and Long 1998).

1.2.1. Life cycle of *C. camelliae*

Infection occurs when ascospores germinate and penetrate the petal tissue. Ascospores are produced in apothecia that arise from sclerotia. The sclerotia develop from the fungal

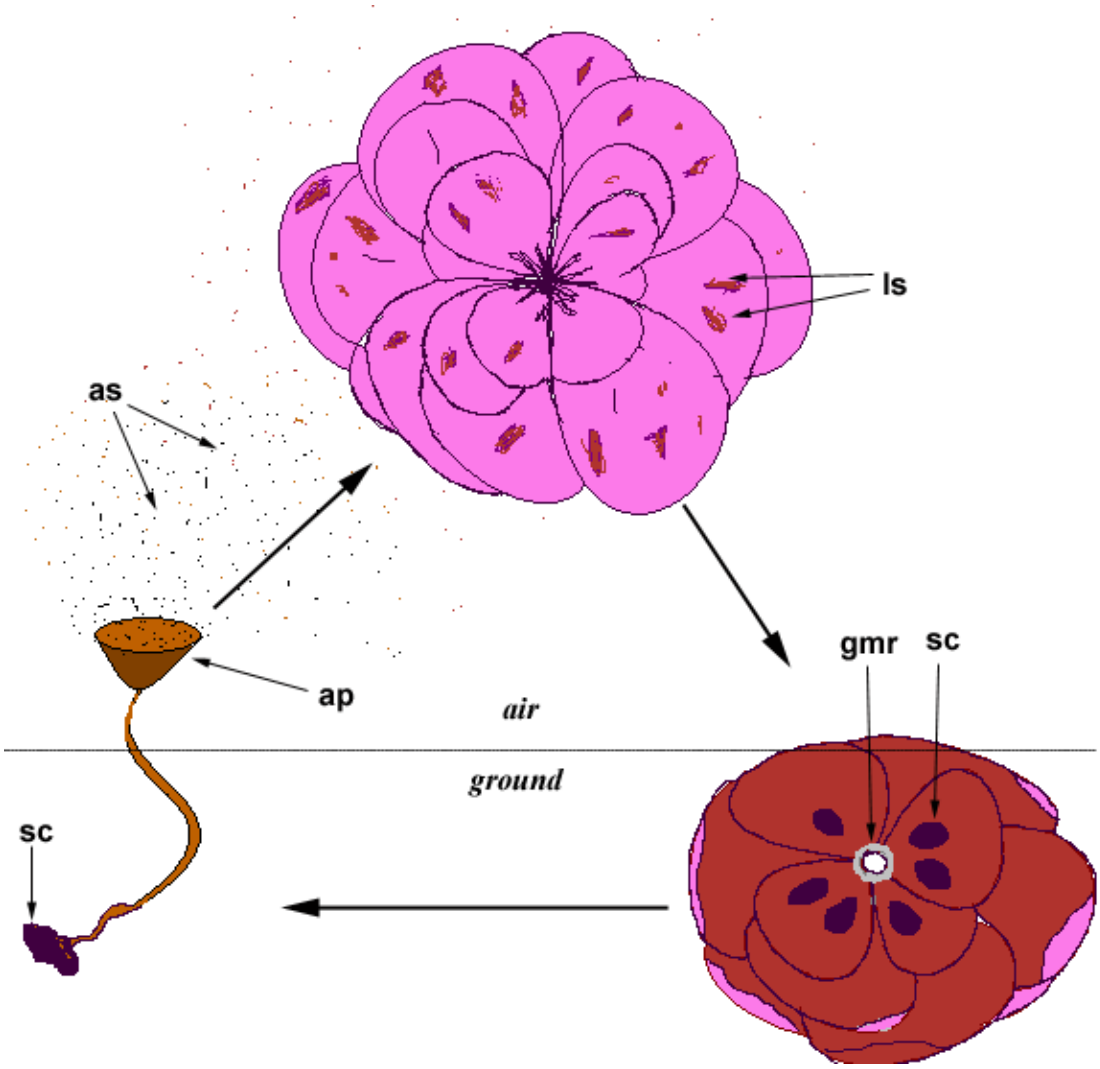


Figure 1.1. Life cycle of *Ciborinia camelliae*. (sc = Sclerotia; ap = apothecia; gmr = grey mycelial ring; as = ascospores; ls = lesions).

pseudoparenychema at the base of the petal and eventually lie dormant after falling to the ground or plant debris. During spring or at the end of winter, the sclerotia begin to germinate and producing apothecia. Sclerotial germination continues for two to three months (Fig. 1.1). Baxter and Thomas (1995) reported that sclerotia remain viable in the soil for at least four years.

1.2.2. Infection process of *C. camelliae*

An understanding of the mode of infection of *C. camelliae* is a prerequisite for developing effective control strategies, particularly those based on host plant resistance. Knowledge of the factors influencing infection processes also provides epidemiologists with information which can be developed into forecasting models, and will aid agronomists developing appropriate agricultural practices, based on crop sanitation and removal of reservoirs of infection. *Ciborinia camelliae* is host-restricted pathogens which infects camellia flower petal tissue (the only host) by ascospores (the only source of infection) (Raabe et al 1978; Kohn and Nagasawa 1984; Dingley 1993) but little other information on the infection process is available.

During the infection process many fungi produce appressoria at specific sites on the leaf surface before penetration (Preece et al. 1967). Ultra-structural studies have shown that both mechanical structures and enzymatic degradation are implicated in penetration (Edwards and Allen 1970; Mc Keen 1974). However, different pathogens have developed different strategies to colonize host plant tissue during the infection process. *Helminthosporium carbonum* in corn leave (Comstock and Scheffer 1973), *B. cinerea* and *B. fabae* in *Vicia fabae* leaves (Cole et al. 1996), and *B. cinerea* in rose petal (Williamson et al. 1995) have been reported to penetrate directly without appressorium formation. Other fungi such as *Colletotrichum* spp. (Bailey et al. 1992; Skipp et al. 1995) may need wounds for germ tube penetration (Boher et al. 1983) and may develop sub-cuticular infection (Walker 1921), intracellular biotrophy (Skipp & Deverall 1972; Mercer et al. 1974; O'Connell et al. 1985) or intracellular hemibiotrophy (Bailey et al. 1990).

C. camelliae needs to be investigated to find whether it could follow one of the above known strategies for the infection process.

1.2.3. Control strategies of *C. camelliae*

Camellia flower blight was initially thought to be an easy pathogen to control. It was thought there was little danger of the disease establishing in parks or in private gardens due to the simple matter of control and eradication by gathering and destroying all fallen flowers (Hansen and Thomas, 1940). Later, because of awareness of sclerotial formation in the soil beneath the tree, it was thought that it could be eradicated through the destruction of infected flowers combined with ground fungicide sprays to kill or inhibit the sclerotia (Hansen and Thomas 1946). However, the disease continued to spread through out the world and the current distribution demonstrates that it is unlikely the disease will be eradicated from well-established areas, as control is problematic. However, sanitation, cultural practice and chemical treatment have been commonly recommended and practiced to minimize the disease.

Sanitation is achieved by collecting all infected flowers and destroying them. To keep the plant free from the infected flowers, Baxter et al. (1983) suggested spreading a plastic sheet underneath a plant to collect all fallen flowers and to hand pick the flowers that lodged in a branch. On the other hand, Hagan (1987) recommended that a layer of 25 mm of fresh bark or pine straw mulch around the base of each camellia after removing the old mulch each spring can interfere with the spread of spores from the apothecia to the flower buds. Further, Stewart (1994) suggested that flowers should not be composted as the sclerotia can survive this process and become a source of substantial infection in an area.

Fungicide spray is widely used to prevent this disease but none of those tested completely controlled this disease. Hagan (1987) recommended that foliar sprays and drenches of selected fungicides may give some protection but this should be used in conjunction with the sanitation and cultural practices to get more effect. Fungicides, applied as a soil drench, will prevent the development of the apothecia on the surface of old mulch or soil beneath the plants. Foliar sprays will provide additional protection from camellia petal blight.

Although combinations of the above strategies are recommended, in practice they currently fail to give total control of this disease. More and more different control strategies for plant disease are being investigated and one of these approaches is using biocontrol agents. The biological control approach has yet to be applied against camellia flower blight disease.

1.2.3.1. Biological control

Plant surfaces provide a habitat for epiphytic microorganism, many of which are capable of controlling the growth of pathogens (Blakeman and Fokkema, 1982). The activity of these microbes and pathogens on plant surface depends on the microclimatological conditions at the surface as well as on the chemical environment such as antifungal properties of diffusible toxins, phytoalexins and surface wax (Blakeman, 1973). Various morphological features on the surfaces of leaves also can have a significant influence on microbial growth. The irregular provision of surface water on leaves results in intermittent growth of microorganisms, particularly bacteria and filamentous fungi, and poses problem of survival during dry periods (Droby and Chalutz 1991). Temperatures that fluctuate widely on leaves over a 24 h period also can cause irregular growth of microorganisms.

Nutrients are important for their direct role as a microbial substrate on the plant surface. Competition for these resources among microbes and pathogens has been observed by many workers and used as a model for a biocontrol system. Dubos (1992) and Elad (1996) argued that this mechanism is the most important for biocontrol. However, Andrews (1992) considered that biotrophic pathogens, e.g. the pathogens causing rusts and mildews, tend to have a relatively short epiphytic phase and typically require few or no exogenous nutrients for penetration. To apply biocontrol at this phase of the cycle, antagonists that act by antibiotics rather than competition should be the most effective (Rytter et al. 1989). In contrast to biotrophs, unspecialized necrotrophs (*Septoria, Cochliobolus*, *Phoma, Alternaria, Botrytis*) tend to grow saprophytically on the phylloplane, taking up exogenous nutrients before they can penetrate. Antagonists operating as nutrient competitors could be effective in this situation (Andrews 1992). Recently a saprophytic fungus *Ulocladium atrum* was examined for it's biocontrol efficacy against *B. cinerea* as a competitor in colonisation of necrotic tissue of pot roses (Kohl and Gerlagh 1999). They found that

Ulocladium atrum effectively reduced sporulation of *B. cinerea* by over 50%. This considerably reduced infection of pruning wounds and contamination of flowers.

Antibiotic production is another mechanism for biocontrol. A number of genera of phylloplane microorganisms, including representatives of filamentous fungi, yeast, and bacteria have been reported to produce antibiotics *in vitro*. They may be active against fungal or bacterial pathogens. For example, one epiphytic bacterial isolate out of 230 from cucumber leaves was found to effectively antagonise *Colletotrichum lagenarium*, the cucumber anthracnose pathogen tested *in vitro* (Leben 1964). Later, Leben and Daft (1965) showed that the isolate produced a peptide antibiotic.

A filamentous fungal group, the *Trichoderma* species, have been studied most extensively for their biocontrol efficacy against various plant pathogens (Papavizas 1985). *Trichoderma* species were well-known for antibiotic production, but some show a less specialized hyperparasitic relationship which restrict development of fungal pathogens by hyphal interaction involving coiling and penetration (Dennis and Webster 1971). Antibiotic substances were extracted from *Trichoderma* spp. and used as biofungicides against various diseases. For example, Trichodermin, a biofungicide obtained from *Trichoderma viride,* is being produced commercially in Russia for use against various diseases of greenhouse-grown vegetable cultivars (tomato, cucumber, capsicum) (Fatueva 1993).

The selection procedures for effective antagonists have been repeatedly tried using procedures such as *in vitro* dual culture plates but with few successful biocontrol agents resulting (Andrews 1990). A new screening procedure for microbes that attach to the fungal hyphae were recently developed by Cook et al. (1997a). The assay was designed to select from mixed populations, bacteria and yeasts capable of attaching to fungal hyphae.

1.2.3.2. Host resistance mechanisms

Plants in their natural environment always encounter large numbers of potentially pathogenic microorganisms, such as fungi, bacteria, virus and viroids, and yet the majority

of plants are resistant and do not suffer in any apparent way from such organisms. Only a few microorganisms have acquired the ability to colonize live plants by evolving from saprophytic organisms to biotrophic or perthotrophic pathogens (Kombrink et al. 1993). The mechanisms conferring resistance to disease differ in detail between plant species and pathogens, but there are also broad similarities between them. Thus resistance is due to a combination of physical and chemical barriers which are either preformed or induced only after infection. A wide range of host responses are induced including phytoalexin synthesis, callose deposition, lignification, oxidative bursts involving free radical production, and production of proteinase inhibitors and lytic enzymes such as glucanases and chitinases (Bell 1981; Kombrink et al. 1993).

The typical preformed, constitutive defense mechanisms are the structural barriers such as waxes, cutin, subarin, lignin, phenolics, cellulose, callose and cell wall proteins which are, however, often rapidly reinforced upon infection. The aerial parts of plants are covered by the cuticle. The cuticle is a continuous layer connected with the epidermal plant cells and consequently the first line of defense. It consists of pectin, cutin and wax layers. Whereas pectin is deposited on the inner side of the cuticle, the wax layer is concentrated on the outside. It acts as a chemical and physical barrier to fungal germination and penetration. Components of wax and cutin are fungistatic to some pathogens (Wang and Pinckard 1973), but are growth supplement to others (Kolattukudy 1980).

Kombrink et al. (1993) stated that the most frequently observed and best characterized active defense reactions are rapid localized cell death, accumulation of phytoalexins, synthesis and deposition of phenolic compounds and protein in the cell wall, and synthesis of Pathogenesis-related (PR) proteins, including the hydrolytic enzymes β-1, 3-glucanase and chitinase. The rapid death of cells (hypersensitive response) (HR) in the vicinity of the fungus may occur in response to intra- and intercellular pathogens. Mostly this mechanism is geared towards biotrophic fungi (Heath 1984). The common association between HR and race-specific resistance to biotrophic parasites led to the assumption that HR cause resistance because biotrophs could not grow in dead cells (Crute et al. 1985). Phytoalexins are lipophilic, low molecular weight antimicrobial compounds comprising many diverse

chemical classes, including isoflavonoids, flavonoids, diterpenes, sesquiterpenes, polyacelylenes, coumariens, isocoumariens, terpenoids, furanoacetylenes and polyenes (Bell 1981; Sequeira 1983). They accumulate locally in large amounts in response to microbial infection or stress but are extremely low concentration in a healthy plant (Albersheim and Valent 1978; De Wit 1985). At high concentrations, phytoalexins are toxic to plant cell and at very high concentration in tissues, necrosis and the symptoms frequently associated with the hypersensitive response appear (Bell 1981). As an antifungal compounds, phytoalexins appear to play an important role in many plant-pathogen interactions (Kokubun et al. 1995). They suppressed *B. cinerea* growth on lettuce (Bennett et al. 1994) and on capsicum (Adikaram et al. 1988), and reduced *Colletorichum destructivum* penetration on cowpea seedlings (Latunde-Dada and Lucas 2001).

Another form of commonly observed responses is the development of a papilla of wall-like material that commonly contains callose at the penetration site (Heath 1980). Callose, a β-1, 3-glucan, is the most commonly identified constituent in papillae but other components include lignin, cellulose, protein, peroxidase, pectin, suberin, gum and silicon also are co-present in papillae (Aist 1976). Using interference contrast microscopy, papilla formation was observed inside host cells at the penetration site before penetration by *Olpidium brassicae* (Aist and Israel 1977a) or *Erysiphe graminis* (Aist and Israel 1977b) and have suggested that the papillae stop the advancing pathogen. Vance and Sherwood (1977) reported lignified papilla formation in reed canarygrass when they inoculated *Helminthosporium avenae* or *Botrytis cinerea*. The role of papillae on resistance was demonstrated by Sherwood and Vance (1980) using cycloheximide to induce blockage of papillae formation that enabled three previously avirulent pathogens to penetrate 12 species of Gramineae. But in contrast, Smart et al. (1986) observed resistance to *Erisiphe graminis* after removal of callose from barley papillae by laminarinase digestion.

Production of pathogenesis related (PR) proteins is suspected as another form of host resistance and can be detected systemically after inoculation of the host. It has been suggested that PR-proteins are involved in induced resistance (De Wit 1985). As such the recent studies showed that PR protein directly involved in induced resistance against fungal

pathogens. Rauscher et al. (1999) induced resistance against the rust fungus *Uromyces fabae* in broad bean plants by applying salicylic acid or 2,6-dichloro-isonicotinic acid. After light-microscopy and scanning electron microscopy studies, and purification of PR protein from inoculated plants they concluded that the inhibition of rust infection hyphae in acquired resistant broad bean plants is mainly due to the anti-fungal activity of induced basic PR-1 protein present in the intercellular space of acquired resistant broad bean leaves.

Hydrolytic enzymes 1,3-β-glucanase and chitinases are also thought to be involved in host resistance. The wide spread distribution of these enzymes in higher plants, their ability to digest major chitin and 1,3-β-glucan constituent in fungal cell walls and their induction by ethylene, pathogen, elicitors and other stresses all suggest their involvement in host resistance (Sahai and Manocha 1993). Chitinases also produced by biocontrol agents e.g. *Trichoderma harzeanum* (Lorito et al. 1993; Harman et al. 1993) and pathogenic fungi. The ability of chitinases to inhibit a pathogen depends on their origin or host pathogen interaction. Chitinases from tobacco, thorn apple and wheat were unable to inhibit *B. cinerea* growth *in vitro* (Broekaert et al. 1988), whereas chitinases from biocontrol agents *Gliocladium virens* (Pietro et al. 1993) and *Trichoderma harzeanum* (Lorito et al. 1993) inhibited this pathogen effectively.

1.3. Confocal microscopy

1.3.1. Confocal microscope and digital images

Advances in computer technology have allowed widespread use of sophisticated imaging techniques. One of the most significant of these techniques is Confocal Laser Scanning Microscopy (CLSM). Confocal microscopy is a modification of fluorescence microscopy that can be used to improve contrast and resolution and to take optical sections (Kwon et al. 1993). In CLSM, a sample is treated with fluorescent dye, and a laser beam is focused on a point in the sample, causing the emission of fluorescent light. This light emitted from the illuminated point is focused on an aperture placed in front of a photodetector. Fluorescent light emanating from outside of the focal plane falls outside the aperture itself and is largely eliminated, resulting in greatly increased contrast and a small gain in resolution compared with conventional microscopy. Kwon et al. (1993) stated that conventional

microscopic images of fungal hyphae are most often taken from a single median focal plane to maximize the information content in the image in spite of the out of focus area which detracts from the focal plane. This results in images with less than optimal contrast and resolution. In confocal images, such areas are covered with optimal contrast and resolution and allow the viewer to clearly see the details in that plane. Such detailed image is obtained by scanning the exciting beam over the sample in raster fashion. The resulting image is an optical section through the sample and stored on a computer in digital form as a two dimensional image. To obtain three-dimensional images, computer software assembles a series of images acquired at defined intervals along the z-axis.

The use of CLSM in the study of plant pathology has many advantages over conventional histopathological techniques because with CLSM whole mounted samples can be scanned and the number and pattern of interior cells or penetrated fungal hyphae in the tissue examined unlike Scanning Electron Microscopy where only surface cells are visible. CLSM data are stored digitally; the data can not only be reconstructed in three dimensions, but also animated or displayed as stereo images, to aid in visual interpretation. The reconstructed sample on a computer can also be made to visualize from any orientation. In addition to these features, a confocal microscopy image provides an opportunity to quantitatively measure organelle distributions (Kwon et al. 1993) and fungal biomass in a volume basis (Vingnanasingam 1998) using an image analysis computer package in appropriate computer system.

1.3.2. Fluorescence staining

Confocal microscopy required fluorescence sources during excitation by a laser beam to produce an imaging fluorescence from the specimen. The amount of light from the imaging fluorescence depends on the fluorescent dye used for the particular specimen. Know et al. (1993) stated that virtually all fluorescent dyes photobleach – immediately following the excitation required for fluorescence – limiting the total amount of light that can be obtain from the fluorescent dye. This photobleaching probability depends on many chemical and physical parameters that affect each type of dye differently. However, several fluorescent dyes have been used for the studies of organelle distribution in fungal

hyphae or fungal-plant interaction by confocal microscopy. Knight et al. (1993) used three dyes (Calcium Green-1, Fura-2 and Indo-1) sensitive to Ca^{2+} for imaging cytosolic free calcium in hyphae of *Basidiobolus ranarum, Neurospora crassa and Uromyces vignae*. Cole et al. (1996) used the monoclonal antibody, BC-KH4 and a secondary antibody-FITC conjugate to immuno-fluoresce *Botrytis* sporelings on infected *Vicia fabae* leaves. Wei et al. (1997) used 0.05% trypan blue in lactophenol and obtained clear images of hyphae of *Colletotrichum gloeosporioides* f. sp. *Malvae* in infected mallow leaves. Schelkle et al. (1996) also used trypan blue and visualized intercellular mycorrhizal hyphae but they pointed out that freshly made stain and immediate observation under CLSM following staining is a must to obtain clear images. Glutaraldehyde, a fixative which can render tissue auto-fluorescent (Bacallao et al. 1995) has been used to visualize fungal hyphae in wood tissue (Singh et al. 1997) and *B. cinerea* hyphae in ornamental and vegetable plant tissue (Vingnanasingam 1998; Vingnanasingam and Long 1998).

The main goal of this thesis is to facilitate developing control strategies against camellia flower blight through investigating the *C. camelliae* infection process, resistance mechanisms of *Camellia* species, and evaluating potential biological control agents against *C. camelliae*.

1.4. Thesis objectives

To:

1. Develop a fluorescent staining technique for confocal microscopy to investigate the infection process of *C. camelliae* and biocontrol efficacy of yeast and bacteria,
2. Describe *C. camelliae* ascospore germination and the infection process on camellia flower petals,
3. Identify the resistance mechanism of some *Camellia* species or cultivars against *C. camelliae*.
4. Isolate and evaluate potential biocontrol agent(s) against *Ciborinia camelliae,*
5. Identify the mode of action of such biocontrol agent(s),

Chapter Two

General Materials and Methods

2.1. *Ciborinia camellia* isolates

2.1.1. Ascospore collection

Apothecia of *Ciborinia camelliae* were collected from the camellia section of Wellington Botanical Garden and from Ben Logi Garden, Wanganui. They were placed in a sealed moist box and held in a cool room at 0-2^{o}C for up to a month as a source of ascospores when required. Ascospores released from apothecia were either allowed to land directly on the experimental camellia petals or were collected within a universal bottle for use as spore suspensions as described below.

2.1.2. Ascospore inoculum preparation

To prepare a spore suspension, a sclerotium and the stipe of its apothecium were wrapped with a wet facial tissue and inverted into the neck of a universal bottle (Fig. 2.1). After 4-6 h, the ascospores released were suspended in to 1-2 ml of sterile distilled water (SDW). A 10 µl aliquot was removed from the suspension and pipetted onto a haemocytometer slide to assess the spore concentration. Two spore counts were taken from five of the 25 inner squares, (the corner squares and the central square) each with a volume of 0.1 µl. After counting, the spore concentration was adjusted with SDW as required.

2.1.3. Ascospore inoculation

Because of early spore germination or of a burst of germination when ascospores were in the water suspension for a long time before inoculation, petal tissue was inoculated with ascospores within 2 h of inoculum preparation. For direct spore deposition of airborne spores, two methods were used to inoculate the petals:

Method 1. A sclerotium and its stipe were wrapped with a wet facial tissue paper and suspended for 1-2 h in an inverted position about 4-5 cm above the petal which was laying in a plastic container. Any spores released were then directly deposited on the petals (Fig. 2.2).

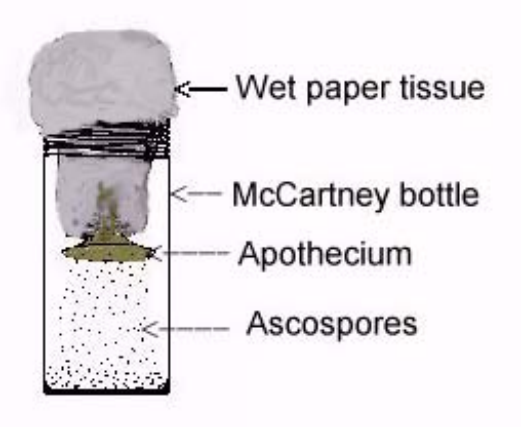


Figure 2.1. Ascospores released from apothecium for inoculum preparation

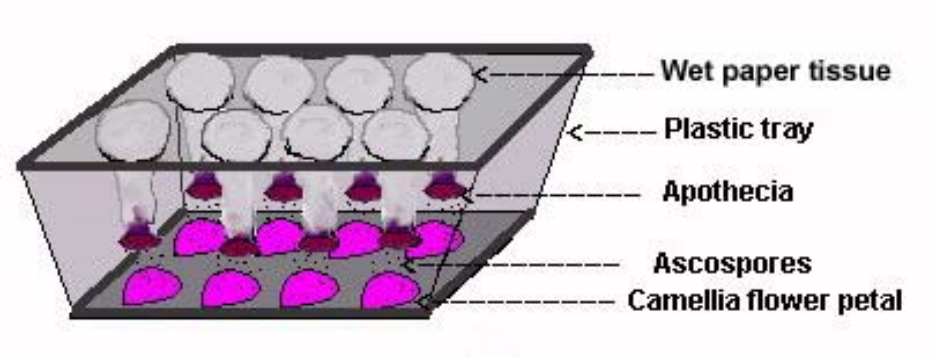


Figure 2.2. Direct ascospore inoculation from apothecia to camellia flower petals

Method 2. This method was adapted from Taylor and Long (1999) where airflow from an aquarium pump was passed over apothecia in a plastic chamber and carried fresh ascospores directly from the apothecia to a settling chamber where they settled on the petals by gravity (Fig. 2.3).

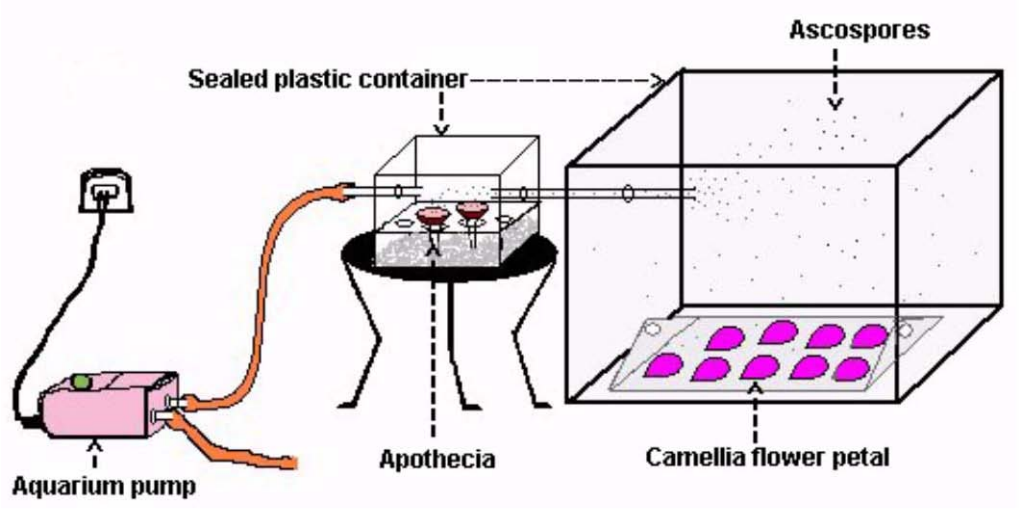


Figure 2.3. Ascospore deposited by gravity inoculation using airflow from an aquarium pump.

2.2. *Botrytis cinerea* isolates

2.2.1. Culture preparation

Botrytis cinerea isolates were collected from infected camellia flowers. Spores from the infected tissue were gently trapped with wet transfer loop (Fig. 2.4) and were transferred on to water agar (WA). They were grown at 20^{o}C for 12-24 h and germinated single spores were transferred to Malt agar (MA) (Malt extract agar -Difco 33.6g/liter; pH 6.5). and

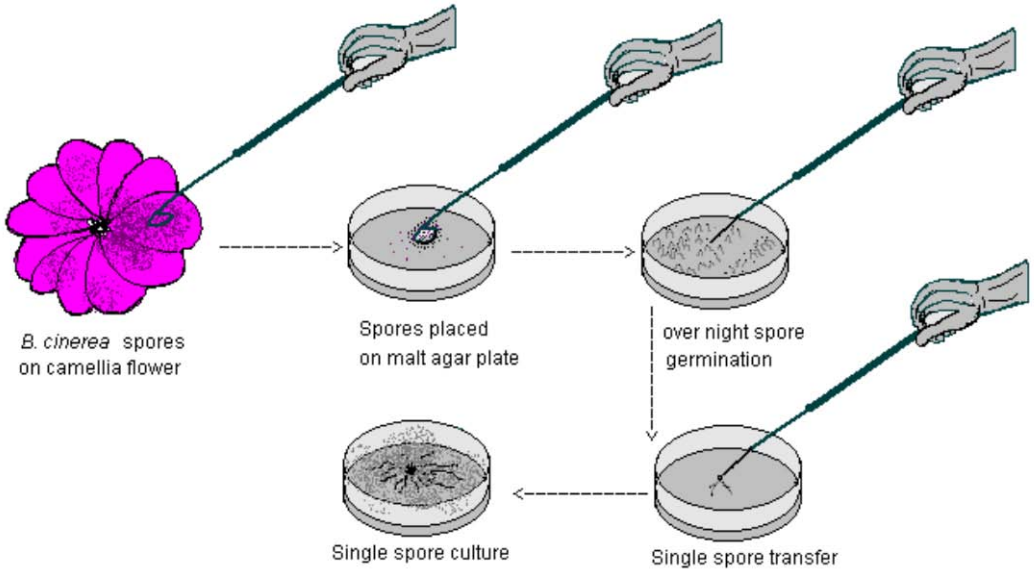


Figure 2.4. Single spore culture of *B. cinerea.*

incubated at 20^{o}C under a 12 h day/night Near Ultra Violet (NUV) regime using a Philips TLD 36W/08, located 40 cm above the cultures, for 5-7 days to induce sporulation. The isolate used in this work was named Bc-C1. It sporulated profusely 5 days after incubation. Plates with sporulation were stored at 4^{o}C for up to 3-4 months for subsequent experiments. After which, fresh flowers were inoculated and the *B. cinerea* re-isolated as a precaution against loss of pathogenicity of the culture.

2.2.2. Preparation of spore suspensions

Fifteen millilitres of sterile distilled water (SDW) were poured over a sporulating colony and spores gently dislodged using a sterilised, bent glass rod. The resulting suspension of conidia and mycelial debris was transferred into a universal bottle and shaken vigorously for a few seconds to disperse clumps of conidia. The contents were transferred to another universal bottle via a sterile 70 μm 'Falcon' cell strainer to remove mycelial debris. Spore concentration was estimated using a haemocytometer as described above.

2.3. Putative biocontrol agents; bacteria and yeast

2.3.1. Culture preparation

All yeast isolates were cultured on nutrient yeast dextrose agar (NYDA) consisting of: 8 g/litre nutrient broth (Gibco-BRL), 15 g / litre bacteriological agar (Gibco-BRL), 5 g / litre yeast extract (Gibco-BRL) and 10 g / litre D glucose (Ajex Chemicals). The pH was adjusted to 4.5 with 6M HCl. Bacterial isolates were cultured in Nutrient Agar (NA) consisting: 28 g / litre nutrient agar (OXOID). All media were sterilised at 121^{o}C for 15 min at 15 psi. All isolates were incubated at 15^{o}C for 2-3 days in total darkness.

2.3.2. Preparation of cell suspensions

After incubation, 3-4 ml of SDW was used to dislodge cells in the colony by repeated pipetting to make a cell suspension. The suspension was transferred to 1.5 ml ependorff tubes and centrifuged at 10,000 rpm for 5 min using a Biofuge A (Heraeus, Spatech) to remove dissolved media in the liquid. The liquid was decanted off and the microbial cell pellet re-suspended in 1.5 ml of SDW by vortexing in the ependorff tubes.

2.3.3. Cells counts

Bacterial cell concentration was determined by counting number of cells in a 10 µl aliquot of cell suspension using a Petroff-Hauser counter (MNK-780-T, Weber Scientific International Ltd., England). Yeast cell concentrations were determined using a haemocytometer.

2.4. Source of test materials

2.4.1. Camellia flowers

Camellia flowers were collected from *C. reticulata* cv. Brian and from a range of varieties of different *Camellia* species located in the Massey University arboretum during the camellia flower season (July to November). Undamaged and healthy flowers or individual petals were used for inoculation with ascospores and /or BCA application.

2.4.2. Lettuce (*Lactuca sativa* L.)

Lettuce leaves were collected from lettuce (*L. sativa* cv Lollo Bionda) grown in the Glass-house in the Agricultural and Horticultural Building Massey University. Lettuce seeds, cv. Ravel-butterhead (Webling and Stewart Ltd. Petone, New Zealand) were planted in 15 cm diameter wells in a tray of 36 wells. A mixture of 3-4 month osmocote, $FeCl_3$, Dolomite and aglime (50:5:40:45) mixed with one unit volume of fine bark medium was used as a potting medium.

2.5. Staining the specimens

2.5.1. Glutaraldehyde

Glutaraldehyde solution (GA) was used to induce fluorescence of fungal hyphae in plant tissue (Singh et al. 1997; Vingnanasingam 1998). GA and buffered sucrose solution were prepared as described by Disbrey and Rack (1970) with a small modification as described below.

2.5.1.1. Glutaraldehyde solution preparation

Phosphate buffer (PB) or Phosphate buffered saline (PBS) was used to prepare glutaraldehyde solutions at concentrations ranging from 0.25% to 4%. PB was prepared by

mixing 83 ml of 1.95% aqueous sodium dihydrogen phosphate (NaH_2PO_4) with 17 ml of 2.52% aqueous sodium hydroxide (NaOH) at pH 7.2 - 7.4. Stock (x10) PBS was prepared by mixing 40 g of NaCl, 1 g of KCl, 5.75 g of Na_2HPO_4 and 1 g of KH_2PO_4 in 500 ml of distilled water and was stored at room temperature. The working concentration was obtained by diluting 50 ml concentrate to 500 ml with distilled water and the pH was adjusted with 0.1M NaOH or 0.1M HCl solution to the required pH level.

2.5.1.2. Buffered sucrose solution preparation

Buffered sucrose was prepared by dissolving 6.5 g sucrose in 100 ml PB or PBS at pH 7.2-7.4 and was stored at 4^oC.

2.5.1.3. Glutaraldehyde treatment

Small pieces (25 mm^2) of lettuce leaf or camellia petal tissues taken from sites inoculated with *B. cinerea* or *C. camelliae* were placed into a universal bottle with approximately 2 ml of glutaraldehyde solution and incubated under dark condition for 6-8 h or overnight at room temperature. After incubation, excess glutaraldehyde was removed from the tissue using two changes of buffered sucrose solution over a period of at least three hours. Washed tissues were mounted on a slide with Citifluor (glycerol/PBS Agar) (ALLTECH, P.O. Box 1000352, Nth Shore MC, Auckland 10, New Zealnad) mounting medium.

2.5.1.4. Clearing of tissue with Chloral hydrate

Two to three millilitres of concentrated chloral hydrate solution (5 g in 2 ml of water) were used to treat the tissues for 2 – 12 h or overnight to clear the pigments and the air bubbles in the tissue.

2.5.2. Trypan blue

2.5.2.1. Preparation of trypan blue

Trypan blue stain was prepared by dissolving 0.05 g of trypan blue in 100 ml of lactophenol solution. Lactophenol solution was prepared as described by Tuite (1969): Twenty gram of phenol crystals were dissolved by gentle heating in a mixer at 20 g of lactic acid, 40 g of glycerine and 20 g of distilled water. Trypan blue stain was added to give a concentration of 0.05%.

2.5.2.2. Staining procedure with trypan blue

For light microscopy examination, all specimens were stained with trypan blue. Small pieces from inoculated tissue, as described above, were placed on glass slides in a few drops of 0.05% trypan blue in lactophenol and heated over a flame for 30 sec. or for just boiling. Excess stain was removed from the specimens by washing several times with distilled water. The stained specimens were mounted in 0.1% $FeCl_3$ in 50% glycerol for microscopic examination.

2.6. Microscopic examination

2.6.1. Light microscopy

Specimens stained with 0.05% trypan blue in lactophenol were examined using phase contrast, nomarski interference contrast, dark field or cross polarised light microscopy (Reichert Nr Austria). Relevant images were photographed using a Nikon FX-35WA camera and exposure system on Fujichrome 64T film or video recorded using a Panasonic F15 CCD video camera and Panasonic NV-FS100 HQ video cassette recorder, and recorded system on VHS Sony super DX tape. Appropriate single video images were captured from live video using the video capture programme in the Media Studio Pro 2.0 advanced multimedia programmes in a computer. The captured images and video sequences were saved on a CD-Rom.

2.6.2. Fluorescence microscopy

Fluorescence microscopy was used to pre examine the specimens treated with glutaraldehyde to confirm fluorescence and the appropriate image location of the specimens to facilitate confocal microscopy.

Specimens treated with Aniline blue, Berberine, Neutral red and Toluidine blue O were examined under an epifluorescent microscope using blue-violet and ultraviolet excitation filters.

2.6.3. Confocal microscopy and image analysis

Pre-examined glutaraldehyde treated specimens were examined under the Leica TCS/NT confocal laser scanning microscope using filters with exciter wavelengths of 488 and 568 nm and imaging wavelengths between 530 and 590 nm. Confocal images were produced by depth scanning, created from 15 to 30 optical sections taken 5 μm apart and combined to form the final image for 2D or 3D view. These images were rotated in various angles to view fungal hyphal development inside the plant tissue. 2D images at appropriate focal points were also obtained from specimens treated with different GA treatment for fluorescence intensity analysis. These images were analysed in an Indy Silicon Grafics Computer System to measure the fluorescence intensity by transferring histogram data into numerical format to facilitate statistical analysis.

2.7. Assessment of infection of *Ciborinia camelliae*

2.7.1. Assessment of visual symptoms from digital images

After symptoms developed, petals were scanned through a "Snapscan 1212" Agfa scanner and images of the petals with infection were obtained and saved as digital images in a computer in 'tiff' file format. The number of infected petal or inoculation sites was counted from the images to measure disease incidence (percent infection). To evaluate disease severity, the infected area was measured by measuring pixel numbers using histograms in the Adobe Photoshop 5.0 computer program. In addition to this method, the appropriate scaling systems for disease incident or severity are described in the materials and methods of individual experiments if needed.

2.7.2. Assessment of infection by counting spore germination

Trypan blue treated specimens were examined under a light microscope using bright field or nomarski interference microscopy with X 10 eyepieces and X 40 objective. Total numbers of spores, germinated spores and germ tube penetration were counted and germ tube and penetrated hyphal length measured under a unit area inoculated by *C. camelliae*.

2.8. Experimental designs and statistical analysis

Generally a Randomised Complete Block (RCB) design and specifically, in some cases, split plot, factorial design and repeated measurement was used with or without sub-sampling in appropriate experiments.

Collected data were analysed by ANOVA using general linear modeling in the SAS system (Version 6.12 PC SAS). Arcsin or root transformation was used whenever necessary to satisfy the requirement of ANOVA.

The appropriate form of experimental design and statistical analysis is described in the materials and methods of individual Experiments or Chapters.

Chapter Three

Glutaraldehyde and confocal microscopy of fungal hyphae in plant tissue

3.1. Introduction

Confocal laser scanning microscopy (CLSM) allows optical sectioning of material and three-dimensional reconstruction of images obtained, both very useful features when studying complex fungal structures within plant tissues (Czymmek et al. 1994; Kobayashi et al. 1994). Fluorescence is the predominant optical mode used for this microscopy since laser-scanning systems are easily adapted to conventional epifluorescence microscopes (Kwon et al. 1993). The use of fluorescence in CLSM has stimulated research in the development of new fluorochromes (Cox 1993) and the exploration of the usefulness of known fluorochromes and dyes used routinely for light microscopy (Schelkle et al. 1996). Many xanthine dyes have been used as stains for light microscopy (Gurr 1965, 1971; Stevens 1974) and as fluorochromes (Thampion et al. 1973; Canny 1987; Oparka 1991; Young and Asford 1992; Melville et al. 1998). Various fluorochromes have been used to study fungal cytology and differentiation with fluorescence microscopy (Butt et al. 1989). The fluorescent probes offer remarkable sensitivity and specificity for the detection and imaging of biological molecules including proteins, nucleic acids, carbohydrates, lipids, and physiological ions in cells or cell free extracts (Wells and Johnson. 1993). This fluorescent probe technology has expanded to include a very wide variety of dyes useful with lasers (Wells and Johnson 1993; Haugland 1992). However, there are limitations to the total amount of light that can be obtained from a fluorescent dye. This is because virtually all fluorescent dyes photobleach.

As a new development, Singh et al. (1997) have tested glutaraldehyde as a fluorescence agent to visualise wood fungi. Since glutaraldehyde was used as a fixative it had a dual role by causing fluorescence of wood fungi in the wood tissue in their experiment. This chemical has also been used in studies on *B. cinerea* where it resulted in bright fluorescing fungal hyphae in vegetable and ornamental plant tissue (Vingnanasingam 1998). However, some components of plant tissue also fluoresce and cause inaccuracies in measurement of hyphal volumes when digitally stored images are quantitatively analysed by an image

analysis computer package. Further improvements on the glutaraldehyde technique to avoid or minimise background fluorescence would be useful for accurate measurement of hyphal volume, to improve contrast of images and to study infection processes of fungi in plant tissue.

3.2. Objectives

1. To improve contrast and resolution of fungal hyphae in plant tissue using modified glutaraldehyde solutions.
2. To remove or minimise any background due to auto-fluorescence of plant tissue by application of quenching agents with glutaraldehyde treatment.

3.3. Materials and Methods

The fluorescence effect of glutaraldehyde was first tested on *B. cinerea* fungal hyphae in lettuce leaf tissues as model experiments since camellia petal tissue was unavailable except for 2-3 months in winter to spring. The technique was then applied to *C. camelliae* and *B. cinerea* on camellia flower tissues.

3.3.1. *Botrytis cinerea* inoculation

Fully developed lettuce leaves were taken from plants grown in a glasshouse as described in Chapter 2, Section 2.4.2. A single leaf was placed in a culture plate cover (13 cm x 8 cm x 4 mm) and inoculated with 2 µl of *B. cinerea* spore suspension containing approximately 100 spores per site using a micro-pipette. All inoculation sites were gently wounded by a sterilised needle and the inoculum suspension was air dried for 1-2 h before incubation in a plastic tray (45 cm x 30 cm x 15 cm) with moistened paper tissue underneath the plates. Each tray was inserted into a clear polythene bag, which was then sealed to ensure a high humidity inside the tray during the incubation period of 24 h at 20^{o}C under 12/12 h light and dark condition.

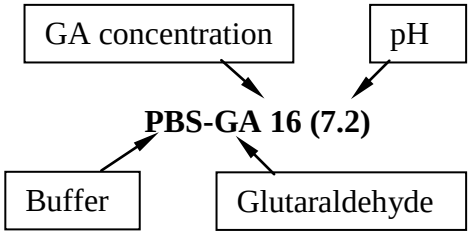
3.3.2. Preview germination examination

After 24 h incubation, a small piece (25 mm^2) of inoculated leaf was cut and stained with 0.05% trypan blue as described in Chapter 2, Section 2.5.2.2. The stained specimens were examined using compound light microscopy to check that there was sufficient spore germination to proceed with preparation for confocal microscopy.

3.3.3. Preparation of glutaraldehyde solutions

Phosphate Buffered Saline-Glutaraldehyde (PBS-GA), Tris buffer-Glutaraldehyde (Tris-GA) or Universal buffer-Glutaraldehyde (Uni-GA) solutions were prepared by mixing 25% glutaraldehyde solution (GA), electron microscopy grade, with PBS, Tris or Universal buffer. A range of concentrations with the required pH level of glutaraldehyde working solution was prepared according to the needs of experiments described below.

Preparation of glutaraldehyde solution for the treatment was based on the buffer, concentration of glutaraldehyde in the solution and pH level of the final solution. For example, PBS-GA 16 (7.2) indicates that the final GA solution prepared for treatment with 16% of 25% glutaraldehyde (electron microscopy grade) in PBS buffer and maintained at pH 7.2. The actual glutaraldehyde concentration was 4%.



3.3.4. Preparation of phosphate buffered saline (PBS)

Concentrated (X10) PBS solution was prepared as described in Chapter 2, Section 2.5.1.1 and normal working concentration of PBS with required pH was obtained as described in Chapter 2, Section 2.5.1.1.

3.3.5. Preparation of Tris (Hydroxymethyl) aminomethene buffer solution (Tris)

Tris solution was prepared by dissolving 12.114 g of Tris (M.wt 121.14) in 1 liter water and the pH was adjusted with 0.1M HCl to the desired level (Dawson et al. 1969).

3.3.6. Preparation of Universal buffer (Uni)

Universal buffer solution was prepared by dissolving 6.008 g of citric acid, 3.893 g of KH_2PO_4, 1.769 g of H_3BO_3 and 5.266 g of diethylbarbituric acid in 1000 ml of distilled water and pH was adjusted with 0.1M KOH to the desired level (Dawson et al. 1969).

3.3.7. Preparation of buffered sucrose solutions

Buffered sucrose was prepared by dissolving 6.5 g. sucrose in 100 ml PBS, Tris or Universal buffer at pH 7.2 - 7.4 to use as a wash solution. This was stored at 4^oC to inhibit microbial growth.

3.3.8. Procedure for glutaraldehyde treatment

Two to three pieces (20-25 mm^2) of lettuce leaf tissue were taken from a *B. cinerea* inoculated site and placed into a universal bottle (6cm x 2.5cm) and treated with about 2 ml of glutaraldehyde solution as described in Chapter 2, Section 2.5.1.3. Washed tissues were mounted on a slide with Citifluor.

3.3.9. Preview prior to confocal examination

Specimens on the slide were pre examined under a fluorescence microscope with a filter that covered a range of wavelength between 550 and 650 nm to confirm that the specimens would fluoresce and to find the appropriate image location to facilitate confocal microscopy image production.

3.3.10. Confocal microscopy

The specimens were examined in a confocal laser scanning microscope (Leica: Model TCS/NT) using a specific wavelength for each treatment. Confocal images were produced with exciter wavelengths of 488 nm and 568 nm and with 530 nm and 590 nm barrier filter

for imaging. Each specimen was observed under 1x40 (immersion) or 1x63 (immersion) objective lenses and the images were created from a single plane of focus or from 15 to 30 optical sections taken 5 μm apart in depth and then recombined to form the final image. All good quality images were transferred to the analyser-computer.

3.3.11. Evaluation of fluorescence intensity

1. Images with fungal hyphae and plant tissue were compared visually for fluorescence brightness and rated as high, medium or low.
2. Fluorescence intensity was also evaluated numerically by measuring pixel grey colour value from the digital image of the fungal hyphae and its background (plant tissue) using a program of "Image Analysis-Densitometry".

3.3.12. Experiment 3.1. Effect of glutaraldehyde solution prepared with different buffers on fungal hyphae and background fluorescence

3.3.12.1. Introduction

Buffer solutions may influence auto fluorescence of cell components. Thus the preparation of glutaraldehyde solution for induced fluorescence with various buffer solutions could affect the auto-fluorescence of plant cells. There is an understanding among researchers that using Tris buffer solution with glutaraldehyde could diminish auto fluorescence of plant or animal tissue rather than using PBS buffer during sample preparation for fluorescence microscopy (Poot 1996).

The objective of this experiment was to evaluate various buffers used with glutaraldehyde for their effect on induced fluorescence of fungal hyphae and background of plant tissue.

3.3.12.2. Materials and Methods

3.3.12.2.1. Preparation of glutaraldehyde solutions with different buffers

PBS-GA, Tris-GA and Uni-GA solutions were prepared by mixing 16 ml of 25% glutaraldehyde (EM grade) with 84 ml of PBS, Tris and Universal buffer solutions and were maintained at pH 7.2 – 7.4 as described above. They are coded as PBS-GA 16 (7.2),

Tris-GA 16 (7.2) and Uni-GA 16 (7.2) respectively. (The actual GA concentration was 4% in each solution)

3.3.12.2.2. Fluorescent treatment

Twenty-four hours after *B. cinerea* inoculation, a number of pieces (20-25 mm^2) of lettuce leave tissue were treated with PBS-GA 16 (7.2), Tris-GA 16 (7.2) or Uni-GA 16 (7.2) solutions as described above. Other pieces were treated with only PBS, Tris or Uni buffers and washed with the respective buffered sucrose solution as control treatments to compare with glutaraldehyde. The washed tissues were further treated with 2-3 ml of concentrated chloral hydrate solution (5 g in 2 ml of water) for 2 – 12 h until the tissues were cleared and free from air bubbles. During chloral hydrate treatment the specimens were placed in a vacuum desiccator to ensure all air bubbles were removed from the tissues. At least 4 specimens from each treatment were mounted on glass slides with Citifluor mounting medium.

The specimens on the slides were first observed under epifluorescence to mark the appropriate hyphal location and were then observed by confocal microscopy to produce confocal micrographs.

3.3.12.3. Results and Discussion

Tris and Universal buffers used in the glutaraldehyde preparation considerably reduced auto-fluorescence of plant cells compared with PBS buffer but also reduced fungal hyphae fluorescence (Plate 3.1). Universal buffer further diminished fluorescence of fungal hyphae even more than Tris buffer (Plate 3.1C). The results indicate that the buffers can influence the intensity of autofluorescence of plant tissue as well as induced fluorescence of fungal hyphae. In fluorescence microscopic studies on plant and animal tissue, many workers recommended using Tris buffer to avoid autofluorescence of the tissue (Poot 1996). However, in this study, Tris buffer diminished fungal hyphal fluorescence along with plant tissue. Hence it could be a disadvantage to use this buffer as a base solution for glutaraldehyde. This buffer was incorporated with other treatments in subsequent experiments for further investigations to increase hyphal fluorescence while keeping background fluorescence low for better contrast and fungal volume measurement.

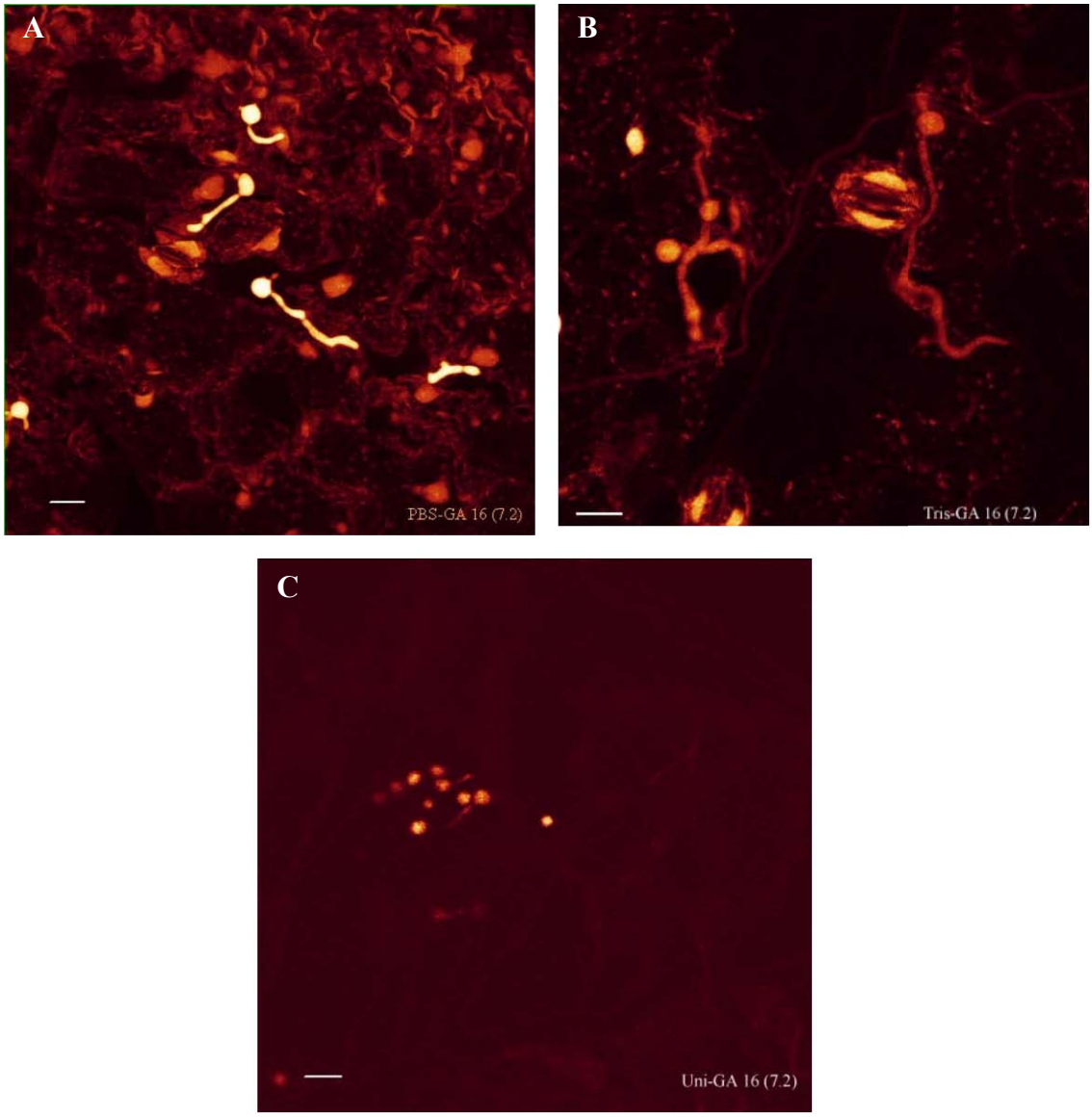


Plate 3.1. Confocal micrograph of *B. cinerea* hyphae in lettuce leaf tissue showing the effect of various buffers on fluorescence. A) GA in Phosphate buffered saline (PBS). B) GA in Tris buffer. C) GA in universal buffer. Bar = 20 µm.

3.3.13. Experiment 3.2. Quenching of background fluorescence of plant tissue

3.3.13.1. Introduction

Quenching is a reduction of fluorescence by a competing deactivating process resulting from the presence of other molecules in the system (Rost 1995). Chlorophyll generally has the ability to autofluoresce, as do other pigments. Dissolving these substances gently with some clearing agents could minimise the whole effect of the background fluorescence of the tissue. Chemicals such as ammonium chloride and sodium borohydrate which have been suggested (Anonymous 1996) for quenching autofluorescence of the tissue, were also tested in this experiment.

3.3.13.2. Materials and Methods

Lettuce leaves inoculated with *B. cinerea* were treated with PBS-GA 16 (7.2) or Tris-GA 16 (7.2) 24 – 36 h after inoculation as described in the previous experiment. GA treated specimens then were treated with either clearing, quenching or brightening agents as described under the respective agents. All specimens were placed in a vacuum desiccator before mounting on a slide to remove air bubbles from the tissues.

1. *Chloral hydrate*

Two to three millilitres of concentrated chloral hydrate solution (5 g in 2 ml of water) was used to treat the tissues for 2 – 12 h.

2. Hydrogen peroxide

Twenty percent (V/V) hydrogen peroxide (6% ai) was used to treat the specimens for 2 h.

3. Ethanol

A series of ethanol concentrations (15, 30, 50, 70, 85, 95 and 100%) was prepared and the tissues were treated for 30 min. at each step except the last (100%) where the tissues were treated three times each for 30 min. to remove any traces of water from the specimen. Another set of tissue also was treated with Ethanol before glutaraldehyde treatment to avoid preservation of fluorescence by glutaraldehyde fixation (Wilde and Fliermans 1979).

4. Triton X 100

0.02% Triton X- 100 solution was used to treat the tissue for 2 h.

5. 2-methoxyethanol

Ten percent (v/v) 2-methoxyethanol was used to treat the tissue for 2 h or overnight.

6. Carbol-Turpentine- Xylene

Carbol-terpentine solution was prepared by melting 40 ml of phenol in 60 ml oil of terpentine. The solution was used to treat the tissue overnight at room temperature. After treatment, the excess clearing solution was removed by xylene.

7. Carbol-Turpentine -Xylene and 2-methoxyethanol

The tissues treated with "Carbol-Turpentine –Xylene" contained excess xylene in the tissue and blocked proper mounting. To avoid this situation, tissues were further treated with 10% (v/v) 2-methoxyethanol to remove excess xylene from the tissue.

8. Ammonium chloride

50 mM (0.134 g /50 ml) ammonium chloride solution was prepared in PBS solution. The solution was used to treat the tissue for 15 min. at room temperature.

9. Sodium borohydrate (NaBH4)

Sodium borohydrate (0.1%) was dissolved in PBS solution and the tissues received two washes of this solution for 5 min each.

10. Ethanolamine

Ethanolamine was diluted with water (485 ml +50 ml H_2O) to give a 0.15 M solution at pH 7.5 and the tissues were treated with this solution for 30 min in ice.

11. Glycine

100 mM (0.75%) glycine solution was prepared in PBS buffer and the tissues were treated with the solution for 5 min at room temperature.

After treatment, all specimens were then mounted on glass slides with Citifluor mounting medium for examination under an epifluorescence microscope or confocal microscope. Specimens were first observed under a fluorescence microscope as described above before confocal microscopy. Visual estimation as illustrated in Plate 3.6 was used to evaluate the effect of the respective quenching agent on background fluorescence or contrast of the image.

3.3.13.3. Results and discussion

Compared with control specimens, those treated with PBS-GA 16 (7.2) Triton X-100 and 2-methoxyethanol treatments gave a quenching effect on background fluorescence of the PBS-GA treated specimens (Plate 3.2C & D). Fungal hyphal fluorescence in Tris-GA 16 (7.2) treated specimens was totally quenched by Triton X 100 (Plate 3.3C). Treatment with Ethanol did not quench background fluorescence markedly in the PBS-GA 16 (7.2) treated specimens but quenched it in Tris-GA 16 (7.2) treated specimens. Ethanol brightened the fungal hyphae in both specimens compared with their respective control treatments (Plate 3.2B & 3.3B). Ethanol seemed to be a fluorescence-brightening agent for fungal hyphae but did not have any marked effect on background fluorescence. Ethanol treatment prior to glutaraldehyde extremely reduced fungal hyphae fluorescence. This indicates that Ethanol may prevent glutaraldehyde to induce fluorescence on fungal hyphae by killing fungal cytoplasm. Hydrogen peroxide increased background fluorescence of both PBS-GA 16 (7.2) and Tris-GA 16 (7.2) treated specimens and also increased hyphal fluorescence in Tris-GA 16 (7.2) treated specimens (Plate 3. 4).

The quenching agents Carbol-Turpentine- Xylene, Carbol-Turpentine -Xylene and 2-methoxyethanol, Ammonium chloride, Sodium borohydrate (NaBH4), Ethanolamine and Glycine tested on PBS-GA 16 (7.2) or Tris-GA 16 (7.2) treated specimens reduced background fluorescence as well as fungal hyphae fluorescence to a low fluorescent intensity. During treatment with Carbol-Turpentine- Xylene or Carbol-Turpentine -Xylene and 2-methoxyethanol, the plant tissue shrank and distorted. These specimens were observed under an epifluorescence microscope and because of the obvious poor results, they were not observed under the confocal microscope.

Chloral hydrate treatment neither quenched nor brightened the background fluorescence or fungal fluorescence but the treatment did facilitate clear observation of specimens by removing all kinds of pigmentation and air bubbles. Therefore, it could be used as a clearing agent after any GA treatment to enhance visibility of specimens under microscopic examination.

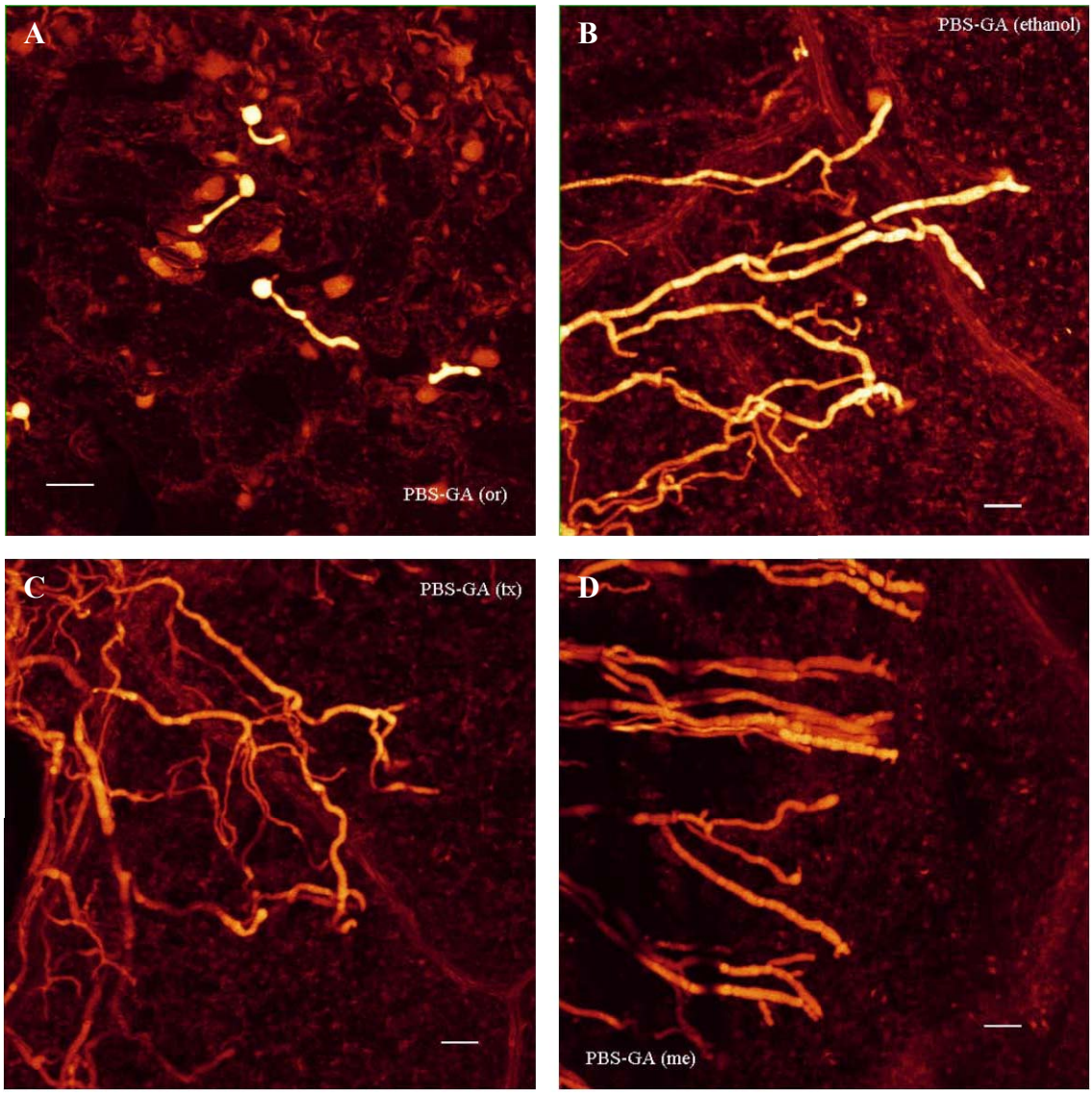


Plate 3.2. Confocal micrograph of *B. cinerea* hyphae in lettuce leaf tissue showing the effect of various quenching agents against background fluorescence after PBS-GA treatment. A) Control – not treated with any quenching agent B) Ethanol treatment. C) Triton X 100. D) 2-methoxyethanol. Bar = 20µm.

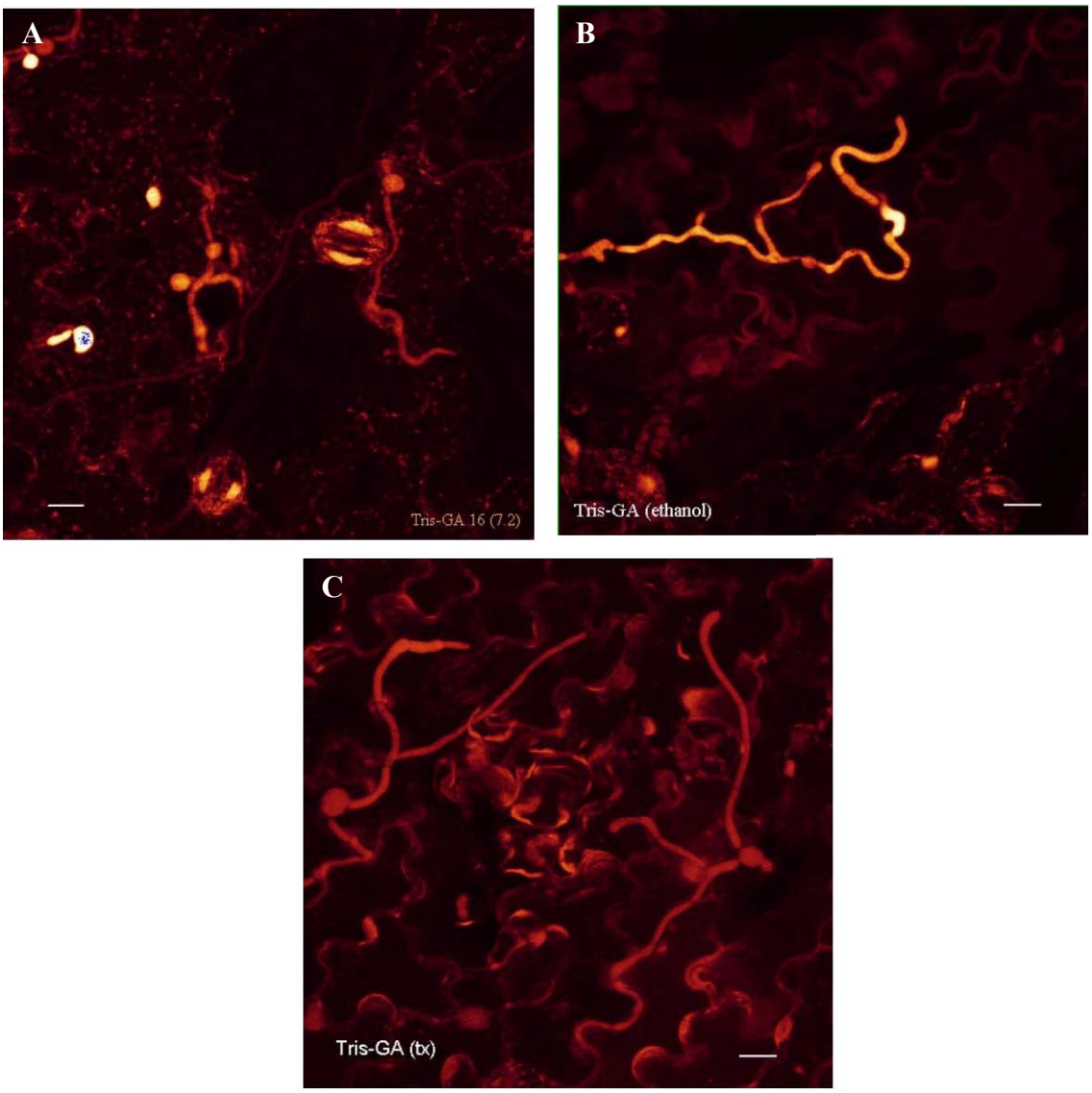


Plate 3.3. Confocal micrograph of *B. cinerea* hyphae in lettuce leaf tissue showing the effect of various quenching agents against background fluorescence after Tris-GA treatment. A) Control – not treated with any quenching agent B) Ethanol treatment. C) Triton X 100. Bar = 20µm.

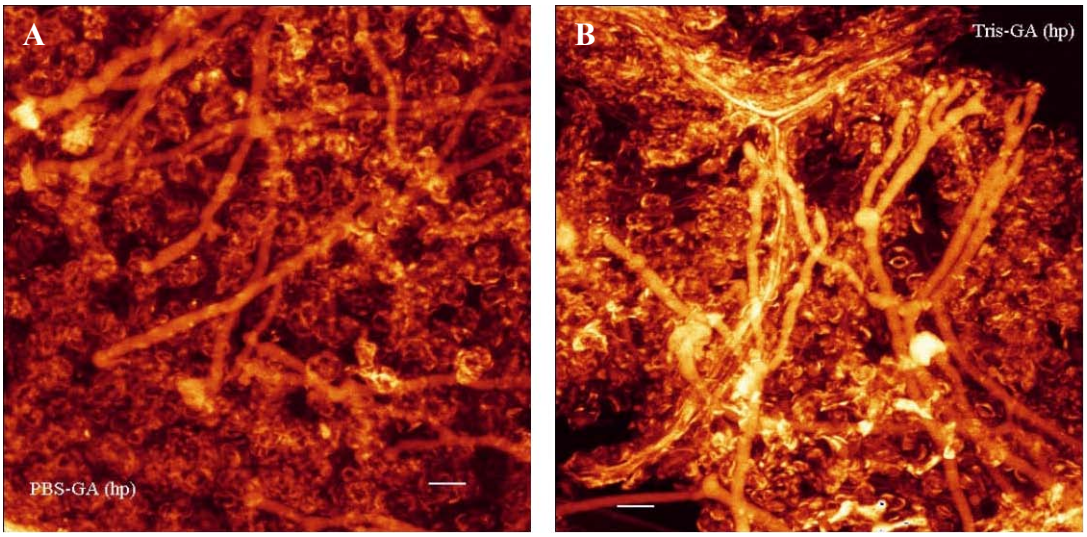


Plate 3.4. Confocal micrograph of *B. cinerea* hyphae in the lettuce leaf tissue showing the effect of hydrogen peroxide after A) PBS-GA and, B) Tris-GA treatment. Bar=20µm.

3.3.14. Experiment 3.3. Effect of the imaging filter on background fluorescence

3.3.14.1. Introduction

After excitation at the correct wavelength the specimens start to fluoresce and the images were obtained by blocking unwanted wavelengths with barrier filters. The Lieca TSC confocal microscope at Massey University has the facility to use two different types of barrier filters: 530 - 590 nm and 585-615 nm. The peak auto-fluorescence of plant cells is below 585 nm so using a 585-615 nm filter can considerably reduce background fluorescence.

3.3.14.2. Materials and Methods

The imaging filter 585-615 nm was used with excitation wavelengths of 488 and 568 nm. The usual filter (530-590 nm) was also used to compare images. Both PBS and Tris buffer GA treated specimens were examined under these filters.

3.3.14.3. Results and discussion

The alternate barrier filter (585-615 nm wave length) used with exciter filters with 488 and 568 nm wavelengths reduced background fluorescence of plant tissue compared with barrier filters of 530 and 590 nm wavelengths (Plate 3.5). However, while this reduction

was not great, it could assist in fungal biomass measurement by helping to avoid adjoining plant cell auto-fluorescence. In some specimens, the alternate barrier filter reduced intensity of fungal fluorescence considerably particularly in the specimens treated with Tris-GA solution. Reduction of background fluorescence when using a barrier filter with wavelength of 585-615 nm indicated that background plant tissue mostly autofluoresced at wavelengths below 585 nm, and that fungal hyphae fluoresce mostly at wavelength above 585 nm.

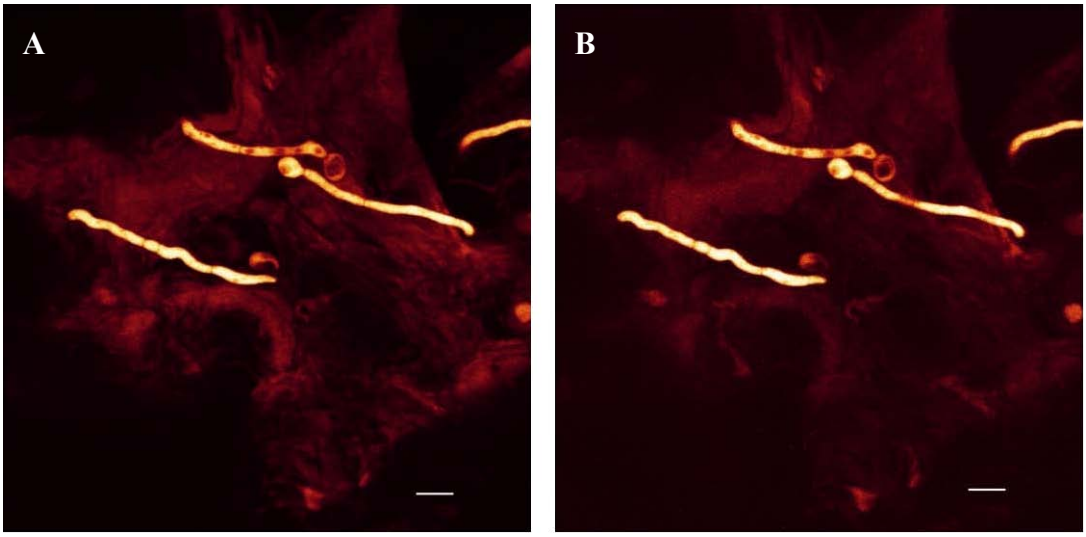


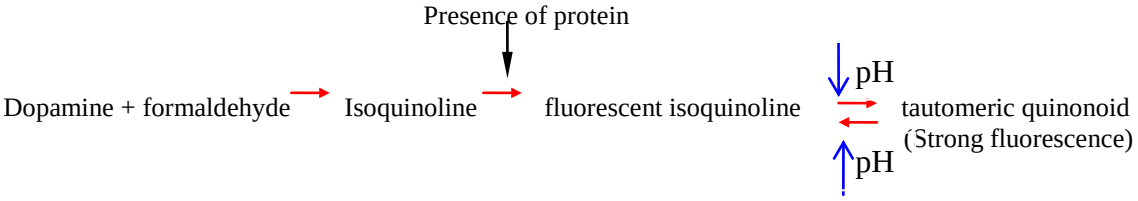
Plate 3.5. Confocal micrograph of *B. cinerea* hyphae in lettuce leaf tissue showing the effect of different imaging filters with wavelength A) 530-590 nm B) 585-615 nm after glutaraldehyde treatment. Bar=20µm.

3.3.15. Experiment 3.4. Effect of pH on background fluorescence

3.3.15.1. Introduction

The principle of this experiment was based on the reversible reaction of fluorescent isoquinoline at different pH. This substance is usually produced when aldehydes react with some biogenic amines, in both plant and animal cells, for example, dopamine and proteins. The level of fluorescence is increased by decreasing the pH level so the isoquinoline changes into quinoid, a strongly fluorescencing substance. The reaction can be reversed by increasing the pH level (Rost 1995).

Example of the fluorescence reaction with amines:



The objective of this experiment was to find the effect of pH on background fluorescence in plant tissue and was based on the assumption of the above reaction.

3.3.15.2. Materials and Methods

3.3.15.2.1. Preparation of GA and washing solutions with different pH ranges

Glutaraldehyde solutions: PBS-GA 16, Tris-GA 16 and washing solutions: PBS-Sucrose 6.5, Tris-Sucrose 6.5 were prepared at a range of pH from 4.0 to 10.0 at 0.5 intervals. Other than the pH adjustment made initially in the buffers using 0.1N to 1N HCl or NaOH, all the procedures were the same as described in the general procedures for preparation of final solutions.

3.3.15.2.2. Treatments

Final glutaraldehyde solutions were prepared with their respective buffers and pH levels as described above.

PBS buffer GA solution

1. PBS-GA 16 (4.0)
2. PBS-GA 16 (4.5)
3. PBS-GA 16 (5.0)
4. PBS-GA 16 (5.5)
5. PBS-GA 16 (6.0)
6. PBS-GA 16 (6.5)
7. PBS-GA 16 (7.0)
8. PBS-GA 16 (7.5)
9. PBS-GA 16 (8.0)
10. PBS-GA 16 (8.5)
11. PBS-GA 16 (9.0)
12. PBS-GA 16 (9.5)
13. PBS-GA 16 (10.0)

Tris buffer GA solution

1 Tris -GA 16 (4.0)
2 Tris -GA 16 (4.5)
3 Tris -GA 16 (5.0)
4 Tris -GA 16 (5.5)
5 Tris -GA 16 (6.0)
6 Tris -GA 16 (6.5)
7 Tris -GA 16 (7.0)
8 Tris -GA 16 (7.5)
9 Tris -GA 16 (8.0)
10 Tris -GA 16 (8.5)
11 Tris -GA 16 (9.0)
12 Tris -GA 16 (9.5)
13 Tris-GA 16 (10.0)

After GA treatment all specimens were treated with chloral hydrate for tissue clearing and to remove air bubbles. The specimens were mounted on a slide with Citifluor mounting medium as in other experiments and examined under a fluorescence microscope. There were at least 4 pieces (each 25 mm^2) of *B. cinerea* inoculated lettuce leaf tissue per treatment.

Fluorescence intensity of fungal hyphae or background was compared among the images by scaling them visually according to their brightness. The criteria for categorisation are low, medium or high fluorescence as illustrated in Plate 3.6.

3.3.15.3. Results and discussion

The two different buffer based GA solutions did not give identical results on fluorescence of plant tissue and fungal hyphae. The background plant tissue fluorescence was bright at pH 6.5 and medium bright at pH 7.0 and above after treatment with PBS-GA 16 while it was medium bright over the entire pH range tested after treatment with Tri-GA 16. On the other hand, hyphae fluoresced brightly over the entire pH range after treatment with PBS-GA 16 whereas they only fluoresced brightly at pH 6.0 and lower after treatment with Tris-GA 16 (Table 3.1).

These results can be explained by the theory of Rost (1995) that the aldehyde reacting with the amine groups produces fluorescent isoquinoline that changed into strongly fluorescent quinoid substances at low pH through reversible reaction.

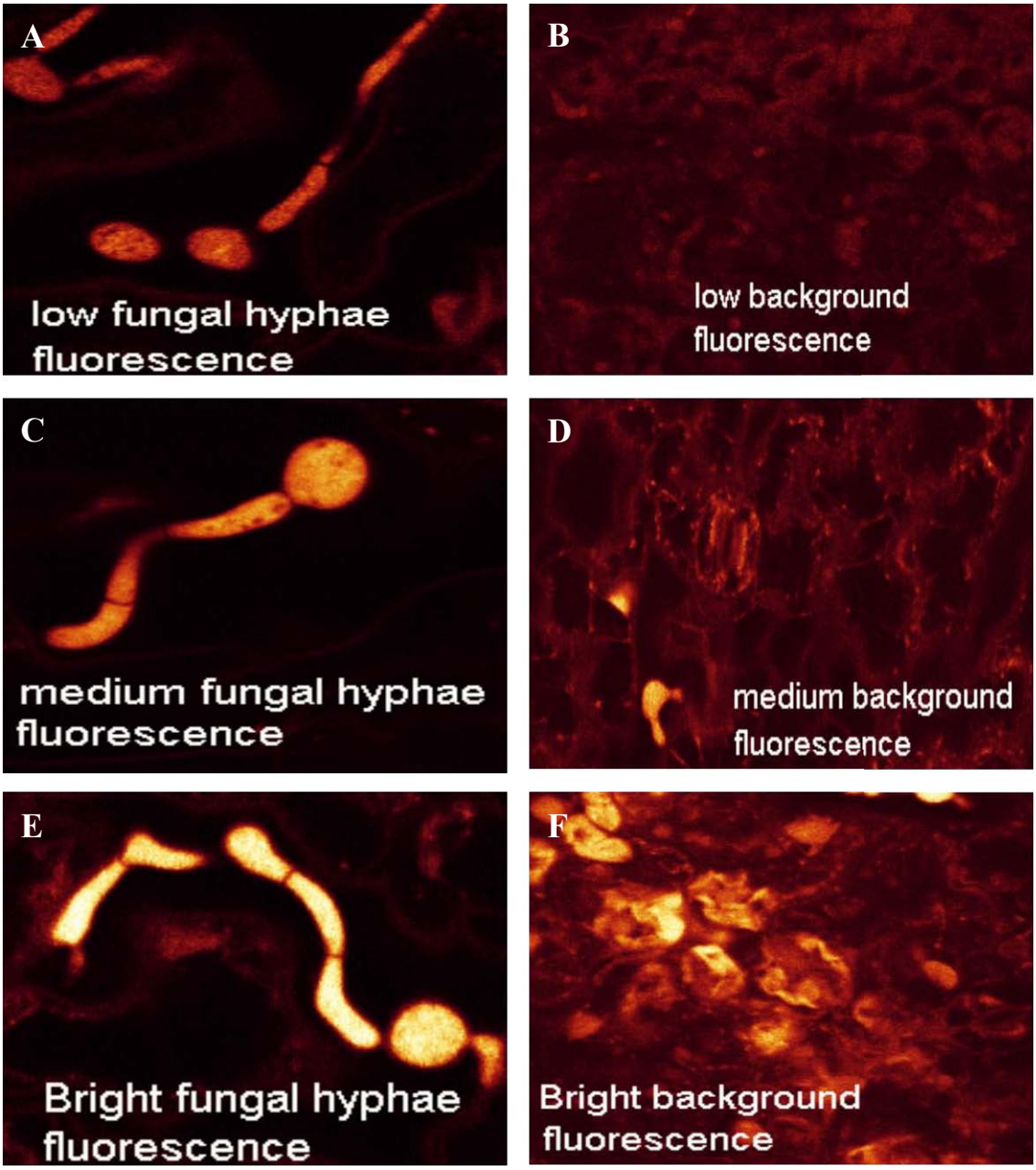


Plate 3.6. Confocal micrograph for fluorescent intensity rating by visual observation. Low (A & B), medium (C & D) and bright (E & F) fluorescence intensity of fungal hyphae (A, C & E) and background plant tissue (B, D & F).

Table 3.1. Effect of pH on glutaraldehyde induced background (plant tissue) and fungal hyphae fluorescence. *R1*, *R2* and *R3* are replicates. Specimens brightness indicated by '+' sign under respected fluorescence brightness categories. B = Bright; M = Medium brightness; L = Low brightness.

GA solution	PH	Background fluorescent									Hyphal fluorescent								
		B			M			L			B			M			L		
		R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
PBS-A16	4.0	+	+	+							+	+	+						
PBS-A16	4.5	+	+	+							+	+	+						
PBS-A16	5.0	+	+	+							+	+	+						
PBS-A16	5.5	+	+	+							+	+	+						
PBS-A16	6.0	+	+	+							+	+	+						
PBS-A16	6.5	+		+		+					+	+	+						
PBS-A16	7.0		+		+		+				+	+				+			
PBS-A16	7.5		+		+		+				+	+				+			
PBS-A16	8.0				+	+	+				+	+	+						
PBS-A16	8.5				+	+				+		+	+	+					
PBS-A16	9.0				+	+	+				+	+	+						
PBS-A16	9.5				+	+	+				+	+	+						
PBS-A16	10.0				+	+	+				+	+	+						
Tris-GA 16	4.0			+	+	+					+	+	+						
Tris-GA 16	4.5				+	+	+				+	+				+			
Tris-GA 16	5.0				+	+	+				+	+	+						
Tris-GA 16	5.5	+				+	+				+	+	+						
Tris-GA 16	6.0				+	+	+				+	+	+						
Tris-GA 16	6.5				+	+	+							+		+		+	
Tris-GA 16	7.0				+	+				+				+	+	+			
Tris-GA 16	7.5					+	+	+						+		+		+	
Tris-GA 16	8.0				+	+	+							+	+	+			
Tris-GA 16	8.5				+	+	+							+	+	+			
Tris-GA 16	9.0				+	+	+							+	+	+			
Tris-GA 16	10.0				+	+	+							+	+	+			

3.3.16. Experiment 3.5. Effect of glutaraldehyde concentration and duration of incubation on fungal hyphae and background fluorescence

3.3.16.1. Introduction

The incubation period and concentration of GA in the buffer may affect the intensity of hyphal and background fluorescence. In the preliminary experiments, autofluorescence was not observed from *B. cinerea* hyphae but plant tissue fluoresced when viewed through the confocal microscope using a 530 – 590 nm exciter wavelength filter. When specimens were treated with glutaraldehyde solutions fungal hyphae fluoresce brightly but the background fluorescence was also enhanced. Glutaraldehyde concentration and incubation

period may influence fluorescence and therefore, the effect of these two parameters needed to be studied.

The objectives of this experiment were to optimise the incubation period and the concentration of GA in the buffer solution to obtain maximum contrast of the image.

3.3.16.2. Materials and methods

3.3.16.2.1. Preparation of GA solution in different concentration

Glutaraldehyde solutions in PBS and Tris buffers were prepared with 1, 2, 4, 8 and 16% of commercially rated 25% glutaraldehyde solution as previously stated. (Actual GA concentration of 1,2,4,8 and 16% are 0.25, 0.5, 1, 2 and 4% respectively)

1. PBS-GA 0 = 0% GA in PBS buffer
2. PBS-GA 1 = 1% GA in PBS buffer
3. PBS-GA 2 = 2% GA in PBS buffer
4. PBS-GA 4 = 4% GA in PBS buffer
5. PBS-GA 8 = 8% GA in PBS buffer
6. PBS-GA 16 = 16% GA in PBS buffer
7. Tris- GA 0 = 0% GA in Tris buffer
8. Tris-GA 1 = 1% GA in Tris buffer
9. Tris-GA 2 = 2% GA in Tris buffer
10. Tris-GA 4 = 4% GA in Tris buffer
11. Tris-GA 8 = 8% GA in Tris buffer
12. Tris-GA 16 = 16% GA in Tris buffer

There were at least 4 pieces of *B. cinerea* inoculated lettuce leaf tissue per GA solution (main treatment).

3.3.16.2.2. Incubation period

The specimens treated with above GA solution were incubated for 1, 2, 4, 6, 8 h and over night (~16 h) as sub-treatments.

3.3.16.2.3. Measurement of fluorescence intensity

All specimens were cleansed with chloral hydrate after treatment and as in the previous experiments were mounted on a slide with Citifluor.

Specimens were examined under the confocal microscope and single view digital images were stored on a diskette. Five places, each consisting of 14 pixels, were randomly marked

from each digital image of fungal hyphae and from the adjoining plant tissue and the grey value of each pixel marked from the image was measured numerologically with a value from 0 to 255 using the "Image analysis-Densitometry" program. The average grey value of the 14 pixels was then calculated as a measure of fluorescence intensity.

3.3.16.3. Results and Discussion

The average fluorescent intensity did not exceed 50 in pixel greyscale of background (plant tissue) in PBS-GA or Tris-GA treated specimens (Fig. 3.1 & 3.3) but was more than 150 in pixel greyscale of fungal hyphae at any incubation period for each concentration of PBS-GA solution studied (Fig. 3.2). The average fluorescent intensity of fungal hyphae in Tris-GA treated specimens was less than that of PBS-GA treated specimens (Fig. 3.3). However, GA concentration and incubation period significantly affected the fluorescence intensity of fungal hyphae as well as of background plant tissue. A polynomial pattern was observed on fluorescent intensity over GA concentration and incubation period in both PBS-GA and Tris-GA treated specimens (Fig. 3.1 & 3.3).

In PBS-GA treated specimens, the average fluorescent intensity significantly increased at 1% and 8% compared with 2% and 4% GA concentration (Fig. 3.1 A). Four hours, 6 h and over night incubation also significantly increased average fluorescent intensity compared with the other incubation periods (Fig. 3.1 B). At 8% GA concentration, the intensity was highest at 4 h and 6 h incubation period (Fig. 3.2). Confocal micrograph of the fungal hyphae treated with 8% GA in PBS buffer and incubated at 4 h (Plate 3.7 A) and 6 h (Plate 3.7 B) showed brighter hyphae compared with the hyphae treated with 2% GA for 4 h (Plate 3.7 C) and with 4% GA for 2 h incubation (Plate 3.7 D). The same trend also was observed for background fluorescence of the same specimens (Plate 3.7).

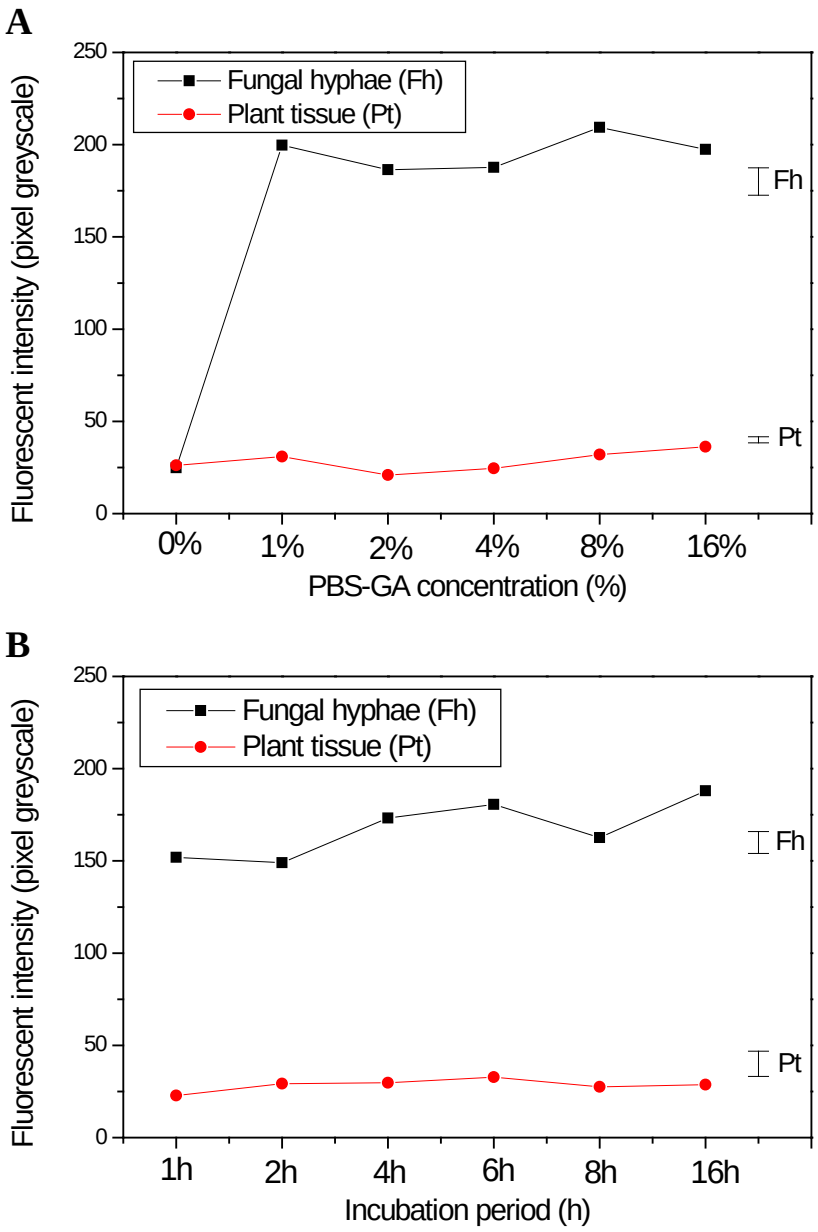


Figure 3.1. Average fluorescent intensity of fungal hyphae and background plant tissue measured by measuring grey value of pixels of the confocal micrograph of the images. The specimens were treated with a range of concentration of glutaraldehyde in PBS buffer and incubated at different incubation periods. A) Effect of PBS-GA concentration on fluorescence. B) Effect of incubation period on fluorescence. Vertical bars represent LSD.

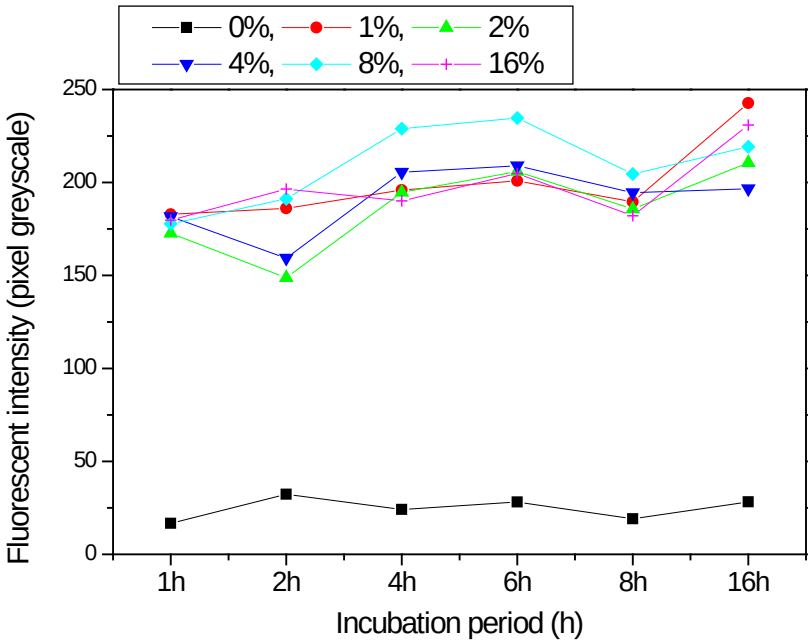


Figure 3.2. Fluorescent intensity of fungal hyphae. The interaction between incubation period and PBS-GA concentrations.

In Tris-GA treated specimens, the average fluorescence intensity of hyphae significantly increased when GA concentration increased up to 8% concentration (Fig. 3.3 A). Incubation period did not affect fluorescent intensity of hyphae significantly after 1h (Fig. 3.3 B). The average fluorescent intensity of background plant tissue was significantly greater after 4 h and 6 h incubation than at other times. Both PBS-GA and Tris-GA treated specimens showed the highest intensity of hyphal fluorescence with 8% GA at 4 h and 6 h incubation periods (Fig. 3.2 and 3.4). Only Tris buffer or 1% GA in Tris buffer did not induce fluorescence of fungal hyphae for up to 6 h incubation but induced fluorescence when the incubation period increased to 8 h or overnight (Fig. 3.4). Confocal micrographs of fungal hyphae treated with 8% GA in Tris buffer and incubated at 4 h (Plate 3. 8A) and 6 h (Plate 3. 8B) showed brighter hyphae compared with the hyphae treated with 2% GA for 4 h (Plate 3. 8C) and with 4% GA for 2 h incubation (Plate 3. 8D). The background fluorescence was relatively low in these specimens (Plate 3.8).

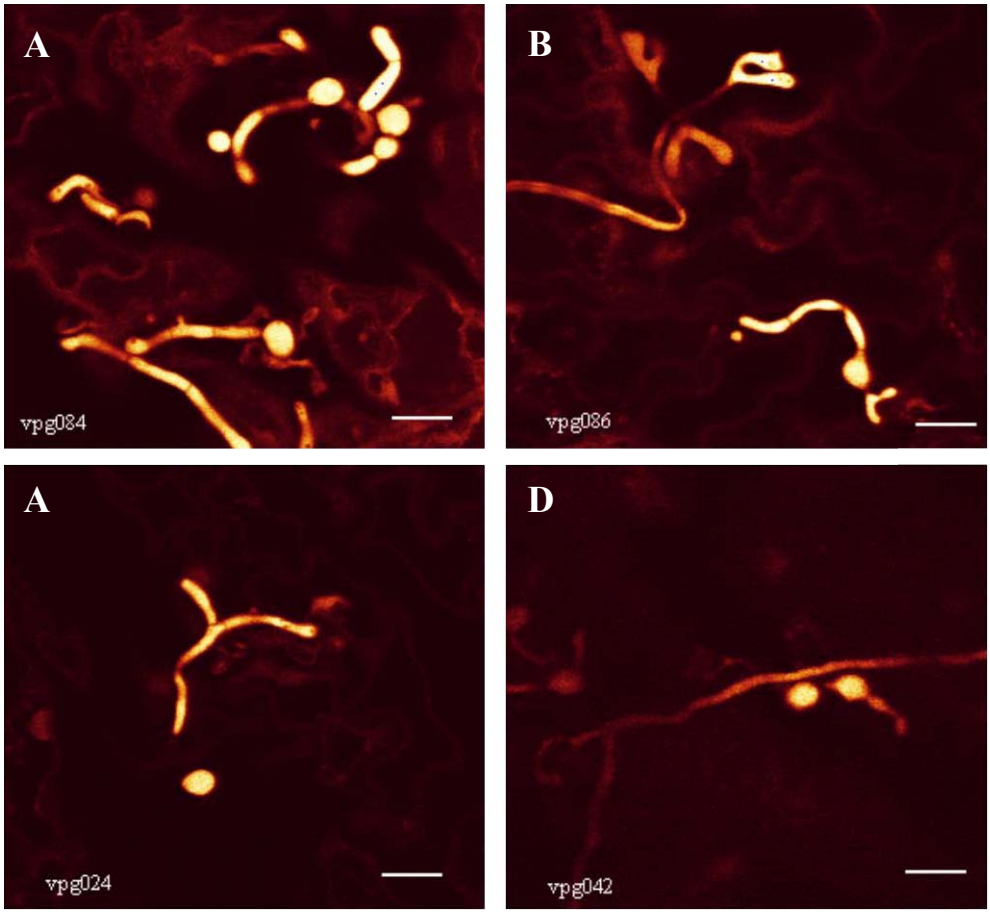


Plate 3.7. Confocal micrographs of single line scanning to measure fluorescence intensity of fungal hyphae and background. The specimen treated with 8% glutaraldehyde in PBS buffer and incubated for (A) 4 h and (B) 6 h, and the specimens treated with (C) 2% glutaraldehyde in PBS buffer and incubated for 4 h and with (D) 4% glutaraldehyde in PBS buffer for 2 h. (bar = 20µm)

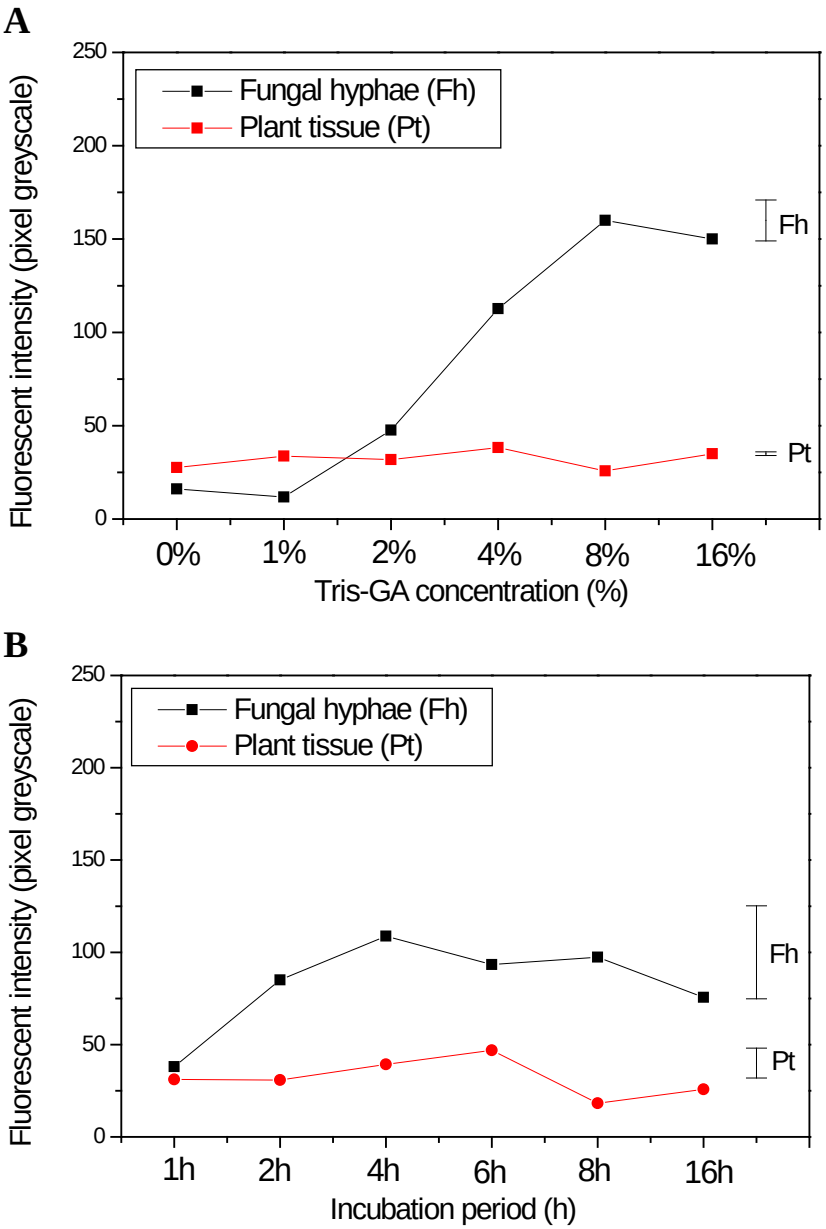


Figure 3.3. Average fluorescent intensity of fungal hyphae and background plant tissue measured by measuring grey value of pixels of the confocal micrograph of the images. The specimens were treated with a range of concentration of glutaraldehyde in Tris buffer and incubated at different incubation periods. A) Effect of Tris-GA concentration on fluorescence. B) Effect of incubation period on fluorescence. Vertical bars represent LSD.

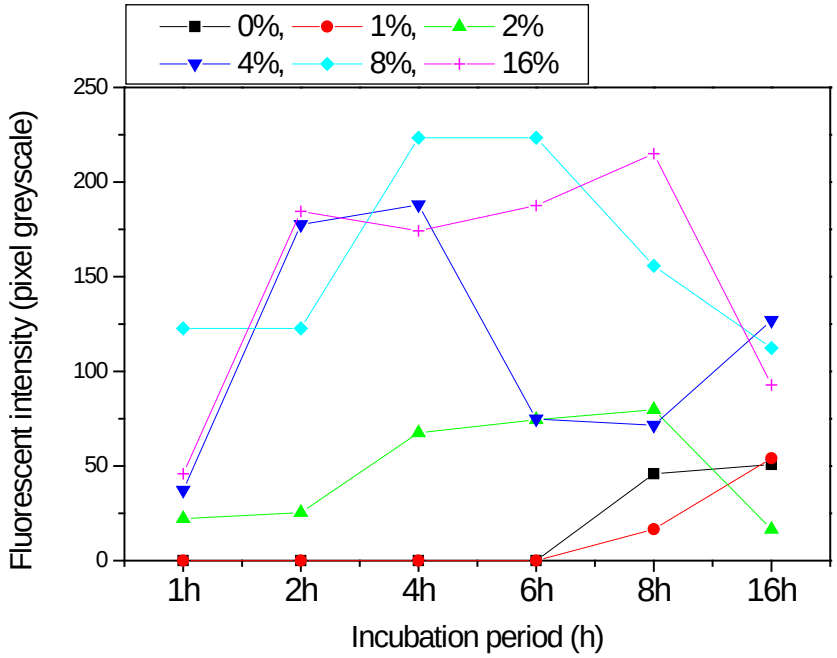


Figure 3.4. Fluorescent intensity of fungal hyphae. The interaction between incubation period and Tris-GA concentrations.

Glutaraldehyde usually reacts with cell proteins and induces autofluorescence (Singh et al. 1997). The aldehyde reaction with protein to induce autofluorescence in the cell is a reversible reaction that depends on pH level and the protein that reacts with aldehyde (Rost, 1995). However, the effect of aldehyde concentration and the duration for incubation on reaction with cell proteins to produce autofluorescence has not been reported. In this experiment, it has been shown that GA concentration and the incubation duration with tissue significantly affect the fluorescence intensity.

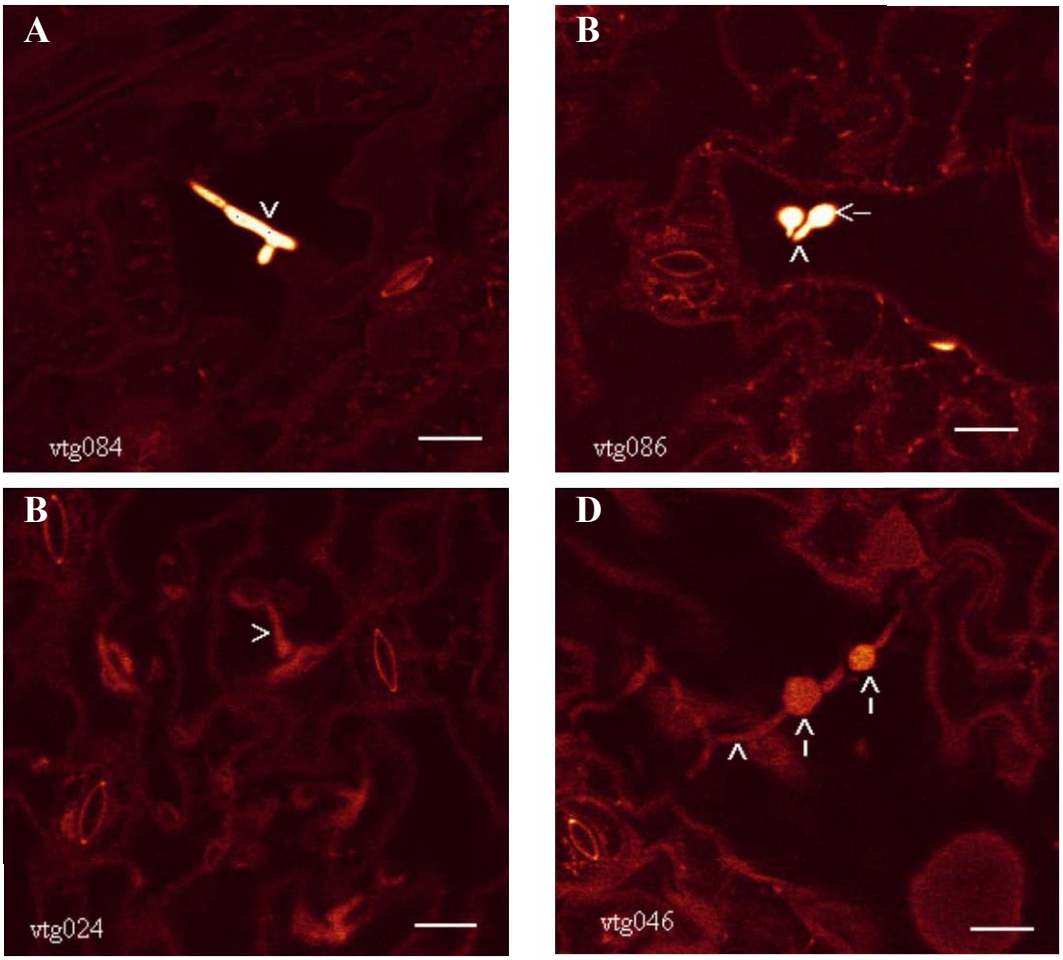


Plate 3.8. Confocal micrographs of single line scanning to measure fluorescence intensity of fungal hyphae and background. Specimens treated with 8% glutaraldehyde in Tris buffer and incubated for (A) 4 h and (B) 6 h, and specimens treated with (C) 2% glutaraldehyde in Tris buffer and incubated for 4 h and with (D) 4% glutaraldehyde in Tris buffer for 6 h. [arrow head (>) = fungal hyphae; arrow (–>) = fungal spore; Bar = 20µm].

Reduction of fluorescence intensity after incubation in 8% GA concentration in both buffers can be explained by saturation that could diminish fluorescence intensity at high concentrations. Dickson and Kolesik (1999) observed bright fluorescence of mycorrhizal fungal hyphae when they were lightly treated with acid fuchsin but the fluorescence was quenched to a sub optimal level when the staining period was lengthened or the staining was repeated. From this current experiment, 8% GA in either buffer, and a 4-6 h incubation period can be recommended as the optimum GA concentration and incubation period to obtain maximum fluorescence of fungal hyphae.

In some cases, fluorescence intensity of fungal hyphae and background plant tissue overlapped (Fig. 3.5A). When incubation time increased, the hyphae and background fluorescence also increased but did not overlap each other (Fig. 3.5B). The differential gap of fluorescence intensity between hyphae and background can facilitate the accurate measurement of hyphal volume.

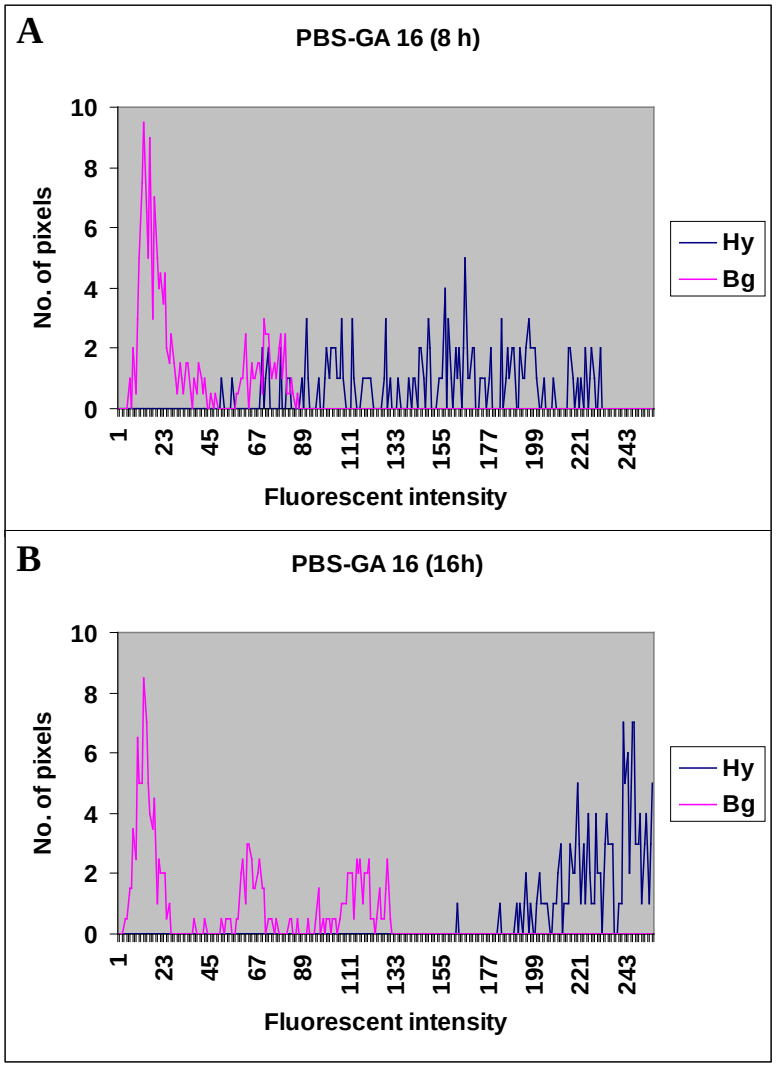


Figure 3.5. Fluorescent intensity measured by pixel greyscale of the image from glutaraldehyde treated specimens. A) Hyphal and background fluorescence overlapping at certain fluorescent intensity level during 8 h incubation. B) Hyphal fluorescence increased and separated from background fluorescence during overnight (16 h) incubation. (Hy = Hyphae; Bg = Background plant tissue).

3.4. Discussion

Confocal Laser Scanning Microscopy provides the facility to produce volumetric images of fungal hyphae that grow within plant tissue. A fluorescent stain specific to *B. cinerea,* for example the BC-KH4 monoclonal antibody in conjunction with fluorescent tagged antimouse antibody, did not fluoresce brightly to produce successful 3D images, because the stain was unable to penetrate deep in to the plant tissue and stain the fungal hyphae (Vingnanasingam 1998). Aldehydes are known to be reagents for induced fluorescence, by reacting with neurotransmitter amines, of animal tissue (Rost, 1995). Glutaraldehyde is one of the aldehyde groups, which reacts with the above amines group or related substances and induces fluorescence. As such, glutaraldehyde has already been tested on wood fungi (Singh et al. 1997) and on *B. cinerea* in vegetable and ornamental crops (Vingnanasingam 1998). It is usually used as a fixative in tissue sample preparation but a disadvantage of its use in general is the induced autofluorescence of untargeted areas and consequent obscuring of areas stained with fluorochrome (Anonymous 1996). However, in this study, glutaraldehyde was targeted to *B. cinerea* hyphae in lettuce leaf tissue and they fluoresce brighter than the plant tissue. In an observation during infection the dead tissue of infected plant material does not fluoresce, but some components of living cells and cell walls shows considerable fluorescence. The cell walls consist of cellulose, hemicellulose and lignin, none of which is reactive to glutaraldehyde. However, lignin, a phenolic compound can autofluorescence (Carver et al. 1996; Sherwood and Vance 1982). The veins of plant leaf tissue and wounded tissue are mainly lignin and show considerable autofluorescence. The latter may be due to autofluorescence of lignin produced during wound repair (Ride 1975).

Autofluorescence of lignin in the parts of the cell walls, veins or wounded tissues is weak and it does not interfere with hyphal fluorescence but it may interfere with measurement of fungal volume in the tissue. This is because, in some cases, the fluorescence intensity of the adjoining cells are almost as high as fungal fluorescence intensity and are not separated into individual entities by the image analysis program.

These background noises were considerably reduced by investigating the effect of buffer in the GA solution, pH, GA concentration, incubation periods and quenching, together with

use of brightening and clearing agents, and suitable imaging filter in the confocal system. Individual or combinations of these factors facilitated high contrast of the image and increased the contrast between fluorescence intensity of fungal hyphae and that of the background to avoid confusion during measurement of fungal biomass within plant tissue. Glutaraldehyde in Tris buffer considerably reduced the background fluorescence as suggested by other fluorescence microscopic studies (Poot 1996). However, the Tris buffer also diminished fungal hyphal fluorescence but this fluorescence was enhanced when the pH of the solution was acidic (< 6.0). When the GA solution was prepared in PBS buffer, the fungal fluorescence did not change over the pH range but background fluorescence increased when the pH decreased to acidic (< 6.5). Aldehydes usually react with amine groups in the tissue and produce fluorescent isoquinoline that changes into strongly fluorescent quinoid substances when the pH decreases through a reversible reaction (Rost 1995). This reaction may occur in plant cells when GA is in PBS buffer and in fungal cells when GA is in Tris buffer.

Glutaraldehyde concentration and the incubation period considerably affected the fluorescence of fungal hyphae as well as background fluorescence and there was generally a big difference observed between fungal hyphae and background fluorescence when the fluorescent intensity was measured by pixel greyscale. However, in a few cases, the same pixel greyscale reading was obtained from both fungal hyphae and the background of digitised image and these could cause confusion in the measurement of hyphal volume. To avoid this situation, a wider gap of pixel greyscale between hyphae and background should be maintained and this was achieved by increasing glutaraldehyde concentration and incubation period. When glutaraldehyde concentration and incubation period were increased fluorescence intensity of both fungal hyphae and background increased but significantly, fungal fluorescence intensity increased more than that of the background (Fig 3-5).

Glutaraldehyde reacted with fungal cell protein differently in different buffer over the incubation period. Glutaraldehyde PBS buffer increased fluorescent intensity of specimens after overnight incubation while GA in Tris buffer decreased it. Saturation may be the

reason for this decrease in fluorescence intensity during overnight incubation when GA was in Tris buffer. Overall 8% GA concentration and a 4-6 h incubation period was the optimum concentration and incubation period to obtain the maximum fluorescent intensity of fungal hyphae in either buffer.

Chlorophyll is one of the autofluorescent components in plant tissue (Krause and Weis 1991) and like other pigments, it can be dissolved in ethanol. A series of Ethanol concentration treatments did not reduce background fluorescence markedly because fluorescence of chlorophyll survives glutaraldehyde fixation (Wide and Fliermans 1979). When specimens were treated with Ethanol before glutaraldehyde, fungal hyphal fluorescence dramatically reduced. The reason may be that the glutaraldehyde is unable to induce fluorescence of dead fungal hyphae. Treating the specimens with the quenching agents Triton X 100 or 2-methoxyethonol also considerably reduced autofluorescence of other components of plant cells. A clearing agent (concentrated chloral hydrate), used after glutaraldehyde treatment bleached the specimens as well as removing the air bubbles and thus avoided any blurry of fluorescing fungal hyphae in the tissue.

The alternate barrier filter (585-615 nm wave length) reduced background fluorescence by reducing the fluorescent intensity of adjoining plant cells of fungal hyphae. It could usefully assist for the fungal biomass measurement. The reduction of background fluorescence by this alternative barrier filter indicated that the background plant tissue mostly autofluoresced below the wavelength of 585 nm, and fungal hyphae fluoresce mostly above the wavelength of 585 nm.

Techniques for the reduction of background fluorescence can assist biomass measurements of fungal hyphae. Nevertheless, complete elimination of background fluorescent would not facilitate studies of the infection process of fungal pathogens because host plant tissue must also fluoresce to some extent in order to visualise the location of the processes.
Use of glutaraldehyde in confocal microscopy, with or with out the modified techniques described here, has been applied only to *Botrytis cinerea* but should be generally applicable to other pathogens in different hosts. This technique was used in subsequent experiments

on camellia flower blight disease to visualise the infection process of *Ciborinia camellia* and to measure biomass after application of biocontrol agents if needed.

Chapter Four

Germination of *Ciborinia camelliae* ascospores and infection of camellia petals

4.1. Introduction

Ascospores are the only propagules of *C. camelliae* that cause infection of camellia flowers. There are no reports on the physiology of *C. camelliae* or on factors that influence infection and disease development under laboratory or field conditions. There are however, numerous studies on effect of temperature, moisture and water on ascospore germination, germ tube growth and penetration of the other pathogens. Temperature has a significant effect on *M. fructicola* ascospore germination and germ tube growth: as temperature increases from 7^{o}C to 15^{o}C, the germination and germ tube length increases but above 15^{o}C they do not (Hong and Michilides 1998). On the other hand, Abawi and Grogan, (1975) reported that *Whetzelinia sclerotiorum* ascospore germination was not drastically affected by temperature. MacHardy (1996) observed optimum temperature for *V. inaequalis* ascospores germination and germ tube growth was between 10-18^{o}C, and germ tube growth was very limited above 22^{o}C.

Generally more ascospores germinated in free water, than in 100% RH but in the absence of free water. When the RH decreased the germination also decreased. Arauz and Sutton, (1989) observed maximum *Botryosphaeria obtusa* ascospore germination in free water and it declined as relative humidity was reduced from 100 to 92% with no germination observed at 88.5% RH. They also observed that the germ tubes were significantly longer in free water at 20^{o} and 28^{o}C than at the corresponding temperature at 100% RH. *Pseudopeziza medicaginis* ascospores germinated best *in vitro* at or near 100% RH but did not germinate at 93% RH and below (Semeniuk 1984). Gadoury and Pearson, (1990) found that ascospores of *Uncinula necator* frequently burst in water but presence of free water is must for germination.

The precise influence of temperature and relative humidity and the present of free water on the developmental stages of *C. camelliae* have not been determined. Investigation of these factors together with possible interactions among them, on *C. camelliae* ascospore

germination was under taken to gain a better understanding of the life cycle and to determine the criteria necessary for petal infection in nature and for the development of inoculation protocols.

4.2. Objectives

1. To investigate the optimum temperature and relative humidity for ascospore germination, germ tube growth and penetration.
2. To investigate the role of free water on ascospore germination and infection on the petal surface.
3. To find how inoculation methods influence the role of free water on ascospore germination and infection on the petal surface.

4.3. Materials and Methods

4.3.1. Plant materials

Undamaged, healthy petals were collected from *Camellia reticulata* cv. Brian as described in Chapter 2.

4.3.2. Fungal materials

Apothecia and ascospores were collected as described in Chapter 2. Ascospores were applied in wet inoculations as a spore suspension prepared as described in chapter 2. Sterilised distilled water was added to obtain an inoculum concentration of $5x10^4$ spores per ml. Fresh inoculum was directly deposited by gravity from an apothecium as described for method 1 in Chapter 2, Section 2.1.3.

4.3.3. Inoculation and incubation

Spore suspension was applied using either a micro-pipette or atomiser. Fresh inoculation was carried using either a paint-brush or directly deposited by gravity from apothecia as described in Experiment 4.2 below. Petals were randomly sampled by removing about 25 mm^2 tissue from an inoculated site on the petal at six hour intervals for 72 h of incubation.

4.3.4. Light microscopy

Specimens were stained with 0.05% trypan blue in lactophenol and examine under a light microscope. Images were photographed or video recorded as described in Chapter 2, Section 2.6.1. Ascospore germination and germ tube penetration were counted and germ tube length was measured with a 400X magnification microscope field using Nomarski's differential interphase contrast technique. Two random microscope fields were examined from each inoculated site on the petal. Germ tubes were considered to have germinated when they reached 3 µm in length.

4.3.5. Experiment 4.1. Effect of temperature and relative humidity on ascospore germination and penetration

4.3.5.1. Introduction

High relative humidity and temperature are important for optimum spore germination as summarized above. Additional example include *Cercospora kikuchii* conidial spore germination, which was to 100% at 100% RH at a range of temperatures (15, 20, 25, 20 and 35^{o}C) but no germination occurred at a RH of 92% (Schuh 1991). On the other hand, low or high temperature may delay spore germination and increase the incubation period. Sharma and Kapoor (1997) observed that ascospores of *Sclerotinia sclerotiorum* started germinating in 4 h within the range of 5-25^{o}C. (Optimum 20^{o}C) but spore germination increased with time even at a sub-optimum temperature at 12^{o}C. The minimum RH and optimal temperature requirement for *C. camellia* ascospore germination, germ tube elongation or penetration has not been investigated.

To determine the effect of relative humidity (RH) on spore germination and infection, a constant RH must be maintained for the duration of the experiment. Elaborate equipment can be used for this purpose but, concentrated salt solution with excess salt in an air-tight container can provide adequate control of RH in the atmosphere above them (Dhingra and Sinclair 1985) since vapour pressure changes little with temperature changes in the salt solution (Winston and Bates 1960).

The objective of this experiment was to determine the influence of temperature and humidity on the germination and infection of ascospores of *C. camelliae*.

4.3.5.2. Materials and Methods

4.3.5.2.1. Calibration of Relative Humidity

A Data logger (Gemini Data loggers - Tiny tag *Plus*) with built in temperature and relative humidity probes was used to calibrate relative humidity of closed containers of saturated salt solutions. The following salt solutions were calibrated to obtain a constant RH.

1. KCl
2. KNO_3
3. $ZnSO_4.7H_2O$
4. NH_4SO_4
5. $MgSO_4.7H_2O$
6. NaCl
7. $Na_2HPO_4.7H_2O$

Saturated solutions were prepared by dissolving salt to saturation in boiling water. when the solution was partially cooled, more salt was added and the mixture of saturated solution and salt was allowed to stand for 2-3 days. After this standing period, about 100 ml of mixture was poured into a plastic container (12 cm x 14 cm on the top and 18 cm x 11 cm in the bottom and 6 cm depth), which was used as a control humidity chamber in subsequent experiments. A stainless steel metal net about 7 cm x 7 cm x 1.5 cm with the corners bent down as legs was placed in the container as a platform. The Data logger was carefully placed on the platform and the container closed with an airtight lid. The data logger was pre-set to record the humidity every five minutes for 6-8 h. Containers were placed in stable temperature cabinets set for 5^oC, 10^oC, 15^oC or 20^oC.

The data logger was removed from the container after 8 h and the data download into the Gemini Logger Manager (GLM) [Gemini Data Loggers (UK) Ltd.] software programme.

The calibration was repeated with the saturated solution until stable readings for RH at assigned temperatures were obtained.

Calibration results and recommendation

The data logger took 30 min to 1 h to give stable and constant temperature and relative humidity reading at the assigned temperatures; 5°C, 10°C, 15°C or 20°C. Fluctuations of 1-2°C in temperature and 2-3% in relative humidity were observed at every temperature tested. Saturated potassium chloride solution gave constant average RH readings in the mid 90s, saturated ammonium sulphate solution in the high 80s and Sodium chloride around 80% at all temperatures tested (Table 4.1). The remaining solutions gave about 100% RH at almost all temperatures.

Table 4-1. Data logger readings of relative humidity from airtight container content saturated salt solution at temperature 5°C, 10°C, 15°C or 20°C .

Chemicals	RH%			
	Temperature			
	5°C	**10°C**	**15°C**	**20°C**
KCl	96.5	94.3	93.8	93.2
KNO3	99.9	>99.9	99.9	98.2
ZnSO4.7H2O	99.9	>99.9	99.3	97.6
NH4SO4	88.4	88.7	87.9	89.5
MgSo4.7H2O	>99.9	>99.9	99.9	96.5
NaCl	82.5	>99.9	80.0	81.6
Na2HPO4.7H2O	99.9	99.9	99.9	99.9

Potassium chloride, ammonium sulphate and sodium chloride solutions were therefore chosen to give a range of relative humidities below 100%.

4.3.5.2.2. Inoculation and incubation

Ascospores were collected in universal bottles as described in Chapter 2 and the inoculum concentration was adjusted to $5x10^4$ spores per ml. The inoculum was applied with a De Vilbis atomiser to camellia petals. In an another set of trial, ascospores were inoculated using fresh spores directly from apothecia, (*Method 2*, described in Chapter 2, Section 2.1.3).

4.3.5.2.3. Incubation and microscopy observation

Wet or fresh spore inoculated petals were placed in containers with saturated salt solutions to maintain 90% or 95% RH as calibrated in Section 4.3.5.2.1 above or with distilled water to maintain 100% RH. They were incubated at 5^{o}C, 10^{o}C, 15^{o}C or 20^{o}C for 48 h.

Eighteen hours after inoculation, four tissue samples (25 mm^2 approx.) were cut from inoculation sites and treated with 0.05% trypan blue in lactophenol. The stained specimens were mounted in 0.1% $FeCl_3$ in 50% glycerol and observed under the light microscope at X 400 magnification to observe spore germination, germ tube growth, appresoria formation and penetration.

4.3.5.3. Results and Discussion

Significant increases over temperature were observed in ascospore germination ($P < 0.0002$) and germ tube penetration ($p = 0.0071$) between 90%, 95% and100% RH, and between 95% and 100% RH respectively (Fig. 4.1A). There were no significant differences between temperatures but an increasing trend was observed from 5^{o}C to 20^{o}C over RH on ascospores germination or germ tube penetration (Fig. 4.1B). Ascospore germination and germ tube penetration occurred in all temperatures / RH combinations except 5^{o}C / 90% RH (Fig. 4.2). These results suggest that a saturated atmosphere is essential for high percentage ascospore germination and germ tube penetration, and temperature should be above 5^{o}C (Fig 4.2).

Penetrated germ tube growth was significantly affected with temperature and RH. The germ tube length was significantly longer at 15^{o}C and 20^{o}C than at 5^{o}C and 10^{o}C when the RH was 100% (Fig. 4.3). These observations suggested that even the spore germination and successful germ tube penetration occurred at low temperature (Fig. 4.2), the growth of penetrated germ tube needs high temperature (Fig. 4.3) to grow further and hence the disease symptoms development can be delayed if the temperature remains low.

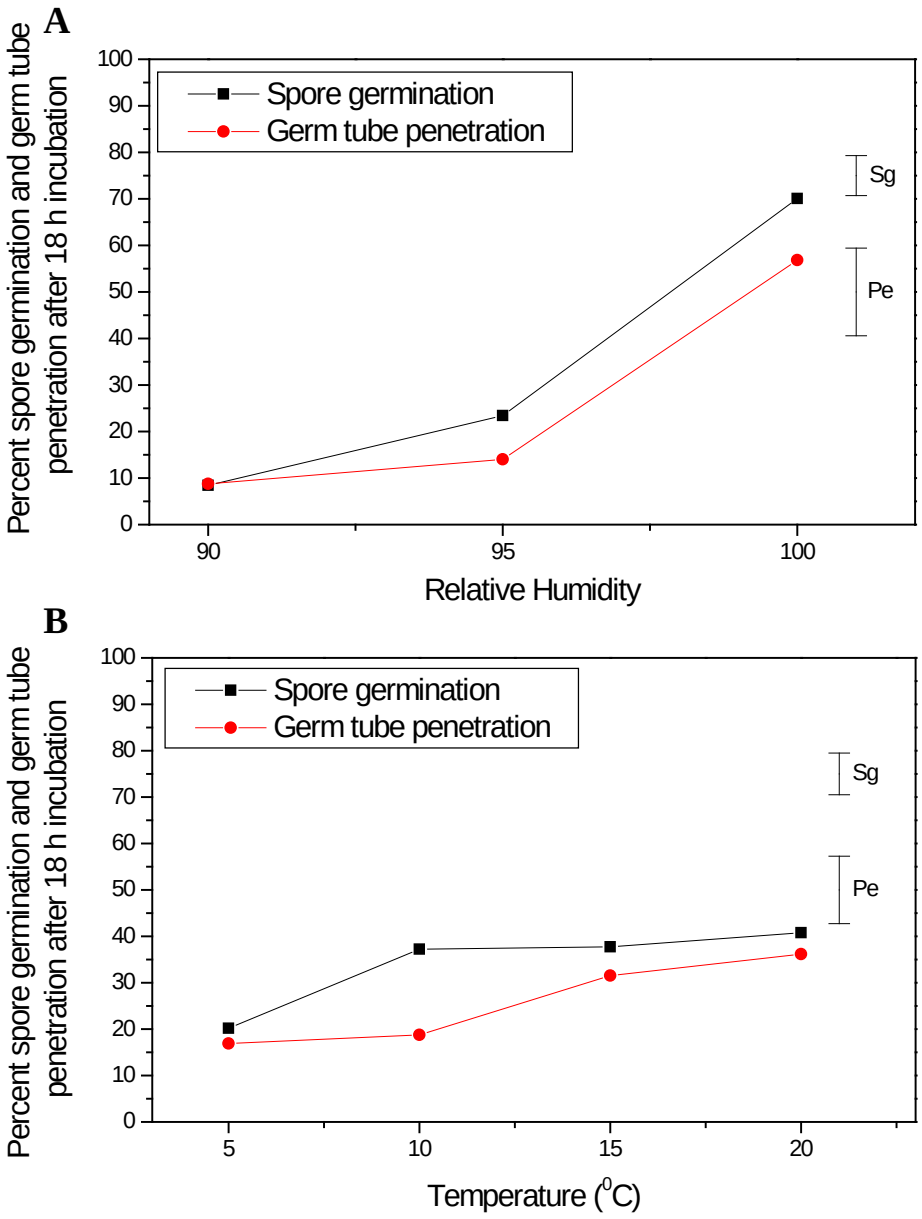


Figure 4.1. Effect of relative humidity (A) and temperature (B) on ascospore germination and germ tube penetration 18 h after inoculation. Vertical bars represent overall LSD of RH and temperature for spore germination (SG) and germ tube penetration (Pe).

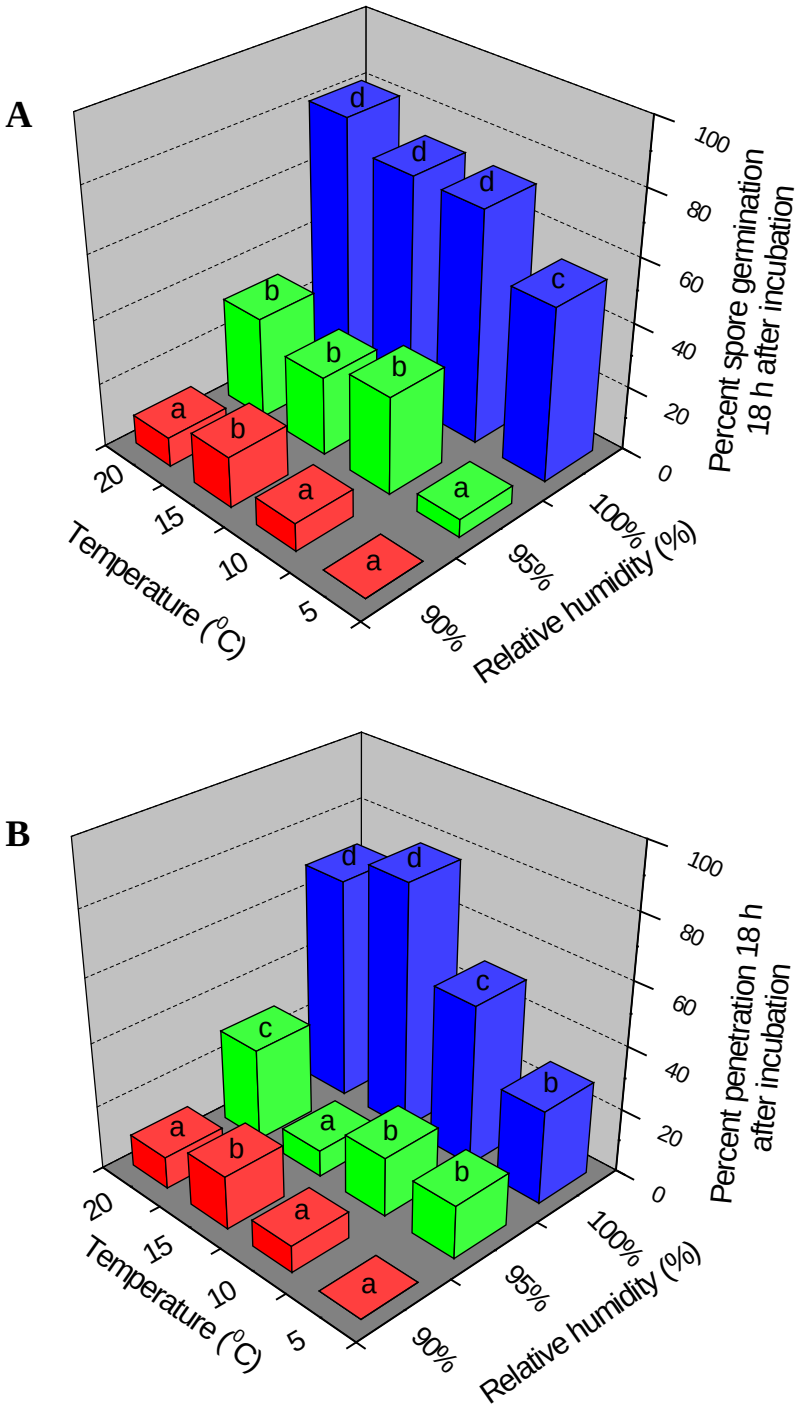


Figure 4.2. Effect of temperature and relative humidity on (A) Ascospore germination, (B) Germ tube penetration after 18 h inoculation. Means with the same letter are not significantly different.

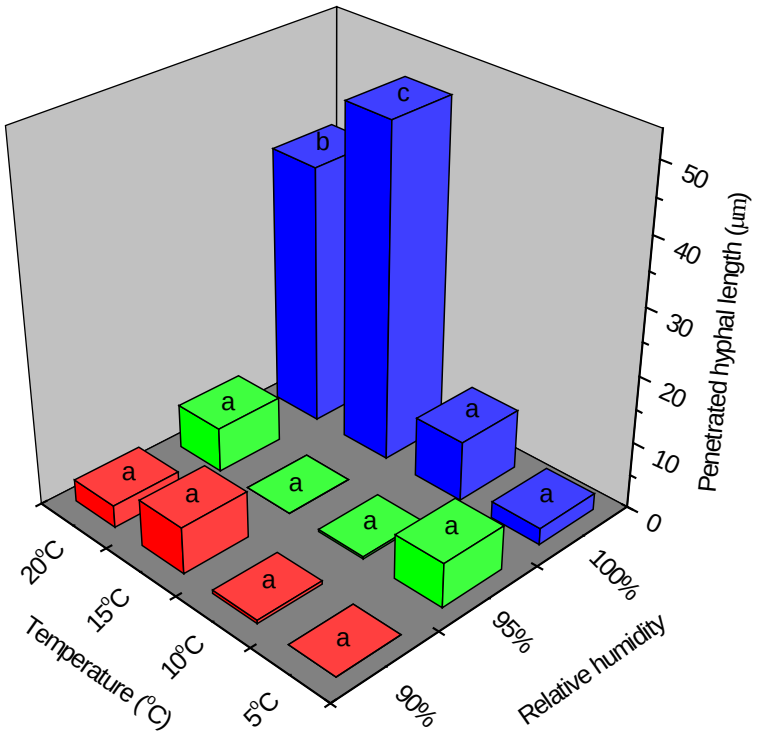


Figure 4.3. Effect of temperature and relative humidity on penetrated germ tube length of *Ciborinia camelliae* after 18 h inocubation. Means with the same letter are not significantly different.

There was some infection of camellia petals after 42 h incubation at 15°C / 100% RH and significantly more at 20°C. There were no infections at 5 or 10°C at any RH level after 42 h incubation but some were found at 15°C in 100% RH and at 20°C in 90% and 95% RH (Fig. 4.4A). By 72 h, infection levels almost reached 100% at 15°C in 90% and 100% RH. However, at 5 and 10°C the only infection were at 10°C in 100% RH (Fig. 4.3B). Infection was observed at 10°C in all three RH when the incubation period was further extended to 90 h (Plate 4.1). These results suggest that low temperature severely restrict both the number of infections and symptom development.

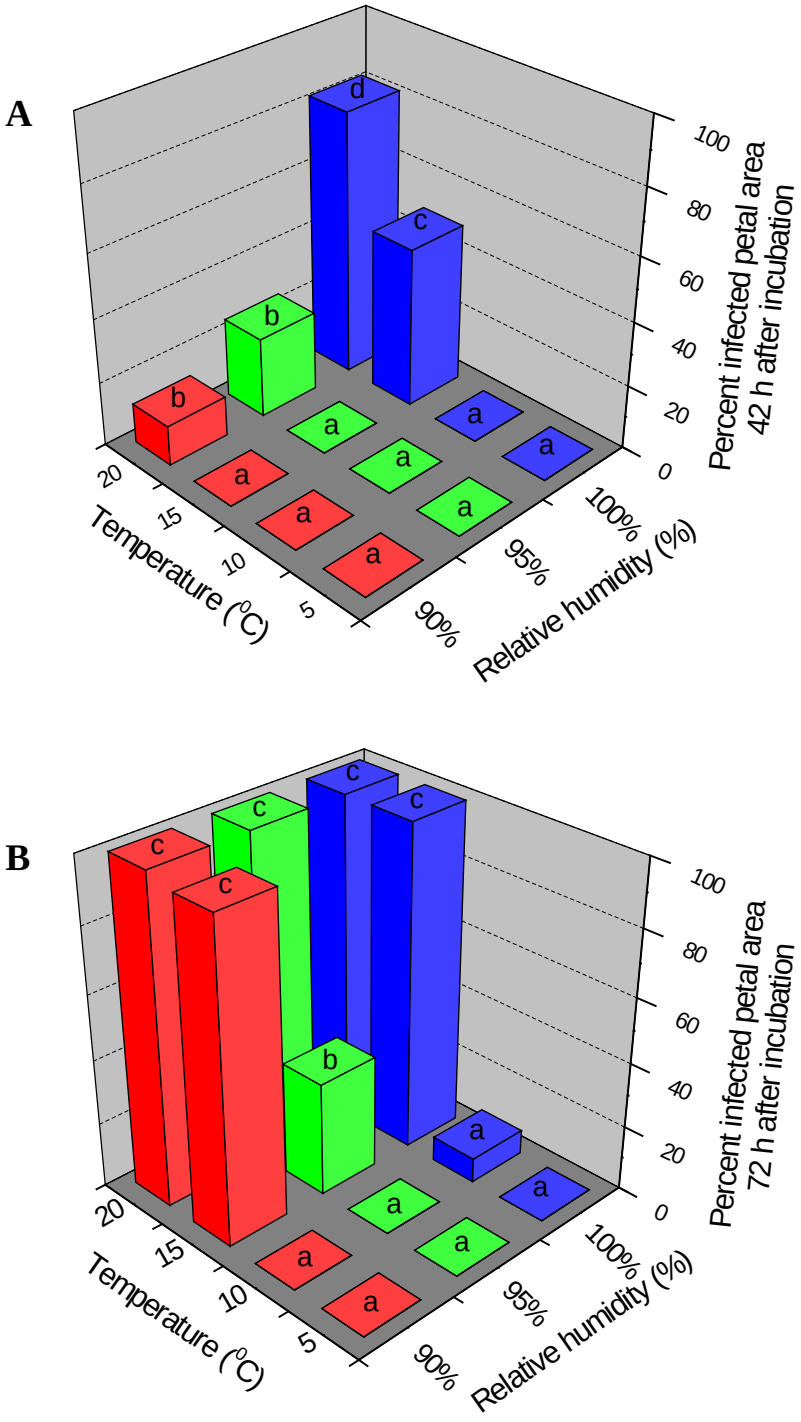


Figure 4.4. Effect of temperature and relative humidity on infection of *Ciborinia camelliae* on camellia petal after 42 h (A) and 72 h (B) inoculation. Means with the same letter are not significantly different.

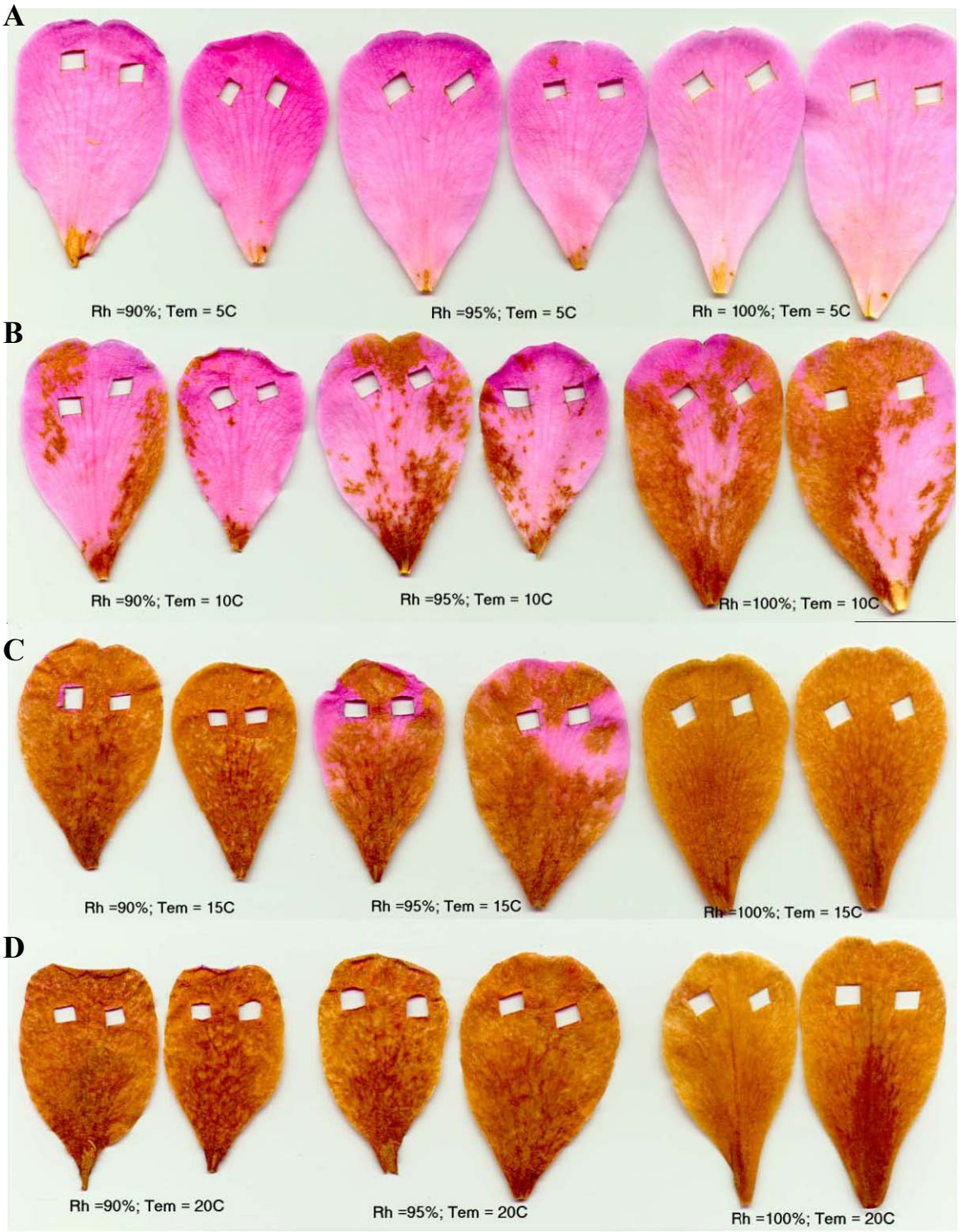


Plate 4.1. Camellia flower petals inoculated with *C. camelliae* and incubated at RH 90%, 95% and 100% in temperatures of 5^0C (A), 10^0C (B), 15^0C (C) and 20^0C (D) for 90 h after inoculation.

4.3.6. Experiment 4.2. Effect of free water and inoculation methods on ascospore germination, penetration and germ tube growth

4.3.6.1. Introduction

Free water maintained continuously at the interface between the inoculum source and the surface of the inoculated tissue is essential for germination of many fungal spores e.g. ascospores of *Venturia inaequalis* on apple (MacHardy 1996) and of *Whetzelinia sclerotiorum* on bean (Abawi & Grogan 1975). Ascospores of *Botryosphaeria obtusa* showed a relatively higher percentage germination and germ tube elongation in free water than without (Arauz & Sutton 1989) as did ascospore of *Mycosphaerella citri* (Whiteside 1974). There are reports of plant infection by dry-inoculated spores under conditions of high humidity. Examples are apple with ascospores of *Venturia inaequalis* (Clayton 1942) and rose petals with conidia of *Botrytis cinerea* (Williamson et al. 1995). The effect of free water on germination of *Ciborinia camelliae* ascospores has not been studied and is important to understand the effect of the presence of free water on spore germination and penetration in order to adopt appropriate inoculation methods in infection studies and screening for resistance to *C. camelliae*.

The objectives of this experiment were to investigate the role of free water on ascospore germination and to assess the success of a range of inoculation methods for achieving high infection rates.

4.3.6.2. Materials and Methods

Apothecia collected from Wellington Botanic Gardens were used as a source of inoculum. Inoculum was generally prepared as a water suspension to facilitate estimation of spore concentration for quantitative studies and ease of application by spraying. Dry spores or fresh spores directly from the source / apothecium was also used as inoculum to get successful infection. Four methods (two ‘wet’ as spores in inoculum suspension, and two ‘fresh’ as spores directly from apothecia) were used to apply ascospores of *C. camelliae* to camellia petals which were then incubated ‘wet’ or ‘dry’.

4.3.6.2.1 Pipette inoculation

Two micro-litre of ascospore suspension ($5x10^4$ spores per ml) were placed at four sites on each of four-flower petal using a micro-pipette (Plate 4.2A). Immediately after inoculation, half the petals were transferred to a humid box and incubated at 20^oC for 36 h. The remaining petals were air dried in a laminar flow cabinet for 1-2 h before placing them in humid boxes and incubating as described above.

4.3.6.2.2. Spray inoculation

A spore suspension at the same concentration as above was sprayed on four camellia flower petals using a fine atomiser (Plate 4.2B). Immediately after spraying, half the petals were placed in a humid box, the other half was air-dried and both sets were incubated as described above.

4.3.6.2.3. Gravity inoculation

Sclerotia with the stipes of their apothecia were wrapped with a wet facial tissue and suspended in an inverted position about 4-5 cm above six petals in a humid plastic container for 2 h (Plate 4.2C). After inoculation, the petals were grouped into three sets. One set was incubated immediately and the other two sets were sprayed with SDW using a fine atomiser. Of those, sprayed with SDW, one was incubated immediately and the other was air dried and incubated as described above.

4.3.6.2.4. Brush inoculation

A paintbrush with fine bristles (15 mm long) was gently touched two or three times onto apothecial hymenia to pickup ascospores. The ascospores on the bristles were then inoculated on to the petal by gently touching the petal surface two to three times (Plate 4.2D). After inoculation, the six petals were grouped into three sets, which were treated as explained in the gravity inoculation method.

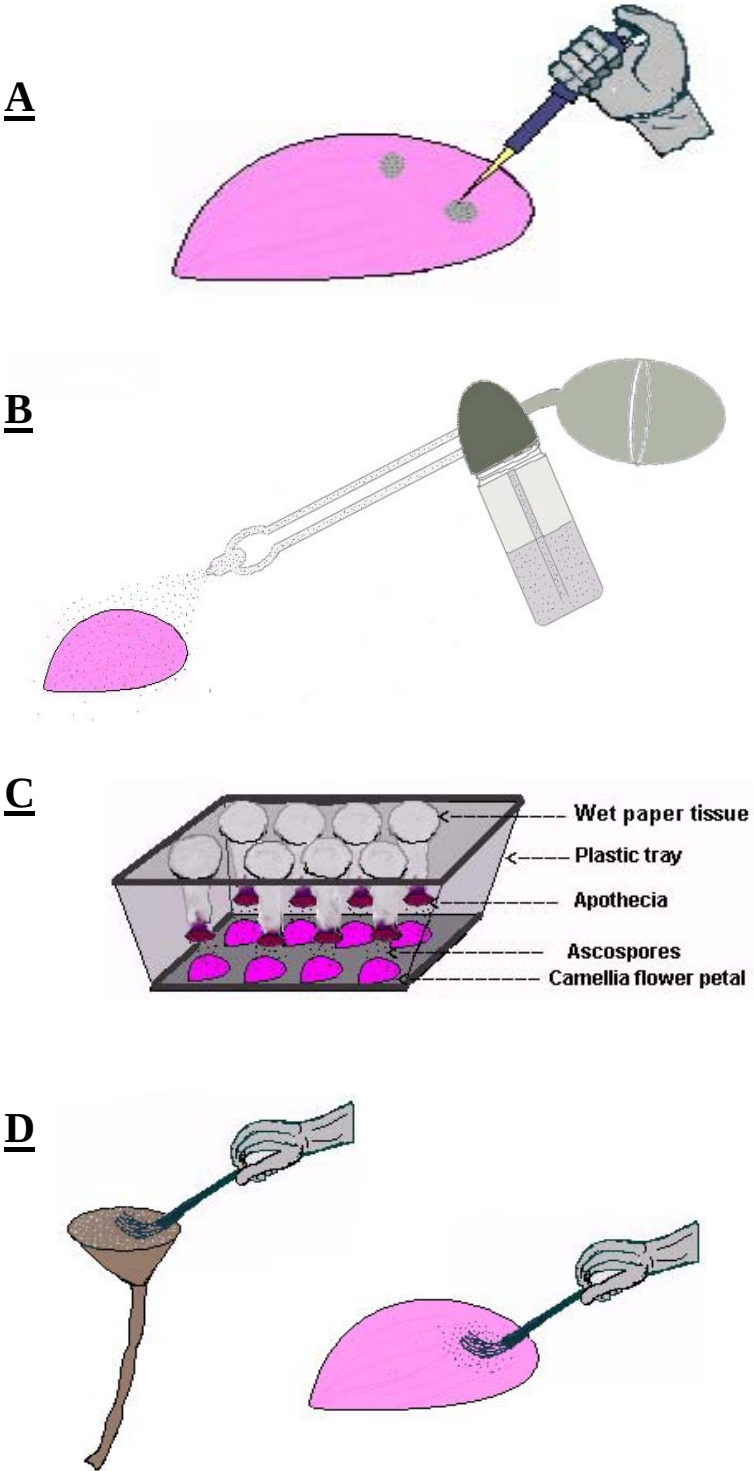


Plate 4.2. Diagrammatic explanation of different inoculation methods. A) Pipette, B) Spray, C) Gravity and D) Brush inoculation.

4.3.6.2.5. Microscopic observation

Pieces of tissues (about 25 mm^2), were cut from inoculated petals (two pieces of tissue from each of the two petals in each inoculation x time treatment) 6 h, 12 h and 24 h after inoculation. They were stained with 0.05% trypan blue in lactophenol on a glass slide over a Bunsen flame for a few seconds until the solution just boiled. The stained specimens were mounted in 0.1% $FeCl_3$ in 50% glycerol and observed under a compound microscope at 400 times magnification. The numbers of germinated and nongerminated spores together with germ tube penetration were counted, and germ tube length was measured. For each treatment, assessments were made from two fields of view at each of two sites on two petals. There were up to 52 spores per site and all hyphae observed were measured.

4.3.6.2.6 Symptoms measurement

All petals were observed for symptom development, which was measured and assessed on a 0 to 4 scale (0 - No lesions; 1 - Slightly visible yellowish brown dots; 2 - Visible yellowish brown pin head size lesions; 3 - 1-2 mm size of brown lesions; 4 - Fully developed > 5 mm size of brown lesions) (Plate 4.3).

4.3.6.2.7. Statistical analysis

The experiment was conducted as a split plot design. Inoculation methods were the main plots and wetness conditions the sub-plots. The experiment was analysed as a generalized linear model using the SAS computer programme.

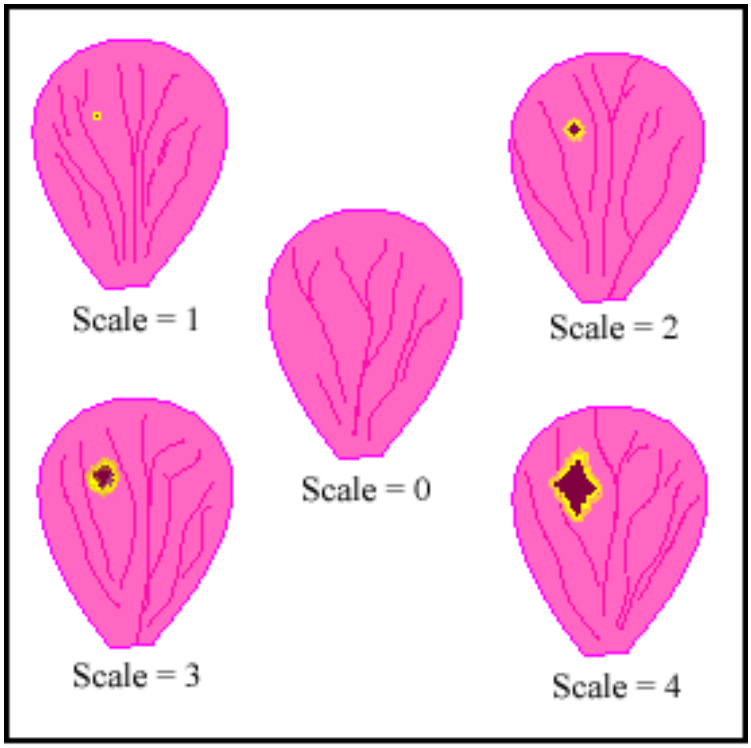


Plate 4.3. A scale (0-4) to measure and assesses the symptom development on camellia petal by *C. camelliae*.

4.3.6.3. Results and Discussion

The effect of water and inoculation method on spore germination and penetration were highly significant ($P<0.001$). Maximum spore germination of *C. camelliae* and germ tube penetration was reached by the 12 h incubation assessment in all treatments except the brush inoculation where germination was low at this time (Fig. 4.5a-h). Both the pipette and gravity inoculations resulted in almost 100% spore germination and penetration but where spore suspensions were allowed to dry before incubation in a humid chamber, then germination and subsequent penetration were dramatically reduced (Fig. 4.5a, b, c and d). When spores were released from an apothecium and allowed to settle on a petal, the addition of water as a fine spray did not affect percentage germination or penetration (Fig. 4.5e and f). If the water was allowed to dry before incubation then germination was reduced at 6 and at 12 h but by 24 h there was no effect on germination. Some spores were allowed to settle on glass slides and microscopic examination showed a small amount of liquid associated with each spore. The associated liquid had disappeared from some spores after an hour but was present on others for longer (Plate 4.4).

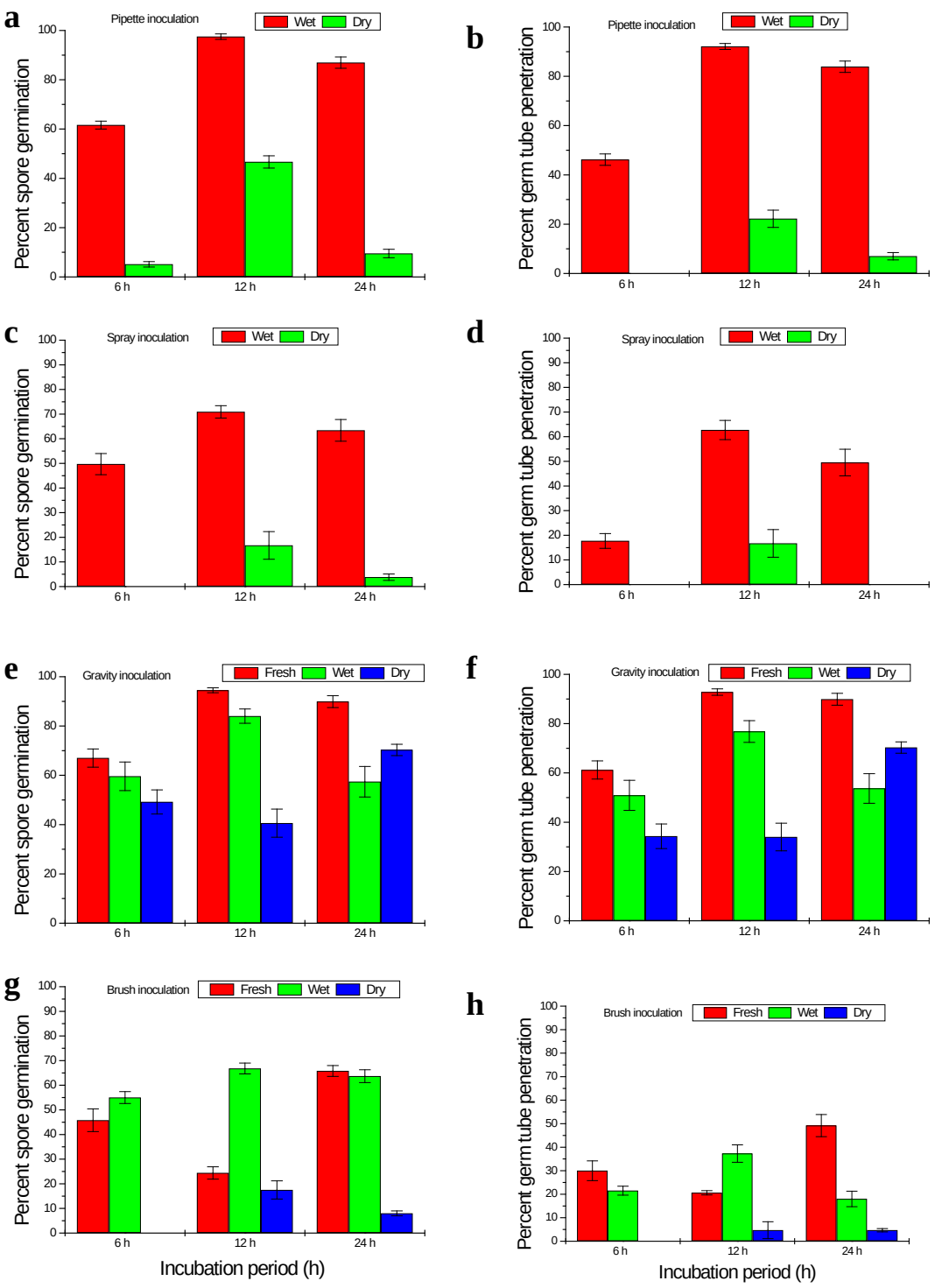


Figure 4.5. Percent germination and penetration of *C. camelliae* ascospore 6 h 12 h and 24 h after incubation. (a) germination and (b) penetration from pipette inoculation, (c) germination and (d) penetration from spray inoculation, (e) germination and (f) penetration from gravity inoculation, and (g) germination and (h) penetration from brush inoculation. Vertical bars represent Standard Error (n=12).

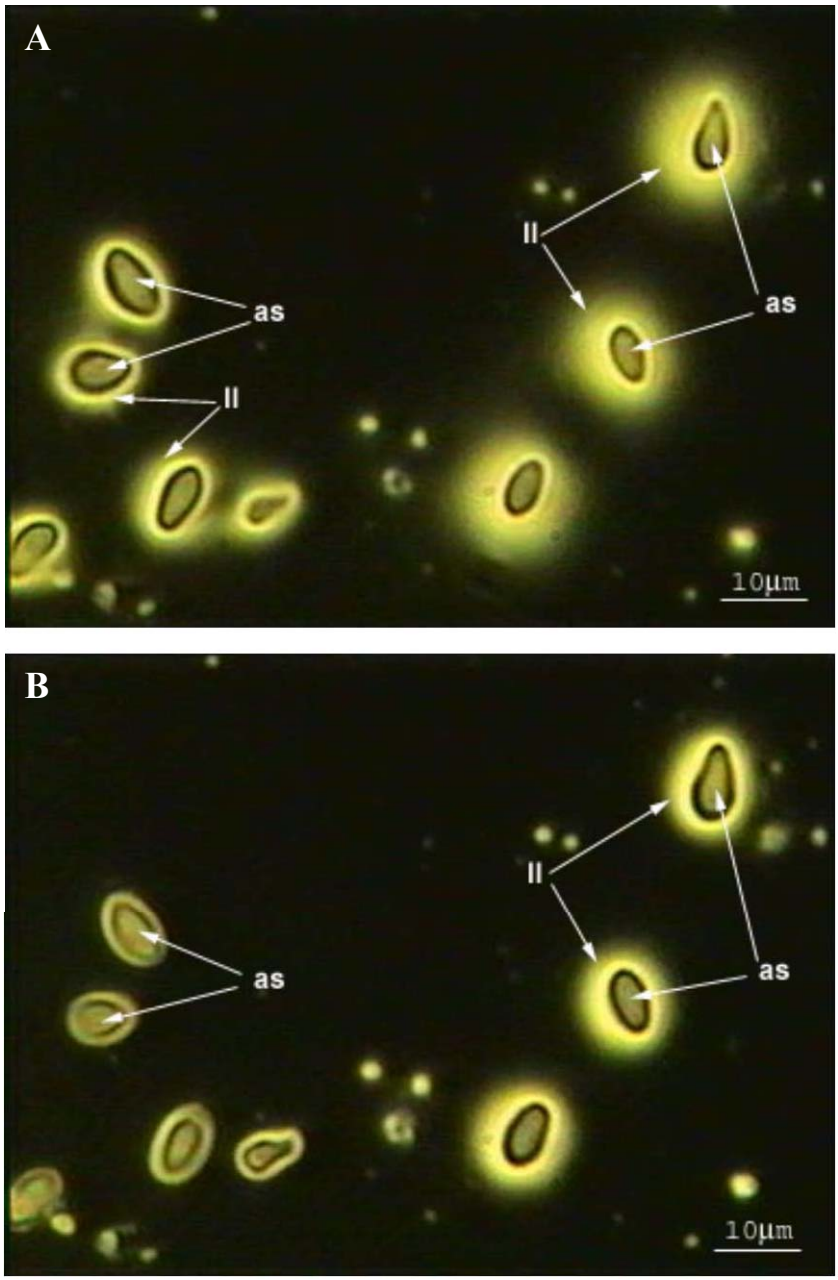


Plate 4.4. Spores deposited by gravitaty on glass slides and observed under dark field illumination. A) A small amount of liquid is associated with each spore, B) The liquid disappeared from some spores after a few minutes. (as = ascospores; ll = liquid layer)

A subsequent spray of water enhanced germination of spores transferred from an apothecium to petals with a brush before incubation but if it was allowed to dry before incubation then there was very little germination or penetration (Fig. 4.5g and h).

Typically, ascospores of *C. camelliae* germinated on the petal surface and the germ tubes penetrated the cuticle almost immediately (Plate 4.5a). In two treatments (pipetted spore suspension and brush applied spores after applying a water spray) where free water was present on the surface of the petals, approximately 10% of the germ tubes grew more than 6 µm over the leaf surface and such germ tubes did not penetrate the cuticle (Plate 4.5b). These germ tubes averaged 16 µ in length in the pipette application and 7.4 µm in the wetted brush application with a maximum of 36 µm. In the latter treatment, these longer germ tubes were usually thicker and there were multiple germ tubes from each ascospore (Plate 4.5c and d).

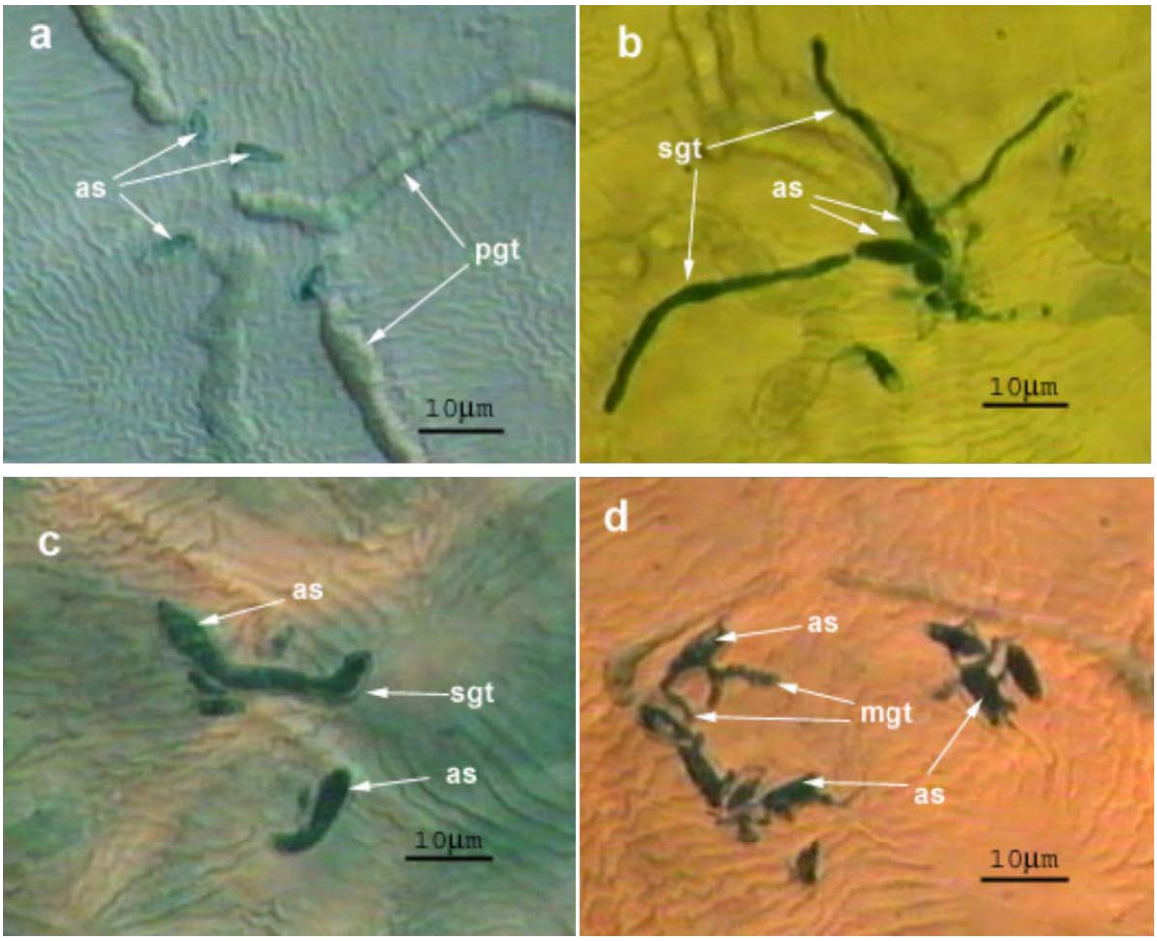


Plate 4.5. Light microscopic image. *Ciborinia camelliae* ascospore germination and germ tube growth. (a) Typical penetration and growth of germ tube underneath the cuticle (b) surface germ tube growth after pipette inoculation, and (c) thicker germ tubes and (d) multiple germ tubes from brush inoculated spores. (as = ascospore; pgt = penetrated germ tube; sgt = surface germ tube; multible germ tube).

Symptoms appeared on petals inoculated by the gravity and brush methods within 24 h and by 36 h disease was extensive in the gravity treatment (Fig. 4.6a). When water was added to petals inoculated by the gravity method, fewer symptoms had appeared by 24 h but by 36 h the maximum disease rating had been reached (Fig. 4.6b). Symptom appearance in the brush treatment was similar whether water was sprayed on the petal or not. When spore suspensions were applied by pipette, symptom development was not as extensive as in the "dry" or "wet" gravity treatments while a spray application of the suspension reached the same disease rating as the gravity method by 36 h (Fig 4.6). Where water was used to make spore suspensions or where "dry" methods were used and then water added, a subsequent drying of the inoculated petal surface both delayed the appearance of, and resulted in a reduced severity of, symptoms (Fig. 4.6c).

4.4. Discussion

C. camelliae infects camellia flowers during July-November in New Zealand, a time when the weather is usually cool and humid. Temperature and relative humidity can have considerable influence on ascospore germination and infection as observed by Arauz and Sutton (1989) for *Botryosphaeria obtuse*. The petals on which ascospores land could be dry or have a coating of water from rain or dew.

Effect of Temperature and Relative Humidity

In this study, ascospores germination and germ tube penetration of *C. camelliae* responded strongly to relative humidity. Maximum spore germination and germ tube penetration occurred at 100% RH at all temperatures (5, 10, 15, 20^{o}C) tested indicating that a saturated atmosphere is critical for spore germination and germ tube penetration. A similar situation was observed *Botryosphaeria obtusa* ascospore germination where relative humidity was more important than temperature although germination was significantly reduced at lower temperatures (Arauz and Sutton 1989). However, penetrated germ tube growth of *C. camelliae* was strongly influenced by temperature. Low temperatures restricted the growth of the penetrated germ tubes even at a high relative humidity. This condition suggested that disease symptom development could be delayed after successful germination and

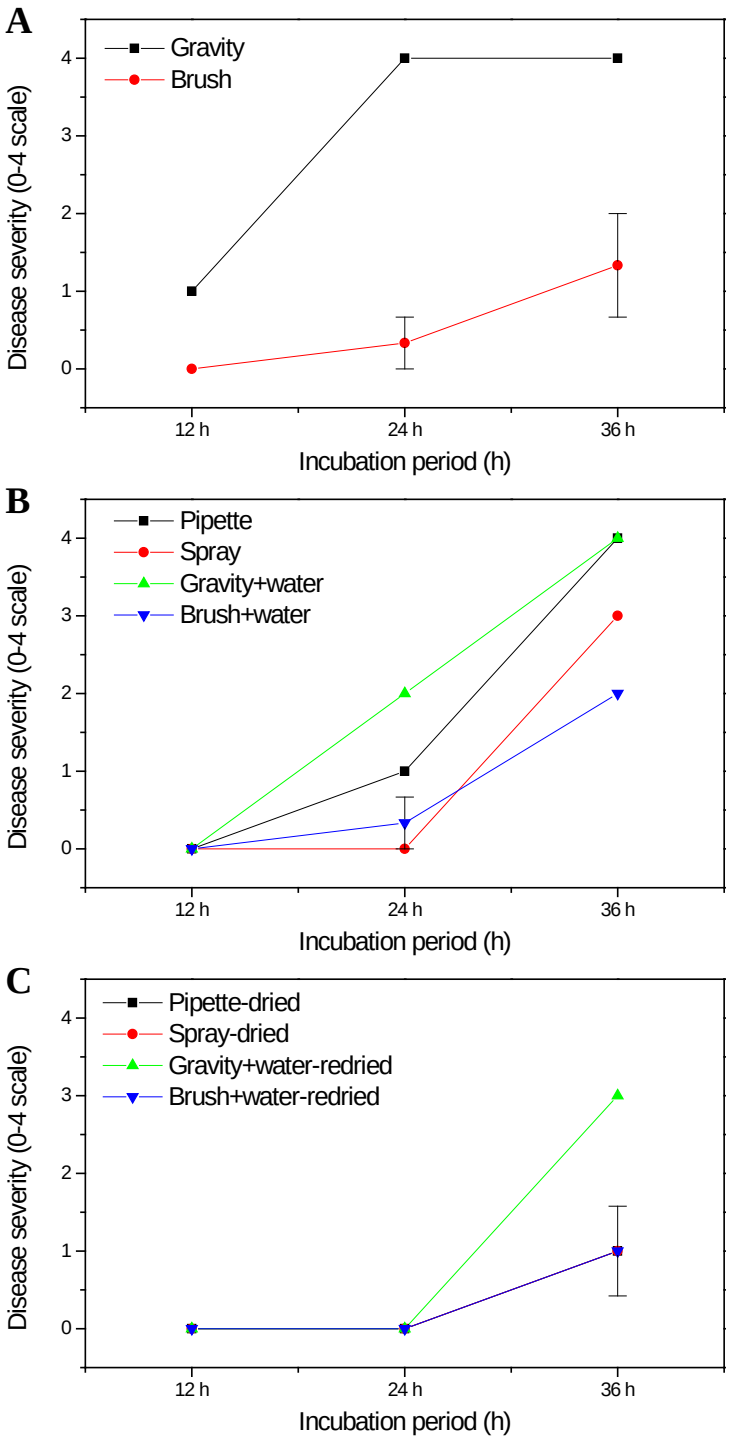


Figure 4.6. Symptom development on camellia flower petals 12 h, 24 h and 36 h after inoculation and incubation (A) without free water (B) with free water and (C) after drying previously wet petal surface. Vertical bars represent Standard Error (n=12).

penetration if the temperature remains low. e.g. if *C. camelliae* ascospores germinated and germ tubes penetrated into the petals during cold weather, there would be a waiting period for symptom development until the environment become warmer. This situation was clearly demonstrated by the observation of percent infection of disease with temperature that correlated with infection hyphal growth.

Effect of free water and inoculation methods

The presence of free water on the infection surface can have a marked effect on the germination behavior of some spores as shown by Cole et al. (1996) for conidia of *Botrytis cinerea* and *Botrytis fabae* on *Vicia fabae* leaves. The effect of free water also has important implications for inoculation studies since inoculum loading is easily controlled in spore suspensions but not in air deposition methods.

Presence or absence of abundant free water was not critical for germination since percentage germination and disease development from spores settling on petals after release from an apothecium were similar to those of the spore suspensions. However, fewer spores transferred by brush germinated than did those obtained direct from an apothecium. Spores released from an apothecium had a small amount of liquid associated with them and this could have provided a reservoir of moisture that was critical for the first hour of germination and which could have been absent from the brush transferred spores. Alternatively, spores transferred by brush could have been damaged by the bristles or could have contained a higher proportion of immature spores released from asci ruptured by the bristles of the brush. Adding water to the petals immediately after transfer of spores by brush increased percentage germination and penetration after12 h incubation but this effect had disappeared by 24 h.

Although addition of free water to spores inoculated by brush or gravity did not have a significant effect on numbers of spores germinating, there was some evidence that it could affect the subsequent growth of germ tubes and penetration. When brush inoculated petals were sprayed with water, germ tubes grew across the surface of the petal and those that grew more than 6 μm did not penetrate the cuticle. They were frequently swollen and many

spores produced multiple germ tubes. In a preliminary observation of *Botrytis cinerea* inoculation to camellia petals, the germ tubes from conidia penetrated petal tissue immediately in the absence of free water but grew across the surface if water was present (Appendix II). The same situation was observed by Cole et al. (1996) when conidia of *Botrytis cinerea* inoculated to bean leaves. They also found that some conidia stood on end in the absence of free water but there was no evidence of this behaviour with ascospores of *C. camelliae*. When germ tubes grow across the surface of the petals there would be a greater probability of them contacting any fungicide deposits than when penetration is adjacent to the spore. The germ tubes of *C. camelliae* usually penetrate adjacent to the spore and this could be one reason why fungicides have failed to give satisfactory control of this disease. The multiple germ tubes in the brush inoculated treatment could result from minor damage to the ascospore walls by the brush bristles while the extended germ tube growth, lack of penetration and swollen hyphae could result from fungitoxic material released from micro damage to the petal surface by the brush. The difference in spore germination and penetration between the pipette and spray ascospore applications is difficult to explain as the same spore suspension was used in each. Possibly the ascospores are delicate and some were damaged by the stress of being forced through a small aperture under pressure.

Overall, either the pipette application of spore suspensions or the use of apothecia suspended over petals would appear to give comparable results when used for inoculation of petals in experimental work. In the field, these results would suggest that there would be little difference between spores landing on a petal with no free water under high humidity conditions and spores landing on a petal wet with dew or rain. However, if the petal dries before penetration has taken place then the number of infections would be reduced.

Chapter Five

Infection process of C*iborinia camelliae*

5.1. Introduction

Ciborinia camelliae causes flower blight disease in camellia and ascospores can only infect camellia flower tissues of camellia (Raabe et al. 1978; Kohn and Nagasawa 1984; Dingley 1993). There are no published studies of the infection process of *C. camelliae*, but other fungi, for example, *Venturia inaequalis* and *Colletotrichum* spp. have been well studied and different strategies of the infection process defined. The various species of *Colletotrichum* have developed different strategies to colonize the tissue of host plants during the infection process (Bailey et al. 1992; Skipp et al. 1995). Some *Colletotrichum* spp. achieves infection through wounds caused by physical damage or insects (Boher et al. 1983). Others penetrate cuticles and establish a benign, but often extensive, sub-cuticular infection prior to development of necrotrophic hyphae which grow through and destroy tissue (Walker 1921). A third group of these pathogens referred to as "intracellular biotrophs" are typified by *C. lindemuthianum* on bean (Skipp & Deverall 1972; Mercer et al. 1974; O'Connell et al. 1985) where a biotrophic relationship is sequentially established in each successive cell colonized by primary hyphae. The fourth strategy is known as "intracellular hemibiotrophy" and is typified by *C. destructivum* in infected cowpea. Hemibiotrophic infections form in viable epidermal cells - hyphae are large and become highly branched, but remain within the initially infected epidermal cells (Bailey et al. 1990), but this intracellular phase lasts only for 48 h in lucerne (Latunde-Dada et al. 1997) and 72 h in cowpea (Latunde-Dada et al. 1996). *C. camelliae* could follow one of the above strategies of the infection process of *Colletotrichum* spp.

This work was undertaken in an attempt to understand the infection process by using techniques such as light and confocal microscopy with glutaraldehyde fluorescence over the time required for the important events of spore germination, germ tube growth, appresorium formation, penetration and infection hyphal growth.

5.2. Objective

To understand the strategy of the infection process of *C. camelliae*, to visualize and measure the physical structures and to measure the timing of important events during the infection process.

5.3. Materials and Methods

5.3.1. Plant materials

Flowers were collected from camellia plants (*Camellia reticulata* cv Brian) as described in Chapter 2 and undamaged and healthy petals were removed from the flowers for inoculation with ascospores.

5.3.2. Fungal materials

Ascospores were collected from apothecia to prepare inoculum for wet inoculation or for direct inoculation as described in Chapter 2.

5.3.2.1. Inoculum preparation

Ascospore inoculum suspension was prepared as described in Chapter 2 and sterilized distilled water was added to obtain an inoculum concentration of $5x10^4$ spores per ml.

5.3.3. Inoculation and incubation

For direct inoculation, method II as described in Chapter 2 was used. For wet inoculation, 2 µl of ascospore inoculum was placed on each of 5-6 sites per petal using a micro pipette. Inoculated petals were placed in a plastic tray with a moist paper towel in the bottom, and covered with a clear polyethylene bag to ensure high humidity. The tray was incubated at 21^oC for 72 h. During incubation, petals were randomly taken and about 25 mm^2 was cut from inoculation sites every six hours for sequence microscopy examination.

5.3.4. Light microscopy

Trypan blue (0.05%) in lactophenol was used to stain *C. camelliae* fungal hyphae in the plant tissue to facilitate light microscopy as described in Chapter 2. Two or three pieces of petal tissue were stained on a glass slide and excess stain was removed by washing with distilled water. Stained pieces of petal were mounted on the same slide with 0.1% $FeCl_3$ in 50% glycerol for light microscopy examination. Cross sections were taken from glutaraldehyde fixed healthy, and infected petals across visible lesion using microtome. The cross sections were stained with toluidine blue and examined under bright field microscopy.

5.3.5. Confocal microscopy

For confocal examination, the samples were incubated with PBS-GA 8 (7.0) (Chapter 3) solution as described in Chapter 2 to induce fluorescence of fungal structures. Chloral hydrate was used to clear the tissue as described in Chapter 2 and to remove air bubbles, thus increasing the contrast of the images. The treated specimens were mounted on a slide with Citifluor mounting media and observed under CLSM using 488 and 568 nm exciter wavelengths and 530 and 590 nm for imaging wavelengths.

5.4. Results

Images of the stages of ascospore germination and hyphal development, from germ tube initiation to primary hyphae or secondary hyphae, were obtained by both light microscopy and CLSM. The cuticle of camellia petals has a very distinctive convoluted surface somewhat like a fingerprint pattern. This surface was a good guide as to which structures were above, and which below, the cuticle. Camellia petals consist about 10 layers of mesophyll cells are in the middle of the petal with vascular bundles (Plate 5.1A). The number of layers were reduced towards edge of the petal and ended with double layer of epidermal cells (Plate 5.1B). The average length and width of ascospores was 7 μm and 3.5 μm respectably. Most ascospores germinated from the side at the sharp end of the spore (Plate 5.2 and 5.7) and a short germ tube (1-3 μm length) formed within six hours. There was no obvious appressorium observed but each germ tube directly penetrated through the cuticle of petal tissue (Plate 5.3) and swelled underneath (Plate 5.4 and Plate

5.6D). In a few cases, a relatively large, multilobed structure developed and showed restricted growth for 30 h (Plate 5.2 and 5.3). A narrow tube (<1 μm length and width) then emerged from the penetrated enlarged germ tube and immediately formed a relatively large, primary hyphae that grew underneath the cuticle towards the junction of the epidermal cells over the next 18 h (Plate 5.5) to 24 h (Plate 5.6 and 5.7). The length of the primary hyphae depends on the place where the spore landed on the petal surface and the direction it grows to nearest epidermal cell wall junction. Upon reaching epidermal cell junction, the primary hyphae branched and grew intercellularly (Plate 5.5 and 5.8). The growth of the fungal hyphae continued intercellularly for at least the next 48 h (Plate 5.9) to 72 h (Plate 5.10). During this period, the plant cell walls became brown and after 48 h a clearly visible brown lesion had developed. Epidermal cell walls were swollen extensively under the cuticle layer in the infected tissue compared with healthy tissue (Plate 5.11). Fungal hyphae were observed underneath the cuticle layer within the swollen cell wall and in between epidermal and mesophyll cell walls in the infected tissue (Plate 5.11B, 5.12). During the first 72 h infection, epidermal and mesophyll cells were shrunken and most mesophyll cells were collapsed, but fungal hyphae was only observed in between the cells walls (Plate 5.11B).

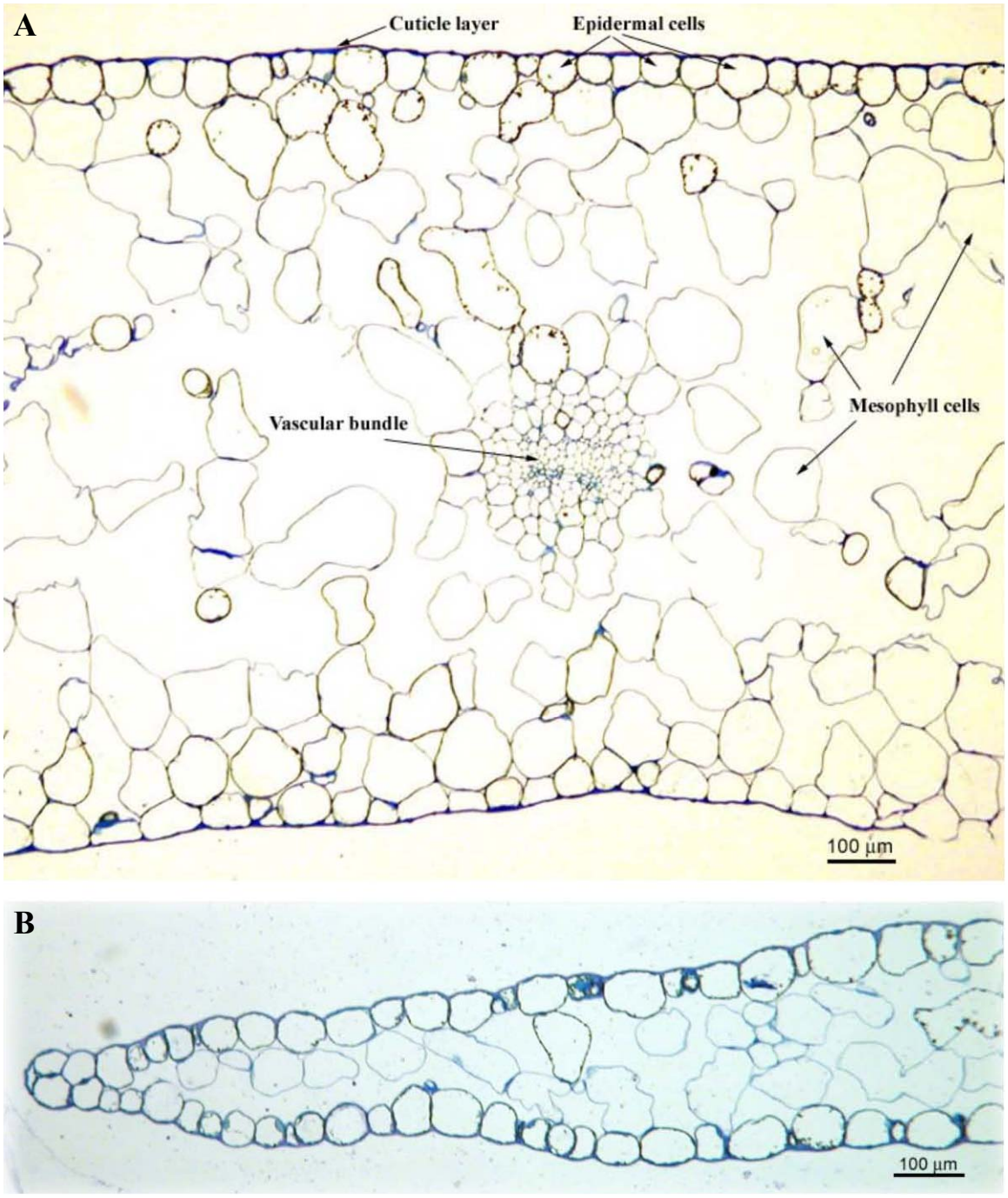


Plate 5.1. Bright field microscopy. Cross-section view of healthy camellia petal. A) Cross section of middle part of the camellia petal. B) Cross section of edge of the camellia petal.

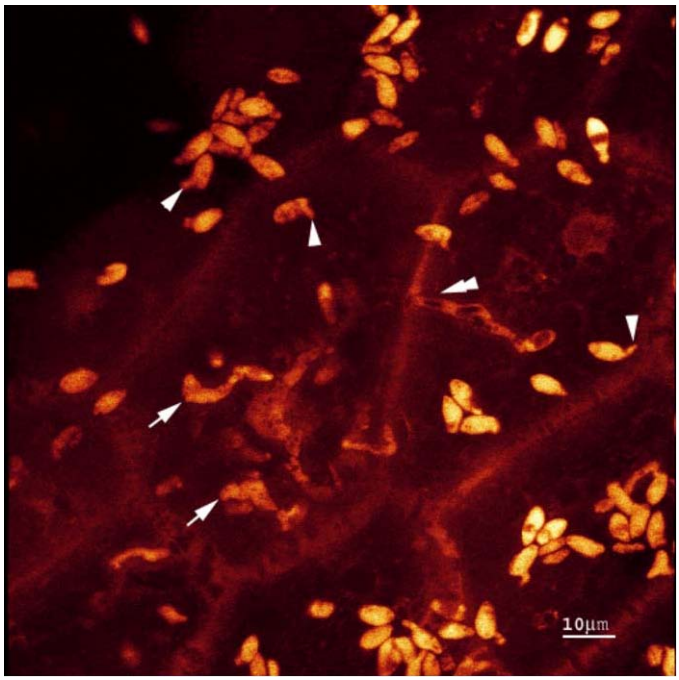


Plate 5.2. Confocal micrograph. *Ciborinia camelliae* ascospore germination (Single arrow head) and formation of mulilobed structure (arrows) after 30 h incubation, A germinated spore from which a hyphae grew towards an epidermal intercellular junction is shown by a double arrow head.

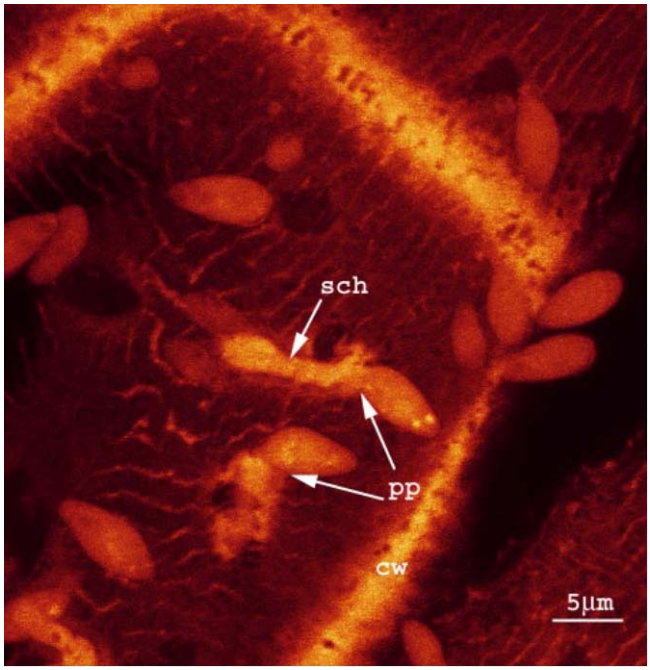


Plate 5.3. Confocal micrograph. *Ciborinia camelliae* ascospore germination and penetration of the cuticle layer of camellia petal tissue at 6 h after inoculation. pp = penetration point; sch = subcuticular hyphae; cw = cell wall.

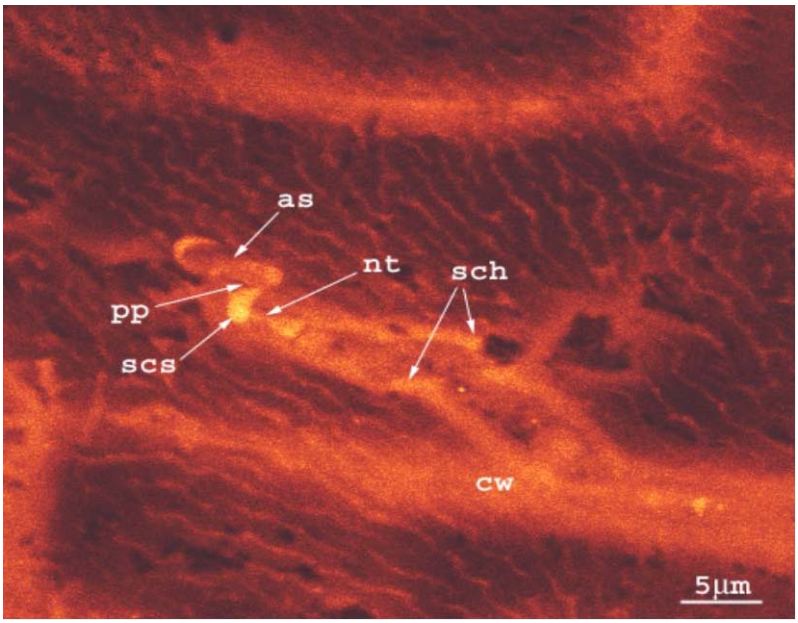


Plate 5.4. Confocal micrograph. *Ciborinia camelliae* ascospore germination and hyphal growth underneath the cuticle and towards the epidermal intercellular junction of camellia petal tissue at 18 h after inoculation. as = ascospore; pp = penetration point; scs = subcuticular swelling; nt = narrow tube; sch = subcuticular hyphae; cw = cell wall.

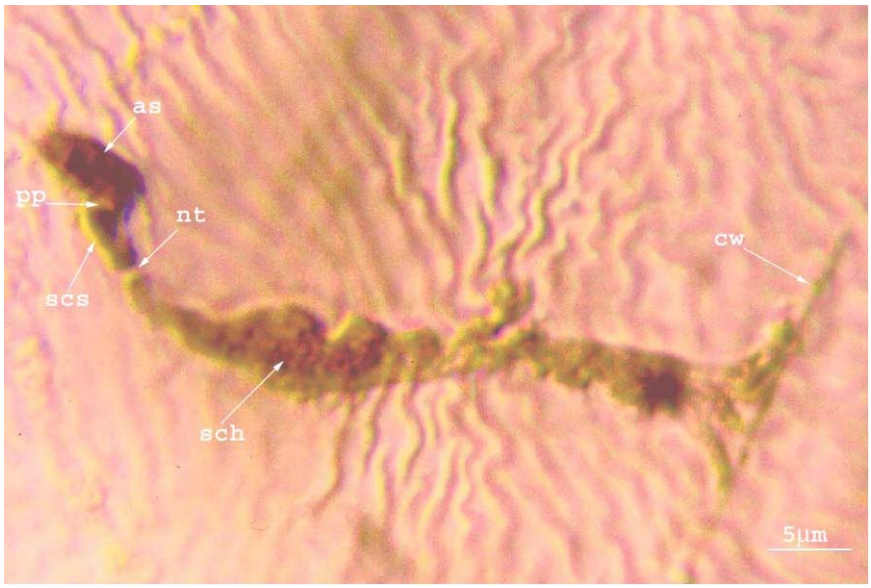


Plate 5.5. Light microscopic image. *Ciborinia camelliae* ascospore germination and hyphal growth underneath the cuticle layer towards the epidermal intercellular junction of camellia petal tissue at 18 h after inoculation. as = ascospore; pp = penetration point; scs = subcuticular swelling; nt = narrow tube; sch = subcuticular hyphae; cw = cell wall.

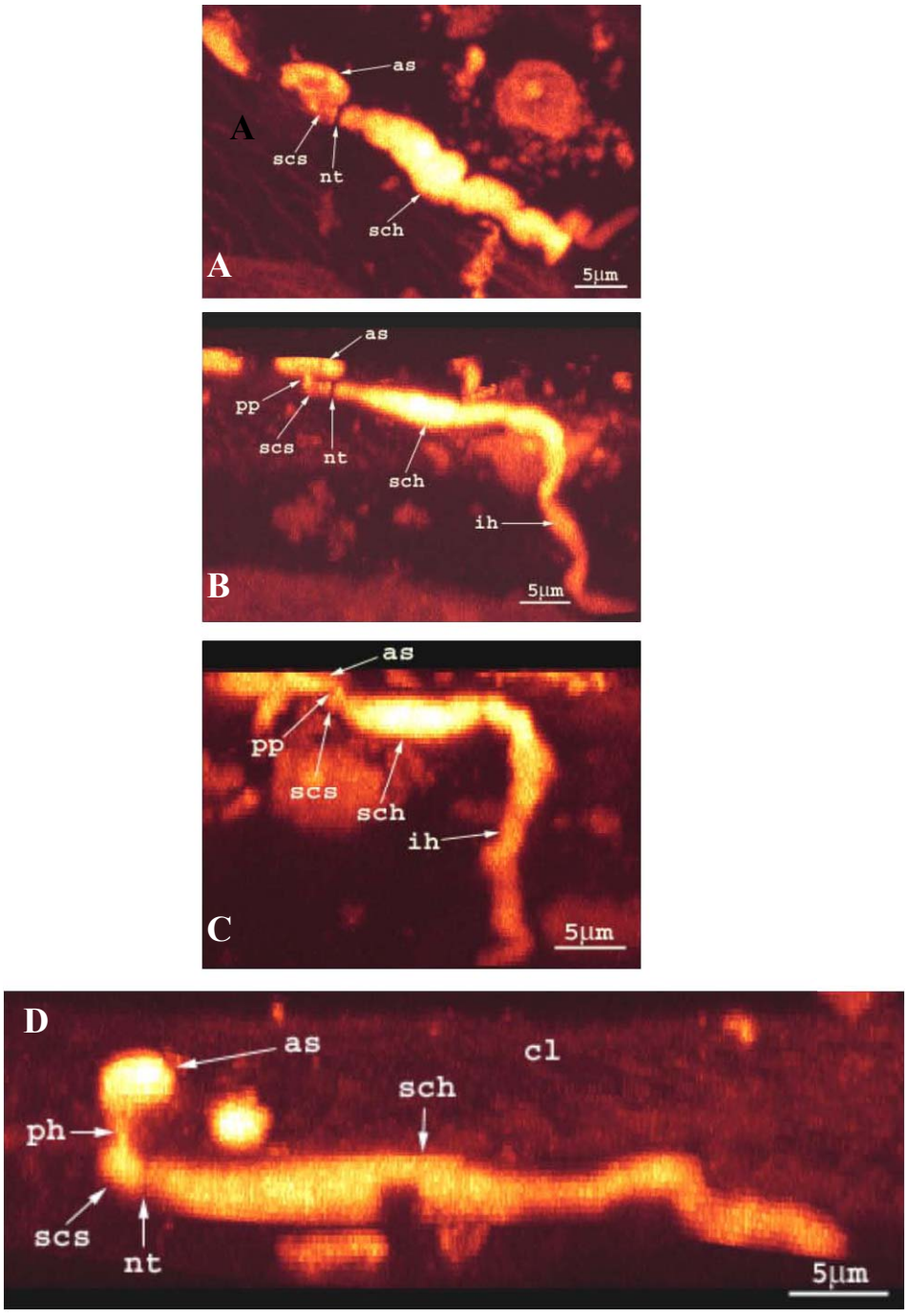


Plate 5.6. Confocal micrograph of *Ciborinia camelliae* ascospore germination and hyphal growth underneath the cuticle and intercellular space 24 h after inoculation. A) Top view of hyphal growth, B) Cross section view of hyphal growth after 80^{o} rotation from bottom to top, C) Further 45^{o} rotation from right to left of view B, D) Another cross section view shows the subcuticular swelling soon after penetration and hyphal growth underneath cuticle. as = ascospore; pp = penetration point; ph = penetration hyphae; scs = subcuticular swelling; nt = narrow tube; sch = subcuticular hyphae; ih = intercellular hyphae; cl = cuticle.

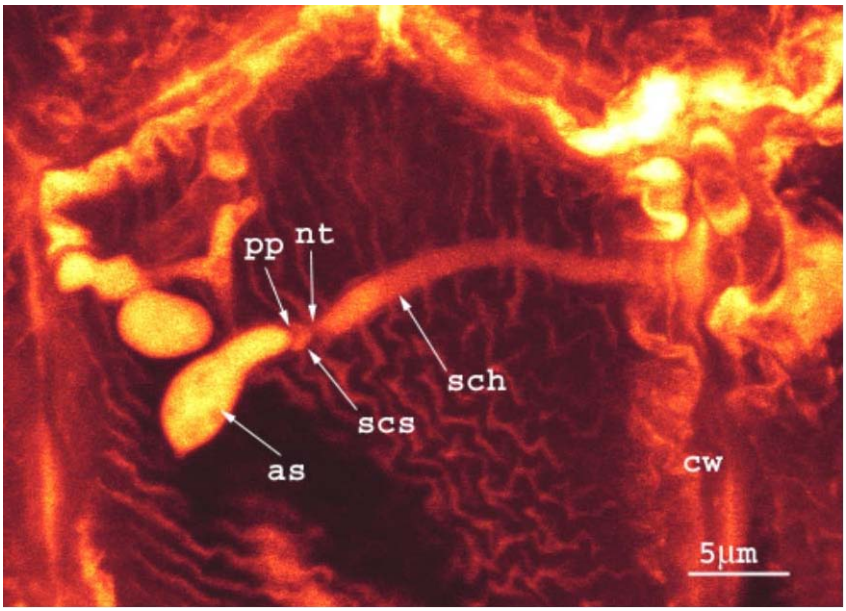


Plate 5.7. Confocal micrograph of *Ciborinia camelliae* ascospore germination and penetration of camellia petal tissue 30 h after incubation. Penetrated germ tubes have grown underneath the cuticle towards epidermal intercellular junctions. as = ascospore; pp = penetration point; scs = subcuticular swelling; nt = narrow tube; sch = subcuticular hyphae; cw = cell wall.

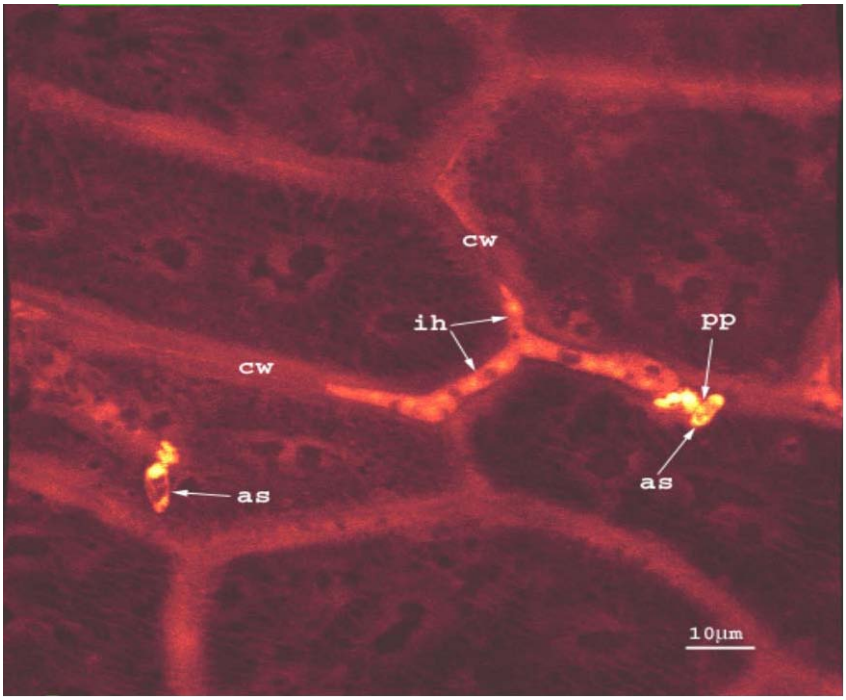


Plate 5.8. Confocal micrograph. *Ciborinia camelliae* ascospore germination and intercellular hyphal growth in camellia flower petal tissue at 18 h after inoculation. as = ascospore; pp = penetration point; ih = intercellular hyphae; cw = cell wall.

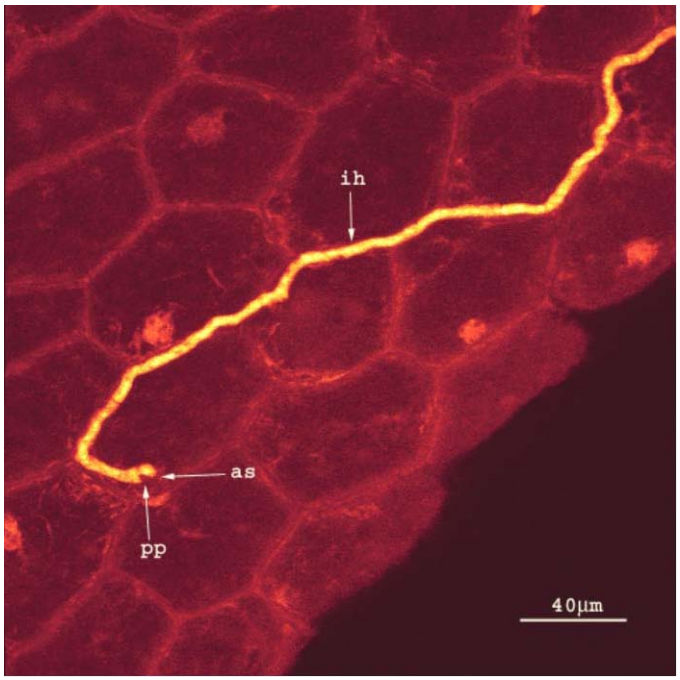


Plate 5.9. Confocal micrograph. *Ciborinia camelliae* intercellular hyphal growth in camellia flower petal tissue at 48 h after inoculation. as =ascospore; pp = penetration point; ih = intercellular hyphae.

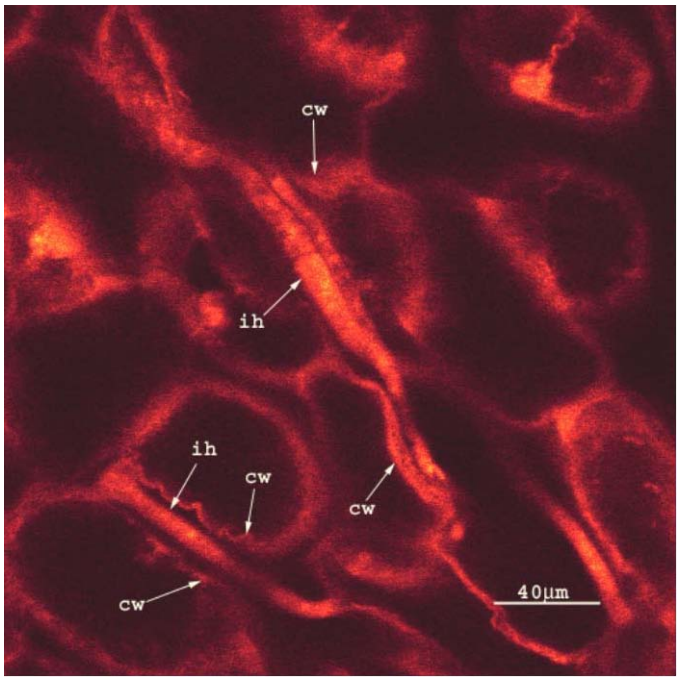


Plate 5.10. Confocal micrograph. *Ciborinia camelliae* intercellular hyphal growth in camellia flower petal tissue at 72 h after inoculation. ih = intercellular hyphae; cw = cell wall.

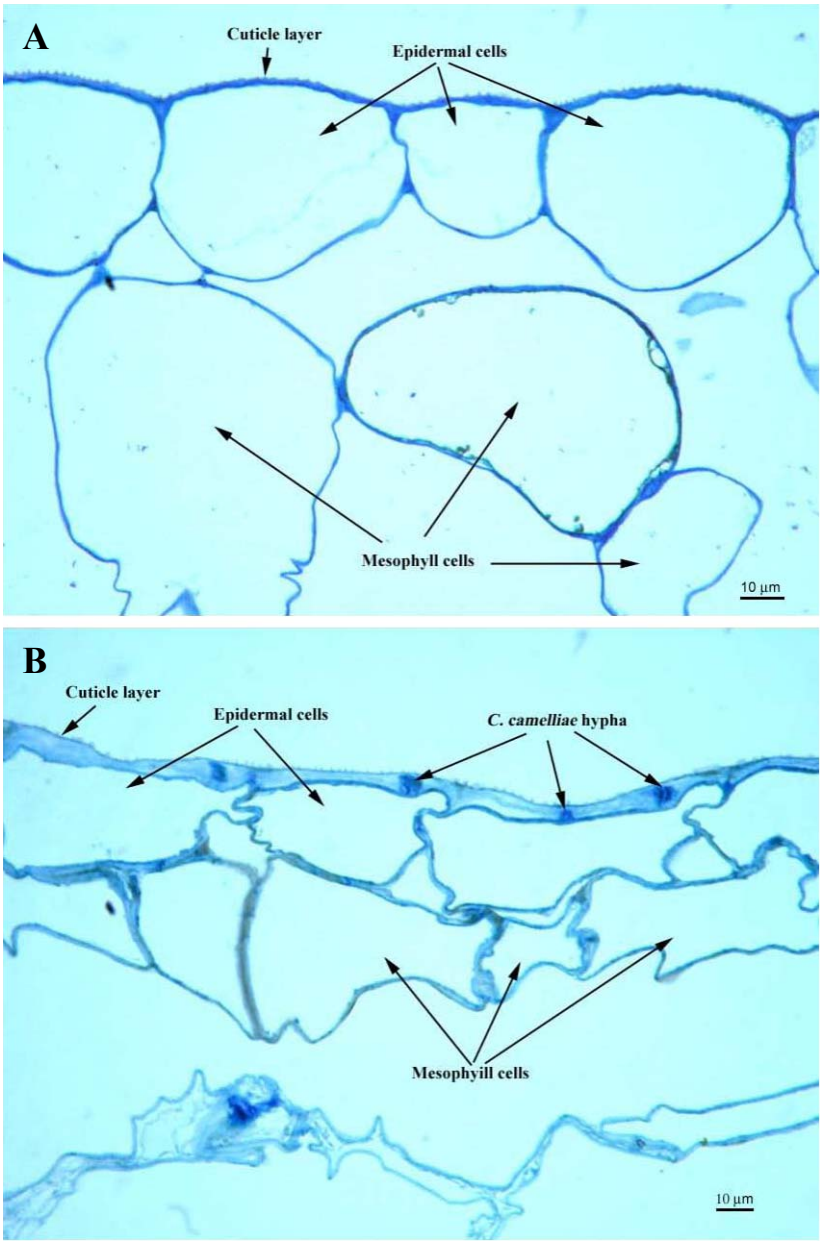


Plate 5.11. Bright field microscopy. Cross-section view of healthy and infected camellia petal tissue. A) Healthy epidermal and mesophyll cells are viewed before *C. camelliae* infection. B) Shrunken epidermal and mesophyll cells are viewed with fungal hyphae in the swollen cell wall after *C. camelliae* infection.

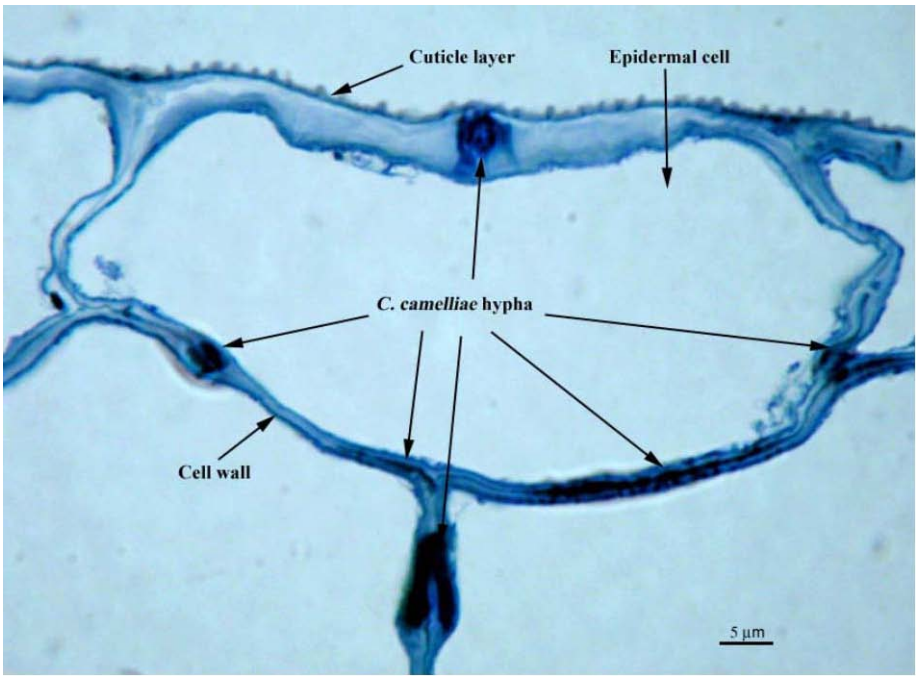


Plate 5.12. Bright field microscopy. Cross-section view of infected camellia petal tissue. Fungal hyphae shown in between cell wall and underneath cuticle layer in the swollen epidermal cell wall.

5.5. Discussion

Light and confocal microscopy of the infection process of *C. camelliae* have revealed differences from other pathogens. In all cases including *C. camelliae*, spores germinate and the host barrier is penetrated for successful infection, but the subsequent growth and behavior of infection hyphae varies between species. Smart (1991) stated that the plant cell wall represents the next physical barrier to a fungal pathogen after breaching the cuticle. In the present study, the ascospores produced a short germ tube with a length of 1-3 µm, which immediately penetrated the cuticle. However, there was no evidence that hyphae penetrated through the epidermal cell walls into cells in the first 72 h of the infection process. This contrasts with the case of *Colletotrichum lindemuthianum* where the fungus first penetrates an epidermal cell via the cuticle and epidermal cell wall then continues as an "intracellular biotrophic" pathogen in several successive cells after it becomes necrotrophic (O'Connell et al. 1985). Similarly, *C. destructivum* penetrates epidermal cells

but remains as an "intracellular hemibiotrophic" pathogen for 48 h in lucerne (Latunde-Dada et al. 1997) and for 72 h in cowpea (Latunde-Dada et al. 1996) in each initially penetrated cell. The initial infection process of *C. camelliae* thus differs from that of these two species of *Colletotrichum*.

During the early stages of infection some pathogens (eg. *Colletotrichum lindemuthianum)* act as intracellular hemibiotrophic pathogen and infect the host cells without killing them. Others (eg. *C. capsici, C. circinans, Venturia inaequalis*) act as subcuticular intramural pathogens that grow exclusively beneath the cuticle and within the walls of host epidermal cells without entering them (Bailey et al. 1992). *Ciborinia camelliae* is clearly comparable with the second group. Bailey et al. (1992) have speculated that *Colletotrichum* spp. that establish hemibiotrophic relationships involving intimate cytoplasmic interaction with their hosts appear to be highly host-specific, while those that grow only in host cell walls (eg. *C. capsici*) have a wide host range. *Ciborinia camelliae* does not fit this categarisation since it grows subcuticular intramurally in the cell walls for the first 24-48 h but has a very restricted host range. Whether this relationship is dependent on the host tissue infected (petals v leaves and fruit) or whether it is characteristic of *C. camelliae* requires further elucidation. The appearance of disease symptoms within 24-48 h even though intracellular inversion does not appear to occur within this time span also requires further investigation.

McKeen (1974) observed that *B. cinerea* infections frequently started from short germ tubes without appresorium formation. Cole et al. (1996) also observed penetration from short germ tubes of *B. cinerea* and *B. fabae* under conditions of dry inoculation but high humidity. However, they also found that long germ tubes were produced when water with glucose was present. In the present study, *C. camellia* produced short germ tubes under both liquid water or dry but humid conditions and readily penetrated petal cuticles. Germ tubes of *Venturia inaequalis,* commonly contact the cuticle and appresoria are formed, but sometimes germ tubes may penetrate the cuticle directly without forming appressoria (MacHardy 1996). Appresoria of *C. camellia* were not as obvious as those of *B. cinerea* (McKeen 1974), and other fungi such as *Sclerotinia sclerotiorum, Rhizoctonia solani, Verticillium albo-atrum, Fusarium* species, and *Cochliobolus carbonum* which have been

recorded invading plant tissue without formation of appresoria (Mendgen and Deising 1993). Although no obvious appresorium was observed in this study, soon after cuticle penetration, the penetration hyphae produced sub-cuticular swellings before development of primary infection hyphae.

Chapter Six

Resistance mechanism of *Camellia* species against *C. camelliae*

6.1. Introduction

During germination of a fungal spore and the infection process, host tissues respond in various ways to defend themselves. Such reactions may be to direct an apposition of substances onto the inner surface of the cellulose walls. Such wall appositions could, by healing or forestall death of injured cells (Aist 1976), contribute to the survival of whole plants from pathogen penetration and subsequent infection. The wall apposition elicited by fungi, termed "papillae" (Smith G 1900 – cited by Israel et al. 1980), may represent a disease resistance mechanism as discussed by Aist (1976) as they intercept some invading pathogens. Papillae commonly contain callose at the penetration site (Heath 1980) and have been observed in a range of plants infected by various pathogens. The timing of papillae formation in relation to growth of *Erysiphae graminis* penetration pegs into living barley coleoptile cells and the growth of *Olpedium brassicae* penetration tubes into living kohlrabi root hairs were monitored by Aist and Israel (1977a,b) using interference contrast microscopy. Resistance due to papillae formation has also been studied in tobacco against *Erysiphe cichoracearum* (Polverari et al. 2000), and in cucumber against *Colletotrichum lagenarium* Siegrist et al. (1994).

Another common response is the rapid death of cells in the vicinity of the fungus: the hypersensitive response (HR). This may occur in response to both intra- and intercellular pathogens. Mostly this mechanism is geared towards biotrophic fungi, which require a living cell for their survival (Heath 1984). The presence of localized, high concentrations of phytoalexins upon fungal infection cause sudden death of cells locally. The study in Chapter 5 showed that the infection process of *C. camelliae* is biotrophic in nature, thus one of the resistance mechanisms of *Camellia* species could be a hypersensitive response.

The presence of other antifungal substance such as pathogenesis-related (PR) protein in the plant might cause inhibition of fungal germination and penetration. The production of PR proteins is suspected to be one of the host resistance mechanisms (Crute et al. 1985).

Until recently, no camellia varieties were known to be resistant to camellia flower blight (Baxter and Segars 1989; Baxter and Thomas 1992), but a recent study indicated that there are such sources of resistance within the genus *Camellia* (Taylor 1999; Taylor and Long 1999) although the mechanisms are not known.

6.2. Objectives

The objectives of this study were to investigate the possible resistance mechanisms in some *Camellia* species possessing resistance to *C. camelliae*. Microscopic techniques were used to observe

1. Physical changes of host cells or tissues at infection sites during and after germination of ascospores.
2. *A*scospore germination and penetration behavior on and under the petal surface.

6.3. Materials and Methods

6.3.1. Host tissues

Camellia flower petals were collected from the species evaluated for resistance by Taylor (1999), and some other species (Table 6.1): *C. transnokoensis, C. lutchuensis, C. grijsii, C. forrestii, C. cuspidata C. yunnanensis, C. pitardii cv Pitardi, C. tricocarpa* and *C. polyodonta*, and commonly cultivated cultivar: *C. reticulata* cv Brain.

6.3.2. Ascospore inoculation and incubation

Ascospores were collected in a McCartney bottle by suspending an apothecium upside down in the bottle and spore suspensions prepared at $1x10^5$ cells/ml as described in Chapter 2. Four or more sites of a petal were inoculated with 2 μl of ascospore inoculum suspension using a micropipette. Inoculated petals were placed in a moist box and incubated at 20^oC for 96 h. During incubation a pair of inoculated sites were cut about 25 mm^2 at six hours interval from 0 h to 96 h for microscopic examination.

Table 6.1. Distribution of Resistance and Susceptibility in the genus *Camellia* (Adapted from Taylor 1999)

Species	Resistance (R) / Susceptible (S)
C. yunnanennsis	R
C. cuspidata	R
C. forrestii	R
C. forreterna	S
C. longicarpa	R
C. lutchensis	R
C. rosiflora	S
C. transnokoensis	R
C. transarisanensis	R
C. euphleobia	S
C. sasanqua	S
C. yuhsienensis	R
C. grijsii	R
C. chekioangaleosa	S
C. japonica	S
***C. pitardii* cv Pitardii**	S
C. polydonta	S
C. reticulata	S
C. saluenensi	S

6.3.3. Staining procedures

6.3.3.1. Trypan blue staining

Samples taken from inoculated petals every 6 h were stained with 0.05% trypan blue in lactophenol on a glass slide as described in Chapter 2. The stained specimens were mounted in 0.1% $FeCl_3$ in 50% glycerol

6.3.3.2. Other staining

Specimens taken at 24 h after *C. camelliae* inoculation were treated with glutaraldehyde solution and mounted on a glass slide as described in Chapter 2.

The following staining procedures were under taken to detect possible suberin formation in the petal tissue upon *C. camelliae* inoculation. The staining procedures are based on the method described by Lulai and Corsini (1998).

6.3.3.2.1. Toluidine blue O or Neutral red

The specimens (25 mm^2 each) from inoculated petals at 36 h after inoculation were paraffin embedded and sliced to 0.2 – 0.5 mm thick using a microtome. After the sections were deparaffinized with xylene the sections were stained 45 min in 0.05% toluidine blue O (CI. 52040 Sigma) in 0.1 M sodium acetate, pH 4.4 then rinsed with water and mounted on a glass slide in water. Other sections were stained for 1 min in a 0.1% solution of neutral red (C.I 50040, Difco Detroit, MI) in 0.1 M potassium phosphate, pH 6.5, followed by rinsing twice in water and were mounted on a glass slide in water. Some sections were stained with toluidine blue O and counterstained with neutral red. The stained sections were mounted on a glass slide in water.

6.3.3.2.2. Berberine / Aniline blue

Deparaffinized sections were stained 45 min in 0.1% berberine in water, rinsed briefly in water and stained 5 min in 0.25% aniline blue. The sections were rinsed again in water and allowed to air dried before mounting on a glass slide in immersion oil.

6.3.4. Microscopic observation

6.3.4.1. Light microscopy

Trypan blue stained specimens were observed at 400 – 1000 X magnification with interference contrast optics. A number of germinated and non-germinated spores were counted and germ tubes measured. Host defense structures, spore germination and germ tube growth were recorded by video as described in Chapter 2.

6.3.4.2. Confocal and Fluorescence microscopy

Glutaraldehyde treated specimens were observed by confocal microscopy and digitised images obtained as described in Chapter 2.

Specimens treated with Toluidine blue O or Neutral red, and Berberine / Aniline blue were examined under an epifluorescent microscope using blue-violet and ultraviolet excitation filters.

6.4. Results

The *Camellia* species rated as resistant to *C. camelliae* did not develop symptoms even 96 h after inoculation with the exception of *C. cuspidata* which showed early stage symptoms (Scale = 1-2) at 48 h but they did not develop further. *C. reticulata* cv Brian, *C. pitardii cv Pitardii* and *C. polyodonta,* developed symptoms with in 48 h after inoculation but *C. polyodonta* had fewer infections than the other two (Plate 6.1).

C. cuspidata and *C. lutchuensis* both showed similar reactions with thickening of the wall of cell at the penetration point (Plate 6.2, 6.3 and 6.4). Necrotic and collapsed cells and cell wall thickening (Plate 6.5) were also observed where spores landed and germ tube penetration had taken place in *C. cuspidata* petal tissue. With *C. transnokoensis,* penetration did not take place but spores germinated and the germ tube grew over the surface of the petal (plate 6.6A). The germ tube growth was restricted and distorted (Plate 6.6B). Most of the spores also showed multiple germination (Plate 6.7). In the species *C. tricocarpa,* the germ tubes of *C. camelliae* produced a large swollen structure underneath the cuticle after penetration. There were considerable amount of single cells microbes were also observed on the petal surface (Plate 6.8). Ascospore germination and germ tube penetration was observed in *C. polyodonta* petal tissue while some spores were distorted and non-viable and surrounded by unidentified celled microbes (Plate 6.9).

Suberin formation in the cell wall was not detected in any of camellia petal by fluorescence microscopy.

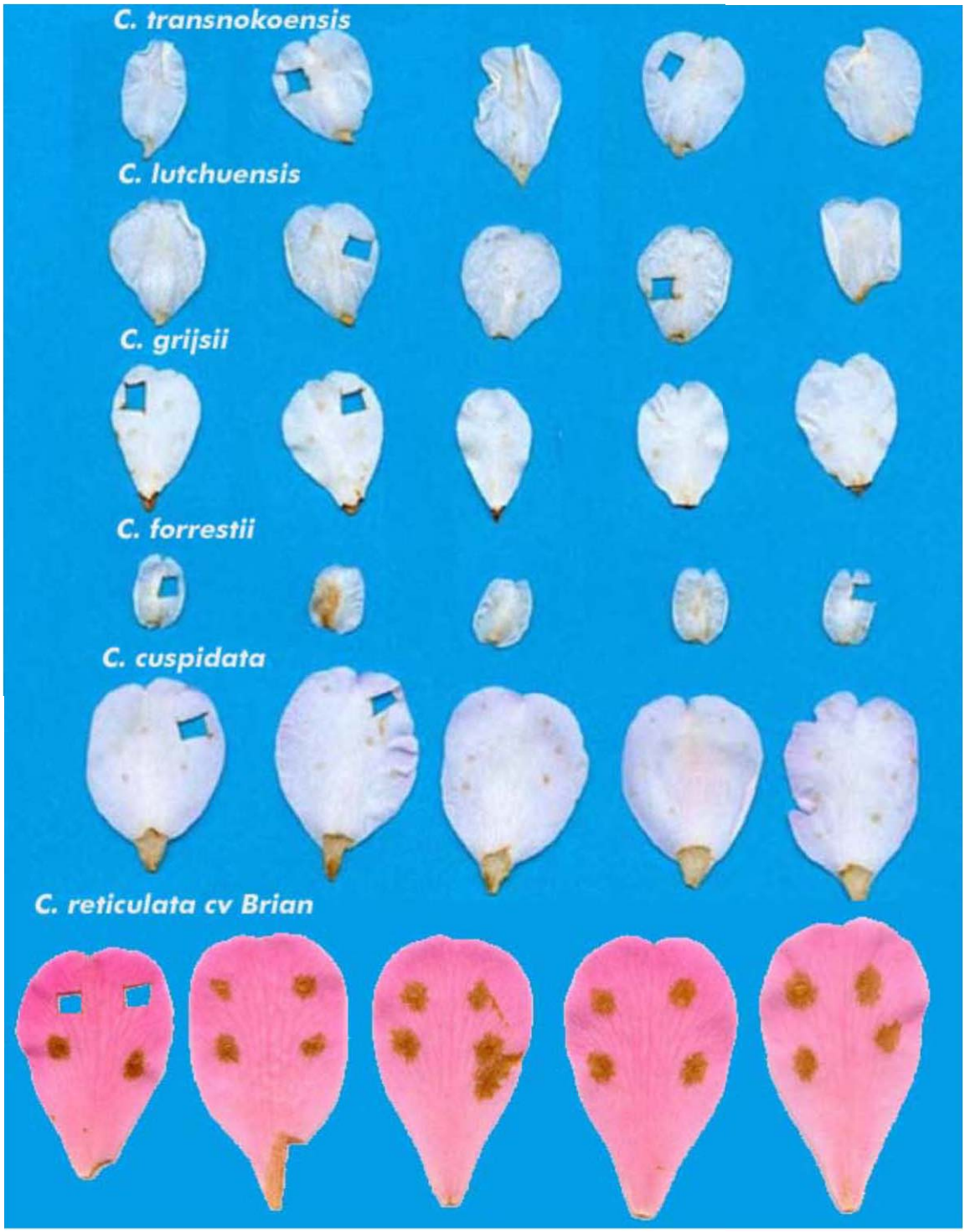


Plate 6.1. Flower petals of *C. transnokoensis, C. lutchuensis, grijsii, C. forrestii, C. cuspidate* and *C. reticulata* cv Brian pipette inoculated with *C. camelliae* ascospore suspension. Symptoms development at 48 h after inoculation.

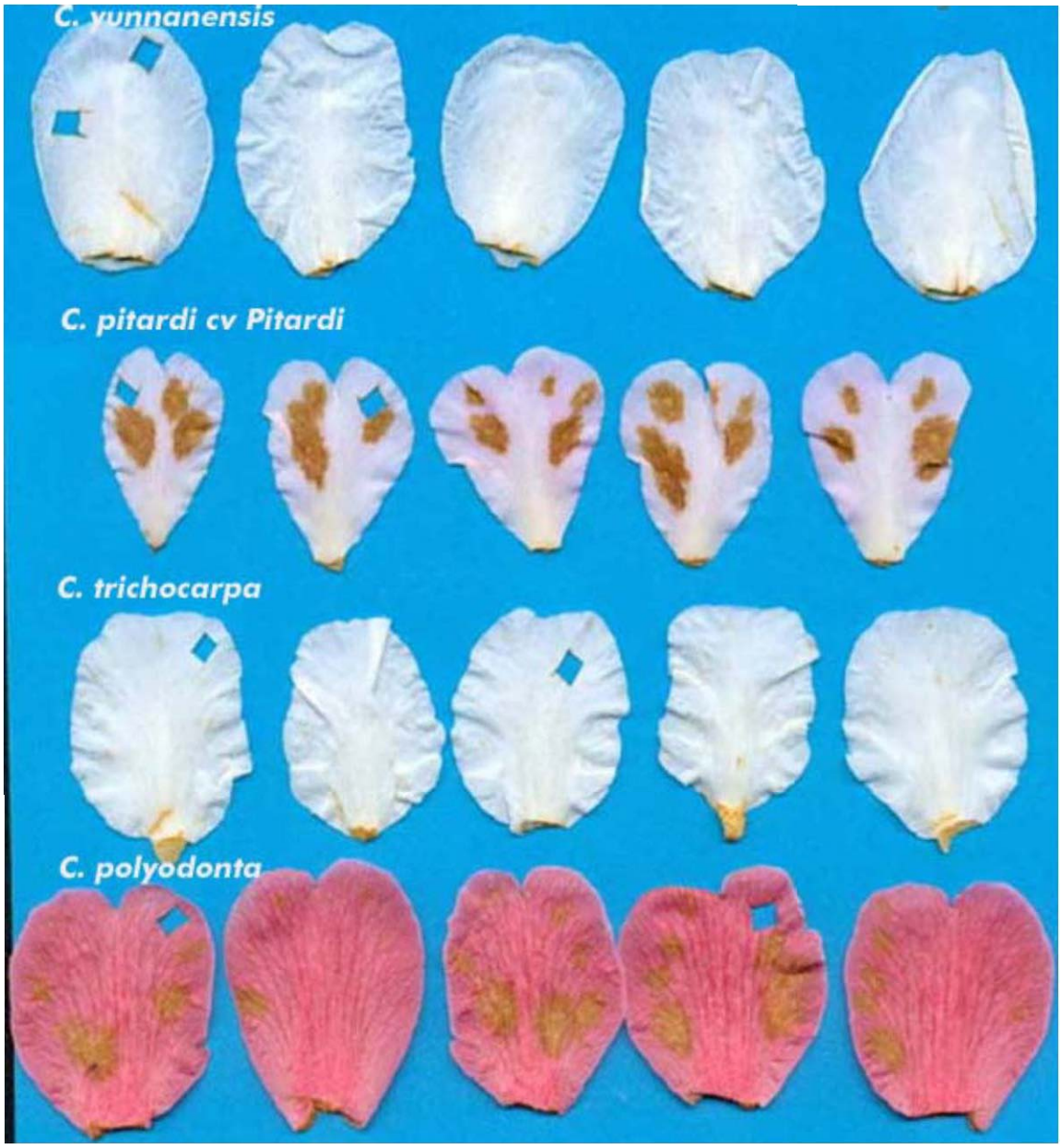


Plate 6.1. Continued:…. *C. yunnanensis, C. pitardi* cv Pitardi, *C. trichocarpa*, and *C. polyodonta.*

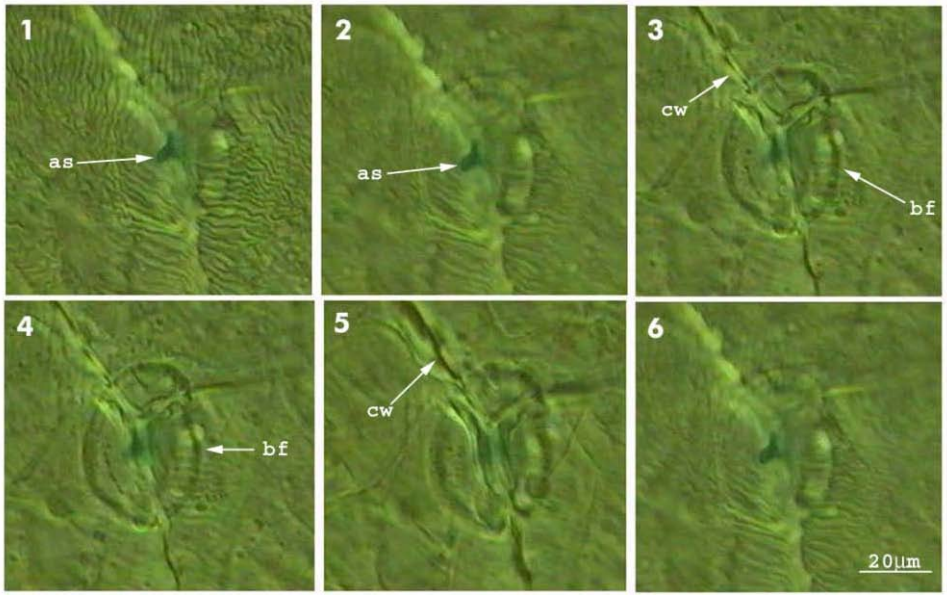


Plate 6.2. Light microscopic image of ascospore germination and the resistant reaction of *C. cuspidata* petal tissue. Decreasingly lower (2-3 µm) focal planes of tissue to show ascospore germination, cell wall thickening and a barrier formed around the penetration point 72 h after inoculation. as = ascospore; cw = cell wall; bf = barrier.

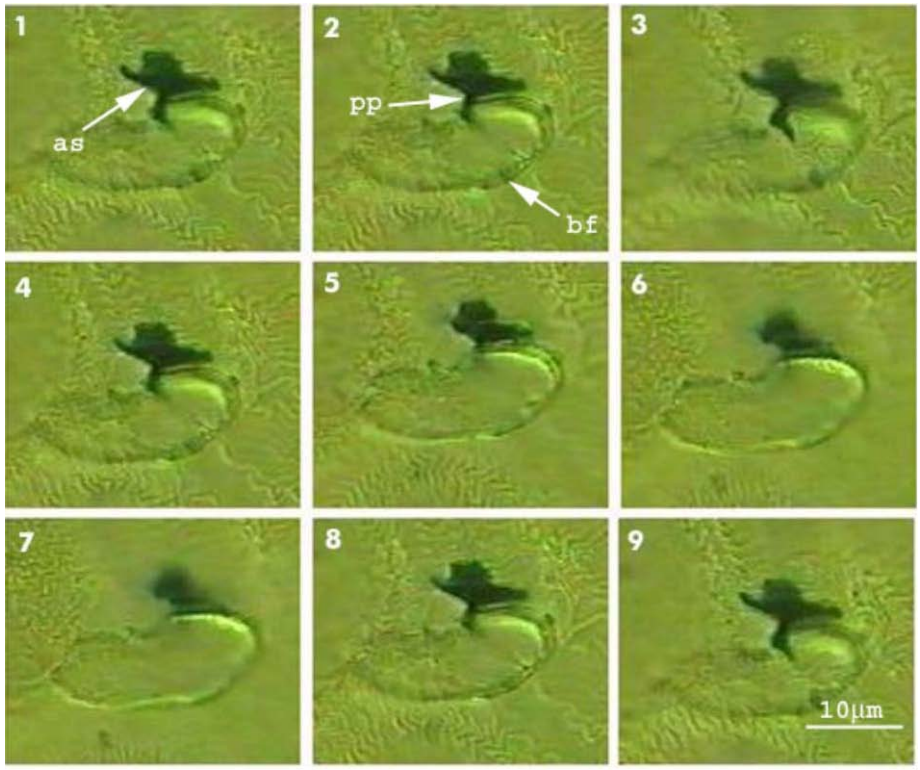


Plate 6.3. Light microscopic image of ascospore germination and the resistant reaction of *C.lutchuensis* petal tissue. Decreasingly lower (1-2 µm) focal plane of tissue to show ascospore germination, penetration and a barrier formed at the penetration point at 30 h after inoculation. as = ascospore; pp = penetration point; bf = barrier.

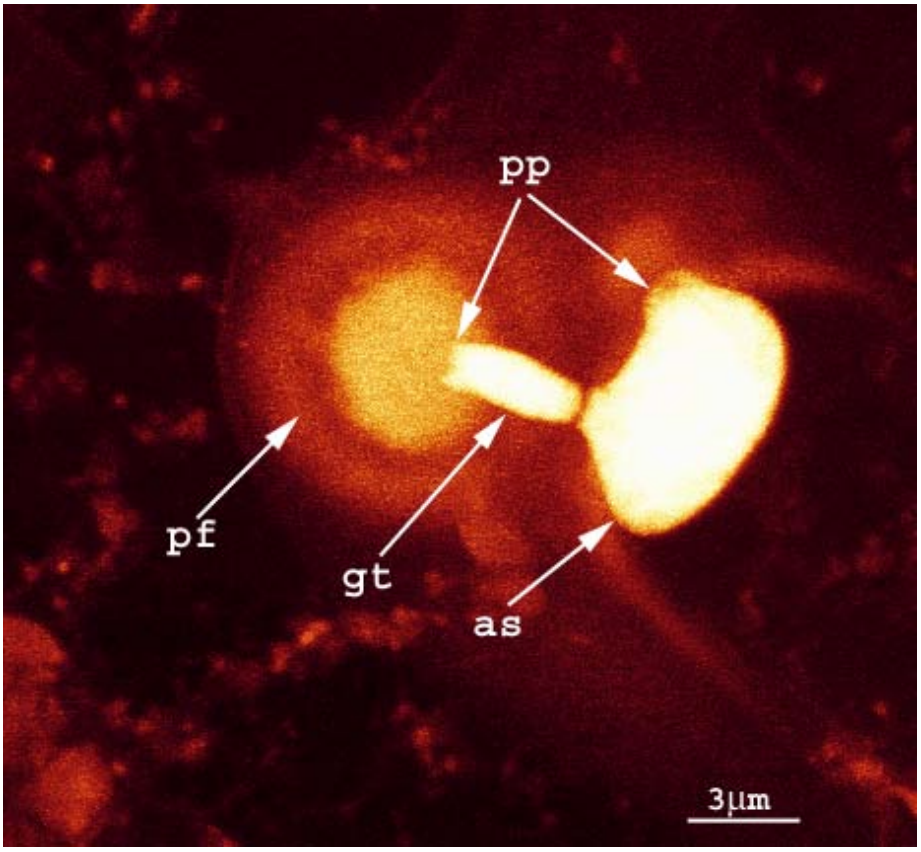


Plate 6.4. Confocal micrograph of *C. camelliae* ascospore germ tube penetration and papilla formation on *C. lutchuensis* petal tissue 20 h after inoculation. as = ascospores; gt = germ tube; pf = papilla; pp = penetration point.

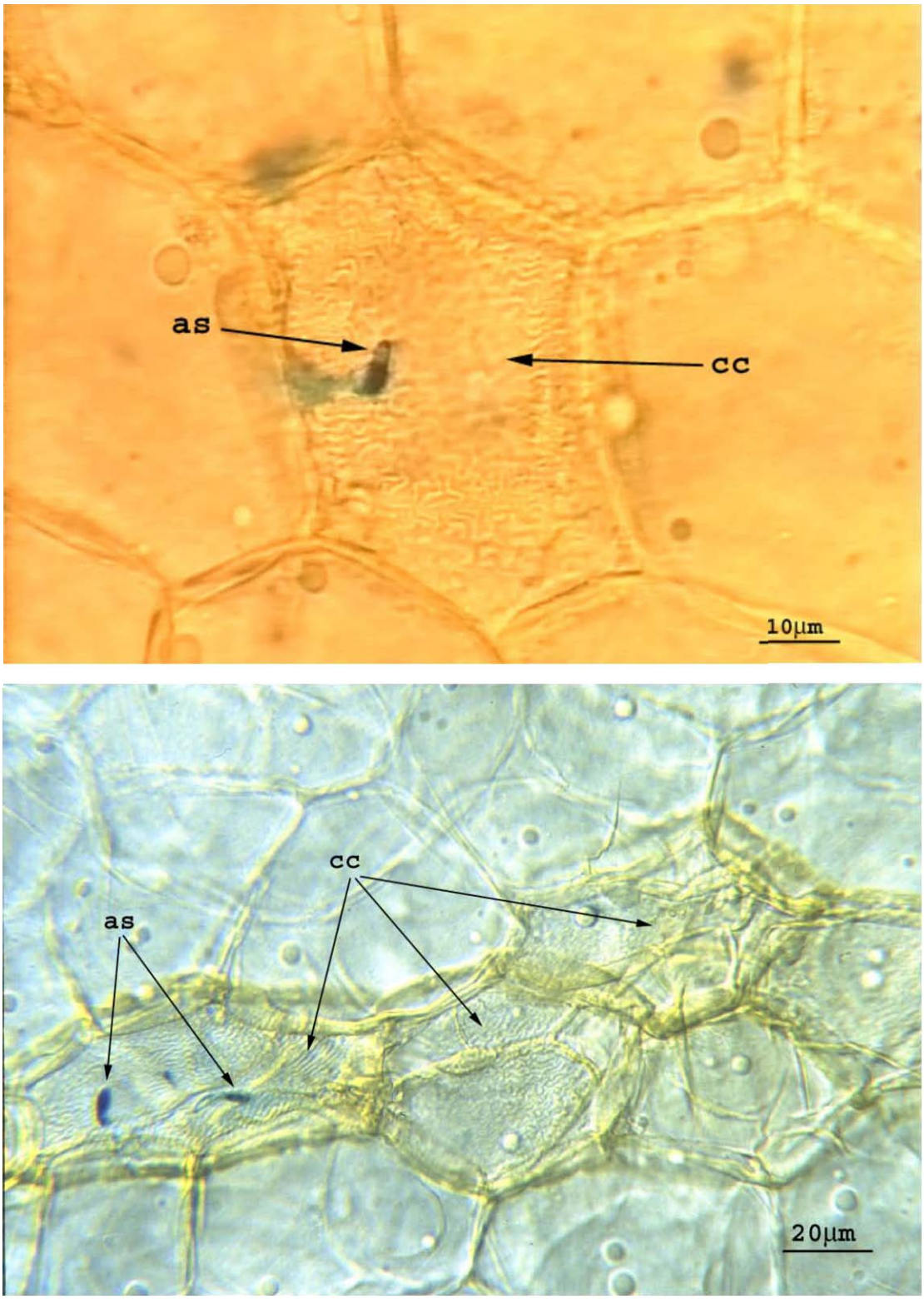


Plate 6.5. Light microscopic image of cell wall thickening and necrotic collapsed cells of *C. cuspidata* petal tissue after *C. camelliae* ascospore germ tube penetration 72 h after inoculation. as = ascospore; cc = collapsed cells.

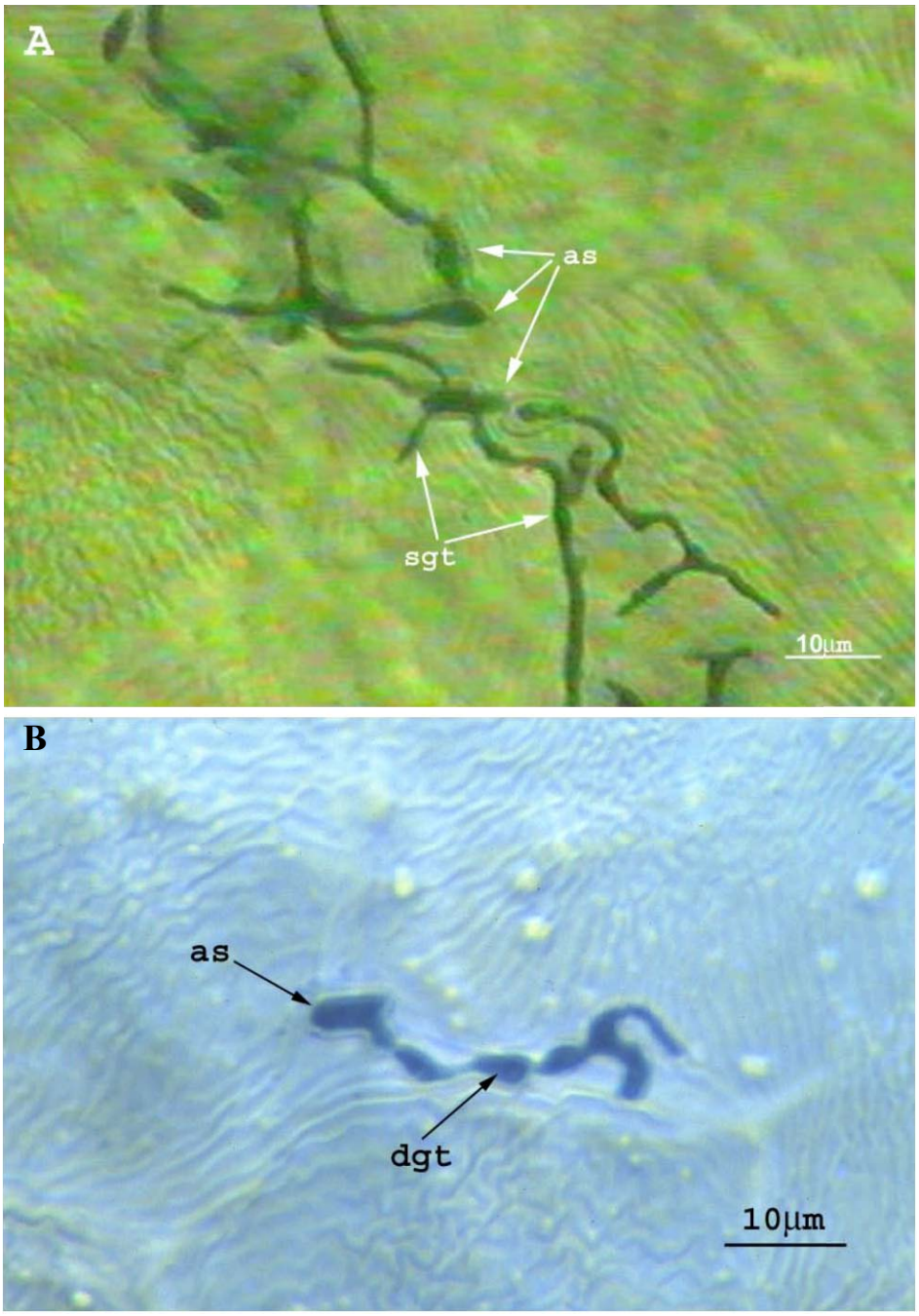


Plate 6.6. Light microscopic image of multiple germination and germ tube growth over the surface with out penetration (A) and restricted growth of distorted germ tubes (B) of *C. camelliae* on *C. transnokoensis* petal tissue 30 h after inoculation. as = ascospore; sgt = surface germ tubes; dgt = distorted germ tube.

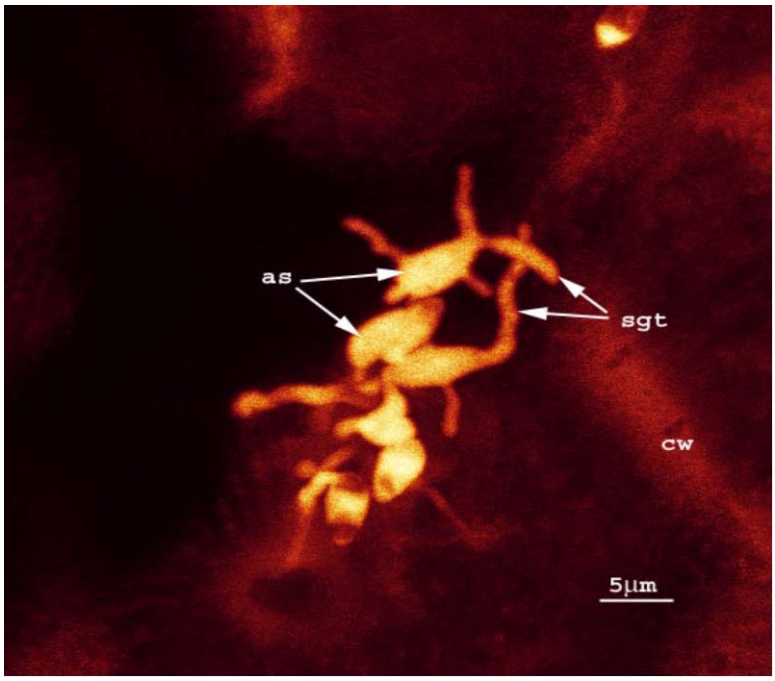


Plate 6.7. Confocal micrograph of multiple germ tubes from germinated *C. camelliae* ascospores on *C. transnokoensis* petal tissue 20 h after inoculation. as = ascospores; sgt = surface germ tubes.

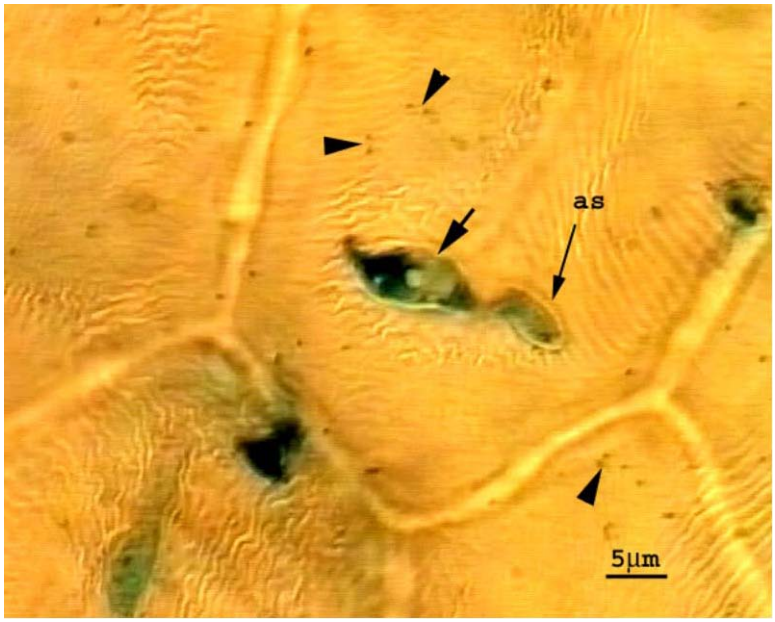


Plate 6.8. Light microscopic image of unusual swelling (short arrow) of penetrated germ tube of *C. camelliae* in *C. tricocarpa* petal 24 h after inoculation. Considerable number of microbes (arrow head) also observed on the petal surface.

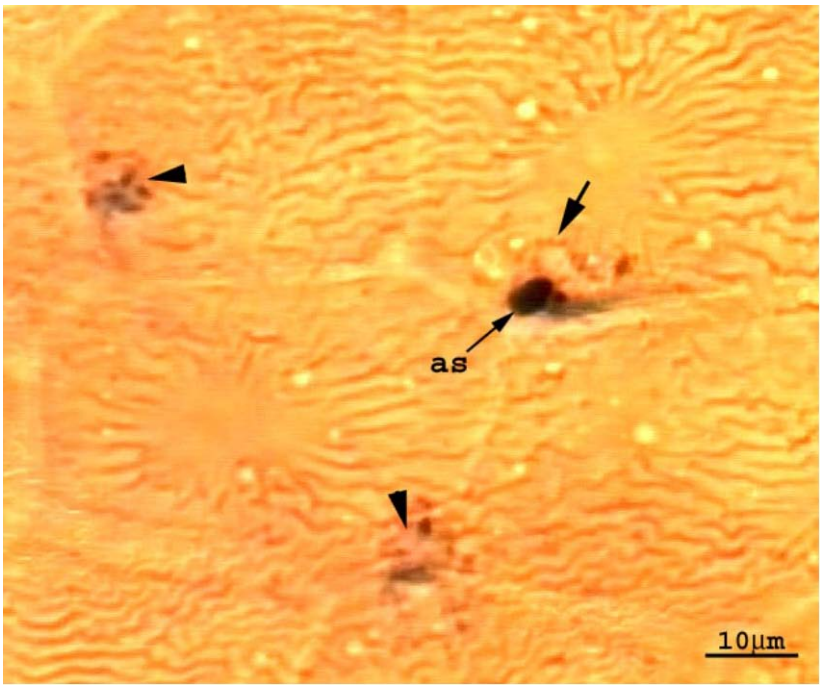


Plate 6.9. Light microscopic image of distorted ascospores of *C. camelliae* on *C. polyodonta* petal tissue 24 h after inoculation (arrow heads). Unidentified microbes observed around ascospores (arrow) and on the surface of the petal. as = ascospores.

6.5. Discussion

There appear to be more than one resistance mechanism functioning within the genus *Camellia*, or even within a single species. There are many reports of papilla formation around penetration sites of fungal pathogens. Sherwood and Vance (1976) and Vance and Sherwood (1976a and 1976b) observed papilla formation on reed canarygrass leaves during the initial stage of penetration by *Helminthosporium avennae*. Cycloheximide treatment inhibits the formation of these lignified papillae with the results that canarygrass tissue becomes susceptible to *Helminthosporium avennae* that normally do not infect the plant (Vance and Sherwood 1976a and 1976b). The evidence for papilla formation in relation to growth of *O. brassicae* penetration tubes (Aist and Israel 1977a) and growth of *E. graminis hordei* penetration pegs (Aist and Israel 1977b) is similar to that presented here for papilla formation in some *Camellia* species as a response to attempted fungal penetration. The papillae found in some camellia petals in this study were not investigated chemically for their composition but there is no reason to think they would differ from the callose and lignin found in other plants. Lignification is a common response to infection or wounding

in plants (Asada and Matsumoto 1967, 1972; Birecka et al. 1975; Ride 1975) and is resistant to degradation by most fungi (Kirk 1971). Further work is requested to conform that lignin and/or suborin is synthesized locally in epidermal cell walls of some *Camellia* species in response to attempted penetration by *C. camelliae* germ tubes as these could be an important selection criterion for camellia breeders.

Localised cell necrosis as a reaction to pathogen infection indicated that some *Camellia* species develop a hypersensitive reaction presumably by accumulating of phytoalexins within the cells. Generally this type of reaction is effective against biotrophic fungi, which need living host cells (Heath 1984). The study on *C. camelliae* infection process in Chapter 5 suggested that *C. camelliae* has biotrophic Characteristics at least in the initial stage of infection. Thus a hypersensitive response could be effective against this pathogen.

In normal conditions, *C. camelliae* ascospores germinate and the germ tubes immediately penetrate the petal cuticle within 6 h and for a further 72 h, growth continues as an intercellular pathogen within the host tissue (Chapter 5). In this investigation, the pathogen formed multiple germ tubes and germ tubes elongated over the petal surface without penetrating in some *Camellia* species. In such cases, the fungal hyphae were distorted. This unusual behaviour on certain *Camellia* species suggests that the cuticle may have cutin and wax layers which strongly prevent germ tube penetration, but may have substances that promote multiple germination of ascospores and germ tube growth on the host surface. Since the germ tubes were usually distorted, such substances are probably fungitoxic.

Large swollen germ tubes of *C. camelliae* were observed when 06L1Y yeast isolate was co-inoculated with ascospore suspensions on susceptible cultivars (Chapter 7). In this experiment, observation of yeast-like microorganisms and the large swollen hyphae of *C. camelliae* germ tubes on the petal surface of *C. tricocarpa* species suggests that the yeast-like organisms may produce antifungal materials that damage the fungal cell wall.

In summery, the some *Camellia* species express resistance to *C. camelliae* infection using different mechanisms: structural defense such as papilla formation, hypersensitive response and impenetrable cuticle layer with antifungal substance to divert the pathogen from penetration. In addition to these mechanisms some species have capability to harbour some microorganism that functions as biocontrol agents.

Chapter Seven

Biological control of camellia flower blight

7.1. Introduction

There is no completely effective control for flower blight of camellia yet. Generally preventive measures: a combination of sanitation, cultural practices, and fungicide treatments are practiced but due to labour requirement of sanitation and cultural practices, and the ineffectiveness of fungicide application, these methods have failed to give adequate control of *Ciborinia camelliae.*

Cultural practices include collection of infected flowers and burning. Baxter et al. (1983) suggested that a plastic sheet laid underneath a plant to collect the fallen flowers and removal of flowers lodged in branches would prevent apothecial development. This procedure may not be practical where the terrain is steep or many camellias are planted, and the growers unable or disinclined to follow the practice. Fungicides have been used in attempts to kill or inhibit sclerotia or apothecia in the soil or ascospores on flower petals but frequent applications are required as both apothecial formation and flowering continue throughout the season. Several fungicide were tested by various workers at different times, but none gave satisfactory control of the disease (Zummo 1960, Parsons 1964; Baxter and Fagan 1975; Baxter and Thomas 1992; Hotchkiss and Baxter 1995; Matsumoto 1995; Baxter 1996; Edwards 1996; Scheibert 1996; Baxter and Thomas 1998; Hotchkiss 1998). Good results obtained in one year may not necessarily be indicative of consistent control since there are fluctuations of disease level throughout the season and between years (Holcomb 1994). Holcomb (1977 and 1997) and Hotchkiss et al. (1997) obtained contradictory results in their fungicide trials due to this variation. Systemic fungicides are not tranlocated to the flower and are no more effective than protectants (Baxter and Thomas, 1994).

There is a growing interest in replacing synthetic chemical pesticides with biocontrol agents and one approach is to manipulate the epiphytic microflora on plant surfaces (Droby and Chalutz 1991). A yeast antagonist, *Debaryomyces hansenii* (Wisniewski et al. 1988) or a mixture of antagonists (Janisiewicz 1987, 1988) has been used to control *Botrytis* rot on

apple. The same yeast (Chalutz and Wilson 1989) and a bacterium, *Bacillus subtilis* (Singh and Deverall 1984) were used as biocontrol agents against citrus fruit disease. Fluorescent *Pseudomonas* isolates reduced disease severity of yam leaf blight (Michereff Filho et al. 1994). Two postharvest cherry diseases were controlled by two antagonistic bacteria, *Bacillus subtilis* and *Enterobacter aerogenes* (Utkhede and Sholberg 1986). Chalutz et al. (1988) used a yeast isolate as a biocontrol agent to control pepper and tomato fruit rot. Nectarine, pear and apricot fruit brown rot was controlled by antagonistic bacteria (Pusey and Wison 1984); *Botrytis* blight of rose was controlled by an antagonistic yeast (Redmond et al. 1987; Hammer and Marois 1989). Several bacteria, predominantly *Bacillus* spp. and yeasts such as *Aureobasidium* spp. were identified as biocontrol agents against *Colletotrichum gloeosporioides* on avocado fruit (Stirling et al. 1995).

An effective biological control agent may inhibit a plant pathogen by producing antibiotics, by successfully competing with the pathogen for nutrients and space, by inducing host resistance, or by interacting directly with the pathogen (Droby and Chalutz 1991). For detection of potential biocontrol agents, *in vitro* techniques in which suspected antagonist and pathogen are co-inoculated in an agar plate have been commonly used (Dhingra and Sinclair 1985). This technique is mostly based on antibiotic production or direct mycoparasitism and many antagonistic organisms have been selected on this basis. Baiting or attachment techniques have also been used to isolate phylloplane microbes. Cook et al. (1997a) developed a method to isolate yeast and bacterial microbes as biocontrol agents against *B. cinerea* using a microbial attachment assay. The microbes isolated by this method may have one or more of the action listed above in addition to attachment to the pathogens (Cook 1997).

Biological control studies suggest there is a time and quantitative relationship between a successful antagonist and pathogen populations. Antagonism has been frequently found when a BCA is co-inoculated simultaneously with the pathogen (Redmond et al. 1987; Roberts 1990; Janisiewicz and Marchi, 1992; Janisiewicz et al. 1994) and has also been observed when the BCA is applied five days ahead of the pathogen (Malathrakis and Kritsotaki 1992). The level of antagonism is increase increasing the antagonist population

(Janisiewicz 1988; Roberts 1990; Leifert et al. 1992a,b; Janisiewicz et al. 1994). Hence, maintenance of a successful BCA population in variable environmental conditions is a critical aspect of successful biocontrol in the field. Thus Leben et al (1965) found an epiphytic bacterium that effectively reduced cucumber anthracnose, early blight of tomato and southern blight of corn in the green house not the field. They demonstrated that this bacterium was not stable and did not withstand drying. Further, they postulated that increased disease control by addition of nutrient and moist treatment is not because of multiplication of bacterial cells but because more cells are viable at the time of pathogen inoculation.

The current work set out to evaluate the potential of microbes on almost surfaces for biocontrol of *Ciborinia camelliae*. In this study, bacteria and yeast were targeted as biocontrol agents against *C. camelliae* because 1) a technique for selective isolation of attacher biocontrol agents had already been developed at Massey (Cook 1997) and 2) there is already much information on industrial scale production of bacteria and yeasts from the brewing and pharmaceutical industries. In addition, the Lincoln University group are investigating the more general isolation of biocontrol agents.

7.2. Objectives

1. To isolate bacteria or yeast biocontrol agents against *C. camelliae* using an attachment assay.
2. To evaluate promising biocontrol agents against *C. camelliae* in the laboratory, in a glasshouse, and in the field.
3. To evaluate the ability of biocontrol agents to survive in various environments.
4. To identify the mode of action of the biocontrol agents.

7.3. Materials and Methods

The experiments in this Chapter were grouped into six Sections: 1) Isolation of biocontrol agents and *in vitro* examination, 2) *In vivo* screening of selected putative BCAs against *C. camelliae,* 3) Evaluation of effective concentration and time of application of selected putative BCAs, 4) Testing of two potential bacterial isolates for mode of action against *C.*

camellia, 5) Evaluation of survival ability of the two potential bacterial isolates in different environmental conditions and in a range of camellia cultivars and 6) Field evaluation of two potential bacterial isolates against *C. camelliae.* The materials and methods are detailed in the respective Sections or Experiments.

7.3.1. Section I. Isolation of biocontrol agents and *in vitro* examination

7.3.1.1. Experiment 7.1. Isolation of biocontrol agents using attachment assay and preliminary observation of biocontrol efficacy

7.3.1.1.1. Introduction

There are various method to isolates phylloplane microbes to use against pathogens. However, baiting techniques have predominantly been used to obtain mycolytic or attacher bacteria or yeast. Old and Patrick (1976) used filter membranes of different pore sizes to exclude all but bacteria-sized organisms. The fungal propagules were enclosed in the membranes and buried in the soil. Toyota & Kimura (1993) and Fradkin and Patrick (1985 a,b,c) used nylon gauzes instead of filter membrane for the same technique. Nesbitt et al. 1981 used agar disc with pre-colonised fungus and submerged them in water containing candidate microbes.

Attacher microbes have been isolated by various methods. Fradkin and Patrick, (1985 a,b) isolated the colonising microbes by vigorously washing the baited fungus in sterile distilled water or saline. Toyota & Kimura (1993) sonicated the colonised fungus and Homma (1984) immersed the baited fungi in liquid media. A pure culture has been obtained by repeated sub-culturing (Homma 1984). However, selection of attacher microbes depends largely on the removal of contaminants that do not attach firmly to the target fungal structure. Cook et al. (1997a) developed an apparatus and protocol for the isolation of bacteria and yeast with the ability of attach to germlings of *B. cinerea*. Their main aim was to minimise the microbial contamination by bacteria or yeast which do not attached to the pathogen, and they found that contamination could be reduced to 1-2%. Because of its efficient and consistent isolation ability this method was chosen to isolate

potential bacteria or yeast biocontrol agents against *C. camelliae*. The effective isolates selected by this method were used in subsequent experiments in this Chapter.

7.3.1.1.2. Materials and Methods

7.3.1.1.2.1 Extraction of microbes from substrate

Four mature, fully expanded camellia leaves and four petals each from individual fully opened flowers were collected from various part of North island of New Zealand. Soil samples were collected from Massey University Arboretum and Wellington Botanical Garden under camellia plants (Table 7.1). The samples (four leaves or four petals) were placed in a plastic bag (15 cm x 10 cm) and washed with 20 ml sterile distilled water (SDW) by shaking and gently massaging for 2 min. The washings were poured into 25 mm diameter filter holders and vacuum filtrated through a 0.2 :m filter (Sartorius SM 113 07 025N). For soil samples, 10 g soil was mixed thoroughly with 50 ml SDW and shaken for 2 min before pre-filtering with Whatman # 1 filter paper to remove large soil and organic debris prior to 0.2 :m filtering as above. The residues were re-suspended in 10 ml SDW to prepare possible microbes suspension from the samples and were stored at 4^{o}C until required.

7.3.1.1.2.2 Co-inoculation of extracted microbes and C. camelliae

Healthy flower petals from fully opened flowers of cultivar 'Brian' were laid in 50 mm diameter glass petri dishes. After autoclaving (5 min at 121^{o}C), 500 µl of SDW was pipetted over the autoclaved petal in the petri dish and a sterile 50 mm diameter disk of cellophane (Jam & Preserve Covers, Caxton Ltd Christchurch) placed over the petal. Three replicate plates of the petal-cellophane media were prepared for each field samples. They were inoculated with 50 µl of *C. camelliae* ascospores suspension prepared as described in Chapter 2 and incubated for 12 h at 20^{o}C before adding 950 µl of microbes extract. Plates were then incubated at 15^{o}C for another 16 h. After incubation, the cellophane with *C. camelliae* and the microbes was suspended in 5 ml McIlvaines buffer (pH 6.0) and agitated with a bend glass rod to release the colony into the buffer solution.

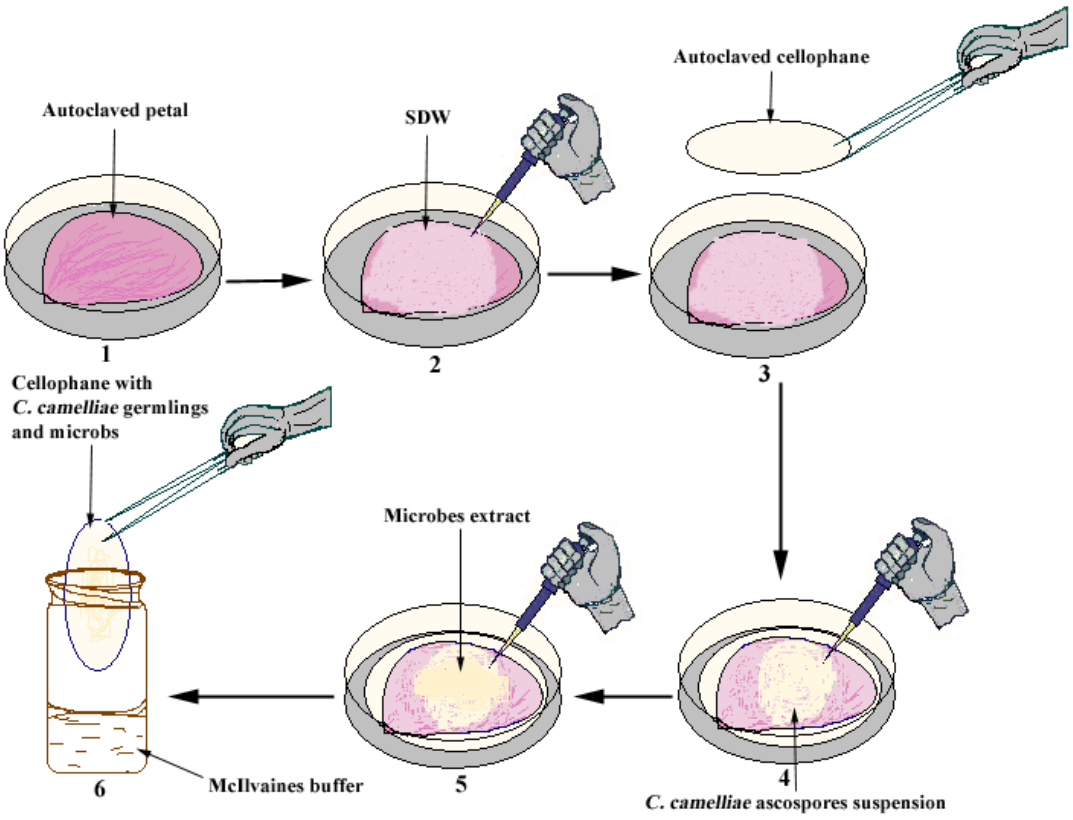


Figure 7.1. Co-inoculation of extracted microbes and *C. camelliae* ascospores. **1**. Autoclaved petri dish with petal. **2**. Pipetted 500 µl of SDW over the autoclaved petal. **3**. Placed 50 mm diameter disk of cellophane over the petal. **4**. Pipetted 50 µl of *C. camelliae* ascospores suspension over the cellophane and incubated for 12 h at 20^{o}C. **5**. Pipetted 950 µl of microbes extract over the inoculated ascospores and then incubated again at 15^{o}C for another 16 h. **6**. Placed Cellophane with *C. camelliae* and the microbes was suspended in 5 ml McIlvaines buffer in a universal bottle.

Table 7.1. Samples of camellia leaf, flower petals and soil from under camellia trees used for BCAs isolation.

Serial No.	ID No.	Year of collection	Place of collection	Cultivars	Plant parts	Condition of flower
1	WLD1	1999	Wanganui	Unknown	leaf	diseased
2	WPD1	1999	Wanganui	Unknown	petal	diseased
3	WLND2	1999	Wanganui	Unknown	leaf	No diseased
4	WPND2	1999	Wanganui	unknown	petal	No diseased
5	WLND3	1999	Wanganui	unknown	leaf	No diseased
6	WPND3	1999	Wanganui	unknown	petal	No diseased
7	WLD4	1999	Wanganui	unknown	leaf	diseased
8	WPD4	1999	Wanganui	unknown	petal	diseased
9	WLND5	1999	Wanganui	unknown	leaf	No diseased
10	WPND5	1999	Wanganui	unknown	petal	No diseased
11	RuLND1	1999	Ruphehu	unknown	leaf	No diseased
12	RuPND1	1999	Ruphehu	unknown	petal	No diseased
13	RoLND1	1999	Roturua	unknown	leaf	No diseased
14	RoPND1	1999	Roturua	unknown	petal	No diseased
15	RoLND2	1999	Roturua	unknown	leaf	No diseased
16	RoPND2	1999	Roturua	unknown	petal	No diseased
17	HLND1	1999	Hamilton	Rebel viney	leaf	No diseased
18	HPND1	1999	Hamilton	Rebel viney	petal	No diseased
19	HLND2	1999	Hamilton	Moshio	leaf	No diseased
20	HPND2	1999	Hamilton	Moshio	petal	No diseased
21	HLND3	1999	Hamilton	Elegans	leaf	No diseased
22	HPND3	1999	Hamilton	Elegans	petal	No diseased
23	HLND4	1999	Hamilton	Nicky Crisp	leaf	No diseased
24	HPND4	1999	Hamilton	Nicky Crisp	petal	No diseased
25	CLND1	1999	Cambridge	unknown	leaf	No diseased
26	CPND1	1999	Cambridge	unknown	petal	No diseased
27	WeLND1	1999	Wellington	unknown	leaf	No diseased
28	WeLND2	1999	Wellington	unknown	leaf	No diseased
29	WePND2	1999	Wellington	unknown	petal	No diseased
30	00L	1999	Massey	Fairy Blush	leaf	No diseased
31	00P	1999	Massey	Fairy Blush	petal	No diseased
32	00S	1999	Massey	Fairy Blush	soil	No diseased
33	01L	1999	Massey	Night Rider	leaf	No diseased
34	01P	1999	Massey	Night Rider	petal	No diseased
35	01S	1999	Massey	Night Rider	soil	No diseased
36	02L	1999	Massey	unknown	leaf	No diseased
37	02P	1999	Massey	unknown	petal	No diseased
38	02S	1999	Massey	unknown	soil	No diseased
39	03L	1999	Massey	*C. salicifolia*	leaf	No diseased
40	03P	1999	Massey	*C. salicifolia*	petal	No diseased

41	03S	1999	Massey	*C. salicifolia*	soil	No diseased
42	04L	1999	Massey	*C.transnokoensis*	leaf	No diseased
43	04P	1999	Massey	*C.transnokoensis*	petal	No diseased
44	04S	1999	Massey	*C.transnokoensis*	soil	No diseased
45	05L	1999	Massey	*C. lutchuensis*	leaf	No diseased
46	05P	1999	Massey	*C. lutchuensis*	petal	No diseased
47	05S	1999	Massey	*C. lutchuensis*	soil	No diseased
48	06L	1999	Massey	*C. grijsii*	leaf	No diseased
49	06P	1999	Massey	*C. grijsii*	petal	No diseased
50	06S	1999	Massey	*C. grijsii*	soil	No diseased
51	07L	1999	Massey	*C. cuspidata*	leaf	No diseased
52	07P	1999	Massey	*C. cuspidata*	petal	No diseased
53	07S	1999	Massey	*C. cuspidata*	soil	No diseased
54	08L	1999	Massey	*C. fraterna*	leaf	No diseased
55	08P	1999	Massey	*C. fraterna*	petal	No diseased
56	08S	1999	Massey	*C. fraterna*	soil	No diseased
57	09L	1999	Massey	*C. forrestii*	leaf	No diseased
58	09P	1999	Massey	*C. forrestii*	petal	No diseased
59	09S	1999	Massey	*C. forrestii*	soil	No diseased
60	Wel1L	2000	Wellington	E.G. Waterhouse	leaf	No diseased
61	Wel1P	2000	Wellington	E.G. Waterhouse	petal	No diseased
62	Wel2L	2000	Wellington	Brian	leaf	No diseased
63	Wel2P	2000	Wellington	Brian	petal	No diseased
64	Wel3L	2000	Wellington	unknown	leaf	No diseased
65	Wel3P	2000	Wellington	unknown	petal	No diseased
66	Wel4L	2000	Wellington	Waiwhetu beauty	leaf	No diseased
67	Wel4P	2000	Wellington	Waiwhetu beauty	petal	No diseased
68	Wel5L	2000	Wellington	Magnoliaeflora	leaf	No diseased
69	Wel5P	2000	Wellington	Magnoliaeflora	petal	No diseased
70	Wel6L	2000	Wellington	Lurie bray	leaf	No diseased
71	Wel6P	2000	Wellington	Lurie bray	petal	No diseased
72	Wel7L	2000	Wellington	*C. transnokoensis*	leaf	No diseased
73	Wel7P	2000	Wellington	*C. transnokoensis*	petal	No diseased
74	Ma1L	2000	Massey	unknown	leaf	No diseased
75	Ma1P	2000	Massey	unknown	petal	No diseased
76	Ma2L	2000	Massey	Highlight 80	leaf	No diseased
77	Ma2P	2000	Massey	Highlight 80	petal	No diseased
78	Ma3L	2000	Massey	unknown	leaf	No diseased
79	Ma3P	2000	Massey	unknown	petal	No diseased
80	Ma4L	2000	Massey	unknown	leaf	No diseased
81	Ma4P	2000	Massey	unknown	petal	No diseased

7.3.1.1.2.3 Extraction of attacher microbes

Microbes attached to germ tubes and ascospores of *C. camelliae* were extracted using the attachment assay procedure of Cook et al. (1997a). A crude mixture of *C. camelliae* germlings and microbes in McIlvaines buffer was poured into a 25 mm diameter polycarbonate filter holder (Sartorius, SM 16517E) fitted with 25 mm diameter sterile nylon mesh with no filter supports. The filter holder had an enlarge top with 10 mm diameter polyethylene syringe barrel which then connected to a 10 mm diameter glass tube. Before pouring the crude mixture, 50 ml of McIlvaines buffer wash solution was pour through the entire apparatus and the eluent collected. The same process was repeated three times. After this, the wash chamber was detached from the glass column and the crude mixture added and immediately drained. With the outlet blocked, 5ml of previously used washed solution was added and the wash chamber was inverted four times, drained-off and re-assembled to the glass column. The remaining 45 ml of wash solution was added to the column and drained through the filter. The remaining buffer was flicked out of the holder then the mesh with microbes attached with *C. camelliae* germlings was aseptically removed and placed into sterile 25 ml nutrient broth (NB) or nutrient yeast dextrose broth (NYDB) in 50 ml conical flask and incubated on an orbital shaker (Certomat M B Braum, Melsungen AG) set at 150 rpm at 15^{o}C for 48 h so that the microbes were released into the media during incubation.

After incubation, the broth was first filtered through 70 µm cell strainers (Falcon 2350) to remove *C. camelliae* germlings and mycelium then filtered through a 0.2 µm filter to trap bacteria and yeast. The trapped bacteria and yeast in the filter were re-suspended in 25% glycerol and stored at –20^{o}C until required.

7.3.1.1.2.4 Microscopic observation of attachment

A portion of bacteria and yeast mixture of each sample on the filter above were re-suspended in 500 µl of SDW and vortexed to co-inoculate with *C. camelliae* to observe microbial attachment. *C. camelliae* ascospores were co-inoculated with 200 µl of mixture from each sample on sterile cellophane laid on NA or NYDA media in a petri plate and incubated as described in Section 7.3.1.1.2.2. After incubation, a piece of cellophane with

C. camelliae germlings and microbes was removed and placed on a slide. They were stained with trypan blue and observed under a light microscope. In another method, the mixture was co-inoculated with *C. camelliae* ascospores to one-tenth strength NB or NYDB. The broth was incubated at 15^{o}C for 24-36 h. After incubation, a drop of broth with *C. camelliae* germlings and microbes was mounted onto a microscope slide and observed under dark field or phase contrast microscopy.

7.3.1.1.2.5. Observation of initial biocontrol efficacy with crude mixture of extract against C. camelliae

The remaining suspension used for attachment microscopic observation was co-inoculated with *C. camelliae* ascospores to camellia flower petals. The microbe concentration was adjusted to 1x10^{6} or 1x10^{7} per ml and the ascospore concentration was 5x10^{4}. Both microbe and ascospore suspensions were inoculated by pipetting 100 µl of each suspension per site. Two sites were inoculated per petal. After inoculation the petals were incubated at 20^{o}C for 48 h to observe any symptom development which was scored on a 0-4 scale as described in Chapter 4.

7.3.1.1.2.6. Isolation of bacteria and yeast from mixed culture of attachment assay

A 500µl aliquot from each sample containing of mixture of attacher microbes was pipetted and spread with a bend glass rod onto NA or NYDA and incubated at 15^{o}C for 3 days. After incubation, individual colonies were examined for colour, colony shape and form. Bacterial and yeast colonies were differentiated by observing the cultures under a compound microscope. Each bacterial and yeast colony was re-isolated until uniform colonies were consistently obtained. Bacterial contaminants in the yeast culture were eliminated by subsequent culture on NYDA amended with 100-200 mg/litre of chloramphenicol (BDH 443042Q, Hagler & Ahearn, 1987). All bacteria and yeast isolates were stored at –20^{o}C in 25% glycerol until required.

7.3.1.1.3. Results

A total of 81 field samples of leaves, petals, and soil from under camellia plants were collected. Attachment microbes were observed in 20 field samples (Plate 7.1), of which, 15

samples were taken from Massey University on October 1999, and 5 from Wellington on September 2000. These mixtures did not show significant control of lesion development but most of the mixtures kept the lesion scale at 3, two mixtures at 2 and one at 1 in the initial test carried out with *C. camelliae* (Table 7.2). None of the microbes from first batch of samples collected from Wanganui, Hamilton, Cambridge, Rupehu, Rotorua or Wellington on September 1999 showed any attachment to *C. camelliae* germlings.

Six yeast and 13 bacterial isolates were isolated from the colonies of the crude mixture that showed attachment to *C. camelliae* germlings and resulted in a reduced lesion size.

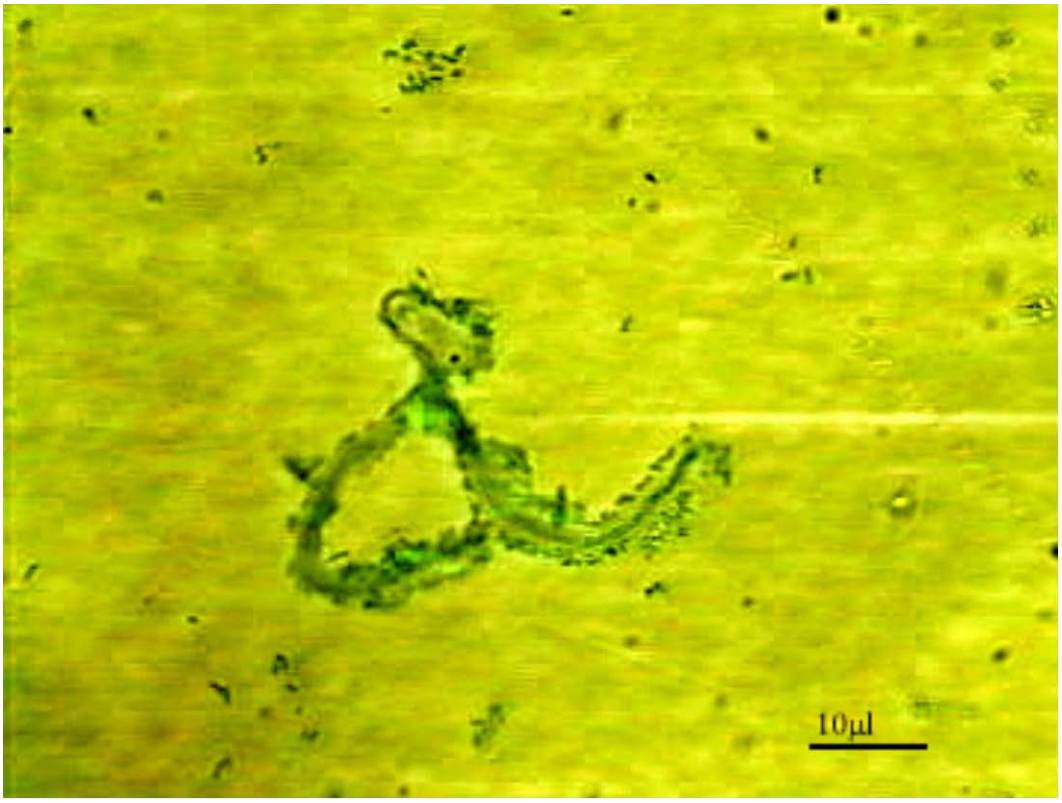


Plate 7.1. Light microscopic image of microbial attachment to ascospore germlings on cellophane cultures observed by light microscopy.

Table 7.2. Attachment observation and disease assessment of microbes isolated from leaves and flower samples of camellia and the soil under camellia trees located at Massey University, Palmerston North and at camellia gardens in Wellington, Wanganui, Ruphehu, Roturua, Hamilton and Cambridge. Samples collected during camellia flowering season in 1999 and 2000. (y = attachment observed, n = no attachment observed, Sample No. corresponding to the sample's serial number in Table 7.1). Lesion assessment was made 48 h after *C. camelliae* was co-inoculated with BCA crude mixture on camellia petals, based on the scale 0-4 described in Chapter 4.

Sample No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Attachment	n	n	n	n	n	n	n	n	n	n	n	n
Disease assessment	4	4	4	3	4	4	3	3	3	4	4	4
Sample No.	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Attachment	n	n	n	n	n	n	n	n	n	n	n	n
Disease assessment	4	4	3	4	4	4	4	4	3	3	3	4
Sample No.	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**
Attachment	n	n	n	n	n	n	y	n	y	n	n	n
Disease assessment	4	3	3	3	3	2	3	4	3	4	3	4
Sample No.	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**
Attachment	n	n	y	y	y	y	y	y	y	y	n	y
Disease assessment	3	3	4	3	3	3	3	2	3	3	3	3
Sample No.	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
Attachment	y	n	y	y	n	y	n	n	y	n	n	y
Disease assessment	3	4	1	2	4	3	4	4	3	3	3	3
Sample No.	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**
Attachment	n	y	y	y	n	n	y	n	n	n	n	n
Disease assessment	4	4	4	4	3	3	4	3	3	4	3	4
Sample No.	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**			
Attachment	n	n	n	n	n	n	n	n	n			
Disease assessment	3	3	4	4	3	3	4	3	3			

7.3.1.2. Experiment 7.2. Observation of biocontrol function(s) of selected bacteria and yeast cultures *in vitro*

7.3.1.2.1. Introduction

To optimise BCA application, it is important to identify the biocontrol functions responsible for plant protection (Deacon and Berry 1993; Wilson and Wisniewski 1989; Blackman and Fokkema 1982) in order to accurately interpret the reasons for success or failure (Nelson and Maloney, 1992; Andrews 1990, 1992). Wilson and Wisniewski (1989) emphasised that such information is required for appropriate and improved future BCA selection and it is also necessary for product registration requirements (Klingauf 1995; Woodhead et al. 1990; Forsyth 1990).

There are a variety of interactions between plants, pathogens and biocontrol agents. Antibiosis: the inhibition of one microbe by the extracellular product of another (Cook and Baker 1983). Siderophore production, the production of compounds that chelate ferric iron and make them unavailable to the pathogen thus resulting in less pathogen infection and some level of biocontrol (Kloepper 1992). Parasitism, characterised by intimate contact between pathogen and antagonist and competition generally for nutrients for survival or other activities of the pathogen. These are the methods generally studied for antagonism *in vitro* using relatively simple methodologies. However, antibiosis, siderophore production and parasitism are easily observed on agar media. The source of competition cannot be observed but is assumed when none of the above is detected. Sutton (1995) suggested the mode of antagonism for an isolate of *Gliogladium roseum* was competition because antibiosis and parasitism were not observed *in vitro* and there were no specific activities exposed by the biocontrol agents of mutant isolates.

Other biocontrol functions such as induced host resistance, plant growth stimulation, cross protection, physical restriction of pathogen growth (Elad 1990) alteration of the wetability of the plant surface and interference with pathogenicity process (Elad 1996) are more difficult to identify and require more complex methodologies. In this experiment the isolated BCAs were tested for putative biocontrol functions detectable on agar media such as anti-*Ciborinia* compounds and siderophore production.

7.3.1.2.2 Materials and Methods

7.3.1.2.2.1 Preparation of cells and spores suspension

Bacterial isolates 01L1B, 04L1B, 05L1B, 06L1B, 07L1B, 08L1B, 09L1B, 00P1B, 03P1B, 04P1B, 05P1B, 07P1B, 04S2B and yeast isolates 04L3Y, 05L1Y, 06L1Y, 06L2Y, 06L3Y and 06L4Y were cultured in NA and NYDA by streaking a loopful of stock suspension of each isolate and incubated at 20^{o}C for 2-3 days. After incubation the cell suspensions were prepared as described in Chapter 2. Cell counts were taken and the concentration was adjusted to 1x10^{7} cells /ml before application. *C. camelliae* ascospores were prepared as described in Chapter 2 and the ascospores concentration adjusted to 1x10^{4} spores/ml.

7.3.1.2.2.2 Antibiotics and parasitism

C. camelliae mycelium only grew successfully on Difco PDA medium (Taylor and Long 1998) and this medium was selected to test BCAs *in vitro*. PDA (15 ml) was poured into a 9 cm diam petri plate and when set, a 5 mm diam agar plug was cut and removed about 15 mm away from the rim of the plate using a cork borer. The hole was filled with NB or NYDB solution and inoculated with a loopful of bacterial or yeast suspension. A 5 mm plug of *C. camelliae* mycelial growth cut from a 10-15 days old culture was placed about 15 mm away from the opposite edge of the plate. There were three replicate plates per isolate. *C. camelliae* alone was used as a control for comparison. The inoculated plates were incubated at 15^{o}C for 5-7 days.

After incubation, each plate was examined for any interaction at the edge of *C. camelliae* and BCA colonies and the distance of any inhibition zone as measured. If any inhibition was observed, the plates were photographed using Nikon camera HFX IIA.

7.3.1.2.2.3 Siderophore production

Bacteria and yeast isolates were pipetted (10 µl) on to King's B medium to detect siderophore production. The plates were incubated at 15^{o}C for 5 days and examined every day under UV light (UVP, san Gabriel, California or UV transilluminator, UVP inc. San Gabriel Ca USA – Cambridge UK) for a fluorescent zone around each colony.

7.3.1.2.3 Results

After 5-7 days incubation at 15°C, diffusible anti-*Ciborinia* substances were not detected from any isolates except 07L1B and 04S2B where a clear inhibition zone was observed between the isolate and *C. camelliae* mycelium (Plate 7.2). *C. camelliae* hyphae grew over the colonies of the other isolates tested without any indication of growth restriction or morphological changes.

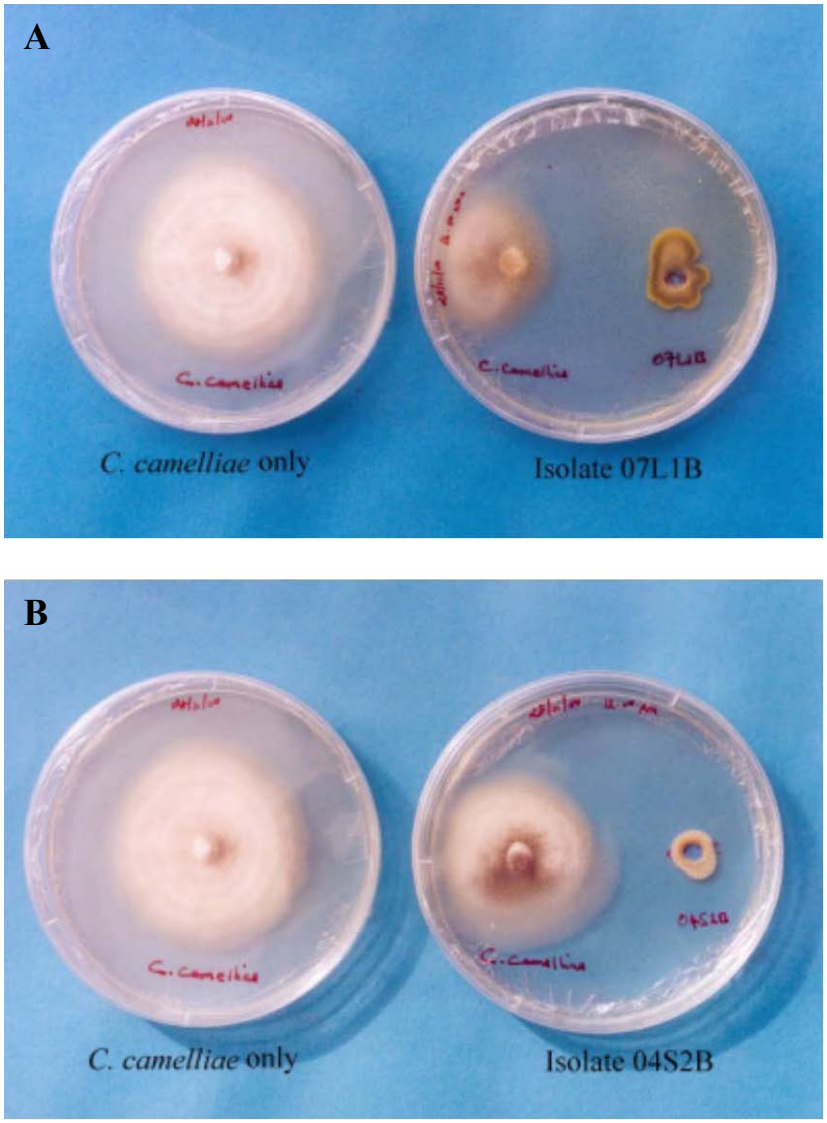


Plate 7.2. *C. camelliae* mycelial growth and inhibition by isolates 07L1B (A) and 04S2B (B) in dual culture on PDA.

Siderophore production was detected in isolates 05P1B, 07L1B and 04S2B within the first 24 h incubation at 15^{o}C (Plate 7.3) but fluorescent zones were not observed from any other isolates even after a further two days incubation.

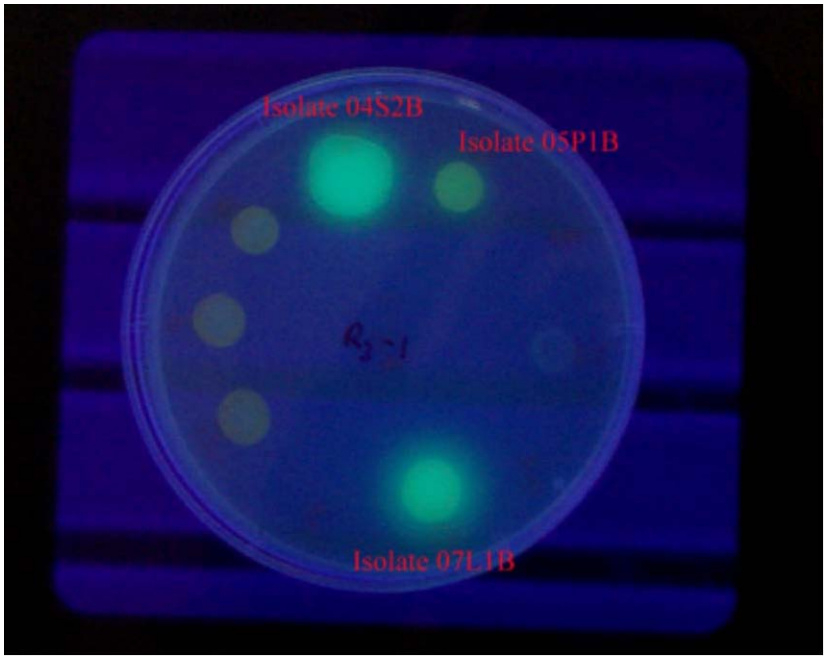


Plate 7.3. Siderophore production in King's B medium. Isolates 04S2B and 07L1B show bright fluorescence and isolate 05P1B fluoresces slightly, and other isolates did not fluoresce. The plates were observed over a UV transilluminator.

7.3.1.3. Preliminary test of isolates 07L1B and 04S2B for identification

Preliminary identification of two isolates (07L1B and 04S2B) was conducted with following test.

1. Dark field microscopy observation
2. Colony formation in nutrient agar plates. Observed colour and shape.
3. Gram's staining test
4. King's medium B cultures for fluorescence

Microscopy observation showed that both isolates are short rods. Smooth white colonies formed on the nutrient agar. Gram's staining test showed that they are gram negative. Both isolates fluoresce on King's medium B culture. These results indicates that they are probably fluorescent pseudomonads.

7.3.2. Section II. *In vivo* screening of selected potential BCAs against *C. camelliae*

7.3.2.1. Introduction

There are no published reports of testing biocontrol agents against *C. camelliae* although a number of fungi, bacteria and yeast have been tested against other phylloplane pathogenic fungi. For example, *B. cinerea* was tested with various biocontrol agents for control of several diseases on a range of hosts *in vivo* (Wilson and Wisniewski 1989, Dubos 1992, Droby and Chalutz 1994 and Cook 1997).

It is easy and economical to screen many antagonists using an *in vitro* agar plate technique but this assay is a poor predictor of biocontrol *in vivo* or in the field (Elard 1990; Andrews 1990). However, the many reports of *in vitro* screening against plant pathogens (Sharma and Saxena 2001; Sarathchandra et al. 1993) reflect its easiness and low cost for screening many antagonists at a time. Some more recent biocontrol experiments have been conducted where phylloplane conditions were reproduced on detached leaves, stems, petals or whole flowers, or whole fruit tissues. For example, Cook et al. (1997b) used tomato stems to screen attachment biocontrol agents against *B. cinerea* and Fiddaman et al. (2000) were used lettuce leaf disc to screen antagonistic bacteria against *B. cinerea* and *Rhizoctonia solani*. However, maintenance of plant tissue turgor, particularly for leaves and petals in this technique is critical (Cheah et al. 1996) thus a high humidity environment must be maintained during the assay (Sutton and Peng 1993; Elad et al. 1994a, b). Kohl et al. (1995) using a wet/dry incubation regime on dead lilly leaves, selected isolates of *Ulocladium atrun* that consistently showed biocontrol against *B. cinerea* in field conditions.

In this section, experiments were conducted with detached camellia petals to screen selected bacteria and yeast biocontrol agents with an attachment assay under high humidity and controlled temperature to evaluate their mechanisms of action and evaluate them under optimum conditions.

7.3.2.2. General Materials and Methods

7.3.2.2.1. Preparation of potential BCA cell and ascospores suspension

Pure cultures of 6 yeast and 13 bacterial isolates, and two bacterial isolates (OX8a and OX2) from Cook (1997) that showed biocontrol against *B. cinerea* were sub-cultured in NYDA and NA media respectively by pipetting a 100 µl aliquot of each culture on to the media and spreading with a sterile bent glass rod. They were incubated at 15^{o}C for 3 days as described in Chapter 2. After incubation, the colonies were removed by repeated pipetting of 5 ml sterile distilled water over them and the resultant cell suspension was collected in a universal bottle. Cell counts were made as described in Chapter 2. An ascospores suspension was prepared as described in Chapter 2 and the concentration was adjusted to $5x10^4$ for pipette inoculation and to $1x10^5$ for spray inoculation.

7.3.2.2.2 Inoculation and incubation

BCA microbes were applied to healthy and to undamaged camellia petals of cultivar 'Brian' using a fine atomizer or a micropipette. *C. camelliae* ascospores were similarly applied (as described in Chapter 4) 1 h after BCA application or when the petals became surface dry. Sprays of ascospores and BCAs covered the entire petals while pipette inoculation was at a specific site. Inoculated petals were placed on a nylon mat (2 mm^2 net size) in a plastic tray moistened with a wet paper towel and were incubated for 72 h.

7.3.2.2.3. Experimental design

Experiments were conducted in a RCB or a split-plot design. All experiments had controls (Ascospores only, SDW only or BCAs only). Each experimental unit consisted of two or more petals and was replicated three times.

7.3.2.2.4. Assessment

After incubation for 20 h, a 25 mm^2 piece of petal tissue was cut from each of two petals in each treatment and stained with trypan blue as described in Chapter 2. The stained tissues were observed by light microscopy and the number of ungerminated and germinated spores, number of germ tube penetrations and the penetrated germ tube length were

recorded from three random microscopic fields piece. The average penetrated germ tube length was categorised as follows:

Scaling system for penetrated germ tube length:

0 = 0 µm	6 = 26-30 µm
1 = 1-5 µm	7 = 31-35 µm
2 = 6-10 µm	8 = 36-40 µm
3 = 11-15 µm	9 = 41-45 µm
4 = 16-20 µm	10 = >45 µm
5 = 21-25 µm	

Lesion size was measured using the 0-4 scale system described in Chapter 4 and the disease severity was estimated as percent petal area infected after incubation.

7.3.2.3. Experiment 7.3. Initial *in vivo* screening of selected 15 bacteria and 6 yeast against *C. camelliae*

7.3.2.3.1. Materials and Methods

Detached camellia petals were co-inoculated with *C. camelliae* ascospores and potential BCAs as described above. Five criteria were used to select effective BCAs against *C. camelliae*. 1) The lesion size (visibility) measured on a 0-4 scaling; 2) Total percent infected area of a petal; 3) spore germination; 4) germ tube penetration and; 5) Penetrated germ tube length (0-10 scale).

7.3.2.3.2. Results

Six bacterial isolates (07L1B, 08L1B, 04P1B, 05P1B, 07P1B and 04S2B), 4 yeast isolates (05L1Y, 06L1Y, 06L2Y and 06L4Y) and OX8a gave control significantly different from non-BCA controls with at least three of the above criteria (Fig. 7.2). These isolates were selected for further evaluation.

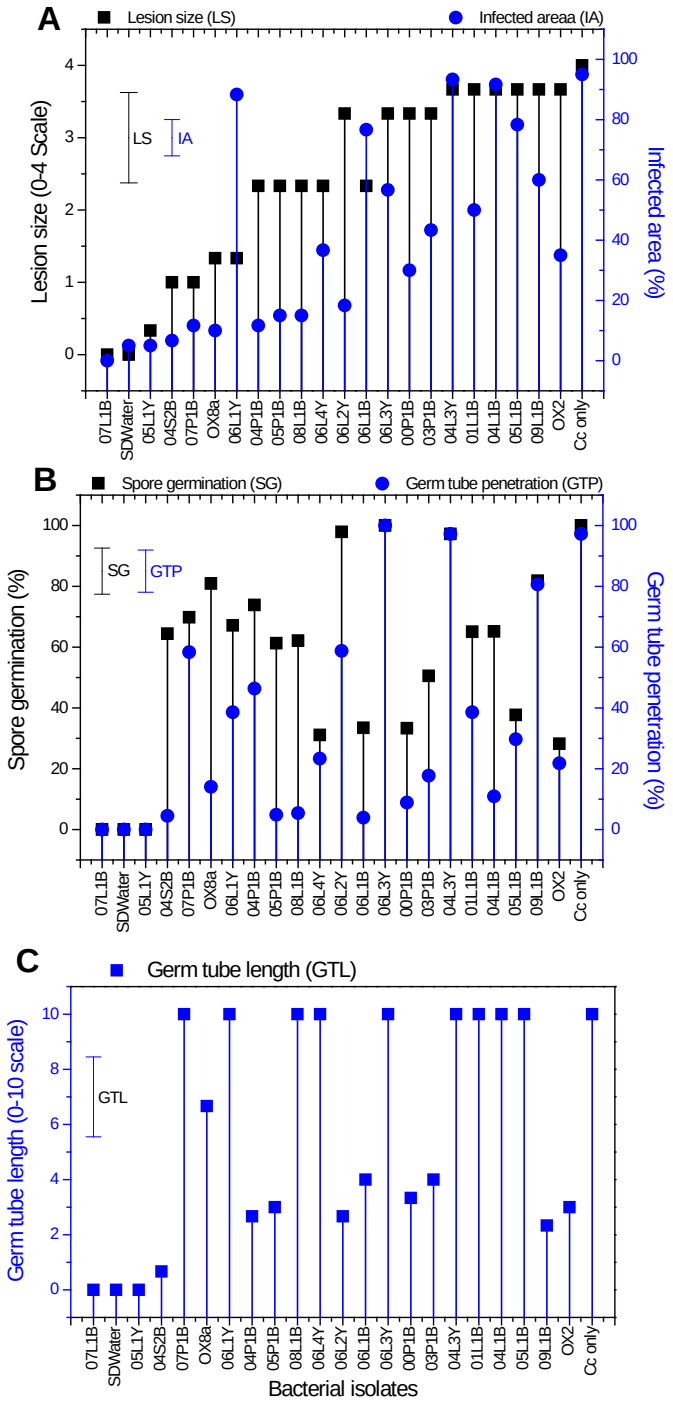


Figure 7.2. Bacterial isolates screened against *C. camelliae in vivo*. A) Lesion size estimated on a 0-4 scale (■) 66 h after incubation and infected petal area measured (●) 94 h after inoculation. B) Percentage spore germination (■) and germ tube penetration (●) calculated 20 h after incubation. C) Germ tube length estimated on a 0-10 scaling system (■) 20 h after incubation. Vertical bars represent LSD.

In addition to the above data, long penetration hyphae was observed, after 20 h incubation, in tissue treated with 3 yeast isolates (04L3Y, 06L3Y and 06L4Y) and 4 bacterial isolates (01L1B, 04L1B, 05L1B and 07P1B). Unusual, large, swollen structure of penetrated hyphae of *C. camelliae* was seen underneath the cuticle of the tissue treated with isolates 06L1Y (Plate 7.4).

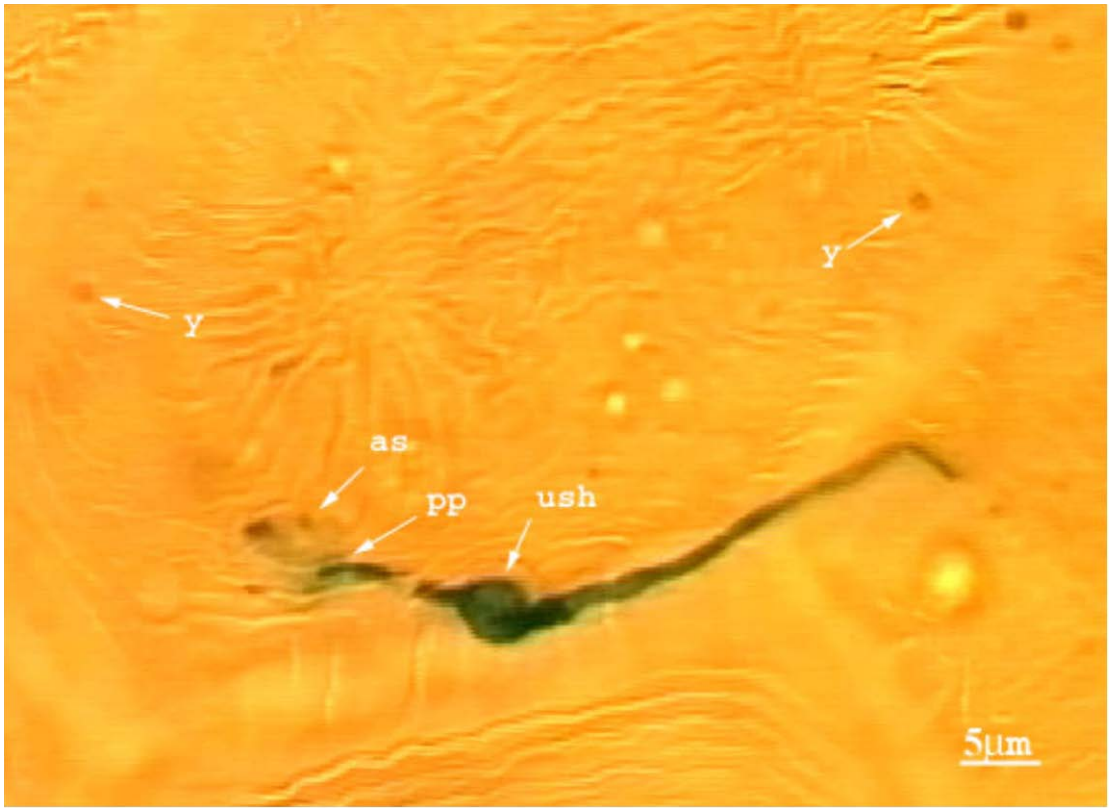


Plate 7.4. Light microscopic image of unusual swelling of *C. camelliae* penetrated germ tube in yeast isolate 06L1Y treated camellia petal tissue. as = ascospore; pp = penetration point; ush = unusual swollen hyphae; y = yeast isolates.

7.3.2.4. Experiment 7.4. A second *in vivo* screening of 7 bacteria including OX8a and 4 yeast against *C. camelliae* by spray and pipette inoculation

7.3.2.4.1. Materials and Methods

The selected isolates and *C. camelliae* ascospore suspension were prepared as described above. Two sites per petal were inoculated by pipette for pipette inoculation while spray

inoculation, petals were fully covered by inoculum droplets. Both inoculations were separately established in RCB design as described above.

Lesion size was estimated using the 0-4 scale, 50 h after inoculation and the maximum length of the lesion was measured 77 h after incubation from pipette inoculated petals, and percent-infected lesion area was estimated from spray-inoculated petals.

7.3.2.4.2. Results

In the pipette inoculation, petals treated with isolates 07L1B and 04S2B did not develop symptoms while those treated with all other isolates had symptom development (Plate 7.5). There was a lower lesion size in diameter 77 h after incubation observed on isolates 06L2Y and 07P1B treated petals but they were not significantly different from others except isolate 05P1B (Fig. 7.3A).

In the spray inoculation treatments, petals treated with 07L1B and 04S2B developed lesions but the percent area infected was significantly less than that in other treatments (Fig. 7.3B).

During BCA inoculation, the droplets of suspension from isolates 07L1B and 04S2B merged or spread over the petal surface soon after spray or pipette inoculation as though a wetting agent was present. This was not observed with other isolates.

The most promising isolates (07L1B and 04S2B) were further investigated on petals inoculated with ascospores by the gravitational method and with other two methods for comparison because under field condition, this is the only way infection takes place.

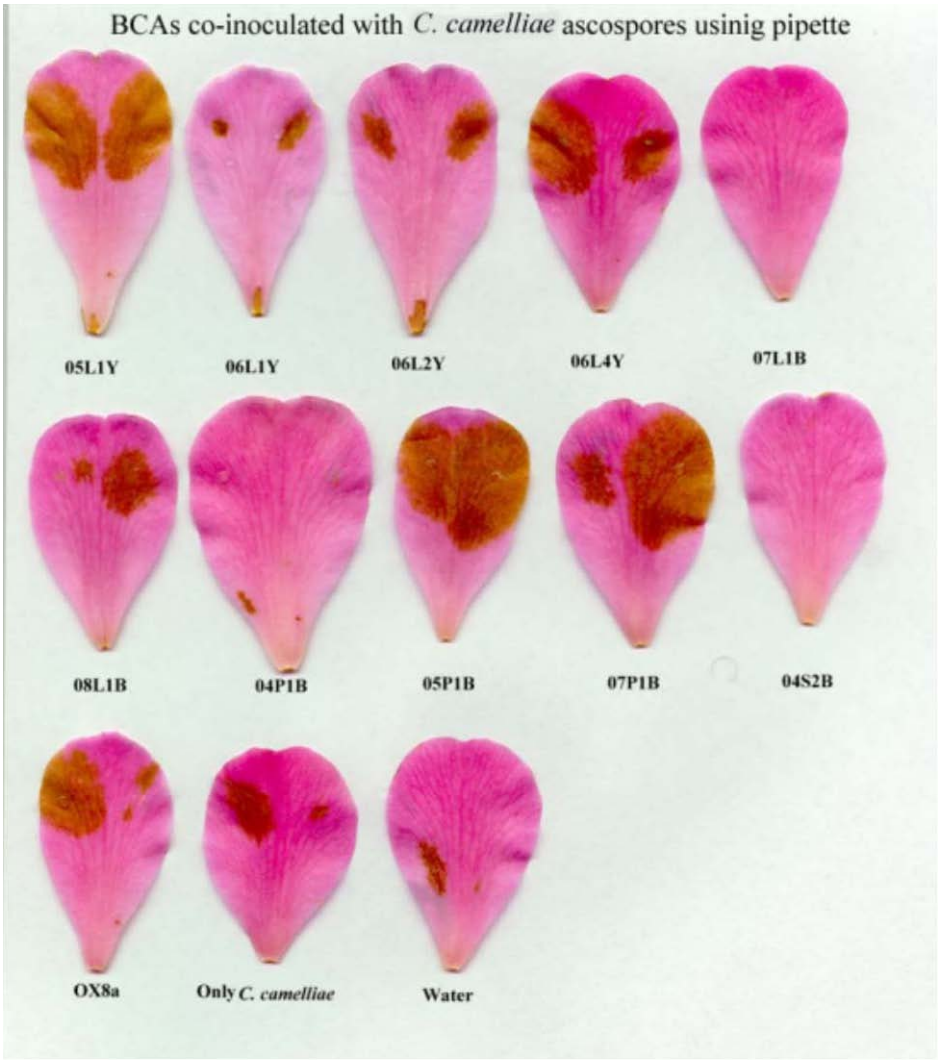


Plate 7.5. Lesion development following co-inoculation of BCAs and *C. camelliae* to camellia petals.

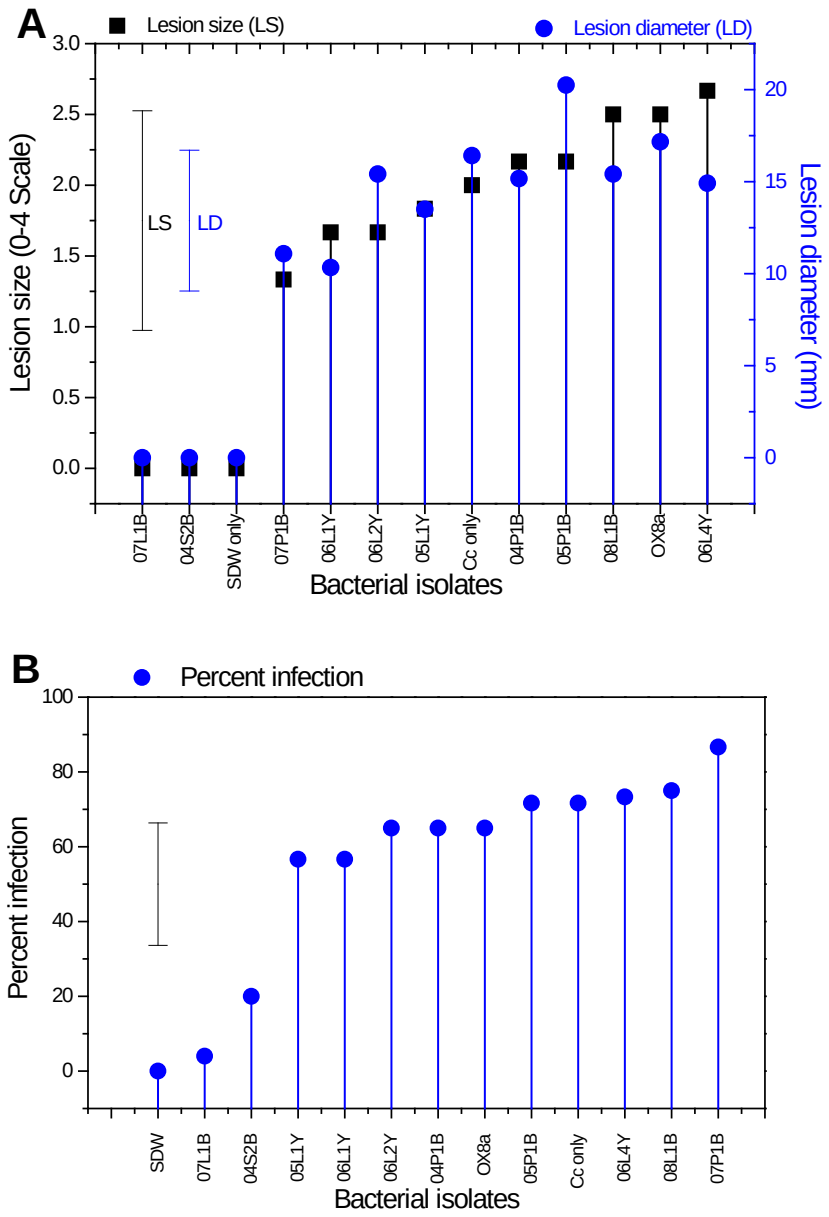


Figure 7.3. *In vivo* screening of biocontrol isolates against *C. camelliae*. A) Lesion size estimated on a 0-4 scale (■) 50 h after incubation and lesion diameter (●) 77 h after incubation on pipette-inoculated petals. B) Infected area (●) measured 50 h after incubation on spray-inoculated petals. Vertical bars represent LSD.

7.3.2.5. Experiment 7.5. Evaluation of bacterial isolates 07L1B and 04S2B on petals inoculated with ascospores by gravitation, spray and pipette

7.3.2.5.1. Materials and Methods

Isolates 07L1B and 04S2B were cultured, inoculum prepared and applied as described in Section 7.3.2.2 in this Chapter. Ascospores were co-inoculated with these isolates by either gravity, spray or pipette inoculation as described in Chapter 4. Lesion size was measured using 0-4 scale 48 h after incubation. Petal tissue samples (25 mm^2) were taken from inoculated sites after 38 h incubation and treated with glutaraldehyde for confocal microscopy as described in Chapter 2.

The experiment was set up as a split plot design inoculation method as main plot. Two potential BCAs (07L1B and 04S2B) were tested and there were three controls (ascospores only, BCAs only and SDW only). Each experimental unit consisted of 5 petals with three replications.

7.3.2.5.2. Results

The average lesion size over three incubation methods was significantly smaller in isolates 07L1B and 04S2B those in the controls but there was no significant different between the two isolates (Fig. 7.4A). Neither isolate induced symptoms showing that they are not phytopathogenic to camellia petals (Fig. 7.4 A&C). The method of inoculating ascospores significantly affected BCA efficiency. Lesion size was significantly lower in pipette and gravity inoculation of ascospores when they were spray inoculated (Fig. 7.4B). Isolate 07L1B gave significantly better control than 04S2B when the ascospores were gravity

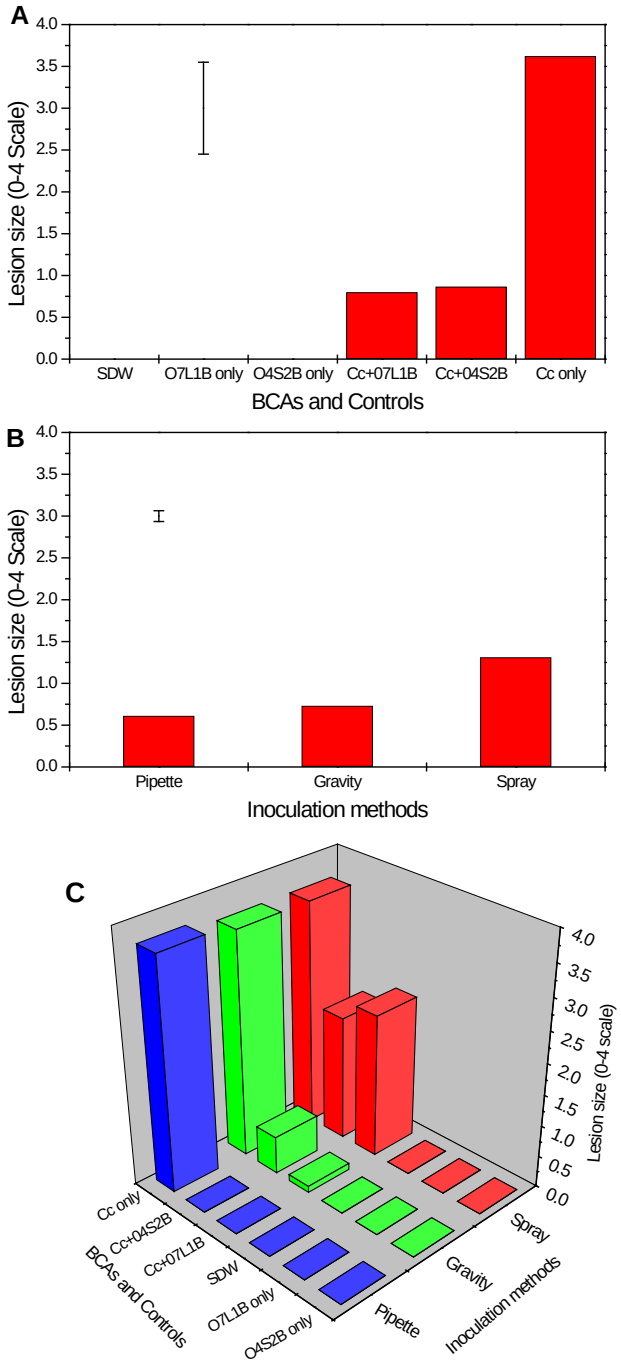


Figure 7.4. *C. camelliae* ascospores co-inoculated with isolates 07L1B or 04S2B by pipette, gravity and spray. A) Average lesion size measured over inoculation methods for BCAs and controls. B) Average lesion size measured over BCA and controls for inoculation methods. C) Interaction between BCAs and inoculation methods on lesion development. Vertical bar represents LSD.

inoculated (Fig. 7.4C) but there was total control of lesion development when ascospores and either BCA was pipette inoculated. Symptoms were more inconsistent on petals

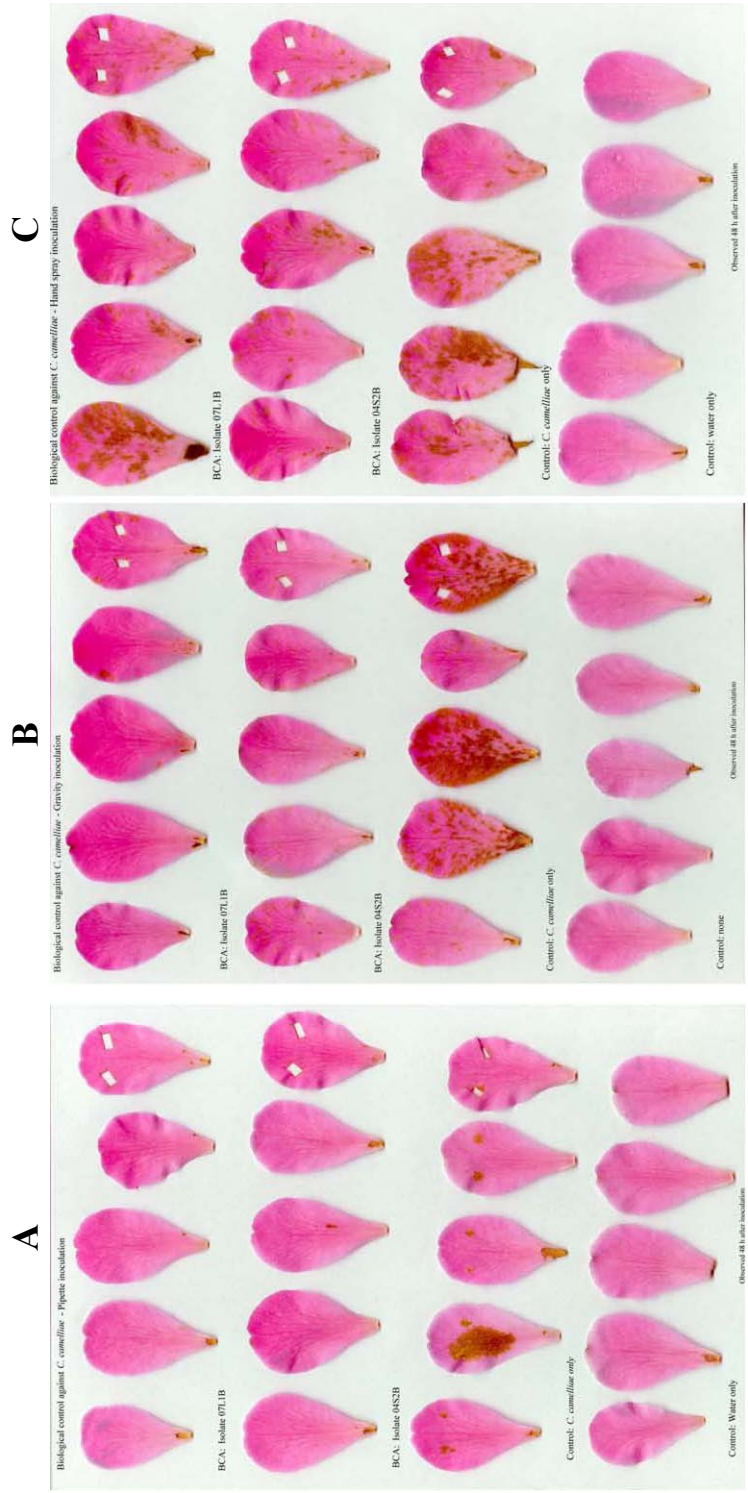


Plate 7.6. Camellia flower petal infections 48 h after *C. camelliae* ascospores and bacterial isolates 04S2B or 07L1B were co-inoculated by A) Pipette inoculation. B) Gravity inoculation. C) Spray inoculation.

inoculated with ascospores by gravity and spray inoculation (Plate 7.6). Confocal microscopic observation found no ascospore germination in the isolate 07L1B treatment while ascospores in the isolate 04S2B treatment germinated but did not penetrate the petals. Both isolates showed sparse attachment to ascospore surfaces or to germ tubes (Plate 7.7).

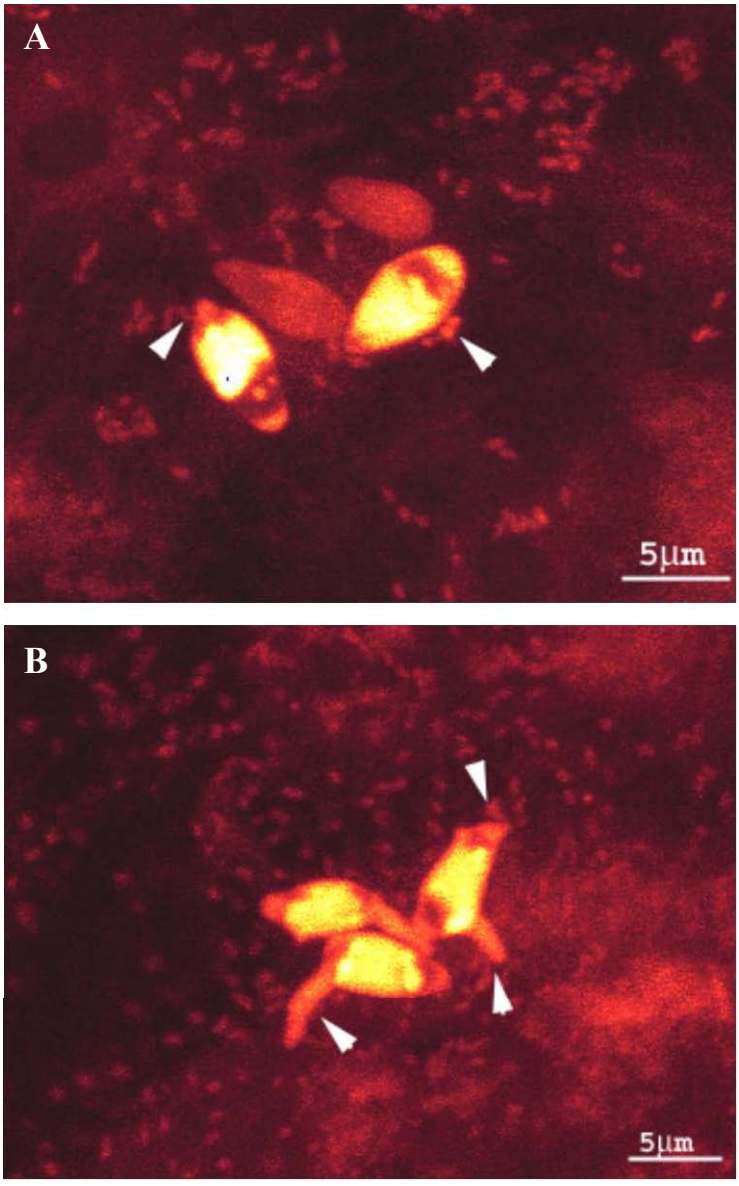


Plate 7.7. Confocal micrograph of ascospores and bacterial biocontrol agents 38 h after inoculation. (A) Bacterial isolate 07L1B - no ascospore germination and sparse bacterial attachment to ascospores and germ tubes. (B) Bacterial isolate 04S2B – ascospore germination but no penetration, and sparse bacterial attachment to ascospores and germ tubes. Arrows show bacterial attachment.

7.3.3. Section III. Evaluation of effective concentration and time of application of selected bacterial isolates

7.3.3.1. Introduction

Successful biocontrol activity may depend on the concentration of biocontrol agents and can differ between different biocontrol agents. Spurr (1981) found that the bacterial antagonists *P. cepacia and B. thuringiensi* required a high concentration ($1x10^8$/ml) to inhibit entire conidial populations of *Alternaria alternata* whereas *B. mycoides* and *P maltophilia* required a very high concentration ($1x10^9$/ml). He also found that their inhibitory activity was more affected by cultural conditions.

In this study the two candidate biocontrol agents were tested activity against *C. camelliae* in water suspension.

7.3.3.2. Experiment 7.6. Effect of bacterial cell concentration on biocontrol activity against *C. camelliae* on petals and intact flowers.

7.3.3.2.1. Materials and methods

Petal inoculation

Bacterial isolates 07L1B and 04S2B were cultured on NA and inoculum was prepared after 2-3 days incubation by adding 10 ml of SDW per plate by repeated pipetting as described in Section 7.3.2.2.1. The suspensions were collected in universal bottles and vortex before cell counts were taken as described in Chapter 2. From this suspension dilution of $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$/ml were prepared using SDW. All suspensions concentration including originals ($1.8X10^8$ for 07L1B; $4.2X10^8$ for 04S2B) were used to co-inoculated with ascospores ($1x10^4$/ml) using a pipette as described above. Four sites on each petal were inoculated with a 2 µl aliquot of BCA and ascospore suspension.

The experiment was conducted as a RCB design with experimental unit of three replication of five petals each.

After twenty hours incubation, two inoculated areas (25 mm^2) from each experimental unit were taken and stained with trypan blue and the number of germinated and none germinated spores, and germ tube penetration were counted under the microscope. Lesion size in mm^2 was measured on the remains tissue of the inoculated area after 44 h incubation using a photo scanner and Photoshop 5.5 program as described in Chapter 2.

Intact flower inoculation

Intact flower inoculation was based on the results of petal inoculation. BCA suspensions were prepared as described above but only 5 ml SDW was added to each culture plate to obtained an increased concentration. The suspension was diluted to $1x10^8$/ml. Both the original and diluted suspensions were used to co-inoculate flowers with ascospores. BCA suspensions were spray inoculated before the ascospore suspensions. Inoculated flowers were incubated in a moist plastic tray covered with polyethylene bag at 20^oC for 48 h. After incubation, flower petals were detached and scanned with a photo scanner to measure the infected area as described in Chapter 2.

7.3.3.2.2. Results

Compared with dilution and controls, both BCA isolates significantly controlled lesion development on petal and intact flowers when they were applied at their highest concentration petal inoculation of isolate 04S2B = $4.2X10^8$; 07L1B = $1.8x10^8$ and whole flower inoculation of 04S2B = $6.8x10^9$; 07L1B = $5.9x10^9$) (Fig. 7.5 and 7.6).

There were no lesions on petals treated with highest concentration of either isolate (Plate 7.8) and lesion size was significantly reduced at a BCA concentration of $1x10^7$/ml. Spore germination was significantly reduced and penetration prevented at the highest concentration of both isolates but germ tube penetration also was significantly reduced at the concentration of $1x10^7$/ml of isolate 07L1B (Fig. 7.5).

On intact flower there was a significant reduction in disease development of undiluted suspensions of both isolates compared with diluted suspension (Fig. 7.6 and Plate 7.9).

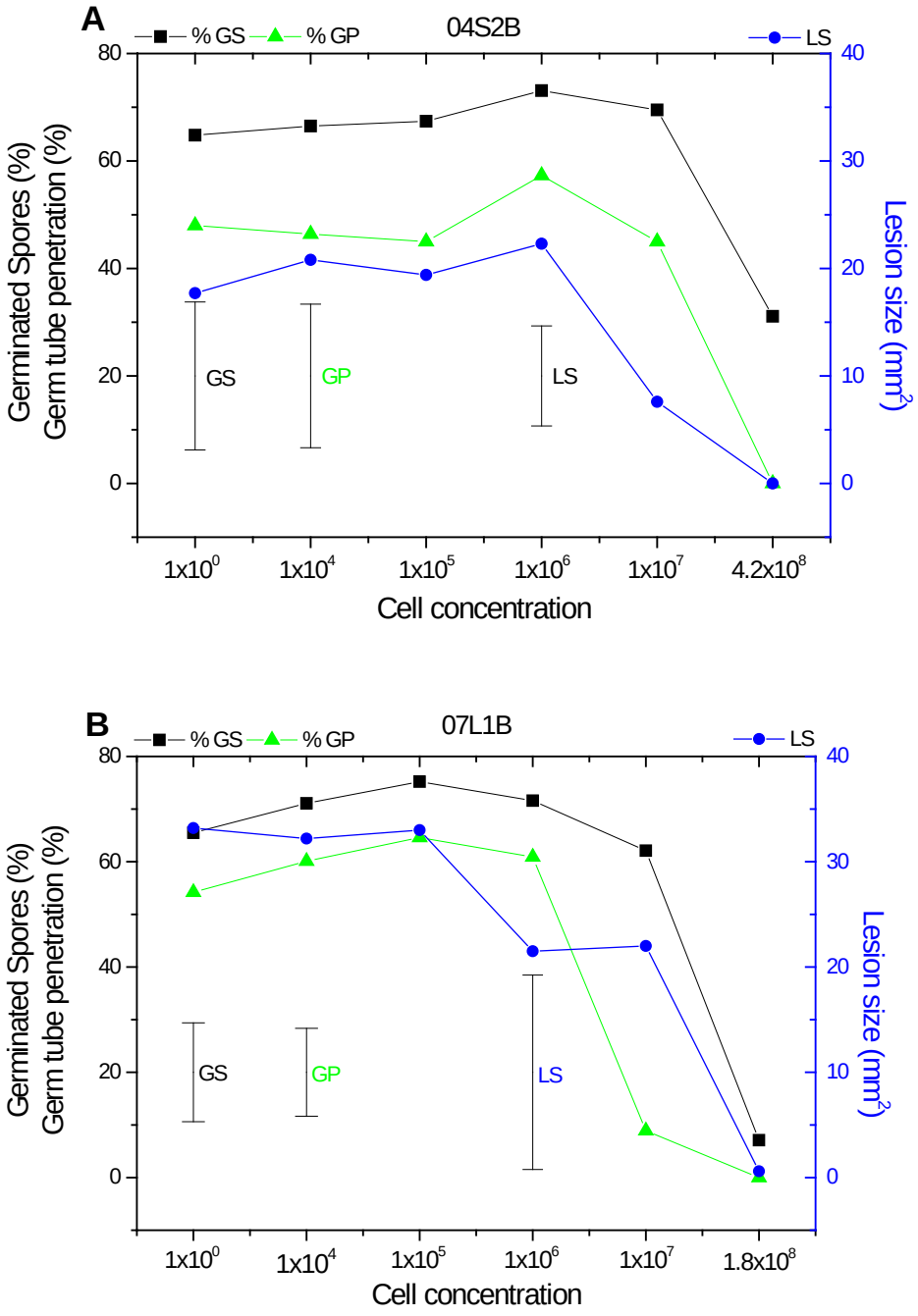


Figure 7.5. Effect on biocontrol of cell concentration of bacterial isolates 04S2B (A) and 07L1B (B) co-inoculated with *C. camelliae* ascospores on petals using pipette inoculation method. Spore germination and germ tube penetration were measured after 22 h incubation, and lesion size measured after 44 h incubation. %GS = Percent germinated spores, %GP = Percent germ tube penetration and LS = Lesion size in mm^2. Vertical bars represent LSD.

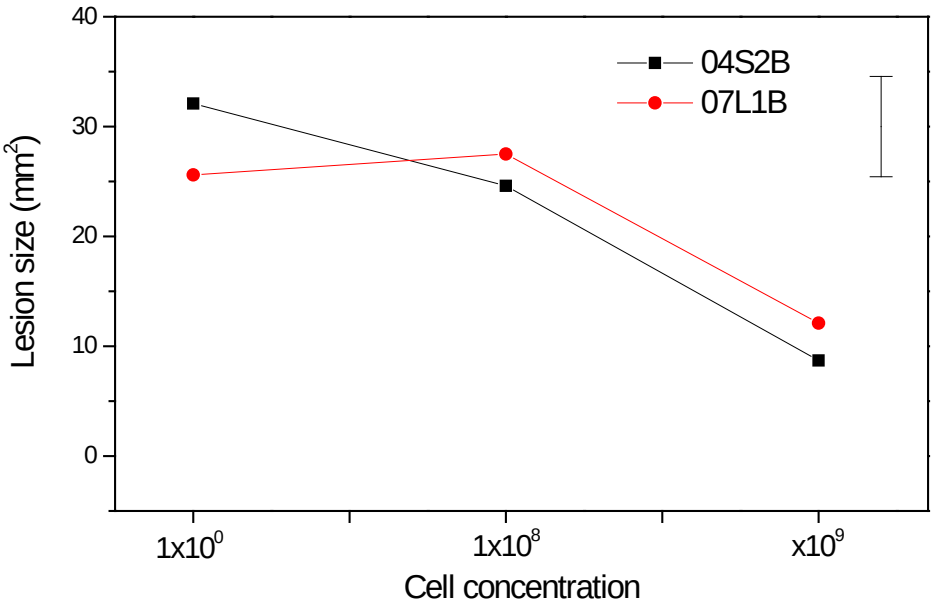


Figure 7.6. Effect on lesion size of cell concentration ($1x10^8$/ml and, $6.8x10^9$/ml for isolate 04S2B and $5.9x10^9$/ml for isolate 07L1B) of two BCAs co-inoculated with *C. camelliae* ascospores on intact camellia flowers. Lesion size measured after 48 h incubation. Vertical bar represents LSD for concentration.

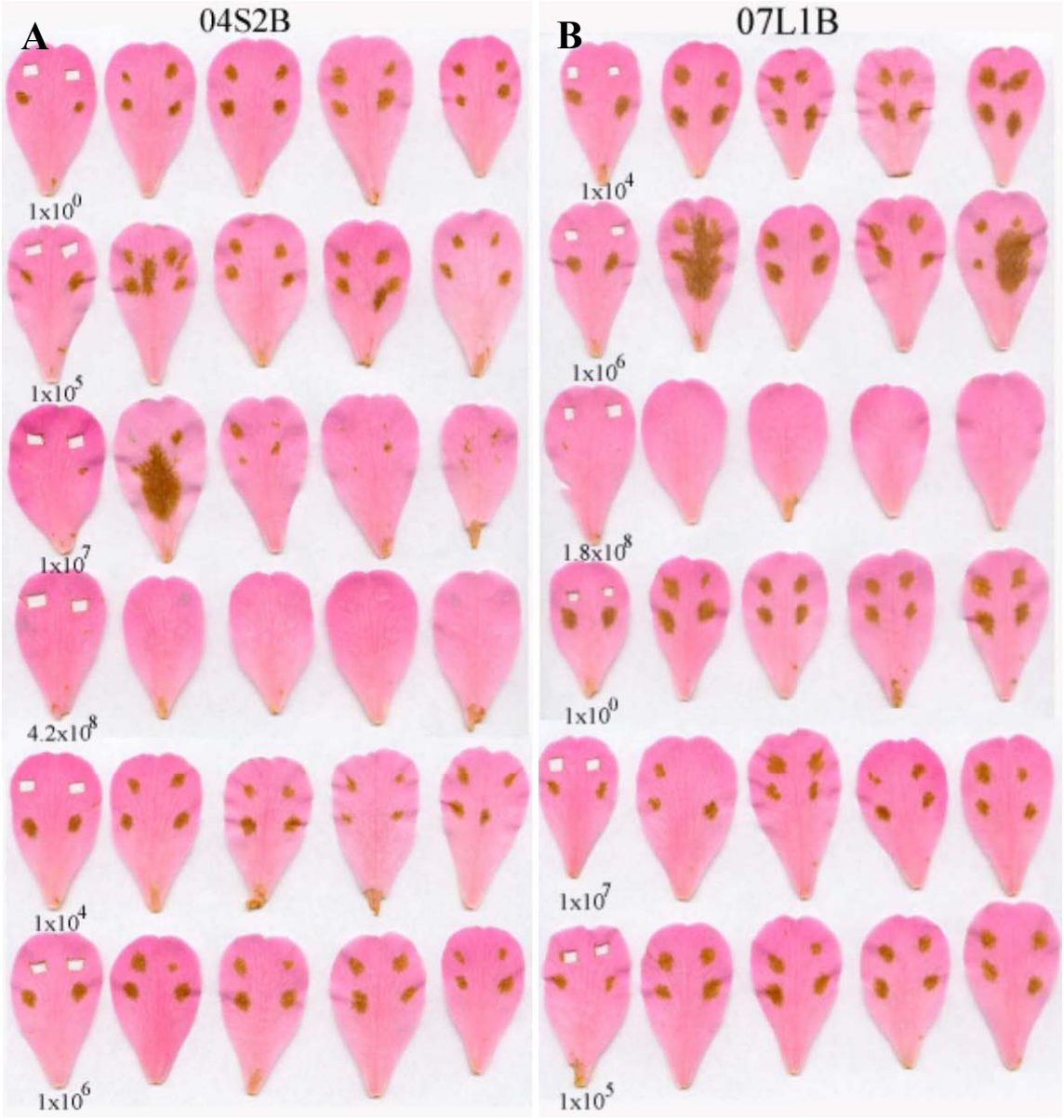


Plate 7.8. Camellia flower petals co-inoculated with *C. camelliae* ascospores and various cell concentrations of isolate 04S2B (A) or isolate 07L1B (B) using the pipette inoculation method. Lesions observed 44 h after inoculation.

Plate 7.9. Petals detached from inoculated intact camellia flowers co- inoculated with *C. camelliae* ascospores and various cell concentrations of isolate 07L1B. The petals were detached from the flowers after 48 h incubation and the infected area scanned by computer scanner. A) Control 1x100; B) 1x108; C) 5.9x109.

7.3.3.3. Experiment 7.7. Time of application of selected bacterial isolates and *C. camelliae* to intact flowers

7.3.3.3.1. Materials and Methods

Suspensions of the selected bacterial isolates were prepared from culture plates without adjusting cell concentration which was $4.3x10^7$ cells/ml for 04S2B and $2.4x10^7$ cells/ml for 07L1B. They were applied as a spray 24 h before, simultaneously, and 24 h after *C. camelliae* inoculation. The *C. camelliae* ascospore concentration was $1x10^5$ and was spray inoculated to intact flowers. Lesion development on a 0-4 scale or by percentage infection was assessed by measuring lesion size using a photocopy scanner and photoshop 5.5 computer program as described in Chapter 2.

Individual flowers were scored for disease severity using a 0-5 scaling system described below.

Disease severity scales on flower infection

Scale	Description
0	= No infection
1.	= 1-5% infected
2.	= 6-10 % infected
3.	= 11-20 % infected
4.	= 21-40 % infected
5.	= >40% infected

Three separate experiments were set up as described below.

Experiment 7.7A. Laboratory condition

Inoculated flowers were placed in a plastic tray which moistened with paper towel and covered with polyethylene bag. The trays were placed in the incubation room at 20^oC for 96 h. Disease severity was measured at 48 h, 60 h and 96 h after *C. camelliae* inoculation.

The experiment was designed for repeated measurement in a split plot design with three replications of three flowers each. BCAs are in main plot. Readings were taken repeatedly at different times.

Results

There was no significant different between the two BCAs. All three times of application significantly reduced disease severity compared with the control. They were also significantly different from each other with the 24 h before ascospore inoculation giving the lowest disease severity. The overall disease development delayed up to 60 h after inoculation due to BCA application but there was an increase observed 60 h after inoculation. The delay was significant when BCAs were applied 24 h before pathogen arrival however, there was an increase at 96 h but much lower than the control (Fig. 7.7).

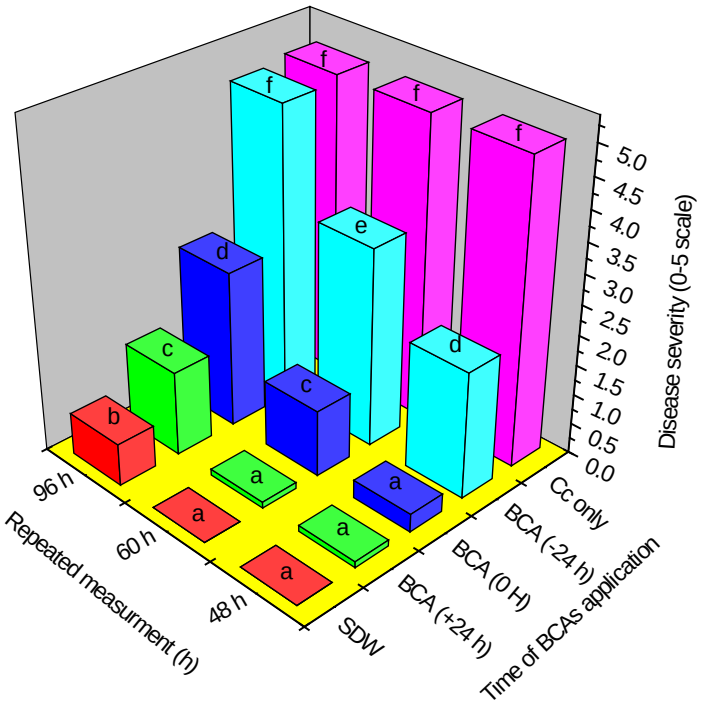


Figure 7.7. Average disease severity of camellia flowers. Interaction between time of application of BCAs and incubation period. BCAs applied 24 h before (+24 h) or 24 h after (-24 h), or at the same time of *C. camelliae* inoculation. Controls = Ascospores only and SDW (Sterile distilled water) only. The inoculated intact flowers were incubated in a moist plastic tray at 20^{o}C. Means with same letters are not significantly different.

Experiment 7.7B. Open environment in controlled incubation chamber

The experiment was conducted partly semi *in situ* - the flowers were collected from the camellia bush with stem (about 10 cm length) still attached. The flower stem was pushed through a hole in the lid of a plastic container full of water to avoid water loss from the flowers over the 3-4 days of the experiment. After co-inoculation with BCAs and ascospores as described in the previous experiment, the intact flowers and stems were incubated in a control chamber where 98 ± 2% RH was maintained at 20^{o}C. Disease severity was assessed 88 h after *C. camelliae* inoculation.

Results

Both isolates significantly reduced the disease severity when they were applied simultaneously and 24 h before the ascospores and there was no significant difference between them (Fig. 7.8).

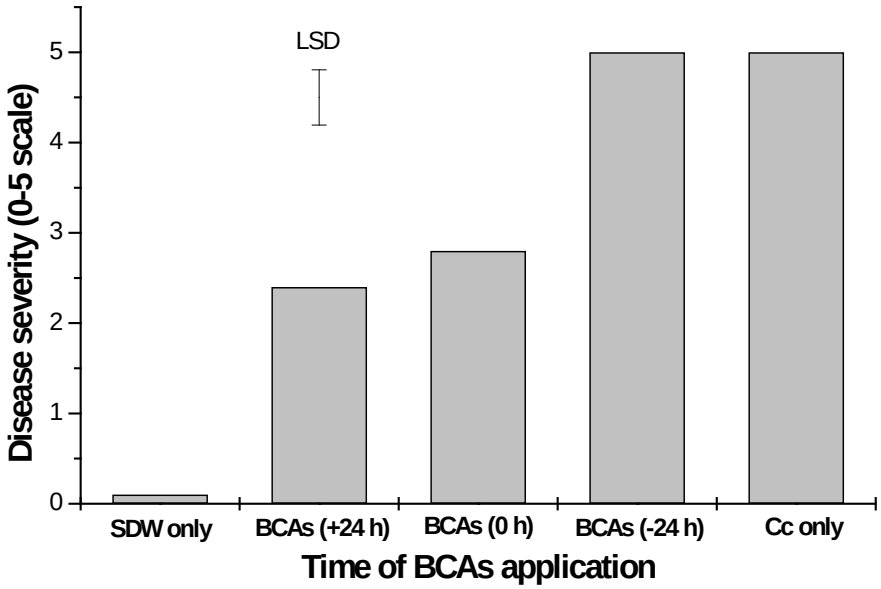


Figure 7.8. Average disease severity of camellia flowers after BCA application at different times. BCAs applied 24 h before (+24 h) or 24 h after (-24 h), or at the same time of *C. camelliae* inoculation. Disease severity was measured 88 h after *C. camelliae* inoculation. Controls = Ascospores only and SDW (Sterile distilled water) only. The inoculated intact flowers were incubated in an incubation chamber at 20^{o}C and 98±2 % RH. Vertical bar represents LSD.

Experiment 7.7C Glasshouse condition

The third experiment was set up in a glasshouse as in the previous experiment. Disease severity was assessed 66 h and 96 h after ascospore inoculation. The glasshouse RH ranged from 35 to 40% and temperature was 24-26^{o}C.

Results

There was no significant difference in biocontrol activity between the two BCAs but time of application had a significant effect on disease control. Simultaneous and 24 h delayed application of BCAs with *C. camelliae* ascospores gave significant control on disease severity compared with the ascospores only control and the control lasted up to 90 h after inoculation (Fig. 7.9). Disease lesions developed from the bottom towards the tip of the petals (Plate 7.10). Because of the low humidity and high temperature in the glasshouse conditions at the petal tip may not have conducive to infection but the microenvironment in the bottom of the flower could have been favourable the ascospores to germinate.

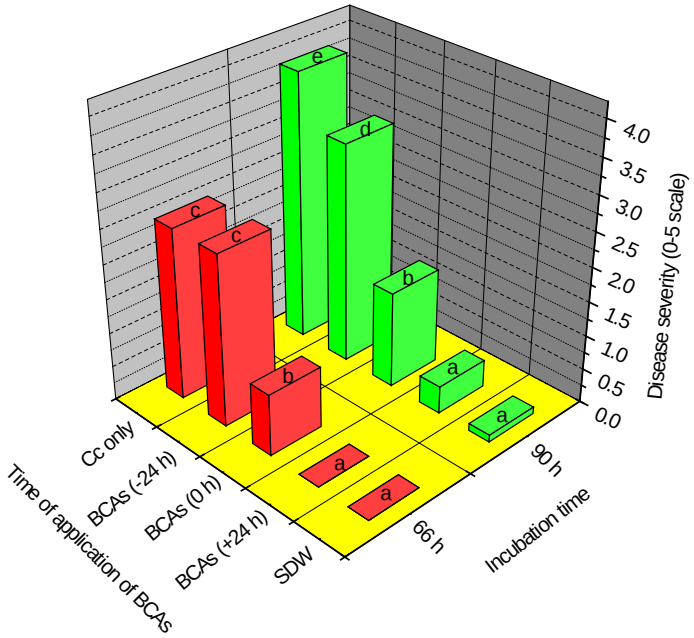


Figure 7.9. Average disease severity of camellia flowers at two different incubation periods in glasshouse condition (35-40% RH and 24-26^{o}C). BCAs applied 24 h before (+24 h) or 24 h after (-24 h), or at the same time of *C. camelliae* inoculation. Controls = Ascospores only and SDW (Sterile distilled water) only. Means with same letters are not significantly different.

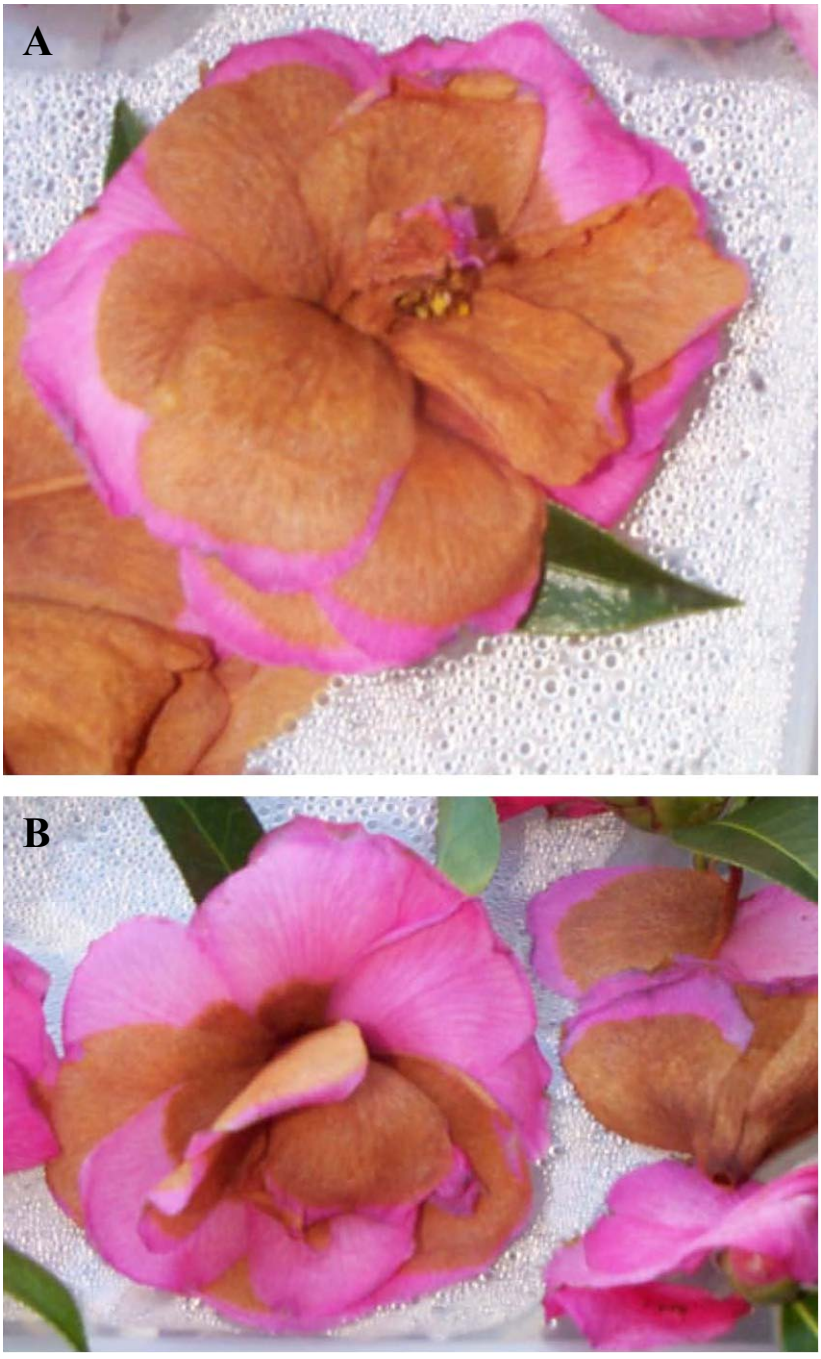


Plate 7.10. Petal lesions developing from the base of the flowers in glasshouse conditions. Photographed 120 h after inoculation. A) Flowers inoculated with *C. camelliae* ascospores only (Control). B) BCA applied 24 h before ascospore inoculation.

7.3.3.4. Experiment 7.8. Evaluation of biocontrol efficacy of two bacterial isolates on a range of camellia cultivars

7.3.3.4.1. Materials and Methods

Six different cultivars: Pagoda, R.L.Wheeler, Nichy Crisp, Lady Loch, Brian and one unknown cultivar were used to observe the activity of two leading biocontrol agents against *C. camelliae* on these cultivars. Two petals healthy in appearance were selected from each cultivar and were co-inoculated with ascospores and BCAs on six sites per petal using a micropipette as described in Section 7.3.3.2.1. As a control treatment each cultivar was also inoculated with ascospores only or with SDW. Inoculated petals were incubated at 20^0C in a closed humid container (100% RH). The BCAs were prepared by culturing them in NA as described in Section 7.3.3.2.1. The cell concentration of 04S2B was $3.2x10^7$/ml and of 07L1B was $2.8x10^7$/ ml. The ascospore concentration was $1x10^4$/ml. The petals were scanned by a photo scanner 48 h after incubation.

7.3.3.4.2. Results

Except for cultivar Nicky Crisp, which showed low rated symptoms (Score No. 1), none of the bacterial isolates treated cultivars showed symptom development. Similar symptoms were observed on petal inoculated with isolate 04S2B and on two petals inoculated with isolate 07L1B (Table 7.3 and Plate 7.11). Some petals developed symptoms from natural infections particularly on the cultivars Nicky Crisp and Lady Loch (Plate 7.11).

Table 7.3. Biocontrol activity of isolates 04S2B and 07L1B co-inoculated with *C. Camelliae* ascospores by pipette on 6 camellia cultivars. A total of 6 sites per petal were inoculated. Cc only = *C. camelliae* ascospores only; SWD = Sterilized distilled water; * = No. of infection per total no. of inoculation.

Cultivars	Pagoda		R.L.Wheeler		Nicky Crisp		Unknown		Lady Loch		Brian	
Petal No.	1	2	1	2	1	2	1	2	1	2	1	2
04S2B	0/6*	0/6	0/6	0/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
07L1B	0/6	0/6	0/6	0/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6
Cc only	6/6	6/6	6/6	6/6	6/6	4/6	5/6	6/6	6/6	?/6	6/6	6/6
SDW	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6	0/6	0/6	1/6

Plate 7.11. Camellia petals. Six cultivars co-inoculated with two lead biocontrol agents (isolates 07L1B and 04S2B) and *C. camelliae* ascospores. Petals were pipette inoculated with 2 µl of both pathogen ($1x10^4$/ml) and BCAs (04S2B = $3.2x10^7$/ml; 07L1B = $2.8x10^7$/ml) suspension per site.

7.3.4 Section IV. Testing of two bacterial isolates for mode of action against *C. camelliae*

7.3.4.1. Introduction

Antibiosis is the inhibition of one microbe by the extracellular products of another (Cook and Baker 1983). Fravel (1988) and, Thomashow and Weller (1996) argued the importance of antibiosis in plant protection with evidence from experiments using cell free extract, direct isolation of antibiotic from the plant host surface and the creation of mutant bacteria with modern genetic methods. Results from the later technique concluding the fact that nutrient are a critical determinant in soil born interactions (Thomashow and Weller 1996). The quantity of phylloplane nutrient resources is considerably lower compared with the rhizosphere (Andrews 1992; Fokkema 1995) therefore, the acquisition of adequate nutrient is very important for the antagonist function in antibiosis mode of action. Competition for nutrients is a widespread phenomenon in the interaction between micoorganisms on the phylloplane. Leben et al. (1965) and Austin et al. (1977) observed presence of additional nutrients increased antagonism against phylloplane pathogen. In some cases adding of nutrient for example D- glucose annulled bacterial inhibition of *B. cinerea* germination (Fraser 1971). Predation or parasitism is characterized by an intimate contact between both microbes. Usually mycoparasites utilize fungal cell-wall degrading enzymes such as chitinase glucanases to dissolve their fungal hosts' cell walls and penetrate the cells (Elad 1996).

In this experiment the two bacterial isolate 04S2B and 07L1B already showed antibiosis in *in vitro* were tested for their mode of action against *C. camelliae* in *in vivo* assay.

7.3.4.2. Materials and Methods

7.3.4.2.1. BCAs culture and ascospores preparation

Bacterial cultures were grown directly from glycerol stocks by pipetting 100 µl of thawed samples into 150 ml conical shape flasks, containing 100 ml of nutrient broth (NB) [8 g/litre (Oxoid CM3), sterilized at 121^{o}C for 15 min]. The cultures were then incubated at 20^{o}C for 72 h on an orbital shaker incubator (Certomat M) set at 150 rpm.

Ascospore suspensions were prepared and concentration adjusted to$1x10^5$ for spray inoculation.

7.3.4.2.2. Preparation of bacterial cell and spore free substrate

The stationary phase cultures derived from NB culture were centrifuged at 15000 rpm for 15 min. The resultant pellet was washed with sterile Ringer's solution and the cells were re-suspended in sterile NB as a nutrient source. The bacterial supernatant was passed through sterile filter paper (Whatman No. 1) and then sterile filtered through 0.2µm syringe tip filters to remove bacterial spores and cells.

7.3.4.2.3. Treatments and inoculation

The following treatments were prepared and applied to flower petals as a spray.

1) Whole nutrient broth culture,
2) Bacterial cells re-suspended in Nutrient Broth (NB)
3) Bacterial cells re-suspended in SDW
4) Bacterial supernatant

They were sprayed using an aerosol spray (Humbrol Aerosol Sray gun, Scientific laboratory Supplies, Nottingham, UK) until the surface of the whole flower petals were thoroughly covered. After a few minutes drying, the petals were either immediately (0 h) inoculated with an ascospores suspension of *C. camelliae* using the same atomiser, or incubated 24 h, 48 h or 72 h before inoculation. Controls included uninoculated petals, petals inoculated with *C. camelliae* alone and petals sprayed with NB solution and subsequently sprayed with *C. camelliae*. Inoculated petals were placed in a plastic humid chamber with wet paper towels and incubated at 20^oC for 72 h.

7.3.4.2.4. Experimental design and disease assessment

The experiment was conducted as a split plot design. Each experimental unit consisted of five petals with three replications. Infected petal area was measured using a photo scanner and computer as described in Chapter 2.

7.3.4.3. Results

Both isolates significantly reduced the disease area when applied to petals as a whole nutrient broth culture, as washed cells resuspended in nutrient broth or as bacterial supernatant (Fig. 7.10 & 7.11). Mean petal infection in both isolate treatments was effectively reduced when petals were incubated with bacteria more than 24 h before *C. camelliae* inoculation than with simultaneous inoculation (Fig. 7.10 & 7.11). The bacterial supernatant of both isolates also gave disease control equal to that of the whole nutrient bacterial culture. This may reflect the production of an antifungal metabolite(s) in culture. Both the resuspended cells and bacterial supernatant initially showed low disease suppression of disease development compared with whole nutrient broth culture but they became as effective as whole nutrient cultures incubated 24 h and 48 h before infection for isolate 07L1B and 04S2B respectively (Fig. 7.10 & 7.11).

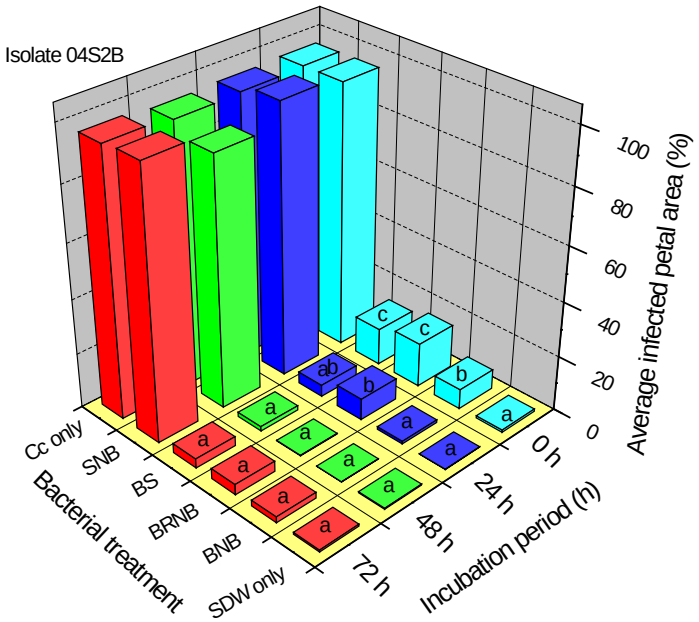


Figure 7.10. Bacterial isolate (04S2B) treatment and longevity against *C. camelliae* infection of camellia petals. Petals were treated with whole nutrient broth culture (BNB), washed bacterial cells resuspended in sterile nutrient broth (BRNB), culture supernatant (BS) or sterile nutrient broth (SNB). They were incubated at different time intervals before challenge with the pathogen. Ascospore only suspension (Cc only) and sterile distilled water (SDW only) were included as control. Means with the same letter are not significantly different.

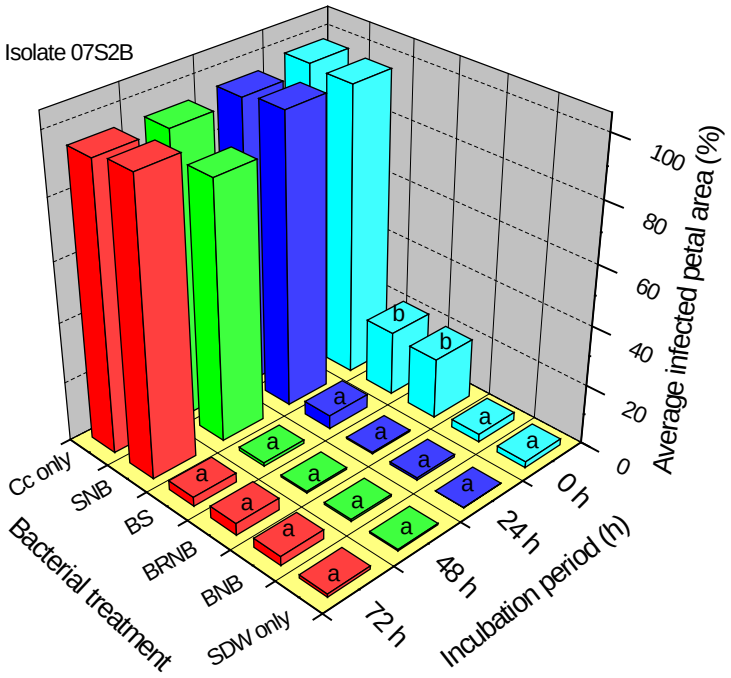


Figure 7.11. Bacterial isolate (07L1B) treatment and longevity against *C. camelliae* infection of camellia petals. Petals were treated with whole nutrient broth culture (BNB), washed bacterial cells resuspended in sterile nutrient broth (BRNB), culture supernatant (BS) or sterile nutrient broth (SNB). They were incubated at different time intervals before challenge with the pathogen. Ascospore only suspension (Cc only) and sterile distilled water (SDW only) were included as control. Means with the same letter are not significantly different.

7.3.5. Section V: Evaluation of survival ability of the two bacterial isolates in different environmental conditions and in a range of camellia cultivars

7.3.5.1. Introduction

A successful antagonist depends on survival, multiplication and colonisation of the plant surface. To achieve these conditions Blakeman and Fokkema (1982) suggested that the phylloplane environment must have high humidity condition or free water on the plant surface for the majority of antagonists. They further indicated that it is not possible to alter the microclimate at the plant surface in the field environment. However, it may be

assumed that the dense petals of camellia flowers may provide a favourable microclimate for the survival of applied antagonists in the field.

In this study, bacterial isolates 04S2B and 07L1B screened through *in vitro* and *in vivo* technique were tested for their survival ability in different environmental conditions in the laboratory, glasshouse and in the field, and in different cultivars.

7.3.5.2. Materials and Methods

The bacterial suspension was prepared in NB medium as described in the previous Sections and 2 ml of prepared bacterial suspension was sprayed per an intact flower which were then incubated in the controlled humidity and temperature chamber, in the glasshouse or in the field as described under each experiment below.

Survival bacterial population counts

Samples were taken every 24 h and colony-forming units (cfu) of the bacteria assessed. This was done by taking 5 cm^2 size of petal tissue from each flower and submerging it in 10 ml of SDW in a universal bottle. They were shaken well for 2-3 min and the suspension was diluted to an appropriate dilution for plating on nutrient agar (NA) by pipetting 100 µl of suspension and spreading it with a sterile bend glass rod.

Before spraying the bacterial suspension, the colony forming units of the suspension were counted by pipetting 100 µl of diluted suspension (1:10^3 or 10^4) to NA plate as described above. The inoculated plates were placed in an incubating chamber set at 25^{o}C for incubation for 2-3 days. After incubation the plates were scanned and the digital images stored in a computer for colony counting. If colonies were dense, the approximate number of colonies was counted by counting the number of pixels of the colony using histogram in the Photoshop 5.5 program.

7.3.5.3. Experiment 7.9. Bacterial isolates survival in the laboratory condition over time

7.3.5.3.1. Materials and methods

The flowers with stems, were collected from cultivar 'Brian' and the intact flowers were set up as described in Experiment 7.7B in Section 7.3.3.3. The flowers were sprayed with 2 ml of NB bacterial suspension until the surface of the whole flower was thoroughly covered. After spraying, the intact flowers and stem were incubated in a control chamber where 98 ± 2% RH was maintained at 20^{o}C for 7 days.

The experiment was conducted in a repeated measurement design with three treatments (isolates 04S2B, 07L1B and SDW as control). Each experimental unit consisted of three intact flowers with three replications. The measurement was taken repeatedly every 24 h from each experimental unit.

7.3.5.3.2. Results

There was a low number of colony forming units (cfu) recovered from unsprayed (control) flowers (10 cfu/cm^2). The high bacterial population recovered from the flowers sprayed with isolate 04S2B (35239 cfu/cm^2) and isolate 07L1B (72105 cfu/cm^2) suggests that the recovered microbial population are probably from the bacterial isolates sprayed onto the flowers, not the residential microbes. There were significant differences between the two isolates in recovered bacterial populations (Fig. 7.12). The population of isolate 04S2B significantly dropped over time while that at isolate 07L1B initially decreased for 24 to 72 h, then significantly increased to more than the initial population at 138 h and 168 h. A significant increase was observed in populations on control flowers at 138 h and 168 h (Fig. 7.13). Petals of most flowers had started to detached from the calyx of the flowers after 72-96 h of incubation.

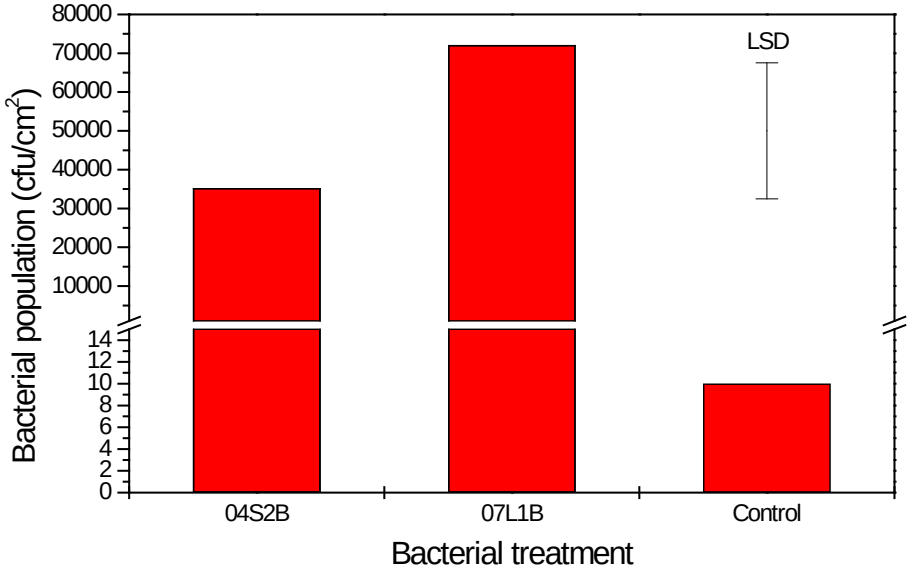


Figure 7.12. Survival of bacteria in camellia flowers. A) Population counts on bacterial sprayed and none sprayed flowers. Vertical bar represents LSD.

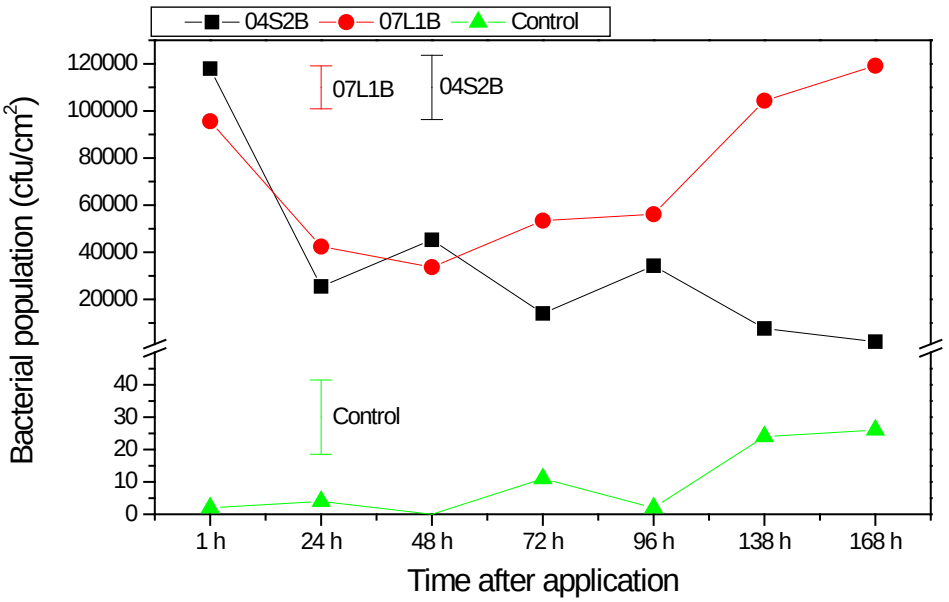


Figure 7.13. Survival of isolates 04S2B and 07L1B in camellia flowers over time. Control represents the microorganism counted in the unsprayed flowers. Vertical bars represent LSD.

7.3.5.4. Experiment 7.10. Effect of RH and temperature on bacterial isolates survival over time

7.3.5.4.1. Materials and Methods

Flowers from cultivar 'Brain were used and the experiment was set up as for Experiment 6.9 but with different combinations of RH and temperature in the incubator. Each set of bacterial sprayed (2 ml per flower) intact flowers as described above were incubated at one of the RH and temperature combinations:

1. 60% RH and 10^{o}C
2. 60% RH and 15^{o}C
3. 60% RH and 20^{o}C
4. 100% RH and 10^{o}C
5. 100% RH and 15^{o}C
6. 100% RH and 20^{o}C

The experiment was designed for repeated measurement in a split split plot design with three replications of three flowers each. Bacterial isolates are in main plot, temperature in subplot and RH is in sub sub plot. Readings were taken repeatedly in different time.

An intact petal was taken from each flower as a sample every 24 h during incubation for 3 days and they were processed as in Experiment 6.9 to count colony-forming units (cfu) of bacteria.

Because only one suitable incubator available each of the above treatments was carried out sequentially. Since bacterial population was estimated only by cfu counting, the applied bacterial population were varied for each treatment. To standardize the reading for comparison the recovery population was calculated in percentage base on their original (inoculated bacterial population) cfu counts.

7.3.5.4.2. Results

Insignificant population was recovered from unsprayed flowers. There was significant difference between BCAs in the percent cfu recovered from sprayed flowers (Fig. 7.14D). RH and temperature affected the population significantly (Fig. 7.14A&B) and the

interaction between two also gave a significant effect (Fig. 7.15A), at 60% RH, bacterial populations were high at 10°C and 15°C but lower at 20°C whereas at 100% RH, were low at 10°C but increased significantly at 15 °C and 20°C. In the first 24 h, the percent cfu

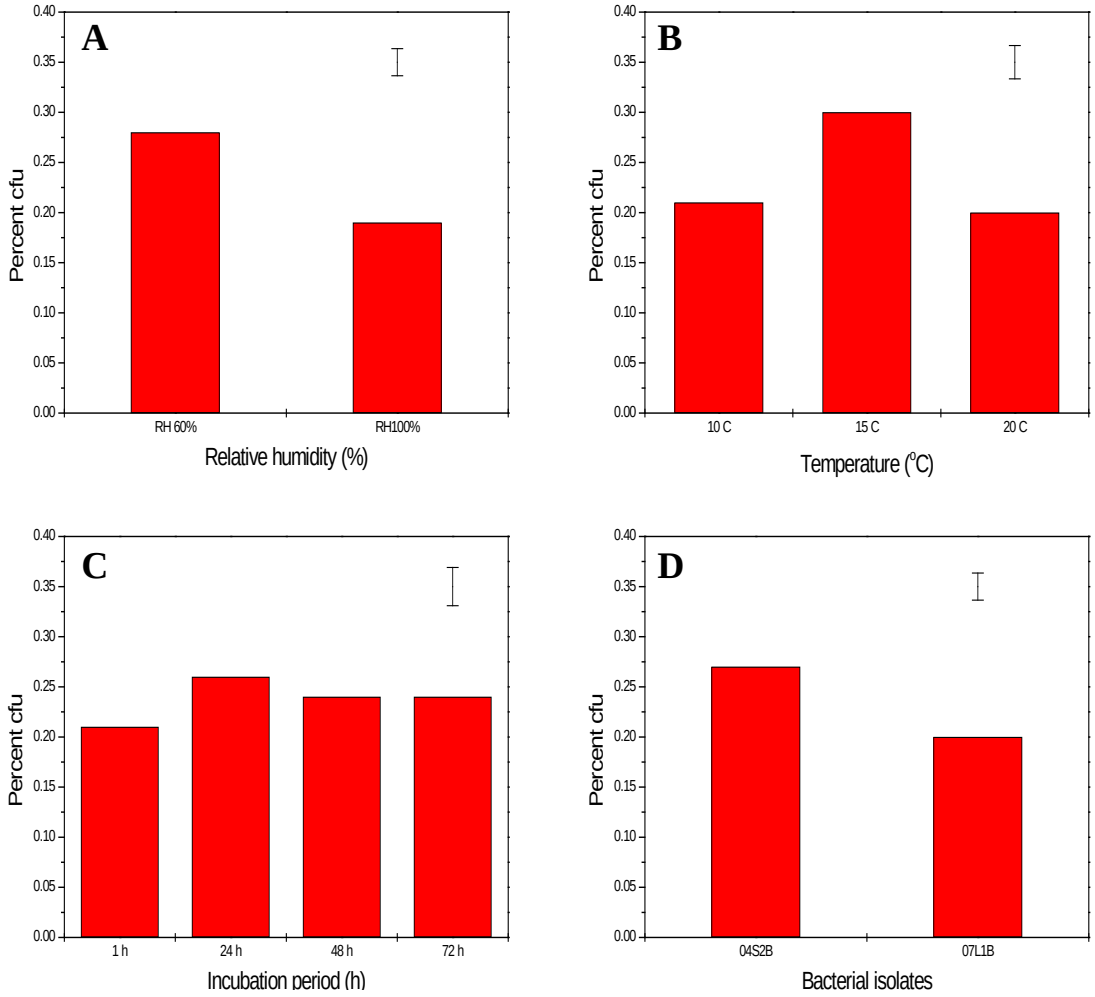


Figure 7.14. Bacterial population recovered from camellia flowers after spray application of potential bacterial BCAs. A) Effect of relative humidity on recovery. B) Effect of temperature on recovery. C) Effect of incubation period on recovery. D) Percent recovery (cfu) of bacteria from flowers treated with isolates 04S2B and 07L1B. The vertical bar represents overall LSD of RH, temperature, incubation period and bacterial isolates.

increased significantly (Fig. 7.14C). At 60% RH the percent cfu decreased with time while percent cfu at 100% RH it also increased, but not significantly (Fig. 7.15B). Percent cfu was significantly increased at 15^{o}C at 24 h incubation than the other two temperatures and were reduced subsequently but maintained higher than others (Fig. 7.15C).

The flowers withered and softened at 60% RH after 24 h incubation at 10^{o}C and 15^{o}C and this could give saprophytic environment by leaching tissue extracts on the surface and that may increased the bacterial populations. At 20^{o}C, petals were stiff and firm but the bacterial populations were low. The desiccated condition due to low RH at high temperature may be the reason for low bacterial populations. On the other hand at 100% RH population increased by increasing temperature and reached highest at 20^{o}C.

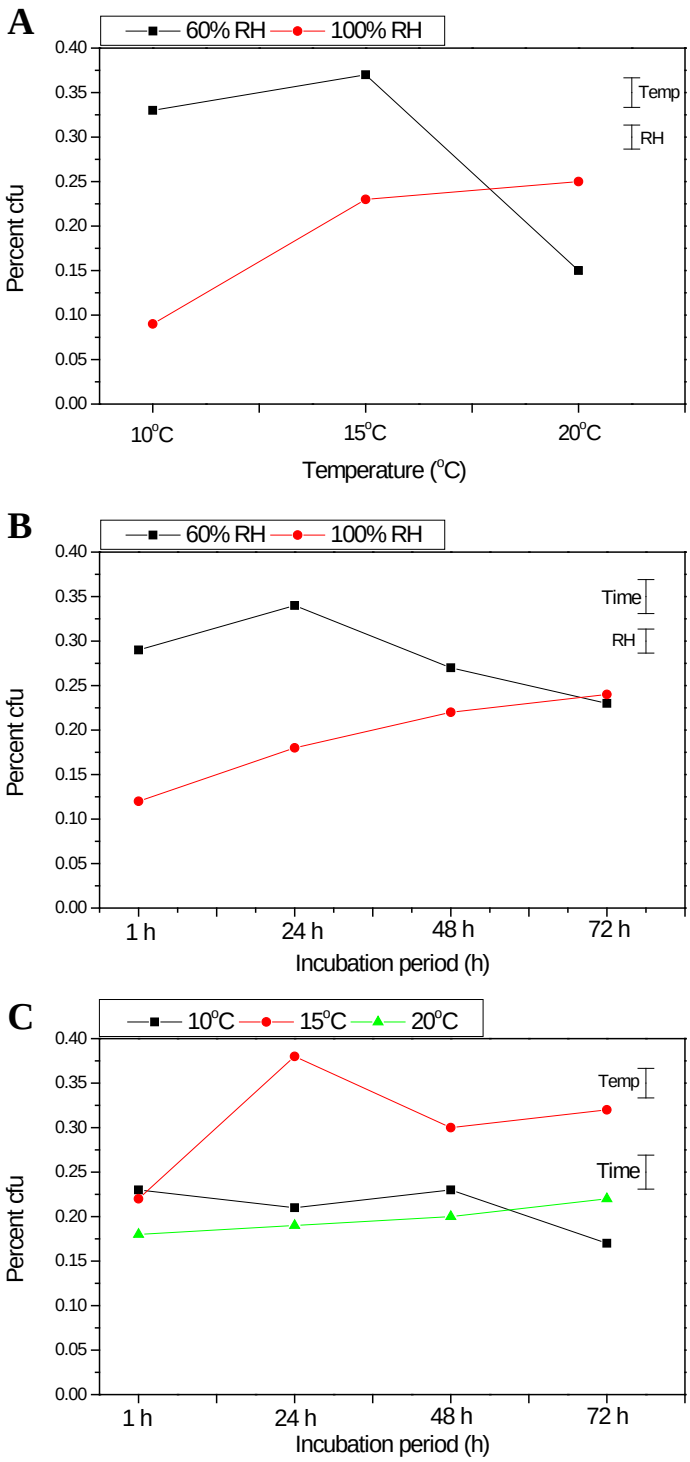


Figure 7.15. Percent recovery of bacteria from camellia flowers treated with potential bacterial BCAs. A) Interaction between relative humidity and temperature on recovery. B) Interaction between relative humidity and incubation period on recovery. C) Interaction between temperature and incubation period on recovery. The vertical bars represent overall LSD of RH, temperature (Temp) and incubation period (Time).

7.3.5.5. Experiment 7.11. Bacterial isolates survival test in greenhouse

7.3.5.5.1. Materials and Methods

The experiment was established in the glasshouse and the sampling process for bacterial population count was carried out as for Experiment 7.9.

7.3.5.5.2. Results

One hour after incubation the average cfu count for isolate 04S2B was $1.3x10^5$ and for isolate 07L1B was $9.1x10^4$ per cm^2 petal area but there were no colonies formed from subsequent samples collected every 24 h for three days. The experiment was repeated twice with the same result. High temperatures (24-28^oC) and low humidity (35-45% RH) were recorded during the experimental period and this could be the reason for the reduction in numbers of bacterial colonies.

7.3.5.6. Experiment 7.12. Evaluation of bacterial isolates survival in different cultivars over time in the field

7.3.5.6.1. Materials and Methods

Four cultivars (Brian, E.G.Waterhouse, Kramer's Supreme and Lady Loch) were used for this experiment in the field. Healthy and undamaged flowers were tagged with the name of bacterial isolate or control. Flowers were sprayed with whole broth bacterial cultures using a fine atomizer. The bacterial cfu were estimated as described in previous experiments.

The experiment was conducted in split plot design with repeated measurement and the cultivars were assigned as main plot.

7.3.5.6.2. Results

Isolate 07L1B has significantly more survival population than isolate 04S2B over four cultivars in the field (Fig. 7.16A). There were significant differences observed among the cultivars with most bacteria obtained from cultivar 'Brian' and least from cultivar E.G.Waterhouse (Fig. 7.16B). There were no significant differences observed between cultivar Kramer's Supreme and Lady Loach. The average population significantly reduced over time (Fig. 7.16C). Cultivars Brian and Lady Loach retained significantly more

population of isolate 07L1B than the other cultivars while cultivar Kramer's Supreme retained significantly more isolate 04S2B (Fig. 7.17).

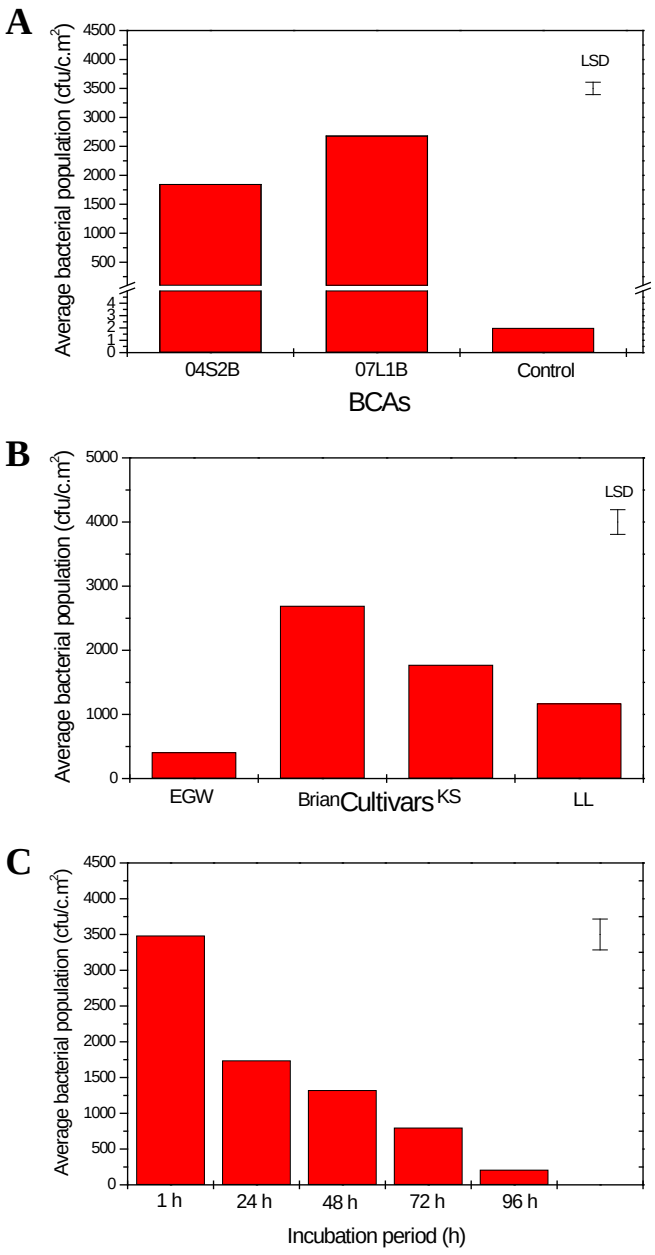


Figure 7.16. Mean microbial populations recovered after isolates 07L1B and 04S2B were sprayed on various camellia cultivars in the field. A) Mean recovery of each isolate and of microbes from none sprayed flowers. B) Mean bacterial population recovered from different camellia cultivars. (EGW = E.G.Waterhouse, KS = Kramer's Supreme, LL = Lady Loach and Brian). C) Mean bacterial population recovered over time. Vertical bar represents LSD.

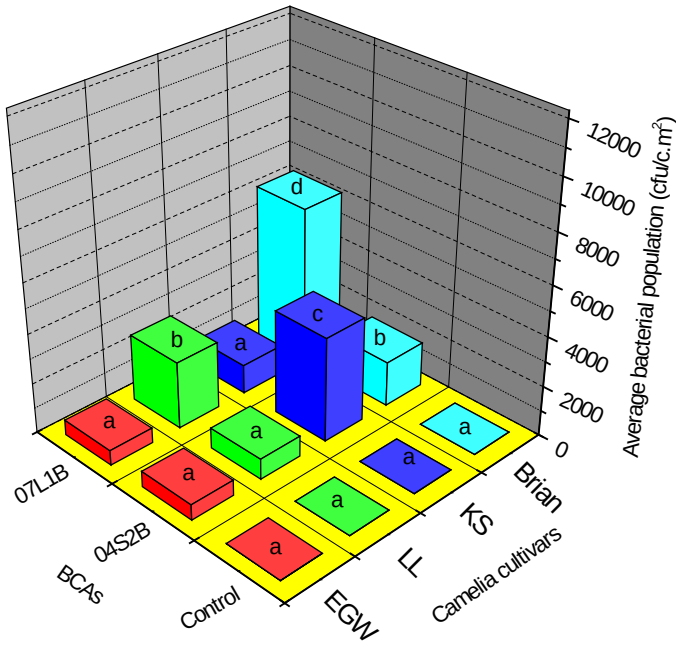


Figure 7.17. Relationship between cultivars (EGW = E.G.Waterhouse, KS = Kramer's Supreme, LL = Lady Loach and Brian) and isolates (07L1 and 04S2) on bacterial population recovery. Control = microbial population recovered from non-sprayed flowers. Means with same letter are not significantly different.

7.3.6. Section VI. Field evaluation of two bacterial isolates against *C. camelliae*

7.3.6.1. Introduction

A number of bacterial and yeast isolates were screened for their biocontrol efficacy against *C. camelliae* by *in vitro* and *in vivo* techniques in the laboratory and in the glasshouse. Controlled lesion development by isolates 04S2B and 07L1B depend on the ascospore inoculation method. The two isolates showed attachment to ascospore germlings as well as production of siderophores and antibiotics. Based on the mode of action experiment, antibiotic production appears to be the main contributor to *C. camelliae* biocontrol by these organisms.

It is seldom that a biocontrol agent, which does well in the laboratory, does the same in the real environment (Andrews 1990). This is probably because the bacterial glycocalyx is lost when bacteria are grown in most laboratory media [Andrews (1990), cited Costerton et al. 1978]. On the other hand, Govan (1975) found that altering culture condition could prevent loss of the glycocalyx. There are reports of BCAs that perform well in the laboratory and do well in field conditions too. *Stenotrophomonas maltophilia* C3 bacterial strain reduced bean rust severity when suspended in buffer with or without chitin amendment equally in the greenhouse and in field conditions (Yuen et al. 2001). Chen et al. (2000) tested 6 bacterial strain that gave over 60% control of *Rhizoctonia solani* causing sheath blight of rice in an *in vitro* test and found that they gave good control of this disease in field conditions. Thus it is possible to find biocontrol agents effective in the field by screening for them *in vitro* and *in vivo*.

The application time of biocontrol agents is important if they are to challenge the pathogen effectively. Fokkema (1991) suggested that in order to provide effective control, the introduced microorganism has to be well established in the phyllosphere before the arrival of the pathogen. If disease is already present, it may be ineffective, because the antagonistic properties may not be diffusible or even because the antagonist supporting nutrient may stimulate the pathogen (Elard et al. 1994a).

In this study, bacterial isolates 04S2B and 07L1B were screened *in vitro* and *in vivo* with a range of times of application to detect any significant effect against *C. camelliae* and to evaluate their survival in field condition.

7.3.6.2. Materials and Methods

7.3.6.2.1. Test flowers in the field

Flowers from cultivars Brian, E G Waterhouse, Kramer's Supreme and Lady Loch were used to conduct field experiments on biocontrol efficacy of the two isolates selected and their survival in field conditions. All four cultivars were located in the Massey University campus. Healthy and undamaged mature buds that were about to open, about 2/3 opened,

or fully opened flowers were tagged as explained below. Experiments were conducted in September and October 2001 during the camellia flowering season.

7.3.6.2.2. Potential bacterial BCA culture preparation

BCA cultures were prepared and cell counts or colony-forming units (cfu) were taken from every culture before they were applied to the flowers.

7.3.6.2.3. Ascospore suspension preparation

Ascospore suspension were prepared in a universal bottle as described in Chapter 2 and the concentration was adjusted to $5x10^5$/ml.

7.3.6.2.4. Inoculation

Flowers were co-inoculated with bacteria and ascospores using a fine atomizer for spray inoculation according to the experimental design.

7.3.6.3. Experiment 7.13. Evaluation of biocontrol of two bacterial isolates against *C. camelliae* by spraying onto unopened flowers.

7.3.6.3.1 Materials and Methods

Cultivar 'Brian' was used for this experiment in the field. The flower buds about to open were tagged with the name of the treatment. The entire bud was sprayed with whole broth bacterial cultures using a fine atomizer until the surface of the whole bud was thoroughly covered. The atomizer was purged between bacterial treatments by spraying SDW into a waste container. The buds were then sprayed with ascospore suspension ($5x10^5$ spores/ml) using the same aerosol spray until satisfactory coverage of bud surfaces was achieved. Ascospores were applied 1 h, 24 h 48 h or 72 h after bacterial suspensions. Control treatments included SDW, buds sprayed with BCA before ascospore inoculation, and non-ascospore BCA inoculated buds.

The experiment was conducted as a factorial design in RCB with ten replicates. Single flower buds were used as the experimental unit.

Four and 7 days after ascospore inoculation the disease severity of flowers was measured on the 0-5 scaling system described in previous experiments. The number of lesion was counted on individual flowers 7 days after ascospore inoculation.

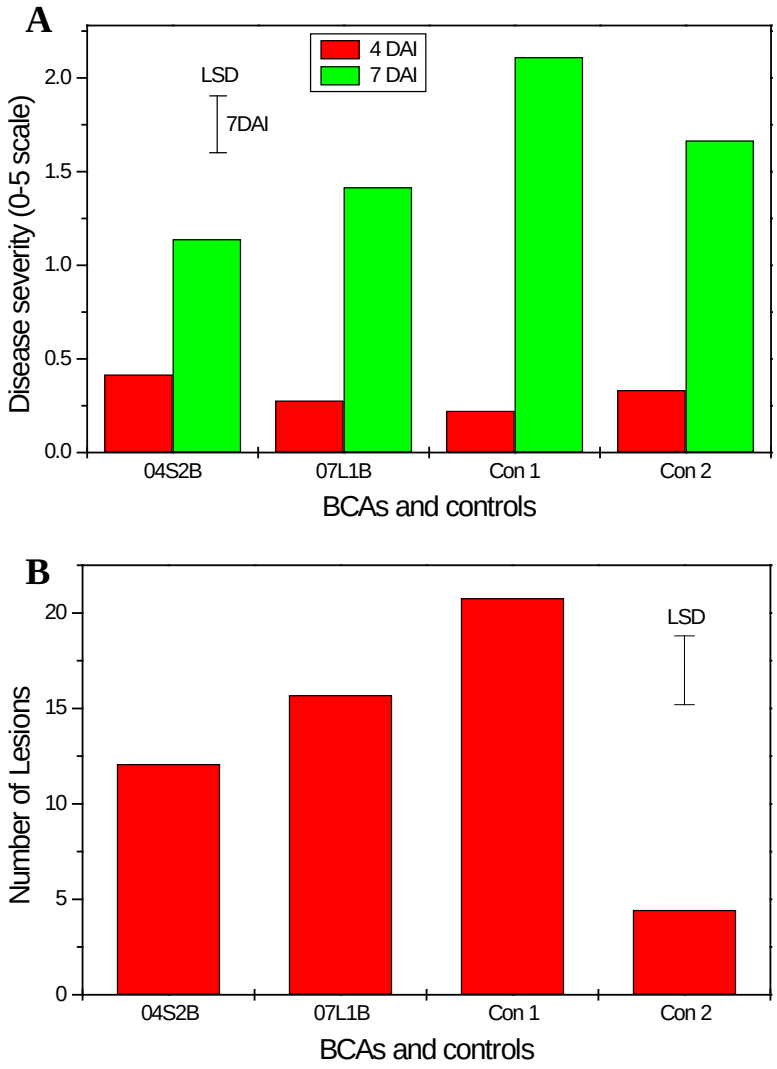


Figure 7.18. Disease evaluations on camellia flowers treated with isolates 04S2B and 07L1B. BCA sprayed at bud stage of the flower. *C. camelliae* inoculated at 1h, 24 h, 48 h and 72 h after BCAs inoculation. Con1 = *C. camelliae* only, Con 2 = No spray. A) Average disease severity at 4 days and 7 days after *C. camelliae* inoculation, B) Average number of lesion 7 days after *C. camelliae* inoculation. Vertical bar represents LSD.

7.3.6.3.2. Results

Both bacteria significantly reduced disease severity and number of lesions 7 days after inoculation compared with the *C. camelliae* inoculated control but there were no significant differences between them (Fig. 7.18).

Isolate 04S2B significantly reduced number of lesions even with a long waiting period (72 h) before challenge by the pathogen, but in contrast, isolate 07L1B significantly reduced number of lesions at earlier (at 1h and 24 h) rather than the long waiting period (at 48 h and 72 h) (Fig. 7.19).

In this experiment the untreated flowers showed high disease severity (percent-infected petal area) and low number of lesions (low disease incident). This is because one or two larger lesions developed, perhaps from a single spore natural infection in some flowers.

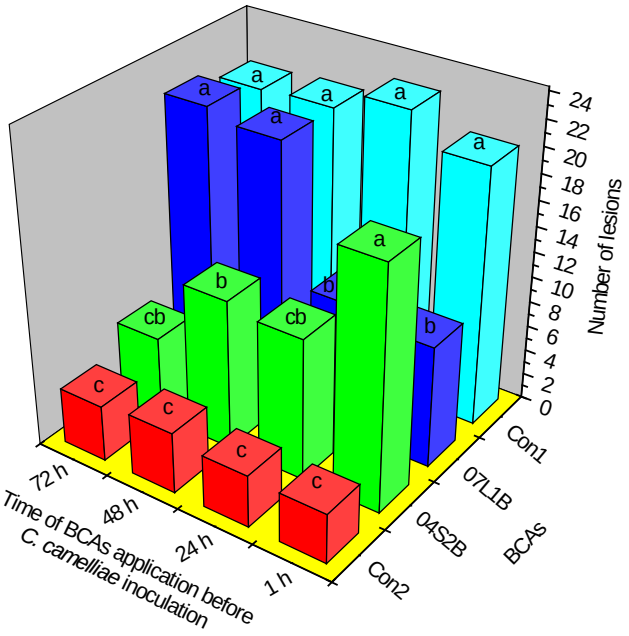


Figure 7.19. Disease evaluations on camellia flowers treated with BCAs (isolates 04S2B and 07L1B) over application time before *C. camelliae* inoculation. BCA sprayed at bud stage of the flower. *C. camelliae* inoculated at 1h, 24 h, 48 h and 72 h after BCAs sprayed. Con1 = *C. camelliae* only, Con 2 = No spray. Means with same letters are not significantly different.

7.3.6.4. Experiment 7.14. Evaluation of biocontrol of two bacterial isolates against *C. camelliae* by spraying to 2/3 opened flowers.

7.3.6.4.1. Materials and Methods

Experiment 7.13 was repeated with 2/3 opened flowers of the same cultivar ('Brian') in the same location.

Disease scoring

Three days after ascospore inoculation, disease severity was measured by percent petal area infected using 0-5 scaling system (Chapter 7, Section 7.3.3.1.2.1) and lesion size using 0-4 scaling system (Chapter 4, Section 4.3.6.2.1). The follwing scaling system was used in addition to the above scales to measure disease incident by counting number of lesions per flower.

Number of lesions rated in scale (0-5):

Scale	Description
0	= No lesions
1.	= 1-10 lesions
2.	= 1-20 lesions
3.	= 1-30 lesions
4.	= 1-40 lesions
5.	= >40 lesions

Bacterial population counts

The first three replicates from every treatment were sampled to estimate survival of bacterial populations by counting cfu. This was done by gently removing one petal from each flower 12 h after *C. camelliae* inoculation, immersing the petals in 10 ml SDW in a universal bottle and plating a 100 µl of aliquot on NA after 10 or 100 fold dilution.

7.3.6.4.2. Results

The isolates did not differ in biocontrol efficacy or average disease severity. Lesion size and number of lesions were significantly reduced compared with the *C. camelliae* inoculated control (Fig. 7.20). The only significant difference between time of BCA application on the above three assessments was that flowers treated with isolate 07L1B showed a significantly larger lesion size 72 h after BCA application compared with other times of inoculation (Fig. 7.21). However, all assessments were much lower than the *C. camelliae* only inoculated control. Many uncountable tiny lesions (< 0.5 mm in diameter) were observed on bacterial isolates treated flowers while well-developed and merged lesions were on untreated flowers (Plate 7.12). Development of tiny lesions in the treated flowers indicated that bacterial suspension effectively prevented number of spores germination and germ tube penetration however, those manage to penetrate the petal surface unable to develop symptoms due to antifungal activity resulted many tiny lesions.

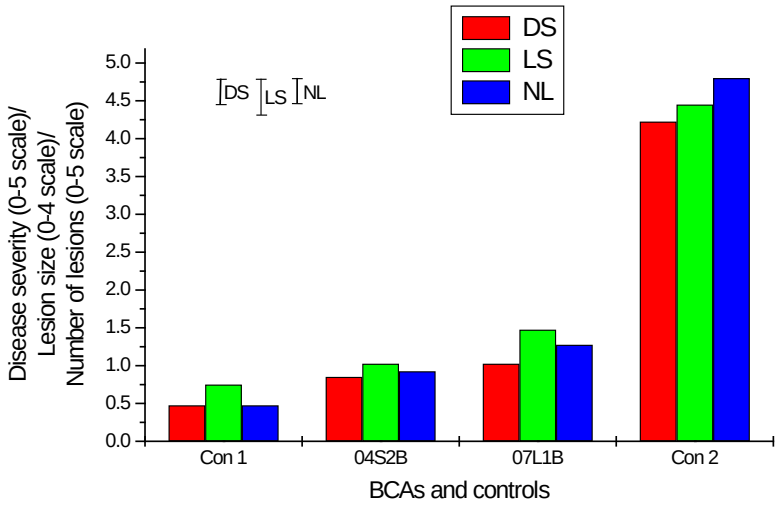


Figure 7.20. Biocontrol evaluation of bacterial isolates 04S2B and 07L1B in the field (Experiment 6.10). Con1 = No spray. Con 2 = *C. camelliae* only. Ds = Disease severity (0-5 scale). LS = Lesion size (0-5 scale). NL = Number of lesions (0-5 scale). Vertical bars represent LSD.

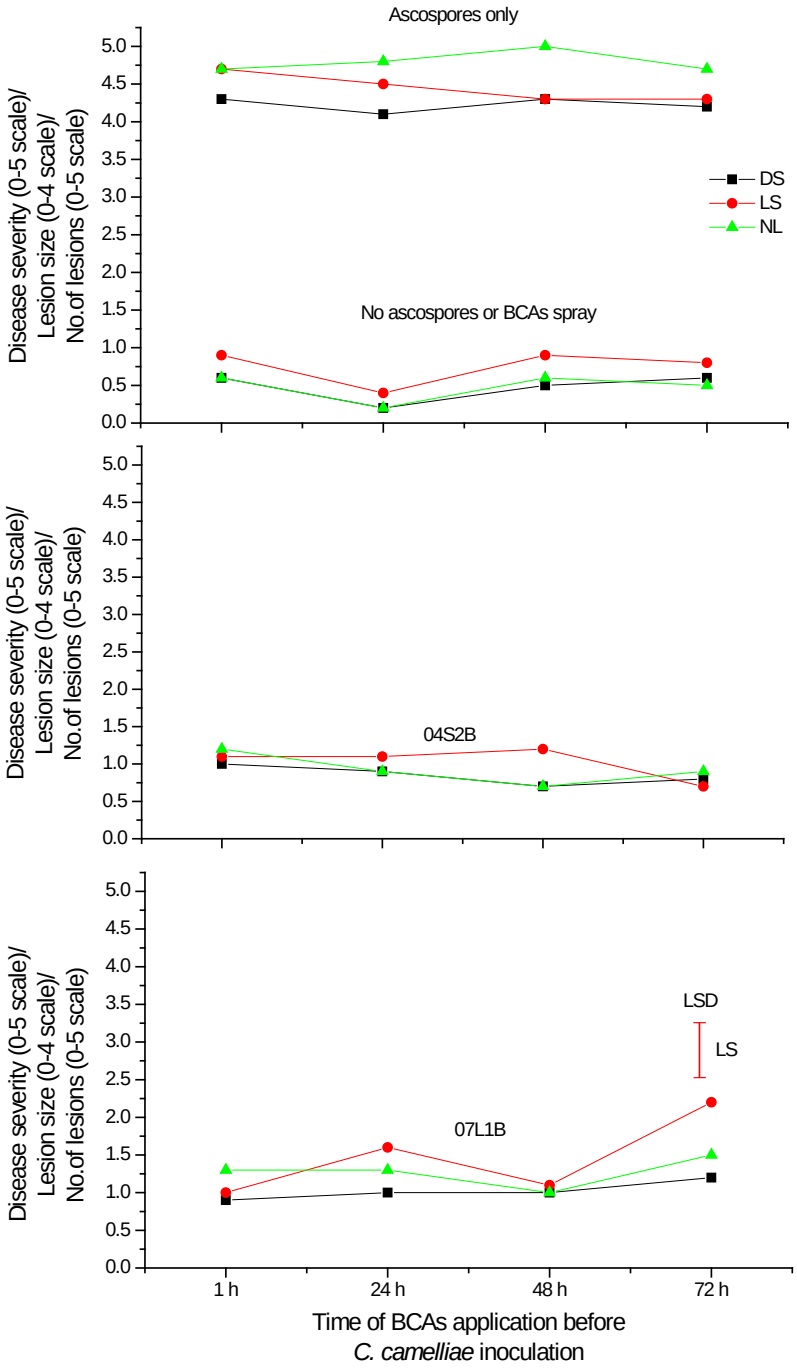


Figure 7.21. Time of BCA application before *C. camelliae* ascospores were inoculation in the field. A comparison of BCA effect over time with ascospore-only inoculated and none inoculated controls. DS = Disease severity, LS = Lesion size, NL = Number of lesions. Vertical bar represents LSD.

Plate 7.12. Reduction of lesion size and disease development of camellia flower blight by spraying BCAs before *C. camelliae* inoculation in the field. A) Flower inoculated with *C. camelliae* ascospores. B) Flower sprayed with isolate 07L1B 72 h before ascospores inoculation. Images captured 3 days after *C. camelliae* inoculation.

The average bacterial population significantly increased on flowers sprayed with BCAs compared with non- sprayed flowers. The bacterial population was significantly higher in isolate 07L1B sprayed flower than in 04S2B sprayed one. The population of bacteria on flowers tested with isolate 04S2B declined significantly 12 h after application from 26000 cfu/cm^2 to 10000 cfu/cm^2, a population size that was maintained for 84 h. In contrast, populations on flowers treated with isolate 07L1B significantly increased after 12 h from 27000 cfu/cm^2 to 42000 cfu/cm^2 at 36 h but the population then declined significantly to 10000 cfu/cm^2 over the next 24 h. After a further 24 h the population again significantly increased to 21000 cfu/cm^2. In the control treatment bacterial population increased to 84 cfu/cm^2 and 150 cfu/cm2 for the *C. camelliae* only sprayed and unsprayed flowers respectively (Fig. 7.22).

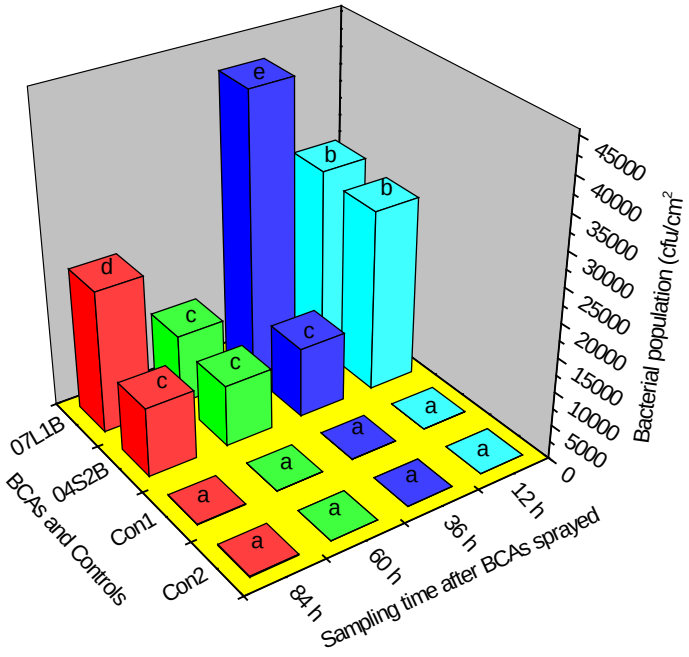


Figure 7.22. Bacterial population of camellia flowers at different times after application. Colony forming unit (cfu) counts of bacterial populations of isolate 04S2B and 07L1B sprayed flowers, and *C. camelliae* ascospore only-sprayed and non-sprayed flowers over time. Con1= C. camelliae only sprayed, Con2 = No spray. Means with same letters are not significantly different.

7.3.6.5. Experiment 7.15. Evaluation of environmental factors affecting on BCAs survival and biocontrol efficacy on *C. camelliae* in the field

7.3.6.5.1. Materials and Methods

Two cultivars (Brian and E.G.Waterhouse) were used to evaluate environmental effect on the survival of two bacterial isolates. The experiment was conducted separately on both cultivars at different times. The bacterial suspension of the isolates was prepared in NB and sprayed as described in previous experiments. The sampling and the cfu counts were processed same as the above experiments.

Flower temperature in between petals, flower petal wetness and, the environment RH and temperature were recorded using Tiny Tag dataloggers (Appendix IV) set to record every 20 min during the experiment.

7.3.6.5.2. Results and Discussion

Cultivar Brian

There was no significant difference observed between petal temperature and environmental temperature during the experimental period. Relative humidity was as high as 100% but petal wetness varied from 20% to 100% throughout the experiment period. During these climatic conditions, the bacterial population dropped about 75% at 24 h, slightly increased at 48 h, and at 72 h it reached about 50% of the initial population counted 1 h after application (Fig. 7.23).

Cultivar E.G Waterhouse

There was no significant difference observed between petal temperature and environmental temperature during the experimental period. Relative humidity (25% to 100%), petal and environment temperatures (7^{o}C to 35^{o}C) were highly variable during day and night. Petal wetness was almost zero through out the experiment except for a few hours when it reached more than 80% on the first day. During these climatic conditions the bacterial population dropped to almost 0% at 24 h from the initial population counted at 1 h after application. This situation also was with slight increase observed at 48 h but the population increased at 72 h to 40% of the initial population at 1 h (Fig. 7.24).

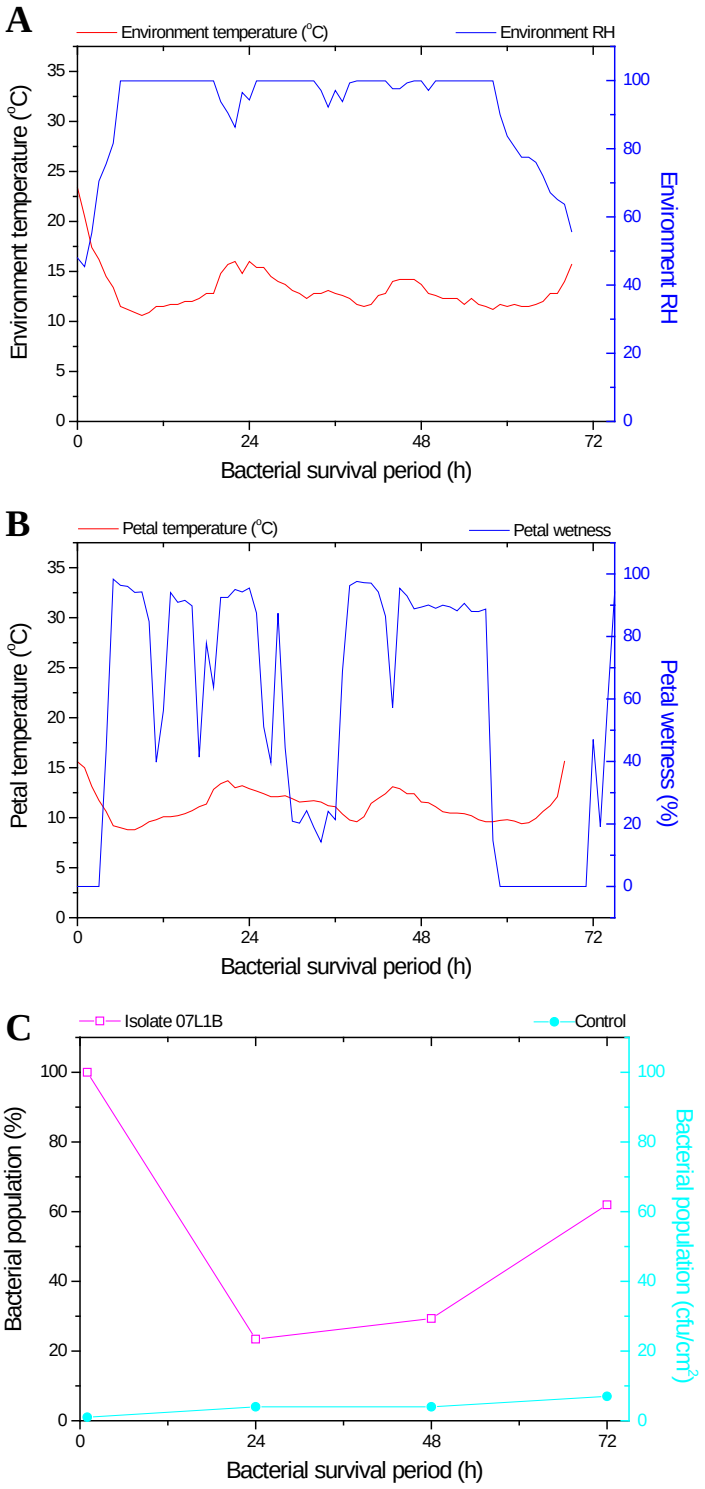


Figure 7.23. Effect of Temperature, Relative Humidity and petal wetness on potential bacterial BCA (07L1B) survival in Brian camellia flowers in the field. A) Environment temperature and Relative Humidity B) Petal temperature and wetness C) Survival of bacterial population in petal over time.

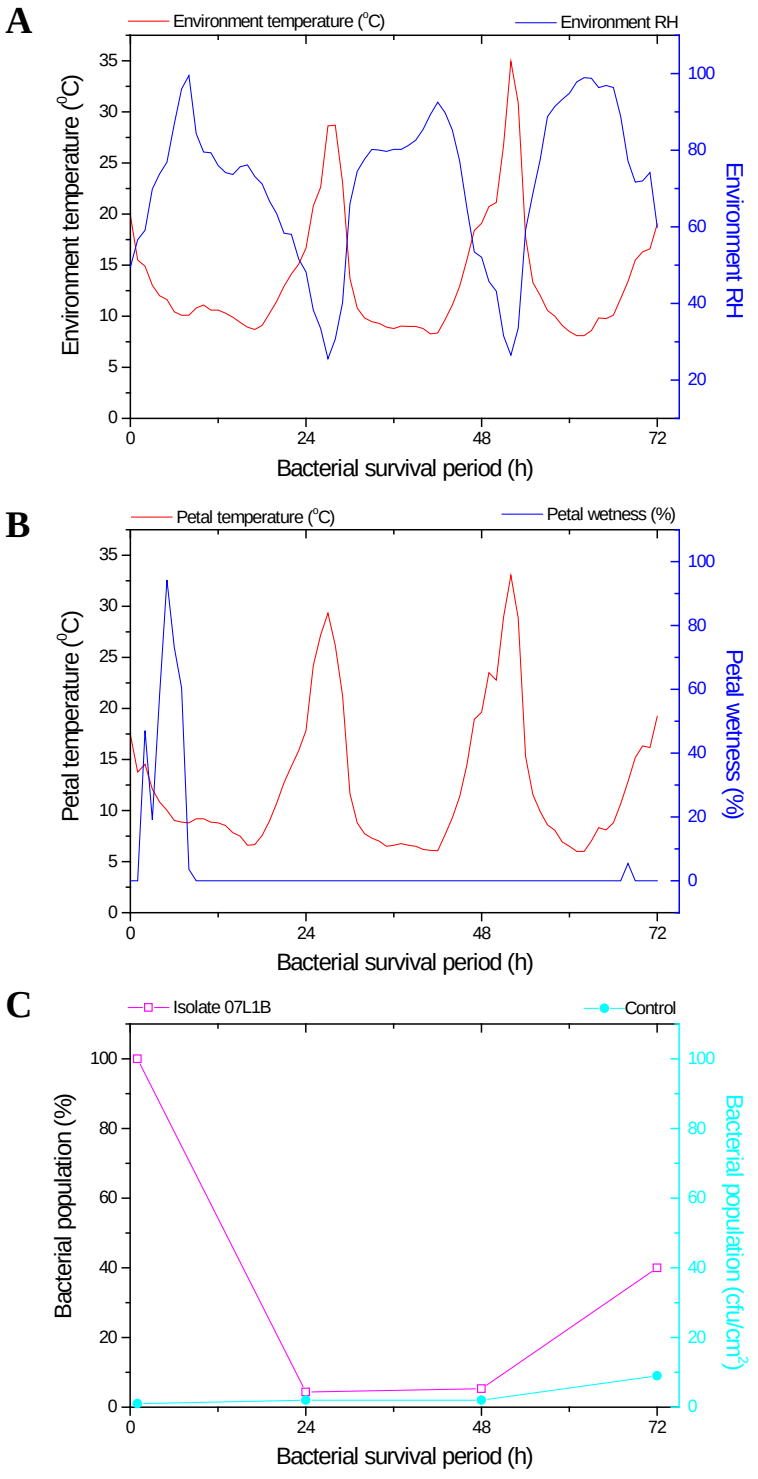


Figure 7.24. Effect of Temperature, Relative Humidity and petal wetness on potential bacterial BCA (07L1B) survival in E.G Waterhouse camellia flowers in the field. A) Environment temperature and Relative Humidity B) Petal temperature and wetness C) Survival of bacterial population in petal over time.

Although bacterial populations behave similarly, the percent population recovered from each cultivar is varied (Fig 7.25) due to environmental condition. High RH and petal wetness, and almost stable temperature ($10^{o}C$ – $15^{o}C$) retain high percent population (Fig 7.23) than the other condition where 0% petal wetness and highly fluctuated RH (25% - 100%) and petal, environmental temperature ($7^{o}C$ - $35^{o}C$) (Fig 7.24). These observations indicate that temperature, RH and petal wetness remarkably influence the survival of bacteria on the petal surface. However, gradual increases in population 48 h after inoculation in both cultivars indicated that the bacteria firmly colonized and start to multiply on the petal surface after some time even in the two different adverse conditions.

Very few residential microbes in the control treatment were obtained from both cultivars during the experimental period (Fig 7.23 and 7.24).

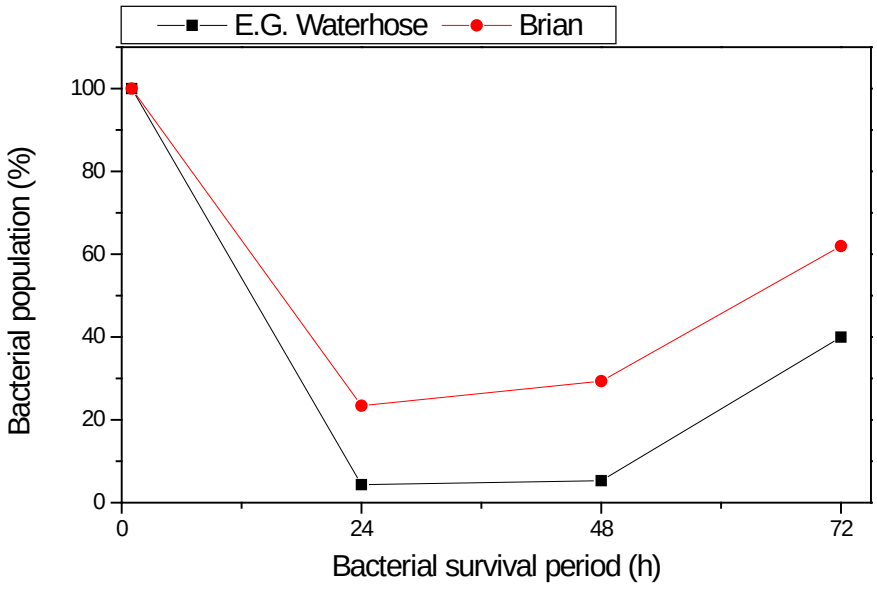


Figure 7.25. Survival of bacterial population in cultivar Brian and E.G.Waterhouse camellia flowers over time in the field.

7.4. Discussion

The early methods to screen for antagonistic microorganisms were based on the mechanisms, which were, believed to be involved in their interaction with pathogens. Antibiotic production was considered to be the main mechanism of antagonism. However, a screening program based on this assumption is likely to discard potential antagonists because of their failure to cause inhibition in culture or select ones that were of no use in the field. Hence a new approach for antagonist selection was adopted: the attachment bioassay developed by Cook et al. (1997a) was used to isolate biocontrol agents against *C. camelliae*. Although this attachment assay mainly targets antagonists that show mycoparasitism through adhesion to fungal hyphae (Cook et al. 1997b), there also other mechanisms, including antibiosis, that could function against the pathogen (Cook 1997). The microbes isolated here attached to *C. camelliae* germlings but only two bacterial isolates consistently gave total inhibition of ascospore germination when they were tested in detached petal bioassays.

Detached petal bioassays were used to screen selected potential BCAs for control of *C. camelliae* on camellia flowers. The validity of using *in vivo* screening, as opposed to *in vitro* methods has been discussed extensively (Campbell, 1986, 1994; Merriman and Russell 1990; Whipps, 1994) and general consensus favours the testing of putative BCAs using as 'natural' an environment as possible. To the initial tests of putative BCA in this study, detached petals were used rather than whole flowers, to permit easy screening of a large number of isolates in terms of space required as well as time to measure entire lesion sizes using a photo scanner in conjunction with the photoshop program in a computer. Earlier work reported the successful use of petal discs to select potential biocontrol bacteria for *B. cinerea* infection of petunia (Gould et al. 1996) but intact petals survive longer than discs. The single petal assays allow for selection of phylloplane competent bacteria, with exogenous nutrients available only from petal exudates and from endogenous reserves, which were accumulated during culture. The screening has conducted in condition fauourable for progress of diseases as free water and temperature conditions ($20^{o}C/20^{o}C$ day/night) were within the range at which infection and natural disease development occurs during the camellia flowering season (P G Long, personal communication). The closed

system adopted was also favourable to the pathogens, as this maintained a high relative humidity. This factor has been shown to be important for the development of *C. camelliae* (Chapter 4 in this thesis). Thus, bacteria, which exhibited good disease suppression in the bioassays, were considered to be of value for further glass house and field trials.

A series of detached petal assay tests with ascospores inoculated by different methods, to screen potential biocontrol agents showed that isolates 07L1B and 04S2B were good potential biocontrol agents against *C. camelliae*. An unusual feature of the suspension of these isolates was the apparent production of a wetting agent that spread the supension over the petal surface. An i*n vitro* test showed that the suspension inhibited *C. camelliae* mycelial growth but the results of dilution and the use of washed bacterial cells prior to application to the petals demonstrated that the disease suppression observed was due to antifungal metabolites produce during culture and that the bacterial cells themselves had little effect on disease progress.

Both isolates 07L1B and 04S2B gave very good control of *C. camelliae* when applied as a whole broth culture. It was shown that although the supernatant did indeed suppress disease progress, suggesting antifungal metabolite production during culture. The bacterial cells resuspended in NB also gave a significant disease reduction. Similar work also carried out by Fiddaman et al. (2000) to determine the mode of action of a promising bacterial isolate against *Botrytis cinerea* on lettuce leave. They obtained the same reaction of this study by reduction of lesion size and suggested that the production of antifungal substance is the cause for the disease reduction. In this study the disease control observed as the petal tissues were incubated under condition conducive for both bacterial and fungal growth, indicating the potential for in situ antagonism of the pathogen.

Control of number of lesions but not of lesion spread indicates control before infection – germination and/or germ tube growth and penetration. In this study, total control on pipette application to ascospores inoculation and minimum level of symptoms development on spray application to spray or gravity ascospores inoculation suggesting inhibition of ascospores germination occurring direct contact with bacterial suspension and there were

no effect once the germ tube penetrate the cuticle and grow inter cellularly. *C. camelliae* ascospores germinate and directly penetrate with short germ within 6 h after landing on the petal surface (Chapter 5). This is why 24 h delay application of these isolates does not control the disease where ascospores germination and germ tube penetration has already been taken place before bacterial suspension arrival. Therefore, the bacterial application should be well ahead before pathogen arrival.

With the exception of Nicky Crisp, these two isolates controlled *C. camelliae* well on a range of different cultivars. This shows the BCAs concerned are not cultivar specific. The symptoms that developed on uninoculated petals of Nicky Crisp indicated that they had natural infections, which would have been established before bacterial isolates application hence the penetrated would have occurred with consequent lack of disease control.

Reisolation of both bacterial isolates from sprayed flower petals was attempted and circumstantial evidence suggested there was a rapid decline in the number of the test isolates on the flower petals in the field. This observation correlates with other bacterial strains (eg. Epiphytic Bacterium isolate A180; *Pseudomonas fluorescence* isolate C37 and C92 and *Xanthomonas campestris* isolate D119) tested with another pathogen in a different host (Leben et al. 1965; Austin et al. 1977). Generally, Fokkema and Schippers (1986) found about 105 residential microorganisms per cm^2 leaf. In this study, only 2-6 residential microorganisms (bacteria or yeast) per cm^2 were found on camellia flower petals. In some cultivars, for example E.G. Waterhouse, colony forming unit survive rarely detected during sampling. Although the bacterial population dramatically reduces in time in the field, the antifungal effectiveness does not reduce significantly for 72 h after bacterial isolate application suggesting that the antifungal microbial substances are remains effective on the petal surface even though the bacteria were not stable and did not withstand drying in the adverse conditions of the field environment. This observation is contrary to many other observations, which indicate that antagonists that perform well in the lab do not do so in the field. However, Leben et al. (1965) postulated that increased disease control by addition of nutrient and moist treatments is not because of multiplication of bacterial cells but because more cells are viable at the time of pathogen inoculation. In this field study, whole nutrient

broth bacterial cultures were used and this may have increased the disease control by maintaining the initial bacterial population that contributes more to the production of antifungal substances.

Colonisation and survival ability of these two bacterial isolates on petal surface varied with cultivars. This may be due to surface morphology and chemistry of the petals possibly the cuticle wax may determine the holding capacity or adhesion of surface microbes. For example, flower petals of cultivar E.G. Waterhouse show a more waxy and smooth shiny surface but retained a lower population of introduced bacterial isolates than cultivar Brian. Loss of bacterial glycocalyx can occur when they are cultured in laboratory media (Andrews 1990) can further contribute loss of attachment with surface. The effect of environmental factors on bacterial survival on both cultivars was tested at different times. However, 24 h after the application bacterial population has been reduced dramatically in both cultivars but Brian retained more population than E.G. Waterhouse. Favourable environment factors may be the reason for this because, petal and environmental temperatures were not varied and both of them are about 10^{o}C and petal wetness and RH are high as about 100% most of the time during experiment period. On the other hand in E.G. Waterhouse, petal and environment temperatures are varied for day and night from about 30^{o}C to 7^{o}C respectively and petal wetness low as 0 and the RH fluctuated as temperature for day and night from about 25% to 100% respectively. This observation indicates that the initial population was lost because of no immediate adhesion with petal surface and washed off by rainwater. However, bacteria that escaped from this situation able to colonize on the surface and increase the population.

The two bacterial isolates considered as the best and the most consistent BCAs for *C. camelliae* came from camellia flower blight resistant species, *Camellia cuspidata* (See Chapter 6). Isolate 07L1B was isolated from *C. cuspidata* leaves and isolate 04S2B isolated from the soil taken 10 cm below from the soil surface under the *C. cuspidata* tree. This observation goes with the widely held belief that biocontrol agents are best isolated from the host plant of the pathogen (Blakeman and Fokkema 1982).

In summery, the bioassay and field results presented in this Chapter indicated that a potential use of biocontrol agents of isolates 07L1B and 04S2B for the control of *C. camellia* causing camellia flower blight. These isolates appear to function by antibiosis and the substance(s) produced are stable in the field for at least 72 h. Because of the petal surface characters, the retention of bacterial population varied with the camellia cultivars and the duration of control effectiveness may depend on the longevity of the antifungal materials than of the bacterial populations that produce them. Application of these isolates must be prior to ascospores arrival on the surface for effective control of the disease.

Further research is needed to ascertain whether or not commercial control is possible with these BCAs.

Chapter Eight

General discussion and conclusion

8.1. Introduction

In recent years camellia flowers in New Zealand have been attacked by flower blight disease caused by *C. camelliae* the most serious disease of camellias worldwide (Raabe et al. 1978). The biology and infection process of the pathogen has not been studied in detail previously. Control strategies have consisted largely of experimenting with fungicides and with cultural practices with little knowledge of the basic biology or physiology of the pathogen. Biological control strategies have been tested for many pathogens but few have been very successful commercially (Andrews 1990) and this strategy has not previously been investigated for this pathogen. Breeding of resistant cultivars was not attempted because no cultivars were known to have resistance (Baxter and Thomas 1992). However, recent research has shown that sources of resistance do exist in the genus *Camellia* (Taylor 1999; Taylor and Long 1999). These findings give high hope for the development of resistant cultivars in the future but there is still a lack of information about resistance mechanisms. In general, breeding for resistance targets a gene or a set of genes that may responsible for single or several components of resistance (Crute 1985). Genes encoding proteins may involve different resistance processes and such defense related genes are potential targets for genetic engineering (Kombrink et al. 1993).

There have been few studies of *C. camelliae* compared with major pathogens of other crops. Host tissue is limited to camellia flowers, ascospores are the only infectious propagules, and the disease is seasonal. It is therefore difficult to study this pathogen throughout the calendar year since there are no alternate hosts. The studies of basic biology; factors affecting ascospore germination, germ tube penetration, symptom development and other infection processes, of testing of control strategies and of resistance mechanisms of camellia must all be investigated within a short period during the camellia plant flowering season and the active period of flower blight disease.

8.2. Glutaraldehyde in confocal microscopy

Prior to investigating the infection process and other biological aspects of the *C. camelliae* pathogen, it was desirable to develop techniques suitable for such investigations. In this thesis, both confocal and light microscopy were extensively used. In particular, CLSM was used to trace the pathway of *C. camelliae* germ tube growth inside the host tissue and to understand the infection process (Chapter 4). CSLM allowed visualization of the fungal structures inside the tissue in three-dimensional or in rotated images but a limitation in biological confocal microscopy is the number of suitable fluorophores that can be tagged onto biological macromolecules to show their location (Tsien and Waggoner 1995). Singh et al. 1997) used glutaraldehyde as a fluorescent agent to detect fungal hyphae in plant tissue. Glutaraldehyde frequently used as a fixative of plant or animal tissue for electron microscopy but it induces fluorescence of untargeted areas of treated specimens, a problem for some fluorescence microscopy (Anonymous 1996). However, Vingnanasingam (1998) has shown that it is possible to obtain clear confocal images of fungal hyphae treated with glutaraldehyde solution although there can be a problem with low contrast and high background fluorescence of some components of plant tissue which make it difficult to measure fungal biomass. Techniques were developed in the current study to eliminate or minimize this obstacle to clear images and allow efficient image manipulation.

A range of buffers, pH, glutaraldehyde concentration and incubation period, using quenching, brightening and clearing agents were examined along with imaging barrier filter manipulation to improve fluorescence contrast. Among these factors, high pH (>7) in PBS buffer, using Triton X-100 and 2-methoxyethanol after glutaraldehyde treatment gave considerably diminished background fluorescence. Glutaraldehyde concentration and incubation period did not reduce fluorescence intensity but widened the gap of fluorescence intensity between fungal hyphae and plant tissue thus increasing contrast of the fungal image.

It is important to note that studies of the infection process of a fungal pathogen need the host plant also to fluoresce to some extent to visualise the location of the processes. Therefore, rather than complete elimination of background fluorescence increasing the

differences of fluorescence intensity between fungal hyphae and plant tissue particularly at adjoining areas would best serve the purpose. The modified technique in this study did not completely eliminate background fluorescence but increase the differences of fluorescence intensity between fungal hyphae and plant tissue at the adjoining area by reducing plant cell fluorescence as well as brightening the fungal hyphae. Other than the original work of Singh et al. (1997) with wood fungi, modified techniques using glutaraldehyde in confocal microscopy has been applied only to *Botrytis cinerea* but should be generally applicable to other pathogens in different hosts.

8.3. Factors affecting spore germination and penetration

There are no previously reported studies on ascospore germination and infection of *C. camelliae* but affects at humidity and temperature on spore germination and infection of other pathogens have been extensively reported elsewhere. The effect of temperature and humidity vary from pathogen to pathogen. For example, Arauz and Sutton (1989) found that temperature and RH equally affect germination of ascospores of *Botryosphaeria obtusa* while Abawi and Grogan (1975) stated that *Whetzelinia sclerotiorum* ascospore germination was not drastically affected by temperature. In this study, *C. camelliae* ascospores germination and germ tube penetration was more influenced by RH than temperature. However, temperature influenced hyphal growth in host tissue, where low temperature (5^{o}C) restricted germ tube growth even at 100% RH (Experiment 4.1, Chapter 4). This observation suggests that cold weather may delay symptom development.

The effect of free water on spore germination and penetration varies with different pathogens. Ascospores of *Botryosphaeria obtusa* (Arauz and Sutton 1989), *Uncinula necator* (Gadoury and Pearson 1990) have maximum germination in free water. On the other hand, with ascospores of *Botryosphaeria dothidea* germination was low in free water but high in 100% RH (Sutton and Arauz 1991). In this study, free water had significant effect on *C. camelliae* ascospore germination and germ tube penetration, but the effect depended on the inoculation method. When spores were inoculated as a suspension, the presence of free water dramatically increased spore germination and germ tube penetration. Spores inoculated directly from apothecia by gravity deposition had as high a percentage

germination and penetration in the absence as in the presence of free water on the petal surface. This situation was possibly explained by the presence of a small amount of liquid associated with freshly released spores that could have provided a reservoir of moisture for spore germination (Experiment 4.2, Chapter 4). This observation, therefore, suggest that in the field, whether petals are wet with dew or rain, or have no free water (but high humidity) should not affect spore germination and germ tube penetration.

Germ tube growth over the surface of plant tissue before penetration is general in many pathogens and it is affected by temperature and moisture. For example, *Botryosphaeria obtusa* ascospore germ tubes were significantly longer in free water compared with the absence of free water in 100% RH. (Arauz and Sutton 1989). In *Botryosphaeria dothidea*, ascospore germ tube lengths declined with lower RH (Sutton and Arauz 1991). Hong and Michilides (1998) observed that *M. fructicola* ascospore germ tubes length increased as temperature increased (7 ^{o}C to 15^{o}C). In this study, *C. camelliae* ascospore germ tubes did not grow over the petal surface but germ tubes germinated with short germ tubes and penetrated immediately whether in the presence or absence of free water. This situation was also observed by Cole et al. (1996) with *Botrytis cinerea* and *Botrytis fabae* where germ tubes penetrated bean leaves immediately in the absence of free water but grew across the surface if water was present. However, in this study, a few spores were germinated and grew over the surface when spores were inoculated by pipette or brush. In brush inoculation, multiple germ tubes with extended germ tube growth and abnormal swollen hyphae were observed. These observations could be explained by the minor damage to the ascospore walls by brush bristles and or the presence of fungitoxic material released from micro damage of the petal surface by the same brush bristles during inoculation (Experiment 4.2, Chapter 4). The production of short germ tubes of *C. camelliae* and immediate penetration adjacent to the spore could be one reason why fungicides have failed to give satisfactory control of this disease since the fungus penetrated the petals without contacting extensive areas of the surface.

8.4. Infection process

A second aspect of this thesis was the infection process of *C. camelliae*. Fungal plant pathogens follow more or less specialized modes of penetration and growth within the host depending upon the range of plants they attack (Wood 1967). For example, *Colletotrichum* spp. achieve infection through wounds caused by physical damage or insects (Boher et al. 1983). Others penetrate cuticles and establish a benign, but often extensive, sub-cuticular infection prior to development of necrotrophic hyphae, which grow through and destroy tissue (Walker 1921). Generally, most fungal pathogens produce appressoria to form infection pegs or hyphae to penetrate the cuticle but in some cases, under different environmental conditions direct penetration with short germ tubes occurs. For example, *B. cinerea* produces short germ tubes and penetrates onion leaves (Clark and Lorbeer 1976), rose petal (Williamson et al. 1995) and bean leaves (Cole et al. 1996), and *B. fabae* also does the same in bean leaves (Cole et al. 1996) under conditions of dry inoculation with high humidity. In *C. camelliae*, the germ tube itself functions as the penetration hypha because no evidence for appressorial formation has been observed in this study. The germ tubes were short and penetrated directly through the cuticle of camellia petals under wet or dry condition within 6 h.

If the fungus grows successfully, the host cell may die rapidly, as in necrotrophic interactions, or after a benign phase, as in hemibiotrophic systems, while obligate biotrophic fungi maintain host cell viability for extended periods of time (Mendgen and Deising 1993). Subcuticular intramural pathogens (eg. *C. capsici, C. circinans, V. inaequalis*) grow exclusively beneath the cuticle and within the walls of host epidermal cells without entering them (Bailey et al. 1992). In this study, *C. camelliae* can be compared with subcuticular intramural pathogens because it behaves in the same way for at least the first 48 h. Bailey et al. (1992) speculated that hemibiotrophic *Colletotrichum* spp. had intimate cytoplasmic interaction with their hosts and appear to be highly host-specific (eg. *Colletotrichum destructivum*), while those that grow sub-cuticularly as well as in host cell walls, the sub-cuticular intramural pathogens (eg. *Colletotrichum capsici*), have a wide host range. Therefore, confusion is created between the relationship of sub-cuticular intramural pathogens and their host range since *C. camelliae* grows sub-cuticularly initially

but has a very specific host range. Further investigation of the infection process and subsequent *inplanta* growth is warranted.

8.5. Resistance mechanisms

Another aspect studied to promote control of flower blight disease was resistance mechanisms of *Camellia* spp. against *C. camelliae*. A wide range of host responses, a combination of physical and chemical barriers both preformed or induced after pathogen infection were recognized and discussed (Chapter 1). These include phytoalexin synthesis, callose deposition, lignification, oxidative bursts involving free radical production, and production of proteinase inhibitors and lytic enzymes such as glucanases and chitinases (Bell 1981; Kombrink et al. 1993). *Camellia* spp., in this study, showed different resistance mechanisms between or even within, species. All species tested inhibit *C. camelliae* ascospore germination or germ tube penetration either partially or completely on the petal surface (Chapter 6) but in susceptible cultivars, germ tubes penetrated within 6 h and colonized the tissue for the next 12 h (Chapter 5). A commonly observed structure is the development of a papilla, usually containing callose or lignin, at the penetration site of the pathogen (Aist 1976; Heath 1980). Such papilla-like structures were observed in this study when *C. camelliae* ascospores germ tubes attempt to penetrate to *C. cuspidata* or *C. lutchuensis* petals. Sherwood and Vance (1976) recognized the papillae formation was due to lignification on reed canarygrass leaves upon *Helminthosporium avennae* infection but in this study, the papilla was not chemically investigated due to shortage of time but studies of other plants have shown they are composed of callose or lignin and there is no reason to think that they would differ in camellia.

Another mechanism observed in this study is the hypersensitive reaction. This kind of reaction generally occurs with biotrophic pathogens (Crute et al. 1985). *C. cuspidata* species, in this study, showed localised cell necrosis indicating that the species have a hypersensitive reaction against *C. camelliae*. This must be an effective reaction because *C. camelliae* is biotrophic at least in the initial stage of infection (Chapter 5). Antifungal metabolites such as chitinases and β-1, 3-glucanases, the pathogenesis related (PR) proteins, have been extensively studied for their antimicrobial activities and are reported

elsewhere. These antifungal materials may be released when plants contact invading fungi or may be present constitutively in the extracellular space and inhibit the fungi (Boller 1993). *C. camelliae* ascospores, in this study, germinated and the germ tube grew over the surface with distorted hyphae without penetration on *C. transnokoensis* petals indicating the activities of antifungal metabolites. The presence of a yeast isolate on camellia petal surface induced abnormal swollen germ tubes (Chapter 7). In this experiment, the same type of large swollen hyphae of *C. camelliae* germ tubes was observed along with yeast-like organisms on the petal surface of *C. tricocarpa* species suggesting antifungal activities of these organisms. Therefore, it could be postulated that the resistance of *C. tricocarpa* may actually be due, at least in plant, to the presence of such yeasts on the petal surface.

8.6. Biological control

Yeast and bacteria have been used as biocontrol agents against many pathogens in different crops and examples are given in Chapter 7. Phylloplane microbes were isolated through the attachment assay used by Cook (1997) with the aim of obtaining microbes that attach to, and suppress *C. camelliae* growth (Chapter 7). Promising BCAs were isolated but their mode of action was antibiosis rather than mycoparasitism. Selected putative BCAs completely or partially suppressed ascospore germination on petals thus reducing disease. The biocontrol results obtained from two bacterial isolates in the field environment (Chapter 7, Section V) were consistent with data obtained from the laboratory (Chapter 7, Section II and III), which included timing of BCA application. In all experiments they conferred plant protection.

Petal discs (Gould et al. 1996) or leaf discs (Fiddaman et al. 2000) have been successfully used to evaluate biocontrol agents against plant pathogens *in vivo*. In this study, detached camellia petals were initially used to evaluate putative BCAs under conditions of free water, temperature (20^{o}C) and relative humidity (~100%) in a closed system adopted to maintain these conditions for *C. camelliae* spore germination and infection (Chapter 4). Two bacterial isolates that gave a total control of ascospore germination were selected for further evaluation of their potential biocontrol activities.

Application of bacteria or yeast biocontrol agents 24 h before *B. cinerea* inoculation to tomato stem gave a higher level of biocontrol than either simultaneous or delayed application (Cook et al. 1997b). In this study, the two-selected bacterial isolates gave significant biocontrol when applied 72 h before or simultaneous until *C. camelliae* arrival but they failed to control ascospore germination or disease development when applied 24 h after *C. camelliae*. Similar results were observed in field trials. *C. camelliae* ascospores germinated and directly penetrated with short germ tubes within 6 h of landing on the petal surface and they did not produce any surface germ tubes (Chapter 5). The bacterial isolates may not have biocontrol efficacy on fungal hyphae already in host tissue and may be effective only against ascospores. In contrast, Cook et al (1997b) observed biocontrol with a 48 h delayed application of BCAs against *B. cinerea* in tomato stems. The current results suggested that the bacterial application should be made at or before the arrival at *C. camelliae* ascospores on the petal surface.

Ascospore germination of *Eutypa lata* was totally inhibited by a strain of *Bacillus subtilis* and reduced infection was observed when they were applied to *Eutypa lata* inoculated grapevines. An antibiotic substance from the *B. subtilis* is the reason for the biocontrol effect (Ferreia et al. 1991). The two isolates tested against *C. camelliae in vitro* and *in vivo,* in the current study, showed antifungal metabolites production during culture (Chapter 7, Section I & IV). Therefore, antibiosis could be the primary mode of action against *C. camelliae* germination and infection and the antifungal substance should be investigated for extract and purification for future reference or possible commercial production.

Decline of BCA populations was observed by Leben et al. (1965) on cucumber leaves and by Austin et al. (1977) on *Lolium perenne*. Decline of bacterial population was also observed in this study after application of both isolates to petals in the field. However, biocontrol effectiveness was observed when bacteria were applied 72 h before pathogen arrival. High longevity of antifungal substance may be the reason for the biocontrol efficacy. Moreover, bacterial isolates were applied as whole nutrient broth cultures that increased antifungal substances by maintaining the initial bacterial population. Leben et al

(1965) postulated that addition of nutrient and moist treatment can maintain viable cells at the time of pathogen inoculation resulting in increased disease control.

Survival ability of these two isolates in different cultivars may vary due to petal, morphological and chemical characters but the effectiveness of these two isolates against *C. camelliae* are not cultivar specific. However, day and night petal temperature, environmental temperature, RH and petal wetness in the field affects the bacterial population survival.

Blakeman and Fokkema (1982) speculated that biocontrol agents are best isolated from the host plant of the pathogen, and both promising BCA isolates used in this study are from a camellia flower blight resistant species, *Camellia cuspidata* (Chapter 6). Isolate 07L1B came from *C. cuspidata* leaves and isolate 04S2B was isolated from soil under the *C. cuspidata* tree.

8.7. Conclusion and recommendation for future studies

Some aspects of infection biology of *C. camelliae* on camellias were investigated and have been documented in this thesis. A technique using glutaraldehyde as a fluorescence inducer in confocal microscopy was developed to effectively investigate the infection process of *C. camelliae.* The technique was first used by Singh et al. (1997) for studying wood decaying fungi and was further developed with *B. cinerea* in lettuce leaves. It has been further refined using to *C. camelliae* in camellia petals and should be useful for other pathogen/plant host combination. The technique can be used to measure fungal biomass in the host tissue since the method considerably reduced the background fluorescence which is usually confusing and misleading in measuring the volume of fungal fluorescence from digital images - use to measure fungal inversion, effect of resistance, fungicide, biocontrol agents etc.

C. camelliae ascospore germination and germ tube penetration, like that of other fungi, is influenced by relative humidity, but temperature has little or no effect over the range encountered in the field during the blight season. Temperature has significant effect on

growth of infection hyphae in the petal tissue and determined the disease development. Ascospore germination and germ tube penetration occurs in the presence or absence of free water if the spores are inoculated as fresh spores deposited by gravity from apothecia under high humidity. The spores inoculated as an aqueous suspension need free water to remain for a high percentage spore germination and germ tube penetration. Absence or presence of free water did not change germ tube growth or mode of penetration but short germ tubes were produced prior to penetration under both conditions. When ascospores or petal the surface were subjected to damage then multiple germination or germ tube growth over the petal surface can occur. Spore germination and petal penetration occurred within 6 h and, ascospores inoculated as a suspension must remain wet for this period for maximum spore germination and germ tube penetration. In field conditions, it can be predicted that if the petal dries before penetration has taken place the number of infections would be reduced.

Confocal microscopy studies revealed the strategy of the infection process as well as some basic features of *C. camelliae* during infection. There was no evidence of appressoria. The fungus behaves as a biotrophic pathogen at least for 48-72 h. Most specifically its behavior fits with subcuticular intramural pathogens such as *Colletotrichum capsici* but, the pathogens in this category have wide host ranges. Because of these two contradictory characters of *C. camelliae,* it must be further investigated in relation to host tissue infected (petals v leaves and fruit) to be confirmed the appropriate category. The appearance of disease symptoms within 24-48 h even though intracellular inversion does not appear to be common within this time span also needs to be considered for future study. Further, a transmission electron microscopic study would be required to understand what occurs to the membrane at the contact point between host cell wall and hyphae during initial stage of infection process of *C. camelliae.* This may shed light on the nutrition transmission required for hyphal growth.

The resistance mechanisms of some camellia species were identified against *C. camelliae. C. cuspidata* expressed resistance to *C. camelliae* by papillae formation and a hypersensitive reaction while *C. lutchuensis* and *C. transnokoensis* express their resistance by formation of papillae and production of antifungal metabolites such as PR proteins

respectively. In addition to these mechanisms some species may have the capability to retain some microorganisms that functions as biocontrol agents on the petal surface. Future work is required for chemical analysis of papillae to determine whether these structures are formed by callose, lignin or other components. The types of PR protein require investigation to determine their role in resistance and their use in possible breeding programmes to incorporate resistance to *C. camelliae* in camellias.

The biocontrol results obtained from two promising bacterial isolates (07L1B and 04S2B) in the field environment were consistent with data obtained from the laboratory. The biocontrol efficacy of these isolates is not cultivar specific but these bacterial isolates are more effective when they contact ascospores directly. They did not show biocontrol activity on already infected flowers. The biocontrol efficacy last for at least 72 h after application and before pathogen arrival although the population declines over time in the field. Possibly the antifungal substance last longer in the field. The potential bacterial isolates and the antifungal substance should be identified for potential commercial production in the future.

This study, answered some basic questions on the biology of *C. camelliae* and provides a basis for evaluating control strategies. For example, systemic fungicides are not tranlocated to the flower and give little disease control (Baxter and Thomas, 1994). The alternative, contact or protectant fungicide must be applied before flowers become infected because there are no extensive surface germ tubes for the fungicide to contact. Further, the fungicide must have the ability to control ascospore germination and the effectiveness must be retained for several days so that flowers can be protected from subsequent arrivals of ascospores. The effective use of both fungicides and biocontrol agents could be coordinated through disease-forecasting systems that predict time of ascospore release from apothecia. Such systems will request more infrastructures on weather conditions in the field and the time frame for apothecial germination and ascospore release from apothecia. A summary chart of development of control strategies is given in Fig. 8.1.

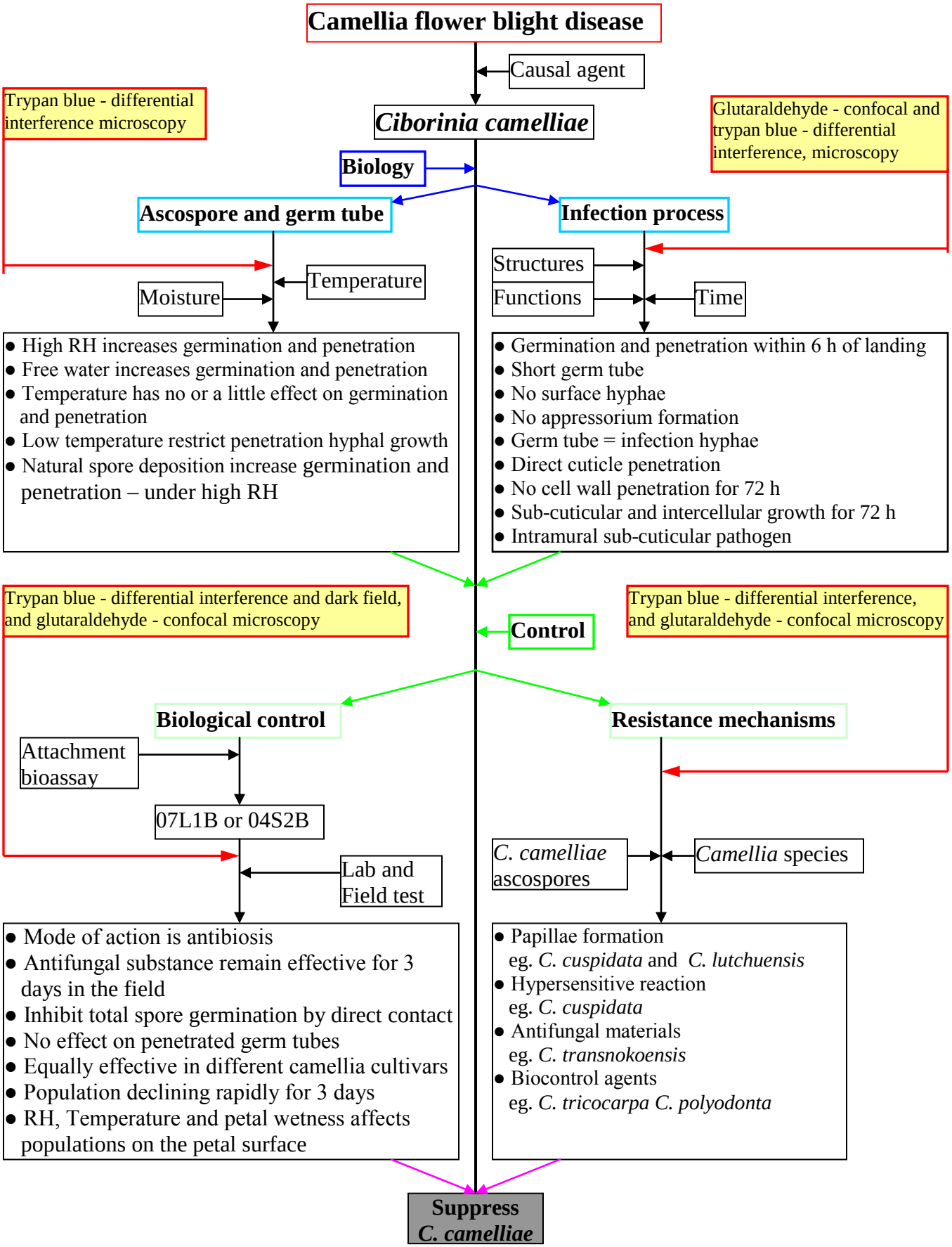


Figure 8.1. Chart of biology and control of *Ciborinia camelliae*

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Appendices

Appendix I. Recipes for buffers, glutaraldehyde solution, culture media and stains used in this study

1. Glutaraldehyde solution for confocal microscopy

I. Buffers

Phosphate buffered Saline (PBS)

Nacl = 40g
Kcl = 1g
Na2HPO4 = 5.75g
(or $Na_2HPO_4.12H_2O$ = 14.4g)
KH2PO4 = 1g
H_2O = 500 ml.

*Solution can be stored at room temparature. For normal working concentration, dilute 50ml concentration to 500 ml with distilled water. PH should be 7.2

Phosphate buffer (PB)

Solution A

NaH_2PO_4 = 1.948g or
$NaH_2PO_4.H_2O$ = 2.26g or
$NaH_2PO_4.2H_2O$ = 2.56g
H_2O = 100ml

Solution B

NaOH = 2.52g
H_2O = 100ml

* Mix 17ml of 2.52 % of NaOH solution with 83ml of 1.948% of NaH_2PO_4 or 2.26% of $NaH_2PO_4.H_2O$ or 2.56% of $NaH_2PO_4.2H_2O$ solution to get the phosphate buffer at pH 7.2-7.4 Adjust pH with 2N NaOH.

Tris buffer (TBS)

Tris (Hydroxymethyl) aminomethene buffer solution

$C_4H_{11}NO_3$ (M. wt 121.14) = 12.114g
H_2O = 1000ml

*Titrate with 0.1M Hcl

Universal buffers (UBS)

Citric acid = 6.008g
KH_2PO_4 = 3.893g
H_3BO_3 = 1.769g
Dimethyl barbituric acid = 5.266g
H_2O = 1000ml

*Titrate with 0.2N NaOH to get the required pH

II. Glutaraldehyde solution for fluorescence

Original

PBS-GA 16 (16% glutaraldehyde in PBS buffer)

25% glutaraldehyde (EM standard) solution = 16ml
PBS buffer = 84ml
*Adjust pH at 7.2 - 7.4 and store at 4^0C
Source:- Desbrey & Rack (1970)

Modified

1. PBS-GA 4 (4% glutaraldehyde in PBS buffer**)**

25% glutaraldehyde solution = 4ml
PBS buffer = 96ml
*Adjust pH at 7.2 - 7.4 and store at 4^0C

2. Tris-GA 16 (16 % glutaraldehyde in Tris buffer**)**

25% glutaraldehyde solution = 16ml
Tris buffer = 84ml
*Adjust pH at 7.2 - 7.4 and store at 4^0C

3. Tris-GA 4 (4 % glutaraldehyde in Tris buffer**)**

25% glutaraldehyde solution = 4ml
Tris buffer = 96ml
*Adjust pH at 7.2 - 7.4 and store at 4^0C

4. Uni-GA 16 (16% glutaraldehyde in universal buffer**)**

25% glutaraldehyde solution = 16ml
Tris buffer = 84ml
*Adjest pH at 7.2 - 7.4 and store at 4^0C

III. Washing solution

Buffered sucrose solution

Buffer = 100ml
Sucrose = 6.5 g
*Adjust pH at 7.2 - 7.4 and store at 4^0C
Source:- Desbrey & Rack (1970)

1. PBS-Sucrose 6.5
2. Tris-Sucrose 6.5
3. Uni-Sucrose 6.5

2. Light microscopy staining

Trypan blue (0.05%) in lactophenol

Phenol (Dissolve by gentile heating)	= 20 g
Lactic acid	= 20 g
Glycerine	= 40 g
Water	= 20 g
Trypan blue	= 0.05 g

Aniline blue

Aniline blue dye	= 0.005g
K_2HPO_4 (Mwt = 174.18)	= 0.0067g
Water	= 10ml

*Adjust pH at 7.2 - 7.4 and store at 4^0C

3. Culture media

Nutrient Agar (1)

Nutrient agar (OXOID)	= 28 g
H_2O	= 1000 ml.

Nutrient Agar (2)

Nutrient broth	= 8g/liter
Biological Agar	= 15g/liter
Distilled water	= 1000 ml.

Nutrient Broth

Nutrient broth	= 8g/liter
Distilled water	= 1000 ml.

NutrientYeast Dextrose Agar (NYDA)

Nutrient broth	= 8g/liter
Biological Agar	= 15g/liter
Yeast extract	= 5g/liter
D Glucose	= 10g/liter
Distilled water	= 1000 ml.

*Adjust pH to 4.5 with 6M HCl.

NutrientYeast Dextrose Broth (NYDB)

Nutrient broth	= 8g/liter
Yeast extract	= 5g/liter
D Glucose	= 10g/liter
Distilled water	= 1000 ml

*Adjust pH to 4.5 with 6M HCl.

Malt agar (1)

Malt extract	= 20g
Agar	= 20g
Distilled water	= 1000 ml

*Adjust the pH to 6.5 using NaOH.

Malt agar (2)

Molt extract agar (Difco)	= 33.6 g
Water (RO)	= 1000 ml.

*Adjust the pH to 6.5 using NaOH.

Malt agar (3)

Maltexo (Maltexo NZ Ltd.) = 30 g
Bacteriological agar = 15 g
Peptone = 5 g
H_2O = 1000 ml.
*Adjust the pH to 6.5 using NaOH.

Water agar

Bacterial agar = 15 g
H_2O = 1000 ml.

Potato Dextrose Agar

Potato dextrose agar (DIFCO) = 39 g
Water = 1000 ml.

4. Other buffer

McIlvaine buffer

Citric acid monohydrate (0.1M) = 21.01 g
Na_2HPO_4 (0.2M) = 28.40 g
PH at 6.0
* To obtain pH 6.0 mix 36.85 ml citric acid with 63.15 ml of Na_2HPO_4.

Appendix II. *Botrytis cinerea* conidiospore germination and germ tube penetration into camellia petals in the presence or absence of free water.

1. Absence of free water

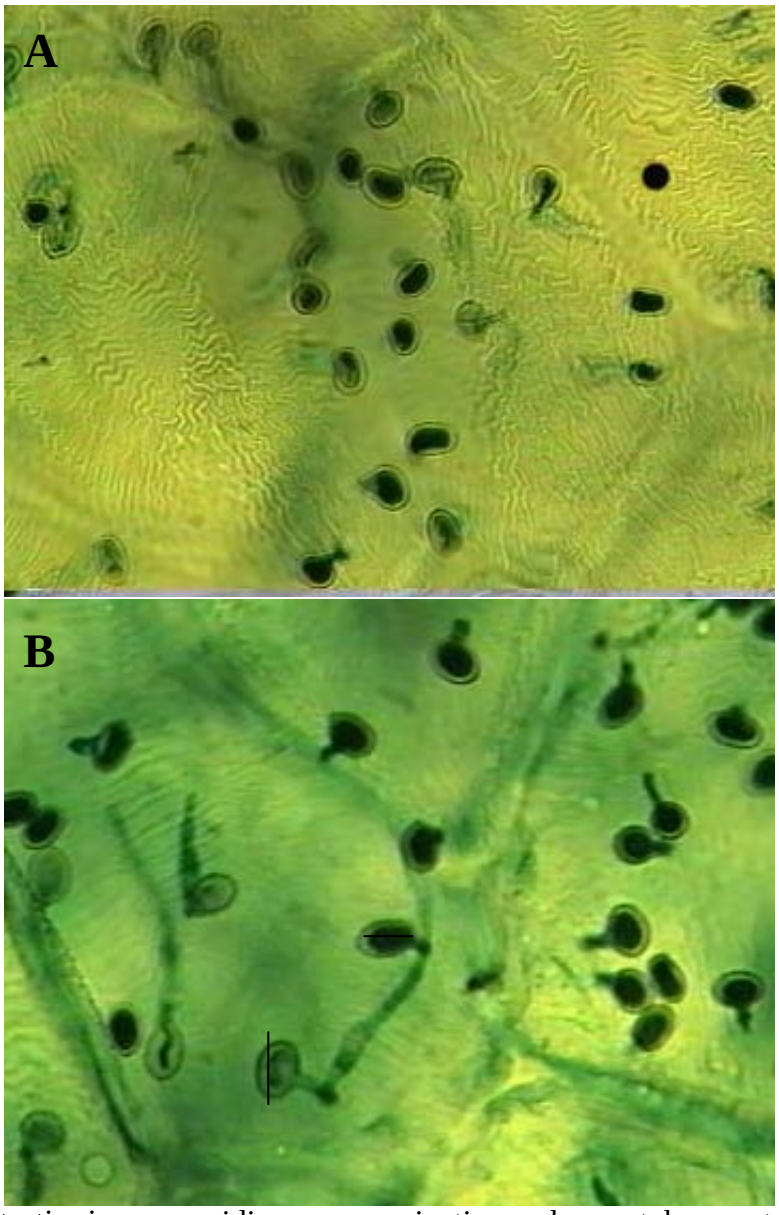


Plate II.1. *Botrytis cinerea* conidiopores germination and germ tube penetration into camellia petals in the absence of free water A) 6 h B) 12 h after inoculation.

2. Presence of free water

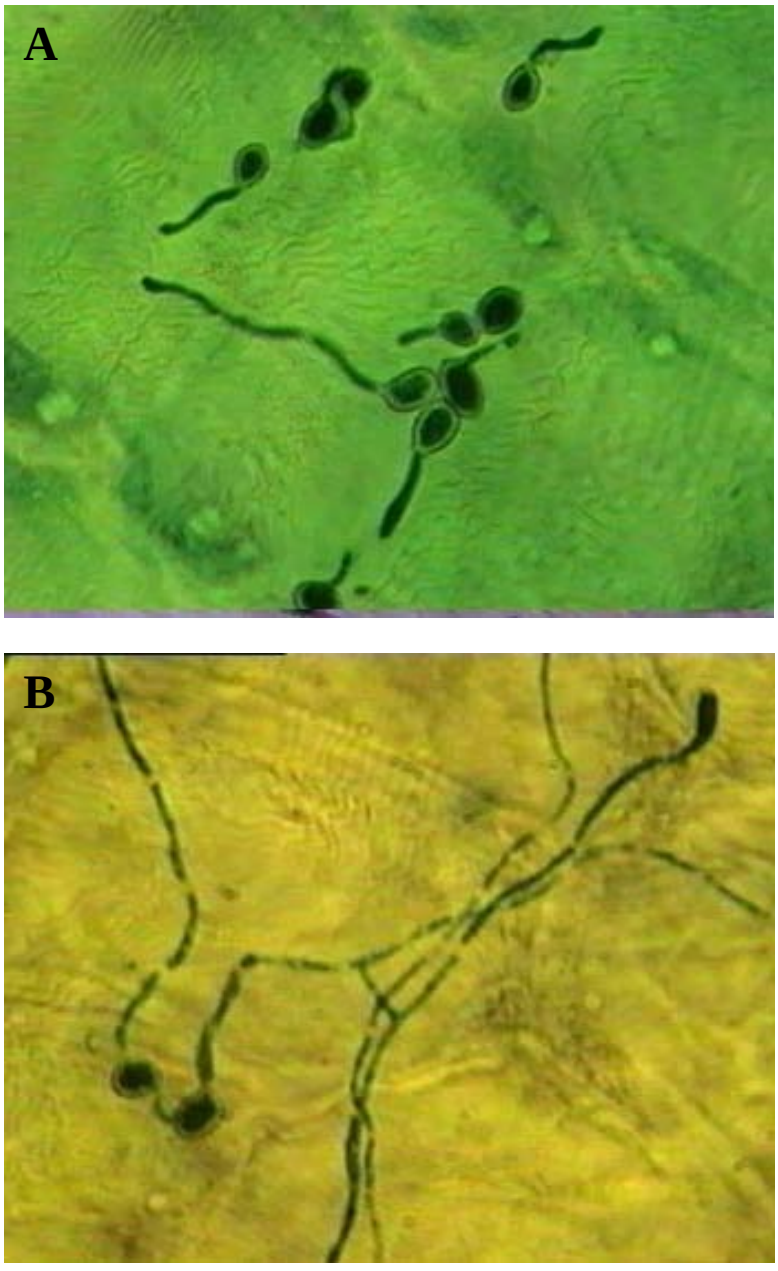
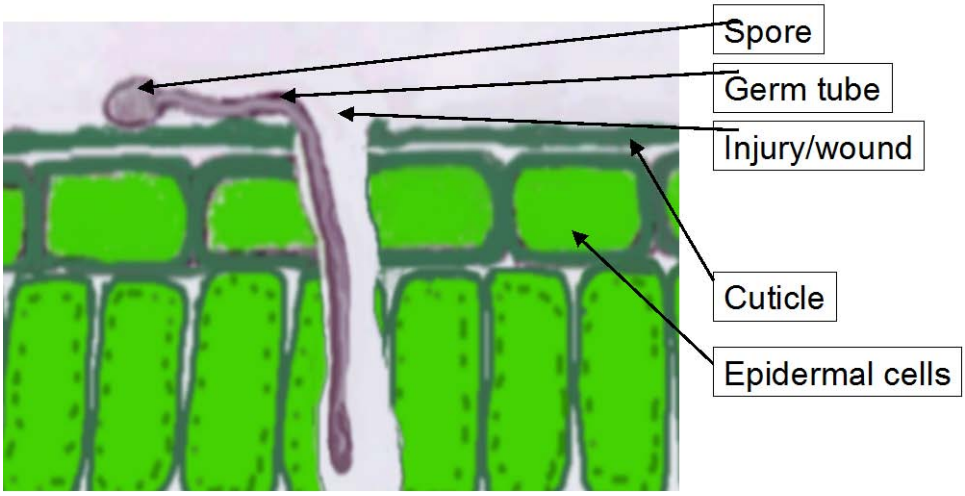


Plate II.2. *Botrytis cinerea* conidiopores germination and germ tube growth on camellia petal surface in the presence of free water A) 6 h B) 12 h after inoculation.

Appendix III. Illustrated diagram for different strategies of infection process of fungal pathogens

Strategies of infection process (1)

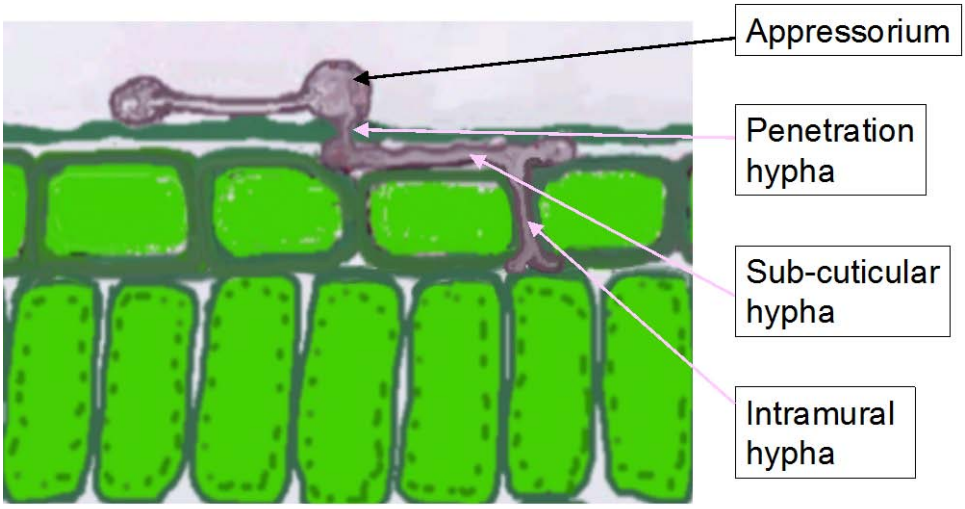
Infection through a wound



eg: *Colletotrichum gloeosporioides*

Strategies of infection process (2)

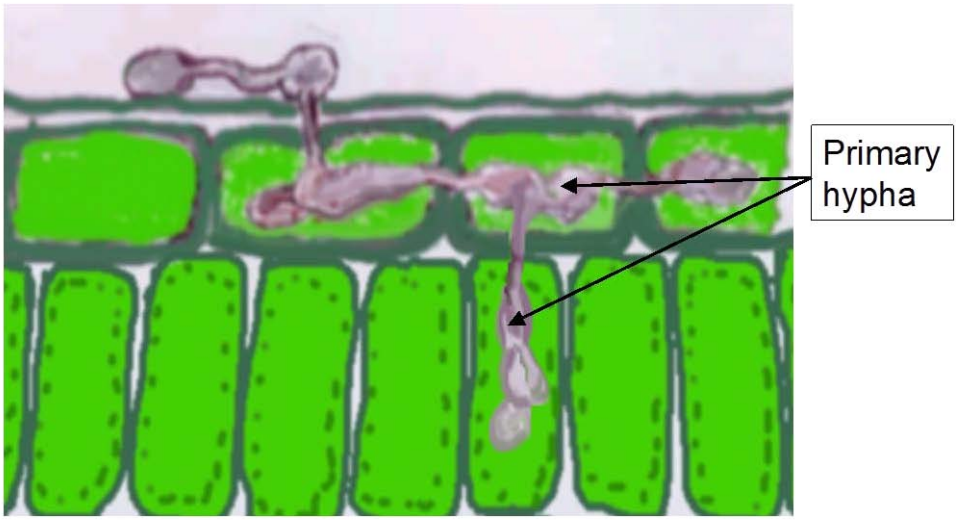
Sub-cuticular intramural



eg: *Colletotrichum capsici*

Strategies of infection process (3)

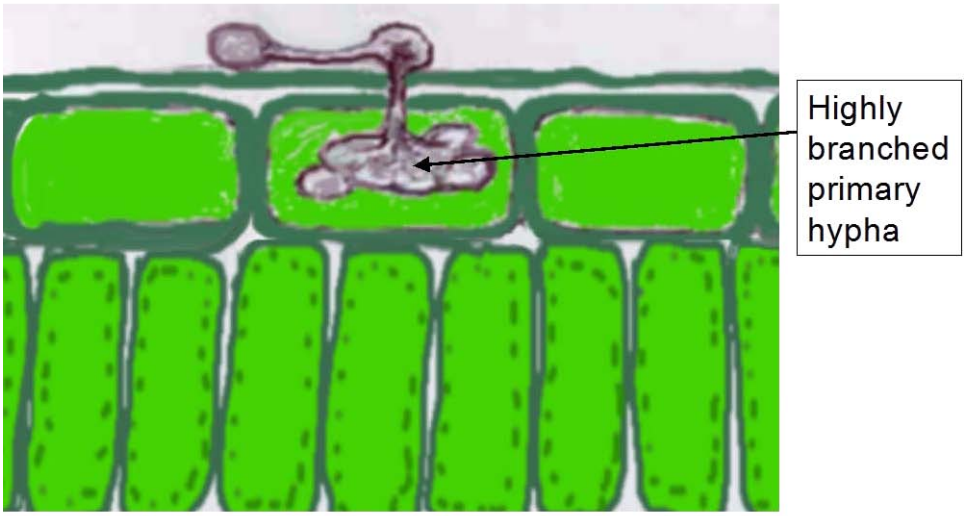
Intracellular biotrophy



eg: *Colletotrichum lindemuthianum*

Strategies of infection process (4)

Intracellular hemibiotrophy



eg: *Colletotrichum destructivum*

Appendix IV. Tinytag *Plus* apparatus

Data loggers manufactured by Gemini Data loggers - Tinytag Plus. Small, powerful, easy to use and offering many advanced features, loggers from the Tinytag Plus range are the ideal choice for most professional logging applications. Able to store more than 16000 data readings, giving over 5 months data at 15 minute intervals, with Trigger Start, 2 programmable alarms, and the ability to offload data while continuing to log all offered as standard.

The dataloggers are launched and offloaded using multi-lingual Software package - GLM. The software is Windows based and the buttons on the toolbar at the top of the screen guide users through the process of setting up loggers and retrieving data. Using this software package it is possible to display the information in any of the three methods: graph, statistics, or tabulated data. Since the screens are dynamically linked, when the user zooms into a narrower range of information on the graph, the data shown on the other screens will reflect the range displayed on the graph. Graphs may be overlaid and manipulated to show the relationship between different events and properties. Data may also be exported (by simple use of one of the buttons) into word-processing or spreads

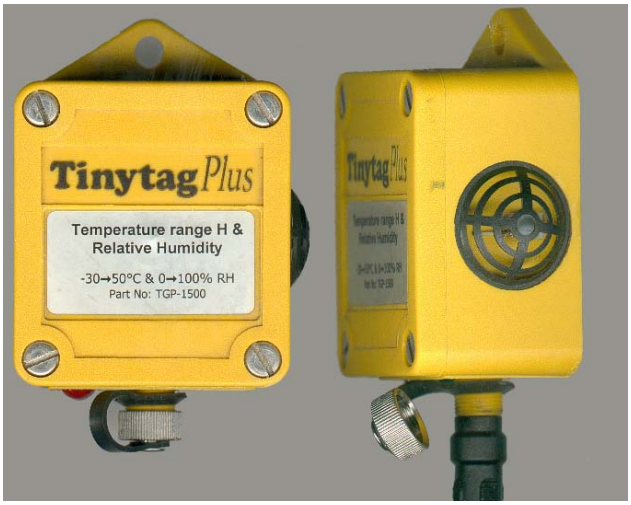


Plate 1. Tinytag Plus data logger with built in temperature and relative humidity probes used to calibrate relative humidity of closed containers of saturated salt solutions, and to measure environmental temperature and relative humidity in the camellia bush during field experiments.

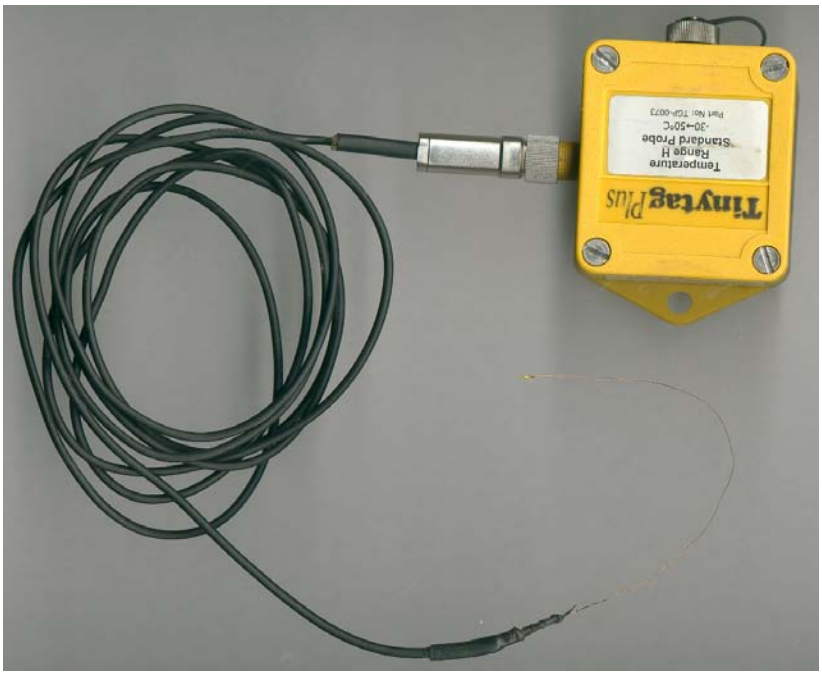


Plate 2. Tinytag P*lus* data logger with external temperature probe used to measure camellia petal temperature during field experiments.

Plate 3. Tinytag Plus data logger with external probe to measure area wetness. This probe was used to measure petal wetness during field experiments.

* Web page address for more details: http://www.geminidataloggers.com