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**TRANSFORMATIONS OF GRAPEVINE PATHOGENS
EUTYPA LATA AND *PHAEOMONIELLA*
*CHLAMYDOSPORA***

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ABSTRACT

A transformation system has been developed for the grapevine pathogenic fungi *Eutypa lata* and *Phaeoemoniella chlamydospora* using a positive selection system based on the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*). The system developed could give large, stable transformants at frequencies between 0.7 and 6.5 transformants per µg of DNA. A second type of colony also grew on the selective media. These were believed to be abortive transformants. The first type of transformants were characterized using classical molecular biological technologies such as PCR and Southern hybridization, and the transformation was shown to be successful.

Plasmids (pBCH-*gfp* and pCT74) containing a *gfp* reporter gene were also transformed into these two fungal species. Expression of the *gfp* gene was checked using a fluorescence microscope and *gfp*-expressing *E. lata* transformants were inoculated onto the host plants blackcurrant and grapevine. Confocal observation of the movement of fungal mycelia in wood tissues was performed but its interaction with host plant was not established in the time available. Purified *gfp*-expressing *P. chlamydospora* transformants were also obtained. A vector containing a fragment of the *P. chlamydospora* putative toxin gene *moxY* was constructed and transformed into *P. chlamydospora*. Putative *moxY* gene disruption transformants were screened with PCR followed by Southern hybridization. The putative *moxY* gene disruption transformants were spore purified and further confirmed with Southern hybridization. Whilst both PCR and Southern hybridization confirmed disruption of the *moxY* gene, clear evidence for the presence of an additional wild type *moxY* was also seen in the same transformants. This led to the suggestions that either *P. chlamydospora* is a natural diploid, or that *moxY* is essential for growth and that selective pressures led to the formation of a wild type: *moxY-hph* diploid.

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1.1 WINE INDUSTRY--A PROMISING INDUSTRY IN NEW ZEALAND**1.1.1 THE HISTORY OF WINE**

According to the recorded wine history, it was the early inhabitants of Asia Minor (more than seven thousand years ago) who first discovered wine. Since that time, wine has been widely used as a safe and healthy beverage to provide calories and essential vitamins required by the body and to get stress relief in daily life (Amerine *et al.*, 1980).

Generally speaking, there are two categories of wine. White wines which result from rapid separation of the juice from the skins and seeds, and do not allow materials such as tannins to remain in the finished wine (Ough *et al.*, 1992), and red wines where the colors and flavors from the skins are allowed to be extracted into the juice (Surico *et al.*, 2000). White and red wines are also made from different grape varieties.

1.1.2 WINE INDUSTRY IN NEW ZEALAND**1.1.2.1 Wine history in New Zealand**

Grapevines were first planted at Kerikeri in New Zealand in 1819. During the second half of the 19th century, only large quantities of cheap and low quality wine were produced (Cooper, 1984). With the encouragement of the removal of the Muller-Thurgau vineyard, and planting of varieties such as Chardonnay, Pinot noir and Merlot etc, New Zealand's reputation as a producer of premium wines has been gradually established (Cooper, 1984).

1.1.2.2 Recent development of wine industry in New Zealand

Recently, New Zealand has improved its wine technology greatly, and obtained a high reputation in the international wine industry, which has led to New Zealand wine exports in excess of NZD\$100 million (Spense *et al.*, 1998) per annum. Recently, the vineyard

area rose to 8,716 hectares in 2000 (an increase of over 40% since 1993), to over 10,000 hectares in 2002 (Spense *et al.*, 1998), and to 15,479 hectares in 2003 with 42%, 26% and 13% of this area situated in Marlborough, Hawkes Bay and Gisborne, respectively (Anonymous *et al.*, 2003). Half the 55 million litres produced in 2003 were exported at a value of NZD\$281 million. Sauvignon Blanc and Chardonnay are the two most commonly grown varieties (4344 ha and 3513 ha, respectively). As for red varieties, they are estimated to be planted in 2549 hectares. It is expected that by 2005, a further 2768 hectares will have been planted (Anonymous *et al.*, 2003).

New Zealand is relatively isolated from the world, but a variety of pathogens have been unconsciously introduced into New Zealand, which has a huge impact on this country in which agriculture and horticulture are of great importance. New strategies must be developed to maintain New Zealand's isolation from further grapevine pests and to research better management of existing pathogens such as *Eutypa lata* and *Phaeomoniella chlamydospora*.

1.2 REASONS WHY NEW ZEALAND CAN PRODUCE PREMIUM WINES WITH HIGH QUALITY

1.2.1 GEOGRAPHICAL REASON

New Zealand is a South Pacific island country with parts of the land uneven and hilly, and with alluvial clay loams in some areas. Thus soil types are generally quite fertile and very suitable for large scale growth of grass and grapevines (Jackson and Schuster, 1994).

1.2.2 CLIMATIC REASON

The average temperature of the main areas of wine-grape production in New Zealand is relatively low (less than 1390 degree days) and it is classed as a cool-climate region. The mechanism by which cool climates generally produce the best quality table wines still

remains elusive, but it is thought that the lower temperatures in autumn are of special significance (Jackson and Schuster, 1994). As warm climates shorten the time of ripening of grapes, and cause rapid development of sugars, rapid loss of acids, and high pHs, the direct consequence is that the juice is often unbalanced with respect to sugar, acid, and pH, and the time for accumulation of essential chemical compounds which add distinction to the wine is insufficient. However, a cool autumn can slow down this development process, thus the juice can be well balanced, and more aroma and flavor constituents are accumulated (Jackson and Schuster, 1994). A very good rainfall in these areas (650-1050 mm per annum) is another advantage for wine grape production (Jackson and Schuster, 1994).

1.3 GRAPEVINE PATHOGEN *E. LATA*

The vine pathogen *E. lata*, the fungus responsible for dying-arm disease in grapevines, and *P. chlamydospora*, the fungus responsible for Petri grapevine decline can both produce toxins. However toxin genes or genes involved in the toxin production have not yet been characterized. Previous studies on *E. lata* have demonstrated that eutypine, a phytotoxin produced by *E. lata*, can only be detected by GC-MS and MS-MS analyses in the crude sap and the inflorescences of diseased plants and can't be detected in healthy material (Tey-Rulh *et al.*, 1991), thus it has been deduced that eutypine is the toxin responsible for the symptoms of dying-arm disease. Further evidence supporting the above conclusion is from Deswarte, *et al.* (1994). Ultrastructural alterations induced by eutypine in leaf cells and protoplasts isolated from plantlets were observed by transmission electron microscopy. The eutypine-induced alterations of the cellular ultrastructure are similar to those previously described *in vivo* in the leaves of diseased grapevines (Deswarte *et al.*, 1996). However a more recently published paper expanded those investigations. A differential production of acetylenic phenol metabolites *in vitro* by three strains of *E. lata* was observed. This evidence suggests that eutypine is not solely responsible for phytotoxicity in grapevines but that dying-arm disease may result from a suite of compounds elaborated by the fungus (Molyneux *et al.*, 2002). With the development of a transformation system, it will be possible to make targeted gene

replacement mutants to determine if the genes are involved in toxin production and if the toxins are involved in the development of disease symptoms.

1.3.1 ROLE OF EUTYPINE

In order to gain a better understanding of the internal mechanism of *E. lata* infection on grapevines, Fallot *et al.*, (1989) successfully isolated an aldehyde compound, i.e. 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl aldehyde, a weak acid, later known as “eutypine”, in the tissues of vines infected by *E. lata*. No such product has been detected in healthy vine wood. After colonization, *E. lata* interacts with host wood, and leads to the production of eutypine, which induces ultra-structural alterations in leaf cells and protoplasts. At the same time, foliage symptoms are often observed (Tey-Rulh *et al.*, 1991). Convincing evidence for a role of eutypine in the disease is the speed of symptom appearance. In addition their intensity was proportional to the eutypine concentration, which further confirmed the functions of eutypine on the disease development (Philippe *et al.*, 1992).

A further study performed *in vitro* indicated an accumulation of eutypine in the cytoplasm through passive diffusion due to an ion trapping mechanism, which lead to proton leakage and uncoupling of mitochondrial oxidative phosphorylation (Deswarte *et al.*, 1996). The direct consequence of this is the modification of the rate of respiration and the energy balance of the grapevine cells. Some genotypes of grapevines have the ability to detoxify eutypine, and therefore show some resistance to its infection (Fallot *et al.*, 1997). Recently, more study focused on derivatives of eutypine, such as eutypinol (4-hydroxy-3-(methyl-3-butene-1-ynyl), but only limited effects have been detected (Amborabe *et al.*, 2001). A gene encoding an aldehyde reductase, which is able to convert the toxic eutypine to its biologically inactive form (eutypinol), have been introduced into *Vitis vinifera* cells, and conferred resistance to the eutypine toxin after expression (Guillen *et al.*, 1998). It is quite possible that new varieties of grapevines could be tested and planted in the near future somewhere in the world.

1.3.2 FORMATION OF ASEXUAL AND SEXUAL STAGES

It usually takes several years for *E. lata* growing on the vines to develop perithecial stromata (Figure 1.1). These are black in colour, and stromatic tissues can be seen on the surface of dead wood. It is perithecial stromata from which ascospores are discharged (Jones, 2001). The anamorph of *E. lata* produces conidiomata, which often exude characteristic single-celled conidia. Based on a previous study performed by Munkvold *et al.*, (1993), it is unlikely that spread of *Eutypa* dieback is mainly caused by conidia as vine-to-vine spread of this disease in vineyards without perithecia was not detected. At present, information about the role of the asexual stage of *E. lata* in the disease cycle is still limited (Jones, 2001).

1.5 EPIDEMIOLOGY OF *E. LATA*

1.5.1 DISSEMINATION OF THE PATHOGEN

1.5.1.1 Production and local dispersal of ascospores

It is said that transfer of viable fragments of mycelium to healthy wood is unlikely to function successfully as inoculum unless they are incubated in a moist environment (Carter, 1991). Carter has demonstrated that when the stromata were dry, a minimum rainfall of about 2 mm sufficed to initiate the liberation of ascospores from the perithecia. Currently, rain is the only known means of release and dispersal of ascospores (Jones, 2001). As vineyards that contain perithecia of *E. lata* have consistently higher rates of disease incidence than those that do not (Hughes *et al.*, 1998; Munkvold *et al.*, 1993), this indicates that internal inoculum sources have a direct correlation with disease incidence. Carter (1965) also found that transport of inoculum of *E. lata* from perithecia to open vessels at pruning wounds during intermittent rainfall is a two-fold process, i.e. deposition from the air to the tree surfaces followed by redistribution of the deposited spores, during subsequent rain showers.

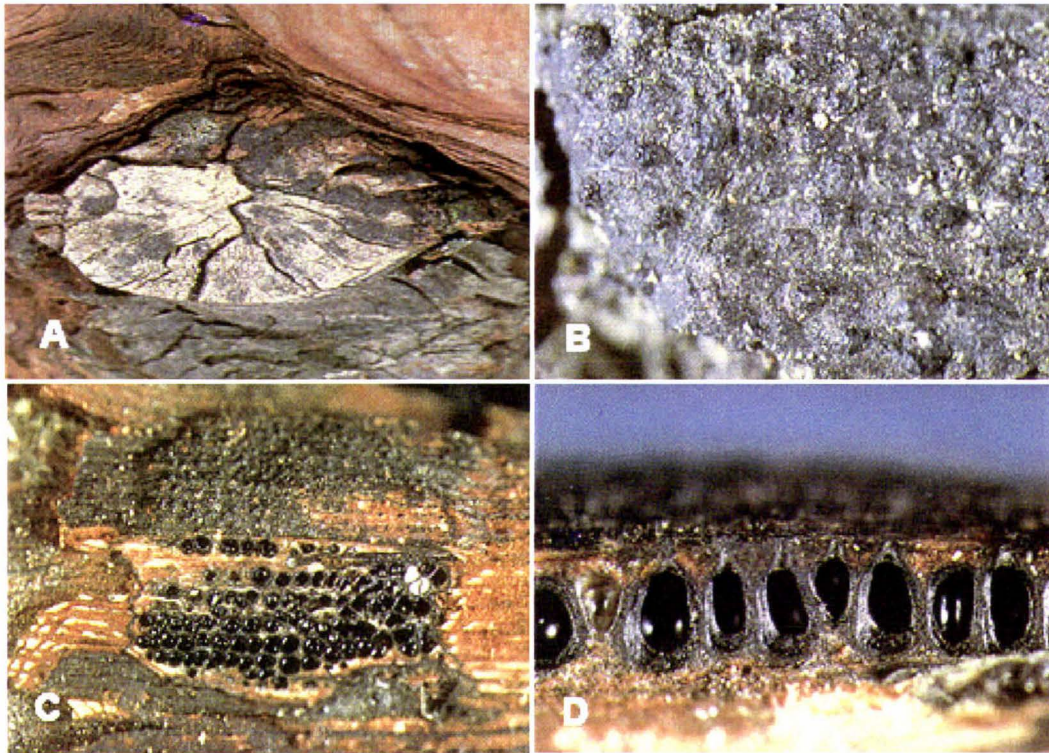


Figure 1.1 Perithecial stroma pictures adapted from Carter, (1991).

A: Perithecial stroma developing on the surface of an old pruning wound on the trunk of a grapevine; **B:** Close-up view of intact perithecial stroma; **C:** Stroma cut tangentially to reveal the locules of the perithecia; **D:** Vertical section through a stroma.

It seems that *E. lata* has evolved a very successful procedure for establishing its mycelium in the vessels of its hosts, and even a single ascospore is able to initiate infection (Ramos *et al.*, 1975).

1.5.1.2 Long range dispersal

Moller & Carter (1965) noted that winds that prevailed during times of rainfall would collect ascospores of *E. lata* liberated from stromata and carry them for long distances to reach the naturally arid areas, and this has been shown by Ramos *et al.*, (1975). This can partly explain the prevalence of *Eutypa* dieback disease in arid localities apparently devoid of perithecia.

1.5.2 ASCOSPORE SURVIVAL

Trese *et al.*, (1980) showed up to 65% germination after holding spores at -20°C for 14 days indicating that the ascospores of *E. lata* are able to survive prolonged periods of freezing after discharge from the perithecia (Carter, 1991). This is consistent with that performed in 1975 by Ramos, Moller & English. A seasonal pattern of ascospore production and release has been revealed in Australia (Moller and Carter, 1965) and in California (Ramos *et al.*, 1975).

1.5.3 PROCESS OF INFECTION

A very interesting phenomenon is the internal etiology of woody trunk diseases. The occurrence of the disease is sometimes attributed to one single or multiple causal agents. As Mugnai *et al.* (1999) noted “it is a complex disease whose symptoms arise from structural and physiological changes that cannot be reduced to a simple scheme of cause and effect”. In terms of classic *Eutypa* dieback symptoms, a number of other fungi, including *Cephalosporium spp.* and *Botryosphaeria spp.* have been isolated in addition to the major causal agent, *E. lata* (Ferreira *et al.*, 1989).

As stated by Carter (1965), the transport of *E. lata* inoculum from the perithecia to the open ends of vessels exposed by pruning wounds during intermittent rainfall is a two-fold process. *E. lata* ascospores usually germinate within the vessels 2 mm or more beneath the wound surface (Moller and Kasimatis, 1978). Carter, (1991) determined the germination rate in vitro, i.e. 11-12 h at the optimal temperature of $20-25^{\circ}\text{C}$.

Newly made wounds are more likely to be infected, and with the extension of time, the opportunity for infection decreases gradually, and after four weeks the wounds are unlikely to become infected (Munkvold and Marois, 1995). The maximum duration of wound susceptibility is still not known, but wounds more than one year old are not likely to be infected (Moller and Kasimatis, 1980). In addition, the temperature is a strong factor affecting the susceptibility of pruning wounds. At a low temperature, infection of

the pruning wounds increased while the growth of other microorganisms is reduced (Munkvold and Marois, 1995; Carter, 1991; Chapuis *et al.*, 1998). The mechanism by which temperatures influence the susceptibility of wounds to infection is partly because it can exert an effect on the accumulation of suberin and lignin, which are common in a wound response reaction. Accumulation of suberin and lignin are linked to the decline in wound susceptibility during the first 28 days after pruning (Munkvold and Marois, 1995).

1.5.4 INTERNAL REASONS FOR INFECTION

When vines are “stressed” from factors such as grafting, water and nutrient deficiencies, severe frosting, overcropping etc, its defense system is unable to cope with the infection caused by a pathogen and unable to combat disease development (Scheck *et al.*, 1999). A typical example was from Ferreira *et al.*, (1999), who found that 70% of artificially inoculated vines exposed to water stress died, compared to 45% in unstressed vines.

1.5.5 SYMPTOM DEVELOPMENT OF *EUTYPA* DIEBACK

The progress of *E. lata* on vines is a chronic process, and usually lasts several years for mature vines until the complete collapse and death of the vines. The external symptoms on leaves and new season shoots are most evident. New shoots appear deformed and discolored. The young leaves are smaller than normal, cupped, chlorotic, and often develop small necrotic spots and tattered margins. Larger areas of necrosis could develop in time coupled with the dwarfing of the internodes (Jones, 2001). Philippe *et al.*, (1992) discovered internal physiological changes and structural alterations in leaf cells, using electron microscopy, they observed cytoplasmic lysis with plasma membrane detachment and complete chloroplast disorganization. On mildly affected shoots, leaves look tattered on the first few nodes (sometimes with immature berries), and this is usually obscured from view by adjacent healthy growth (Jones, 2001).

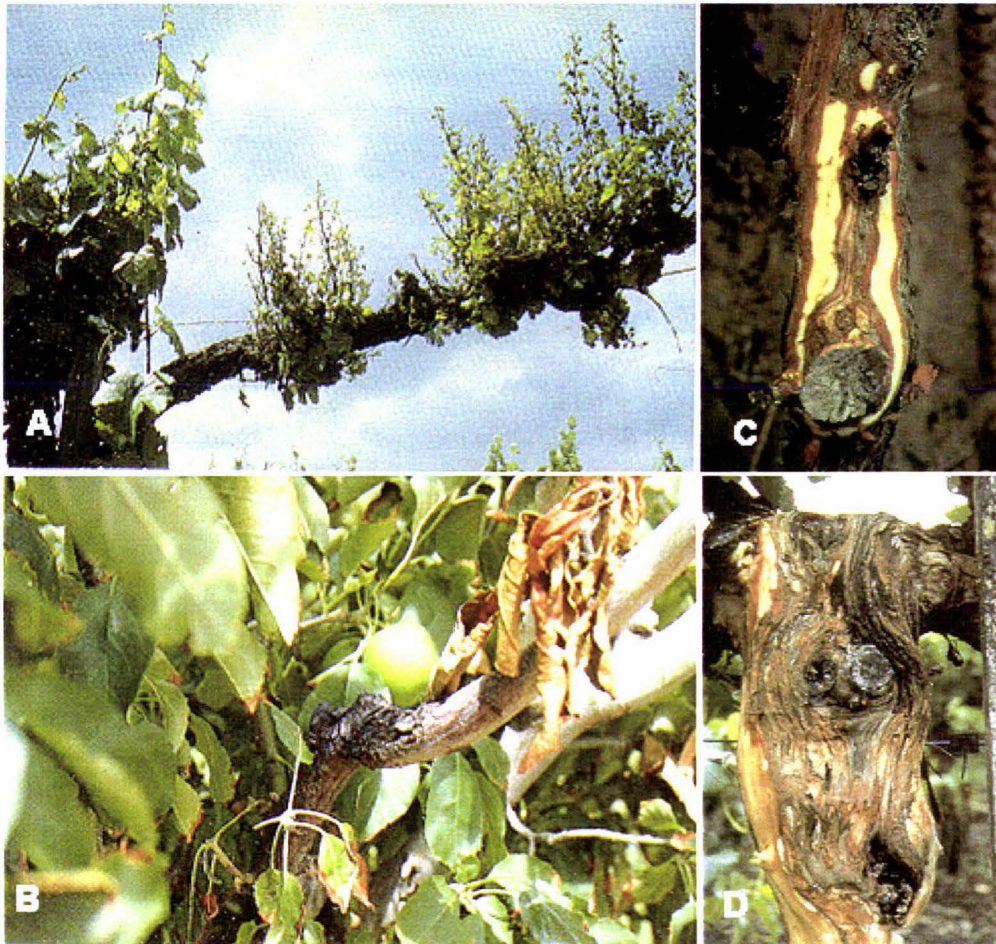
The symptoms on foliage of diseased arms are increasingly extensive with the development of this disease, even the shoots could not be produced on the diseased arm

in the spring when normal shoots are usually developed. Trunks are developed with imbalance, i.e. one side of the vine dead while the other side appears healthy until the whole vine is completely dead (Jones, 2001).

Trunk cankers are often found inside the bark connecting to shoots bearing foliar symptoms, sometime with only a narrow strip of live wood observed through the canker. The cankers occurring in the trunk tend to penetrate towards the center of the arm or trunk with the presence of a wedge shaped zone of necrotic sapwood (Figure 1.2). Due to the chronic feature of the infection process, early stage treatment or prompt remedial surgery seems unlikely to be of value (Jones, 2001).

1.5.6 PATHOGENIC DIFFERENCES IN *E. LATA* ISOLATES

Cultivars of grapevines vary in susceptibility to *E. lata* infection, and some isolates of the pathogen do not cause the stem and foliar symptoms typical of the disease on grapevine cuttings (Munkvold and Marois, 1995). Peros *et al.*, 1997 demonstrated that 55 *E. lata* isolates, each collected from a different vine in a single vineyard showed a large variation in pathogenicity after testing in a greenhouse. In order to examine the internal differences, molecular markers such as isozymes and random amplified polymorphic DNA sequences (RAPDs) were tested. RAPDs appear to be more useful than isozymes to describe the genetic variation of the fungus as RAPD analyses identify more polymorphisms and the uniqueness of each isolate was confirmed (Peros *et al.*, 1999). However, the shortage of these markers limited the ability to group the isolates according to their pathogenicity (Jones, 2001). In addition, random mating between *E. lata* populations in two vineyards was also confirmed using RAPD (Peros and Larignon, 1997; Peros and Larignon, 1998). In 1999, DeScenzo *et al.* identified two clades of *Eutypa* species, i.e. *E. armeniaca* and *E. lata*, through Amplified Fragment Length Polymorphism (AFLP) analysis and sequence analysis of the Internal Transcribed Spacer



**Figure 1.2 Symptoms of *Eutypa dieback* adapted from Carter, (1991).
 A: acute foliage symptoms on grapevine; B: Typical symptoms of *Eutypa dieback* in apple; C: Longitudinal slice of an infected grapevine arm, showing invasion of sapwood below an old pruning wound; D: Grapevine trunk, showing an advanced canker centred on an old pruning wound.**

(ITS) region of the ribosomal DNA, hence molecular markers have proven to be useful tools in evaluation the genetic systems and structures of *Eutypa* population (Jones, 2001). Serological identification of *E. lata* was investigated in the early 1970's, but the results of this method have not been promising (Jones, 2001).

1.5.7 SENSITIVITY OF GRAPE VARIETIES TO *E. LATA*

The issue of susceptibility of grape varieties to *E. lata* has been controversial, and there are no known grape cultivars immune to *E. lata* infection. However it is still possible that

the susceptibility of cultivars to *E. lata* could be variable depending on individual cultivars. Two years later, this point of view was challenged by the evidence observed in France, i.e. Cabernet Sauvignon was susceptible while Merlot was tolerant (Carter, 1991). This also was supported by a report from Hawkes Bay vineyards in New Zealand. The reason for argument is partly attributed to the word “susceptibility” itself, how serious the external or internal symptoms are defined as “infected” or “normal”. There is no international standard for this at the moment. The conclusion made by Chapuis, *et al.*, 1998 is widely accepted at present: there are no differences in susceptibility to infection between Cabernet Sauvignon and Merlot cultivars, although their symptom expressions are markedly different. Some “infection symptoms”, such as foliage, are just a kind of defense reaction of the plant cells toward fungal invasion, and it is inappropriate to put them into the category of infection.

1.6 EFFECT ON YIELD

Undoubtedly, *Eutypa* dieback has caused a significant reduction in the yield of infected grapevines, and has gradually become a major threat in the vine industry. The direct yield losses resulted from it have been estimated between 11% and 100% in Washington State, USA and Greece respectively according to the extent of severity (Johnson and Lunden, 1985). Yield reduction is primarily due to a diminished number of clusters per vine although its effect on mean cluster weight is not always significant because of the mechanism of compensation by producing more fruit on shoots that arise from the remaining healthy buds (Lider *et al.*, 1975). As the chronic characteristic of disease process, economic losses due to *Eutypa* dieback may be minor in early years, and its impact on yield thus increases with age. In addition to the direct losses, this disease can also exert an effect on vineyard longevity (Munkvold *et al.*, 1994), wine quality reduction, and the long-term loss of productivity (Jones, 2001).

Australian vineyards have gradually suffered more from *Eutypa* dieback, and this has seriously affected their sustainability (Pascoe and Cottral, 2000). In New Zealand, this kind of threat is increasing. Hence, it is urgent to have an in-depth study to gain a better understanding and to find an ideal method of treatment of this disease.

1.7 GRAPEVINE PATHOGEN *P. CHLAMYDOSPORA* AND PETRI DISEASE

1.7.1 THE HISTORICAL EVOLUTION OF THE NAME FOR *P. CHLAMYDOSPORA*

It was Lionello Petri who first discovered the characteristic symptom of Petri disease in 1912 (Chiarappa, 1999). For a long time, the causal agent for Petri disease remained elusive. *Cephalosporium spp* were initially regarded as the agent of Petri disease; however, this fungus has also been isolated from other trunk diseases such as esca (Chiarappa, 1959) and dieback (Ferreira *et al.*, 1989). Thus the direct relationship between Petri disease and its causal agent could not be established. Later, other fungi, similar but morphologically different that caused dieback symptoms, were isolated. From their morphology and genetic study, they were ascribed to a new genus *Phaeoacremonium*, and renamed as *P. chlamydosporum* and *P. aleophilum*, respectively (Crous *et al.*, 1996). *P. chlamydosporum* was unique in the *Phaeoacremonium* genus. For example, the mycelium is initially like yeast and darkens with age, it has chlamydospore-like cells and microsclerotia and unimorphic conidia. In addition, genetic analysis revealed it is unique in conservative gene sequences. Based on this, *P. chlamydosporum* was renamed as *Phaeomoniella chlamydospora*.

As the disease caused by *Phaeomoniella(Pm) chlamydospora* (Adalat *et al.*, 2000; Wallace *et al.*, 2003) has a wide range of names and different areas have their own names, it was resolved at the 2nd International Workshop on Grapevine Trunk Diseases, that the current name of “Petri disease” be used (Surico, 2001).

1.7.2 SYMPTOMS OF PETRI DISEASE

The effects of *P. chlamydospora* on the growth of vines are different according to their ages. For young vines, the external symptoms include slow establishment, poor growth and smaller rootstocks (Pascoe and Cottral, 2000). The parameters for growth also include above and below ground dry weight and the number of internodes and roots. After inoculation, these parameters are all reduced (Adalat *et al.*, 2000). A survey performed by

Pascoe and Cottral (2000) in Australia showed that nearly half of the plants showed a problem in establishment after *P. chlamydospora* infection.

As for older vines, the external effects include poor or late budburst, weak and stunted shoot growth, reduced internode length, reduced leaf size and leaves that are occasionally chlorotic and/or necrotic (Mugnai *et al.*, 1999). The mechanism underlying this is probably toxin production from these pathogenic fungi. Toxins and/or toxic secondary metabolites can cause damage to host cells, including damage to cell membranes, cellular transport systems, or enzymatic reactions involved in biological pathways (Tabacchi *et al.*, 2000). Some similar metabolites, such as phytotoxins have been extracted from *P. chlamydospora* and *P. aleophilum*, and their functions on host vines have been demonstrated by Sparapano *et al.*, 2000. After direct application, a 57% reduction in grapevine callus dry weight, chlorosis and necrosis in leaves and other typical Petri disease symptoms were observed.

The internal symptoms of vines with Petri disease include extensive browning of the vascular tissue, and excretion of a tar-like substance (Morton, 1995). This browning is attributed to the production of peroxidase by *P. chlamydospora*, which leads to the transformation of resveratrol and related compounds (Mugnai *et al.*, 1999). Some other symptoms, such as brown/black streaking, and formation of tyloses, were also observed on the cut section of infected vines (Pascoe and Cottral, 2000).

1.7.3 DISSEMINATION OF THE PATHOGEN

Some researchers have suggested that the main source of infection from *P. chlamydospora* is the use of material from diseased mother-vines (Larignon and Dubos, 1999; Mugnai *et al.*, 1999). This has been demonstrated by Chicau *et al.*, (2000) after isolation of *P. chlamydospora* pathogen from rootstock mother-vines, rootstock cuttings (Fourie and Halleen, 2001) and the rootstock section of young grafted vines (Bertelli *et al.*, 1998; Zanaotto *et al.*, 2001). In contrast, many researchers have consistently failed to isolate the pathogen from the scion part of grafted vines (Bertelli *et al.*, 1998; Zanzotto *et*

al., 2001). Pascoe and Cottral (2000) suggested that movement into the current season's growth occurs through movement of conidia in the vascular system. However this has been argued by Edwards *et al.* (2003), who suggested that movement may occur through active mycelial growth of *P. chlamydospora*.

Like the infection progress of *E. lata*, it is possible the infection by *P. chlamydospora* results from the aerially disseminated conidia that persist on the surface of canes, when rehydration of material, disbudding, making of grafting cuts and root forcing (Mugnai, 2000; Zanaotto *et al.*, 2001), it infects wounds, inoculation experiments from Scheck *et al.*, 1998 and Larignon and Dubos, (2000) have demonstrated this.

The effects of the presence of *P. chlamydospora* and *P. aleophilum* on grafting have been proved to be significant. In 1994, Ferreira *et al.* demonstrated that callus formation rate was reduced from 76% (controls) to 29% (grafted vines dipped in conidial suspensions of *P. parasitica*, which was later reclassified as *P. chlamydospora*). Recently, Adalat *et al.*, 2000; Wallace *et al.*, 2003 demonstrated that when *P. chlamydospora* was inoculated, the callus formation at the base of cutting had been dramatically reduced.

Another possibility of infection is from soil (Bertelli *et al.*, 1998; Surico *et al.*, 2000). This is consistent with the result from Adalat *et al.*, 2000, who reported that 7% of single bud cuttings of Chardonnay planted in sand artificially infested with *P. chlamydospora* were later found to be infected.

1.7.4 INTERNATIONAL DISTRIBUTION

Petri disease has been observed worldwide. In New Zealand, Jaspers *et al.*, 2000 reported that 84% of vines showing external symptoms of Petri disease had internal staining and *P. chlamydospora* was the fungus most commonly isolated.

While *P. chlamydospora* was first isolated on grapevines; this does not mean other plants are not susceptible to its infection and colonization. In 2000, Di Marco *et al.* reported that

P. chlamydospora as well as other similar fungi including *P. aleophilum* were also isolated from kiwifruit vines with a similar esca disease.

1.7.5 DISEASE MANAGEMENT

1.7.5.1 Cultural control

To control Petri disease, selection of resistant cultivars seems necessary. However the view of “no apparent difference in varietal susceptibility” (Pascoe and Cottral, 2000) has been widely accepted. Hence selecting high quality cuttings that are stress-free, disease-free and injury-free has been highly recommended (Morton, 1999). Other methods to prevent Petri disease infection include avoiding cultivar preparation during wet periods (Larignon and Dubos, 2000), reducing stress of cutting materials (Pascoe *et al.*, 2000), application of hot-water treatment (although negative and positive views are co-existing) (Edwards *et al.*, 2003; Fourie and Halleen, 2003), and use of fungicides (Jaspers, 2001).

From a variety of experiences obtained from controlling of other plant diseases, application of chemicals (fungicides) is likely to be a feasible method during the grafting process and the time when vines are getting infected. However no products are currently available specifically against *P. chlamydospora*. The near future of this seems to be bright, a number of systemic products have been tested *in vitro*, and shown to be very promising (Groenewald *et al.*, 2000; Jaspers, 2001), with a reduction in mycelial growth of the pathogen and conidial germination (Jaspers, 2001) have been observed *in vitro*. Some *in vivo* tests have been performed in the glasshouse with fungicides, such as thiabendazole (Fragoeiro pers. Comm.), Prochloraz, managanese (Laukart *et al.*, 2001), and various phosphorous based products (Di Marco *et al.*, 1999; Khan and Gubler, 2001; Laukart *et al.*, 2001). Large scale practical applications still need serious consideration although the potential as a treatment has been demonstrated. Further, as these chemicals usually are hazardous to the environment, some countries have banned their use.

1.7.5.2 Sanitation methods

It is believed that regular pruning of grapevines each year provides a multitude of entry points for the pathogen. Because of the slow growth of the pathogen, manifestation of the infection symptoms is usually delayed; this makes recognition of the disease difficult, and in time measures for controlling this infection seems unrealistic (Jones, 2001). Thus the time for pruning should avoid wet weather due to ascospore dispersal during this period (Chapuis *et al.*, 1998; Moller and Kasimatis, 1980).

1.7.5.3 Fungicide control

Although no ideal fungicides are available to control the disease at present, some chemicals, such as benomyl and flusilazole do provide barriers against the invasion of the pruning wood if these chemicals are flooded to the exposed vesicles at the surfaces of the pruning woods before the spores arrive (Moller and Kasimatis, 1980).

1.7.5.4 Biological control

Biological control seems to be a good idea for controlling this disease based on the mechanism that these biological organisms can serve as competitors. However the prerequisite is that the organisms must be able to grow under field conditions (Jones, 2001) and are not harmful to the environment. As for economic considerations, it is dependent on the biological agents themselves and the labor cost during application of these agents (Jones, 2001).

In order to control and or prevent infection, vineyard management, such as good vineyard sanitation to remove diseased material and maintain good soil structure is highly recommended to maintain a healthy environment for vines (Emmett and Magarey, 1994; Mugnai *et al.*, 1999).

1.8 DETECTION

1.8.1 DETECTION BY MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF PATHOGEN

It has been believed that *P. chlamydospora* and *P. aleophilum* are the two major causal agents of Petri disease. In term of the power to infect grapevines, *P. chlamydospora* shows higher aggressiveness than *P. aleophilum* or *P. inflatipes* (Adalat *et al.*, 2000).

More recently, callus formation has been reduced in the *P. aleophilum* inoculated vines rather than in *P. inflatipes* ones (Wallace *et al.*, 2003).

The common feature of these pathogens is that they all are Petri disease causing agents; hence it is necessary to distinguish them from each other before application of treatments. The typical morphological features and characteristics of *P. chlamydospora* have been described by Crous *et al.*, (1996) and Pascoe, (1999), such as deep olive color with a white slimy margin (on potato dextrose agar and malt agar); grow slowly; green/brown mycelium; large number of chlamydospores; global brown pycnidia, etc.

1.8.2. DETECTION OF FUNGI USING THE ITS REGION

Based on the information on morphology of colonies described by Cater and Moller, 1977, culturing the pathogen from diseased wood and comparing with a reliable reference culture transferred at the same time may be another way for the diagnosis of *E. lata*. However due to the interference from other fungi that are co-existing on the diseased wood (infected vines are more vulnerable to other fungi), it may be difficult to confirm its presence through this method. Application of the ITS region by PCR using universal DNA primers specific for the conserved 18S and 28S elements (White *et al.*, 1990) followed by direct sequencing has been successfully used to detect fungal plant pathogens (Jones, 2001). Fungi detected using this method include *Armillaria* species (Harrington and Wingfield, 1995) and *Phaeoacremonium* species (Tegli *et al.*, 2000).

Whiteman *et al.*, (2002) reported using species-specific PCR for the detection of *P. chlamydospora* in soil. Using a nested PCR, this method preamplified a 600 bp region of the ribosomal DNA, followed by amplification of a 360 bp species-specific region. This assay was able to detect 10^2 conidia/ml when a spore suspension was added to sterilized soil samples and 50 fg when genomic DNA was added directly to the reaction. Compared to traditional method (agar plate isolation), this method is faster and more precise.

1.9 *gfp* AS A REPORTER GENE

1.9.1 DISCOVERY OF THE *gfp* AS A REPORTER GENE

In 1978, Prendergast and Mann isolated the green fluorescent protein (*gfp*) from *Aequorea forskalea*. In the following year, Ward and Cormier, (1979), characterized the Renilla green-fluorescent protein, and found that it is an energy transfer protein in coelenterate bioluminescence. However, studies at the molecular level on this protein were not performed until 1992. Prasher *et al.* reported the cloning and sequencing of cDNA and genomic clones of *gfp* from the cnidarian, *Aequorea victoria*. The *gfp10* cDNA encodes a 238-aa-residue polypeptide with a calculated Mr of 26,888. The great discovery from Chalife *et al.*, 1994 and Inouye and Tsuji, 1994 demonstrated that when the *gfp* gene was introduced into heterologous organisms, it could generate fluorescence upon UV or blue light excitation. This discovery made the widespread usage of *gfp* as a reporter gene possible and thus led to the worldwide application in biological studies at a molecular level.

Green-fluorescent proteins (*gfps*) can be used as energy-transfer acceptors in bioluminescence. Upon receiving energy from either a luciferase-oxyluciferin excited-state complex or a Ca^{2+} -activated phosphoprotein, *gfps* emit green fluorescence *in vivo*. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is comprised of modified amino acid (aa) residues within the polypeptide (Prasher *et al.*, 1992).

This gene or derivatives have been successfully expressed and conferred fluorescence to a variety of organisms such as bacteria (Chalfie *et al.*, 1994); yeast (Flach *et al.*, 1994; Niedenthal *et al.*, 1996), filamentous fungi (Spellig *et al.*, 1996); mammals (Pines, 1995), *Drosophila* (Wang and Hazelrigg, 1994), and plants (Haseloff and Amos, 1995; Sheen *et al.*, 1995).

This unique expression of *gfp* makes it very useful in detection of expression of specific tissue *in vivo* or monitoring the interactions between organisms, such as the interaction between pathogenic fungi and their host plants (this project is based on this consideration). *gfp* serves as a reporter mainly because of the unique qualities of this 238-amino-acid, 27-kDa protein which absorbs light at maxima of 395 and 475 nm and emits light at a maximum of 508 nm (Lorang *et al.*, 2001).

1.9.2 CHARACTERISTICS OF GFP

GFP is extremely stable *in vivo*, and is not affected by destructive sampling, cell permeabilization, or leakage of products. GFP itself is not able to cross most membranes, with the exception of the nuclear membrane through the nuclear pores (Grebencok *et al.*, 1997). Furthermore, the fusion of the GFP to the C or N terminus of many cellular and extracellular proteins make it more stable and will not lead to a loss of activity, thereby permitting the tagging of proteins for gene regulation analysis, protein localization, or specific organelle labeling (Lorang *et al.*, 2001). The mature protein resists many proteases and is stable up to 65°C and at pH 5 to 11, in 1% sodium dodecyl sulfate or 6 M guanidinium chloride (Cubitt *et al.*, 1995; Ward, 1998), and in tissue if fixed with formaldehyde, methanol, or glutaraldehyde.

1.9.3 THE ADVANTAGES AND DISADVANTAGES OF USING *gfp* AS A REPORTER

The popularity of *gfp* as a biomarker in biological research has its reasons. As DNA sequences encoding specific secretory proteins or signals can be fused to *gfp*, usually without altering their targeting, it is a useful alternative to conventional dyes previously

used to investigate compartments *in vivo* (Brandizzi *et al.*, 2004). Compared with other traditional reporters such as β -glucuronidase, β -galacturonidase, chloramphenicol acetyltransferase, and firefly luciferase, which rely on cofactors or substrates for activity, the fluorescence of GFP requires only UV or blue light and oxygen, and therefore, *in vivo* observation of *gfp* expression is possible with individual cells, with cell populations, or in whole organisms interacting with symbionts or environments in real time (Lorang *et al.*, 2001).

On the other hand, GFP has its disadvantages, for example, pH values can affect GFP fluorescence, the optimal pH range for *gfp* expression is from pH 7.2 to 8.0 (Ward, 1998). GFP will not function as a biomarker in methanol-acetic acid (3:1) as a result of an absence of fluorescence, and it can be masked by autofluorescent aldehyde groups in tissue fixed with glutaraldehyde (Lorang *et al.*, 2001). Other limitations on *gfp* as a reporter for some applications include its low turnover rate, 2-h lag time for autoactivation of its chromophore, improper folding at high temperatures (37°C), which results in nonfluorescence and insoluble forms of the protein, and requirement for oxygen, which is not present in equal concentrations in all subcellular locations or cell types (reviewed by Cubitt *et al.*, 1995 and Ward, 1998).

1.9.4 RECENT DEVELOPMENT OF *gfp* AS A REPORTER MARKER

In order to overcome the problems posed by these limitations, a variety of *gfp* derivatives have been produced and demonstrated high promise. Most of these derivatives are mutant forms of *gfp*, which have a higher ability to resist the influences of high temperature and can still maintain a good conformation. In addition, these modifications can increase solubility and fluorescence, reduce photobleaching (Crameri *et al.*, 1996, Cubitt *et al.*, 1995, Siemering *et al.*, 1996), and reduce half-lives (Andersen *et al.*, 1998). Combined with fluorescence-activated cell sorting, confocal microscopy or quantitative image analysis techniques, *gfp* technology can be used to isolate transformed cells or specific cell types from populations of cells (Cormack *et al.*, 1996), and to quantify gene expression of individual cells within whole organisms (Brand, 1995).

1.9.5 *gfp* UTILIZATION IN FUNGAL STUDY

gfp as a reporter has been widely used in fungal studies, including yeast and filamentous fungi. *Ustilago maydis* was the first filamentous fungus for which successful expression of *gfp* was reported (Spellig *et al.*, 1996), followed by *Aspergillus nidulans* (Fernández-Ábalos *et al.*, 1998) and *Aureobasidium pullulans* (Vanden Wymelenberg *et al.*, 1997). In a review from Lorang *et al.*, (2001), *gfp* expression has been reported for 16 species comprising 12 genera of filamentous fungi, including *Colletotrichum* (Dumas *et al.*, 1999), *Mycosphaerella* (Skinner *et al.*, 1998), *Magnaporthe* (Kershaw *et al.*, 1998), *Cochliobolus* (Maor *et al.*, 1998), *Trichoderma* (Bae and Knudson., 2000), *Podospora* (Berteaux-Lecellier *et al.*, 1998), *Sclerotinia* (Vautard-Mey *et al.*, 1999), *Schizophyllum* (Lugones *et al.*, 1999), *Aspergillus* (Du *et al.*, 1999) and *Phytophthora* (Bottin *et al.*, 1999).

Expression of *gfp* in filamentous fungi requires a well-developed transformation system, and a fungal promoter that can drive the expression of *gfp*. A new vector containing the designed *gfp* gene and a promoter (prefer a strong promoter) is required to be constructed prior to fungal transformation. A new fungal transformation vector that expresses *Sgfp* under the control of the *ToxA* gene promoter from *Pyrenophora tritici-repentis* was developed by Ciuffetti *et al.*, (1997) who demonstrated its use in plant pathogens belonging to eight different genera of filamentous fungi (*Fusarium*, *Botrytis*, *Pyrenophora*, *Alternaria*, *Cochliobolus*, *Sclerotinia*, *Colletotrichum*, and *Verticillium*).

1.9.6 WILD-TYPE AND ENGINEERED *gfp* GENES

The prerequisite of *gfp* utilization in fungal transformation is its successful expression. However the wild-type *A. victoria gfp* gene does not confer appreciable fluorescence in many transformed fungi due to the translation problem, hence the wild-type *gfp* gene is required to be modified with optimized codon usage. After modification, the *gfp* variants express proteins (SGFP, yEGFP, and EGFP1) that also contain a serine-to-threonine substitution at amino acid 65 (S65T), that causes a "red shift" from excitation maxima of 395 and 470 nm to a maximum of 488 nm (Lorang *et al.*, 2001).

After comparison of the expression of four *gfp* variants driven by a common promoter in *A. nidulans*, Fernández-Ábalos *et al.*, (1998) concluded that SGFP (Chiu *et al.*, 1996) conferred the highest GFP concentration and level of fluorescence to transformants. SGFP contains the S65T mutation as well as plant-optimized codon usage that also delete a cryptic intron splice site reported to reduce *gfp* expression in *Arabidopsis* (Haseloff *et al.*, 1997). *Sgfp* (Blue-*Sgfp*-TYG) has been the *gfp* gene most often used for transformation of filamentous fungi. *Egfp1* (Clontech, Inc., Palo Alto, Calif.) is similar to *Sgfp* in that it contains the S65T mutation and 190 silent base mutations corresponding to human codon usage preferences (Yang *et al.*, 1996). Examples of *Egfp1* utilizations have been reported in *Aspergillus flavus*, *A. pullulans*, *Magnaporthe grisea*, and *Podospora anserina* (Berteaux-Lecellier *et al.*, 1998; Du *et al.*, 1999; Liu and Kolattukudy, 1999, van West *et al.*, 1999).

1.9.7 THE UTILITY OF *gfp* AS A REPORTER GENE

Unlike other reporters, usually with destructive sampling that limit observations to a moment in time, *gfp* affords detection of gene expression and protein localization that is continuous in time and development within a single living specimen. In addition, *gfp* does not require additional substrates or fixing of tissue (Lorang *et al.*, 2001). Furthermore, *gfp* gene expression is easily quantified in whole cultures via fluorometry (Fernández-Ábalos *et al.*, 1998) or in individual cells or subcellular compartments with confocal microscopy (Liu and Kolattukudy, 1999; Spear *et al.*, 1999).

1.9.8 INVESTIGATING CELL DYNAMICS WITH *gfp*

The emergence of *gfp* has created a milestone in the study of cellular biology of filamentous fungi *in vivo*. Filamentous fungi are regarded as an ideal organism for cell dynamics study due to their simple anatomy and rapid growth rates. Transformation of filamentous fungi with *gfp* expressing vector enables this study to be more delicate and more dynamic. One example is from *A. nidulans* transformed with *gfp* vector (Suelmann and Fischer, 2000), a model system for investigating the molecular basis of eukaryotic,

cellular morphogenesis. Nucleus-targeted GFP in *A. nidulans* allowed real-time visualization of nuclear migration and mitosis, and the behavior of specific nuclei at various developmental stages (Fernández-Ábalos *et al.*, 1998, Suelmann *et al.*, 1997).

1.9.9 VISUALIZING FUNGI IN THEIR ENVIRONMENTS: HOST-PATHOGEN INTERACTIONS, MYCOPARASITISM, AND THE PHYLOSOPHERE

Filamentous fungi that have been transformed with *gfp* so far are mainly plant pathogens or residents of plant surfaces, and genes of interest in these systems are those that play roles in host-fungus interactions. The merits of *gfp* in elucidating regulation of genes and the cellular location of their protein products are obvious. Tracking fungal strains that carry mutations for or that overexpress such genes in plants is also of great interest (Lorang *et al.*, 2001).

In order to detect *gfp* expression in fungi, track fungi in plants, monitor their distribution, and to estimate their biomass, laser scanning confocal microscopy and epifluorescence microscopy using a computer-controlled, z-stepper motor and filters in conjunction with a video camera were used by Spear *et al.*, (1999) for the fungus *A. pullulans*. Data gathered by either method was analysed with image analysis software (Optimas v6.2 for PC; Media Cybernetics, Del Mar, Calif.). The advantages and disadvantages of Laser scanning confocal microscopy were compared with conventional epifluorescence microscopy. Compared with conventional epifluorescence microscopy, the optical sectioning capability of confocal microscopy affords clear visualization of GFP despite the autofluorescence and light-scattering properties of plant cell walls (Haseloff and Amos, 1995).

Labelling whole fungi with *gfp* generally results in a cytoplasmically located protein occurring in all fungal morphotypes (hyphae, spores, appressoria, etc.) with no obvious effects on fungal growth or pathogenicity (Maor *et al.*, 1998; Spellig *et al.*, 1996). Of *gfp* tagging of plant pathogenic fungi, *Sgfp* was easily detected with epifluorescence microscopy (Leica DMRB and Endow *gfp* filter cube, exciter HQ470/40, and emitter HQ525/50 with beamsplitter Q495LP) (Lorang *et al.*, 2001).

1.9.10 VECTORS FOR *gfp* TAGGING OF FILAMENTOUS FUNGI

A variety of *gfp* or modified *gfp* expression vectors have been developed for all major classes of filamentous fungi. Each individual fungus has its own unique properties; hence selection of vector will depend on the fungus itself. In addition to modification of the *gfp* gene itself, modification of the gene promoter to get a strong promoter is an alternative way to enhance GFP fluorescence. Limited numbers of fungal promoters and the variability of the strength of promoters in heterologous fungi have posed a problem for *gfp* application in fungal studies. In addition, not only *gfp* but also selectable markers need to be taken into consideration prior to transformation. One commonly used *gfp* vector is pCT74, which contains a *Sgfp* driven by *ToxA* promoter (appendix 3F).

1.9.11 *gfp* AND THE FUTURE OF FUNGAL BIOLOGY

Recently the complete sequences of the *S. cerevisiae* and *S. pombe* genomes were determined, and genome projects for several filamentous fungi, including *A. nidulans*, *Neurospora crassa*, *M. grisea*, and *Candida albicans* are well under way (Lo *et al.*, 2000, Lim *et al.*, 2004). The promoter sequences for every gene in all of these organisms will gradually be available. Coupled with the rapid developments in Microarray, digital imaging, and bioinformatics technologies, and with high-throughput systems that detect, store, and display complex fluorescent images, *gfp* technology is increasingly powerful (Lorang *et al.*, 2001).

Using *gfp*-tagged fungi for tracking fungal distributions in natural systems seems to be a good idea. However, a big concern is that a large number of easily dispersible spores produced by many filamentous fungi tend to accumulate to very high concentrations in the air, hence contained environments for working with these engineered fungi should be used, and the ecological implications of introducing these organisms into the environment must be seriously considered (Lorang *et al.*, 2001).

The utilization of *gfp* and its spectral variants have changed the approach to studying the dynamics of the plant secretory pathway. *gfp* technology has shed new light on secretory

events by allowing bioimaging *in vivo* right to the heart of a plant cell (Brandizzi *et al.*, 2003).

Although *gfp* has become an invaluable tool for *in vivo* investigation, *gfp* technology has to be used wisely to avoid artefacts and data misinterpretations. In certain cases, the expression of *gfp* may involve protein overexpression, and this overexpression could exert effects on internal systems in the organisms, such as on the plant endomembrane system. There, the value of controls on transformation levels and ensuring that the labelling with GFP fusions is due to correct targeting should not be underestimated. In addition, GFP tagging of proteins may result in loss of a specific function or, worse, acquisition of new function (Brandizzi *et al.*, 2003).

Further misfolding of proteins and malfunction caused by *gfp*, and sensitivity to pH values should be taken into serious consideration. GFP may accumulate but its fluorescence may be easily quenched by the acidic pH and specific experimental procedures may need to be adopted to visualize GFP fluorescence. In addition, reporter systems could have an influence on *gfp* expression (Tamura *et al.*, 2003).

Further, the methods through which fungi grow and spread in their host plant still remain unknown. Currently, the use of the green fluorescent protein (*gfp*) as a biomarker for observation of fungal spreading in plants has been widely used, such as for *sapstain* fungi (Lee *et al.*, 2002). *gfp* is introduced into a vector containing an *hph* gene, which will subsequently be introduced into a fungal protoplast, therefore, after incubation; any movement of the transfected fungus in the plant can be monitored through tracking the expression of *gfp* by confocal microscopy. In order to achieve these it is necessary to develop a transformation system for these fungi.

In conclusion, recent advances in *gfp* molecule engineering, fluorescence detection, and imaging analysis are opening the door for us to gain considerable information about fungal genomes. A clever utilization of these technologies and information is likely to lead to a bright future for fungal biology.

1.10 TARGETED GENE REPLACEMENT

Gene targeting ('knock-out') technology is now widely used in the basic science of all disciplines of pathology and is the wilful introduction of precise mutations into the genome of organisms, affecting the function of a single gene or genes (Riminton, 2002).

1.10.1 OVERVIEW OF GENE TARGETING TECHNIQUE

To successfully disrupt a gene *in vivo* a number of tasks need to be completed. A targeting vector needs to be designed using knowledge of the genomic sequence containing the genes of interest. Design is governed by three principles. First, to disrupt one or more exons of the target gene by the interruption/excision of sequence, or by the introduction of a stop codon. Second, to incorporate a selectable marker, that enables the identification of transfected cells in culture. Third, to provide adequate sequence homology in flanking regions enabling homologous recombination (Riminton, 2002). After transfection by protoplasts/PEG method or electroporation, the vector sequence may be incorporated into the host genome randomly, or by the homologous replacement of endogenous sequence. Thus the normal function of the genes can be determined through observation of any alterations in comparison with wild-type organisms.

1.10.2 ADVANCES IN GENE TARGETING TECHNIQUES

Recent advances in gene targeting technology enable the specific inactivation of genes restricted both in time and anatomic location. The characterization of tumor necrosis factor (TNF), the ubiquitously expressed and most studied of cytokines, provides an ideal illustration of the effectiveness of gene targeting experiments in the investigation of inflammatory biology. The complexities of TNF have halted definitive progress in TNF biology. Gene targeting enabled precise inactivation of TNF and definition of its roles (Riminton, 2002).

This technique developed includes the use of the recombinase protein Cre, and the Cre-specific recognition sequence *LoxP*. The targeting construct is designed with the targeted allele flanked by two *LoxP* sites ('floxed'), with site-specific excision of the intervening sequences achieved by Cre expression. The Cre-*LoxP* system is used in 'knock-ins' where the neomycin resistance cassette is floxed and deleted from the replaced gene.

Gene targeting is now established as a key experimental technology for the identification of participating genes in the complex disease processes, which can be introduced into the current project for identifying those genes and factors involved in the grapevine disease black goo and Petri grapevine decline.

As this project was designed for a one year MSc, the convenient "one step" gene disruption method through homologous recombination was chosen for disruption of the *P. chlamydospora* putative toxin gene *moxY* (cloned by Hayley Ridgway, Lincoln University). A vector containing part of the *moxY* gene and selective marker *hph* will be constructed. After transformation of this vector into *P. chlamydospora* protoplasts, transformants selected based on hygromycin resistance are screened through PCR for targeted integration and concurrent disruption of the toxin genes or genes involved in toxin production. Therefore these genes' functions can be determined by comparing with wild-type organisms. However the prerequisite of this is the successful development of a highly efficient transformation system.

Fungal genomics and the remarkable improvements in transformation technology, together with the achievements in higher homologous recombination efficiencies (Garcia-Maceira *et al.*, 2000), and possibly targeted PCR (Wach *et al.*, 1997), make development of genomic approaches a reality for filamentous fungi. These developments would allow elucidation of fungal gene (particularly for those putative toxin genes) functions on a massive scale (Lorang *et al.*, 2001).

1.11 TRANSFORMATION OF FUNGAL PLANT PATHOGENS

At present, *E. lata* and *P. chlamydospora* are still poorly understood, partly due to a lack of molecular analyses that could characterize pathogenicity and virulence factors of these fungi. The need for a transformation protocol was emphasized in several studies as a necessary instrument for the investigation of disease-causing mechanisms. This transformation system is essential to the genetic analysis of gene regulation and biochemical features of these fungal species and the expression of recombinant proteins in them. Gene transformation of filamentous fungi is a relatively new area. With the rapid development of molecular biology this area has undergone unbelievable improvements.

The literature available on the subject illustrates a diverse range of strategies and procedures which work for specific fungal species, but unfortunately not one of the approaches can be universally applied to all fungi. The first description of such a system was in *N. crassa* (Case *et al.*, 1979), followed soon after with gene transfer in another ascomycete, *A. nidulans* (Ballance *et al.*, 1983). Many other transformation systems have been developed for a range of commercially and agriculturally important fungal species (Punt and van den Hondel, 1992).

Currently, the mainly existing methods that could be used for transformation are protoplast/PEG method, electroporation, biolistic transformation, and *Agrobacterium tumefaciens*-mediated transformation; the most popular one is the classical protoplast/PEG method using hygromycin B resistance gene (*hph*) as a dominant selectable marker.

1.11.1 FUNGAL PROTOPLASTS

Bacteria, fungi, and plants have rigid, mainly polysaccharide cell walls that give a characteristic morphology to the cells and provide support and protection to the enclosed protoplast (Peberdy & Ferenczy, 1985). They described 'protoplast' as a "naked cell completely devoid of cell wall residues". They still represent the organized entities of the

living components of cells, which are able to carry out active metabolism and energy transfer. Fungal protoplasts have been used and still are used extensively in cell wall synthesis studies and are providing a means of studying a wide range of cellular, biochemical, and genetic processes.

1.11.2 SELECTABLE MARKERS

The markers that have been selected for transformation include not only genes encoding antibiotic resistance enzyme but also those encoding special enzymes, even unique fluorescent proteins that can be easily detected. Most recently developed transformation systems are mainly focused on marker selection and vector construction. Both markers that are capable of complementing a mutation such as auxotrophic markers (negative selection), and those that can confer a new property to the host cell, like antibiotic resistance (positive selection), are useful. Each has its own advantages and disadvantages, such as a wild-type transformant (thus harmless to the environment) would be obtained after using a negative selectable marker, rather than acquiring a mutant with an additional characteristic. However the advantage of using positive selectable marker is that transformation does not need a mutant.

However the transformation frequencies based on the protoplast/PEG method are not always high. As obtaining a large number of transformants is essential to the eventual aim (i.e. to determine genes' function through gene targeting), any factors beside the selective markers involved in this process are all required to be seriously considered. To optimize those conditions and find a specific optimal protocol for the establishment of the transformation system is crucial for making successful targeted gene replacement.

Environmental consideration for these genetic modified organisms is essential. Techniques for eliminating selectable markers from transformed organisms have been developed (Komari *et al.*, 1996), but research that addresses the fitness, epidemiology, and possible ecological implications of releasing transformed fungi into ecosystems is lacking.

1.12 AIMS AND OBJECTIVES

As there is no single all-encompassing method to transform fungal cells, many factors including protoplast isolation, shuttle vector, selection system etc, can affect transformation success. In addition, transformation protocols will vary between different fungi and different laboratories. Therefore, transformation systems for *E. lata* and *P. chlamydospora* will be developed to find a method which is reliable to obtain expected transformants.

Aim 1: *Development of transformation systems for E. lata and P. chlamydospora.*

To develop transformation systems for *E. lata* and *P. chlamydospora*. The transformation method will be based on classic PEG/protoplast. Fungal protoplasts will be transformed with plasmid pAN7-1 and *gfp*-containing plasmids pBCH-*gfp* and pCT74.

Aim 2: *Molecular characterization of transformants.*

To characterize transformants with PCR and Southern hybridization. The characteristic genes (*hph*, *gfp* and *moxY*) in each plasmid will be amplified with PCR and confirmed with Southern hybridization. Check of the *gfp* expression will also be performed with a UV microscope.

Aim 3: *Establishment of a relationship between E. lata and host blackcurrants.*

To inoculate a purified *E. lata* transformant containing an expressed *gfp* gene onto blackcurrants, one of its host plants, followed by observation of its colonization and penetration in the wood tissue through detection of GFP fluorescence. Therefore, an internal interaction between *E. lata* and blackcurrants will be established.

Aim 4: *Obtaining a putative toxin gene moxY disrupted P. chlamydospora transformant.*

To construct a plasmid containing a *moxY* gene homologous fragment, and transform *P. chlamydospora* based on the previously developed transformation system. The expected

moxY gene disrupted transformants through homologous recombination will be screened through PCR, followed by Southern hybridization.