Phosphite induces morphological and molecular changes in *Phytophthora* species

This thesis is submitted to Murdoch University for the degree of Master of Philosophy

by

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary educational institution.

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(Mee-Hua Wong)
Abstract

The influence of the chemical phosphite on Phytophthora species was investigated by studying the morphological and molecular changes induced by phosphite.

In vitro experiments were conducted to study the effects of phosphite on five isolates of each of five species of Phytophthora grown in low phosphate defined medium. Sensitivity to phosphite varied greatly among the five isolates of each species and resulted in a significant interaction between isolate and phosphite effect. The EC$_{50}$ values ranged from less than 5 to 10 µg/ml for P. cinnamomi, to 13 µg/ml for P. nicotianae, to 27 µg/ml for P. citricola, to 24 µg/ml for P. palmivora and to 49 µg/ml for P. capsici.

Phosphite concentrations from 5 to 100 µg/ml caused different degrees of morphological changes. Mycelial growth of all species was significantly suppressed by phosphite at 5 µg/ml while at 100 µg/ml there was hyphal lysis. Swelling of hyphae with stunted side-branches and shrinking of cytoplasm from hyphal tips and hyphal walls were characteristic changes observed. Phosphite also retarded the development and caused distortion and lysis of chlamydospires, sporangia and zoospores. Zoosporogenesis was also adversely affected.

Differential display reverse transcription-PCR was used to study changes in gene expression in P. cinnamomi induced in response to phosphite stress. The differential conditions were simulated by growth on a defined medium with and without phosphite amendment. This technique resulted in the isolation of 34 putative differentially expressed
cDNA fragments which were cloned and sequenced. Nucleotide sequences of 26 of these cDNA clones were generated. BLASTX analysis of these nucleotide sequences against the NCBI database revealed that 18 exhibited homology to gene sequences encoding known proteins involved in various biological processes. The remaining eight showed homology to either hypothetical or unknown or unnamed proteins.

The expression level of four of these cDNA clones were further analysed by real-time quantitative RT-PCR using SYBR Green 1 assay. Three candidate endogenous reference genes namely, tubulin, cyclophilin and actin were evaluated to determine their expression level under the influence of phosphite. None of these genes were significantly regulated by phosphite. As tubulin had the highest expression among the three, it was chosen as the endogenous reference gene. Amplification efficiencies between the reference gene and each of the target genes were validated and found to be approximately equal or within 5% of each other. The relative gene expression between the phosphite-treated and untreated samples can thus be determined using the comparative $C_T$ ($\Delta\Delta C_T$) method. One of the cDNA clones, CP6 which showed differential expression of three-fold was up-regulated. The remaining three were constitutively expressed. CP6 which encodes 1564 nucleotides showed sequence homology, at the amino acid level with proteophosphoglycans from *Leishmania major*.

This study demonstrated the growth inhibition and morphological deformities caused by phosphite in *Phytophthora* species. It also illustrated the use of a modified DDRT-PCR method to study genes expressed in phosphite stress regulation. The application of real-time
quantitative RT-PCR with SYBR Green I assay facilitated the quantification of the expression level of some of these genes.
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Abbreviations

aa            amino acid
bp            base pair
0°C           degree centigrade
cDNA          complementary deoxyribonucleic acid
cm            centimeter
Cₜ            threshold cycle
ΔCₜ           change in threshold cycle
CV            coefficient of variation
DDRT-PCR      differential display reverse transcription-polymerase chain reaction
DEPC          diethyl-pyrocarbonate
DNA           deoxyribonucleic acid
3’            hydroxyl-terminus of DNA molecule
5’            phosphate-terminus of DNA molecule
DNase I       deoxyribonuclease I
dNTPs         deoxyribonucleoside triphosphates
dsDNA         double-stranded DNA
DTT           dithiothreitol
EC₅₀          effective concentration 50 percent
ED₅₀          effective dose  50 percent
EDTA          ethylenediamine-tetracetic acid
FA            formaldehyde
f. sp.        formae speciales
g             gram
hr            hour
kbp           kilobase pair
L             litre
LB            Luria-Bertani
M             molar
mg            milligram
min           minute
ml            millilitre
mm            millimeter
mRNA          messenger ribonucleic acid
nM nanomolar
nm nanometer
nt nucleotide
NTP no-template control
PCR polymerase chain reaction
pmol picomole
R² correlation coefficient
rcf relative centrifugal force
Rₙ fluorescence signal
∆Rₙ change in fluorescence signal
RNA ribonucleic acid
RNase ribonuclease
rpm revolution per minute
RT reverse transcription
RT-PCR reverse transcription-polymerase chain reaction
SD standard deviation
sec second
TAE tris-acetate-EDTA
Taq Thermus aquaticus
TBE tris-borate-EDTA
U enzyme unit
UV ultra violet
µg microgram
µL microlitre
µM micromolar
µm micrometer
V volt
W Australia Western Australia
wt weight
Chapter 1

Introduction and Literature Review

1.1 Biology of Phytophthora

The genus *Phytophthora* contains some of the most destructive plant pathogens, causing a large number of diseases on a wide range of plant species worldwide. *Phytophthora* is derived from the Greek words ‘phyto’ (plant) and ‘phthora’ (destroyer) or plant destroyer. There are about 60 species in the genus, all of them plant pathogens. Most species have wide host range, and each can cause a myriad of diseases on different species (Erwin and Ribeiro, 1996).

*Phytophthora* species cause diseases with symptoms ranging from root rot, collar rot, stem canker, leaf blight and fruit rot. They are responsible for some of the most devastating diseases such as the jarrah dieback on *Eucalyptus marginata* (jarrah) and understorey species in the jarrah forest caused by *P. cinnamomi* in Australia (Podger, 1972; Dell and Malajczuk, 1989; Shearer and Tippett, 1989), the late blight of potato caused by *P. infestans* in Ireland, black pod of cocoa due to *P. palmivora*, foot rot in black pepper due to *P. capsici* and many other diseases of economic importance. The estimated losses due to *Phytophthora* diseases amount to billions of dollars annually (Erwin and Ribeiro, 1996).

The genus *Phytophthora* belongs to the Class Oomycetes. It has traditionally been placed under the Family Pythiaceae in the Kingdom Chromista. (Erwin and Ribeiro, 1996).
However, current knowledge suggests that the Oomycetes are closely related to heterokont algae; both of which come under the monophyletic Kingdom Stramenopila. Though Oomycetes are not true fungi, they share many characteristics with the true fungi such as an absorptive mode of nutrition, growth by polarized hyphal extension and reproduction involving the formation of spores (Money, 1998).

*Phytophthora* species have both sexual and asexual cycles (Figure 1.1). These cycles result in the production of the sexual oospores and three types of asexual propagules namely sporangia, zoospores and chlamydosporos (Erwin and Ribeiro, 1996; Goodwin, 1997). Oospores are formed from oogonia and antheridia. They germinate to give rise to sporangia. Chlamydosporos are formed from mycelium. Oospores have thick walls and those of chlamydosporos may be thick or thin. While oospores are considered to function as structures to survive unfavorable environmental conditions, there is some dispute about the role of chlamydosporos as long term survival structures (McCarren *et al.*, 2004). The most abundant asexual propagule is the sporangium. Sporangia either germinate directly by producing germ tubes or indirectly releasing motile zoospores in free water. The encysted zoospores germinate to germ tubes and mycelium. These asexual spores are the infective propagules of the pathogen.
1.2 Major diseases caused by *Phytophthora* species

1.2.1 *Phytophthora cinnamomi*

*Phytophthora cinnamomi* which has nearly 1,000 hosts (Zentmyer, 1980) is an important pathogen in Australian forests and sandplain flora. A recent report showed that approximately 41% of the 5710 plant species in the South-West Botanical Province of Western Australia are susceptible or highly susceptible to *P. cinnamomi* (Shearer *et al.*, 2004). It is the cause of jarrah dieback on *Eucalyptus marginata* (jarrah), an important forest tree in Western Australia. The disease has caused death of extensive area of jarrah
forest resulting in considerable loss in timber production (Wills, 1992). Jarrah is the only Eucalyptus species of the jarrah forest killed by P. cinnamomi (Podger, 1972). The susceptibility of Eucalyptus species varies greatly; marri (Corymbia calophylla) are more tolerant while yarri (E. patens), bullich (E. megacarpa), karri (E. diversicolour) and tuart (E. gomphotocephala) are resistant. Many species of the forest understorey and shrubs are susceptible (Dell and Malajczuk, 1989; Shearer and Tippett, 1989; Wills, 1992) while grasses and sedges are resistant (Shearer and Tippett, 1989; Laidlaw and Wilson, 2003).

Symptoms of P. cinnamomi in the forest are indicated by the yellowing, chlorosis and death of the susceptible understorey species (Figure 1.2). The infected jarrah show crown decline with leaf chlorosis and dieback of primary branches. Leaves on epicormic shoot are smaller, resulting in unthriftiness of the crown. With time, epicormic production and leaf area decline. The infected trees may die or survive in a declining state for many years (Podger, 1972; Dell and Malajczuk, 1989; Shearer and Tippett, 1989).

Apart from the biological and economic impact in the jarrah forest, the disease has also caused loss of forest and sandplain biodiversity (Weste and Law, 1972; Wills, 1992). Wills (1992) reported that disease caused by P. cinnamomi has significantly changed the floristic and structural diversity of the Stirling Range area in Western Australia. Similar observations were made by Laidlaw and Wilson (2003) in the eastern Otway Ranges, Victoria. The loss of floristic diversity and structural change in the habitat has adverse implications on the associated fauna in the area and its scenic and tourism values (Wills, 1992; Laidlaw and Wilson, 2003).
As *P. cinnamomi* cannot be eradicated once it has infected an area, integrated management control strategies are necessary to minimize spread and development of the disease. Hardy *et al.* (2001) have outlined various management strategies for the control of *P. cinnamomi* in natural ecosystem. These include road access control to restrict movement of vehicles to non-infested areas, cleaning of vehicles and equipment to remove adhering soil or plant debris, preventing the movement of infested water into disease-free areas and minimizing road building, mining and forestry activities during wet period when conditions are favorable for spread of the pathogen.

In recent years, the chemical phosphite has been used to reduce the incidence and inhibit the spread of *P. cinnamomi* in natural ecosystems (Shearer and Tippett, 1989; Hardy *et al.*, 2001). The application of phosphite significantly reduced disease front extension in
**Banksia** woodland (Shearer et al., 2004). Studies by Pilbeam et al. (2000) and Tyan et al. (2001) have shown phosphite treatment to successfully reduce colonization of *P. cinnamomi* in native plant communities in Western Australia.

### 1.2.2 *Phytophthora palmivora*

*Phytophthora palmivora* is one of the most destructive *Phytophthora* species attacking more than 140 plant species worldwide (Lim, 1990). Its ability to infect such a wide range of species and almost every part of the plant makes it one of the most important plant pathogen in the genus. *P. palmivora* is deemed the most devastating pathogen of cocoa tree (*Theobromae cocoa*); responsible for a 20 - 30% loss of the world’s cocoa crop. It is endemic in all cocoa-producing countries (Erwin and Ribeiro, 1996).

The disease affects all parts and all growth stages of the plant causing black pod, stem canker and seedling blight but majority of the losses are due to black pod (Anderson and Guest, 1990). Pod infection starts on the surface of the pod with a well-defined brownish-black lesion progressing over the entire pod within a short period of time. As the disease develops, a whitish bloom of mycelia and sporangia is formed on the necrotic lesion (Opeke and Gorenz, 1974) (Figure 1.3). Stem canker is characterized by brown necrotic bark around the trunk with watery brownish-grey tissues underneath the bark (McMahon and Purwantara, 2004).
Integrated approaches to control the disease include cultural practices, resistant varieties and pesticide applications. Cultural practices such as sanitation, pruning of lower branches and canopy contribute to disease reduction. Planting of certain tolerant genotypes (McMohon and Purwantara, 2004) is likely to be of benefit. Apart from conventional pesticides such as copper and metalaxyl, the chemical phosphite has also been found to be effective in controlling the disease. Trunk injection of potassium phosphonate (Anderson and Guest, 1990; Holderness, 1992) significantly reduced stem canker and black pod incidence of cocoa.

1.2.3 Phytophthora capsici

Foot rot caused by Phytophthora capsici is one of the most devastating disease of black pepper (Piper nigrum). It occurs in all pepper producing countries. Losses were
estimated at 5 - 20% annually in Malaysia, Indonesia and Vietnam (Drenth and Sendall, 2004).

Symptoms are leaf yellowing and wilting and branches appearing to droop. The rootstock and roots have brownish-black lesions (Figure 1.4). Berries turn brown, become sunken and drop. Sometimes characteristic fimbriate edge lesions may observe on the leaves. As the disease progresses, leaves and branches turn brown and defoliation occurs resulting in a skeletal plant (Wong, 2004).

Figure 1.4  Foot rot of black pepper infected with *P. capsici* showing brownish-black lesion on the rootstock. (Photo courtesy Dept. of Agriculture, Sarawak).
An integrated approach with emphasis on prevention is needed to manage the disease. Cultural practices include field hygiene, pruning of lower branches, improving soil drainage and avoiding planting materials from high-risk areas. The amendment of soil with organic matter encourages development of antagonistic microorganisms (Manohara et al., 2004). Apart from metalaxyl which is commonly used to control the disease, phosphite, applied through root infusion protects plants against *Phytophthora* infection in black pepper (Wong 2004).

### 1.3 Phosphite

The chemical, phosphorous acid ($\text{H}_3\text{PO}_3$) which in solution is in equilibrium with its tautomeric form, phosphonic acid is at present the cheapest and most widespread pesticide in use for many *Phytophthora* diseases. The corresponding esters of phosphonic and phosphorous acids are termed phosphonates and phosphates, respectively (Cohen and Coffey, 1986; Dunhill, 1990).

Phosphonates or phosphite, the anionic form of phosphorous acid is used in formulations containing either the aluminum salt of ethyl-phosphonate (Fosetyl-Al) or the potassium salt of phosphite (Foli-R- Fos) (Guest, 1984; Cohen and Coffey, 1986; Grant et al., 1990; Dercks and Buchenauer, 1987; Dunhill, 1990; Martin et al., 1998). These compounds are reported to be effective for the control of diseases caused by Oomycetes in which *Phytophthora* has been shown to be particularly susceptible (Coffey and Joseph, 1985; Cohen and Coffey, 1986; Martin et al., 1998; McDonald et al., 2001). One of the
biological properties which made phosphite so unique is its ability to translocate in both the xylem and phloem of the plant (Schwinn, 1983; Guest and Bompeix, 1990; Erwin and Rebeiro, 1996).

1.4 **Efficacy of phosphite on Phytophthora species and isolates**

*Phytophthora* species vary markedly in their sensitivity to phosphite. It exerts a moderate antifungal activity against some *Phytophthora* species *in vitro* but has low or no activity against some other *Phytophthora* species (Coffey and Bower, 1984).

Coffey and Bower (1984) tested mycelial growth of nine species of *Phytophthora* for their sensitivity to phosphite and found that the ED$_{50}$ values ranged from 5.2 µg/ml for *P. citrophthora* to 224 µg/ml for *P. infestans*. *P. citrophthora*, *P. citricola*, *P. cinnamomi*, *P. cactorum* and *P. capsici* are very sensitive while *P. megasperma* and *P. infestans* are tolerant (Coffey and Bower; 1984; Fenn and Coffey, 1984; Derck and Buchenauer, 1987).

Phosphite also targets other stages of the life cycle of *Phytophthora* species, especially sporulation. Production of oospores, chlamydomspores, sporangia and zoospore release were adversely affected; with *P. citricola* more sensitive than *P. cinnamomi* (Coffey and Joseph, 1985). However, Wilkinson *et al.* (2001) showed sporangia and zoospore release from *P. cinnamomi* colonized plants that had been treated with phosphite.

Sensitivity to phosphite may also vary between different isolates of a species. Coffey and Bower (1984) reported the inhibition of radial growth by phosphite ranged from 0 - 45%
and 0 - 83% among isolates of *P. cinnamomi* and *P. megasperma* respectively from different hosts. In addition, they also found significant inhibition by phosphite for isolates of *P. infestans* from different countries. This variation was also observed by Bashan *et al.* (1990). Of 71 Australian isolates of *P. cinnamomi* tested, over 80% were classified as intermediate while the remaining were classified as sensitive or tolerant with EC$_{50}$ values ranging from 4 - 148 µg/ml phosphite (Wilkinson, *et al.*, 2001). However, the results of Coffey and Bower (1984), Bashan *et al.* (1990) and Wilkinson *et al.* (2001) were based on measurement of radial growth rather than dry weight of mycelia may compromises the accuracy of the above conclusions.

### 1.5 The role of phosphate

Phosphate plays a vital role in influencing the uptake of phosphite in *Phytophthora* species. The antifungal activity of phosphite is highly dependent on the concentration of phosphate present in the *in vitro* medium or in the soil (Smillie, *et al.*, 1989; Griffin *et al.*, 1993; Darakis *et al.*, 1997).

An inverse relationship between fungal sensitivity to phosphite and phosphate concentration has been established (Varadarajan *et al.*, 2002). Growth of *P. palmivora* was inhibited at phosphite concentration of 10 mM in medium with high phosphate rate of 10 mM whereas 0.1 mM phosphite was sufficient to cause inhibition at low phosphate rate of 0.1 mM (Griffin *et al.*, 1989). In another study on *P. palmivora* isolates, ED$_{50}$ was 30 mM phosphite in the presence of 7 mM phosphate and 1 mM phosphite when there was
only 0.1 mM phosphate present (Griffin et al., 1993). These results corroborate with Fenn and Coffey (1984) who showed that increasing the phosphate concentration 100-fold caused a significant decrease in the efficacy of phosphite against *P. cinnamomi*.

The antagonistic effect of phosphate on phosphite activity *in vitro* towards mycelial growth of *Phytophthora* species appears to be due to competition between both anions for the common transport system for phosphate and phosphite uptake (Barachietto et al., 1989; Grant et al., 1990; Darakis et al., 1997).

### 1.6 Mode of action of phosphite

#### 1.6.1 Direct effect on the pathogen

As mentioned above, the direct adverse effect of phosphite on *P. cinnamomi* was demonstrated by Coffey and Joseph (1985) who showed that the application of phosphite can inhibit mycelial growth, oospores, sporangia and zoospore release and chlamydomospore production. Grant *et al.* (1990) using *P. palmivora* as a model showed that the phosphonate anion can act directly on the fungus by reducing growth. Phosphite resistant mutants of *P. capsici* can be generated by treatment with mutagens and tolerant mutants of *P. capsici* and *P. palmivora* showed the same enhanced level of resistance to phosphite both *in vitro* and *in vivo* (Bower and Coffey; 1985; Fenn and Coffey, 1985; Dolan and Coffey, 1988). These results supported the contention that the chemical acts directly on the pathogen.
1.6.2 Indirect effect by stimulating plant defence mechanism

The indirect effect of phosphite was observed by Guest and Bompeix (1990) who reported that the chemical causes the pathogen to release elicitor-active metabolites which in turn increase the host defense response. An enhanced defense response in phosphite-treated plants following pathogen challenge is well established (Grant et al., 1990). For example, low phosphite concentrations within the roots of *Eucalyptus marginata* stimulated host defense enzymes (4-CL and CAD) and caused accumulation of phenolics whereas high concentrations inhibited the pathogen’s growth directly (Jackson et al., 2000).

The control of *Phytophthora* infection in capsicum and tobacco by Fosetyl-Al was associated with enhanced hypersensitivity and increased phytoalexin accumulation; similar to that found in resistant cultivars (Guest, 1984). Phosphite treatment increased the synthesis and accumulation of phytoalexin in the inoculated cowpea and this resulted in growth restriction of *P. crytogeae* (Saindrenan et al., 1988). Smillie et al. (1989) reported that the protection provided by phosphite against *P. cinnamomi*, *P. nicotianae* and *P. palmivora* in lupin, tobacco and papaya was due to the plant defense system acting in concert with the direct effect of the chemical. Phosphite application increases the concentration of phytoalexin in *Citrus* species inoculated with *P. citrophthora* whereas in healthy plants, its production was not induced (Afek and Sztejnberg, 1989). Nemestothy and Grant (1990) demonstrated the indirect mode of action of Fosetyl-Al in *P. nicotianae* inoculated plant was associated with an increase in phytoalexin and
ethylene accumulation and lignin deposition. Recent findings showed that phosphite-treated *Xanthorrhoea australis* seedlings infected with *P. cinnamomi* produced accumulations of phenolic and lignin-like compounds around infection hyphae and in cortical and vascular cell walls of infected tissues, another indication of defence-associated host responses (Daniel *et al.*, 2005).

Further evidence of plant response to phosphite was shown by Molina *et al.* (1998) who demonstrated that the chemical induced the expression of system acquired resistance (SAR) marker gene, PR-1 in *Arabidopsis* plants. The indirect mode of action of phosphite was further proved by Chuang *et al.*, (2003) who identified the expression of a broad spectrum of defence-related genes up-regulated in response to Fosetyl-Al treatment in phosphite-treated *Arabidopsis* plants.

### 1.7 Sites of action

Several studies have proposed mechanisms by which phosphite might induce damaging changes in structural components and metabolic intermediates in *Phytophthora* species. Specific sites of action of phosphite in *P. palmivora* could be the synthesis of adenylate and the polyphosphorylated nucleotides as their levels were reduced in the presence of phosphite (Griffin *et al.*, 1990). A similar observation on a reduction in the adenylate pool of *P. citrophthora* with phosphite treatment was reported (Barchietto *et al.*, 1992). Niere *et al.* (1990) used $^{31}$P NMR to show that phosphite perturbs phosphorous metabolism in *P. palmivora*. This corroborated with the observation that phosphite alters phosphorous
metabolism in *P. palmivora* and *P. melonis* (Martin *et al*., 1998). It appears that the metabolism of pyrophosphate is the primary site of phosphite inhibition as it caused an increased accumulation of phosphorous in both pyrophosphate and polyphosphate (Niere *et al*., 1994). A recent study has shown that phosphite inhibited several enzymes involved in the glycolytic and phosphogluconate pathways, indicating that it may inhibit several enzymes rather than acting at a single unique site (Stehmann and Grant, 2000). The availability of molecular techniques may give new insight into the site of action of phosphite.

### 1.8 Differential display reverse transcription-PCR

The major biological processes of living organisms are driven by changes in the levels and patterns of gene expression. The identification and analysis of these genes provide information on how they influence and regulate biological processes and also help to understand the underlying molecular mechanisms of a particular biological system.

The traditional methods of detecting differentially-expressed genes such as subtractive and differential hybridization involved the construction, screening and probing of the cDNA libraries. These techniques, apart from being time-consuming and labour-intensive also required large amounts of RNA. Furthermore, only one pair of RNA population can be compared at any given time (Bauer *et al*., 1993; Liang and Pardee, 1993; Sturtevant, 2000).
Differential display or DDRT-PCR which was developed by Liang and Pardee is a powerful tool in the analysis and identification of genes that are differentially expressed at the mRNA level (Liang and Pardee, 1992; Liang et al., 1995). The principle of this technique is described in Chapter 3. DDRT-PCR has been widely used in the study of differential gene expression especially in the field of medical research. It has also been extensively adopted in biological research to study differential gene expression in plant-pathogen interactions (Benito et al., 1996; Munoz and Bailey, 1998; Seehaus and Tenhaken, 1998; Sanchez-Torres and Gonzalez-Candelas, 2003; Wang et al., 2003) or other developmental or physiological changes (Crauwels et al., 1997; Gross and Watson, 1998; Melin et al., 1999) in both plants and microorganisms.

Many of the limitations of the hybridization-based methodologies are overcome by the polymerase chain reaction-based techniques. The DDRT-PCR technique only requires small amounts of RNA and it allows the comparison of several RNA populations simultaneously (Bosch and Lohmann, 1997; Jorgensen et al., 1997). Though effective, the technique does have some drawbacks. The major limitation is the high incidence of false positives. In addition, the use of radioactive labeling would hinder laboratories that are not equipped to handle radioisotope work which require special handling and disposal.

Since the 1990’s, studies based on DDRT-PCR procedures but with further refinements and improved efficiency have been reported. Various aspects of the technique have been modified and optimized to improve the identification of differentially expressed genes (Bauer et al., 1993; Liang et al., 1993). The use of modified anchored oligo-dT and
arbitrary primer sequences improved the efficiency of the method (Ito and Sakaki, 1997). The addition of two bases MN at the 3’ end provided the oligo-dT primers with specificity for anchoring to the beginning of the poly(A) tails (Liang et al., 1993). Later it was found that one-based anchored oligo-dT primer minimized the redundancy and under-representation of certain RNA species and its use further reduced the number of reverse transcription reactions needed for each RNA sample (Liang et al., 1994). The introduction of a restriction site at the 5’ end of both the anchored and arbitrary primers increases the primer length and efficiency of cDNA amplification. This also facilitates cloning and sequencing (Liang et al., 1994).

Modification of the PCR thermal cycling profile by using an initial low stringency step followed by high stringency steps have been adopted in DDRT-PCR (Ito and Sakaki, 1997; Wang et al., 2003) to help priming efficiency and increased reproducibility of the technique. Running duplicate samples is recommended to minimize false positive bands (Liang et al., 1993).

To avoid radioisotope labeling, Ito and Sakaki (1997) used a fluorescent image analyzer and a fluorescent dye-staining method to analyse the cDNA bands generated. Bosch and Lohmann (1997) also successfully adopted the method without the use of radioisotopes. They visualized the amplified cDNA bands by using silver staining. The sensitivity and resolution were comparable to the radioactive procedures and even low abundance PCR products were detected. The fact that agarose gel has been used successfully in place of
polyacrylamide gel to detect the DDRT-PCR products (Rompf and Kahl, 1997; Ahmed et al., 2000) has made the technique more attractive and less expensive.

Though the efficiency of DDRT-PCR has been in dispute, many researchers have demonstrated the feasibility of using the technique to identify differentially expressed genes. When a putative differentially expressed cDNA has been identified, further confirmation of the candidate cDNA is carried out using Northern blotting (Crauwel et al., 1997; Gross and Watson, 1998; Munoz and Bailey, 1998; Seehaus and Tenhaken, 1998; Sanchez-Torres and Gonzalez-Candelas, 2003), dot or slot blots (Mou et al., 1994; Zhang et al., 1996; Chalifour, 1997) and RNase protection assays (Hummel et al., 1997). Northern blot hybridization or RNase protection assays though effective, are laborious and require a large quantity of RNA. In addition, these techniques lack the sensitivity required to analyse low abundance transcript (Simpson et al., 2000; Rajeevan et al., 2001; Kim et al., 2003). More recently, real-time quantitative RT-PCR technique has been used to analyse and quantify differential gene expression (Rajeevan et al., 2001; Wang et al., 2003).

1.9 Real-time quantitative PCR

The development of real-time PCR technique has expedited the process of quantifying PCR product. Unlike other earlier techniques which require intensive and laborious post-PCR manipulation, real-time PCR monitors the product accumulation in real time concomitant with PCR amplification requiring no PCR post-manipulation. The two widely used assays
are the fluorogenic 5’-exonuclease and SYBR green I assays. The principles of these two assays are described in Chapter 4.

The technology to detect PCR product in real time has been widely used for different applications, such as clinical diagnostic (Winer et al., 1999; Simpson et al., 2000; Giulietti et al., 2001; Ponchel et al., 2003), analysis and quantitative gene expression studies in insects, plant-microorganism interaction (Paton et al., 2000; Wang et al., 2003) and detection and quantification of pathogen in plant tissues (Vandemark and Barker, 2003; Silva et al., 2005). It is also ideally suited for the validation of differentially expressed genes identified by cDNA arrays and differential display (Rajeevan et al., 2001).

Real-time PCR, which combines the advantages of conventional PCR with quantitative capability (Simpson et al., 2000) is, at present one of the most sensitive and accurate method for the detection and quantification of gene expression (Giulietti et al., 2001). Its high sensitivity allows the quantification of rare transcripts and small changes in gene expression (Kim et al., 2003). This technique is rapid and requires a thousand-fold less RNA than conventional assays (Rajeevan et al., 2001). Though real-time PCR is a powerful technique, it requires optimization of various reaction components and conditions.

The many advantages of real-time PCR in terms of accuracy, sensitivity, broad dynamic range, high throughput, minimum amount of RNA, without use of radio-isotopes and absence of post PCR manipulations has made it the preferred choice over other conventional methods for the detection and quantification of gene expression (Schmittgen, 2001; Vandesompele et al., 2002; Radonic et al., 2004).
1.10 Aims of the thesis

Phosphite is currently an effective chemical to control *Phytophthora* species *in-planta*. However, continuous use on native vegetation and on commercial crops poses the risk of the selection of *Phytophthora* strains with tolerance to the chemical which would constitute a serious threat to natural ecosystems, timber and horticultural industries.

Though studies on the efficiency of phosphite on *Phytophthora* species and its mode of action have been reported extensively, little is known about the molecular mechanisms involved when the pathogen is exposed to phosphite. A better understanding and knowledge on the underlying molecular events occurring in the pathogen is therefore needed.

The overall aim of this research was to study the morphological and molecular changes in *P. cinnamomi* induced by phosphite. The information on the phenotypic alteration and gene regulation will assist in the development of improved biochemicals against the pathogen. This would ultimately contribute to better management strategies for more effective control of diseases caused by *Phytophthora* species.

To achieve this aim, objectives were set:

- To study the effects of phosphite on different *Phytophthora* species and to examine the morphological changes induced by phosphite.
• To isolate and identify the gene(s) that is/are responsive to phosphite treatment by adopting and modifying the DDRT-PCR technique.

• To analyse and quantify gene expression by using the real-time quantitative RT-PCR technique.
Chapter 2

*In vitro* effects of phosphite on isolates of different *Phytophthora* species

2.1 Introduction

Phosphite, the anion form of phosphonic acid is an effective control agent for crop diseases caused by Oomycetes, particularly *Phytophthora* species (Guest, 1984; Coffey and Joseph, 1985). It is the active metabolite of several formulations of pesticides that are widely used to control plant infection by *Phytophthora* species (Cohen and Coffey, 1986).

Most fungicides act by inhibiting the energy metabolism, blocking biosynthesis or altering the cell wall of the fungus. They may also affect the development and formation of various morphological structures such as germinal tubes, appressoria or spores (Garcia *et al.*, 2003).

One of the modes of action of phosphite is its direct effect on the pathogen (Guest and Bompeix, 1990). It has been demonstrated *in vitro* that mycelial growth and spore production of *Phytophthora* species were inhibited by phosphite (Coffey and Joseph, 1985). Variations in sensitivity were observed between and within *Phytophthora* species (Coffey and Bower, 1984). Within *P. cinnamomi*, growth inhibition for different isolates ranged from 0 – 44.8% at 5 µg/ml phosphite. Response to phosphite based on *formae speciales* (f. sp.) were observed in *P. megasperma* species with ED$_{50}$ values of 90 µg/ml and 20 µg/ml for *P. f. sp. medicaginis* and *P. f. sp. glycinea* isolates respectively. On the other hand, six
isolates of *P. citrophthora* from *Citrus* had a narrow range of inhibition from 80.3 – 89.3% (Coffey and Bower, 1984). There was great variation in phosphite sensitivity among 11 isolates of *P. infestans* from different geographical locations with ED$_{50}$ values ranging from 4 – 281 µg/ml (Bashan, 1990). A similar observation was made for 71 Australian isolates of *P. cinnamomi* which had EC$_{50}$ values ranging from 4 – 148 µg/ml (Wilkinson *et al*., 2001).

Apart from its inhibitory effect on the growth of mycelium, phosphite was also reported to cause adverse morphological changes of mycelium in *Phytophthora* species (Dercks and Buchenauer, 1987). In phosphite-treated *Xanthorrhoea australis* seedlings, convolution of cytoplasm and rupture of the cell wall of the invading hyphae of *P. cinnamomi* was observed (Daniel *et al*., 2005).

This chapter aimed to establish the efficacy of phosphite on five species of *Phytophthora* and to examine the variability of different isolates within each of the species towards phosphite. As *Phytophthora* species require phosphate for optimum growth and the efficacy of phosphite is highly dependent on the concentration of phosphate in the growth medium, an initial experiment was carried out to determine the minimum level of phosphate required by each *Phytophthora* species for optimal growth. The effect of phosphite was then tested using a level of phosphate in the medium which would have minimal effect on phosphite uptake.
2.2 Material and Methods

2.2.1 Phytophthora species isolates

Five isolates each of Phytophthora cinnamomi, P. nicotianae, P. citricola, P. palmivora and P. capsici were used (Table 2.1).

Table 2.1 Sources and details of isolates of five species of Phytophthora used.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Phytophthora species</th>
<th>Source</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP9448</td>
<td><em>P. cinnamomi</em></td>
<td><em>Eucalytus marginata</em></td>
<td>Willowdale, W. Australia</td>
</tr>
<tr>
<td>MP62</td>
<td><em>P. cinnamomi</em></td>
<td><em>Eucalytus marginata</em></td>
<td>Jarrahdale, W. Australia</td>
</tr>
<tr>
<td>MP9418</td>
<td><em>P. cinnamomi</em></td>
<td><em>Corymbia calophylla</em></td>
<td>Willowdale, W. Australia</td>
</tr>
<tr>
<td>MP9411</td>
<td><em>P. cinnamomi</em></td>
<td><em>Eucalytus marginata</em></td>
<td>Willowdale, W. Australia</td>
</tr>
<tr>
<td>MU33</td>
<td><em>P. cinnamomi</em></td>
<td>Soil</td>
<td>Cape Arid, W. Australia</td>
</tr>
<tr>
<td>MP5</td>
<td><em>P. nicotianae</em></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>MP9</td>
<td><em>P. nicotianae</em></td>
<td><em>Chamelaucium sp.</em></td>
<td>South Perth, W. Australia</td>
</tr>
<tr>
<td>MU7</td>
<td><em>P. nicotianae</em></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>MU317</td>
<td><em>P. nicotianae</em></td>
<td><em>Banksia brownie</em></td>
<td>Woodland, W. Australia</td>
</tr>
<tr>
<td>MP95014</td>
<td><em>P. nicotianae</em></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>MP1</td>
<td><em>P. citricola</em></td>
<td><em>Pinus radiata</em></td>
<td>Baudin Plantation, W. Australia</td>
</tr>
<tr>
<td>MP2</td>
<td><em>P. citricola</em></td>
<td>Soil</td>
<td>Nannup, W. Australia</td>
</tr>
<tr>
<td>MP3</td>
<td><em>P. citricola</em></td>
<td>Soil</td>
<td>Walpole, W. Australia</td>
</tr>
<tr>
<td>MP4</td>
<td><em>P. citricola</em></td>
<td><em>Banksia attenuata</em></td>
<td>Yanchep, W. Australia</td>
</tr>
<tr>
<td>MP41</td>
<td><em>P. citricola</em></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>MU128</td>
<td><em>P. palmivora</em></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>94111</td>
<td><em>P. palmivora</em></td>
<td><em>Durio sp.</em></td>
<td>Not available</td>
</tr>
<tr>
<td>PM006</td>
<td><em>P. palmivora</em></td>
<td><em>Durio sp.</em></td>
<td>Sg. Paon, Sarawak</td>
</tr>
<tr>
<td>PM007</td>
<td><em>P. palmivora</em></td>
<td><em>Theobroma cocoa</em></td>
<td>Sarikei, Sarawak</td>
</tr>
<tr>
<td>PM012</td>
<td><em>P. palmivora</em></td>
<td><em>Theobroma cocoa</em></td>
<td>Tarat, Sarawak</td>
</tr>
<tr>
<td>PP014</td>
<td><em>P. capsici</em></td>
<td><em>Piper nigrum</em></td>
<td>Skrang, Sarawak</td>
</tr>
<tr>
<td>PP019</td>
<td><em>P. capsici</em></td>
<td><em>Piper nigrum</em></td>
<td>Mile 31, Penrissen, Sarawak</td>
</tr>
<tr>
<td>PP021</td>
<td><em>P. capsici</em></td>
<td><em>Piper nigrum</em></td>
<td>Mile 9, Penrissen, Sarawak</td>
</tr>
<tr>
<td>PP026</td>
<td><em>P. capsici</em></td>
<td><em>Piper nigrum</em></td>
<td>Batu Lintang, Sarawak</td>
</tr>
<tr>
<td>PP027</td>
<td><em>P. capsici</em></td>
<td><em>Piper nigrum</em></td>
<td>Serian, Sarawak</td>
</tr>
</tbody>
</table>
These isolates were maintained on commeal agar (Oxoid) (Appendix I) under water and kept at room temperature. Prior to use, isolates were subcultured onto cornmeal agar and grown at 26 °C +/- 1 °C in an incubator in the dark.

2.2.2 Preparation of fungal inoculum

Inoculum was prepared by culturing each isolate on modified Ribeiro’s agar medium (Appendix I). Each isolate was grown for five days in the dark at 26 °C +/- 1 °C in an incubator. A mycelium plug of 6 mm diameter was taken from the actively growing edge of each colony for use as inoculum.

2.2.3 Preparation of phosphite solution

The phosphite used was phosphorous acid (H₃PO₃, 99%, Aldrich). A stock solution was prepared with distilled water. The pH of the solution was adjusted to 6.5 with 6M potassium hydroxide. The solution was sterilized by filtering through a 0.2 µm Millipore filter (Schleicher and Schuell) before storing in the dark at 4 °C.

2.2.4 Condition for mycelial growth

20 ml of Ribeiro’s aqueous medium was dispensed into each 9 mm diameter Petri plate. One 6 mm diameter piece of the five-day-old inoculum was placed onto the medium in each plate. The inoculated plates were incubated in the dark at 26 °C +/- 1 °C for 7 days at which time the mycelium was harvested.
2.2.5 Harvesting of mycelium and determination of mycelial dry weight

The mycelium was harvested by vacuum filtration onto a pre-weighed 47 mm diameter glass microfibre filter paper (Filtech) lining a Buchner funnel and then rinsed twice with sterilized distilled water. The filter paper containing the mycelium was dried overnight at 100 °C (Smillie et al., 1989) before weighing.

2.2.6 Growth response of Phytophthora species to phosphate

*P. cinnamomi* (MP62), *P. palmivora* (MU128), *P. capsici* (PP027), *P. nicotianae* (MP9) and *P. citricola* (MP2) were used in this experiment (Table 2.1).

Modified Ribeiro’s aqueous medium incorporated with one of six concentrations of phosphate: 0, 0.1, 0.5, 1, 5 and 10 mM were prepared by adjusting the concentration of phosphate accordingly (Appendix 1). Each plate was inoculated with a mycelium plug from the stock cultures growing on modified Ribeiro’s agar medium containing no phosphate. Mycelia were harvested as described in Section 2.2.5. There were three replicates per treatment and the experiment was repeated once.

2.2.7 Responses of Phytophthora species to phosphite

All five isolates from each of the five *Phytophthora* species were used (Table 2.1). The concentration of phosphate used in the modified Ribeiro’s medium was 0.1 mM as derived from the phosphate growth curve in the preliminary experiment in Section 2.2.6. This level
was selected as it was the minimum level that allowed good growth of the fungus and would thus least interfere with phosphite uptake.

Modified Ribeiro’s aqueous medium amended with either 0, 5, 10, 25, 50 or 100 µg/ml phosphite was dispensed into each 9 mm diameter Petri plate. A piece of the five-day-old inoculum growing in modified Ribeiro’s agar medium containing 0.1 mM orthophosphate was placed in each plate. Harvesting of mycelia was carried out as described in Section 2.2.5. There were three replicates of each phosphite concentration and each experiment was repeated once.

2.2.8 Sporangium production

An isolate of *P. cinnamomi* (MP9448) was used (Table 2.1). Sporangia were produced according to Coffey and Joseph (1985) with modifications. Three pieces of five-day-old mycelium plug grown on V8 agar (Appendix I) were placed in each 9 mm diameter Petri plate. About 20 ml of V8 broth (Appendix I) was dispensed into the plate until they were almost covered. The plates were incubated in the dark at 26 °C +/- 1 °C incubator for four days. V8 broth was decanted and the mycelium plugs were rinsed three times with sterile distilled water. For each rinse, the mycelium plugs were left standing in the water for 30 min to allow V8 broth to diffuse into the water. After the final rinse, 20 ml of 10% soil extract (Appendix 1) amended with either 0, 5, 10, 25, 50 or 100 µg/ml phosphite was dispensed into each plate. The plates were incubated under illumination at 25 °C for two days.
2.2.9 Microscopic examination

Mycelia grown in various concentrations of phosphite as described in Section 2.2.7 was examined microscopically and photographed after staining with 0.1% aniline blue in lactic acid.

Spores of *P. cinnamomi* prepared as described in Section 2.2.8 were examined unstained. Differential interference contrast (DIC) images taken using a compound microscope with Nomarski optics.

2.2.10 Data analysis

The results were expressed as percentage growth inhibition of mycelium. Results were calculated as \((y1 – y2)/y1 \times 100\) where \(y1 = \) mean dry weight of phosphite-free mycelium and \(y2 = \) mean dry weight of phosphite-treated mycelium.

Linear regression lines were obtained by computing the percentage inhibition of mycelial growth against the log concentration of phosphite used. The EC\(_{50}\) value for each isolate was calculated from these linear regression lines (Coffey and Bower, 1984; Darakis *et al.*, 1997).

For statistical analysis, each species was analysed separately by two-way analysis of variance (ANOVA), with isolates and phosphite concentration as the main effects and percent inhibition as the dependent variable. Prior to analysis, data were reflected and square root transformed to correct for heteroscedascity and a non-normal distribution.
2.3 Results

2.3.1 Growth response of Phytophthora species to phosphate

This preliminary experiment aimed to determine the minimum level of phosphate required by each Phytophthora species for optimal growth without exerting a profound detrimental influence on phosphite uptake in subsequent phosphite experiments.

All five Phytophthora species were responsive to phosphate and each species responded differently to phosphate concentration (Figure 2.2). Over the range of 0 to 0.5 mM phosphate, *P. cinnamomi* and *P. nicotianae* showed a positive response to phosphate concentration. This growth increase continued for *P. cinnamomi* up to 10 mM. However, no further increase in growth was observed at higher phosphate concentration for *P. nicotianae*. The remaining three species showed a positive response to phosphate over the range of 0 to 0.1 mM. *P. palmivora* and *P. capsici* showed no further response as the phosphate concentration was increased up to 10 mM. Increasing phosphate from 10 mM to 50 mM caused a decrease in mycelial growth (data not shown). The response of *P. citricola* was intriguing in that although it showed the same response as *P. palmivora* and *P. capsici* over the range of 0.1 mM to 1 mM, there was an exponential rise in growth between 1 mM and 5 mM and no further increase was observed at 10 mM.

As the addition of 0.1 mM phosphate is sufficient to cause a positive growth response in all five Phytophthora species, this level of phosphate was chosen as the appropriate level to be used in the growth media in subsequent phosphite experiments.
2.3.2 Within species variation in response to phosphite

The mycelial growth of all five isolates of *P. cinnamomi* were significantly (P<0.001) inhibited by phosphite from 5 µg/ml through to 100 µg/ml with the percentage inhibition increasing as phosphite levels increased (Figure 2.3). However, the extent of inhibition and the rates at which it increased with phosphite concentration were not equal across all isolates, causing significant interactions.
Figure 2.3  Percentage growth inhibition of five isolates of *P. cinnamomi* grown in modified Ribeiro’s aqueous medium amended with phosphite. Each point represents mean of three replicates. (□) MP62, (■) MP9418, (▲) MP9448, (Δ) MP9411, (●) MU33. 

\[ y = 16.91x + 32.98 \]
\[ y = 12.52x + 56.56 \]
\[ y = 25.46x + 38.39 \]
\[ y = 15.85x + 44.14 \]
\[ y = 29.85x + 29.71 \]

Figure 2.4  A representative dose-response curve of five *Phytophthora* species isolates to phosphite. Figure shown is the response of five isolates of *P. cinnamomi* to phosphite with their respective linear regression line. Each point represents mean of three replicates. (□) MP62, (■) MP9418, (▲) MP9448, (Δ) MP9411, (●) MU33. The correlation coefficient ($R^2$) ranged from 0.91 to 0.99.

Isolate MU33 was the most tolerant isolate with an EC$_{50}$ of 10.2 µg/ml. The remaining four isolates were sensitive with EC$_{50}$ of less than 5µg/ml (Figure 2.4) (Table 2.2).
A similar trend to *P. cinnamomi* was observed for five isolates of *P. nicotianae* (Figure 2.5) with significant (P<0.001) phosphite, isolate and the interaction between phosphite and isolate effects.

Apart from isolate MP317 which was the most tolerant *P. nicotianae* isolate with an EC$_{50}$ of 13.0 µg/ml, the rest of the isolates, MU7, MP5, 95014 and MP9 were all very sensitive with EC$_{50}$ values of less than 5 µg/ml (Table 2.2).

In *P. citricola*, a significant interaction on the percent inhibition (P<0.001) was observed between isolate and phosphite. The percent inhibition increased with increasing phosphite levels for all isolates, but the rate of increase was different. Isolate MP1 was moderately inhibited at low levels and inhibition increased very slowly at higher levels. Growth inhibition was especially rapid in isolate MP2, which was not heavily inhibited at lower
levels but substantially inhibited at higher levels. Isolates MP3, MP4 and MP41 were all strongly inhibited at low phosphite levels (Figure 2.6).

The sensitivity to phosphite of *P. citricola* isolates can be divided into two groups; isolates MP1 and MP2 were tolerant with EC$_{50}$ values of 27.3 µg/ml and 27.0 µg/ml respectively whilst the remaining three isolates, MP3, MP4 and MP41 were phosphite sensitive with EC$_{50}$ values of less than 5 µg/ml were the more susceptible ones (Table 2.2).

**Figure 2.6** Percentage growth inhibition of five isolates of *P. citricola* grown in modified Ribeiro’s aqueous medium amended with phosphite. Each point represents mean of three replicates. (□) MP1, (■) MP2, (▲) MP3, (∆) MP4, (●) MP41.

The response of *P. palmivora* isolates to phosphite was similar to *P. citricola*. There was a significant interaction (P<0.001) on the percent inhibition between isolate and phosphite. For all isolates, the percentage inhibition increased with increasing phosphite concentrations, but the rates of increase were different between isolates. Isolate MU128 which was the least inhibited at low phosphite concentrations but was inhibited rapidly at
higher levels. Isolate PM006 was moderately inhibited at lower concentrations and substantially inhibited at higher concentrations but the inhibition rate slowed down at the highest level. The remaining three isolates PM007, PM012 and 94111 were all heavily inhibited from the start but the rates of inhibition slowed at higher levels (Figure 2.7).

As in *P. citricola*, inhibition in *P. palmivora* could be assigned to two groups; the tolerant isolates were MU128 and PM006 with EC$_{50}$ of 24.4µg/ml and 16.6 µg/ml respectively whereas the sensitive isolates were PM012, PM007 and 94111 with EC$_{50}$ values of less than 5 µg/ml (Table 2.2).

![Figure 2.7 Percentage growth inhibition of five isolates of *P. palmivora* grown in modified Ribeiro’s aqueous medium amended with phosphite. Each point represents mean of three replicates. (■) PM006, (□) PM007, (▲) PM012, (△) MU128, (●) 94111.](image)

A comparison of the five isolates of *P. capsici*, all from the same host plant species revealed a significant interaction (P<0.001) on the percent inhibition between phosphite and isolate. All five isolates were inhibited by phosphite and the percentage inhibition
increased at higher phosphite concentrations for all isolates. Apart from isolate PP027, all the isolates were strongly inhibited at low phosphite concentrations and the inhibition continued with increasing concentrations. The trend of inhibition for isolate PP027 was different; it was least inhibited at lower concentrations but inhibition increased substantially at higher concentrations (Figure 2.8).

Among the five isolates, PP027 was the most tolerant with a high EC₅₀ value of 48.6 µg/ml whilst the remaining four were all very sensitive with EC₅₀ values of less than 5 µg/ml (Table 2.2).

**Figure 2.8** Percent growth inhibition of five isolates of *P. capsici* grown in modified Ribeiro’s aqueous media amended with phosphite. Each point represents mean of three replicates. (■) PP014, (□) PP019, (▲) PP021, (Δ) PP026, (●) PP027.
Table 2.2 The EC$_{50}$ value of each isolate of *Phytophthora* species.

<table>
<thead>
<tr>
<th><em>Phytophthora</em> species</th>
<th>Isolate no.</th>
<th>EC$_{50}$ value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cinnamomi</em></td>
<td>MP9448</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td><em>P. cinnamomi</em></td>
<td>MP62</td>
<td>&lt;5.0</td>
</tr>
<tr>
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2.3.3 Microscopic examination

All concentrations of phosphite added to Ribeiro’s aqueous medium distorted the hyphal and spore morphology of each *Phytophthora* species.

Hyphae were swollen, short-branched and stunted (Figure 2.9 C,D). Hyphal tips were swollen, distorted with abnormal growth protrusions (Figure 2.10 A,B,C). The cell contents of hyphae were disorganized with the cytoplasm shrinking from either the hyphal wall or hyphal tips (Figure 2.10 F,G). Lysis occurred at the tips or along the hyphae with the cytoplasm exuding from the hyphae (Figure 2.10 D,E). In addition, spherical intercalary hyphal swellings were found in *P. palmivora* (Figure 2.9 F). The formation of these hyphal swellings is an indication of abnormal growth or growth under subnormal conditions (Blackwell, 1949).

*P. palmivora* was the only species that produced sporangia readily *in vitro*; without stimulation. In this species, sporangia and chlamydospores produced in the medium amended with 10 µg/ml phosphite were distorted with the cytoplasm shrinking from the sporangial or chlamydospore wall (Figure 2.11 C,F). Lysis of sporangia occurred and resulted in vacuolation in the sporangium and the cytoplasm exuding from the papilla (Figure 2.11 D). A small number of sporangia were produced in *P. capsici* and were also adversely affected by the phosphite treatment (Figure 2.11 E).
In *P. cinnamomi*, the adverse effects of phosphite on the morphology of both sporangia and chlamydospores were similar to that observed in *P. palmivora* with distortion and lysis of spores (Figure 2.13 B,C,D,E). Based on visual assessment, the production of sporangia and chlamydospores were reduced in the presence of phosphite. Zoosporogenesis of sporangia were affected; the collapse of cytoplasm resulting in abortive sporangia or cleavage of cytoplasm in an undifferentiated state (Figure 2.12 B,F). For those few sporangia that produced and released zoospores, the encysted zoospores were swollen with a non-defined cyst wall (Figure 2.12 D). These encysted zoospores may have lost their infectivity or been non-viable. Knot-like swellings along the hyphae (Figure 2.13 F) was another characteristic observed at higher phosphite concentrations which concurred with observation by Dercks and Buchenauer (1987) in three *Phytophthora* species they have studied.
Figure 2.9 Light micrographs showing the effects of phosphite on the hyphal morphology of Phytophthora species grown in modified Ribeiro's aqueous medium. (A) Normally developed mycelium of P. palmivora grown in medium with no phosphite amendment. (B) Normally developed mycelium of P. nicotianae. (C) Short-branched, stunted and swollen hyphae of P. cinnamomi grown in media amended with 100 µg/ml phosphite. (D) Distorted and swollen hyphae of P. nicotianae exposed to 50 µg/ml phosphite. (E) Swollen hyphae of P. nicotianae exposed to 100 µg/ml phosphite. (F) Spherical, intercalary hyphal-swellings with radiating hyphae of P. palmivora grown in the presence of 50 µg/ml phosphite. Scale bar indicates 20 µm.
Figure 2.10 Light micrographs showing the effects of phosphite on the hyphal morphology of Phytophthora species grown in modified Ribeiro’s aqueous medium. (A, B, C) Swollen and distorted tip with abnormal growth protruding from the hyphal tip of P. nicotianae, P. cinnamomi, P. palmivora grown in media amended with 50 µg/ml, 100 µg/ml and 5 µg/ml phosphite respectively. (D) Lysis of hyphal wall along the hyphae of P. cinnamomi with exudation of cytoplasm (arrow) exposed to 100 µg/ml phosphite. (E) Lysis of hyphal tip with the cytoplasm exuding from the tip of P. palmivora (arrow) exposed to 100 µg/ml phosphite. (F) Cytoplasm of hyphae shrinking from the hyphal wall of P. palmivora (arrow) exposed to 10 µg/ml phosphite. (G) Cytoplasm shrinking from the hyphal tip of P. palmivora (arrow) in the presence of 100 µg/ml phosphite. Scale bar indicates 20 µm.
Figure 2.11 Light micrographs showing the effects of phosphite on the spore morphology of *Phytophthora* species grown in modified Ribeiro’s aqueous medium. (A) Normally developed sporangium of *P. palmivora* grown in medium with no phosphite amendment. (B) Normally developed chlamydospore of *P. palmivora*. (C) Distorted sporangia of *P. palmivora* (arrow) grown in medium amended with 10 µg/ml phosphite. (D) Lysis of sporangia of *P. palmivora* resulting in vacuolation and exudation of cytoplasm from the papilla (arrow) under the influence of 10 µg/ml phosphite. (E) Cytoplasm shrinking from the sporangial wall of *P. capsici* (arrow) exposed to 100 µg/ml phosphite. (F) Cytoplasm shrinking from the chlamydospore wall of *P. palmivora* (arrow) grown in the presence of 10 µg/ml phosphite. Scale bar indicates 20 µm.
Figure 2.12 Differential interference contrast (Nomarski optics) image of spore morphology of *P. cinnamomi* affected by phosphite. (A) Normally developed sporangium with zoospores before their release. (B) Abortive sporangium showing collapse of cytoplasm (arrow) grown in the presence of 50 µg/ml phosphite. (C) Normally developed zoospores after encystment. (D) Encysted swollen zoospores lacking a well-defined cyst wall (arrow) under the influence of 100 µg/ml phosphite. (E) Normally developed sporangium before zoosporogenesis. (F) Sporangium with expulsion of undifferentiated cytoplasm (arrow) under the influence of 100 µg/ml phosphite. Scale bar indicates 50 µm.
Figure 2.13 Differential interference contrast (Nomarski optics) image of spore morphology of P. cinnamomii affected by phosphite. (A) Normally developed sporangium unexposed to phosphite. (B) Distorted sporangia in the presence of 10 µg/ml phosphite. (C) Lysis of a sporangium resulting in exudation of cytoplasm from the papillum under the influence of 5 µg/ml phosphite. (D) Lysis of chlamdospore with exudation of cytoplasm under the influence of 10 µg/ml phosphite. (E) Abnormally developed sporangia on mycelium exposed to 50 µg/ml phosphite. (F) Effect of 25 µg/ml phosphite resulting in knot-like swellings along the hyphae. Scale bar indicates 50 µm.
2.4 Discussion

Phosphite significantly reduced in vitro growth of *P. cinnamomi*, *P. nicotianae*, *P. citricola*, *P. palmivora* and *P. capsici*. The sensitivity of the *Phytophthora* species to phosphite was evident as 5 µg/ml of phosphite in the medium was sufficient to cause significant inhibition in isolates from all five species. This adverse effect of phosphite was further reflected in the morphological studies which identified damage to mycelium, sporangia, zoosporces and chlamydospores. The interaction between isolate and phosphite was significant, indicating variations in the extent of the inhibition and the rate at which inhibition was increased across all isolates.

All five isolates from each of the five species were collected from different geographical locations. It seems, therefore that this could be one of the factors contributing to this variation. This observation concurred with the results of Bashan *et al.*, (1990) who used isolates of *P. infestans* from different geographical locations and showed variations in the sensitivity to Fosetyl-Al and phosphite by factors of up to 16.

The inverse relationship between phosphate and phosphite could be another contributing factor. The differential sensitivity displayed by the various isolates or species of *Phytophthora* towards phosphite was due to the variation in capacity to discriminate between phosphite and phosphate and to exclude phosphite in the presence of phosphate (Griffith *et al.*, 1993; Stehmann and Grant, 2000). Differences among isolates may also be attributed to differences in the uptake of phosphite as a more sensitive isolate was found to
accumulate more phosphite than resistant ones (Griffith et al., 1993). This is in contrast to Barchietto et al. (1989) who showed that differences in the rates of phosphite uptake was not a contributing factor in the differential response among isolates.

Host plants or sources from which these isolates were collected did not seem to affect the susceptibility of isolates to phosphite as evident from P. capsici isolates. Despite all five isolates of P. capsici being from black pepper (Piper nigum), they differed significantly in their response to phosphite. Since none of these P. capsici isolates had ever been exposed to phosphite before, this wide variation was possibly due to differences in inherent tolerance or attributed to mutation occurring among them.

The phosphate concentration of 0.1 mM used in this study was comparatively lower than concentrations used in previous studies. A higher phosphate level of 0.84 mM and 7.35 mM was used by Coffey and Bower (1984) and Wilkinson et al. (2001) respectively. On the other hand, a lower concentration of 0.084 mM was used by Coffey and Joseph (1985). However, in the present study comparison of EC$_{50}$ values with these studies was not attempted because of different concentration of phosphate used and radial growth rather than mycelial dry weight was used as a parameter for measurement of phosphite inhibition in these earlier studies. As phosphate affects the in vitro efficacy of phosphite (Griffith et al., 1989), for meaningful comparison of results, phosphate concentration in the growth media must be taken into consideration. In the present study, determination of inhibition was based on mycelial weight in liquid media instead of measurement of radial growth on solid media. The latter provides a more accurate assessment of growth as under suboptimal
conditions on agar, growth of mycelium is sparse and its density is reduced though the
diameter of the colony is not necessarily reduced.

The experiments in this chapter clearly demonstrated that phosphite acts directly on
*Phytophthora* species; by inhibiting mycelial growth and spore formation and causes
deformation and lysis of these structures. To determine the site of action or the metabolic
pathway of phosphite action in *Phytophthora* species, studies on its effects at the molecular
level was carried out in subsequent experiments.
Chapter 3

Differential display reverse transcription-PCR analysis of phosphite responsive gene in \textit{P. cinnamomi}

3.1 Introduction

Phosphite, a systemic pesticide used widely to control \textit{Phytophthora} species exhibits various biological effects on the pathogen. It induces numerous morphological, structural and metabolic changes in \textit{Phytophthora} species (Coffey and Joseph, 1985; Dercks and Buchenauer, 1987; Griffith \textit{et al.}, 1990; Niere \textit{et al.}, 1994; Stehmann and Grant, 2000).

Although phosphite has long been established as an effective control agent for \textit{Phytophthora} diseases, the mechanism for its activity is still not fully elucidated. The inhibitory effect of phosphite may involve the activities of enzymes related to phospholipid metabolism (Dercks and Buchenauer, 1987). This view was supported by observations that phosphite perturbs polyphosphate and pyrophosphate synthesis in \textit{Phytophthora} species (Niere \textit{et al.}, 1990). It was also shown that phosphite interferes with the synthesis of adenylate, reducing the level of polyphosphorylated nucleotides in \textit{P. palmivora} (Griffin \textit{et al.}, 1990). In \textit{P. citrophthora}, the levels of glucose-6-phosphate and 6-phosphogluconate dehydrogenase, two key enzymes of the pentose phosphate pathway were increased in the presence of phosphite (Barchietto \textit{et al.}, 1992). The levels of several enzymes involved in the glycolytic and phosphogluconate pathways in \textit{P. palmivora} were found to be affected
by phosphite (Stehmann and Grant, 2000). According to Varadarajan et al. (2002), the toxicity of phosphite against Phytophthora species may be due to differing sensitivities of phosphorylating enzymes, and/or metabolic pathways regulated by phosphate.

To gain further insight into the relationship between phosphite and the pathogen, gene expression in P. cinnamomi induced by phosphite stress was examined through the differential display (DDRT-PCR) technique. This technique employs the sequential use of two different primers, an anchored oligo-dT primer and an arbitrary primer. The anchored oligo-dT is first used to anneal to the poly(A) tail-3’ end of the mRNA permitting initiation of reverse transcription of this subpopulation of mRNA in the first strand cDNA synthesis. The generated cDNAs are amplified using an arbitrary primer of defined sequence along with the anchored oligo-dT primer by PCR. The amplified radio-labeled products are resolved on polyacrylamide gel (Liang and Pardee, 1992) (Figure 3.1).

This chapter describes the use of a modified differential display method to isolate and identify gene(s) in P. cinnamomi whose expression is affected by phosphite.
Figure 3.1 Schematic diagram of the differential display reverse transcription-PCR. Arrowhead indicates a putative differentially displayed band. After differential display, cloning followed by sequencing is carried out to characterize the gene. Adapted from Colonna-Romano et al. (1997) with modifications.
3.2 Materials and Methods

3.2.1 Growth curve of *P. cinnamomi* in response to phosphite

A preliminary experiment was carried out to determine the optimum harvest time of mycelium during the growth cycle of *P. cinnamomi* for the extraction of its total RNA.

*P. cinnamomi* isolate MP9448 was used. The isolate was grown in modified Ribeiro’s medium containing 0.1 mM phosphate (Appendix I) and either 0 or 5 µg/ml phosphite. The concentration of phosphite used was determined from Chapter 2, section 2.2.7. At daily intervals, for a period of seven days, mycelium from three replicate plates in each treatment were harvested, dried and weighed as described in Chapter 2, section 2.2.5.

To obtain mycelium for RNA extraction, isolate was grown, the mycelium was harvested, rinsed twice with sterilized distilled water, blotted with filter paper and snap-frozen in liquid nitrogen before storing at -77 °C.

3.2.2 Extraction of total RNA

Total RNA of phosphite-treated and untreated mycelium was extracted according to Logemann *et al.* (1987) with some modifications. One gram of mycelium was frozen in liquid nitrogen and ground to powder with mortar and pestle. The powder was homogenized in 8 ml of guanidine buffer (8M guanidine hydrochloride, 20 mM MES (4-morpholineethansulfonic acid), 20 mM EDTA, pH 7). Immediately prior to applying
guanidine buffer, 3.4 µl mercaptoethanol per ml buffer was added. The guanidine hydrochloride extract was centrifuged in a pre-cooled 4 °C centrifuge (RC-5B Refrigerated Superspeed Centrifuge, Sorvall) for 10 min at 11,950 x g. The supernatant was poured into a clean tube and 0.5 volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Fluka BioChemika) was added. After the extraction, the mixture was centrifuged for 45 min at 11,950 x g at 4 °C. The supernatant was then transferred to a clean tube and 0.2 volume of 1M sodium acetate and 0.7 volume of pre-cooled -20 °C ethanol was added, mixed and left to precipitate at -20 °C overnight.

The precipitate was collected by centrifuging at 11,950 x g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 0.75 volume of sterilized 3M sodium acetate. The pellet was collected by centrifuging at 11,950 x g for 5 min at 4 °C. The wash was repeated with 3M sodium acetate and pellet dissolved in DEPC-treated water. The RNA was stored at -77 °C for later use.

3.2.3 Quantification of total RNA

Total RNA was quantified using a spectrophotometer (QuantaGene, The Australian Chromatography Company). The sample was diluted 1:10 in Tris-Cl (10 mM Tris-Cl, pH 7) and the diluted sample used to determine the absorbance at 260 nm. The concentration of RNA was calculated based on an absorbance of 1 unit at 260 nm corresponding to 40 µg of RNA per ml.
The absorbance at 280 nm was also measured to determine the purity of RNA which was based on the absorbance ratio of 260 nm / 280 nm.

3.2.4 Determination of RNA integrity

The integrity of RNA from both samples was checked by electrophoresis on a formaldehyde (FA) agarose gel according to Sambrook et al. (1989). The 1.2% FA agarose gel was prepared by dissolving agarose in 3.5 parts DEPC water. After cooling, 1.1 parts 5x FA gel-running buffer (0.1 M MOPS (3 - N - morpholino propane - sulfonic acid), 40 mM sodium acetate, 5 mM EDTA) and 1 part FA was added to the agarose and the gel was left to set in a fumehood. The gel was placed in 1x FA gel-running buffer in the electrophoresis tank for about 30 min to equilibrate. The RNA samples were prepared by mixing 2.7 μg total RNA in 2 μl FA gel-running buffer, 3.5 μl FA and 10 μl formamide and heated at 65°C for 15 min and immediately chilled on ice. 1μl ethidium bromide (10 mg/ml) and 2 μl FA gel-loading buffer were then added. The gel was pre-run for 5 min before samples were loaded and electrophoresis at 6.8 V/cm for two hr. The gel was viewed under a UV transilluminator (TFX-35M, Gibco BRL) and photographed (DC 120 Camera, Kodak Digital Science).

3.2.5 DNase I treatment of total RNA

The extracted RNA was treated with deoxribonuclease I (DNase I, Amplification grade, Invitrogen) according to the manufacturer’s instructions. DNase I (1 U/μl) was added to the RNA sample (1 μg) and incubated for 15 min at room temperature. 1 μl of a 25 mM EDTA
solution was added to the reaction mixture and heated for 10 min at 65 °C to inactivate the reaction.

3.2.6 cDNA synthesis

The first-strand synthesis was carried out using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacture’s instructions. Equal amounts of RNAs from phosphite-treated and untreated mycelium were reverse transcribed in four subsets with the respective two-base anchor primers, ET12VA, ET12VC and ET12VG and ET12VT (E is an EcoR1 site, 5’-GAATTC-3’ and V is a degenerate base). To synthesize first-strand cDNA, a 20 µl reaction consisted of 0.5 µg RNA, 5 µM of each respective anchor primer, 0.5 mM dNTPs, 5 mM MgCl2, 10 mM DTT, 40 units RNase inhibitor and 200 units of SuperScript III reverse transcriptase. The reaction was first carried out by incubating the RNA, anchor primer and dNTPs at 65 °C for 5 min before chilling on ice for at least 1 min. The remaining components were then added and the mixture incubated at 50 °C for 60 min before inactivating the reaction by heating at 70 °C for 15 min. The sample was digested with 2 units of ribonuclease H at 37 °C for 20 min. Both phosphite-treated and untreated samples of cDNA synthesized were kept at -20 °C until used.

3.2.7 DDRT-PCR

The differential display analysis was conducted using anchor primers and deoxyoligonucleotide primers of length 18-24 mers with arbitrary sequences. 19 arbitrary
primers were screened with each of the four subsets of cDNA synthesized. The sequences of the four anchor primers and 19 arbitrary primers used are as listed in Table 3.1.

PCR amplification of the synthesized first-strand cDNA was carried out in duplicates in a 20 µl reaction. The composition of the reaction mixture was; 2 µl (1:20 dilution) of cDNA, 1x PCR buffer (670 mM Tris-HCl (pH 8.8), 166 mM (NH₄)₂SO₄, 4.5 % Triton X-100, 2 mg/ml gelatin), 1.5 mM MgCl₂, 0.2 mM dNTPs (Fisher Biotech), 2.5 µM of the respective anchor primer, 0.5 µM of the respective arbitrary primer, 1 unit (U) Taq (Thermus aquaticus) DNA polymerase (Fisher Biotech) and PCR-grade water (Fisher Biotech) to 20 µl volume. The concentrations of both MgCl₂ and dNTPs used were determined from the optimization experiments.

Amplifications were carried out in duplicates for each treatment along with a negative control in which PCR-grade water was used as a template. PCR was performed on a thermal cycler (Corbett Research) with two steps of thermal cycling profile as follows: 2 cycles of 94 ⁰C for 3 min, 42 ⁰C for 4 min, 72 ⁰C for 3 min, followed by 35 cycles of 94 ⁰C for 30 sec, 60 ⁰C for 30 sec, 72 ⁰C for 2 min and a final extension cycle of 72 ⁰C for 5 min.

Amplification products were analysed by gel electrophoresis as described in Section 3.2.9. The amplified cDNA bands from both the phosphite treated and untreated samples were compared and PCR products corresponding to differentially displayed bands were excised from the gel with a sharp scalpel. The excised gels were stored at -20 ⁰C before the extraction of cDNA from the gel pieces.
3.2.8 Gel electrophoresis of DDRT-PCR products

Amplification products were analysed by electrophoresis in 2% high resolution agarose gel (Progene). 7 µl of the product that had been mixed with 1.5 µl of 6x loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was loaded. 100 bp DNA ladder (Gene Ruler, Fermentas) was used as the marker. The gel was electrophoresed at 5 V/cm for 3.5 hr in 1x TBE (45 mM Tris-borate, 1 mM EDTA pH 8). The gel was stained in ethidium bromide (0.5 µg/ml) (Sigma) for 20 min. The gel was viewed under a UV transilluminator (TFX-35M, Gibco BRL) and photographed (DC 120 Camera, Kodak Digital Science).
Table 3.1 List of the anchored and arbitrary primers used for DDRT-PCR.

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3.2.9 Gel extraction and purification

The amplified cDNA bands from the excised gels were extracted and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The extraction and purification was carried out according to the manufacturer’s instructions. Briefly, one volume of Membrane Binding Solution was added to the gel slice and the mixture was vortexed and incubated at 60 °C for 10 min. The mixture was transferred to a SV Minicolumn assembly and incubated for 1 min at room temperature before being centrifuged for 1 min at 16,000 x g. The flow-through was discarded. 700 µl of Membrane Wash Solution was added and the Minicolumn was centrifuged for 1 min at 16,000 x g. The flow-through was discarded. The wash was repeated with 500 µl of the same wash solution but centrifuged at 16,000 x g for 5 min. The flow-through was discarded and the Minicolumn was centrifuged for another 1 min at the same relative centrifugal force (rcf). The Minicolumn was transferred to a 1.5 ml microcentrifuge tube. 50 µl of Nuclease-free water was added to the membrane and incubated at room temperature for 1 min before centrifuged at 16,000 x g for 1 min to elute the DNA. The purified cDNA was stored at -20 °C until re-amplification.

3.2.10 Re-amplification of cDNA extracted from gel

The purified cDNA from each gel fragment was re-amplified using the same anchor and arbitrary primers which were used in the DDRT-PCR to generate the band. The re-amplification was conducted in a 20 µl PCR reaction. 2 µl of the purified cDNA was used as template and the other components of the reaction remain the same as in DDRT-PCR. The thermal cycling profile was denaturation at 94 °C for 3 min followed by 35 cycles of
high stringency step with an annealing temperature of 60 °C and a final extension at 72 °C for 5 min. Five reactions were carried out for each cDNA extract to generate 100 µl of PCR product. After re-amplification, the five reactions were pooled.

### 3.2.11 Gel electrophoresis of re-amplified products

The re-amplified products were analysed by electrophoresis in 1.5% agarose gel (Bio-Rad Laboratories) at 9 V/cm for 1 hr in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). 10 µl of the product and 2 µl of 6x loading dye was mixed and loaded. The gel was stained in ethidium bromide (0.5 µg/ml) for 15 min. The gel was viewed and photographed as described in Section 3.2.9.

### 3.2.12 Purification of PCR products

After re-amplification, PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purification was carried out following the manufacturer’s instructions. An equal volume of Membrane Binding Solution was added to the PCR reaction. The prepared PCR product was transferred to the SV Minicolumn assembly and incubated for 1 min at room temperature. The remaining part of the protocol was the same as described in Section 3.2.10. The concentration of the purified PCR product was quantified using a fluorometer as described in Section 3.2.14. The cDNA was stored at –20 °C until cloning and transformation.
3.2.13 DNA quantification

DNA concentration was determined with a fluorometer (Hoefer DyNA Quant 200, Pharmacia Biotech). DNA sample was added to the Hoechst 33258 fluorescent dye (0.1 µg/ml) in 1x TNE (100 mM Tris, 10 mM EDTA, 2M NaCl) which binds to double-stranded DNA and emits fluorescence that is proportional to the DNA concentration. The fluorescence emitted at 460 nm was compared to the calf thymus DNA standard (100 µg/ml) (Sigma).

3.2.14 Cloning and transformation

TOPO TA Cloning Kit for Sequencing (Invitrogen) was used for the cloning and transformation of amplified cDNA fragments generated from DDRT-PCR.

3.2.14.1 Ligation of PCR products into the plasmid vector

The cloning system consists of a plasmid vector, pCR4-TOPO. The ligation was set up in a final volume of 6 µl consisting of an insert : plasmid ratio of 6:1, 1 µl of salt solution (1.2 M NaCl, 0.06 M MgCl₂), 1 µl of sterilized water and 1 µl of the TOPO vector (10 ng/µl) according to the manufacturer’s recommendations.

The amount of insert used was calculated based on the following equation:

\[
\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{insert}}{\text{molar ratio of vector}} = \text{ng of insert}
\]
The reaction was mixed and incubated at room temperature for 20 min before placing it on ice.

3.2.14.2 Transformation of chemically competent *E. coli*

The pCR4-TOPO construct was transformed into the *Escherichia coli* using the One Shot Chemically Competent *E. coli* strain TOP10. The reaction was performed according to the manufacturer’s instructions. 2 µl of the ligation mixture product was added to a vial of One Shot Chemically Competent *E. coli* and the reaction was incubated on ice for 20 min. The cells were heat shocked for 30 sec at 42 °C and the tubes immediately transferred to ice. 250 µl of SOC medium (2% trytone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added and the tube shaken (225 rpm) at 37 °C for an hr. Two different volumes, 50 µl and 75 µl from each transformation was spread onto pre-warmed Lauria-Bertani (LB) plates containing kanamycin (50 µg/ml) (Sigma) and spread with X-gal (1 mg) (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside) (Progene). The plates were incubated at 37 °C for 16 to 24 hr.

3.2.15 Analysis of positive transformants by PCR

PCR was used to identify the correct recombinant clone with the insert of expected size. Ten white colonies from each transformation were selected for analysis. Each single colony was randomly picked with a pipette tip and resuspended in 15 µl sterile distilled water. 4 µl of the bacterial suspension was used as template for the PCR. The primers used were T3 (5’ ATTAACCCTCACTAAAGGGA 3’) and T7 (5’ TAATACGACTCACTA...
TAGGG 3’). PCR was carried out in a 20 µl reaction consisting of 1x PCR buffer, 4 mM MgCl₂, 0.2 mM dNTPs, 10 pmol of each of the primer and 1 U Taq polymerase. The thermal cycling profile was initial denaturation at 94 °C for 3 min followed by 25 cycles of denaturing at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The amplified product was analysed by agarose gel electrophoresis as described in Section 3.2.12. The clone with the right sized insert was then selected for sequencing.

3.2.16 Isolation of plasmid DNA

After verification of the insertion of PCR product of interest into the plasmid by PCR, the colony was grown in 10 ml LB broth supplemented with kanamycin (50 µg/ml) for 16 hr at 37 °C with agitation at 225 rpm. Cells were harvested by centrifuged at 6,000 x g for 15 min and the supernatant was decanted.

DNA was extracted from the cell using Wizard Plus SV Minipreps DNA Purification System (Promega). The purification was carried out following the manufacturer’s instructions. The cell pellet was resuspended in 250µl of Cell Suspension Solution by vortexing. 250 µl of Cell Lysis Solution was added and incubated for 5 min until the cell suspension cleared. 10 µl of Alkaline Protease Solution was added and the mixture was incubated for 5 min at room temperature. 350 µl of Neutralization Solution was added and the bacterial lysate was centrifuged at 16,000 x g for 10 min. The cleared lysate was transferred to the spin column and the supernatant was centrifuged at 16,000 x g for 1 min. The flow-through was discarded and 750 µl of Column Wash Solution was added to the
spin column and centrifuged at the same speed for 1 min. The flow-through was discarded and the wash was repeated using 250 µl of Column Wash Solution and centrifuged at the same speed for another 2 min. 100 µl of Nuclease-Free Water was added to the spin column to elute the plasmid DNA. The concentration of purified plasmid DNA was quantified using fluorometer as described in Section 3.2.14.

### 3.2.17 Sequencing

Purified plasmid DNAs (Section 3.2.17) were sequenced using ABI PRISM Big Dye Terminator kit (Applied Biosystem) and an ABI 3730 automated DNA sequencer (Applied Biosystems).

Both strands of each clone were sequenced. The reaction consisted of 4 µl of the dye terminator mix (Big Dye version 3.1, Applied Biosystems), 3.2 pmoles of either T3 or T7 sequencing primer, 300 ng of plasmid DNA and sterile distilled water to make up to 10 µl final volume. Sequencing reactions were performed using a thermal cycler (Corbett Research) with the following conditions; initial denaturation at 96 °C for 2 min followed by 25 cycles of 96 °C for 10 sec, 55 °C for 5 sec and extension at 60 °C for 4 min. After the reaction, the re-amplified product was purified by ethanol precipitation. This was carried out by transferring the 10 µl sequencing reaction to a 0.5 ml tube containing 2.5 volume of 100% ethanol, 1/10 volume of 3M sodium acetate (pH 5.2) and 1/10 volume of 125 mM EDTA. The mixture was left for 20 min at room temperature before being centrifuged at 16,000 x g for 30 min. The supernatant was discarded and 12.5 volume of 70% ethanol was added to rinse the pellet and the tube was centrifuged for 5 min at room temperature.
The supernatant was discarded. The pellet was dried in a vacuum concentrator (SpeedVac, Savant) for 15 min before storing at -20°C.

Sequencing was conducted at the State Agricultural Biotechnology Centre, Murdoch University.

3.2.18 Sequence analysis and Blast search

Sequence data were analysed using the software programme, Sequence Editor V 1.03 (Applied Biosystems). Sequences were edited manually to remove vector sequences and to amend ambiguous bases by comparison with the chromatograms from the programme.

BLAST (Basic Local Alignment Search Tool) programme was used to search the GenBank database of the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) for possible sequence homology. Each nucleotide sequence was subjected to BLASTX analysis which translates the query nucleotide sequence in six reading frames and compares the translational products to protein database.

3.3 Results

3.3.1 Growth curve of *P. cinnamomi* in response to phosphite

The growth curve of *P. cinnamomi* can be divided into two distinct phases; the growth phase where the growth increases exponentially and the stationary phase when there is no further net increase in biomass (Figure 3.2). The growth of *P. cinnamomi* was adversely
affected by phosphite treatment. Inhibition was evident at 24 hr. The growth of the phosphite-treated sample slowed down after the third day and entered the stationary phase by the fourth day. After the sixth day, it entered the phase of decline. In contrast, the growth of the untreated sample was exponential until the fifth day when it entered the stationary phase and the mycelium weight remained constant. Based on the results of this growth curve, three-day-old mycelium was harvested for RNA extraction.

![Figure 3.2](image)

**Figure 3.2** Time course of mycelial growth of *P. cinnamomi* in modified Ribeiro’s aqueous medium. (□) no phosphite, (■) with 5 µg/ml phosphite. Each point represents mean of three replicates. Vertical bars represent standard deviation.

### 3.3.2 RNA extraction

The yields of total RNA obtained were 689 µg and 594 µg per g for phosphite-untreated and treated mycelium respectively.

The two RNA samples extracted were pure as evident from the absorbance ratio of 260/280
nm values of 2.06 and 2.07 for total RNA extracted from phosphite-untreated and treated samples respectively.

The denaturing formaldehyde agarose gel electrophoresis showed two distinct ribosomal bands in both RNA samples (Figure 3.3).

![Figure 3.3](image)

**Figure 3.3** Formaldehyde agarose gel electrophoresis of total RNA extracted from *P. cinnamomi* grown in modified Ribeiro’s aqueous medium. Lanes: (1) no phosphite; (2) with 5 µg/ml phosphite. Arrowheads indicate the two ribosomal bands.

### 3.3.3 Optimization of DDRT-PCR

To determine the concentrations of magnesium chloride and nucleotides to be used in DDRT-PCR, optimization of these two components were carried out. cDNA from untreated mycelium was used as template and amplification was carried out using the ET12VA anchor primer and Arp 2 arbitrary primer. The optimization series include MgCl₂ at 0.5 mM increments from 1.0 mM to 3.0 mM and dNTPs at 0.1 mM increment from
0.1 mM to 0.5 mM. The rest of the PCR components and reaction profiles were as described in Section 3.2.8.

Except for the MgCl₂ concentration of 1.0 mM, 1.5 mM to 3.0 mM vary slightly in band patterns but 1.5 mM was found to produce bands of relatively higher intensity at 0.2 mM dNTPs (Figure 3.4).

![Figure 3.4](image.png)

**Figure 3.4** Optimization of magnesium chloride concentration. Lanes: (M) 100 bp molecular ladder; (1) 1.0 mM; (2) 1.5 mM; (3) 2.0 mM; (4) 2.5 mM; (5) 3.0 mM.

At MgCl₂ concentration of 1.5 mM, dNTPs concentrations of 0.1 to 0.3 mM produced a comparable number of bands but 0.2 mM showed the clearest pattern while 0.4 mM resulted in less bands and no product was seen in the reaction containing 0.5 mM (Figure 3.5). The optimal concentrations of MgCl₂ and dNTPs to be used were determined to be 1.5 mM and 0.2 mM respectively.
3.3.4 DDRT-PCR

The differential display analysis was performed with 76 primer pair combinations. The selection of candidate band was based on a comparison of duplicate band patterns generated from the two different RNA samples, obtained from phosphite-treated and untreated \textit{P. cinnamomi} mycelium (Figure 3.6). Differential bands were excised from the gel. The selection also requires that the excised bands have to appear in both the duplicated lanes. This procedure resulted in the identification of 35 putative differentially displayed bands between the two different RNA samples.
Figure 3.6 A representative differential display gel pattern of cDNA from *P. cinnamomi* mycelium grown in modified Ribeiro’s aqueous medium. The fingerprint was derived from amplification of ET12VA anchor primer and Arp 12 arbitrary primer. Lanes: (1, 2) no phosphite; (3, 4) with 5 µg/ml phosphite; (5) negative control. Arrowhead indicates a putative differentially expressed cDNA band with an approximate size of 1200 bp.

### 3.3.5 Re-amplification

Re-amplification was carried out to obtain sufficient DNA from differential display bands for downstream analysis. cDNA from each gel fragment was extracted and re-amplified. All the cDNA fragments except one were re-amplified. About half of the candidate bands generated single target PCR product corresponded to the size observed on the original display. However, the remaining candidate bands generated more than one PCR product but in this case; the target PCR product appeared more intense than the non-target product on
the gel (Figure 3.7). Cloning was therefore carried out to identify the differentially expressed cDNA.

![Image: Figure 3.7 Re-amplification of cDNA fragments. Lanes: (M) 100 bp molecular ladder; (3, 5, 9, 10, 11, 12, 13, 14, 15) single PCR product was recovered; (1, 2, 4, 6, 7, 8) multiple PCR products were recovered. The estimated size of cDNA bands ranged from 220 bp to 1600 bp.]

### 3.3.6 Cloning and transformation

Colonies harbouring the recombinant plasmid have a disrupted lacZ gene and appeared white while colonies with non-recombinant vectors were blue. White colonies were selected directly from the LB plate and screened by PCR for the presence of insert using T3 and T7 primers flanking the cloning site. Both the T3 and T7 primers anneal to sites in the pCR 4-TOPO vector 52 bp upstream or downstream respectively from the cloning site. Therefore, colonies that contained plasmids with inserts gave a band of 104 bp plus the size of the insert. Based on the PCR results, clones with the correct insert size were selected (Figure 3.8).
All the 34 cloning and transformation reactions yielded recombinant clones. However, the success of obtaining clones with the right insert size varied from 10 to 100%. Positive clones were purified and plasmid DNA isolated for sequencing.

![Image](image.png)

**Figure 3.8** PCR screening of recombinant clones. Lanes: (M) 100 bp molecular ladder; (1, 2, 3, 4, 9, 10) clones with the right insert size of 750 bp; (11) negative control.

### 3.3.7 Sequence analysis of cDNA fragments

Clones from all the 34 cDNA fragments were sequenced. Out of these, 28 produced readable sequences. Sequence length varied from 238 bp to 1564 bp. The size of these cDNA fragments were relatively close to the estimated size from the original display from which these fragments were obtained. Two of the cDNAs were found to be redundant. All the cDNA fragments were each amplified with their respective arbitrary primer on both the 5’ and 3’ ends as determined by DNA sequencing. In all cases, a match was found between the arbitrary primer sequence and the nucleotide sequence generated except for one, CP34 where there was one nucleotide missing. Nucleotide sequences of these cloned cDNA fragments are presented in Appendix II.
BLASTX searches of the remaining 26 nucleotide sequences against NCBI databases identified homologies to 18 known proteins, six hypothetical proteins and one each of unknown or unnamed proteins of various species. The sequence identities ranged from 23% to 100% over 58 to 306 amino acid residues. Sequence analysis of these amplified cDNA fragments indicated that they encoded gene products of various functions. The sequence similarities and details of each cDNA clones are shown in Table 3.2.
### Table 3.2 BLASTX results of *P. cinnamomi* cDNA sequences identified

<table>
<thead>
<tr>
<th>Clone</th>
<th>Origin of band</th>
<th>Length (nt)$^a$</th>
<th>Sequence homology (source species)$^b$</th>
<th>Accession no.$^c$</th>
<th>Sequence identity$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1</td>
<td>Arp 1 ET$_{12}$VA</td>
<td>1413</td>
<td>Conserved hypothetical protein (<em>Coccidiodes immitis</em>)</td>
<td>EAS30199</td>
<td>59% (112 aa)</td>
</tr>
<tr>
<td>CP2</td>
<td>Arp 3 ET$_{12}$ VA</td>
<td>1381</td>
<td>Glycerate kinase II (<em>Escherichia coli</em>)</td>
<td>NP415047</td>
<td>100% (141 aa)</td>
</tr>
<tr>
<td>CP4</td>
<td>Arp 8 ET$_{12}$ VA</td>
<td>746</td>
<td>ABC transporter, ATP-binding protein (<em>Brucella abortus</em>)</td>
<td>EAS30199</td>
<td>87% (240 aa)</td>
</tr>
<tr>
<td>CP5</td>
<td>Arp 8 ET$_{12}$ VA</td>
<td>806</td>
<td>Hypothetical protein mil7828 (<em>Mesorhizobium loti</em>)</td>
<td>NP108064</td>
<td>39% (146 aa)</td>
</tr>
<tr>
<td>CP6</td>
<td>Arp 12 ET$_{12}$ VA</td>
<td>1564</td>
<td>Proteophosphoglycan ppq4 (<em>Leishmania major</em>)</td>
<td>AAZ14280</td>
<td>23% (210 aa)</td>
</tr>
<tr>
<td>CP7</td>
<td>Arp 18 ET$_{12}$ VA</td>
<td>290</td>
<td>Cation transporting ATPase (<em>Thermoplasma voltae</em>)</td>
<td>BAB604061</td>
<td>32% (103 aa)</td>
</tr>
<tr>
<td>CP8</td>
<td>Arp 18 ET$_{12}$ VA</td>
<td>715</td>
<td>Carbohydrate transporter/sugar porter (<em>Arabidopsis thaliana</em>)</td>
<td>NP174313</td>
<td>28% (177 aa)</td>
</tr>
<tr>
<td>CP9</td>
<td>Arp 19 ET$_{12}$ VA</td>
<td>458</td>
<td>Twin-arginine translocation pathway signal (<em>Solibacter usitatus</em>)</td>
<td>ZP00520570</td>
<td>37% (143 aa)</td>
</tr>
<tr>
<td>CP11</td>
<td>Arp 19 ET$_{12}$ VA</td>
<td>607</td>
<td>CyID, ABC transporter ATP-binding protein (<em>Brucella abortus</em>)</td>
<td>YP223481</td>
<td>84% (197 aa)</td>
</tr>
<tr>
<td>CP12</td>
<td>Arp 3 ET$_{12}$ VC</td>
<td>341</td>
<td>Methyl-accepting chemotaxis protein (<em>Xanthomonas campestris vesicatoria</em>)</td>
<td>YP363433</td>
<td>53% (107 aa)</td>
</tr>
<tr>
<td>CP13</td>
<td>Arp 3 ET$_{12}$ VC</td>
<td>333</td>
<td>Hypothetical protein (<em>Chaetomium globosum</em>)</td>
<td>EAQ83457</td>
<td>33% (83 aa)</td>
</tr>
<tr>
<td>CP15</td>
<td>Arp 9 ET$_{12}$ VC</td>
<td>1255</td>
<td>Cys/Met metabolism pyridoxal-phosphate-dependent enzymes (<em>Acidivorox sp.</em>)</td>
<td>ZP01381365</td>
<td>74% (215 aa)</td>
</tr>
<tr>
<td>CP16</td>
<td>Arp 9 ET$_{12}$ VC</td>
<td>1272</td>
<td>Phytoene synthase (<em>Brucella melitensis</em>)</td>
<td>NP539991</td>
<td>77% (161 aa)</td>
</tr>
<tr>
<td>CP19</td>
<td>Arp 3 ET$_{12}$ VG</td>
<td>238</td>
<td>Hypothetical protein mlr1095 (<em>Mesorhizobium loti</em>)</td>
<td>NP102760</td>
<td>72% (58 aa)</td>
</tr>
<tr>
<td>CP21</td>
<td>Arp 3 ET$_{12}$ VG</td>
<td>557</td>
<td>TonB-dependent receptor (<em>Barkholderia sp. 383</em>)</td>
<td>YP367005</td>
<td>29% (112 aa)</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Accession</td>
<td>Description</td>
<td>Length</td>
<td>Identity (aa)</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-------------</td>
<td>--------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>CP22</td>
<td>Arp 6 ET12VG</td>
<td>Protein kinase domain containing protein (Oryza sativa)</td>
<td>1316</td>
<td>34% (146 aa)</td>
<td></td>
</tr>
<tr>
<td>CP24</td>
<td>Arp 8 ET12VG</td>
<td>Short-chain dehydrogenase/reductase SDR (Mesorhizobium sp. BNC1)</td>
<td>258</td>
<td>60% (75 aa)</td>
<td></td>
</tr>
<tr>
<td>CP25</td>
<td>Arp 8 ET12VG</td>
<td>Hypothetical protein (Dictyostelium discoideum)</td>
<td>665</td>
<td>29% (129 aa)</td>
<td></td>
</tr>
<tr>
<td>CP26</td>
<td>Arp 11 ET12VG</td>
<td>Hypothetical protein (Rhizobium etli)</td>
<td>1213</td>
<td>44% (238 aa)</td>
<td></td>
</tr>
<tr>
<td>CP28</td>
<td>Arp 18 ET12VG</td>
<td>Spermidine/putrescine ABC transporter, permease protein, putative (Brucella suis)</td>
<td>311</td>
<td>91% (99 aa)</td>
<td></td>
</tr>
<tr>
<td>CP29</td>
<td>Arp 18 ET12VG</td>
<td>Unknown protein (Arabidopsis thaliana)</td>
<td>701</td>
<td>28% (235 aa)</td>
<td></td>
</tr>
<tr>
<td>CP30</td>
<td>Arp 18 ET12VG</td>
<td>PH domain containing protein (Tetrahymena thermophila)</td>
<td>750</td>
<td>37% (69 aa)</td>
<td></td>
</tr>
<tr>
<td>CP31</td>
<td>Arp 18 ET12VG</td>
<td>Type I polyketide synthase AVES 2 (Streptomyces avermilitis)</td>
<td>902</td>
<td>26% (126 aa)</td>
<td></td>
</tr>
<tr>
<td>CP32</td>
<td>Arp 3 ET12VT</td>
<td>UDP-N-acetylgalactosamine pyrophosphorylase (Xanthomonas axonopodis pv. citri)</td>
<td>938</td>
<td>74% (306 aa)</td>
<td></td>
</tr>
<tr>
<td>CP34</td>
<td>Arp 15 ET12VT</td>
<td>Thiamine ABC transporter, permease protein (Brucella suis)</td>
<td>1401</td>
<td>82% (140 aa)</td>
<td></td>
</tr>
<tr>
<td>CP35</td>
<td>Arp 18 ET12VT</td>
<td>Unnamed protein product (Tetraodon nigroviridis)</td>
<td>853</td>
<td>26% (220 aa)</td>
<td></td>
</tr>
</tbody>
</table>

* Length of cDNA clones include primer sequence.
* Using the BLASTX programme against NCBI database.
* GenBank accession number or relevant reference as stated in the BLASTX results.
* Percent identity at the amino acid level, length of match is shown in parentheses.
3.4 Discussion

In these experiments, the DDRT-PCR technique was modified to omit the use of radio-isotope labeling and polyacrylamide gel was replaced by high resolution agarose gel. The use of this modified method resulted in the identification of 34 putative differential display bands between phosphite-treated and untreated mycelium of *P. cinnamomi*.

Re-amplification of some of the cDNA bands extracted from the gels resulted in the production of multiple bands. This has been encountered by other researchers and is ascribed to the presence of multiple species of DNA within a single band on the gel (Li *et al.*, 1994; Goormachtig *et al*. 1995; Vogeli-Lange *et al*., 1996; Smith *et al*., 1997; Jurecic *et al*., 1998; Seehaus and Tenhaken, 1998). Another contributing factor is the possibility of band contamination during recovery. Despite great care taken during excision of band of interest, neighbouring bands might have been isolated as well resulting in generation of multiple bands when re-amplified. Contamination of other co-migrating cDNA fragments (Zhang *et al*., 1996) and excision of multiple fragments from the gel (Mou *et al*., 1994) are contributing factors in obtaining multiple bands in the re-amplified products.

The nucleotide sequences generated showed that the cDNA fragments contrary to expectation were amplified with the arbitrary primer on both sides. The expectation is that they would be amplified with the arbitrary primer and the anchored primer on either side. cDNA fragments generated by arbitrary primers alone were also observed by other researchers (Liang *et al*., 1995; Benito *et al*., 1996; Mou *et al*., 1994; Gross and Watson,
amplification with the arbitrary primer is more likely than with the anchored primer
because of the higher GC content of the arbitrary primer. Mou et al. (1994) and Jurecic et
al. (1998) attributed this to internal priming. As the arbitrary primes are closer together,
amplification between them is more efficient and therefore the predominant amplification
product will be the sequence located between the arbitrary primers.

More than 80% of the cDNA clones produced readable sequences. A BLASTX search of
the nucleotide sequences of these cDNA clones revealed that the majority of the amino acid
sequence of the genes identified showed significant sequence identity with known proteins.
Generally, sequences that share 25% identity for at least 100 residues or at least 20%
identity for more than 200 amino acid residues are considered as homologous and likely to
have related functions (Doolittle, 1990; Agros et al., 1991; Pearson, 1996). The inferred
proteins are involved in a range of cellular functions including transport, metabolism,
biosynthesis, regulation, transcription and signal transduction.

From the results of BLASTX search, one notable feature is the proportions of clones found
to encode ABC transporters. The results showed significant sequence identity with ABC
transporter proteins of bacteria (82-91% sequence identity over 99-240 amino acid
residues). Most of the ATP-binding cassette (ABC) protein superfamily are membrane
proteins (ABC transporters) that play important roles in the transport of a wide range of
compounds across membranes and cellular detoxification (Sanchez-Fernandez et al., 2001;
Wolflger et al., 2004). One of the clones, CP2 showed a very significant homology to
glycerate kinase II of *Escherichia coli* with 100% sequence identity over 141 amino acid residues. Glycerate kinase, an enzyme of the glycerate pathway converts glycerate into 3-phosphoglycerate, an intermediate within the glycolytic pathway (Cusa *et al.*, 1999). Clone CP15 shared 74% sequence identity over 215 amino acid residues with cys/met metabolism pyridoxal-phosphate-dependent enzymes from *Rhodoferox ferrireducens*. Another clone, CP32 showed significant homology to UDP-N-acetylglucosamine pyrophosphorylase of *Xanthomonas axonopodis* with 74% sequence identity over 306 amino acid residues. UDP-N-acetylglucosamine pyrophosphorylase is involved in the synthesis of UDP-N-acetylglucosamine, an essential metabolite serving as a precursor of cell wall components in bacteria and fungi (Yamada-Okabe *et al.*, 2001).

The use of this modified differential display method enabled the isolation of 26 putative differential cDNA clones induced by phosphite in *P. cinnamomi*. To analyse whether these cDNA clones were differentially expressed, further tests to quantify their expression level by real-time quantitative PCR was carried out in Chapter 4.
Chapter 4

Real-time quantitative RT-PCR analysis of phosphite-induced gene expression in *P. cinnamomi*

4.1 Introduction

The analysis of gene expression requires sensitive and precise measurements of gene transcripts. There are a number of techniques available to quantify gene expression but at present, real-time quantitative RT-PCR is the most sensitive, accurate and reproducible of the quantification methods, especially for rare transcripts and genes that are expressed at low levels (Giulietti *et al.*, 2001; Kim *et al.*, 2003). Two of the most widely used real-time PCR assays are SYBR Green I and fluorogenic 5’-exonuclease.

In SYBR Green I assay, the intercalating dye, SYBR Green I binds directly to double-stranded DNA (dsDNA). As the dsDNA accumulates during the course of PCR, the dye emits a fluorescence signal that is directly proportional to the amount of PCR product generated. This increase in fluorescence emission is read in real time and the resulting data are graphed as amplification plots; which are plots of cycle number versus change in fluorescence (Figure 4.1). Threshold cycle (*C*ₜ) values are then calculated based on the cycle number at which the fluorescence emission increases beyond a threshold level. As a *C*ₜ value is proportional to the logarithm of initial amount of target cDNA in a sample, the relative concentration of one target cDNA with respect to another is reflected in the
difference in cycle number necessary to achieve the same intensity of fluorescence (Simpson et al., 2000; Giulietti et al., 2001; Ponchel et al., 2003).

For the fluorogenic 5’-exonuclease assay, the 5’ nucleolytic activity of the Taq polymerase enzyme cleaves the dual-labeled probe, the TaqMan probe and on nuclease degradation of the probe, the 5’ reporter dye and the 3’ quencher dye are separated which results in an increase in the emission of the reporter dye fluorescence. The increase in fluorescence emission is measured during the course of the reaction and is proportional to the amount of PCR product present (Winer et al., 1999; Giulietti et al., 2001). As in SYBR Green I assay, the data are graphed as amplification plots.

Figure 4.1 A typical amplification plot obtained with a real-time PCR assay. An amplification plot is the plot of fluorescence signal vs. cycle number. During initial cycles of the PCR reaction, the fluorescence signal is below the detection threshold and is defined as the baseline. As the reaction progresses, an increase in fluorescence above the baseline detects accumulated PCR product. The threshold cycle (C\text{T}) is defined as the cycle number at which the fluorescence passes the threshold (Bustin, 2003). (Adapted from DNA/RNA Real-Time Quantitative PCR, Perkin-Elmer Biosystems).
Compared to fluorogenic 5'-exonuclease assay, SYBR Green I-based assay is gaining popularity as it is relatively fast, does not require a specific probe for each amplicon under investigation and is therefore more economical. On the other hand, it is less specific due to its characteristics of binding to any dsDNA; detecting both specific and non-specific PCR products. However, the use of well-designed primers, DNase treatment of RNA samples and optimization of the reaction conditions can reduce non-specific binding (Giulietti et al., 2001; Vandesompele et al., 2002; Ponchel et al., 2003). In addition, the incorporation of Hot Start PCR which entails withholding the activity of the enzyme polymerase until the denaturation step helps to circumvent non-specific amplification (Bogetto et al., 2000).

There are two commonly used methods to analyse data from real-time PCR experiments; the absolute quantification and relative quantification (Livak and Schmittgen, 2001; Bustin, 2003). One of the relative quantification methods, the comparative $C_T$ ($\Delta\Delta C_T$) method uses a single sample termed the calibrator sample, for comparison of gene expression level with the unknown sample. The calibrator sample, usually the non-treated sample is chosen to represent 1x expression of the gene of interest. The data are then presented as the fold change in gene expression relative to the non-treated sample (Winer et al., 1999; Livak and Schmittgen, 2001).

To ensure accuracy and precise measurements of gene transcripts, the measurements in each sample are typically normalized to a reference gene. The use of an endogenous reference allows the normalization of differences in the amount of amplifiable cDNA in test samples generated by different quantity and quality of starting materials and differences in
RNA extraction and cDNA synthesis (Livak and Schmittgen, 2001; Radonic et al., 2004; Nicot et al., 2005). Various housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GADPH), actin, ribosomal genes, cyclophilin and tubulin are commonly used as endogenous references. However, the endogenous reference gene of choice is one whose expression should only be minimally regulated under the conditions used in the experiment (Giulietti et al., 2001; Nicot et al., 2005) and this has to be established for each experiment.

This chapter described the application of real-time quantitative RT-PCR using SYBR Green I assay to analyse the change in expression of a number of P. cinnamomi genes in response to phosphite stress.

4.2 Materials and methods

4.2.1 Primer design

Both the forward and reverse primers for 13 cDNA clones were designed based on the nucleotide sequence of the respective target gene generated from the sequencing results. Three primer pairs for candidate endogenous reference genes, tubulin, cyclophilin and actin were designed based on the known nucleotide sequences of P. cinnamomi tubulin gene (accession number AY766221), P. infestan cyclophilin gene (accession number AF424658) and P. palmivora actin gene (accession number AY729846) obtained from the GenBank database.
In designing these primers, one of the most important criteria to take into account was the relatively small amplicon size of not more than 250 bp (SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green Instruction Manual, Invitrogen). Vector NTI Advance 10 (Invitrogen) and Primer Express V.3 (Applied Biosystems) softwares were used to design the primers. The designed primers were synthesized by GeneWorks, Australia.

4.2.2 Two-step real-time quantitative RT-PCR

4.2.2.1 cDNA synthesis

SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen) was used in the reverse transcription and real-time PCR.

Total RNA from both phosphite-treated (+P) and phosphite-untreated (–P) *P. cinnamomi* mycelium was DNase treated prior to cDNA synthesis as described in Chapter 3, section 3.2.5. Five hundred nanogram of RNA from each of the treatments was reverse transcribed according to the manufacturer’s protocol. The 20 µl reaction consisted of 2x RT Reaction Mix (2.5 µM Oligo(dT)$_{20}$, 2.5 ng/µl random hexamers, 10 mM MgCl$_2$ and dNTP), 2 µl RT Enzyme Mix (SuperScript III RT and RNaseOUT) and DEPC-treated water. All the components were mixed and incubated at 25°C for 10 min. A further incubation at 42°C for 50 min was carried out. The reaction was terminated by incubating at 85°C for 5 min and then chilled on ice. Two units of *E. coli* ribonuclease H was added and incubated at 37°C for 20 min. Both samples of cDNA synthesized were kept at -20°C until used.
4.2.2.2 Real-time PCR

Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detector (Applied Biosystems).

Both cDNA samples synthesized were diluted 1:10 prior to amplification. Each reaction was performed in a 25 µl volume. The reaction components were 12.5 µl Platinum SYBR Green qPCR SuperMix UDG (SYBR Green I, Platinum Taq DNA polymerase (60U/ml), 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM MgCl₂, 400 µM each of dGTP, dATP, dCTP, dUTP and 40U/ml UDG), 0.2 µM of each forward and reverse primer, 0.5 µl ROX (5-carboxy-X-rhodamine, succinimidyl ester (25 µM) in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, 0.01% Tween 20) and PCR-grade water to make up the volume to 22.5 µl. Finally, 2.5 µl of diluted cDNA template was added to the reaction mix.

Amplification for each sample was carried out in triplicate along with a no-template control (NTC) in which PCR-grade water was used as template. The thermal cycling conditions consisted of one cycle at 50 °C with a 2 min hold, 1 cycle at 95 °C with a 2 min hold for denaturation and ‘hot-start’, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec. Data were collected and amplification plots of \( \Delta R_n \) versus cycle number were generated for analysis.

To confirm that only one PCR product of the expected size was amplified in the phosphite-treated and untreated samples, all the reaction products were further analysed by agarose gel electrophoresis as described in Chapter 3, section 3.2.11.
4.2.3 Checking amplification and specificity of primers

Prior to carrying out real-time PCR, the amplification and specificity of each primer pair was checked by PCR to ensure the designed primers amplified a single band of expected size.

cDNAs synthesized were diluted 1:10 and 2.5 µl was used as a template for the reaction. The PCR assay and the thermal cycling profile was the same as described in Section 4.2.2.2 except that the reaction was carried out in a PCR thermal cycler (Cobett Research).

The amplified product was analysed by agarose gel electrophoresis as described in Chapter 3, section 3.2.11. The primer pair that generated a single band of expected size was used in the real-time PCR.

4.2.4 Optimization of PCR conditions

PCR conditions were optimized for primers that did not amplify a single band using the conditions in Section 4.2.2.2. For primers that generated multiple bands, the primer concentration was reduced to 100 nM while the annealing temperature was increased from 55 °C to 61 °C with two degree increment using the temperature gradient. For primers with no amplification, the MgCl₂ concentration was increased to 4 and 5 mM while the annealing temperature was reduced from 55 °C to 49 °C with two degree diminution. The rest of the PCR components and thermal cycling conditions remained the same.
4.2.5 Data analysis

The data were analysed and amplification plots were generated using the software program, Sequence Detector V 1.7 which is part of the ABI PRISM 7700 Sequence Detection System. The standard curves were generated using both the software programs, Sequence Detector V 1.7 and Microsoft Excel.

4.3 Results

4.3.1 Primer design and specificity

Of the 16 designed primers tested, primers for four of the target genes and the three endogenous reference genes produced single band of expected size (Table 4.1). The remaining primers either did not amplify or amplified more than one band. Further optimizations were carried out on those primers.

4.3.2 Optimization of PCR conditions

Initial attempts to amplify cDNA with the remaining primers either resulted in multiple bands or no amplification. Subsequent PCR optimization which included: i) reducing the primer concentration, ii) increasing the MgCl₂ concentration or iii) increasing or decreasing the annealing temperatures did not improve the results.
Table 4.1 Primer sequences of target genes and endogenous genes used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1</td>
<td>Scp1</td>
<td>AATCGCTACGAGGTTCCGCCGGCAGCGCAAAAGTTGTCTGA</td>
<td>189</td>
</tr>
<tr>
<td>CP6</td>
<td>Scp6</td>
<td>TTGATCTCGTCTGCTGCTGGGCGGTGGTCTGAATCGTGTA</td>
<td>106</td>
</tr>
<tr>
<td>CP22</td>
<td>Scp22</td>
<td>CGCTCCCAATCAGATGTTAGCACCCCGATGAGATATGG</td>
<td>117</td>
</tr>
<tr>
<td>CP29</td>
<td>Scp29</td>
<td>GTGCATCACTTCGTCGCTGCTTCACCCCGATGAGATATGG</td>
<td>110</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Hk1</td>
<td>TCGTGCTGCTTTTGGATGCGCATACACGCTCCGCGGATCA</td>
<td>144</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>Hk2</td>
<td>TAGATGCGCAACCCGCGAGGTCTGTGCAAAGAGACACGCGGGAAG</td>
<td>127</td>
</tr>
<tr>
<td>Actin</td>
<td>Hk3</td>
<td>TTTGACTGAAGCCCGCCCTCAATCGCGTCCAGGCAGGTTC</td>
<td>242</td>
</tr>
</tbody>
</table>

4.3.3 Selection of the endogenous reference gene

Real-time PCR was carried out to measure and compare the transcription level of three candidate endogenous reference genes; tubulin, cyclophilin and actin subjected to phosphite stress.

An amplification plot was generated for each of the endogenous reference genes and their C_T values were then obtained (Figure 4.2). The difference in transcript level between the phosphite-treated and untreated samples was then calculated based on the formula, \( \Delta C_T = C_T (+P) - C_T (-P) \). The difference in expression level between the two RNA samples of each of the genes is shown in Table 4.2.
The calculated $\Delta C_T$ values indicated little variation in the expression of all three candidate endogenous reference genes between the phosphite-treated and untreated samples. Of the three genes, tubulin was the most highly expressed as indicated from its comparatively low $C_T$ values. It was chosen as the endogenous reference for normalization in subsequent experiments. The results also demonstrated specificity of each of the primers and fidelity of each of the assays by the absence of specific signal in the NTC which showed a $C_T$ value of 40 and the intra-assay coefficient of variation (CV) of 0.1% to 2.3%.

![Image](image.png)

**Figure 4.2** A representative linear view of the amplification plot generated to determine the expression of candidate endogenous reference genes under the influence of phosphite. Figure shown is the amplification plot of cDNA of phosphite-treated and untreated samples for the endogenous reference gene, tubulin showing the change in fluorescence ($\Delta R_n$) of SYBR Green I dye plotted versus cycle number ($C_T$). Threshold was set at 10 standard deviations from the baseline fluorescence. Each sample point represents the mean of triplicate $C_T$ values.
Table 4.2 $\Delta C_T$ values indicating the level of gene expression of tubulin, cyclophilin and actin between phosphite-treated (+P) and untreated (–P) mycelium of *P. cinnamomi*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>$C_T$ (+P)</th>
<th>$C_T$ (–P)</th>
<th>$\Delta C_T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td>24.35</td>
<td>24.13</td>
<td>0.22</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>26.05</td>
<td>25.90</td>
<td>0.15</td>
</tr>
<tr>
<td>Actin</td>
<td>34.20</td>
<td>34.02</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Each $C_T$ value represents mean of three replicates.

4.3.4 Determination of amplification efficiency

The comparative $C_T$ ($\Delta \Delta C_T$) method was used to calculate the relative gene expression (User Bulletin #2, Applied Biosystems, 1997). For the $\Delta \Delta C_T$ method to be valid, the efficiency of amplification between the target gene and the endogenous reference gene must be approximately equal (Livak and Schmittgen, 2001) or within +/- 10% of each other (Relative Quantification ($\Delta \Delta C_T$) Reaction Efficiency, i Science, Applied Biosystems).

A 10-fold dilution series (1:10, 1:100, 1:1,000, 1:10,000) of cDNA from the phosphite-untreated sample were prepared and used as template in the real-time PCR. The amplification was carried out for each pair of endogenous reference gene and target gene to be studied. Amplification for each dilution was carried out in duplicate. For each dilution, the change in fluorescence ($\Delta Rn$) was measured and plotted against the cycle number ($C_T$) in the amplification plot. A standard curve for both endogenous reference and target genes was then constructed by plotting the $C_T$ values against the logarithm of the 10-fold serial dilutions of cDNA. From the slope of the standard curves generated, PCR efficiencies for
each pair of endogenous reference gene and target gene were calculated (Figure 4.3), based on the formula, Efficiency (E) = (10 \(^{-1/slope}\) – 1) x 100% (Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR, Applied Biosystems; Radonic et al., 2004).

All the PCR assays displayed an efficiency of 83% to 95% (Table 4.3). For every pair of endogenous reference and target genes compared, the difference in efficiency was less than 5%. Therefore, the \(\Delta\Delta C_T\) method could be used for calculating the gene expression level.

There was minimal variation among replicated samples as shown from the intra-assay CV of 0.03% to 2.8%.

**Table 4.3** PCR amplification efficiency of each of the target gene and the endogenous reference gene assays

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Primer</th>
<th>Reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>E (%)</td>
<td>(R^2)</td>
</tr>
<tr>
<td>Scp1</td>
<td>-3.821</td>
<td>82.7</td>
<td>0.988</td>
</tr>
<tr>
<td>Scp6</td>
<td>-3.670</td>
<td>87.3</td>
<td>0.999</td>
</tr>
<tr>
<td>Scp22</td>
<td>-3.512</td>
<td>92.6</td>
<td>0.999</td>
</tr>
<tr>
<td>Scp29</td>
<td>-3.648</td>
<td>88.0</td>
<td>0.986</td>
</tr>
</tbody>
</table>

The values of slope and correlation coefficient (\(R^2\)) were derived from the standard curve generated.
Figure 4.3 Representative standard curves generated to determine the amplification efficiency of endogenous reference gene and target gene. The two standard curves shown are tubulin (bottom) and CP22 (top). Each point represents the mean of duplicate $C_T$ values.

4.3.5 Quantification of gene expression

Real-time PCR were carried out by using primers that produced single bands. For amplification of each target gene, the tubulin gene was also included as an endogenous reference.

An amplification plot was generated for each target gene and tubulin gene pair and $C_T$ values were then obtained (Figure 4.4). Based on the $C_T$ values, the $\Delta C_T (+P)$ and $\Delta C_T (–P)$ values which have been normalized to the tubulin gene were determined. The $\Delta \Delta C_T$ value which represents the difference in transcript level between the phosphite-treated and untreated mycelium was then calculated. The amount of transcript in the phosphite-treated mycelium, normalized to an endogenous reference and relative to the untreated mycelium is
expressed as $2^{\Delta\Delta CT}$. A positive $\Delta\Delta CT$ value indicates down-regulation while a negative $\Delta\Delta CT$ value indicates an up-regulation of gene transcription (Radonic et al., 2004). In the present study, a gene is considered differentially expressed if its relative expression is two fold or greater (Rajeevan et al., 2001).

The fold difference in the expression level of normalized phosphite-treated mycelium relative to untreated mycelium of four cDNA clones are shown in Table 4.4. Of these, one gene, CP6 showed differential expression with a three-fold induction. The remaining three genes did not exhibit an induction of expression. The intra-assay CV of 0.2% to 3.6% indicated little variation among replicated samples.

**Table 4.4** The level of gene expression between phosphite-treated (+P) and untreated (–P) mycelium of *P. cinnamomi* determined by the comparative $\Delta\Delta CT$ method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>$\Delta C_T$ (+P)</th>
<th>$\Delta C_T$ (–P)</th>
<th>$\Delta\Delta C_T$</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1</td>
<td>-8.94</td>
<td>-8.29</td>
<td>-0.65</td>
<td>1.6</td>
</tr>
<tr>
<td>CP6</td>
<td>-5.26</td>
<td>-3.63</td>
<td>-1.63</td>
<td>3.1</td>
</tr>
<tr>
<td>CP22</td>
<td>1.83</td>
<td>1.55</td>
<td>0.28</td>
<td>1.2</td>
</tr>
<tr>
<td>CP29</td>
<td>-0.76</td>
<td>-0.25</td>
<td>-0.51</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Each $C_T$ value represents the mean of three replicates. The $\Delta C_T$ (–P) or (+P) values are each represented as, $\Delta C_T(+P) = C_T$ target (+P) – $C_T$ tubulin (+P) or $\Delta C_T(–P) = C_T$ target (–P) – $C_T$ tubulin (–P). The $\Delta\Delta C_T$ value is represented as, $\Delta\Delta C_T = \Delta C_T(+P) – \Delta C_T(–P)$. The fold difference is calculated from the formula, $2^{\Delta\Delta CT}$. 

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Figure 4.4  A linear view of amplification plot of cDNA of phosphite-treated (+P) and untreated (–P) for target gene, CP6 and the reference gene, tubulin (Hk1) showing the change in fluorescence of SYBR Green I dye plotted versus cycle number. Ten standard deviations above the baseline fluorescence was used as the threshold. Each sample point represents the mean of triplicate $C_T$ values.

Figure 4.5 The same reaction products for the target gene, CP6 (left) and the reference gene, tubulin (right) were further analysed by agarose gel electrophoresis. Both CP6 and tubulin showed a single amplification product of the expected size of 106 bp and 144 bp respectively Lanes: (M) 100 bp molecular ladder; (1 to 3) –P; (4 to 6) +P; (7) NTC.
4.4 Discussion

The use of real-time PCR with SYBR Green I assay has shown to be a good method of quantifying differential gene expression. The real-time assay is non-radioactive, requires only small amounts of cDNA and the progress of the reaction can be monitored in real-time. Additionally, the relative quantification using the comparative $\Delta \Delta C_T$ method is well-suited to the present experimental system.

In this study, three endogenous reference genes were evaluated to ascertain the most appropriate one to use. The assessment of the transcription level of all three candidate endogenous reference genes showed little regulation under the influence of phosphite, fulfilling the general requirement for an endogenous reference. However, the tubulin gene was chosen in favour of cyclophilin and actin as the endogenous reference because its expression level was the highest among the three genes tested. Conversely, actin which had the lowest expression level among the three genes was unsuitable for quantitative assay as it has been reported to be affected by some biological factors (Nicot et al., 2005). The choice of an ideal housekeeping gene as an endogenous reference is not easy as there is no gene in a living cell whose transcription is totally invariant to cell cycle fluctuation or environmental conditions (Radonic et al., 2004). GADPH, which is widely used as an endogenous reference was not considered in the current study as it is a glycolytic enzyme (Giulietti et al., 2001) and several enzymes involved in the glycolytic and phosphogluconate pathways were reported to be affected by phosphite (Stehmann and Grant, 2000). It is therefore important to validate for a particular experiment an endogenous
reference that is not significantly regulated when subjected to the experimental treatment (Winer et al., 1999; Radonic et al., 2004).

Of the primers designed for real-time PCR, seven yielded a single band suitable for further analysis. Although the reason for the failure of the remaining to yield an amplicon of the expected size is not known, there are two possible explanations. Firstly, the designed primers were not specific and were able to hybridize to multiple sites on the template, giving rise to multiple PCR products. According to Rajeevan et al. (2001), certain primer pairs may show multiple peaks in the melt curve analysis despite optimizing the annealing temperature and holding time. They attributed this to non-specificity of primers or complex transcriptional regulation such as alternate splicing events or detection of novel gene family members. Secondly, during cDNA synthesis, longer mRNA transcripts may not have been transcribed to allow both forward and reverse primers or either one of the primers to bind efficiently; resulting in amplification failure. Though redesign and retesting new primers may increase the chance of obtaining some primers that generate a single band, it was not possible to continue pursuing this option due to time constraints.

The real-time PCR results demonstrated the specificity of the primer pairs and absence of genomic DNA contamination or non-specific amplification product. All the primers gave a \( C_T \) value of 40 in the no-template control except for CP6 which gave a \( C_T \) value of around 38 after the exponential phase towards the end of the PCR cycles. Subsequent analysis of agarose gel electrophoresis of the post PCR products showed that this was due to the formation of primer-dimers (Figure 4.5). The use of SYBR Green I which binds to dsDNA
can lead to the detection of non-specific product and primer-dimers at low template concentration or excess primer concentration (Paton et al., 2000). Despite the fact that the formation of primer-dimers may have an effect on the accuracy of absolute quantification, their presence would not compromise the precision of relative quantification as used in the present study. This is because primer-dimers were formed in both the investigated samples, so that the result expressed as a ratio of the amount of amplified phosphite-treated sample relative to untreated sample remained constant. In addition, the quantification which was based on $C_T$ values was determined early in the exponential phase of the reaction at around $C_T$ of 19 to 21 while primer-dimers were only detected at $C_T$ of around 38 towards the end of the amplification cycles.

Of the four putative differential genes analysed, one, CP6 showed differential expression between phosphite-treated and untreated mycelium with a three-fold difference. This manifested the influence of phosphite in up-regulating this gene. The remaining three genes which presented comparable expression in both treatments did not exhibit induction of expression. These were most probably constitutively expressed genes. It has been shown that some differential cDNA clones obtained during mycorrhizal colonization of bean roots contained a mixed population of constitutively and differentially expressed cDNAs (Vogeli-Lange et al., 1996). It could not be excluded that the weakly expressed differential cDNAs are masked by more abundant constitutive ones (Zegzouti et al., 1997).
This study has shown that differential gene expression can be detected unequivocally by real-time PCR with SYBR Green I assay. It also demonstrated the sensitivity of the assay and its ability to detect subtle changes in gene expression.
Chapter 5

General Discussion

5.1 Overview

The chemical, phosphite is an effective control agent for plant diseases caused by Phytophthora species. The main aim of this research was to study morphological and molecular changes in Phytophthora species when the pathogen was exposed to phosphite. Progress was made towards designing experiments and applying techniques to achieve this aim. Five Phytophthora species, P. cinnamomi, P. nicotianae, P. citricola, P. palmivora and P. capsici were studied for their growth inhibition and morphological alterations induced by phosphite. P. cinnamomi was further investigated for its molecular changes when subjected to phosphite treatment using the modified DDRT-PCR and real-time quantitative RT-PCR techniques. Phosphite was shown to have a direct and possibly an indirect mode of action on Phytophthora species.

5.2 Efficacy of phosphite on Phytophthora species

This study clearly showed that phosphite has a direct mode of action on Phytophthora species. The chemical adversely affected the growth, development and morphology of both mycelium and spores of the five Phytophthora species studied. Though there are several previous reports on the suppressive effects of phosphite on the mycelial growth of various Phytophthora species (Coffey and Bower, 1984; Fenn and Coffey, 1984; Bashan et al.,
1990; Wilkinson et al., 2001), very few have described the effects of phosphite on the morphology of hyphae and spores and the differences between species. This study has provided new insight into the extent of damage inflicted on the pathogen by phosphite. This is of fundamental importance as both hyphae and spores are infective propagules which play a critical role in the epidemiology of diseases caused by Phytophthora species. Therefore, understanding the mechanism(s) by how phosphite disrupts these infective propagules will be critical to the control of Phytophthora species.

This study also highlights the differential phosphate requirements for maximum growth for each Phytophthora species; with the optimum concentration of phosphate ranging from 0.1 mM to 5 mM among the five species. The large difference among these species is intriguing and warrants further investigation. Additionally, it poses the question of whether this differential phosphate requirement also applies to different isolates within a species. This would be an interesting subject to explore and is likely to unravel the mechanism(s) behind the differential sensitivity of isolates to phosphite.

A relatively low concentration of phosphate was incorporated into the growth medium in this study which might explain why 5 µg/ml of phosphite caused such strong growth inhibition. However, the relationship between phosphite and phosphate is unresolved and needs further investigation as previous work is contradictory with some reports that the antifungal activity of phosphite was highly dependent on the phosphate concentration present (Smillie et al., 1989; Griffith et al., 1993; Darakis et al., 1997) while others report that it hardly had any effect (Fenn and Coffey, 1984). This may have practical implications
as the common practice of increasing the phosphate levels in the plant or soil by applications of phosphate fertilizers might well result in less effective control of the pathogen. Hence, phosphate levels in both plant and soil may need to be taken into account when applying phosphite to control the pathogen in the field. However, emphasis should be placed on phosphate in the plant and readily available phosphate in the soil, as many soils bind phosphate making it unavailable to the plant.

5.3 Challenges of DDRT-PCR

The DDRT-PCR technique offers an opportunity whereby the RNA transcripts present in both phosphite-treated and untreated mycelium can be compared simultaneously. Additionally, the technique only requires small amounts of RNA. Though it was shown to be a good alternative to other methods such as subtractive and differential hybridization for this experimental system, there were problems with its application. For example, there was the inherent problem of contaminating cDNA species co-migrating with the cDNA of interest. A single, unique band on the display gel may actually contain a composite of identically sized but distinct cDNA sequences (Vogeli-Lange et al., 1996; Smith et al., 1997; Jurecic et al., 1998). Using a DNA sequencer, Smith et al. (1997) demonstrated the extent of cDNA contamination with the complexity of individual cDNA bands ranging from six to eleven peaks. Although cloning was carried out, the complexity of the cDNAs raises the issue of which clone to select or the number of clones that need to be screened to find one with differential expression. Despite screening at least two colonies with the correct insert, Corton and Gustafsson (1996) attributed their low success rate to an
insufficient number of clones screened. Whilst Jurecic et al (1998) sequenced two to three clones to exclude the possibility of unrelated cDNAs of the same size being present in the re-amplified product. Therefore, in the present study, as only one clone with the right sized insert from each ligation was sequenced, the chances of identifying the clone with differentially transcribed cDNA as opposed to constitutively expressed ones would have been reduced.

Despite the widespread use and success of the technique in some experimental systems, the results obtained by many researchers have indicated a significant incidence of false positives with the percentage of confirmed altered regulation ranging from less than 10 to 30% (Liang et al., 1993; Mou et al., 1994; Goormachtig et al., 1995; Blanchard and Cousins, 1996; Corton and Gustafsson, 1996; Seehaus and Tenhakens, 1998; Melin et al., 1999). The main contributing factor was the presence of contaminating cDNAs with the differential transcribed cDNA (Vogeli-Lange et al., 1996; Smith et al., 1997; Jurecic et al., 1998). However, Bertioli et al. (1995) singled out the strong bias of the technique to detect higher abundance transcripts as opposed to less abundant transcripts for their failure to detect differential gene expression despite their use of 65 primer pair combinations. Ledakis et al. (1998) also observed that the technique precludes the detection of less abundantly expressed transcript in favor of highly expressed ones. This could be of importance as transcripts corresponding to regulatory genes are often rare (Zegzouti et al., 1997).
5.4 Biological significance of the differentially expressed cDNA clone

The differentially expressed cDNA clone, CP6 which encodes 1564 nucleotides was found to share sequence identity (23% over 210 amino acids residues) with the proteophosphoglycan gene from Leishmania major. Proteophosphoglycans are members of phosphoglycan-containing glycoconjugates which play important roles in the survival, development and virulence of this parasitic Leishmania species. Proteophosphoglycan molecules are made up of phosphoglycan chains linked to the polypeptide backbone via phosphodiester linkages to serine, a protein modification termed phosphoglycosylation (Gopfert et al., 1999; Ilg et al., 1999; Klein et al., 1999).

There is no report on the functions of proteophosphoglycan in Phytophthora species. However, the induction of a gene homologous to proteophosphoglycan from L. major has been observed during cleavage of sporangial cytoplasm in P. infestans (Tani et al., 2004) and a proteophosphoglycan gene with similarity to the L. major gene has been identified in P. sojae (Qutob et al., 2002). Recently, it has been shown that a bioadhesive glycoprotein, proteophosphoglycan secreted by zoospores of the green algae Ulva linza is involved in the attachment of the spores to various substrata onto which they settle (Stanley et al., 2005). In Phytophthora species, the secretion of an adhesive glycoprotein by zoospores has been demonstrated (Sing and Bartnicki-Garcia, 1975; Gubler et al., 1989; Hardham, 2001). The adhesive ensures firm attachment of the zoospores to the host and subsequent successful infection. Various proteins that are associated with adhesion in Phytophthora, notably PcVsv1 protein of P. cinnamomi (Robold and Hardham, 2005) and mucin-like proteins of
*P. infestans* (Gornhardt *et al.*, 2000) produced during zoospore encystment and cyst germination respectively have been reported. Based on the phylogenetic tree by Van de Peer and de Wachter (1997) which suggested a close evolutionary relationship between Oomycetes and green algae, it can be speculated that CP6 may play a role as an adhesive for the attachment of zoospores to plant surfaces. However, it is difficult to envisage a function for mycelium similar to that of zoospore. As CP6 was isolated from mycelium of *P. cinnamomi*, it is not unreasonable to speculate that it is a hyphal adhesive. This view was supported by reports that a cell wall glycoprotein isolated from mycelium of *P. parasitica* var. *nicotianae* plays a role in the adhesion of hyphae onto cellulose substrates. This hyphal adhesive is also an elicitor of plant defence (Mateos *et al.*, 1997; Gaulin *et al.*, 2002) thus consistent with the widely reported notion that an indirect mode of action of phosphite is the activation of plant defence system through production of elicitsins by the pathogen (Guest and Bompeix, 1990; Grant *et al.*, 1990; Perez *et al.* 1995; Jackson *et al.*, 2000). An increased in the production of elicitor glycoconjugates when *Phytophthora* species was exposed to phosphite has been shown by Rouhier *et al.* (1993).

### 5.5 Future work

The CP6 gene identified in this study was identified on the basis of transcriptional activation by phosphite treatment of *P. cinnamomi*. It remains to be demonstrated whether the changes in RNA levels are mirrored by corresponding changes in protein levels. This would require further analysis to measure the protein levels in phosphite-treated and untreated mycelium.
Further experiments must also be directed at confirming the role of this gene in determining sensitivity of *P. cinnamomi* to phosphite. It is as yet unknown whether the differential expression reflects a direct or indirect effect of phosphite. Effects on gene expression may be direct on gene regulation or indirect caused by a stress response triggering a cascade of gene expression (Melin *et al.*, 1999). Studies with *Saccharomyces cerevisiae* have shown that inhibition of a key regulatory protein in the phosphate starvation response results in the transcriptional activation of more than two hundred genes (Carroll *et al.*, 2001), many of which are part of a generalized environmental stress response and are not directly involved in the phosphate starvation response. The role of a gene could be confirmed either by knockout to disrupt the gene or knockdown of expression using RNA interference (Cogoni, 2001).

### 5.6 Conclusion

The main aim of this study was achieved and has contributed to new knowledge on morphological alterations and molecular changes in *Phytophthora* species in response to phosphite. Morphological examinations coupled with molecular techniques are opening windows to our understanding of the complex modes of action of phosphite in *Phytophthora* species. The information generated will lead to a better understanding of the underlying mechanism(s) of action of phosphite in the future. This in turn will contribute to more effective control strategies for diseases caused by *Phytophthora* species.
References


DNA/RNA Real-Time Quantitative PCR, Perkim-Elmer Biosystems


Pilbeam, R.A., Colquhoun, I. J., Shearer, B.L. and St J. Hardy, G.E. (2000) Phosphite concentration; its effect on phytotoxicity symptoms and colonization by *Phytophthora*
cinnamomi in three understory species of Eucalyptus marginata forest. Australasian Plant Pathology 29: 86 – 95.


Relative Quantification (∆∆ Cₜ) Reaction Efficiency, i Science, Applied Biosystems.


Shearer, B.L. and Tippett, J.T. (1989) Jarrah Dieback: The dynamics and management of Phytophthora cinnamomi in the Jarrah (Eucalytus marginata) forest of South-Western Australia. Research Bulletin No. 3 Dept. of Conservation and Land Management Como Western Australia.


Appendix I

Media and buffers preparation

Modified Ribeiro’s medium

Basal medium

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>4.5 g</td>
<td>BDH</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>100 mg</td>
<td>Sigma</td>
</tr>
<tr>
<td>KNO₃</td>
<td>150 mg</td>
<td>AJAX Chemical</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>see below</td>
<td>BDH</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>500 mg</td>
<td>AJAX Chemical</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>100 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>Microelement stock solution</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Ferric stock solution</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td></td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>1 ml</td>
<td></td>
</tr>
</tbody>
</table>

For 0.1 mM phosphate, KH₂PO₄ 13.6 mg
For 0.5 mM phosphate, KH₂PO₄ 68.0 mg
For 1.0 mM phosphate, KH₂PO₄ 136 mg
For 5.0 mM phosphate, KH₂PO₄ 680 mg
For 10.0 mM phosphate, KH₂PO₄ 1.36 g

The pH of the medium was adjusted to 6.2 with 6M KOH before autoclaved at 15 psi for 20 min.
Thiamine-HCl was added after the autoclaved medium was cooled to approximately 55 °C.
(Based on Ribeiro, 1978)
Microelement stock solution

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
<td>41.1 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>87.8 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>7.85 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>MnSO$_4$·H$_2$O</td>
<td>15.4 mg</td>
<td>APS</td>
</tr>
<tr>
<td>Na$_2$B$_4$O$_7$·10H$_2$O</td>
<td>0.5 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Ferric stock solution

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$·6H$_2$O</td>
<td>50 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.6 g</td>
<td>AJAX Chemical</td>
</tr>
<tr>
<td>KOH</td>
<td>1.5 g</td>
<td>BDH</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Thiamine-HCl stock solution

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine-HCl</td>
<td>0.1 g</td>
<td>Sigma</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

The solution was filtered sterilized before used.
Modified Ribeiro’s agar medium

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribeiro’s medium</td>
<td>1000 ml</td>
<td></td>
</tr>
<tr>
<td>Agar, Grade A</td>
<td>17 g</td>
<td>BBL</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>1 ml</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

The mixture was autoclaved at 15 psi for 20 min. Thiamine-HCl was added after the autoclaved medium was cooled to approximately 55 °C.

V8 broth

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8 juice</td>
<td>50 ml</td>
<td>Campbell Soup</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.1 g</td>
<td>AJAX Chemical</td>
</tr>
<tr>
<td>β-sitosterol</td>
<td>0.02 g</td>
<td>Sigma</td>
</tr>
<tr>
<td>Distilled water</td>
<td>950 ml</td>
<td></td>
</tr>
</tbody>
</table>

V8 juice was centrifuged at 6,000 x g for 15 min and the supernatant was decanted for used. The mixture was autoclaved at 15 psi for 20 min. (Based on Hardham et al., 1991)

V8 agar

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8 juice</td>
<td>100 ml</td>
<td>Campbell Soup</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.1 g</td>
<td>AJAX chemical</td>
</tr>
<tr>
<td>β-sitosterol</td>
<td>0.02 g</td>
<td>Sigma</td>
</tr>
<tr>
<td>Agar, Grade A</td>
<td>17 g</td>
<td>BBL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>900 ml</td>
<td></td>
</tr>
</tbody>
</table>

The mixture was autoclaved at 15 psi for 20 min.
**Corn meal agar**

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal agar</td>
<td>17 g</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

The mixture was autoclaved at 15 psi for 20 min.

**Luria-Bertani broth**

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
<td>BBL</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
<td>BBL</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
<td>AJAX Chemical</td>
</tr>
<tr>
<td>Distilled water</td>
<td>top up to 1000 ml</td>
<td></td>
</tr>
<tr>
<td>Kanamycin (10 mg/ml stock solution)</td>
<td>5 ml</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

The pH of the solution was adjusted to 7.0 with NaOH and the volume was brought up to 1 L. Kanamycin was added after the autoclaved medium was cooled to approximately 55 °C.

**Luria-Bertani agar medium**

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB medium</td>
<td>1000 ml</td>
<td>BBL</td>
</tr>
<tr>
<td>Agar, Grade A</td>
<td>15 g</td>
<td>BBL</td>
</tr>
<tr>
<td>Kanamycin (10 mg/ml stock solution)</td>
<td>5 ml</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

The mixture was autoclaved at 15 psi for 20 min. Kanamycin was added after the autoclaved agar medium was cooled to about 55 °C.
**Soil extract**

<table>
<thead>
<tr>
<th>Soil</th>
<th>100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The mixture was left to stand overnight, filtered through Whatman No. 1 filter paper. Freshly prepared soil extract was used. (Based on Erwin and Ribeiro, 1996 with modifications).
Appendix II

Nucleotide sequences of cDNA clones

CP1
TGTGCTTCTGTTGCGATCTGCACATCGCCAGAAGCAACTTCACCCGATAGGCTAAAGATGGAAGAG
TGCGAGGAAGTTTCGCTCATCCGAGCTAGTGTGCTTTTAATCCCTTGCTTATCTTCTACTGCGT
TCACACAGATGCTTGTATGCTGCTGGGATGCATTTTATGCTGCTGTGGCATCTC

CP2
CATCGTATTACACGCGTCGACATCGCCAGAAGCAACTTCACCCGATAGGCTAAAGATGGAAGAG
TGCGAGGAAGTTTCGCTCATCCGAGCTAGTGTGCTTTTAATCCCTTGCTTATCTTCTACTGCGT
TCACACAGATGCTTGTATGCTGCTGGGATGCATTTTATGCTGCTGTGGCATCTC
GTGCCGACCAGCCACGATAGGCTGGCGCAGGTATAATCATCCTGCCATTC
TGCCAAACGGGCAAGGGCAAGCATACCTTGAAAGAAGCATAGTGTCAGCTGCGGCCACCATCAA
CATAAACAAGACCACCAAGGGGACGTTGAAATACGATG

CP4

AGAAGACCCCGTCCTTGGCTTGAGGCTCACCAGATTTGGGAGCAAGACCACATTTGGGGGAGGC
CAGGGGTTCGCTTGGTGTGGGTTGAAGATGTGTAATACGATG

CP5

AGAAGACCCCGTCCTTGGCTTGAGGCTCACCAGATTTGGGAGCAAGACCACATTTGGGGGAGGC
CAGGGGTTCGCTTGGTGTGGGTTGAAGATGTGTAATACGATG

CP6

CAGCTTCACCATGCTTGGGCTTCCTCGCTTCGCTTCATATGCTTGCTTGCTCCTTCAGCTTTAGGTCTG
GGAGTCCACCTCCTCCCAATCCTCGTCTGGGCTAAGACTGGTCTGCTGGGCTTGGGGGAGCAAGACCAC
CAGGGGTTCGCTTGGTGTGGGTTGAAGATGTGTAATACGATG
TTTTCTGTTCAGTCTGCTGGGCTTGCAAAATCTGCAAGTGTAAATGCAGTCGGTATGACCGCAACACGTCTCCGCGATAATGCCGAGACGTTGCGCAGCAAGACAATGGTTGTTCTGCGTATCGCTTTCTTCATATCCATGTTGCC

CP12
CATCCTATTTAACGCCGTGCTGGACACCTAGCGGTTGCGGATTACCAGGCGGAGAAGGGCAGCCTGACCTGCTGCCATGCTCGTG
GAAGCGCTTGGGGGAGCTAGGCTGCTTTTGCAGCGCTCTTGGCCTGCCTGGGACATAAGCTGGGAGCCCTGGAGGCTTTTTCATATCCATGGTGCC
CP13
CATCCTATTTAACGCCGTGCTGGACACCTAGCGGTTGCGGATTACCAGGCGGAGAAGGGCAGCCTGACCTGCTGCCATGCTCGTG
GAAGCGCTTGGGGGAGCTAGGCTGCTTTTGCAGCGCTCTTGGCCTGCCTGGGACATAAGCTGGGAGCCCTGGAGGCTTTTTCATATCCATGGTGCC
CP15
GGCCCTCCTTTGAGACCGCGTCACAATTGATCAAGGAGTAACGGCAGACTTTTACATCAACTCCACAGCGCTG
CTGCCCAATCCTCCTGGCTCTGGGACCTGGTCGCTCTGTGGCTCCTGGCTCGTCCATGCGGCAAGACCGAAGACGCCTGACCTGCTGCCATGCTCGTG
GAAGCGCTTGGGGGAGCTAGGCTGCTTTTGCAGCGCTCTTGGCCTGCCTGGGACATAAGCTGGGAGCCCTGGAGGCTTTTTCATATCCATGGTGCC
CP16
GGCCCTCCTTTGAGACCGCGTCACAATTGATCAAGGAGTAACGGCAGACTTTTACATCAACTCCACAGCGCTG
CTGCCCAATCCTCCTGGCTCTGGGACCTGGTCGCTCTGTGGCTCCTGGCTCGTCCATGCGGCAAGACCGAAGACGCCTGACCTGCTGCCATGCTCGTG
GAAGCGCTTGGGGGAGCTAGGCTGCTTTTGCAGCGCTCTTGGCCTGCCTGGGACATAAGCTGGGAGCCCTGGAGGCTTTTTCATATCCATGGTGCC

AATGCGCCATTGAAACGCGTACATCTGGAATGACACGACTTGAAGACAATCGCATCATCGGGCTTGTTCGGGCGACATCTTAACCGTCTGGTAAAACTCGGTGAGTAGCGTGTTCAAATCGCGAGAACAGTGTCGGGTGGTGGATTGCCTTTTGCAGGCGTTCGTAACGATCTTGAGCTCGCCAAATGCTCGGCGATACTCTACATTGCCACACTTCTCCATATTGTCTCGAGTTTTACCCATGCTTCTGTAGCTTTCCACATTCGTAATCGCCAAAGGGAGTCGCAGGCTCGAGCTCAGGCTGGGATGCTGTTGCGCTCTCTTTTGCTACCAAACGCTCTAGTTCATGGACTATAGATGGTAGACAGGCACGCTTGTGGGGGCTTGGCAGCAAATTGCGGA

CP24
AGAAGACCCCGCCTTGGCAACCAAAATAGCCCGGGGAAAAACTTCTGGCTGATCCTTTTTTGGCAATGAGCTGCGCACAAGCGGCCGAACAGGGCGGGCCAGGCGCTGGTGAA
GGCCTGGGCCGACGAGATCGAAAGCACCCCTGTCCGCGTCAGCATCGTCGATCCGGGCCGGATGCGCACGGCCATGCGCGCCCAGGCCTATCCGGGTGAAGATCCGTCGGTCCTGCCCCATCCCTCCGAGATCGGCCCTATGGTCGTCGACCTGGCCCGCCCAGACGCCACGCCGCCCAGAAGGCGGGGTCTTCT

CP25
AGAAGACCCCGCCTTGGCAACCAAAATAGCCCGGGGAAAAACTTCTGGCTGATCCTTTTTTGGCAATGAGCTGCGCACAAGCGGCCGAACAGGGCGGGCCAGGCGCTGGTGAA
GGCCTGGGCCGACGAGATCGAAAGCACCCCTGTCCGCGTCAGCATCGTCGATCCGGGCCGGATGCGCACGGCCATGCGCGCCCAGGCCTATCCGGGTGAAGATCCGTCGGTCCTGCCCCATCCCTCCGAGATCGGCCCTATGGTCGTCGACCTGGCCCGCCCAGACGCCACGCCGCCCAGAAGGCGGGGTCTTCT

CP26
GTGCCAGAAATCCGGTTGTCAAAATCCATAGGCCGTTGCAGCTCTGGCGTGAACTC
TGCAAAATCAAAATAGAGCAAGCTGCTTAAAGCAAGTTGGTCTGACCTTTCAATTTCACCCGCCCAAGGCTGAATACTGCAATCAGGCTGTTCAGTTCATCGGAAAACAACTGATTTTTGCAGTTGCTGGTGAATGGGGATGGATGGTGGCAATCGCGCTCGACTTATGGATGCAAACCCCGATTTGTGCGCTACGCCCAACCGCTCATGACCCGCGAGGCTCTGCATAACAGCGATTGGTGCAGACCTGTGGCGAGCATCGCAAATTTTACGCGCGCAGAATCTGGAAATAATATCCTGCACGATGTTGCATCA
CGCGCGCCCTGGGAGTTTTCCGGTGATCGGATATGGATCGGGCAGGTAACCTCAAATGGTATATCAAGTCGATATCGCATGTCACAAAGCAACACAACACGGATTCTCGCTCGTGCTCGTGACGCTGGCCTTATGGGCTGGGAGCGCCCCGGCAATCGTGGCGACAACCGGATTCTGCCAC
GTAGGGCTCTGCACCAGATTCACGCGCCATCGCTCGTACAGCACCAGTACCTGCGCAGCATCGGG
AACCTGCGGACATCGCTCGTACAGCACCAGTACCTGCGCAGCATCGGG

CP34
GGTGAAAGGCGGCTTATCCGGAAGGCAATTACCTTCAGATTGAACTTGCT
GGCGAAGAATGTCGATCTGCGTACATGCGCTCATTCTTGCTCTCGGCGTACACGC
CTGAGCTGAGCTGAGCTGACATGCTGCGTACATGCGCTCATTCTTGCTCTCGGC

CP35
TTTAGAAGAGCGTCTCGCTCCTCAGATTTAGGATTTATCTGCTCTGCTCTGCTCTG

Flanking primer sequence is underlined.