REAL-TIME PCR ASSAY FOR β-1, 3-GLUCANASE GENE EXPRESSION IN BLACK PEPPER (*Piper nigrum* L.)

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Real-time PCR assay for β-1,3-glucanase gene expression in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Real-time PCR assay for β-1,3-glucanase gene expression in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done independently by **Mr. Dukare Kiran Shanker** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ABBREVIATIONS

β	Beta
Δ	Delta
µl	Micro litre
Act	Actin
AR	Analytical reagent
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAM	Carrot agar medium
CCD	Charged coupled device
cDNA	Complementary DNA
COH	College of Horticulture
CP	Crossing point
CPBMB	Centre for Plant Biotechnology and Molecular Biology
C_T	Threshold cycle
CTAB	Cetyl trimethyl ammonium bromide
ddCt	Delta delta threshold cycle
DEPC	Diethyl pyrocarbonate
DIC	Distributed Information Centre
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
DNS	Dinitrosalicylic acid
dNTP	Deoxyribo nucleoside triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gluc	β-1,3-glucanase
GPX	Glutathione peroxidase
GST	Glutathione-s-transferase
h	Hour
ha	Hectare
HR	Hypersensitive response
HRGP	Hydroxyproline-rich glycoproteins
IP	Imaging plate
ISR	Induced systemic resistance
JA	Jasmonic acid
kg	Kilogram
LED	Light emitting diode

M	Average expression stability
M	Molar
ml	Milli litre
mM	Milli molar
M-MuLV	Moloney murine leukaemia virus
MOPS	3-(N-Morpholino)-propanesulfonic acid
mRNA	Messenger RNA
NAC	No amplification control
NCBI	National Centre for Biotechnology Information
ng	Nano gram
nM	Nano molar
NTC	No template control
OAS	Oral allergy syndrome
°C	Degree Celsius
OD	Optical density
P	Pairwise variation
PAL	Phenylalanine ammonia lyase
PC	*Piper colubrinum*
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PMSF	Phenyl methane sulphonyl fluoride

PN	*Piper nigrum*
PR	Pathogenesis-related
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal RNA
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sequence detection system
SSC	Standard Saline Citrate
ssDNA	Single stranded DNA
t	tonne
TAE	Tris acetate EDTA
TE	Tris HCl EDTA
TMV	Tomato mosaic virus
tRNA	Transfer RNA
UV	Ultra violet
V	Pairwise variation
v/v	volume/volume

List of tables

List of Plates

List of Figures

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), renowned as "King of spices," is one of the important spice crops of the world and is an export oriented crop in India with a substantial share in foreign exchange earnings.

Black pepper occupies an area of 2,36,000 ha with production of 50,000 t during 2006-2007 (Spices Board, 2008). However, in productivity, India occupies the last position among the leading black pepper producing countries in the world and it is only 310 kg/ha. One of the major reasons for this peculiar situation is the high incidence of disease especially the *Phytophthora* rot, caused by *Phytophthora capsici*. It is prevalent in all pepper growing areas causing heavy crop loss every year

Sarma *et al.* (1991) reported yield loss up to 50 per cent in black pepper in India, due to *Phytophthora* foot rot disease. Similar reports are also there from other pepper growing countries indicating the seriousness of the disease.

Piper colubrinum, is a wild, exotic species from South America, reported to be resistant to *Phytophthora* rot. Parab (2000) confirmed its resistance to the disease and reported higher level of β-1,3-glucanases to be the main factor contributing to resistance.

β-1,3-glucanases, are the pathogenesis-related proteins, reported in various plant species. During fungal infection, it acts upon the β-1,3-glucan component of fungal cell wall. This enzyme is reported to have a positive role in the defence mechanism against *Phytophthora* rot in black pepper (Nazeem *et al.*, 2008).

Since conventional breeding programmes for this perennial spice crop are complex, time consuming and less effective in obtaining the resistant genotypes, an attempt was made to unravel the mechanism for disease tolerance at the molecular level though real-time PCR assay.

Real-time PCR has emerged as a method of choice for fast, affordable and efficient estimation of gene copy number. It is a highly sensitive and accurate method for determination of gene expression through detection of changes at transcript level. It is based on the detection and quantification of fluorescence during each cycle of PCR (Higuchi *et al.*, 1993) and the increase in fluorescence in each cycle is directly proportional to the amount of product formed in each cycle.

The work was taken up in a multiphased manner. The resistant and susceptible genotypes were challenged with the fungal pathogen for initiating the response. The variation in glucanase enzyme levels in the plant at different time intervals, the gene copy number in the genome and the relative expression at transcriptional level was worked out to confirm and assign the actual role of β-1,3-glucanases in the defence mechanism in black pepper.

The study was intended to elucidate the β-1,3-glucanase gene copy number and gene expression level using real-time PCR technique with the following objectives:

1. To evaluate the β-1,3-glucanase gene copy number in *Piper nigrum* & *Piper colubrinum* through Southern blotting and real-time PCR assay.
2. To evaluate the level of expression of the gene in *Phytophthora* infected and healthy black pepper plants in relation to *P. colubrinum*.

Review of Literature

2. REVIEW OF LITERATURE

β-1,3-glucanases and chitinases are hydrolytic enzymes that are abundant in plant species, located either in the vacuole or secreted into extracellular spaces of plant cells. They are also known as pathogenesis-related (PR) proteins (Meins *et al.*, 1992; Simmons, 1994). β-1,3-glucanases are involved in plant defence responses and the higher activity enhances resistance to disease in resistant cultivars (Netzer *et al.*, 1979).

2.1 Black pepper (*Piper nigrum* L.) and *Phytophthora* foot rot

Black pepper (*Piper nigrum* L.), rewarded as "King of spices," is one of the important export oriented spice crops of India with a substantial share in foreign exchange earning of the country. It occupies an area of 2,36000 ha with production of 50,000 t during 2006-2007 (Spices Board, 2008). However, in productivity, India occupies the last position among the leading black pepper producing countries in the world (Alagappan and Manoharan, 2001). One of the major reasons for this peculiar situation is the high incidence of disease especially the dreadful *Phytophthora* foot rot disease. It is prevalent in all pepper growing areas and causes heavy crop loss every year (Anandraj and Sarma, 1991; Sarma *et al.*, 1994).

In India, this disease was first reported by Barber (1902) from Waynad region of Kerala. Butler (1906) coined the term 'wilt,' due to rapid death of the plant. Later, Muller (1936) reported a similar type of disease from Dutch East Indies and coined the term 'foot rot.' Nambiar and Sarma (1977) referred the disease as 'quick wilt disease' of black pepper based on sudden death and wilting of vines. However, the terminologies of the disease have been changed to *Phytophthora* foot rot of black pepper and to *Phytophthora* rot of black pepper (Vijayaraghavan, 2003).

The destruction of vines in India due to *Phytophthora* rot disease has been recorded to be 40 to 50 per cent, which indicates seriousness of the disease infestation (Dewaard, 1979). Harper (1974) has reported a yield loss of 50 per cent in Indonesia due to this disease. Crop losses due to 25-30 per cent vine deaths have been reported from Kerala (Nambiar and Sarma, 1977). Sastry (1982) and Dutta (1982) recorded heavy incidence of the disease in Uttara Kannada and Shimoga districts of Karnataka causing 100 per cent death of vines in some gardens.

There is no report regarding highly effective control measure to tackle this disease and all the cultivated types have been found susceptible to the disease. Keeping in view of the economic losses caused by fungal pathogen, conventional breeding programmes are in operation to develop disease resistant cultivars. However, this approach has not been very successful. Biotechnological approaches could be utilized to compliment conventional breeding as well as to develop transgenic lines of this crop by incorporating disease resistance genes.

2.1.1 Causal Organism

Muller (1936), first identified the *Phytophthora* isolates from black pepper as *P. palmivora* var. *Piperis*. However, various workers treated it as *P. palmivora* and was later renamed as *P. capsici* by Tsao and Alizadeh (1988).

The fungus shows umbellate sporangial ontogeny with caducous sporangia with long pedicels. Sporangial shape varies from ovoid to pyriform with a tapering base. The fungus grows luxuriantly at 25-30 ^{0}C on carrot agar medium. Sporangial production was found to be abundant under continuous light and zoospores germinated within 15-20 minutes after encystment (Sastry and Hegde, 1987).

2.1.2 Symptomatology

According to Muller (1936), all parts of the plant at all stages of growth

were susceptible to the disease and showed rotting symptoms in collar region, leaf, root , vine and spikes.

Holliday and Mowat (1963) from Sarawak, and Nambiar and Sarma (1977) from India observed zonate lesions with fimbriate margins on the infected leaves. In addition, Turner (1973) observed rapid development of symptoms within 36 to 48 h of leaf inoculation.

Appearance of water soaked lesions on the leaves and stems of infected vines and brown discolouration of infected fine roots were reported by Mammootty *et al.* (1980). They also observed flaccidity of young and mature leaves followed by yellowing of younger leaves. Anandraj and Sarma (1991) reported that being a soil borne pathogen, the fungus gained entry into main roots through fine roots and reached the foot and collar of the vine and culminated into foot rot. Further, rotting of roots impeded transportation of water and minerals, thus brought physiological drought in plants.

2.1.3 Sources of resistance to *Phytophthora* foot rot

The study conducted for screening cultivated types against *Phytophthora* foot rot, could not find any resistant cultivar (Holliday and Mowat, 1963; Ruppel and Almeydor, 1965; Turner, 1973; Kuch and Khew, 1980; Sarma *et al.*, 1982; Vilasini, 1982). However, they found that wild species like *P. colubrinum* and *P. obliqum* showed certain resistance. Sarma *et al.* (1991) also reported the immunity of *P. colubrinum*, a wild relative of pepper from Amazon basin. Parab (2000) has confirmed the resistance behaviour of *P. colubrinum* against *P. capsici* and reported that the activity of β-1,3-glucanases was higher in *P. colubrinum* as against cultivated ones after challenging with *P. capsici.*

2.2 The plant defence mechanism

Resistance is the ability of an organism to exclude, overcome completely or to some extent the effect of pathogen or other damaging factor (Agrios, 1997).

Disease resistance is manifested in plants as limited symptoms, reflecting the inability of the pathogen to grow or multiply. It often takes the form of hypersensitivity response (HR) in which the pathogen remains confined to necrotic lesions near the site of infection. Hypersensitive response is the localized and rapid death of one or few cells at the infection site to restrict further invasion by pathogen. This may give resistance for biotrophic pathogens, which obtain their energy from living cells. In contrast, cell death may benefit to necrotrophs, which obtain their energy from dead cells (Kumar *et al.*, 2002). Plants can activate separate defence pathways depending on the type of pathogen encountered (Garcia-Brugger *et al.*, 2006). Necrotrophic pathogen initiates jasmonic acid (JA) and ethylene dependent responses, whereas biotrophic pathogen initiates salicylic acid (SA) dependent responses.

Leaves and roots are the main target organs for several pathogens. To survive under such circumstance of biotic stresses, plants have developed number of coordinated defence responses relying on extensive changes in gene activity (Yang *et al.*, 1997).

The defence is based on preformed barriers and induced responses (Bryngelsson and Collinge, 1992). Preformed barriers include the cuticle, host cell walls and antimicrobial compounds. The induced responses start after the adherence of pathogen onto a host surface. It involves recognition of specific signal molecules originated either from the pathogen or from degradation products of the host cell walls. Based on their origin, these specific signal molecules are termed as either exogenous or endogenous elicitors (Collinge *et al.*, 1993; Fujita *et al.*, 2004). Elicitors trigger a network of signaling pathways to coordinate a succeeding defence response (Yang *et al.*, 1997).

The earliest defence responses are the opening of specific ion channels responsible for creating ion flux across the plasma membrane, the rapid production of reactive oxygen species (ROS) such as superoxide (O^{-}_{2}) and hydrogen peroxide (H_2O_2), and phosphorylation and dephosphorylation of specific

proteins. Production of reactive oxygen species (ROS) such as superoxide (O^{-}_2) and hydrogen peroxide (H_2O_2) is known as oxidative burst (Doke *et al.*, 1996; Conrath *et al.*, 1997). The oxidative burst helps in cross-linking reactions and activation of enzymes involved in strengthening the plant cell walls and the accumulation of secondary metabolites such as phytoalexins. Accumulation of compounds such as lignin, suberin, thionins, hydroxyproline-rich glycoproteins (HGP) and polysaccharides such as cellulose, callose and pectins give rise to formation of thickened cell wall and specific papilla at the site of penetration (Collinge *et al.*, 1993; Brisson *et al.*, 1994; Baker and Orlandi, 1995).

The production of reactive oxygen species (ROS) is the first signal of hypersensitivity response (HR). H_2O_2 would be toxic to microbes and cause considerable damage to activate protective mechanism. Moreover, Leon *et al.* (1995) has indicated that the H_2O_2 increases the expression of the enzymes involved in biosynthesis of salicylic acid (SA) such as glutathione-s-transferase (GST) (Levine *et al.*, 1994) and glutathione peroxidase (GPX) (Avsian-Kretchmer *et al.*, 2004).

Plants develop systemic acquired resistance (SAR) following activation of HR. Systemic acquired resistance (SAR) is the phenomenon whereby uninfected distal parts of the plant develop resistance to further infection, involving several pathogenesis-related proteins such as chitinase (PR-3) and glucanases (PR-2), either antifungal or antibacterial in function (Ryals *et al.*, 1996; Sticher *et al.*, 1997).

Induced systemic resistance (ISR) is the second type of systemic resistance, which is mediated by jasmonate and ethylene dependent pathways and does not involve PR proteins (Pieterse *et al.*, 1998; van Loon *et al.*, 1998). Tazum (2001) has shown that ISR is non-specific against a broad spectrum of pathogens. It depends on timely accumulation of multiple gene products such as hydrolytic enzymes, peroxidase or other defence-related gene products.

Somssich and Hahlbrock (1998) have observed that various combinations

of available defence mechanisms can create an efficient defence against most phytopathogens. All these pathways are associated with enhanced transcription of numerous defence genes and thereby with their translated protein products. Pathogenesis-related proteins are a major class of these proteins, which are induced both locally around infection site and systemically in the uninfected portion of the plant (van Loon *et al.,* 2006).

2.3 Pathogenesis-related proteins

The term pathogenesis-related (PR) proteins were first used by Antoniw *et al.* (1980) to indicate, "Proteins encoded by host plant but induced only in pathological or related situations."

Pathogenesis-related proteins are the proteins coded by the host plant but induced specifically in pathological or related situations, not only accumulate locally in the infected leaf, but also induced systemically and associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses (van Loon and van Strien, 1999).

A protein, newly expressed upon infection but not necessary in all pathological conditions, is included in pathogenesis-related proteins group. Pathological situations not only refer to the hypersensitive response to pathogen attack in which PR proteins are most common but also include parasitic attack by nematodes, insects and herbivores (van Loon, 1999).

Pathogenesis-related proteins, initially named as 'b' proteins, were discovered in tobacco leaves, hypersensitively reacting to tomato mosaic virus (TMV) by two independent groups, Gianinazzi *et al.* (1970), and van Loon and van Kammen (1970). These proteins have focused an increasing research interest in view of their possible involvement in plant resistance to pathogens. This assumption came from initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. However later it was found that

these proteins are not only induced in resistant but also susceptible plant pathogen interactions, as well as in plants subjected to abiotic stress (van Loon, 1985).

PR proteins accumulate in high quantity in response to HR. They are present at low concentrations or absent in healthy plants, but may account up to 10 per cent of the soluble proteins in leaves within few hours of infection (van Loon *et al.*, 1987).

Since the discovery of PR proteins, seventeen families have been identified (Table 1) based on amino acid sequence, serological relationship and enzymatic or biological activity (van Loon *et al.*, 2006). Within each PR family, there are several classes comprising of different isoforms with either high or low pI values. Most of the families were originally identified from tobacco, but from other plant species including monocotyledons such as barley, wheat, rice and maize have also been identified. The families are numbered in the order in which they were discovered and new PR proteins identified in different species are assigned to the existing recognized families and, if no similarity exists, a new family is created (Santen, 2007).

Various PR proteins have potential antimicrobial activity and are involved in defence mechanisms against some fungal pathogens (van Loon, 1997). Niderman *et al.* (1995) have observed that different isoforms of PRs, mainly basic have capacity to exhibit antifungal activity and target specificity *in vitro,* especially when different PRs are combined to create synergistic effects. Since PRs within different families are closely related and their mRNA and protein may readily cross hybridize, it is difficult to clarify a specific role in resistance for a single isoform (van Loon *et al.*, 2006).

2.3.1 Significance of various PR protein families in plant defence

The analysis of specific role of different PR protein families in limiting pathogen activity revealed the PR-2 family as β-1,3-endoglucanases and the PR-3, 4, 8 and 11 as endochitinases, which could act against fungi. The chitinases as

Table 1. Recognized families of pathogenesis-related proteins (modified from van Loon *et al.*, 2006)

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr3 [Gns2('Glb')]*
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*YPR3, Chia*
PR-4	Tobacco 'R'	Chitinase type I, II	*Ypr4, Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato inhibitor I	Proteinase-inhibitor	*Ypr6, Pis ('Pin')*
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8, Chib*
PR-9	Tobacco"Lignin-forming peroxidase"	Peroxidase	*Ypr9, Prx*
PR-10	Parsley "PR-1"	Ribonuclease-like	*Ypr10*
PR-11	Tobacco "Class V" chitinase	Chitinase type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13, Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

well as the proteinase inhibitors (PR-6) could target nematodes and herbivorous insects. Members of PR-8 family also possess lysozyme activity and may be directed against bacteria, whereas defensins (PR-12) (Lay and Anderson, 2005) and thionins (PR-13) (Epple *et al.*, 1997) have broad antimicrobial and antifungal activity.

Members of PR-1 and thaumatin-like PR-5 families are associated with activity against oomycetes while some lipid transfer proteins (PR-14) have shown antifungal and antibacterial activities (Garcia-Olmedo *et al.*, 1995).

PR-7, an endoproteinase, is the most conspicuous PR in tomato. It might aid in microbial cell wall dissolution (Jorda *et al.*, 2000). PR-9 is a specific type of peroxidase, which could act in cell wall reinforcement by catalyzing lignifications and enhance resistance against multiple pathogens (Passardi *et al.*, 2004). PR-10 shows homology to ribonucleases while some members have weak ribonuclease activity (Bufe *et al.*, 1996). It has been assumed that ribonuclease activity of PR-10 type proteins points to the role in defence against viruses (Park *et al.*, 2004). However recently, Caporale *et al.* (2004) have found that the ribonuclease activity of wheat PR-4 type protein is antifungal in function.

The families PR-15, 16 and 17 have been added recently. PR-15 and PR-16 are typical of monocots and comprise families of germin-like oxalate oxidase and oxalate oxidase like proteins respectively with superoxide dismutase activity (Bernier and Berna, 2001). These proteins generate hydrogen peroxidase that can be toxic to different types of attackers or could directly or indirectly stimulate plant defence responses (Donaldson *et al.*, 2001; Hu *et al.*, 2003). PR-17 proteins have been found as an additional family of PRs in infected tobacco, wheat and barley, and contain sequences resembling the active site of zinc-metallo proteinases (Christensen *et al.*, 2002), but have remained uncharacterized so far.

A putative novel family PR-18 comprises fungus and salicylic acid (SA)-inducible carbohydrate oxidases, as exemplified by proteins with hydrogen peroxide-generating and antimicrobial properties from sunflower (Custers *et al.*,

2004). Not all families are represented in all plant species, and occurrence as well as properties of different members within a family may differ strongly.

2.3.2 Role of pathogenesis-related (PR) proteins other than plant defence

Multifunctional proteins have been detected during plant development and senescence (Hanfrey *et al.*, 1996; Liljeroth *et al.*, 2005; van Loon *et al.*, 2006). This could be explained with the cell, tissue, organ and development specific expression pattern of PRs.

Vogeli-Lange *et al.* (1994) have revealed that basic tobacco glucanase PR-2 functions developmentally in seed germination by weakening the endosperm, thus allowing the radical to protrude. Chitinases, homologous to PR-3 and PR-4, act as morphogenetic factors in carrot embryogenesis (Kragh *et al.*, 1996). It has been shown that several PRs accumulate upon the transition of plants to flowering and senescence, suggests the developmental role (Fraser, 1981; Hanfrey *et al.*, 1996). Basic PR-5 contributes to osmotic adaptation by inducing abundantly in tobacco and tomato cells in response to osmotic stress (Singh *et al.*, 1987).

Several of the plant defence related proteins have been proven to be latex allergens and cross reactive allergens in fruits, vegetables and pollens. As such, it is probable cause of increase in allergenicity, in response to the creation of stress resistant varieties with gene recombination techniques, responsible for oral allergy syndrome (OAS) or similar syndromes (Hoffman-Sommergrubber, 2001; Vieths *et al.*, 2002; Tashpulatov *et al.*, 2004).

These findings raise the question as whether PR genes evolved primarily to limit damage by invading pathogens or were adapted from other functions to serve an accessory protective role (van Loon and van Strien, 1999).

However due to presence of β-1,3-glucans as well as chitin as a major component of plant pathogenic fungal cell wall (Wessel and Sietsma,1981), more intension has been given to investigate about β-1,3-glucanases and chitinases, as

result of possible role in plant defence against pathogenic fungi (Abeles *et al.*, 1971; Pegg, 1977; Boller, 1985).

β-1,3-glucanases are particularly interesting among the proteins having known enzymatic function because they are hormonally and developmentally regulated in uninfected plants (Felix and Meins, 1987; Mohnen *et al.*, 1985) and are thought to protect plants from fungal infection (Boller, 1988).

2.4 β-1,3-glucanases

The role of β-1,3-glucanases in plant defence is suggested by the observation that β-1,3-glucanases and endochitinases are co-ordinately induced as a part of the hypersensitivity response to pathogen infection (Vogeli-Lange *et al.*, 1988; Meins and Ahl, 1989). Chitinase and β-1,3-glucanases, when used in combinations, exhibit fungicidal activity *in vitro*, when used at concentrations comparable to those found in extracts from induced plants (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). In contrast, it has been found that not only plants (Abeles *et al.*, 1971) but also phytopathogenic fungi (Bell, 1981) produce glucanases.

2.4.1 Mode of action

β-1,3-glucans are important structural components of fungal cell walls (Wessels and Sietsma, 1981). Plant β-1,3-endoglucanases (PR-2) are believed to act primarily on β-1,3-glucans present in the cell wall of most fungal pathogens to release oligosaccharides (Mauch and Staehelin, 1989). β-1,3-glucanases catalyze hydrolytic cleavage of β-1,3-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger and Meins, 1999). The oligosaccharide fragments thus released may act as elicitors, which serve to trigger further defence response.

Hwang *et al.* (1989) have observed that *P. capsici* initially grows in intercellular space of pepper (chilli) plants and thereby comes in contact with β-1,3-glucanases and chitinase, located probably in the middle lamella. Upon

contact, β-1,3-glucanases release oligosaccharides from the β-1,3-glucan in the fungal cell wall. These oligosaccharides act as elicitors for phytoalexin production, which is important in deciding resistance to *P. capsici* (Kim and Hwang, 1994).

2.4.2 Occurrence

β-1,3-glucanases accumulate to high concentrations (up to four per cent of soluble proteins) in lower leaves and roots of healthy plants (Felix and Meins, 1986). These enzymes can also be found in specialized tissue types such as leaf epidermis (Keefe *et al.,* 1990) and in stylar tissues of the flower (Lotan *et al.,* 1989).

β-1,3-glucanases induced by ethylene has been observed in the vacuoles of lower epidermal cells and parenchyma cells adjacent to vascular bundle, and over the middle lamella in the intercellular space in bean leaves (Mauch and Stachelin, 1989; Mauch *et al.,* 1992).

In an incompatible reaction in wheat leaves against *Puccinia recondita,* PR-2 was mainly recovered in the domain of the host cell wall closer to plasmalemma, intercellular space, guard cells and secondary thickening of xylem vessels as well as in the hyphal cytoplasm and cell wall (Hu and Rijkenberg, 1998).

Tomato roots infected with *Fusarium oxysporum* showed PR-2 predominantly localized in the cell walls and vacuoles of the host, and in the cell wall and septa of the fungus (Benhamou *et al.,* 1989).

β-1,3-glucanases have been studied in floral organs of barley, where it is developmentally regulated. It has been noted in the anther and pistil tissues, including the stigmatic hairs. Besides cell walls, glucanases have also been recovered in plastids and in the cells of the style (Liljeroth *et al.,* 2005).

2.4.3 Classes of β-1,3-glucanases

The β-1,3-glucanases have been well characterized in different plant species (Castresana *et al.,* 1990; Beerhues and Kombrink, 1994). They exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization and pattern of regulation (Meins *et al.,* 1992).

The PR-2 family is divided into three structurally distinct classes of β-1,3-glucanases, with acidic and basic counterparts that significantly differ in their specific enzymatic and antifungal activity (Kauffmann *et al.,* 1987; Sela-Buurlage *et al.,* 1993).

2.4.3.1 Class I β-1,3-glucanases

Class I β-1,3-glucanases, basic isoforms, can be induced by pathogens, accumulate primarily in vacuoles, and are thought to be involved in normal development of healthy plants during seed germination, growth and flower development (Simmons, 1994). Several *in vitro* experiments have demonstrated that antifungal effects are mainly by basic class I β-1,3-glucanases against a wide range of fungi, either alone or in combination with PR-3 (Mauch *et al.,* 1988). The synergistic effect between PR-2 and PR-3 has also been shown in transgenic plants (Zhu *et al.,* 1994).

2.4.3.2 Class II β-1,3-glucanases

These are represented by three subgroups, PR-2a, 2b and 2c, which are acidic extracellular proteins. The style specific SP41a and SP41b are acidic extracellular proteins (Ori *et al.,* 1990) while subgroup PR-2c proteins, GL153 and GL161, are either neutral or basic vacuolar proteins (Ward *et al.,* 1991).

2.4.3.3 Class III β-1,3-glucanases

Class III includes a pathogen induced, acidic extracellular β-1,3-glucanases.

Other than above mentioned three classes, a class IV isoform of β-1,3-glucanase has also been observed, which is non-responsive to pathogen infection (van Eldik *et al.,* 1998; Thanseem *et al.,* 2003).

2.4.4 Structural organization of β-1,3-glucanase gene

Almost all β-1,3-glucanase genes isolated from various plant species contain a single intron, with the exception of a gene isolated from *Nicotiana plumbaginifolia,* which contains two (Castresana *et al.,* 1990). The position of the intron is the same in genes for acidic and basic glucanases, indicating that the structure of these glucanase genes is highly conserved, and they may have arisen from a single common ancestor. These genes differ from each other in upstream, downstream and intron sequences, although similar in their coding region (Linthorst, 1991).

2.4.5 Factors influencing β-1,3-glucanase gene expression

In healthy plants, β-1,3-glucanases have been found in higher concentrations in roots and older leaves relative to younger leaves (Vogeli-Lange *et al.,* 1994). The expression of β-1,3-glucanases is up-regulated in response to the stress hormone ethylene (Felix and Meins, 1987) and down-regulated by treatment with abscisic acid (Rezzonico *et al.,* 1998) and combination with auxin and cytokinin (Vogeli-Lange *et al.,* 1994). They are also induced by viral (Vogeli *et al.,* 1988), bacterial (Meins and Ahl, 1989), and fungal infections (Edington *et al.,* 1991).

2.4.6 Significance of β-1,3-glucanases in plant defence

Plants have developed a variety of constitutive and inducible defence mechanisms to resist the colonization of potential pathogen. Induction and accumulation of PR proteins including β-1,3-glucanases in the infected tissue has often been observed in incompatible plant-pathogen interactions (Egea *et al.,* 1999; Jabekumar *et al.,* 2001).

β-1,3-glucanases catalyze the endotype hydrolytic cleavage of β-1,3-glycosidic linkages in β-1,3-glucans, the major cell wall component in many pathogenic fungi. *In vitro* studies have shown that the purified protein can effectively promote the lysis of hyphal tips and thereby inhibit the fungal growth (Sela-Buurlage *et al.,* 1993). β-1,3-glucanases can also act indirectly by releasing certain oligosaccharides from cell wall of pathogens, which can act as elicitors to activate further defence reactions (Boller, 1995).

It was noted that β-1,3-glucanases had inhibitory activity against chitin negative *Phytophthora capsici* but shown no antifungal activity against the chitin containing fungi *Alternaria mali, Colletotrichum gloeosporiodes, Magnaportha grisea* and *Fusarium oxysporum* f. sp. *Cucumerinum* (Kim and Hwang, 1994).

In addition, it has been observed that transgenic plants overexpressing β-1,3-glucanase have shown higher resistance against pathogens (Yoshikawa *et al.,* 1993; Jongedijk *et al.*, 1993).

2.4.7 Role of β-1,3-glucanases in physiological and developmental processes in plants

The β-1,3-glucanases play important roles in various physiological and developmental processes in healthy plants. These include cell division (Fulcher *et al.,* 1976), microsporogenesis (Worrall *et al.,* 1992), germination (Casacuberta *et al.,* 1992), flowering (van Eldik *et al.,* 1998) and senescence (Hanfrey *et al.,* 1996).

In vitro plant cell culture studies have shown that β-1,3-glucanases have associations with callus emergence. During callus formation, auxin induced elongation of cell wall involves the degradation of cell wall β-1,3-glucans by glucanase (Yoshida, 1995). In addition, high levels of β-1,3-glucanase activity has been reported in embryonic cultures, compared with the non-embryonic cultures of *Cichorium* (Helleboid *et al.,* 2000).

During somatic embryogenesis, the callose deposition on the cell walls of embryonic cells gradually disappears once the cells start to divide and develop into somatic embryos. Since callose (a mixture of β-1,3-glucans) is a potential substrate for β-1,3-glucanases, it has been hypothesized that the enzyme plays an important role in somatic embryogenesis (Dong and Dunstan, 1997; Helleboid *et al.*, 2000).

2.5 Analysis for β-1,3-glucanase gene expression

2.5.1 Real-time PCR

Real-time PCR is a technique, which collects data throughout the PCR process. It allows quantification of the initial amount of nucleic acid template most specifically, sensitively and reproducibly, combining amplification and detection into a single step; based on the detection of a fluorescence accumulation proportional to the PCR product at each cycle of PCR (Raeymaekers, 2000; Wong and Medrano, 2005; Dussault and Pouliot, 2006; Espy *et al.*, 2006).

The invention of polymerase chain reaction (PCR) by Karry Mullis in 1984 revolutionized the field of science (Saiki *et al.*, 1985; Mullis and Faloona, 1987). Real-time PCR, which is based on polymerase chain reaction, is at present, the most sensitive method for the detection of low abundance mRNAs (Bustin, 2000). It can be used for different applications, such as clinical diagnosis (Bustin and Dorudi, 1998), for the analysis of tissue specific gene expression (Bustin *et al.*, 2000) and for the determination of gene copy number (Mason *et al.*, 2003).

Higuchi *et al.* at Roche Molecular Systems and Chiron accomplished the first demonstration of real-time PCR (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993). They could visualize and record accumulation of DNA with a video camera by including a common fluorescent dye, ethidium bromide (EtBr) in the PCR and running the reaction under ultraviolet light, which causes EtBr to fluoresce. Subsequently, this technology quickly matured into a competitive market, becoming commercially widespread and scientifically influenced.

Applied Biosystems (USA) first made real-time PCR instrumentation commercially available in 1996, after which several other companies added new machines to the market. Presently, Applied Biosystems, Biogene, Bioneer, BioRad, Cepheid, Corbett Research, Idaho Technology, MJ Research, Roche Applied Science and Stratagene offer instrumentation lines for real-time PCR (Valasek and Repa, 2005).

2.5.1.1 Principle

The real-time PCR system is based on the detection and quantification of fluorescence during each cycle of PCR (Higuchi *et al.*, 1993; Livak, 1997). The increase in fluorescence in each cycle is proportional to the amount of product formed in each cycle. In the initial cycles of PCR, there is little change in fluorescent signal, which defines baseline. An increase in fluorescence above the baseline indicates the detection of accumulated target. Reactions are characterized by threshold cycle (C_T), the PCR cycle at which fluorescence emitted by target passes the threshold. Threshold is the line whose intersection with the amplification plot defines the threshold cycle (C_T). The threshold level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The resulting fluorescence emission is detected either with charged coupled device (CCD) camera or photomultiplier tubes. Consequently, greater the quantity of target DNA in the starting material, faster a significant increase in fluorescent signal will appear, yielding lower C_T and *vice versa* (Heid *et al.*, 1996; Dorak, 2006).

2.5.1.2 Theory of real-time PCR

PCR can be broken into four major phases, the linear ground phase, early exponential phase, log linear (exponential) phase and plateau phase (Tichopad *et al.*, 2003). During the linear ground phase (usually the first 10-15 cycles), fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. Baseline fluorescence is the fluorescence

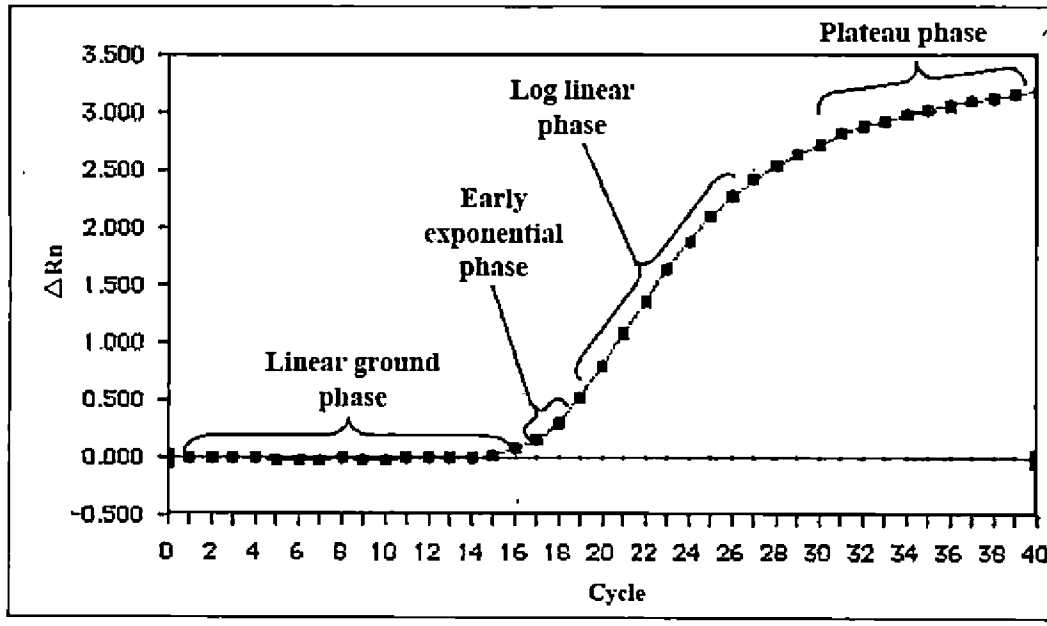


Fig. 1. Phases of the PCR amplification curve

available at the initial cycles of PCR, in which there is little change in fluorescence signal.

At the early exponential phase, the amount of fluorescence reaches a threshold, where it is significantly higher (usually ten times the standard deviation of the baseline) than the background levels. The cycle at which this occurs is known as threshold cycle (C_T) or crossing point (CP). This value is representative of the starting copy number in the original template and is used to calculate experimental results (Heid *et al.*, 1996).

During the log linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions.

Finally, plateau stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation (Bustin, 2000).

2.5.1.3 One-step versus two-step real-time PCR

When quantifying mRNA, real-time PCR can be performed as either a one-step reaction, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single tube, or as a two-step reaction, where reverse-transcription and PCR amplification occur in separate tubes. Although one-step real-time PCR reaction minimizes experimental variation due to occurrence of both enzymatic reactions in a single tube, this method may not be suitable in situations where the same sample is assayed on several occasions over a period of time. This is mainly because this method uses RNA as a starting template, which is prone to rapid degradation if not handled properly. In addition, one-step reactions are less sensitive than two-step reactions.

Two-step real-time PCR reaction separates the reverse transcription reaction from the real-time PCR assay, allowing several different real-time PCR assays on dilutions of a single cDNA. Since the process of reverse-transcription is

notorious for its highly variable reaction efficiency (Mannhalter *et al.*, 2000), using dilutions from the same cDNA template ensures that reaction from subsequent assays have the same amount of template as those assayed earlier. Data from two-step real-time PCR is quite reproducible with Pearson correlation coefficients ranging from 0.974 to 0.988. A two-step protocol is preferred while using a DNA binding dye such as SYBR® Green I, because of elimination of primer-dimers through the manipulation of melting temperatures. However, it is prone for increased opportunities of DNA contamination in real-time PCR (Vandesompele *et al.*, 2002a; Wong and Medrano, 2005).

2.5.1.4 Sequence detection chemistries

Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using variety of fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (Higuchi *et al.*, 1993).

2.5.1.4.1 DNA binding dyes

DNA binding dyes emit fluorescence when bound to dsDNA. As the double stranded PCR product accumulates during each subsequent cycle, more dye can bind and emit fluorescence. Thus, the fluorescent intensity increases proportionally to dsDNA.

DNA binding dyes include two types, intercalators such as ethidium bromide and minor grove binders such as SYBR® Green I.

SYBR® Green I dye shows increased sensitivity to detection without PCR inhibition over ethidium bromide and thus more useful. It binds to the minor grove of dsDNA and emits thousand-fold greater fluorescence than when it is free in solution (Wittwer *et al.*, 1997). Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of dsDNA binding by SYBR® Green I producing detectable amount of fluorescent signal. Thus, any

amplification of DNA in the reaction tube is measured. Other dyes such as BEBO, YEYO-1, TOTO-1 etc. have been described, but are not as widely used (Valasek and Repa, 2005).

The major concern with the usage of DNA binding dye is non-specificity, since it can detect dsDNA including non-specific reaction. To ensure specificity, the dissociation curve of the amplified product can be analyzed to determine the melting point. If there are two or more peaks, it suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target.

The major benefits for using these dyes include flexibility of the dye, since one dye can be used for different gene assays, and it is inexpensive compared to other chemistries (Wong and Medrano, 2005).

2.5.1.4.2 Hydrolysis probes

Hydrolysis probes, exemplified by the TaqMan® chemistry, also known as 5' nuclease assay, fluoresce upon probe hydrolysis by 5'→3' nuclease activity of Taq DNA polymerase during amplification. The fluorescence generated is proportional to the accumulated PCR product. Holland *et al.* (1991) were the first to demonstrate the cleavage of a target probe during PCR by 5' nuclease activity of Taq DNA polymerase, which could be used to detect amplification of the target specific product.

The sequence specific probe is labelled with a reporter dye on the 5' end and a quencher dye on the 3' end (Gibson *et al.*, 1996). When the probe is intact, the quencher reduces the fluorescence intensity of the reporter by fluorescence resonance energy transfer (FRET), where the fluorophore transfers its energy to the quencher (Clegg, 1992). The reporter (fluorophore) and quencher are separated by up to 40 bp to achieve quenching. However because FRET is distance dependent, it is important to design dual label probes to be as short as possible (Bustin, 2002). The annealed and quenched probe is degraded by the

5'→3' nuclease activity of Taq DNA polymerase during the extension step of the PCR. Probe degradation allows separation of the reporter from the quencher dye resulting in increased fluorescence emission (Heid *et al.*, 1996; Gibson *et al.*, 1996).

Common quencher flourophores include TAMRA, DABCYL and BHQ, while reporters include FAM, VIC, NED etc. Hydrolysis probes give greater insurance regarding the specificity because only sequence specific amplification is measured (Applied Biosystems, USA). In addition, hydrolysis probes allow simple detection of point mutation within the amplicon using melting curve analysis (Valasek and Repa, 2005).

2.5.1.4.3 Hybridization probes

Hybridization probes can be utilized in either a four or a three oligonucleotide manner (Bernard and Wittwer, 2000). The four oligonucleotide method consists of two PCR primers and two sequence-specific probes that bind adjacent to each other in head-to-tail arrangement; the upstream probe is labelledwith an acceptor dye on the 3' end, and the downstream probe with a donor dye on the 5' end (Bernard *et al.*, 1998). This allows the donor and acceptor flourophores to experience an increase in florescence resonance energy transfer (FRET) when bound. The three oligonucleotide method is similar to the four oligonucleotide method, except that the upstream PCR primer is labelled with an acceptor dye on the 3' end, and thus replaces the function of one of the probes from the four oligonucleotide method.

2.5.1.4.4 Hairpin probes

There are several other variations on the reporter-quencher theme, including molecular beacons, scorpions, sunrise primers, lux fluorogenic primers. They keep the reporter and quencher together before amplification while separating them and generating the fluorescence signal during amplification (Valasek and Repa, 2005).

2.5.1.5 The instrumentation of real-time PCR

A critical requirement for real-time PCR technology is the ability to detect fluorescent signal and record the progress of the PCR. Because fluorescent chemistries require both a specific input of energy for excitation and a detection of a particular emission wavelength, the instrumentation must be able to do both simultaneously and at the desired wavelengths. Thus, the chemistries and instrumentation are intimately linked.

At present, there are three basic ways in which real-time instrumentation can supply the excitation energy for flourophores, which include lamp, light emitting diode (LED) and laser. Lamps are classified as broad spectrum emission devices, whereas LEDs and lasers are narrow spectrum. To collect data, the emission energies must also be detected at the appropriate wavelength. Detectors include charge coupled device (CCD) cameras, photomultiplier tubes or other types of photo-detectors.

Another portion of the instrumentation consists of a thermocycler to carry out PCR. The particular importance given for real-time PCR is the ability of the thermocycler to maintain consistent temperature among all sample wells, as any difference in temperature could lead to different PCR amplification efficiencies. This is accomplished by using a heating block (Peltier based), heated air or combination of two. Heating blocks generally change temperature more slowly than heated air, resulting in longer thermocycling times.

To analyze real-time PCR data, real-time PCR systems are provided with computer hardware and data-acquisition and analysis software. Software platforms try to simplify analysis of real-time PCR data by offering graphical output of assay results including amplification and dissociation curves. The amplification curve gives data regarding the kinetics of amplification of the target sequence, whereas the dissociation curve reveals the characteristics of the final amplified product (Valasek and Repa, 2005).

2.5.1.6 Types of real-time PCR quantification

2.5.1.6.1 Absolute quantification

The absolute quantification assay is used to quantify unknown samples by interpolating their quantity from a standard curve. It uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between C_T and initial amounts of gDNA or cDNA, allowing the determination of the concentration of unknowns based on their C_T values (Heid *et al.*, 1996). This method assumes all standards and samples have approximately equal amplification efficiencies (Souaze *et al.*, 1996).

The PCR standard is a fragment of double stranded DNA (dsDNA), single stranded DNA (ssDNA), or cDNA bearing the target sequence. A standard used must be pure species. DNA standards have been shown to have a larger quantification range and greater sensitivity, reproducibility, and stability than RNA standards (Pfaffl *et al.*, 2004). However, a DNA standard can not be used for a one-step real-time RT-PCR due to the absence of a control for the reverse transcription efficiency (Giulietti *et al.*, 2001).

2.5.1.6.2 Relative quantification

A relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample.

During relative quantification, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator (Livak and Schmittgen, 2001). When using a calibrator, the results are expressed as a target/reference ratio.

There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantification assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error (Liu and Saint, 2002; Muller *et al.*, 2002).

2.5.1.6.2.1 Standard curve method for relative quantification

The quantity of each experimental sample is first determined using a standard curve and then expressed relative to a single calibrator sample (Livak, 1997). The calibrator is designated as one fold, with all experimentally derived quantities reported as an n-fold difference relative to the calibrator. Because sample quantity is divided by calibrator quantity, standard curve units are eliminated, requiring only the relative dilution factors of the standards for quantification. This method is often applied, when the amplification efficiencies of the reference and target genes are unequal (Liu and Saint, 2002). It is also the simplest method of quantification because it requires no preparation of exogenous standards, no quantification of calibrator sample, and is not based on complex mathematics. However, because this method does not incorporate an endogenous control, usually a housekeeping gene, result must still need to be normalized.

2.5.1.6.2.2 Comparative C_T ($2^{-\Delta\Delta CT}$) method

The comparative C_T method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample. While this method includes a correction for non-ideal amplification efficiencies, the amplification kinetics of the target gene and reference gene assays must be approximately equal (Medhurst *et al.*, 2000), because different efficiencies will generate errors when using this method (Liu and Saint, 2002).

Consequently, a validation assay must be performed, where serial dilutions are assayed for the target and reference gene, and the results plotted with the log input concentration for each dilution on the X-axis, and the difference in C_T (Target C_T – Reference C_T) for each dilution on Y-axis. If the absolute value of the slope of the line is less than 0.1, the comparative C_T method may be used (Livak and Schmittgen, 2001). The PCR product size should be kept small (less than 150bp) and the reaction rigorously optimized (Marino *et al.*, 2003). Since the comparative C_T method does not require a standard curve, it is useful when

assaying a large number of samples, since all the wells could be filled with sample reactions rather than standards.

2.5.1.6.2.3 Pfaffl method

The Pfaffl model combines gene quantification and normalization into a single calculation. This model incorporates the amplification efficiencies of the target and reference gene to correct for difference between two assays (Pfaffl, 2001). The relative expression software tool, REST, which runs in Microsoft Excel, automates data analysis using this model (Pfaffl *et al.*, 2002).

2.5.1.6.2.4 Q-Gene method

Q-Gene is a fully comprehensive Microsoft Excel based software application that aids in the entire process of real-time PCR experiment, form experimental planning and setup through data analysis and graphical presentation (Muller *et al.*, 2002).

Q-Gene calculates the mean normalized gene expression with standard errors using two different mathematical models, both correcting for amplification efficiencies. The calculated expression values are then compared between two matched groups to determine the expression of a sample relative to a calibrator.

2.5.1.6.2.5 Gentle *et al.* method

Gentle *et al.* (2001) designed one of the first models in which both fold changes between samples and amplification efficiencies of experimental versus control samples are calculated without the use of standard curves.

2.5.1.6.2.6 Liu and Saint method

Liu and Saint (2002) developed a sigmoidal mathematical model to quantify and normalize gene expression. Similar to Gentle *et al.* method, this method calculates amplification efficiencies from the actual slope of the amplification plot rather than a standard curve.

Table 2. Characteristics of relative quantification methods (modified from Wong and Medrano, 2005)

Sr. No.	Methods	Amplification efficiency correction	Amplification efficiency calculation	Amplification efficiency assumption	Automated Excel-based programme
1.	Standard curve (Livak, 1997)	No	Standard curve	No experimental sample variation	No
2.	Comparative C_T ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001)	Yes	Standard curve	Reference= target	No
3.	Pfaffl method (Pfaffl, 2002)	Yes	Standard curve	Sample= control	REST
4.	Q-Gene method (Muller *et al.*, 2002)	Yes	Standard curve	Sample= control	Q-Gene
5.	Gentle *et al.* method (Gentle *et al.*, 2001)	Yes	Raw data	Researcher defines log-linear phase	No
6.	Liu and Saint method (Liu and Saint, 2002)	Yes	Raw data	Reference and target genes can have different efficiencies	No
7.	DART-PCR (Peirson *et al.*, 2003)	Yes	Raw data	Statistically defined log-linear phase	DART-PCR

2.5.1.6.2.7 Amplification plot method

The amplification plot method uses a simple algorithm to calculate the amplification efficiency of every sample individually within the real-time PCR assay. These data are used in the calculation for expression quantification. Peirson *et al.* have developed a Microsoft Excel workbook entitled Data Analysis for Real-Time PCR (DART-PCR), which quickly calculates all results from raw data (Peirson *et al.*, 2003).

2.5.1.7 Amplicon design

The optimal amplicon length for real-time RT-PCR is less than 100 bp, but amplicon more than 400 bp have been used for TaqMan® systems (Bustin *et al.*, 1999). Shorter amplicons amplify more efficiently than longer ones and, are more tolerant of reaction conditions. This is because they are more likely to be denatured during the 92-94^{0}C step of the PCR, allowing the probes and primers to compete more effectively for binding to their complementary targets. As the extension rate of Taq DNA polymerase is between 30 and 70 bases per second (Jeffreys *et al.*, 1988), it also means that polymerization times, as short as 15 seconds, are sufficient to replicate the amplicon, making amplification of genomic DNA contaminants less likely and reducing the time it takes to complete the assay. The length of primers and probe required to generate a specific product defines the minimum practical length of the amplicon, which in our hands is 63 bp (Bustin *et al.*, 1999).

2.5.1.8 Primer design

Primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. If the intron-exon boundaries are unknown, or when targeting an intronless gene, it is necessary to treat the RNA sample with RNase free DNase. Use of Mn^{2+} rather than Mg^{2+} minimizes any problems caused by amplification of re-annealed DNA fragments (Bauer *et al.*, 1997). Primer selection is based on estimated Tm, the desire for small

amplicon size and the location of the probe. The TaqMan® system provides its own primer/ probe design programme, Primer Express (Applied Biosystems, USA).

2.5.1.9 Selection of endogenous control gene for real-time PCR normalization

Normalization of gene expression is used to correct sample to sample variation. Starting material obtained from different individuals usually varies in tissue mass or cell number, RNA integrity or quantity, or experimental treatment. Under ideal conditions, mRNA levels can be standardized to cell number, but when using whole tissue sample, this type of normalization is impossible (Vandesompele *et al.*, 2002b). Therefore, real-time PCR results are usually normalized against a control gene that may also serve as positive control for the reaction. The ideal control gene should be expressed in an unchanging fashion regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment. Because there is no one gene that meets these criteria for every experimental condition, it is necessary to validate the expression stability of a control gene for the specific requirements of an experiment prior to its use for normalization (Schmittgen and Zakrajsek, 2000).

According to Thellin *et al.* (1999) and Vandesompele *et al.* (2002b), at least two or three housekeeping genes should be used as internal standards because the use of single gene for normalization could lead to relatively large errors. Many studies have shown that internal standards, mainly housekeeping genes used for the quantification of mRNA expression could vary with the experimental conditions (Thellin *et al.*, 1999; Warrington *et al.*, 2000; Sturzenbaum and Kille, 2001; Radonic *et al.*, 2004). The most commonly used reference genes are housekeeping genes including genes for 18S rRNA, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (Suzuki *et al.*, 2000; Bustin, 2002).

Currently at least nine housekeeping genes have been well described for the normalization of gene expression signals (Sturzenbaum and Kille, 2001). The

most common are actin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal genes, cyclophillin and elongation factor 1-α (ef1α) (Sturzenbaum and Kille, 2001; Bezier *et al.*, 2002; Dean *et al.*, 2002; Thomas *et al.*, 2003). Adenosyl phosphoribosyl transferase (aprt) (Orsel *et al.*, 2002) and tubulin (Ozturk *et al.*, 2002; Williams *et al.*, 2003) may also be used. Many studies on housekeeping gene expression mainly deal with human tissues (Thellin *et al.*, 1999; Schmittgen and Zakrajsek, 2000; Warrington *et al.*, 2000; Radonic *et al.*, 2004), bacteria and viruses (Savli *et al.*, 2003; Stocher *et al.*, 2003).

As far as known, only a few have concerned plants, for example, barley (Burton *et al.*, 2004), rice (Kim *et al.*, 2003), poplar (Brunner *et al.*, 2004), *Arabidopsis* and tobacco (Volkov *et al.*, 2003). Nicot *et al.* (2005) have shown that ef1α and 18S rRNA are the most stable under biotic stress in potato. GAPDH has been found to be most stably expressed under biotic stress in grape (Shi, 2005).

2.5.1.10 Advantages of real-time PCR

There are many benefits of using real-time PCR over other methods to quantify gene expression as well as to estimate gene copy number.

- Real-time PCR assays are 10,000 to 1,00,000 fold more sensitive than RNase protection assays (Wang and Brown, 1999), 1000 fold more sensitive than dot blot hybridization (Malinen *et al.*, 2003), and can even detect a single copy of a specific transcript (Palmer *et al.*, 2003).

- Real-time PCR assays can reliably detect gene expression differences as small as 23 per cent between samples (Gentle *et al.*, 2001) and have lower coefficient of variation (SYBR® Green I at 14.2 per cent, TaqMan® at 24 per cent) than end point detection such as band densitometry (44.9 per cent) and probe hybridization (45.1 per cent) (Schmittgen *et al.*, 2000)

- Real-time PCR can also discriminate between mRNAs with almost

identical sequences, requires much less RNA template than other methods of gene expression analysis, and can be relatively high throughput given the proper equipment (Wong and Medrano, 2005).

- Real-time PCR is considered as high throughput approach for estimating gene copy numbers and zygosity over other approaches (Callaway *et al.*, 2002).

2.5.2 Evaluation of gene copy number

Pollack *et al.* (2002) have shown that there is strong correlation between gene expression and gene copy number. It was observed that Hsp70 concentration was higher in the extra copy strain of *Drosophila* than in the excision strain at most of the time (Feder *et al.*, 1996). However, Niswender *et al.* (1997) observed that there was reciprocal relationship between glucokinase gene copy number and blood glucose concentration.

There are two methods, most commonly used for determining gene copy number, Southern hybridization and real-time PCR.

2.5.2.1 Southern hybridization

Southern hybridization is most commonly used method for determining gene copy number. In this method, the blot of digested genomic DNA is hybridized with a radioactive DNA probe corresponding to the gene of interest, which in turn produces a characteristic banding pattern. Presence of clear and distinct bands corresponds to the gene copy number.

Thimmapurum *et al.* (2001) and Thanseem *et al.* (2003) have used Southern hybridization to determine β-1,3-glucanase gene copy number in peach and rubber respectively.

Although Southern analysis is reliable, it is time consuming and laborious, and requires large amount of high quality DNA. Furthermore, in the case of

restriction site loss or concatamers, the number of bands does not correspond to the gene copy number (Bubner and Baldwin, 2004).

2.5.2.2 Evaluation of gene copy number though real-time PCR

Real-time PCR has emerged as the method of choice for fast, affordable and efficient estimation of copy number (Ingham *et al.*, 2001; Mason *et al.*, 2003; Bubner and Baldwin, 2004). Various platforms and efforts have been proposed to improve the accuracy of real-time PCR for this application using both the external standard curve based method and the ΔC_T method involving an internal reference gene (Ingham *et al.*, 2001; Ballester *et al.*, 2004; Yang *et al.*, 2005).

Despite the obvious advantage of real-time PCR, the accuracy and detection limit of real-time PCR was found to be controversial during transgene copy determination. Mason *et al.* (2003) had found that only 70 per cent of the real-time PCR based transgene copy determination results could be verified with Southern blot analysis.

Recent advances in real-time PCR statistical analysis have provided an opportunity for improving real-time PCR based transgene copy number estimation with vigorous quality control and robust hypothesis testing. In fact, one of the most prominent factors that are ignored in real-time PCR based gene copy determination is amplification efficiency, for which a significant deviation from 100 per cent can drastically alter copy number estimation. Amplification efficiency is therefore one aspect of quality control that must be addressed by statistical models (Yuan *et al.*, 2006; Yuan *et al.*, 2008).

2.5.3 Gene expression analysis

2.5.3.1 Northern hybridization

Northern blot analysis and reverse-transcription (RT) PCR have been used to detect β-1,3-glucanase gene transcripts. Northern blot analysis has also been

used to compare the levels of transcripts.

Northern hybridization of a wheat β-1,3-glucanase gene in a normal wheat line and a mutant line were different. The level of transcript reached the maximum point at or before 24 h after infection in a normal wheat line, but in the mutant line, the peak values were not reached until 48 h or later, indicating a slower defence response to infection by *F. graminearum* in the mutant (Li *et al.*, 2001).

Northern blot analysis of tobacco β-1,3-glucanase gene (NbPR2) in an infected tobacco plant showed increasing levels of the transcript throughout the infection by *Colletotrichum destructivum* and a faster rate of increase appeared to occur 48 h of post infection, while the level of a control gene (NbEF-1α) transcript showed no significant change (Dean *et al.*, 2002).

Northern blot analysis of a pepper β-1,3-glucanase gene transcript showed that the expression pattern of the infected plants dependent on plant growth stage. At the four leaf stage, accumulation of the pepper β-1,3-glucanase gene transcript was strongly induced at 48 h after infection with *Colletotrichum coccodes*. In contrast, strong induction of the β-1,3-glucanase transcript occurred 24 h after fungal infection at the eight leaf stage (Hong and Hwang, 2002).

2.5.3.2 Real-time PCR for β-1,3-glucanase gene expression studies

Real-time PCR has been used in quantifying the expression levels of β-1,3-glucanase genes in variety of plants.

The expression of two chitinases genes *FaChi2-1* and *FaChi2-2* in strawberry plants inoculated with *Colletotrichum fragariae* was monitored with real-time PCR. The results indicated rapid induction of *FaChi2-1* transcript, which occurred within 2 to 6 h of inoculation. However, induction of *FaChi2-2* transcript occurred slower, at 24 to 48 h after inoculation (Khan and Shih, 2004).

Shi (2005) observed β-1,3-glucanase gene expression in strawberry and reported that expression of FaBG2-3 was much greater than FaBG2-1 in both the uninfected and the infected plants.

2.5.4 β-1,3-glucanase gene expression studies in black pepper

Parab (2000) studied the expression pattern of PR protein in black pepper in relation to the *Phytophthora* foot rot disease. It was found that β-1,3-glucanases activity was relatively higher in *P. colubrinum* than *P. nigrum.* However, phenylalanine ammonia lyase (PAL) activity increased substantially in *P. nigrum* immediately after infection with the fungal pathogen and the rate of increase later declined. In *P. colubrinum*, the PAL activity was found to be low. Chitinases activity was almost nil in healthy and infected plants of both *P. nigrum* and *P. colubrinum*. Thus, it was concluded that, among pathogenesis-related (PR) proteins in black pepper, β-1,3-glucanase and PAL showed positive role in the defence mechanism of black pepper in relation to *Phytophthora* foot rot disease. Achuthan *et al.* (2002) confirmed the accumulation of β-1,3-glucanases in relation to *Phytophthora* foot rot in black pepper.

With this background, the present study was taken up to confirm the role of β-1,3-glucanases in the defence mechanism of black pepper and to unravel the mode of its expression in comparison with the resistant genotype *P. colubrinum.*

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Real-time PCR assay for β-1,3-glucanase gene expression in black pepper (*Piper nigrum* L.)," was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture (COH), Vellanikkara during the period from December 2007 to June 2009. The chemicals used in this study were of good quality (AR grade), and obtained from different reputed firms like, Ambion Inc., USA; Applied Biosystems, USA; Bangalore Genei, India; GE Healthcare, USA; Merck, Germany; Invitrogen, USA; Sigma Aldrich, USA and Sisco Research Laboratory(SRL), India. Plastic wares and glass wares used in the study were obtained from Applied Biosystems, USA; Axygen Biosciences, USA; Borosil and Tarsons India Ltd. Instruments such as PCR thermal cycler (Eppendorf), gel documentation system (UVP), electrophoresis system (Biorad), real-time PCR system (Applied Biosystems 7300 Real-Time PCR system) available at CPBMB were used for the work. The software necessary for gene sequence analysis were accessed at Distributed Information Centre (DIC). The details of work carried out are presented in this chapter.

3.1 Maintenance of source plants

Cuttings required for the study were procured from the pepper garden of CPBMB. Fifty rooted cuttings each of *Piper nigrum* var. Panniyur 1 and *Piper colubrinum* were maintained in mud pots, filled with potting mixture and kept under greenhouse condition.

3.2 Maintenance of the pathogen

Pure culture of *Phytophthora capsici*, obtained from Department of Plant Pathology, COH, was inoculated on carrot agar medium in sterilized Petri plates and incubated at room temperature, 26 ± 2^{0}C. In order to maintain the virulence of

the pathogen, it was inoculated on the rooted cuttings of susceptible genotype of black pepper (Panniyur 1) and then re-isolated.

The artificial inoculation was carried out by adopting culture disc method. The lower surface of the recently matured leaves were inoculated with the mycelia disc (5 mm) of the pathogen after giving pin pricks @ 8 to 10 per 5 mm^2 and the surface of the disc was covered with the moistened cotton. The mycelial disc from seven day old culture of *Phytophthora capsici* was used for inoculation.

The pathogen was re-isolated after three days of inoculation. Infected leaves were washed with tap water and dried using blotting paper. Small bits (5 mm^2) of infected leaf portion along with some healthy areas were surface sterilized with 0.1 per cent mercuric chloride for 15 sec and washed with three changes of sterile water. The bits were then transferred aseptically to a sterile Petri dish containing carrot agar medium amended with the streptomycin sulphate (200 mg/l^2) and carbendazim (800 mg/l^2). After five days of incubation, these leaf bits were again transferred to sterile carrot agar medium without antibiotic/ fungicide for the growth of the pathogen. The re-isolated pathogen was purified by single hyphal tip method and was maintained on carrot agar slants for further studies. Pure cultures were subcultured periodically to maintain the viability of the pathogen.

The rooted cuttings of *P. colubrinum* were inoculated with the culture of *P. capsici* as mentioned above. The inoculated plants were kept for one month under green house condition for the disease development.

3.3 Estimation of β-1,3-glucanase activity

The total β-1,3-glucanase enzyme activity was colourimetrically assayed by the laminarin dinitrosalicylate method of Abeles and Forrence (1970) with the modifications suggested by Pan *et al.* (1991). Parab (2000) used this method for studying β-1,3-glucanase enzyme activity in black pepper with slight modifications in original protocol.

3.3.1 Reagents required for enzyme assay

The details regarding preparation of reagents are provided in Appendix I.

1. Sodium acetate buffer (0.05 M, pH 5)
2. Laminarin (4 per cent)
3. Sodium hydroxide (4.5 per cent)
4. Dinitrosalicylic acid (DNS)
5. Cystein HCl (0.05 M)
6. Phenyl methane sulphonyl fluoride (PMSF) (0.1 M)
7. Ascorbic acid (5 mM)

3.3.2 Preparation of crude enzyme extract

Leaf samples were collected on ice at 0, 2, 6, 12, 24, 48 h intervals each from *P. nigrum* and *P. colubrinum* following inoculation with *P. capsici*. Leaf sample, each of about 500 mg, was taken from the area surrounding the inoculation site and used for preparation of crude enzyme extract.

The samples were ground to fine powder with liquid nitrogen and then homogenized each with 1 ml of sodium acetate buffer using mortar and pestle. It was followed by addition of 50 µl each of ascorbic acid, PMSF, β-mercaptoethanol and 100 µl of cystein HCl. The homogenate was centrifuged at 15000 rpm for ten min at 4^0C. The resultant supernatant was used as source of enzyme for subsequent assay of activity.

3.3.3 Assay for β-1, 3-glucanase

The crude enzyme extract (62.5 µl) was taken followed by addition of equal volume of (62.5 µl) 4 per cent laminarin. The reaction mixture was incubated at 40^0C for 10 min. The reaction was stopped by adding 375 µl of DNS and heating the reaction mix for 5 min at 100^0C. The coloured solution so

obtained was chilled on ice for 5 min and diluted to 10 ml with distilled water. The absorbance was measured at 500 nm using spectrophotometer (Spectronic® 20 Genesys, USA). The blank was the crude enzyme extract mixed with laminarin with zero time incubation.

One unit of enzyme activity was defined as the amount of enzyme, which produce reducing sugar equivalent to 1 µmol of glucose equivalent per 10 min at 40^0C (Pan *et al.*, 1991).

3.4 Isolation of DNA

The plant DNA was isolated from young and tender leaf samples of *P. nigrum* as well as *P. colubrinum* using CTAB extraction method suggested by Rogers and Bendich (1994) with slight modifications.

3.4.1 Reagents

The procedure for preparation of reagents required for DNA isolation is given in Appendix II.

1. CTAB extraction buffer
2. CTAB 10 per cent
3. β-mercaptoethanol
4. Chloroform : isoamyl alcohol (24:1)
5. Chilled isopropanol
6. Ethanol (70 per cent)
7. RNase A (DNase free)
8. TE buffer

3.4.2 Procedure for DNA isolation

- 1 g of leaf sample was ground to a fine powder along with 50 µl of

β-mercaptoethanol in liquid nitrogen and then homogenized with 5 ml of CTAB extraction buffer using mortar and pestle.

- The homogenate was transferred into an Oakridge centrifuge tube and incubated at 65^0C for 20 min in hot water bath with intermittent shaking.
- Following incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the homogenate and mixed briefly by inverting the tubes. This was followed by centrifuging the content at 10000 rpm for 15 min at 4^0C.
- The upper aqueous phase was then separated and mixed with $1/10^{th}$ volume of 10 per cent CTAB and equal volume of chloroform: isoamyl alcohol. It was then centrifuged at 10000 rpm for 15 min at 4^0C.
- The resultant upper aqueous phase was separated and mixed gently with 0.6 volume of chilled isopropanol with slight inversion and incubated overnight at -20^0C to allow the precipitation of DNA.
- The precipitated DNA was pelleted at the bottom of the tube by centrifuging at 10000 rpm for 15 min at 4^0C.
- The pellet was washed with 70 per cent ethanol, air dried and stored at -20^0C after dissolving with 100 µl of TE buffer.

3.4.3 RNase treatment

RNase treatment was carried out to remove RNA from the isolated genomic DNA. Large amount of RNA in the sample can chelate Mg^+ and reduce the efficiency of the PCR (Padmalatha and Prasad, 2006).

- RNase A (10 mg/ml) of about 10 µl, free from DNase, was added to DNA sample diluted up to 400 µl and incubated at 37^0C for 1 h.
- The reaction mix was mixed with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 10000 rpm for 10 min at 4^0C.
- The DNA present in aqueous phase was re-precipitated with 0.6 volume of chilled isopropanol and pelleted out by centrifuging at 10000 rpm for 10 min at 4^0C.
- The pellet was air dried and stored at -20^0C after re-suspending in 50 µl of TE buffer.
- The quality of DNA was checked by Agarose gel electrophoresis and the concentration was determined using Nanodrop spectrophotometer (Nanodrop Technologies, USA).

3.5 Southern hybridization

Southern hybridization was carried out with the protocol suggested by Sambrook and Russel (2001) with slight modifications. The genomic DNA isolated from leaf samples of *P. nigrum* and *P. colubrinum* was restricted with the enzyme *Eco*RI and *Hind*III (BangaloreGenei, India) individually and separated by agarose gel electrophoresis. The electrophoresed samples were further blotted on nylon membrane and hybridized with the β-1,3-glucanase gene specific radio-labelled probe.

3.5.1 Restriction digestion of plant genomic DNA

Following components were added to the microcentrifuge tube to make up the reaction volume to 100 µl.

Plant genomic DNA : 20 µl (10 µg)

10X Assay buffer : 10 µl

Distilled water : 65 µl

*Eco*RI : 5 µl

The reaction mix was incubated overnight at 37^{0}C to allow restriction digestion of genomic DNA. Same reaction was set with another sample of DNA with the enzyme *Hind*III for both *P. nigrum* and *P. colubrinum.*

3.5.2 Agarose gel electrophoresis

The restricted DNA sample was then electrophoresed on 0.8 per cent agarose gel. The details of procedure are as follows.

- Agarose (1.2 g) was dissolved in 150 ml 1X TAE buffer by heating in a microwave oven.
- The solution was then allowed to cool up to 65^{0}C and then added 10 µl of ethidium bromide.
- The dissolved agarose was poured into a clean gel casting tray containing positioned comb and allowed to gel. The gel was then submerged into a 1X TAE buffer in a buffer tank.
- The restricted DNA samples were incubated at 55^{0}C for 5 min and mixed with sample loading dye and loaded into wells.
- The electrophoresis was performed at 20 V till the dye ran 70 to 75 per cent of the total gel (approximately 8 to10 h).
- The gel was viewed over UV transilluminator (Vilber Lourmat Deutschland GmbH, Germany) to observe progress of the run.
- After sufficient run, the gel was trimmed to the required size and used for pre-treatment.

3.5.3 Pre-treatment of the gel

3.5.3.1 Reagents

The procedure for preparation of reagents required for gel pre-treatment is given in Appendix III.

1. Depurination solution
2. Denaturation solution
3. Neutralization solution
4. SSC (Standard Saline Citrate, 20X)

3.5.3.2 Depurination

Depurination was done by soaking the gel in two volumes of depurination solution (0.2 M HCl) with constant gentle agitation until the bromophenol blue turned yellow and xylene cyanol turned yellowish green (approximately 5 to 10 min). Depurination was followed by 3 to 4 washings with sterile distilled water.

3.5.3.3 Denaturation

Denaturation was allowed for 1 h in adequate amount of denaturation solution with constant gentle agitation, replacing the denaturation solution with fresh one after 30 min. Gel was rinsed 3 to 4 times with sterile distilled water following denaturation.

3.5.3.4 Neutralization

Gel was neutralized for 30 min with neutralization solution followed by 3 to 4 washings with sterile distilled water.

3.5.3.5 Pre-treatment

Finally, gel was pre-treated with SSC (6X) for 10 to 20 min before blotting.

3.5.4 Pre-treatment of nylon membrane (Hybond XL, GE Healthcare, USA)

Nylon membrane was carefully cut to a size slightly larger than the gel and soaked in sterile distilled water for 5 min followed by 2X SSC for 10 min.

3.5.5 Transfer of DNA to nylon membrane (Blotting)

The neutral transfer buffer, 10X SSC was used for transfer of DNA from gel onto a nylon membrane through capillary movement. The membrane was handled carefully with gloves and sterile forceps.

3.5.5.1 Setting of blotting assembly .

The unit for Southern capillary transfer was assembled as follows.

- A glass plate was placed on the tray and the tray was filled with 10X SSC buffer up to a level just below the glass plate.
- A wick made of 3 sheets of Whatman No. 3 filter paper slightly wider than the gel was placed on the glass plate overhanging two ends into a transfer buffer. The wick was made wet with 10X SSC and the air bubbles trapped underside were smoothed out with a glass rod.
- The Pre-treated gel was placed on the wick in upside down position such that the portion of gel facing well would be downside. The air bubbles trapped underside the gel were smoothed out with the glass rod. One of the corners of the gel was trimmed with a sharp blade to indicate the orientation of the gel.
- The gel was surrounded with stripes of parafilm from all the sides to prevent short circuiting the transfer buffer directly from the reservoir to paper towels placed on the top of gel.
- The pre-wetted nylon membrane was then placed on the gel and air

bubbles trapped underside the membrane were smoothed out with glass rod. The corner of the nylon membrane, facing to the trimmed corner of the gel, was cut in similar fashion.

- The nylon membrane was then covered with 3 pieces of pre-wetted Whatman No. 3 filter paper and a pipette was rolled over them to remove air bubbles entrapped underside.

- A stack of 6 to 8 pieces of blotting papers followed by a 5 to 8 cm thick stack of paper towels was placed on filter paper to form a blot.

- A small glass plate was kept on the blot along with a weight of 500 g. This allowed the continuous flow of liquid from the reservoir through the gel towards the blot with capillary action. This enabled transfer of denatured DNA from the gel onto the membrane (Plate 1).

- The transfer was allowed for 16 to 24 h.

- Following the transfer, the stack of papers was separated from the blotting assembly to free the membrane.

- The membrane was labelled with a soft lead pencil according to well positions, separated from the gel and air dried for about 30 min.

- The transfer of restricted DNA was confirmed by re-staining the gel with ethidium bromide and observing over UV transilluminator.

3.5.5.2 Immobilization of DNA onto the nylon membrane

The transferred DNA was immobilized on the membrane by exposing air

Plate 1 . Blotting assembly for Southern hybridization

dried nylon membrane to UV light (254 nm) for 7 min inside a UV crosslinker. The membrane is now ready for hybridization. For long term storage, the membrane was dried thoroughly, wrapped loosely in filter paper and stored at room temperature under vacuum for further use.

3.5.6 Probe preparation and radiolabelling

3.5.6.1 Preparation of probe

The β-1,3-glucanase gene specific probe was prepared from PCR amplified product of β-1,3-glucanase gene specific primers (GluF3- ATAGGTG TT/CTGCTATGGA/CATG and GluR3- CCAGCCGAT/CGGCCAC/ACCACTC TC). The amplified product was purified after separating on agarose gel using AxyPrep DNA gel extraction kit (Axygen Biosciences, USA). The eluted amplicon was then analysed for quality and quantity using Nanodrop spectrophotometer and then stored at -20^{0}C.

3.5.6.2 Probe labeling

The β-1,3-glucanase gene specific probe was radiolabelled using Megaprime DNA labelling system (GE Healthcare, USA).

3.5.6.2.1 Reagents

The details of the reagent component are explained in Appendix III.

- Klenow fragment (3 units/µl)
- Random primer
- Reaction buffer
- dGTP, dCTP, dTTP
- Nuclease free water

- α-^{32}P labelled dATP
- Probe DNA

3.5.6.2.2 Probe labelling procedure

1. Amplified and eluted PCR product of section 3.5.6.1 was diluted to a final concentration of 5 ng/µl in nuclease free water.
2. 25 µl of diluted probe DNA was taken into a sterile Eppendorf tube holed at cap followed by addition of 5 µl random primer.
3. The content was denatured by heating in boiling water bath for 5 min.
4. It was followed by brief spinning to recollect the content at the bottom of the tube and kept at room temperature.
5. The reagents were added to the above content one by one as follows
    a. Unlabelled nucleotides 4 µl each of dGTP, dCTP, dTTP
    b. Reaction buffer 5 µl
    c. Klenow fragment 2 µl
    d. Nuclease free water 16 µl (to make final volume 50 µl)
6. The reaction mix was spinned to recollect the content at the bottom.
7. Finally, 5 µl of α-^{32}P labelled dATP was carefully added and mixed briefly by pipetting in and out.

8. The reaction mix was spinned briefly and incubated for 1 h at room temperature.

9. Following incubation, the reaction was stopped by adding 5 µl of EDTA (0.2 M).

10. Before adding into hybridization solution, the radiolabelled probe was denatured by heating in boiling water bath for 5 min followed by chilling on ice.

3.5.7 Hybridization

Hybridization was divided into two steps- prehybridization and hybridization.

3.5.7.1 Prehybridization

Membrane blot of section 3.5.5.2 was pre-wetted in 6X SSC for 5 min and transferred to a plastic box containing 50 ml of pre-warmed 1X prehybridization solution (Bangalore Genei, India). The box was sealed with a lid and incubated at 62^0C for 2 h in hybridization oven (Amersham Biosciences, USA) with a constant agitation.

3.5.7.2 Hybridization

The prehybridization solution in the plastic container was replaced by 50 ml of pre-warmed 1X hybridization solution (Bangalore Genei, India) of same quantity. The heat denatured radiolabelled probe of section 3.5.6.2.2 was recollected at the bottom of the tube by brief spinning and then added to the pre-warmed hybridization solution in the box. The solution was agitated to distribute the probe evenly into the solution. The prehybridized nylon membrane was placed into the box containing above solution and incubated overnight at 62^0C with constant agitation in hybridization oven after resealing the box with the lid.

3.5.8 Post hybridization washings

Post hybridization washings were given to remove the non-specifically bound probes from the membrane and to minimize the background signal interference. Each of the stringency wash was monitored with a radioactivity monitor to retain sufficient activity on the membrane. The washings were given as follows.

- The membrane was taken out of hybridization and placed in a solution containing 2X SSC and 0.5 per cent SDS for 5 min at room temperature with intermittent shaking.
- Second washing was given with a solution containing 2X SSC and 0.1 per cent SDS for 15 min at room temperature with intermittent shaking.
- The membrane was then transferred to a solution containing 0.1X SSC and 0.5 per cent SDS and incubated at 62^0C for 30 min. If necessary, this was repeated once again.
- Finally the membrane was rinsed with 0.1X SSC at room temperature and allowed to air dry on a pad of filter paper for about 30 min to 1 h.

3.5.9 Image analysis by phosphorimager

The air dried membrane was exposed to an erased Imaging plate (IP) (Fugifilm Life Sciences, USA) for about 2 h. The membrane was removed from the IP and stored in dry desiccators for further use.

The Phosphorimager (Fugifilm Life Sciences, USA) was switched on 1 h before the image analysis to warm it up.

The invisible image of the nylon membrane imitated on IP was analyzed with the Phosphorimager using FLA-5100 image analysis software (Fugifilm Life Sciences, USA) for Windows®.

3.6 Isolation of RNA

The TRIzol® (Invitrogen, USA) method described by Chomczynski and Sacchi (1987) was used for isolation of RNA with slight modifications. Young and tender leaf samples from *P. nigrum* and *P. colubrinum*, both control as well as inoculated with the pathogen, were used for RNA isolation. RNA was isolated from both the genotypes at healthy stage and at different time intervals (1, 2, 6, 12 and 24 h) after inoculation with *P. capsici*.

3.6.1 Reagents

The details of the reagents are given in Appendix II.

- TRIzol® reagent (Invitrogen, USA)
- Chloroform
- Chilled isopropanol
- Ethanol 80 per cent
- DEPC (Sigma-Aldrich, USA)
- RNaseZap (Ambion, USA)
- DEPC treated double autoclaved water (nuclease free water)

All reagents were prepared with DEPC treated double autoclaved water.

3.6.2 General precautions

RNases, being highly stable, special care was taken to avoid contamination, while doing RNA isolation.

All glasswares, mortars and pestles, microtips and microcentrifuge tubes were treated with DEPC treated water (0.025 per cent) and autoclaved twice. Reagents were prepared by dissolving them in double autoclaved DEPC treated water. Electrophoresis unit and pipettes were cleaned with RNaseZap (Ambion, USA) followed by rinsing with double autoclaved DEPC treated water. Gloves were used throughout the experiment to avoid RNase contamination resulting from skin secretions.

3.6.3 Procedure

1. Leaf sample (100 mg) was ground to fine powder with liquid nitrogen using mortar and pestle.

2. To the powdered mass, 1 ml of TRIzol® reagent was added and allowed to thaw with intermittent mixing of the content.

3. Chloroform (200 µl) was added to the above homogenate and mixed by vigorous shaking. It was allowed to stand for two min at room temperature.

4. The content was centrifuged at 13000 g for 15 min at 4^0C.

5. The upper aqueous phase of about 50 per cent of total was carefully transferred to a fresh microcentrifuge tube.

6. Chilled isopropanol (500 µl) was added to the supernatant and mixed gently with slight inversion to precipitate the RNA. The content was centrifuged at 13000 g for 15 min at 4^0C to pellet out the precipitated RNA.

7. The supernatant was discarded and pellet was washed with 80 per cent ethanol.

8. The pellet was air dried for about 10 min and dissolved in 20 µl of nuclease free water.

3.6.4 Quality analysis of total RNA

The quality of RNA was checked with Agarose gel electrophoresis using MOPS buffer. The RNA samples were run in 0.8 per cent agarose gel with MOPS buffer keeping the voltage at 90 V. The gel was allowed to run for about 70 per cent of total and observed under UV light.

3.6.5 Spectrophotometric analysis of total RNA

The quality and quantity of RNA was estimated using Nanodrop spectrophotometer (NanoDrop Technologies Inc., USA). The concentration and quality of total RNA was generated automatically by the instrument. The ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} were recorded to assess the quality of RNA. A ratio of OD_{260}/OD_{280} between 1.8 and 2, and OD_{260}/OD_{230} above 1.0 indicates good quality of RNA.

3.7 cDNA synthesis

cDNA was synthesized from total RNA using Genei M-MuLV RT-PCR kit (BangaloreGenei, India).

3.7.1 Materials

- RNA sample
- Random hexamer (1 μg/μl)
- 5X M-MuLV RT buffer
- 30 mM dNTP mix (7.5 mM each)
- DTT (0.1 M)
- M-MuLV reverse transcriptase (50 units/μl)
- Autoclaved DEPC treated water

3.7.2 Procedure

1. 9 µl total RNA (~1500 µg RNA) was added to a 0.2 ml microcentrifuge tube.
2. 1µl of random primer was added and the reaction mix was incubated at 65^0C for 10 min and then at room temperature for 2 min to remove any secondary structure present in the RNA sample.
3. The reaction mix was spinned briefly to collect the content at the bottom of the tube and the reagents were added one by one as follows.
    a. 1 µl RNase inhibitor
    b. 1 µl DTT (0.1 M)
    c. 4 µl RT buffer (5X)
    d. 2 µl dNTP mix (30 mM)
    e. 0.5 µl M-MuLV reverse transcriptase
    f. 1 µl nuclease free water
4. The content was mixed well, spinned and incubated at 37^0C for 1 h.
5. Final incubation was done at 95^0C for 2 min to denature RNA-cDNA hybrids.
6. The reaction mix was spinned briefly and transferred immediately on ice.
7. cDNA was then stored at -20^0C for long term storage and was later used for real-time PCR assay.

8. In order to detect contamination with genomic DNA, NAC (No Amplification Control) was included in the reaction without adding reverse transcriptase.

3.8 Northern Hybridization

3.8.1 Dot Blot for total RNA

Dot blot was performed with total RNA from both *P. nigrum* and *P. colubrinum*, isolated at different intervals after inoculation with *P. capsici*. The dot blot analysis was done as per the manufacturer's protocol (Amersham Hybond XL membrane product booklet, GE Healthcare, USA). The reagents were prepared in nuclease free water and care was taken to avoid RNase contamination.

3.8.1.1 Preparation of RNA sample

1. The denaturation reaction (total 30 µl) for total RNA sample was set in a microcentrifuge tube as follows.

    a. RNA (upto 20 µg) : 1.0 µl (1 µg total RNA)

    b. 10X MOPS buffer : 1.5 µl (0.5X)

    c. Formaldehyde : 5.5 µl (2.2 M)

    d. Formamide : 15.0 µl (50 per cent)

    e. Nuclease free water : 7.0 µl

2. The reaction mix was incubated for 15 min at 55^0C.

3. Separate reaction mixes were prepared for RNA sample isolated at different intervals after inoculation with the pathogen both from *P. nigrum* and *P. colubrinum*.

3.8.1.2 Dot blotting by manual procedure

The RNA samples prepared in section 3.8.1.1 were used for blotting manually on Hybond XL nylon membrane (GE Healthcare, USA). Details of the procedure are as follows.

1. The Hybond XL nylon membrane was cut to an appropriate size and marked with pencil to form grids, keeping the area for each grid 1 cm^2.
2. The membrane was pre-wetted with 2X SSC and used for blotting.
3. The denatured RNA sample (2 µl) was carefully applied at the centre of the grid with micropipette without touching the end of the tip to the membrane. The sample was allowed to dry and again applied with the same sample with the equal quantity. This was repeated to finish the entire RNA sample.
4. The above procedure was repeated for all RNA samples, leaving 1 cm space in between two different samples.
5. The damp membrane was placed on a piece of filter paper and irradiated at 254 nm for 1 min 45 seconds at 1.5 J/cm^2 in an UV crosslinker.
6. The UV crosslinked membrane was dried thoroughly, wrapped loosely in filter paper and stored at room temperature under vacuum.

3.8.1.3 Probe preparation and radiolabelling

3.8.1.3.1 Preparation of probe

The β-1,3-glucanase gene specific probe was prepared from RT-PCR amplified product with β-1,3-glucanase gene specific primers from cDNA sample. The amplified product was purified after separating on agarose gel using AxyPrep DNA gel extraction kit (Axygen Biosciences, USA). The eluted probe was then

analysed for quality and quantity using Nanodrop spectrophotometer. The probe was then stored at -20^{0}C.

3.8.1.3.2 Probe labelling

The β-1,3-glucanase gene specific cDNA probe was radiolabelled using Megaprime DNA labeling system (Amersham Biosciences, USA). The procedure described in section 3.5.6.2 was also followed for labelling to cDNA probe.

3.8.1.4 Hybridization

Hybridization was divided into two steps- prehybridization and hybridization. The protocol for hybridization and prehybridization was repeated as per section 3.5.7 with little modification. The incubation temperature was decreased to 58^{0}C for both steps and hybridization was carried out using β-1,3-glucanase specific cDNA probe.

3.8.1.5 Post hybridization washings

Post hybridization washings were performed as per the section 3.5.8.

3.8.1.6 Image analysis by phosphorimager

Image analysis was done for the hybridized nylon membrane as per the section 3.5.8.

3.8.2 Northern Hybridization for total RNA

Northern hybridization was performed for RNA samples isolated at different intervals after inoculation with the pathogen both from *P. nigrum* and *P. colubrinum* as per the manufacturer's protocol (Amersham Hybond XL membrane product booklet, GE Healthcare, USA).

3.8.2.1 Agarose gel electrophoresis

3.8.2.1.1 Reagents

All reagents were prepared with DEPC-treated water. Details of preparation of each component are given in Appendix II.

- Ethidium bromide (200 µg/ml)
- Formaldehyde (12.3 M)
- Formamide (Invitrogen, USA)
- 10X formaldehyde gel-loading buffer
- 10X MOPS buffer

3.8.2.1.2 Preparation of agarose gel containing 2.2 M formaldehyde

Agarose (1.5 g) was added to 130 ml of sterile water and dissolved by boiling in a microwave oven for preparation of 1.0 per cent agarose gel. The solution was cooled to 55^0C. Formaldehyde (7.9 ml) and 10X MOPS buffer (15 ml) were preheated to 55^0C and added to the above agarose solution. Agarose gel was casted in a gel casting tray with slots formed by a comb with at least four more teeth than the number of RNA samples under test. The gel was allowed to set for at least 1 hour at room temperature and covered thereafter with Saran wrap until the samples were ready to be loaded.

3.8.2.1.3 Protocol for agarose gel electrophoresis

1. The denaturation reaction (total 30 µl) for total RNA sample was set in a microcentrifuge tube as follows.

a. RNA	:	4.0 µl (20 µg total RNA)
b. 10X MOPS buffer	:	1.5 µl (0.5X)

c. Formaldehyde : 5.5 µl (2.2 M)

d. Formamide : 15.0 µl (50 per cent)

e. Nuclease free water : 4.0 µl

2. The reaction mix was incubated for 15 min at 55^0C.

3. 3 µl of 10X formaldehyde gel-loading buffer was added and again the reaction mix was transferred to an ice box.

4. The agarose formaldehyde gel was installed in a horizontal electrophoresis unit. The buffer tank was filled sufficiently with 1X MOPS buffer to cover the gel to a depth of nearly 1 mm.

5. The gel was run for 5 min at 5 V/cm, and then the RNA samples were loaded into wells, leaving the two outermost lanes on each side of the gel empty.

6. The gel was run submerged in 1X MOPS buffer at 5 Vcm^{-1} until the bromophenol blue migrated nearly 50 per cent (approximately 2 h). Higher voltage during electrophoresis leads to smearing of bands.

7. The electrophoresis buffer was replaced manually every hour with new one, to avoid fluctuations in pH during the run.

8. The RNA was visualized by placing the gel on UV transilluminator.

9. The gel was then trimmed to an appropriate size with a sharp blade.

3.8.2.2 Pre-treatment of the gel

1. The gel was placed in a suitable tray containing plenty of DEPC-treated water and incubated for 15 min with gentle agitation.

2. The DEPC-treated water was replaced with sterile 10X SSC and incubated for 15 min. It was repeated once more.

3.8.2.3 Pre-treatment of the nylon membrane (Hybond XL)

The Hybond XL nylon membrane was cut to the size slightly larger than the gel (1 mm) with a sterile scalpel. The membrane was handled carefully with gloves at each and every step.

The membrane was floated on the surface of deionized water in a glass dish for 5 min followed by treatment with 2X SSC for 10 min.

3.8.2.4 Transfer and fixation of denatured RNA to the nylon membrane

The denatured RNA sample was transferred onto a Hybond XL nylon membrane (Amersham Biosciences, USA) with neutral transfer method (Sambrook and Russel, 2001).

3.8.2.4.1 Reagent

- 20X SSC (Prepared in DEPC treated water)

3.8.2.4.2 Assembly of the apparatus and blotting

The apparatus for Northern blotting was assembled as follows. Care was taken during each step to avoid any contamination with RNases.

- A glass plate was placed on the tray and the tray was filled with 10X SSC buffer up to a level just below the glass plate.
- A wick made of 3 sheets of Whatman No. 3 filter paper slightly wider than the gel was placed on the glass plate overhanging two ends into a transfer buffer. The wick was made wet with 10X SSC and the air bubbles trapped underside were smoothed out with glass rod.
- The Pre-treated gel was placed on the wick in upside down position such that the portion of gel facing well would be downside. The air bubbles trapped underside the gel were smoothed out with glass

rod. One of the corners of the gel was trimmed with a sharp blade to indicate the orientation of the gel.

- The gel was surrounded with strips of parafilm from all the sides to prevent short circuiting the transfer buffer directly from the reservoir to paper towels placed on the top of gel.

- The pre-wetted nylon membrane was then placed on the gel and air bubbles trapped underside the membrane were smoothed out with glass rod. The corner of the nylon membrane, facing to the trimmed corner of the gel, was cut in similar fashion.

- The nylon membrane was then covered with 3 pieces of pre-wetted Whatman No. 3 filter paper and a pipette was rolled over them to remove air bubbles entrapped underside.

- A stack of 6 to 8 pieces of blotting papers followed by a 5 to 8 cm thick stack of paper towels was placed on filter paper to form a blot.

- A small glass plate was kept on the blot along with a weight of 500 g. This allowed the continuous flow of liquid from the reservoir through the gel towards blot with capillary action. This enabled transfer of denatured RNA from the gel onto the membrane.

- The transfer was allowed for overnight.

- Following the transfer, the stack of papers was separated from the blotting assembly to free the membrane.

- The membrane was labelled with a soft lead pencil according to well positions, separated from the gel and used for UV crosslinking.

- Complete transfer of RNA was confirmed by re-staining the gel with ethidium bromide and observing on UV transilluminator.

3.8.2.4.3 Immobilization of RNA to the membrane

The damp membrane was placed on a piece of blotting paper and irradiated at 254 nm for 1 min 45 seconds at 1.5 J/cm^2 in a UV crosslinker.

The UV crosslinked membrane was dried thoroughly and wrapped loosely in aluminium foil or filter paper. The covered membrane was stored at room temperature preferably under vacuum.

3.8.2.5 Probe preparation and radiolabelling

The β-1,3-glucanase gene specific cDNA probe was prepared and used for Northern hybridization after radiolabelling with α-^{32}P dATP as per the section 3.8.1.3.

3.8.2.6 Hybridization

Hybridization was divided into two steps- prehybridization and hybridization. The protocol for hybridization and prehybridization was repeated as per section 3.5.7 with little modification. The incubation temperature was decreased to 58^{0}C for both steps and hybridization was carried out using β-1,3-glucanase specific cDNA probe.

3.8.2.7 Post hybridization washings

Post hybridization washings were performed as described in the section 3.5.8.

3.8.2.8 Image analysis by phosphorimager

Image analysis was done for the hybridized nylon membrane as per the section 3.5.8.

3.9 Real-time PCR assay

Real-time PCR assay was performed with the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, USA).

The assay was performed both with genomic DNA and cDNA, derived from total RNA samples isolated at different intervals after infection with *P. capsici*, both from *P. nigrum* and *P. colubrinum*. The primers specific to β-1,3-glucanase and housekeeping genes, to be used for standardization and selection endogenous control, were designed and used for amplification. The absolute quantification and relative quantification was performed to determine gene copy number and relative gene expression respectively for β-1,3-glucanase gene in both *P. nigrum* and *P. colubrinum*.

3.9.1 Primer designing

Primers were designed for the assay according to criteria available for real-time PCR. The primer designing was done with Primer Express® Software Version 3.0 (Applied Biosystems, USA).

3.9.1.1 Criteria for designing primers

- Amplicon length – 50-150 bp for optimum PCR efficiency.
- Optimal primer length- 20 bases.
- Tm (melting temperature)- 58 to 60^{0}C (Optimum Tm- 59^{0}C).
- GC per cent- 30 to 80 per cent.
- The last five nucleotides at the 3' end contain no more than two G + C residues.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.

- In case of gene expression studies with real-time PCR, the amplicon should span one or more introns to avoid amplification of the target gene from the contaminated genomic DNA.

- If the gene does not contain intron, then run RT minus control to observe contamination of RNA with genomic DNA.

3.9.1.2 Procedure

Primers were designed for the housekeeping genes to be used as endogenous control and for the β-1,3-glucanase gene. Since there is no report for the reference gene to be used for normalization of β-1,3-glucanase gene expression in black pepper against *Phytophthora* rot disease, three housekeeping genes commonly used in plants for normalization of gene expression under biotic stress were evaluated in the present study. They include 18S rRNA, actin and GAPDH.

- Conserved sequences for β-1,3-glucanase, 18S rRNA, actin and GAPDH were downloaded from NCBI GenBank and used for primer designing. These details about the sequence have been given in Appendix V.

- The protocol was followed as given below in the Primer Express® Software Version 3.0,

  File > New >TaqMan® MGB Quantification or TaqMan® Quantification > OK

- The sequence for which primer had to be designed was copied in the sequence tab.

- Parameters for primers were set according to the requirement (as detailed in section 3.9.1.1) and primers were obtained by clicking "Find Primers /Probe" option in the "Tools" menu bar.

- Primers were selected with a low penalty score and low amplicon length.
- The primers designed were ordered in lyophilized form from Applied Biosystems, USA.

3.9.2 Screening of primers for amplification

The primers were screened to check the amplification with genomic DNA as well as cDNA. The protocol was followed as given by the manufacturer (Applied Biosystems, USA). Each reaction was set in triplicate leaving one without template in order to treat as control (NTC) to monitor the formation of primer dimer. The basic protocol was set as follows.

3.9.2.1 Real-time PCR reaction mix (20 µl each)

➢ SYBR® Green master mix (Applied Biosystems, USA)	:	10.0 µl
➢ Forward primer	:	2.0 µl (1000 nM)
➢ Reverse primer	:	2.0 µl (1000 nM)
➢ Sample DNA	:	2.0 µl (100 ng)
or cDNA sample	:	2.0 µl (10 ng)
➢ Nuclease free water	:	4.0 µl

3.9.2.2 Operating procedure for real-time PCR machine

The operating procedure for creating a document in real-time PCR was followed as per the guidelines of manufacturer (Applied Biosystem's Absolute Quantification getting started guide). Details of the procedure are as follows together for both absolute and relative quantification.

1. Switch on the computer followed by real-time PCR system.
2. Select **Start > Programs > Applied Biosystems > 7300 SDS Software** (🔍) to start SDS Software 1.3.1.
3. Select **File > New.**
4. In the assay drop-down list of the New Document Wizard, select **Absolute Quantification (Standard Curve) / Relative Quantification (ddCt) Plate / Relative Quantification (ddCt) Study.**
5. Accept the default setting for Container and Template (**96-Well Clear** and **Blank Document).**
6. Enter a name in the default Plate Name field or accept the default name and click **Next >.**
7. Select detectors to add to the plate document and click **Add>** to add the detectors plate document, then click **Next>.**
8. Specify detectors and task for each well.
    a. Click on a well to select it.
    b. Click on the detector name to select the detector for the well.
    c. Click under the Task column to assign the detector task.
    d. Enter a quantity for wells that contain standards.
    e. Click **Use**
    f. Click **Finish.**

    The SDS Software 1.3.1 creates the plate document.
9. Enter the sample names.

10. Verify the information on each well in the Setup tab.

11. Select **Instrument** tab. By default, the standard PCR conditions for the two-step RT-PCR method are displayed.

12. Enter the sample volume.

13. While using SYBR® Green master mix, click **Add Dissociation Stage** to know about presence of contamination.

14. Select **File > Save As** and save the file as SDS document (**.sds**) by clicking **Save** after entering the name.

15. Load the plate or eight tube stripes into the instrument.

16. Click **Start.**

As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence emissions. After the run, the status values and buttons are grayed out, the Analysis button is enabled (▷), and a message indicates whether or not the run is successful.

3.9.2.3 The thermal profile for real-time PCR

The thermal profile was set as per Table 3 and assay was performed as absolute quantification in 7300 System SDS software for real-time PCR (Applied Biosystems, USA). Dissociation stage was added to detect the formation primer dimer and non-specific product.

3.9.3 Absolute quantification for estimation of β-1,3-glucanase gene copy number

Absolute quantification for estimation of gene copy number for β-1,3-glucanase gene was done using standard curve method.

3.9.3.1 Standardization of primer concentration

The standardization of optimum primer concentration was done by screening with different concentrations of primers keeping all the other reagents constant. The different concentrations screened for forward and reverse primer includes 50, 150, 300, 450, 600, 750, 900 nM concentration for each primer. Each of the reaction was set in triplicate leaving one for NTC. The thermal profile was set as per Table 3 and assay was performed as absolute quantification in 7300 System SDS software for real-time PCR (Applied Biosystems, USA). The reaction set up was done as per the Table 4.

3.9.3.2 Standardization of DNA concentration

Different concentrations of genomic DNA ranging from 10 ng to 100 ng were tried to standardize the assay for optimum DNA concentration (Table 5). The thermal profile was set as per Table 3 and assay was performed as absolute quantification in 7300 System SDS software for real-time PCR (Applied Biosystems, USA).

3.9.3.3 Preparation of standards

For absolute quantification by standard curve method, β-1,3-glucanase gene specific standard was prepared from PCR amplified product with β-1,3-glucanase gene specific primers. The amplicon was purified after separating on 2 per cent agarose gel using AxyPrep DNA gel extraction kit (Axygen Biosciences, USA).

Concentration of the purified PCR product was determined with Nanodrop spectrophotometer and its corresponding concentration was converted into copies per microlitre by using the Avogadro constant (6.023×10^{23}) and its molecular weight (number of bases of the PCR product multiplied by the average molecular weight of a pair of nucleic acids, which is 660 Da) (Sambrook and Russel, 2001).

Table 3. The thermal profile for real-time PCR

Stage	Function	Temperature	Time	Cycles
1	Hotstart	50^0C	2 min	1
2	Initial Denaturation	95^0C	10 min	1
3	Denaturation/Melt	95^0C	15 sec	40
4	Annealing/Extension	60^0C	1 min	
5	Dissociation	95^0C	15 sec	1
		60^0C	30 sec	1
		95^0C	15 sec	1

Table 4. Standardization of primer concentration for absolute quantification

Sr. No.	Primer conc. (nM)	SYBR (µl)	FP (µl)	RP (µl)	DNA (µl)	H_2O (µl)	Total (µl)
1.	50*50	10	0.2	0.2	2	7.6	20
2.	150*150	10	0.6	0.6	2	6.8	20
3.	300*300	10	1.2	1.2	2	5.6	20
4.	450*450	10	1.8	1.8	2	4.4	20
5.	600*600	10	2.4	2.4	2	3.2	20
6.	750*750	10	3	3	2	2	20
7.	900*900	10	3.6	3.6	2	0.8	20

Primer conc. – Primer concentration

FP - Forward primer, RP - Reverse primer

Primer stock concentration - 5 µM

SYBR - SYBR® Green master mix-2X

H_2O - sterile MilliQ water

DNA concentration final-100 ng per reaction

Table 5. Standardization of DNA concentration for absolute quantification

Sr. No.	DNA conc. (ng)	SYBR (µl)	FP (µl)	RP (µl)	DNA (µl)	H_2O (µl)	Total (µl)
1.	10	10	1.2	1.2	2	14.4	20
2.	20	10	1.2	1.2	2	14.4	20
3.	40	10	1.2	1.2	2	14.4	20
4.	50	10	1.2	1.2	2	14.4	20
5.	60	10	1.2	1.2	2	14.4	20
6.	80	10	1.2	1.2	2	14.4	20
7.	100	10	1.2	1.2	2	14.4	20
8.	NTC	10	1.2	1.2	0	16.4	20

FP - Forward primer, RP - Reverse primer

Primer stock concentration - 5 µM

SYBR - SYBR® Green master mix-2X

H_2O - sterile MilliQ water

Primer concentration final – 300 nM each of forward and reverse primer

Tenfold dilutions were made for the β-1,3-glucanase gene specific standard and stored in aliquots at -20^{0}C and used throughout the assay.

3.9.3.4 Estimation of gene copy number

To quantify gene copy number, tenfold serial dilutions were made for standards ranging from 1×10^{12} to 1×10^{1}. The reaction setup was carried out as given below for each standard dilution and unknown sample. Each reaction was set in triplicate leaving one for NTC.

Real-time PCR reaction mix (20 µl)

- SYBR® Green master mix : 10.0 µl
- Forward primer : 1.2 µl (300 nM)
- Reverse primer : 1.2 µl (300 nM)
- Sample DNA : 2.0 µl (100 ng)
- Nuclease free water : 4.0 µl

The thermal profile was set as per Table 3 and assay was performed as Absolute Quantification in Applied Biosystems's SDS 1.3.1 system software for real-time PCR (Applied Biosystems, USA).

Standard curve was generated by plotting C_T (threshold cycle) values corresponding to each standard dilution against corresponding log number of the standard concentration (expressed as copies μl^{-1}). By knowing the C_T value for unknown sample, the copy number was calculated based on standard curve (Chini *et al.*, 2007).

3.9.4 Relative quantification for detection of β-1,3-glucanase gene expression activity

The expression of β-1,3-glucanase gene was determined with relative quantification normalizing the data with the stable housekeeping gene.

3.9.4.1 Standardization of primer concentration

The standardization of optimum primer concentration was done by screening with different concentrations of primers keeping all other reagents constant. The different concentrations screened for forward and reverse primer for β-1,3-glucanase, 18S rRNA, actin and GAPDH includes 50, 300, 600, 900nM concentration for each primer in various combinations. Each of the reaction was set in triplicate leaving one for NTC. The thermal profile was set as per Table 3 and assay was performed as Absolute Quantification in 7300 System SDS software for real-time PCR. The reaction set up was done as discussed in Table 6.

3.9.4.2 Standardization of cDNA concentration

Different dilutions were made for cDNA obtained from total RNA isolated at 12^{th} hour interval from *P. nigrum*. These dilutions were amplified with β-1,3-glucanase gene specific primer in real-time PCR to get optimum cDNA concentration for relative quantification. The reaction was set as per Table 7 and the thermal profile was set as per Table 3. The real-time PCR assay was performed as Absolute Quantification plate document.

3.9.4.3 Selection of reference gene for relative quantification

The standardization of reference gene was performed in order to obtain stably expressed housekeeping gene to normalize gene expression data. Three genes viz., 18S rRNA, actin and GAPDH were used for standardization of reference gene along with β-1,3-glucanase. The cDNA obtained from total RNA isolated at different intervals (0, 1, 2, 6, 12, 24 h) after infection with *P. capsici* from *P. nigrum* was used for amplification. The reaction was set up separately for

Table 6. Standardization of primer concentration for relative quantification

Sr. No.	Primer conc. (nM)	SYBR (µl)	FP (µl)	RP (µl)	DNA (µl)	H_2O (µl)	Total (µl)
1.	50*50	10	0.2	0.2	2	7.6	20
2.	50*300	10	0.2	1.2	2	6.6	20
3.	50*900	10	0.2	3.6	2	4.2	20
4.	300*50	10	1.2	0.2	2	6.6	20
5.	300*300	10	1.2	1.2	2	5.6	20
6.	300*900	10	1.2	3.6	2	3.2	20
7.	900*50	10	3.6	0.2	2	4.2	20
8.	900*300	10	3.6	1.2	2	3.2	20
9.	900*900	10	3.6	3.6	2	0.8	20

Primer conc. – Primer concentration

FP - Forward primer, RP - Reverse primer

Primer stock concentration - 5 µM

SYBR - SYBR® Green master mix-2X

H_2O - sterile MilliQ water

cDNA dilution - 1:1

Table 7. Standardization of cDNA concentration for relative quantification

Sr. No.	cDNA dilutions	SYBR (µl)	FP (µl)	RP (µl)	DNA (µl)	H_2O (µl)	Total (µl)
1.	1	10	1.2	1.2	2	14.4	20
2.	1:1	10	1.2	1.2	2	14.4	20
3.	1:5	10	1.2	1.2	2	14.4	20
4.	1:10	10	1.2	1.2	2	14.4	20
5.	1:20	10	1.2	1.2	2	14.4	20
6.	1:50	10	1.2	1.2	2	14.4	20
7.	1:80	10	1.2	1.2	2	14.4	20
8.	1:100	10	1.2	1.2	0	16.4	20

FP - Forward primer, RP - Reverse primer

Primer stock concentration - 5 µM

SYBR - SYBR® Green master mix-2X

H_2O - sterile MilliQ water

Primer concentration final – 300 nM each of forward and reverse primer

each cDNA sample and each gene as per the standardization. The thermal profile was set as per Table 3 except dissociation stage. The assay was performed as Relative Quantification (ddC_T) plate document in 7300 System SDS software.

The data obtained after relative quantification was analyzed with Relative Quantification (ddC_T) Study in 7300 System SDS software for threshold cycles as well as to see gene expression relative to each of the housekeeping gene amplified. The C_T (threshold cycle) values, obtained after analysis, were analyzed using MS Excel based NormFinder (Molecular Diagnostic Laboratory, Aarhus University Hospital, Denmark) and geNorm (PrimerDesign Ltd., USA) software tool for Windows® platform.

3.9.4.4 Relative quantification for β-1,3-glucanase gene expression assay using comparative C_T ($2^{-\Delta\Delta CT}$) method

The cDNA representing the total RNA, isolated from *P. nigrum* and *P. colubrinum* at 0, 1, 2, 6 and 12 h after inoculation with *P. capsici* was used for relative quantification of β-1,3-glucanase gene expression with real-time PCR.

Each of the reaction was set in triplicate with 20 µl volume. The amplification was carried out for both β-1,3-glucanase gene and GAPDH as a reference. The primer concentration used for β-1,3-glucanase gene was 300 nM for both of the primers while that of GAPDH it was 900 nM for forward primer and 300 nM for reverse primer. 4 µl of each 1:5 diluted cDNA sample was used for each reaction. The reaction set up was done according to standardized protocol and the thermal profile was set as per Table 3 except dissociation stage. The assay was performed as Relative Quantification (ddC_T) plate in 7300 System SDS software for real-time PCR.

The data obtained after relative quantification was analyzed using the comparative C_T method ($\Delta\Delta C_T$) using Applied Biosystems Relative Quantification (ddC_T) study software (Livak and Schmittgen, 2001).

3.9.5 Analysis of real-time PCR data

Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. Threshold is the line whose intersection with the amplification plot defines the threshold cycle (C_T). The threshold cycle value is inversely proportional to the logarithm of the initial copy number. For ideal amplification, the threshold cycle value should fall within 15 to 35.

3.9.5.1 Amplification pattern

The amplification pattern observed was scored as poor, average and good, based on graphical representation obtained.

1. Good- Threshold cycle value at or before 30^{th} cycle with Rn (Reporter normalized) value higher than four.

2. Average- Threshold cycle value between 30 and 35 with Rn value between two and four.

3. Poor- threshold cycle value over 35 and Rn value below two.

3.9.5.2 Dissociation curve

Dissociation curve indicates the optimum melting temperature for denaturation of real-time PCR product at the end of the reaction. The nature of dissociation curve was categorized into

1. Satisfactory- Single sharp peak near the expected melting temperature.

2. Unsatisfactory- Multiple peaks or peaks much earlier to expected melting temperature.

3.9.5.3 Standard curve

The standard curve was obtained by plotting log value of different dilutions of standard DNA against the threshold cycle (C_T). Standard curve obtained after amplification in real-time PCR for different dilutions of DNA was categorized into two based on its slope and R^2 value.

1. Good- $-3.6 < \text{Slope} < -3.1$ and R^2 value > 0.99.
2. Poor- $-3.6 > \text{Slope} > -3.1$ and R^2 value < 0.99.

Slope of the curve and R^2 value ideally indicates the efficiency of the reaction.

The efficiency of the reaction was calculated with the formula-

$$\textbf{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Results

4. RESULTS

The results generated during the investigations related to real-time PCR assay for β-1,3-glucanase gene in black pepper (*Piper nigrum* L) are presented in this chapter.

4.1 Maintenance of source plants

Cuttings of *P. nigrum* and *P. colubrinum* showed vigorous and healthy growth under the management practices described (Plate 2A and 2B). The cuttings started sprouting within 12 to 18 days of planting. Sprouting was observed earlier in *P. colubrinum* (12 DAP) compared to *P. nigrum* (18 DAP).

It is also observed from the Table 8 that, sprouting percent and survival were higher in *P. colubrinum*, which recorded 100 and 90 per cent against 92 and 80 per cent in *P. nigrum.*

4.2 Maintenance of pathogen

P. capsici culture was maintained on carrot agar medium. *P. capsici* showed profuse growth with uniform dense, cotton wool like aerial mycelium on carrot agar medium. The mycelial growth was observed within 24 h of inoculation and took 14 to 15 days to cover the entire plate when maintained under 26 ± 2^0C (Plate 4A).

On artificial inoculation of *P. capsici* on the healthy leaves of black pepper, pale water soaked lesions appeared within 18 h of inoculation which later turned black. Lesions gradually enlarged covering large areas of leaves (Plate 4B). The leaves became flaccid and got detached from plant within five days after infection. The virulent pathogen re-isolated from the inoculated leaves was used for the study (Plate 4D).

Artificial inoculation of *P. capsici* on *P. colubrinum* did not give any symptom even up to 30 days of inoculation.

Table 8. Details of sprouting and survival recorded in *P. nigrum* and *P. colubrinum* cuttings

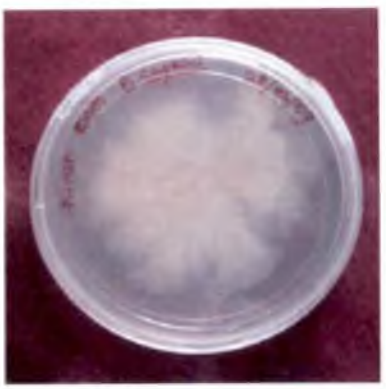
Species	No. planted	Days for sprouting (Mean)	Sprouting		Survival rate	
			No. (30 DAP)	Percentage (30 DAP)	No. (60 DAP)	Percentage (60 DAP)
P. nigrum	50	18	46	92	40	80
P. colubrinum	50	12	50	100	45	90

DAP-Days after planting

A. *Piper nigrum* var. Panniyur 1

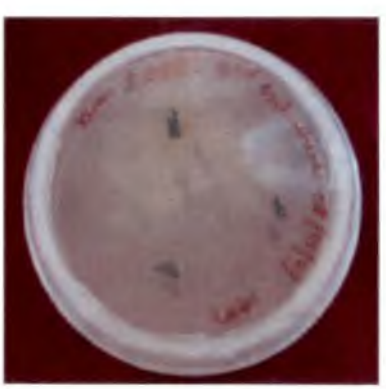
B. *P. colubrinum*

Plate 2. Rooted cuttings used as source material in *Piper nigrum* and *Piper colubrinum*



A. Growth of *Phytophthora capsici* on carrot agar medium

B. Inoculation with the pathogen and development of foliar symptoms



C. Re-isolation of *P. capsici* from infected leaf bits

Plate 3. Maintenance of the pathogen and disease infection

4.3 Estimation of β-1,3-glucanase activity

The β-1,3-glucanase enzyme activity assayed in leaf samples of *P. nigrum* var. Panniyur 1 and *P. colubrinum* is presented in Table 9 and graphically presented in Fig. 2.

The activity was observed to be 26.29 enzyme units in *P. nigrum* and it was 88 enzyme units in *P. colubrinum* before inoculation with the *P. capsici*. Following inoculation with the fungus, the enzyme activity in *P. nigrum* increased significantly upto 76.86 units at 24 h interval and thereafter decreased upto 48 units at 48 h interval. The enzyme activity recorded in *P. colubrinum* at different intervals after inoculation was almost similar to the normal values.

4.4 Isolation of plant genomic DNA

The agarose gel electrophoresis of genomic DNA isolated both from *P. nigrum* and *P. colubrinum* revealed that DNA sample was free from RNA contamination and discreteness of the band indicated the presence of good quality of DNA free from any degradation and shearing (Plate 4).

The values recorded for the spectrophotometric analysis of DNA isolated from both *P. nigrum* and *P. colubrinum* are indicated in Table 10. The absorbance ratio of 260/280 ranged between 1.84 and 1.90 while 260/230 ranged between 1.28 and 1.45. The quality could be interpreted as good and the quantity was above 1000 ng/µl.

The isolated DNA was further used for Southern blotting and real-time PCR assay.

4.5 Southern hybridization

Agarose gel electrophoresis for the DNA sample digested with restriction enzymes showed well separated smear on the gel both in the case of *Eco*RI and

Table 9. β-1,3-glucanase activity at different intervals after inoculation with the pathogen

Interval	Enzyme activity (enzyme unit/mg protein/10 min)	
	P. nigrum	*P. colubrinum*
Control	26.29	88.00
2 h	30.43	90.57
6 h	37.29	95.43
12 h	41.14	87.86
24 h	76.86	90.50
48 h	48.00	84.29

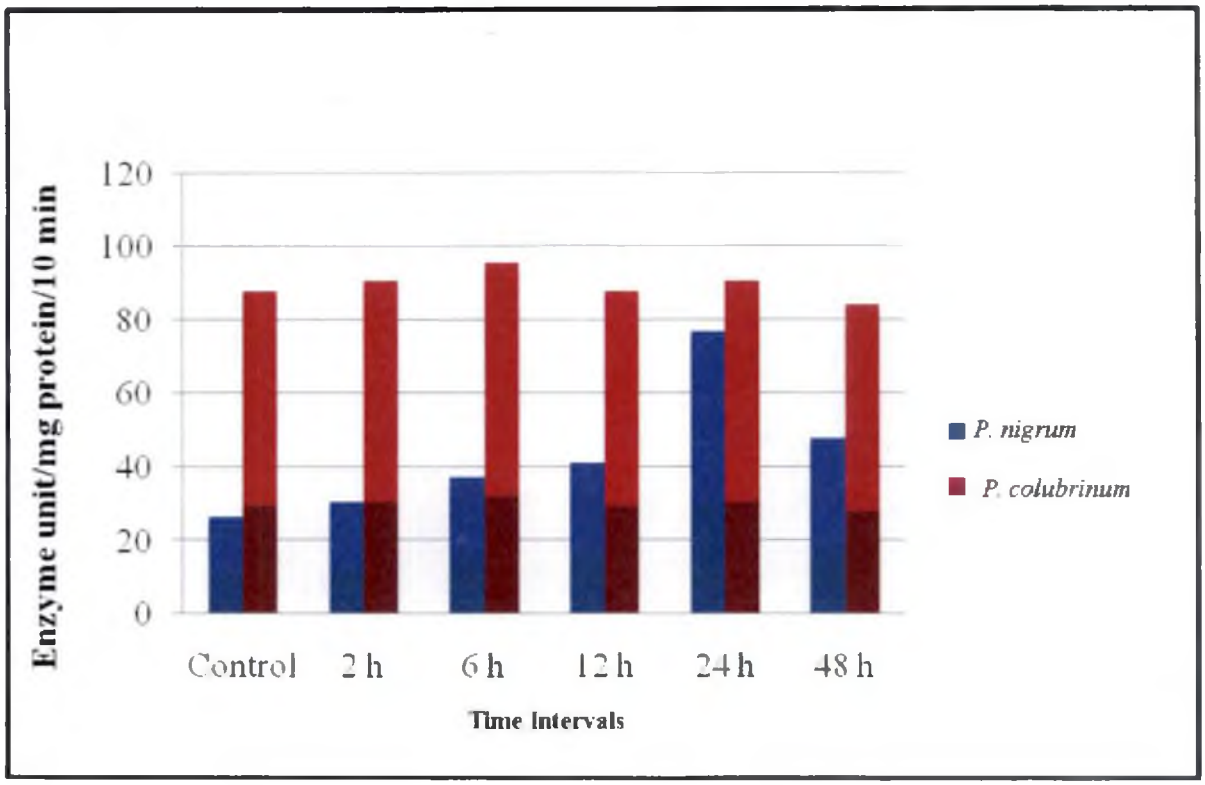


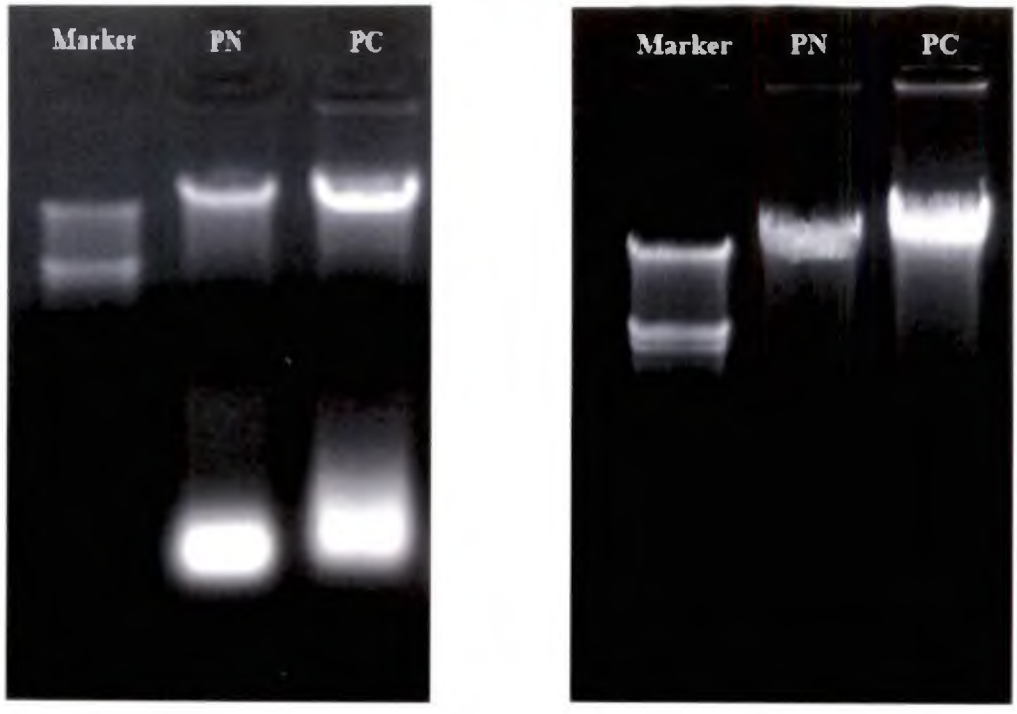
Fig. 2. β-1,3-glucanase activity in *P. nigrum* and *P. colubrinum* at different intervals after inoculation the pathogen

Table 10. Qualitative and quantitative analysis of plant genomic DNA, the probe* and the standard **

Sr. No.	DNA Source	Absorbance Ratio		Quantity (ng/µl)	Quality
		260/280	260/230		
1.	*P. nigrum*	1.84	1.28	1036.8	Good
2.	*P. colubrinum*	1.90	1.45	1226.4	Good
3.	β-1,3-glucanase gene specific probe	1.90	1.16	146.6	Good
4.	β-1,3-glucanase gene specific standard	1.86	1.19	21.28	Good

*The probe DNA used for blotting described in section 4.5 and 4.9.

** The standard used in absolute quantification described in section 4.10.2.



A. Before RNase treatment

B. After RNase treatment

Plate 4. DNA isolated from leaf samples of *P. nigrum* (PN) and *P. colubrinum* (PC)

(Marker-*Eco*RI/*Hind*III λ DNA double digest)

*Hind*III. This indicated that the restriction of DNA was complete (Plate 5A and 5B).

Following the blotting, the gel was re-stained with ethidium bromide. The re-stained gel was free from DNA smear, which clearly indicated that there was transfer of DNA during Southern blotting (Plate 5A and 5B).

The genomic DNA when amplified with the gene specific probe, it gave an amplicon of size 700 bp. The amplicon was eluted and the DNA re-isolated to use as a probe in Southern hybridization. The absorbance ratio of the eluted amplicon at 260/280 was 1.9 with concentration of about 146.6 ng/µl, which indicated good quality and quantity (Table 10 and Plate 6).

The blotted nylon membrane was hybridized with β-1,3-glucanase gene specific probe and analyzed using phosphorimager. The results are indicated in Plate 7. More number of signals were observed in *P. colubrinum* compared to *P. nigrum*. The *Eco*RI digested sample indicated that, there were three bands in *P. colubrinum* as against only one in *P. nigrum* (Plate 7A). The DNA samples digested with *Hind*III, clearly indicated six signal bands in *P. colubrinum* as against only one in *P. nigrum* (Plate 7B). This in turn suggested that the β-1,3-glucanase gene copy number was higher in *P. colubrinum* compared to *P. nigrum*.

4.6 Isolation of total RNA

The RNA samples isolated from both the species, after agarose gel electrophoresis are presented in Plate 8A. The results indicated that the samples were free of DNA contamination and integrity of bands indicated the presence of good quality of RNA free from any degradation. The bands observed for 28S, 18S, 5S and tRNA were intact and distinct.

The values for spectrophotometric analysis of total RNA isolated from both the species are given in Table 11. The absorbance ratio of 260/280 ranged between 1.91 and 1.92, while 260/230 ranged between 0.96 and 1.08, which

Before blotting **After blotting**

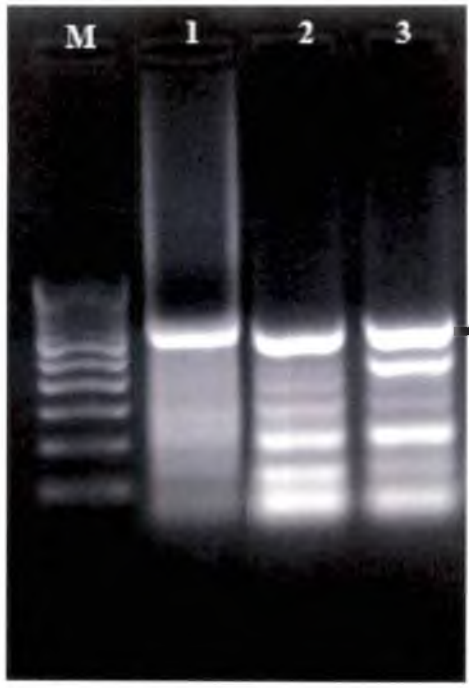
A. Digestion with enzyme- *Eco*RI

Before blotting **After blotting**

B. Digestion with enzyme- *Hind*III

Plate 5. Agarose gel before and after blotting for the DNA samples digested with different restriction enzymes

(Marker-*Eco*RI/*Hind*III λ DNA double digest, PN- *P. nigrum*, PC- *P. colubrinum*)



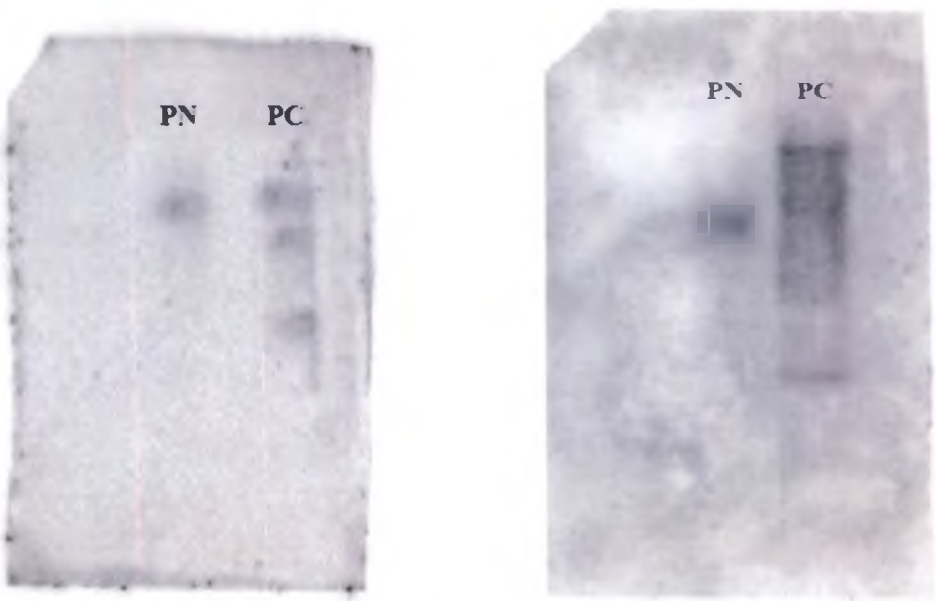
β-1,3-glucanase gene specific band (700 bp)

M- 100bp ladder marker.

1- Sample re-amplified with gene specific primer

2 and 3 – Initial amplification with genomic DNA

Plate 6. Agarose gel electrophoresis for probe DNA



A. DNA digested with *Eco*RI **B. DNA digested with *Hind*III**

(*P. nigrum* - PN and *P. colubrinum* - PC)

Plate 7. Southern hybridization of genomic DNA with β-1,3-glucanase gene specific probe

(The black signals indicate presence of the gene n different fragments)

Table 11. Qualitative and quantitative analysis of total RNA

Sr. No.	RNA Source	Absorbance Ratio		Concentration (ng/µl)	Quality
		260/280	260/230		
1.	*P. nigrum*	1.91	0.96	4072.0	Good
2	*P. colubrinum*	1.92	1.08	5134.0	Good

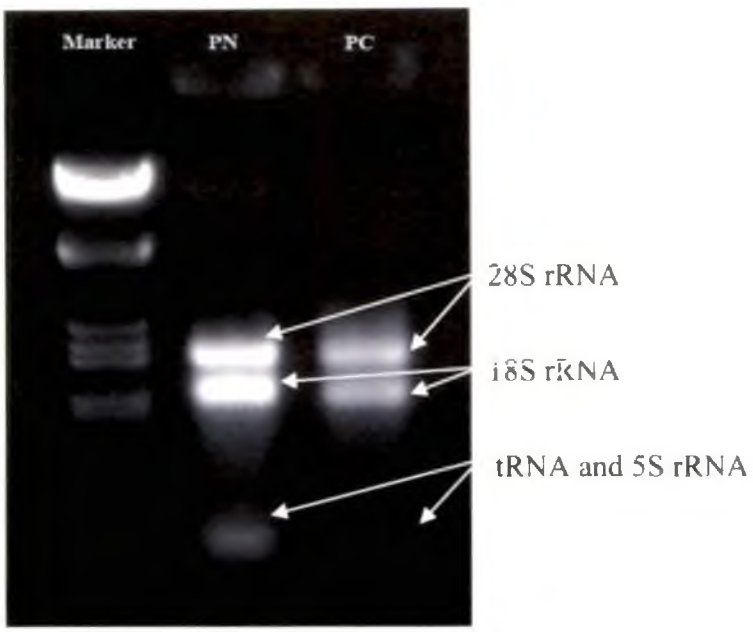


Plate 8A. Total RNA isolated from leaf samples of *P. nigrum* (PN) and *P. colubrinum* (PC)

(Marker-*Eco*R1/*Hind*III λ DNA double digest)

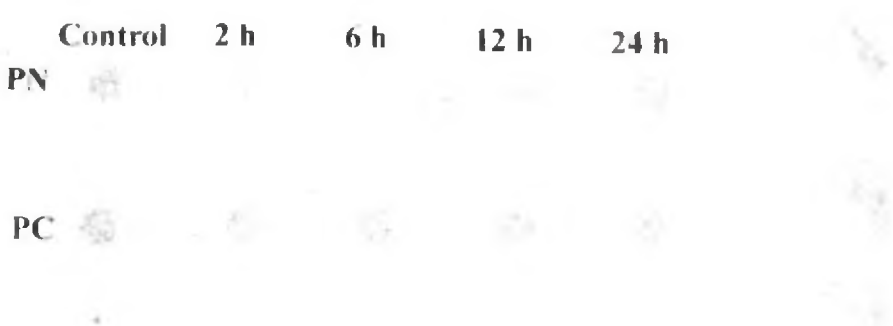


Plate 8B. Dot blot analysis of RNA isolated at different intervals after infection

indicated good quality of RNA. The quantity of total RNA ranged between 4072.0 ng/µl and 5134.0 ng/µl. The total RNA isolated was used for RNA dot blot analysis, Northern blotting and real-time PCR assay.

4.7 Northern hybridization

4.7.1 Dot blot for transcript detection in total RNA

The dot blot analysis performed for detection of RNA transcripts for the β-1,3-glucanase gene indicated the presence of gene transcripts at all the intervals stated. This confirms the presence of gene expression under healthy and diseased condition in both the species upto 24 h after infection (Plate 8B).

4.7.2 Northern blot analysis for detection of transcript level

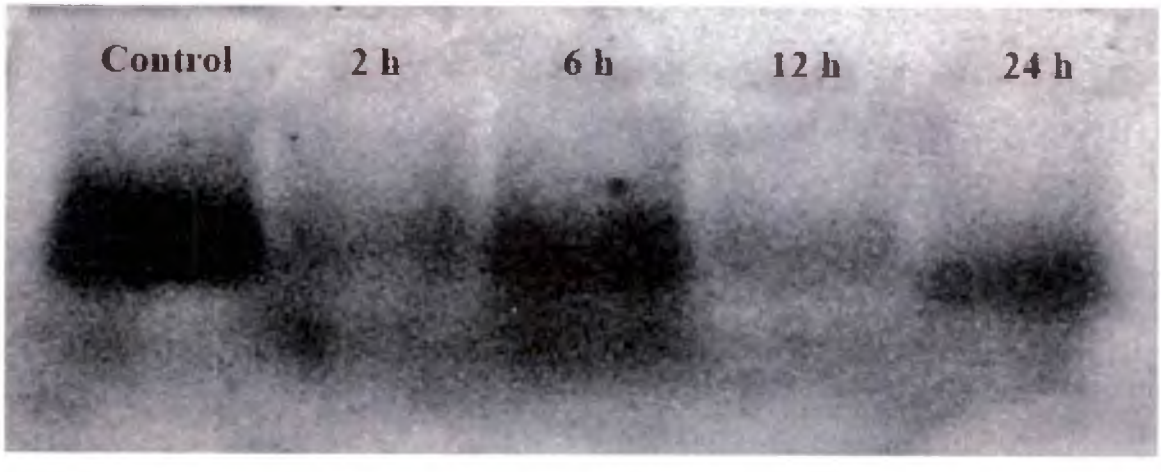
Results of Northern blot analysis carried out for *P. colubrinum* and corresponding agarose gel electrophoresis indicating 18S rRNA are presented in Plate 9. The blotting results were compared with 18S rRNA, to indicate the equal loading of total RNA. The results indicated that the β-1,3-glucanase gene specific transcript was observed in all the treatments at varying intensities. When compared with the gel electrophoresis for 18S rRNA sample, the quantity of RNA for glucanase gene was more in control and 24 h treatment sample in Northern blot. However the intensity of band was much higher in 6h treatment sample when compared with equivalent amount of 18S rRNA in the gel.

However the results did clearly indicate that the transcription of β-1,3-glucanase gene is constitutive, irrespective of infection with the pathogen, in the resistant species *P. colubrinum*.

4.8 Real-time PCR assay

Both absolute and relative quantification were carried out in this study. The primers were designed for the β-1,3-glucanase gene. Primers were also

A. Agarose gel picture showing 18S rRNA in the total RNA sample at different intervals



B. Northern blot after hybridization with the probe

(The black signals indicate presence of the gene in different fragments)

Plate 9. Northern blot analysis for detection of β-1,3-glucanase gene transcript in *P. colubrinum* at different time intervals after infection

designed for different housekeeping genes for selecting the one as endogenous control (reference gene).

For absolute quantification, a standard graph was plotted with the known sample and the copy numbers of unknown sample of *P. nigrum* and *P. colubrinum* were detected.

In relative quantification, the gene expression at different time intervals after infection with the pathogen was studied relative to control after normalizing it with the expression of reference gene.

4.8.1 Primer designing and screening for amplification

The details of primers designed for the β-1,3-glucanase gene and housekeeping genes (18S rRNA, Actin and GAPDH) to be used as endogenous control are provided in Table 12. The designed primers were screened with genomic DNA as well as cDNA for amplification through real-time PCR.

The dissociation curves obtained for different sets of primers are provided in Fig. 3 to 7. The dissociation curve indicates the unique melting temperature for the amplicon obtained with the particular set of primers.

The first set of primers (GlucI) though gave amplicon of expected size (100 bp), the dissociation curve of *P. nigrum* and *P. colubrinum* indicated different pattern for genomic DNA. Three peaks were observed for *P. colubrinum* while there was single peak for *P. nigrum*. However, the pattern was uniform with respect to cDNA (Fig. 3).

The second set of primers (GlucII) designed for glucanase gene gave expected amplicon size (125 bp) and single peak in the dissociation curve for both genomic DNA and cDNA in both the species (Fig. 4). Hence second set of primer was selected for further studies.

Table 12. Primers designed for gene of interest and reference gene

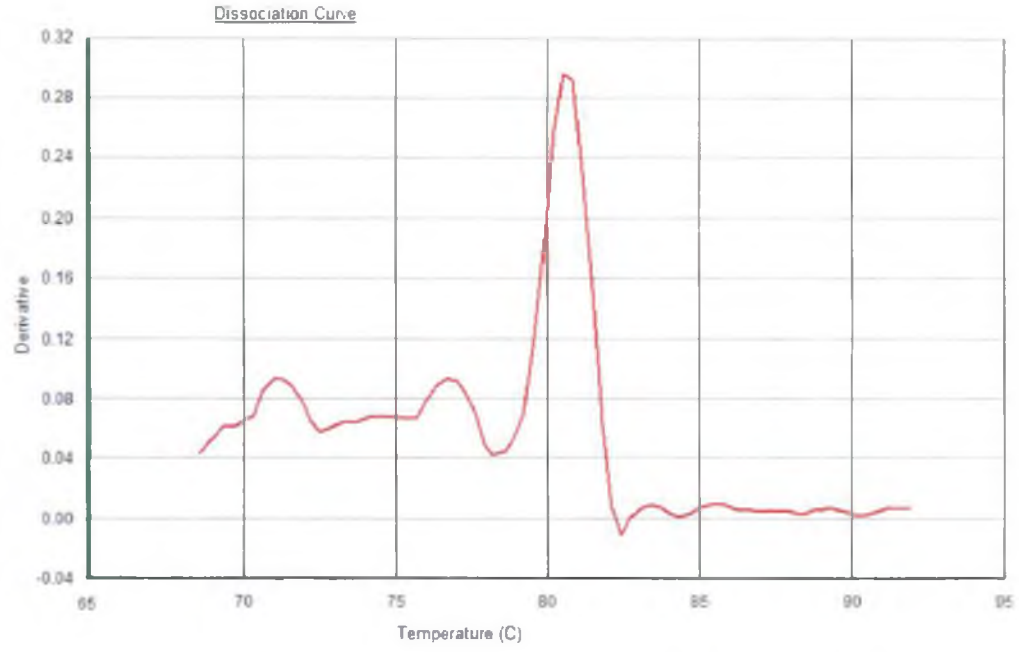
Gene targetted	Primer code	Primers set	Primer sequence	Length (bases)	GC%	Tm (^{0}C)	EAS (bp)	OAS (bp)	NDC		PAC	Remarks
									PN	PC		
B-1,3-glucanase	GlucI	PnGlucRTF1	5'GAGGCAATGCTCAGGCACTACT3'	22	55	59.5	100	~100	S	M	Good	Unsatisfactory
		PnGlucRTR1	5'CACGGCAGTTGACACCTTGA3'	20	55	59.3						
B-1,3-glucanase	GlucII	PnGlucRTF2	5'CCGTCGGAAACGAGGTCAT3'	19	58	59.5	125	~125	S	S	Good	Satisfactory
		PnGlucRTR2	5'CCACGGCAGTTGACACCTT3'	19	58	58.2						
GAPDH	GAPDH I	PnGAPDHF1	5'CATGCAGCTACCCAGAAAACC3'	21	52	58.3	61	~61	S	S	Average	Satisfactory
		PnGAPDHR1	5'CCCTTCCGCCTCTCCAAT3'	18	61	58.6						
18S rRNA	18S I	Pn18SF1	5'CGAAAGCATTTGCCAAGGAT3'	20	45	58.8	100	~100	S	S	Good	Satisfactory
		Pn18SR1	5'TCGGCATCGTTTATGGTTGA3'	20	45	58.6						
Actin	Act I	MdActF1	5'GCCACACCGTCCCAATCTAT3'	20	55	58.3	135	~135	M	M	Poor	Negotiable
		MdActR1	5'CAGCAGTGGTGGTGAACGAGTA3'	22	55	59.8						

EAS – Expected amplicon size, OAS - Observed amplicon size, PAC – Pattern of amplification curve

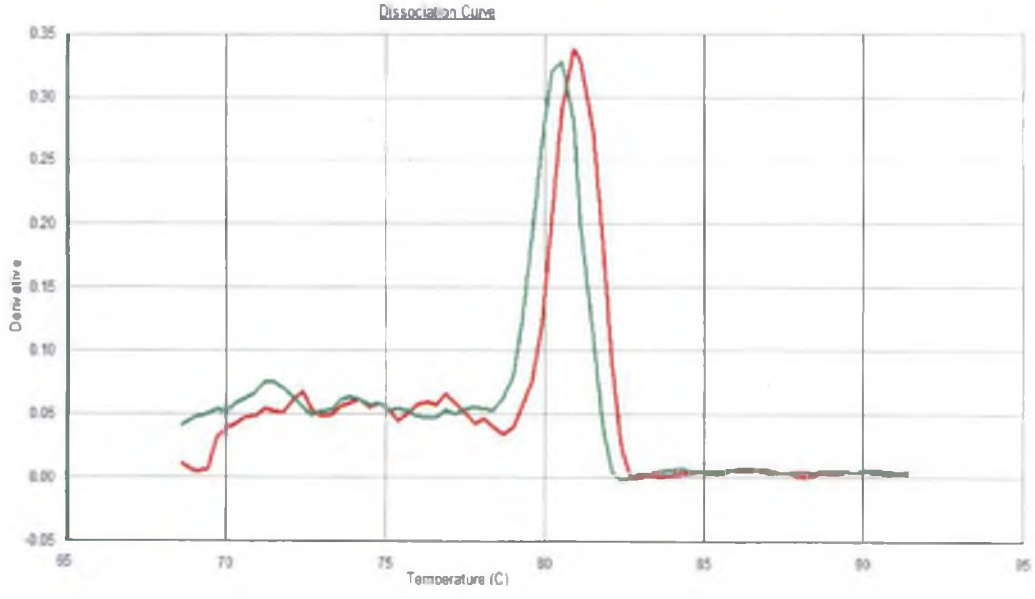
NDC – Nature of dissociation curve: expressed as number of peaks observed in dissociation curve

PN – *P. Nigrum*, PC- *P. Colubrinum*

S - Single peak and M - Multiple peaks

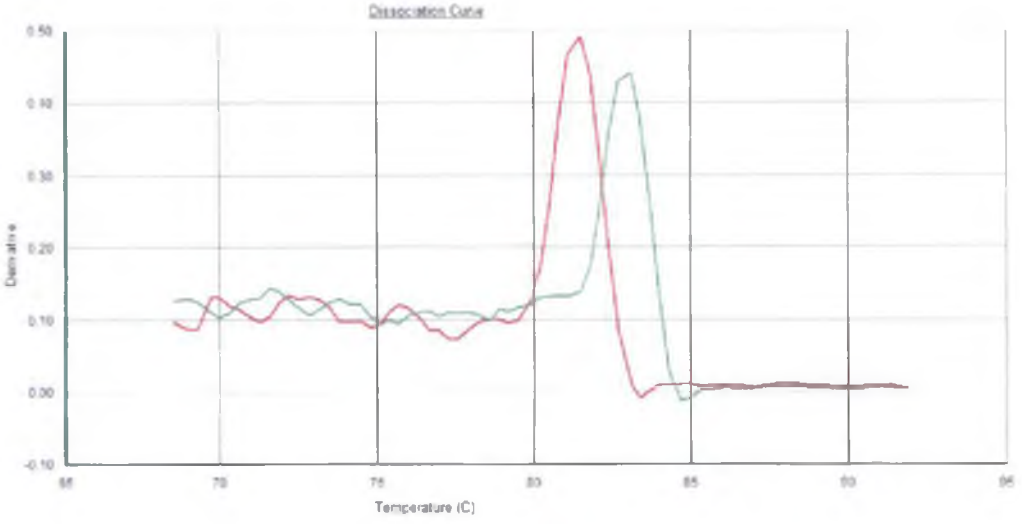


A. Amplification with genomic DNA in *P. colubrinum*

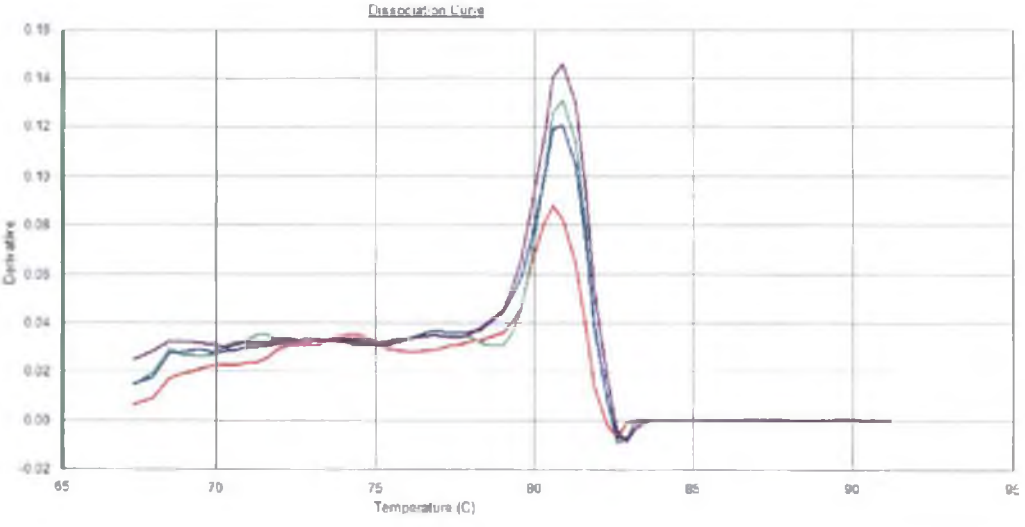


B. Amplification with cDNA ('—' *P. nigrum* & '—' *P. colubrinum*)

Fig. 3. Dissociation curve for real-time PCR amplified β-1,3-glucanase gene product with GlucI primer set in *P. nigrum* and *P. colubrinum*



A. Amplification with genomic DNA ('—' *P. nigrum* & '—' *P. colubrinum*)



B. Amplification with cDNA ('—' & '—', *P. nigrum* & '—' '—' *P. colubrinum*)

Fig. 4. Dissociation curve for real-time PCR amplified β-1,3-glucanase gene product using GlucII primer set in *P. nigrum* and *P. colubrinum*

The primers designed for the housekeeping genes, viz, 18S rRNA, actin and GAPDH, gave the expected amplicon size (Table 12). The dissociation curve observed for all the three genes with respect to the primers designed was also uniform for *P. nigrum* and *P. colubrinum* (Fig. 5, 6 and 7). However three peaks were observed in the case of primer set for actin gene (Fig. 7). This indicated suitability of the primer and need for further standardization of primer concentration.

4.8.2 Absolute quantification for estimation of β-1,3-glucanase gene copy number

4.8.2.1 Standardization of primer concentration

Primer concentration was standardized for amplification of β-1,3-glucanase gene in order to obtain minimum concentration of primer pair giving optimum amplification of DNA. The details of least threshold cycle and the dissociation curve obtained with different combinations of the selected primers are presented in Table 13.

The real-time PCR reactions are characterized by threshold cycle, the cycle number at which there is significant increase in fluorescence over the threshold. Threshold is the line whose intersection with the amplification plot defines the threshold cycle (C_T).

Among the six concentrations tried for the second primer set (GlucII) selected for the glucanase gene, the average threshold cycle (C_T) observed was the least (18.93) for 300 nM each of forward and reverse primer. The melting temperature observed was 81.4^{0}C. Based on observations, this concentration was selected for further studies.

4.8.2.2 Standardization of DNA concentration

With different concentrations of DNA amplified with the selected primer concentration (GlucII_300×300 nM), the threshold cycle (C_T) value was the same

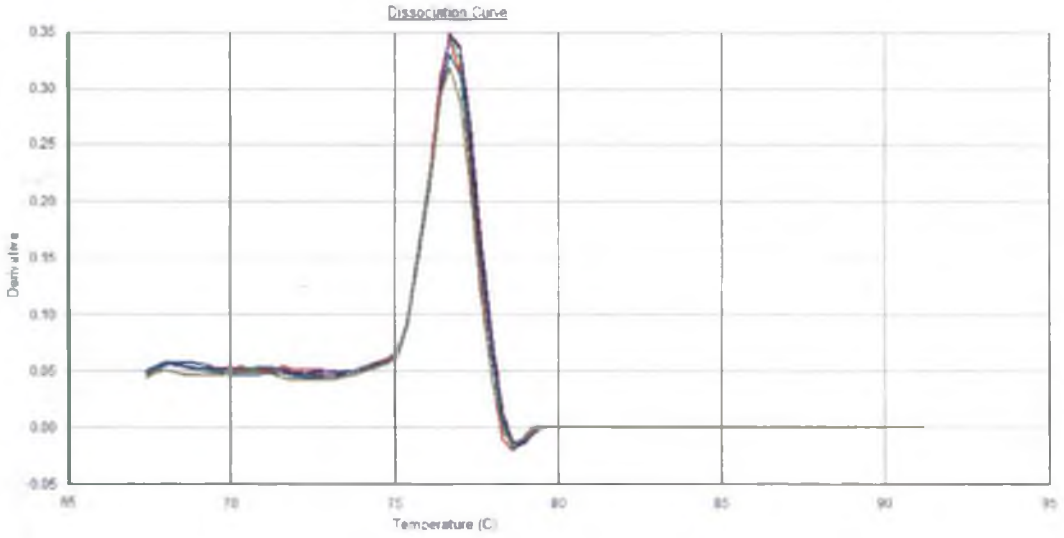


Fig. 5. Dissociation curve for real-time PCR amplified 18S rRNA gene product with cDNA using 18S I primer set in *P. nigrum* and *P. colubrinum*

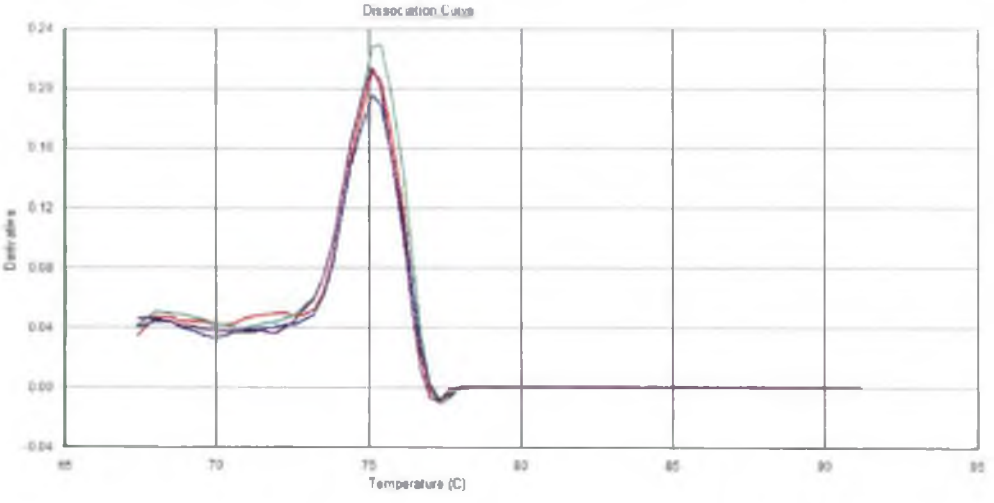


Fig. 6. Dissociation curve for real-time PCR amplified GAPDH gene product with cDNA using GAPDH I primer set in *P. nigrum* and *P. colubrinum*

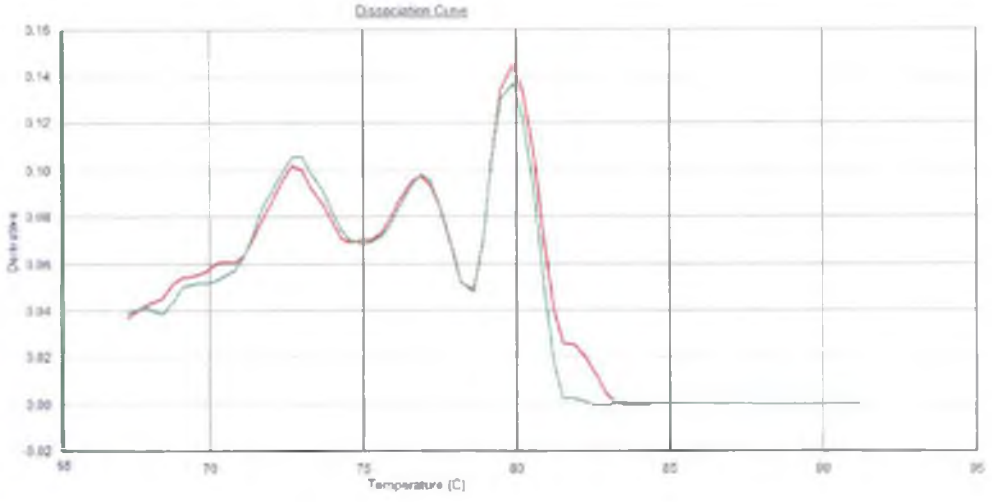


Fig. 7. Dissociation curve for real-time PCR amplified Actin gene product with cDNA using Act I primer set in *P. nigrum* and *P. colubrinum*

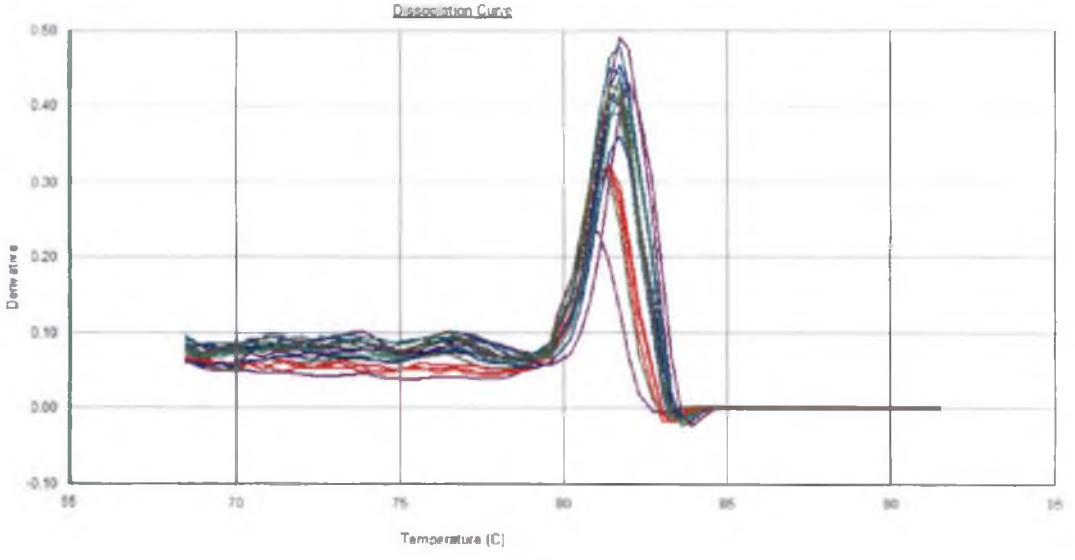


Fig. 8. Dissociation curve observed for different concentrations of genomic DNA when amplified with ß-1,3-glucanase gene specific primer

Table 13. Standardization of primer concentration for absolute quantification

Sr. No.	Primer concentration (nM)	Average C_T	Standard deviation	Melting temperature (^{0}C)	NDC	PAC
1.	Gluc II_150×150	19.14	0.055	81.1	S	A
2.	**Gluc II_300×300**	**18.93**	**0.111**	**81.4**	**S**	**G**
3.	GlucII_450×450	19.02	0.235	81.7	S	G
4.	Gluc II_600×600	19.29	0.182	81.5	S	G
5.	GlucII_750×750	19.14	0.021	81.7	S	A
6.	Gluc II_900×900	19.21	0.174	81.4	S	A

The primer concentration is given for forward and reverse primers.

NDC – Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good and A – Average

for 40 ng and 60 ng of DNA concentration (18.19) while it was the least (17.59) for 100 ng and highest (21.34) for 10 ng DNA concentration. Since the threshold cycle value was constant for both 60 ng and 40 ng of DNA sample, the minimum concentration 40 ng was selected for real-time PCR assay (Table 14).

The dissociation curve observed with the selected primer concentration was satisfactory at different concentrations of genomic DNA tried. All the samples gave single peak as revealed in the Fig. 8.

4.8.2.3 Preparation of standards

The genomic DNA for *P. nigrum* was amplified with the gene specific primers and the amplicon was purified for use as standard. The spectrophotometric analysis of the purified amplicon showed absorbance ratio of 260/280 as 1.84 with a DNA concentration of 21.28 ng/µl (Table 10). This indicated that quality as well as quantity of the standard was good.

The amplification of the standard with real-time PCR indicated single peak in dissociation curve with good amplification (Fig. 9). The graph plotted for threshold cycles against log values of corresponding serially diluted DNA, to be used as standard in real-time PCR assay, is provided in Fig 10. The standard curve obtained was a straight line with a slope of -3.250 and R^2 value of about 0.9935, which revealed that amplification was good with maximum efficiency for use as standard. The amplification pattern of different concentrations of DNA used as standard is presented in Fig. 11.

The standard was diluted in tenfold dilutions such that the final concentrations were ranged from 2×10^5 to 2×10^1 copeis/ µl. The dilutions were maintained in separate aliquot and stored at -20^0C.

4.8.2.4 Absolute quantification

The absolute quantification was carried out to detect the gene copy number. The optimum quantity of DNA (40 ng) from both *P. nigrum* and *P.*

Table 14. Standardization of DNA concentration for absolute quantification

Sr. No.	DNA concentration	Average C_T	Standard deviation	Melting temperature	NDC	PAC
1.	10 ng	21.34	0.079	82.1	S	P
2.	20 ng	19.18	0.060	82.8	S	A
3.	**40 ng**	**18.19**	**0.077**	**82.8**	**S**	**G**
4.	60 ng	18.19	0.602	82.6	S	A
5.	80 ng	18.875	0.179	82.8	S	A
6.	100 ng	17.59	0.701	82.6	S	A
7.	120 ng	17.74	0.408	82.4	S	A

NDC -- Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good and A – Average

Final primer concentration for both forward and reverse primer - 300×300 nM

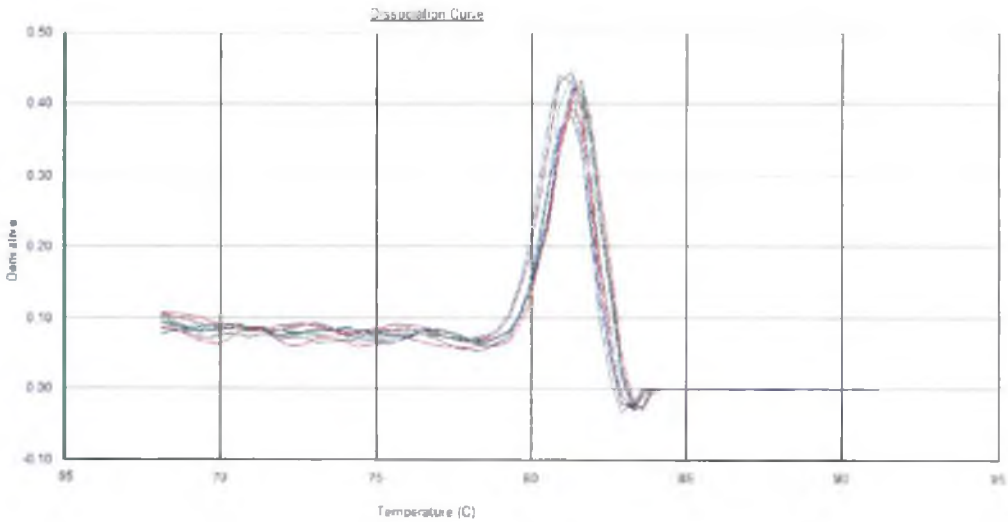


Fig. 9 Dissociation curve for amplification of different dilutions of standard DNA with GlucII primer set

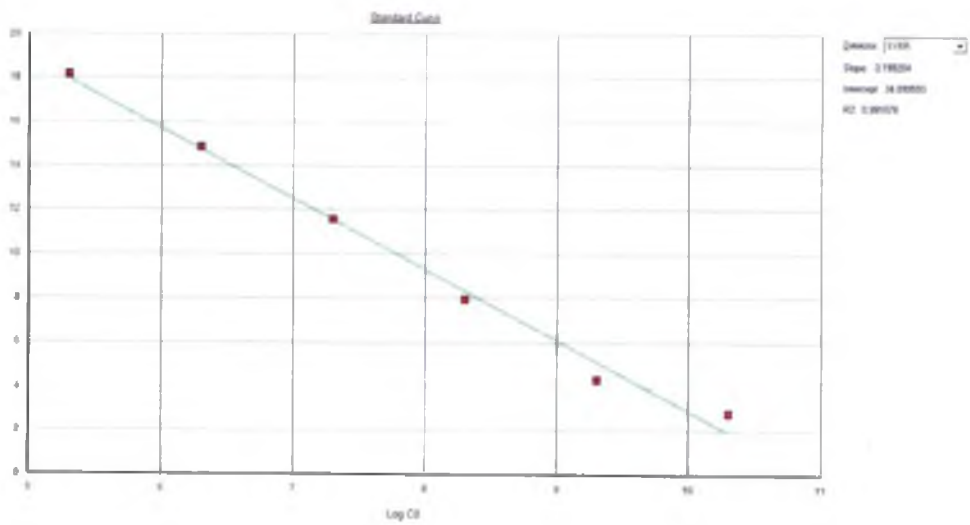


Fig. 10 Standard curve for absolute quantification

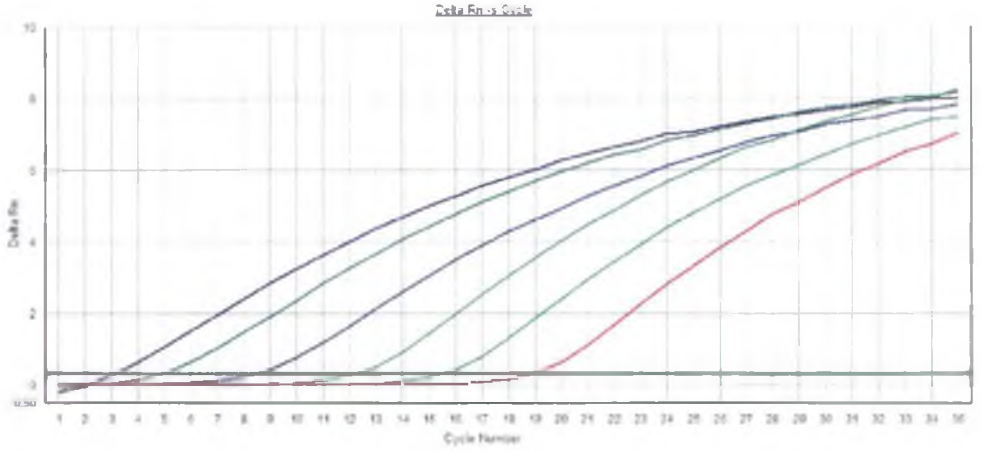


Fig. 11 Amplification curve for different dilutions of DNA used as standard

colubrinum was amplified with the β-1,3-glucanase gene specific primers using real-time PCR; along with six different dilutions of DNA taken as standard. Three different samples were used for the assay in each of the species. The results obtained are presented in Table 15. The dissociation curve obtained is presented in Fig. 12 and the amplification pattern is provided in Fig. 13.

The mean glucanase gene copy in *P. nigrum* was 57438, while it was 96422 in *P. colubrinum*. There was no primer dimer formation or non-specific amplification as indicated by dissociation curve.

The amplification pattern observed for the two species was also different relative to gene copy number. *P. colubrinum* genomic DNA was observed to get amplified at an early stage (low C_T value) than *P. nigrum*, when amplified with glucanase gene specific primers in real-time PCR assay.

4.8.3 Relative quantification for detection of β-1,3-glucanase gene expression

In relative quantification, the changes in β-1,3-glucanase gene expression in *P. nigrum* relative to the reference gene was analyzed. The reference gene, to be used as the endogenous control, was selected from three housekeeping genes, viz; 18S rRNA, actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Since it is the study at expression level, cDNA synthesized out of total RNA (described in section 3.7) was used for real-time PCR amplification. The primers and cDNA concentrations to be used for the assay were standardized before relative quantification.

4.8.3.1 Standardization of primer concentration

4.8.3.1.1 Primer set for β-1,3-glucanase

In order to standardize the minimum concentration of primer pair giving

Table 15. Absolute quantification of gene copy number (PN- *P. nigrum*, PC- *P. colubrinum*)

Sample Name	Gene copy number
PN 1	48781.81
PN 2	63864.53
PN 3	59667.36
PN mean	**57437.90**
PC 1	100265.82
PC 2	77970.33
PC 3	111030.41
PC mean	**96422.19**

PN – *Piper nigrum* and

PC - *Piper colubrinum*

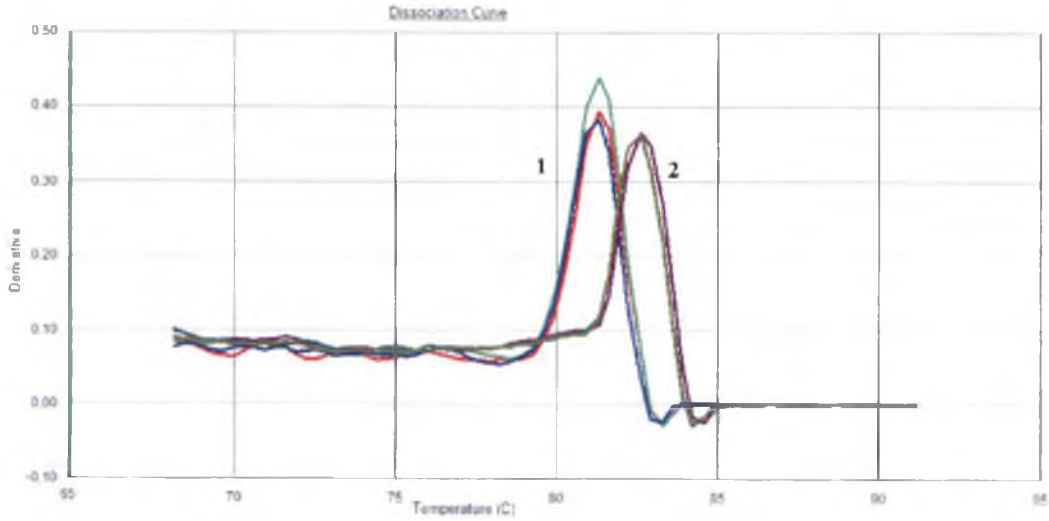
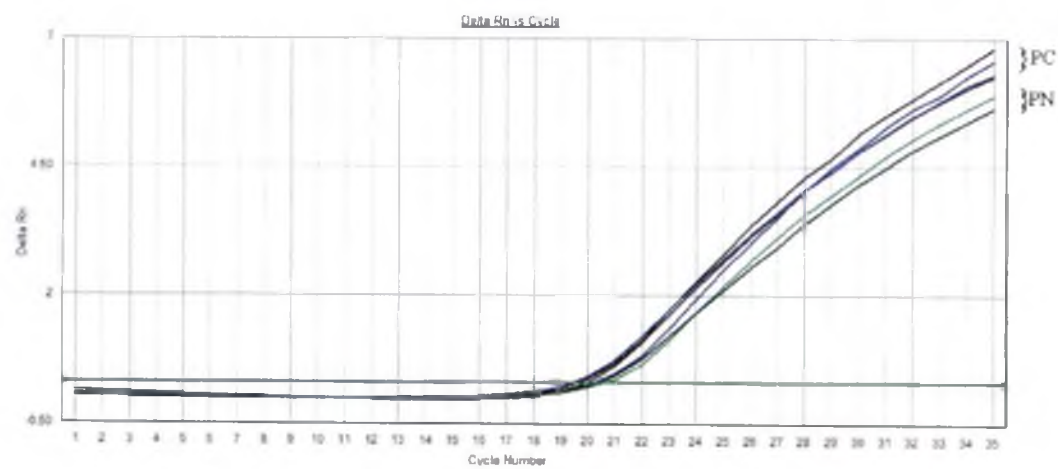


Fig. 12 Dissociation curve obtained for absolute quantification

(1- *P. nigrum*, 2- *P. colubrinum*)



(PN- *P. nigrum*, PC- *P. colubrinum*)

Fig. 13 Amplification curve for genomic DNA with β-1,3-glucanase gene specific primers (GlucII) in absolute quantification

optimum amplification for β-1,3-glucanase in the cDNA samples, different combinations of forward and reverse primers were tried and the results are presented in Table 16. The primer concentrations were selected as per the protocol provided by the manufacturer (Applied Biosystems, USA). Based on different parameters like average C_T value, melting temperature, nature of dissociation curve and amplification pattern, the forward and reverse primer concentration of 300×300 nM was selected for amplifying the cDNA, synthesized from the total RNA isolated at different intervals from *P. nigrum and P. colubrinum* after infection with the pathogen.

The dissociation curve and the amplification pattern with the selected primer concentration are presented in Fig. 14A and 14B.

4.8.3.1.2 Primer set for 18S rRNA

The threshold cycle values, melting temperature, nature of dissociation curve and amplification pattern observed for different concentrations of primers designed for 18S rRNA are presented in Table 17.

The primer concentration of 30×30 nM was found to be ideal with a C_T value of 18.02 and melting temperature 76.5^{0}C. This combination also gave good amplification pattern and the dissociation curve was satisfactory. The dissociation curve and amplification pattern for the selected concentration of primers is provided in Fig. 15A and 15B.

4.8.3.1.3 Primer set for actin

The threshold cycle values, melting temperature, nature of dissociation curve and amplification pattern observed for different concentrations of primers designed for actin are presented in Table 18.

The primer concentration of 300×900 nM was found to be ideal with a C_T value of 36.63 and melting temperature 81.6^{0}C. This combination also gave good amplification pattern and the dissociation curve was satisfactory. There was no

Table 16. Standardization of β-1,3-glucanase gene specific primer (GlucII) concentration for relative quantification

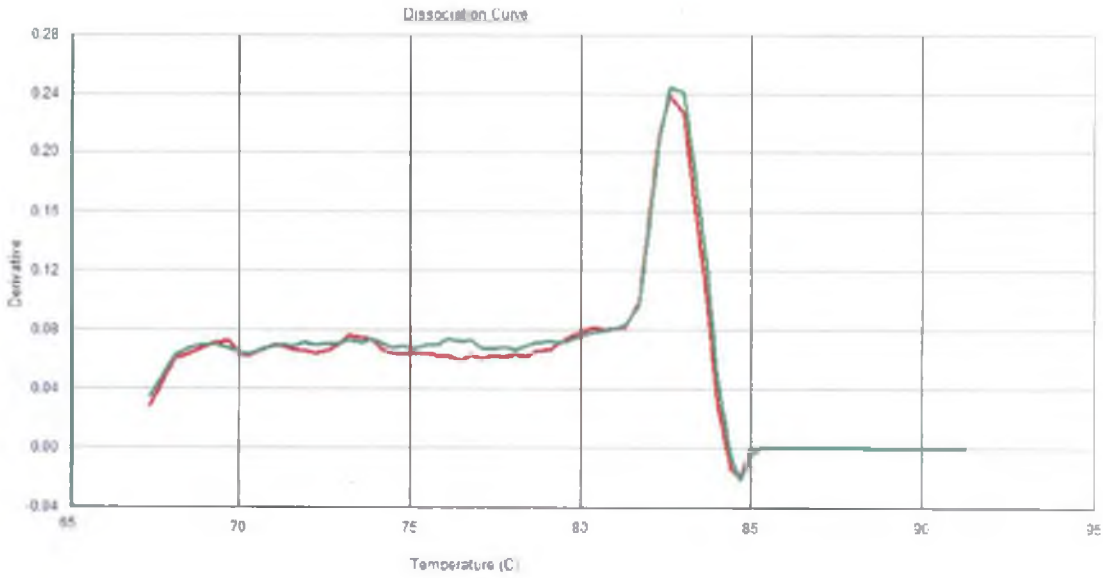
Sr. No.	Primer concentration	Average C_T	Standard deviation	Melting temperature	NDC	PAC
1.	50×50	27.94	0.27	82.15	S	P
2.	50×300	27.02	0.097	82.30	S	P
3.	50×900	27.075	0.032	82.30	S	A
4.	300×50	26.255	0.009	82.15	S	A
5.	**300×300**	**25.33**	**0.039**	**82.60**	**S**	**G**
6.	300×900	25.18	0.006	83.8	S	A
7.	900×50	25.405	0.023	82.6	S	A
8.	900×300	24.54	0.06	82.6	S	A
9.	900×900	24.995	0.08	83	U	A

The primer concentration is given for forward and reverse primers.

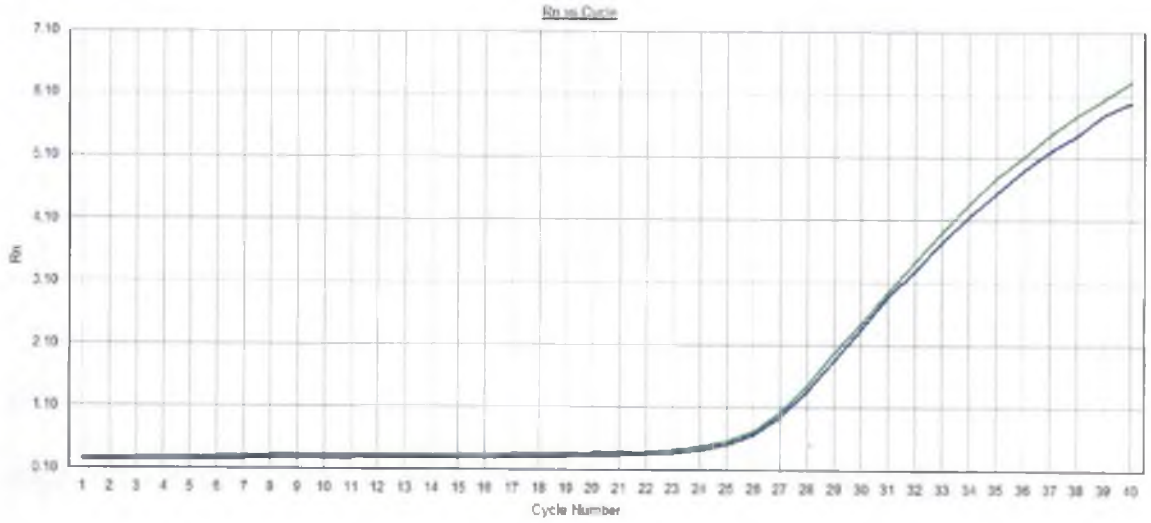
NDC – Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good, A – Average and P - Poor



A. Dissociation curve for 300×300 nM GlucII primer combination



B. Amplification curve for amplification with 300×300 nM GlucII primer concentration

Fig. 14. Amplification of GlucII primers with cDNA of *Piper spp.*

Table 17. Standardization of 18S rRNA gene specific primer concentration for relative quantification

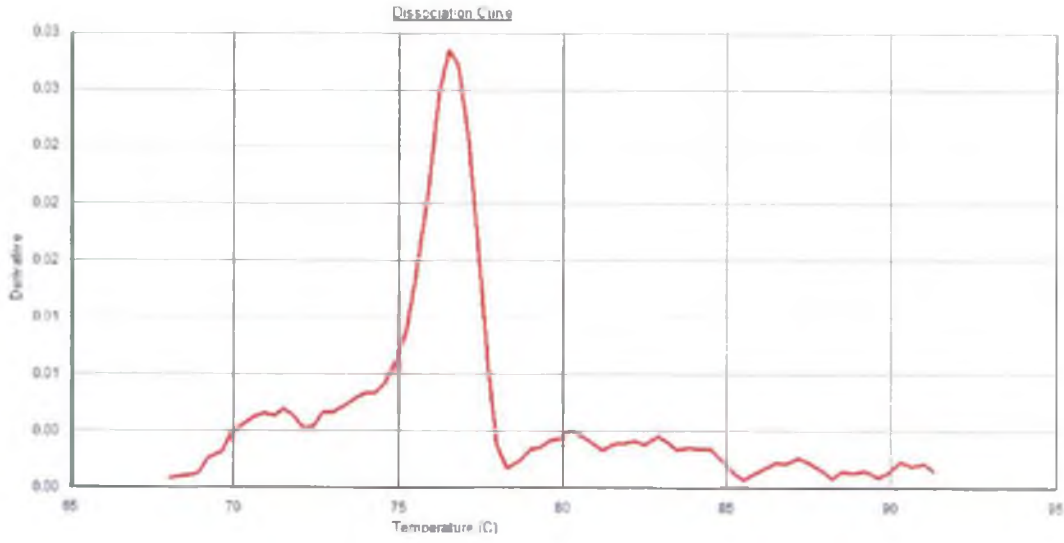
Sr.No.	Primer concentration	Average C_T	Melting temperature	NDC	PAC
1.	10*10	16.61	73	S	A
2.	10*30	25.82	76.8	S	A
3.	10*90	13.8	76.5	S	A
4.	30*10	28.39	76.5	S	A
5.	**30*30**	**18.02**	**76.5**	**S**	**G**
6.	30*90	14.03	76.5	S	G
7.	90*10	14.54	76.2	S	G
8.	90*30	13.39	76.2	S	G
9.	90*90	12.73	76.8	U	G

The primer concentration is given for forward and reverse primers.

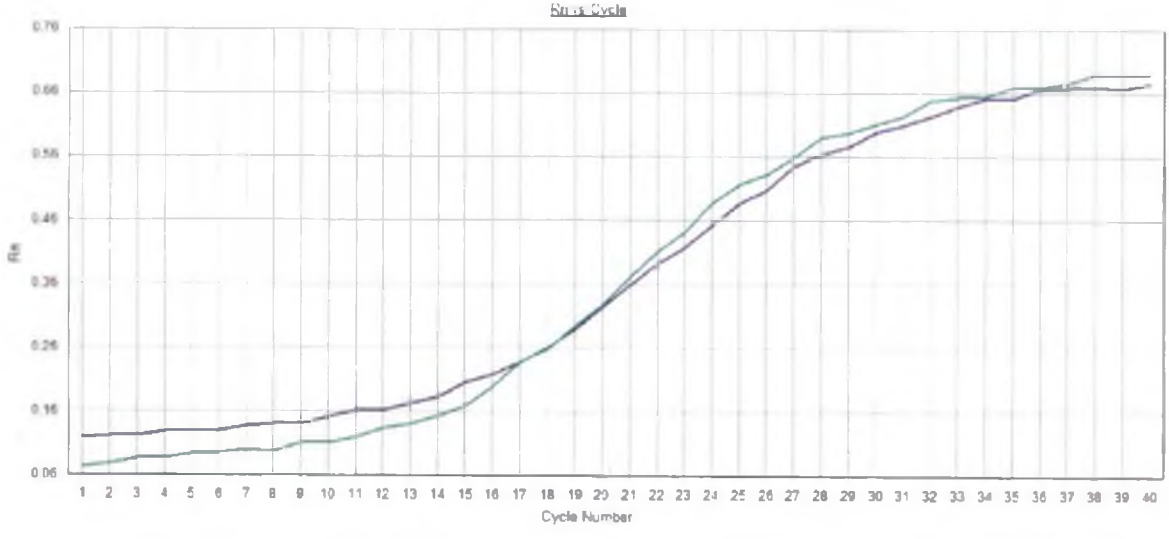
NDC – Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good, A – Average and P - Poor



A. Dissociation curve for 30×30 nM 18S rRNA primer combination



B. Amplification curve for amplification with 30×30 nM 18S rRNA primer concentration

Fig. 15. Amplification of 18S I primers with cDNA of *Piper spp.*

Table 18. Standardization of Actin gene specific primer concentration for relative quantification

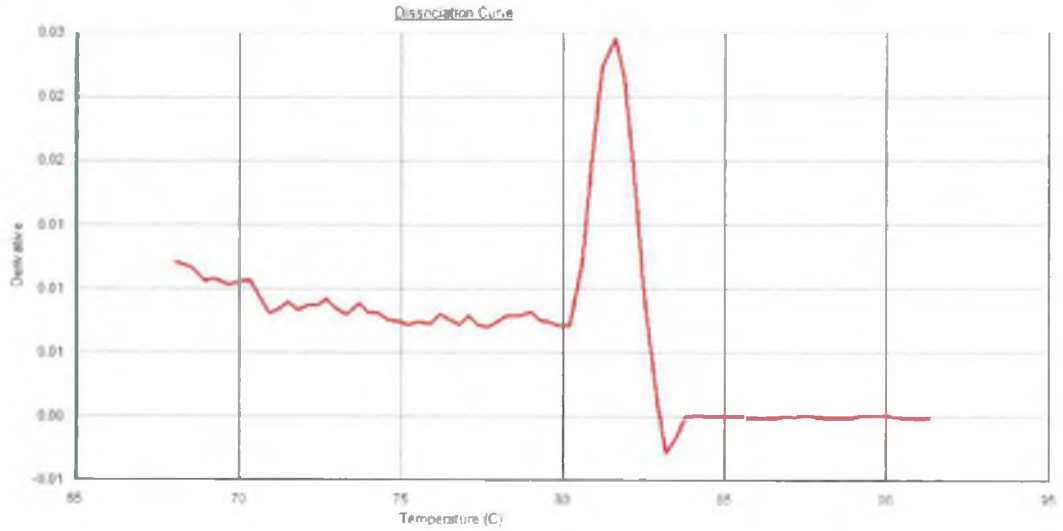
Sr.No.	Primer concentration	Average C_T	Melting temperature	NDC	PAC
1.	50×50	Undetermined	73	U	P
2.	50×300	Undetermined	73	U	P
3.	50×900	Undetermined	74.9	U	A
4.	300×50	Undetermined	73	U	A
5.	300×300	Undetermined	75.9	U	P
6.	**300×900**	**37.63**	**81.6**	**S**	**A**
7.	900×50	Undetermined	72.7	U	A
8.	900×300	34.1	84.2	U	A
9.	900×900	37.35	79.6	U	A

The primer concentration is given for forward and reverse primers.

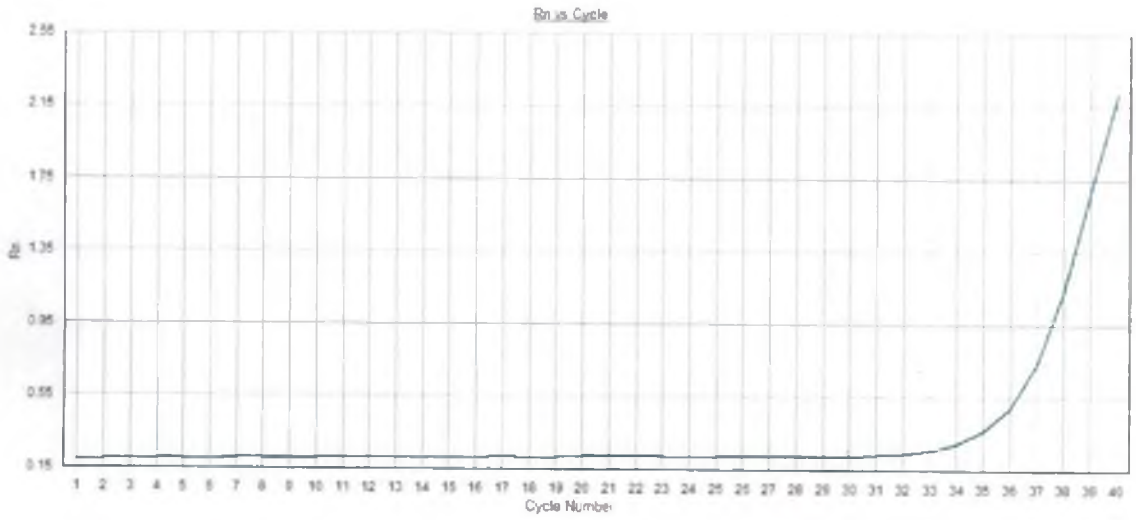
NDC – Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good, A – Average and P - Poor



A. Dissociation curve for 300×900 nM Actin primer combination



B. Amplification curve for amplification with 300×900 nM Actin primer concentration

Fig. 16. Amplification of ACT I primer set with cDNA of *Piper spp.*

amplification for all other primer concentrations except 900×300 and 900×900, which showed non-specific product formation. The dissociation curve and amplification pattern for the selected concentration of primers is provided in Fig. 16A and 16B.

4.8.3.1.4 Primer set for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

The threshold cycle values, melting temperature, nature of dissociation curve and amplification pattern observed for different concentrations of primers designed for GAPDH are presented in Table 19.

The primer concentration of 900×300 nM was found to be ideal with a C_T value of 27.13 and melting temperature 75.2^{0}C. This combination also gave good amplification pattern and the dissociation curve was satisfactory. The dissociation curve and amplification pattern for the selected concentration of primers is provided in Fig. 17A and 17B.

4.8.3.2 Standardization of cDNA concentration

Different dilutions of cDNA were tried for real-time PCR amplification with the selected β-1,3-glucanase gene specific primer set GlucII. The best dilution was selected based on average threshold cycle value, nature of dissociation curve and amplification pattern. The details are provided in Table 20.

The cDNA dilution of 1:5 gave the best amplification and expected dissociation curve and C_T value. The dissociation curve and amplification pattern obtained for the selected concentration of cDNA are provided in Fig 18A and 18B.

4.8.3.3 Selection of reference gene for relative quantification

The evaluation of expression stability and selection of best housekeeping

Table 19. Standardization of GAPDH gene specific primer concentration for relative quantification

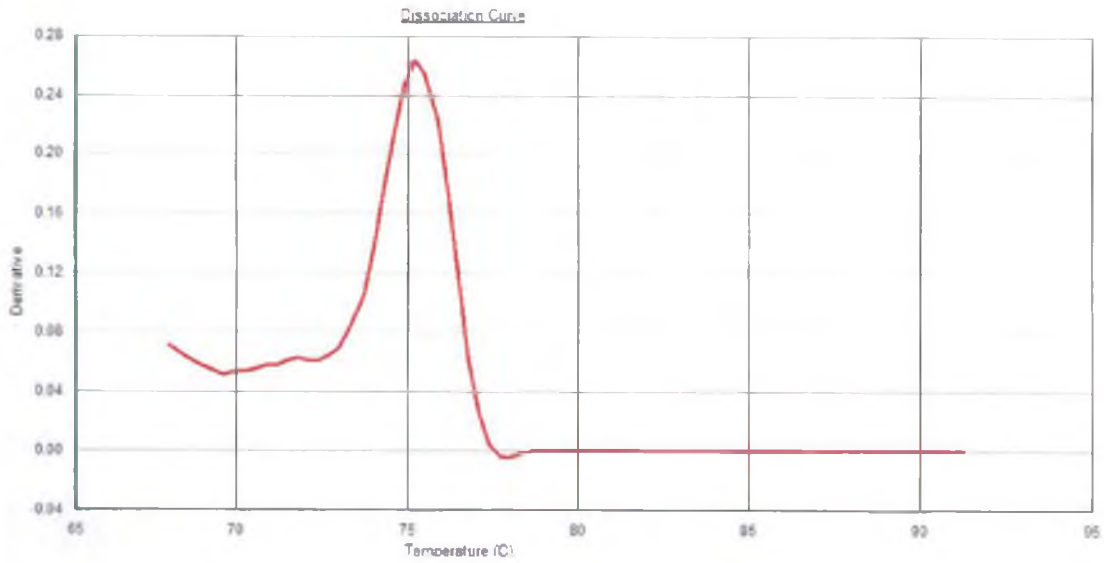
Sr.No.	Primer concentration	Average C_T	Melting temperature	NDC	PAC
1.	50×50	Undetermined	79	U	P
2.	50×300	29.97	75.2	U	P
3.	50×900	30.17	75.2	U	A
4.	300×50	29.72	74.9	S	A
5.	300×300	28.3	75.2	S	A
6.	300×900	28.54	74.9	S	A
7.	900×50	28.71	74.9	G	A
8.	**900×300**	**27.13**	**75.2**	**S**	**G**
9.	900×900	27.29	75.5	U	G

The primer concentration is given for forward and reverse primers

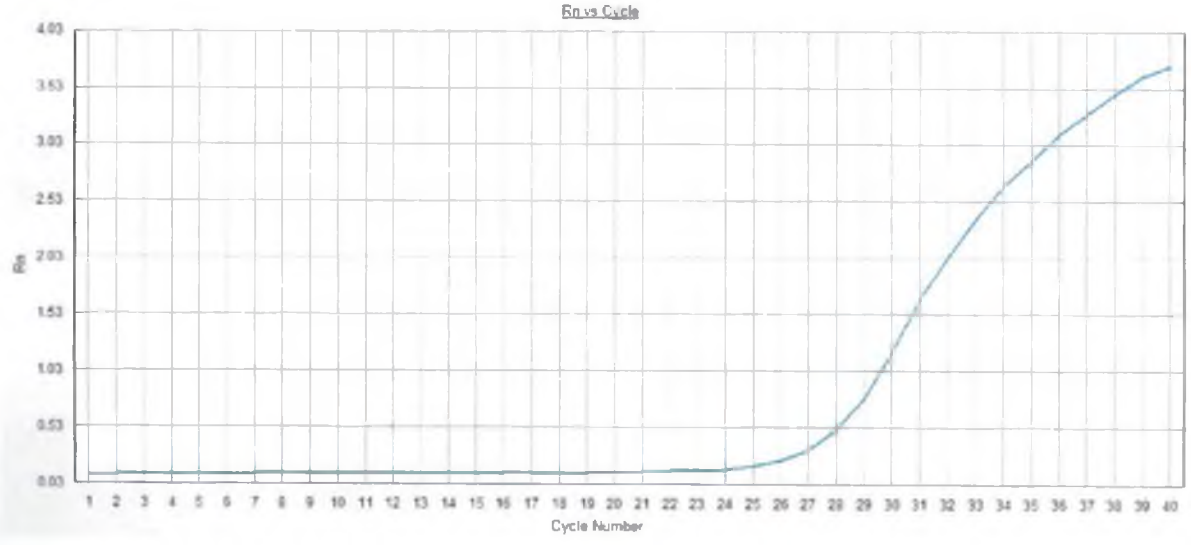
NDC – Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good, A – Average and P - Poor



A. Dissociation curve for 900×300 nM GAPDH primer combination



B. Amplification curve for amplification with 900×300 nM GAPDH primer concentration

Fig. 17. Amplification of GAPDH I primer set with cDNA of *Piper spp.*

Table 20. Standardization of cDNA concentration for relative quantification

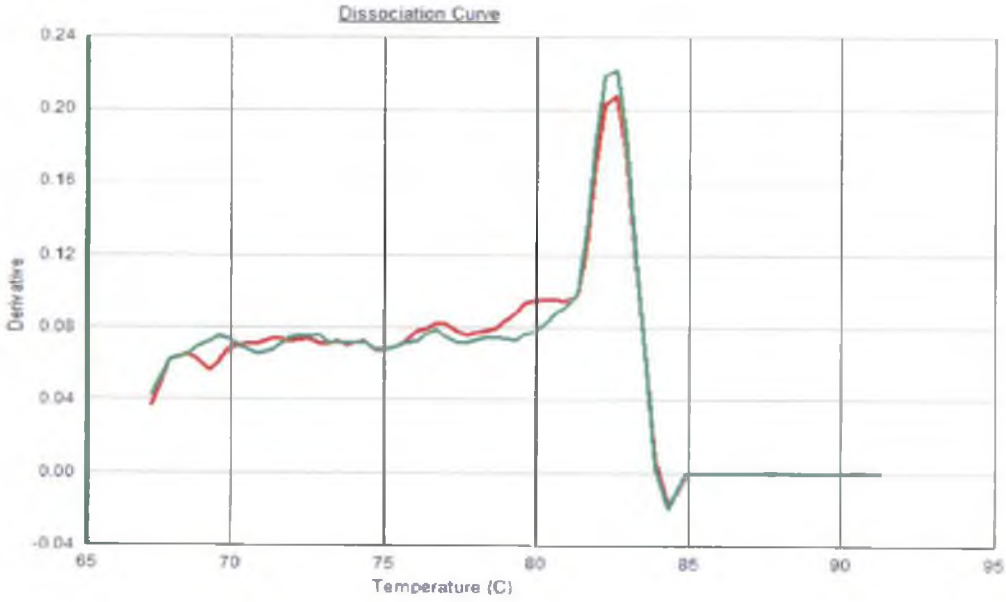
Sr. No.	cDNA dilution	Average C_T	Standard deviation	Melting temperature	NDC	PAC
1.	1	24.18	0.286	82.60	S	P
2.	1:1	24.94	0.128	82.60	S	A
3.	**1:5**	**25.97**	**0.062**	**82.60**	**S**	**G**
4.	1:10	26.84	0.451	82.05	S	A
5.	1:20	28.37	0.435	82.05	S	A
6.	1:50	28.32	0.266	81.75	S	P
7.	1:80	28.80	0.258	81.60	S	P
8.	1:100	29.52	0.668	81.10	S	P

The primer concentration given for forward and reverse primers are 300 nM each.

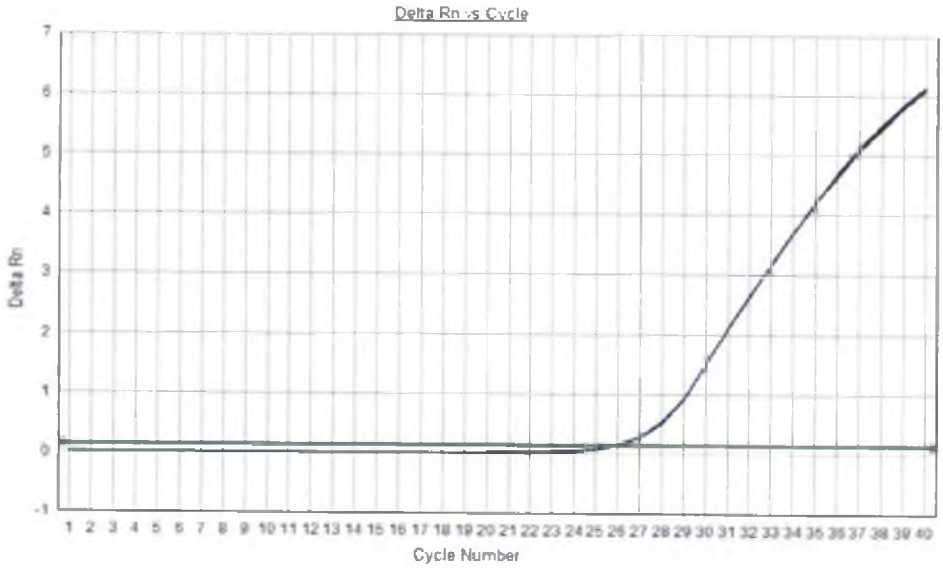
NDC -- Nature of Dissociation curve

PAC – Pattern of amplification curve

S – Satisfactory, G - Good, A – Average and P - Poor



A. Dissociation curve for 1:5 cDNA dilution with glucanase gene specific primer



B. Amplification curve obtained with 1:5 cDNA dilution using glucanase gene specific primers

Fig. 18. Amplification of GlucII primer set for cDNA for standardization of cDNA concentration

gene was done based on geNorm analysis (Vandesompele *et al.*, 2002b) and Norm-Finder (Anderson *et al.*, 2004).

The results obtained are provided in Table 21 and Table 22. The relative expression, when analyzed with the software geNorm at different intervals after infection with the pathogen; the expression was observed to be relatively stable in GAPDH with the least average expression stability (M) (0.282) and it was the highest for 18S rRNA gene (0.384).

GAPDH was identified as the most stable gene, also in the analysis with the NormFinder. The stability value observed for GAPDH was the least (2.071), while it was the highest for actin gene (3.232).

Thus GAPDH was selected as reference gene for normalization of gene expression data in relative quantification.

4.8.3.4 Relative quantification of β-1,3-glucanase gene expression

Relative quantification in real time PCR assay was carried out after normalization of data with GAPDH as endogenous control. . The amplification curve obtained for relative quantification in real-time PCR assay is provided in Fig. 19A and 19B, which indicated that samples in both the species gave good amplification. The relative quantification data was calculated by analyzing the threshold cycle values obtained after real-time PCR for both target and reference gene with comparative C_T ($2^{-\Delta\Delta CT}$) method. The relative quantification data was plotted against the treatments given for both *P. nigrum* and *P. colubrinum* (Fig. 20). The data is represented as gene expression relative to control sample in both the species.

In *P. nigrum*, relative to healthy condition (control) and when normalized with the endogenous control, the β-1,3-glucanase gene expression was found to get elevated upto the sixth hour. The gene expression was found elevated three times over the control during the second hour and later reverted back to normal level. At the sixth hour, it was found elevated upto four times after inoculation

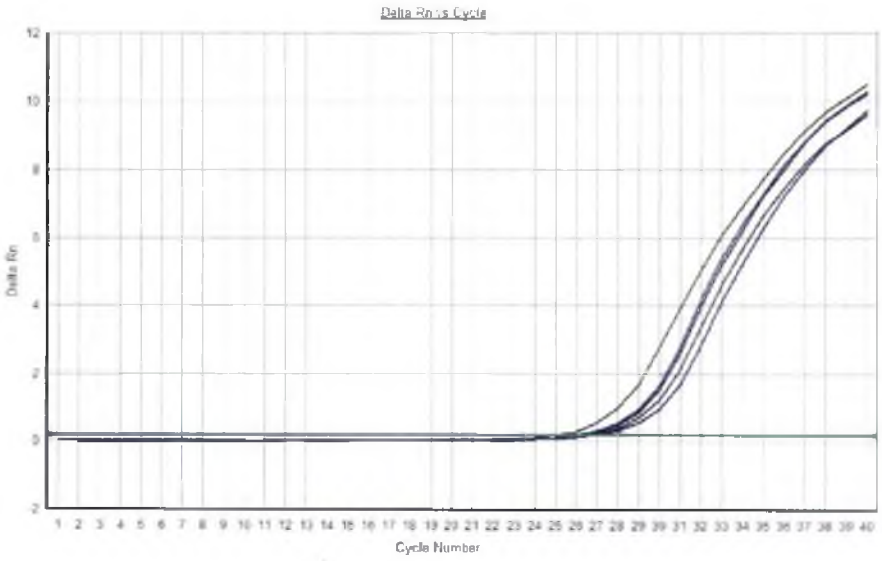
Table 21. Relative gene expression stability derived through geNorm analysis

Treatment	18 S RNA	Actin	GAPDH	Normalization Factor
0 h	1.04E+01	2.52E+01	2.66E+01	**0.9172**
2 h	1.10E+01	2.82E+01	2.77E+01	**0.9832**
6 h	1.80E+01	3.27E+01	2.70E+01	**1.2081**
12 h	8.46E+00	3.32E+01	2.51E+01	**0.9206**
24 h	1.12E+01	2.99E+01	2.66E+01	**0.9969**
P	11.4334103	29.6794928	26.5761266	
M < 1.5	0.384	0.295	0.282	

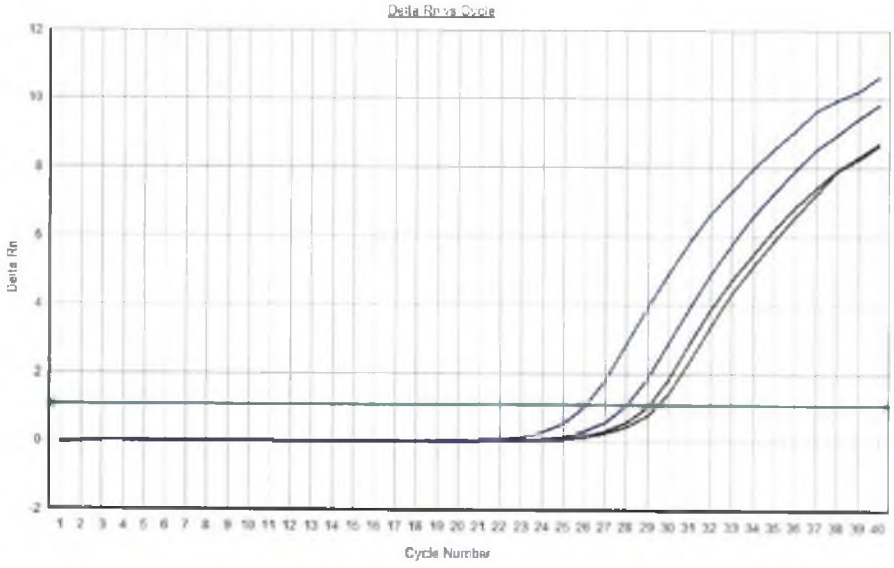
M - Average expression stability P- Pair wise variation

Table 22. NormFinder analysis for selection of reference gene

Sr. No.	Treatment	0 h	2 h	6 h	12 h	24 h	Stability value	Standard error	Best gene
1.	18s	10.402	10.991	18.000	8.464	11.217	2.528	1.463	**GAPDH**
2.	Actin	25.188	28.160	32.717	33.177	29.912	3.232	1.440	
3.	GAPDH	26.562	27.697	27.000	25.060	26.633	2.071	1.600	



A. *P. nigrum*



B. *P. colubrinum*

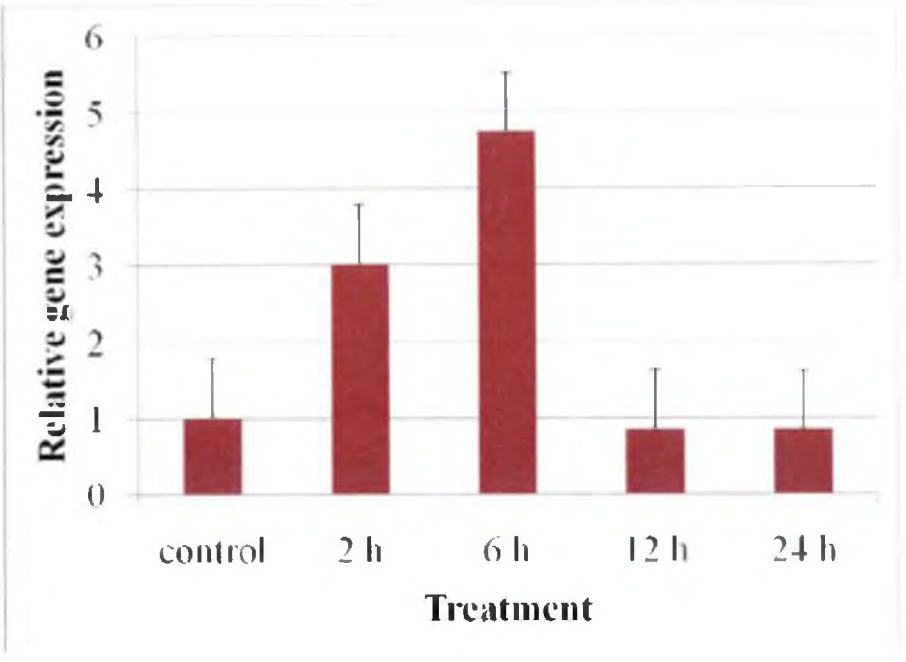
Fig. 19 Amplification curve for β-1,3-glucanase gene for relative quantification

with the pathogen, when compared to the normal level in healthy condition. The values were comparable to the normal condition after 12 h even under infected condition (Fig. 20A).

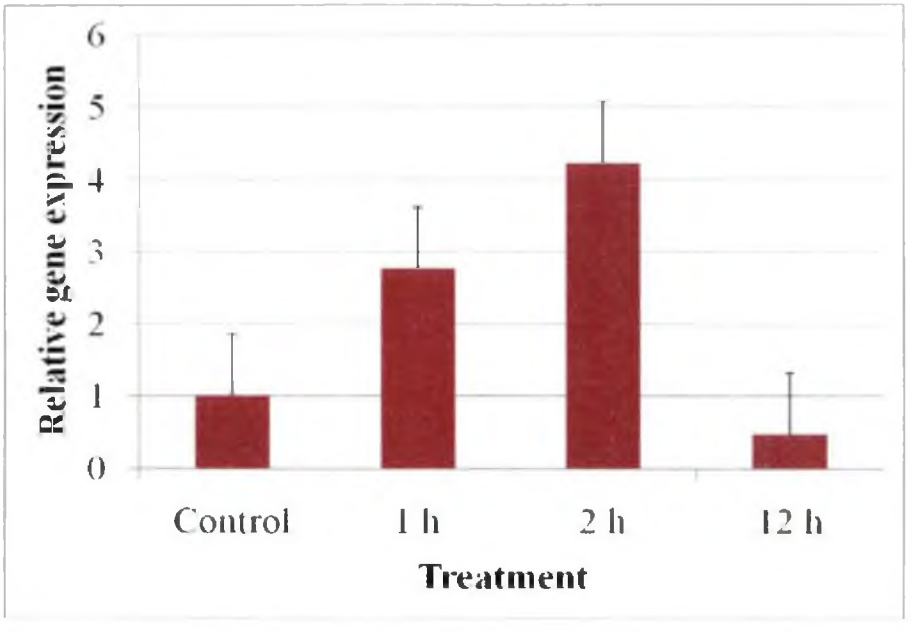
In case of *P. colubrinum*, the response was quick and the gene expression level was found elevated above four times at the second hour after infection compared to healthy (control) plants (Fig. 20B).

The amplification curve showed that the detection of transcript level started much earlier in *P. colubrinum* compared to *P. nigrum*. In case of *P. nigrum*, the transcript detection started at around 26th cycle (Fig. 19A), while it started around 23rd cycle in *P. colubrinum*. This indicated the presence of more number of β-1,3-glucanase transcripts in *P. colubrinum* compared to *P. nigrum*.

The results also indicated that the rate of increase in transcript level was gradual in *P. nigrum* and it was faster in *P. colubrinum*, immediately after infection.



A. *P. nigrum*



B. *P. colubrinum*

Fig. 20. Relative quantification of β-1,3-glucanase gene expression in *Piper spp.* normalized with GAPDH as endogenous control

Discussion

5. DISCUSSION

The results obtained in the study carried out to investigate the role of β-1,3-glucanase gene expression in *Phytophthora* rot resistance in black pepper are discussed in this chapter.

5.1 Source plants

The growth of *Piper colubrinum* was observed to be vigorous and early as compared to *Piper nigrum* when grown under green house conditions. It has been reported that *P. colubrinum* is resistant, while *P. nigrum* is highly susceptible to *Phytophthora* rot. *P. nigrum* is reported to be an allopolyploid, while *P. colubrinum* is a diploid (Ravindran, 2000).

5.2 Maintenance of the pathogen

The original culture of *Phytophthora capsici*, obtained from the Department of Plant Pathology, College of Horticulture, was maintained on carrot agar medium. Since there is a chance of loosing the virulence in continuous *in vitro* culture, the pathogen was artificially inoculated on *P. nigrum*, in which the symptoms could be observed within 18 h of inoculation. The pathogen was re-isolated from the inoculated leaves to regain the virulence and further maintained on carrot agar slants. Such reports of loosing the virulence through repeated culturing and regaining it through re-isolation already exist in various cases (Kashino *et al.*, 1990).

On artificial inoculation of a *P. capsici* on *P. colubrinum*, no symptoms could be observed even after one month of inoculation. This study also confirmed the immunity of *P. colubrinum* and susceptibility of *P. nigrum* to *P. capsici*. Earlier reports are also in conformity with the present findings (Alconero *et al.*, 1971; Sarma and Nambiar, 1982; Vilasini, 1982 and Sarma *et al.*, 1991).

5.3 β-1, 3-glucanase activity

Upon infection with the pathogen, the glucanase enzyme activity was found to increase upto the 24th hour in *P. nigrum* and thereafter started decreasing (Fig. 21). In *P. colubrinum,* the enzyme activity was almost stable throughout the treatment indicating constitutive expression at translational level. However the glucanase activity was significantly higher in *P. colubrinum* compared to *P. nigrum* before and after inoculation with the pathogen. This could be the reason for the resistance of *P. colubrinum* to the disease. The difference in glucanase activity in *P. nigrum* and *P. colubrinum* has been reported by previous workers (Parab, 2000; Achuthan *et al.*, 2002 and Nazeem *et al.*, 2008) and are in conformity with the results obtained in the present study.

5.4 Isolation of nucleic acids

Black pepper is a perennial climber with high content of polysaccharides and phenol, along with high amount of nucleases. This restricts the scope for isolation of nucleic acids with good quality and quantity. In order to isolate nucleic acids from leaf samples of black pepper, the CTAB method suggested by Rogers and Bendich (1994) was used for DNA isolation while TRIzol® method (Chomczynski and Sacchi, 1987) was used for RNA isolation from both the species- *P. nigrum* and *P. colubrinum*.

In DNA isolation, the detergent CTAB disrupts cell membrane to release nucleic acids into the buffer. Along with EDTA, it binds with magnesium ions, a cofactor for most nucleases and thus prevents degradation from endogenous nucleases. The chloroform in presence of isoamyl alcohol removes the pigments, denatures and separates the proteins from DNA. The pH and osmotic pressure of the reaction were maintained with Tris HCl. In addition, CTAB along with NaCl facilitate the separation of polysaccharides based on their differential solubilities.

In this study, it was observed that, when the time of incubation with extraction buffer was reduced, the quantity of DNA observed was high and of

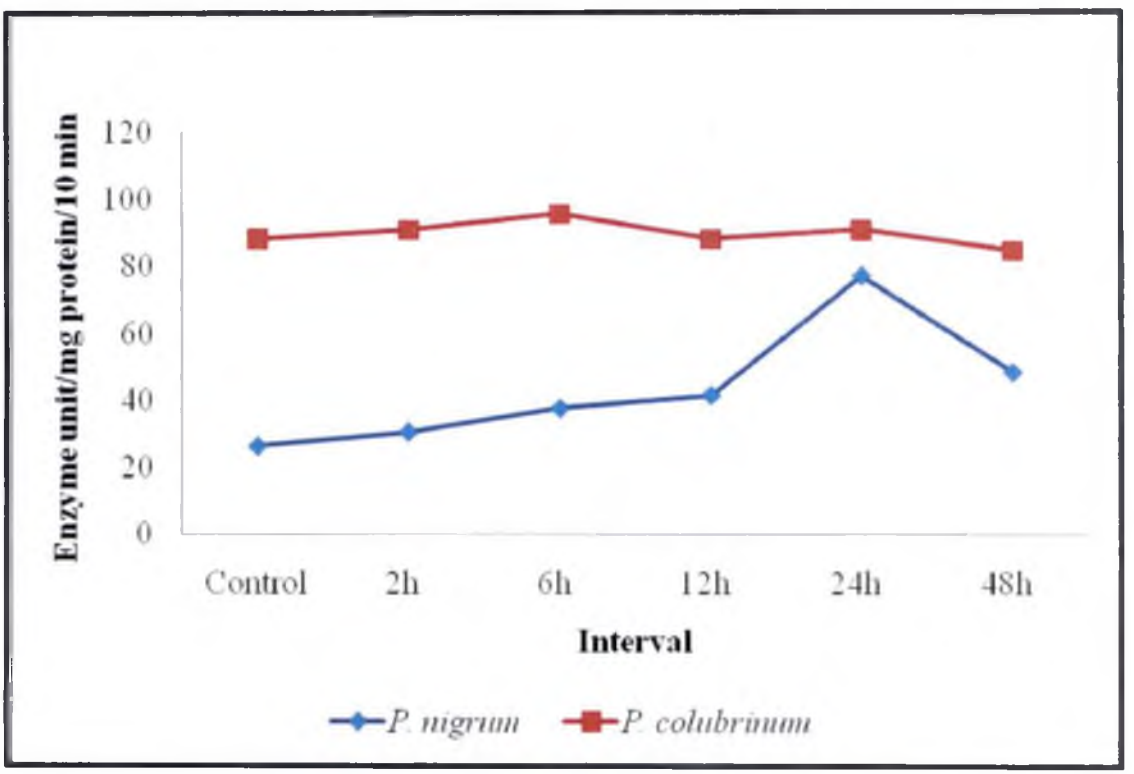


Fig. 21 Comparison of β-1,3-glucanase enzyme activity in *P. nigrum* and *P. colubrinum*

good quality without much degradation. The pellet obtained after DNA isolation was white with tender leaf, while it turned brownish with increase in leaf maturity. This was because of higher phenol interference in black pepper. Tender leaf sample when used with increased concentration of β-mercaptoethonol and polyvinyl pyrrolidone (antioxidants) was found to give better quality DNA. This was also earlier reported by Babu (2000), while isolating DNA from black pepper.

The total RNA was isolated from *Piper spp.* with TRIzol® method as suggested by Chomczynski and Sacchi (1987). This method is a single step RNA isolation method based on guanidine isothiocynate/ phenol/ chloroform extraction. It requires less time compared to conventional RNA isolation methods and is less prone to RNA degradation.

TRIzol® reagent is a monophasic solution of phenol and guanidine isothiocynate. It maintains the integrity of the RNA, while disrupting cells and solubilizing cell components during the sample homogenization. Addition of chloroform, followed by centrifugation, separates the solution into aqueous phase and organic phase. The RNA remains in the aqueous phase and is recovered by precipitation with isopropanol. The DNA and proteins remain in the organic phase and can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropanol yields proteins from the organic phase.

TRIzol® method was reported to be effective in isolation of total RNA from black pepper by Kushwah (2008). This method was thus used for isolation of total RNA at different intervals after inoculation with the pathogen in both the species. It was observed that careless handling of RNA reduces the yield as well as the quality of RNA. Care was taken to have minimum treatments for purification of RNA. Tender leaf sample with minimum incubation with the reagent and prevention of homogenization during thawing gave good quality opaque to transparent RNA pellet.

The quality and quantity of nucleic acids was assessed by agarose gel

electrophoresis and spectrophotometric analysis of samples. The presence of intact and distinct bands indicates good quality for both DNA and RNA samples. However, the RNA quality was also analyzed with presence of bands for 28S, 18S, 5S rRNA and tRNA over the gel. The ratio obtained after spectrophotometric analysis of nucleic acid samples indicated the quality and quantity of the respective sample. The absorbance ratio of 260/280 between 1.8 and 2.0 is considered to be good for both DNA and RNA. The ratio below 1.8, indicate that the sample contains higher amount of proteins, while the ratio above 2.0 indicates the quality of the sample is not good due to possible contamination with polysaccharides and polyphenols (Sambrook and Russel, 2001).

The agarose gel electrophoresis was carried out with TAE buffer for DNA samples in both the species. The DNA samples were often found contaminated with RNA. This RNA contamination was removed with further purification of genomic DNA samples with RNase treatment. RNase A is an endoribonuclease that specifically attacks ssRNA to 3' end pyrimidine residues and cleaves the phosphate linkage to the adjacent nucleotides (Sambrook and Russel, 2001).

For RNA samples, the agarose gel electrophoresis was carried out using MOPS buffer. The results indicated that the RNA was free from DNA and protein contamination. The presence of bands for 28S, 18S, 5S rRNA and tRNA over the gel indicated that the quality of RNA was good. The quantity determined was about 1 $\mu g\mu l^{-1}$ for DNA and about 5 $\mu g/\mu l$ for RNA in the respective samples.

The poor quality DNA and RNA may carry the inhibitors of PCR such as phenols, polysaccharides as well as proteins, which could also inhibit restriction digestion of DNA by restriction enzymes (Sambrook and Russel, 2001). In order to avoid such problems, highly pure quality DNA as well as RNA were used throughout the experiment.

5.5 Southern Hybridization

Southern hybridization was carried out as a prelude to detection of gene

copy number. The samples digested with restriction enzyme upon electrophoresis are expected to separate out and the signals obtained, when hybridized with a radio-labelled probe, would be proportional to the gene copy number.

In Southern hybridization, *P. colubrinum* samples gave higher signals than *P. nigrum*, when hybridized with β-1,3-glucanase gene specific probe (Plate 7). The results were almost similar with the DNA samples digested with the two different restriction enzymes, *Eco*RI and *Hind*III. This difference in banding pattern indirectly highlighted variation in genetic makeup of the two species. These results indicated that the gene copy number may be higher in *P. colubrinum* compared to *P. nigrum*. Similar reports also exist in other crop plants like peach (Thimmapurum *et al.*, 2001) and rubber (Thanseem *et al.*, 2003). This could be the reason behind why the glucanase gene expression was higher in resistant genotype *P. colubrinum* compared to susceptible one *P. nigrum*. Real-time PCR analysis was done to further confirm the results of Southern hybridization.

5.6 Northern hybridization

The dot blot was done for total RNA from *P. nigrum* and *P. colubrinum* with β-1,3-glucanase specific probe to detect the presence of transcript. The results obtained after hybridization suggested that the glucanase transcript was present in all the samples indicating glucanase gene expression throughout the treatment. However, the dot blot was not good enough to analyse the relative gene expression in each treatment. This may be because the samples are not separated out in dot blot assay and DNA contamination is another possible factor which can interfere with the results.

In order to observe the relative quantity of glucanase gene specific transcript, Northern hybridization was carried out with total RNA of *P. colubrinum* at different intervals after electrophoresis and blotting, and using β-1,3-glucanase specific probe. The results clearly indicated that the signal strength was much more varying at different intervals after inoculation with *P. capsici*. Differential expression of glucanase gene during stress condition has been

confirmed through Northern hybridization in various other crops (Li *et al.*, 2001; Dean *et al.*, 2002)

5.7 Real-time PCR assay

Real-time PCR is a technique which record data during each cycle of the amplification process as it occurs and allows quantification of the initial amount of nucleic acid template most specifically, sensitively and reproducibly. It is based on the detection and quantification of fluorescence during each cycle of PCR (Higuchi *et al.*, 1993, Raeymaekers, 2000; Wong and Medrano, 2005; Dussault and Pouliot, 2006 and Espy, 2006). The increase in fluorescence in each cycle is proportional to the amount of product formed. In the initial cycles of PCR, there is little change in fluorescent signal, and it defines baseline. An increase in fluorescence above the baseline indicates the detection of accumulated target. Reactions are characterized by threshold cycle (C_T), the cycle at which fluorescence emitted by target passes the threshold. Threshold is the line whose intersection with the amplification plot defines the threshold cycle (C_T). The threshold level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. Consequently, greater the quantity of target DNA in the starting material, a significant increase in fluorescent signal will appear, yielding lower C_T and *vice versa* (Heid *et al.*, 1996 and Dorak, 2006).

SYBR® Green I is a DNA binding dye which binds to the minor grove of double stranded DNA and emits fluorescence thousand-fold greater than when it is free in solution (Wittwer *et al.*, 1997). Therefore, greater the amount of dsDNA present in the reaction tube, greater the binding by SYBR® Green, producing detectable amount of fluorescent signal. Thus, any amplification of DNA in the reaction tube is measured.

In the present study, real-time PCR assay was performed to determine the gene copy number and expression pattern for β-1,3-glucanase gene from the two

Piper spp. The assay was divided into absolute quantification and relative quantification

Absolute quantification was carried out to determine the β-1,3-glucanase gene copy number using serially diluted standard DNA derived from PCR amplified product from the genomic DNA.

Relative quantification was carried out with cDNA samples synthesized from total RNA isolated at different intervals after inoculation with the pathogen. A stable housekeeping gene was identified as an endogenous control for normalization of data. The gene expression pattern was derived from both susceptible and resistant genotypes after normalizing the data with the expression of endogenous control.

During the present study, the SYBR® Green master mix (Applied Biosystems, USA) was used for detection of amplification in real-time PCR in both absolute and relative quantification. This dye is non-specific in binding to DNA and hence can detect any double stranded DNA present in the reaction mix. To ensure the specificity of the reaction, dissociation curve of amplified product was obtained during the real-time PCR amplification. The single peak indicates uniqueness of amplified product, while the multiple peaks indicate the presence of non-specific product as well as primer dimers.

In order to rule out primer dimer formation, in addition to the reactions with template DNA, one extra reaction was included as NTC (no template control). Absence of amplification in NTC indicated that the amplification was free from primer dimer formation. If there was primer dimer formation, amplification would have been observed as presence of peak in dissociation curve as well as late detection of amplification on amplification curve.

In case of amplification with cDNA, in order to avoid contamination with the genomic DNA in the sample, one NAC (No amplification control) was included in the reaction. NAC is the cDNA synthesis reaction without addition of reverse transcriptase. NAC was maintained while preparing cDNA from total RNA without adding reverse transcriptase keeping all other reagents constant.

5.7.1 Absolute quantification

Absolute quantification is based on the amplification of the unknown sample of DNA with the serially diluted standard DNA, specific to the same gene, whose concentration is known. The standard DNA is the purified PCR product whose concentration is known. The concentration of the standard DNA was assessed though spectrophotometric analysis and using Avogadro's constant. Further, the dilution of standard was done tenfold with respect to each other. A minimum of three different dilutions are needed to carry out absolute quantification. However, five or more dilutions are considered ideal for detection of gene copy number. Six dilutions were used to detect copy number in the present study as instructed by the manufacturer.

The standard curve was obtained after plotting threshold cycle against log of respective DNA concentration in terms of copy number/µl. The results obtained for the unknown sample are interpolated with the standard curve and given as the copy number/µl (Heid *et al.*, 1996). During amplification, the efficiency for both unknown and standard DNA amplification is considered as equal (Souze *et al.*, 1996). For the higher accuracy and sensitivity of the reaction, the slope of the standard curve should be around -3.3, while for higher efficiency the R^2 value should be more than 0.99, as per the instructions of the manufacturer (Applied Biosystems, USA).

In the present study, for absolute quantification, primers were designed for the gene of interest, β-1,3-glucanase. The sequence information available on NCBI and the software 'Primer Express 3' were utilized for the purpose.

The amplicon obtained from genomic DNA as template with the primers designed was of the expected size (Table 12). The threshold cycle values obtained upon real-time PCR assay of the serially diluted sample when plotted against the dilution provided almost a straight line, fit for use as standard curve. The slope and R^2 values of the curve were optimum (Fig. 10).

Gene copy number was determined based on this standard curve for the unknown samples of genomic DNA from *P. nigrum* and *P. colubrinum,* through real-time PCR amplification. It was interesting to observe high copy number in *P. colubrinum* than in susceptible genotype *P. nigrum*. In this context, it is worthwhile to analyze the ploidy level of both the species. *P. colubrinum* is reported to be a diploid and *P. nigrum* as a polyploid (Ravindran, 2000).

Earlier workers have reported the ploidy level to influence disease reaction. Diploids are reported to be more tolerant than polyploids (Craenen and Ortiz, 1996; Andreas *et al.*, 2006). However, it is controversial to observe the gene copy number for β-1,3-glucanase to be higher in the diploid species *P. colubrinum* than in the polyploid studied. In the present study higher copy number was observed in *P. colubrinum* with Southern blotting. More number of signals in the restricted DNA of *P. colubrinum* is indicative of presence of the gene in different fragments of the genome suggesting higher copy number. Amplification of genomic DNA through real-time PCR confirmed the high copy number of the said gene in *P. colubrinum* and it was quantified as double than that of *P. nigrum*. So there could be chances for presence of multiple loci as revealed by Southern blot, governing the gene expression. This might have happened during the course of evolution through duplication and translocation of genes (Gardner *et al.*, 1991).

5.7.2 Relative quantification

The relative quantification was used to analyze the gene expression at transcript level relative to another reference sample as a control. During real-time PCR, changes in sample gene expression are measured based on either an external standard or a reference sample.

In the present study, the relative quantification was carried out using Comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). This method does not require a standard curve and is thus useful when assaying a large number of samples. The comparative C_T method calculates changes in gene expression as a relative difference between an experimental and calibrator sample. Calibrator is the control sample which is maintained without the treatment or which is stable throughout the treatment. The threshold cycle data obtained after relative quantification for gene expression in the study was normalized with the threshold cycle data for expression of an endogenous control gene. Endogenous control is the reference gene, mostly housekeeping gene whose expression is stable throughout the treatment. The normalization of gene expression is used to correct the sample to sample variation. Due to lack of any one of the stable gene for a particular experimental condition, validation of the expression stability of an endogenous control gene for the specific requirement is carried out prior to its use for normalization (Schmittgen and Zakrajsek, 2000).

For relative quantification in the present study β-1,3-glucanase gene specific primers 'GlucII' which were standardized for absolute quantification were used after optimization with the cDNA.

In order to standardize the reference gene for use as an endogenous control, three genes viz, 18S rRNA, actin and GAPDH were selected. Studies carried out for relative quantification by earlier workers also revealed these housekeeping genes to be stable under different biotic and abiotic stress conditions and are thus commonly used as endogenous control in real time PCR assay (Suzuki *et al.,* 2000; Bustin, 2002). Primers were designed based on conserved sequences present for these genes using software 'Primer Express 3'. The designing of primers was carried out considering various parameters described for real-time PCR by the manufacturer.

These gene specific primers were first screened for amplification with cDNA. The amplification data revealed that the amplification was satisfactory for

all the primers. The primers were further standardized to get the optimum concentration for amplification with cDNA. The optimum primer concentration is one which gives the minimum threshold cycle value with maximum efficiency and with single peak in dissociation curve and maximum Rn value. The cDNA concentration was also standardized using different dilutions of cDNA. The optimum concentration is the one, which gives maximum amplification with minimum threshold cycle value.

To identify the stable endogenous control gene, amplification of three housekeeping genes viz, 18S rRNA, actin and GAPDH was carried out with selected primer and cDNA concentration in real-time PCR. The cDNA synthesized from total RNA isolated at different intervals after infection with the pathogen in *P. nigrum* was used for the study. The evaluation of expression stability and selection of best housekeeping gene was done based on geNorm (Vandesompele *et al.*, 2002b) and NormFinder (Anderson *et al.*, 2004) software.

Vandesompele *et al.* (2002b) defined two parameters to quantify the housekeeping gene stability: M (Average expression stability) and V (Pairwise variation). A low M value is indicative of a more stable expression. Vandesompele *et al.* (2002b) proposed 0.15 as a cut-off value for the pairwise variation below which the inclusion of an additional control gene is not required. Depending upon the stress imposed the stable housekeeping genes in a species may vary and this signifies the need for standardization in each experiment (Bustin, 2000).

Among the three housekeeping genes studied, GAPDH showed least (0.163) average expression stability (M) compared to others revealing high stability of the gene during infection with *P. capsici* (Table 17 and Fig. 22). GAPDH was identified as the most stable gene, also in the analysis with 'NormFinder'. The stability value observed for GAPDH was the least (2.071), while it was 3.232 for actin gene.

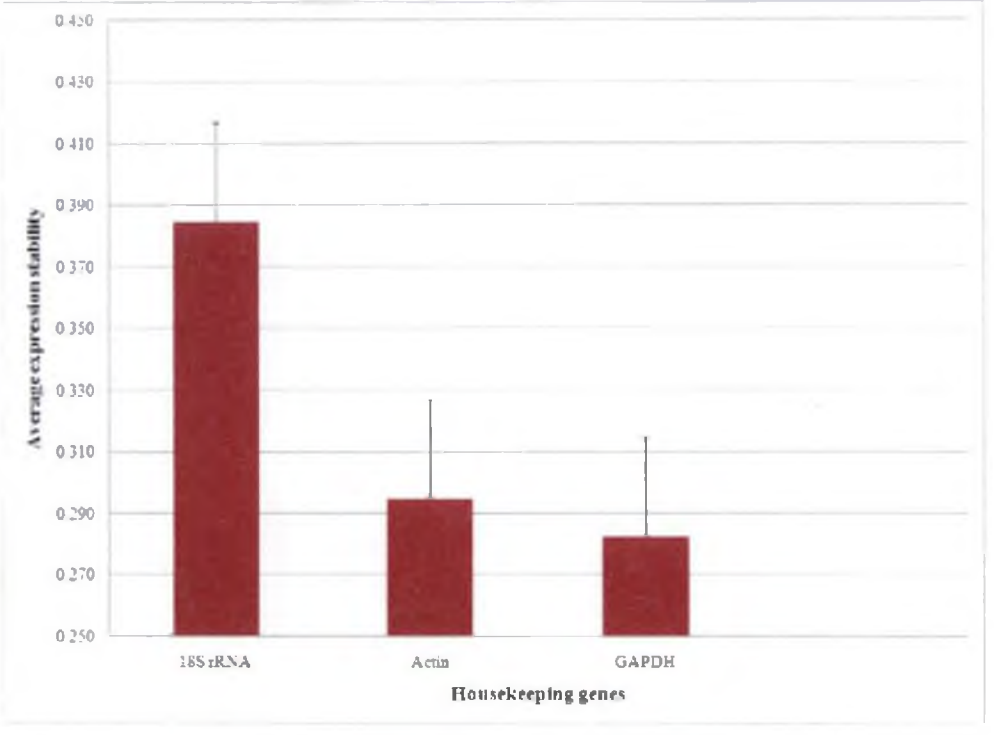


Fig. 22. geNorm analysis for selection of reference gene

Glyceraldehyde-3-phosphate dehydrogenas (GAPDH) in an enzyme involved in the glycolysis in the respiratory pathway. It is also involve in other metabolic processes such as transcription activation, initiation of apoptosis, etc. and it has been used as reference gene in various other crops (Tarze *et al.*, 2007; Nelson and Cox, 2007). Thus GAPDH was selected as reference gene for normalization of gene expression data in the present study.

The relative quantification was carried for β-1,3-glucanase gene by amplifying it along with endogenous control with similar reaction pattern using cDNA synthesized from total RNA isolated at different intervals from both the *Piper spp.* The threshold cycle values obtained for glucanase gene were normalized with that of endogenous control. The data was expressed as gene expression relative to control (healthy) sample.

The relative quantification for β-1,3-glucanase gene in both the species clearly indicated the differential pattern of expression in both the species. The gene expression was found to be four times higher to its initial level at healthy condition in *P. colubrinum*, while it was only three times higher in *P. nigrum* within 2 h after inoculation.

The amplification curve showed that the detection of transcript level started much earlier in *P. colubrinum* compared to *P. nigrum*. In case of *P. nigrum*, the transcript detection started at around 26th cycle (Fig. 19A), while it started around 23rd cycle in *P. colubrinum*. This indicated the presence of more number of β-1,3-glucanase transcripts in *P. colubrinum* compared to *P. nigrum*. The results also indicated that the rate of increase in transcript level was gradual in *P. nigrum* and it was faster and higher in *P. colubrinum*.

The results of the study gives a clear picture of involvement of β-1,3-glucanase on the defence mechanism in *Piper spp.* The susceptible species, *Piper nigrum* recorded low enzyme activity compared with *P. colubrinum* under healthy conditions. Upon inoculation with the pathogen, the enzyme activity got elevated in *P. nigrum*, but did not reach the level of native activity in *P. colubrinum*, in

which it was almost stable even after inoculation. The gene copy number determined was relatively high in the resistant genotype and was almost double than that of susceptible one. At transcript level also, *P. colubrinum* showed better reaction with respect to the β-1,3-glucanase gene confirming its role in the defence mechanism.

The results obtained could be well exploited for disease management in the valuable spice crop, black pepper. Enhancing the expression level using elicitors during the peak season of infection would be a wise option. Techniques to increase the gene copy number and placing the gene under a strong promoter for better expression are the other options suggested.

Summary

6. SUMMARY

The present study entitled "Real-time PCR assay for β-1, 3-glucanase gene expression in black pepper (*Piper nigrum* L.)," was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture (COH), Vellanikkara during the period from December 2007 to June 2009. The study was intended to find out β-1,3-glucanase gene expression level and gene copy number in black pepper in comparison with *Piper colubrinum* when challenged with the pathogen *Phytophthora capsici*. Salient findings of the study are summarized in this chapter.

1. Plant material (rooted cuttings) for both *P. nigrum* and *P. colubrinum* was maintained under greenhouse condition in mud pots filled with potting mixture. Both *P. nigrum* and *P. colubrinum* showed vigorous and healthy growth.

2. The pathogen, *P. capsici*, showed luxuriant growth with uniform dense cotton wool like aerial mycelium on carrot agar medium. On artificial inoculation, the pathogen developed disease symptoms on susceptible genotype of *P. nigrum* by developing lesions in the area surrounding the inoculation within 18 h. In order to regain virulence, the pathogen was further re-isolated from the infected plant. No disease symptom was observed on the resistant genotype, *P. colubrinum*.

3. The leaf samples from infected plants were further used for β-1,3-glucanase enzyme assay. Laminarin was used as glucanase substrate and the activity was assessed as enzyme unit/mg protein/10 min at 40^0C. The enzyme activity was found to increase upto 24^{th} hour in *P. nigrum* and thereafter reverting back to the normal level. In contrast, the enzyme activity was almost stable throughout the treatment in resistant species *P. colubrinum*. The native enzyme activity in *P. colubrinum* ranged between 84 and 95 units while the

maximum activity observed in *P. nigrum* was only 76 units when challenged with the pathogen.

4. To isolate the genomic DNA from black pepper, CTAB method as suggested by Rogers and Bendich (1994) was used with slight modifications. The method was found to give good quality and quantity DNA as revealed by agarose gel electrophoresis and spectrophotometric analysis. This DNA was used to perform Southern blotting and real-time PCR assay.

5. The total RNA was isolated from *Piper spp.* using TRIzol® method as suggested by Chomczynski and Sacchi (1987) with slight modifications. The leaf samples inoculated for different intervals with the pathogen were used for total RNA isolation from both the species. The agarose gel electrophoresis and spectrophotometric analysis revealed that the total RNA isolated was of good quality containing high amount of RNA. This RNA was further used for Northern hybridization and cDNA synthesis to carry out real-time PCR assay.

6. Southern hybridization was performed as per the protocol suggested by Sambrook and Russel (2001) with slight modifications. The genomic DNA digested with restriction enzymes *Eco*RI and *Hind*III was blotted on to Hybond XL nylon membrane. The blotted DNA was hybridized to β-1,3-glucanase gene specific probe with overnight incubation at 62^0C. More number of signal bands were observed in *P. colubrinum* compared to *P. nigrum* in both the samples. This indicated that the gene copy number was more in *P. colubrinum* compared to *P. nigrum*.

7. Absolute quantification was carried out in real-time PCR, to detect gene copy number for β-1,3-glucanase using gene specific DNA as standard. Primers were designed for the β-1,3-glucanase gene from the conserved sequences available at NCBI, according to specifications suggested for real-time PCR (Applied Biosystems, USA). These primers were further standardized and used for real-time PCR amplification. The DNA concentration was also

standardized to obtain optimum real-time PCR amplification. The glucanase gene specific standard was developed from PCR purified product.

8. The absolute quantification was performed for unknown samples of *Piper spp.* with the serially diluted standard DNA. The result was expressed as copy number/µl. The results showed that the copy number was 57438.00 in *P. nigrum,* while it was 96422.00 in *P. colubrinum.* This data clearly supported the conclusion that the gene copy number was higher in *P. colubrinum* as revealed by Southern hybridization and also indicated that it was almost double than that of *P. nigrum.*

9. Northern hybridization along with dot blot was performed with total RNA to assess the glucanase gene expression level after inoculation with the pathogen. Dot blot was carried out by loading equal quantity of total RNA on the grids drawn on the nylon membrane. The RNA blotted was immobilized and hybridized with glucanase gene specific probe. Similarly, Northern hybridization was performed after agarose gel electrophoresis of the RNA sample. The dot blot for total RNA detected the presence of β-1,3-glucanase gene specific transcript throughout the treatment in both of the species. However, the Northern blot analysis when compared with 18S rRNA, showed differential level of gene expression at transcriptional level.

10. The relative quantification was carried out to detect the level β-1,3-glucanase gene expression at transcriptional level in both the genotypes following inoculation with the pathogen. To normalize the data with the expression of stably expressed endogenous control, primers were designed for three commonly used housekeeping genes, viz, 18S rRNA, actin and GAPDH. Optimum concentrations were standardised for the primer set and cDNA for real-time PCR assay.

11. The endogenous control genes were amplified with the cDNA samples, prepared from total RNA isolated from *P. nigrum* at different intervals after infection with the pathogen. The threshold cycle values were analyzed with

the softwares, geNorm and NormFinder. The results obtained indicated that the GAPDH was the most stable gene in the present study. Thus, GAPDH was selected as an endogenous control (reference gene) for relative quantification.

12. The relative quantification was performed with the cDNA samples synthesized from total RNA, isolated at different intervals after inoculation with the pathogen, from both the *Piper spp.* The amplification was performed for glucanase gene as the target and GAPDH as an endogenous control (reference gene). The reaction conditions were maintained same for both the target and the endogenous control. The data was expressed as gene expression relative to the control (healthy) sample.

13. The level of gene expression was high in *P. colubrinum* under normal conditions and it was found elevated over 4 times within 2 h after inoculation with the pathogen. The level of expression was low in *P. nigrum* in healthy condition and was found elevated only upto 3 times within 2 h when challenged with the pathogen. The rate of increase was faster and higher in *P. colubrinum* than in *P. nigrum.*

14. The high copy number of the genes and its better expression levels would have contributed much to the tolerance of *P. colubrinum* to *Phytophthora* rot disease.

15. The findings can be well exploited for *Phytophthora* rot management in *P. nigrum.*

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Appendices

Appendix I

Reagents required for β-1,3-glucanase enzyme assay

A. Sodium acetate buffer (0.05 M, pH 5)

Composition

Sodium acetate	:	0.41 g
Distilled water	:	100 ml

Sodium acetate of about 0.41g was dissolve in 50 to 70 ml of distilled water followed by adjusting the pH to 5 with 0.1 N HCl. The final volume was made to 100 ml with distilled water.

B. Laminarin (4 per cent)

Composition

Laminarin	:	40 mg
Distilled water	:	1000 µl

The laminarin was dissolved in distilled water by heating briefly in a boiling water bath just before use.

C. Sodium hydroxide (4.5 per cent)

Composition

Sodium hydroxide	:	4.5 g
Distilled water	:	100 ml

D. Dinitrosalicylic acid (DNS)

Composition

Dinitrosalicylic acid	:	0.88 g
Na^+K^+ tartarate	:	25.5 g
Sodium hydroxide	:	30 ml
Distilled water	:	88 ml

DNS of about 0.88 g was dissolved in 88 ml of distilled water at 50^0C. This was followed by dissolving Na^+K^+ tartarate with drop by drop addition of 30 ml NaOH (4.5 per cent). The resultant solution was cooled at room temperature and stored in dark at room temperature.

E. Cystein HCl (0.05 M)

Composition

Cystein HCl	:	0.439 g
Distilled water	:	50 ml

F. Phenyl methane sulphonyl fluoride (PMSF) (0.1 M)

Composition

PMSF	:	20 mg
Isopropanol	:	1 ml

The solution was prepared fresh just before use.

G. Ascorbic acid (5 mM)

Composition

Ascorbic acid	:	0.05 g
Distilled water	:	100 ml

Appendix II

Reagents required for nucleic acid isolation

A. Reagents required for DNA isolation

1. C-TAB extraction buffer (100 ml)

CTAB	:	2 g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1 g

Adjusted the pH to 8 and made up the final volume upto 100 ml.

2. CTAB (10 per cent, 100 ml)

CTAB	:	10 g
NaCl	:	4.09 g

3. Chloroform- isoamyl alcohol (24: 1)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0^0C and was used for the study.

5. Ethanol (70 per cent)

To the 70 parts of absolute ethanol (100 per cent), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

7. TAE buffer (50 X)

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8)	:	0.5 ml

The solution was prepared , autoclaved and stored at room temperature.

B. Reagents required for total RNA isolation

1. DEPC treated water (0.025 per cent)

DEPC (250 µl) was added to water (1000 ml) and allowed to swirl on magnetic shaker for 2 h. The water is then autoclaved twice and stored at room temperature. It was used for preparation of solutions required for RNA isolation and for Northern blotting.

2. Preparation of MOPS (3-(N-Morpholino)-propanesulfonic acid) buffer (10X, 500 ml)

MOPS (0.2 M)	:	20.9 g
4 M Sodium acetate (80 mM)	:	10 ml
0.5 M, pH-8, EDTA (10 mM)	:	10 ml

The pH was adjusted to 7 and the final volume made to 500 ml with DEPC treated water.

Appendix III

A. Reagents required for Southern and Northern blotting

1. Depurination solution (0.2 M HCl, 400 ml)

   HCl : 7.2 ml

   The final volume was made to 100 ml with sterile distilled water.

2. Denaturation solution (400 ml)

   NaCl (1.5 M) : 35.06 g

   NaOH (0.5 M) : 8.0 g

3. Neutralization solution (pH 7.4, 400 ml)

   NaCl (1.5 M) : 35.06 g

   TrisHCl (1 M) : 63.04 g

4. SSC (Standard Saline Citrate) (20X, 1000 ml)

   Sodium citrate : 88.23 g

   NaCl : 175.32 g

   Sodium citrate was dissolved in 800 ml of sterile distilled water followed by dissolving NaCl. The pH was adjusted to 7 and the final volume was made to 1000 ml. The buffer was autoclaved and stored at 4^0C.

All the reagents, required for Northern Blotting, were prepared in DEPC treated water.

B. Details of the reagent provided with Megaprime DNA labeling system (Amersham Biosciences, USA)

1. Primer solution

   Random nonamer primers in an aqueous solution

2. Nucleotide solutions

   dATP, dCTP, dGTP and dTTP in Tris HCl, pH- 8, 0.5 M EDTA

3. Reaction buffer

   A 10X concentrated buffer containing Tris HCl, pH-7.5, β-mercaptoethonol and $MgCl_2$

4. Enzyme solution

   1 unit/μl DNA polymerase 1 Klenow fragment cloned in 100 mM potassium phosphate, pH 6.5, 10 mM β-mercaptoethanol and 50 per cent glycerol

C. Composition of prehybridization and hybridization solution

1. Prehybridization solution (Bangalore Genei)

   Sodium citrate, denhardt's reagent, SDS, sonicated salmon sperm DNA.

2. Hybridization solution (Bangalore Genei)

   Sodium citrate, denhardt's reagent, SDS, sonicated salmon sperm DNA.

Appendix IV

Composition of carrot agar medium

Carrot	:	200 g
Agar	:	20 g
Distilled water	:	1000 ml

APPENDIX V

Sequence information used for primer designing

A. β-1,3-glucanase

> *Piper nigrum beta 1,3-glucanase-like mRNA, complete sequence*
GCCGCGGGAATTCGATTATGGGAACAATCTATATACAGATCAAGAAACATCCAA
AGAATGAGAATCTATTGGCCAAGGCCAGAGGCACTCCAAGCATTGAGAGGCTCC
AACATCCAAGTCCTCCTTGGTGTCCCCAACGACTGGCTCCAAGAGCTAGCCAGCA
ACCCCTCCGCGGCAAGCCGGTGGGTGCAAGACCACGTCCGGGCATACTGGCCGG
CTGTTCGATTCCGGTACATCGCCGTCGGAAACGAGGTCATCCCCAGAGGCAATGC
TCAGGCACTACTCCCGGCCATGCGAAATGTTTACAACGCAATTGCTGCCGCCGGC
CTACAGAATGACATCTCAAGGTGTCAACTGCCGTGGACACCGGGGTCCTCCTCCC
TCCGAGCGCGGTTCCTGGCCAACACCGGAGCTCCCCTCCTAGCCAACGTCTACCC
GTATTTTAGCTACAAGGGCAATCCTGCGGACATCCCACTGTCGTATGCACTGTTTA
CTTACCCATCGGTTA

> *Piper colubrinum*, β-1,3-glucanase-like gene, partial sequence

GGGCATACTGGCCGGCGGTTCGATTCCGGTACATCGCCGTCGGCAACGAGGTCAT
CCCCCGAGGCAATGCTCAGGCAGTGCTCCCGGCCATGCGAAATGTTTACAACGCC
ATTGTTGCCGCCGGCCTACAAAATGACATCAAGGTGTCGACTGCCGTGGACACCG
GGGTCCTAGGCACATCCTACCCTCCCTCTGCCGGTGCATTCTCCGGCGCGGCTGA
CGGGTACTTGCGGCCAATAGCACGGTTCCTCGCCGACACCGGAGCTCCCCTTCTA
GCCAACGTTTACCCGTATTTTAGCTACAAGGGCAATCCTGCCGACATCCCATTGTC
ATATGCACTGTTCACGTACCCATCGGTTAGAGTGCAGGATGGGGCTAACCAATAT
AGGAACCTCTTTGATGCCATCTTGGATGCTATGTACGCTGCCTTGGAGGAGGTTG
GGACACCCAATGTTAGATAGTTGTATCGGAGAGTGGTTGGCCGTCGGCGGGGCAT
TCTGCGGCCACCATTGACAATGCAAGGACTTACAACCAGAATCTGATCAGGCATG
TTGGGCAGGGGACTCCAAAAAGGCCAAATCGAATTCCCGCGGCCGCCA

B. Actin

> *Magnolia denudata* actin mRNA, complete cds

ATGGCTGATGGTGAAGATATTCAACCCCTTGTCTGTGACAATGGAACTGGAATG
GTGAAGGCTGGATTAGCTCCCTATGTATGTCCCAGGGCAGTGTCCCCAATTTTTG
TCGGTCGGCCTCGTCACACTGGTGTTATGGTTGGGATGGGACAAAAAGATGCTTA
TGTTGGTGATGAGGCCCAATCCAAGAGAGGTATTCTCACTTTGAAATACCCCATT
GAGCATGGAATTGTGAGTAACTGGGATGACATGGAGAAGATCTGGCATCACACC
TTCTACAATGAGCTCCGTGTAGCTCCTGAAGAACATCCCGTCCTCCTTACTGAGG
CCCCTCTCAACCCCAAGGCCAACAGGGAGAACATGACACAGATCATGTTTGAAA
CCTTCAATGTCCCTGCCATGTATGTTGCTATTCAGGCTGTCCTCTCCCTCTATGCC
AGTGGTCGTACCACAGGTATTGTGCTTGATTCCGGTGACGGAGTCAGCCACACCG
TCCCAATCTATGAAGGTTATGCGCTCCCCCATGCTATCCTCCGGCTTGGACTTGCT
GGACGGGACTCGACCGATGCCCTGATGAAGATCCTCACAGAGAGAGGGTACTCG
TTCACCACCACTGCTGAGCGGGAAATTGTCCGGGATATGAAAGAGAAGCTTGCC
TATGTGGCCCTTGACTATGAACAAGAGCTTGAGACTGCCAAGAGCAGCTCCTCG
ATTGAGAAGAGCTATGAGCTGCCTGACGGGCAGGTGATCACCATTGGAGCTGAG
AGGTTCCGTTGCCCAGAAGTGCTCTTCCAGCCTTCTCTCATTGGGATGGAAGCTG
CTGGTATCCACGAGACCACTTACAACTCGATCATGAAGTGTGATGTCGATATCAG
GAAGGATTTGTATGGCAACATCGTGCTTAGTGGTGGGTCTACCATGTTCCCGGGC
ATTGCTGATCGTATGAGCAAGGAGATCACTGCCCTTGCTCCTAGCAGCATGAACA
TCAAGGTGGTGGCTCCACCAGAGAGGAAGTACAGTGTCTGGATTGGAGGGTCTA
TCCTGGCCTCTCTCAGCACCTTCCAGCAAATTTGGATGTCCAAGGGGGAATATGA
CAAACCCGGTCCATCCATTGTCCACAGGAAATGCTTCTAA

C. GAPDH

> *Piper nigrum* glyceraldehyde 3-phosphate dehydrogenase gene, partial sequence

TGGTAATGATTATTAACATTACGCAGCAAACATGGTCGTCTTTGGTAAATCCGTC
AGCTTGTGTAAGGGTTGATAGTTTTGATTTTTTCATCCTGTGACATGCAGCTACCC
AGAAAACCGTTGATGGACCTTCAATGAAAGATTGGAGAGGCGGAAGGGCTGCTA
GCTTCAACATCATTCCTAGCAGCACGGGAGCTGCCAAGGTAAAATCTGCCTGTTG
TATTTTTGCTTGTGTATGTTGGTTTTTTACCATGACTCCCAGTTATACAAGCTCTTT
TTGTATTGTGATACCTGCCCGATAAACCATTTGCTTTAGTATCAGGTAGGTATATA
TTGATTAATGTATTTCTATTGAATTTAATGTAGTGAGTGTTTTTTTAACTTGGATTT
CGCAGGCTGTTGGTAAGGTGCTTCCTTCTTTGAATGGAAAGCTTACTGGAATGTC
ATTCCGTATTCCTACCGTCGATGTTTCTGTGGTTGACCTTACTGTGAGGCTTGAAA
AAGCCGCAACATACGAGCAGATTAAGGCGGCCATCAAGTAAGTGGTGACTTCTG
TCGTGTTTTTCAAGTTGAGTTGGTGAATGTTGAAAGATGGTAATTTGGTACTTTTG
TGACTTGTGAAATTTTGTATATTCTTTATTTTTGTCAAAAGAGCAATTGTCTCATC
CGTTCGTCTTTTGGTACGACAGGGAGGAGGCTGCGGGTAAATTGAAGGGCATTCT
TGGATATACCGAAGATGATGTTGTTTCCTCTGACTTCGTCGGTGACAATAGGTAA
GCTGATATCATGCATTTTTTTTTGCCCATCTGTGATGAAAAGAAAAACCCATAAA
GACGTTTTGTCCGTCCATAACGATCATTTTCCATCTCAGATGAGAGAAAAAAAAA
ACAGAGATTTTGTTTTACAGGTATTTTCTTGTTGTTGGGCTTTGTCTTACCATTTTG
TCTGCTTTCTCTGCAAACAGG

D. 18S rRNA

> *Piper nigrum* 18S ribosomal RNA (18S rRNA)

CGAATGGCTCATTAAATCAGTCATAATTTGTTTSATGGTATTCACTACTCGGATAA
CCGTAGTAATTCTAGAGCTAATACGTGCACCAAACCCCGACTTCTGGGTAGGGAT
GCATTTATTAGATAAAAGGTCAGCCCGTTGAACTGATGATTCATGATAACTTGAC
GGATCGCACGGCCTTTGTGCTGGGATAGTGGCCTACCATGGTGGTGACGGGTAA
CGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATC
CAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGGGAGGTAGTG
ACAATAAATAACAATACCGGGCTCTTCGAGTCTGGTAATTGGAATGAGNACAAT
CTAAATCCCTTAACGAATTCTTGGATTTATGAAAGACGAACAACTGCGAAAGCA
TTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGA
TCAGATACCGTCCTAGTCTCAACCATAAACGATGCCGACCAGGGATCGGCGGAT
GTTGCTTTTAGGACTCGCCGGCACCTTATGAGAAATCAAAGTTTTTGGGTTCCGG
GGGGAGTATGGTCGAAACTTACCGGTCCAGACATAGTAAGGATNGACAGACTGA
TAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGC
GATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTAC
GTGGAGGCNACCCTCCACGGCCAGCTTCTTAGAGGGACTATGGCCGTTTAGGCC
ACTTAAATTTCATCGTGATGGGGATAGATCATTGCAATTGTTGGTCTTCAACAAG
GAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGACTACGTCCCTGCCCTTTG
TACACACCGCCCGTCGCTCCTACCGATTGGATGGTCCGGTGAAGTGTTTGGAGAC
GTCGCGAGAAGTCCACTGAACCTTATCATTTAGAGG

REAL-TIME PCR ASSAY FOR β-1, 3-GLUCANASE GENE EXPRESSION IN BLACK PEPPER (*Piper nigrum* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Cultivated black pepper (*Piper nigrum*) varieties are susceptible to *Phytophthora* rot, caused by *Phytophthora capsici*, resulting in yield losses upto 50 per cent. Since conventional breeding programmes for this perennial spice crop are complex and time consuming, an attempt was made to unravel the mechanism for disease tolerance at the molecular level.

β-1,3-glucanases are the pathogenesis-related proteins reported in black pepper. The present study was carried out to confirm the role of β-1,3-glucanases in the defence mechanism and to unravel the mode of expression in comparison with the resistant genotype, *Piper colubrinum*.

Real-time PCR (polymerase chain reaction) assay and northern blotting were performed to determine the expression pattern of the gene encoding β-1,3-glucanases in both susceptible and resistant genotypes after infection with the fungus *Phytophthora capsici*. The data was normalized with a stably expressing housekeeping gene. Southern hybridization was carried along with real-time PCR to determine the gene copy number in both *P. nigrum* and *P. colubrinum*.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as the reference gene (endogenous control), since it was more stable than 18S rRNA and actin genes studied. The real-time PCR assay indicated marked difference in β-1,3-glucanase at transcript level in the genotypes studied, when the data was normalized with endogenous control. The rate of increase in transcript level after infection with the pathogen was low and gradual in *P. nigrum* while it was much higher and faster in *P. colubrinum*. The glucanase enzyme activity at healthy stage in *P. colubrinum* was 88.00 unit/mg protein/10 min, while it was only 26.29 unit/mg protein/ 10 min in *P. nigrum*. Upon inoculation with the fungus, the gene expression was elevated over four times within two hours after inoculation in *P. colubrinum* while it was much lower in *P. nigrum*. The northern blot analysis also

indicated differential expression of the gene. Southern hybridization indicated that the gene copy number varied in two species, while real-time PCR assay confirmed that it was almost double in *P. colubrinum* than in *P. nigrum*.

The results clearly indicate the positive role of β-1,3-glucanases in the defence mechanism and highlight the differential gene expression in susceptible and resistant genotypes.