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DIFFERENTIAL EXPRESSION OF PATHOGENESIS-RELATED PROTEINS DETERMINES THE DISEASE TOLERANCE AND SENSITIVITY IN TEA CULTIVARS DURING GREY BLIGHT DISEASE DEVELOPMENT

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A B S T R A C T

Tea is one among the non-alcoholic beverages consumed by people across the continents. As tea is a monocrop, it serves a haven for numerous pests and pathogens. Being a timeless plant, the agronomical practices and stable microclimate favours the disease development. Among the diseases affecting the leaves, grey blight – courtesy of *Pestalotiopsis theae- is* one of the most vital and detrimental diseases in tea. Plant modifies its arsenal system against the pathogens to produce higher levels of antimicrobial products which includes pathogenesis proteins. Understanding the expression pattern of these proteins will help in developing disease tolerance cultivars. All the three pathogenesis-related proteins exhibited higher expression in the impaired cultivars, while the sensitive cultivars showed lesser degree of enzyme concentration. Expression pattern of pathogenesis-related proteins like Phenylalanine ammonia-lyase, β-1,3 glucanases and chitinase were reported and discussed greatly.Activity of all three enzymes were found to be increased in resistant cultivars than the sensitive cultivars. The enzyme activity was slightly higher in the infected plants when compared with the uninfected plants.

Keywords: *Pestalotiopsis theae, Camellia sinensis,* Pathogenesis related proteins, grey blight

INTRODUCTION

Tea is the most demanded, alcohol free liquid consumable produced from the softer leaves consisting of two leaves and a bud. Tea belongs to the family Theaceae, which includes 30 genera and 500 species. Being a perennial plant, the agronomical practices and stable microclimate favors the disease development. The ecological niche includes a number of pathogens which attack different parts of the plant. Most prominent diseases in tea are a result of fungal pathogens, and some are due to bacteria. About 400 pathogens are reported in tea ecosystem (Chen and Chen, 1990).

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Grey blight disease of tea leaves caused by *Pestalotiopsis theae* is regarded one among the most detrimental ailments. This affliction has been recorded in most of the tea –cultivating countries around the world (Koh et al., 2001). It was known to exist in north eastern India since 1872, and Massee (1898) confirmed the identity of the fungus responsible for the disease as *Pestalotiopsis.* Tea industry is fully dependent on leaves, therefore, any disease affecting leaves negatively influences the production of tea. The *Pestalotiopsis* mainly infects the mature leaves, and thus the lethality of the plant is limited unless a large number of leaves are affected along with defoliation. The infection limits both the quality and quantity of the crop. The pathogen also attacks young stem and bare stalk left after plucking. When the young stem is impaired, the health of the plant is more severely affected, as the impaired stem falls off, retarding growth and thus reduces the production of the

crop. Though, chemical fungicides show some good results in controlling the grey blight pathogen, potential fungicide residues and phytotoxicity are considered as major problems apart from the environmental pollution, health hazards to human mankind, and high cost in disease management. Plant genes have many important enzymes which lead to the atrophy of pathogenic cell walls, especially chitinase, which have been used to induce plant imparity to fungal pathogens. The plant also synthesizes some proteins called as defense-related proteins in response to defensive reaction (Bowles, 1990). Proteins which are secreted in host cells during infestation or similar circumstances are regarded as "pathogenesis-related proteins" (PR-proteins) which have been extensively explored from an agricultural outlook. These types of response mechanisms of higher plants against pathogens are found to be conserved throughout the process of evolution. In the same way, majority of the plants secrets and stores same kind of defense related proteins in the pathological conditions, immaterial of the differences in their morphology.

Phenols are mostly synthesized through shikimic acid pathway. Phenylalanine ammonia-lyase (PAL) is one of the enzymes take part in the biosynthetic roadmap of phenol. The deamination of phenylalanine to trans-cinnamic acid and ammonia is catalysed by PAL. Numerous investigations have showed an increase in levels of PAL action in an assortment of plant tissues retaliating pathogen infection. Action of PAL in rust impaired leaves was higher when in relation to healthy leaves in pea (Reddy and Rao, 1978). Increased PAL action was also noticed in *Phytophthora capsici* impaired black pepper leaves (Jebakumar *et al.,* 2001) which shows that PAL has an important role during disease development in plants.

Chitinases and β-1,3 glucanases take part in plant imparity against pathogenic fungal organisms (Kim and Hwang, 1994). Chitinases probably has an active role in the development of healthy plants, and β-1,3 glucanases are also similarly expressed in unstressed plants (Garcia-Garcia *et al.,* 1994). β-1,3 glucan and chitin, are reported to be a polymer of N-acetylglucosamine, which are considered to be the major fungal cell-wall components (Bartnicki-Garcia,1968). The enzyems β-1,3 glucanases and chitinases are recorded as defense related enzymes due to its fungal cell degrading property (Boller *et al.,* 1983). Plant cells possesses chitin-degrading enzymes which degrade the fungal cell walls and thus the released chitin fragments are perceived by the plants (chito oligosaccharides) (Wan *et al.,* 2008) which gives signal to the plants for expression of defense related genes.

MATERIALS AND METHODS

The enzymatic action of phenylalanine ammonia lyase (PAL) was estimated in concordance to the devises of Sadasivam /Manickam, (1996). One gram of leaf illustrative was introduced into liquid nitrogen, powdered, and amalgamated for 5 min in 2 ml of ice cold 25 mM borate-HCL buffer (pH-8.8) consisting o five mM β-mercaptoethanol (0.4ml L^{-1}). The resultant homogenous mixture was spun at 12000 rpm for 20 min. Using 0.2 ml of supernatant as unrefined enzyme extract, the assay additionally comprised of buffer (0.5 ml borate-HCL; pH 8.8; 1.1M), and 1ml of L-phenylalanine (0.1M) - all incubated at 32 ℃ for 60 min. 0.5ml of 1M trichloroacetic acid was used to quench the reaction. A standard curve was plotted using the action of transcinnamic acid by measuring absorbance at 290 nm, the enzyme action in the illustrative being represented as µmol t-cinnamic acid produced min^{-1} mg^{-1} protein.

The action of β, 1, 3 glucanase was estimated in concordance with the method of Pan et al. (1991). Two gram leaf illustrative ground in 3.0 ml of 0.1M sodium phosphate buffer (pH 7.0) was spun at 10000 rpm for 15 min at 4 ^{o}C. 0.5 ml supernatant used as enzyme extract was transferred to a 2ml centrifuge tube with the mixture consisting of, additionally, 0.5 ml substrate (0.5% laminarin dissolved in 0.1M phosphate buffer pH 6.5) and 1 ml 0.1 M phosphate buffer (pH 6.5). The mix was spun at 1000 rpm for 3 mins. One ml of the supernatant was diluted to 3.0 ml using distl. water, to which 3 ml of dinitro salicylic acid (DNS) reagent was added, and the overall reaction incubated for 5 min in a boiling water bath . One ml of 40% potassium sodium tartarate (dissolved in distilled water) was introduced to terminate the reaction. Upon measuring absorbance at 510 nm, specific action (1 unit of β, 1,3-glucanase) was described as the quantitiy of the enzyme that released 1 µmol of glucose mg^{-1} protein h^{-1}

The action of chitinase was estimated in concordance to the method of Ano et al., (2000). Two g leaf illustrative was ground in 3.0 ml of 0.1M sodium phosphate buffer (pH 7.0) and spun at 10000 rpm for

15 mins at 4 ^{o}C. Using 0.5 ml supernatant was used as enzyme extract, the mix consisted additionally of 0.5 ml of chitin (1% chitin dissolved in Mcilvaine buffer (pH – 5.8) and1 ml of Mcilvaine Buffer (pH – 5.8). The volume of the resultant mix was diluted to 3 ml and incubated at 36 ^{o}C for 30 min in distilled water. The result was then introduced into a water bath (boiling) for 10 min prior to spinning at 10000 rpm for 3 min. One ml of supernatant was diluted to 3 ml in distl. water. Once 3 ml of DNS reagent was added to the supernatant, the mixture was further subjected to 5 mins of boiling water bath incubation. 40% potassium sodium tartarate was used to quench the reaction. Upon measuring the absorbance at 540 nm, one unit (U) of chitinase action was described as the quantity of enzyme needed to release 1µmol of *N*-acetyl-_-d-glucosamine min^{-1} from 0.5% of dry colloidal chitin solution under controlled, assay conditions.

Temperature of all assays was tediously maintained at 25 ^{o}C. Bradford (1976) methodology was used promptly to estimate the degree of soluble proteins from the extracted unrefined enzymes using a standard BSA control. Ultrospec 2100 Pro, Amersham Bioscience, USA UV/Visible spectrophotometers were used to conduct all spectrometric evaluations. Lastly, each experiment comprised of 5 illustrative duplicates, and 3 overall experimental duplicates.

RESULTS

Effect of three crucial pathogenesis-related proteins which includes PAL, chitinase and β,1,3-glucase were estimated within leaves impaired by grey blight disease and in healthy leaves consisting of both impervious and prone cultivars.

Impervious cultivars showed heightened action of PAL. This is generally irrespective of the impervious or prone nature of cultivars. Among impervious variants, UPASI 10 registered the highest PAL action in the impaired illustrative, followed by UPASI 3 and UPASI 6. Among prone cultivars, however, UPASI 1 showed highest PAL action in the impaired illustrative, followed by UPASI 4 and UPASI 7 (Figure 1).

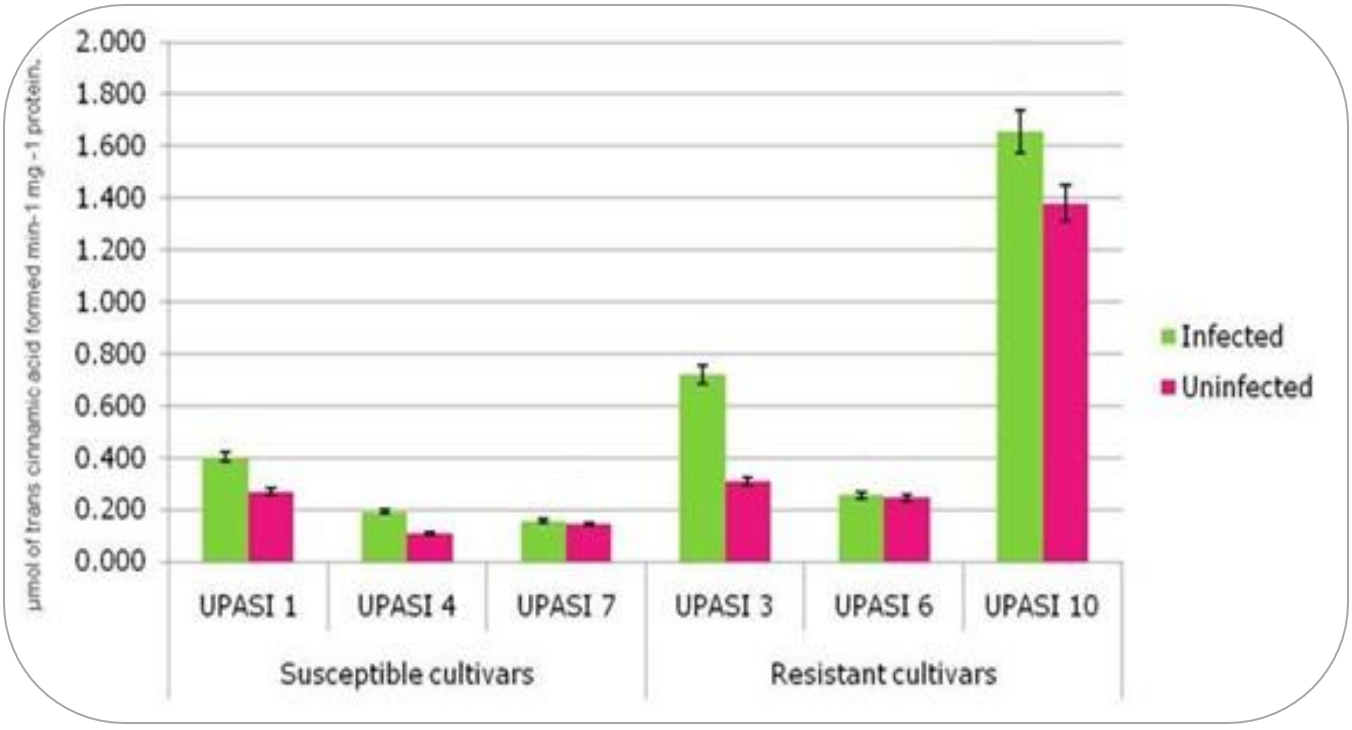

Figure 1. Differential action of PAL in *P. theae* impaired and unimpaired leaves of tea cultivars. (Values represent means ± SE of five replications)

Impervious cultivars showed heightened action of β, 1, 3 glucanase. Generally, higher β, 1, 3 glucanase action was exhibited in relation to the unimpaired illustrative, again, irrespective of the variant (impervious or prone) of cultivar. Among the impervious cultivars, UPASI 3 registered highest β, 1, 3 glucanase action in the impaired illustrative, followed by UPASI 6 and UPASI 10. In prone cultivars, however, UPASI 7 registered highest β, 1, 3 glucanase action, followed by UPASI 1 and UPASI 4 (Figure 2).

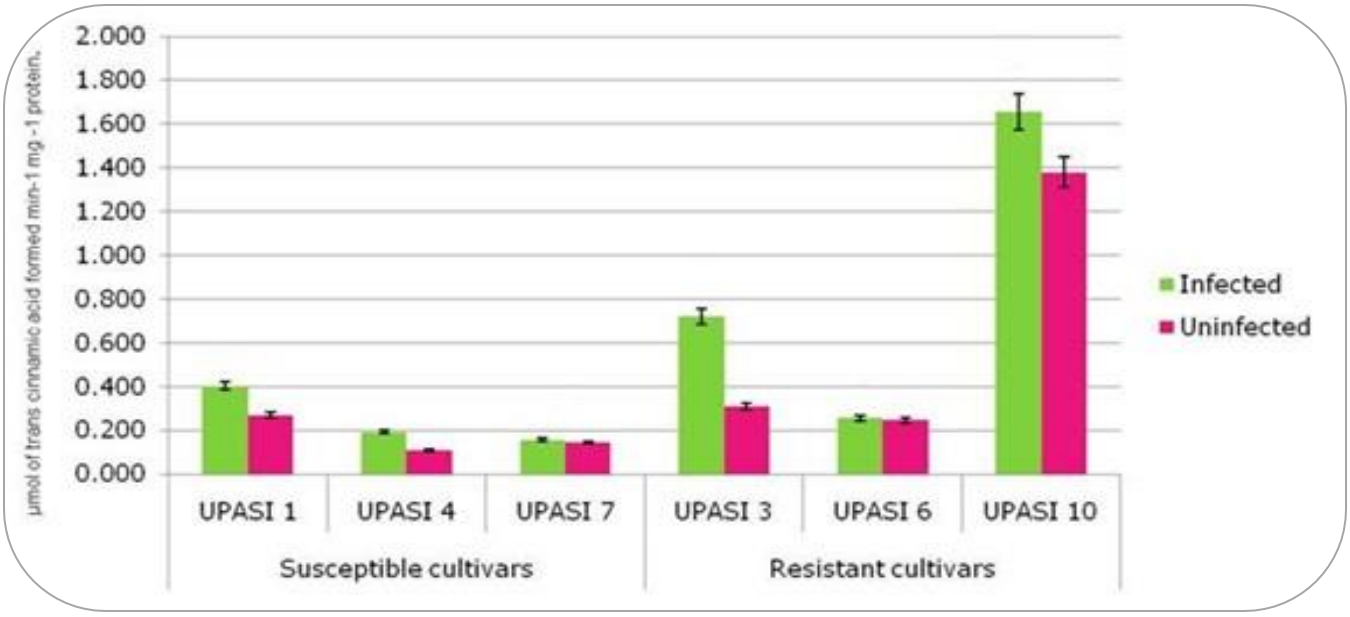

Figure 2. Differential action of β, 1, 3 Glucanase in *P. theae* impaired and unimpaired leaves of tea cultivars. (Values represent means ± SE of five replications)

Impervious cultivars showed the highest action of chitinase. Irrespective of the imparity and sensitivity of the cultivars, the impaired illustrative exhibited higher chitinase action in relation to unimpaired illustrative. Among the impervious cultivars, UPASI 3 registered highest chitinase action in the impaired illustrative, followed by UPASI 6 and finally UPASI 10. As for sensitized cultivars, UPASI 1 registered highest chitinase action in the impaired illustrative, followed by UPASI 7 and UPASI 4 respectively. Slight variation was identified between the impaired and unimpaired illustrative in the prone cultivars in relation to the impervious cultivars (Figure 3).

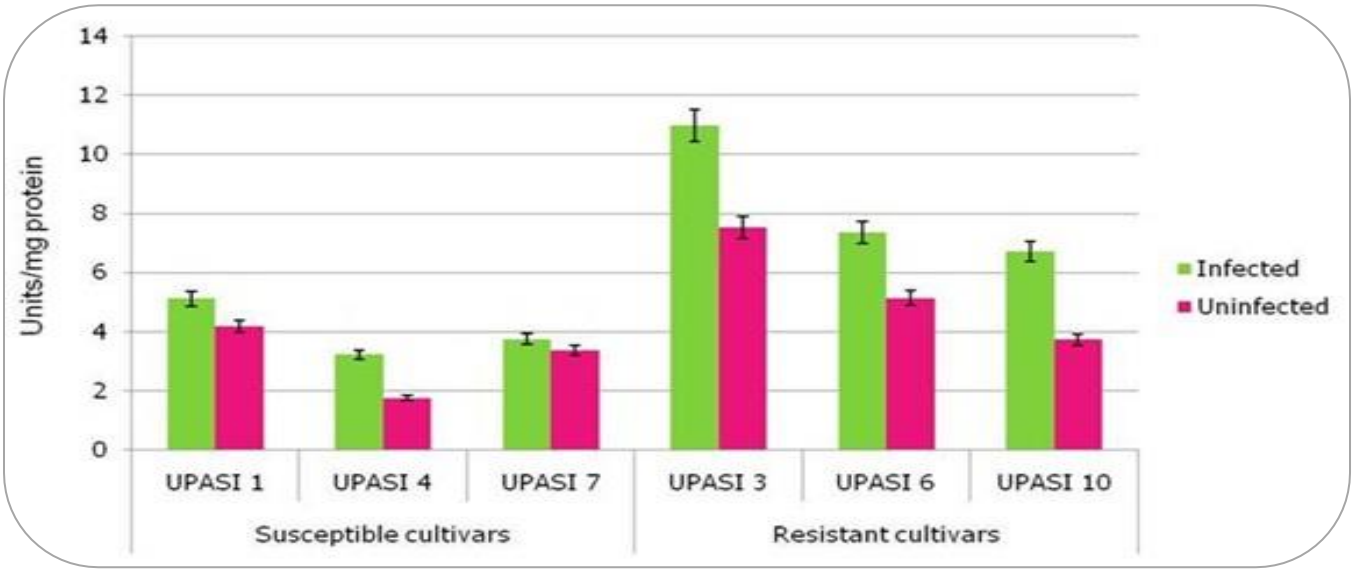

Figure 3. Differential action of chitinase in *P. theae* impaired and unimpaired leaves of tea cultivars. (Values represent means ± SE of five replications)

DISCUSSION

Higher action of chitinase was shown in the impervious when in relation to the prone variants. Irrespective of the imparity and sensitivity, the impaired leaves showed higher action when in relation to the healthy leaves in general. Punja and Zhang (1993) reported that the chitinase shows constitutive expression at low levels which can be enhanced by a variety of abiotic agents (hormones, minerals, radiations, etc) and by biotic factors (bacteria, fungi, viruses, etc). Wan *et al*., (2008)

uncovered that plant cells harbor chitin-lysing enzymes to break the cell wall of the fungal pathogen. Moreover, plants perceive the chitin fractions as chito oligosaccharides which are released from fungal cell walls. Recognition of chitin ends up in inducing the defense signaling pathways in host plants.

Chitinolytic enzymes in plant, including those in tea, are vast and greatly variable. The enzymes are organized in various divisions and into endo- and exo-acting types according to Shoresh and Harman's (2010) domain analyses and sequence motifs. Through the present study, we have attempted to document the differential action of chitinase enzyme in the impaired and healthy leaves in both impervious and prone cultivar varieties. The data concludes that the chitinase enzymes in tea has an extensive regulatory control, and grey blight disease development hampers this regulation. The differential action of the enzyme in the impaired and healthy leaves is a direct consequence of systemic induction by *P. theae*. Multiple other studies on chitinase strengthen the fact that the chitinolytic enzymes play an important role in pathogenesis (Huynh *et al.*, 1992; Ponath *et al.*, 2000). High levels of chitinolytic enzyme expression alone or in conjunction with other antifungal proteins frequently lead to host defense against pathogen (Lorito *et al*., 1998; Bolar *et al*., 2001). Boller (1988). Metraux *et al.*, (1988) also stated that in pathogenic conditions the enzyme is strongly triggered. This discovery, the realty that no chitinase substrate was discovered in plants, and the conception that chitinases had antifungal properties (Shlumbaum *et al.*, 1986; Broekaert *et al*., 1988) contributing to the assumption that pathogens are targeted by plant chitinases.

The differential action of another enzyme β-1, 3-glucanase was also documented. The trend found in chitinase action was further observed in β-1, 3-glucanase. The impervious cultivars exhibited higher β-1, 3-glucanase action in relation to the prone cultivars. Irrespective of the imparity and sensitivity of the cultivars, the impaired leaves showed higher action when in relation to the healthy variants. Activation of plant hydrolases, especially of chitinase and β-1, 3-glucanase after pathogenic infection, play a crucial role in plant imparity (Boller, 1987; Fink *et al*., 1988). Mazau *et al*., (1987) established that the 1,3-glucans in the fungal cell walls were degraded by the enzyme and it lead to a distinct grade of imparity.

Increased activity of β-1, 3-glucanase was observed in the impervious cultivars supports the concept that the enzyme take part in pathogen defense which was further supported by Nasser *et al*., (1988) documented higher accumulation of β-1, 3-glucanase in leaves of maize upon infection with brome mosaic virus. Ferraris *et al*., (1987) reported that infection with *Fusarium oxysporum* f. sp. *lycopersici* induced the secretion of β-1, 3-glucanase action in prone and imparity cultivars. Saikia *et al*., (2005) distinguished the antifungal action of purified β-1, 3-glucanase enzyme as a sign of the direct action of the enzyme on the pathogens. Mauch *et al*., (1988) demonstrated that β-1, 3-glucanase isolated from the tissues of pea has a synergistic effect in degrading the fungal cells walls partially. In addition, Ryan (1987) and Vidhyasekaran and Velazhahan (1996) further strengthened that the enzyme also indirectly plays a role in inducing host defense mechanisms through the release of oligosaccharides from their walls by enzymatic functions that serve as enhancers or inducers of several defense related genes. Anguelova *et al.*, (1999) associated high constitutive levels of β-1, 3-glucanase action to leaf rust imparity in wheat. Likewise, substantial levels of β-1, 3-glucanase action were recorded in impervious muskmelon plants in relation to prone plants by Ward *et al*., (1991). Ren and West (1992) stated that accumulation of chitinase and β-1, 3-glucanase not only have the ability to degrade cell components such as chitin and β-1, 3-glucan but also to release fungal elicitors, which in turn induce various plant defense responses. This concept was proved and reported in our previous studies on differential gene expression during grey blight disease development in tea (Senthilkumar *et al.,* 2012)

While demonstrating the differential action of PAL in impaired and healthy leaves of the impervious and prone cultivars, trends same as the chitinase and β-1, 3-glucanase were observed. Impervious cultivars established higher action, irrespective of the imparity and sensitivity, all the cultivars showed higher enzyme action in the impaired leaves than the healthy ones. This shows that PAL was induced heavily during the disease development.

Kervinen *et al*., (1998) established that PAL genes are induced in barley in the course of fungal infection. PAL is the primary enzyme in the phenylpropanoid pathway. Ramamoorthy *et al*., (2002) demonstrated that this enzyme contributed to enhanced imparity

against invasion of *Pythium* in tomato and hot peer. PAL has an impeccable action in the phenolic phytoalexin biosynthesis (Daayf *et al*., 1997). When groundnut plants were treated with *Pseudomonas. fluorescens*, substantial action of PAL was uncovered (Meena *et al*., 2000). Cucumber plants sprayed with *Pseudomonas corrugata* had raised levels of PAL initially, following which levels were lowered after being challenged by *Pythium aphanidermatum* (Chen *et al*., 2000). Increase in mRNAs corresponding to PAL and chalcone synthase were observed in the starting stages of the assocication between bean roots and various rhizobacteria (Zdor and Anderson, 1992).

The action of the pathogenesis-inducing proteins chitinase, β-1, 3-glucanase and PAL, haven shown nearly concordant trends. Furthermore, it is observed that these enzymes were heavily induced in the impaired leaves than in unimpaired variants. The activities of these enzymes were found to be substantial in the impervious cultivars in comparison to prone cultivars. This demonstrates that the aforementioned enzymes have a direct role in inhibiting the disease, and allowing for imparity within the host. All these enzymes were also labelled in our cDNA and SSH library, and are available as ESTs (Senthilkumar and Mandal, 2012). Complete sequencing of these enzymes will definitely prove worthy in future endeavors relevant to crop improvement programmes.

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Palanisamy Senthilkumar	:	Lay out and conduct experiment, Manuscript write up
Muthusamy Suganthi	:	Help in write up and research
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