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Expression of the pathogenesis related proteins during sesame-*Macrophomina phaseolina* interaction

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Abstract

Sesamum indicum L. is highly vulnerable to biotic stresses in which charcoal rot disease caused by *Macrophomina phaseolina* is one of the major constraints of sesame yield. In response to pathogen infection plant produces a large amount of secondary metabolites like PR proteins, phenolics and other signaling compounds. In present investigation the role PR proteins β -1,3 glucanases and chitinases during host-pathogen interaction was studied at different time interval i.e. 0, 24, 48 and 72 hours post inoculation. Maximum induction of β , 1-3 glucanases was found at 48hpi in resistant genotypes than the susceptible. However maximum chitinase induction was noted in susceptible genotypes at 48 hpi. The expression of both genes was studied by quantitative real time PCR which was also supported the biochemical enzyme assay.

Keywords: Pathogenesis related proteins, *Macrophomina phaseolina*, *Sesamum indicum* L.

Introduction

Sesame is widely known oil seed crop belongs to *Pedaliaceae* family having oblong capsules as fruit containing small and oval seeds which show white, red, brown or black color. It is one of the oldest, ancient crops cultivated for its high nutritional and flavorsome oil (Pathak *et al.*, 2014) [22]. Sesame crop is affected by many diseases like *Alternaria* leaf blight, *Cercospora* leaf spot, wilt, stem blight, powdery mildew, bacterial leaf spot and phyllody etc. Among all the diseases, root rot caused by *Macrophomina phaseolina* is a major problem in India and one or more significant economic constraints to sesame production worldwide (Khan, 2007) [18]. The host range of this pathogen is wide that it can attack more than 500 plant families. In sesame it causes charcoal rot which is highly devastating disease, causing the yield loss up to 50% (Chattopadhyay and Sastry, 1999) [6].

To cope with these stresses plants, produce pathogenesis-related (PR) proteins. It is one of the most important and effective plant defense mechanism and classified into 17 families on the basis of their amino acid sequence data and biochemical functions (Van Loon *et al.*, 2006) [32]. They are low molecular weight, acid-soluble, protease resistant which accumulates in higher plants during incompatible interactions with fungi, viruses and bacteria (Adam, 2004) [2]. Among all PR proteins β -1,3 glucanase (EC 3.2.1.39) and chitinase (EC 3.2.2.1.14) are two important hydrolytic enzymes that are reported in many plant species during various biotic stresses. The PR-2 class includes β -1,3-glucanases, disassemble fungal cell walls by catalysing the hydrolysis of β -1,3-d-glucosidic linkages in β -d glucans, which is the major cell wall component of most pathogenic fungi. Apart from biotic stresses this enzyme is also involved in several physiological and developmental process such as seed and pollen germination, bud dormancy, flower growth and fruit ripening (Su *et al.*, 2016) [29]. Some groups of PR proteins such as PR-3, PR-4, PR-8, and PR-11 have chitinase activity and so can impact on the growth and survival of many fungi because of β -1,3-glucans or chitin as the major structural component of their cell wall (Kauffmann *et al.* 1987) [17]. The main substrate of chitinase is chitin, a natural homopolymer of β -1,4-linked N-acetyl-D-glucosamine (NAG) residues (Kasprzewska 2003) [16]. Plant chitinase-mediated hydrolysis of fungal chitin contributes to two major plant defense responses: the first is degradation of the fungal cell wall, which

results in to the inhibition of hyphal growth (Schlumbaum *et al.*, 1986) [27]; the second, is that cleaved chitin fragments released from the fungal cell wall serve as elicitors for plant immune responses (Shibuya & Minami, 2001; Kaku *et al.*, 2006) [28]. β -1,3-glucanases disassemble fungal cell walls by catalysing the hydrolysis of β -1,3-D-glucosidic linkages in β -D-glucans, which is the major cell wall component of most pathogenic fungi.

In present work, induction of PR proteins and their expression level by qRT PCR was investigated during interaction of resistant and susceptible sesame genotypes with pathogen at various infection stages.

Materials and Methods

Plant material, preparation of *M. phaseolina* inoculum and fungal infection

Five biological replications of GT-10, Rama (resistant) and RT-373, AT-306 (susceptible) were sown in pots, contain sterile soil during summer 2018. Seeds were procured from Oilseed Research Station, Junagadh Agricultural University, Amreli. Pots were kept at field condition and 15-20 plants were maintained in each pot. The pathogen *M. phaseolina* was multiplied on sorghum grains. Polypropylene bags were filled up with about 200 grams of grains. Open end of these bags was packed with 4 cm piece of PVC pipe and nonabsorbent cotton. Then it was autoclaved at 121 °C at 15 psi for 20 min. for sterilization. Than after inoculated with 2-3 discs of size 5 mm diameter from 6 days old culture of pathogen, put in BOD incubator after seven days of incubation. After 60 days of sowing at the post flowering stage plants were more vulnerable to *M. phaseolina*. So, at the 60 days after sowing, the post flowering stage the plants were inoculated with *M. phaseolina*. Root and leaf tissues were collected from both resistant and susceptible genotypes at four post inoculation stages (PIS) i.e. 0 hpi (hours post infection), 24 hpi, 48 hpi and 72 hpi on the basis of external disease symptoms.

Determination of pathogenesis-related (PR) proteins

The activities of two PR proteins, β -1,3 glucanase and chitinase were assayed as described by Abeles *et al.*, (1970).

Determination of β -1,3-glucanase and chitinase activity

The following reagent / buffers were used:

0.1 M sodium acetate buffer (pH 5)

0.1 M sodium citrate buffer (pH 5)

2% solution of laminarin (w/v)

0.5% chitin (w/v)

Extraction of β -1, 3-glucanase and chitinase

0.1-gram leaf and root tissues were taken from control and inoculated sesame plants of all the four genotypes namely, GT-10 and Rama (resistant), and RT-373 and AT-306 (susceptible) at different time intervals (0, 24, 48 and 72 h) of fungus inoculation. The tissue was homogenized in a prechilled mortar pestle in 1 ml of 0.1 M of sodium acetate buffer (pH 5) for β -1,3-glucanase and 0.1 M sodium citrate buffer (pH 5) for chitinase. The homogenate of β -1,3-glucanase and chitinase was filtered through two layers of cheese cloth premoistened in 0.1 M of sodium acetate buffer (pH 5) and 0.1 M sodium citrate buffer (pH 5), respectively. Then filtrate was centrifuged at 10,000 rpm in a cooling centrifuge for 10 min at 4 °C. The supernatant was collected and used for the assay.

Assay of β -1,3-glucanase (EC 3.2.1.6)

The extract from both, control and inoculated plants was taken for the assay. β -1,3-glucanase activity was assayed spectrophotometrically with laminarin as a substrate (Sigma L-9634) using dinitrosalicylic acid (DNSA) method (Miller 1959) with some modifications. The reaction mixture containing 500 μ L of crude protein extract was mixed with 500 μ L of 2% (w/v) laminarin and incubated at 50 °C for 1 h. The reaction was terminated by addition of 1 mL 1% (v/v) staining DNS reagent and boiling for 5 min. After cooling to room temperature, the contents were diluted 1:10 in water and the absorbance was measured at 500 nm. A standard curve of D-glucose was prepared at 500 nm for the unit activity of the enzyme. The unit of enzyme was defined as the amount of enzyme required to liberate glucose unit/hr/gm protein.

Assay of chitinase (EC 3.2.1.14)

Colloidal chitin was prepared according to Berger and Reynolds (1958). Chitinase activity was measured by the release of NAG using colloidal chitin as a substrate according to the method of Reissig *et al.* (1955) [25]. The chitinase activity (unit/hr/gm) was calculated using the standard curve of NAG.

Determination of protein

The protein content of β -1,3-glucanase and chitinase was determined using the method of Lowry *et al.* (1951) [20].

RNA extraction, cDNA synthesis and qRT PCR

Total RNA was extracted from the root tissue of both GT-10 (resistant) and RT-373 (susceptible) sesame genotypes using RNeasy plant mini kit (Qiagen, GmbH) with minor modifications. The cDNA was synthesized by using Quantitect Reverse Transcription Kit according to manufactures instruction. RT PCR mix which is a blend of oligo-dT and random primers that allows high cDNA yields from all regions of RNA transcripts, even from 5' regions random primer scheme for initiating cDNA synthesis. The 20 μ L of reaction mixture included gDNA Wipeout Buffer, 7X 2 μ L, template RNA 1 μ g, water nuclease free (variable), Quantiscript Reverse Transcriptase 1 μ L, Quantiscript RT Buffer, 5x 4 μ L and RT PCR mix 1 μ L. PCR was then performed using 1 μ L (\leq 100 ng/reaction) aliquot of the first strand cDNA in a final volume of 10 μ L containing 10 pM of specific primers (forward and reverse) (As an Endogenous control, 18S rRNA primer) was used as control to normalize each sample for variations in the amounts of RNA used. PCR was carried out using 5 μ L 2x QuantiFast SYBR Green PCR Master Mix (Genetix, USA), Primer F and R both were 0.5 μ L, Template cDNA 1 μ L and RNase free water 3 μ L to final volume 10 μ L in a thermal cycler (ABI-7500 Fast Real-Time PCR System) programmed as follows An initial denaturation for 5 min at 95 °C, 35 amplification cycles [5 min 95 °C (initial denaturation), 10 s at 95 °C (denaturation), 30 s at 58 °C/ 60 °C (annealing)] for primers. Each sample was tested in triplicate for all primers. Melting curve analysis was performed on all samples to ensure amplification of a single product with the expected melting temperature and the absence of primer-dimers. Relative RNA quantities were determined with delta-delta ($\Delta\Delta$) Ct, according to the following formula (Dussault and Pouliot, 2006) comparing the data for each gene of interest with the data for mock-inoculated control samples at each time point. The data was normalized by comparison to 18S rRNA gene.

$\Delta\Delta Ct = [(Ct \text{ G.O.I Ctr} - Ct \text{ Ref. Ctr}) - (Ct \text{ G.O.I infected} - Ct \text{ Ref. infected})]$

Where: G.O.I. = Gene of Interest

Ref: reference gene (18S rRNA)

Ctr = Control (non-infected)

Fold increase = $2^{-\Delta\Delta Ct}$

Table 1: Primer sequence used in real time PCR

Gene name	Primer Sequence	Product size	Gene accession No.
18S	3'AGAAACGGCTACCACATCCA 5' 3'CCAACCCAAGGTCCAACACTAC 5'	251	AJ236041.1
Chitinase	3'AAACTGGAAGCCCACCAA 5' 3'GACCAACACCCATCAAATCA 5'	167	NC_026147
Beta 1,3 glucanase	3'CGCTGATTTTGGTTTTGGA 5' 3'GACGGGCATAGTTTGAGTTTG 5'	182	NC_026159

Result and Discussion

In order to investigate an early change in enzyme activity, the leaves and roots were analyzed at the 24, 48 and 72 h after pathogen inoculation. After 24 h of infection, enzymes activity of β -1,3 glucanase were slightly changed in all the four genotypes. While, at 48 hpi enzyme activity was increased to 2.25-, 2.06-, 1.79- and 2.29- fold in leaves whereas 1.6, 1.09-, 1.89-, 1.41- fold in roots of GT-10, Rama, RT-373 and AT-306, respectively compared to their non-infected leaves and roots (Fig. 1 and 2). The enzyme activity was decline sharply in infected and uninfected tissues of both resistant and susceptible genotypes at 72 hpi compared to 48 hpi. In all the varieties, infected cultivars were showed higher values in comparison with healthy tissues. However, leaf of resistant varieties had higher induced activity at 48 hpi and root of RT-373 had higher induced activity at 48 hpi among all.

These results agree with Radhajejalakshmi *et al.* (2009) [23], who observed the higher glucanase activity in suspension

cultured cells and leaves of tomato plants after inoculation of *A. solani* and its elicitor. Previously, it was shown by Forslund *et al.* (2000) [8] that β 1, 3-glucanases were induced in barley upon attack by *Rhopalosiphum padi*. β -1,3 Glucanases are usually associated with plant defense against pathogen invasion. For example, Glu-1 from jujube (*Ziziphus jujube* Mill) fruit and MpGlu from plantain (*Musa paradisiaca*) were highly induced by *Cryptococcus laurentii* and *Fusarium oxysporum* infection, respectively (Jin *et al.*, 2007; Tian *et al.*, 2007) [13, 29]. It was found that there was a coordinated induction of the expression of one β -1,3 glucanase and three chitinase isoforms in maize seedlings in response to infection by the fungus *Fusarium moniliforme* and this observed expression induction correlates with an increase in the level of translatable mRNA for this particular β -1,3-glucanase isoform (Cordero *et al.*, 1994) [7]. Mahatma *et al.* (2008) [20] also found the higher activity of β -1,3 glucanase enzyme in leaves of downy mildew resistant pearl millet genotypes.

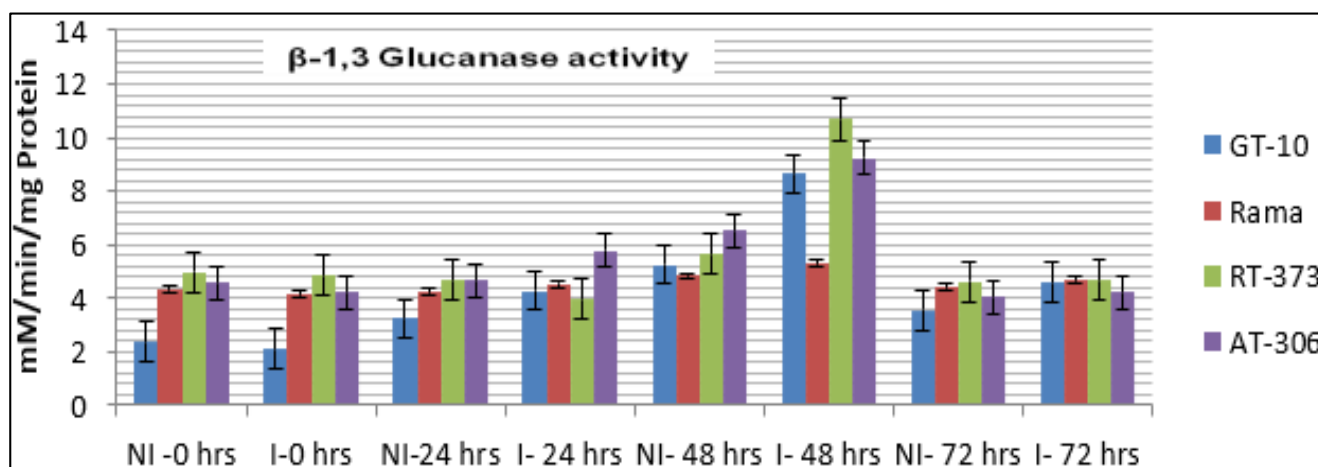


Fig 1: Changes in activity of β -1,3 Glucanase at different post inoculation stages in root.

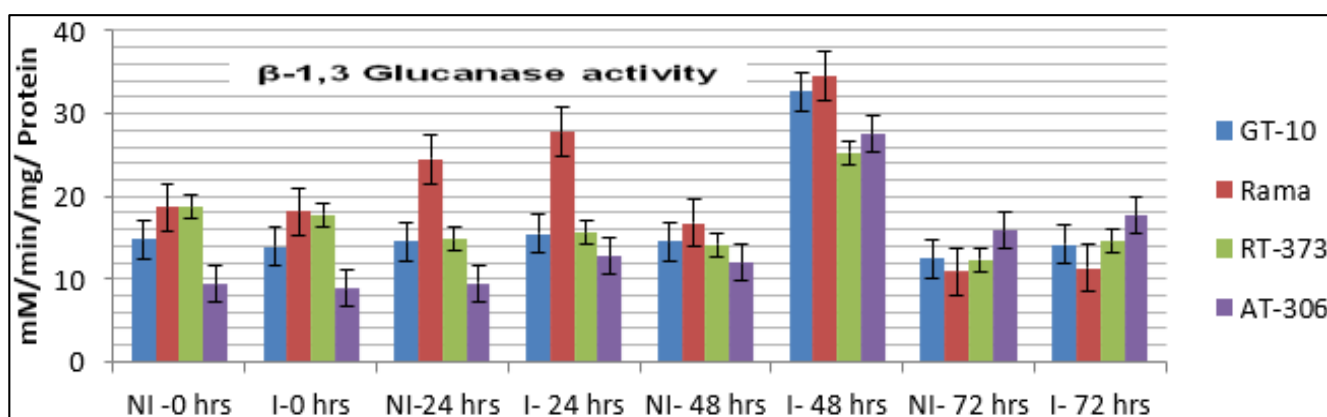


Fig 2: Changes in activity of β -1,3 Glucanase at different post inoculation stages in leaf.

A significant increase in chitinase activity in all the four genotypes of sesame was observed at 48 h after pathogen inoculation. Thereafter a gradual decrease in activity was observed. The chitinase activity was 5.59-, 4.61-, 2.27- and 2.13-fold higher in leaves of GT-10, Rama, RT-373 and AT-306, respectively, then their control (Fig. 3 and 4). The maximum enzyme activity was recorded at 48 h after the infection and thereafter at 72 h after infection gradual decline in enzyme activity was noticed. The activation of chitinase was more rapid and higher in plants of resistant genotypes than in susceptible genotypes.

PR proteins accumulate rapidly at the intracellular and extracellular level under various biotic and abiotic treatments (Graham *et al.*, 2003) [10]. The PR proteins such as β -1,3-glucanases and chitinases play multiple roles in plant self-defense (Ham *et al.*, 1991) [11].

Among the most studied PR proteins are the chitinases (PR-3) and β -1, 3 glucanases (PR-2), both with hydrolytic activity depolymerizing structural saccharides present in the wall of

pathogens. Enzymes can act alone or in synergism (Cavalcanti, 2006) [5]. These enzymes are important determinants of the resistance of plants to fungal diseases (Funnell *et al.*, 2004) [9]. It has been demonstrated that genetically engineered over-expression of PR proteins can increase resistance in plants (Velazhahan and Muthukrishnan 2004). The activation of defense mechanisms in plants is considered to be consequent upon an initial recognition event in which the host plant detects molecular components of the pathogen, known as elicitors (Van't Slot and Knogge, 2002) [32]. These elicitor molecules bind to a receptor(s) on the plasma membrane of plant cells and activate the signaling events required for the onset of the defense responses (Umemoto *et al.*, 1997) [30]. Some PR proteins are constitutively expressed in plants at low levels, but the expression of most of the PR proteins is turned on in response to a pathogen attack. Induction of PR proteins is a consequence of the activation of plant defensive pathways, which limit the entry, or the further spread of the pathogen.

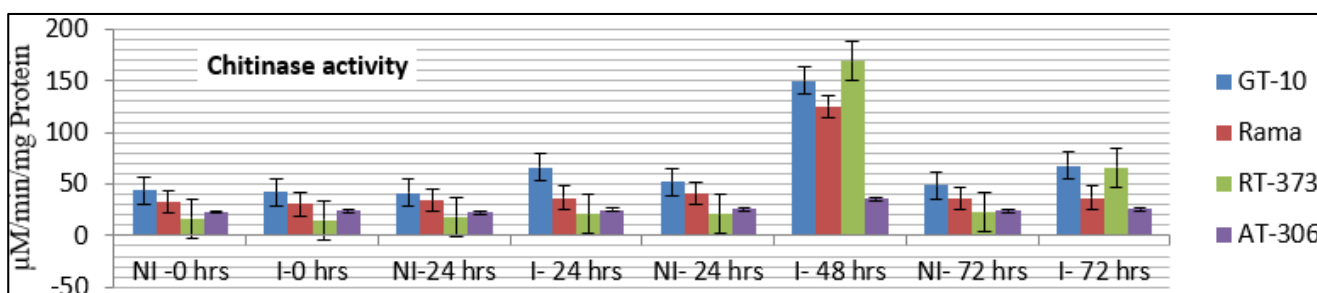


Fig 3: Changes in the activity of chitinase at different post inoculation stages in root.

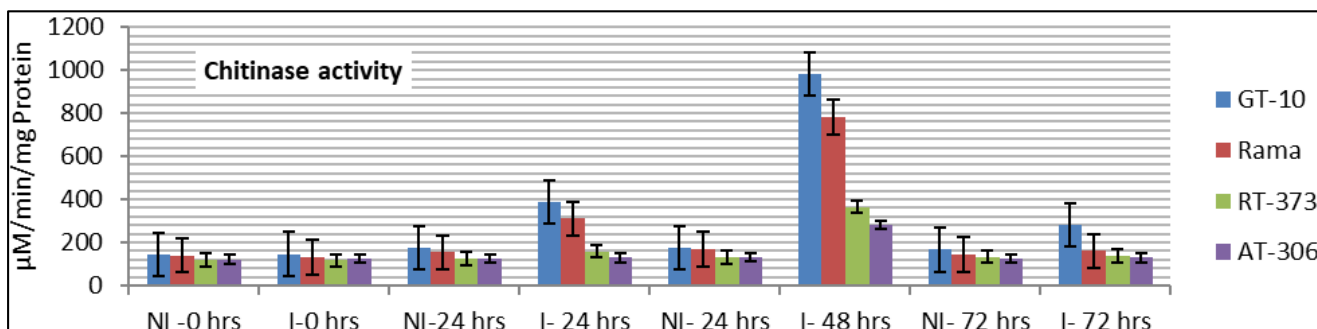


Fig 4: Changes in the activity of chitinase at different post inoculation stages in leaf.

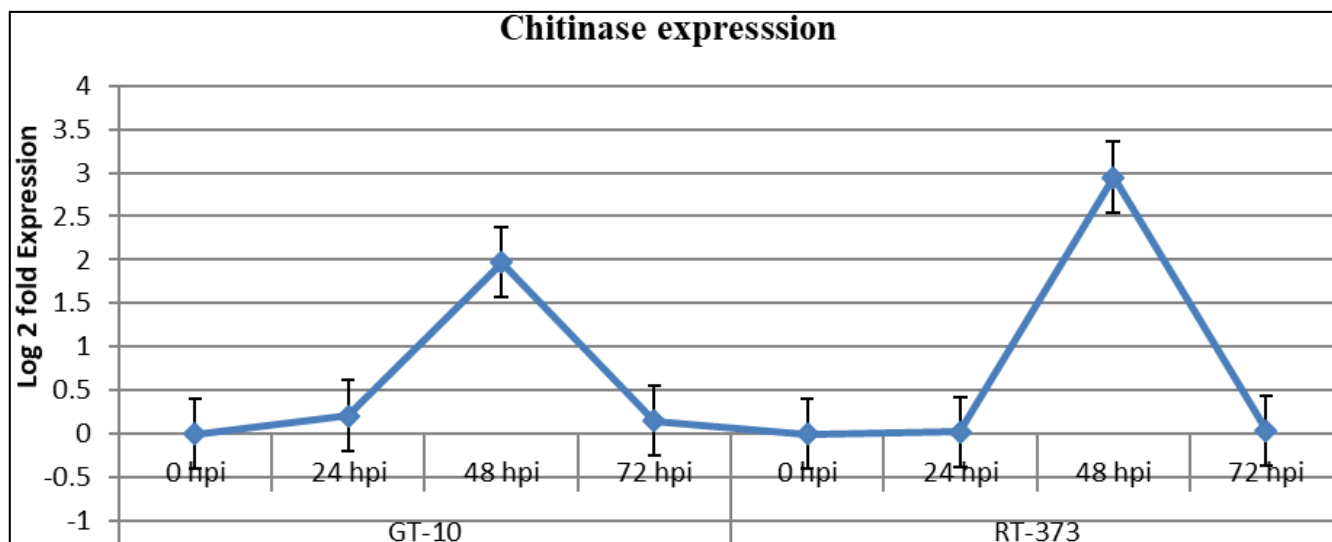


Fig 5: Changes in the expression of chitinase gene at different post inoculation stages in root

To understand expression profile of chitinase and β -1,3 glucanase qRT PCR was carried out. Results show that expression level of chitinase gene in GT-10 was increased with the disease development. The highest expression was noted on 48 hpi (1.97 fold) and after that it was declined at 72 hpi (0.15 fold) compare to control condition (Fig. 5). Here, 0 hpi was taken as control as no disease development was observed at this time. The same pattern of expression was observed in RT-373 however the expression level was increased at the 48 hpi (2.95 fold) as compared to GT-10. Sayari *et al.* (2014) [25] found increased level of chitinase class-1 (PR 3) transcript at 24 hpi in resistant rice cultivar Tarom against *Rhizoctonia solani*, the causal agent for rice

sheath blight disease. They did not observe noticeable expression of this gene in susceptible rice cultivar. Contrarily, they found 14-fold higher expressions in resistant cultivar compared to the control and after that it was declined. Rawat *et al.*, (2017) [23] isolated and characterized class IV chitinase gene and its promoter. They found inducible expression of this gene after *Alternaria* infection in local and distal tissues. This result is also supported by Islam *et al.*, (2010) [12] who found the up regulation of chitinase genes in *Pseudotsuga menziesii* with in 12 h of inoculation with fungus *Phellinus sulphurascens*, a causal agent for laminated root rot followed by down regulation within 2 to 3 days post inoculation.

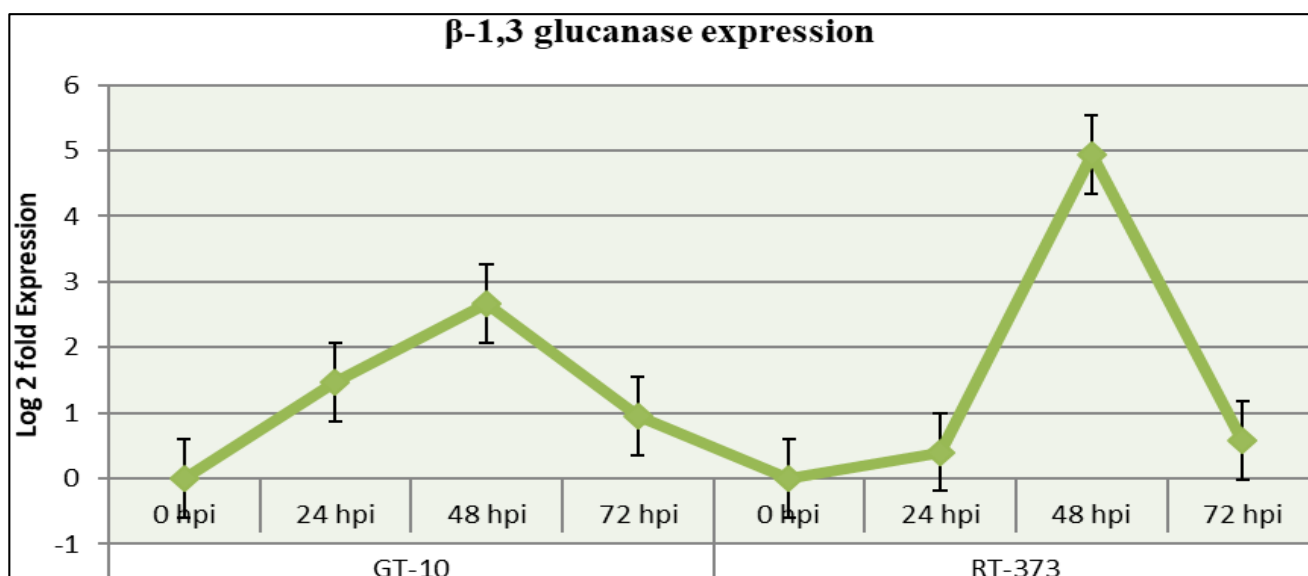


Fig 6: Changes in the expression of β -1,3 glucanase at different post inoculation stages in root.

The induction in expression of β -1,3 glucanase gene was found at 24 hpi (1.465 fold) and after that it elevate at 48 hpi (2.66) in GT-10. At 72 hpi the expression level was found to decline at 0.95-fold (Fig. 6). In RT-373 expression level was found 0.4-fold at 24 hpi and raised to 4.94-fold at 48 hpi. Then after at 72 hpi sudden decrease (0.57 fold) was observed. β -1,3 glucanase belonging to PR 2 group, plays a major role during plant-pathogen interaction. β -1,3 glucanase family in *Arabidopsis* comprised of 50 genes having up regulated transcript during pathogen infection (Levy *et al.*, 2007) [18]. Moreover, enhancement in PR 2 gene expression was found in apple shoots at 24 to 48 hpi with the apple pathogen, *Erwinia amylovora*. However, these proteins were not induced in young apple shoots when it treated with known elicitors. So, this finding suggests that PR 2 plays major role in elicitor stimulation and signaling in systemic acquired resistance (Bonasera *et al.*, 2006) [4]. It has been also reported that transformed tobacco lines with β -1,3 glucanase plastid showed resistance against *R. solani*, *Peronospora hyoscyami* f.sp. *tabacina* and *Phytophthora nicotianae* in green house condition and *P. hyoscyami* f.sp. *tabacina* and *P. nicotianae* in field condition under high inoculum pressure (Boccardo *et al.*, 2019) [3].

Conclusion

This study revealed the role of β -1,3 glucanase and chitinase in sesame during infection of *Macrophomina phaseolina*. Highest activity of these enzymes in susceptible genotypes may impart tolerance to the infection at some level. After decline of the activity plants are started to wilt and ultimately

died. But in case of resistance genotypes the level of enzyme activity is not more than susceptible one. So, in resistance genotypes some other proteins of PR family, phenolics and other secondary metabolites may involve in imparting resistivity. Based on our study it can be concluded that the increased level of enzyme activity and its gene expression with time of post infection suggests that PR proteins may have some role in host-pathogen interaction and in plant defense.

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