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Defense genes expressed by chemical elicitors and *Rhizoctonia solani* infection in mungbean (*Vigna radiata*)

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ABSTRACT

The effects of chemical elicitors salicylic acid (SA) and jasmonic acid (JA) on expression of defense genes PR 10, epoxide hydrolase (EH), catalase and calmodulin alone and with infection by *Rhizoctonia solani* were analysed using qPCR at 1–4 days post inoculation/application (dpi) in highly susceptible (HS; Ratna) and moderately resistant (MR; HUM1) varieties of mungbean [*Vigna radiata* (L.) Wilczek] during 2015 and 2016 at Indian Agricultural Research Institute, New Delhi and National Bureau of Plant Genetic Resources, New Delhi. In general, JA application in presence of *R. solani* upregulated the mean expression of these genes in both the varieties ranging from 1.8–11.9-fold, whereas SA application in presence of *R. solani* upregulated the mean expression from 1.2–3.4-fold. The JA and SA alone, also upregulated the expression of these genes in both HS and MR varieties with higher expression in MR variety. The expression of calmodulin and PR10 was higher at 1 dpi in HS variety while EH and catalase was higher at 4 dpi. In MR variety, PR10 was higher at 1 dpi and EH, catalase and calmodulin were over expressed at 4 dpi. The results clearly indicated that both JA and SA were responsible for upregulation of the defense genes in both HS and MR varieties of mungbean. The genes expression was accelerated in presence of *R. solani* at early stage of plant growth which delayed the infection caused by *R. solani* and helped to reduce disease development, hence may be used for the management of the disease.

Keywords: Defense genes, Induced resistance, Methyl jasmonate, Salicylic acid

India is the largest pulse producer as well as consumer accounting for 25% of world's pulse production from 35% global area under pulses. During 2020–21, the pulse production in India has been estimated as 25.72 million tonnes (MT) from 28.83 million hectare (Mha) area with 892 kg/ha productivity. Mungbean [*Vigna radiata* (L.) Wilczek] is an important pulse crop sustaining soil fertility by fixing atmospheric nitrogen. Web blight or wet root rot caused by *Rhizoctonia solani* Kühn [teleomorph - *Thanatephorus cucumeris* (Fr.) Donk] is responsible for substantial reduction in mungbean production in India (Dubey *et al.* 2014). The pathogen has ability to infect large number of cultivated crops including weeds, produces several symptoms and is difficult to manage using fungicides as they offer little protection (Dubey 2003, Dubey *et al.* 2014).

Plants protect themselves by inbuilt defense mechanism involving complex defence signalling networks (Gough and Sadanandom 2021) and due to pathogen invasion, they are induced to express defense related genes. Plant resistance can be induced by systemic resistance (ISR) and systemic acquired resistance (SAR). In SAR, the resistance develops systemically either in response to pathogens attack as hypersensitive reaction or necrosis development or by the application of salicylic acid (SA) and other synthetic compounds. ISR is commonly induced by bio-agents or rhizospheric bacteria, and is facilitated by jasmonate and ethylene, without immediate expression of PR proteins (Kouzai *et al.* 2016).

Both, JA and SA are important for the pathogen signaling pathways to induce expression of genes related to pathogenesis-related (PR) protein. JA played an important role in growth of plant as well as induction of defense responses to biotic stresses (Santino *et al.* 2013, Meena *et al.* 2022) and importing immunity in plants against fungal infections (Yamada *et al.* 2012). JA application upregulated expression of several pathogenesis-related (PR) genes in plants (Peng *et al.* 2012, Yang *et al.* 2013) indicating that JA plays an important role in resistance against pathogens. The pathogen effectors can utilize complex hormonal crosstalk mechanism of a plant for their own benefit, ensuring low level of induction of defense response (Caarls *et al.* 2015). The proposed study was aimed to distinguish the responsiveness of different defense related genes against various phytohormones in moderately resistant (MR) and

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highly susceptible (HS) mungbean varieties with and without infection of *R. solani*.

MATERIALS AND METHODS

The present study was carried out during 2015 and 2016 at Indian Agricultural Research Institute, New Delhi and National Bureau of Plant Genetic Resources, New Delhi to analyse the effects of chemical elicitors salicylic acid (SA) and jasmonic acid (JA) on expression of defense genes PR 10, epoxide hydrolase (EH), catalase and calmodulin alone and with infection by *Rhizoctonia solani*.

Plant materials: The seeds of mungbean (*Vigna radiata*) varieties Ratna and HUM-1 earlier proved highly susceptible and moderately resistant, respectively to *R. solani* Kühn used in the present study. The seeds of these varieties were taken from Pulse Laboratory, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. Sodium hypochlorite (2%) was used for surface-sterilization of the seeds for 1 min and washed twice with sterile distilled water prior to sowing. These surface sterilized seeds were sown in sterile pro-mix soil (peat-moss: sand: vermiculite; 1:1:1) filled in 15 cm dia pots at 10 seeds/pot during 2015 and 2016 at Indian Agricultural Research Institute, New Delhi and National Bureau of Plant Genetic Resources, New Delhi. The pots were kept at 32°C temperature and 80% humidity in a controlled environmental condition available at Phytotron/control facilities.

Rhizoctonia solani *inoculum*: An isolate of *R. solani* (RDLM-1) being maintained in Pulse Laboratory, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi was used. The inoculum of *R. solani* was multiplied on medium (potato dextrose broth) at 25 ± 1°C for 10 days. The medium having mycelial growth was gently mashed in pre-sterilized mortar and pestle and each pot was inoculated with 25 ml of such inoculum having 10^5 cfu/ml by drenching in soil.

Pre-treatment: The seedlings (at 2–3 leaves stage) of mungbean varieties were treated with either 50 ml/pot of 100 µM salicylic acid or 100 µM methyl jasmonate (both from Sigma-Aldrich, UK) at 4 days before inoculation (*R. solani*) by drenching on the soil. There were 6 treatments for each variety as plants inoculated with *R. solani*, treated with salicyclic acid and inoculated with *R. solani*, treated with salicyclic acid only, treated with methyl jasmonate and inoculated with *R. solani*, treated with methyl jasmonate only, and control (uninoculated, untreated). Each treatment was replicated six times. The plants of two replications were used for gene expression analysis and four replications were continued for disease scoring up to 20 days. One to four days post inoculated plants were uprooted and stored at -80°C for experimentation.

Reverse transcription PCR: The stem and leaves of the plants were used for isolation of total RNA using RNASure kit (Nucleo-pore, UK) and quantified after isolation. Verso cDNA kit (Thermo Scientific, UK) was used to synthesize cDNA using total RNA (1 µg) following the manufacturer's instructions and it was used as templates in PCR reactions. PCR reaction was consisting of gene-specific primers10 pmol, dNTPs 0.2 mM and *Taq* polymerase 1 U (Bangalore Genei, India). Already available sequences of mungbean defense genes were used to design the primers from NCBI database (Table 1). Conventional and quantitative PCR experiments were conducted using these designed gene-specific primers.

Conventional PCR: PCR reaction mixture (25 µl) consisting of cDNA 30 ng, $MgCl_2$ 2.5 mM, dNTPs 150 µM, each gene-specific primers 5 pmol and *Taq* polymerase 1.5 U (Bangalore Genei, India) in 1X *Taq* buffer was used. Initial denaturation for 5 min at 95°C, followed by 35 cycles including denaturation for 45 sec at 94°C, annealing for PR 10, epoxide hydrolase and catalase for 45 sec at 60°C, whereas for calmodulin at 62°C, extension for 30 sec at 72°C and final extension for 5 min at 72°C were used as PCR conditions.

Quantitative real time PCR: Real time PCR system (MiniOpticon, BioRad, USA**)** was used to perform quantitative expression analysis. PCR strips (8 well) were used with reactions volume (20 µl per well) in a reaction buffer with 1X Evagreen supermix (BioRad Laboratories, USA), primer (2.5 pmol each) and cDNA (20 ng). The real time PCR conditions were as initial denaturation for 3 min at 95°C followed by 45 cycles of denaturation for 10 sec at 95°C,

Table 1 Gene specific primers designed and used to analyze gene expression by conventional and real-time PCR

Gene	NCBI accesion no.	Primer sequence (5' to 3')	Amplicon size (bp)
PR-10	AY792956.1	F- GACGAGGCAAACTTGGGATA	217
		R- CAGCCTTGAAAAGTGCATCA	
Epoxide hydrolase	HQ316148.1	F- AACTGGGGGCCTCAACTACT	243
		R- TCCTCTGCAGCTTCTTGGTT	
Catalase	D13557.1	F- AGTTCCCCATACCTCCTGCT	219
		R- GAGAACGGTCAGCCTGAGAC	
Calmodulin	DQ778070.1	F- AACAAGGAGGTCGTGGTGTC	300
		R- ATGCCGATCACAAAACAACA	
Actin	AF143208.1	F- TCGTGTGGCTCCTGAAGAAC	230
		R- AGATTGCATGTGGAAGGGCA	

annealing for 20 sec at 60°C, extension for 20 sec at 62°C and plate read at 62°C for catalase, PR10 and epoxide hydrolase genes. The reaction conditions for calmodulin gene were as initial denaturation for 4 min at 95°C followed by 45 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 60°C extension and plate read at 62°C. For single PCR product verification, melting curves 65–95°C with readings every 0.5°C and 5 sec holds between readings were performed. To determine the specificity of primers to mungbean genes, *R. solani* DNA and its reverse transcribed RNA were used as templates. Non-template control was used to check the non-appearance of primer-dimer and each sample was replicated twice. A non-regulated reference gene (Actin) was used in similar copy numbers per cell to normalize the relative expression ratios of the defense genes. Differences in crossing point [$C_{(T)}$ values] of every single sample against a control sample (un-inoculated and untreated mungbean cultivars) was used to calculate the relative expression. Differential expression of various genes as mRNA abundance in treated samples and control was measured using CFX Manager (BioRad Laboratories, USA).

Disease development: The observation of disease development was continued up to 20 dpi as it was very less up to 4 dpi. The number of diseased plant were counted in each pot and the incidence was measured using formula as disease incidence (%) = plants showing symptoms (in number) × 100/total plant stand at 1, 2, 3, 4 and 20 dpi separately (Dubey and Singh 2013).

Statistical analysis: The completely randomized design (CRD) factorial was used to analyse the data for significant ANOVA using the SAS Software (SAS Institute, version 9.1, Cary, NC). Mean values of two years experimentations (2015 and 2016) were used after analysis. Fisher's Protected Least Significant Differences was used to determine the variations in the mean values (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Expression of defense related genes: In susceptible variety Ratna, the highest expression of PR 10 was in the plants treated with JA and *R. solani* inoculated at 3 dpi (3.4-fold) followed by SA treated and *R. solani* inoculated at 1 dpi and JA treated and *R. solani* inoculated at 1 dpi. In general, the expression was

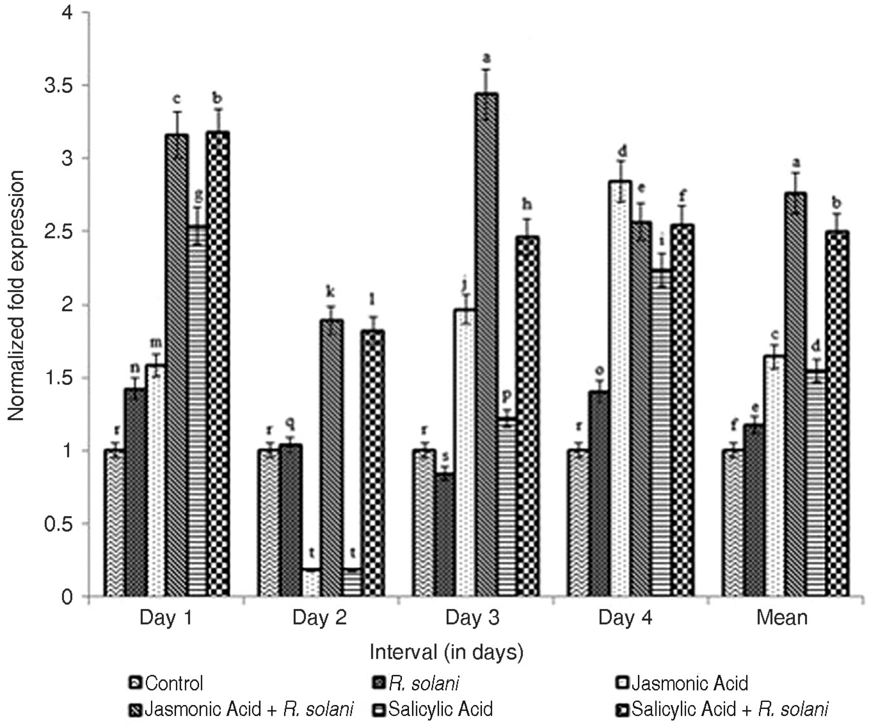

Fig 1 Expression of PR10 gene in highly susceptible mungbean variety Ratna at different days of post inoculation/application with *Rhizoctonia solani,* Jasmonic Acid, Jasmonic Acid + *R. solani*, Salicylic Acid, Salicylic Acid + *R. solani* and un-inoculated (control).

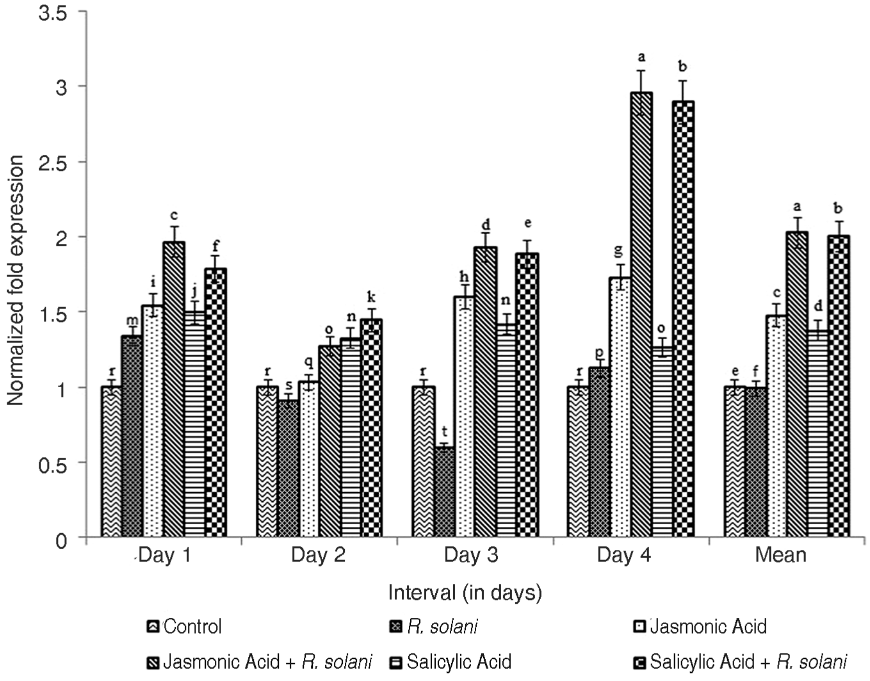

Fig 2 Expression of epoxide hydrolase gene in highly susceptible mungbean variety Ratna at different days of post inoculation/application with *Rhizoctonia solani,* Jasmonic Acid, Jasmonic Acid + *R. solani*, Salicylic Acid, Salicylic Acid + *R. solani* and un-inoculated (control).

higher at 1 dpi and decreased at 2 dpi and further increased at 3 dpi and 4 dpi for all the treatments. The mean value of expression was the highest in the plants treated with JA and *R. solani* inoculated (2.8-fold) (Fig 1). In variety HUM-1, the highest expression of PR10 was at 4 dpi (8.1-fold) in the plants treated with JA and inoculated with *R. solani* followed by plants treated with similar combination at 3 dpi (7.4-fold) and SA treated and *R. solani* inoculated at 4 dpi (5.3-fold). Plants treated with only JA showed the highest upregulation at 4 dpi (3.6-fold) followed by 3.0-fold increase at 3 dpi. The mean value of expression indicated maximum upregulation in JA treated and *R. solani* inoculated plants (5.2-fold) trailed by SA treated and inoculated with *R. solani* (3.5-fold) (Supplementary Fig 1).

The expression of EH in variety Ratna was the highest at 4 dpi in JA treated and inoculated with *R. solani* plants (3.0-fold) followed by SA treated and *R. solani* inoculated plants (2.9-fold). The value of mean expression indicated that the highest expression was measured in JA applied plants and inoculated with *R. solani* (2.03-fold) followed by SA treated and pathogen inoculated (2.0-fold) (Fig 2). The expression of EH was the highest in the JA treated and pathogen inoculated plants with at 4 dpi (2.8-fold) trailed by 1 and 3 dpi in variety HUM-1. Overall, the plants treated with JA and inoculated with *R. solani* showed the highest mean expression of EH (2.3-fold) followed by the plants treated with JA (1.6-fold) (Supplementary Fig 2).

In variety Ratna, the plants treated with JA along with *R. solani* inoculation showed the highest increase at 1 dpi (2.4-fold) followed by 4 dpi (2.3-fold). The mean expression of catalase was down regulated in the plants inoculated while upregulated in the other treatments with the highest increase in JA treated and pathogen inoculated plants (1.8-fold) (Fig 3). In variety HUM-1, the plants treated with SA and *R. solani* inoculated exhibited the maximum upregulation (3.6-fold) at 1 dpi followed by JA treated and pathogen inoculated at 4 dpi (3.4-fold). Only JA treated plants at 4 dpi (3-fold) followed by 1 dpi showed the next levels of expression. The mean value of expression was maximum in the plants applied with JA and inoculated with *R. solani* (2.5-fold). SA treated and pathogen inoculated plants followed by the plants treated with SA were subsequently ranked in respect of expression levels (Supplementary Fig 3).

In variety Ratna, the plants treated with JA and inoculated with *R. solani* exhibited the maximum upregulation of calmodulin at 1 dpi (9.1-fold) followed by 4 dpi (4.6-fold) and 3 dpi (4.5-fold). Only JA treated plants showed upregulation (3.1-fold) at 1 dpi and only SA treated plants showed (2.5-fold) upregulation at 1 dpi. The value of mean expression was the maximum in the plants treated with JA and inoculated with *R. solani* (4.7-fold) trailed by JA treated plants. The plants treated with SA and inoculated with *R. solani* trailed by the plants treated with SA showed 1.6-fold and 1.5-fold upregulations, respectively (Supplementary Fig 4). In variety HUM-1, the plants treated with JA and inoculated with *R. solani* exhibited the maximum up regulation of calmodulin at 4 dpi (29.4-fold) followed by only JA treated plants at 4 dpi (27.0-fold). The value of mean expression was maximum in the plants treated with JA and inoculated with *R. solani* (11.9-fold) followed by the plants treated with JA. SA treated and inoculated plants showed 2.2-fold upregulation followed by SA treated plants with 1.7-fold increase (Supplementary Fig 5).

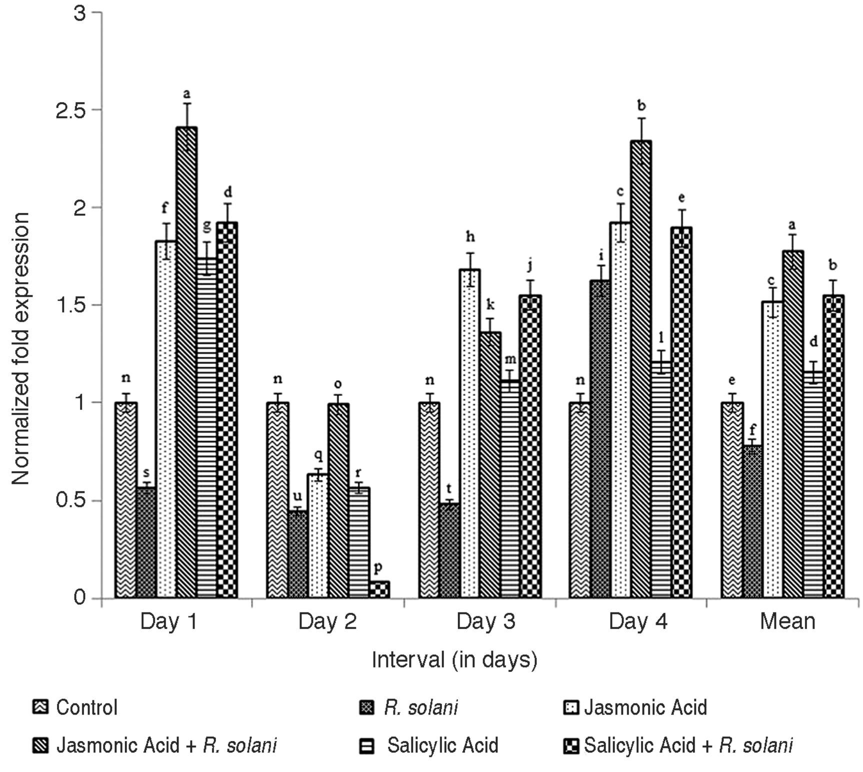

Fig 3 Expression of catalase gene in highly susceptible mungbean variety Ratna at different days of post inoculation/application with *Rhizoctonia solani,* Jasmonic Acid, Jasmonic Acid + *R. solani*, Salicylic Acid, Salicylic Acid + *R. solani* and un-inoculated (control).

The application of chemical elicitors namely, JA and SA raised the upregulation of the genes included in the present study and related to defense in both the varieties either alone or in presence of *R. solani* infection, but the upregulation was higher for *R. solani* infection comparatively with single one. Similarly, Zambounis *et al.* (2012) observed the relative expression of PR-10 between highly susceptible and a partially field-resistant cultivar treated with SA and JA prior to inoculation and after inoculation with an isolate of *Fusarium oxysporum* f. sp. *vasinfectum*. In present study, SA and JA treatments upregulated the expression of genes related to defense including PR 10, but their expression varied in respect to varieties and durations. Elicited expression of SA marker gene in reaction to *F. oxysporum* f. sp. *lycopersici* infection in tomato was observed (Di *et al.* 2017). The present results in respect to EH expression were supported by earlier findings. The expression of epoxide hydrolase was induced in rough lemon inoculated with *Alternaria alternata.* The active oxygen species may act as toxin against the pathogen directly and is involved in various defense genes activation (Gomi *et al.* 2003).

Disease development: JA and SA treated plants along with un-inoculated plants did not show any disease symptoms caused by *R. solani*. Both the varieties were infected with *R. solani* and showed variable disease incidence. The susceptible variety Ratna showed 2.8–94.5% disease incidence for 1–20 dpi. The plants treated with JA and inoculated with *R. solani* showed 5.6% disease incidence at 1 dpi that gradually increased and reach 11.1% at 4 dpi and further at 20 dpi it was 27.8%. SA treated and *R. solani* inoculated plants initially caused 5.6% disease incidence at 1 dpi and gradually reached 13.9% at 4 dpi, further at 20 dpi, it was 30.5% (Supplementary Fig 6). The moderately resistant variety HUM 1 showed 2.8–33.3% disease incidence under JA treatment and *R. solani* inoculated conditions from 3–20 dpi, whereas in case of SA treated and *R. solani* inoculated conditions, the disease incidence varied from 5.6–11.1% at 3–20 dpi. The mean data of treatments indicated that in the presence of pathogen *R. solani*, JA treatment supported less disease development (3.3%) followed by SA treatment (5%) (Supplementary Fig 7).

The overexpression of these genes by application of JA and SA at early stage of infection delayed the establishment of the disease in both HS and MR varieties with more prominent effect with MR variety. Earlier reports also showed that JA plays major role in importing immunity in plants against infection of pathogens. External application of JA induced resistance in rice against bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) and blast (*Magnaporthe grisea*) (Yamada *et al.* 2012) and upregulated PR genes expression (Yang *et al.* 2013). The upregulation of OsWRKY30 gene expression in transgenic rice activated JA biosynthetic genes, which increased JA accumulation, and improved the ability of rice plants to resist against *R. solani* causing sheath blight and *M. grisea* causing blast (Peng *et al.* 2012). The application of analogue of SA and JA are able to activate plant defense response and may be used for management of plant diseases (Faize and Faize 2018). The results of the study clearly indicated that the infection of *R. solani* induced the genes related to defense in plants as epoxide hydrolase, calmodulin, catalase and PR10 in various levels at different dpi. However, the level of regulations was not uniform at all the times. Even, their expression was variable in moderately resistant and highly susceptible varieties of mungbean.

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