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Comparative study of induction of pathogenesis related proteins in pigeon pea varieties against *Phytophthora drechsleri* f. sp. *cajani* infection

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ABSTRACT

Plants encountered various infections in their natural environments, and they evolved a variety of defence systems to fend against the pathogen. One of them is the induction of pathogenesis-related proteins. In this study, the induction of pathogenesis-related proteins in four pigeon pea varieties, such as IPAC-02, ICPL 11260, Shrawani and Shweta were studied; upon *Phytophthora* blight infection. The comparative study was done by morphological and biochemical analysis of all pigeon pea varieties against the *Phytophthora* infection. The result of morphological data indicates that the infection ICPL 11260 and Shrawani had significantly decreased root length; shoot length, number of leaves, fresh weight, and dry weight, compared to IPAC-02 and Shweta. The induction of PR protein as beta,1-3-glucanase, chitinase, phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase with their respective non-infected varieties at various stages was analysed from pre-infection to 1st day after infection up to 16th day after infection and the result showed higher PR activity in IPAC-02 and Shweta and peaked on the fourth and seventh day after infection respectively, whereas they were lowest activity in ICPL 11260 and Shrawani starting from the first day after infection. These results imply that the pattern of pathogenesis-related protein induction may play a crucial role in plant defence mechanisms, contributing to the selection of pigeon pea varieties' resistance to Phytophthora infections.

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Introduction

The *Cajanus cajan* (L.) Mill sp. (pigeon pea) is the second most edible legume crop after chickpea and offers a significant source of vitamins and protein. Due to its tolerance to drought, pigeon peas are one of the most extensively grown and consumed pulses in the world. Because of their high nutritional content, pigeon peas offer vegetarians a well-balanced diet. Malnutrition and nutritional deficiencies are common among Indians, who account for 26.8% of the population living below the national poverty line. The higher yield and better quality of pigeon peas are crucial to fulfil the protein requirement of the growing Indian population.

Pigeon pea productivity in India is low due to different types of abiotic and biotic stresses like plant diseases and a lack of new cultivars. Many biotic factors influence the production of pigeon peas, like fusarium wilt, sterility mosaic disease, Phytophthora blight disease, among which Phytophthora blight disease caused by *Phytophthora drechsleri* f. sp *cajani*, is the most devastating biotic stress in India and like other Phytophthora species-related diseases it is responsible for complete yield loss during the *Phytophthora*

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drechsleri f. sp *cajani* epidemic has been reported by several scientists for massive economical loss (Chauhan et al. 2002; Sharma et al. 2006).

There is a continuous decline in pigeon pea production in the Indian subcontinent due to the increased vulnerability of pigeon peas to Phytophthora blight (PB) is one of the primary reasons as reported by Pande et al. (2011). *Phytophthora* blight infection may be possible in any stage of pigeon pea growth, but it is more prominent in the seedling stage and leads to plant mortality (Chand et al. 2017).

Management of this disease is very important for the optimum yield, and the use of chemical and biological control tools can act as disease management strategies, but it has its pros and cons. So, cultivating resistant varieties is the cheapest, safest and economically viable method. However, understanding the mechanism responsible for providing resistance to the plant is essential. Many diseases assault plants over their life cycle and in response to it, plant develop a variety of extremely sophisticated defence mechanisms to overcome all such stress by combining constitutive and induced variables to develop resistance/tolerance as defensive mechanisms of plants.

These defensive mechanisms induce structural and biochemical defence through an intricate communication network. Plant defence against pathogenic restrictions and general adaptation to stressful situations depend heavily on a group of proteins called "pathogen-related proteins" (PRs), which are encoded by the plants and produced in response to a variety of stress stimuli (Appu et al. 2021). These PRs can upregulate in response to infection and serve as the plant's initial line of defence. PRs are essential for early defensive processes that reduce the disease severity and mortality caused by pathogens.

Plants are known to employ pattern-triggered immunity (PTI) in conjunction with rapid immune system responses as their first line of defence against pathogen invasion and effector-triggered immunity (ETI) typically results in plant resistance against biotic stress. Effector-triggered immunity can activate other defence components if the initial defence response is in effective. The group of PR proteins that function as an early defence mechanism in response to pathogen invasion includes beta,1-3-glucanase, chitinase, phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase. These enzymes play a crucial role in the plant defence system, coordinating with each other to cease pathogen growth and progression in the plants.

Plant resistance is mostly reliant on early pathogen identification and defence signalling, which enables the plants to initiate their defensive mechanism. The primary objective of this investigation was to examine the role of PR protein in the pre-and post-infection of pigeon pea varieties against *Phytophthora drechsleri* f.sp. *cajani*.

Materials and Methods

Plant Materials and fungal pathogen

The seeds of pigeon pea varieties; ICPL 11260, IPAC 02 and fungal pathogen *Phytophthora* f. sp. *cajani* were procured from the Indian Pulse Research Centre Kanpur, Uttar Pradesh, India. The seeds of the pigeon pea varieties, Shrawani and Shweta, were purchased from a local Agroshop at Anand, Gujarat, India.

Pot cultivation of pigeon pea and study of percent disease incidence after Phytophthora infection

The earthen pots having 10.5 inches diameter and 10 inches depth were filled with sterilised sandy loam soil used in this experiment. Pigeon pea seeds of ICPL 11260, IPAC-02, Shrawani, and Shweta varieties were surface sterilised with 0.1% mercuric chloride for 2 minutes, followed by washing with sterile distilled water and planted in the pots with 10 seeds per pot in 3 replications under a net house under favourable environmental conditions for fungal growth.

The pigeon pea varieties ICPL 11260, IPAC 02, Shrawani and Shweta were screened for resistance and susceptibility against Phytophthora blight disease using four different inoculation methods mycelial mesh, node inoculation, zoospore suspension and detached leaf method. On the basis of most effective method for manual infection, zoospore suspension was selected for this study. Preparation of Fungal Zoospore suspension for inoculation was carried out using the method described by Sharma et al. (2015). The average percent disease incidence was determined based on the range of percent disease incidence.

Effect of fungal pathogen Phytophthora drechsleri f. sp. cajani on plant morphology

The earthen pots containing ten-day-old seedlings were infected with zoospores suspension containing 1.5 x 10^5 zoospores/ml was used (10ml/seedling) to infect the seedlings, while sterile distilled water was used as inoculant for the control plants. Morphological traits were recorded for 45 days, first at five-day intervals and subsequently at ten-day intervals, from the fifth day after inoculation (three replications). The root length, shoot length, fresh weight and dry weight were manually done and collected data were subjected to the Univariate analysis of variance, mean, standard deviation and posthoc tests were performed using the Duncan test.

Biochemical analysis of induction of PR proteins in the pigeon pea varieties upon Phytophthora infection.

The fifteen-day-old pigeon pea varieties were inoculated with 10 ml of a 10^5 zoospore/ml suspension of *Phytophthora* f. sp. *cajani*. Fresh leaf samples of both non-infected and infected plants were used as samples for various biochemical analyses and samples were collected at seven different time points: pre-infection, 1st, 4th, 7th, 10th, 13th, and 16th days after infection (DAI) for assessing the activity of the pathogen-related proteins such as beta-glucanase, chitinase, phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase activity due to the fungal infection in the plant.

Estimation of Beta-glucanase activity from the plant leaf extract

The beta-glucanase enzyme activity was measured using the laminarin-dinitro salicylic acid approach (Pan et al. 1991). The assay mixture (62.5µl of 4% laminarin and 62.5µl of plant extract) was incubated at 40 °C for 10 minutes. Then 375 µl of the dinitro salicylic acid reagent was added to stop the reaction and the reaction mixture was placed boiling water bath for five minutes. The 0.5 mL of the dark brown reaction mixture was diluted with 4.5 mL of distilled and absorbance at 500 nm was measured. The beta-glucanase enzyme activity is expressed in the U/mg protein.

Estimation of Chitinase activity from the plant leaf extract

The chitinase enzyme activity was measured using the Boller and Mauch technique (1988). The reaction mixture comprised 0.2mL enzyme, 0.2 mL of 10 mM sodium acetate buffer, and 0.2mL of 0.05% chitin (Sigma) dissolved in boiling water. The reaction mixture was incubated for an hour at 50°C and the synthesis of sugar N-acetyl glucosamine was assessed using the DMAB technique, followed by 2 minutes centrifugation of the assay mixture at 2000 rpm (Reisig et al. 1955). The enzyme activity of chitinase was quantified in the U/mg protein.

Estimation of phenyl ammonia-lyase (PAL) activity from the plant leaf extract

Phenylalanine ammonia-lyase (PAL) activity was determined according to the method of Dickerson et al. (1980). One gram of leaves was homogenized in a pre-chilled mortar and pestle in 5 ml of extraction buffer containing 50 mM Tris-HCl buffer (pH 8.5) with 0.04% ß-mercapto-ethanol. The homogenate was centrifuged at 10,000 rpm for 15 min and supernatant was used as an enzyme source for the determination of enzyme activity. The reaction mixture containing 0.1 ml of plant extract, 1 ml of 50 mM TrisHCl (pH 8.8), and 0.5 ml of 1mM L-phenylalanine was incubated for 60 min at 30 °C and the reaction was terminated by adding 0.5 ml of 1N HCl. The reaction mixture with extracted with 1.5 ml of toluene for 30 sec by vortexing and then the reaction mixture was centrifuged at 1300 g for 5 min for the recovery of toluene extract. The activity was measured by taking absorbance at 290 nm and pure toluene was used as a blank. The enzyme activity was expressed as µmol trans-cinnamic acid released/min/g fresh weight.

Estimation of polyphenol oxidase (PPO) activity from the plant leaf extract

Polyphenol oxidase (PPO) activity was measured by the method of Mayer et al. (1965). One gram of leaves was homogenised in 5 ml of 0.1M sodium phosphate buffer pH 6.0. The homogenate was centrifuged at 10,000 rpm at 4°C for 15 min and the supernatant was used to determine the enzyme activity. The reaction mixture contained 1ml of catechol and 4.5ml of 0.1M phosphate buffer and the reaction was initiated by the addition of 0.5 ml of enzyme extract. The changes in the colour were measured at 490 nm at an interval of 30 seconds up to 3 minutes. The enzyme activity was expressed as the change in O.D/min/g protein.

Estimation of Peroxidase (PO) activity from the plant leaf extract

One gram of pigeon pea leaf tissue was homogenised with cold 0.1M phosphate buffer at pH 7.0 and the extract was centrifuged at 16,000 g at 4°C for 15 min. The supernatant was used as an enzyme source to determine enzyme activity. The reaction mixture had 0.5 ml of 0.01M O-dianisidine, 0.5 ml of H_2O_2, 1 ml of 0.1M phosphate buffer, 2.4 ml of distilled water and 0.2 ml of enzyme extract added into the test tube. The reaction was stopped by adding 1ml of 2 N H_2SO_4 and enzyme activity was measured by taking the absorbance at 430 nm. The activity was expressed as a change in the absorbance/min/g protein and in blank H_2O_2 was excluded by adding equal volume water.

Statistical analysis

The data were analyzed statistically by SPSS 21 statistical software (SPSS Inc.). Mean values were statistically compared by Duncan's multiple range test (DMRT). It was significant at 0.05% level. The data reported in the graphs are means of 3 replications and all the treatments were repeated three times. The visual representation data in the form of graphs was made by using GraphPad Prism.

Results and Discussion

Pigeon pea is one of the most important legume crops of the subtropics and tropics, whose productivity is adversely affected by a group of biotic stressors. The substantial crop

losses caused due to such biotic stress need to be reduced for the sustainable food supply required for the growing population. Among these *Phytophthora* blight is recognised as a potential devastating disease for farmers. All pigeon pea varieties were manually infected with Phytophthora zoospores suspensions at favorable growth conditions of the pathogen. The pigeon pea varieties were infected with the fungal pathogen, *Phytophthora drechsleri* f. sp. *Cajani* showed a notable difference in their growth traits. The data of the experiment indicates that the infected Shrawani and ICPL11260 had significant lower growth parameters at 30 days of growth. The minor variation in the number of leaves ICPL11260 and IPAC 02 had been observed across the groups on the 20th day of infection which may be related to the slow rate of pathogen infection progression toward leaves. This indicates that Shweta and ICPL11260 had some sort of defence mechanism that halted the infection from further progression as shown in Table-1. Jain et.al (2019), also reported that the fungal-infected plants are expected to have reduced resources for growth and reproduction as defence is costly and pathogens withdraw nutrients from the host. Fungal pathogens are reported to produce non-enzymatic toxins, which are responsible for altering plant physiology to utilize plant resources for its growth and establishment in the plant host, causing reduced plant growth in susceptible varieties (Peng et al. 2021).

The biotic stress-induced limitation of pigeon peas production poses a significant challenge to meeting the fulfilment of nutrients-rich diet to prevent malnutrition in a growing population. To sustain the pigeon peas production, farmers have to cultivate already known resistant varieties or there is a requirement for the development of new resistant varieties with higher yields. An important window into plant defence mechanisms is provided by the plant's mode of resistance to pathogens (Hönig et al. 2023). Immediate recognition of plant pathogens is needed to stimulate plant protection enzymes.

The induction of PR proteins during the early stages of pathogen infections is one of the plant defence mechanisms against pathogen invasion. The plant enzyme beta-1,3-glucanase is activated in response to pathogen invasion. This enzyme catalyses the hydrolysis of the pathogen's hyphal cell wall, resulting in the release of beta-1,3-glucan oligosaccharides. These oligosaccharides function as elicitors and microbe-associated molecular patterns responsible for the activation of signalling cascades (Jha et al. 2022). This process leads to the production of antimicrobial compounds like phytoalexins, which offer a variety of local and systemic defence responses and ultimately prevent the pathogen's progression in the plants. (Perrot et al. 2022). The result of the present study reveals a significant ($p<0.05$) difference in beta-glucanase activity found in the infected pigeon pea varieties upon fungal infection. The beta-glucanase activity of infected ICPL-11260 and Shrawani showed 0.38- and 0.39-fold reductions, while the infected IPAC 02 and Shweta had a notable increase of an average of 1.80- and 1.96-fold, during the experimental period, with a maximum increase of 2.19- and 2.07 -folds on the 4th day after infection (DAI), respectively (Fig 1). Gupta et al. (2013) reported that the induction of beta-1-3 glucanase and chitinase was higher in *Erucasativa's* resistant plant compared to the susceptible plant in response to the fungal pathogen *Alternaria brassicicola*.

Chitinases belong to the family of glycosyl hydrolases that catalyse the hydrolysis of chitin glycosidic bonds. Chitinases were among the earliest recognised proteins to exhibit a significant role in innate immunity. It catalyses the fungal cell wall component chitin, which, upon hydrolysis, acts as biological elicitor and provides the signals to the cell membrane receptors for defence activity. As a result of the fungal infection in the pigeon pea varieties a significant decrease in chitinase activity in infected ICPL-11260 and Shrawani was recorded, while a noticeable increase in infected IPAC-02 and Shweta. Chitinase activity in the infected ICPL-11260 and Shrawani showed a notable reduction of an average of 0.42- and 0.48-fold, respectively.

On the other hand, compared to non-infected counterparts, infected IPAC-02 and Shweta had a significant rise that peaked 2.24- and 1.97-fold on the 4th DAI, respectively (Fig.2). Divya et al. (2020) studied the induction of PR protein in rice plants treated with chitosan nanoparticles upon the infection of *Rhizoctoniasolani* (Kuhn), which revealed the higher amount of chitinase, peroxidase, beta 1-3 glucanase, polyphenol oxidase, and phenylalanine ammonia-lyase in rice plants attributed to the lowest disease incidence compared to other chemically treated plants.

The lignin pathway, which started with l-phenylalanine, depends heavily on PAL for its production. This is the first enzyme of the phenylpropanoid metabolism pathway and catalyses the conversion of l-phenylalanine's ammonia to trans-cinnamic acid, which in turn triggers the biosynthesis of lignin. In addition, PAL is essential for the synthesis of phytoalexins and phenol. As a result, the increase in PAL activity following Phytophthora infection points to the activation of several defence mechanisms, including lignification of the cell wall and the production of toxic substances like phytoalexins and phenolics, which ultimately kill the pathogen and prevent its proliferation within the plant cells. PAL activity was observed to differ significantly ($p<0.05$) across the infected pigeon pea varieties. (Fig. 3), the PAL activity of the infected ICPL-11260 and Shrawani

Table 1 Effect of *Phytophthora drechsleri* f. sp. *cajani* on plant growth parameter in one month old pigeon plants 20 days after Infection

Pigeon plants 20 days after Infection							
Pigeon pea varieties	Treatment	Root length (cm)	Shoot length (cm)	Number of leaves	Plant spread (cm)	Fresh weight (gm)	Dry weight (gm)
CPL-11260	Non-Infected	5.71 ± 0.08^{b}	22.87 ± 0.23^{c}	10.67 ± 0.58^{c}	43.32±1.12^{b}	2.20 ± 0.08^{bc}	0.61 ± 0.04^{b}
	Infected	5.16 ± 0.26^{a}	18.05 ± 0.19^{a}	8.33 ± 0.58^{b}	39.32±0.14^{a}	1.14 ± 0.07^{a}	0.24 ± 0.02^{a}
IPAC-02	Non-Infected	6.46 ± 0.58^{c}	23.39 ± 0.25^{d}	13.00 ± 1.01^{d}	38.42±0.11^{b}	2.27 ± 0.09^{c}	0.95 ± 0.05^{d}
	Infected	6.26 ± 0.10^{c}	22.61 ± 0.23^{c}	10.67 ± 0.58^{c}	16±0.07^{a}	1.19 ± 0.09^{a}	0.82 ± 0.03^{c}
SHRAWANI	Non-Infected	5.82±0.15^{b}	23.88±0.08^{b}	9.67±0.58^{b}	40.12±0.22^{b}	2.15±0.02^{b}	0.61±0.02^{b}
	Infected	4.81±0.07^{a}	18.4±0.32^{a}	6.33±0.58^{a}	23.14±0.11^{a}	1.18±0.04^{a}	0.24±0.03^{a}
SHWETA	Non-Infected	5.76±0.11^{b}	23.54±0.3^{b}	11.00±1.00^{b}	46,24±0.06^{b}	2.16±0.04b	0.93±0.02^{d}
	Infected	4.78±0.18^{a}	21.8±0.46^{b}	9.67±0.58^{b}	28.4±0.14^{a}	1.12±0.07^{a}	0.8±0.02^{c}
	Mean Square	0.989	18.880	11.889	35.212	1.002	0.275
	F (df = 3,8)	54.458	185.569	23.778	121.11	500.911	734.304
	Significant	0.000	0.000	0.000	0.000	0.000	0.000

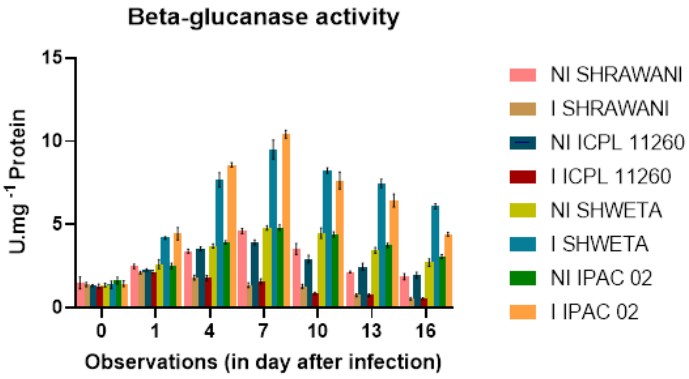

Fig 1. Beta-glucanase activity of pigeon pea varieties on pre-infection and post-infection days. Values represent mean ± S.D. (NI- control, I- Infected), at a significant level p<0.05, (replication =3)

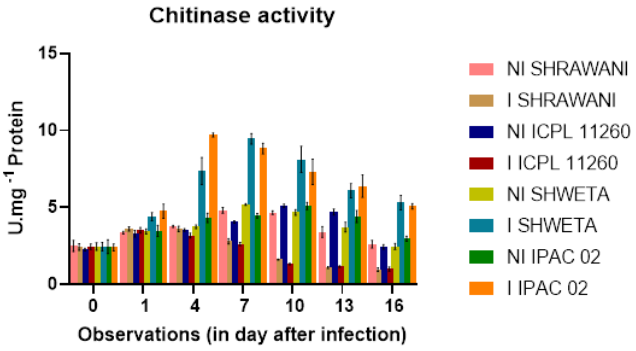

Fig 2. Chitinase activity of pigeon pea varieties on pre-infection and post-infection days. Values represent mean ± S.D. (NI- control, I- Infected), at a significant level p<0.05, (replication =3)

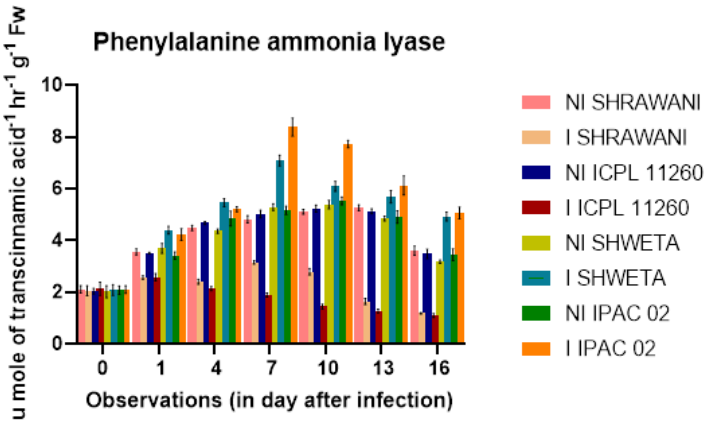

Fig 3. Phenylalanine ammonia-lyase activity of pigeon pea varieties on pre-infection and post-infection days. Values represent mean ± S.D. (NI- control, I- Infected), at a significant level p<0.05, (replication =3)

was reduced to 0.35- and 0.47-folds compared to their non-infected counterparts. Conversely, infected IPAC-02 and Shweta showed a noticeable rise of a maximum of 1.62- and 1.34-fold increase at the 7th Dai. Koç et al. (2011), researched on three pepper cultivars that were inoculated with different concentrations of zoospores of *Phytophthora capsici-22*, to analyse the time course of phenylalanine ammonia-lyase (PAL) activity, and the result revealed that the PAL activity was higher in the highly resistant cultivar compared to the non-infected one on the 2nd DAI.

During microbial invasion, Polyphenol Oxidase (PPO) takes part in the oxidation of polyphenols into quinones and the lignification of plant cells. The PPO lies dormant until it is released by harmful agents such as wounds, senescence, or invasion by pathogens or insect pests. The production of quinines by PPO can alkylate essential amino acids and reduce the quality of plant nutrition; it can cause oxidative stress. The result of the fungal infection in the pigeon pea varieties, (Fig. 4) showed a notable increase in polyphenol oxidase (PPO) activity in infected IPAC-02 (1.37) and Shweta (1.20) folds and a considerable decrease in infected ICPL-11260 (0.78) and Shrawani (0.77). In comparison to their non-infected counterparts, PPO activity in the infected ICPL-11260 and Shrawani consistently declined from the 1st DAI to the 16th DAI. Infected IPAC-02 and Shweta, on the other hand, showed a notable increase, peaking at 1.57 and 1.52-fold on the 7th DAI, respectively, and a gradual reduction by the 16th DAI was recorded. Mohammadbagheri et al. (2021) studied polyphenol oxidase (PPO) activity in the resistant *C. annuum* showed 2-fold enhanced activity compared to the susceptible genotypes infected with *P. capsica*.

The peroxidase (POD) enzyme is essential for either triggering defence mechanisms or mitigating the negative consequences of oxidative stress during a pathogen attack (Kidwai et al. 2020). It is a crucial enzyme in the plant's biochemical defence system because it offers a wide range of protection against biotic and abiotic stressors (Devi et al. 2020, Abo Nouh et al. 2021, 2024). The fungal attack had a major impact on the peroxidase activity in the infected pigeon pea varieties. (Fig.5) Infected IPAC 02 and Shweta showed a prominent increase of 1.37- and 1.35-folds in peroxidase activity upon fungal infection, respectively, which was a maximum of 1.80- and 1.71-folds in IPAC 02 and Shweta on 4th DAI. Where as, POD activity in the infected ICPL-11260 and Shrawani consistently declined from 1st DAI to 16th DAI, and on average the reduction in the POD activity of 0.62- and 0.66-folds was recorded in ICPl-11260 and Shrawani, respectively. The early induction of POD suggests a significant role in the oxidative burst mechanisms, which is the first line of defence mechanism to prevent pathogen invasion.

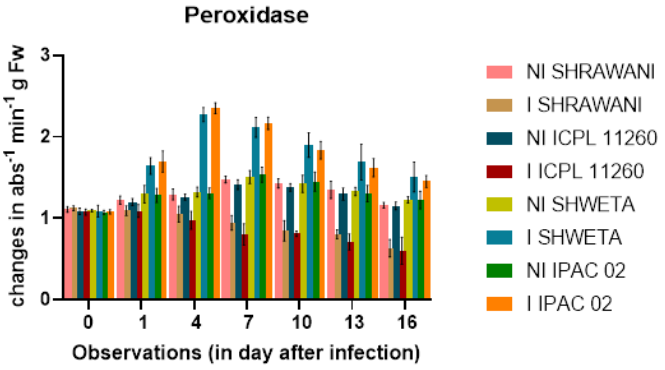

Fig 4. Polyphenol Oxidase activity of pigeon pea varieties on pre-infection and post-infection days. Values represent mean ± S.D. (NI- control, I- Infected), at a significant level $p<0.05$, (replication =3)

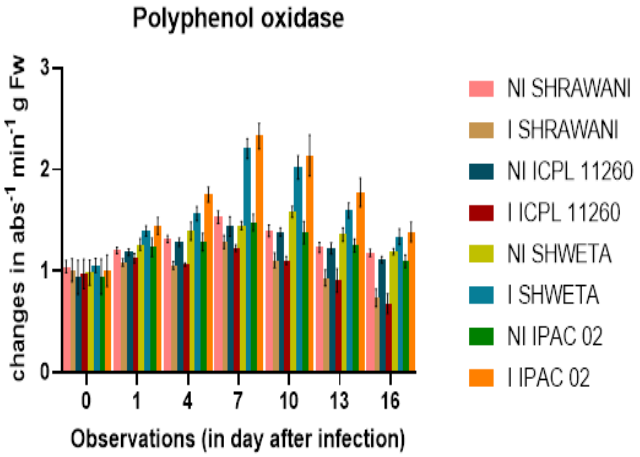

Fig 5. Peroxidase activity of pigeon pea varieties on pre-infection and post-infection days. Values represent mean ± S.D. (NI- control, I- Infected), at a significant level $p<0.05$, (replication =3)

Conclusion

In the present study, distinct pre- and post-infection stages of Phytophthora infection were examined about the induction of different PR proteins in four pigeon pea varieties. The research findings indicate that PR-proteins play a complex and coordinated role in the plant defence system, ultimately leading to resistance against Phytophthora blight disease in infected IPAC 02 and Shweta. Specifically, the induction of PR proteins, such as beta-1-3-glucanase, chitinase, phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase, was higher and induced earlier in plants than in non-infected ones. However, the induction of PR proteins was significantly lower in infected ICPL 11260 and Shrawani than in non-

infected counterparts, indicating that the pathogen progressed more rapidly in these plants and eluded the first line of defence, making them susceptible to *Phytophthora* infections. The differential activity of PR proteins in the resistant and susceptible varieties suggests these PR proteins could be utilised as biomarkers in the plant breeding program to develop the resistant varieties against Phytophthora blight disease.

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Conflict of interest
The authors have no conflicts of interest to declare.

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