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Utkarsh Upadhyay
Chandrashekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Raj K Mishra
ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Sonika Pandey
ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Mukesh Srivastava
Chandrashekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Saurabh Saini
Chandrashekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh, India

Corresponding Author:
Raj K Mishra
ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Activation of phenylpropanoid and oxidative defense enzymes (PAL, POX, PPO) in field pea (*Pisum sativum* L.) by *Trichoderma*-based biocontrol treatments against powdery mildew (*Erysiphe pisi* DC.)

Utkarsh Upadhyay, Raj K Mishra, Sonika Pandey, Mukesh Srivastava and Saurabh Saini

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Abstract
Biocontrol agents (BCAs) not only antagonize pathogens but also prime host defensive metabolism. This study quantified the induction of three defense enzymes phenylalanine ammonia-lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) in field pea (*Pisum sativum* cv. Arkel) following seed treatments with *Trichoderma afroharzianum* (T_3), *Trichoderma asperellum* (T_6) and a bioconsortia (SHEP-6 + *T. asperellum* Th-31, T_5), under challenge by *Erysiphe pisi* DC. Leaves sampled 15 days post-inoculation were assayed for enzyme activities. All BCA treatments significantly (ANOVA, $p \leq 0.05$) increased PAL, POX and PPO activities relative to the inoculated untreated control. PAL peaked at 3.12 n mol cinnamic acid min^{-1} g^{-1} fresh tissue in T_3/T_5 (≈2.6× control), POX reached 0.058 ΔA_{420} min^{-1} mg^{-1} protein in T_5/T_3 (≈2.3× control), and PPO attained 0.042 ΔA_{420} min^{-1} mg^{-1} protein in T_5 (≈2.3× control). The strong co-induction of PAL with oxidative enzymes suggests coordinated activation of the phenylpropanoid pathway and oxidative cross-linking reactions that reinforce cell walls and produce antimicrobial phenolic quinones. The bioconsortia (SHEP-6 + *T. asperellum*) elicited the highest responses, indicating potential synergism. These biochemical changes likely contribute to the reduced powdery mildew development observed in other parts of the study. Results support integrating Trichoderma-based bioformulations into pea disease management to induce durable resistance via metabolic priming.

Keywords: *Pisum sativum*, *Erysiphe pisi*, *Trichoderma*, PAL, Peroxidase, Polyphenol oxidase, Induced resistance

Introduction
Powdery mildew, caused by *Erysiphe pisi* DC., is one of the most destructive foliar diseases of field pea (*Pisum sativum* L.), leading to premature senescence, defoliation, and poor pod filling that ultimately reduce yield and seed quality (Davidson *et al*. 2004; Fondevilla and Rubiales 2012) [20, 22]. Management through fungicides provides only partial and transient control and raises environmental concerns, prompting the search for sustainable alternatives. Among biological options, biocontrol agents (BCAs) such as *Trichoderma* spp. are widely recognized for their dual mode of action direct antagonism through mycoparasitism and antibiosis, and indirect induction of host defense mechanisms (Harman *et al*. 2004; Shoresh *et al*. 2010; Vinale *et al*. 2014) [23, 15, 19].

A hallmark of *Trichoderma*-mediated induced resistance is the activation of key enzymes associated with the phenylpropanoid and oxidative pathways. Phenylalanine ammonia-lyase (PAL) serves as the entry-point enzyme in phenylpropanoid metabolism, catalyzing the conversion of L-phenylalanine to trans-cinnamic acid, which is the precursor of lignin, flavonoids, and a wide array of phenolic phytoalexins (Dixon *et al*. 2002; Mauch-Mani and Slusarenko 1996) [21, 24]. Peroxidase (POX) and polyphenol oxidase (PPO) are oxidative enzymes that polymerize phenolics, cross-link cell wall components, and generate quinones toxic to invading pathogens (Mayer and Harel 1991; Passardi *et al*. 2005) [13, 25]. Together, these enzymes enhance the plant's structural and biochemical resistance to pathogen ingress.

Understanding the magnitude and coordination of PAL, POX, and PPO induction by *Trichoderma*-based BCAs is therefore essential for optimizing bioformulation efficacy and designing effective application strategies. The present study investigates the changes in these three defense-related enzymes in field pea following seed treatment with *T. afroharzianum*, *T. asperellum*, and a bioconsortia (SHEP-6 + *T. asperellum* Th-31) under *E. pisi* challenge, aiming to elucidate the biochemical basis of *Trichoderma*-induced resistance in pea.

Materials and Methods

Plant material, biocontrol agents, and experimental layout

Field pea (*Pisum sativum* L.) cultivar Arkel was used as the host plant. Seeds were treated with different biocontrol formulations prior to sowing. The treatments included:

- **T_1:** Untreated inoculated control (baseline comparison)
- **T_2:** *Trichoderma asperellum*
- **T_3:** *Trichoderma afroharzianum*
- **T_4:** Bioconsortia (SHEP-6 + *T. asperellum* Th-31)

Plants were raised in 20 cm diameter pots filled with sterilized soil:sand:FYM (2:1:1 v/v) under glasshouse conditions. Environmental parameters were maintained to favor powdery mildew development, with mean daytime temperature of 22-24 °C and relative humidity of 70-75%.

At the early flowering stage, plants were uniformly sprayed with a conidial suspension of *Erysiphe pisi* (1×10^4-10^5 conidia mL^{-1}). Enzyme assays were conducted using leaves collected at peak symptom expression, approximately 15 days post-inoculation (dpi). Each treatment was replicated three times, and the experiment was arranged in a completely randomized design (CRD).

Sampling and enzyme extraction

Five to six fully expanded upper leaves were harvested from each replicate at 15 dpi. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Leaf tissue (1.0 g) was homogenized in 5 mL of ice-cold 0.1 M phosphate buffer (pH 6.8) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) to neutralize phenolic interference. The homogenate was centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatant (crude enzyme extract) was used for assays.

Enzyme activities were expressed per gram fresh weight (for PAL) or per milligram protein (for POX and PPO), following conventions used in previous biochemical studies of legume defense responses (Hammerschmidt 1989; Sadeghi *et al*. 2018) [2, 4].

Enzyme assays

Phenylalanine ammonia-lyase (PAL) activity

PAL activity was assayed following the formation of trans-cinnamic acid from L-phenylalanine (Zucker 1965) [5].

The 3 mL reaction mixture contained 1.0 mL of 0.05 M L-phenylalanine, 1.0 mL of enzyme extract, and 1.0 mL of 0.1 M Tris-HCl buffer (pH 8.5). The mixture was incubated at 30 °C for 30 min, and the reaction was terminated by adding 0.5 mL of 6 N HCl or by rapid cooling on ice. Absorbance of the released trans-cinnamic acid was measured at 290 nm (ε = 9630 M^{-1} cm^{-1}), and activity was calculated as:

$$\text{PAL activity} = \frac{(\mu mol \text{ cinnamic acid formed}) \times 1000}{\text{min} \times g \text{ fresh tissue}}$$

and expressed as n mol cinnamic acid min^{-1} g^{-1} FW. When a standard curve was used, the formula of Zucker (1965) [5] was applied for quantification.

Peroxidase (POX) activity

POX activity was determined using guaiacol as substrate following Chance and Maehly (1955) [1]. The reaction mixture (3 mL) consisted of 2.5 mL of 50 mM phosphate buffer (pH 6.0), 0.1 mL of 20 mM guaiacol, 0.1 mL of 40 mM H_2O_2, and 0.3 mL of enzyme extract. The increase in absorbance at 420 nm was recorded for 3 min, and activity was expressed as:

$$\text{POX activity} = \frac{\Delta A_{420}/\text{min}}{\text{mg protein}}$$

and reported as ΔA_{420} min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO) activity

PPO activity was estimated using catechol as substrate according to Mayer *et al*. (1965) [3]. The reaction mixture (3 mL) contained 2.5 mL of 50 mM phosphate buffer (pH 6.5), 0.2 mL of 0.1 M catechol, and 0.3 mL of enzyme extract. The rate of increase in absorbance at 420 nm was recorded for 3 min, and activity was expressed as:

$$\text{PPO activity} = \frac{\Delta A_{420}/\text{min}}{\text{mg protein}}$$

and reported as ΔA_{420} min^{-1} mg^{-1} protein.

Statistical analysis

All enzyme assays were performed in triplicate, and results were expressed as mean ± standard error (SE). Data were subjected to one-way analysis of variance (ANOVA) to evaluate treatment effects on enzyme activities. Treatment means were separated using Tukey's Honest Significant Difference (HSD) test at $p \leq 0.05$.

Pearson's correlation coefficients (r) were computed to assess associations between enzyme activity levels and disease severity (Percent Disease Index, PDI). Statistical analyses were conducted using SPSS v.26 or R v.4.3.0. Significance levels were interpreted at $p \leq 0.05$ unless otherwise indicated.

Results and Discussion

Induction of defense-related enzymes

The activities of three key defense enzymes phenylalanine ammonia-lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO) were significantly influenced ($p \leq 0.05$) by different *Trichoderma*-based treatments in field pea following *Erysiphe pisi* inoculation (Table 1).

All biocontrol treatments showed higher enzyme activities than the untreated inoculated control, indicating biochemical activation of host defense mechanisms.

Phenylalanine ammonia-lyase (PAL) activity

PAL activity increased substantially in all *Trichoderma*-treated plants compared to control (Table1)

The highest PAL activity (3.12 n mol cinnamic acid min^{-1} g^{-1} FW) was recorded in the bioconsortia treatment (SHEP-6 + *T. asperellum* Th-31), followed by *T. afroharzianum* (2.98) and *T. asperellum* (2.82), whereas the untreated inoculated control exhibited the lowest PAL activity (1.22).

This represented approximately a 2.5-fold enhancement over

control levels.

The observed increase in PAL activity suggests that *Trichoderma* formulations activate the phenylpropanoid pathway, leading to the synthesis of lignin, flavonoids, and phenolic phytoalexins that strengthen plant cell walls and inhibit pathogen spread.

Similar enhancement of PAL activity has been reported in *Trichoderma*-treated chickpea and pigeonpea plants challenged with fungal pathogens (Sadeghi *et al*. 2018; Singh *et al*. 2020) [4, 16].

The induction of PAL by biocontrol agents is associated with signal amplification through salicylic acid and jasmonic acid pathways (Shoresh *et al*. 2010; Contreras-Cornejo *et al*. 2016) [15, 9].

Thus, the elevated PAL activity in bioconsortia-treated plants indicates an accelerated phenolic metabolism contributing to disease suppression.

Peroxidase (POX) activity

POX activity, measured as guaiacol oxidation at 420 nm, showed significant increases across all treated plants (Table 1).

The maximum POX activity (0.058 ΔA_{420} min^{-1} mg^{-1} protein) was recorded in the bioconsortia (T_4) and *T. afroharzianum* (T_3) treatments, while *T. asperellum* (T_2) recorded 0.051 and the control 0.025.

These results represent an approximate twofold increase over control.

Peroxidases are key enzymes involved in the oxidative polymerization of phenolic monomers, lignin formation, and cross-linking of cell-wall proteins that provide mechanical resistance to pathogen invasion.

Enhanced POX activity following *Trichoderma* application has been reported in pea, chickpea, and lentil plants infected with various foliar pathogens (Bisen *et al*. 2015; Kumar *et al*. 2021) [7, 12].

The higher POX levels in bioconsortia-treated plants suggest synergistic activation of the oxidative burst and lignification processes, thereby strengthening the structural defense barrier against *E. pisi*.

Polyphenol oxidase (PPO) activity: PPO activity, which catalyzes the oxidation of phenolics to quinones, also increased markedly in *Trichoderma*-treated plants.

The highest PPO activity (0.042 ΔA_{420} min^{-1} mg^{-1} protein) was observed in bioconsortia-treated plants, followed by *T. asperellum* (0.038) and *T. afroharzianum* (0.036).

The control recorded only 0.018, indicating a twofold increase in treated plants.

The elevation of PPO activity reflects an enhanced capacity of the host to generate antimicrobial quinones that can inhibit pathogen hyphal growth (Mayer and Harel 1991; Arfaoui *et al*. 2005) [13, 6].

Similar induction patterns of PPO have been documented in *Trichoderma*-treated chickpea and soybean during compatible pathogen interactions (Singh *et al*. 2022; Jogaiah *et al*. 2018) [17, 11].

These oxidized phenolics are toxic to invading fungi and contribute to the hypersensitive response (HR)-like necrosis at infection sites, thereby restricting pathogen establishment.

Overall enzyme dynamics and correlation with resistance

Collectively, the coordinated increase of PAL, POX, and PPO activities in *Trichoderma*-treated pea plants demonstrates the activation of both phenylpropanoid and oxidative defense pathways.

Correlation analysis ($r \geq 0.85$, $p \leq 0.05$; data not shown) indicated a strong negative association between enzyme activity and disease severity, suggesting that biochemical activation contributed directly to reduced powdery mildew incidence.

These findings align with earlier reports that *Trichoderma* species act as potent inducers of systemic and local resistance by upregulating key defense-related enzymes (Harman 2011; Vinale *et al*. 2014; Sood *et al*. 2020) [10, 19, 18].

The enhanced defense enzyme activities in the bioconsortia treatment highlight the additive or synergistic effect of combining bacterial and fungal BCAs, which is consistent with previous reports of consortium-induced resistance in legumes (Raghavendra *et al*. 2019; Choudhary *et al*. 2021) [14, 8].

Such dual activation of metabolic and enzymatic defense mechanisms provides a sustainable alternative to chemical fungicides for managing powdery mildew in pea.

Table 1: Effect of biocontrol treatments on defense enzyme activities in field pea infected with *Erysiphe pisi*

Treatment	PAL activity (n mol cinnamic acid min^{-1} g^{-1} FW)	POX activity (ΔA_{420} min^{-1} mg^{-1} protein)	PPO activity (ΔA_{420} min^{-1} mg^{-1} protein)
T_1 - Control	1.22±0.08	0.025±0.003	0.018±0.002
T_2 - *T. asperellum*	2.82±0.10	0.051±0.004	0.038±0.003
T_3 - *T. afroharzianum*	2.98±0.12	0.058±0.005	0.036±0.003
T_4 - Bioconsortia (SHEP-6 + *T. asperellum* Th-31)	3.12±0.11	0.058±0.004	0.042±0.004

Values represent mean ± SE (n = 3). Means followed by different letters (not shown) differ significantly according to Tukey's HSD ($p \leq 0.05$).

Conclusions

Seed treatments with *Trichoderma afroharzianum*, *T. asperellum* and the SHEP-6 + *T. asperellum* bioconsortia significantly induced PAL, POX and PPO in field pea infected with *E. pisi*. The bioconsortia produced the strongest overall induction, suggesting synergistic priming of host defenses. These metabolic changes likely underpin reduced disease progress and improved yield components in treated plants. Integration of such bioformulations into crop protection programs can reduce reliance on chemical fungicides and contribute to sustainable pulse production.

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