

Seed treatment with *Trichoderma* on yield and defense gene in maize (*Zea Mays L.*) against *Rhizoctonia solani f. sp. sasakii*

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The present research investigates how different strains of *Trichoderma spp.* stimulate maize yield and activate defense gene expressions (Phenylalanine ammonia lyase – *pal1*, *pal2*, and salicylic acid gene) against *Rhizoctonia solani f. sp. sasakii*, the causal agent of banded leaf and sheath blight. In addition to enhancing yield, the results indicate that *Trichoderma spp.* induce the expression of defense genes such as *pal1*, *pal2* and salicylic acid genes, which play a role in biological control against *Rhizoctonia solani* by activating systemic acquired resistance-related gene expressions in maize plants. This was evidenced through biochemical and qRT-PCR analyses, which showed higher PAL enzyme activity and expression levels of defense genes *pal1* and *pal2*, as well as salicylic acid gene in *Trichoderma*-treated maize plants. These findings suggest that *Trichoderma spp.* have the potential to stimulate defense genes, thereby protecting maize plants against *Rhizoctonia solani* infection.

Keywords : Phenyl alanine ammonia lyase (*pal*), *Rhizoctonia solani*, salicylic acid (*sal*). *Trichoderma*

INTRODUCTION

Among the cereal crops, maize (*Zea mays L.*) is highlighted as a crucial crop providing food, industrial products, and animal feed, with a global average yield of 57.2 quintals per hectare (Anonymous, 2023). Numerous fungi, bacteria, and viruses attack the maize crop, with banded leaf and sheath blight caused by *Rhizoctonia solani f. sp. sasakii* being particularly destructive. This disease results in substantial yield losses of 10-40% annually, amounting to economic losses in the millions. The disease results in breakage of the stalk, premature death and rotting of the ear as direct losses. The disease reduces the yield and quality of maize kernel as indirect losses.

As it is a soil-borne disease that encompasses a wide range of hosts, it becomes difficult to manage. The management practices followed were not effective as the pathogen produces a resting structure (sclerotia) that can persist in soil

for a minimum of two years. It was noticed that most of the commercially cultivated composites and hybrids of maize are moderately susceptible to the disease. Due to the lack of durable resistance, variability was observed among the maize populations may be one of the prime reasons that obstruct for development of resistant varieties (Zhang *et al.* 2012). When there are no resistant cultivars, farmers mostly depend on systemic fungicides for managing the disease. Surplus and long-term use of chemical pesticides pollute the atmosphere, and water and leave hazardous residues in soil. In recent years, the public has become aware of the hazardous and harmful effects of chemical pesticides. As a result, research was started for measures towards sustainable plant protection which were non-hazardous and environmentally safe.

Among the sustainable plant protection management practices, the most relied one is biological control which is an important alternative for managing plant diseases. Biological control, notably through the use of *Trichoderma spp.*, emerges as a promising alternative. *Trichoderma spp.* are beneficial fungi known for their rapid

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colonization of soil and ability to persist in the root rhizosphere. They enhance plant growth, induce systemic resistance, and produce organic compounds that combat pathogens. *Trichoderma spp.* are primary colonizers, proliferate rapidly in soil, colonize and survive on the root rhizosphere for longer periods thus they became an effective group in biological control of plant diseases. It promotes the growth of plants and secrete organic compounds that are capable of inducing local or systemic resistance in plants (Harman *et al.* 2004; Benitez *et al.* 2004; Vinale *et al.* 2008). Induction of systemic resistance is due to the increased production of defense substances that consist of enzymes related to phenylpropanoid pathway. Phenylalanine ammonia-lyase and chalcone synthase are some of the enzymes involved in phenylpropanoid pathway (Yedidia *et al.* 2003; Hanson *et al.* 2004). Seed treated with *Trichoderma harzianum* enhanced the activity of PAL enzyme in maize seedlings (Madhavi *et al.* 2018). Overall, the research underscores the potential of *Trichoderma spp.* not only in disease management but also in boosting crop yields under field conditions. Their ability to provide effective, environmentally safe protection aligns with the growing global interest in sustainable agriculture practices. Therefore, further exploration into the bio-stimulant activity of different *Trichoderma spp.* strains against *Rhizoctonia solani* infection in maize could pave the way for more resilient and productive maize cultivation methods.

MATERIALS AND METHODS

Under greenhouse conditions a research was conducted to evaluate the efficacy of seed-treated *Trichoderma spp.* for their abilities to increase the defensesignaling genes in maize plants. Maize hybrid, DKC 9081 seeds were rinsed five times with double distilled water to remove chemicals. These seeds were treated with different strains of *Trichoderma* formulation (@10g/Kg seed and planted in experimental field. The plot size was 5.0 mX4.0 m with randomly blocked design. For enzyme assay, seeds were sown in the trays and arranged on greenhouse benches in a Completely Randomized Design (CRD) with four treatments and replicated five times (Table 1). Suitable

agronomic cultural practices were followed throughout the study. The Banded leaf and sheath blight (BLSB) pathogen, *Rhizoctonia solani f. sp. sasakii* was inoculated by matchstick method on the basal leaf sheath of the maize plant. Absorbent cotton with a few drops of sterile water wrapped with aluminium foil was placed on the inoculated sheath for the maintenance of the moisture. Plants were incubated and grown under greenhouse conditions at 26 ± 2 °C and leaf samples were collected at 0, 24, 48 and 72 hrs after inoculation of leaf sheaths with *Rhizoctonia solani*. Flag leaf samples collected were dipped in liquid nitrogen and stored at -80°C until further use for estimation of the PAL enzyme activity and gene expression studies.

Assay for phenylalanine ammonia-lyase activity

The enzymatic activity estimation was carried out by following the method of Sadasivam and Manickam (1996) with slight modifications. 500 mg of plant leaves were collected and homogenized it in 5 ml of cold 25 mM borate-HCL buffer (pH 8.8) containing 5mM mercapto-ethanol (0.4ml/L). The homogenate was centrifuged at 12,000g for 20 min, supernatant was collected and used as an enzyme source. Later in a test tube, pipette out 0.5 ml of borate buffer, 0.2 ml of enzyme solution and 1.3 ml of water, add 1 ml of 0.1 M L-phenylalanine solution to start the reaction and incubate it at 32°C for 30 to 60 mins. 0.5 ml of 1 M trichloroacetic acid was added to stop the reaction. The absorbance was measured at 290 nm with a spectrophotometer. A standard curve was prepared for Trans-cinnamic acid at a concentration of 1mg ml⁻¹. The enzymatic activity was expressed as micromole trans-cinnamic acid formed per gram of fresh weight per min.

Analysis of gene expression by quantitative real-time PCR

Total RNA was isolated using the Trizol method following the manufacturer's instructions (Invitrogen, Thermo-Fisher Scientific, India) and reverse-transcribed with the help of M-MLV Reverse transcriptase enzyme (NEB). SYBR

Green assays were done using SYBR qPCR kit (Thermo Fisher Scientific, India) in qRTPCR machine (Quantstudio 5, Applied Biosystems, USA) where β -actin served as the endogenous control and gene-specific primers are listed in (Table 2). The delta-delta CT ($\Delta\Delta CT$) method was used for analysis of data (Pandey *et al.* 2010). Gene expression levels in plants corresponding to time point 0 HAI (control) were taken as 1 (as reference) and relative expressions of genes at various other time points 24, 48 and 72 HAI were determined. Using the formula $2^{-\Delta\Delta Ct}$ (comparative C_T technique), the fold change was calculated where $\Delta\Delta Ct = (C_T \text{ target} - C_T \text{ reference gene}) \text{ infected} - (C_T \text{ target} - C_T \text{ reference gene}) \text{ healthy}$.

RESULTS AND DISCUSSION

Effect of seed treatment with *Trichoderma* on yield parameters of maize

Initially, the maize seeds of cv. DKC 9081 were treated with different strains of *Trichoderma* @ 10 g/kg of seed and planted in the experimental field of the university. The result on maize yield, shoot dry matter and harvesting index is presented in Table 3. All the *Trichoderma* species showed significant yield enhancement over the untreated. Among them, seed treatment with *T. harzianum* showed highest harvest index (41.9) which was closely followed by *T. ernacium* and *T. virens* however, the differences are statistically non-significant.

Expression of phenylalanine ammonia lyase enzyme activity

Analysis of PAL enzyme activity of different *Trichoderma*-treated maize plants on inoculation with *Rhizoctonia solani* at different time intervals viz., 0, 24, 48 & 72 HAI. At 0 HAI, shows that the enzyme activity was lower in different *Trichoderma* treated maize plants when compared to control (105.97 mg trans Cinnamic acid $\text{min}^{-1}\text{g}^{-1}$ fresh weight of leaf sample) initially. But after 24, 48 & 72 HAI, there was an increased PAL enzyme activity in different *Trichoderma* treated plants whereas the activity was subsequently reduced in control. Among all the *Trichoderma* strains, the highest PAL enzyme activity was recorded with *Trichoderma*

ernacium strain after 48 HAI (Table 4). In general, the PAL enzyme activity was recorded highest from 24 to 72 HAI in all treated maize plants whereas in pathogen inoculated control, it shows a declining trend after 24 HAI.

Expression of *pal* and *salicylic acid* gene by *Trichoderma*

The *pal1*, *pal2* and *sal* gene activity of different *Trichoderma*-treated maize plants on inoculation with *Rhizoctonia solani* were given at different time intervals viz., 0, 24, 48 & 72 HAI. The *pal1* and *pal2* gene expressions increased from 0 to 48 HAI and at 48 HAI the highest fold change of gene expression was recorded with *Trichoderma ernacium* strain. In the case of *sal* gene, the highest activity was observed at 72 HAI. In both cases, the gene expression was highest in the *T. ernacium* strain (Table 5)

The increased PAL enzyme activity simultaneously led to augmented levels of the defense signalling molecules such as the phenolic compounds and salicylic acid in the plant thereby resulting in resistance to pathogenic diseases (Klessig and Malamy, 1994). Earlier studies indicate that the simultaneous induction of systemic acquired resistance (SAR) as well as induced systemic resistance (ISR) related genes during root colonization by *Trichoderma spp.* that protect the various lifecycles of the pathogen *Rhizoctonia solani* (Salas-Marina *et al.* 2011).

Application of biocontrol agents and their metabolites induces the phenylpropanoid pathway for the synthesis of chemical substances that produce higher amounts of phenolics, precursor molecules for lignin biosynthesis (De Ascensao and Dubery, 2000). There was a significant increase in Phenylalanine ammonia-lyase (*pal*), chalcone synthase (*chs*) and Hydroxycinnamoyl-CoA quinate transfer (*hqt*) defense genes in *Trichoderma pubescens* treated tomato plants when inoculated with *Rhizoctonia solani* (Behir *et al.* 2023).

Madhavi *et al.* (2018) reported that the PAL enzyme activity gradually increased from 24 h to 96 h in seed treatment and foliar application of *Pseudomonas fluorescens* and *Trichoderma harzianum* in combination treatment.

Table 1: Details of treatment used in the experiment are given below.

Treatment	Details
T1	Untreated (control)
T2	<i>Trichoderma virens</i> mutant G2 (collected from BARC, Mumbai)
T3	<i>Trichoderma ernacium</i> (collected from ICAR-NRRI, Cuttack)
T4	<i>Trichoderma harzianum</i> (collected from UBKV, Department of Plant Pathology)

Table 2: Primers used for this experiment

Gene name	Forward Primer	Reverse Primer	Annealing Temperature (°C)	References
<i>pal 1</i>	TCGATCGAGCGAGAGA TCAA	CGATAGGTGTGCCCTGGAA GT	54	Sahu <i>et al.</i> (2016)
<i>pal 2</i>	CAACGGTCTTCCTTCC AACCT	TCCGCACTTTGGACATGGT	52	Sahu <i>et al.</i> (2016)
<i>Sal</i>	CGAAATAATGTTCCATG GTGTT	TGTACTACGGATCGGTGCA A	46	Jiang <i>et al.</i> (2020)
<i>actin</i>	GCTGGACGTGACCTAA CTG	GCAGTTTCAAGCT CCTGCTC	52	Jiang <i>et al.</i> (2020)

Table 3: Effect of seed treatment with *Trichoderma* on harvesting index of Maize (cv. DKC9081)

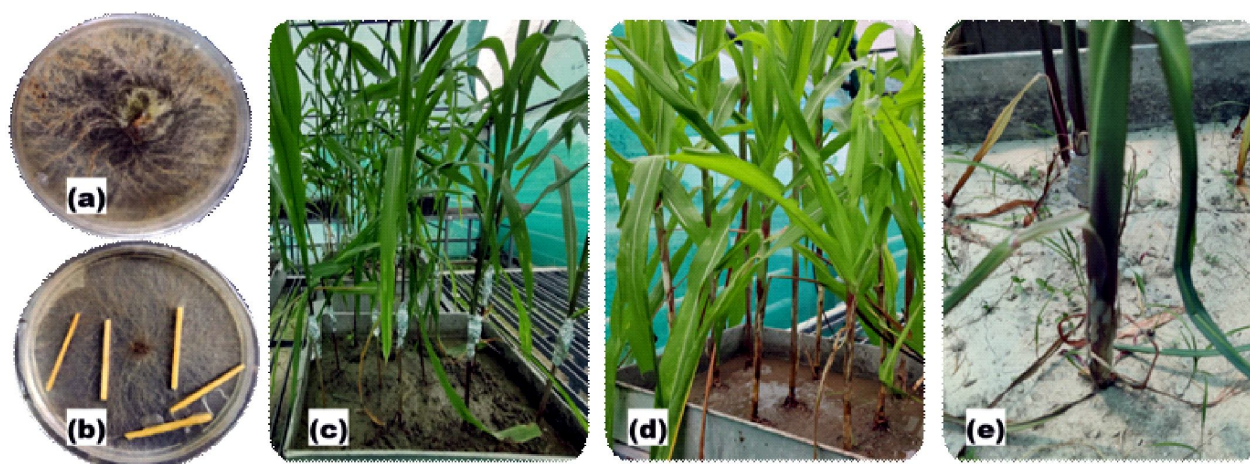
Treatment	Grain Yield (Kg/ 20 sq. m.)	Total shoot dry matter (Kg/ 20 sq. m.)	Harvest Index (%) of Maize
1. Control	11.4 ^B ± 4.99	30.72 ^A ± 3.35	26.26 ^B ± 6.23
2. <i>Trichoderma virens</i> mutant G2	15.58 ^{AB} ± 0.13	22.86 ^B ± 0.18	40.52 ^A ± 2.18
3. <i>Trichoderma ernacium</i>	16.9 ^A ± 0.4	24.84 ^B ± 2.88	40.66 ^A ± 2.24
4. <i>Trichoderma harzianum</i>	17.85 ^A ± 0.55	24.84 ^B ± 2.16	41.9 ^A ± 1.37

Table 4 : Phenyl alanine ammonia lyase enzyme activity in *Trichoderma* treated maize plants inoculated with *Rhizoctonia solani*

Treatment	PAL enzyme activity (mg trans Cinnamic acid min ⁻¹ g ⁻¹ fresh weight)			
	0 HAI	24 HAI	48 HAI	72 HAI
Control	105.97 ^a ± 8.30	80.39 ^c ± 1.87	81.42 ^a ± 9.29	76.16 ^a ± 8.03
<i>T. virens</i> mutant G2	88.65 ^b ± 0.56	92.59 ^b ± 2.46	85.42 ^a ± 10.71	82.11 ^a ± 10.04
<i>T. ernacium</i>	61.17 ^c ± 4.55	90.55 ^b ± 3.41	90.74 ^a ± 10.03	82.21 ^a ± 10.05
<i>T. harzianum</i>	84.70 ^b ± 3.71	103.33 ^a ± 1.63	85.74 ^a ± 11.28	82.44 ^a ± 8.86

Table 5 : Gene expression analysis of defensesignaling genes (*pal* and *sal*) in *Trichoderma* treated maize plants inoculated with *Rhizoctonia solani*

Treatment	Relative fold expression of defensesignaling genes											
	<i>pal-1</i> (HAI)				<i>pal-2</i> (HAI)				<i>sal</i> (HAI)			
	0	24	48	72	0	24	48	72	0	24	48	72
<i>T.virens</i> mutant G2	0.5	0.43	6.86	0.03	0.04	0.36	2.48	0.03	2.08	0.45	0.18	5.26
<i>T. ernacium</i>	0.06	1.9	7.96	0.57	0.06	0.66	3.02	1.86	0.83	0.84	0.17	5.52
<i>T. harzianum</i>	1.75	0.42	5.93	2.35	1.64	0.33	2.36	1.54	1.56	0.61	0.77	4.56

**Fig.1:** *Rhizoctonia solani* pathogen culture and inoculation on Maize plants (a) *Rhizoctonia solani* culture on PDA media, (b) Mycelial culture of *Rhizoctonia solani* on match sticks, (c) Artificial inoculation of cultured *Rhizoctonia solani*, (d) BLSB Symptoms and (e) Sheath blight symptom on maize plant.

Reduced levels of SA endogenously makes rice plants susceptible to *R. solani* infection (Kouzalet *et al.* 2018). Koley *et al.* (2022) reported that salicylate priming in tomato plants provide better tolerance to *R. solani* infection during initial phase by activating SA-mediated defense genes followed by jasmonate priming at later stages. Moreover, salicylic acid is produced by phenylpropanoid pathway from benzoic acid, phenylalanine and other conjugates related.

CONCLUSION

Trichoderma shows potential as a biocontrol agent to enhance maize yield and provide resistance against *Rhizoctonia solani* through mechanisms involving PAL enzyme activity, SA gene expression, and possibly other pathways. Continued research will help clarify these mechanisms and optimize *Trichoderma*-based strategies for agricultural applications.

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DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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