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Seed-Borne Probiotic Yeasts Foster Plant Growth and Elicit Health Protection in Black Gram (*Vigna mungo* L.)

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Abstract: Black gram is one of the most indispensable components of the world food basket and the growth and health of the crop get influenced by biotic and abiotic factors. Beneficial phyto-microbes are one among them that influence the crop growth, more particularly the seed borne microbes are comparatively beneficial, that they pass from generation to generation and are associated with the plants from establishment to development. In the present study, twenty seed-borne yeasts were characterized and tested for growth promotion of black gram and their antagonism against black gram phytopathogens. Two yeasts, *Pichia kudriavzevii* POY5 and *Issatchenkia terricola* GRY4, produced indole acetic acid (IAA), siderophore, 1-amino cyclopropane-1-carboxylic acid deaminase (ACCD), and plant defense enzymes. They solubilized phosphate and zinc and fixed atmospheric nitrogen. Inoculation of these two yeast isolates and *Rhizobium* BMBS1 improved the seed germination, physiological parameters and yield of black gram. Inoculation of *Rhizoctonia solani*-challenged plants with plant growth-promoting yeasts, resulted in the synthesis of defense-related enzymes such as peroxidases (POD), chitinases, catalase (CAT), and polyphenol oxidases (PPO). Thus, the seed-borne yeasts, *Pichia kudriavzevii* POY5 and *Issatchenkia terricola* GRY4, could be used as plant probiotics for black gram.

Keywords: defense enzymes; plant growth promotion; multi-functional yeast; pathogen challenging; plant probiotics; seed-borne yeast; siderophores

1. Introduction

The rhizosphere is colonized by microbes that may be beneficial, pathogenic, or neutral in effect. The beneficial microorganisms and their bioactive molecules play a significant role in sustaining and improving soil fertility. They act as indices of plant and soil health. Inoculating plants with beneficial microorganisms influences plant growth by controlling plant pathogens, increasing inorganic fertilizer use efficiency, and improving resistance to abiotic stresses such as drought and salinity [1–3]. These plant growth-promoting characteristics and the immunity power to cope with the biotic and abiotic stresses due

to climatic changes could be further enhanced by altering the rhizospheric microbial community with beneficial microbes and their bioactive molecules.

While bacteria and fungi are being exploited widely to promote plant growth, yeast also seems promising because of its flexibility in metabolism, wide adaptability, and plant health-promoting potential to a greater extent. Despite many industrial applications of yeasts, the potential role in plant growth promotion and combating various biotic stresses is under-exploited. More particularly, seed-borne yeasts have been the least studied. Since yeasts require simple nutrients, their population is generally higher in the rhizosphere than in the bulk soil [4,5]. Yeasts of the Ascomycota and Basidiomycota classes are symbiotic or mutualistic with the plant's endosphere. They utilize plants' sugars and amino acids and protect plants from stresses [6]. Yeasts are more suitable than bacterial and fungal bio-control agents for cultivation, storage duration, and survival inside the plant's endosphere [7]. A diverse range of yeasts exhibit plant growth-promoting and bio-control characteristics [8–10]. They produce siderophores [10] and phytohormones [11]; solubilize inorganic phosphate [12,13]; oxidize nitrogen and sulfur [12]; stimulate mycorrhizal root colonization [13,14]. Organic phosphorus solubilization is mediated by phytases [15] and plant growth promotion with polyamines [16]. Yeasts also mitigate biotic stress by inducing systemic resistance (ISR) via the production of peroxidase (POD) and catalase (CAT) [17]. *Candida* sp., *Pichia* sp., *Rhodotorula* sp., *Debaryomyces* sp., and *Metschnikowia* sp. are known as antagonists to fungal phytopathogens [18]. Plant growth promoting yeasts from rice rhizosphere [16], *Drosera* rhizosphere [19] legume rhizosphere [20], maize rhizosphere [21], vine-yard soil [22] have been reported.

Black gram (*Vigna mungo* var., *mungo* (L.)) is a food legume crop predominantly grown in tropical and subtropical countries. India is the largest producer and consumer globally, followed by Myanmar and Pakistan [23]. However, black gram is exposed to many biotic and abiotic stresses that hinder its productivity. Key biotic constraints in the production are fungal, bacterial, viral, and nematode pathogens, which cause significant yield losses. *Rhizoctonia bataticola* (Pycnidial stage: *Macrophomina phaseolina*) is a highly destructive and major seed–soil-borne pathogen with a broad host range due to its aggressive nature. Common symptoms are root rot, seedling blight, leaf blight, stem canker, damping-off, and seed decay in black gram. This fungus produces various cell wall degrading enzymes, hydrolytic enzymes, and phytotoxins such as botryodiplodin and phaseolinone for damaging plant tissues [24]. The diseased plants can be easily pulled out. The affected plant carries black-colored microsclerotia that overwinters in the soil for up to 15 years as a saprophyte for infecting the next season's crops [25]. Since it is a seed and soil-borne pathogen, the management of *Rhizoctonia bataticola* is complex with toxic agrochemicals [26].

In this present investigation, we address the plant growth promotion by probiotic yeasts and the bio-control of *Rhizoctonia* root rot in black gram by seed-borne yeasts as an alternative to fungal and bacterial bio-agents. We hypothesized that the seed-borne yeasts isolated from different sources have multifarious plant growth promotion effects through direct and indirect mechanisms. Secondly, yeast-mediated Induced Systemic Resistance (ISR) could minimize root rot incidence by producing extracellular enzymes, antioxidant enzymes, and defense-related enzymes, which help to improve a plant's physiological parameters during stress.

2. Materials and Methods

2.1. Isolation of Seed-Borne Yeasts

Seed-borne yeasts were isolated from the seeds of grapes, pomegranate, tomato, and black gram. The seed samples were surface sterilized with 0.1% $HgCl_2$, washed thrice with sterile distilled water, and ground with mortar and pestle. The ground samples were serially diluted (10^{-2}, 10^{-3}, 10^{-4}) and were spread onto solid Yeast Extract Peptone Dextrose (YEPD) agar plates added with 250 µg/mL chloramphenicol with pH 6.5–6.7 [11]. The plates were incubated at 30 ± 2 °C for three days.

2.2. Morphological and Biochemical Characterization of the Seed-Borne Yeast Isolates

The morphological and physiological traits such as colony morphology, cell morphology, Gram staining, budding pattern, growth in liquid medium were studied as described by Kurtzman et al. [27]. The characteristics like texture, color, surface, elevation, margin, cell shape, and agar-grown colony were recorded. Gram's reaction was performed as described by Gerhardt [28]. Further to this, Gram's staining reaction was confirmed through the KOH-string test. The yeast isolates were tested for the budding pattern by smearing the cultures onto a clean glass slide and observing under 40× magnification. Budding was classified as polar, monopolar, bipolar, multilateral, fission, arthroconidia formed by fission, chlamydospores, endoconidia produced by budding, and blastoconidia formed on elongated conidiophores [29]. The pellicle and the sediment formation characteristic of the yeast isolates were studied in Yeast Extract Petone Dextrose (YEPD) broth as mentioned by Kreger-van Rij [30]. The yeast isolates were subjected to biochemical characteristics such as carbohydrate utilization [31], starch hydrolysis [32], citrate utilization [33], and carboxy methyl cellulose hydrolysis [34].

The activities of the extracellular enzyme such as chitinase and protease were evaluated. The chitinase activity of yeast was assessed as mentioned in Hsu and Lockwood [35]. The chitinolytic activity of yeast isolates was tested by preparing the chitinolytic agar medium supplemented with 0.1, 1.0, and 1.5 percent colloidal chitin. The yeast isolates were spot inoculated onto the medium and incubated at 30 ± 1 °C for seven days. The zone formation and colony growth were measured 3 and 7 days after inoculation.

Protease activity was quantified as per the procedure of Tsuchida et al. [36] with slight modification using 2 percent casein (Hammerstein casein, Merck, Germany) in 0.2 M carbonate buffer (pH 10) as substrate. One unit enzyme activity was correlated as the amount of enzyme that released 1 µg of tyrosine/mL/min under assay conditions.

2.3. Molecular Characterization of Selected Seed-Borne Yeasts Isolates

The molecular characterization was performed by sequencing ITS 1/ITS 4. The genomic DNA from GRY4 and POY5 was isolated using the Hi-PurATM Yeast Genomic DNA Purification Kit (Hi-Media, Mumbai, India). Genomic DNA of the yeast isolates was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR-amplified product was excised, purified from the gel, and sequenced using the fluorescent dye terminator method (ABI prism equipment and a Bigdye TM Terminator cycle sequencing ready reaction kit V.3.1). Sequences were aligned and checked for closest neighbors using NCBI Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 24 March 2022). Two promising isolates, one from seed surface of grapes GRY4 and one from seed surface of pomegranate POY5, were subjected for molecular characterization. The resulting sequence of GRY4 showed 98 percent similarities to the *Issatchenkia terricola* (363 bp) (NCBI accession No. MG547741), and POY5 showed 93 percent similarities to *Pichia kudriavzevii* (506 bp) (NCBI accession number MG547742) and were thus identified.

2.4. Functional Characterization of Seed-Borne Yeasts for Plant Growth Promotion Activity

2.4.1. IAA Production

Yeast isolates were inoculated in Yeast Extract Peptone Dextrose (YEPD) broth (one flask added with 1 percent tryptophan and another without tryptophan), incubated for 48 h at 30 ± 1 °C on a rotary shaker, and centrifuged at 10,000 rpm for 20 min. Further to this, 50 µL of ortho-phosphoric acid and 2 mL of Salkowski's reagent were mixed with 2 mL of supernatant and incubated at 30 ± 1 °C for 25 min under dark conditions. The developed reddish-pink color indicated IAA production, and the absorbance was measured at 530 nm spectrophotometrically (UV-160 A, Shimadzu, Japan) against control. The quantitative estimation of IAA was conducted using a standard graph [37].

2.4.2. Phosphate Solubilization

One mL of yeast culture was inoculated to Pikovskaya's broth and incubated at 30 ± 1 °C for three days. The contents were filtered, and the supernatant was analyzed for the phosphate content [38]. An uninoculated medium was used as a control.

2.4.3. Zinc Solubilization

A quantitative assay for zinc solubilization was performed in Bunt and Rovira medium [39] supplemented with 0.1 percent ZnO. One percent of overnight grown yeast cultures were grown in this medium for 72 h at 120 rpm at 28 ± 2 °C. Following the incubation, samples were withdrawn at different time intervals such as 24, 48, 72, 96, and 120 h and centrifuged at 10,000 rpm for 10 min. The concentration of Zn in the supernatant was quantified through Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

2.4.4. 1-Aminocyclopropane-1-carboxylate Deaminase (ACCD) Activity

The yeast isolates were screened for their ability to metabolize 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of nitrogen. The yeast isolates were spotted onto Petri plates of the modified nitrogen-free Dworkin and Foster medium (MDF) containing 0.3% ACC. Control plates without ACC were also spot inoculated. The plates were incubated for five days at 30 ± 2 °C. The ACC deaminase activity was estimated by measuring the amount of α-ketobutyrate produced and expressed as µm of α-ketobutyrate/mg/protein/h [40].

2.4.5. Biological Nitrogen Fixation Potential

The nitrogen fixation efficiency of the isolates was evaluated by acetylene reduction assay (ARA) in a gas chromatograph (GC). It was expressed as moles of ethylene formed/h/mg of protein [41].

2.4.6. Hydrogen Cyanide (HCN) Production

The yeast isolates were streak plated on Kings B medium supplemented with glycine (4.4 g/L). Whatman No.1 filter paper dipped in picric acid (2% sodium carbonate in 0.5% picric acid) was placed inside the lid of each Petri plate. The plates were sealed airtight with parafilm paper, incubated at 30 ± 1 °C for 4 days, and observed for change in filter paper color from deep yellow to reddish-brown [42].

2.4.7. Siderophore Production

The modified Chrome Azurol Sulphonate (CAS) agar assay was employed to test the ability of yeast isolates to produce siderophores [43]. CAS blue agar and YEPD were solidified onto Petri plates. YEPD agar was cut into halves, and one half was replaced by CAS blue agar. Yeast isolates were spotted onto YEPD agar halves near the borderline between the two media and were incubated in the dark at 30 ± 1 °C for 10 days. An uninoculated CAS agar plate was maintained as a control. Siderophore production was confirmed based on the color change of the CAS blue agar to yellow between the borderline [44].

2.5. Screening of Yeast Isolates for Antagonistic Activity against Phytopathogenic Fungi by Dual Culture Plate Method

The yeast isolates were screened for direct antagonism against *Colletotrichum dematium* MTCC 8652, *Rhizoctonia solani* MTCC 4633, *Alternaria alternata* MTCC 3656, *Aspergillus niger* 2724, and *Fusarium oxysporum* MTCC 2106 on Potato Dextrose Agar (PDA) plates by placing the pathogen in one half and yeast in the other half of the Petri plate and allowing to grow for seven days. The percent inhibition on the growth of the test pathogen was calculated using the formula as described in Rabindran and Vidhyasekaran [45].

2.6. Test for Compatibility of Seed-Borne Yeast Isolates with Rhizobium sp. BMBS1

Selected yeast isolates were tested with the *Rhizobium* strain for compatibility in terms of growth by cross streak assay in nutrient agar medium [46]. A single yeast colony was

streaked vertically on a Petri plate containing nutrient medium followed by streaking *Rhizobium* sp. BMBS1 perpendicular to the yeast isolates. A control plate was maintained without *Rhizobium.*

2.7. Pot Culture Assay to Assess the Potential of the Seed-Borne Yeast Isolates in the Induction of Systemic Resistance against Rhizoctonia Solani MTCC 4633

A pot culture experiment was conducted in black gram (var. MDU. 1) with Completely Randomized Block Design (CRBD) with seven treatments replicated three times. The pots containing 10 kg soil (soil + FYM @ 4:1), had 183.02 kg N, 18.21 kg P_2O_5, and 342 kg K_2O content with a pH 7.1 and EC 0.21 dSm^{-1}. The black gram (var. MDU. 1) seeds were surface sterilized with 1% mercuric chloride for 3 min. and washed thrice with sterile distilled water. The rhizobial and yeast cultures were used at 5 mL each/kg of seed. Four seedlings were maintained per pot. One ml of *R. solani* fungal spore suspension (10^6 CFU/mL) was applied to the soil in each pot as per the treatments 20 days after sowing (DAS). The treatments were imposed as follows:

T1—*Issatchenkia terricola* GRY4 + *Rhizobium* sp. BMBS 1 + *Rhizoctonia solani* MTCC 4633
T2—*Pichia kudriavzevii* POY5 + *Rhizobium* sp. BMBS 1 + *Rhizoctonia solani* MTCC 4633
T3—*Issatchenkia terricola* GRY4 + *Rhizoctonia solani* MTCC 4633
T4—*Pichia kudriavzevii* POY5 + *Rhizoctonia solani* MTCC 4633
T5—*Rhizobium* sp. BMBS1 + *Rhizoctonia solani* MTCC 4633
T6—*Rhizoctonia solani* MTCC 4633, and
T7—Uninoculated control

Germination percent in each pot was calculated 10 days after sowing (DAS). Bio-metric observations were recorded periodically, and yield was recorded at the time of harvest (65 DAS) and expressed in g/plant.

2.8. Effect of Seed-Borne Yeasts on the Induction of Defense-Related Enzymes in Blackgram upon Challenge Inoculation

Leaves from three-week-old plants infected with *R. solani* were collected on 0, 1, 2, 3, 4, and 5 days after challenge inoculation. The collected samples were analyzed for the defense enzymes. Peroxidase activity (POD) was assayed spectrophotometrically through an increase in optical density due to guaiacol's oxidation to tetra-guaiacol. It was expressed as µmol tetra-guaiacol formed/min/g fresh weight [47]. Spectrophotometric polyphenol oxidase (PPO) enzyme assay measured pheomelanin formation from different phenolic substrates. It was expressed as a change in absorbance/min/g of leaf tissue [48]. Catalase (CAT) enzyme assay was performed as per Chaparro-Giraldo et al. [49]. Plant chitinase enzymes hydrolyze the chitin polymer consisting of *N*-acetyl glucosamine (GlcNAc) units and play antifungal activities against many fungi. They were expressed as nmol GlcNAc equivalent/min/g of leaf tissue [50].

2.9. Statistical Analysis

All the experiments were performed in triplicate and the average was subjected to further statistical analysis. Data were analyzed by SPSS version 26 and expressed as mean ± standard errors. Tukey's HSD multiple comparisons were carried out to see the response of yeast isolates on each parameter [51].

3. Results

3.1. Morphological and Biochemical Characterization of the Seed-Borne Yeast Isolates

Twenty morphologically different yeast isolates from the seeds of grapes, pomegranate, tomato, and black gram were selected. Most isolates from black gram, grapes, and tomato showed a sedimented growth in liquid medium. The majority of the yeast isolates showed a bipolar budding pattern except for the three from tomato TOY1, TOY 3, and TOY 4. The margin was irregular or entire in different isolates. Except for pomegranate isolate POY 1, which is elongate, all the cells were spherical. Biochemical characterization showed that all

the isolates produced catalase. Most of them were positive for the starch test, and some isolates could utilize citrate. The sugar fermentation studies showed positive results with sucrose and glucose utilization, thus producing acid and gas. Only three isolates of black gram namely BGY2, 3, and 4, tested positive for lactose utilization.

3.2. Selection of Efficient Seed-Borne Yeasts through Evaluation of Different Plant Growth Promotion Traits

Among the twenty isolates, higher IAA production was recorded in POY5 (21.62 ± 0.061 µg/mL). Siderophore production was positive in POY4, POY5, TOY4, GRY4, and BGY2. Eight yeast isolates (POY1, POY5, TOY1, TOY4, TOY5, GRY4, GRY5, and BGY4) showed HCN production. ACCD activity was observed only in two yeast isolates, POY5 (2.69 ± 0.014 nmol α-ketobutyrate released/min/mg protein) and GRY4 (2.4 ± 0.002 nmol α-ketobutyrate released/min/mg protein) (Table 1).

Table 1. Plant growth-promoting metabolites produced by the seed-borne yeast isolates.

Seed-Borne Yeast Isolates	IAA Production (µg/mL)	IAA without Tryptophan (µg/mL)	Siderophore Production	HCN Production	ACCD Activity (nmol α-Ketobutyrate Released /min/mg Protein)
POY1	6.81 ± 1.838 ^{ij}	5.59 ± 0.259 ^{d}	−	+	ND
POY2	13.54 ± 2.590 ^{defg}	10.03 ± 0.059 ^{a}	−	−	ND
POY3	15.69 ± 1.928 ^{bcde}	6.89 ± 0.021 ^{c}	−	−	ND
POY4	18.22 ± 2.943 ^{abc}	3.25 ± 0.026 ^{f}	+	−	ND
POY5	21.62 ± 0.061 ^{a}	9.50 ± 0.214 ^{ab}	+	+	2.69 ± 0.014
TOY1	13.93 ± 2.333 ^{def}	9.42 ± 0.733 ^{ab}	−	+	ND
TOY2	15.23 ± 3.570 ^{cde}	2.33 ± 0.087 ^{g}	−	−	ND
TOY3	13.83 ± 2.673 ^{def}	2.36 ± 0.063 ^{g}	−	−	ND
TOY4	6.32 ± 3.452 ^{j}	4.81 ± 0.013 ^{e}	+	+	ND
TOY5	10.16 ± 2.836 ^{ghi}	3.17 ± 0.016 ^{f}	−	+	ND
GRY1	13.11 ± 2.614 ^{efg}	2.61 ± 0.004 ^{g}	−	−	ND
GRY2	16.81 ± 5.108 ^{bcd}	2.44 ± 0.060 ^{g}	−	−	ND
GRY3	12.43 ± 3.051 ^{bcd}	2.61 ± 0.075 ^{fg}	−	−	ND
GRY4	18.81 ± 4.644 ^{ab}	7.44 ± 0.200 ^{c}	+	+	2.4 ± 0.002
GRY5	10.45 ± 1.055 ^{fgh}	2.33 ± 0.009 ^{g}	−	+	ND
BGY1	16.81 ± 5.108 ^{bcd}	2.36 ± 0.034 ^{g}	−	−	ND
BGY2	11.18 ± 0.932 ^{fg}	9.25 ± 0.075 ^{b}	+	−	ND
BGY3	12.59 ± 3.505 ^{efg}	4.25 ± 0.122 ^{e}	−	−	ND
BGY4	12.43 ± 3.172 ^{efg}	2.28 ± 0.076 ^{g}	−	+	ND
BGY5	7.17 ± 0.867 ^{hij}	9.06 ± 0.097 ^{b}	−	−	N.D.
SEd	2.06	0.08			1.07
CD (0.05)	4.14	0.16			

Values are average of triplicates. ± standard deviations. Figures with different letters are statistically different at the p 0.01 level. ND = Not detected. IAA = Indole-3-acetic acid, HCN = hydrogen cyanide, ACCD = 1-aminocyclopropane-1-carboxylate deaminase.

Isolate POY5 exhibited higher nitrogen fixation potential compared to other isolates. All the 20 isolates showed phosphate (P) solubilization potential in the range of 0.19 to 0.42 g/L. Isolate POY5 showed the highest P release (0.46 ± 0.02 g/L). Tukey's multiple comparisons showed no difference among the yeast isolates in P solubilization activity within 24 h, 48 h, and 72 h of inoculation. Tukey's multiple comparisons tests showed that the yeast isolate POY5 recorded significantly higher Zn solubilization (181.40 ± 1.79 mg/L) on the fifth day of incubation. The amount of zinc solubilized increased with an increase in incubation time; the maximum Zn solubilization was recorded on the fifth day.

3.3. Extracellular Enzyme Production and Antagonistic Activity against Phytopathogenic Fungi of the Seed-Borne Yeast Isolates

Antifungal activity was tested in yeast isolates *Issatchenkia terricola* GRY4 and *Pichia kudriavzevii* POY5 against phytopathogenic fungi namely, *Colletotrichum dematium* MTCC 8652, *R. solani, A. alternata, A. niger*, and *F. oxysporum*. In this study, most yeast isolates exhibited chitin utilization and were higher at 1.5 percent colloidal chitin. All the tested isolates except GRY1 showed growth at all levels of colloidal chitin. All the isolates exhibited protease activity, and maximum activity was observed in POY5 (0.330 ± 0.035 U/mL) and GRY5 (0.348 ± 0.049 U/mL) against the lowest activity in GRY3 (0.135 ± 0.022 U/mL). However, the protease production among the isolates was non-significant at a 95 percent confidence interval. This study did not reveal any growth inhibition by the yeast isolates GRY4 and POY5 on the test pathogens. No inhibition in the growth of any of the tested fungal pathogens was noticed in dual culture with both the yeast isolates (POY5 and GRY4).

3.4. Reflections of the Seed-Borne Yeast Isolates on Growth and Yield of Black gram

In the experiment conducted with various treatments, in which plants were challenged with the pathogen *Rhizoctonia solani* MTCC 4633, maximum root length was observed in the treatment that received *Pichia kudriavzevii* POY5 (10.90–20.30 cm/plant), followed by *Issatchenkia terricola* GRY4 (8.90–16.90 cm/plant) with an increase of 53.50–81.30 percent and 25.30–50.90 percent, respectively, over the uninoculated control. The combined inoculation of *I. terricola* GRY4 recorded the maximum shoot length with an increase of 47.3, 70.2, and 31.80 percent over the uninoculated control at 30, 45, and 60 days of growth, respectively. *P. kudriavzevii* POY5 inoculation exhibited a higher number of leaves (43.7 ± 2.40) followed by GRY4 (40.9 ± 2.09) on 60 DAS than the control. The least number of leaves was recorded with the uninoculated control (32.5 ± 1.70) and *R. solani*-challenged plants (31.2 ± 0.30). The single inoculation with *Pichia kudriavzevii* POY5 (24.3 ± 0.55) and combined inoculation with *Rhizobium* sp. were among the yeast isolates. BMBS1(21.9 ± 0.01) resulted in a higher number of nodules in plants challenged with the pathogen *R. solani,* increasing 50.9 and 36.0 percent compared to the uninoculated control. The maximum pod yield (31.4 ± 0.22 pods/plant) and grain yield (13.5 ± 0.25 g/plant) were recorded in the single inoculation of *Pichia kudriavzevii* POY5. However, there were no significant differences among GRY4 co-inoculated with *Rhizobium* sp. BMBS1 (11.5 ± 0.15 g/plant), POY 5 co-inoculated with *Rhizobium* sp. BMBS 1 (10.8 ± 0.12 g/plant), and GRY4 alone (11.1 ± 0.14 g/plant) for the grain yield. The inoculation of *Rhizobium* sp. BMBS1 and yeast isolates increased the pod yield and grain yield of black gram significantly over the uninoculated control. In general, a single inoculation with *I. terricola* GRY4 or *P. kudriavzevii* POY5 combined with *Rhizobium* sp. BMBS1 recorded more pods and higher grain yields compared to the inoculation with *Rhizobium* sp. BMBS1 alone. A higher grain yield of 13.5 ± 0.25 g/plant was recorded with the inoculation of *Pichia kudriavzevii* POY5 challenged with *Rhizoctonia solani* MTCC 4633 followed by the inoculation with *Issatchenkia terricola* GRY4 + *Rhizobium* sp. BMBS1 was challenged with *Rhizoctonia solani* MTCC 4633 with a recorded yield of 11.5 ± 0.15 g/plant (Figure 1).

3.5. Effect of Seed-Borne Yeast Isolates on the Induction of Defense-Related Enzymes in Black Gram upon Challenge Inoculation

Higher peroxidase activity with absorbance change of 2.58 ± 0.07 units/min/g of leaf tissue was observed in the combined inoculation of yeast GRY4 (*I. terricola*) + *Rhizobium* sp. BMBS1 with challenged pathogen inoculation. This was a 55% increase in plants challenged with pathogen alone after four days of challenging (Figure 2). The PPO activity was higher on four days in plants that received *Pichia kudriavzevii* POY5 + *R. solani* MTCC 4633 compared to other treatments (Figure 3).

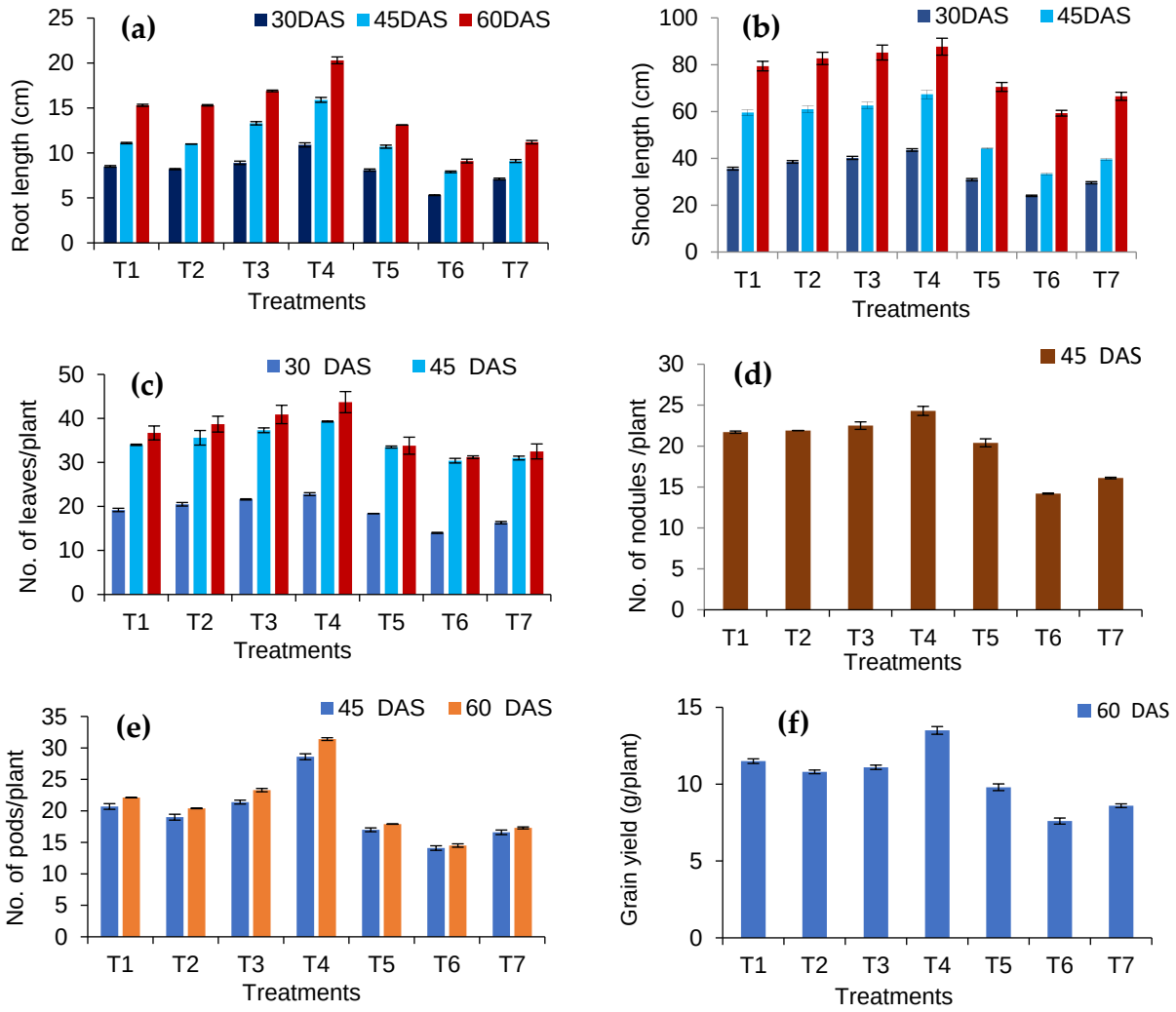

Figure 1. Reflection of seed-borne yeast isolates on growth and yield of black gram treated with *Rhizobium* sp. BMBS 1 and challenged with *R. solani*. (**a**) Shoot length; (**b**) root length; (**c**) number of leaves; (**d**) number of nodules; (**e**) number of pods; (**f**) grain yield (values are the average of triplicates). DAS = Day after sowing T1—*I.terricola* GRY4 + *Rhizobium* sp. + *R. solani* MTCC 4633, T2—*P. kudriavzevii* POY5 + *Rhizobium* sp. + *R. solani*, T3—*I. terricola* GRY4 + *R. solani*, T4—*P. kudriavzevii* POY5 + *R. solani* MTCC 4633, T5—*Rhizobium* sp. BMBS1 + *R. solani*, T6—*R. solani*, and T7—uninoculated control.

Catalase activity was higher in the combined inoculation of *Pichia kudriavzevii* POY5 + *Rhizobium* sp. BMBS1 along with *R. solani* (0.29 µM/min/g leaf tissue) compared to *R. solani* (0.27 µM/min/g leaf tissue) at day 4 of the challenge inoculation. The catalase enzyme activity was enhanced by yeast inoculation and a combination of yeast and *Rhizobium* sp. BMBS1 and pathogen challenge inoculation to the tune of 3.7–7.4% over inoculation of pathogens alone (Figure 4).

Maximum chitinases (PR proteins) were observed with the combined inoculation of yeast POY5 (*P. kudriavzevii*) and *Rhizobium* sp. BMBS1, which recorded a 6.3 percent increase over pathogen inoculation on day 4 (Figure 5). These defense enzymes were observed to be higher on day four and decreased on day 5 of the challenge inoculation.

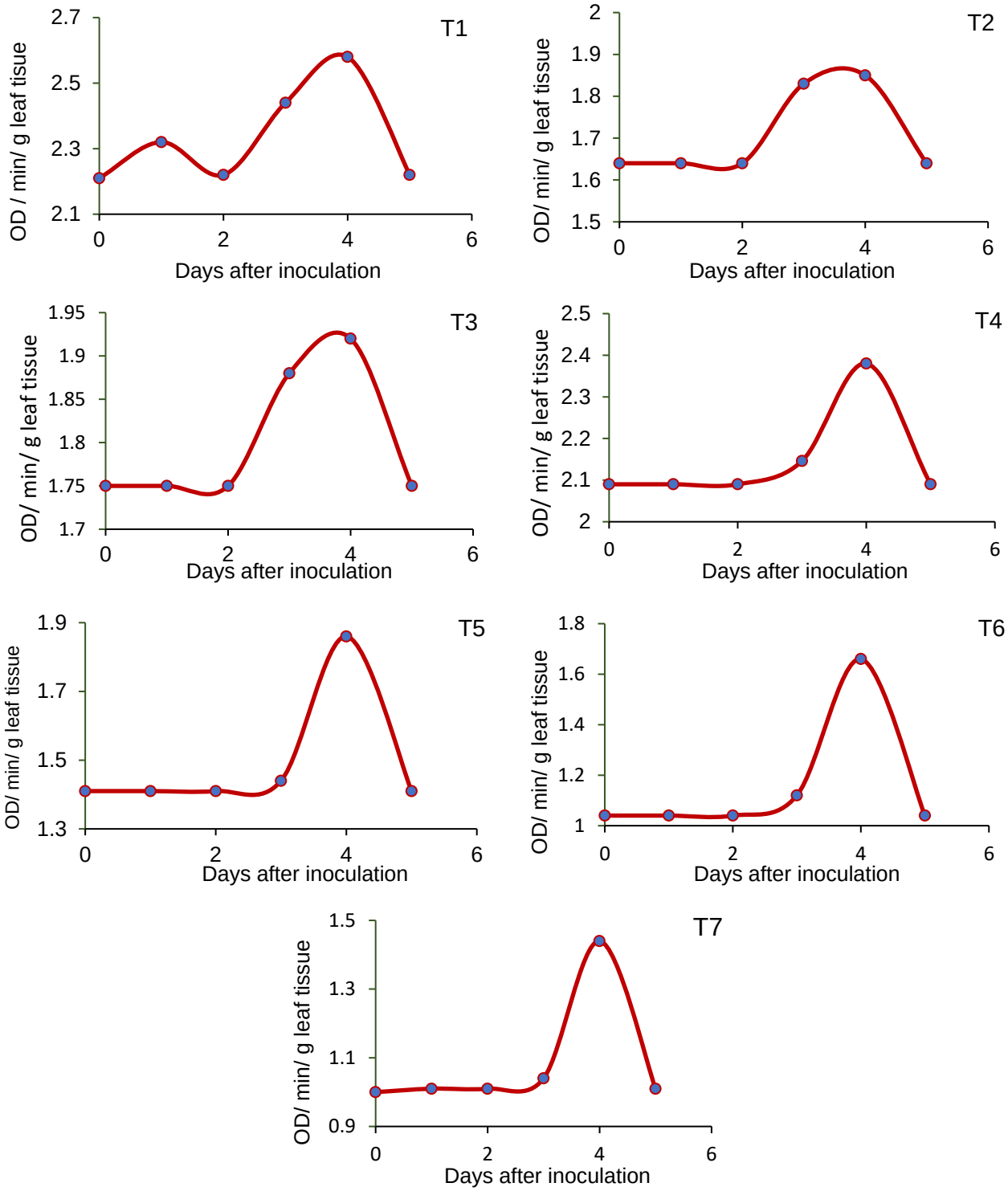

Figure 2. Induction of POD activity in black gram inoculated with yeast, Rhizobium sp. and challenge inoculated with R. solani. T1—Issatchenkia terricola GRY4 + Rhizobium sp. + R. solani MTCC 4633, T2—P. kudriavzevii POY5 + Rhizobium sp. + R. solani, T3—I. terricola GRY4 + R. solani MTCC 4633, T4—Pichia kudriavzevii POY5 + R. solani MTCC 4633, T5—Rhizobium sp. BMBS1 + R. solani MTCC 4633, T6—R. solani MTCC 4633, and T7—uninoculated control.

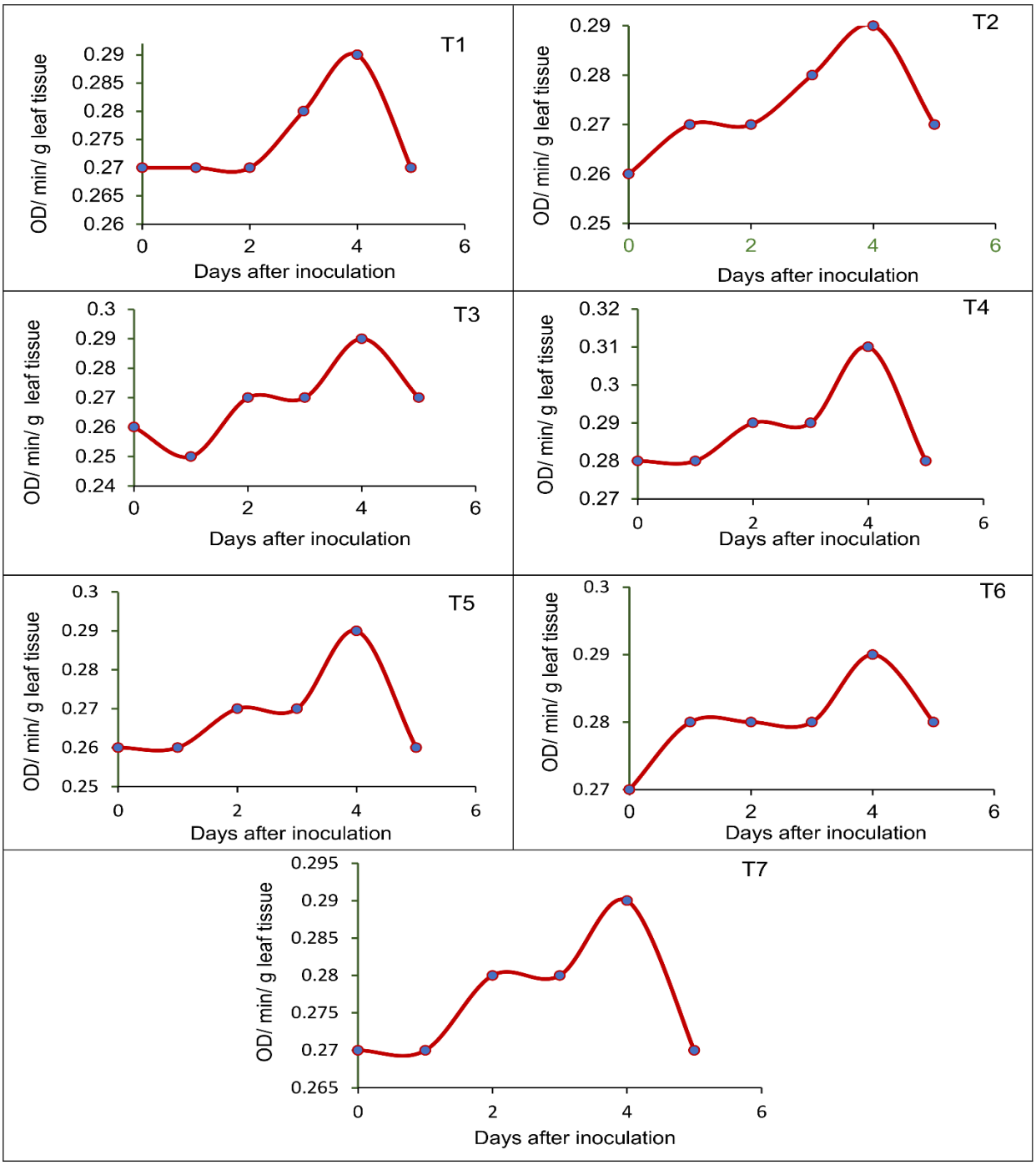

Figure 3. Induction of polyphenol oxidase in black gram inoculated with yeast, *Rhizobium* sp., and *R solani*. T1—*Issatchenkia terricola* GRY4 + *Rhizobium* sp. + *R. solani* MTCC 4633, T2—*P. kudriavzevii* POY5 + *Rhizobium* sp. + *R. solani*, T3—*I. terricola* GRY4 + *R. solani* MTCC 4633, T4—*Pichia kudriavzevii* POY5 + *R. solani* MTCC 4633, T5—*Rhizobium* sp. BMBS1 + *R. solani* MTCC 4633, T6—*R. solani* MTCC 4633, and T7—uninoculated control.

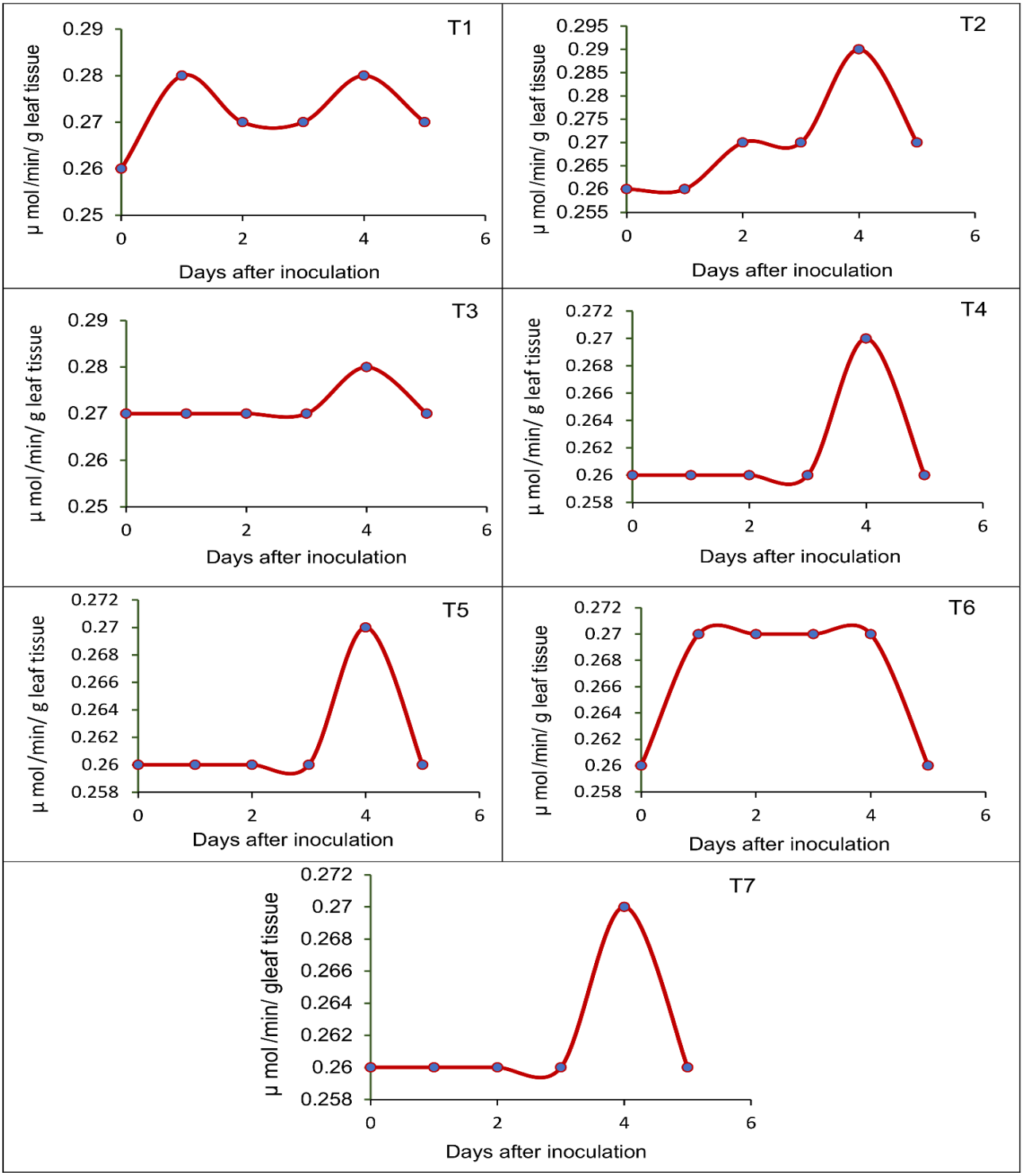

Figure 4. Induction of CAT in black gram inoculated with yeast, *Rhizobium* sp. and challenge inoculation of *Rhizoctonia solani*. T1—*Issatchenkia terricola* GRY4 + *Rhizobium* sp. + *R. solani* MTCC 4633 T2—*P. kudriavzevii* POY5 + *Rhizobium* sp. + *R. solani*, T3—*I. terricola* GRY4 + *R. solani* MTCC 4633, T4—*Pichia kudriavzevii* POY5 + *R. solani* MTCC 4633, T5—*Rhizobium* sp. BMBS1 + R. solani MTCC 4633, T6—R. solani MTCC 4633, and T7—uninoculated control.

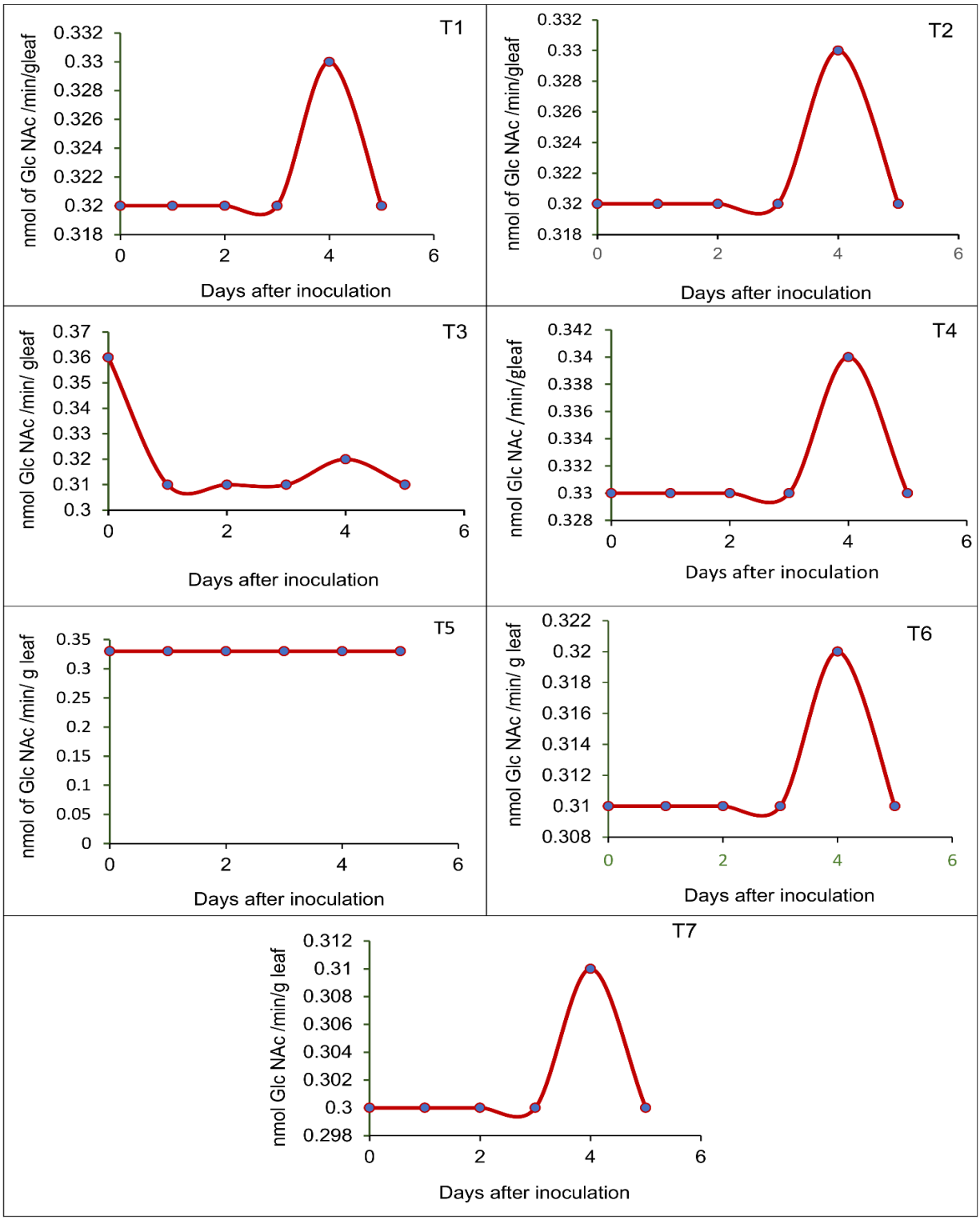

Figure 5. Induction of chitinase activity in black gram inoculated with yeast, *Rhizobium* sp. and *R. solani*. T1—*Issatchenkia terricola* GRY4 + *Rhizobium* sp. + *R. solani* MTCC 4633, T2—*P. kudriavzevii* POY5 + *Rhizobium* sp. + *R. solani*, T3—*I. terricola* GRY4 + *R. solani* MTCC 4633, T4—*Pichia kudriavzevii* POY5 + R. solani MTCC 4633, T5—*Rhizobium* sp. BMBS1 + *R. solani* MTCC 4633, T6—*R. solani* MTCC 4633, and T7—uninoculated control.

However, the PPO and chitinase activities were non-significant among all the treatments. In general, the enzyme activities were enhanced in all inoculated treatments com-

pared to the uninoculated control. Inoculation of both the yeast isolates GRY4 and POY5 exhibited higher enzyme activities with and without *Rhizobial* inoculation under the conditions of *Rhizoctonia solani* inoculation.

4. Discussion

Identifying potential microbes from natural resources and deploying effective microbes for plant growth promotion is one of the underlying concepts for promoting next generation and sustainable agriculture. This research showed the multifarious potential of two seed-borne yeasts, *Issatchenkia terricola* GRY4 and *Pichia kudriavzevii* POY5, with plant growth and health-promoting potential. Plant growth-promoting yeasts namely, *Kluyveromyces esculenta*, *Pichia caribbica*, *Candida tropicalis*, *Saccharomyces cerevisiae* from *Manihot esculenta*, *Zea mays*, *Cola acuminate,* and *Sorghum bicolor* have been identified by earlier workers [52]. About 1035 epiphytic and endophytic yeast isolates from rice and sugarcane [53] tested for plant growth-promoting traits exhibited different morphological characteristics, and morphological variations in colony size, color, pigmentation, forms, margin, and elevation were observed among the other yeast isolates. The source of isolation may contribute to variations in cultural characteristics. Previous work reported different colony forms, dimorphic growth, hyphal development, and taxonomic traits [54]. The yeast colonies from palm wine varied from whitish cream to pink, and the surface texture was dry and wrinkled [55]. *Saccharomyces cerevisiae* strains exhibited morphological and physiological differences with rough and smooth colonies [56]. Biochemical characterization of yeast isolates indicated differences in carbon utilization patterns. Earlier reports explored the morphological and biochemical characteristics of yeast isolates namely, *Candida ipomoeae, Candida famata, Candida succiphila, Rhodotorula mucilaginosa, Kodamaea anthophila, Debaryomyces hansenii,* and *Pichia lachancei* from Jamun's fruit surface. The yeast isolates were reported to utilize starch and gelatin and growth at 10 percent sucrose [57]. All the 81 yeast isolates were obtained from sugar silage fermented glucose, galactose, sucrose, methyl α-d glucosides, and raffinose. A few isolates fermented maltose and none fermented cellobiose. About 65 percent of isolates fermented lactose [58]. Most of the isolates from palmyra palm and sugar palm showed good growth at high concentrations of ethanol [59].

Earlier workers performed screening and characterization of yeast for various plant growth promotion traits. Most PGP organisms utilized L-tryptophan, which is secreted in root exudates as a precursor of IAA production. Hence, the ability to produce IAA varied with strains or types of microorganisms. IAA production was comparatively higher in yeast isolates than fungi and actinobacteria [60]. The yeast isolates from rice and sugarcane leaves were screened for plant growth-promoting traits, and *Rhodotorula paludigenum* DMKU-RP301 was found to be a better IAA producer, whereas *Torulaspora globosa* DMKU-RP31 was found to be a plant growth promoter by direct and indirect mechanisms and antagonistic to pathogens by producing volatile antifungal compounds [53].

Similarly, the soil yeast *Candida tropicalis* HY (CtHY) isolated from the rice rhizosphere showed little IAA production, higher ACC deaminase activity, polyamine production, and phytase production. It colonized rice seedlings and enhanced germination percentage, root and shoot length, and vigor index [16]. Endophytic yeast, *Williopsis saturnus*, isolated from maize seedlings, produced indole-3 acetic acid (IAA) and indole-3-pyruvic acid (IPA) acid in a medium amended with tryptophan. The yeast inoculation with indole precursor, tryptophan, modulated plant IAA, and IPA levels significantly increased root and shoot length and dry weight under glasshouse and gnotobiotic conditions [11]. Soil yeast *Meyerozyma guilliermondii* CC1 was an IAA producer, P solubilizer, and chitinolytic. IAA production in *Rhodosporidium paludigenum* DMKU RP301 was influenced by factors like carbon and nitrogen sources, temperature, presence of growth factors and tryptophan [61] Two strains of *Rhodotorula mucilaginosa* isolated from stems of hybrid poplar plants produced IAA hormone when amended with tryptophan and promoted plant growth [62]. *Aureobasidium melanogenum* SDBR-CMU-S1-10 and *Papilio tremalaurentii* SDBR-CMU-S1-02 isolates from Assam tea soil produced siderophore and exhibited plant growth promotion

activity [63] The yeast *Wickerhamomyces anomalus* MSD1 from marine algae was reported to possess atmospheric nitrogen-fixing potential, solubilization of insoluble phosphate and zinc, siderophore production, and ACC deaminase activity [64]. With the result of the present and earlier works, it is well documented that yeast influences the growth of plants by nutrient solubilization and biomolecule production.

Soil yeast and yeast-like fungi produce diverse biologically active compounds, including an antimicrobial substance that reduces phytopathogenic infections [5,65]. Plant growth-promoting traits of grapes seed-borne *Issatchenkia terricola* GRY4 and pomegranate seed-borne *Pichia kudriavzevii* POY5 included chitinase and protease activity. The secretion and excretion of chitinase are some of the inherent characteristics of bio-control agents because of their potential to degrade the fungal cell wall [66]. Yeasts namely, *Metschnikowia* sp. [67], *Tilletiopsis* sp. [66], *Saccharomycopsis schoenii* [68] were potent bio-control agents through chitinase activity. The soil yeast *Lachancea kluyveri* SP132 exhibited antifungal activity by producing chitinase cellulase against *Rhizoctonia solani* [69]. *Wickerhamomyces anomalus* SDBR-CMU-S1-02 isolate from Assam tea plantation soil possessed protease activity [63]. *Candida oleophila* showed the ability to secrete chitinase, protease, and glucanase effectively against *Penicillium digitatum* [70]. Yeast isolates from grapes namely, *Candida intermedia*, *Candida lusitaniae*, and *Debaryomyces hansenii* were reported as active chitinase producers [22].

The reflections of these multifarious yeast isolates were realized with the plant assay wherein *Issatchenkia terricola* GRY4 and *Pichia kudriavzevii* POY5 influenced root length, shoot length, and yield of *Rhizoctonia solani*-challenged black gram. *Pichia kudriavzevii* POY5-inoculated black gram plants managed the pathogen challenge by increasing the root length by 123.07% and shoot length by 47.89%. The root structure of rice was greatly influenced by plant growth-promoting bacteria–Protist interaction, resulting in enhanced lateral root growth by 272.08–380.41% [71]. The increased root length is attributed to the IAA production by the yeast isolates, which is in line with the observations of earlier workers that lower concentrations of IAA stimulated primary root elongation and that of higher promoted lateral roots and root hairs [72,73]. An increased seed vigor index was observed in maize by replacing half of the chemical fertilizer with yeast inoculation, thus resulting in improved plant growth, biomass, cob yield, and uptake of calcium, magnesium, iron, manganese, and zinc [74]. Similarly, yeasts isolated from the phyllosphere of the carnivorous plant *Drosera indica* increased the number of lateral roots and root hairs due to IAA production via the up-regulation of auxin-inducible gene expression [75]. The bio-control agents activate plant defense genes of the phenylpropanoid pathways, namely encoding peroxidase (POX.), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) [76]. They are involved in lignin's biosynthesis, which acts as a protective barrier for pathogen entry into plant tissues [77]. This study proved the activation of defense enzymes in *R. solani*-challenged black gram with the beneficial yeast isolates *I. terricola* GRY4 and *P. kudriavzevii* POY5. Co-inoculation of *I. terricola* GRY4 and *Rhizobium* sp. BMBS1 significantly activated peroxidase, whereas *P. kudriavzevii* POY5 and *Rhizobium* sp. BMBS1 enhanced catalase activity. As a powerful antioxidant, catalase plays a major role in signaling processes in plants during adverse conditions [78]. Furthermore, recently it was revealed that yeast assisted *Rhizobium* sp. in nodulation by producing unique signaling molecules that triggered the lectin pathway [79,80]. PGPRs namely, *Lysinibacillus fusiformis*, *Bacillus subtilis*, and *Achromobacter xylosoxidans* increased peroxidase and polyphenol oxidase in *Alternaria solani*-challenged tomato plants [81]. Though many reports stated defense enzyme productions by the bacterial antagonists [82–88], this may be the first report regarding enhancing defense enzymes through yeast inoculations, particularly seed-borne yeasts.

In summary, these two plant growth-promoting yeasts, *Pichia kudriavzevii* POY5 and *Issatchenkia terricola* GRY4, which possessed the capacity to release auxin, to transform fixed phosphorus and zinc to an available form, to fix atmospheric nitrogen, to produce siderophore, hydrogen cyanide, ACC deaminase, various extracellular enzymes, and defense-related enzymes for the plant growth promotion of black gram, could be further

explored for their endophytic association. They could be developed into a formulation for promoting the growth and health of black gram.

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