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ORIGINAL PAPER

Evaluating Lignification, Antioxidative Defense, and Physiochemical Changes in Soybean Through Bio-Priming Under Graded Soil Fertilization

Subhadip Paul[1] · **Amitava Rakshit**[1]

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Abstract
Our objective was to evaluate the *Trichoderma viride* BHU-2953 seed priming on stem lignification, antioxidant enzyme activity, and changes in soybean physiological attributes under graded nitrogen (N), phosphorus (P), and potassium (K) application. Pot experiment was conducted by allocating six treatments in completely randomized design. Stem lignification, enzyme activities of cinnamyl alcohol dehydrogenase (CAD), guaiacol peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX), H_2O_2, lipid peroxidation, and different plant physiochemical parameters were evaluated at different growth stages. Bio-priming enhanced the stem CAD and POD activities, causing vascular lignin deposition as a result of more root colonization by *T. viride* BHU-2953. Inflated leaf APX activities in primed treatments reduced the harmful effect of H_2O_2, generated from increased leaf SOD activities. Similarly, lipid peroxidation was minimized in bio-primed leaves, alleviating the stress from graded NPK doses. Longer root lengths in bio-primed soybeans improved the physiological use efficiency (*PUE*) of N and P. Higher *PUE* in *T. viride* BHU-2953-treated soybeans were able to enhance plant height, dry matter accumulation, and leaf area as compared to untreated ones. Seed yields of bio-primed soybeans were found higher than control but similar as compared to treatment only received recommended doses of fertilizers. Inoculation of *T. viride* BHU-2953 has positively influenced activities of APX, SOD, CAD, POD, and root length, triggering antioxidant defense responses, vascular lignification, and *PUE*s of nutrients, respectively. Thus, application of our findings can be highly encouraged by low input sustainable crop production.

Keywords Ascorbate peroxidase · Cinnamyl alcohol dehydrogenase · Guaiacol peroxidase · Superoxide dismutase · *Trichoderma viride* BHU-2953

1 Introduction

Soybean [*Glycine max* (L.) Merr.] is a highly valued crop, cultivated commercially as an oilseed. Reports have shown a decline in its productivity as a result of inadequate nutrition, abiotic, and biotic stresses (Sepat et al. 2017; Paul et al. 2019). Major focuses have been given to alleviate stresses, caused by soil-borne pathogens, drought, flood, and salinity (Mutava et al. 2015; Zhang et al. 2015; Zhang et al. 2017). However, the nutrient stress of soybean has been neglected under low soil fertility status. Low soil fertility under intensive cultivation practices has been reported to reduce the potential yield of soybean varieties (Sepat et al. 2017; Paul et al. 2019). Generally, these deficiencies were refined by applying mineral fertilizers, but to sustain the long-term productivity microbial augmentation is much needed (Rao and Reddy 2010; Paul et al. 2019). The *Bradyrhizobium japonicum* is generally used to enhance the nitrogen (N) economy in soil, while increasing phosphorus (P) uptake can be achieved by inoculating phosphorus-solubilizing microbes (Argaw 2012). However, the high mortality of *B. japonicum* upon seed inoculation makes it a less effective cultural practice to supply plant-N (Salema et al. 1982; Streeter 2007). In a general term, consortia are more preferred over single inoculation. In this regard, potential effect of other microbial cultures needs to be evaluated.

✉ Amitava Rakshit
amitavar@bhu.ac.in

[1] Department of Soil Science and Agricultural Chemistry, Institute of Agricultural Sciences, Banaras Hindu University, Uttar Pradesh, Varanasi 221005, India

The *Trichoderma* is primarily considered a bio-control agent and plant abiotic stress alleviator but in recent studies, these soil-inhabiting fungal genera were also found to increase the nutrient uptake in different field crops (Singh et al. 2014a, b; Meena et al. 2016; Paul and Rakshit 2021). The genus was also found to promote plant growth (Singh et al. 2013a; Gajera et al. 2016). Auxin-mediated root elongations in *Trichoderma*-treated plants were also delineated by earlier researchers (Druzhinina et al. 2011). Meena et al. (2016) had found more plant heights and nitrogen uptake in wheat. Higher root length, plant dry matter, and yield were obtained by Yadav et al. (2018) through bio-priming maize seeds with *T. viride*. John et al. (2010) showed that *Trichoderma viride* reduced the pathogenicity of *Fusarium oxysporum* and *Pythium arrhenomanes* in soybean. Stem rot (*Sclerotinia sclerotiorum*) of soybean can be successfully controlled by *Trichoderma harzianum* isolate (Zhang et al. 2015). Salt tolerance in soybean was attained by inoculating *T. harzianum* that increased higher proline contents in roots and leaves under salinity stress (Khomari et al. 2017). Their primary mechanisms within plants are xylem lignification and the triggering of antioxidant enzymes (Singh et al. 2013a). When treated with *Trichoderma* species, activities of lignifying enzymes such as cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) and guaiacol peroxidase (POD; EC 1.11.1.7) were reported to increase (Singh et al. 2013a; Singh et al. 2013b; Gajera et al. 2016). The fungal genera were also experimented to enhance the activities of plant defense enzymes, i.e., ascorbate peroxidase (APX; EC 1.11.1.11) and superoxide dismutase (SOD; EC 1.15.1.1) under stress conditions (Singh et al. 2013b; Gajera et al. 2016; Zhang et al. 2017). Thus, a single inoculation potential of *Trichoderma* needs to be assessed to supplement soybean nutrition alongside the induction of plant systemic defense responses under nutrient stress.

Singh et al. (2014a, b) have prepared 2% W.P. formulation of *Trichoderma viride* BHU-2953 that was successfully controlled tomato wilt (*Fusarium oxysporum*) and damping-off of chili (*Pythium aphanidermatum*). The formulation was also found effective in enhancing plant height and yield of chili and tomato. Still, no information is available regarding its inoculating effect on host physiochemical changes i.e., regulation of plant antioxidant enzymes, lignification, and physiological use efficiency of essential plant nutrients. This particular strain was also found to be compatible with other rhizospheric beneficial microbes (Singh et al. 2014a, b). Hence, we have selected *Trichoderma viride* BHU-2953 as a potent seed inoculant for our experiment. Previously, we have discovered high soil P solubilization in the rhizosphere of soybean via inoculation of *Trichoderma viride* BHU-2953 under graded nutrient application (Paul and Rakshit 2021).

In plant-cellular organelles, nitrogen (N), phosphorus (P), and potassium (K) play additive roles that regulate photosynthesis, respiration, enzyme activation, transport of assimilates, osmoregulation, protein synthesis, and ion homeostasis (Marschner 2012). Thus, cutting down the levels of these three nutrients becomes essential for inducing cumulative nutrient stress. To alleviate this stress, the soybean seeds were inoculated with *T. viride* BHU-2953. Considering the reports of earlier researches, we hypothesized that *T. viride* BHU-2953 inoculation will improve (i) stem lignification, (ii) leaf antioxidant enzyme activities, and (iii) changes in plant physiological parameters of soybean under graded NPK application.

2 Material and Methods

2.1 Site Description

The experiment was conducted in the Department of Soil Science and Agricultural Chemistry, Institute of Agricultural Sciences (IAS), Banaras Hindu University (BHU). Soils were collected from Agricultural Research Farm (25° 15′ 19″ N, 82° 59′ 31″ E), situated at 80.7 m above mean sea level. Initial soil characters are mentioned in the Table 1.

2.2 Fungal Talc Preparation and Seed Bio-Priming

The *Trichoderma viride* BHU-2953 was collected from the Department of Mycology and Plant Pathology, IAS, BHU. The fungus was then cultured in potato dextrose agar (*PDA*) under a sterilized environment and incubated for 5 days. The spore was harvested with sterile distilled water. The optical density (600 nm) of this spore suspension was found to be 1.073 which represents 2.11×10^7 spore mL^{-1}. It was further diluted to 2×10^5 spore mL^{-1} and added to 10 g sterile talc with 0.2% peptone and 1% carboxy-methyl cellulose. The soybean variety JS 95–60 is chosen as it is an early maturing (75–82 days) type (Dixit et al. 2009). Approximately, 500 g

Table 1 Physical, chemical, and biological parameters of initial soil

Parameters	Values
Depth of soil	0 – 15 cm
Bulk density	1.52 Mg m^{-3}
Soil textural class	Sandy loam
pH	7.4
Electrical conductivity	0.44 dS m^{-1}
Organic carbon (Walkley and Black 1934)	0.43%
Available soil N (Subbiah and Asija 1956)	0.058 mg g^{-1}
Available soil P (Olsen et al. 1954)	0.016 mg g^{-1}
Available soil K (Hanway and Heidel 1952)	0.072 mg g^{-1}
Trichoderma spp.	4.2×10^3 CFU g^{-1}

CFU colony-forming unit

soybean (*Glycine max* cv. JS 95–60) seed was sterilized in sodium hypochlorite (10% NaOCl) solution for 10 min, washed repeatedly with distilled water, and shade-dried thereafter. Dried seeds were then blended with the talc formulation and left for shade drying.

2.3 Experimental Details

A set of eighteen polythene-lined pots were filled with collected soil (10 kg pot^{-1}) after air drying and sieving through a 2-mm sieve. Soils were not fumigated to preserve the activities of beneficial soil microbes. Completely randomized design (CRD) was followed in the experiment, taking three replications and six treatments: *C* (control; without bio-priming + no fertilization), *RDF* (recommended doses of fertilizers; 290.2 mg N pot^{-1}, 178.6 mg P and K pot^{-1}; without bio-priming), *T90* (90% *RDF*; 261.18 mg N pot^{-1}, 160.8 mg P and K pot^{-1} + bio-priming), *T80* (80% *RDF*; 232.2 mg N pot^{-1}, 142.9 mg P and K pot^{-1} + bio-priming), *T75* (75% *RDF*; 217.7 mg N pot^{-1}, 134 mg P and K pot^{-1} + bio-priming), and *T70* (70% *RDF*; 203.2 mg N pot^{-1}, 125 mg P and K pot^{-1} + bio-priming). Soybean seeds, bio-primed with *T. viride* (strain no. BHU-2953), were sown only in *T90*, *T80*, *T75*, and *T70* pots, whereas untreated seeds were sown in control and *RDF*. Urea, di-ammonium phosphate, and sulfate of potash were applied in soil (except for control). As inoculation of seeds with *Bradyrhizobium* spp. or *Rhizobium* spp. were averted in this experiment, higher N doses were applied (except control).

2.4 Stem Lignification Study

A lignification study was carried out on 21 days after sowing (21 *d*) because the stem succulence starts to decline after 3 weeks of soybean (JS 95–60) growth. Transverse sectioning of soybean base stem (0.5 cm from the soil surface) was done on glass slides and immersed in 20% (w/v) aqueous solution of phloroglucinol (Merck Millipore, India). Reddish-pink coloration of lignin near xylem was visualized under a light microscope (Nikon DS-fi1, Japan) in 10× magnification (Mishra et al. 2014).

Stem lignin content was quantified with the acetyl bromide method after exhaustive removal of protein from the cell wall (Moreira-Vilar et al. 2014). To remove the protein, dried stems (0.3 g) were homogenized in a pre-chilled mortar with 0.05 M K-phosphate buffer (pH 7) and centrifuged repeatedly at 1400 × g for 5 min with K-phosphate buffer at pH 7 (two times), 1% (v/v) Triton X-100 buffer (Merck Millipore, India) at pH 7 (three times), 1 M NaCl (two times), distilled water (two times), and acetone (two times). Final residue was dried overnight at 60 °C in a hot-air oven to obtain a protein-free fraction. This protein-free pellet (0.02 g) was digested with acetyl bromide (Merck Sigma-Aldrich, India) solution (25% v/v in glacial acetic acid) at 70 °C for half an hour, cooled in ice bath, and added with 2 M NaOH, 5 M $NH_2OH.HCl$, and glacial acetic acid (4 mL). The mixture had undergone spectrophotometric lignin determination at 280 nm (μController Based UV–Visible Spectrophotometer Type-117, Systronics India Ltd., India) after centrifugation at 1400 × g for 5 min.

The CAD (EC 1.1.1.195) activity was estimated with the method as described by Morrison et al. (1994). Cut pieces of soybean stem (0.25 g) were homogenized in a pre-chilled mortar, using 0.1 M Tris–HCl buffer (Merck Millipore, India) pH 7.6, 0.5% polyethylene glycol (Merck Millipore, India), and 0.02 M 2-mercaptoethanol (Merck Millipore, India), and stored at 4 °C overnight. The ice-cooled homogenate was centrifuged at 25,000 × g on the next day. The supernatant (0.6 mL) was added with 0.1 M K-phosphate buffer (pH 7.6), 0.001 M cinnamaldehyde (Merck Sigma-Aldrich, India), 0.02 M $ZnSO_4.7H_2O$, and 0.001 M nicotinamide adenine dinucleotide phosphate hydrogen (NADPH; Merck Millipore, India). Readings (340 nm) were taken before incubation (0 min) and after incubation (15 min) at 30 °C, against control.

Fresh soybean stems (0.25 g) were homogenized with 0.2 M phosphate buffer (pH 7.8) in a pre-chilled mortar, and centrifuged (25,000 × g for 15 min) at ⁻ 4 °C, and activity of POD (EC 1.11.1.7) was studied using Goliber (1989) method. The collected supernatant (0.04 mL) was mixed with 0.02 M guaiacol (Merck Sigma-Aldrich, India) solution and 0.01 M H_2O_2 (Merck Emsure® ACS, ISO, Reag. Ph Eur, India) in presence of 0.1 M phosphate buffer (pH 7). The spectrophotometric enzyme activity was measured at 470 nm.

2.5 *Antioxidant Enzyme Activities, H_2O_2 Content, and Lipid Peroxidation Assay of Leaves*

Leaf samples were collected for determining H_2O_2 content, SOD (EC 1.15.1.1) and APX (EC 1.11.1.11) activities, and extent of lipid peroxidation at three different growth stages: early growth stage (25 *d*), peak vegetative stage (40 *d*), and POD maturity stage (70 *d*).

Leaf H_2O_2 concentration was determined by the method, discussed by Zhang et al. (2015). Powered (in liquid N_2) leaf samples (0.5 g) were homogenized with chilled (⁻4 °C) acetone (90% v/v) in a mortar and centrifuged (14,500 × g for 10 min) at ⁻4 °C. Supernatant (1 mL) was added with $TiCl_4$ (20% v/v in concentrated HCl) (Merck Millipore, India) solution, and concentrated liquid NH_3 (Merck Millipore, India) and left for 5 min for completion of reaction. The precipitate was washed repeatedly with precooled acetone (90% v/v), unless became colorless and then dissolved in 2 M H_2SO_4. Concentration of H_2O_2 (μmol g^{-1} FW) was determined with spectrophotometer at 410 nm.

Sampled leaves (0.25 g) were powdered in liquid-N_2 to arrest proteolytic activity, added in 0.05 M phosphate buffer (pH 7) with polyvinyl pyrrolidone (1% v/v) (Merck Millipore, India), grinded in a pre-chilled mortar, and centrifuged (⁻4 °C) at 15,000 × g for 10 min, and the supernatant was mixed with 50 µM phosphate buffer (pH 7.8), 10^{-4} M EDTA, 0.013 M methionine (Merck Millipore, India), 75 µM nitro blue tetrazolium chloride (NBT; Merck, India), and 2 µM riboflavin (Merck Sigma-Aldrich, India). The mixture was shaken vigorously and placed under fluorescent lamp (15 W) for 10 min. After switching off the lamp, the absorbance of reaction mixture was taken at 560 nm against a control, and the SOD activity was expressed in U g^{-1} FW (Dhindsa et al. 1981).

Activity of APX was assessed by Kausar et al. (2012) method. The grinding powdered (with liquid N_2) soybean leaves (0.25 g) were done with 0.025 M K-phosphate (pH 7.9), containing 4×10^{-4} M EDTA, 0.001 M ascorbic acid (Merck Emsure® ACS, ISO, Reag. Ph Eur, India), and polyvinyl pyrrolidone (2% v/v). The mixture was centrifuged for 20 min at 15,000 × g (⁻4 °C). The supernatant was added with 0.025 M K-phosphate (pH 7), 2.5×10^{-4} M ascorbic acid, 4×10^{-4} M EDTA, and 10^{-4} M H_2O_2, and APX activity (U g^{-1} FW) was measured by decrease in absorbance at 290 nm.

Lipid peroxidation was determined using 2-thiobarbituric acid (Merck Sigma-Aldrich, India) in acidic medium (Heath and Packer 1968). Leaf samples (0.3 g) were homogenized in 20% (v/v) trichloroacetic acid (Merck Emsure® ACS, ISO, Reag. Ph Eur, India). The mixture was centrifuged (4000 × g) for 20 min and the supernatant was added with 4% (v/v) butylated hydroxytoluene (Merck, India), containing thiobarbituric acid (0.5% w/v), and trichloroacetic acid (20% v/v) in ethanol. The reaction mixture was then heated (95 °C) in water bath for half an hour, cooled in refrigerator, and again centrifuged (12,000 × g) for 15 min. The pink color intensity of supernatant was determined (absorbance) in spectrophotometer at 532 nm wavelength (subtracting absorbance at 600 nm) and the peroxidation of lipid was expressed as µmol of thiobarbituric acid reactive substances (*TBARS*) formed per gram of fresh weight (FW).

2.6 Physiological Nutrient Use Efficiency

Plant (root + stem + leaf) and seed samples were digested in digester (Jackson 1973). The N-content in the digest was measured in the Kjeldahl apparatus (Kjeldhal Automatic Nitrogen Distillation System, KEL PLUS, Pelican, India) using boric acid mixed indicator, followed by titration against 0.02 N H_2SO_4. Using the yellow color method, P-content was measured in a spectrophotometer (at 420 nm), while the K content was determined flame photometer (µController Based Flame Photometer Type-128, Systronics India Ltd., India). The obtained values were converted to physiological use efficiencies (*PUE*) of N, P, and K using the following equation as given by Fageria and Baligar (2005):

$$PUE\left(\mathrm{kgkg}^{-1}\right) = \frac{(BY_{Fert} - BY_{Cnt})}{(NU_{Fert} - NU_{Cnt})}$$

where $(BY_{Fert} - BY_{Cnt})$ is the differences in root + stem + leaf + grain yield (kg) between fertilized pot and control and $(NU_{Fert} - NU_{Cnt})$ is the differences in nutrient accumulation in root + stem + leaf + grain (kg) between fertilized treatment and control one. The *PUE* compares the biological yield differences between treatment plants and control per unit nutrient application and implied the extent of nutrient use efficiency under graded soil NPK.

2.7 Root Colonization Assay

Soybean root colonization by *T. viride* (BHU-2953) was studied at 21 *d* (during lignification study) and 70 *d* using a modified method of Zhang et al. (2015). Surface sterilized (with 10% NaOCl) root cut pieces (1 g) were crushed with 0.05 g L^{-1} streptomycin sulfate (Hi-Media® Laboratories Pvt. Ltd., India) and 9 mL agar suspension (0.05% w/v in water). The content was diluted 10^{-4} times using serial dilution technique, plated on Trichoderma specific medium (*TSM*), and incubated (27 °C) for 5 days. The fungal densities were counted as colony-forming units (CFU) formed onto TSM, multiplied with respective dilution factors per gram of roots.

2.8 Plant Physiological Attributes and Yield

Plant heights were recorded at 25 *d*, 40 *d*, and 70 *d*. Tennant (1975) method was used for root length (in 1 cm × 1 cm grid size) measurement at three different growth stages with the following formula: root length (cm) = (11/14) × no. of root interceptions with grid × 0.7857. Leaf area (cm^2) was measured with a portable leaf area meter (LI-3000C, LI-COR Biosciences, Elron Instrument Company Pvt. Ltd., India). An average of randomly selected ten leaves per pot (fully expanded leaves from top to bottom) was measured for estimating leaf area. Arnon (1949) method was used for chlorophyll (*chl.*) determination in leaves. Color intensity was measured at 663 nm and 645 nm for *chl. a* and *chl. b*, respectively. Relative leaf water contents were also estimated taking composite leaf samples (1 g) from different plant heights as explained by Smart and Bingham (1974). Relative leaf water (*RLW*) percentage was calculated with the following formula: *RLW* (%) = {weight of (fresh leaf − dry leaf)}/{weight of (turgid leaf − dry leaf)} × 100. Yield values and plant dry matter were obtained at 70 *d*.

2.9 Statistical Analysis

Comparison between treatment means was performed using Duncan's multiple range test (DMRT) at $P < 0.05$. Test of significance as well figures, drawn, were carried out using analysis of variance (one-way ANOVA; $P < 0.05$) in MS Excel (version 2010). Principal component analysis (*PCA*; $P < 0.05$) was performed in the R software (version 4.0.3) to reduce the dimensions (*Dim*) during the comparison of multiple treatments.

3 Results

3.1 Effect on Stem Lignification

Phloroglucinol under acidic medium stained the vascular lignin deposits around xylem as reddish-pink, clearly visible under the light microscope (Fig. 1). Peculiar rectangular-type lignification patterns were clearly visible in bio-primed treatments (Fig. 1c, 1d, 1e, 1f), while circular lignification patterns were observed in untreated soybean stems (Fig. 1a, 1b) and it is clearly visible that lignification around the xylem was wider in bio-primed soybeans than untreated ones. The acetyl bromide method of lignin content estimation had shown similar results as in the case of histochemical staining. Comparatively higher lignin contents were found

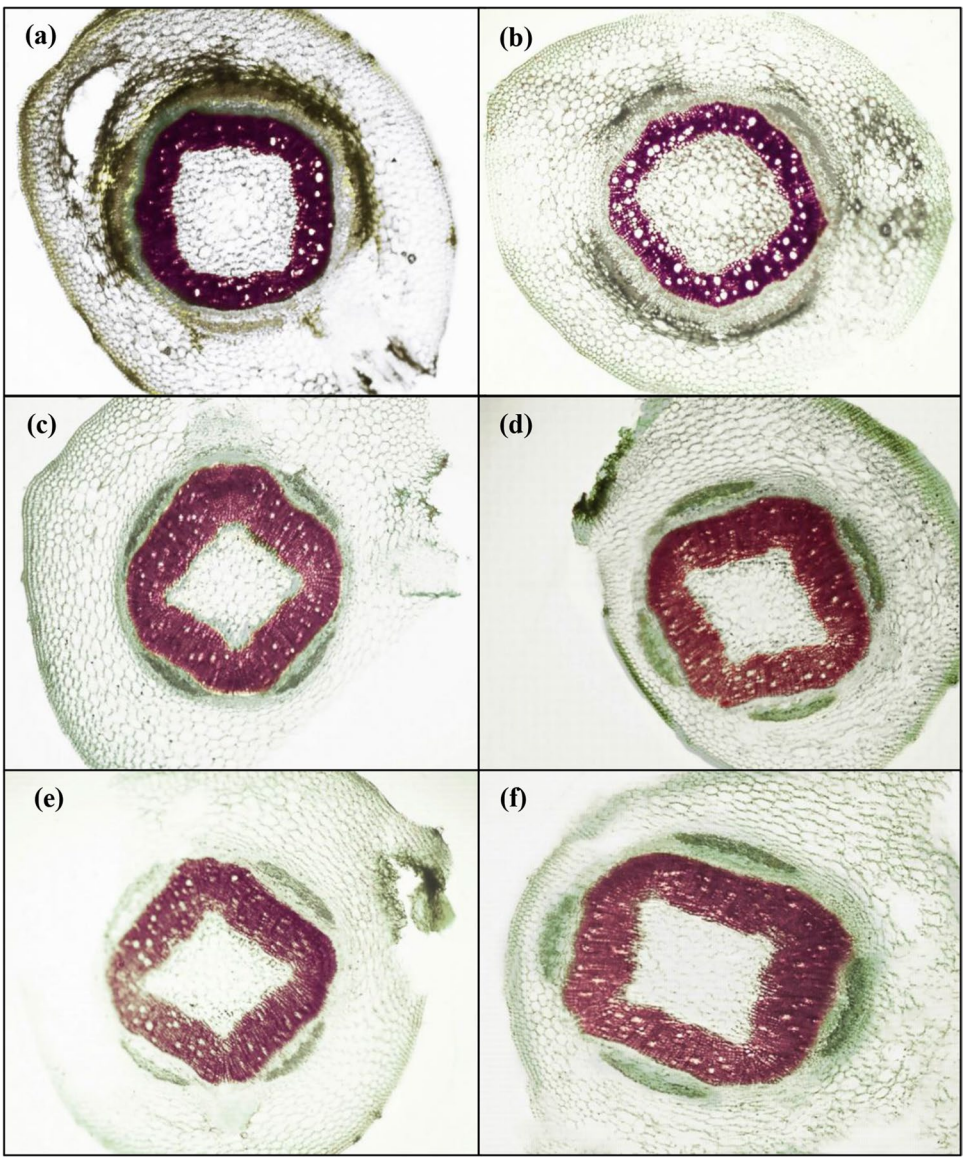

Fig. 1 Vascular lignification of **a** control (*C*), **b** recommended doses of fertilizers without seed bio-priming (*RDF*), **c** 90% *RDF* + bio-priming (*T90*), **d** 80% *RDF* + bio-priming (*T80*), **e** 75% *RDF* + bio-priming (*T75*), and **f** 70% *RDF* + bio-priming (*T70*) at 21 *d*. *d*, days after sowing

in the base stems of bio-primed soybeans, while significantly lower lignin contents ($P < 0.05$) were observed in *C* and *RDF* (Fig. 2a). The highest lignin content was recorded from *T70* and the lowest was found in *RDF*. Bio-priming soybean seeds with *T. viride* had increased the stem lignin content by 90.35% in *T70*, 84.35% in *T75*, 81.53% in *T90*, and 78.37% in *T80* in comparison to *RDF*. However, control had 9.03% more stem lignin content over *RDF* as assessed by acetyl bromide method.

Activities of CAD in bio-primed soybeans were found higher than untreated ones (Fig. 2b). The lowest CAD activity was recorded from *RDF*, while the highest was found in *T70* (1.76 times higher than *RDF*). From Fig. 2c, distinguishable activities of POD were obtained higher in bio-primed treatments (*T90*, *T80*, *T75*, and *T70*), compared to *C* and *RDF*. Significantly higher POD activities ($P < 0.05$) were found in *T70* (2.88-fold higher than *RDF*) and *T75* (2.82-fold higher than *RDF*), whereas *T90* was found 2.66-fold, and *T80* 2.6-fold higher POD activities than *RDF* (Fig. 2c). Decrement in lignifying enzyme activities followed similar trend in relation to lignin content around vascular tissues: *T70* > *T75* > *T90* > *T80* > *C* > *RDF*.

3.2 *Effect on Antioxidant Enzyme Activities, H_2O_2 Content, and Lipid Peroxidation of Leaves*

During each sampling over the progression of soybean growth stages, we had found that the concentration of H_2O_2 had been increased in leaf tissues (Fig. 3a). At 25 *d*, no significant differences in H_2O_2 levels were observed between all treatments, but with the advancing plant growth stages, controls were recorded with the highest leaf H_2O_2 contents than other treatments. The lowest levels of H_2O_2 were recorded from *RDF* (40 *d*) and *T90* (70 *d*). Among bio-primed treatments, highest H_2O_2 contents (40 *d*) were associated with leaves of *T70* followed by *T80*, *T75*, and *T90*. However, significantly no differences in H_2O_2 levels were obtained among bio-primed treatments and *RDF* on 70 *d*.

The SOD activity of *T70* was recorded highest on 40 *d* (Fig. 3b). No significant difference was found among *T80*, *T90*, and *T75*. Similarly, no significant differences were observed among bio-primed treatments at 25 *d*. During this period, leaves of bio-primed treatments were recorded to have nearly 1.41 times higher SOD activities than *RDF*. At 40 *d*, on an average 1.39-fold higher SOD activities were found in bio-primed soybean leaves compared to *RDF*. Significant differences ($P < 0.05$) in leaf SOD activities existed among *T90*, *T75*, and *T70* at 40 *d* and between *T90* and *T75* at 70 *d*. Among non-primed treatments, comparatively higher activities of SOD were recorded from *C* on 25 *d* (1.17 times higher than *RDF*) and 40 *d* (1.24 times higher than *RDF*), but at 70 *d*, *RDF* was seen to show higher SOD activity (1.16-fold high) than control. Unlike leaf peroxide contents, SOD activities were found to increase up to 40 *d* but decreased at 70 *d*.

We found non-significant leaf APX activities among bio-primed treatments during all three stages (Fig. 3c). Significant activities ($P < 0.05$) existed between non-primed soybean leaves at 25 *d* and 40 *d*. In the first two stages of sampling, APX activities were recorded higher in bio-primed than non-primed soybeans, but at 70 *d*, *RDF* was found statistically at par with *T75*. Similar to SOD, leaf APX activities were found to increase up to 40 *d* and decreased thereafter (70 *d*).

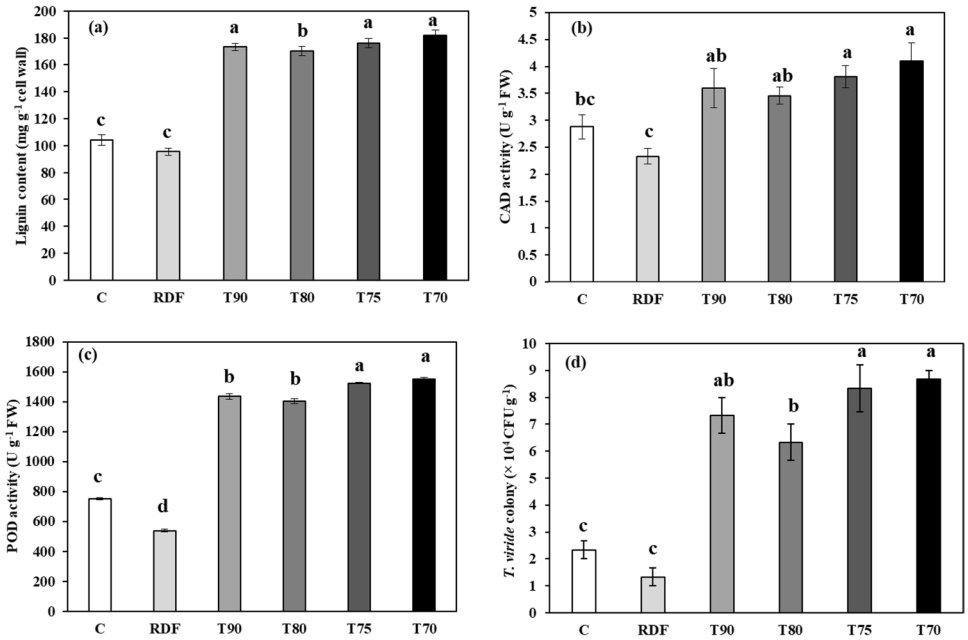

Fig. 2 Effect of *T. viride* bio-priming on **a** lignin contents, **b** CAD activities, **c** POD activities in stem, and **d** colony formation of *T. viride* in roots in soybean under graded fertilizer doses at 21 *d*. *C*, control; *RDF*, recommended doses of fertilizers without seed bio-priming; *T90*, 90% *RDF* + bio-priming; *T80*, 80% *RDF* + bio-priming; *T75*, 75% *RDF* + bio-priming; *T70*, 70% *RDF* + bio-priming. Small alphabets over bars (mean ± SE) indicate significant differences ($P < 0.05$) between treatment means as per Duncan's multiple range test. CAD, cinnamyl alcohol dehydrogenase; CFU, colony-forming unit; FW, fresh weight; POD, guaiacol peroxidase; SE, standard error

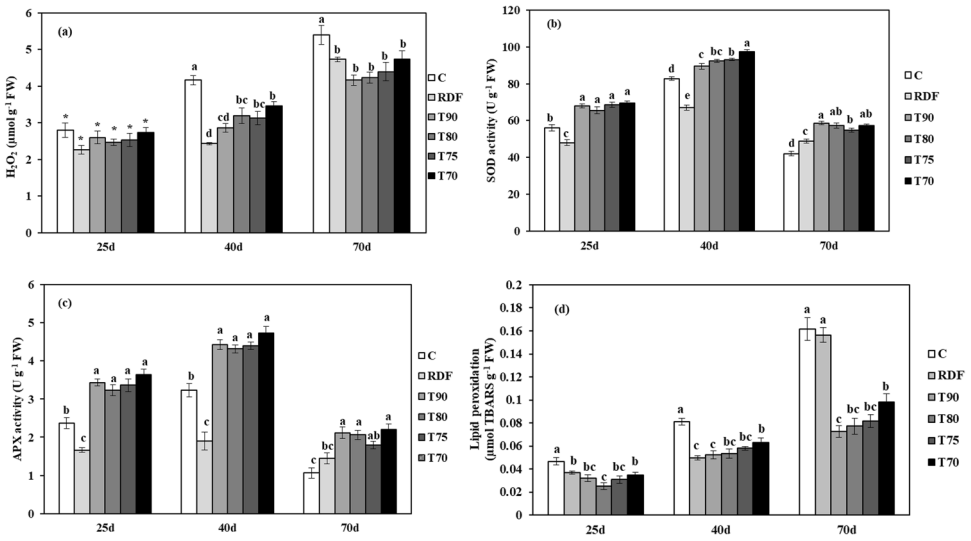

Fig. 3 Effect of *T. viride* bio-priming on **a** leaf H_2O_2 content, **b** SOD activities, **c** APX activities, and **d** lipid peroxidation (*TBARS* values) in soybean leaves under graded fertilizer doses at 25 *d*, 40 *d*, and 70 *d*. *C*, control; *RDF*, recommended doses of fertilizers without seed bio-priming; *T90*, 90% *RDF* + bio-priming; *T80*, 80% *RDF* + bio-priming; *T75*, 75% *RDF* + bio-priming; *T70*, 70% *RDF* + bio-priming. Small alphabets over bars (mean ± SE) indicate significant differences ($P < 0.05$) between treatment means as per Duncan's multiple range test. Asterisks (*) on bars at particular *d* indicate non-significant values ($P < 0.05$) between treatment means. APX, ascorbate peroxidase; *d*, days after sowing; FW, fresh weight; SE, standard error; SOD, superoxide dismutase; *TBARS*, thiobarbituric acid reactive substances

The highest *TBARS* values were found in *C* during all stages and *RDF* at 70 *d* (Fig. 3d). Lowest values were obtained from *T80* (25 *d*), *RDF* (40 *d*), *T90* (40 *d*), and *T90* (70 *d*) which were nearly 46%, 38.6%, 35.3%, and 55.05% lower than control during their respective growth periods. The *TBARS* values in *RDF* were found at par with bio-primed soybeans at 25 *d* and 40 *d*, but during 70 *d*, the values were found significantly higher than bio-primed soybeans. The *TBARS* values were found to increase with time and reached a maximum during 70 *d*. Significance of bio-priming can be observed at 70 *d* when comparatively lower *TBARS* values were recorded as compared to *C* and *RDF*.

3.3 Effect on Nutrient Use Efficiency

Data on *PUE* of N, P, and K are presented in Table 3. The effects of priming were very distinct in the case of N and P use efficiencies which were found to be significantly higher ($P < 0.05$) in bio-primed treatments as compared to *RDF*. However, in the case of K use efficiency, *RDF* was found to be statistically at par with *T90* and *T80* which were comparatively higher than *T75* and *T70*. Average values of total N, P, and K contents are presented in supplementary materials (Online Resource 1; Table S8).

3.4 Effect on Root Colonization

Soybean root colonization by *T. viride* is presented in Fig. 2d (21 *d*) and Table 3 (70 *d*). From both data, it was clearly observed that comparatively higher root colonies were established in the roots of bio-primed soybeans. Numbers of colonies in bio-primed roots were found nearly 5.75-fold and 4.18-fold higher than *RDF* at 21 *d* and 70 *d*, respectively, while these values were nearly 3.29-fold and 4.5-fold higher than *C*. Fungal colonies of *Trichoderma* spp. in the roots of non-primed soybeans were assumed to be native in origin, as we did not sterilize the soils.

3.5 Plant Physiological Attributes and Yield

Chlorophyll contents of leaves are presented in Table 2. Over the progression of soybean age, leaves of *RDF*, *T90*, and *T80* were found to have the higher *chl. a* content. In case of *chl. b*, *RDF*, *T90*, and *T80* were found with significantly higher values than other treatments. At 40 *d* and 70 *d*, *T75* also showed statistically higher values. In both cases, leaf chlorophyll contents were found to decrease over progressive soybean age. Relatively higher leaf areas were recorded ($P < 0.05$) in *T90* and *T80* during all growth stages along with *RDF* at 25 *d* (Table 2). Control was found to bear the lowest leaf area per plant. At 25 *d*, *T90* was found with a 15.6% higher leaf area than *C*. However, the leaf area of bio-primed treatments became > 60% (except *T70*) and > 100% higher than *C* at 40 *d* and 70 *d*, respectively. Length of roots was found very prominent in our study (Table 2). Bio-primed soybeans were found with significantly higher ($P < 0.05$) root lengths as compared to *C* and *RDF*. Treatment *T90* was found superior to other bio-primed treatments.

Table 2 Root length, plant height, leaf area, chlorophyll contents, and relative leaf water of different treatments at different soybean growth stages (25 *d*, 40 *d*, and 70 *d*) (replication = 3, mean ± SE)

Treatments	Root length (cm)	Plant height (cm)	Leaf area (cm^2 $plant^{-1}$)	*chl. a* (mg g^{-1})	*chl. b* (mg g^{-1})	*RLW* (%)
25 d						
C	63.3 ± 1.3^{e}	29.3 ± 1.76^{c}	72.9 ± 1.77^{c}	1.5 ± 0.06^{c}	0.66 ± 0.01^{c}	$66.1 \pm 2.03^{*}$
RDF	74.1 ± 1.1^{d}	45 ± 2.08^{ab}	83.5 ± 1.86^{ab}	2.2 ± 0.11^{a}	0.84 ± 0.01^{a}	$66.3 \pm 2.19^{*}$
T90	102.5 ± 1.71^{a}	49.3 ± 2.03^{a}	84.3 ± 2.71^{a}	2.1 ± 0.11^{a}	0.82 ± 0.03^{ab}	$67.7 \pm 1.76^{*}$
T80	90.4 ± 2.25^{b}	40.7 ± 1.45^{b}	80.1 ± 1.28^{ab}	1.9 ± 0.17^{ab}	0.8 ± 0.02^{ab}	$67.3 \pm 2.85^{*}$
T75	76.1 ± 0.82^{d}	43.3 ± 1.86^{b}	78.1 ± 2.11^{bc}	1.8 ± 0.08^{bc}	0.78 ± 0.02^{b}	$66.7 \pm 2.96^{*}$
T70	83.6 ± 1.41^{c}	30 ± 1.16^{c}	72.3 ± 1.26^{c}	1.8 ± 0.04^{bc}	0.77 ± 0.02^{b}	$66.3 \pm 1.76^{*}$
40 d						
C	70 ± 1.26^{e}	33.5 ± 2.02^{c}	136 ± 4.63^{d}	1.5 ± 0.06^{c}	0.61 ± 0.02^{c}	$60.8 \pm 1.33^{*}$
RDF	82.2 ± 1.58^{d}	49.2 ± 2.32^{b}	198.5 ± 8.23^{c}	2.1 ± 0.11^{a}	0.82 ± 0.01^{a}	$65.4 \pm 2.11^{*}$
T90	112.2 ± 2.13^{a}	55.3 ± 1.17^{a}	252 ± 6.15^{a}	2 ± 0.21^{ab}	0.81 ± 0.03^{a}	$68.3 \pm 0.98^{*}$
T80	99.4 ± 2.46^{b}	46.2 ± 1.48^{b}	246.4 ± 11.4^{ab}	1.7 ± 0.16^{abc}	0.78 ± 0.01^{ab}	$65.1 \pm 2.26^{*}$
T75	81.4 ± 1.62^{d}	46.3 ± 2.09^{b}	219.1 ± 12.21^{bc}	1.6 ± 0.08^{bc}	0.77 ± 0.02^{ab}	$65.8 \pm 1.74^{*}$
T70	89.5 ± 1.51^{c}	37.3 ± 1.45^{c}	195.2 ± 12.81^{c}	1.6 ± 0.12^{bc}	0.74 ± 0.02^{b}	$65.6 \pm 3.71^{*}$
70 d						
C	82 ± 0.77^{e}	42.3 ± 2.33^{c}	71.7 ± 9.6^{c}	0.5 ± 0.03^{c}	0.24 ± 0.01^{c}	48 ± 0.58^{b}
RDF	101.4 ± 0.91^{d}	53.7 ± 1.76^{abc}	128.7 ± 9.9^{b}	0.7 ± 0.07^{a}	0.39 ± 0.02^{a}	60.7 ± 1.65^{a}
T90	139.3 ± 1.68^{a}	64.3 ± 4.98^{a}	179.9 ± 9.27^{a}	0.6 ± 0.05^{ab}	0.38 ± 0.02^{a}	60.8 ± 1.62^{a}
T80	121.3 ± 1.43^{b}	57.3 ± 6.12^{ab}	153.3 ± 5.11^{ab}	0.6 ± 0.03^{ab}	0.37 ± 0.01^{a}	58.9 ± 2.25^{a}
T75	98.8 ± 2.02^{d}	53.3 ± 2.91^{abc}	147.4 ± 7.52^{b}	0.58 ± 0.02^{bc}	0.35 ± 0.02^{a}	59 ± 2.08^{a}
T70	109.5 ± 2.15^{c}	52 ± 2.89^{bc}	147.1 ± 11.9^{b}	0.53 ± 0.03^{bc}	0.29 ± 0.02^{b}	59.6 ± 2.9^{a}

Different small letters in each column at different soybean growth stages indicate significant differences among treatment means using Duncan's multiple range test at $P < 0.05$ level. Asterisk (*) denotes non-significant values at $P < 0.05$

C control; *RDF* recommended doses of fertilizers without seed bio-priming; *T90* 90% *RDF* + bio-priming; *T80* 80% *RDF* + bio-priming; *T75* 75% *RDF* + bio-priming; *T70* 70% *RDF* + bio-priming; *chl.* chlorophyll; *d* days after sowing; *RLW* relative leaf water; *SE* standard error

Table 3 Root colonization by *T. viride*, plant dry matter, seed yield, and physiological use efficiencies of nitrogen, phosphorus, and potassium of soybean at 70 *d* (replication = 3, mean ± SE)

Treatments	*T. viride* colony (CFU g^{-1}) × 10^4	Plant dry matter (g pot^{-1})	Seed yield (g pot^{-1})	*PUE* (kg kg^{-1})		
				N	P	K
C	4.3 ± 0.88^{c}	51.8 ± 3.03^{d}	14.9 ± 0.3^{b}	-	-	-
RDF	4.7 ± 1.45^{c}	75.1 ± 3.93^{b}	17.3 ± 0.13^{a}	32.4 ± 1.6^{b}	253.8 ± 3.58^{d}	41.1 ± 1.07^{a}
T90	18.3 ± 1.76^{ab}	88.2 ± 2.27^{a}	18 ± 0.36^{a}	37.9 ± 1.21^{a}	301.8 ± 5.34^{a}	43.3 ± 1.97^{a}
T80	14.7 ± 1.33^{b}	77.3 ± 3.32^{b}	17.3 ± 0.55^{a}	37.9 ± 0.92^{a}	296.2 ± 4.98^{ab}	37.9 ± 1.74^{ab}
T75	21.7 ± 2.4^{a}	73.7 ± 2.34^{bc}	16.6 ± 0.63^{a}	36.8 ± 0.68^{a}	282.5 ± 3.96^{bc}	35.3 ± 2.23^{b}
T70	23.3 ± 2.19^{a}	64.6 ± 2.46^{c}	16.6 ± 0.63^{a}	35.7 ± 1.23^{a}	277.9 ± 5.35^{c}	29.1 ± 2.04^{c}

Different small letters in each column at different soybean growth stages indicate significant differences among treatment means using Duncan's multiple range test at $P < 0.05$ level

C control; *RDF* recommended doses of fertilizers without seed bio-priming; *T90* 90% *RDF* + bio-priming; *T80* 80% *RDF* + bio-priming; *T75* 75% *RDF* + bio-priming; *T70* 70% *RDF* + bio-priming; *CFU* colony-forming unit; *d* days after sowing; *K* potassium; *N* nitrogen; *P* phosphorus; *PUE* physiological use efficiency; *SE* standard error

Control was found with lowest root length followed by *RDF*. Plant heights from the soil are presented in Table 2. There was no significant difference between *RDF* and bio-primed treatments with exceptions *T70* at 25 *d* and *T90* at 40 *d*. The highest dry matter was produced by *T90* (Table 3). Dry matter produced by soybeans follow a trend of decrement: *T90* > *T80* > *RDF* > *T75* > *T70* > *C*. There were no significant differences in leaf relative water contents at 25 *d* and 40 *d* but lowest *RLW%* was recorded in *C* at 70 *d* (Table 2). The bio-primed treatments and *RDF* showed statistically similar seed yields, while control exhibited significantly lower ($P < 0.05$) values than others (Table 3).

3.6 Principal Component Analysis

The lignification-associated variables were reduced into four dimensions (Online Resource 1; Table S1). The first two dimensions (*Dim 1* and *Dim 2*) accounted 99.679% of the total variance. All four variables i.e., lignin content, CAD activity, POD activity, and *T. viride* colonies in root have shown high-quality loadings and their contributions are almost similar in constructing *Dim 1* (Fig. 4a, Table S3). In constructing *Dim 2*, CAD activity has shown the highest contribution (61.992%); however, both loadings and treatment scores are found to vary more ($P < 0.05$) along the *Dim 1* (Figs. 4a and 5a, Tables S2 and S3). Near unity squared cosine values of these variables on *Dim 1* indicate high positive correlations among them. The highest score was found in *RDF*, followed by *C* at the negative side of *Dim 1* (Fig. 5a), while bio-primed treatments have shown high scores on the positive coordinate of *Dim 1* (Fig. 5a). Results infer that non-treated seed had strong negative influences on the lignification but opposite fact can be attributed to bio-primed soybeans.

PCA was performed on antioxidant enzyme activities, H_2O_2 content, and extent of lipid peroxidation (*TBARS*), parting growth stages into three groups. A total of eighteen treatments i.e., six treatments in three different growth stages (25 *d*, 40 *d*, and 70 *d*) were adjusted in four orthogonal dimensions, among which the first two dimensions (*Dim 1*, 2) together explained a total variation of 96.452% (Figs. 4b and 5b, Table S4). The variables were found to vary more significantly ($P < 0.05$) along with *Dim 1* (Fig. 4b). Higher loadings and squared cosine values (*Dim 1*) indicate that SOD and APX activities were highly correlated with each

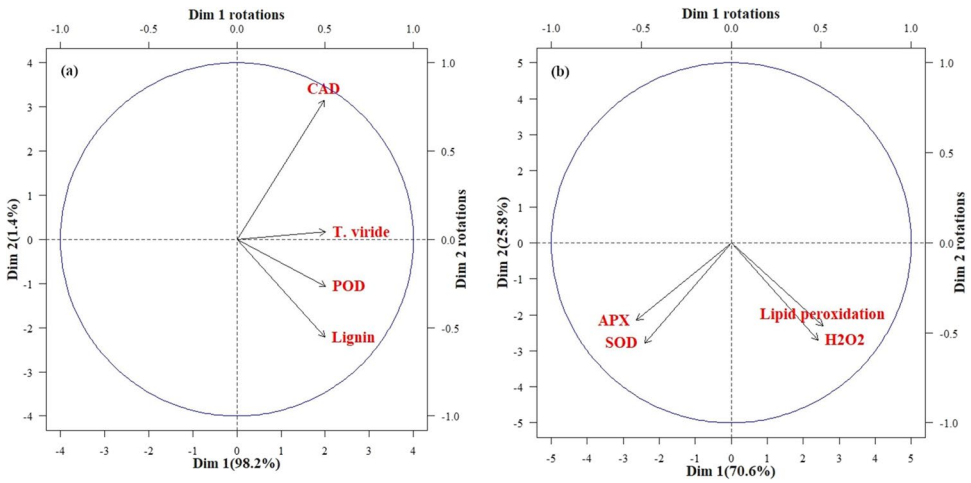

Fig. 4 Principal component loading plots of **a** stem lignification study (lignin content, CAD, POD, and *T. viride* colonies) and **b** growth stage-wise leaf H_2O_2 content, lipid peroxidation, and antioxidant enzyme (APX and SOD) activities. APX, ascorbate peroxidase; CAD, cinnamyl alcohol dehydrogenase; CFU, colony-forming unit; Dim, dimension; POD, guaiacol peroxidase; SOD, superoxide dismutase

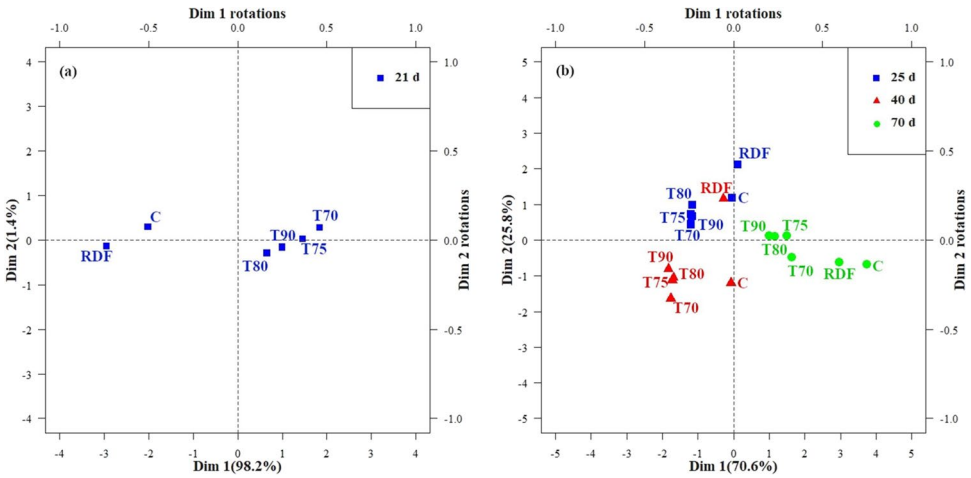

Fig. 5 Principal component score plots of **a** stem lignification study (lignin content, CAD, POD, and *T. viride* colonies), and **b** growth stage-wise leaf H_2O_2 content, lipid peroxidation, and antioxidant enzyme (APX and SOD) activities. *C*, control; *RDF*, recommended doses of fertilizers without seed bio-priming; *T90*, 90% *RDF* + bio-priming; *T80*, 80% *RDF* + bio-priming; *T75*, 75% *RDF* + bio-priming; *T70*, 70% *RDF* + bio-priming. APX, ascorbate peroxidase; CAD, cinnamyl alcohol dehydrogenase; CFU, colony-forming unit; *d*, days after sowing; Dim, dimension; POD, guaiacol peroxidase; SOD, superoxide dismutase

other at the negative side of the *Dim 1* (Fig. 4b, Table S6). On the positive side of *Dim 1*, H_2O_2 and lipid peroxidation (*TBARS*) were found to correlate positively. At 25 *d*, no treatments were found influencing the lipid peroxidation and H_2O_2 strongly (Fig. 4a, Tables S5 and S7). At 40 *d*, bio-primed treatments were found to be highly influencing the SOD and APX activities (Figs. 4b and 5b, Tables S5 and S7). However, on 70 *d*, all treatments were found in the positive coordinates of *Dim 1*, signifying more influence on H_2O_2 and lipid peroxidation (Figs. 4b and 5b, Tables S5 and S7). Effects of these variables at three different growth stages (groupings) have shown that changes in SOD and APX activities were strongly influenced by the bio-primed treatments at 40 *d* followed by 25 *d*, whereas *C* and *RDF* were found to influence lipid peroxidation and H_2O_2 contents at 70 *d*.

4 Discussion

Lignin is a complex, highly branched, water-insoluble organic compound, made up of phenylpropanoid units, and linked together by ether bonds. Microbial infections in roots were reported to assist the vascular lignification via activating the phenylpropanoid pathway (Singh et al. 2013a). At the last step of phenylpropanoid pathway, CAD converts cinnamaldehydes into cinnamyl alcohols which are subsequently oxidized by POD to facilitate lignin polymerization (Singh et al. 2011). Thus, activities of CAD and POD are taken as good indicators of site-specific vascular tissue lignification in response to microbial infection. In our study, inflated colony formations of *T. viride* BHU-2953 were responsible for the activation of phenylpropanoid pathway that has remarkably triggered the CAD and POD activities in the vascular bundles of bio-primed soybean. These in fact resulted in higher stem lignin deposition which is further confirmed by phloroglucinol staining (Fig. 1c, d, e, f, Fig. 2a). Comparatively lower nutrient dose in *T70* had led to nutrient starvation which was responsible for the highest lignification in this treatment. Lignin deposition can strengthen soybean stem against lodging as well as pathogen entry into host under the field conditions. As the soils were not sterilized, colonies of indigenous *Trichoderma* isolates were also recorded from the roots of untreated *C* and *RDF*. However, these values were not significant as compared to bio-primed treatments. This suggests enhancement of root colonization through *T. viride* seed priming. Several works have highlighted the effect of *Trichoderma* seed priming on enhanced stem lignification, activities of CAD, and POD that supports our findings (Singh et al. 2013a; Singh et al. 2013b; Gajera et al. 2016).

Under NPK limiting conditions, the balance between incoming solar radiation and its utilization within chloroplast gets hampered (Cakmak 2005; Ohyama 2010; Hernández and Munné-Bosch 2015). In order to dissipate this excess energy, reactive oxygen species (*ROS*) are generated (Muñoz and Munné-Bosch 2018). The SOD and APX are two important defense enzymes that guard plants against these *ROS*-mediated oxidative damages. Higher activity of leaf SOD was triggered by *T. viride* BHU-2953 in bio-primed soybeans. The SOD primarily converts *ROS* into H_2O_2. If not quenched simultaneously, H_2O_2 will oxidize the membrane lipids. This H_2O_2 is further reduced to water by induced APX activities within bio-primed leaves (Singh et al. 2011; Gajera et al. 2016). That is why minimum lipid peroxidation of leaves was recorded from bio-primed soybeans. However, contrasting leaf H_2O_2, SOD, APX, and lipid peroxidation between non-primed *C* and *RDF* was depended upon the external NPK fertilization. The higher NPK dose in *RDF* without bio-priming has maintained the optimum nutrient concentration within the plant body and their cell functionality, alleviating the nutrient stress, while completely opposite phenomena can be ascribed to *C*. During plant aging, senescence disrupts normal cell functions to an extent that plant cannot maintain its enzymatic activities and ion homeostasis (Dhindsa et al. 1981). This increases the H_2O_2 level. That is why at 70 *d*, low SOD and APX activities could not cope with the photo-oxidative damage of lipids. Numerous works have reported the effect of *Trichoderma* inoculation on enhancing plant defense enzymes that support our findings (Singh et al. 2011; Singh et al. 2013b; Zhang et al. 2015). Singh et al. (2013a) found higher leaf SOD activities in chickpea when seeds were treated with *Trichoderma* isolates. Higher SOD and APX activities in groundnut leaves were found by Gajera et al. (2016) who treated seeds with *T. viride*. Zhang et al. (2017) found elevated SOD activities in *Trichoderma*-treated soybean seeds that reduced the pathogenicity of *Fusarium* rot. These induced systemic defense responses can protect soybean from abiotic and biotic stresses, maintaining plant performance under adverse situations in the field condition.

Trichoderma was reported to produce auxin and α-1-amino cyclopropane-1-carboxylate deaminase within hosts, elongating their roots (Druzhinina et al. 2011; Zhang et al. 2017; Yadav et al. 2018). Higher root length increased the *PUE*s, especially for N and P in bio-primed plants as compared to the *RDF*. This suggests better soil nutrient acquisition in hosts upon *T. viride* inoculation. Previously, we have discussed that higher soil phosphatase activity and more root lengths enhanced P-acquisition in bio-primed soybeans (Paul and Rakshit 2021). Velmourougane et al. (2017) have discovered higher soil available N in the rhizosphere of chickpea along with longer roots when *T. viride* and *T. viride* + *Azotobacter chroococcum* were applied as bio-film. However, dose-dependent *PUE* of K was quite observable in all treatments irrespective

of bio-priming. This is because diffusion of soil K to plant roots is much faster as compared to N and P (Marschner 2012). The *PUE* not only gives about the idea of a plant's ability to accumulate soil nutrients but also tells about the biomass produced per unit of nutrient(s). Enhanced *PUE*s in turn increased the leaf areas of bio-primed soybean. More leaf area boost up the net photosynthesis which resulted in more plant dry matter and seed yield. Decreased chlorophyll content in *C* was resulted from low soil N application that enhanced the peroxide accumulation and loss of leaf pigments. Our results are also supported by Meena et al. (2016) who found higher root length, nitrogen use efficiency, plant height, chlorophyll content, and grain yield in wheat, bio-primed with *Trichoderma*. As ample watering was provided to plants, no difference in *RLW%* was observed in soybeans during 25 *d* and 40 *d* but control was found with low *RLW%* 70 *d*. Higher H_2O_2 content in control during leaf senescence enhanced the lipid peroxidation of the leaf membranes that destroyed the leaf water conductivity (Li-Ping et al. 2006). Absence of external K application might also have reduced the stomatal conductance of *C*, resulting in reduced seed yield. Higher use efficiencies of N, P, and K in response to *T. viride* bio-priming can withstand the starvation from low plant available nutrient status in soil.

5 Conclusions

To the best of our knowledge, this study was the first of its kind that has augmented different soybean physiochemical changes with *T. viride* BHU-2953 inoculation under graded macronutrients. Induced activities of cinnamyl alcohol dehydrogenase and guaiacol peroxidase in bio-primed soybeans have resulted in higher stem lignification than untreated soybeans. Leaf defense enzymes were also triggered by *T. viride* inoculation that has minimized the photo-oxidative damages of lipid membranes from hydrogen peroxide. However, the damages could not cope with the 70-day-old plants. The bio-primed soybeans were also found with increased root lengths that have explored more soil volume to increase the physiological use efficiency of nitrogen, phosphorus, and potassium. This increased nutrient use efficiency has resulted in more leaf areas, dry matter, and seed yields in bio-primed soybeans. These traits make the interactions between soybean and *T. viride* more convenient for field application.

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Declarations

Conflict of Interest The authors declare no competing interests.

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