Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-023-10929-w

Bio-priming with *Trichoderma* Enhanced Faster Reserve Mobilization in Germinating Soybean Cotyledons under Graded Macronutrients

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

Received: 4 August 2021 / Accepted: 22 January 2023
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2023

Abstract
The study highlighted the effect of seed bio-priming with *Trichoderma viride* upon degradation of stored foods, total phenol content, and uptake of nitrogen (N), phosphorus (P), and potassium (K) in cotyledons of germinating soybean (*Glycine max* var. JS 95-60) under graded NPK application. The germination was carried out in Petri dish and soil, separately. Observations were monitored for two days (day 3 and day 5). We estimated hydrolysis of starch, lipid, and protein by measuring the activities of α-amylase [EC 3.2.1.1], β-amylase [EC 3.2.1.2], lipase [EC 3.1.1.3], and leucine Aminopeptidase [EC 3.4.11.1], respectively. Total NPK in cotyledons were obtained by acid-digestion. The free radical scavenging activity and lipid peroxidation were performed using 2, 2-diphenyl-1-picrylhydrazyl and thiobarbituric acid reactive substances, respectively, to perceive anti-oxidant potential of phenol. We found strong influence of bio-priming on the activities of lipase, α-amylase, and β-amylase that degraded stored lipid and transient starch faster than non-primed seeds. Protein hydrolysis was moderately affected by induced leucine aminopeptidase activities and found significantly higher in bio-primed seeds on day 5. Elevated phenols ($P < 0.05$) in bio-primed cotyledons were able to diminish the lipid peroxidation with higher ($P < 0.05$) radical scavenging activities. During both days, higher K contents were found in bio-primed cotyledons but the concentrations of N and P were found significantly higher in bio-primed treatments only on day 5. The combined effect from all these has resulted in faster and vigorous germination of *Trichoderma viride* treated soybean seeds than non-primed ones.

Keywords α-amylase · β-amylase · Bio-priming · Leucine aminopeptidase · Lipase · *Trichoderma viride*

Introduction

Soybean (*Glycine max* [L.] Merr.) is one of the most sensitive oilseed crop, grown worldwide at a commercial scale. Germination performance of soybean is very poor among oilseed crops (Rathore et al. 1981). It performs epigeal germination in which cotyledon forces out of soil with the help of hypocotyl. Having high oil and protein contents, seeds of soybean is most susceptible to biotic and abiotic stresses at the time of germination (Brown and Huber 1988; Begum et al. 2010; Mastouri et al. 2010; Tančić et al. 2013). Productivity of this oilseed is solely depended upon management practices that can alleviate the disease severity and different environmental stresses. Previously, major focus has been given to biotic stress management using synthetic pesticides but chemical treatment also carries potential risks to cause ecosystem hazards (Schulz and Thelen 2008; Nettles et al. 2016). Hence, eco-friendly management practices should be highly encouraged for sustaining soybean productivity in long run. Seed priming is a process of hydrating seeds with different agents that boost up seed metabolic processes, influencing early germination (Begum et al. 2010; Ali and Elozeiri 2017; Sharma et al. 2020; Rajput et al. 2021). The genus *Trichoderma* is a very prominent seed priming agent, inducing plant systemic defence, metabolite production, enzyme modulation, and nutrient acquisition alongside the germination (Mastouri et al. 2010; Ali and Elozeiri 2017; Paul and Rakshit 2021; Rajput et al. 2021). Due to its promising bio-control efficacy, faster and more vigorous germination can be observed in seeds, inoculated with both pathogen and the *Trichoderma* spp. (Zheng and Shetty 2000; Mastouri et al. 2010; Singh et al. 2010; Jain et al. 2012; Tančić et al.

Handling Editor: Thomas Roitsch.

✉ Amitava Rakshit
amitavar@bhu.ac.in

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

2013; Rajput et al. 2021; Sreenayana et al. 2021). However, some treatments, inoculated with *Trichoderma* spp. but non-inoculated with disease causing agents, have also shown faster germination rate as compared to control. The exact mechanisms, imposed by *Trichoderma* during germination still require scientific explanations. Moreover, it is a well-established fact that food reserves in the cotyledons nourish seedling emergence during germination until onset of photosynthesis (Brown and Huber 1988; Gonzalez and Vodkin 2007). Being an oilseed leguminous crop, soybean cotyledons contain an appreciable amount of lipids and proteins along with sugars (Brown and Huber 1988). As different species of *Trichoderma* were evidenced to secrete number of hydrolytic enzymes, responsible for degradation of starch, protein, and lipids, there is a possibility that *Trichoderma* seed priming can enhance the storage mobilization within germinating soybean cotyledons (Kashmiri et al. 2006; Mahmood and Rahman 2008; Coppola et al. 2019; Sharma et al. 2020). Activities of amylase, lipase, and aminopeptidase are of great concern among these hydrolytic enzymes. Study needs to be carried out based on seed-inoculation of *Trichoderma* and its effect on hydrolytic mobilization of cotyledonary reserve during soybean germination.

Cells generate a good amount of free radicals at the time of seedling emergence (Singh et al. 2010). The oxidative damage, caused by these radicals, can totally stumble the seed germination process through disrupting the functions of membrane-bound organelles (Ali and Elozeiri 2017). Level of these free radicals is minimized by activities of different anti-oxidants (Xu and Chang 2007). Phenols and phenolic compounds are important anti-oxidant compounds that possess free radical scavenging activities (Xu and Chang 2007; Sharma et al. 2019; Tanase et al. 2019). *Trichoderma* has also been reported to enhance phenol contents, reducing the peroxide injuries inside different plant parts (Zheng and Shetty 2000; Begum et al. 2010; Singh et al. 2010; Jain et al. 2012; Rajput et al. 2021). Still, radicle scavenging activities of *Trichoderma* and its effect on membrane stability during seed germination remain unexplored. There is a chance that priming with *Trichoderma* might be able to suppress the oxidative damage of soybean during germination.

During these metabolic changes, cotyledons exhaust mineral nutrients, especially nitrogen (N), phosphorus (P), and potassium (K) to a great extent (Hanway and Weber 1971; Gonzalez and Vodkin 2007; Marschner 2012). Supply of these three macro-nutrients is essential during any seed germination process. Nitrogen is assimilated by soybean for synthesizing new amino acids, phenolic compounds, and development of embryonic axis; while P are required for generation of ATP (adenosine triphosphate), glyoxylate cycle, and biosynthesis of phenols via phenylpropanoid pathway (Hanway and Weber 1971; Marschner 2012). Similarly, K activates the enzymes responsible for starch biosynthesis, membrane stability, and cell osmoregulation (Hanway and Weber 1971; Gonzalez and Vodkin 2007; Marschner 2012). Several reports are there regarding the necessity of mineral nutrition during seed germination, yet impact of nutrient exhaustion during this process has been ignored over time (Hanway and Weber 1971; Singh et al. 2010; Marschner 2012; Ali and Elozeiri 2017; Rajput et al. 2021; Sreenayana et al. 2021). Since intake of mineral elements through seed coat is an obtuse process, most experiments have withdrawn the addition of nutrient solution in Petri dish studies (Chrispeels and Boulter 1975; Adams et al. 1980, 1981; Hildebrand and Hymowitz 1981; Rathore et al. 1981; Brown and Huber 1988; Singh et al. 2010; Rajput et al. 2021). To simulate natural soil emergence in the laboratory, inclusion of nutrient should be performed in further germination studies. Researchers have revealed that *Trichoderma* can escalate nutrient acquisition at different stages of crop growth (Mastouri et al. 2010; Ali and Elozeiri 2017; Paul and Rakshit 2021). Hence, introducing *Trichoderma* might be helpful to boost up nutrient intake inside seeds at the time of germination. Considering these multifunctional traits of *Trichoderma*, we hypothesized that seed priming with *Trichoderma* will ensure early germination and higher uptake of applied nutrients without incurring severe oxidative damages within soybean seed. Thus, aims of the present experiment were to investigate single inoculation of *Trichoderma viride* on (i) hydrolytic degradation of stored lipid, starch, and protein, (ii) total phenol content as well as its free radical scavenging activity, and (iii) uptake of N, P, and K inside the cotyledons to boost up the soybean germination.

Materials and Methods

Fungal Spore Suspension Preparation and Seed Priming

The *Trichoderma viride* BHU-2953 strain (Accession No. NAIMCC-F-02976) was collected from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University. It was first isolated by Prof. H. B. Singh and Prof. B. K. Sharma from the rhizosphere of chickpea (*Cicer arietinum*), grown in Varanasi, Uttar Pradesh, India. It has a dark green colony on potato dextrose agar (PDA) media with wavy edge, floccose to arachnoid mycelial form, sour coconut like smell, uncrowned phialides, and branched-flexuous conidiophores. Previously, Singh et al. (2014) have successfully controlled tomato wilt (*Fusarium oxysporum*) and damping-off of chilli (*Pythium aphanidermatum*), using *T. viride* BHU-2953 (2% W.P.) alongside crop growth promotion. In our previous studies, we also have obtained greater soil-P acquisition, higher vascular lignification in stem, and enhanced activities

of plant defense enzymes in soybean through inoculating the *T. viride* BHU-2953 strain under graded nutrient application (Paul and Rakshit 2021, 2022). However, no information is available on its effect upon seed germination. Thus, *T. viride* BHU-2953 has been selected in our experiment as a potent priming agent.

The fungus was incubated in PDA plates (Fig. 1) for five days and harvested with sterile saline water (0.85% NaCl). The spectrophotometric spore counts (2.08×10^7 spores mL^{-1}) were taken at 600 nm (optical density: 1.103). The suspension was diluted to 2.08×10^5 spore mL^{-1} by mixing 1.5% carboxy-methyl cellulose to the spore suspension (Jain et al. 2012). A total of 500 soybean (*Glycine max* var. JS 95-60) seeds were surface sterilized with 1% NaOCl solution (1 min), washed carefully with sterilized distilled water, and shed dried for next 120 min. The dried seeds were then dipped in the fungal spore suspension (500 mL) at two instalments (250 seeds in each case) for 120 min and after that incubated for 24 h with 98% humidity before starting of the experiments. The non-inoculated seeds were only soaked in carboxy-methyl cellulose suspension devoid of fungal spores.

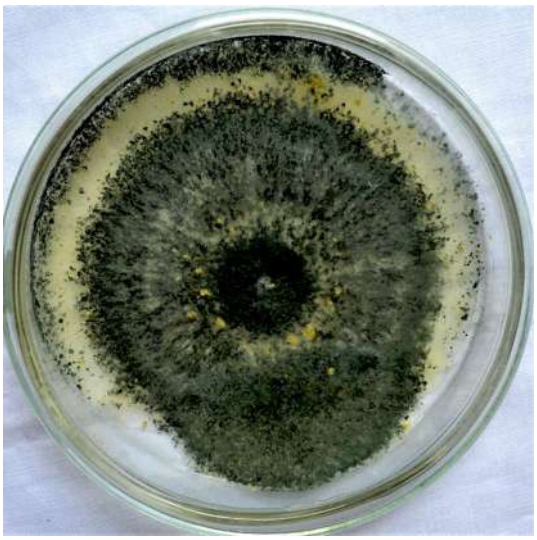


Fig. 1 The cultured *Trichoderma viride* BHU-2953

Germination

Seed germination was recorded on day 3 and 5. The treatment details are mentioned in Table 1. Urea is used for N-source; while, di-hydrogen orthophosphate and potassium sulfate is used for P- and K-source, respectively.

Study-I

This study was carried out in Petri dishes (19 cm diameter), taking 3 replications and 6 treatments. Twenty five (25) seeds in each plate were placed between triple-layered Whatman grade-1 (thickness: 180 µm) filter papers (ISTA 1985). Control (L0) is moistened with sterile distilled water. One time liquid NPK solutions (10 mL) were added to the rest of the treatments according to their treatment details (Table 1). Then, all the treatments were moistened with sterilized distilled water and incubated at 25 °C in dark. Observations were monitored on day 3 and day 5.

Study-II

Pot culture was performed in 3.5 kg sterile soils in 3 replications (15 seeds in each). Soil was collected from Research Farm, Banaras Hindu University (25° 16′ N, 82°

Table 1 Treatment details for study-I and study-II

Treatment names	Description
Study-I	
L0	No fertilization + no seed bio-priming (control)
L100	29 µg N mL^{-1} + 18 µg P and K mL^{-1} + no seed bio-priming
LT90	26 µg N mL^{-1} + 16 µg P and K mL^{-1} + seed priming with *T. viride*
LT80	23 µg N mL^{-1} + 14.4 µg P and K mL^{-1} + seed priming with *T. viride*
LT75	21.8 µg N mL^{-1} + 13.5 µg P and K mL^{-1} + seed priming with *T. viride*
LT70	20.3 µg N mL^{-1} + 12.6 µg P and K mL^{-1} + seed priming with *T. viride*
Study-II	
V0	No fertilization + no seed bio-priming (control)
V100	29 mg N kg^{-1} + 18 mg P and K kg^{-1} + no seed bio-priming
VT90	26 mg N kg^{-1} + 16 mg P and K kg^{-1} + seed priming with *T. viride*
VT80	23 mg N kg^{-1} + 14.4 mg P and K kg^{-1} + seed priming with *T. viride*
VT75	21.8 mg N kg^{-1} + 13.5 mg P and K kg^{-1} + seed priming with *T. viride*
VT70	20.3 mg N kg^{-1} + 12.6 mg P and K kg^{-1} + seed priming with *T. viride*

Table 2 Physiochemical parameters of initial soil sample

Initial parameters	Recorded values
Bulk density (g cm^{-3})	1.5
Texture	Sandy loam
pH	7.25
EC (dS m^{-1})	0.46
Organic carbon (%)	0.427
Available N (mg kg^{-1})	56.2
Available P (mg kg^{-1})	15.4
Available K (mg kg^{-1})	70.7

59′ E) and sterilized with chloroform fumigation. The soil physiochemical parameters were mentioned in the following Table 2. The NPK solutions were mixed with the soils in their respective treatments (Table 1). Germination percentages were recorded with the formula:

$$\text{Germination (\%)} = \frac{\text{(Number of seeds germinated or emerged)} \times 100}{\text{Total number of seeds}}$$

Starch, Protein, and Lipid Contents

Dried, powdered (passing through 1 mm sieve) cotyledons (0.1 g) of different treatments were placed in centrifuge tubes (10 mL) along with 80% ethanol (5 mL), heated (80 °C) on water bath for half an hour, centrifuged for 5 min at 2000 g (Refrigerated Universal Centrifuge T-60, Laby Instruments Industry, India), and supernatants were discarded. The ethanol extraction was repeated three times and residues were freed from ethanol through evaporation, followed by per-chloric acid ($HClO_4$) digestion, and colorimetric (620 nm) starch determination with anthrone (Merck Millipore, India) in acid medium (Kuai et al. 2014).

Dried powder of cotyledons (weighed previously) were added with methanol: chloroform in test tube at 1:2 ratio (v/v) and mixed thoroughly in shaker for 60 min at room temperature. The extract was centrifuged for 30 min (three times at 5000 g) at 25 °C. This removes the lipid from the pellets. Lipid content was calculated by measuring reduction in dry weight of the pellet before and after the extraction (Marques et al. 2013).

Remaining dried pellets (used in lipid measurement) were digested IR-digester (KES 20L RAL-TS, KEL PLUS, Pelican, India) with concentrated H_2SO_4 and catalyst mixture. Total nitrogen content in the digest was measured in Kjeldahl apparatus (DISTYL EM VA, Kjeldhal Automatic Nitrogen Distillation System, KEL PLUS, Pelican, India) (Jackson 1973). Total protein content was measured by multiplying the value with 6.25.

Amylolytic Enzyme Activities

Cotyledons (1 g) of germinated soybeans were homogenized with 10 mL sterilized distilled water (4 °C) in a pre-chilled mortar and centrifuged at 16,000 g for 30 min. The supernatants were collected for assessing amylase activities.

Heating (70 °C for 5 min) of supernatants (5 mL) with calcium chloride di-hydrate (0.003 M $CaCl_2$. $2H_2O$) was done to inactivate β-amylase. The α-amylase [EC 3.2.1.1] activity was measured then with Tárrago and Nicolás (1976) method. The hot supernatant was immediately added with 0.1 M citrate buffer (Merck Sigma-Aldrich, India) at pH 5 followed by 2% (w/v) soluble starch (Merck Millipore, India) and heated (30 °C) for 5 min. Di-nitro salicylic acid (Sigma® Life Science, India) were added after and again heated (50 °C) subsequently for 5 min for color development. After making the final volume (10 mL), α-amylase activity was measured (at 540 nm) in spectrophotometer (μController Based UV–Visible Spectrophotometer Type-117, Systronics India Ltd., India) as maltose released in the reaction.

Activity of β-amylase [EC 3.2.1.2] was assessed after inactivating α-amylase with 0.5 mL 0.1 M disodium salt of ethylene di-amine tetra-acetic acid (EDTA; Merck, India) (Tárrago and Nicolás 1976). The method was same as α-amylase activity measurement except that 0.1 M citrate buffer was maintained at lower pH (pH 3.4). Both α- and β-amylase activities were expressed in U g^{-1} cotyledon.

Leucine Aminopeptidase Assay

Fresh cotyledons were homogenized in 0.025 M K-phosphate (pH 7) with 0.002 M 2-mercaptoethanol (Merck Millipore, India). The homogenate was filtered, centrifuged for 20 min (12,000 g; 4 °C), and supernatant was added with 0.025 M K-phosphate buffer at pH 7 and 0.001 M 2-mercaptoethanol.

L-leucine-4-nitroanilide (0.025 g) (Thermo Fisher Scientific, India) was dissolved in dimethyl sulfoxide (Merck Sigma-Aldrich, India) and volume make-up was done with 0.1 M phosphate buffer at pH 7. Enzyme extract (0.1 mL) was incubated with 2 mL of the above solution at 40 °C (20 min). Then 1 mL of 1.5 N $HClO_4$ was added to stop the reaction. The absorbance of the turbid supernatant was taken at 410 nm for leucine aminopeptidase (LAP) [EC 3.4.11.1] activity (Chrispeels and Boulter 1975).

Lipase Assay

Enzyme was extracted by homogenizing cotyledons in chilled 0.1 M Tris–HCl buffer (Merck Millipore, India) (pH 7.6) with 0.001 M phenylmethylsulfonyl fluoride (Merck Sigma-Aldrich, India), 0.001 M EDTA, glycerol (20%;

v/v), 0.001 M $MgCl_2$, 0.002 M dithiothreitol (Merck Sigma-Aldrich, India), and 0.01 M KCl. Then the mixture was centrifuged for 20 min at 16,000 g (4 °C) and supernatant was used as enzyme extract.

Buffer for lipase [EC 3.1.1.3] activity was prepared by dissolving *p*-nitrophenyl palmitate (Merck Sigma-Aldrich, India) in 70% (v/v) in phosphate buffer (pH 7), containing isopropyl alcohol (Merck Sigma-Aldrich, India), sodium deoxycholate (Merck Sigma-Aldrich, India), and gum Arabic (Thermo Fisher Scientific, India). Enzyme extract (0.1 mL) was taken in 2.4 mL of this buffer-solution and the spectrophotometric (at 410 nm) lipase activity was determined by amount of *p*-nitrophenol, released from *p*-nitrophenyl palmitate hydrolysis (Gupta et al. 2002).

Free Radical Scavenging Assay

Cotyledons were homogenized with acetone, evaporated at 45 °C, cooled down to − 4 °C, and used crude extracts. Final volume was made up to 25 ml during analysis.

Free radical scavenging activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Merck Sigma-Aldrich, India). Cotyledon solution was mixed with 0.0001 M DPPH in ethanol and left in dark place for 20 min. The decrease in absorbance (from purple to yellow color) was measured at 517 nm against calibration curve, made by 6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox; ACROS Organics™, Thermo Fisher Scientific India Pvt. Ltd., India) and expressed it in µmol of Trolox equivalent (TE) per gram of cotyledon (Xu and Chang 2007).

Total Phenolic Assay

Cotyledons were grinded in pre-chilled mortar (− 4 °C) using acetone: water (50:50; v/v), centrifuged at 1200 g (− 4 °C; 10 min) and the supernatant was stored. The procedure was repeated to collect the remaining supernatants.

Total phenolic content was measured with the method of Xu and Chang (2007) by mixing Folin-ciocalteu reagent (Merck Sigma–Aldrich, India), Na_2CO_3 (7%; w/v), and double distilled water with cotyledon extract. The mixture was incubated for 10 min (25 °C), added with double distilled water. The absorbance (765 nm) was taken after 120 min with gallic acid as standard and a blank. Total phenol content was obtained from standard curve and expressed as mg of gallic acid equivalent (GAE) per gram of cotyledon.

Lipid Peroxidation Assay

According to the method of Heath and Packer (1968), peroxidation of lipid was measured with 2-thiobarbituric acid (Merck Sigma–Aldrich, India) medium. Cotyledons of 0.3 g were grinded in trichloroacetic acid (20%; v/v) (Merck Emsure® ACS, ISO, Reag. Ph Eur, India), centrifuged (4000 g) for 20 min, supernatant was separated, and added with butylated hydroxytoluene (4%; v/v) (Merck, India), 0.5% (w/v) thiobarbituric acid, and trichloroacetic acid, dissolved in ethanol. The solution was heated for 30 min (95 °C) in water bath, cooled to 4 °C, and centrifuged again at 10,500 g (15 min). Lipid peroxidation was calculated by µmol of thiobarbituric acid reactive substances (TBARS) present per g cotyledon, subtracting absorbance at 600 nm from 532 nm.

Total Nitrogen, Phosphorus, and Potassium Contents

Cotyledons are washed, dried at 65 °C in oven, powdered, and passed through 2 mm sieve. For N estimation, 0.5 g cotyledons were predigested overnight with concentrated H_2SO_4 and catalyst mixture (K_2SO_4: $CuSO_4$. $5H_2O$: Se powder). The mixture was then heated to 370 °C in IR-digester. The clear digest after filtration was distilled with 40% NaOH, and 4% H_3BO_3-mixed indicator in Kjeldahl apparatus. The solution was then titrated against 0.02 N H_2SO_4 until pink color appeared (Jackson 1973).

For P and K, 0.2 g powdered cotyledon was digested in di-acid mixture (HNO_3: $HClO_4$), filtered, and made up the volume to 50 mL. The P content in digest was measured by vanado-molybdate yellow color method at 420 nm; while K content was measured in flame photometer (µController Based Flame Photometer Type-128, Systronics India Ltd., India) (Jackson 1973).

Statistics

One way analysis of variance (ANOVA; $P < 0.05$) was performed to find out significant differences between treatment means in each study, separately at different sampling periods. The mean differences were compared with Duncan's multiple range test (DMRT) at 5% significance level. The R application (version 4.0.3) was used to analyse principle component (PC) analysis. Figures were drawn in MS Excel (version 10) and in R. Validity of study-I and study-II data were justified with Pearson's correlation test ($P < 0.05$) on day 3 and day 5 basis (Supplementary information; Table S6).

Results

Effect on Germination

Higher germination percentage, presented in Table 3, was associated with *T. viride* priming under graded NPK doses rather than full recommended dose. At day 3, more than two

Table 3 Changes in starch, protein, lipid, total phenol content, lipid peroxidation, DPPH radical scavenging activity, nitrogen, phosphorus, and potassium contents in cotyledon and germination percentage (mean ± SE)

Treatment	Germination (%)	Starch (mg g^{-1})	Protein (mg g^{-1})	Lipid (mg g^{-1})	Phenol content (mg of GAE g^{-1})	DPPH (µmol TE g^{-1})	Lipid peroxidation (µmol TBARS g^{-1})	N (%)	P (%)	K (%)
Day 3										
Study-I										
L0	28 ± 2.3^{d}	16.2 ± 0.2^{ab}	$26.3 \pm 0.7^{*}$	50 ± 1^{a}	0.9 ± 0.02^{b}	0.31 ± 0.02^{b}	1.26 ± 0.013^{a}	$2.8 \pm 0.1^{*}$	$0.32 \pm 0.012^{*}$	0.91 ± 0.02^{b}
L100	46.7 ± 1.3^{c}	16.6 ± 0.1^{a}	$24.7 \pm 0.4^{*}$	48.9 ± 0.6^{a}	0.9 ± 0.02^{b}	0.31 ± 0.002^{b}	1.21 ± 0.013^{b}	$2.94 \pm 0.09^{*}$	$0.32 \pm 0.012^{*}$	0.94 ± 0.01^{b}
LT90	72 ± 2.3^{a}	15.3 ± 0.2^{c}	$24.9 \pm 0.8^{*}$	39 ± 1.5^{b}	1.3 ± 0.03^{a}	0.62 ± 0.004^{a}	0.77 ± 0.012^{d}	$3.12 \pm 0.05^{*}$	$0.35 \pm 0.007^{*}$	1.42 ± 0.02^{a}
LT80	66.7 ± 1.33^{a}	15.5 ± 0.1^{c}	$25.7 \pm 0.9^{*}$	39.3 ± 0.7^{b}	1.24 ± 0.02^{a}	0.61 ± 0.013^{a}	0.77 ± 0.009^{d}	$3.05 \pm 0.1^{*}$	$0.33 \pm 0.009^{*}$	1.41 ± 0.01^{a}
LT75	66.7 ± 3.5^{a}	15.8 ± 0.1^{bc}	$24.3 \pm 1.2^{*}$	39.7 ± 0.7^{b}	1.24 ± 0.02^{a}	0.58 ± 0.007^{a}	0.83 ± 0.012^{c}	$3 \pm 0.12^{*}$	$0.33 \pm 0.007^{*}$	1.41 ± 0.02^{a}
LT70	57.3 ± 2.7^{b}	15.97 ± 0.2^{b}	$25 \pm 0.7^{*}$	40 ± 1.1^{b}	1.2 ± 0.02^{a}	0.58 ± 0.033^{a}	0.83 ± 0.01^{c}	$3 \pm 0.12^{*}$	$0.33 \pm 0.006^{*}$	1.41 ± 0.01^{a}
Study-II										
V0	0^{b}	16.3 ± 0.2^{a}	$26 \pm 1.5^{*}$	51.7 ± 1.3^{a}	0.9 ± 0.02^{b}	0.33 ± 0.01^{b}	1.3 ± 0.01^{a}	$2.94 \pm 0.19^{*}$	$0.3 \pm 0.019^{*}$	0.88 ± 0.18^{b}
V100	0^{b}	16.7 ± 0.2^{a}	$24.7 \pm 2.9^{*}$	49.9 ± 0.7^{a}	0.96 ± 0.01^{b}	0.38 ± 0.01^{b}	1.21 ± 0.02^{b}	$3.01 \pm 0.21^{*}$	$0.34 \pm 0.003^{*}$	1 ± 0.08^{b}
VT90	53.3 ± 3.9^{a}	15.1 ± 0.3^{c}	$25.5 \pm 0.5^{*}$	40.3 ± 1.8^{b}	1.3 ± 0.01^{a}	0.69 ± 0.03^{a}	0.81 ± 0.02^{d}	$3.12 \pm 0.09^{*}$	$0.36 \pm 0.008^{*}$	1.42 ± 0.03^{a}
VT80	48.9 ± 4.4^{a}	15.2 ± 0.2^{c}	$25.1 \pm 0.8^{*}$	40.7 ± 1.2^{b}	1.3 ± 0.03^{a}	0.67 ± 0.02^{a}	0.8 ± 0.02^{d}	$3.14 \pm 0.05^{*}$	$0.35 \pm 0.007^{*}$	1.43 ± 0.02^{a}
VT75	46.7 ± 6.7^{a}	15.6 ± 0.2^{bc}	$24.3 \pm 1.3^{*}$	41.6 ± 0.7^{b}	1.3 ± 0.03^{a}	0.65 ± 0.03^{a}	0.9 ± 0.02^{c}	$3.01 \pm 0.13^{*}$	$0.34 \pm 0.012^{*}$	1.41 ± 0.04^{a}
VT70	44.4 ± 2.2^{a}	16.2 ± 0.2^{ab}	$25 \pm 0.9^{*}$	41.8 ± 1.2^{b}	1.3 ± 0.02^{a}	0.64 ± 0.03^{a}	0.87 ± 0.01^{c}	$2.99 \pm 0.16^{*}$	$0.34 \pm 0.007^{*}$	1.41 ± 0.01^{a}
Day 5										
Study-I										
L0	54.7 ± 4.8^{c}	18.3 ± 0.3^{a}	24.2 ± 0.6^{a}	44.2 ± 1.4^{a}	0.61 ± 0.02^{c}	0.21 ± 0.02^{b}	1.45 ± 0.01^{a}	2.56 ± 0.012^{c}	0.24 ± 0.003^{e}	0.53 ± 0.02^{d}
L100	72 ± 4^{b}	17.4 ± 0.1^{b}	23.3 ± 0.7^{a}	42.3 ± 1.2^{a}	0.74 ± 0.02^{c}	0.26 ± 0.02^{b}	1.48 ± 0.03^{a}	2.63 ± 0.042^{c}	0.25 ± 0.003^{d}	0.82 ± 0.01^{c}
LT90	100^{a}	12.7 ± 0.1^{d}	20.9 ± 0.5^{c}	30 ± 1^{b}	1.9 ± 0.25^{a}	0.58 ± 0.02^{a}	1.16 ± 0.02^{b}	2.84 ± 0.021^{a}	0.28 ± 0.002^{a}	1.21 ± 0.03^{a}
LT80	98.7 ± 1.3^{a}	13.2 ± 0.4^{d}	22.7 ± 0.3^{ab}	31.2 ± 0.9^{b}	1.24 ± 0.03^{b}	0.55 ± 0.03^{a}	1.17 ± 0.01^{b}	2.8 ± 0.032^{a}	0.27 ± 0.003^{ab}	1.14 ± 0.03^{ab}
LT75	97.3 ± 1.3^{a}	15.1 ± 0.2^{c}	21.2 ± 0.7^{bc}	32.3 ± 1.2^{b}	1.21 ± 0.02^{b}	0.55 ± 0.03^{a}	1.19 ± 0.01^{b}	2.72 ± 0.022^{b}	0.27 ± 0.002^{b}	1.02 ± 0.08^{b}
LT70	96 ± 2.3^{a}	15.4 ± 0.3^{c}	21.1 ± 0.4^{bc}	32.7 ± 0.9^{b}	1.21 ± 0.03^{b}	0.54 ± 0.02^{a}	1.17 ± 0.01^{b}	2.71 ± 0.012^{b}	0.26 ± 0.003^{c}	1.02 ± 0.08^{b}
Study-II										
V0	51.1 ± 4.4^{c}	18.6 ± 0.6^{a}	23.9 ± 0.8^{a}	43.3 ± 0.7^{a}	0.6 ± 0.03^{c}	0.25 ± 0.02^{b}	1.52 ± 0.05^{a}	2.58 ± 0.02^{c}	0.25 ± 0.002^{c}	0.52 ± 0.02^{c}
V100	68.9 ± 4.4^{b}	17.8 ± 0.5^{a}	23.7 ± 0.3^{a}	41.9 ± 1.5^{a}	0.8 ± 0.02^{b}	0.27 ± 0.02^{b}	1.59 ± 0.04^{a}	2.71 ± 0.01^{b}	0.26 ± 0.009^{bc}	0.81 ± 0.02^{b}
VT90	97.8 ± 2.2^{a}	12.9 ± 0.2^{c}	22 ± 0.4^{ab}	29.9 ± 1.3^{b}	1.3 ± 0.03^{a}	0.61 ± 0.02^{a}	1.19 ± 0.02^{b}	2.93 ± 0.01^{a}	0.29 ± 0.002^{a}	1.22 ± 0.03^{a}
VT80	95.6 ± 4.4^{a}	13.5 ± 0.6^{c}	22.3 ± 0.3^{ab}	31.1 ± 0.9^{b}	1.3 ± 0.02^{a}	0.6 ± 0.01^{a}	1.2 ± 0.01^{b}	2.73 ± 0.03^{b}	0.27 ± 0.003^{ab}	1.18 ± 0.05^{a}
VT75	82.2 ± 4.4^{ab}	15.5 ± 0.6^{b}	21.2 ± 1.1^{b}	32.8 ± 1.1^{b}	1.2 ± 0.04^{a}	0.59 ± 0.05^{a}	1.25 ± 0.01^{b}	2.73 ± 0.01^{b}	0.27 ± 0.001^{ab}	1.09 ± 0.04^{a}
VT70	86.7 ± 10.2^{a}	15.7 ± 0.6^{b}	20.9 ± 0.5^{b}	32.8 ± 1.5^{b}	1.2 ± 0.04^{a}	0.58 ± 0.02^{a}	1.28 ± 0.01^{b}	2.71 ± 0.02^{b}	0.26 ± 0.012^{bc}	1.07 ± 0.11^{a}

Letters in superscript denote significant differences ($P < 0.05$) between treatment means within each column of study-I and study-II (on day 3 and day 5, separately) as obtained by DMRT

Single asterisk (*) within different study periods indicate non-significant values ($P > 0.05$) in each column

times higher germination was observed in bio-primed treatments (study-I); while no germinations were observed in V0 and V100 during study-II (Figs. 5, 6; Table 3). About one and half fold higher germinations were seen in bio-primed soybeans (in both studies) as compared to controls (L0 and V0) during day 5.

Starch, Protein, and Lipid Contents

Food reserves (starch, lipid, and protein contents) of cotyledon were presented in Table 3. Lowest ($P < 0.05$) starch contents were obtained from LT90 and LT80 from study-I and VT90 and VT80 from study-II. On day 3, starch contents in bio-primed LT75 and LT70 were found statistically at par with control. Similar result was obtained from VT70, V0, and V100 (day 3). On day 5, L100 was found to possess lower starch value than L0. Moving from day 3 to day 5, there were increase in starch levels in L0 (13.4%), V0 (14.05%), L100 (5.2%), and V100 (6.4%); whereas, completely opposite phenomena were observed in bio-primed seeds. During day 5 of study-I, decrement in starch contents were found to be 17.15%, 14.6%, 4.4%, and 3.8% in LT90, LT80, LT75, and LT70, respectively, as compared to day 3. Similarly decrease in starch contents were observed in VT90 (14.7%), VT80 (11.03%), VT75 (0.83%), and VT70 (3.15%) on day 5 in study-II.

Lipid contents were decreased bio-primed treatments (Table 3). The decrement values were nearly 23% (LT90), 20.6% (LT80), 18.5% (LT75), and 18.4% (LT70) in study-I; whereas, the values were 25.8% (VT90), 23.6% (VT80), 21.2% (VT75), and 21.5% (VT70) in study-II. The lipid content of L0/V0 was found statistically at par with L100/V100. The bio-primed treatments did not vary significantly among them in respect to lipid content.

No significant differences in protein content were observed during day 3 (Table 3). Meanwhile, the differences became prominent at day 5 when maximum protein contents were found in L0, L100, and LT80 (study-I) and in V0, V100, VT90, and VT80 (study-II).

Amylolytic Enzyme Activities

The activities of α- and β-amylase in cotyledons were found highest in LT90/VT90 which was about two-folds higher than L0/V0 (Figs. 2a, b, 3a, b). In case of β-amylase, the activities were found statistically at par among bio-primed treatments. In each study, bio-primed soybean showed

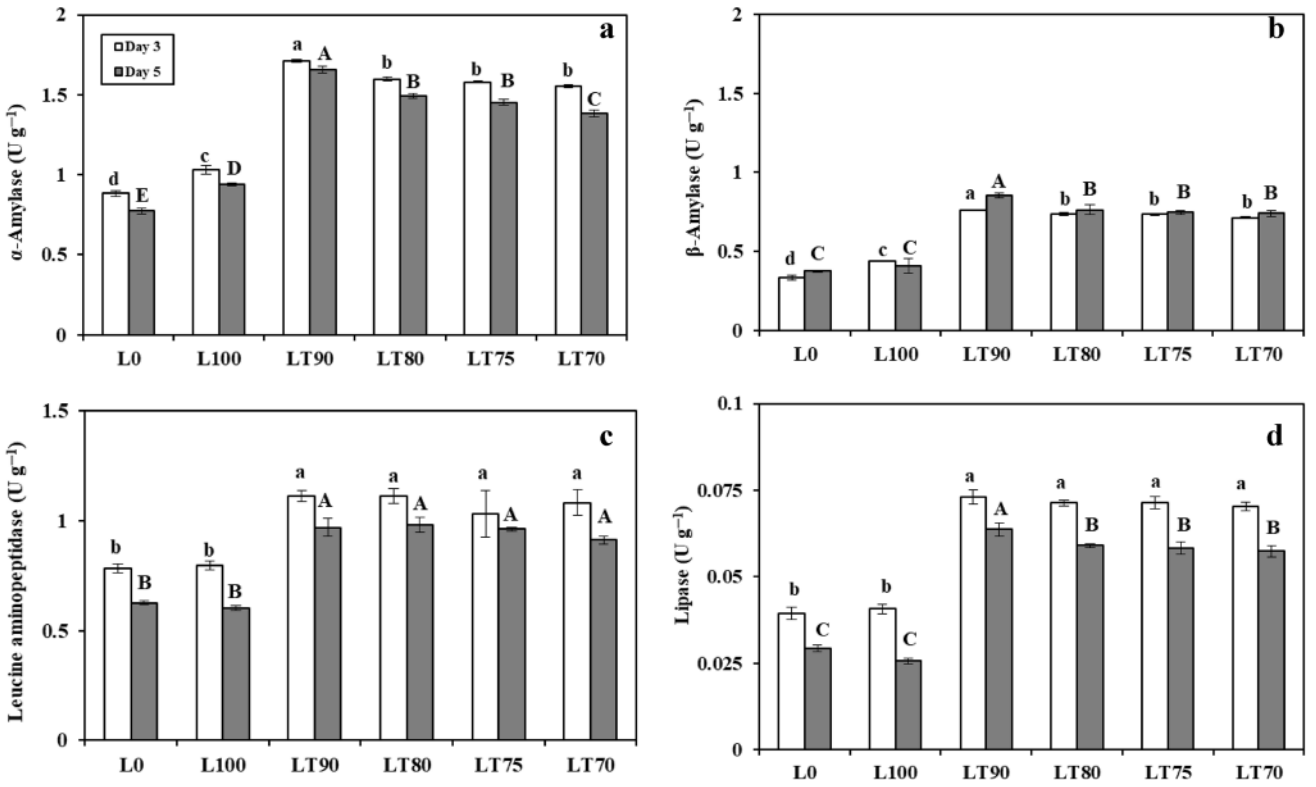


Fig. 2 Activities of **a** α-amylase, **b** β-amylase, **c** leucine aminopeptidase, and **d** lipase in germinated soybean cotyledons of study-I. Lower case alphabets indicate significant difference ($P < 0.05$) in treatment means on day 3; whereas, upper case alphabets indicate significant difference ($P < 0.05$) in treatment means on day 5 as obtained from DMRT

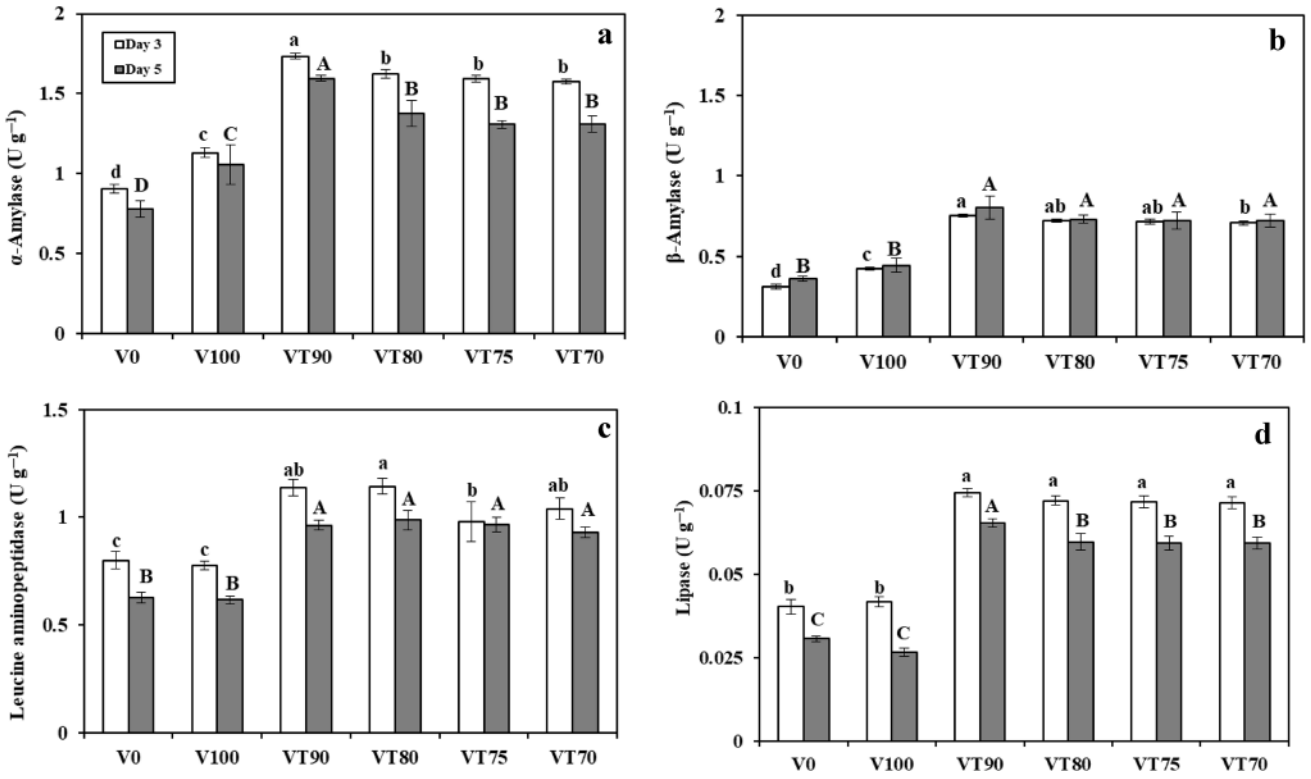


Fig. 3 Activities of **a** α-amylase, **b** β-amylase, **c** leucine aminopeptidase, and **d** lipase in germinated soybean cotyledons of study-II. Upper case alphabets indicate significant difference ($P < 0.05$) in treatment means on day 5; whereas, lower case letters indicate significant difference ($P < 0.05$) in treatment means on day 3 as obtained by DMRT

higher amylolytic activities than non-treated cotyledons. A general amylolytic activity trend was found to follow: LT90/VT90 > LT80/VT80 ~ LT75/VT75 ~ LT70/VT70 > L100/V100 > L0/V0.

Leucine Aminopeptidase Activity

On day 3, 1.4-times higher LAP activities were found in cotyledons of LT90, VT90, LT80, and VT80; while during day 5, all bio-primed treatments showed nearly 1.5-times higher LAP activities as compared to the their respective controls (Figs. 2c, 3c). LAP activities were found to decrease on day 5 than day 3. The activities of L0 and L100 did not vary significantly on both days (study-I). Similar result can be observed in case of V0 and V100 (study-II). All bio-primed treatments did not vary among themselves significantly on each day of sampling with an exception of study-II on day 3 when VT80 was found higher ($P < 0.05$) than VT75 (Fig. 3c).

Lipase Activity

Activities of lipase are depicted in Fig. 2d (study-I) and Fig. 3d (study-II). The activities of lipase in bio-primed cotyledons were found about 1.8-times and 2-times higher than controls (L0 and V0) but nearly 1.75- and 2.2-times higher than fertilized treatments without seed priming (L100 and V100) on day 3 and 5, respectively. Maximum activity was found in LT90 (0.064 U g^{-1}) and VT90 (0.064 U g^{-1}) on day 5 among all treatments.

Free Radical Scavenging Activity

The DPPH is a free radical compound which is used in biochemical procedures to represent activities of free radicals. The DPPH radicle scavenging activities were always found higher in case of bio-primed treatments (Table 3). Compared to control, scavenging activities of bio-primed cotyledons got almost doubled on day 3; while, compared to L100, it was 1.9-times higher. Compared to V100 (study-II), the activities were recorded nearly 1.7-times more in bio-primed cotyledons (Table 3). On day 5, the activities of treated cotyledons were nearly 2.6-times higher than L0 (study-I) and about 2.1-times higher than L100. The day 5

scavenging activities of bio-primed soybeans were found nearly 2.4-times higher than V0 and nearly 2.2-times higher than V100 (study-II).

Total Phenolic Compounds

Phenol contents of bio-primed treatments did not vary significantly among them but found higher ($P < 0.05$) than non-primed treatments on day 3 (Table 3). The LT90 (study-I) was found to be 3.13-times higher than L0 and 2.6-times higher than L100 on day 5 but rest treated seeds were found almost two-folds higher than L0 and 1.6-times higher than L100. Lowest phenolic activities were found in V0 (study-II) on day 5. During that time, about 2-times higher phenol were found in bio-primed treatments (study-II) than V0 but 1.6-times higher than V100.

Lipid Peroxidation

Compared non-treated soybeans, lipid peroxidation were found lower in bio-primed treatments (day 5) but during day 3, some differences were there among them (Table 3). Higher ($P < 0.05$) lipid peroxidations were obtained from LT75/VT75 and LT70/VT70 (day 3). Rest four treatments (LT90, LT80, VT90, and VT80) showed significantly lower TBARS values. On day 3, lipid peroxidation followed a trend: L0 (V0) > L100 (V100) > LT75 (VT75) ~ LT70 (VT70) > LT90 (VT90) ~ LT80 (VT80).

Total Nitrogen, Phosphorus, and Potassium Contents

Mineral nutrient contents in cotyledons were presented in Table 3. Statistically non-significant N and P contents were obtained on day 3 but significant K increment was observed in LT90 (VT90) to LT70 (or VT70) cotyledons than non-primed ones, attributing bio-priming mediated higher K uptake. In study-I, N contents were found highest ($P < 0.05$) in LT90 (2.84%) and LT80 (2.8%) and during study-II, maximum was found in VT90 (2.93%) on day 5. In case of P, LT90 (0.28%) and LT80 (0.27%) in study-I and VT90 (0.29%), VT80 (0.27%), and VT75 (0.27%) of study-II were found with highest ($P < 0.05$) phosphorus concentrations on day 5. Similarly, highest ($P < 0.05$) K concentrations on day 5 were obtained from LT90 (1.21%) and LT80 (1.14%) in study-I; while all *T. viride* treated cotyledons of study-II were found with statistically similar K concentrations but significantly higher than V0 (0.52%) and V100 (0.81%) at the same time.

Principle Component Analysis

Principle component analysis divided twelve treatments (six from study-I and six from study-II) and fourteen variables into eleven orthogonal components carrying 100% variances (Online Resource 2, Table S1, S3). First two components together explained 90.2% and 94.3% variances on day 3 and

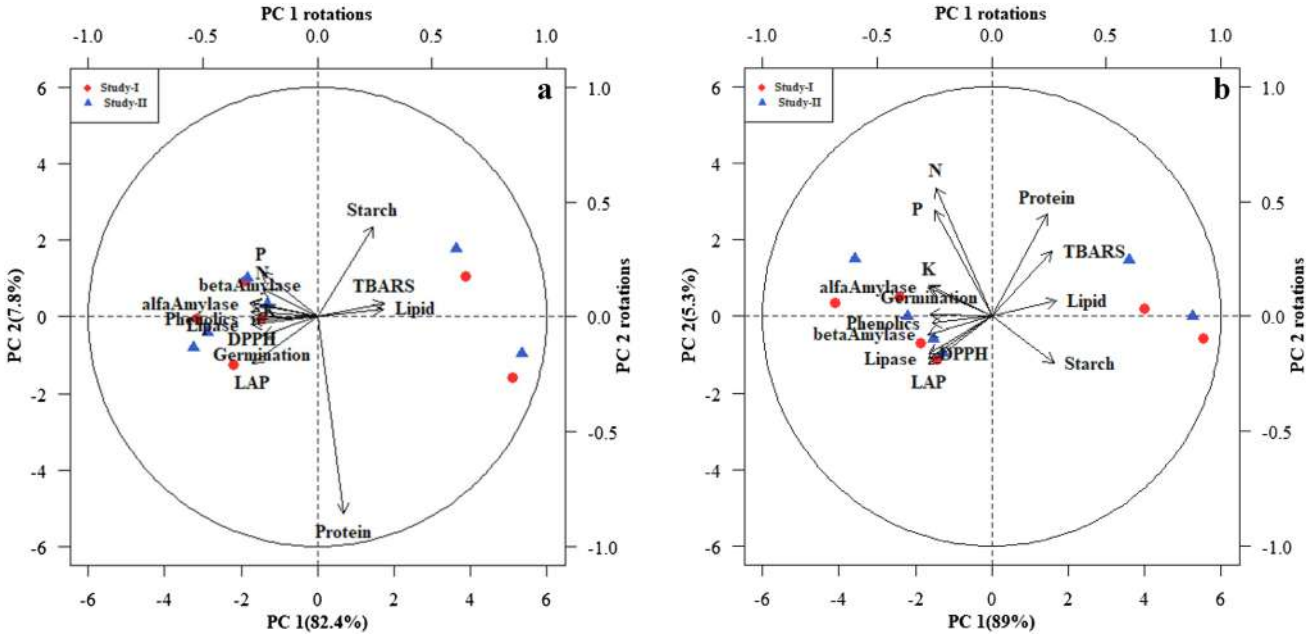


Fig. 4 Biplots of principle compont analysis ($P < 0.05$) at **a** day 3 and **b** day 5. alfaAmylase, α-amylase activity; betaAmylase, β-amylase activity; DPPH, free radicle scavenging activity; Germination, germination percentage; LAP, leucine aminopeptidase activity; Lipase, lipase activity; Lipid, lipid content; N, nitrogen; K, potassium; P, phosphorus; Phenolics, total phenol content; Protein, protein content; Starch, starch content; TBARS, lipid peroxidation

day 5, respectively (Fig. 4a, b). Variables other than protein contributed almost equally to construct PC 1 on day 3 (Table S2). Higher scores of non-primed treatments on the positive coordinate of PC 1 have a positive influence on lipid peroxidation, starch, protein, and lipid contents; while, bio-primed soybeans positively influenced rest parameters (Fig. 4a; Table S2, S5). On day 5, all the variables have contributed equally in constructing PC 1 but protein, N, P contents, LAP activity, and lipid peroxidation possess major loadings on PC 2 (Table S4). Except for protein, N, and P contents, rest parameters established a good quality presentation on PC1 (Table S4). As compared to day 3, scores of day 5 on PC 1 have similarity (Table S5). Thus, we can infer that non-primed treatments have put a positive influence on food reserves of cotyledons; whereas, bio-primed treatments have positively influenced the rest variables (Fig. 4b; Table S4, S5). There were very minute deviations in eigenvectors of germination (on both days) from PC 2 origin along the negative coordinate of PC 1 (Fig. 4a, b; Table S2, S4). Variables (α-amylase, β-amylase, LAP, lipase, K, phenol contents, and free radicals scavenging activity) with higher squared cosine values (near unity) on the negative coordinate of PC 1 have very strong positive correlations on soybean germination; while variables (lipid and its peroxidation) on the positive coordinate of PC 1 possessed strong negative correlations on germination (Fig. 4a, b; Table S2, S4).

Discussion

Current investigation revealed that *T. viride* BHU-2953 has boosted up soybean germination. The imbibed seeds had initiated the activities of different hydrolytic enzymes, i.e., lipase, amylases, and leucine aminopeptidase that are responsible for degrading lipid, starch, and protein, respectively. As soybean seed contains more lipid substances than sugars and proteins, inoculation of *Trichoderma* primarily enhanced the lipase activity in bio-primed cotyledons (Brown and Huber 1988; Kashmiri et al. 2006; Roveda et al. 2010; Canseco-Pérez et al. 2018; Mendes et al. 2019). The mobilization of storage lipids during oilseed germination is directly associated with glyoxylate cycle (Gonzalez and Vodkin 2007; Marques et al. 2013). Lipid is stored within soybean cotyledons in form of triacylglycerol which is mobilized by the activities of lipase at pre-germinative stage (Gonzalez and Vodkin 2007). In the present study, bio-primed cotyledons showed a reduction in lipid content ($P < 0.05$) due to induced activities of lipase by *T. viride* BHU-2953, enhancing faster lipid mobilization within these treatments in comparison to untreated ones. This is also supported by Kashmiri et al. (2006) who have shown increased secretion of lipase by *T. viride* in presence of oil as sole carbon nourishment. Consequently, in previous reports, Roveda et al. (2010), Canseco-Pérez et al. (2018), and Mendes et al. (2019) have also found a wide range of lipases, produced by *Trichoderma* isolates that invoked lipid hydrolysis when cultured with oil containing media. As the endosperm of soybean contains low amount of carbohydrates, anaerobic β-oxidation and gluconeogenesis convert the lipid hydrolysis products into low molecular weight carbohydrates to form starch (Adams et al. 1981; Brown and Huber 1988; Gonzalez and Vodkin 2007). This increase in transitory starch can be observed up to day 5 as in case of untreated cotyledons in the present experiment (Adams et al. 1980; Hildebrand and Hymowitz 1981). On the contrary, *T. viride* BHU-2953 inoculated cotyledons showed a decrease in starch values on day 5 in comparison to day 3 (Table 3). The results are found consistent in both studies (significant R^2 values at $P < 0.05$; Table S6). Enhanced amylase activities, especially α-amylase in bio-primed soybeans have resulted in faster degradation of transient starch. However, biplots clearly showed a higher β-amylase activity which enhanced the starch hydrolysis in germinating cotyledons of bio-primed treatments (Fig. 4a, b). The β-amylase releases maltose units from non-reducing ends of the starch (Adams et al. 1981; Hildebrand and Hymowitz 1981). It implies that *Trichoderma viride* BHU-2953 can either secrete extracellular α- and β-amylases or trigger de novo synthesis of amylolytic enzymes for degradation of recently synthesized starch in germinating cotyledons. The finding is also supported by previous reports. Schellart et al. (1976) have seen faster degradation of starch into maltose units by the action of extracellular α-amylase, secreted by *Trichoderma viride*. Marques et al. (2003) has evidenced the production of extracellular amylase by *Trichoderma viride*. The results are also corroborated by Mahmood and Rahman (2008) who have found that *T. viride* secretes extracellular amylase enzymes when plated with starch-agar medium. Again, Sharma et al. (2020) have found higher amylase activity in germinating maize seeds that was applied with *T. viride*-cyanobacteria biofilm rather than control. Applied-N along with *T. viride* has also reported to influence higher amylase activities in bio-primed treatments than untreated cotyledons (Schellart et al. 1976; Mahmood and Rahman 2008). LAP, an exopeptidase, detaches leucine from N-terminal ends of polypeptides, i.e., proteins. It is primarily responsible for soil N-cycling and hydrolysis of stored protein when *Trichoderma* proliferation dominates in a system (Stasz et al. 1988; Galletti et al. 2015). From biplots, it was clear that protein degradation was not influencing the germination on day 3 although LAP activities were found quite higher in *T. viride* treated seeds. According to Marques et al. (2013) and Ali and Elozeiri (2017), protein hydrolysis takes longer time to initiate but increases as the germination progresses for development of embryonic axis. However, *Trichoderma* mediated LAP secretion has triggered the protein hydrolysis on day 5

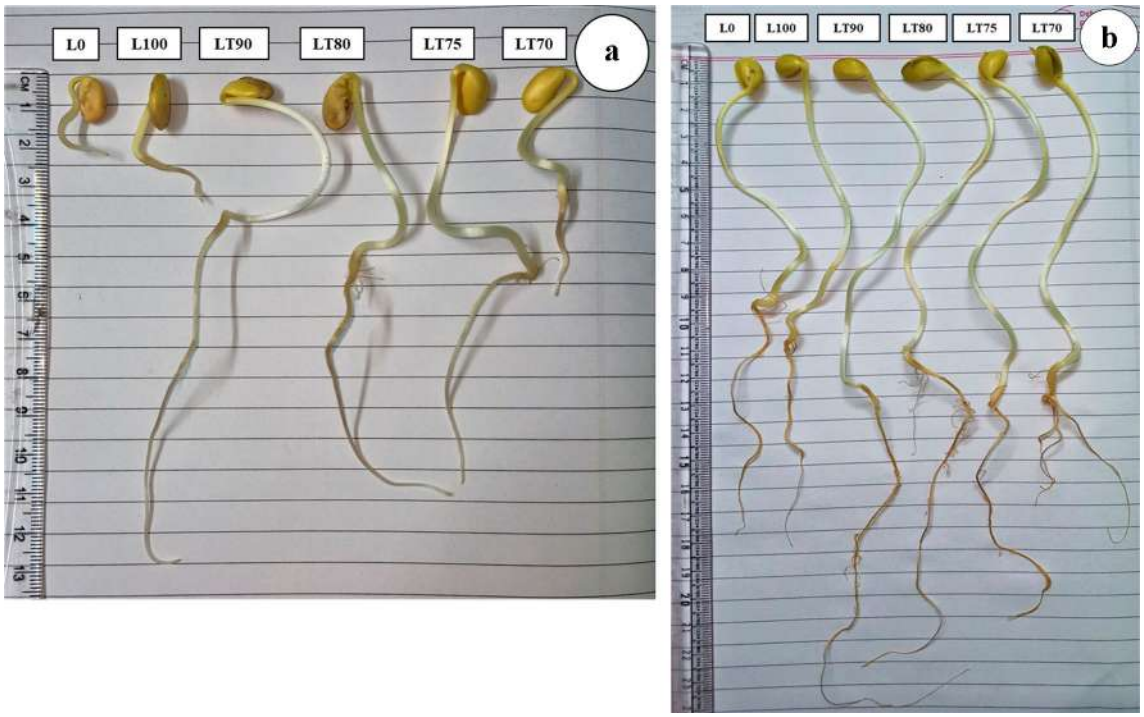


Fig. 5 Germinated *Glycine max* var. JS 95-60 in study-I on **a** day 3 and **b** day 5

(Galletti et al. 2015). The hydrolysed products must have been used to lengthen the hypocotyl (Figs. 5, 6), providing energy for its emergence from soil (Rathore et al. 1981).

Sudden enhancement of these metabolic changes generates free radicals within the seeds (Ali and Elozeiri 2017; Singh et al. 2010). Free radicles are responsible for peroxidation of membrane-bound lipids (TBARS), destabilizing membrane integrity at the time of germination. Soybean germination stage is most susceptible to take oxidative damage, causing phytotoxicity at cellular level. The impaired membrane either hampers or completely staggers the germination process if those radicles are not quenched properly (Ali and Elozeiri 2017). Moreover, the anti-oxidant defense mechanisms in soybean cells can only prevent this damage up to certain level (Singh et al. 2010; Tančić et al. 2013; Ali and Elozeiri 2017). This is the sole reason that we have documented relatively higher lipid peroxidation in untreated seeds as compared to bio-primed ones (Table 3). Hence, external supply of anti-oxidants becomes necessary to suppress the cytotoxicity. In this experiment, higher radical scavenging activities (DPPH) in bio-primed seeds were closely associated with total phenol contents which have lessened the lipid peroxidation to a minimum as compared to untreated seeds (Fig. 4; Table 3, S2, S4, S5). This indicates that *T. viride* priming can alleviate the oxidative stress at the time of germination, enhancing viability of soybean. Mastouri et al. (2010) found reduction in peroxide damage of tomato seedling when primed with *Trichoderma* at the time of germination. Jain et al. (2012) used a microbial consortium, using *Trichoderma* isolate with plant growth promoting bacteria, in pea seeds to elicit the phenol content during germination. Some previous reports also confirmed that phenols can raise free radicle scavenging activity in soybean seed-matrix when inoculated with *Trichoderma* (Begum et al. 2010; Singh et al. 2010; Tančić et al. 2013). Begum et al. (2010) also suggested that higher phenol content infers *Trichoderma* infection on soybean seed coats. Similarly, Zheng and Shetty (2000) have found a positive relationship among phenol content, root length, and seedling vigour in peas through *T. viride* inoculation at the time of germination. Not only phenol content, the strain, *Trichoderma viride* BHU-2953, has been reported to increase superoxide dismutase and ascorbate peroxidase activities that have reduced the lipid peroxidation in soybean leaves via H_2O_2 scavenging (Paul and Rakshit 2022).

The nitrogen and phosphorus nutrition plays important role for production of hydrolytic enzymes as well maintenance of carbon to nitrogen ratio within fungal body (Schellart et al. 1976; Mahmood and Rahman 2008). Most of reserve-N in cotyledons is lost during protein hydrolysis which is considered responsible for N-deficit during germination (Ali and Elozeiri 2017). In this study, this deficiency might have been aided by *T. viride* BHU-2953 mediated higher N-uptake (day 5) when reserve protein hydrolysis became faster within bio-primed cotyledons (Harman et al. 2004a; Ali and Elozeiri 2017). That is why N content of



Fig. 6 Germinated *Glycine max* var. JS 95-60 in study-II at **a** day 3 and **b** day 5

L100 were found significantly lower than *T. viride* treated seeds with graded N-doses (study-I; day 5); while V100 was found statistically at par with VT80, VT75, and VT70 (study-II). However, non-significant P-content on day 3 might be the result of slow diffusion rate of phosphate ions (Hanway and Weber 1971; Marschner 2012; Paul and Rakshit 2021). Energy requirement for necessary metabolic changes is primarily met by ATP during germination (Hanway and Weber 1971; Marschner 2012). Phosphorus nutrition is essential for synthesis of ATP. Priming enhances protrusion of *Trichoderma* hyphae out of seeds to uptake more amount of applied phosphorus (Harman et al. 2004a, 2004b;

Begum et al. 2010). Thus, bio-primed cotyledons were found with significantly higher P-content on day 5 (Table 3). In previous study, we have found that increased rhizospheric phosphatase activity coupled with longer root lengths had raised the P-content in *T. viride* BHU-2953 treated soybeans (Paul and Rakshit 2021). In contrast, K contents in bio-primed cotyledons were always recorded higher ($P < 0.05$) than untreated L100/V100 although L100/V100 received more amount of potassium dose (Table 3). This is because diffusion of K^+ ion is more than N and P (Marschner 2012). As starch biosynthesis is also regulated by K^+ concentration, transient starch synthesis might have assisted the potassium uptake during germination (Brown and Huber 1988; Marschner 2012). Besides, both studies were carried out with addition of NPK-solution. Thus, a hike in salt concentration can be expected in those systems, especially in case of study-I. *Trichoderma* might have enhanced the potassium uptake to maintain solute neutrality and execute faster starch hydrolysis within bio-primed cotyledons. Similar inference was drawn by Yildirim et al. (2006) who discovered K^+ uptake in squash was enhanced by *Trichoderma* isolate to ameliorate harmful effect of salt stress. Bakhshandeh et al. (2020) have also documented higher K^+ uptake in *Trichoderma*-primed soybean seeds under both optimum and salt stress conditions. Apart from this, phenol was reported to reduce seed-integument thickness, helping N, P, and K diffusions into cotyledons along with water (Hanway and Weber 1971; Sharma et al. 2019). As bio-primed seeds showed more phenolic activities, *Trichoderma viride* BHU-2953 might be considered responsible for higher nutrient uptake during germination of soybean seeds.

Although germination requires sequence of physiochemical episodes to occur within seed, reserve food mobilization plays principal role to provide energy and nutrition for radicle emergence. *Trichoderma viride* augmented these metabolic changes in addition to enhancement of phenol content, radicle scavenging activities, and nutrient uptake for escalating vigorous germination in bio-primed soybeans (Figs. 5, 6; Table 3). The effect of *T. viride* priming is very distinguishable in study-II (day 3) where no emergence has occurred in case of untreated soybeans; while treated seeds shown ~ 44% to ~ 53% emergence (Fig. 6a; Table 3). Strong correlation coefficients (R^2; $P < 0.05$) between two studies append the uniformity of our result (Table S6). The results are also supported by different researchers (Zheng and Shetty 2000; Mastouri et al. 2010; Tančić et al. 2013; Bakhshandeh et al. 2020). Faster and uniform germinations in pea seeds, bio-primed with fermented apple pomace extracts using different *Trichoderma* isolates, were reported by Zheng and Shetty (2000). Begum et al. (2010) have discovered that combined application of *Trichoderma* isolates in association with *Pseudomonas* can trigger the soybean germination process. Mastouri et al. (2010) have bio-primed tomato seeds with *Trichoderma* and obtained superior germination against abiotic (chilling, heat, and salinity) and biotic (*Pythium ultimum*) stresses. Tančić et al. (2013) have also found enhanced soybean germination when primed with *Trichoderma* isolates. Using two different *Trichoderma* isolates, separately, Bakhshandeh et al. (2020) have found superior germinations in treated soybean seeds under both abiotic stress and optimum conditions that elucidates our interpretation.

Conclusion

Based on the present findings, we can conclude that *Trichoderma viride* BHU-2953 can enhance faster germination in inoculated soybeans along with graded doses of macro-nutrients' solution. The fungus not only triggered the activities of hydrolytic enzymes responsible for faster seed-reserve mobilization but also enhanced the nitrogen, phosphorus, and potassium uptake for nourishing soybean during emergence. In addition, increased phenolic activities by *Trichoderma viride* BHU-2953 have promoted good seedling vigour during germination through mitigating oxidative membrane damages in bio-primed soybeans. These traits make *Trichoderma viride* a potent seed priming agent for its application in low cost sustainable agriculture. Further research is desirable at molecular level for better understanding of *Trichoderma viride* interactions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00344-023-10929-w.

Acknowledgements Authors would like to thank Prof. H.B. Singh, Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi for the *Trichoderma viride* BHU-2953 fungus and necessary provisions for this study.

Author Contributions SP carried out the experiment, manuscript drafting, and interpretation of statistical data. AR designed the research, statistical analysis of the data, and figure preparation.

Funding Not applicable.

Data Availability All data of this experiment are presented in this article and within supplementary material.

Declarations

Conflict of interest Subhadip Paul and Amitava Rakshit declare that they have no conflict of interest.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

References

Adams CA, Rinne RW, Fjerstad MC (1980) Starch deposition and carbohydrase activities in developing and germinating soya bean seeds. Ann Bot 45:577–582. https://doi.org/10.1093/oxfordjournals.aob.a085863

Adams CA, Broman TH, Rinne RW (1981) Starch metabolism in developing and germinating soya bean seeds is independent of β-amylase activity. Ann Bot 48:433–440. https://doi.org/10.1093/oxfordjournals.aob.a086147

Ali AS, Elozeiri AA (2017) Metabolic processes during seed germination. Adv Seed Biol 6:141–166. https://doi.org/10.5772/intechopen.70653

Bakhshandeh E, Gholamhosseini M, Yaghoubian Y, Pirdashti H (2020) Plant growth promoting microorganisms can improve germination, seedling growth and potassium uptake of soybean under drought and salt stress. Plant Growth Regul 90:123–136. https://doi.org/10.1007/s10725-019-00556-5

Begum MM, Sariah M, Puteh AB, Abidin MZ, Rahman MA, Siddiqui Y (2010) Field performance of bio-primed seeds to suppress *Colletotrichum truncatum* causing damping-off and seedling stand of soybean. Biol Control 53:18–23. https://doi.org/10.1016/j.biocontrol.2009.12.001

Brown CS, Huber SC (1988) Reserve mobilization and starch formation in soybean (*Glycine max*) cotyledons in relation to seedling growth. Physiol Plant 72:518–524. https://doi.org/10.1111/j.1399-3054.1988.tb09159.x

Canseco-Pérez MA, Castillo-Avila GM, Chi-Manzanero B, Islas-Flores I, Apolinar-Hernández MM, Rivera-Muñoz G, Gamboa-Angulo M, Sanchez-Teyer F, Couoh-Uicab Y, Canto-Canché B (2018) Fungal screening on olive oil for extracellular triacylglycerol lipases: selection of a *Trichoderma harzianum* strain and genome wide search for the genes. Genes. https://doi.org/10.3390/genes9020062

Chrispeels MJ, Boulter D (1975) Control of storage protein metabolism in the cotyledons of germinating mung beans: role of endopeptidase. Plant Physiol 55:1031–1037. https://doi.org/10.1104/pp.55.6.1031

Coppola M, Cascone P, Lelio ID, Woo SL, Lorito M, Rao R, Pennacchio F, Guerrieri E, Digilio MC (2019) *Trichoderma atroviride* P1 colonization of tomato plants enhances both direct and indirect defense barriers against insects. Front Physiol 10:813. https://doi.org/10.3389/fphys.2019.00813

Galletti S, Fornasier F, Cianchetta S, Lazzeri L (2015) Soil incorporation of brassica materials and seed treatment with *Trichoderma harzianum*: effects on melon growth and soil microbial activity. Ind Crops Prod 75:73–78. https://doi.org/10.1016/j.indcrop.2015.04.030

Gonzalez DO, Vodkin LO (2007) Specific elements of the glyoxylate pathway play a significant role in the functional transition of the soybean cotyledon during seedling development. BMC Genom 8:1–22. https://doi.org/10.1186/1471-2164-8-468

Gupta N, Rathi P, Gupta R (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. Anal Biochem 311:98–99. https://doi.org/10.1016/S0003-2697(02)00379-2

Hanway JJ, Weber CR (1971) N, P, and K percentages in soybean (*Glycine max* (L.) Merrill) plant parts1. Agron J 63(2):286–290. https://doi.org/10.2134/agronj1971.00021962006300020027x

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004a) *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56. https://doi.org/10.1038/nrmicro797

Harman GE, Petzoldt R, Comis A, Chen J (2004b) Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. Phytopathol 94:147–153. https://doi.org/10.1094/PHYTO.2004.94.2.147

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198. https://doi.org/10.1016/0003-9861(68)90654-1

Hildebrand DF, Hymowitz T (1981) Role of β-amylase in starch metabolism during soybean seed development and germination. Physiol Plant 53:429–434. https://doi.org/10.1111/j.1399-3054.1981.tb02726.x

ISTA (1985) International seed testing association. Seed Sci Technol 13:299–513

Jackson ML (1973) Soil Chemical Analysis. Prentice Hall of India Pvt Ltd, New Delhi

Jain A, Singh S, Sarma BK, Singh HB (2012) Microbial consortium–mediated reprogramming of defence network in pea to enhance tolerance against *Sclerotinia sclerotiorum*. J Appl Microbiol 112:537–550. https://doi.org/10.1111/j.1365-2672.2011.05220.x

Kashmiri MA, Adnan A, Butt BW (2006) Production, purification and partial characterization of lipase from *Trichoderma viride*. Afr J Biotechnol 5:878–882

Kuai J, Liu Z, Wang Y, Meng Y, Chen B, Zhao W, Zhou Z, Oosterhuis DM (2014) Waterlogging during flowering and boll forming stages affects sucrose metabolism in the leaves subtending the cotton boll and its relationship with boll weight. Plant Sci 223:79–98. https://doi.org/10.1016/j.plantsci.2014.03.010

Mahmood S, Rahman SR (2008) Production and partial characterization of extracellular α-amylase by *Trichoderma viride*. Bangladesh J Microbiol 25:99–103. https://doi.org/10.3329/bjm.v25i2.4870

Marques S, Pala H, Alves L, Amaral-Collaco MT, Gama FM, Girio FM (2003) Characterisation and application of glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viride* CCMI 84 for enzymatic deinking of mixed office wastepaper. J Biotechnol 100:209–219. https://doi.org/10.1016/S0168-1656(02)00247-X

Marques EC, de Freitas PA, Alencar NL, Prisco JT, Gomes-Filho E (2013) Increased Na+ and Cl− accumulation induced by NaCl salinity inhibits cotyledonary reserve mobilization and alters the source-sink relationship in establishing dwarf cashew seedlings. Acta Physiol Plant 35:2171–2182. https://doi.org/10.1007/s11738-013-1254-5

Marschner H (2012) Marschner's mineral nutrition of higher plants, 3rd edn. Academic Press, London

Mastouri F, Björkman T, Harman GE (2010) Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. Phytopathol 100:1213–1221. https://doi.org/10.1094/PHYTO-03-10-0091

Mendes DB, Da Silva FF, Guarda PM, Almeida AF, de Oliveira DP, Morais PB, Guarda EA (2019) Lipolytic enzymes with hydrolytic and esterification activities produced by filamentous fungi isolated from decomposition leaves in an aquatic environment. Enzyme Res. https://doi.org/10.1155/2019/8182425

Nettles R, Watkins J, Ricks K, Boyer M, Licht M, Atwood LW, Peoples M, Smith RG, Mortensen DA, Koide RT (2016) Influence of pesticide seed treatments on rhizosphere fungal and bacterial communities and leaf fungal endophyte communities in maize and soybean. Appl Soil Ecol 102:61–69. https://doi.org/10.1016/j.apsoil.2016.02.008

Paul S, Rakshit A (2021) Effect of seed bio-priming with *Trichoderma viride* strain BHU-2953 for enhancing soil phosphorus solubilization and uptake in soybean (*Glycine max*). J Soil Sci Plant Nutr 21:1041–1052. https://doi.org/10.1007/s42729-021-00420-4

Paul S, Rakshit A (2022) Evaluating lignification, antioxidative defense, and physiochemical changes in soybean through biopriming under graded soil fertilization. J Soil Sci Plant Nutr. https://doi.org/10.1007/s42729-022-00809-9

Rajput RS, Singh J, Singh P, Vaishnav A, Singh HB (2021) Influence of seed biopriming and vermiwash treatment on tomato plant's immunity and nutritional quality upon *Sclerotium rolfsii* challenge inoculation. J Plant Growth Regul 40:1493–1509. https://doi.org/10.1007/s00344-020-10205-1

Rathore TR, Ghildyal BP, Sachan RS (1981) Germination and emergence of soybean under crusted soil conditions. Plant Soil 62:97–105. https://doi.org/10.1007/BF02205029

Roveda M, Hemkemeier M, Colla LM (2010) Evaluation of lipase production using different strains of microorganisms isolated from dairy effluent through submerged fermentation. Food Sci Technol 30:126–131. https://doi.org/10.1590/S0101-20612010000100019

Schellart JA, Visser FM, Zandstra T, Middelhoven WJ (1976) Starch degradation by the mould *Trichoderma viride* I. The mechanism of starch degradation. Antonie Van Leeuwenhoek 42:229–238. https://doi.org/10.1007/BF00394119

Schulz TJ, Thelen KD (2008) Soybean seed inoculant and fungicidal seed treatment effects on soybean. Crop Sci 48(5):1975–1983. https://doi.org/10.2135/cropsci2008.02.0108

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B (2019) Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Mol 24:2452. https://doi.org/10.3390/molecules24132452

Sharma V, Prasanna R, Hossain F, Muthusamy V, Nain L, Das S, Shivay YS, Kumar A (2020) Priming maize seeds with cyanobacteria enhances seed vigour and plant growth in elite maize inbreds. 3 Biotech 10:1–5. https://doi.org/10.1007/s13205-020-2141-6

Singh HB, Singh BN, Singh SP, Nautiyal CS (2010) Solid-state cultivation of *Trichoderma harzianum* NBRI-1055 for modulating natural antioxidants in soybean seed matrix. Bioresour Technol 101:6444–6453. https://doi.org/10.1016/j.biortech.2010.03.057

Singh HB, Singh A, Sarma BK, Upadhyay DN (2014) *Trichoderma viride* 2% WP (Strain No. BHU-2953) formulation suppresses tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* and chilli damping-off caused by *Pythium aphanidermatum* effectively under different agroclimatic conditions. Int J Agric Environ Biotechnol 7:313–320. https://doi.org/10.5958/2230-732X.2014.00249.6

Sreenayana B, Vinodkumar S, Nakkeeran S, Muthulakshmi P, Poornima K (2021) Multitudinous potential of *Trichoderma* species in imparting resistance against *F. oxysporum* f. sp. *cucumerinum* and *Meloidogyne incognita* disease complex. J Plant Growth Regul. https://doi.org/10.1007/s00344-021-10372-9

Stasz TE, Weeden NF, Harman GE (1988) Methods of isozyme electrophoresis for *Trichoderma* and *Gliocladium* species. Mycologia 80:870–874. https://doi.org/10.1080/00275514.1988.12025741

Tanase C, Bujor OC, Popa VI (2019) Phenolic natural compounds and their influence on physiological processes in plants. In: Watson RR (ed) Polyphenols in Plants: Isolation, Purification, and Extract Preparation, 2nd edn. Academic Press, London, pp 45–58

Tančić S, Skrobonja J, Lalošević M, Jevtić R, Vidić M (2013) Impact of *Trichoderma* spp. on soybean seed germination and potential antagonistic effect on *Sclerotinia sclerotiorum*. Pestic Phytomed (belgrade) 28:181–185. https://doi.org/10.2298/PIF1303181T

Tárrago JF, Nicolás G (1976) Starch degradation in the cotyledons of germinating lentils. Plant Physiol 58:618–621. https://doi.org/10.1104/pp.58.5.618

Xu BJ, Chang SK (2007) A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J Food Sci 72:S159–S166. https://doi.org/10.1111/j.1750-3841.2006.00260.x

Yildirim E, Taylor AG, Spittler TD (2006) Ameliorative effects of biological treatments on growth of squash plants under salt stress. Sci Hortic 111:1–6. https://doi.org/10.1016/j.scienta.2006.08.003

Zheng Z, Shetty K (2000) Enhancement of pea (*Pisum sativum*) seedling vigour and associated phenolic content by extracts of apple pomace fermented with *Trichoderma* spp. Process Biochem 36:79–84. https://doi.org/10.1016/S0032-9592(00)00183-7

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.