

Protein Profiling and Biochemical Analysis of Chickpea Seedling Treated with *T. viride* (49CP)

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In vitro effect on chickpea seedling induced by biopriming of *Trichoderma viride* ie. the treatment T₁ (*T. viride* 5g/kg seed) was excelled overall significant superior performance by contributing 68%, 12.07cm, 16.37cm, 28.44cm, 0.592g, 1933.92 and 40.26 in germination, root length, shoot length, seedling length, dry weight, vigour index I and vigour index II, respectively. The highest protein content was observed in chickpea seedling that's bioprimed by *Trichoderma viride* formulation (*T. viride* @5g/kg seeds) was estimated through Kelpus analyzer was 0.41%. The protein quantification of chickpea seedlings was estimated through Lowry and Bradford method were 1.06 and 1.08 mg/ml. The protein profiling of chickpea seedling on SDS PAGE were observed maximum five bands, first band at 250 kda, second band at 100 kda, third band was lying between 75 to 50 kda, and fourth band was at 20 kda.

Keywords: Chickpea seedling, biopriming, *Trichoderma viride* and vigour index.

Chickpea, commonly known as Gram or Bengal gram is the most important pulse crop in India. It's probable place of origin lies in south western Asia. The important gram growing countries are India, Pakistan, Ethiopia, Burma and Turkey. India ranks first in the world in respect of production as well as acreage followed by Pakistan. In India the major chickpea producing states are Madhya Pradesh, Rajasthan, Uttar Pradesh, Haryana, Maharashtra and Punjab. Chickpea (*Cicer arietinum* L.) is an important leguminous crop grown in diverse soils and agro-climatic conditions. Chickpea occupies about 38 per cent of area under pulses and contributes about 50 per cent of the total pulse production of India. In India Chickpea

is cultivated on area 9.60 million ha with annual production (grains) of 8.83 million tonnes and average yield level is 920 kg/ha, (FAO STAT, 2013).

Chickpea is a good source of energy, protein, minerals, vitamins, fibre, and also contains potentially health-beneficial minerals and vitamins. The protein content ranged from 18.46 to 24.46 g/100g, oil content ranged from 5.68 to 9.01 g/100g and ash from 3.55 to 4.46 g/100g (Nobile *et al.* 2013). It is used for human consumption as well as for feeding to animals. It is eaten both whole fried or boiled and salted or more generally in the form of split pulse, which is cooked and eaten. Both husks and bits of the '*dal*' are valuable cattle feed. Fresh green leaves are used as vegetable (sag). Straw of chickpea is an excellent fodder for cattle. The grains are also used as vegetable (chhole). Chickpea flour (besan) is used in the preparation of various types of sweets. Recent studies have also shown that

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they can assist in lowering of cholesterol in the bloodstream (Murty *et al.*, 2010). *Trichoderma* sp. are well known as bio-priming agent for biological suppression of several soil borne diseases. Thus the application of bio-control agents (BCAs) for plant disease are currently being examined as alternatives to the synthetic pesticides due to their perceived increased level of safety and minimal environmental impacts (Cotxarrera *et al.*, 2002; Brimmer and Boland, 2003).

MATERIALS AND METHODS

Freshly harvested seed of chickpea (Uday) was obtained from seed processing plant of C. S. Azad university of Agriculture & Technology, Kanpur. 50 seeds for each treatment were counted, weighted and soaked overnight in 100 ml water with different doses of biocontrol agent (*T. viride* (49CP), fungicide and pathogen in three replications. Treatments were as follows:

Treatment details

T₁ : Seed biopriming @ 5g *T. viride* formulation/ kg of seed.

T₂ : Seed biopriming @ 10g *T. viride* formulation/ kg of seed.

T₃ : Seed biopriming @ 15g *T. viride* formulation/ kg of seed.

T₄ : Seed biopriming @ 20g *T. viride* formulation/ kg of seed.

T₅ : Seed biopriming @ 0.2% of Bavistin/ kg seed.

T₆ : Seed inoculated @ 10 % *Foc* / kg seed.

T₇ : Control (without Treatment).

Experimental details

Biocontrol agent used-	<i>Trichoderma viride</i> (49CP)
Substrates used-	crystal sand
Plastic tray size	20x15"
Crop	chickpea
Variety	Uday
Design	Completely Randomized Design (CRD)
No. of Treatments	7
No. of Replications	3
No. of plastic tray	21
Date of sowing	31-01-2015

Sterilization of crystal sands

Crystal sand was sterilized by placing it

in poly bags (of 5kg. capacity) at 121.6 °C (1.1kg/cm²) temp. for 30 minutes, in an autoclave.

Tray filling with sands and sowing of chickpea seeds

After sterilization, crystal sand was left overnight for proper cooling. Next day 21 plastic trays were filled with sterilized crystal sand @ 1kg per tray. In each tray 50 (10×5) bioprimed chickpea seeds were sown and covered with one thin layer of crystal sands.

Assessment of germination, root length, shoot length and vigour index of chickpea.

After sowing of chickpea seed watering/ spraying were done regularly at 3 days of interval through hand sprayer for maintained moisture in crystal sand. Number of germinated seeds was counted after 10 days of sowing and calculated germination percentage as following formula

$$\text{Germination \%} = \frac{\text{No. of germinated seeds} \times 100}{\text{No. of total sown seeds}}$$

Seedling length of the seed was recorded 20 days after treatments in all the experiments. Shoot and root lengths of the seeds were recorded on the basis of randomly selected ten plants per treatment in lab experiment (Dubey *et al.* 2011).

The dry weight of the seedling were measured on the basis of randomly selected 10 germinated seeds per experiment were placed in hot air oven at 60° C for 36 hours.

Two recommended methods were used as described by (Abdul Baki and Anderson 1973) for calculation of vigour index I and vigour index II was

Vigour index I = Germination per cent × seedling length

Vigour index II = Germination per cent × dry weight

Protein analysis of chickpea seedling through Kjeldahl method

Total protein content of chickpea seedling of different treatments were estimated by Kel plus (Pelican –ultima Deo Dist.) nitrogen analyzer. Total protein and nitrogen estimated by this method, multiplied by a factor for estimating the total protein content. About 1g dried and well-powdered sample was accurately weighting on a piece of filter paper and transfer along with the filter paper to 30ml micro Kjeldahl digestion tube. Then 15ml of conc. H₂SO₄ and 3g digestion mixture (C₄SO₄ K₂SO₄ 5:1)

added to digestion tube. Sample was digestion an electric heater at 400°C for 2h.

After cooling, the digest transferred to micro Kjeldahl distillation apparatus using successive small quantities of water. 20ml of 2.5 per cent NaOH solution poured in it and NH₃ liberated by steam distillation was collected in 100ml conical flask containing 20ml of 40 per cent boric acid solution in which few drops of mixed indicator added. Boric acid containing ammonium borate was titrated against N/10 standard HCL until the first appearance of pink colour at the endpoint. A reagent blank with filter paper (no sample, only digestion mixture and H₂SO₄) also run and titrated value for blank recorded. Percent N in the sample calculated by using following formula:

$$\text{Nitrogen \%} = \left(\frac{1.4 \times N \times V}{W} \right) \times 100$$

$$\text{Protein \%} = \text{Nitrogen \%} \times 6.25$$

Where, N = Normality of HCL, V = Titre value of sample – Titre value of blank

W = Weight of the sample

Protein estimation of chickpea seedling by Bradford method

The 4mg/ml of the BSA was used as standard. Different dilutions of the standard were made (0.5, 1, 2, 3 and 4mg) into tube 1 aliquot 100µl of D.W., this was served as blank. To each tube of standard and sample add 1ml of the Bradford reagent was added and mixed. All tubes were incubated at RT for 2min. After incubation period optical density was taken at 595nm by using spectrophotometer (Bio-Rad). Calibration curve was constructed by plotting absorbance reading on Y axis against standard protein concentration (mg/ml). Sample conc. was calculated using standard graph as a reference.

Protein estimation of chickpea seedling by Lowry method

The 4mg/ml of BSA standard was used. Different dilutions of the standard were made (0.5, 1, 2, 3 and 4mg). To each tube of standard and sample 2ml of complex forming reagent was added and kept for 10 min. at RT. After 10 minutes of incubation period, 0.2ml of Folin-Ciocalteu reagent solution was added to each tube and incubates for 20-30 minutes. After incubation period sample absorbance was taken at 660nm by using spectrophotometer (Bio-Rad). Calibration curve was constructed by plotting absorbance reading on Y axis against standard protein concentration (mg/ml). Sample concentration was calculated using standard graph as a reference.

Protein profiling of chickpea seedling through SDS-PAGE

Total protein was extracted using developed method by (Goggin *et al.* 2011). 0.5 g of Plant leaves were frozen by liquid nitrogen, grinding to a fine powder using mortar and pestle then transferred to a fresh centrifuge tube. Add two ml of extraction buffer (Tris-HCl 1M, pH 8, EDTA, 0.25), SDS, 10%, glycerol, 50%) and mix well. Centrifuge the content of the tubes at 12000rpm for 20 min at 4°C after centrifugation process completes discard the supernatant. Mix the pellets with 1ml of sample buffer (80% Acetone, 0.07% β-mercaptoethanol and 2mM EDTA) and centrifuge it at 12000 rpm for 15 minutes. Repeat this process until chlorophyll removes. When clear pellet comes mix this with mili Q water and store it at -20 Protein concentration of samples were determined using Bradford assay (Bradford,1976) and Lowry assay (Lowry *et al.*1951). BSA was used as a standard.

Table 1. *In vitro* effect on chickpea seeds induced by biopriming of *T. viride* (49CP)

Treatment	Germination %	Root length(cm)	Shoot length(cm)	Seedling length(cm)	Dry weight of seedling(g)	Vigour index I	Vigour index II
T ₁	68	12.07	16.37	28.44	0.592	1933.92	40.26
T ₂	58	11.25	15.25	26.50	0.550	1537.00	31.90
T ₃	57	10.00	14.50	24.50	0.518	1396.50	29.53
T ₄	56	8.70	14.35	23.05	0.495	1290.80	27.72
T ₅	49	8.68	14.85	23.53	0.484	1151.50	23.72
T ₆	45	8.09	13.15	21.24	0.499	955.80	22.46
T ₇	47	8.11	15.60	23.71	0.539	1114.370	25.33
CD@ 5%	5.455	2.711	1.345	-	0.061	-	-

RESULTS

Chickpea seeds were bioprimered with bioagent fungicide and pathogen, for assessment of germination per cent, root and shoot length, seedling length, dry weight, vigour index I and vigour index II. The data recorded are presented in Table 1.

The treatment T_1 was found to be significantly superior and effective in maximum germination (68%) of chickpea followed by T_2 (58%), T_3 (57%), T_4 (56%), T_5 (49%) and T_7 (47%) whereas T_6 (45%) has given the lowest germination per cent. Similarly, the beneficial impact of seed bioprimering was also recorded for root length, shoot length, seedling length and dry weight vigour index-I and vigour-II in which T_1 treatment excelled overall significant superior performance by contributing 12.07cm, 16.37cm, 28.44cm, 0.592g, 1933.92 and 40.26 in root length, shoot length, seedling length, dry weight, vigour index I and vigour index II, respectively. Whereas, T_6 treatment (10% *Foc*) were recorded for all these

lowest physiological attributes by contributing 8.09cm, 13.15cm, 21.24cm, 0.499mg, 955.80 and 22.46.

Germination and seedling length along with seedling dry weight are important attributes, which determine the quality of seed of any seed lot. Besides quality seed parameters, seed vigour index also plays very crucial role in predicting the fate of any seed lot under biotic and abiotic stress conditions.

Data presented in Table 1 also revealed that over all superior performance was contributed by T_1 treatment achieving the higher vigour index-I as well as vigour index-II. Whereas T_7 treatment (control) showed least superior impact than T_6 treatment (10% *Foc*) on germination per cent, root length, seedling length as well as vigour index-I and vigour index-II.

Total protein content was determined by multiplying a factor with the observed nitrogen values and presented in Table 2. A perusal of the data presented in Table 2, revealed that T_1 treatment showed having the highest nitrogen content (0.067%) followed by T_2 (0.063%), T_3 (0.057%), T_4 (0.051%), T_5 (0.046%), T_7 (0.044%) and T_6 (0.040%). Similarly, well as the highest protein content in T_1 (0.41%) followed by T_2 (0.39%), T_3 (0.35%), T_4 (0.31%), T_5 (0.28%), T_7 (0.27%) and T_6 (0.25%).

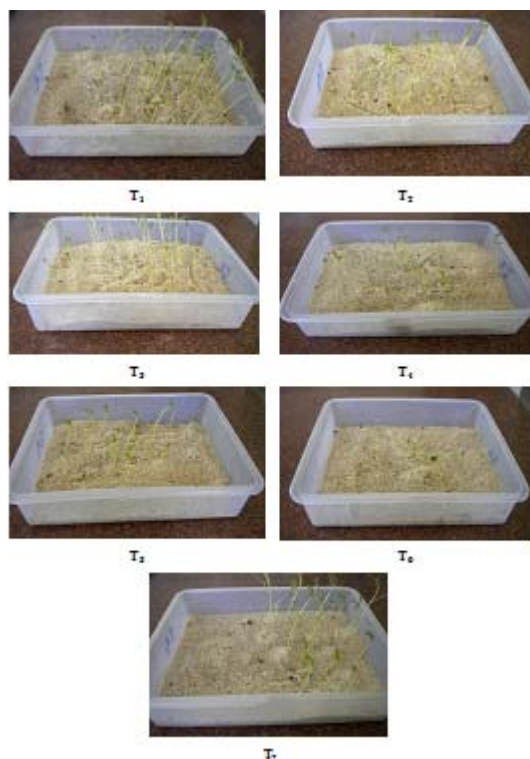


Fig.1. *In vitro* germination of chickpea seeds

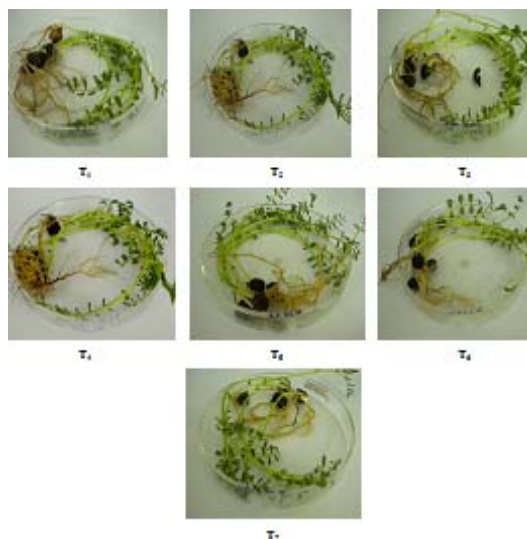


Fig. 2. *In vitro* seedlings of chickpea

Protein quantification of chickpea seedling by Lowry and Bradford method

The total protein content analyzed by Lawry and Bradford method showed (Table 3) variation in all the studied treatments. The higher levels of protein content recorded in the treatment T₁ that was 1.06 and 1.08 mg /ml. whereas the lowest protein content was observed in treatment T₆ was 0.73 and 0.63. Quantitative estimation clearly showed that the highest amount of protein content is present in Treatment T₁ in which chickpea seedlings were treated with bioformulation of *T. viride* @ 5g/kg seed.

Protein profiling of chickpea seedling on SDS PAGE

The chickpea protein profiles are shown in Fig. 3. In treatment T₁ five bands were observed, first band at 250 kda, second faint band at 100 kda, third band was lying between 75 and 50 kda, and fourth band was at 20 kda. In treatment 2 to 7 first band was occurred at 250 kda and second band

between 50 and 37. In treatment T5 a faint band occurred at 50 kda. From this study we can conclude that the application of formulation of *Trichoderma viride* @ 5g /kg seeds has induced some PR proteins in the chickpea seedlings. These PR proteins play an important role during abiotic and biotic stress conditions.

DISCUSSION

In vitro effect on chickpea seedling induced by biopriming of *Trichoderma viride* ie. the treatment T₁(*T. viride* 5g/kg seed) excelled overall significant superior performance by contributing 68%, 12.07cm, 16.37cm, 28.44cm, 0.592g, 1933.92 and 40.26 in germination, root length, shoot length, seedling length, dry weight, vigour index I and vigour index II, respectively. Similar results find with Amna *et al.* (2014) investigate the effect of *Trichoderma* sp. on growth activity of chickpea seedling. Germination

Table 2. Protein analysis of chickpea seedling) through Kelplus analyzer

Treatment	Titration reading	Nitrogen %	Protein%
T ₁	0.740	0.067	0.41
T ₂	0.710	0.063	0.39
T ₃	0.670	0.057	0.35
T ₄	0.655	0.051	0.31
T ₅	0.590	0.046	0.28
T ₆	0.550	0.040	0.25
T ₇	0.580	0.044	0.27
CD@ 5%	0.061	-	-

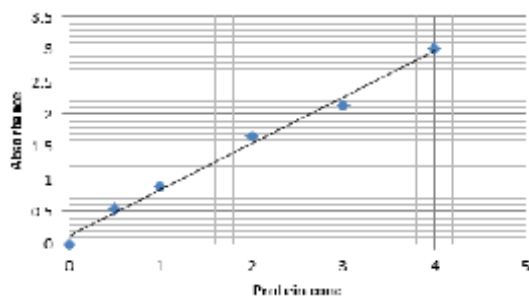


Fig.3: BSA standard curve of Lowry method

Table 3. Protein quantification of chickpea seedling Lowry and Bradford method

Treatment	Lowry method (660nm)Protein content (mg/ml)	Bradford method (595nm)Protein content (mg/ml)
T ₁	1.06	1.08
T ₂	1.02	1.01
T ₃	1.01	1.00
T ₄	1.00	0.89
T ₅	0.98	0.82
T ₆	0.63	0.73
T ₇	0.96	0.80

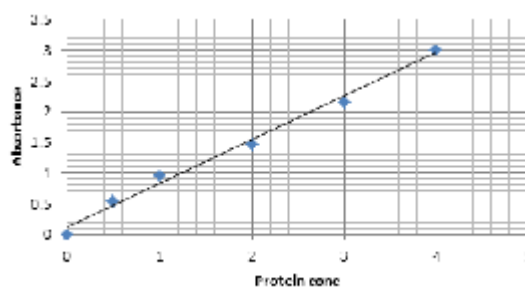


Fig.4: BSA standard curve of Bradford method

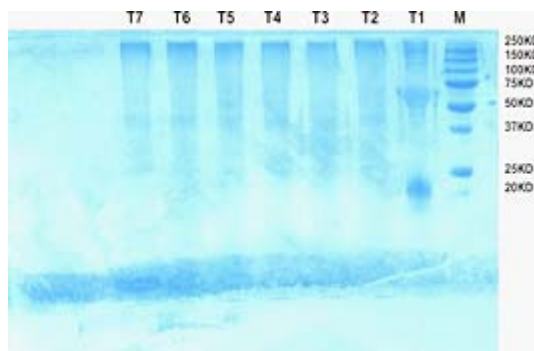


Fig. 5. Protein profiling of chickpea seedling on SDS PAGE

percentage (90, 70) and vigour index-I (1657.8, 1364.3) were enhanced by *T. harzianum* and *T. viride* respectively.

Kumar *et al.* (2014) Chickpea seeds were treated with different concentrations of *Trichoderma* bioformulation such as 5%, 10%, 20% gm/kg seed followed by treatment with 0.2% Bavistin. As a result, the percentage of seed germination was found to be higher in *T. harzianum* (*Th. azad*) & *Trichoderma viride* (01PP) treated seeds with 5% bioformulation as compared to the other concentrations. Various attributes with their observations include seed germination (92% and 90%), root length (12.38 and 12.19 cm) shoot length (4.97 and 4.32 cm) seedling length (17.38 and 16.50 cm), dry weight (1.19 and 1.88 cm), vigour index I (5197.12 and 1485) and vigour index II (109.48 and 169.20). Among all treatments, control showed the poorest performance for all seven seed quality attributes.

The highest nitrogen and protein content was observed in chickpea seedling that's bioprimered by *Trichoderma viride* formulation was estimated through Kelplus analyzer showed treatment T₁ (0.067%) nitrogen and protein (0.41%) compared to control T₇ (0.044%) nitrogen and (0.27%) protein. Similar finding supported with Shahid *et al.* (2015) studied that Biochemical effect of mycolytic enzymes produced by *Trichoderma* which enhanced nitrogen (0.304%) and protein content (1.89%) of chickpea seedlings compare to control, when seeds were treated with *Trichoderma*. *Trichoderma* treated plant showed a significant stimulatory effect on all the tested eight parameters (germination %, root length shoot length dry

weight, vigour index I and vigour index II) as compared to control.

Lowry and Bradford method showed variation in all the studied treatments. The higher levels of protein content recorded in the treatment T₁ that was 1.06 and 1.08mg/ml. whereas the lowest protein content was observed in treatment T₆ was 0.73mg/ml and 0.63mg/ml. Quantitative estimation clearly showed that the highest amount of protein content is present in Treatment T₁ in which chickpea seedlings were treated with bioformulation of *Trichoderma viride* @ 5g/kg seeds.

The protein profiling of chickpea seedling on SDS PAGE were observed, in treatment T₁ five bands, first band at 250kda, second band at 100kda, third band was lying between 75 to 50kda, fourth band was at 20 kda. From Treatment 2 to 7 the banding pattern is same. From this study we can conclude that the application of formulation of *Trichoderma viride* @ 5g/kg seeds has induced some PR proteins in the chickpea seedlings. These PR proteins play an important role during abiotic and biotic stress conditions. Rajik *et al.*, (2012) found maximum increase in soluble protein content was in (T₁) *T. harzianum* (Kan.) treated plant showing 35.04 mg/g of fresh leaves against 20.49 mg/g of fresh leaves in case of control-II. A high content of phenols which are the indication of first stage of defense mechanism was also recorded in treated plant with *T. harzianum* (Kan.) treatment representing 2.62 g/mg of fresh leaves against 1.38 in control-I at 20 days of inoculation. The disease severity showed negative correlation with soluble protein ($r = -0.6364$) and total phenol ($r = -0.7653$). Protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the soil treated with *T. harzianum* (Kan.) to synthesize some new protein representing maximum number of 22 bands.

Yadav *et al.* (2015) reported 14 per cent disease incidence was recorded from salicylic acid treated plants. Challenge inoculation with abiotic inducers sensitized the seedlings to produce increased level of soluble proteins. Maximum increase of soluble protein content (24.26 and 24.72mg/g of fresh leaves) was found with salicylic acid treated plant at 5 and 10 days after pathogen inoculation. Similarly, phenol content was (24.26

and 24.72mg/g of fresh leaves) also found to be maximum in salicylic acid treated plant at 5 and 10 days of inoculation. Protein profiling by SDS-PAGE revealed that foliar spray with salicylic acid induced the synthesis of new proteins; however, such new proteins were not recorded in control.

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