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Plant growth promoting activities of a fungal strain *Penicillium commune* MCC 1720 and it's effect on growth of black gram

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Abstract

In the present study Plant Growth Promoting Fungi (PGPF) were isolated from the rhizospheric soil of rice. Five PGPF were isolated and different plant growth promoting traits were screened. Among them PS2 showed the best PGP traits like phosphate solubilisation, IAA, HCN, ammonia, siderophore production etc. PS2 was identified as *Penicillium commune* by 18S r DNA sequencing. Further experiments were carried out with PS2. For pot culture assay healthy Black Gram- *Vigna mungo* (L.) Hepper seeds were inoculated with PS2. The PS2 treated seeds exhibited better germination percentage and seedling vigour index than the untreated control. After 30 days of treatment plant showed better root, shoot length, biomass production and photosynthetic efficacy over the control. In present day modern agricultural system is trying to find out the alternatives to reduce the detrimental effects of chemical fertilizers. So, PS2 may be used as a potent biofertilizer to overcome this problem.

Keywords: Plant growth promoting fungi (PGPF), *Penicillium commune*, black gram, *Vigna mungo*, biofertilizer

1. Introduction

Fertilizers and pesticides are the major part in the modern agricultural system. Indiscriminate use of pesticides herbicides and fertilizers create a great threat in agricultural practices resulting in poor soil quality and reduced crop production. So this is the high time to find an alternate green strategy to solve this problem. Efforts should be given on the strategies based on nontoxic or less hazardous use of alternatives for plant growth and nutrient management. Plant growth promoting microorganisms (PGPM) draws attention to agricultural scientists and environmentalist to solve this burning issue. Rhizosphere is the most complex and biologically active hot spot for rhizospheric microflora [1]. They flourish and nourished with the help of rhizodeposits secreted by plants and create a beneficial healthy relationship with plant [2]. Rhizospheric microflora are of various kinds such as viruses, nematodes, bacteria, fungi, oomycetes, algae etc. Among them Plant Growth Promoting Rhizobacteria (PGPR) and Plant Growth Promoting Fungi (PGPF) are the two major important groups which directly and indirectly promote plant growth in several ways. Hyakumachi (1994) [3] documented the beneficial role of multipurpose PGPF for plant growth promotion. Several heterogenous nonpathogenic fungi documented as PGPF by many researchers but among them *Aspergillus*, *Penicillium*, *Phoma*, *Trichoderma*, *Piriformospora*, *Fusarium* etc. are well recognized [4-6]. Hyakumachi (1994) [3] revealed that about 44% of rhizospheric fungi are PGPF and according to Khalmuratova *et al.* (2015) [7] *Fusarium* (25%) is the most dominant genera followed by *Penicillium* (12.5%) and *Alternaria* (7.5%). Recent researches have revealed that PGPF secretes many growth promoting compounds such as hormones and increase seed germination, photosynthetic efficacy, root, shoot growth, flowering, crop yield etc [8, 9]. So the objectives of the present work is the isolation of PGPF strains from the rhizospheric soil of rice field of Burdwan district, characterization of plant growth promoting traits like-phosphate solubilization, production of indole acetic acid (IAA), HCN, ammonia, siderophore, exopolysaccharides and Nitrogen fixation etc. under *in vitro* condition, isolation of the PGPF exhibiting the best plant growth promoting traits and it's identification by 18S rDNA sequencing followed by it's application for improvements of growth of Black gram - *Vigna mungo* (L.) Hepper.

2. Material and methods

2.1 Isolation of fungi from rhizospheric soil

Rhizospheric soil of rice were collected from rice field of agricultural farm, Golapbagh Campus, Burdwan University (Latitude- N 23°14'20.86", Longitude- E 87°51'45.743"). Soils were kept aseptically in a sterilized plastic bag. For serial dilution technique about 1 gm of soil was taken and dissolved into 10 ml of double distilled water in a test-tube and serially diluted upto 10 times. PDA media [Infusion from potatoes 200 g/l Dextrose (Glucose) 20 g/l Agar 15 g/l pH after sterilization (at 25 °C) 5.6±0.2] was used for this purpose. The petri-plates were incubated in BOD incubator at 30 °C. 5 different fungal isolates with distinct morphology were selected and further pure cultured and maintained in PDA slants for further use.

2.2 Characterization of Plant Growth Promoting Traits (PGP) *in vitro* condition

2.2.1 Estimation of phosphate solubilisation

For qualitative estimation of phosphate solubilisation fungal isolates were inoculated into PKV Agar (Yeast extract 0.5 g/l Dextrose 10 g/l Calcium phosphate 5 g/l Ammonium sulphate 0.5 g/l Potassium chloride 0.2g/l Magnesium sulphate 0.1g/l Manganese sulphate 0.0001g/l Ferrous sulphate 0.0001g/l Agar 15 g/l) plates and kept in BOD incubator at 30 °C for 3days. Clear halo zone surrounding the colony is the positive indication for phosphate solubilization. PSI (phosphate solubilisation index) was determined using following formula^[10] (Table-2)

$$\text{PSI} = (\text{Colony diameter} + \text{Halozone diameter}) / \text{Colony diameter.}$$

For quantitative estimation fungal isolates were grown in PKV broth in a shaker incubator at 180 rpm for 5 days. Then the culture was centrifused at 10000 rpm for 10 minutes. Quantity of phosphorus in the supernatant was estimated by vanado-molybdate-yellow colour method. 0.5ml of supernatant was added to 2.5 ml of Barton's reagent and volume made upto 50ml with distilled water and O.D value was taken at 430nm after 15 minutes. Concentration of solubilising phosphate was estimated using a standard curve of KH_2PO_4 ^[11]. (Fig 1)

2.2.2 Estimation of IAA production

Fungal strains were inoculated in 0.5% Tryptophan supplemented PDA plates. Then the plates were incubated for 3 days on BOD incubator at 30±2°C. After 72 h of growth culture plate was flooded with Salkowski's reagent (35% of Perchloric acid, 1 ml 0.5 M FeCl_3) solution, and extra solution was decanted. After 5 to 10 minutes if red or pinkish red zone appeared surrounding the fungal colonies then it was a positive indication of IAA production^[12].

For quantitative estimation fungal strains were grown in 0.5% Tryptophan supplemented PD broth and was kept for 3 days on a rotary shaker incubator at 180rpm, 30±2°C. After 72 h of growth, the culture was centrifuged at 10000 rpm for 15 minutes and 1 ml of supernatant was taken in a test tube mixed with equal volume of Salkowski's reagent (35% of perchloric acid, 1 ml 0.5 M FeCl_3) solution. Then the reaction mixture was kept at dark condition for 20 minutes. Appearance of pink color in the test-tube indicated the production of IAA. After that absorbance was taken at 533 nm. Production of IAA was estimated using a standard curve of Auxin^[12]. (Fig 2)

2.2.3 Quantitative estimation of Exopolysaccharide (EPS) production

Exopolysaccharide production was assayed by following the method of Dubois *et al* (1956)^[13]. The fungal isolates were grown in PKV broth and kept in a shaker incubator at 180 rpm and 30 °C for 3 days. After that growth culture was centrifuged at 8000 rpm for 20 minutes. Then 3 ml of acetone was added to 1 ml of supernatant and recentrifuged at 6000 rpm for 10 minutes. 3 ml of Acetone was added to the pellet and again centrifuged. This process was repeated for 3-5 times. After that, this precipitate was suspended in de ionized water. 1 ml of 5% aqueous phenol, 1 ml of this suspension, 5 ml concentrated Sulphuric acid was added and the mixture and kept for 20 minutes. Appearance of orange red colour indicates the presence of polysaccharide. Optical density (O.D) was taken at 490 nm and the concentration was determined by using the standard curve of glucose. (Fig 3)

2.2.4 Production of Ammonia

Production of ammonia was determined by following the method of Cappuccino *et al*, (1992)^[14]. 48 hrs old fungal cultures were inoculated in 10 ml peptone water and incubated for 2-3 days at 30±2 °C. 0.5 ml of Nessler's reagent was added with each experimental set. The transformation of brown to yellow colour was positive indication for ammonia production. (Table-1)

2.2.5 Production of HCN

Screening of fungal isolates for hydrogen cyanide (HCN) production was done as per methodology described by Lorck H (1948)^[15]. Fungal isolates were streaked into 4.4 g/l glycine supplemented PDA medium. A no 1 whattman filter paper was soaked into 2% sodium carbonate, 0.5% picric acid solution. After that it was placed into the upper lid of petriplate. Petridishes were sealed with parafilm and incubated for 4 days at 30±2 °C. Transformation of yellow to orange red colour of filter paper confirmed HCN production. (Table-1)

2.2.6 Production of Siderophore

Siderophore production was detected by the method of Schwyn and Neilands (1987)^[16] using blue agar plates containing the dye chrome azurol S (CAS). Siderophore production was confirmed by appearance of orange halo zone around the colonies on blue plates. (Table-1)

2.2.7 Selection of best performing fungal strain

On the basis of plant growth promoting traits the isolate showing the maximum traits was selected as the best performing strain. Microscopic Characters was determined. Pot culture assay was performed with the selected fungal strain. (Fig 5 & 6)

2.2.8 Identification of the selected fungal isolate

The best performing fungal strain (PS2) was sent to NCMR-NCCS, Pune for molecular identification using 18S rRNA gene sequencing method. Phylogenetic tree was prepared using MEGA 7.0 software. (Table -3; Fig 4)

2.3 Evaluation of different growth parameters of Black gram- *Vigna mungo* (L.)Hepper. in pot culture

2.3.1 Seed inoculation with fungal strain

Seed inoculation with strain was done by following the methods of Yadav *et al* (2011)^[17]. Healthy seeds of Black

gram (cultivar Tau-1) seeds were surface sterilized with 0.1% HgCl₂ for 5 min and then 70% ethyl alcohol for 2 min. Then the seeds were rinsed for five to six times by sterile distilled water. Fungal strain was grown in PD broth on shaking incubator (180 rpm) at 28±2 °C for 24 h. The healthy surface sterilized seeds of Black gram were soaked into the spore suspension for 30 minutes with an adhesive agent (16% Arabic gum). After that the seeds were air dried. Pots were filled with 250gm of autoclaved soil. Each pot received five seeds and were sown at 5 cm depth. Seeds treated with distilled water were recognized as control and was also maintained. Pots were kept in to plant growth chamber for 30 days and irrigated with distilled water after regular interval.

2.3.2 Evaluation of Germination percentage of seed

Germination percentage (GP) was calculated using this formula (Fig 7)

$$GP = \frac{\text{Total no. of seed germinated}}{\text{total no of seeds in all replicates}} \times 100$$

2.3.3 Evaluation of Seedling vigour index (SVI)

SVI was determined following this formula^[18] (Fig 8)
 SVI= [(mean root length + mean shoot length) × GP]

2.3.4 Assessment of root-shoot length, fresh weight and dry weight of plant

After harvesting the shoot and root length were recorded in centimetre and fresh and dry weight were recorded in gram of each plant. For estimation of dry weight plants were dried in a hot air oven at 65 °C for 3 days and weighed. (Table-3)

2.3.5 Estimation of chlorophyll from leaves

2g of fresh leaf tissue was taken from each replicates and homogenized with 80% acetone and after that centrifuged at 10,000 rpm for 8 minutes, this process was repeated for several times until the precipitate became colourless. Then OD value of supernatant was recorded at 645 nm and 663 nm^[19].

The amount of chlorophyll was calculated by this formula:

Chlorophyll a: 12.7(A663) – 2.69(A645)

Chlorophyll b: 22.9(A645) – 4.68(A663)

Total Chlorophyll: 20.2(A645) + 8.02(A663) (Table-3)

2.3.6 Statistical analysis

The data obtained were analyzed using Student “t” test for analysis comparison. The values were represented as mean ±SD (n=3). Probability value (p value) less than 0.05 was considered as statistical significant.

3. Results

Table 1: Plant growth promoting traits of fungal isolates

Name of the isolate	Phosphate Solubilization	Production of IAA	Production of Ammonia	Production of siderophore	Production of Exo-polysaccharide	Product-ion of HCN
PS2	+++	+++	++	+	+++	+
PS5	++	+	++	-	++	-
PS7	-	+	+	-	+	-
PS10	-	++	+	+	++	+
PS12	++	+	++	-	+	+

Results of qualitative tests done for the isolated PGPF where vigorous is represented by ‘+++’, good growth by ‘++’, poor growth by ‘+’ and ‘-’ represents no growth

Table 2: Phosphate Solubilization Index (PSI) of five fungal Isolates

PSI	PS2	PS5	PS7	PS10	PS12
	2.23±0.11	1.40±0.06	-	-	1.56±0.09

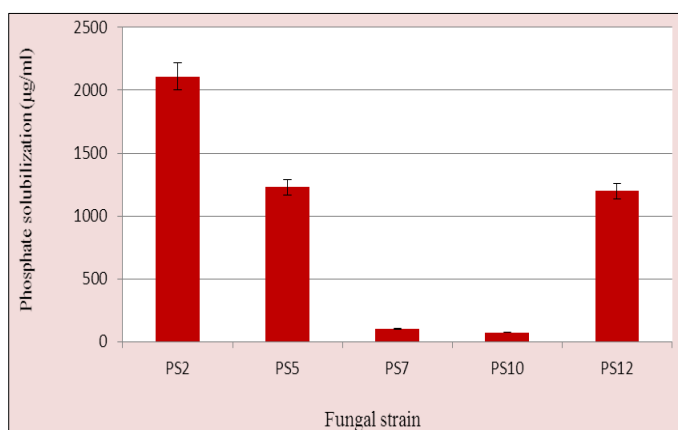


Fig 1: Quantitative estimation of phosphate solubilization

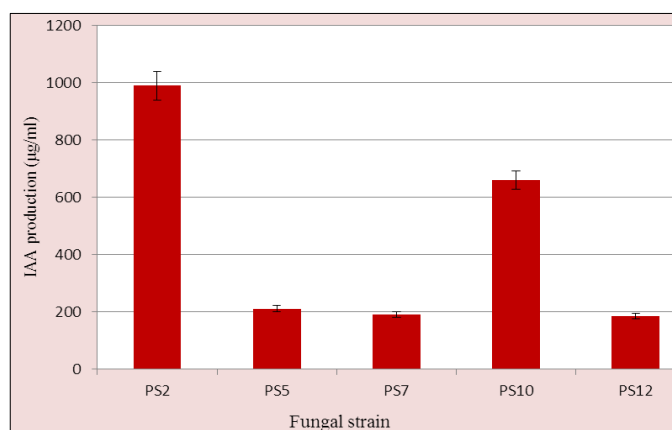


Fig 2: Quantitative estimation of IAA production

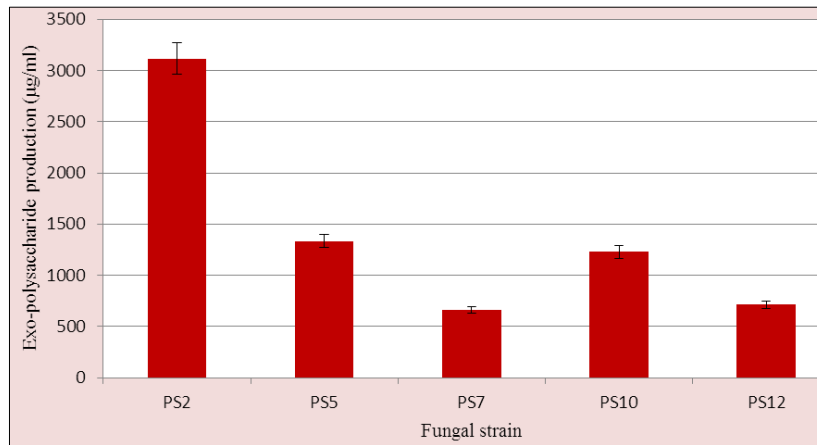


Fig 3: Quantitative estimation of Exo-polysaccharide production

Table 2: Molecular identification of fungal strain PS2

Strain designation	Closest neighbour	% similiarity	Accession number
PS2	<i>Penicillium commune</i> CBS 343.51	99	MCC 1720

Sequence in FASTA format

```
>GCTCTGGGGGTCGTTGCCAACCCTCCTGAGCCAGTCCGAAGGCCTCACTGAGCCATTCAATCGGTAGTAG
GACGGGCGGTGTGTACAAAGGGCAGGGACGTAATCGGCACGAGCTGATGACTCGTGCCTACTAGGCATTCTCGTT
GAAGAGCAATAATTGCAATGCTCTATCCCAGCACGACAGGGTTTAAACAAGATTACCCAGACCTCTCGGCCAAGGT
GATGTACTCGCTGGCCCTGTCAGTGTAGCGCGCGTGGCGCCAGAACATCTAAGGGCATCACAGACCTGTTATTGC
CGCGCACTTCCATCGGCTTGAGCCGATAGTCCCCcTAAGaAGCCAGCGGCCCGCaAATGCGGACCGGGCTATTTAAG
GGCCGAGGTCTCGTTTCGTTATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCATC
CAAAAGATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTTTGTCTGGACCTGGTGAGTTTCCCCGTGTTGAGTCA
AATTAAGCCGAGGCTCCACGCCCTTGTGGTGCCTTCCGTCAATTTCTTTAAGTTTCAGCCTTGCGACCATACTCCCC
CCAGAACCCAAAACTTTGATTTCTCGTAAGGTGCCGAACGGGTCATCATAGAATCCCGTCCGATCCCTAGTCGGC
ATAGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCCCTGATTAATGAAAACA
TCCTTGGCGAATGCTTTCGCAGTAGTTAGTCTTCAGCAAATCCAAGAATTCACCTCTGACAGCTGAATACTGACGC
CCCCGACTATCCCTATTAATCATTACGGCGGTCTAGAAACCAACAAAATAGAACCGCACGTCTTATTCTATTATTC
CATGCTAATGTATTTCGAGCAAAGGCCTGCTTGAACACTCTAATTTTTTTCACAGTAAAAGTCTGTTCCCCCACA
GCCAGTGAAGGCCATGAGGTTCCCCAGAAGGAAAGGTCCAGCCGGACAGTACTCGCGGTGAGGCGGACCGGCCAG
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CTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATTCCTCCGTTACCCGTTGCCACCATGG
TAGGCCACTATCCTACCATCGAAAGTTGATAGGGCAGAAATTTGAATGAACCATCGCCGGCGCAAGGCCATGCGAT
TCGTTAAGTTATTATGATTCACCAAGGAGCCCCGAAGGGCGTTGGTTTTTTTATTTAATAAATACACCCCTTCTGAA
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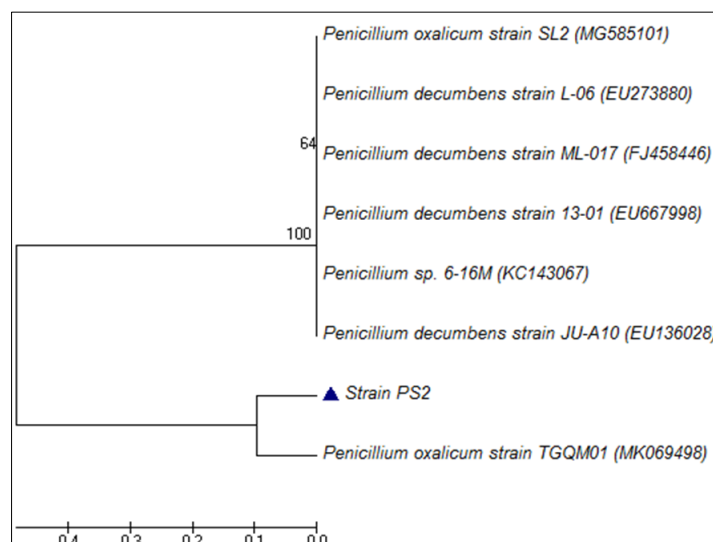


Fig 4: Phylogenetic tree of the strain PS4

The phylogenetic tree showing the position of Strain PS2 (Blue marked) among the related taxa based on 18S rRNA gene sequences. Bootstrap values expressed as percentages are given at branch points and strain PS2 formed a tight cluster with *Penicillium oxalicum* Strain TGQM1 with 100 % bootstrap confidence. Accession numbers are given in parentheses.



Fig 5: Pure culture of PS2

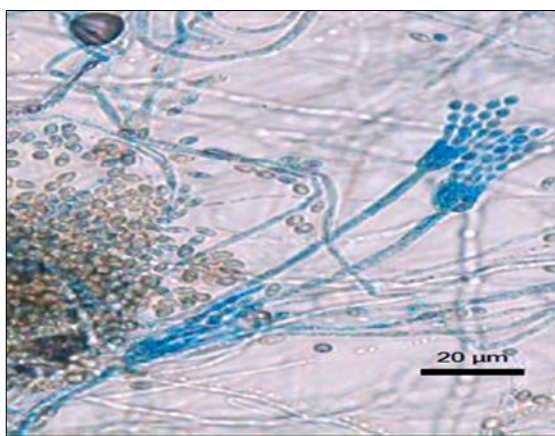


Fig 6: Microscopic View of *Penicillium commune*

3.1. Microscopic characteristics of *Penicillium commune*

Hyphae: entire, smooth, hyaline with width 1.39-2.05 (1.79) μm . Conidiophore: width 1.72-2.71 (2.25) μm . Phialospore: length 3.37-5.54 (4.3) μm and width 2.39-3.65 (3.06) μm . Phialide: length 11.4-13.7 (12.81) and width 1.72-2.72 (2.09) μm .

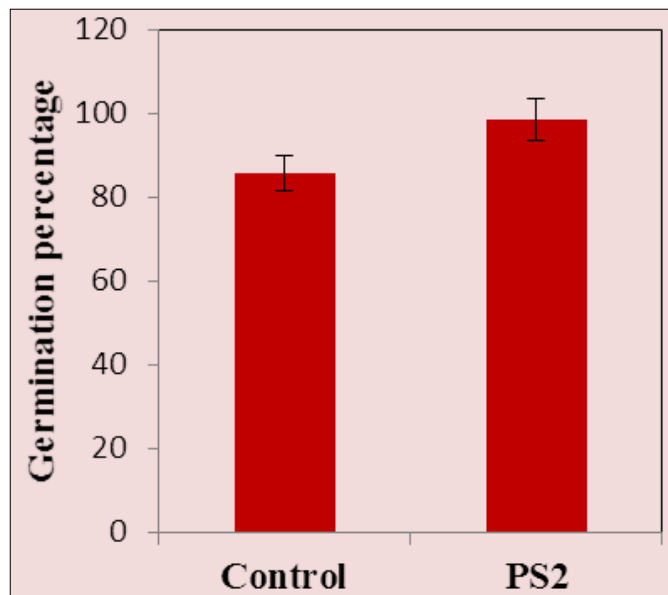


Fig 7: Percentage of seed germination in control and PS4 treated Black gram plant

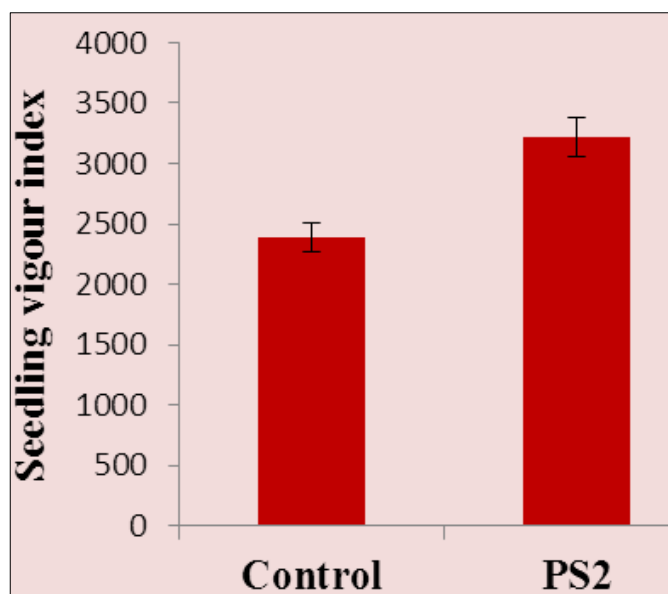


Fig 8: Seedling Vigour index of Control and PS4 treated Black gram plant

Table 3: Different growth parameters of control and treated Black gram plant

Growth parameters	Control		Treated	
	15 days	30days	15days	30 days
Root length (cm)	7.26±0.31	12.85±0.57	13.09±0.51*	20.11±0.83*
Shoot length (cm)	10.95±0.42	23.05±0.94	15.34±0.69*	30.24±1.31*
Plant fresh Weight (gm)	3.8±0.13	10.78±0.41	8.60±0.52	25.09±1.06*
Plant dry Weight (gm)	0.45±0.05	0.97±0.04	0.90±0.06	3.06±0.14*
Chl a (unit/g fr. weight)	0.67±0.03	0.71±0.06	0.99±0.04*	1.96±0.11
Chl b (unit/g fr. weight)	0.24±0.02	0.32±0.03	0.74±0.05	1.23±0.07
Total Chl (unit/g fr. weight)	0.98±0.03	1.03±0.03	1.87±0.08*	3.19±0.12*

The values are represented as mean \pm SD (n=3) $p < 0.05$ as compared to control

4. Discussion

PGPF generally colonize in the vicinity of root region of plant and create a beneficial relationship with wide range of host plants. PGPF exhibited many PGP traits which directly or indirectly increase the vigour and productivity of plant. In this

study five fungal strains were isolated from rhizospheric soil. Different PGP traits like phosphate solubilization, IAA production, HCN, ammonia, siderophore production etc were examined *in vitro* condition. Among five isolates PS2 showed the best performance. PS2 was identified as *Penicillium*

commune by 18s rDNA sequencing method. PS2 established itself as a good phosphate solubilizer in broth culture and showed highest phosphate solubilizing index. Mittal *et al* (2008) [20] also find the similar result with *Aspergillus* sp. and *Penicillium* sp. after six days of inoculation. Result revealed that PS2 is a potent IAA and EPS producer. When Black gram seeds inoculated with PS2 it showed higher germination percentage and seedling vigour index than uninoculated control. Doni *et al.* (2014) [21] find similar result with *Trichoderma* inoculated rice seed germination and SVI. Mushtaq *et al.* (2012) [22] revealed that presoaking of seeds with culture filtrates of the nine *Penicillium* isolates effectively increase seed germination in tomato than the control seeds. Vujanovic and Goh (2012); Islam *et al.* (2014a, b) [23-25] and many other researchers found that better germination and seedling vigour in PGPF treated plants. In case of root, shoot growth and biomass production PS2 inoculated Black gram plant showed higher root, shoot length, biomass and chlorophyll production than control plant after 15 and 30 days. Mittal *et al.* (2008) [20] has been reported the similar result with six phosphate-solubilizing fungi (two strains of *Aspergillus awamori* and four of *Penicillium citrinum*) isolated from rhizospheric soil of various crops, in pot assay of chickpea plants (*Cicer arietinum* L. cv. GPF2). Saber *et al.* (2009) [26] also reported that the similar result in mung bean inoculated with *A. niger* and *Penicillium* sp. where significant increase in growth, seed yield, photosynthetic efficacy and nodulation status was observed in comparison to control.

5. Conclusion

It can be concluded from above study that PS2 established itself as a very efficient PGPF and has a significant role in Black gram plant growth promotion under laboratory condition. So as an inoculants PS2 can be effectively use in the formulation of biofertilizers and provide an alternative ecofriendly strategy in modern agricultural system.

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