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Impact Assessment of Varied Agroclimatic Conditions on Phosphate Solubilization Potential of Fungi in Fermentation and Soil-Plant System

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In this work, two phosphate solubilizing fungi *viz., Aspergillus tubingensis S33* and *A. niger* S36 were studied under different physiochemical and nutritional parameters in the lab, and in vitro under soil-plant experiments at two very distinct agro-climatic conditions *viz*., Banasthali, Tonk (Rajasthan), and Dwarahat, Almora (Uttarakhand), India. Phosphate-solubilizing capability was checked with different carbon and nitrogen sources. Maltose, glucose, and fructose were optimal carbon source in *A. tubingensis* S33 while fructose in the case of *A. awamori* S33. Amongst nitrogen sources, S33 showed maximum phosphate solubilization with ammonium sulfate while, S36 with ammonium sulfate and sodium nitrate. Ammonium was more stimulating than nitrate as the chief nitrogen source. In vivo experiments revealed that solubilization was noticeable at all the temperatures, but optimal temperature was 25–35°C. The optimal initial pH for Tricalcium Phosphate (TCP) solubilization was 8.0. The ideal concentration of TCP for solubilization was 7.5 $g{\cdot}l^{-1}$. The application of both strains in two different geographical sites exhibited a significant ($p<0.05$) rise in wheat growth, grain yield, and available Phosphorus (P). Fungal inoculation with TCP amendment exhibited a more notable effect on growth, yield, and soil fertility than control. This study support that these isolates will be able to work efficiently in varied climatic conditions and will show consistent efficiency on field application.

Keywords: *Aspergillus niger*, *Aspergillus tubingensis*, Bio-inoculant, Solubilizing microbes, Wheat

Introduction

Excessive application of chemical fertilizer not only affects the environment, soil health, and economy but also the availability of nutrients. When mineral phosphate fertilizer is applied to soil, only 10–25% is available to plant, and the rest is changed to the unavailable form by precipitation with a cation such as Fe^{3+}, Mg^{2+}, Al^{3+}, and Ca^{2+}. In nature, many microbes can remobilize this unavailable form of P and are called phosphate solubilizing microbes (PSMs). These microbes are present in small numbers, so they are isolated from the environment, multiplied, and further artificially re-inoculated in high numbers in the soil and called biofertilizers.[1–5]

There are several reports where isolates showed high phosphate-solubilizing potential in labs, but sporadic literature on successful consistent field application.[1,2,6] The possible reason was the climate that produced different biotic and abiotic stresses in varied geographical region.[7,8] Rashid *et al.*[9] stated that treatment of wheat with PSMs in the different ecological zone of the same state (Punjab) showed variation in growth and yield. Abiotic stress includes varied nutritional and mineralogical conditions *viz*., type of carbon, nitrogen, un-available phosphorus sources, and their concentration; physical conditions *viz*., pH, temperature, humidity, etc. that differ from soil to soil and with geographical regions. All these abiotic with biotic stress make the performance of PSMs inconsistent. Therefore, strains with broad-spectrum performance are more suitable for development of biofertilizers. Keeping the above points in mind, the current study aimed to characterize the phosphate-solubilizing fungal (PSF) strains *viz*., *A. tubingensis* S33 and *A. niger* S36 to varied physiochemical and nutritional condition. In addition, the assessment of these strains as potential biofertilizers for wheat was done at two different agro-climatic locations *viz*., Banasthali, Tonk, Rajasthan, and Dwarahat, Uttarakhand, during the same period to compare the effectiveness of these bio-inoculants.

Materials and Methods

Microorganisms

The microorganisms *viz*., *A. tubingensis* S33, and *A. niger* S36 were previously separated from rhizosphere of

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Vigna radiate cultivated at Banasthali, Rajasthan, India. They were initially recognized on the basis of colony morphology and staining methods. Further, affirmation was done by molecular methods by amplifying the fungal DNA (ITS1-5.8S-ITS4 region) by using universal primers ITS-1 and ITS4. The PCR reaction mixture comprised of DNA (100–200 ng), Tris–HCl (pH 8.4) (20 mM); dNTPs (0.1 mM); $MgCl_2$ (1.5 mM); primer (0.3 μM); and Taq polymerase (1.5 U) (Bangalore Genei, India) with final volume 25 μl. The reaction mixture was kept in a Mastercycler (BIO-RAD Gene Cycler™) with initial denaturation at 95°C for 4.5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension phase at 72°C for 30 seconds, followed by final extension phase at 72°C for 3 minutes. PCR products were extracted on agarose gel (1.2%) and cleaned by Geneaid Biotech. Ltd. Gel/PCR DNA fragment extraction kit. The PCR product was sequenced at Banglore Genei.[1]

The comparative analysis of fungal ITS1-5.8S-ITS2 region by the Basic Local Alignment Search Tool (BLAST) confirmed S36 as A. niger and S33 as A. tubingensis. These sequences were deposited under accession numbers JF313460 (S33) and JF313461 (S36) in the National Center for Biotechnology Information (NCBI).[1] Both strains were sub-cultured every month on potato dextrose agar (PDA) slants and stored at 4°C.

Effect of Carbon and Nitrogen Source

The effect of carbon source on phosphate solubilization was studied by replacing the glucose (1%) present in Pikovaskaya's (PVK) broth with maltose, fructose, mannitol, sucrose and sorbitol. In the second experiment, ammonium sulfate (AS; 0.5%) was replaced with varied nitrogen (N) sources viz., Ammonium Nitrate (AN), Ammonium Chloride (AC), Potassium Nitrate (PN), Sodium Nitrate (SN), and urea (U) in PVK broth. One ml spore suspension (5×10^5 CFU·ml^{-1}) was inoculated in all the flasks and incubated at 130 rpm and 30 ± 2°C in a incubator shaker for 6 days. Uninoculated broth was treated as control and samples were withdrawn at each 48 h.

Sixth-day culture broth of different carbon sources was filtered and the filtrate was used for organic acid examination by high-performance liquid chromatography (HPLC; SCL-10AVP Shimadzu), using ODS-3 column (Whatman).[10]

Effect of Different Concentrations of Tricalcium Phosphate (TCP)

The impact of TCP concentrations on soluble P was studied by varying TCP concentrations viz., 1, 2, 3, 4, 5, 7.5, and 10 g·l^{-1} in PVK broth. For inoculation and incubation, same method was used as above.

Effect of Different Physical Factors

To study the impact of pH, the initial pH of PVK broth was adjusted at 6, 7, 8, 9, and 10. To study the P solubilization efficiency at various temperatures, spore inoculated flasks were kept at distinct temperatures viz., 15, 25, 35, and 45 ± 2°C on a shaker (130 rpm, 12 days). All experiments were executed in triplicate.

Chemical Analysis of Culture Broth

The qquantitative soluble P determination was done by molybdenum-blue method[11] and the pH was measured by digital pH meter.

Inoculum Preparation

For inocula preparation, both strains were cultured on PVK agar media at 30°C for 5 days. The spores were collected from the plate using autoclaved distilled water, and 5×10^8 CFU·ml^{-1} adjusted by hemocytometer.

Soil- Plant Experiment

Pot experiments were conducted at two different agro-climatic sites *viz.*, the semi-arid climate of Banasthali, Rajasthan (26.38ºN 75.87ºE, 315 m altitude) and the subtropical climate of Dwarahat, Uttarakhand (29.78ºN 79.43ºE, Himalayan hilly terrain, 1467 m altitude) (Table 1). Soil-plant experiment was conducted in Plastic pots (2 kg) with unsterile soil in an entirely randomized block design. The soil was sieved (mesh, 2 mm), mixed with 0.1% TCP, and then filled in pots. The experiment consisted of nine treatments (with three replication

Table 1 — Climatic and physio-chemical properties of soils of two experimental sites

Site	Agro-climatic region	Soil type	Altitude/latitude	Climate	pH	Available P (kg·ha^{-1})	Organic C
Dwarahat, District Almora, Uttarakhand	Hills, Western Himalayas	Loamy	29·7833/ 79·4333 1467 M above from the sea level	30–70 mm rain fall; 12–22°C in summer; 1–5°C in winter	6.5	9	0.322%
Banasthali, District Tonk, Rajasthan	Semi-arid Eastern plain region	Sandy loam	26.4083 / 75.8649 289 M above from sea level	500–700 mm rain fall 32–45°C in summer; 12–15°C in winter	8.5	5.08	0.78%

each): (1) control (only soil), (2) soil+TCP (19.97 $mg \cdot g^{-1}$ P); (3) soil + SSP; (4) soil + S33; (5) soil + S36; (6) soil + S33 + S36; (7) soil + TCP + S33; (8) soil + TCP + S36; (9) soil + TCP + S33 + S36. Soil and soil + TCP were taken as controls.

Surface sterilization of seeds was done with 0.1% NaOCl solution treatment followed by distilled water washing. Then the exterior soil was removed and ten seeds were arranged evenly in pots. One ml inoculum was evenly sprinkled on the seeds and then covered by soil. After an hour, watering was performed and then periodically. After germination, thinning was performed (five plants per pot).

Plants were uprooted on the 110th day of sowing, and their root and shoot lengths, dry weights, and total P in root and shoot were recorded. The total P was determined by the vanadomolybdophosphoric acid method.[12] The tightly adhering soil of roots was collected in sterilized bags and further used for the determination of available soil P by colorimetric method.[13]

Statistical Analysis

The data attained was processed by analysis of variance (ANOVA) and the difference among parameters was done by Duncan tests employing SPSS software (version 16.0).

Results and Discussion

Many workers have demonstrated the advantageous effects of PSF on several crops *viz*., *P. oxalicum* on wheat and maize[6]; *A. awamori* and *P. citrinum* on chickpea[14]; *P. expansum*, *Mucor ramosissimus*, and *Candida krissii* on wheat[15]; *P. oxalicum* I1 on maize[16]; *P. oxalicum* P4 and *A.niger* P85 on maize[17]; *A. niger* and *P. brevicompactum* on coffee[18]; *Penicillium* sp. EU-DSF-10 on *Sorghum bicolour*[19]; *Penicillium rugulosum* on maize.[20] However, sporadic literature is available on *in vivo* as well as *in vitro* characterization of different physical, nutritional, and agro-climatic conditions to understand the adaptability behaviour of bio-inoculants in actual field conditions. Cakmakci *et al*.[21] studied the growth improvement of sugar beet by plant growth-promoting rhizo bacteria in two soil types having different organic matters and observed that *Bacillus* RC07 had great possibility to be developed as bio fertilizer. Similarly, Kaur & Reddy[22] studied PSB-3 and PSB-5 as bio-inoculants for the improvement of maize and wheat crops at three discrete agro-climatic zones *viz*., central plain, sub-mountain undulating central and sub-mountain undulating zone. In the current research, the potential of *A. niger* S33 and *A. tubingensis* S36 was not only studied in broth by varying the physical and nutritional factors, but also in the soil-plant experiment with wheat in two different agro-climatic conditions *viz*., Banasthali, Rajasthan (semi-arid region) and Dwarahat, Uttarakhand (hilly western Himalayan region).

Effect of Carbon and Nitrogen Source

Both strains were solubilized the inorganic phosphate with all tested C sources (Fig. 1a). The strain *A. awamori* S33 showed a significant preference for maltose, glucose, and fructose, followed by sorbitol. The significantly lowest pH values were found with maltose and fructose. In the case of *A. awamori* S36, fructose was a significantly better C source, followed by sorbitol > maltose ≥ sucrose ≥ glucose > mannitol. Different authors stated that different strains preferred distinct carbon sources *viz*., *A. awamori* S29 used maltose[1]; *A. niger* preferred maltose and mannitol[23]; *A. aculeatus* preferred arabinose and glucose.[24]

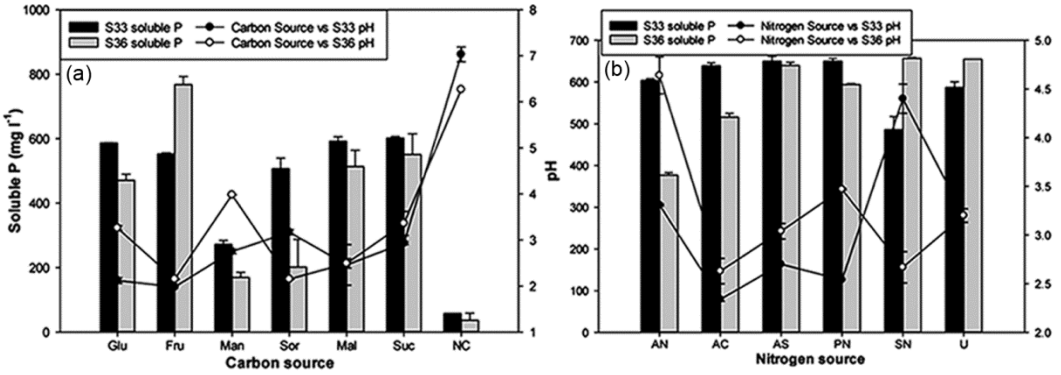

Fig. 1 — Effect of different substrate carbon on amount of soluble P and pH: (a) Carbon sources (Glu = glucose, Fru = fructose, Man = mannitol, Sor = sorbitol, Mal = maltose, Suc = sucrose, NC = no carbon), and (b) Nitrogen sources (AN = ammonium nitrate, AC = ammonium chloride, AS = ammonium sulphate, PN = potassium nitrate, SN = sodium nitrate, U = urea)

Table 2 — Organic acid produced by S33 and S36 strains in the presence of 6 different carbon sources

Carbon source	Oxalic acid ($g·l^{-1}$)	Malic acid ($g·l^{-1}$)	Citric acid ($g·l^{-1}$)	Succinic acid ($mg·l^{-1}$)	Fumaric acid ($g·l^{-1}$)	Total acid ($g·l^{-1}$)
			A. tubingensis S33			
Glucose	0.69	2.50	22.84	—	0.02	26.05
Fructose	3.20	4.15	26.43	16.52	—	50.30
Maltose	0.72	—	4.80	0.52	—	6.04
Sucrose	4.14	32.45	11.81	5.90	0.28	54.58
Mannitol	5.42	2.19	2.81	1.45	0.02	7.02
Sorbitol	2.76	9.10	16.71	14.85		43.43
			A. niger S36			
Glucose	1.08	3.89	35.55	—	0.02	40.55
Fructose	1.08	3.90	35.44	—	0.02	40.44
Maltose	0.70	11.63	15.88	—	—	28.21
Sucrose	2.84	18.64	28.77	10.50	0.05	60.80
Mannitol	0.54	1.02	3.31	2.23	0.02	7.11
Sorbitol	2.45	3.04	15.68	10.38	0.02	31.57

In all cases, a significant negative correlation was noted between phosphate solubilzation and pH. The possible reason for the pH decrease was organic acid production by fungi. Further, to determine organic acids, six-day-old culture filtrate was used for organic acid analysis by HPLC. Both strains produced the highest total organic acid in the presence of sucrose (Table 2) which was 54.58 and 60.8 $g·l^{-1}$ for S33 and S36, respectively. That is high in comparison to reported by Li *et al*.[25] for NJDL-03 (~4000 $mg·l^{-1}$) and NJDL-12 (~10,000 $mg·l^{-1}$). The most commonly produced acids was oxalic and citric and other acids are malic succinic and fumaric. Many authors reported that PSFs secrete varied organic acids, *viz*., butyric, citric, fumeric, gluconic, malic, oxalic, succinic, and tartaric.[25–27]

S36 produced the highest amount of citric acid i.e. 35.55 $g·l^{-1}$ and 35.44 $g·l^{-1}$ in the presence of glucose and fructose, respectively. In the presence of sucrose, the major acids were citric, malic, and succinic in both strains. There are also reports where citric and oxalic acids were two major organic acid that solubilize inorganic P through the release of acidic protons or by chelate metal ions.[15,16,24–28]

In the case of S33, maltose showed the lowest acid production, but significantly higher phosphate solubilization which points out the existence of some other phosphate solubilization mechanisms.

Both strains can solubilize the TCP in the presence of all tested nitrogen sources (Fig. 1b). In S33 strains, significantly highest phosphate solubilization was observed with AS followed by AC > PN > AN > U > SN. While *A. awamori* S36 exhibited significantly higher amount of soluble P when SN and AS were used as nitrogen sources. Hence, it can be inferred that these strains can solubilize TCP efficiently with both forms of nitrogen. Similar results were reported by Reyes *et al*.[29] for *P. rugulosum*.

Effect of pH

Both the strains can grow and solubilize the phosphate at a wide range of pH 6–10 (Fig. 2a). Dhakar and Pandey[30] reported that microbes isolated from extreme conditions showed wide pH tolerance. When phosphate solubilization at varied pH was analyzed, it was found that both the strains attained significantly higher soluble P at pH 8.00, while minimum soluble P was noted at pH 10.00. It can be inferred that the strains isolated from alkaline soil have the potential to solubilize phosphate at high pH. Similar findings were quoted by Jain *et al*.[10,31] for PSF and by Nautiyal *et al*.[32] in the case of PSM. S36 was able to solubilize P (122 $mg·l^{-1}$) even at pH 10.00.

In general, phosphate solubilization went up with the dropping pH. Both strains showed significantly lower pH at pH 8.00. Maximum pH drop due to acid production was attained within 48 hours of inoculation when the initial media pH was adjusted at 6.00, 7.00, and 8.00. Whereas for initial pH 9.00 and 10.00, the maximum drop in pH values was shifted to later days of growth. Primarily, there was a significant negative correlation noted between Phosphate solubilization and pH at all tested pHs, except at 10. Singal *et al*.[33] found that pH 6.00 was most

conducive for rock phosphate solubilization by *A. japonicus* and *A. foetidus*. Xiao *et al.*[34] found the optimum pH for RP solubilzation as 5.5 for *C. krissii*, 7.5 for *M. ramosissimus* and 7.0 for *P. expansum*.

Effect of TCP Concentration

A saturation study was performed by growing these strains with different concentrations of TCP (Fig. 2b). The objective was to check the impact on phosphate solubilization with TCP concentration rise. Overall, a significant lift in available P was observed with the rise in TCP (1 to 10 $g \cdot l^{-1}$). However, an insignificant rise was observed in soluble P content with 7.5 and 10 $g \cdot l^{-1}$ TCP. Maximum soluble P i.e. 818 $mg \cdot l^{-1}$ was observed in S33, followed by 558 $mg \cdot l^{-1}$ in S36 at 7.5 $g \cdot l^{-1}$ TCP concentration. Further, the media with lesser quantities of TCP was more acidic in comparison to the media with greater amounts. Gaur & Sachar[35] also made similar observations with rock phosphate.

Effect of Temperature

The impact of temperature on soluble P efficiency, four temperatures *viz*., 15, 25, 35, and 45°C were selected. The results are depicted in Fig. 3 (a–d). All inoculated flasks showed significant change in pH and phosphate solubilization over control. Uninoculated flasks remained almost consistent with regard to pH during 12 days study period.

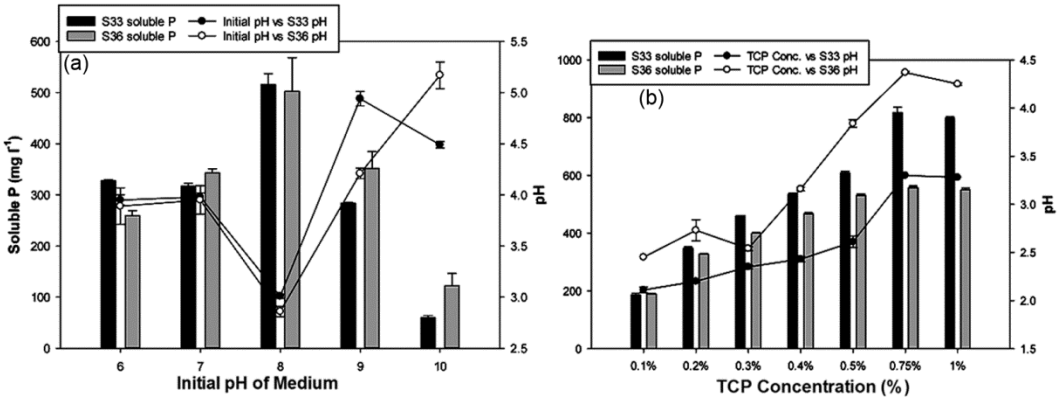

Fig. 2 — Effect of initial conditions on amount of soluble P and pH: (a) pH, and (b) TCP concentration

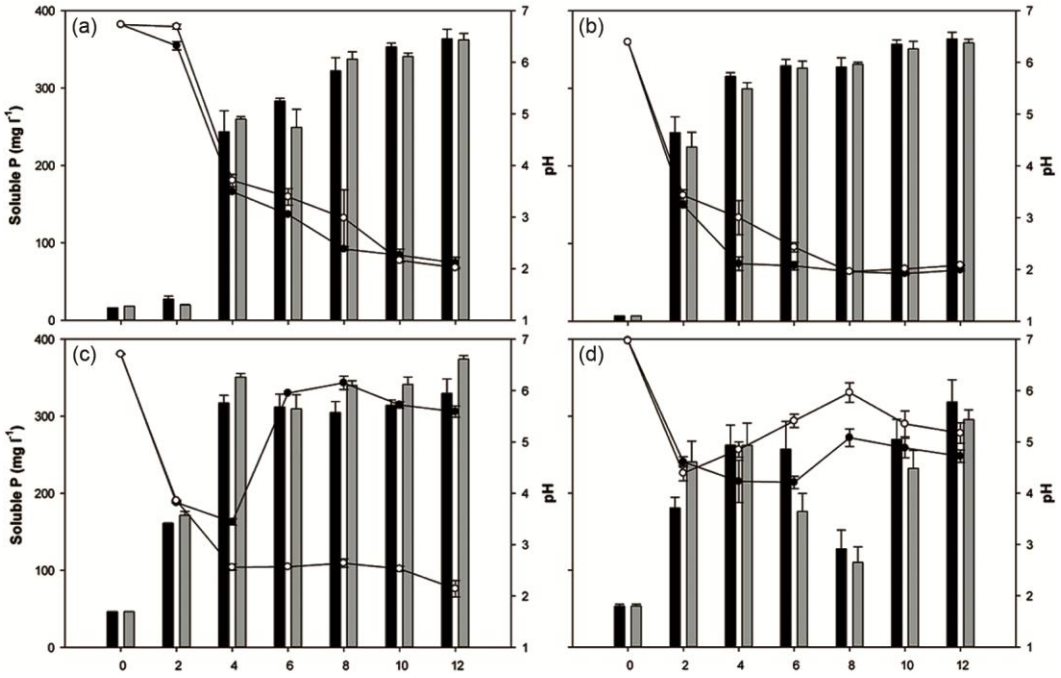

Fig. 3 (a–d) — Tri-calcium phosphate solbilization (bar graph) and changes in pH (line graph) of broth by *A. tubingensis* S33 (dark coloured bar and line graph) and *A. niger* S36 (grey coloured line with empty circle and bar graph) at four different temperature *viz*., (a) 15°C, (b) 25°C, (c) 35°C, and (d) 45°C

Overall, S36 performed significantly ($P<0.05$) better than S33, except at 15°C. A negative correlation was seen between soluble P and pH at every tested temperature, except 45°C. The ideal temperature for phosphate solubilization was found 25 and 35°C. Rawat & Tewari[36] reported optimal temperature for *Trichoderma viride* at 28°C and Barroso *et al*.[23] for *A. niger* at 30°C.

Two solubilization patterns were observed during the experiment. The first one, observed at 15 and 25°C, showed the initial sharp increase in phosphate solubilization and then it became gradual. On the other hand, at 35 and 45°C phosphate solubilizing capacity reached at peak before decreasing and then increasing second time. Many authors also described such correlation.[31,37–39] There are reports where pH drop was found directly related to organic acid production in medium.[31,40,41]

Effect of Fungal Inoculants on Plant Growth and Yield

Both fungi exhibited a significant growth and yield hike in wheat plants than uninoculated control plants

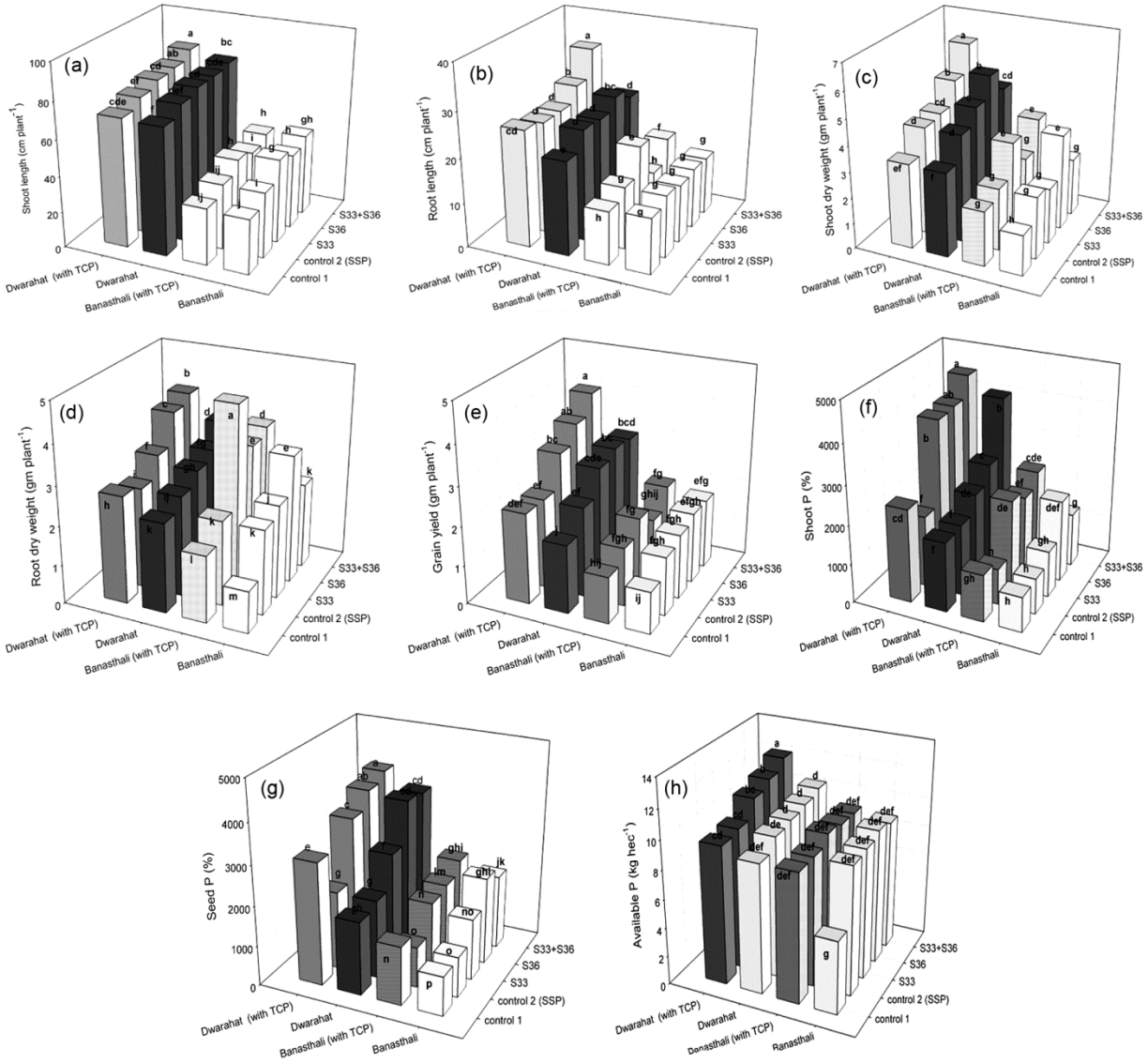

Fig. 4 (a–h) — Effect on *Triticum aestivum* plant growth and yield after inoculating with *A. tubingensis* S33, *A. niger* S36 and S33 + S36 consortiumin TCP amended and unamended soil: (a) shoot length (b) root length (c) shoot dry weight (d) root dry weight (e) grain yield (f) shoot P (g) Seed P, and (h) Available P; Data are means of five replicates; Mean values with the same letters do not differ significantly by Duncan's multiple range test at $P \leq 0.05$

Plate 1 — Wheat plants without inoculation, amended with soluble super phosphate (SSP), inoculated with *A. tubingensis* S33, *A. niger* S36 and S33 + S36 consortium along with tri-calcium phosphate (TCP) fertilization after 30 days of sowing at Dwarahat, Almora (Uttarakhand) India

(Fig. 4 a–h; Plate 1) at both agro-climatic regions. Fungal inoculated plants performed either comparable or significantly better in comparison to chemical fertilizer at both agro-climatic conditions. As depicted in Fig. 4 a–e, the consortium showed significant hike in all growth and yield variables after 110 days of growth in Dwarahat region. The enhancement in shoot length, root length, shoot dry weight, root dry weight and grain weight was 9, 30, 86, 48 and 75%, respectively. The performance of consortium was better in second half of the growth cycle. The possible reason could be that these fungal isolates were not the native of this region, therefore faced more competition when inoculated singly. In consortium, the strains helped each other to overcome the stress, resulting in significantly good results in a second half of growth. This also supports the views expressed by earlier studies[42–45] that the joint application of fungal inoculants might improve efficacy and performance over inoculation with individual fungal inoculants.

On the contrary, in Banasthali region, both joint and individual strain inoculations, performed comparable. The reason may be that the strains are native of that environment, hence face less competition with other microbial community. Consortium inoculated plants showed increase in shoot dry weight (70%), grain yield (65%) and shoot length (27%) in comparison to uninoculated control. When the plant growth was compared between plants grown in hilly western Himalayan and semi arid plain regions, the hilly climate had significant stimulatory effect on plants with respect to length and dry weight of shoot. There are many reports of yield upliftment due to treatment of PSF with external P source.[1,6,14] Kaur & Reddy[21] recorded significant improvement in soil fertility and crop yield on three geographical locations with PSFs and external P in contrast to uninoculated control.

Effect of Fungal Inoculants on Nutrient Uptake by Plant

The results presented in Fig. 4 (f) and (g) deduced that total P assimilation was significantly increased in wheat plants by treatment of S33 and S36 in comparison to uninoculated control at both agro-climatic conditions. When pots contained soil enriched with TCP, highest shoot and seed P was shown by consortium at both agro-climatic conditions i.e. 92% and 43% at Banasthali region and 89% and 32% at Dwarahat region, respectively. There are many reports where increase in P uptakes was observed by inoculation of PSF.[6,14]

Effect of Fungal Inoculants on Available P

Available P in wheat's rhizosphere soil significantly increased in contrast to sowing (Fig. 4 h). In the Banasthali region, S33 + TCP showed a significant increase in available P (9.6 $kg \cdot ha^{-1}$). In the Dwarahat region, the highest available P was observed in the consortium (12.2 $kg \cdot ha^{-1}$) with TCP enriched soil. There are reports of increases in the soil available P content *viz*., *Penicillium oxalicum* increased in wheat and maize[6]; *A. awamori* and *P. Citrinum* raised soil P in chickpeas.[14] It raised soil fertility for the next crop.

Conclusion

In conclusion, *A. tubingenesis* S33 and *A. niger* S36 successfully stimulated the growth of wheat in the pot experiments at two different geographical sites. They are accustomed well to the stress conditions *in vivo* as well as *in vitro*. That indicates the potential of both fungi to be further developed as a successful bio-fertilizer for commercialization.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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