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Response of aerobic rice to *Piriformospora indica*

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Rice cultivation under aerobic condition not only saves water but also opens up a splendid scope for effective application of beneficial root symbionts in rice crop unlike conventional puddled rice cultivation where water logged condition acts as constraint for easy proliferation of various beneficial soil microorganisms like arbuscular mycorrhizal (AM) fungi. Keeping these in view, an *in silico* investigation were carried out to explore the interaction of hydrogen phosphate with phosphate transporter protein (PTP) from *P. indica.* This was followed by greenhouse investigation to study the response of aerobic rice to *Glomus fasciculatum*, a conventional P biofertilizer and *P. indica*, an alternative to AM fungi. Computational studies using ClustalW tool revealed several conserved motifs between the phosphate transporters from *Piriformospora indica* and 8 other *Glomus* species. The 3D model of PTP from *P.indica* resembling "Mayan temple" was successfully docked onto hydrogen phosphate, indicating the affinity of this protein for inorganic phosphorus. Greenhouse studies revealed inoculation of aerobic rice either with *P. indica*, *G. fasciculatum* or both significantly enhanced the plant growth, biomass and yield with higher NPK, chlorophyll and sugar compared to uninoculated ones, *P. indica* inoculated plants being superior. A significantly enhanced activity of acid phosphatase and alkaline phosphatase were noticed in the rhizosphere soil of rice plants inoculated either with *P. indica*, *G. fasciculatum* or both, contributing to higher P uptake. Further, inoculation of aerobic rice plants with *P. indica* proved to be a better choice as a potential biofertilizer over mycorrhiza.

Keywords: Dock, *Glomus fasciculatum*, Phosphate transporter, *Piriformospora indica*, P uptake, 3D model.

Piriformospora indica, alternatively referred to as AM like fungi[1], play a significant role in the plant growth and development[2-4]. It has been proved that *P. indica* is involved in P uptake to the host plants[5,6]. Investigations on wheat plants inoculated with *P. indica* revealed that gene expression level of phosphate transporter of the fungi was higher especially under P deficit condition[6].

AM fungi—Arbuscular mycorrhizae are soil fungi having symbiotic association with higher plants, which enhances the uptake of diffusion-limited nutrients such as P, Cu, K, Zn and S[7-9]. Mycorrhizal fungi are known to influence plant growth and development, through enhancement of wager uptake and by the production of biochemical compounds that may confer disease resistance[10-12]. Unlike *Rhizobia* and *Azolla* which have achieved tremendous success as biofertilizer agents to meet nitrogen demand for the plants, application of mycorrhiza on large scale basis to improve the soil phosphorus availability to the plants has not been successful yet. This is primarily attributed to the difficulty experienced in the cultivating AM fungal inoculum on synthetic media. In the present scenario, AM fungal inoculum is prepared by growing them on roots of an appropriate host plant[13]. Since, the current protocol for the production of AM fungal inoculum is tedious as it involves more space and more time, it is inevitable to find a suitable alternative, which can augment the nutrient deficiency especially phosphorus.

Piriformospora indica is a mutualistic fungus which mimics majority of the beneficial characteristics of AM fungi such as broad host spectrum, growth promotion, enhanced P uptake, increased biomass and yield of host plants[1,6,14,15]. The most notable advantage of *P. indica* over AM fungi is that it is a facultative symbiont which can be easily cultivated axenically on a variety of synthetic media[16,17], which opens up a wider scope for developing *P.indica* as a potential biofertilizer agent.

Phosphate transporter proteins (PTP)—Phosphate transporter genes in various AM fungi as well *P.indica* plays an important role in P uptake[6,18,19,20].

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Although interaction of *P. indica* with rice plants has been investigated earlier by Prajapati *et al*[21], their studies do not reveal the role of PTP in P uptake from soil. Also, 3D model of PTP from *P. indica* constructed by Yadav *et al*[6] does not say anything about interaction of P_i with the protein. Therefore, in the present study an attempt has been made at *in silico* level to understand interaction between P_i and PTP of *P. indica*, followed by greenhouse investigations to study the influence of *P. indica* as well as *Glomus fasciculatum* on aerobic rice.

Materials and Methods

Investigations were carried out at Department of Plant Biotechnology, UAS, Bangalore to study the influence of *P. indica* and *G. fasciculatum* on the growth and yield of aerobic rice (MAS 946). *Glomus fasciculatum* was also included in the study to evaluate the relative performance of *P. indica* as a biofertilizer agent for aerobic rice.

In silico studies—PTP sequence from *P. indica* and other AM fungi (*G.versiformae*, *G. proliferum, G. intraradices, G. diaphanum, G. aggregatum, G. irregular*, *G. clarum*, *G. custos*) along with 1PV6 and 1PW4, the PDB templates used for modelling PTP by Yadav *et al* [6], were submitted to CLUSTALW tool[22] to identify the conserved amino acid residues. To understand the evolutionary relationship of PTP from *P. indica* with other AM fungi, phylogenetic tree was created using PHYLIP package[23]. Output generated was also used for selecting the appropriate template for 3D structure prediction of PTP from *Pirifomospora*. The 3D structure of PTP from *P. indica* was predicted by I TASSER server[24], a web based programme for protein structure and function prediction. The initial PTP model was subjected to loop refinement based on ERRAT report which displayed the region to be refined. This information was later used to identify the loop regions of the modelled PTP structure using DeepView package[25]. MODLOOP[26] server was accessed for loop refinement of PTP model and was done so by specifying the loop regions. Final loop refined model was once again validated through ERRAT[27] and PROCHECK tool[28]. Structural homologs for the predicted PTP structure was later searched using DaliLite server[29]. To understand the interaction of P with PTP from *P. indica*, 3D conformer of HPO_4 (CID:3681305) from PubChem compound database was downloaded and subjected to geometry optimization studies using Gaussian package[30] installed on SGI Altix UV10. For optimizing the structure, B3LYP theory was used with 6-31G as the basis set. The standard orientation of the optimized structure generated was visualized using ARGUS lab package and the structure was saved in PDB format. Geometrically optimized HPO_4 structure was subsequently docked onto PTP model from *Piriformospora*. Docked conformations and interaction energies were obtained using HEX (v6.3) software[31], a protein-protein docking program. Free energies were calculated based on shape and electrostatics using default grid spacing of 0.6 Å. Among large number of docked outputs generated, the best orientation was selected based on the lowest dock energy.

Greenhouse and laboratory investigations—Fresh soil for greenhouse experiment was collected from regional research station, GKVK campus. Polybags were later filled with this soil along with farm yard manure and sand in a ration of 3:1:1

Fungal inoculum (*P. indica* and *G. fasciculatum*) and aerobic rice planting material—Fungal inoculum used in the study included pure culture of *P. indica* (supplied by Dr. Ajit Varma, Professor, Amity University, Noida) and *G. fasciculatum* (supplied by the host department). *Piriformospora indica* was initially cultured using Kaefer agar medium[32]. Subsequently, actively growing mycelia of the fungus upon attaining full growth was transferred to Kaefer's broth by punching out 8 mm of agar discs from the agar plates using sterilized cockborer[16,17]. The liquid fungal culture maintained in 500 mL conical flask containing 100 mL liquid medium broth was used for inoculation of aerobic rice plants. *Glomus fasciculatum* culture was maintained with *Sorghum bicolor* as the host using sterilized soil under greenhouse condition[33]. Root fragments of *Sorghum bicolor* and rhizosphere soil constituted *G. fasciculatum* inoculum.

Experimental layout—Experiment in the form of completely randomized design (CRD) consisted of 24 treatments resulting from a combination of 4 different inoculation types, each having 6 replications. Treatment details are: (T1) Uninoculated plants; (T2) Plants inoculated with *P. indica*; (T3) Plants inoculated with *G. fasciculatum* and (T4) Plants dually inoculated with *G. fasciculatum* and *P. indica.*

Treatment details—Inoculation of *G. fasciculatum* to the polybags of T3 and T4 treatments were carried

out as per the procedure suggested by Pandey and Banik[34]. *Glomus* inoculum was added at the rate of 50 g per polybag. *Piriformospora indica* was inoculated to rice plants following the methodology suggested by Achatz *et al*[35]. For T2 and T4 treatments, *P. indica* inoculum was prepared using freshly harvested 7 days old broth culture diluted upto 30% with distilled water. Rice seeds were subsequently dipped into the beaker containing *P. indica* inoculum for 30 min prior to sowing into the polybags. Soil substrate for T2 and T4 polybags were treated with *P. indica* by harvesting actively growing fungal mycelium which was mechanically crushed, followed by soil mixing (2 g/300 g soil substrate) and finally incorporating them into the potting mixture for sowing. After 15 days, soils of these polybags were again treated with the *P. indica* inoculum by adding the culture into the root zone of the potted plants. All the treatments received adequate irrigation at regular intervals to maintain required field capacity of rice plants.

Observations recorded after 120 days of sowing included root colonization, NPK content, soil enzyme activities, chlorophyll and sugar content, plant biomass and yield. Plant growth parameters (plant height, number of leaves and number of tillers) were recorded at 30, 60, 90 and 120 days after sowing.

Percent root colonization—Root colonization by *G. fasciculatum* and *P. indica* were carried out by gridline intersect method[36,37]. Before assessment of % root colonization, harvested roots were stained using the method suggested by Phillips and Hayman[38].

Nutrient uptake (NPK) studies—Nitrogen content in the plant tissue was carried out by Micro–Kjeldahl method[39]. Root and shoot samples (100 mg each) of rice were digested with conc. H_2SO_4, using K_2SO_4 and $HgSO_4$ as catalysts. Digested samples were distilled after addition of 10 mL of 40% NaOH. Ammonia liberated was collected into 2% boric acid with methyl red and methylene blue as indicators. Ammonium borate by-product solution was titrated against 0.02N H_2SO_4 to calculate total N content (%) of the plant.

Plant P concentration was estimated colorimetrically based on vanadomolybdate yellow colour method[40]. Oven dried shoot and root samples were digested using 10 mL of tri-acid mixture (nitric acid; perchloric acid and sulphuric acid) in the ratio 6:3:1 (v/v/v) and diluted to 100 mL. An aliquot (10 mL) was taken to which 10 mL vanadomolybdate reagent was added and diluted to 50 mL. Reaction mixture was shaken for a while and allowed to stand for 20 minutes. Based on the intensity of yellow colour, plant P content (%) was calculated at 420 nm using spectrophotometer.

Potassium concentration in plant tissues was estimated by using flame photometer[40]. The tri-acid digested plant sample solution was fed in to the flame photometer and the readings were recorded. The readings were compared with standard curve of KCl solution and % K was calculated in the plant sample.

Biochemical analysis (chlorophyll, total sugars and soil enzyme activities)—Chlorophyll content in aerobic rice leaves was determined 90 days after planting[41]. Leaf tissue (100 mg) was placed in a vial containing 7 mL of dimethyl sulfoxide (DMSO) and chlorophyll was extracted in to the fluid by incubating at 65 °C overnight. The extract was then transferred to a graduated tube and made up to a total volume of 10 mL with DMSO. Assay was done by recording the OD values in spectrophotometer both at 645 and 663 nm. Chlorophyll a (*chl* a) and chlorophyll b (*chl* b) of the leaves were finally computed using the formula suggested by Arnon[42].

Phenol sulphuric acid method was followed for estimating the total plant sugar[43]. Dried powdered shoot and root samples (100 mg) were extracted with 10 mL of hot 80% of alcohol overnight with constant stirring using magnetic stirrer. Extract (100 µL) was taken in test tubes and the volume was made up to 1 mL water to which 1 mL of phenol solution and 5 mL of 96% H_2SO_4 were added. The tubes were placed in a water bath at 25-30 °C for 20 min and the brick red colour was measured at 490 nm. The total amount of carbohydrate (%) present in the sample was calculated using standard graph.

Activity of soil enzymes (acid and alkaline phosphatase) was analyzed by the method suggested by Eivasi and Tabatabai[44]. One gram of soil sample collected from root zones of aerobic rice plants was placed in 50 mL volumetric flask containing 0.2 mL of toluene and 4 mL of MUB (Modified Universal Buffer *p*H 6.5 for assay acid phosphatase or *p*H 11 for assay of alkaline phosphatase). To this, 1 mL of p-nitrophenyl phosphate solution was added. Flasks were swirled, stoppered and incubated at 37 °C for 1 h. This was followed by addition of 1mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH and then filtered. Intensity of yellow colour was measured at 420 nm using spectrophotometer. The p-nitrophenol content

of filtrate was measured by referring to a calibration graph with standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

Plant biomass and yield—Harvested rice plants (shoot and root) were dried in an oven at 60 °C for 4 days to record shoot and root dry weight. Before harvest (i.e. 120 days after planting), number of panicles and grain yield was recorded for individual plant. Data were statistically analyzed by ANOVA at 5% level of significance.

Results

Multiple sequence alignment of PTP sequence of *P.indica* with eight different species of *Glomus*, 1PW4_A and 1PV6_A suggest that residues towards the C-terminal region appear to be more conserved than N –terminal region. Among various residues, pro232, lys332 and phe425 were conserved with the remaining set of sequences (Fig. 1). Residues between "241thr – his257" were not getting aligned with any of the remaining PTP sequences (Fig. 1).

Conserved residues and sequence motifs between PTP of P.indica and Glomus sp.—A total of 10 residues located at various positions of PTP sequence in *P.indica* were conserved with the PTP sequences of 8 *Glomus* species considered in this study. And these include: ala266, 358, asp258, 381, arg447, glu234, 440, gln389, gly180, 216, 368, phe300, 467, lys354, thr365, 376, trp297 and val187, 263. Based on the alignment results, 26 distinct sequence motifs spread across at different sites were identified in the PTP sequence of *P. indica* (Fig. 1), whose length varied from two to seven amino acid residues. Among these motifs, "384GRK386" and "391MGF393" are of significance, as they are part of *signature tag* of major facilitator superfamily (MFS) (from amino acid 376 to 393), identified by the PROSITE server (Fig. 1). Also, these two regions, along with T376, D381, Q389 were completely conserved among all the PTP sequences of *Glomus* species, but not in1PW4_A and 1PV6_A (Fig. 1).

Conserved residues and sequence motifs between PTP of Piriformospora indica, 1PW4_A and 1PV6_A—Comparison of PTP sequence of *P. indica* with 1PW4_A and 1PV6_A revealed a total of 5 residues ($gly^{93,143}$, asp^{97}, lys^{111}, ile^{290} and glu^{515}) being conserved. In the sequence motif "$^{165}RRG^{167}$" of PTP from *P.indica*, only the second and third residues were conserved with the glycerol 3 phosphate sequence of *E. coli* (1PW4_A) (Fig. 1).

Phylogenetic analysis showed that, despite PTP sequence from *P.indica* having a long branch length with the PTP sequence of *Glomus* species, it shared close ancestral relationship only with PTP sequences of *G. irregulare* and *G. intraradices*. Based on the nodes shared by the PTP sequences, 2 distinct clades could be recognized; while PTP from *P. indica* with *G. irregulare* and *G. intraradices* formed one clade, remaining PTP sequences from other *Glomus* species constituted the second clade group (Fig. 2).

Molecular modelling and docking—Using 1PW4_A as the template, I-TASSEER server was able to generate a 3D model for the PTP sequence from *P. indica* (Fig. 3). The predicted structure had a strong resemblance to the shape of a "Mayan temple". Residues "245ala to lys255" of the model lying in between 241thr – his257 which did not get aligned with any of the remaining PTP sequences, was predicted as helix by I-TASSER server. Upon loop refinement, the quality of the 3D model of PTP improved. Upon energy minimization using DeepView package, the energy value recorded for PTP model was -9185.86 KJ/mol. Data for Ramachandran plot obtained through PROCHECK server, shows that 89.1% of residues in the core region, followed by 6.1% in the favoured, 2.2% in generously allowed and 2.6 % in disallowed region. For 1PW4_A, 85.4% of residues in the core region, followed by 13.5% in the favoured, 1.1% in generously allowed and 0.0 % in disallowed region. Overall G-factor for the model of -0.44 was well within the acceptable threshold value of -0.5, suggesting that the generated structure was satisfactory. Dali server generated several structural homologs for the theoretical structure of PTP. Among these homologs, 1PW4_A, template used for building PTP model, happens to be the top hit with the highest Z score (44.5) and the lowest RMSD value (1.6 Å). The Cα backbone of PTP model got well superimposed onto the PTP structure (G3P: *E. coli*, 1PW4_A) as well as other closely related proteins from diverse group of organisms such as lactose permease from *E.coli* (1PV6_C) and proton/peptide symporter family protein from *Shewanella oneidensis*. Hydrogen orthophosphate upon optimization using B3LYP/6-31G level of theory got converged into a global minimum (Fig. 4 (i)). The molecule got docked onto the PTP model with dock energy of -109.7 KJ/ mol (Fig. 4 (ii)). Binding site analysis using Deep View

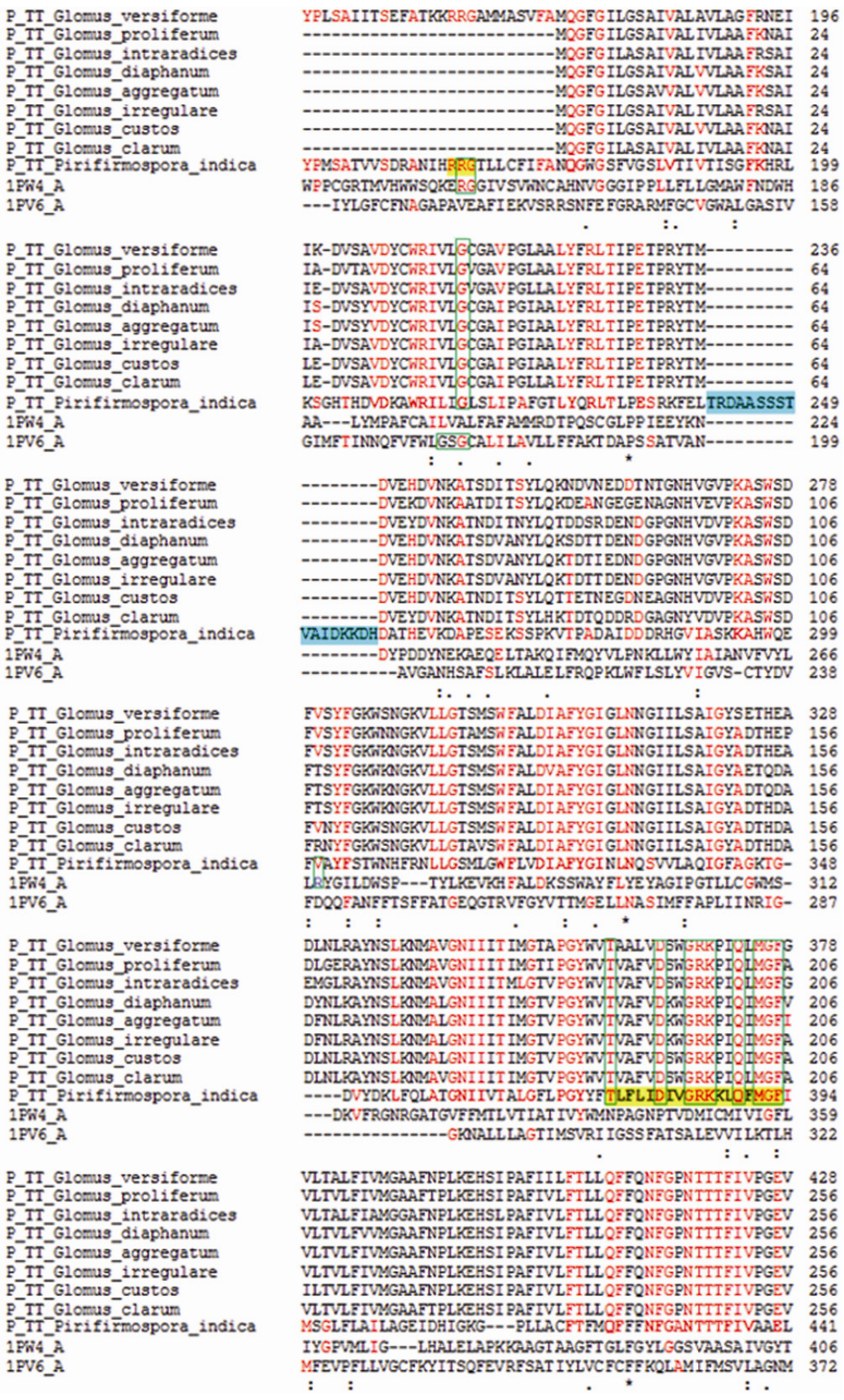

Fig. 1—Multiple sequence alignment of phosphate transport protein (PTP) sequence from *P. indica* with PTP sequences from other AM fungi, along with 1PW4_A and 1PV6_A

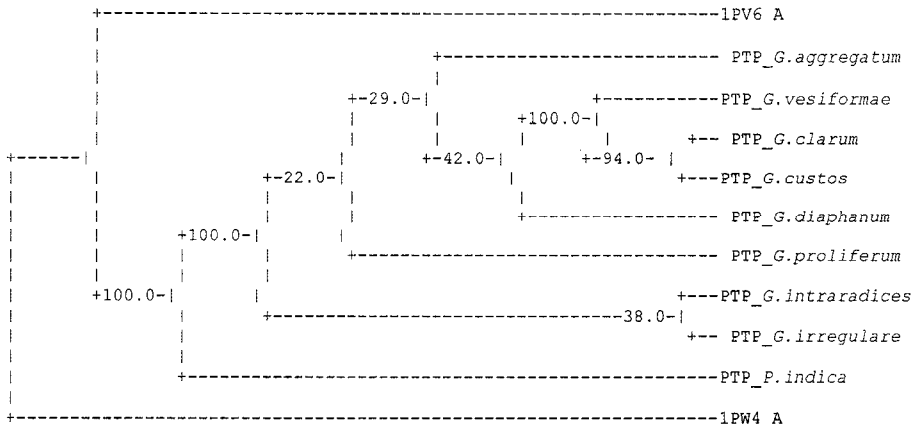

Fig. 2—Phylogenetic analysis of phosphate transport protein sequence of *P. indica* with PTP sequences of other AM fungi along with 1PW4_A and 1PV6_A

package showed that 50phe – met58, ser185, thr188, ile213, ile215, ser335, phe355 and thr359 of PTP model were interacting with HPO_4 (Fig. 4 iii).

Fungal root colonization—Rice plants inoculated with either *P. indica* or *G. fasciculatum* alone or both showed significantly higher percentage of colonization compared to untreated ones. Nevertheless, root colonization was significantly higher in those plants inoculated with *P. indica* compared to remaining treatments (Table 1). Soil enzyme activity─Assay for soil acid and alkaline phosphatase revealed, activity of these enzymes were much higher in the rhizosphere of rice plants inoculated with *P. indica* and *G. fasciculatum* compared to control plants (Table 1). Rhizosphere of rice plants inoculated with *P. indica* recorded maximum acid phosphatase activity which was significant compared to remaining treatments. Though alkaline phosphatase activity was noticed maximum in the rhizosphere of rice plants inoculated with *Glomus fasciculatum*, it did not differ significantly in treatment that received dual inoculation.

Nutrient content (NPK) of rice plants—The NPK content in shoot and root portions of rice plants were significantly higher when inoculated either with *P. indica, G. fasciculatum* or both compared to control plants (Table 2). Though no significant differences in shoot and root P content could be noticed among rice plants treated with fungal inoculum, there was marked variation in N and K content in these treatments (Table 2). Nitrogen and potassium content between the rice plants treated with *P. indica* as well as dual inoculation appeared to show similar trend, wherein the rice plants inoculated with *P. indica* showed significantly higher percentage of these two nutrients compared to rest of the treatments (Table 2). The N content was nearly 1.3 times higher in the rice plants treated with *P.indica* compared to those treated with *Glomus fasciculatum*. Again, there was further significant reduction in the N and K content of the shoot portion of the rice plants inoculated with *Glomus fasciculatum* alone (Table 2). Though K content differed significantly in the root portion of the rice plants that received dual inoculation and *G. fasciculatum* alone, the plants treated with *P. indica* did not show any marked increase.

Chlorophyll and soluble sugar content—Both, *chl* a and *chl* b content differed significantly in rice plants when inoculated either with *P. indica* or *G. fasciculatum* or both compared to uninoculated plants (Table 3). Though there was not much variation noticed in the *chl a* content of rice plants inoculated with *P. indica* as well as dual inoculation, it was significantly less in those that were treated with *G. fasciculatum* alone (Table 3). Whereas, analysis for *chl b* in the rice plants inoculated with *P. indica* suggests, it was significantly higher compared to the plants that received dual inoculation. In relation to these treatments, plants inoculated with *G. fasciculatum* alone did not show any significant increase in *chl b* content. Rice plants inoculated with either *P. indica* or *G. fasciculatum* alone or both showed similar trend with respect to soluble sugar content. Maximum amount of soluble sugar content noticed in shoot and root portions of rice plants inoculated with *P. indica* decreased significantly in plants that received dual inoculation, followed by further significant reduction in those plants treated with *G. fasciculatum* (Table 3).

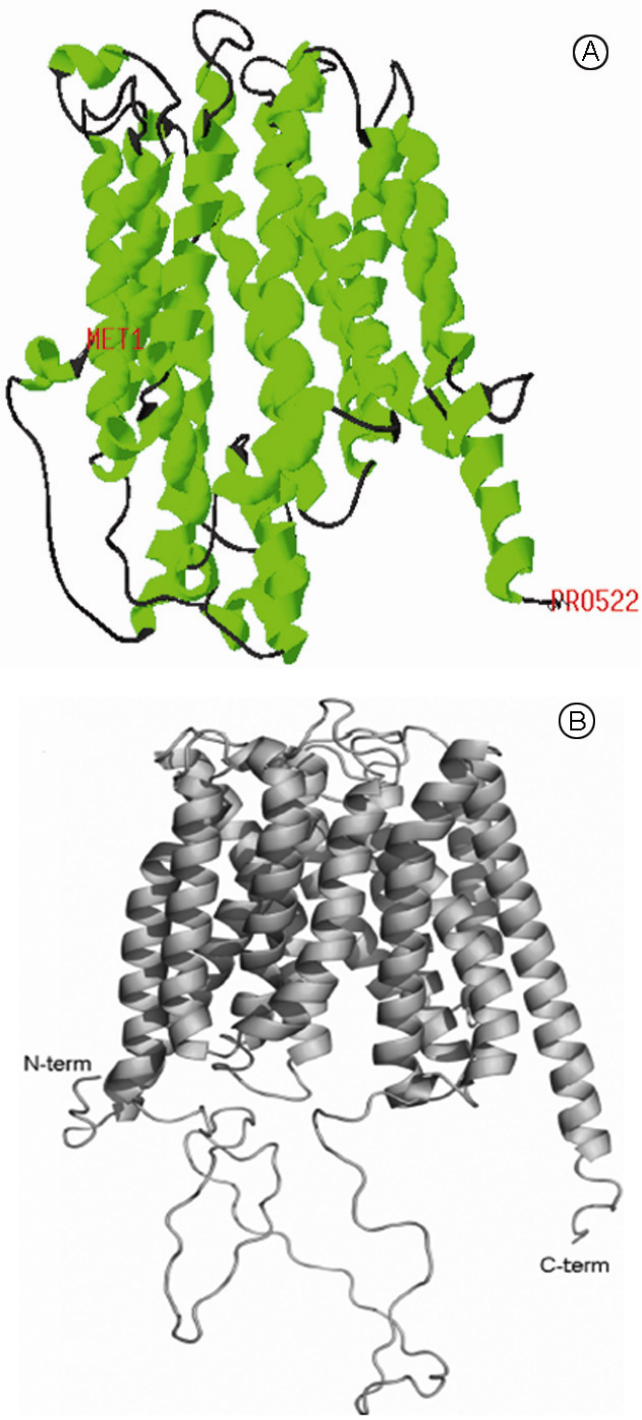

Fig. 3—Comparison of (A) 3D model of phosphate transporter protein predicted by the I-TASSER with (B) homology modelled phosphate transporter protein from *P. indica* (Yadav *et al*., 2010). Both the models have used 1PW4_A as the template

Plant growth parameters—Inoculation of rice with either *P. indica* or *G. fasciculatum* alone or combination of both showed significant improvement in plant growth parameters (plant height, number of leaves and tillers) compared to uninoculated (Table 4). Among all the treatments, plants inoculated with *P. indica* were superior since they had maximum plant height (87.50 cm), maximum number of leaves (123) and tillers (38) after 120 days of planting. Rice plants inoculated with *P. indica* after 30 days of sowing were 1.25 times taller than dual inoculation and 1.14 times taller than *G. fasciculatum* treated rice plants (Table 4). However, observation taken at 60, 90 and 120 days after sowing revealed that variation in plant height among all the three fungal treatments appeared to be diminishing gradually. Similar type of trend could be noticed with remaining two parameters (number of leaves and tillers) of rice plants inoculated with *P. indica* after 30 days of sowing.

Plant biomass and yield—Compared to untreated rice plants, significant increase in shoot and root dry weight, number of panicles per plant and grain yield were noticed when inoculated either with *P. indica* , *G. fasciculatum* or both. Although shoot dry weight did not differ significantly between the plants treated with *P.indica* and dual inoculation, it was significantly higher compared to the plants inoculated with *Glomus fasciculatum*. Similar trend was noticed for number of panicles per plant and grain yield (Table 5). Maximum grain yield (42.53 g/plant) was recorded in rice plants that received dual inoculation (Table 5).

Discussion

Results from multiple sequence alignment showed high degree of variation of PTP sequence from *P. indica* with respect to PTP of eight other AM fungi and PDB templates (1PV6 and 1PW4); variation being more prominent towards the N–terminal region. It is interesting to note that residues between 241thr–his257 of PTP from *P.indica* were not getting aligned with any of the remaining PTP sequences. This suggests a new functional role for this region for P transport in *P. indica*, which is absent in AM fungi.

Analysis of the whole length of PTP sequences showed only 3 amino acid residues conserved towards the C–terminal end (pro232, lys332 and phe425). However, comparison of PTP sequences between *P. indica* and *Glomus* sp., showed conservation of 10 individual residues and 26 sequence motifs at various locations. Among the sequence motifs, "384GRK386" and "391MGF393" which are part of signature tag of "Major Facilitator Superfamily", is in agreement with the observation made by Yadav

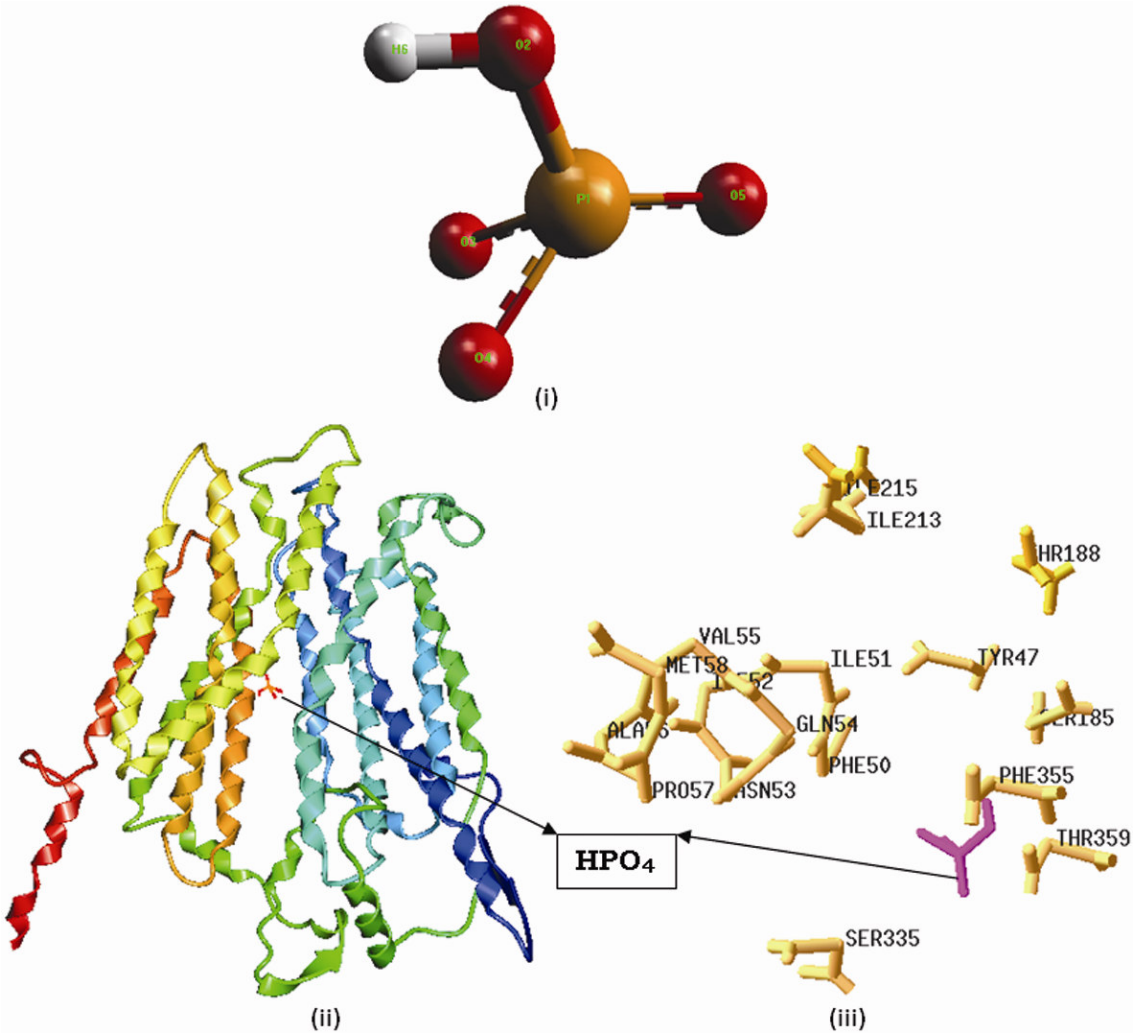

Fig. 4—(i) Optimized structure of hydrogen phosphate [HPO_4] computed by B3LYP/6-31G level of theory using GAUSSIAN package. Energy (RB3LYP)=-642.78 AU (at the end of 14 cycles). Image was generated using ArgusLab package (ii) Docking of geometrically optimized hydrogen phosphate (HPO_4) onto the 3D model of phosphate transporter protein of *P. indica*. While the 3D model was predicted by I- TASSER server, docking was carried out by HEX software. [Dock energy of HPO_4=-109.7 Kj/mol]. Image was generated using ArgusLab package (iii) Active site residues of phosphate transporter protein from *P. indica* interacting with the HPO_4 (magenta coloured)

et al[6], who have noticed similar type of signature tag present in PTP sequence of *Piriformospora indica*. PROSITE documentation describes MFS as single polypeptide secondary carriers capable of transporting small solutes in response to chemiosmotic ion gradients. Comparison of PTP sequence of *P. indica* with glycerol 3 phosphate transporter sequence of *E. coli* (1PW4_A) revealed, second and third residue of "165RRG167" motif of PTP from *P. indica* got conserved at the corresponding site of PTP sequence of 1PW4_A. According to the studies made by Yadav *et al*[6], "RRG" motif in PTP of *P. indica* represents phosphorylation site for cAMP and cGMP dependant protein kinase.

Long branch length for PTP of *P. indica* generated by PHYLIP is suggestive of the fact that this fungi might have evolved earlier compared to other eight mycorrhizal species taken into the present study. This is in accordance with the observations made by Zuccaro *et al*[45], who have suggested *P. indica* might have evolved much earlier, while comparing its evolutionary relatedness with AM fungus, *Laccaria bicolour* and saprotrophic fungus, *Coprinopsis cineria*. As pointed out by Zuccaro *et al*[45], *P. indica* represents a missing link between a saprotrophic and mycorrhizal fungi.

Despite 1PV6_A showing closer evolutionary relationship with PTP of *P.indica*, 1PW4_A, displayed

Table 1—Effect of fungal (*P. indica* and *Glomus fasciculatum*) inoculation on the fungal root colonisation percentage and root zone enzyme activity in aerobic rice (var. MAS 946)

Treatments	Root colonisation (%)	Acid phosphatase (µg PNP g^{-1} soil hr^{-1})	Alkaline phosphatase (µg PNP g^{-1} soil hr^{-1})
Ct	26.30^{c}	32.39^{d}	8.63^{c}
Pi	83.72^{a}	53.34^{a}	12.54^{b}
PG	74.96^{a}	48.41^{b}	15.16^{a}
Gf	55.97^{b}	43.32^{c}	17.71^{a}
SEM +/-	2.02	1.29	1.01
CD @ 5 %	5.96	3.81	2.97

Ct: Uninoculated plants/control; Pi: Plants inoculated with *P. indica*; PG: Plants dually inoculated with *P. indica* and *Glomus fasciculatum*; Gf: Plants inoculated with *Glomus fasciculatum*;

Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.

Table 2—Effect of fungal (*P. indica* and *Glomus fasciculatum*) inoculation on percentage NPK content of aerobic rice (var. MAS 946-1)

	N		P		K	
Treatments	Shoot	Root	Shoot	Root	Shoot	Root
Ct	1.77^{d}	1.69^{c}	0.15^{b}	0.11^{b}	2.56^{d}	1.71^{c}
Pi	2.72^{a}	2.31^{a}	0.23^{a}	0.15^{a}	3.16^{a}	2.12^{ab}
PG	2.45^{b}	2.22^{b}	0.23^{a}	0.16^{a}	3.01^{b}	2.09^{b}
Gf	2.11^{c}	2.19^{b}	0.20^{a}	0.15^{a}	2.80^{c}	2.17^{a}
SEM +/-	0.04	0.02	0.01	0.01	0.04	0.02
CD @ 5 %	0.12	0.06	0.03	0.02	0.13	0.06

The treatments details Ct: Uninoculated plants/control; Pi: Plants inoculated with *P. indica*; PG: Plants dually inoculated with *P. indica* and *Glomus fasciculatum*; Gf: Plants inoculated with *Glomus fasciculatum*; Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.

Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.

Table 3—Effect of fungal (*P. indica* and *Glomus fasciculatum*) inoculation on chlorophyll (mg/g fresh weight) and soluble sugar content of aerobic rice (var. MAS 946)

	Chlorophyll (mg/g fresh weight)		Soluble sugar (%)	
Treatments	Chl a	Chl b	Shoot	Root
Ct	0.99^{c}	0.43^{c}	5.87^{d}	2.96^{d}
Pi	2.03^{a}	1.05^{a}	9.53^{a}	6.09^{a}
PG	2.19^{a}	0.90^{b}	8.83^{b}	5.73^{b}
Gf	1.22^{b}	0.98^{ab}	7.23^{c}	4.48^{c}
SEM +/-	0.07	0.03	0.03	0.04
CD @ 5 %	0.20	0.09	0.09	0.12

The treatments details Ct: Uninoculated plants/control; Pi: Plants inoculated with *P. indica*; PG: Plants dually inoculated with *P. indica* and *Glomus fasciculatum*; Gf: Plants inoculated with *Glomus fasciculatum*; Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.

Means followed by the same letter between the treatment levels do not differ significantly at P = 0.05

as an out group by PHYLIP, was selected as the template to build the PTP model from *Piriformospora indica*. This is because PTP model from *P. indica* constructed using 1PW4_A as the template by Yadav *et al*[6] had lesser gaps in the helical regions compared to the model obtained using 1PV6_A as the template. Tertiary structure of PTP was very much similar to the model generated by Yadav *et al*[6] and showed a strong resemblance to the shape of a Mayan temple. This is in conformation with the studies conducted by Huang *et al*[58] on glycerol -3 phosphate transporter which had similar type of architecture. The spot distributions of amino acids for the PTP model displayed in Ramachandran plot closely resembled to

Table 4—Effect of fungal (*P. indica* and *Glomus fasciculatum*) inoculation on plant height, number of leaves and number of tillers of aerobic rice (var. MAS 946)

Treatments	Days After Sowing 30	60	90	120
	Plant height (cm)			
Ct	39.58[b]	63.43[c]	80.33[b]	80.17[b]
Pi	55.18[a]	83.00[a]	89.17[a]	87.50[a]
PG	44.27[b]	76.83[b]	87.50[a]	85.83[ab]
Gf	47.60[b]	71.83[b]	84.33[a]	85.33[ab]
SEM +/-	1.84	2.05	2.23	2.27
CD @ 5 %	5.43	6.05	6.58	6.71
	Number of leaves/plant			
Ct	11[c]	43[d]	72[b]	101[b]
Pi	23[a]	81[a]	106[a]	123[a]
PG	15[b]	71[b]	97[a]	118[a]
Gf	12[c]	60[c]	89[b]	113[b]
SEM +/-	0.66	1.75	3.34	4.78
CD @ 5 %	1.95	5.15	9.84	14.09
	Number of tillers/plant			
Ct	3[b]	14[c]	23[c]	29[b]
Pi	6[a]	21[a]	31[a]	38[a]
PG	4[b]	18[b]	28[ab]	37[a]
Gf	4[b]	17[b]	26[bc]	37[a]
SEM +/-	0.36	0.78	1.37	2.06
CD @ 5 %	1.07	2.30	4.03	6.08

The treatments details: Ct: Uninoculated plants/control; Pi: Plants inoculated with *P. indica*; PG: Plants dually inoculated with *P. indica* and *Glomus fasciculatum*; Gf: Plants inoculated with *Glomus fasciculatum*; Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.

Means followed by the same letter between the treatment levels do not differ significantly at P = 0.05.

1PW4_A. And the overall G-factor calculated by PROCHECK for the model was well above the accepted threshold of -0.5, indicating the model prediction to be reasonably good. Lee and Briggs[62] and Yadav *et al*[6] have used similar kind of structure assessment tools for validating the 3D models of proteins. The Cα backbone of PTP model got well superimposed onto PTP structure as well as other closely related proteins from diverse group of organisms.

The 3D structure of PTP model got successfully docked with geometrically optimized hydrogen orthophosphate molecule (HPO_4). Analysis of the binding site revealed that phosphate binding pocket of the PTP model was predominantly occupied by hydrophobic residues. Though Huang *et al*[58] have identified asp45,269 to be the active site residues for phosphate binding site in glycerol -3 phosphate transporter protein, similar type of residues could not be found at the active site of the predicted PTP model. Instead, the alignment output shows that these regions were substituted by gln54 and val301 in the PTP sequence of *Piriformospora indica*. Further, val301 was not present in the close vicinity of the ligand, HPO_4, as revealed by the DeepView package. The docking results thus indicate that new types of residues are involved in binding the phosphate moiety. Despite Yadav *et al*[6] effort in understanding only the 3D structure of PTP for *P. indica*, the present study was successful in exploring the interaction between PTP and inorganic phosphorus. Outcome from the computational studies clearly shows the role *P. indica* can play as an efficient P transporter.

To prove PTP of *P. indica* is indeed involved in phosphate transport, greenhouse investigation with aerobic rice as the host plant treated with *P. indica*, *G. fasciculatum* as well as dual inoculation was

Table 5—Effect of fungal (*P. indica* and *Glomus fasciculatum*) inoculation on dry weight of shoot and root as well as panicle and grain yield of aerobic rice (var.MAS 946)

Treatments	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Number of panicles per plant	Grain yield (g/plant)
Ct	21.50[c]	11.83[b]	10.33[c]	29.15[c]
Pi	33.17[a]	18.50[a]	21.17[a]	42.13[a]
PG	32.33[a]	17.83[a]	19.33[a]	42.53[a]
Gf	27.17[b]	16.67[a]	15.00[b]	35.87[b]
SEM +/-	1.46	0.79	1.11	1.22
CD @ 5 %	4.31	2.32	3.27	3.61

The treatments details: Ct: Uninoculated plants/control; Pi: Plants inoculated with *P. indica*; PG: Plants dually inoculated with *P. indica* and *Glomus fasciculatum*; Gf: Plants inoculated with *Glomus fasciculatum*; Means followed by the same letter between the treatment levels do not differ significantly at $P = 0.05$.
Means followed by the same letter between the treatment levels do not differ significantly at P = 0.05

taken up. During the course of the investigation, several parameters were recorded and these include: % root colonization, NPK content, soil enzyme activity, chlorophyll and sugar content followed by plant growth and yield.

Root colonization was significantly higher in rice plants inoculated with *P. indica*, compared to remaining treatments. This could be due to the unique pattern of colonization strategy followed by *P. indica* while colonizing root cells of rice plants. During colonization process, some of the matured root cells of rice plants might be dead, thereby facilitating *P. indica* to colonize these root cells with ease. Deshmukh *et al*[53] have reported expression of *BAX inhibitor- I*, a gene responsible for preventing plant cell death, is attenuated in barley due to *P. indica* colonization without impairing root vasculature and functions. According to Qiang *et al*[68], *P.indica* mediated endoplasmic reticulum stress is a contributing factor for the attenuation of *BAX inhibitor-I*, leading to plant cell death. Further, Zuccaro *et al*[45] were able to demonstrate that mature cells of barley were readily susceptible for *P. indica* proliferation. Based on these evidences, it is possible to conclude that similar type of mechanism might also be operating in rice plant. Another reason for promoting better colonization by *P. indica* could be the suppression of plant innate immunity, as suggested by Deshmukh *et al*[53].

On the other hand, roots of rice plants that received dual inoculation had lesser colonization by *P. indica* and *G. fasciculatum* compared to the plants that were treated with *P. indica* alone. This could be due to the fact that *G. fasciculatum* being an AM fungus, are obligate biotrophs that require live host cells for proliferation[45]. This being the case, plant defense mechanism might play a role in restricting the proliferation of *G. fasciculatum* during its initial phase of colonization. In case of dual inoculation, there could be a competition between the two participating fungal symbionts to find a suitable niche, either in the form of live or dead tissues, eventually diminishing the extent of root colonization compared to the invasion of the root tissues by *P. indica* alone. Had longer duration crop been taken for the study, there is a possibility for the aforesaid scenario of root colonization to change.

There was no significant difference in shoot and root P content among rice plants treated with any of the three fungal inocula. This could be due to the fact both *P. indica* and *G. fasciculatum* might be having an efficient phosphate transporter protein capable of transporting P from soil. And therefore, cannot be taken as a sole criterion for selecting the efficacy of *Piriformospora* over *Glomus*. Yadav *et al*[6] were able to isolate and characterize a high affinity phosphate transporter gene from *P. indica* involved in the enhanced P uptake of host plant. Similar type of P transporter has also been reported from several *Glomus* species and plays active role in P transport from soil to host plant[18,20].

There was marked variation in N and K content of rice plants treated with *P. indica* compared to remaining treatments. Maximum N content was noticed in rice plants inoculated with *Piriformospora*. This could be due to elevated expression of NADH dependent nitrate reductase, a key enzyme for nitrate assimilation in plants as reported out by Sherameti *et al*[73]. *Piriformospora* also stimulates production of phosphatidic acid in host plants and plays role in nitrogen uptake and plant growth[57]. All these factors might have contributed for an increased uptake

of N by rice plants inoculated with *Piriformospora* compared to plants treated with *Glomus*. Since ammonium transporter has been reported to play a role in N uptake in the plant treated with AM fungi treatment[56], this could be one of the reasons for observing a higher N content in rice plants inoculated with *Glomus* compared to control. Rice plants inoculated with all the three different types of fungal inoculum had higher K content. As suggested by Abbot and Robson[46] and Varma *et al*[77], this could be due to the increased absorbing surface area of rice roots caused by extensive network of mycelium produced by both the fungal symbionts. Higher accumulation of K noticed in rice plants associated with these fungi is due to the fact that these root endophytes also triggers secretion of growth regulators, hastening K uptake[65]. Reduced level of root colonization in rice plants treated with *Glomus* alone and dual inoculation may be one of the contributing factors for lower N and K content compared to *Piriformospora* treatment.

Activities of acid and alkaline phosphatase were much higher in rhizosphere of rice plants inoculated with *P. indica* as well as *G. fasciculatum* compared to control. This could be the reason for rice plants inoculated with either *P. indica*, *G. fasciculatum* or both to have high P content. Fries *et al*[55] have demonstrated acid phosphatase and alkaline phosphatase activity in soil is associated with P uptake in plants. Archana *et al*[47] have reported that *P. indica* as a plant growth promoting fungus triggers secretion of acid phosphatases and thereby mobilizing complex forms of phosphate from rhizosphere. This facilitates host plant to have better accessibility of soil phosphorus. Since acid and alkaline phosphatases are prevalent in plant roots after mycorrhizal colonization, as proposed by Tisserant *et al*[75], it can be considered as a marker for analyzing the symbiotic efficiency of mycorrhizal colonization.

Chlorophyll content differed significantly in rice plants when inoculated either with *P. indica* or *G. fasciculatum* or both compared to uninoculated rice plants. This may be due to an increased stomata conductance, photosynthesis and number of chloroplasts with larger sized mesophyll cells in rice plants treated with fungal inoculum[59,69]. Moreover, presence of higher concentration of cytokinin in plants treated with AM fungi or *P. indica* could be one of the reasons for superior photosynthetic apparatus[51,67], thereby increasing chlorophyll content and photosynthetic rate.

When compared to untreated plants, higher concentration of total sugar in shoot and root was recorded in rice plants treated with *Glomus* and *Piriformospora* as well. This is in agreement with the findings of Borah and Phukan[49] who concluded that *Solanum melongena* inoculated with AM fungi significantly increased total sugar content compared to uninoculated plants. It is evident from the present study that compared to the untreated rice plants, plants treated with either *P. indica*, *G. fasciculatum* or both are capable of accumulating higher chlorophyll content. This may be responsible for abundance of photosynthates[66] and thereby contributing for higher concentration of total sugar in shoot. Although roots are devoid of chlorophyll, there is a considerable amount of sugar. This could be due to carbohydrate sink established by the symbionts colonizing the roots of host plants[48,52]. Similar analogy can be extended to the studies carried out by Sherameti *et al.*[73] who observed stimulation of starch degrading enzyme in *Nicotiana* and *Arabidopsis* inoculated with *P. indica*; this can be correlated with our findings which show an enhanced concentration of total soluble sugar in rice plants inoculated with *Piriformospora*.

Among all the treatments, rice plants inoculated with *P. indica* were superior as they showed maximum plant height, maximum number of leaves and tillers after 120 days of planting. This increase may be due to an enhancement in P uptake as well as growth promoting activities by *Piriformospora* and *Glomus*. *Piriformospora* as well as mycorrhiza improves host plant growth and development through acquisition of P and other mineral nutrients from soil and increased plant height may be due to increased plant growth promoting rhizosphere activity[21,50,64].

Shoot and root dry weight, number of panicle per plant as well as grain yield were significantly higher in all the fungal treated rice plants compared to uninoculated. These observations are in accordance with earlier findings[21,59] which demonstrates increased shoot and root dry weight of rice plants inoculated with *Glomus* and *Piriformospora*. Several past investigations reveals increased level of auxin concentration in plant roots associated with AM fungi and *P.indica*, leading to more number of lateral roots[53,63,74]. This also can lead to an increase in root biomass of plants treated either with *Glomus*, *Piriformospora* or both. Between the rice plants inoculated with *P. indica* and dual inoculation, the

former had higher shoot dry weight than the latter. Probable reason for this could be due to higher root colonization of rice plants treated with *P. indica* compared to any other treatments leading to higher secretion of phytohormones which ultimately boosts plant growth.

Outcome from theoretical studies suggest that the phosphate transporter protein from *P. indica* has an affinity for HPO_4 leading to an increased P uptake in rice plants treated with this fungus. This has also been confirmed by carrying out greenhouse experiments which clearly demonstrates the role of *Piriformospora* in P metabolism. Further, inoculation of aerobic rice plants (MAS 946) with *P. indica* proved to be a better choice as a potential biofertilizer over mycorrhiza.

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