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Study of *Piriformospora indica* as bioinoculant for nutrient management in Calcareous soil

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Abstract: Calcareous soil with high pH and nutrient deficiency has always been a threat to agricultural productivity. Adequate crop productivity and its supply is a need of the hour to meet increasing demand of food for growing population worldwide. Another major concern is loss of soil fertility due to over use of chemicals and other environmental factors which lead to serious nutrient deficiency. These nutrient deficiencies eventually limit the scope of agriculture in those areas and causing loss of productivity. Organic agriculture can be an alternative source for restoring soil nutrients content and their physicochemical properties. Microbial reclamation of nutrient deficient calcareous soil is a novel approach towards sustainable agriculture without causing any environmental disturbances. *Piriformospora indica* a root endophytic fungus already proved to be a beneficial influence on plant growth and were studied for its role in creating a channel for nutrient supply to plants at the same time reducing the burden of chemicals usage. In-vitro study reveals that the fungus can tolerate an alkaline pH up to 11.0 and remain metabolically active. The study also revealed that fungus has high affinity towards insoluble Calcium Carbonates and Phosphates which enhances the Cell Dry Biomass upto 30 to 40 percent. Further studies revealed that the fungus was tolerant and physiologically active at increasing concentration of Calcium Carbonate. *P. indica* in present study proves to be a potent source of bio-inoculants for nutrient management in Calcareous soil.
Key Words: Calcareous Soil, Nutrient Deficiency, *Piriformospora indica*, Alkali tolerant, Nutrient Management.

Introduction:

Crop cultivation in calcareous soil always has been a challenge when considered overall growth and productivity. Calcareous soil has high pH making the soil alkaline due to presence of calcium carbonate as a main component. The major factors affecting the yield are failure of the crop to absorb enough nutrients from soil, non-availability of essential nutrient elements in soil, unfavourable environmental conditions, incidence of insect pests and diseases, etc[9]. The most commonly used chemical for treatment of calcareous soils has traditionally been sulphur and gypsum, due to availability and low cost. Conventionally, these chemicals are applied to the surface of the soil and then incorporated by employing normal cultivation practices. Eventually the chemical dissolved either by the native soil moisture, by irrigation water or by natural precipitation, or a combination thereof.

There is however a number of disadvantage regarding the performance of chemicals in reclaiming soil condition. Though chemical amelioration has ensured crop cultivation in calcareous soil, still deficiency has been observed in crops. This may be dueto the improper assimilation of applied chemical which gets tied up in highly insoluble compounds, rendering the added nutrient only sparingly available for plant uptake. High concentration of Calcium and Carbonates inhibits the uptake of nutrients by plants[8]. Also cost of restoring alkali land by chemical use is virtually huge for permanent cure. In the present scenario, food security and nutrient management are two most important aspect need to be addressed in modern agricultural practices. Soil origin, natural condition and improper farming practices have transformed huge areas of agricultural potent soil into barren land. There is an immediate need to address this issue and bring these soils under agriculture. One potential remediation method involves the stimulation of microbial population in calcareous soils to mobilize nutrients like Phosphate and enhancing the other beneficial Rhizospheric microbial population which eventually improve soil quality and at the same time supply nutrients to plants. In sustainable agricultural, horticultural and forestry ecosystems, different beneficial microbes were already explored to enhance crop production and tolerance of plants to different biotic stress conditions[9]. Many micro-organisms including rootendophytes establish symbiotic relationships with plants and play an essential role in maintaining a better soil and plant health[10].

Piriformospora indica, a root endophytic fungus already proved to be beneficial influence on plant growth especially under water scarcity and salt stress[11]was discovered by Verma *et al*[3].*P. indica* a root colonizing fungus, has positive influence on a wide range of plants of agriculture, forestry and flori-horticulture. The fungus has a wide-host root-colonizing endophytic fungus which allows the plants to grow under extreme physical and nutrient stresses[4]. The fungus promotes nutrient uptake, allows plants to survive under water, temperature and salt stresses, and confers systemic resistance to toxins, heavy metal ions, insects and pathogenic organisms[5]. In the present study *P. indica* was tested for its growth under in-vitro calcareous condition at high alkaline pH.*P indica* was also subjected to different soluble and insoluble forms of Calcium and Phosphates. The percentage was varied to study the maximum tolerance as compared to the composition of calcareous soil. In the present research,*P. indica* was studied for its role and potency of growth under alkaline pH and high concentration of Calcium carbonate. The present study thus conducted can hereby explore the possibilities of *P. indica* as an excellent bio-inoculant for nutrient management in calcareous soil considering its past references.

Experimental:

Collection of Culture:

Pure culture of *Piriformospora indica* Verma et al. was obtained from Amity institute of Microbial Technology, Amity University. *P. indica* was subcultured and prepared in Hill Kaefer Agar media[6] and PDA media and were incubated at 28^{o}C± 2^{o}C for 5-7 days. It was maintained and preserved on Hill Kaefer's medium[6]. *P. indica* was cultured as described previously[3,7]in Petri dishes on a modified Kaefer's medium (KM: $NaNO_3$, 7.0mM; KCl, 7.0mM; $MgSO_4$, 2.1mM; KH_2PO_4, 9.2mM; $ZnSO_4$, 0.77mM; H_3BO_4, 0.18mM; $MnSO_4$, 0.02mM; $CoCl_2$, 0.007mM; $CuSO_4$, 0.0065mM; $FeSO_4$, 0.02mM; EDTA, 0.02mM; ammonium molybdate, 0.001mM; thiamine, 0.003mM; gylcine, 0.005mM; nicotinic acid, 0.002mM; pyridoxine, 0.0004mM; glucose, 110mM; peptone, 2g/l; yeast extract, 1g/l; casein hydrolysate, 1g/l, pH 6.5) with 1% (w/v) agar. The plates were preserved in replicate at 4^{o}C for future studies.

To study the growth of *Piriformospora indica* at pH range 4.0-11.0.

P. indica was studied in vitro for its growth at acidic and alkaline pH, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. The cultures were subjected to different pH in solidi.e. Agar as well as in liquid media. The inoculation was done in PDA and PDB medium at different pH rangei.e. 4.0 -11.0. The plates were incubated at 28^{o}C± 2^{o}Cfor 5-7 days and broth were incubated at Shaker incubator at 28^{o}C± 2^{o}C at 180 RPM for 4-7 days. Radial growth, Cell Dry Biomass, was checked after 5 days and 7 days of incubation. All the experiments were conducted with 5 replicates.

To study the growth of *Piriformospora indica* under different soluble and insoluble forms of Calcium and Phosphate.

P.indica was studied in-vitro for its growth and metabolism at different types of soluble and insoluble Calcium and phosphates. Potato Dextrose Agar and Potato Dextrose Broth were supplemented with different Calcium and Phosphorous containing soluble and insoluble compounds. The different sources were Calcium Carbonate, Calcium Chloride, Tri-Calcium Phosphate, Potassium dihydrogen phosphate, dipotassium hydrogen phosphate. The plates were incubated at 28°C± 2°C for 5-7 days and broth were incubated at Shaker incubator at 28°C± 2°C and at 180 RPM for 5-7 days. Radial growth, Cell Dry Biomass, was checked after 4 days and 7 days of incubation. All the experiments were conducted with 5 replicates.

To study of growth of *Piriformospora indica* under different concentration of Calcium Carbonate:

P.indica was studied in-vitro for its growth and tolerance at different concentration of Calcium Carbonate. Potato Dextrose Agar and Potato Dextrose Broth were supplemented with different concentration of Calcium Carbonate viz., 0.25 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 % and 3.5 %. The Calcium Carbonate at different concentrations as above was added after pH was adjusted to pH 6.0.the experiment was conducted in both liquid i.e. broth and solid i.e. in Agar media. The autoclaved medium was poured in sterile petri plates and broth was kept as it is for inoculation. The inoculation was done from freshly prepared *P. indica* plate. The inoculated plates were incubated at 28°C± 2°C for 5-7 days and broth were incubated at Shaker incubator at 28°C± 2°C at 180 RPM for 5-7 days. Radial growth, Mycelia Biomass, and amount of chlamydospore formation were checked after 4 days and 7 days of incubation. All the experiments were conducted with 5 replicates.

Statistical Analysis.

The collected data were statistically computed using Graph Pad Prism 6 software. Data were subjected to analyses of variance and treatment means were compared at significant at $P < 0.05$.

Results:

Collection of culture.

A pure culture of *Piriformospora indica* was obtained and microscopically observed for its mycelia structure and pattern. *P. indica* was sub-cultured and prepared in Hill Kaefer Agar media and PDA media. Mycelia were recorded as white and almost hyaline, but conspicuous zones (rhythmic growth) are observed in older cultures. The mycelium was observed as mostly flat and submerged into the substratum. Chlamydospores appear singly or in clusters and were distinctive due to their pear shaped structure[1].

To study the growth of *Piriformospora indica* at pH range 4.0-11.0.

P. indica in present study were tested for its efficiency to grow at both acidic and alkaline pH, especially with respect to calcareous soil.*P. indica* shows considerable variation in growth in terms of radial growth and cell dry biomass (CDB) when allowed to grown at pH range of 4.0-11.0.The medium was analyzed for pH after 5th days and 7 days of incubation, but no fall in pH was observed.

Table 1: Cell Dry Biomass (g/ 100 ml of broth) and Radial diameter in cm of *P. indica* when grown at pH 4-11 after 5 days of incubation (5 DAI)

pH4.0		pH5.0		pH6.0		pH7.0		pH8.0		pH9.0		pH10.0		pH11.0	
Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)	Cell Biomass (g/100ml broth)	Radial Diameter (cm)
1.07 ± 0.12	4.0 ± 0.21	1.09 ± 0.13	4.0 ± 0.19	1.10 ± 0.13	4.4 ± 0.19	1.28 ± 0.14	4.9 ± 0.20	1.15 ± 0.11	4.7 ± 0.18	1.18 ± 0.14	4.8 ± 0.21	1.09 ± 0.12	4.5 ± 0.19	0.97 ± 0.10	3.7 ± 0.18

*Values are mean ± SD of five replicates

Table 2: Cell Dry Biomass (g/ 100 ml of broth) and Radial diameter of *P. indica* when grown at pH 4-11 after 7 days of incubation (7 DAI)

pH 4.0		pH 5.0		pH 6.0		pH 7.0		pH 8.0		pH 9.0		pH 10.0		pH 11.0	
Cell Biomass (g/100ml)	Radial Diameter(cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)	Cell Biomass (g/100ml)	Radial Diameter (cm)
1.25 ± 0.12	4.5 ± 0.19	1.32 ± 0.11	4.7 ± 0.20	1.45 ± 0.14	5.3 ± 0.22	1.58 ± 0.15	5.5 ± 0.23	1.55 ± 0.12	5.61 ± 0.24	1.51 ± 0.15	5.32 ± 0.20	1.21 ± 0.13	4.92 ± 0.18	1.17 ± 0.12	4.5± 0.19

*Values are mean ± SD of five replicates

Study revealed that there was increase in biomass and radial growth at pH range 7.0 -10.0. A maximum of Cell Dry Biomass (CDB) was recorded ie1.45 ± 0.14 g/100 ml to 1.51 ± 0.15 g/100 ml at pH 6.0 and 9.0 respectively. At pH 10, the cell dry biomass was 1.21± 0.13 g/100ml which was significantly moderate compared to the values recorded for other pH. Cell Dry Biomass was recorded for pH 11.0i.e. 1.17 ± 0.12 g/100 ml which was comparativelyless than the neutral pH.The maximum radial growth was recordedas 5.3± 0.22 cm to 4.92 ± 0.18 at pH range 6.0 to 10.0 respectively. A slight decrease in radial growth was observed and recorded forpH 11.0i.e. 4.5 ± 0.19 cm.Though there was gradual decrease in biomass and radial growth, from the above data, *P. indica*Verma*et.al.* Isolate can be considered to be alkali tolerant. The microscopic analysis confirmed the presence of healthy mycelia and chlamydospore formation without any stress at pH 6.0-10.0. At pH 11.0 though the mycelia was found to be healthy, there was less formation of peer shaped chamydospores, which is the characteristics of *Piriformosporaindica.*

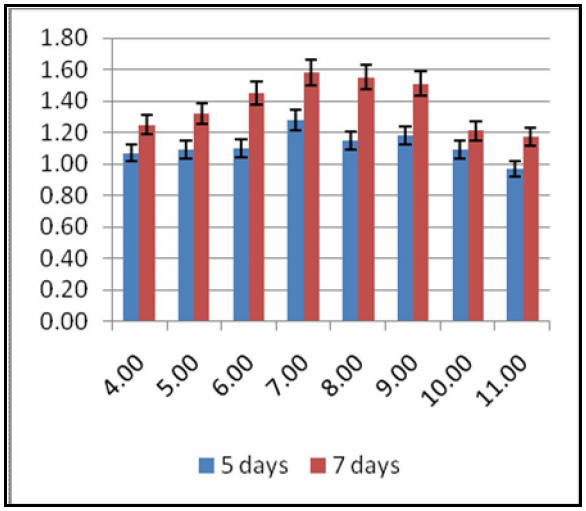

Fig 1: Cell Dry Biomass of *P.indica*(g/100 ml)at pH range of 4.00 to 11.00

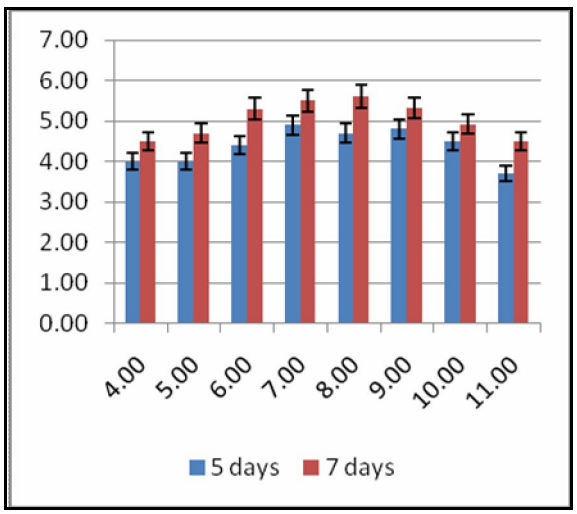

Fig 2: Radial cell growth of *P.indica*in cm at pH range of 4.00 to 11.00

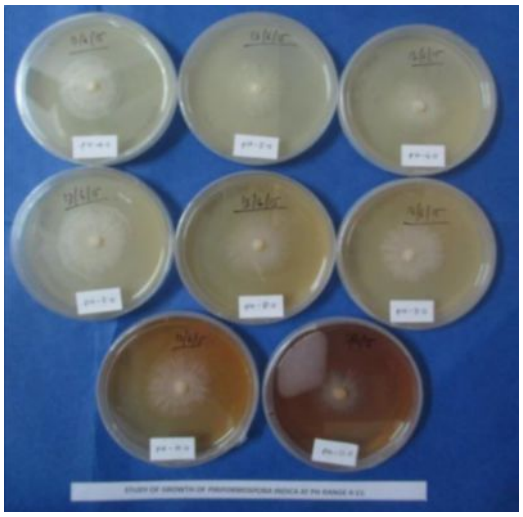

Fig 3: Fig: Radial cell growth of *P.indica*at pH range of 4.00 to 11.00 after 5days.

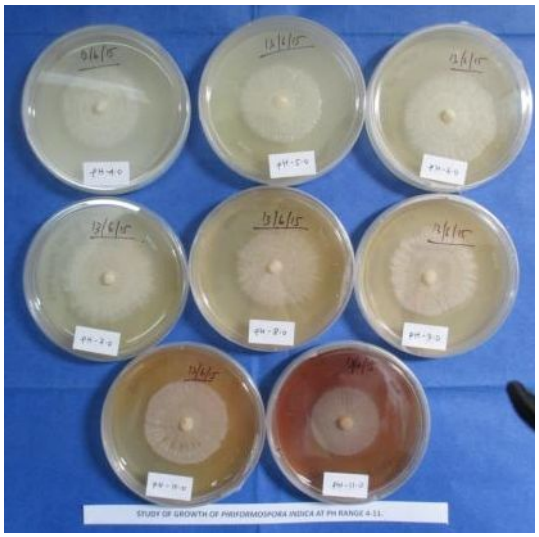

Fig 4: Fig: Radial cell growth of *P.indica* at pH range of 4.00 to 11.00 after 7 days.

To study the growth of *P.indica* under different soluble and insoluble forms of Calcium and Phosphate.

Piriformospora indica was studied for its growth in different sources of soluble and insoluble Phosphate and Calcium. *P indica* shows prominent and varied growth and biomass production at different calcium and Phosphate sources. *P.indica* when supplemented with Calcium carbonate and Tri-calcium Phosphate recorded a remarkable increase in biomass and radial growth compared to other components. The insoluble calcium and Phosphate induces the growth to a tune of 25 to 30 %. The insoluble compounds used in the study were found to be completely solubilised in the absence of any organic acids production. This maybe due to the presence of Phosphate transporter system of *P. indica* that facilitates the disappearance of turbidity of the broth to clear transparent liquid[2].

Table 3: Cell Dry Biomass, Radial growth and pH after 5 days and 7 days of incubation when grown in different media supplemented with different sources of soluble and insoluble forms of Calcium and Phosphates.

S.No	Compounds	pH		Cell Dry Biomass gm/100 ml		Radial Growth in plate (cm)	
		5 days	7 days	5 days	7 days	5 days	7 days
01	Calcium Carbonate	6.6±0.21	6.59±0.19	1.46±0.14	1.72±0.12	4.3±0.16	6.3±0.18
02	Calcium Chloride	5.7±0.19	4.9±0.18	0.94±0.11	1.10±0.13	2.8±0.14	3.4±0.15
03	Tri Calcium Phospahte	6.5±0.22	6.2±0.18	1.52±0.15	1.88±0.15	4.6±0.18	6.9±0.15
04	Potassium dihydrogenPhospahte	6.7±0.21	6.5±0.20	0.97±0.12	1.15±0.12	2.9±0.15	3.5±0.13
05	Dipotassiumhydrogen Phosphate	6.4±0.9	6.2±0.19	1.11±0.15	1.25±0.15	2.7±0.16	4.0±0.17
06	Ferric Phosphate	6.5±0.22	6.3±0.21	0.62±0.10	0.75±0.12	2.5±0.15	3.0±0.13
07	Control (PDA/PDB)	6.6±0.19	6.4±0.19	1.12±0.11	1.28±0.13	4.3±0.17	4.8±0.17

*Values are mean ± SD of five replicates

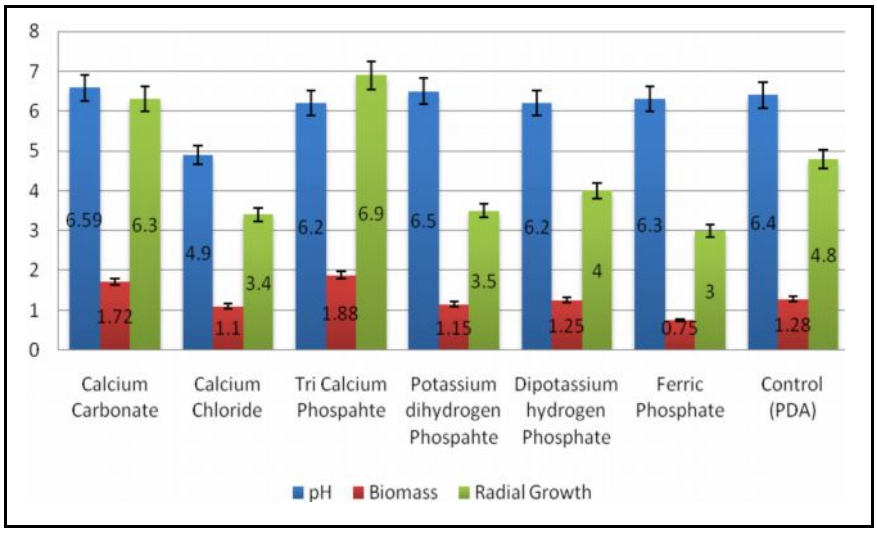

Fig 5: Biomass, Radial Growth and pH of *P.indica* when grown under different soluble and insoluble forms of Calcium and Phosphate.

The results obtained were mean of 3 replicates. The results were recorded after 5 days and 7 days of incubation. There was no as such variation in pH in all the supplemented broth. Significant yield in bio mass and radial growth of *P. indica* was recorded when supplemented with 0.5 % Calcium Carbonate and Tri-Calcium Phosphate respectively. Microscopic observation of these two samples shows maximum number formation of pear shaped chlamydospores[3]. The mycelia were also observed to be more rigid and healthy in Calcium carbonate and TCP supplemented broth compared to other supplemented sources including Control (Potato Dextrose Media).

To study the growth of *Piriformospora indica* under different concentration of Calcium Carbonate:

Piriformospora indica was studied for its growth in different concentration of Calcium Carbonate. *P. indica* shows optimum growth and biomass production at different calcium carbonate percentages i.e. 0.5% to 3.5 %. *P. indica* when supplemented with Calcium carbonate and Tri-calcium Phosphate recorded a remarkable increase in biomass production and radial growth compared to other components. The insoluble calcium and Phosphate increased the growth to a tune of 25 to 30 %. The insoluble compounds used in the study were found to be completely solubilised without any production of organic acids. This may be due to the presence of Phosphate transporter system of *P. indica* that facilitates the disappearance of turbidity of the broth to clear transparent liquid[2].

Table 4: Growth, pH, Cell Dry Biomass and Radial Growth of *P. indica* when grown in different concentration of Calcium Carbonate.

S.No	Calcium Carbonate %	pH		Cell Dry Biomass gm/100 ml		Radial Growth in plate (cm)	
		5days	7 days	5 days	7 days	5 days	7 days
1	0.25	6.54±0.21	6.45±0.18	1.35±0.14	1.76±0.14	3.77±0.12	5.66±0.14
2	0.50	6.53±0.22	6.51±0.19	1.51±0.15	1.81±0.17	4.01±0.13	5.89±0.12
3	1.00	6.66±0.23	6.56±0.21	1.55±0.16	1.88±0.16	4.06±0.13	6.01±0.14
4	1.50	6.71±0.23	6.58±0.20	1.52±0.15	1.89±0.15	4.09±0.12	6.08±0.13
5	2.00	6.73±0.21	6.63±0.19	1.49±0.13	1.86±0.15	4.09±0.12	6.06±0.16
6	2.50	6.76±0.20	6.66±0.18	1.50±0.16	1.88±0.16	4.11±0.11	6.12±0.15
7	3.00	6.78±0.22	6.69±0.22	1.51±0.14	1.87±0.14	4.10±0.13	6.11±0.15
5	3.50	6.76±0.21	6.66±0.23	1.41±0.12	1.65±0.15	4.01±0.12	5.99±0.13

*Values are mean ± SD of five replicates

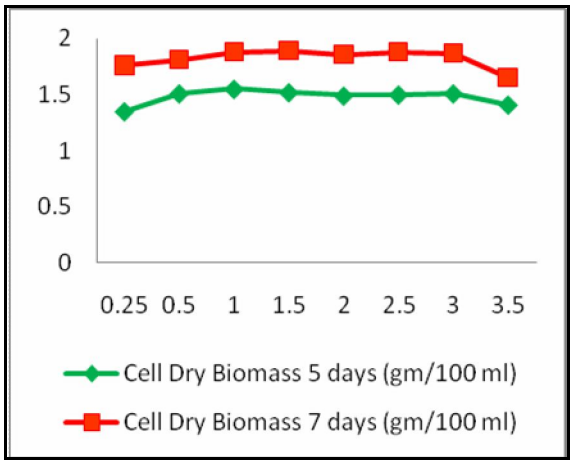

Fig 6: Cell Dry Biomass of *P.indica* when grown under different soluble and insoluble forms of Calcium and Phosphate.

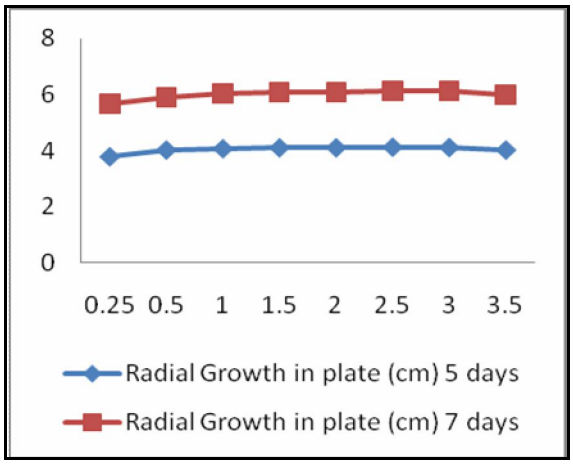

Fig 7: Radial Growth of *P.indica* when grown under different soluble and insoluble forms of Calcium and Phosphate.

The result obtained was mean of 5 replicates. There was no significant fall in pH when *P.indica* was grown at different concentration of Calcium Carbonate. There was no production of acid while complete dissolution of insoluble Calcium carbonate which was a significant observation in the study. Calcareous soil has high percentage of Calcium carbonate which alters soil pH and also affects the availability of nutrients to plants. Present study envisages a possibility of adopting *P indica* for nutrient management in Alkaline Calcareous soil. Significant yield in bio mass and radial growth of *P. indica* was recorded when supplemented with different concentration of Calcium Carbonate. At Calcium Carbonate concentration of 0.25 % to 3.5%, a steady production of cell dry biomass (CDM) 1.76±0.14 to 1.65±0.15 and Radial growth of 5.66±0.14 to 5.99±0.13 was recorded.No as such inhibition was recorded due to increasing concentration of the carbonates. Microscopic observation also shows more number of pear shaped chlamydospores[3].

Discussion:

Piriformospora indica is an endophytic fungus that colonized monocots as well as dicots[15]. *P. indica* has been termed as plant probiotic because of its plant growth promoting activity and its role in enhancement of the tolerance of the host plants against abiotic and biotic stresses[13]. *P. indica* is already reported to be involved in high salt tolerance, disease resistance and strong growth-promoting activities leading to enhancement of host plant yield[12, 14]. Present study was conducted to understand *P. indica* capacity to withstand high alkaline pH.*P indica* was also subjected to stress of different concentration of Calcium Carbonate with respect to Calcareous soil. This study has great significance as the fungus is being exploited and tested for bioremediation of calcareous soil. From the study, it is concluded that *Piriformspora indica* Vermaet.al, can with stand alkaline

pH upto 11.0. The fungus found to have optimum growth at pH 6.0-10.0. Increase in cell dry biomass and radial growth along with presence of significant amount of chlamydospores at a wide range of pH 4.0-11.0. The fungus can tolerate and shows prominent growth and metabolism at low acidic pH 4.0 to high alkaline pH 11.0. *P. indica* was also studied in-vitro for its activity to solubilize and utilizes different sources of insoluble and soluble form of Calcium and Phosphate. *P indica* when supplemented with Calcium Carbonate and Tri-Calcium Phosphate showed remarkable growth. To understand the tolerance of *P. indica*, on different concentration of Calcium Carbonate, a study was conducted and it was concluded that Calcium Carbonate promotes the growth of *P.indica*. Considering the present research, *P indica* Verma *etal*. Isolate is a promising agent for use in organic agriculture to manage nutrient deficiency in Calcareous soil.

This also provides further scope to study the interaction of *P.indica* with other beneficial microbial inoculants which are presently used in the bioremediation of calcareous soils.

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