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Studies on effect of *in vitro* co-cultivation of [*Arachis hypogaea* (L.)] with *Piriformospora indica* on plant growth

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Abstract
Piriformospora indica, a novel, axenically cultivable, endophytic fungus belongs to recently defined family Sebacinaceae, colonizes the roots of variety of plants and shows significant plant growth promotion, increased biomass and other multi-functional activities. Groundnut [*Arachis hypogaea* (L.)] is the important oil-seed crop in India. The present study was performed aiming to co-cultivate *P. Indica* with groundnut plant roots under *in vitro* conditions and results were analyzed by Two-sample analysis test. The growth parameters under *in vitro* study of *P. indica* treated and controlled plants such as plant biomass (fresh weight, dry weight), shoot length, root length, protein content were significant and found non- significant in sugar content and total chlorophyll content. The staining and the microscopic observations confirmed the persistence of chlamydospores of *P. indica* intracellularly in treated roots. The PCR reaction performed to monitor the existence of *P. indica* in plant roots, the band size 220 bp was observed in treated plant root and fungal mycelia where it was absent in control and untreated root sample. The results of this study indicated the growth enhancement of plant under co-cultivation, so the *P. indica* could be exploited to boost up the growth enhancement effects in *A. hypogaea*.

Keywords: *Piriformospora indica*, [*Arachis hypogaea* (L.)], co-cultivation, growth enhancement, *PiTef*

Introduction
Groundnut [*Arachis hypogaea* (L.)], belonging to the family fabaceae and sub family papilionaceae is an important food and oil crop in tropical and subtropical regions. It is also known as peanut, monkey nut, pig nut and goober nut. The name of the genus "Arachis" in greek means underground and the species name "hypogaea" means underground. It is categorized as grain, oil seed and legume crop. It is one of the fore most important oilseed crops of the world. Most of the wild species of the ground nut are diploid (2n=20) but the cultivated species are tetraploid (2n=40).

Ground nut is the native of South America and is widely grown in the tropical and subtropical region of the world. India ranks second in the world in groundnut cultivation with around 26.20 million hectares under cultivation with production of 25.25 million tons and productivity of 968 kg per hectare. In state of Maharashtra it is cultivated at around 0.24 million ha area with production 0.24 million tons and productivity is around 988 kg/ha. (Anonymous, 2016) [1].

The fungus is named *Piriformospora indica* as the chlamydospores of the fungus are pear shaped (piriformo: *pear shaped*) and discovered in India. This fungus belongs to recently defined family sebacinaceae and order sebacinales (Verma and Arya, 1998) [36]. *P. indica* shares similarity with arbuscular mycorrhiza in terms of colonization with majority of (80%) vascular plant roots (Newman and Reddel 1987) [22] but such arbuscular mycorrhizas are obligate biotrops, needs a host to grow. In contrast most of ericoid and ectomicorizal fungi can be grown on artificial media but their root colonization is limited to Ericaceae or woody plants. *P. indica* shows ease in cultivation on artificial media and versatility in terms of interaction with plants such as mono- and dicotyledonous plants (Pham *et al.* 2004, Verma and Arya 1998) [25, 36]. *P. indica* can be a promising tool for significant plant growth promotion and multifunctional activities which increased the scope for the study of plant-fungi interactions. *P. indica* interaction with plants attributes several growth promoting effects such as increased average seed weight (Rai *et al*., 2001) [27], raised grain yield in spring barley (Waller *et al*., 2005) [38], promoted nutrient uptake (Kumar *et al*., 2011) [16], stimulated resistance against biotic (Lin, *et al* 2019) [19] and abiotic stresses in rice (Jogawat *et al*., 2013) [13], imparts enhanced production of biomass in economically important crops (Franken 2012) [10], early flowering in

arabidopsis (Pan *et al*., 2017) [23], acts as biofertilizer and bioprotector in its host plants (Singh *et al*., 2003) [32] further induced seed germination in Orchid (Blechert *et al*., 1999) [6] and helped in biological hardening for micropropagated tobacco (Sahay NS. and Varma A., 1999) [29].

A perusal of literature related to *P. indica* revealed that very little work has been carried out on interaction of *P. indica* with *A. hypogaea* which is economically important food and oil seed crop. In this study, we investigated the effect of *P. indica* on the significant growth enhancement in *A. hypogaea.*

Materials and methods Cultivation of *P. indica*

The mother culture of endophyte *P. indica* was cultured in a modified *Kaefer Medium* (Hill and Ka¨fer 2001) [12]. The slightly acidic KM medium is a good source of sugar and protein. The growth parameters for fungal growth such as pH, temperature and incubation time were standardized and the fungus was grown in dark, under optimum growth conditions (pH of media: 6.5, temperature: 22–24 ºC, incubation time: 4 weeks). The circular disc having spore and fully grown hypae of *P. indica* (5 mm in diameter) is subcultured to the center of a fresh PNM medium dish in the dark in an inverted position for 7 days at 22 ºC. (Johnson *et al.,* 2011 b) [14].

***In vitro* seed germination**

To eliminate the naturally occurring surface microorganism, under a laminar airflow cabinet the seeds were surface-sterilized sequentially with sterile distilled water for three times and 0.1% $HgCl_2$ (1 min) and finally rinsed for three times with sterile distilled water. These seeds were aseptically transferred to 1/2 MS medium (Murashige, T and Skoog F, 1962) [21] medium supplemented with 3% (w/v) sucrose and pH 5.8. The proper root growth was achieved by adding 1.0 mg/l naphthalene acetic acid (NAA) along with controlled conditions at 25±1 ºC with a light intensity of 80 l E/m^2 /s and 16 h light/8 h dark cycle.

***In vitro* co-cultivation of *P. indica* and ground nut plant** Co-cultivation procedure was carried out according to the model system described by Johnson *et al.,* (2011b) [14] to study the plant beneficial traits. The 12 days old morphologically identical seedlings were transferred to modified PNM medium which have the previously grown mycelia of *P. indica*, which facilitate proper growth of fungus and the seedlings. (Pesˇkan-Bergho¨fer *et al*., 2004; Shahollari *et al*., 2007b; Sherameti *et al*., 2008) [24, 30, 31].

Colonization of fungus: Microscopic observation were taken to ensure and monitor root colonization of *P. indica* fungus with the roots of forty five days old *in vitro* seedling. five root samples were collected randomly, cut into 1 cm length pieces and washed thoroughly in running tap water to remove traces of media. The roots were softened in 10% KOH solution for 15 min, acidified with 1 M HCl for 10 min and stained with 0.02% of trypan blue in lactophenol for overnight (Dickson, S 1998, Phillip and Hayman, 1970) [8, 26]. The roots were destained with lactic acid: glycerol: water solution in the ratio of 14:1:1. The roots were mounted for microscopic examination.

The presence or absence of chlamydospores was considered as an index for the presence or absence of colonization. The quantification of colonized roots was assessed by percentage colonization (Giovannetti and Mosse, 1980) [11].

$$\text{Percentage of colonization} = \frac{\text{Number of root segments colonized}}{\text{Total number of root segments observed}} \times 100$$

Growth analysis of *in vitro* cultured plants: The *P. indica* inoculated plants and control plants were observed at 15 to 45 days at the intervals of 0, 15, 30, and 45 days to study the growth parameters. The growth parameters recorded under study were root lengths, shoot lengths and dry weight. The length of roots and shoots were measured in cm with the help of scale. The total soluble sugar (Yoshida, *et al* 1972) [40], soluble protein (Lowry, *et al* 1951) [20], the total chlorophyll (Arnon, 1949) [3] were estimated. The plant was taken out of the media and washed off to remove any loose media to measure fresh weight of the plant. The plants were blot to remove any free surface moisture and weighed immediately. The dry weight was measured by washing and blotting the plant removed from media; further the plant was dried in an oven, at 100° F overnight, cooled it in a dry environment and weighed.

PCR (Polymerase chain reaction) assay to ensure the persistence of *P. indica* in groundnut roots

The 100 mg of 45 days old *P. indica*-colonized roots and controlled roots were used for genomic DNA isolation. The DNA also isolated from the mycelia grown in the liquid culture (Fig. 4) The DNA isolation was performed from all the sample by cetyltrimethylammonium bromide (CTAB) method with slight modifications (Rogers and Bendich 1994) [28].

In the present investigation the annotated primer *P. indica* transcription elongation factor (*PiTef*, GenBank accession no: AJ249911, Buetehorn *et al*. 2000) [7] was used to perform PCR. Primers had the sequences 5′- TTCTGGGAAGTCGTCTCTG-3′and 5′-AGCCAACCATGA-AGAAGTG-3′. The final volume of 25 µl master mix contained 2.5µl PCR buffer 10X, 0.8µl MgCl2(500 mM), 0.8µl dNTPmix(10mM), 1µl of each 10 pM primers (forward and reverse primers), 0.3µl taq DNA polymerase (5U/µl), 16.6µl deionized water. Cycling conditions were 94°C for initial denaturation at for 3 min followed by 35 cycles of 94 °C for 2 min, 50 °C for 1 min, and 72 °C for 2 min and the final extension of 72 °C for 8 min. The PCR products were visualized and analyzed by agarose (2%) gel electrophoresis. In the lane M ladder of 50 bp was loaded however next three lanes (1, 2 and 3) were used to load the PCR products from *P. indica* treated, non-treated and fungal mycelium respectively; the last lane (4) was used as a water control.

Results

Growth promotory effects of *P. indica* inoculation to host *A. hypogaea*

The Effect of fungi colonization resulted in the enhanced growth characteristics in treated plants compared to controlled plants grown under *in vitro* condition. The regular observation from 1st day of co-culture to 45th days revealed that the considerable growth enhancement was started after 13 days of co-cultivation. The growth characteristics of treated and controlled plants under study were analysis by statistically by paired comparison t- test (Sokal and Rohlf 1981) [33] and biometric data calculated with SAS (6.12).

The treated and controlled plants showed significant difference in plant biomass (fresh weight, dry weight), shoot length, root length, protein content and found non-significant in sugar content and total chlorophyll content.

In vitro co-cultured plants were analyzed after 45 days, the data observed and recorded for the growth parameters of plants were significantly affected by *P. indica* interaction after 45 days. (Table No.1). The biometric observation reveled the differences in morphological characters, in case of shoot length, plants colonized with *P. indica* (6.86 cm) compared with control plants (5.03 cm). Likewise, measurements recorded of root length were

longest (6.06 cm) in *P. indica* inoculated plants and shortest in controlled roots (4.13 cm). Additionally, *P. indica* co-cultured plant showed the potential of increasing the biomass (dry weight, 0.93 g) and biomass (fresh weight, 5.9 g) compared to controlled plants biomass (dry weight, 0.74 g) and biomass (fresh weight, 3.66 g). The biochemical analysis of treated and controlled plants showed significant change in protein concentration however there was no significant change in the leaf morphology and sugar content of plants under comparison.

Staining of colonized roots of host plant

The proper colonization of *P. indica* in roots of groundnut plant was studied after 45 days of co-culture by Trypan blue staining and microscopic observations. The fully developed intra cellular spear shaped chlamydospores was found in single, double or tetrad chain in the co-cultured roots of host plant while such spores were absent in controlled plant roots. The root colonization was ranged from 50-60% (Fig 3).

PCR assay

The PCR analysis was carried out by amplifying genomic DNA from treated roots, untreated roots and fungual mycelia by using *P. indica*-specific *PiTef* gene primers. The pcr products were electrophoresed to investigate the gene specific bands in the agarose. The amplicon of size 220 bp was observed in the *P. indica* colonized roots of plant and such type of amplicon was absent in controlled and untreated sample.

Discussion

The significant growth promoting effects of plant and *P. indica* interactions were corroborated in earlier studies in various crops. In the present investigation the percentage of root colonization noted in the range of 50-60% which was reported 60- 70% in *Curcuma longa* (Bajaj *et al.,* 2014) [5], 68% in *B. Oleracea* (Doltabadi and Goltapeh, 2013) [9]. The higher biomass production observed in present co-cultivation study of plant fungus interaction which may be resulted due to increase in leaf surface area (Achatz *et al.,* 2010) [1] or may be due to more number of leaves along with increased shoot length causes the increased rate of photosynthesis (Kungu *et al.,* 2004) [17]. The overall enhanced biomass has reported earlier in various plants after plant-fungus interaction (Varma *et al.,* 2001) [35]. The leaf morphology was similar in both treated and controlled plants, same observed by Bajaj *et al.,* (2014) [5]. There was no significant difference found in chlorophyll content which was found significant by earlier worker Jogawat, *et al.,* (2013) [13]. The non-significant results were found in total chlorophyll content, the sugar content also shown similar data. There is close relationship of chlorophyll contents with photosynthetic rate (Susheelamma *et al*., 2002) [34], which may have effected on the total sugar content.

The confirmation of plant interaction was analyzed by molecular technique i.e. PCR aasay by *PiTef* gene The amplicon size of 220 bp was observed for the *PiTef* gene in the present investigation was in coherence with the observations made by (Lakshmipriya, *et al*., 2017) [18].

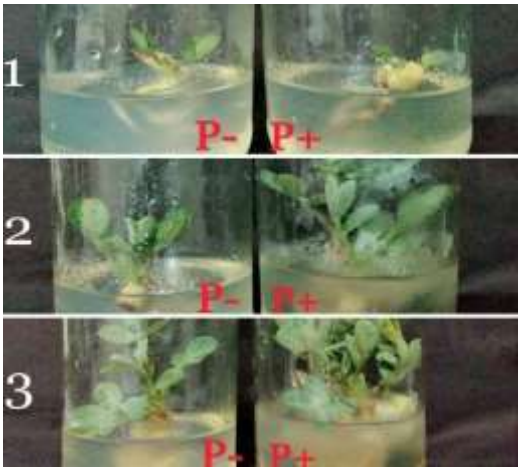

Fig 1: Effect of *In vitro P. indica – A. hypogaea co-cultivation on growth* of plant. Picture (1): 12 Days old *A. hypogaea plants, Picture* (2): plants after 25 days, Picture (3): plants after 45 days; Experiment was conducted by paired comparison t- test statistical method with 3 replication.

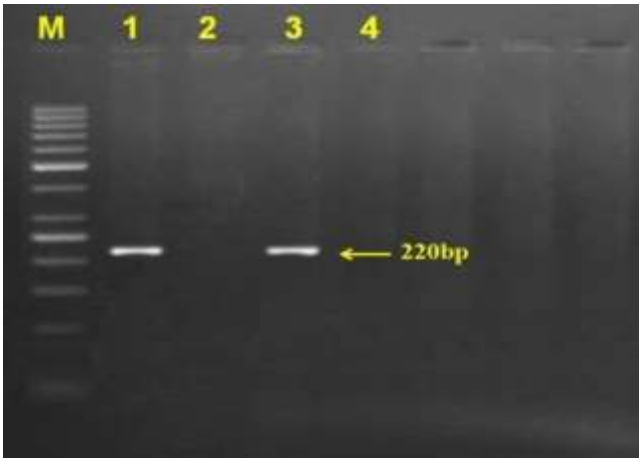

Fig 2: Confirmation of *P. indica* colonization by PCR using *PiTEF1* primers. M: 50bp marker, Lane 1: Amplification from *P. indica* co-culture plant roots, Lane 2 =controlled roots, Lane 3 mycelia of *P. indica* and lane 4: Water control

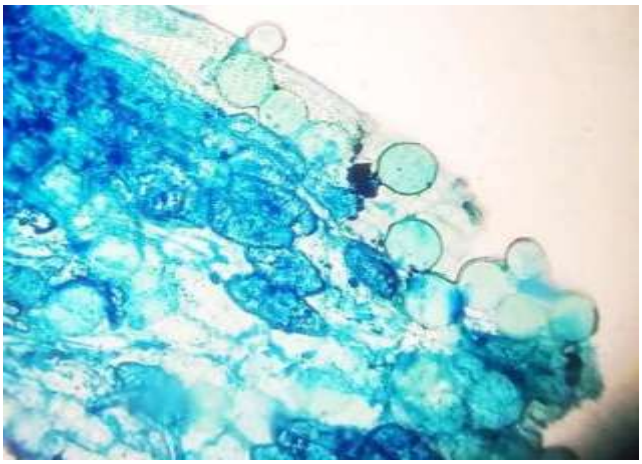

Fig 3: The Microscopic view of the Histochemical analysis of the *P. indica* co-cultured roots of *A. hypogaea*, chlamydospores in cortical region of plant root after 20 days of colonization.

Fig 4: Broth Culture of *P. indica* (20 days old)

Table 1: Effect of *P.indica* on the growth characteristics of *in vitro* grown *A. Hypogaea* plants.

Parameter	Controlled Mean±SD	Treated Mean±SD	T-test
Biomass, Fresh weight (g)	3.66 ± 0.56	5.9 ± 0.4	**
Biomass, Dry weight (g)	0.74 ± 0.14	0.93 ± 0.17	**
Shoot length (cm)	5.03 ± 0.20	6.86 ± 0.40	*
Root length (cm)	4.13 ± 0.4	6.06 ± 0.25	*
Protein (mg/g)	86.86 ± 7.0	155.53 ± 8.1	**
Sugar (mg/g)	127.3 ± 15.05	163.13 ± 56.51	NS
Chl content (mg/g)	4.067 ± 0.153	4.167 ± 0.493	NS

T-test: *$P \leq 0.05$; **$P \leq 0.001$; NS not significant.

Conclusion

On the basis of results obtained in the present investigation, It could be finally concluded the endophytic fungus, *P. indica* possess all the potential characteristics to be well utilized as a growth enhancer in *in vitro* cultured *A. hypogaea*. It was noticed that *P. indica* co-cultivation model affected positively in most of the growth parameters and biochemical componetns of the plant. The *in vitro* co-culture can be used strategically for growth promotion in *A. hypogaea*.

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