

## Comparative Study of Growth of *Piriformospora indica* by using Different Sources of Jaggery

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<http://dx.doi.org/10.22207/JPAM.12.2.56>

(Received: 30 March 2018; accepted: 15 May 2018)

*Piriformospora indica* is a root endophytic fungus that build a symbiotic association with the roots of a vascular host plants and induces overall plant growth. The *Pindica* can grown on a variety of synthetic and complex media. But these readymade medium consist of about 20-25 expensive chemicals. To minimise the cost for *Pindica* production (Arora et al 2014 and Varma et al, 2015) have already standardized a cost effective Jaggery medium (4% w/v) as a energy nutrient source for mass cultivation of *P. indica* with no compromise on quality. But growth of fungus varies substantially with different sources of Jaggery. So we collect Jaggery from four different sources i.e from 1. Local shop, 2. Indian Institute of Sugarcane Research, Lucknow, 3. Safal, New Delhi and 4. Organic Jaggery to check its impact on fungal growth and also check its efficacy on Spinach (*Spinacia oleracea*). The data observed from the present study shows that maximum growth of *Pindica* in terms of dry cell weight, radial diameter and spore count was recorded on Safal Jaggery medium as compared to other Jaggery medium. Moreover, fungal biomass grown on this medium showed positive effect on growth of spinach as it leads to increase in plant height and its dry and fresh weight. This finding suggest that Safal Jaggery medium is a cheaper and best source for commercial cultivation of *Pindica*.

**Keywords:** *Pindica*, Safal Jaggery, Spinach, cultivation, growth.

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*Piriformospora indica* is a root endophyte which has all properties of a typical arbuscular mycorrhizal fungus<sup>1</sup>. But unlike other AM fungus it can easily cultivate in laboratory on a variety of synthetic submerged and solid media like Hill and Kaefer, Potato Dextrose, YMP, MS etc<sup>2</sup>. The fungus isolated from the soil of thar desserts of north western Rajasthan, India<sup>1</sup>. It belongs to phylum Hymenomycetes and order Sebaciales (Basidiomycota). It can colonize with roots of variety of plants and resistant to many fungal pathogens so it is capable to enhance overall plant growth<sup>3,4</sup>. This fungus has a multifarious activities as it can form a symbiotic association with variety of plants including legumes, flowering plants<sup>5</sup>, thale cress<sup>6</sup>, variety of grasses<sup>7</sup>, tobacco<sup>8</sup>. Due to

this symbiotic association it has a strong potential to use as a biofertilizer in the field of agriculture<sup>9</sup>.

The fungus can grow inter- and intracellularly and produces auto fluorescent and pear shaped chlamyospores with thick walled hypahe with length and width of 15-24 and 11-16 µm respectively. Its shelf life is about 3 months at room temperature. The chlamyospores can be present in clusters of 2-3 or as a single spore also<sup>9,10</sup>. The fungus enters in root cortex of inoculated plant but it does not enter in the aerial part and endodermis of plants<sup>11,12</sup>. The fungus helps in nutrient uptake, protect plants from salt, water and temperature stresses and provide complete resistance to acidity, heavy metal toxins, pathogens etc<sup>13</sup>. It can use as a plant regulator, bio fertilizer, bioregulator because it promotes early flowering, seed production, excessive production of biomass. On solid media *Pindica* forms round ring like structures which are formed by chlamyospores or mycelium<sup>14</sup>.

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Hill and Kaefler was most optimum media out of all other tested media used for growth and maintenance of *P.indica*<sup>15</sup>. But this synthetic media is consist of very expensive chemicals so in previous study cheap and easily available media was used for the mass cultivation of fungi<sup>16</sup>. In this study, we report about the mass production of *P.indica* by using a easily available, cost effective and indigenous nutritional source of energy which is known as Safal Jaggery medium. It is commercially and quantitatively competitive to Hill and Kaefler. Further its efficacy is also checked by inoculating a plant of Spinach (*Spinacea oleracea*) with the fungal biomass obtained on a Safal Jaggery medium.

Spinach which is popularly known as “palak” is belongs to a family chenopodiaceae family.

It is a nutritive plant which is rich in riboflavin, ascorbic acid, vitamin A, thiamine and also essential minerals like calcium and iron. So, it is an important and common part of human food for daily intake as it fullfill all the requirements of healthy nutrients. Moreover, this plant has a broad surface area and fast growth rate. Because of all these unique features *S. oleracea* is used in a number of scientific researches<sup>17,18</sup>.

## MATERIAL AND METHODS

### Culturing and maintenance of mycobiont *P. indica*

The *p. indica* was cultivated on Hill-Kaefler medium<sup>19</sup>. One *P.indica* disc of 8mm in diameter from 8 days old *P.indica* plate is cut by using sterilized cork borer and then placed in the centre of fresh hill and kaefler agar medium plate. Now, after this the inoculated plates were kept in an incubater at  $28 \pm 2$  °C for about 7-10 days. Then, these plates were stored at 4°C for further use in experiments.

### Growth of *P. indica* on different sources of jaggery

*P. indica* can be grown on variety of complex synthetic media for example Potato Dextrose, Hill and Kaefler, YPG media but all these complex synthetic medium consisting of about 20-25 expensive chemicals. So, to minimize the use of these expensive nutrients we have standardized Jaggery (Gur) as energy nutrient source obtain

from Sugarcane (*Saccarum officanarum*). With no compromise on its quality. But Growth of Fungus varies substantially with different sources of Jaggery. For this purpose we collected 4 different types of Jaggery from different places i.e From local shop, Indian Institute of Sugarcane Research, Lucknow, Safal, New Delhi and Organic Jaggery (Fig 1). Now, jaggery obtained was convert into powder form and dissolved in distilled water at 4% (w/v). This concept is based on preliminary success of the fungus on 4 % (w/v) using identical parameters<sup>20</sup>. Hill-Kaefler medium is taken as a control in all experiments.

To check growth pattern of fungus on solid media one *P.indica* disc having live spores of 8mm in diameter from 8 days old *P.indica* plate is cut by using sterilized cork borer and then placed in the centre of freshly prepared Jaggery agar medium plate. Now, after this the inoculated plates were kept in a incubater at  $28 \pm 2$  °C for about 7-10 days.

For mass multiplication *P.indica* was grown in shaking flasks containing Jaggery medium without agar. Media with pH 6.5 was sterilized at 15 psi pressure for 20 minutes in an autoclave. The focus was on excessive production of chlamydo spores for better formulation and storage for transfer to field at room temperature. For this puepose the bottles containing liquid broth media were inoculated with *P. indica* disc and then kept on rotator shaker at 120 rpm in dark at 28°C for about 10days.

### Measurement of cell and radial growth

The *P. indica* growth was measured in terms of DCW i.e dry cell weight in per liter of culture Broth. After about 9-10 d of growth a known volume of biomass was filtered by using Whatmann filter paper No. 1 and then dried in a hot air oven for overnight at about 80°C. After removing all moisture the weight of dry mass was determined. To check the DCW 3 flasks were removed everyday. The scale was used to measure the mycelium radial growth from inoculated disc and it was measured every alternate day.

### Spore counting of fungus

To count the pear shaped chlamydo spores of *P.indica* culture first separate these spores from mycelium. For this separation in case of solid media, 1 ml of tween 20 was spread on the agar plate and swirled gently by using sterilized spatula on the surface of agar plate. Now, the already detached

spores are scratched and collected in a centrifuge tube. These spores are centrifuged at 3000 rpm for about 8 mins which is further followed by washing with distilled water containing sterilized tween 20. Number of spores is observed by using Haemocytometer with microscope.

#### Estimation of residual glucose

A 1ml sample diluted with 1ml of sterilized water was taken in a test tube, 3ml DNS reagent was added and heated for 15 min in a boiling water bath. Then the hot tube was supplemented with 1ml Rochelle salt solution and cooled to room temperature. The absorbance was measured at 575nm using Spectrophotometer (Systronics-20).

**Table 1.** Growth of *P.indica* on solid and liquid media

Medium	DCW (g/l)	Radial diameter (cm)
Hill and Kaefer	13.26	5.9
Local	14.11	6.8
Indian Institute of Sugarcane Research, Lucknow	13.81	6.9
Safal, New Delhi	16.81	7.1
Organic	12.16	5.6

**Table 2.** Growth and sporulation of *P.indica*

Medium	Spore count	Day of start of Sporulation
Hill and Kaefer	$1 \times 10^8 - 1 \times 10^9$	6
Local	$1 \times 10^9 - 1 \times 10^{10}$	6
Indian Institute of Sugarcane Research, Lucknow	$1 \times 10^8 - 1 \times 10^9$	7
Safal, New Delhi	$1 \times 10^9 - 1 \times 10^{10}$	5
Organic	$1 \times 10^8 - 1 \times 10^9$	8

**Table 4.** Different Growth Parameters after 20days

Characters	Control	Treated	Changes (%)
Seed germination	66.6	88.8	13.8
Plant height	5.49±0.06	8.02±0.01	46.08
Root length	1.44±0.01	2.26±0.02	56.94
Shoot dry weight	0.72±0.05	0.92±0.05	27.77
Root dry weight	0.36±0.02	0.53±0.02	47.22

#### Confocal microscopy

*P. indica* spores grown on Safal Jaggery medium were examined under Confocal laser scanning microscope LSM-780 ((Carl-Zeiss, Inc., Jena, Germany). First these spores were separated from mycelium by the method explained in section 4. Now, these pure spores were placed on a glass slide and trichloroacetic acid/ethanol in the ratio of 1/0.15% v/w were used for the fungal biomass fixation. The spore sample was washed with distilled water and then with PBS (phosphate buffered saline) which contains 50 µg/mL WGA-AF488 and 0.2% Silwet L-77. Now, these samples were observed under confocal microscope.

#### Separation of Culture filtrate

To prepare fungal formulation the fungal biomass was grown on liquid Safal Jaggery medium containing live chlamydo spores and mycelium were collected through filtration after 9 days by using sterilized muslin cloth (400nm). It can be preserved at 4 °C if not required immediately.

#### Co-cultivation of *P.indica* biomass with plants

Efficacy of *P.indica* spores grown on Safal Jaggery medium was checked on Spinach (*Spinacia oleracea*). Seeds were procured from National Seed

**Table 3.** Sugar consumption rate by *P.indica*

Days	Spore count	Residual Sugar g/l
0.	-	
1.	-	
2.	$1 \times 10^2 - 1 \times 10^4$	0.124
3.	$1 \times 10^3 - 1 \times 10^5$	0.117
4.	$1 \times 10^4 - 1 \times 10^5$	0.112
5.	$1 \times 10^5 - 1 \times 10^7$	0.110
6.	$1 \times 10^9 - 1 \times 10^{10}$	0.104
7.	$1 \times 10^8 - 1 \times 10^{10}$	0.103
8.	$1 \times 10^8 - 1 \times 10^{10}$	0.101
9.	$1 \times 10^4 - 1 \times 10^5$	0.100

Corporation, IARI, New Delhi, India. Seeds were sterilized and germinated by method mentioned in Arora *et al* 2014. For this experiment :1 mixture of Soil and peat with pH 6.8 was used.

For Mycorrhizal inoculation the 2gm of fungal mycelium is mixed carefully with 100g of sterile soil (w/w). Now, the upper surface of soil was covered with a thin layer of fungal inoculums which is further covered by a very thin layer of soil i.e forming a sandwich like model. Pre germinated seeds were transplanted in each pot. Each pot is consist of 3 seeds and were kept in biohardening lab at 28°C. Plants were fertilized with 1/10 Hoagland solution on every alternative week.

#### Estimation of plant growth parameters

The plants inoculated with fungus were harvested after about 20 days to check changes in its growth parameters like root and shoot length, root and shoot fresh and dry weight. Shoots and roots were separated from each other and then washed under running tap water to remove traces of soil and other materials. Now, root and shoot length was measured by using scale. Fresh weight was measured immediately and dry weight was measured by drying the root and shoot sample in an hot air oven at 80°C for 24h.

#### Root colonization

To check the presence of *P.indica* spores in plants the root sample of each inoculated and uninoculated plants were collected and boiled with 10% potaasium hydroxide to make it soft and then treated with 2% hydrochloric acid for atleast 4 mins. These samples were further stained with 0.5% cotton-blue dye and then observed under a light microscope at 40X magnification.

#### Statistical analysis

Each experiment was performed in triplicate and results presented as a mean±standard error (SE) and differences were considered to be significant at  $p < 0.05$ .

## RESULTS

#### Selection of media for *Pindica* cultivation on the basis of Dry cell weight and radial growth

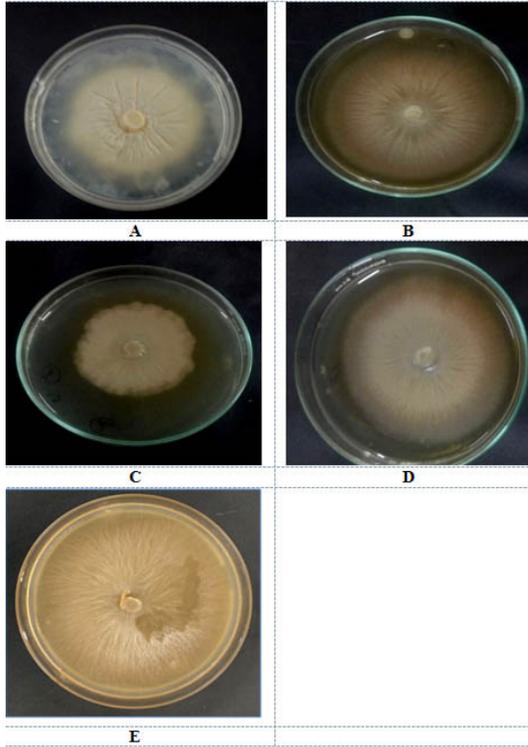
Four different types of jaggery were used for *P.indica* cultivation. Hill and kaefer served as a control. *P.indica* was grown on both solid and liquid media to find a appropriate media for sporulation and growth of fungus. *The P.indica* growth in submerged and in solid media after 10d is shown in fig 2(a-e) and 3 (a-e) respectively.

Maximum DCW i.e dry cell weight of *P.indica* was observed in Safal Jaggery medium



**Fig. 1.** Four different sources jaggery : A. Local B. Indian Institute of Sugarcane Research, Lucknow C. Safal, New Delhi D. Organic

i.e 16.81g/l as compared to control. DCW of *P. indica* was relatively less in other Jaggery medium in comparison to Safal (Table 1). Now, diameter

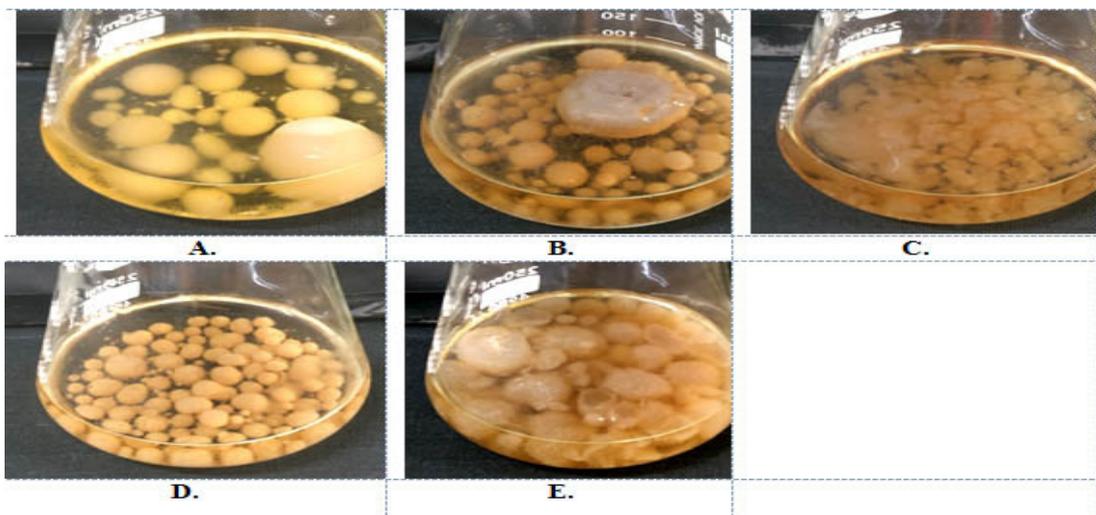


**Fig. 2.** Growth of *P.indica* on solid media A. Hill and kaefer B. Local C. Organic D Indian Institute of Sugarcane Research, Lucknow E. Safal, New delhi

of leading hyphae of *P.indica* colonies growing on different Jaggery medium and Hill and Kaefer medium was also observed. This observation shows that again *p.indica* disc which was inoculated in the centre of Safal Jaggery medium plate leads to a significant increase in leading hyphae with diameter of 7.1 cm (Table 1) which is maximum out of all other media. This analysis of diameter of radial growth of fungus was measured every alternative day until the growth of fungus touched the edges of petriplate (Fig 2a-e). In terms of morphology of *P.indica* hyphae it was observed that the fungus showed same growth pattern of packed fungus with coral morphology in jaggery medium this pattern confirms that this is suitable media<sup>1,21</sup>.

#### Spore count by using haemocytometer

Out of all five media, Safal Jaggery medium produced much higher spore yield of  $1 \times 10^9$ - $1 \times 10^{10}$  spores/ml. Similarly there is early start in sporulation i.e on 6<sup>th</sup> day. (Table 2). The least spore count of  $1 \times 10^6$ - $1 \times 10^7$  (spores/ml) is obtained from Organic Jaggery and fungus start sporulated at 8<sup>th</sup> day. Fig 4 showed morphogenesis of chlamydospores grown on Hill and Kaefer and Safal Jaggery media. This figure depicts that in case of Hill and Kaefer the spores were small with thin walled hyphae and less in number on the other hand Safal Jaggery medium has more bigger spores with thick walled hyphae. Overall, Safal Jaggery media was found to be best medium as it gives highest number of bigger spores.



**Fig. 3.** Growth of *P.indica* in liquid media A. Hill and kaefer B. Local C. Organic D Indian Institute of Sugarcane Research, Lucknow E. Safal, New delhi

### Sugar consumption rate of Jaggery Medium from 0hr to 9<sup>th</sup> day

The spore count and substrate consumption of *P.indica* in Safal Jaggery medium was observed and shown in Table 3 and Fig 5. Initially there is a lag phase for about 25h because the adaption of fungus to this environment. Now, there is exponential increase in spore count for next 7days as maximum spore count is of  $1 \times 10^8$

$-1 \times 10^{10}$  at 7<sup>th</sup> day of cultivation. Then there is decrease in spore count after day 7 which is due to complete utilization of glucose. Due to this quick consumption of glucose colonies start getting aggregate and form clusters that's why there is increase in biomass but decrease in spore count.

### Confocal microscopy

*P.indica* spores grown on different jaggery sources were observed under CLSM (Confocal

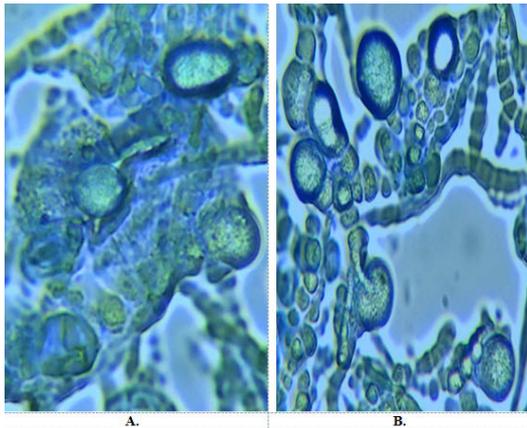


Fig. 4. Morphogenesis of Chlamydospores on A. Hill and Kaefer B. Safal jaggery medium

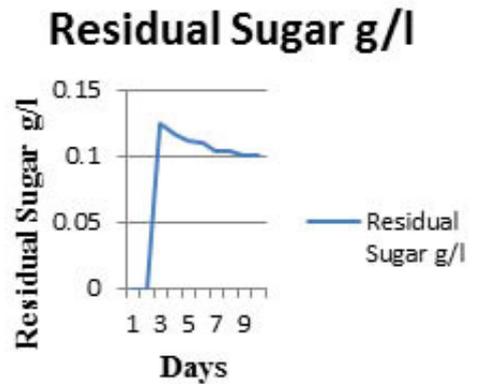


Fig. 5. Sugar consumption rate of Safal Jaggery medium

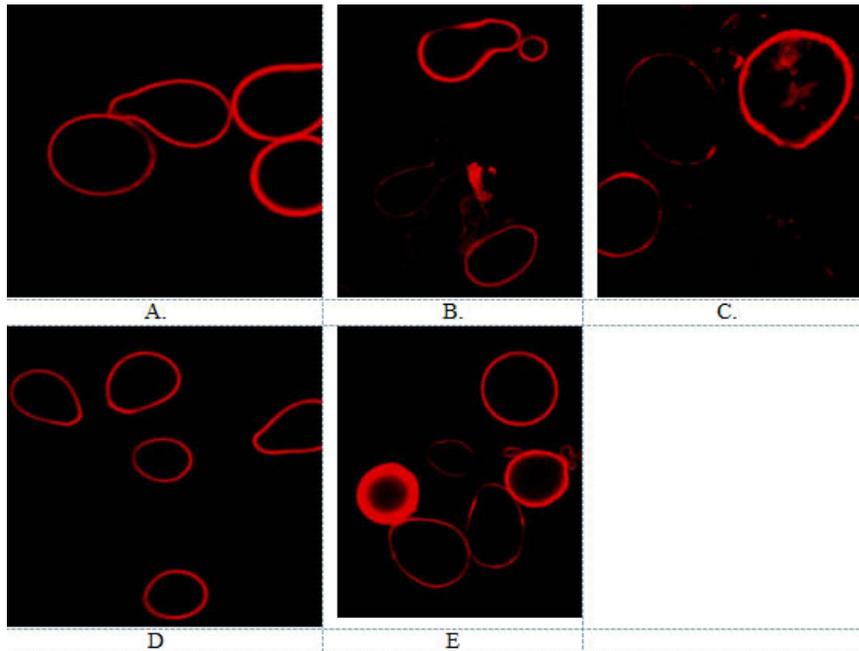


Fig. 6. Confocal Microscopy of *P.indica* A. Hill and kaefer B. Local C. Organic D Indian Institute of Sugarcane Research, Lucknow E. Safal, New delhi

laser scanning microscopy) by using trypan blue dye (Fig 6 a-e). Laser excitation at 488 nm resulted in giving out in the observable range. In Safal Jaggery medium spores were bigger in size with thick and hyalin hyphae (Fig 6e). Moreover, it was observed that fungal spores are more in numbers with smooth surface as compare to spores grown on other media in which spore count was less with morphological deformities and disaggregation.

**Comparison of growth parameters**

The *P.indica* biomass grown on Safal Jaggery medium was used to check its efficacy on Spinach (*Spinacia oleracea*). Germination rate of seeds inoculated with fungal biomass is increased by 13.8 % as compare to control (Fig 7 and Table 4). A visual representation of the plants shows the early flowering in case of treated as compare to control (Fig 8). The data observed indicates that fungal culture has positive effect on different

growth parameters of plant as mentioned in Table 4 and Fig 9. This treatment results in an increase in root and shoot length, root and shoot dry and fresh weight by 56.08, 46.08, 27.77 and 47.22 % respectively as compare to plants which were not inoculated with *P.indica*.

**Root colonization**

Root sample of treated plant stained were collected and stained with with lactophenol cotton blue and then observed under microscope. Microscopic observation showed the presence of inter and intracellular spores which proves that fungus enters in roots through root hairs and then grows in root cortex (Fig10).

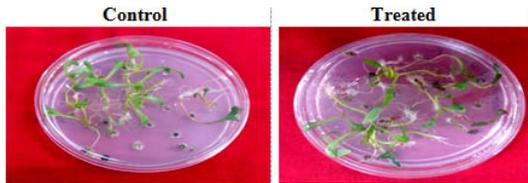


Fig. 7. Seed germination in petriplate



Fig. 8. Plant growth after 20 days of inoculation

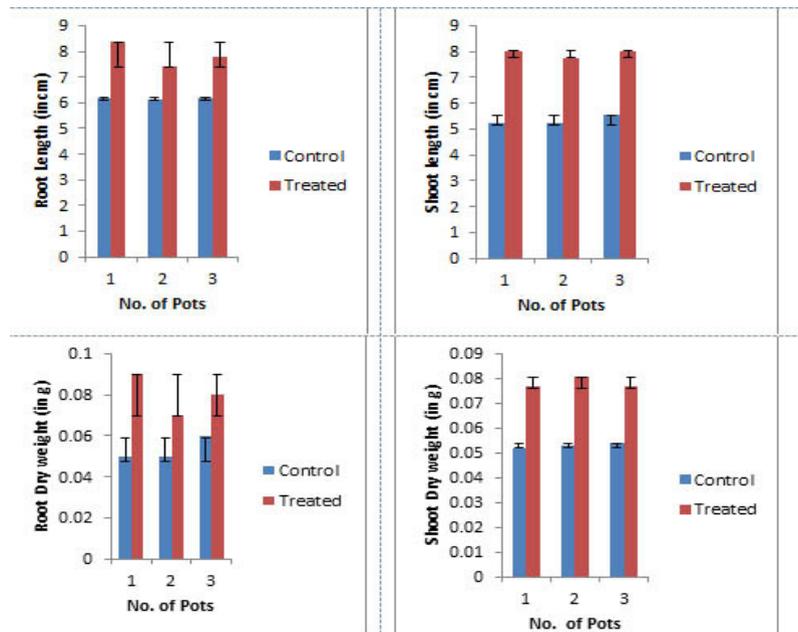


Fig. 9. Different Growth Parameters of Spinach (*Spinacea oleracea*) after 20days



Fig. 10. Root colonization

## DISCUSSION

In literature there are a variety of different media to grow different groups of fungus, but there is not any previous work which shows the axenic production of obligate symbiotic fungus as they can not grown on synthetic media without any plant host<sup>22</sup>. So, to grow AM fungi without a symbiotic association is a serious concern. But, *P. indica* is capable to grow axenically under laboratory conditions on a variety of readymade media. But these media is very expensive for mass production of fungus. As *P. indica* greatly improves the overall plant biomass and growth of different economically and medicinally important plants. Due to these beneficial properties of *P. indica*, there is a need to standardized a best possible nutrient medium for fungal growth<sup>23</sup>. This optimized media should be capable of providing all the nutrients for better growth<sup>24</sup>. To commercialize this magic fungus we have to focus on standardized the cost effective media. An advanced cost reduction technology is achieved by improving the better consumption of resources<sup>25</sup>. This cost effective method should not effect the quality and quantity of fungal spores and its positive effect on plant growth.<sup>23</sup> explain that substrate availability and consumption can drastically effect the spore count and biomass. Our results are also match with findings in all previous reports. In current work, the growth and spore count of fungus is different on different Jaggery media. But Safal Jaggery medium gives high number of effective spores.

The results of experiment of Co-cultivation of *P. indica* biomass with Spinach (*Spinacia oleracea*) indicates that *P. indica* biomass grown on Safal Jaggery medium has positive effect

on different growth parameters of plant. As in treated plant there is increase in root and shoot length and weight as compare to uninoculated one. It means there is no change in the Viability of *P. indica* spores. This matches with the observations mentions in previous investigations<sup>5,27,28,4</sup>.

On the basis of all observations it is clear that Safal Jaggery medium is a highly cost effective energy source which is easily available. As cost of one litre Hill and Kaefer media is about 450/- rs on the other hand same quantity of Safal Jaggery medium is cost less than 2/- rs. Therefore, Safal Jaggery medium is a best and cheap energy source which provide all nutrients to fungus to produce highly active spores to induces plant growth.

## CONCLUSION

*P. indica* is a well known endophytic fungus which is beneficial for a variety of plants as it increases plant growth rate. The present work has a aim to standardized an improved and cost effective energy source for mass cultivation of this magic fungus. Safal Jaggery medium which is a derivative of sugar cane was the cheap and cost effective source to grow *P. indica* on a large scale. It is a low priced substarte which is ecofriendly and appropriate to use as a biofertilizer to improve agriculture.

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