Mycopath (2017) 15(1): 33-39 **Research Article**

Metabolic profiling of rhizosecretion from root exudates of *Piriformospora indica* endophytic fungi colonized plants through hydroponic cultivation

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Abstract

Plant molecular farming has made an extensive development and is an attractive and widely accepted powerful technology for extraction of pharmaceutical products from plants. The present research work focuses on the study of root exudates induced in the host plant by root colonizing endophytes. The fungus *Piriformospora indica* imparts growth promotion and uptake of various nutrients by host plant. The fungus also mediates production of bioactive components during colonization and the active compounds are released out by host plants through root exudates, which is evidenced during hydroponic cultivation. The root exudates from hydroponic study were subject to GC-MS assay. The *P. indica* colonization in *Setariam italica* (L.) P. Beauvois plant has been observed and exudated compounds were found to include bioactive compounds identified through GC-MS analysis peaks showed production of compounds *viz.*,m-Hydroxyphenylacetic acid , Oxime-methoxy phenyl, 4-Ethylbenzoic, Cyclopentylester, 4-Nitrocinnamaldehyde, Benzaldehyde;4(1,3-dioxolanzyl). Endophyte colonized plants secreted compounds related to various biological activities; increase in hormone production, antipathogenic activity, metabolic activity, increase the uptake of nutrients, and antioxidant property. The endophyte may be used for production of plant bioactive compounds through hydroponics and root exudate extraction.

Keywords: Endophytic fungi, hydroponic cultivation, metabolic profiling, *Piriformospora indica*, rhizosecretion, root exudates analysis.

Introduction

The use of plants for production of pharmaceutical compounds/ is a mounting business, evidenced by the increasing number of companies investing in this field worldwide. Plants, seeds and cultured plant cells are potentially one of the most economical system for large-scale production of bioactive drug molecules for the biochemical, veterinary and pharmaceutical industries (Twyman *et al*., 2005; Kermode, 2006; Boehm, 2007; Floss *et al*., 2007; Pujol*et al*., 2007; Streatfield, 2007).

Root-inhabiting or endophytic fungi were greatly focused for deeper research because their hyphae connect to the challenges and resources of the surrounding with the plant in ideal manner. An example for such root endophytes is presented by the basidiomycete fungus *Piriformospora indica*. The fungus possesses a broad host plant spectrum for colonization and positively affects different aspects required to apply this root endophyte in agri- and horticultural practices relating to plant growth, nutrition and defence or tolerance thus explaining the facts about biological basis for the observed effects on host plants (Saxena *et al*., 2002). At the present time, the availability of novel techniques for the characterization of microbial communities with high through put sequencing approaches, metabolomics and the development of non-destructive, localized exudates sampling techniques (Neumann *et al*., 2009; Bakker *et al*., 2012; Chaparro *et al*., 2013) paves way for a more comprehensive look on the interactions between rhizosphere microbiomes and roots of their host plants. During the last two decades massive advancements were achieved in the characterization of factors determining root exudation, which exhibits high variability with in different plant species, even cultivars, with in different root zones, developmental stages of individual plants and in response to various biotic and abiotic stress factors (Neumann, 2007; Neumann and Romheld, 2007; Badri and Vivanco, 2009).

The secretion of phytochemicals from roots is an important way for plants to respond to and alter their environment. Over the last several years, research and technical advancements have provided

a better understanding of how exudates mediate communication between plants and other microorganisms. Exciting trends are emerging from different but interconnected strands of research in the field of rhizosphere biology (Dayakar and Vivanco, 2009). However, efforts should now focus on decoding the chemical dialogues between organism in the multifarious rhizosphere. In addition, there is a need to understand the mechanism and regulation of root exudation to better utilize phytochemical production for enhanced agricultural benefit. A major challenge for researchers is to identify new transport system and regulatory mechanism involved in the root-secreted phytochemicals and their role in the rhizosphere. Complete characterization of the chemical components of root exudates involved in favouring disease resistance and facilitating more beneficial associations with microbes of root exudates is yet another challenge, but it is important to understand the role of phytochemical compounds secreted as root exudates to complete the scenario. In addition, it is very clear that research on root behavior is needed to provide ecological and evolutionary data to understand multitrophic interactions and the link between ecological diversity for the benefit of balanced ecosystem.

Piriformospora indica, a novel endophytic root colonizing fungi significantly increases plant biomass, flowering and yield, nutrient value etc., during its colonization and proliferation in the roots of the host plants and also confers resistance against root and shoot pathogens (Waller *et al*., 2005). The endophyte *P. indica* is a wonder fungi bringing about a vast array of physiological changes in the plants upon colonization. The effect of colonization; result in production of various root exudate compounds. This kind of molecular farming studies will enhance the understanding of various secondary metabolites and bioactive compounds released through rhizosecretion. The present research work aimed to inoculate the endophytic fungi *P. indica* in study plants and check for their root colonization; and to perform hydroponic cultivation and screen the rhizosecreted metabolites from endophyte colonized study plants that is compared to non-colonized control plant and to perform metabolic profiling of root exudates by GC-MS analysis.

Materials and Methods

P. indica cultures were obtained from Dr. Varma's laboratory at Department of Microbial Biotechnology, Amity University, Noida, India. (Varma *et al*., 2001). The fungi have been already proven as effective endosymbiotic mycobiont, mediating plant growth promotion. The fungal strain was obtained and formulations were made for further studies with Hill and Kafer medium (Hill and Kafer, 2001).

Hydroponic studies

Hydroponic cultivation allows the growth of plants in liquid nutrient medium, and the liquid is then analysed for rhizosecreted root exudates. The *Setaria italica* seeds were taken, surface sterilized in 2.5% mercuric chloride for 2 minutes and immersed in sterile distilled water for 5 min. Seedlings were allowed to germinate and reach a length of 5 cm of both control and endophytic fungi colonized, then transferred to beaker containing 100 mL of sterile liquid half-strength MS medium. Plants were grown aseptically for 2 weeks at 24 ^{o}C under a 16/8 h day/night photoperiod. After two weeks the half-strength MS medium of both control and endophytic fungi inoculated medium were verified with GC-MS analysis and the peak results obtained were analyzed for the components and various metabolites (Kamachi, 1991).

Identification of fungal colonization of plant roots

After 2 weeks of hydroponic cultivation, the foxtail millet plant roots were taken for staining to verify the colonization of endophyte. Both control plant and test plants were observed under microscope. The wet mount preparation of the root was done for the identification of the structure of root. It was done by using Trypan blue stain. The structure of the root and endophytic fungi colonization were observed under bright field microscope (Deshmukh *et al*., 2006).

To identify the colonization of *P.indica* in the randomly chosen plant roots, the stained root samples were kept in clean glass slide and mounted with cover slip with gentle pressing. The slide was viewed under microscope and percentage of colonization inside the roots was calculated by using the formula given by Giovannetti and Moss (1980):

$$\text{Root colonization (\%)} = \frac{\text{No of root segments colonized}}{\text{Total No of segments observed}} \times 100$$

Metabolic profiling of rhizosecretion from root exudates by GC-MS

The rhizo secretions from root exudates of endophytic fungi colonized plant and the control plant from Hydroponic studies were subject to GC-MS analysis. The endophytic fungal biomass at the end of 1st week and 2nd week after inoculation into hydroponic media was subjected to GC-MS analysis and the peak results obtained were analyzed for the components and validate the various metabolites produced by endophytic fungi *P. indica* (Nasrallah *et al*., 2013).

Results and Discussion

P. indica have already been proven as effective endosymbiotic mycobiont, mediating plant growth promotion. The endophytic fungi were cultured on Hill and Kaefer agar medium and the cultural characteristics of *P. indica* showed puffed white

chalky powdered appearance with pointed tip at the center of each colony in the selective agar medium. This fungus is organ specific and it predominantly grows in root. Lacto phenol cotton blue staining of *P. indica* showed the morphology of pear shaped chlamydospores at hyaline hyphae was examined under microscope at 45X.

Germination studies of fungi in host plants

Germination studies of *P. indica* have revealed better plant growth promotion which is evidenced by comparing the physical parameters of endosymbiotic plant with the control plant. In all the two types of plants the growth rate was excellent in *P. indica* colonized *S. italica* plants in soil culture. *P. indica* inoculated/colonized seeds germinated on the third day after inoculation, whereas control plant took 5 days for seed germination, which reveals that *P. indica* colonization involves activating plant growth promotional measures in host plant.

Hydroponic cultivation

Hydroponic cultivation allows the growth of plants in liquid nutrient medium, and the liquid is then analyzed for rhizosecreted root exudates. After two weeks of growth of plants in hydroponic medium, the half-strength MS medium of both control and endophytic fungi inoculated medium was subjected to GC-MS analysis and the peak results obtained were analyzed for the components and release of various metabolites through plant roots. It has been determined that the endophytic fungi colonized plants were different from the control plants of *S. italica*. Those plants were taken and put inside the hydroponic medium for checking the rhizosecretion by root exudates. The differences in the growth observed between control and treated plants were suggested to be caused by greater absorption of water and nutrients due to extensive colonization of root by *P. indica* (Rai *et al.,* 2001).

Microscopic observation of root colonization by fungus

The stained root samples were observed under light microscope (40X). The slide was viewed under microscope and percentage of colonization inside the roots was calculated (Giovannetti and Mosse, 1980). The endophytic fungi have colonized 79% of foxtail millet roots. As said the growth of the study plants was higher when compared with control study plants of (*S. italica*). Root colonization of the study plant showed presence of *P. indica* at 79% in *S. italica* plant.

Metabolic profiling of rhizosecretion from root exudates by GC-MS analysis

The fungi were inoculated in the Hill &Kaefer medium and allowed to grow. After a week of inoculation the fungal biomass was collected and subjected for gas chromatography and Mass spectrometry.

The GC-MS results show that most of primary metabolites required for the fungi were produced during the log phase whereas stationary phase enhances production of secondary metabolites with antimicrobial properties and also help for conferring resistance to pathogenic infection to host plants (Fig. 1&2). The peaks referred in GC-MS have been identified as different compounds produced by the root exudates, the molecules and their functions were referred in earlier literature and characteristics, with therapeutic properties of these compounds has been predicted (Table 1&2).

The GC-MS results shows that among the various metabolites produced by the fungi, there are many medically important compounds that have been referred to show ant-bacterial, anti-fungal, anti-parasitic, anti-cancer, insecticidal, nematicidal properties (Liu *et al*., 2009). Analysis of the metabolites produced by *P. indica* after a week of inoculation by GC-MS showed that many anti-fungal, anti-bacterial, anti-leishmanial activity, anti-microbial activity, herbicidal activity, High analgesic activity, Antioxidant, hypoglycemic activity compounds in secondary metabolites analysis (Table 1&2).

Similar GC-MS analysis of root exudate analysis during hydroponic cultivation of *Setariaitalica* has been performed to identify the bioactive compounds induced by *P. indica* colonization in the host plant, which is well evidenced through root exudates from hydroponic studies (Neumann and Romheld, 2007; Badri and Vivanco, 2009).

The bioactive compounds produced by endophyte colonized plants were more compared to the number of compounds released by control plants (Table 3&4). The endophyte colonized plants secrete compounds related to various biological activities; increase in hormone production, antipathogenic activity, metabolic activity, increase the uptake of nutrients, and antioxidant property (Neumann, 2007)

The GC-MS results showed that crotonic acid; 4-ethylbenzoic and cyclopentyl ester were Setaria control plant exudates, and Oxime-methoxy phenyl, 4-Ethylbenzoic, Cyclopentylester, 4-Nitrocinnamaldehyde, Benzaldehyde;4(1,3-dioxolanzyl), 1-Ascorbic acid 2,6-dipalmitate, Hexylacrylate, Nitroglycerin-1,2,3-propanetriol, trinitrate, 3-(Octadecyloxy)propyl stearate, and Adipic acid were Piriformospora fungi colonized Setaria plant exudates, showing the difference in compounds exudated by the colonized plant, which may enhance host plant growth promotion and assume host plant protection. Similar studies on root exudates metabolite profiling has been reported by (Hossein *et al.,* 2011) in their research article that reported increase in oil yield and production of anethole metabolite in *Foeniculum vulgare* co-

colonized by *P. indica* and *Sebacina vermifera* analysed by GC-MS. Nadine *et al.* (2016) have reported an article in which GC-MS analysis of root exudates ion profiling has been studied in *Arabidopsis thaliana* colonized by *P. indica*.

Conclusion

The molecular farming studies will enhance the understanding of various secondary metabolites and bioactive compounds released through rhizosecretion. The root exudate compounds produced during endophytic fungal colonization in host plant represent the bioreactive properties of endophyte that enhance their symbiotic relationship with host plants.

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Table 1: Analysis of the metabolites produced by *Piriformospora indica* in culture broth after a week inoculation by GC-MS.

Sr. No	Compound Name	Formula	Molecular weight	Comments
1	Dodecane	$C_{12}H_{26}$	170	TBT (Tributyl phosphate) in plant reprocessing.
2	1,2-Dimethyl Hydrazine	$C_2H_8N_2$	60	Herbicides
3	Pyridinamide	$C_5H_5N_3O_2$	139	Plant phospholipids, inhibit neurological damage.
4	Fosmidomycin	$C_4H_{10}NO_5P$	183	Anti-bacterial agent
5	m-Hydroxyphenylacetic acid	$C_{14}H_{24}O_3Si_2$	296	Used in Plant-Microbe interaction.
6	1H-Imidazole	$C_4H_4IN_3O_2$	253	Anti-bacterial agent
7	Caprylic acid	$C_8H_{16}O_2$	144	Improving natural gas liquid.
8	Decyl alcohol	$C_{10}H_{22}O$	158	Used as fatty acids in plants.
9	Hexadecanoic acid, Palmitic acid	$C_{17}H_{34}O_2$	270	Used as fatty acid by plant tissues

Table 2: Analysis of the metabolites produced by *Piriformospora indica* in culture broth after two weeks of inoculation by GCMS.

Sr. No	Compound name	Formula	Molecular weight	Comments
1	1,2-dimethoxycyclopentane	$C_7H_{14}O_2$	130	Antiviral activity.
2	*n*-Pentadecanoic acid	$C_{15}H_{30}O_2$	242	Antioxidant, Antimicrobial activity.
3	*cis*-9,*cis*-12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280	Antibacterial, Antiviral and Antioxidant activity.
4	2-Chlorocycloheptanone	$C_9H_{15}ClO_2$	190	Antiproliferative activity.
5	Benzoic acid, 3-formyl-2,4-dimethoxy-6-methyl	$C_{27}H_{26}O_8$	478	Antimicrobial, Antibacterial, Antifungal activity.
6	Pentanedioic acid, 3-oxo-, dimethyl ester	$C_7H_{10}O_5$	174	Antioxidant, Antimicrobial activity.
7	Propanedioic acid, methyl,ethyl ester	$C_6H_{10}O_4$	146	Antileshmanial activity.
8	Benzenemethanol	$C_{12}H_{19}N_{24}$	191	High analgesic activity, Antioxidant, hypoglycemic activity.

Table 3: Analysis of the metabolites produced by *Setaria italica* control plants through GC-MS.

Sr. No.	Compound Name	Formula	Molecular weight	Comments
1	Oxime-methoxy phenyl	$C_8H_9NO_2$	151	Antioxidant and Antimicrobial activity
2	4-Ethylbenzoic, Cyclopentylester	$C_{14}H_{18}O_2$	218	Antimicrobial, Antioxidant, Biological activities
3	Benzaldehyde; 2-5 bis (Trimethylsilyl)	$C_{13}H_{22}O_3S_{12}$	282	Antitumor, Biological activity.
4	Crotonic acid	$C_8H_{12}O_2$	140	Antifungal, Allelopathic activity, Biological activity

Table 4: Analysis of the metabolites produced by *Piriformospora indica* colonized *Setaria italica* plant.

Sr. No.	Compound Name	Formula	Molecular weight	Comments
1	Oxime-methoxy phenyl	$C_8H_9NO_2$	151	Antioxidant and Antimicrobial activity
2	4-Ethylbenzoic, Cyclopentylester	$C_{14}H_{18}O_2$	218	Antimicrobial, Antioxidant, Biological activities
3	4-Nitrocinnamaldehyde	$C_9H_7NO_3$	177	Biologcal activities, Antidiabetic activity, Exact structure of auxin action.
4	Benzaldehyde;4(1,3-dioxolanzyl)	$C_{10}H_{10}O_0$	178	Antitumor, Biological activity.
5	1-Ascorbic acid 2,6-dipalmitate	$C_{38}H_{68}O_8$	652	Antioxidant, Antimicrobial Pro-oxidant activity
6	Hexylacrylate	$C_9H_{15}O_2$	190	Biological activity
7	Nitroglycerin-1,2,3-propanetriol, trinitrate	$C_3H_5N_3O_9$	227	Antifungal activity, Vasodilator
8	3-(Octadecyloxy)propyl stearate	$C_{39}H_{78}O_3$	594	Antianginal activity
9	Adipic acid	$C_6H_{10}O_4$	146	Antacids and jelling agents

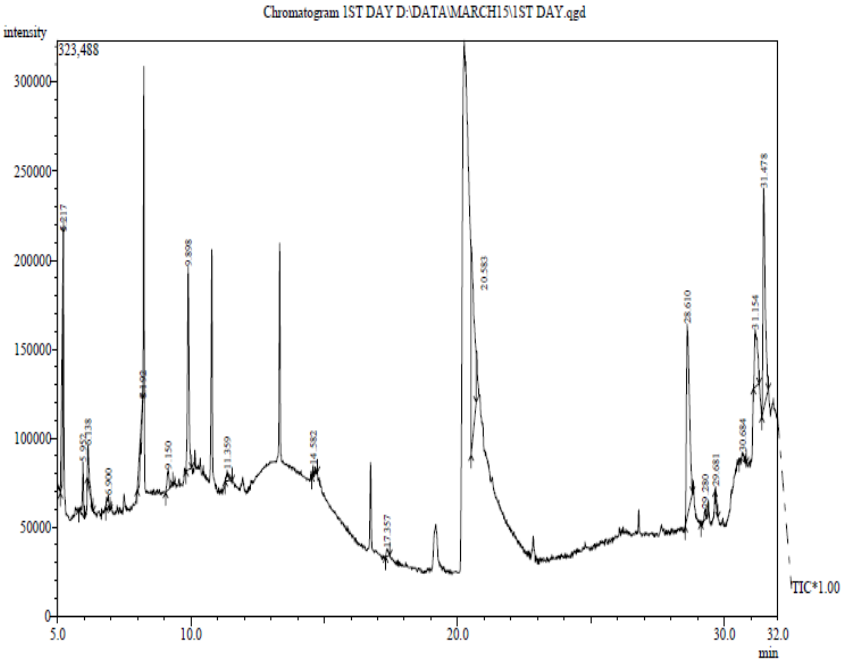

Fig. 1: Analysis of the metabolites produced by *P. indica* in culture broth after a week's inoculation by GC-MS.

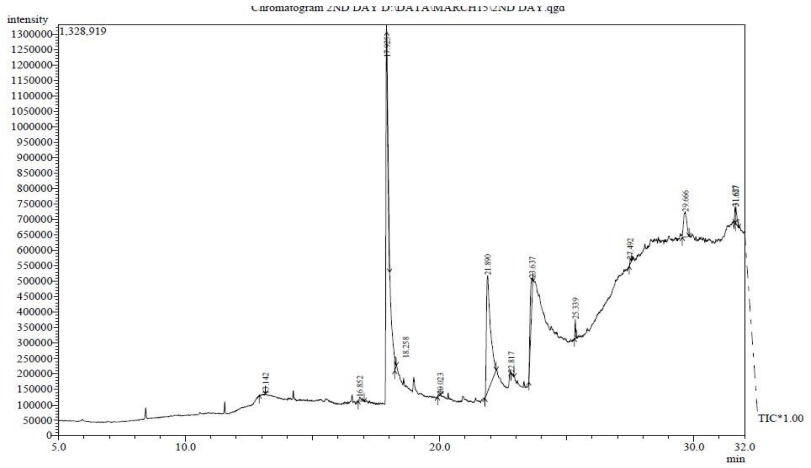

Fig. 2: Analysis of the Metabolites Produced by *Piriformospora indica* after two weeks of inoculation by GC-MS.

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