Promotion of seed germination of Green gram and Chick pea by *Penicillium verruculosum* RS7PF, a root endophytic fungus of *Potentilla fulgens* L.

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Abstract

The culture broth of a fungal endophyte associated with the roots of *Potentilla fulgens* L. showed ability to promote seed germination in *Vigna radiata* (Green gram) and *Cicer arietinum* (Chick pea) under assays in *in-vitro* conditions. The culture broth of the endophytic fungus *Penicillium verruculosum* RS7PF (Genbank accession number: EU579531) was further tested for the presence of Indole Acetic Acid (I.A.A.) using spectrophotometric analysis. The growth characteristics of the fungi in potato dextrose agar medium was compared with its simultaneous release of plant growth promoter and results showed that the concentration of IAA in the culture broth peaked during the 10th day of growth phase. Our results indicate that the endophytic fungi is capable of producing I.A.A by its own metabolic machinery which may be a crucial step in its establishment inside the plant host and its subsequent promotion of seed germination.

Keywords: *Potentilla fulgens,* fungal endophyte, *Penicillium verruculosum*, indole acetic acid, seed germination, green gram, chick pea.

Running Title: Seed germination promotion by an endophyte

Introduction

Endophyte-infected plants often grow faster than non-infected ones. This effect is at least in part due to the endophyte's production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances, and/or partly owing to the fact that endophytes could have enhanced the host's uptake of nutritional elements such as nitrogen and phosphorus, for instance endophytic fungi such as *Piriformospora indica* have also been proven to initiate positive growth responses in the medicinal plants like *Spilanthes calva* and *Withania somnifera* (Rai *et al.,* 2001).

Potentilla fulgens L. (Hindi Vernacular name: Bajradanti) of the Rosaceae family, commonly found at higher altitudes (1500-2000 m MSL) in the Khasi hills of Meghalaya, (India) has been used as a folk remedy for a variety of ailments by the ethnic tribal population. Traditionally, pieces of tap roots of *P. fulgens* are chewed along with raw areca nut (*Areca catechu* L.) and betel leaves (*Piper betel* L.). *P. fulgens* also finds applications in Ayurveda (Indian traditional system of medicine) primarily as a medicine to cure toothache, diarrhea and liver disorders. Recently, the root extracts of this plant have also presented a marked anti-diabetic activity (Syiem *et al.,* 2002) and protective activity against UV and hydrogen peroxide induced DNA damage in laboratory conditions (Bhagobaty & Joshi., 2008). Because of its high medicinal value and its newly discovered anti-diabetic property, its demand in the domestic as well as in the international market is likely to increase even more in the near future. Thus, enhancement of the growth and bioactive agents of this plant species is desirable. The first step towards achieving this goal may be the inoculation of the plant roots with endophytic fungi which have plant growth promoting activities.

The present study was therefore undertaken to study the effect of the metabolites of a root endophytic fungi isolated from the tap roots of *P. fulgens* on promoting the germination of *Vigna radiata* (Green gram) and *Cicer arietinum* (Chick pea) seeds.

Materials and methods

Fungal growth characteristics:

The fungal growth of was measured spectrophotometrically using a protocol modified from Meletiadis *et al.,* (2001) at 450 nm in a UV visible spectrophotometer.

Preparation of the fungal culture broth crude extract:

1 ml of each of the fungal cultures in liquid culture medium (potato dextrose broth medium,Himedia laboratories Pvt.Limited ,Mumbai, India) were aseptically transferred under laminar flow to 1.5 ml microfuge tubes and centrifuged at 13000 rpm for 5 mins at 4ºC in an Haraeus Biofuge refrigerated ultra-centrifuge. The supernatants free from the culture media was then filtered through 0.2 micron syringe filter and transferred to a new 1.5 ml microfuge tubes and kept at 4ºC until, they were used.

Seed germination assay:

100 seeds of *Vigna radiata* and *Cicer arietinum* were allowed to germinate on petri dishes with 5 ml aliquots of the crude fungal culture

broth. Control seeds were germinated with only the fungal culture broth medium used to grow the fungus. The dishes were incubated in the dark at 25° C. (modified from Hasan, 2002). The germination percentage and length of shoot were determined after 24 hours of incubation and continued unto 5 days from the initial date of incubation.

Detection and quantification of IAA in the fungal culture broth crude extract:

An 1-ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) and allowed to stand at room temperature for 20 min before the absorbance at 535 nm was measured (Patten & Glick, 2002). The concentration of IAA in each culture medium was determined by comparison with a standard curve. This estimation was carried out for a period of two weeks (15 days) to correlate I.A.A. production by the endophyte with its growth characteristics.

Results and Discussion

Fungal growth characteristics:

The endophytic fungus showed a sigmoid type of growth pattern when inoculated in potato dextrose broth medium at 27 °C and 150 rpm BOD shaker. The fermentation broth turned reddish in color on the 10th day of the growth cycle. The stationary phase was achieved more or less around the same time and it continued till 15th day (Figure 1). This type of growth is very common in fungi and other microorganisms.

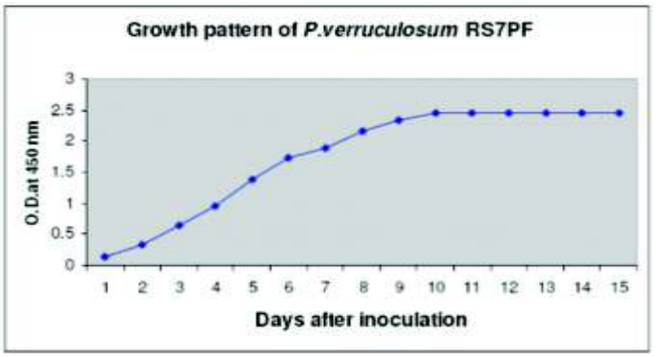


Figure 1: Growth characteristics of the endophytic fungus

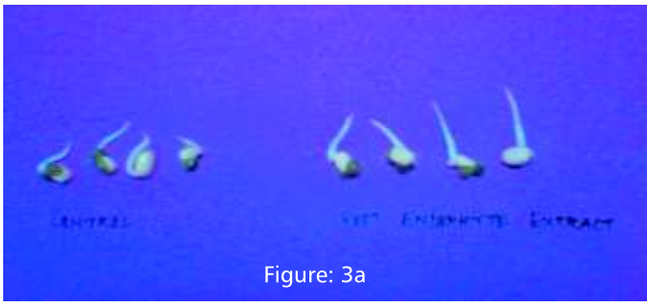
Seed germination assay:

The crude culture broth of the endophytic fungi enhanced the germination percentage of both *Vigna radiata* and *Cicer arietinum* i.e. 95 and 87 percent respectively as compared to the controls (Table 1.and Figure 3a-b). The elongation of shoots was observed at every 24 hours interval from the start of the germination assay and results show that the endophytic fermentation broth enhanced / promoted the elongation of shoots in both the plant species i.e. 38 and 23 mm respectively, after 96 hours from the start of the incubation. The growth of *Vigna radiata* shoots in case of the culture broth media controls was only 15 mm and 9 mm for the *Cicer arietinum* seedlings. (Table 1)

Table 1: Effect on seed germination of the crude fermentation broth of *P.verruculosum* RS7PF.

Time after germination in hours	Shoot elongation after incubation with fermentation broth of *P. Verruculosum* RS7PF (in mm)*			
	Vigna radiata (Green gram)	Control	*Cicer arietinum* (Chick pea)	Control
24	24 (95)$	8 (73)	14 (87)	2 (55)
48	36	13	17	6
72	38	15	18	9
96	38	15	23	9

* The values are mean of 3 replications.
$The germination percentage is given under parentheses.



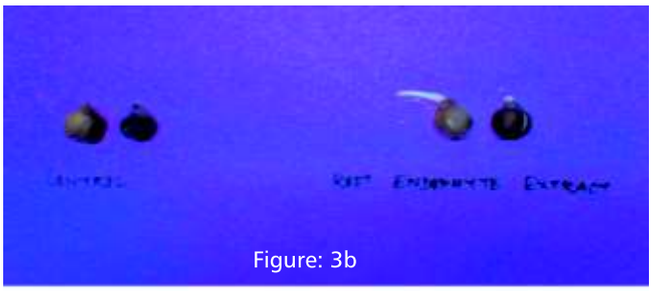


Figure 3a and 3b: Promotion of seed germination of *Vigna radiata* (a) and *Cicer arietinum* (b) after 24 hours by the metabolites of endophytic fungus *Penicillium verruculosum* strain RS7PF.

Detection and quantification of IAA in the fungal culture broth crude extract:

Indole acetic acid (I.A.A.) was detected after 24 hours of incubation of the fungus in its liquid growth medium by Salkowski's reagent which gives a pink reddish color in the presence of I.A.A. The concentration of I.A.A. peaked on the 10th day of the fungal growth cycle i.e. 100 ng/ml of the culture broth extract (Figure 2). It is therefore likely that the endophyte starts producing the plant growth promoters in a nutrient limiting environment (in stress condition) since by 10th day it also reaches a near about stationary phase of growth in liquid potato dextrose

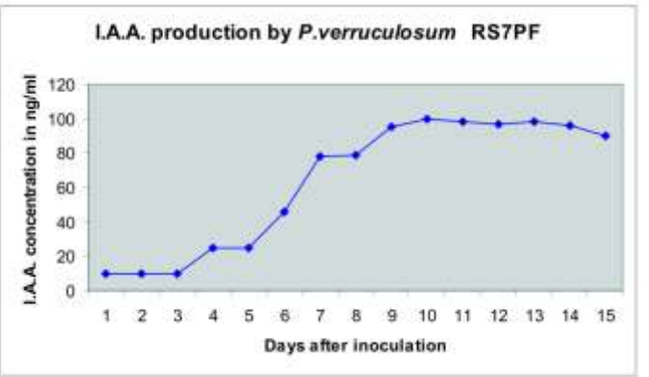


Figure 2: Indole acetic acid production by the endophyte.

medium under the given set of conditions in the laboratory. But the fungus also produces I.A.A. after 24 hours of inoculation in the growth medium suggesting the fact that it is genetically programmed to produce low but useful amounts of plant growth promoters This may have importance from the evolutionary point of view of the plant-fungal symbiosis and may be crucial for the initial establishment of the fungus inside the plant host and its subsequent promotion of plant growth.

Conclusions

From our investigation it can be logically extrapolated that the endophytic fungus may have the metabolic machinery to produce plant growth regulators and thereby promote seed germination in crop plants. However the exact purpose of producing the same by the endophytic fungus in natural conditions i.e. either to increase its plant root infection ability or to solely promote the plant growth will only be clear after looking at the molecular aspects of the plant fungal symbiosis and the genes responsible for the phenomenon.

References

Bhagobaty, R.K. and Joshi, S.R. 2008. DNA damage protective activity of the crude metabolites of endophytic fungi isolated from two ethno-pharmacologically important medicinal plants of the Khasi Hills of Meghalaya, India. *Pharmacologyonline* **3**: 882-888.

Hasan, H.A.H. 2002. Gibberellin and auxin production by plant root-fungi and their biosynthesis under salinity-calcium interaction. *Rostlinna Vyroba*, **48** (3): 101-106.

Meletiadis,J., Mouton, J.W., Meis, J.F.G.M., Bouman , B.A., Donnelly, J.P., Verweij, P.E., and Network, E. 2001. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. *Journal of Clinical Microbiology,* **39**(9): 3402-3408.

Patten, C. L., and Glick, B.R. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology,* **68**(8): 3795-3801.

Rai, M., Acharya, D., Singh, A., and Varma, A. 2001. Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. *Mycorrhiza*, **11**:123-128.

Syiem, D., Gareth, S., Khup, P.Z., Khongwir, B.S., Kharbuli, B. and Kayang, H. 2002. Hypoglycemic effect of *Potentilla fulgens* L. in normal and alloxan-induced diabetic mice. *Journal of Ethnopharmacology,* **83**: 55-56.

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Smith, D.L., D.A. Starrett and K.C. Gross. 1998. A gene coding for tomato fruit β- galactosidase II is expressed during fruit ripening cloning, characterization and expression pattern. *Plant Physiol*., **117:** 417-423.

Sree Vidhya, C.S., M. Manoharan and G. Lakshmi Sita. 1999. Cloning and characterization of salicylic acid induced, intracellular pathogenesis related gene from tomato (*Lycopersicon esculentum*). *J. Biosci.*, **24**: 287-293.

Takken, F.L., D. Schipper, H.J. Nijkamp, J. Hille. 1998. Identification and Ds-tagged isolation of a new gene at the Cf-4 locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. *Plant J*, **4:** 401-11.

Taylor, J and L.A. Harrier. 2003. Expression studies of plant genes differentially expressed in leaf and root tissues of tomato colonized by the arbuscular mycorrhizal fungus *Glomus mosseae*. *Plant Molecular biology,* **51**: 619-629.

Traut, T.W. 1994. The functions and conserved motifs of nine types of peptide segments that form different types of nucleotide binding sites. Eur *J Biochem*., 229: 9-19.

Vidavsky, F., S. Leviatov, J. Milo, H.D. Rabinowitch, N. Kedar, H. Czosneck, 1998. Response of tolerant breeding lines of tomato, Lycopersicon esculentum, originating from three different sources (L. *peruvianum,* L. *pimpinellifolium*, and L. *chilense*) to early controlled inoculation by tomato yellow leaf curl virus (TYLCV). *Plant Breed.,* **117:** 165169.

Vincente, J.G. and G.J. King. 2001. Characterization of disease like sequences in *Brassica oleracea* L. *Theor. Appl. Genet*., **102**: 555-563.

Woodgett, J.R., K.L. Gould and T. Hunter. 1986. Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur. *J. Biochem*., **16**: 177-184.

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