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Endophytic communities from *Phyllanthus amarus* with special reference to *Aureobasidium pullulans*

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Endophytic assemblage of *Phyllanthus amarus* was studied during monsoon, winter and summer seasons in Bhadra river project area of Malnad region, Western Ghats, Southern India. %). The colonization rate (%) and colonization frequency (%) were high during the winter than monsoon and summer seasons. *Aureobasidium pullulans* (27.0 %), *Nigrospora oryzae* (13.83 %), *Curvularia lunata* (11.33 %), *and Alternaria alternata* (7.5 %) were dominant endophytic fungal species isolated from leaf tissues *of P. amarus.* A. Pullulans was frequently isolated during all the seasons. In dual culture antagonistic studies *A. pullulans* inhibited the growth of some phytopathogens effectively. The shoot length, root length and leaf area were increased significantly over the control seedlings of wheat and chilli cropsinoculated with *A. pullulans*. Overall growth parameters were near equal to the seedlings supplemented with the chemical fertilizers.

Keywords: *Aureobasidium pullulans, endophyte*, antagonism, growth promotion.

Introduction

Foliar fungal endophytes are fundamental aspect of plant biology and found in all plants examined till date (Stone *et al*., 2000). The endophyte plant interaction ranged from mutualism to antagonism. These microbes play a major role in physiology and metabolism of host plants with the production of alkaloids, phytohormones and protect the host from herbivores, insects, and various phytopathogens (Omacini *et al.*, 2001; Shankar Naik *et al.*, 2006).

Endophytes are the major components of fungal diversity and also affect the structure of host plant community (Gilbert and Strong, 2007) and may produce substances of potential use to modern medicine, agriculture and

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industry such as novel antibiotics, antimycotics, immunosuppresants and anticancer compounds (Tan and Zou, 2001; Strobel and Daisy, 2003). *Phyllanthusamarus* is an herb with anti viral effect used for treatment of hepatitis, jaundice, gonorrhea, and diabetes by the local people. In the present study we documented the distribution of fungal symbionts in *P. amarus*and bioefficacy of *A. pullulans in vitro*

Materials and methods

Apparently healthy looking leaf samples of *Phyllanthusamarus* growing in Bhadra river project area of Malnad region were collected during different seasons and brought in sterile polythene bags to the laboratory and processed within 24 h of collection. Surface sterilization of samples was done by cleaning leaves under running tap water and cutting them into ≤ 1 cm segments followed by stepwise washing with 70% ethanol for 2 min, Sodium hypochlorite solution for 5 min and 70% ethanol for 30 s followed by two rinses in sterile distilled water, then allowed to surface dry under sterile conditions (Arnold *et al.*, 2000).

Leaf segments were placed on 9 cm Petri plates containing potato dextrose agar (PDA, Hi Media Laboratories, Mumbai, India) medium amended with streptomycin 250 (mg/L^{-1}) to suppress bacterial growth. The efficacy of surface sterilization was confirmed by pressing the sterilized leaf segments on to the surface of PDA medium. The absence of growth of any fungi on the medium confirmed that the surface sterilization procedure was effective (Schulz *et al.*, 1993). Petri plateswere incubated at 28± 1º C with a 12 h photoperiod, and sporulation was induced by incubation in a light chamber under near UV light for 1 to 12 d. Fungi growing out from the leaf segments /seeds were subsequently transferred onto fresh PDA plates. Pure cultures were spread on fresh PDA slants. Endophytic fungal species were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores. Cultures that failed to sporulate were recorded as sterile form. Two collections were made in each season. Two hundred segments were randomly selected from the leaves/season. All the isolates were numbered and maintained in Culture Collection Centre of Department of Applied Botany, Kuvempu University, Shankaraghatta, India. The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by total number of segments incubated in that sample x 100. Frequency of colonization by individual taxa was calculated similarly.

Dual culture technique was applied to test the antagonistic efficacy of *A. pullulans*. Five days old mycelial discs (5mm diam.) of test pathogens were placed on four corners of petri plates containing PDA medium. Spores of *A. pullulans* were inoculated at the center of PDA plates. In control studies, the

plates were inoculated with selected plant pathogens for comparison of diameter growth with dual culture plates. Plates were incubated at 28±1º C for 5 days and per cent inhibition was calculated using the formula C-T/C x 100 where C is the colony growth of test pathogens in control and T is the colony growth of test pathogens in dual culture. Experiment was carried out in triplicate.

The strain of *A. pullulans* used for plant growth promotion studies in vitro. The starter cultures *A. pullulans* was grown on petri plates containing PDA medium for 5 days. After 5 days, discs of size 5 mm of these fungi were inoculated into 500 ml conical flasks containing 200 ml of Potato Dextrose Broth (Dextrose–20 g; Potato–100 g; Distilled Water–1000 ml) and incubated at 30±2º C under constant shaking conditions (100 rpm) in the dark for 10 days. Fungal mycelium was harvested and washed several times with sterile water. Surface sterilized seeds wheat and chilli were mixed with a mechanically homogenized culture of *A. pullulans* in sterile water. In the second set of experiment, one chemical fertilizer N: P: K (17:17:17) was applied at the rate of 60 mg. The uninoculated control and treatments were maintained separately in three replications.

Soil sample was prepared by adding mixture of Soil: Sand: Farmyard manure (3:1:1). This mixture was disinfected with 1% formalin for 48 h and left for 5 days to aerate to eliminate excess formalin [Rai *et al.*, 2001].

Treated and untreated seeds were sown in pots (9 inch height by 10 inch diameter) containing soil prepared by adding mixture of Soil: Sand: Farmyard manure. The pots were kept under the normal growth conditions approximately 75% relative humidity, 22º C to 26ºC, 12 h daylight. The emerged plants were irrigated on every second day with equal amount water to pots containing control plants and pots with treatments. The plantlets were harvested on 45 days after sowing. Growth parameters such as seed germination, leaf area, shoot length, root length, fresh weight, and dry weights were estimated. Root colonization was assessed by the method of Philips and Hayman (1970).

Results

Total 537 fungal isolates belonged to 13 different species and a sterile form were obtained from 400 leaf segments incubated during monsoon winter and summer seasons. These fungal species represented Ascomycete (8.6 %), Coelomycete (3.6 %), Hyphomycete (81.6 %), Zygomycete (1.1 %), and Sterile forms (2.6 %). The colonization rate (%) (Fig 1) and colonization frequency (%) were high during the winter than monsoon and summer seasons. *A. pullulans*(27 %),*Nigrosporaoryzae*(13.83 %), *Curvularialunata*(11.33 %), and *Alternaria alternate* (7.5 %)were dominant endophytic fungal species isolated

fromleaf tissues *of P. amarus* (Table 1). *A. pullulans* was isolated as dominant endophytic fungi throughout the year (Fig2). The fungal species like *A. pullulans, Botryosphaeria subglobosa, Cladosporium cladosporioides, Nigrospora oryzae, Sphaeropsis sapenea* were frequently isolated during the monsoon season. *A. alternata, A. pullulans, B. subglobosa, C. lunata, N. oryzae*, was frequently recovered from winter season. Similarly *A. pullulans, C. lunata, N. oryzae, G. roseum* were recovered during summer season (Table 1). In antagonistic studies maximum inhibition was observed against *Alternariaalternata* (71.0 %), and least against *Rhizopus stolonifer* (58.3 %) (Fig 3). The shoot length, root length and leaf area were increased significantly over the control seedlings in both the crop plants and overall growth parameters were near equal to the seedlings supplemented with the chemical fertilizers (Table 2).

Table 1. Endophytic fungi isolated from *Phyllanthus amarus* during different seasons from Bhadra river project area, Western Ghats India

Endophytic fungi	***Fungal group***	**Colonization frequency (%)**			***Mean total***
		monsoon	**winter**	**summer**	
Alternariaalternata	Hyphomycetes	-	16.5	6.0	7.50
Aspergilluscandidus	Hyphomycetes	7.0	-	-	2.33
Aureobasidiumpullulans	Hyphomycetes	30.0	36.0	15.0	27.00
Botryosphaeriasubglobosa	Ascomycetes	16.0	10.0	-	8.67
Cladosporiumcladosporioides	Hyphomycetes	12.0	-	-	4.00
Curvularialunata	Hyphomycetes	-	19.5	14.5	11.33
Gliocladiumroseum	Hyphomycetes	-	-	7.0	2.33
Gliomastixmurorum	Hyphomycetes	-	-	2.0	0.67
Humicolafuscoatra	Hyphomycetes	4.5	5.5	-	3.33
Nigrosporaoryzae	Hyphomycetes	12.0	19.5	10.0	13.83
Rhizopusstolonifer	Zygomycetes	-	3.5	-	1.17
Sphaeropsissapenea	Coelomycetes	11.0	-	-	3.67
Sterile form- white(AL-1)	Mycelia sterilia	-	-	8.0	2.67
Verticilliumchlamydosporium	Hyphomycetes	2.5	3.5	-	2.00
Total colonization frequency (%)		95	97.5	56.5	83.00
Total colonization rate(%)		66.5	70.0	50.5	62.3

Data based on 200 leaf segments/season

Table 2. Associative effect of fertilizer, endophyte treatment on growth parameters of chilli and wheat after 7th, 15th, 30th, 45th days of sowing.

	7th day						15th day					
	Chilli			Wheat			Chilli			Wheat		
	Control	NPK	*A.pul*	Control	NPK	*A.pul*	Control	NPK	*A.pul*	Control	NPK	*A.pul*
Seed germination (%)	81	89	86	74	93	94	-	-	-	-	-	-
Shoot length (cm)	6.5 a	12.1 b	8.9 a	11.0 a	31.4 c	27.6 c	6.8 a	13.8 b	9.5 a	19.7 a	40.8c	41.9 c
Root length (cm)	5.1 a	7.5 b	7.2 b	5.4 a	13.0 b	9.3 a	5.4 a	7.8 a	7.8 a	6.3 a	15.9 b	13.4 b
Fresh weight (g)	0.13 a	0.44 a	0.22 a	0.2 a	0.41 a	0.33 a	0.18 a	0.59 a	0.26 a	0.21 a	0.7 a	0.73 a
Dry weight (g)	0.001 a	0.01 a	0.001 a	0.01 a	0.07 a	0.06 a	0.02 a	0.11 a	0.04 a	0.05 a	0.31 a	0.31 a
Leaf area (cm)	0.5 a	1.1 a	0.6 a	6.8 a	11.7 a	13.1 b	0.6 a	1.5 a	1.0 a	9.1 a	19.0 b	17.5 b
Root colonization (%)	-	-	53	-	-	63	-	-	74	-	-	74

	30th day						45th day					
	Chilli			Wheat			Chilli			Wheat		
	Control	NPK	*A.pul*	Control	NPK	*A.pul*	Control	NPK	*A.pul*	Control	NPK	*A.pul*
Shoot length (cm)	7.5 a	14.0 b	10.2 a	28.1 a	52.0 c	51.8 c	8.3 a	15.8 b	11.3 a	39.0 a	55.0 b	55.1 b
Root length (cm)	5.8 a	8.8 b	8.9 b	10.9 a	20.9 b	18.1 b	6.3 a	9.0 b	9.1 b	14.0 a	22.2 b	18.9 b
Fresh weight (g)	0.20 a	0.61 a	0.32 a	0.41 a	1.2 a	1.22 a	0.43 a	1.23 a	0.50 a	0.6 a	2.82 a	3.2 a
Dry weight (g)	0.03 a	0.15 a	0.08 a	0.13 a	0.21 a	0.39 a	0.05 a	0.11 a	0.58 a	0.08 a	0.28 a	0.41 a
Leaf area (cm)	1.2 a	2.7 a	2.0 a	12.7 a	23.4 b	22.1 b	2.3 a	3.7 a	3.0 a	15.8 a	27.3 b	25.0 b
Root colonization (%)	-	-	67	-	-	79	-	-	68	-	-	91

Data based on average of three replications; Different letters following means in rows indicate a significant difference among treatments based on DMRT (P≤ 0.05)

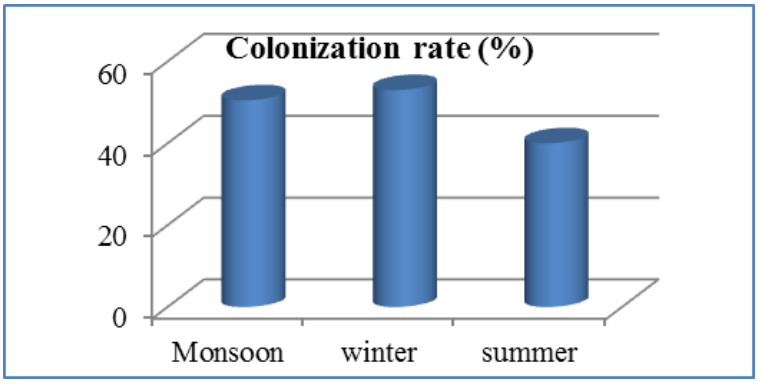

Fig. 1. Colonization rate (%) of endophytic fungi in leaves of *P. amarus*

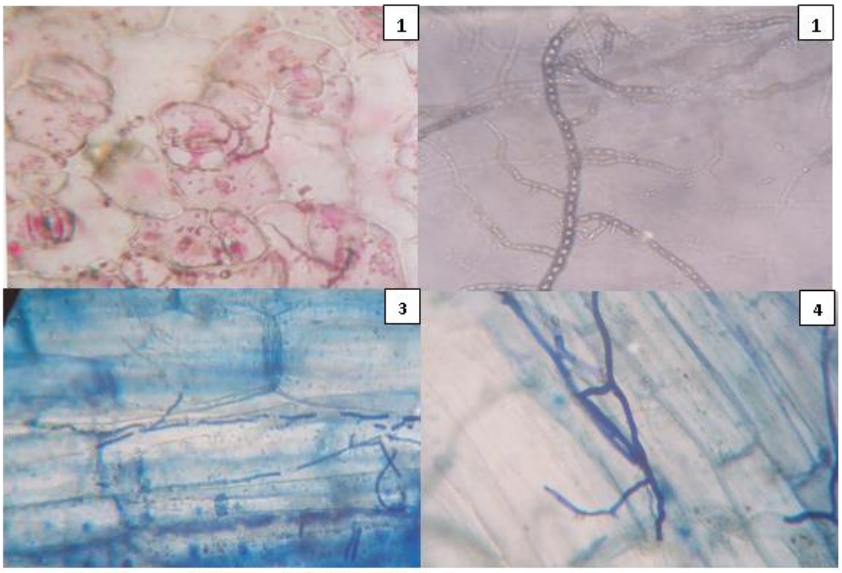

Fig. 2. Photographs showing colonization of *A. pullulans*

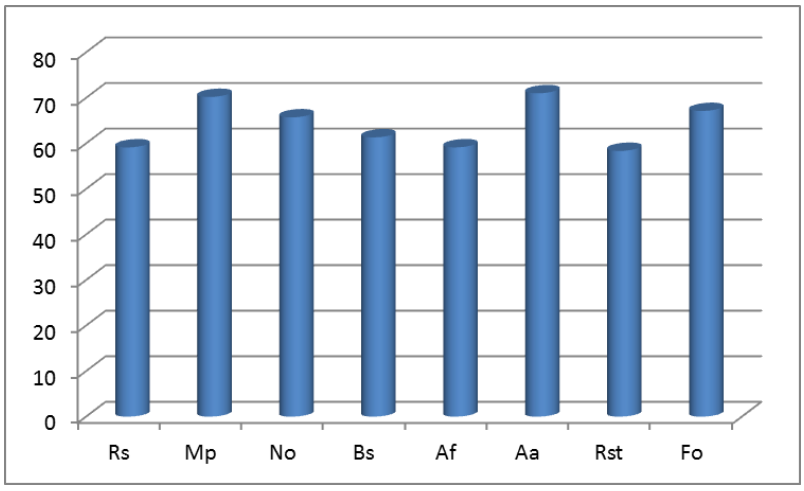

Fig. 3. Percentage inhibition of *A. pullulans* against some phytopathogens in dual culture method

Data are mean of three replication; ± = Standard deviation

Rs- *Rhizoctoniasolani*; Mp- *Macrophominaphaseolina*; No- *Nigrosporaoryzae*; Bs- *Bipolarissorokiniana*Af- *Aspergillusflavus*; Aa- *Alternariaalternata*; Ast- *Rhizopusstolonifer*; Fo- *Fusariumoxysporum*

Discussion

Colonization frequency (%) fungal endophyte in this study was within the range of many host plants studied in the tropics (Suryanarayanan*et al.,* 2003,).The high colonization frequency during the wet season than the dry season suggested that the infection was positive correlated with the precipitation ((Bills, 1996; Osono and Mori, 2005). *Aureobasidiumpullulans* (de bary) Arnaud is well known as ubiquitous on aerial plant surfaces usually found associated with plants from temperate and tropical plants (Buckly and Pugh, 1971). It shows a varied type of phenotypic plasticity transition from homogenous to sectored, yeast to mycelial and to giant to micro colonial forms.

The plasticity is might be due to leaf age, carbon substrates, growth medium and light, temperature and other climatic factors (Slepecky and Starmer, 2009). Presence of *A. pullulans* in the *P. amarus* host tissues in throughout the seasons indicated that it is well adapted for the host chemistry. Antagonism of *A. pullulans*against phytopathogens is might be due to production of biological substances in the culture medium. Similar antagonism is reported against fungi such as *Penicillium digitatum, Botrytis cinerea,Monilia laxa* and various pathogens (Schena *et al.,* 2002; Andrews *et al.,* 1983). Strains of *A. pullulans* produces many metabolites like Poly galacturonase, B-1-3-glucanase, exochitinase, endochitinase, (Oliver*aet al.,* 2009; Zhang *et al.,* 2010) and are considered as possible bio control agents of post harvest diseases (Leibinger*et al.,* 1997; Vero *et al.,* 2009). The growth promoting efficacy of *A. pullulans* has already been documented in our previous studies on rice, jowar, groundnut and fingermillet (Shankar Naik *et al.,* 2008). It has been also reported that auxin is produced by isolates of *A. pullulans* (Buckley and Pugh, 1971). Hence further studies on this fungi helps to understand the ecology and production of novel bioactive compounds of potential use in agriculture, medicine and industries.

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