ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Influence of Microbial Inoculants on Production of Andrographolide and Control of Root Rot in *Andrographis paniculata* (Burm . F. Nees).

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ABSTRACT

The root rot of *Andrographis paniculata* is a very serious soil borne disease caused by *Fusarium chlamydosporum.* The role of *Glomus fasciculatum*, *Gliocladium virens* and *Bacillus subtilis* as biocontrol agents was studied in green house conditions by using these organisms either single or in combination. The seedlings of *A.paniculata* were raised in pots filled with sterile sand and soil (1:1 v/v) mixture. Plants treated with *G.fasciculatum* and *G.virens* showed a disease severity index of 32.46% compared to the uninoculated control plants which had a disease severity index of 84.5%. The same treatment also resulted in the maximum growth, yield and andrographolide content in the *A.paniculata* plants. The fungicide captan (0.25%) was not as effective as the microbial inoculants in controlling the pathogen.

Keywords: *Bacillus subtilis*, biological control, *Fusarium chlamydosporum*, *Gliocladium virens*, *Glomus fasciculatum*.

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ISSN: 0975-8585

INTRODUCTION

Andrographis paniculata (Burm.f.Nees), a well-known herb belonging to the family *Acanthaceae* and is widely distributed in tropical areas of Asia like India, Pakistan and Sri Lanka and commonly known as "Kalmegh". It is known as the "King of Bitters" [1]. This plant rose into prominence by virtue of its lactone andrographolide, diterpene present in tubers [2]. This compound is hepatoprotective, antihelmenthic and also a blood purifier [3]. Andrographolide acts well as an anticancer agent and as a stimulant of immune response [4]. Because of the continuous collection of these tubers from wild sources, this plant has been included in the list of endangered species. Recently farmers have started to grow it as a crop because of its economic potential [5]. But the plant is susceptible to many diseases of which root rot caused by *Fusarium chlamydosporum* is the most important disease which occurs in severe form. Biological control of plant pathogen is considered as a potential control strategy in recent years as the chemical control results in accumulation of harmful residues which may lead to serious ecological problems. The literature on biological control of soil borne pathogens of medicinal plants is very limited. The Fungi belonging to the genus *Gliocladium* are the most promising biocontrol agents against a range of plant pathogens under a variety of environmental conditions [6]. *Bacillus subtilis* and vesicular arbuscular mycorrhizal (VAM) fungi are also considered as an important biocontrol agents known to suppress a wide range of plant pathogens [7-8]. The present study was carried out to evaluate the effect of three microbial inoculants viz. *Glomus fasciculatum*, *Gliocladium virens* and *Bacillus subtilis* in controlling *Fusarium* wilt of *A.paniculata*.

MATERIALS AND METHODS

Microbial inocula

The isolate of *G.fasciculatum* was obtained from Biotechnology Research Center, Tirupati, Andhra Pradesh, India which was maintained as a pot culture in sterilized sand and soil (1:1 v/v) mixture on *Sorghum* cultivar CV40. The air dried inoculum contained VAM hyphae, spores and root particles. The number of infective propagules in the inoculum was 110/g. *G.virens* was obtained from Regional Center of Organic Farming, Bangalore, India was inoculated into potato dextrose broth and incubated at 28±2 $^{\circ}$C for 7 days. After one week the mycelial mat was separated, macerated using a homogenizer and the fungal mass was suspended in 0.1 M $MgSO_4$ solution. The inoculum contained 7×10^5 colony forming units (cfu) of *G.virens*/ml suspension. The culture of *B.subtilis* was obtained from Regional Center of Organic Farming, Bangalore, India. It was grown in nutrient broth for 48 hr on a shaker. The bacterial cells were harvested by centrifugation at 10,000 rpm for 5 min and suspended in 0.01 M $MgSO_4$ solution. The cfu(s) of *B.subtilis* was 21×10^5/ml inoculum.

Isolation and identification of *Fusarium chlamydosporum*

The *F.chlamydosporum* was locally isolated from diseased root fragments of *A.paniculata* and the identity was confirmed by Plant Pathology Division, Regional Agricultural College, Andhra Pradesh, India. The pathogen was maintained on potato dextrose agar medium.

***In vitro* studies *of Fusarium chlamydosporum* antagonists**

The microbial interactions between *G.fasciculatum*, *G.virens*, *B.subtilis* and *F.chlamydosporum* were observed by measuring the inhibition zone between the pathogen and antagonist. The control was maintained without antagonist for comparison

Pot experiments

The seeds of *A.paniculata* were surface sterilized using 0.1% $HgCl_2$ for 5 min. After thorough washing 3 to 4 seeds were sown in each pot (20 cm × 10 cm) containing 250 g of sterile potting mixture (soil and sand, 1:1 v/v ratio). After germination the seedlings were thinned to one per pot. The inoculum of *G.fasciculatum* (10g/pot), *G.virens* and *B.subtilis* (10 ml/pot) was added to the planting hole as per the treatment before sowing the seeds. The pots were watered with 250 ml of water on every alternate day. The following treatments were established with six replications.

- Uninoculated control
- Inoculation with *G.fasciculatum*
- Inoculation with *G.virens*
- Inoculation with *B.subtilis*
- Supplementation with 0.25% captan to the potting mixture
- Inoculation with *G.fasciculatum+G.virens*
- Inoculation with *G.fasciculatum+B.subtilis*
- Inoculation with *G.virens+B.subtilis*

Two more doses of inoculum were added on the 4^{th} and 12^{th} day after transplanting.

Disease severity index

The disease index was computed by adopting 0-4 scale to cover all the broad symptomological criteria [9]. 0-no symptoms, 1-slight dropping of leaves and/or vascular browning in root region and no plant mortality, 2-wilting of leaves and/or vascular browning extended into root region and no plant mortality, 3-severely wilted, withered except terminal bud, 4 dead plant.

The percent disease index (PDI) was determined by the formula

$$PDI = \frac{\text{Summation of individual scores}}{\text{Maximum grade} \times \text{Total number of plants}} \times 100$$

Morphological and nutrient concentration (P) analysis

The plant height and number of branches were recorded once in 30 days up to the day of harvest (90 days) after planting. After harvest, the length, thickness and fresh weight of tuberous roots were measured. To determine the dry weight of shoot and root, the plant material was dried at constant temperature of 70°C for 12 hr in a hot air oven. The

phosphorous content was estimated calorimetrically by the vandomolybdate yellow color method [10].

Estimation of andrographolide

Andrographolide in roots of *A.paniculata* was determined by high performance liquid chromatography (Agilent Technologies Series 1100) and quantified according to [3].

Microbial analysis

Mycorrhizal root colonization was determined by the grid line intersect method outlined by [11]. Extramatricular chlamydospore of VAM fungi in the root zone soil was estimated by wet sieving and decantation method [12]. *G.virens* and *B.subtilis* population in root and soil were determined by serial dilution using potato dextrose agar and nutrient agar media respectively.

STATISTICAL ANALYSIS

The data obtained was subjected to Duncan's Multiple Range Test (DMRT) using SPSS Ver. 11.5.

RESULTS AND DISCUSSION

Of the various treatments studied, co-inoculation with *G.fasciculatum* and *G.virens* enhanced the plant height, number of branches, length and thickness of tubers to the maximum (Table 1). The next best treatment was inoculation with *B.subtilis* and *G.virens* followed by the inoculation with *G.fasciculatum* and *B.subtilis* as co-cultures. Single inoculation with *G.virens, B.subtilis* or *G.fasciculatum* enhanced plant height, number of branches and tuber characteristics compared to un-inoculated control. Among these three, inoculation with *G.virens* resulted in the best response followed by the inoculation with *G.fasciculatum* and *B.subtilis* alone. The growth promoting effect of *G.fasciculatum* on *A.paniculata* [13] and other medicinal plants [14] were reported earlier. Plant growth stimulation by *B.subtilis* has also been reported by earlier workers [15]. The synergistic effect of *G.virens* and *G.fasciculatum* resulted in the maximum growth and dry matter yield of *A.paniculata* (Table 2). This type of synergism between *Trichoderma* sp and *G.fasciculatum* has been reported in case of marigold [16]. Other two dual inoculations such as *B.subtilis* and *G.virens*, *G.fasciculatum* and *B.subtilis* also enhanced the growth and yield of *A.paniculata* compared to all the single inoculants and treatment with the fungicide captan. Application of captan 0.25% resulted in better growth with respect to the control, but it was less effective when compared to single or dual inoculation with microbial inoculants. Phosphorous concentration in shoot and root was found maximum in the co-culture treatment with *G.fasciculatum+B.subtilis* followed by *G.fasciculatum+G.virens* and *B.subtilis+G.virens* (Table 2). Among single inoculations, *G.fasciculatum* treated plants had significantly high phosphorous concentration than other inoculations and control plants. These findings are in accordance with the [17-18]. Maximum shoot and root P concentration was found in the plants inoculated with *G.fasciculatum+B.subtilis* which was significantly higher compared to treatment with *G.fasciculatum* alone. Increased 'P' uptake and dry matter production by *G.fasciculatum* in presence of bacterial inoculants is reported earlier [19]. Andrographolide concentration in roots of *A.paniculata* was maximum in plants

inoculated with *G.fasciculatum+G.virens*, which was significantly more compared to all other treatments (Table 2). All the treatments with single microbial inoculants improved andrographolide concentration of roots compared to control. This is the first report of an increase in andrographolide concentration in the roots of *A.paniculata* by inoculation with microorganisms. There are reports on increased ajmalicine [20], rosmarinic acid [21] and artemisinin [22] inoculated with microbial inoculants. Plants inoculated with *G.fasciculatum* have significantly higher mycorrhizal root colonization compared to other monoinoculated and control plants (Table 3). A similar trend was also noticed in mycorrhizal spore numbers in the root zone soil. Such an increase in root colonization levels of plants grown in unsterile soil inoculated with VAM fungi has been observed earlier [23]. Of the dual inoculations, inoculation with *G.fasciculatum+B.subtilis* showed a maximum percent of VAM colonization compared to other co-culture treatments. The chemical capton did not show any adverse effect on mycorrhizal root colonization. The spore count was found maximum in the treatment with *G.fasciculatum+B.subtilis* followed by *G.fasciculatum+G.virens*. The percent of VAM colonization and number of spores in rhizosphere of *G.fasciculatum* treated plants was higher than *G.virens+B.subtilis* treatment. The increased number of *G.mosseae* spores in presence of *T.viridae* is observed [24].

A synergistic interaction between *T.viridae* and *G.intraradices* with marked beneficial effect on tomato cultivars has also been reported [25]. Population of *G.virens* in the root zone soil increased in all treatments from the date of planting to the date of harvest (Table 4). Maximum cfu of *G.virens* was obtained in the treatment *G.fasciculatum+G.virens* followed by the treatments *B.subtilis+G.virens* and *G.virens* alone. Similarly the *B.subtilis* population increased in all the treatments starting from the date of planting to the date of harvest. The *B.subtilis* population was maximum in the presence of *G.virens* followed by *G.fasciculatum.* There was an increase in the population of rhizosphere bacteria in pea plants inoculated with *G. intraradices* [26]. The synergestic interactions of *T.harzianum* with *G.mosseae* resulted in high colonization and plant growth response [27]. At the time of harvest, G.*fasciculatum+G.virens* treatment showed the lowest disease index, followed by the *B.subtilis+G.virens* and *G.fasciculatum+B.subtilis* treatments (Table 5). Among single inoculations, *G.virens* offered more specific protection against the pathogen.

Disease suppressiveness by *G.virens* is attributed to the production of chitinase, volatile and nonvolatile compounds [28]**.** Although *B.subtilis* showed better inhibition of *Fusarium* compared to *G.virens* under *in vitro* conditions, this was not true in green house conditions. The inefficiency of *B.subtilis* may be due competition by indigenous soil microflora or due to the repeated culturing *Bacillus in vitro* resulted in loss of efficiency. *G.fasciculatum* protected the plants much better than *B.subtilis* against the pathogen. VAM fungi are known to increase the resistance of plants to pathogens by modification of cell wall, production of antimicrobial compounds and altered rhizosphere microflora [29]. VAM fungi alleviating the severity of disease caused by root pathogenic fungi have been reported by several workers [30,31].

ISSN: 0975-8585

Table 1: Effect of VAM, *G.virens* and *B.subtilis* on growth and yield of *Andrographis paniculata* raised in wilt sick soil.

Treatment	height of plants (cm)	no. of branches	length of tubers (cm)	diameter of tubers (cm)
Control	18.5 [a]	6.81 [a]	5.82 [a]	1.79 [a]
G.fasciculatum	24.67 [abc]	9.43 [ab]	8.25 [ab]	3.57 [b]
G.virens	25.94 [bc]	9.84 [bc]	8.01 [ab]	4.10 [bc]
B.subtilis	22.13 [ab]	8.16 [a]	7.70 [ab]	3.32 [bc]
Captan	20.13 [a]	7.98 [a]	6.51 [a]	3.01 [bc]
G.fasciculatum+G.virens	29.14 [c]	13.04 [c]	10.96 [b]	4.01 [bc]
G.fasciculatum+B.subtilis	28.14 [bc]	11.63 [bc]	9.48 [ab]	3.57 [c]
B.subtilis+G.virens	28.79 [bc]	12.81 [c]	9.54 [ab]	3.72 [bc]

Means having same superscript do not differ significantly at p<0.05. The values are average of 20 observations.

Table 2: Effect of VAM, *G.virens* and *B.subtilis* on shoot and root dry weight, 'P' concentration and andrographolide concentration of *Andrographis paniculata* raised in Fusarium wilt sick soil.

Treatment	shoot dry weight (gm)	root dry weight (gm)	shoot 'P' concentration (%)	root 'P' concentration (%)	andrographolide concentration (%)
Control	7.15 [a]	0.04 [a]	0.12 [a]	0.11 [a]	0.29 [a]
G.fasciculatum	9.78 [ab]	0.12 [ab]	0.35 [f]	0.26 [d]	0.40 [d]
G.virens	10.77 [cd]	0.18 [bc]	0.19 [c]	0.13 [b]	0.36 [c]
B.subtilis	6.93 [b]	0.13 [ab]	0.21 [c]	0.15 [d]	0.38 [c]
Captan	8.17 [b]	0.09 [ab]	0.15 [b]	0.14 [ab]	0.33 [b]
G.fasciculatum+G.virens	19.14 [e]	0.19 [c]	0.27 [e]	0.22 [cd]	0.49 [f]
G.fasciculatum+B.subtilis	11.98 [d]	0.16 [bc]	0.36 [g]	0.32 [e]	0.37 [dc]
B.subtilis+G.virens	12.59 [d]	0.24 [c]	0.25 [d]	0.23 [c]	0.35 [e]

Means having same superscript do not differ significantly at p<0.05. The values are average of 20 observations

Table 3: Effect of VAM, *G.virens* and *B.subtilis* on mycorrhizal root colonization and spore numbers in the root zone soil of *Andrographis paniculata* raised in *Fusarium* wilt sick soil.

Treatment	Mycorrhizal colonization (%)	Spore numbers/100g soil
Control	38.29	102
G.fasciculatum	64.92	267
G.virens	43.9	129
B.subtilis	47.10	132
Captan	41.28	111
G.fasciculatum+G.virens	68.00	306
G.fasciculatum+B.subtilis	72.25	336
B.subtilis+G.virens	48.90	168

The values are average of 20 observations.

ISSN: 0975-8585

Table 4: Influence of microbial inoculants on population of *G.virens* and *B.subtilis* in the root zone soil of *Andrographis paniculata* raised in *Fusarium* wilt sick soil.

Treatment	Days After Planting					
	G.virens population (cfu X 10^4)			*B.subtilis* population (cfu X 10^4)		
	30	40	90	30	40	90
Control	3.00 ^{a}	2.57 ^{a}	3.52 ^{a}	32.4 ^{a}	38.00 ^{ab}	40.25 ^{b}
G.fasciculatum	3.25 ^{a}	3.94 ^{d}	4.10 ^{a}	36.50 ^{ab}	35.11 ^{a}	40.12 ^{b}
G.virens	20.00 ^{d}	21.96 ^{c}	23.00 ^{e}	40.00 ^{a}	41.76 ^{ab}	42.98 ^{c}
B.subtilis	3.60 ^{ab}	3.28 ^{a}	4.53 ^{b}	68.00 ^{c}	74.00 ^{e}	79.63 ^{d}
Capton	4.00 ^{b}	4.28 ^{ab}	5.93 ^{b}	36.15 ^{ab}	35.0 ^{ab}	33.00 ^{a}
G.fasciculatum+G.virens	24.46 ^{a}	26.78 ^{d}	27.01 ^{a}	39.00 ^{ad}	42.00 ^{b}	41.00 ^{bc}
G.fasciculatum+B.subtilis	7.50 ^{c}	7.53 ^{b}	8.48 ^{c}	70.10 ^{a}	73.82 ^{c}	80.15 ^{d}
B.subtilis+G.virens	20.00 ^{a}	21.69 ^{c}	24.21 ^{d}	71.14 ^{c}	74.68 ^{c}	82.10 ^{e}

Means having same superscript do not differ significantly at $p<0.05$. The values are average of 20 observations

Table 5: Influence of microbial inoculants on percent disease index of *Andrographis paniculata* raised in *Fusarium* wilt sick soil.

Treatment	Days After Planting		
	45	90	120
Control	10.52	65.25	83.50
G.fasciculatum	7.63	58.28	69.23
G.virens	6.00	57.84	66.56
B.subtilis	8.56	62.96	70.86
Captan	9.02	63.00	78.63
G.fasciculatum+G.virens	5.60	30.28	34.08
G.fasciculatum+B.subtilis	7.96	47.86	57.00
B.subtilis+G.virens	6.56	43.92	55.53

The values are average of 20 observations

CONCLUSION

The *A.paniculata* plant is the only source of andrographolide and attempts are on to increase the *in planta* content of andrographolide to reduce its cost of production. Towards this end, the use of microbial inoculants is precisely aimed in this direction. To conclude, our present study clearly indicated that root rot of *A.paniculata* caused by *F.chlamydosporum* can be controlled with *G.fasciculatum+G.virens* inoculation. The present study also showed that inoculation with microbial inoculants not only increased the tuber yield but also the andrographolide content. Our streamlined approach will be particularly useful under organic farming conditions, especially for medicinal plants where the use of chemicals has to be reduced because they are not only hazardous to human health but also soil health.

ISSN: 0975-8585

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