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Association of Dual Mycorrhizal Species with Leucas Aspera Enhances Growth and Total Biomass

Anubrata Paul[1], Jayashree D. R[2]

[1,2] Department of Biotechnology, M.S. Ramaiah College of Arts , Science & Commerce, MSRIT POST, Bangalore University, Bangalore-560054, Karnataka, India

Abstract: ***Leucas aspera is a common weed known as "Thumbai ",is a widely distributed throughout India and is used in various fields of medicine and agriculture. There have a few studies conducted on the effects of co-inoculated of AM fungi in various crop plants for their better inoculation responses. The present study was carried out to investigate the effect of dual inoculation of different species of AM fungi Aculospora lacunose and Glomus mossae for enhancement of growth parameters and plant biomass. Significant increase over control in plant height ,number of leaves, internode length, fresh and dry weight , stomatal index , leaf area index, chlorophyll content ,percent root colonization and spore count re recorded . The result confirms the feasibility of utilizing this strategy for their systematic cultivation and provides useful information for management agricultural systems.***

Keywords: Fumigation process, root colonization, Spore count. Dual inoculum of Mycorrhizal species, Plant growth parameter and total biomass of Leucus aspera

1. Introduction

AMF are among the most ecologically significant organisms on the plant (Fitter et al. 2011). "Mycorrhiza" the term used to describe the symbiotic association between a fungus and a root of higher plant (Frank, 1885). Both of the host plant and fungal member, benefited potentially from this association (Powell and Bagyaraj, 1984).

Medicinal plants are considered one of the major sources of drugs in modern as well as traditional medicinal systems throughout the world. India with its mega-biodiversity and knowledge of rich ancient traditional systems of medicine (Ayurveda, Siddha Unani, Amchi and local health traditions) provide a strong base for the utilization of a large number of plants in general healthcare and alleviation of common ailments of the people (Pandey; Rastogi and Rawat).

Symbiotic association between fungus and roots of medicinal plants are very much significant in terms of nutrition supplies, plant protection and hence the growth and yield of the medicinally important biochemical of these plants.

Among 42 AM fungi species, in a survey 5 genera like *Glomus, Aculospora, Scutellospora and Gigaspora* were discovered from the rhizosphere soil associated with 32 selected medicinal plant species. Among the Am fungal colony, *Glomus* was found to be the most dominant genera and has been reported earlier in the rhizospheres of medicinal plants (Allen *et al.*1995; Francis and Read, 1994 and Selvaraj *et al.* 2001). The main reason for their abundance may be their ability to sustain wide range of temperatures and pH for spore germination. Also, *Aculospora* found in abundance in the rhizosphere samples of A. Aspera are often associated with acidic soils (Abbott and Robson, 1991).

AMF are reported to be widespread in medicinal and aromatic plants Udea *et al*. (1992 and BuKhari et al. (2003). Inoculation of AMF during early stage of acclimatization process has become an alternate strategy for better establishment by improving the plant growth. The AM fungi association had not only enhanced the growth of medicinal plants but also improve the productivity of medicinal compounds.

AMF are known to improve plant growth in different ways like increased phosphorous uptake, increase in biomass of plants (Fattah and Gamal, 2001 and Javot *et al*. 2007)) and resistance to climatic and edaphic stresses, pathogens and pests (Raman and Mahadevan, 1996). AM fungi have been found to increase chlorophyll content as reported by Demir, 2004.

Fumigation of soil to control soil borne pathogens and weeds is a common practice in agricultural fields (Haas *et al*. 1987; Merge 1982a, 1982b). Fumigation of soil eliminates or greatly reduces VAM populations (Afek *et al*. 1981; Buttery *et al*. 1988; Haas *et al*. 1987; Kleinschmidt and Gerdemann.1972). Therefore, reintroduction of suitable VAM fungi into fumigated soil before planting is often of primary important for plant growth (Adams *et al*.1990; Afek *et al*.1991; Kormanik *et al*. 1980 and South 1977).

Leucas aspera (Willd.) Linn. (Lamiaceae) commonly known as 'Thumbai' (Rai *et al*.2005). This is a common way side weed, locally abundant in crop fields, wasteland and fallow fields. The herb is erect, branched containing glandular hairs secreting volatile oils, which are scented. It is also called as 'chota halkusa', distributed throughout India from the Himalayas down to Ceylon (Nadkarni, 1976). The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, expectorant, aperients, diaphoretic, insecticide and emmenagogue. Leaves are considered useful in chonic rheumatism, psoriasis and other chronic skin eruptiohns. Bruised leaves are applied locally in snake bites (Rai et al. 2005 and Shirazi, 1947). The entire plant revealed presence of triterpenoids (Kamat and Singh, 1994).

The plant has numerous medicinal values such as analgesic

and anti-inflammatory activities (Raddy *et al*.1993), anti-allergic, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effect (Kahkohen *et al*. 1999). The decoction of whole plant with equal amount of leaves of tulsi (Ocimum sativum) as a dose of 10ml TDS x 5 days is used to cure malarial fever (Varghese, 1996).

In recent times, attention has been focused on dual inoculation involving different AM fungi species. The inoculum mixtures of two species were significantly more effective than single species inoculums, as supported by Mamta and Tilak (1987). The objective of the study aims at dual inoculation of different AM fungi species with *Leucas aspera* L. to enhance the growth and yield of plant biomass. Global priorities in developing new drugs with good economic returns for the farmers.

2. Materials and Method

Sample collection, experimental site, fumigation and experimental design.

2.1 Experimental Site

A field experiment was conducted in Biotechnology department, M.S. Ramaiah College of Arts, Science and Commerce, MSRIT Post, MSR nagar, Bangalore-54.

2.2 Experimental Design

The experimental design consisted of Randomized Block Design (RBD) with seven treatments of three replicates during the summer season. Two blocks measuring (3 x 4 feet) were designed.

2.3 Sample collection

The seed samples were collected from the college premises in the garden area. These seeds were sterilized in 2% sodium hypochloride and thoroughly washed in distil water for 4-5 times, soaked in distill water for 1 hours, the floating seeds were discarded and viable seeds were used for further sowing in field. The efficient strains of AM fungi were selected for the present study were *Aculospora bireticulata, A.lacunosa, A.laevis, Glomus aggregatum and G.mosseae*, collected from department of Agricultural Microbiology, GKVK, University of Agricultural Sciences, Bangalore-65.

2.4 Fumigation of Soil

Each Plot measuring (3 x 4 feet) were digged around $1^{1/2}$ feet deep, filled with Red sand loamy soil (1:1) and fumigated with 1% formaldehyde (100 ml in 1L of distill water), sprayed on the soil, covered with polytene cover and every alternate day tested for pH and AM spores colonization. On 5^{th} day refumigation with 1% formaldehyde was repeated and covered with polytene cover and every two days once tested for pH and AM spores. This was maintained for 15 days. Fumigation of the soil reduces AMF populations (Afek *et al*. 1991).

2.5 Field Test

Field test was designed in Randomized Block Design and was carried out to determine the compatibility between AMF load and Medicinal herbs. The AM fungi 0.4 g (2.8 x 106 propogules) along with seeds (4 no.) were applied 2 cms below the fumigated soil surface. The experiment was conducted with dual inoculums of 6 treatments, 1 uninoculated control in triplicates.

2.6 Treatments

T_1 – Control T_2 – *A.bireticulata* + *G.aggregatum*
T_3 – *A.lacunosa* + *G.aggregatum* T_4 – *A.laevis* + *G.aggregatum*
T_5 – *A.bireticulata* + *G.mosseae* T_6 – *A.lacunosa* + *G.mosseae*
T7 – *A.laevis* + *G.mosseae*

Samples were collected for each treatment at 90^{th} days after inoculation with the treatments and analyzed for different morphological parameters.

2.7 Plant growth parameters:

Three seedlings per treatment were harvested with rhizosphere soil at 90^{th} days after inoculums. Roots were washed thoroughly with tap water to remove adhering soil particles.

The plant height, number of branches, number of leaves and root length were recorded at 90^{th} days after inoculation and measured in cms.

2.8 Total Biomass

The shoot and root portions of the uprooted plants were separated and oven dried for 72 hrs at 60^0C. The dry weights were then recorded separately for shoots and roots and average of the three plants were expressed in grams per plant.

2.9 Spore Count

Wet Seiving and Decanting Technique (Gerdemann and Nicolson, 1963). Mix a volume of 25 gm of soil in 500ml of tap water, allow standing for 10 minutes and Wet sieve under 450um, 300um, 250um, 150um & 45um (placing Whatmann paper in the 45um) sieve plates. Transfer the Whatmann paper with AM spores on petridish (with grids) and examined under a Stereoscopic Binocular for Spore Count.

2.10 Mycorrhizal root colonization: Rapid Clearing and Staining Technique' (Phillips & Haymann 1970)

The roots were carefully uprooted at the time of harvesting & thoroughly washed under running tap water. Fresh root samples were cut into 1 cm segments. The root bits were cleared by placing them in plastic vials containing 10 ml of 10% KOH for 48 hrs at room temperature **(Phillips & Haymann 1970)**. After decanting the alkali, the residual alkali was neutralized by immersing them in 10 ml of 10%

HCl for 15 minutes. The acid solution was decanted, washed in fresh water & root segments were stained with 0.1% Tryphan Blue stain **(Phillips & Hayman 1970)** in Lactoglycerol for 24 hrs. The stained roots was determined by Gridline intersect method **Giovannetti and Mosse (1980).**

AM Colonization percent was calculated using **Nicolson's** simple formula **(1995).**

$$\%Root\ Colonization = \frac{No.\ of\ segments\ colonized\ with\ AM}{Total\ no.\ of\ segments} \times 100$$

3. No. of Plates

4. Result and Discussion

Field experiment was carried out to study the effect of AM fungi (*A.biretticulata, A.lacunosa, Alaevis, G.aggregatum and G. moseae*) as dual inoculums in Leucas aspera. The effect of AM fungi with dual inoculums on growth parameters (plant height, number of leaves and number of branches) were presented in Table 1.The inoculums mixture of two species was significantly more effective than single species inoculums. These results are supported by Mamta and Tilak, (1987).

The *L. aspera* seed germinated within a week compared to control. On 3rd day of inoculum, the seed germination was found in *G. mosseae (T5 – A.bireticulata + G.mosseae, T6 – A.lacunosa + G.mosseae, T7 – A.laevis + G.mosseae)* compared to *G. aggregatatum (T2 – A.bireticulata + G.aggregatum, T3 – A.lacunosa + G.aggregatum, T4 – A.laevis + G.aggregatum)* dual inoculums. This again proves for the previous single inoculums in *L. aspera* seed with *G. mosseae, as* reported by T and J (2013) and also this may be due to the phytohormones secretion by AM fungi (Azcon-aguilar and Barea , 1978) . However, Jalaluiddin and Hamid (2011) did not find the promotaryeffect of AM Fungi on germinationin the varieties of Sunflower.

Here in the study as per the result obtained the pH was between 6.5 - 6.8 . Sankaranarayanan and Sundarababu (2001), stated that for the mycorrhizal development the pH was 6 -7. This variation of pH in the study could be attributed to the host mediated changes in the rhizosphere of plants Aditya Kumar et al. (2010) .

Among the 7 treatments, the maximum plant height, both root and shoot was recorded in T_6 (*A.lacunosa + G.mosseae*) as 26.00 & 67.67 cms. , followed by dual inoculums treatments T_5,T_4,T_3,T_7,T_2 (Table 1) whereas, least plant height was observed in uninoculated in control T_1 root 7.33 cms and shoot 16.67 cms. The highest total biomass was observed in*T6 (A.lacunosa + G.mosseae)* root fresh and dry weight as 2.71 & 1.29 cms, whereas, shoot fresh and dry weight as 30.01 & 7.54 cms. The control revealed less total biomass i.e., fresh root and dry weight 0.35 & 0.19 g/plant while, shoot fresh and dry weight 4.34 & 0.80 g/plant. This has also been supported by Khan *et al*. (2008), in *Medicago sativa* with dual AM inoculation with *Glomus radices* +

Gigaspora rosea and *Glomus etunicatum* + *Glomus intraradices* on the growth and nutrients uptake and significantly increased the root and shoot dry weight.

The % root colonization was high, 100% in T5 & T7 Glomus species co-inoculated with Acaulospora species *(A.bireticulata* + *G.mosseae* and *A.laevis* + *G.mosseae*) and 99.20% in T6 (*A.lacunosa* + *G.mosseae*). While, the spore count was high in T4(*A.laevis* + *G.aggregatum*) as 22.00, T6(*A.lacunosa* + *G.mosseae*) as 20.00 and as such, AM fungal spores were absent in the control (Table 2). This is due to the effect of the fumigants which has inhibited during the initial 15-20 days, after treatment and then this deleterious effect decreased. Aggarwal *et al*. (2009) worked on the effects of fumigants on non-target micro-organisms including mycorrhizal fungi and has stated that fumigants must be assessed carefully and should be intelligently used in agricultural system. Considerable variation in percent root colonization and number of different AMF spores associated with plant rhizosphere was observed but no definite correlation could be established between them (Kalita *etal.* 2002). However contradictory results were reported by Mutabaruka et al. (2002), as a significant positive correlation and by Lowis and Lim (1987), as a negative correlation between percent root correlation and AMF spores.

Glomus and *Acualospora* are the dominant mycospecies in the roots of Medicinal plants , already reported by many workers like Wang *et al*. (1997); Allen *et al*.(1995); Morton *et al*.(1991); Selvaraj *et al*.(2001); Francis and Read,(1994) and Muthukumar *et al*.(2001). Even the whole plant morphologic parameter and total Biomass showed least values in T1 controland T6 reveal. The higher promiser the dual inoculum to be best as state by earlier report (table 3) .This once again proves the dual inoculation of AM species which upholds the observation made earlier in Phyllanthus amarus as stated byAnusuya and Senthil Kumar, (2003).

The knowledge of tribal (folk) medicine is anormous but has been lying dormant due to various reason and is worthy of exploration (Arfan khan and Atiya Khanum,2000). L. aspera being one of the folk medicinal herbs requires exploration and scientific evaluation as the efficacy of a drug depends upon various factors such as the genuinity, preparation, administration and dosage of the drug. Further, investigation is necessary to enhance the biochemical compounds from this medicinal herb by AM fungi dual inoculums.

5. Summary and Conclusion

The overall objective of this project was to investigate how the dual inoculums of arbuscular mycorrhizal symbiosis can affect the growth responses and enhance the total biomass in the herbal plant 'Thumbai' (Leucas aspera L.). As a result of an increasing interest in natural/herbal medicines, more effort is now needed to produce herbal products of better quality. Thus, it was hypothesized that the naturally occurring AM fungi (AMF) could play an important role in improving the growth parameters and increase the total biomass, so as to increase phytochemical concentrations in medicinal herbs such as 'Thumbai', as organic methods of cultivation are increasingly sought after to grow such plants.

Despite a reasonable amount of information available in the literature on the changes of the growth performance in the roots of host plants following AM colonisation, very little is known about such processes in the aerial part of such plants. Leucas aspera has hardly been studied as a host plant in AM research and very little is known of its responsiveness to AM colonization. However, recently, single inoculums of AM fungi Glomus and Aculospora species has been reported to increase the growth responses in L.aspera by Tejavathi and Jayashree (2013).

In order to understand the dual inoculums of AMF mechanism the experiment was set-up to investigate the effect of AM development stage with respect to two different species of AM fungi compared with the normal host plant. The result showed that inoculation with G. mosseae and three different Acualospora species improved plant growth and biomass compared to G.aggregatum and Acualospora as species and comparatively least growth in uninoiculated control .

In the present study 100% mycorrhizal colonization in G. mosseae+A.bireticulata, G.mosseae+A.lacunosa and G.mosseae+A.laevis was recorded but it was more efficient in enhancing the growth performance compared to other three dual inoculums of Glomus and Acaulospora and no colonization was found in the control plants.

It can be concluded that the key results presented in this project do indicate that inoculating 'chota halkusa' with AMF can be beneficial to improve the growth efficiently as reported by earlier workers. Here the dual inoculum of different AM rungal species has been more beneficial compared to single inoculums. Such results could be of potential interest to 'Thumbai' grower who wishes to cultivate this medicinal herb commercially. It thus emerges that in order to provide strong scientific base. Indian traditional medicinal herbs need to be explored using modern scientific tools. Also, rapid steps are to be taken for the export of herbal medicines.

Table 1: Dual Effect of AM Fungi on Growth Parameter in L. aspera. (90 days after inoculation)

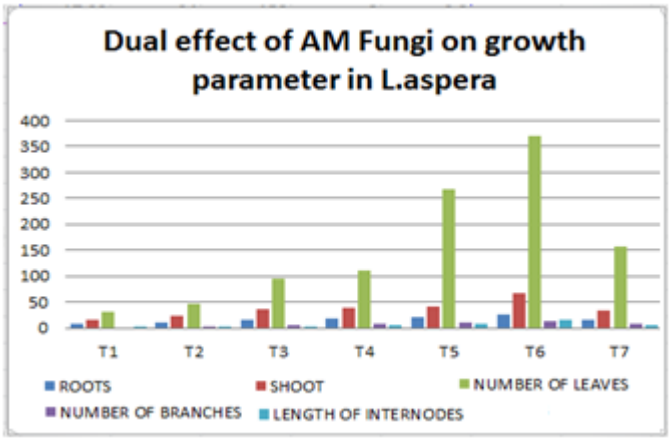
Trails	*Growth Parameters*		*Number of Leaves*	*Number of Branches*	*Length of Internodes*
	(cm)	(cm)			
T1	7.33	16.67	31	0	1.5
T2	10.33	23	47	4	2.8
T3	15	35.67	96.33	6	3.6
T4	18	39.33	111.33	8	6.8
T5	21.67	41.33	267.67	10	9.4
T6	26	67.67	370	14	15.1
T7	17.33	34	158	8	6.2

Table 2: Dual Effect of AM Fungi on Total Biomass in L. aspera. (90 days after inoculation)

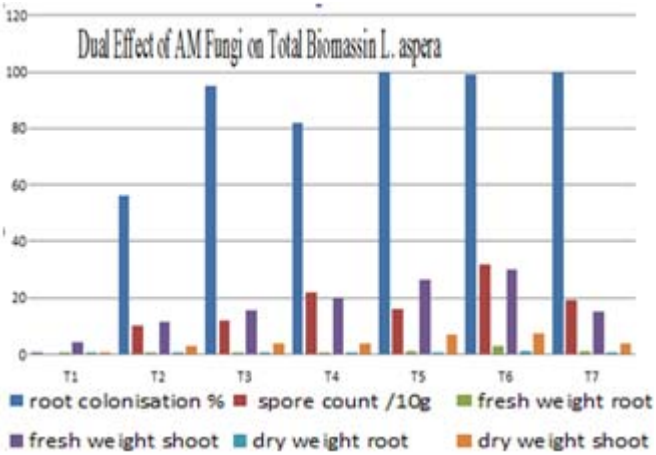
	Root colonization	*Spore count*	*Fresh weight*		*Dry weight*	
Trials	%	No. Spore/10g)	Root (g)	Shoot (g)	Root (g)	Shoot (g)
T1	0.8	0	0.35	4.34	0.19	0.8
T2	56.2	10	0.41	11.55	0.28	2.89
T3	95.2	12	0.48	15.71	0.3	3.67
T4	82	22	0.65	20.03	0.44	3.71
T5	100	16	1.03	26.51	0.6	6.77
T6	99.2	32	2.71	30.01	1.29	7.54
T7	100	19	0.94	15.3	0.67	3.84

Table 3: Dual Effect of AM Fungi on Whole Plant in L. aspera (90 days after inoculation)

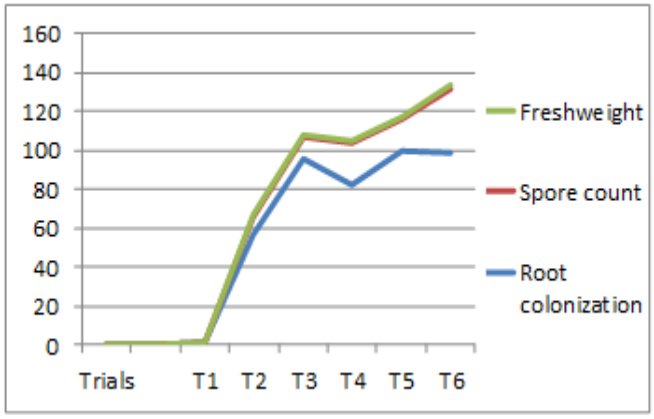
Trials	*Growth*	*Fresh Weight*	*Dry Weight*
T1	24	0.5	0.99
T2	33.33	0.47	3.17
T3	50.67	0.66	3.97
T4	57.33	1.14	4.15
T5	289.34	2.6	7.37
T6	93.67	4.27	8.83
T7	51.33	2.55	4.51



Graph 1: Dual Effect of AM Fungi on Growth Parameter in L. aspera (90 days after inoculation)



Graph 2: Dual Effect of AM Fungi on Total Biomass in L. aspera (90 days after inoculation)



Graph 3: Dual Effect of AM Fungi on Whole Plant in L. aspera (90 days after inoculation)

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Author Profile

Anubrata Paul is a master degree student of M.S. Ramaiah College Of Arts, Science &Commerce, MSRIT POST, Bangalore-560054, Karnataka, India, Bangalore University, and Department of Biotechnology.

Jayashree D.R. is working as Assistant Professor in M.S. Ramaiah College of Arts, Science & Commerce, MSRIT POST, Bangalore-560054, Karnataka, India, Bangalore University. Department of Biotechnology.