Inoculum Production of Endophytic Mycorrhiza Using Mustard Seed Waste as Substrate

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ABSTRACT
A pot culture experiment was conducted to study the effect of soil amendment with mustard seed (member of Brassicaceae) waste on population and root infection of indigenous Arbuscular Mycorrhizal (AM) fungal species (*Acaulospora laevis* and *Glomus mosseae*) under polyhouse conditions. Two monocot plant species viz. *Hordeum vulgare* and *Triticum aestivum* were also examined for mycorrhization in the study. Observations were made 75 DAS (Days After Sowing) in terms of percent root colonization, spore density of AM fungi and the effectiveness of AM fungi on shoot and root biomass of both host plants. The results indicated that AM fungal spore population and colonization levels were substantially enhanced by the application of mustard seed waste as substrate over control. Among growth parameters, shoot and root biomass were recorded more in wheat than barley. On the whole, mycorrhization was reported the highest with maximum concentration of waste. Also, wheat appeared to be a better host than barley.

Key Words: AM fungi, Mass production, Mustard seed waste, Brassicaceae, Wheat, Barley.

INTRODUCTION

The continuing increase in global population coupled with the limitations in the world's supply of natural resources and widespread degeneration of the environment presents a major challenge to the agricultural scientists today. The developmental research addresses this problem by looking for sustainable solutions causing least damage possible to the ecosystem. In order to implement such a plan, the judicious use of nature's own biofertilizers such as arbuscular mycorrhizal fungi (AMF) appears to be one of the suitable alternatives to this problem. But, the mass production of inoculum is one of the hindrances in the large scale application of AM fungi as these are obligate symbionts and cannot complete their life cycle without a living host. The methods of culture and inoculum production of AM fungi have progressed from the relatively simple pot culture technique to the currently used techniques such as nutrient film technique, aeroponics, hydroponics, *in vitro* root organ culture and axenic culture. Even though many kinds of techniques are available for inoculum production of AM fungi, the method followed most common in practice is the traditional pot-culture method (Selvaraj and Chellappan 2006) where the fungi are usually maintained and multiplied in conjunction with suitable host plant roots.

However, due to increasing urbanization and industrialization different wastes are discharged into rivers and other areas which could be applied to the soil intentionally to evaluate their effect on flora and fauna. Several workers have introduced waste substrates in addition to soil-sand mixture to promote the AM fungal growth (Douds *et al*. 1997; Alguacil *et al*. 2009) earlier. Evidently, there have been no published reports concerning the use of mustard seed waste for mass production of AM fungal inoculum. Considering the vast potentiality of different kinds of wastes in enhancing the growth of AM fungi, a systematic investigation was undertaken to assess the mass production of two AM fungi i.e. *Acaulospora laevis* and *Glomus mosseae* using wheat and barley as hosts and mustard seed waste as substrate.

MATERIALS AND METHODS

Inoculum carrier and plant hosts: Mustard seed waste was used for mass production of both AM fungi. Two hosts namely barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) were selected for the mass multiplication of both AM fungi, as these are fast growing and produce extensive root systems in a relatively short time.

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Mycobiont: Dominant efficient strains of AMF i.e. *Acaulospora laevis* [Gerdemann & Trappe] and *Glomus mosseae* [(Nicolson & Gerdemann) Gerdemann & Trappe] were isolated from the rhizospheric soil of wheat and barley and identified with the help of keys as given by Walker (1986), Schenck and Perez (1990), Morton and Benny (1990), Mukerji (1996) and Kumar *et al.* (2009) for the mass production of AM inoculum.

Preparation of starter culture and potting experiment: Mother inoculum of each AM fungus was prepared by 'Funnel Technique' (Menge and Timmer 1982). Earthern pots (15x15 cm) were filled with sand:soil (1:3) and different concentrations of the substrate to make the final weight. Ten percent of inoculum having AM spores and colonized root pieces was added to each pot except control.

Surface disinfection and sowing of seeds: Healthy seedsof each selected host (Wheat and Barley) were surface sterilized and then washed before sowing. Each treatment with different hosts was replicated thrice. Plants were watered as needed and fertilized with Hoagland solution (Hoagland and Arnon 1950).

Mycorrhizal Assessment: AMF spores were extracted from the soil by 'Wet Sieving and Decanting method' (Gerdemann and Nicolson 1963). Quantitative estimation of AM spores was done by 'Grid Line Intersect Method' (Adholeya and Gaur 1994). Root colonization was assessed by 'Rapid Clearing and Staining Technique' (Philips and Hayman, 1970). Presence of infection and the percent infection was calculated by the following equation (Giovannetti and Mosse 1980):

$$\text{Root Colonization (\%)} = \frac{\text{No. of root segments}}{\text{Total no of root segments observed}} \times 100$$

Effectiveness of AM fungi: Effectiveness of AM fungi on both plant hosts was determined by taking shoot (fresh and dry) and root (fresh and dry) biomass 75 days after inoculation.

Statistical analysis: Data was statistically subjected to interpret by one way as well as two way analysis of variance (ANOVA) followed by post hoc test using SPSS 16.0 software. Means were then ranked at P=0.05 level of significance using Duncan's Multiple Range Test (DMRT) for comparison. All the variables were then correlated using Pearson Correlations at different significance levels.

RESULTS AND DISCUSSION

Results clearly feature that inoculum production of AM fungi (*A.laevis* and *G.mosseae*) varied considerably with different hosts when mustard seed waste was used as substrate. The sporulation and root colonization of *A.laevis* as well as biomass of the seedlings were found the highest at maximum concentration of the waste (Table 1). The spore number was recorded more with wheat (73.33±3.51) as host than barley (53.00±3.00). But, the increment in spore count was more in case of barley (297.60%) than wheat (260.70%). Similar were the observations made with reference to the status of root colonization. Abundance of mycelium, vesicles as well as arbuscules of *A.laevis* was reported maximal at 180 gm concentration of the substrate but more in wheat than barley. Likewise, plant biomass (shoot and root) was registered maximum at concentration of 180 gm followed by 120 and 60 gm with both the trap plants with higher in wheat than barley (Table 1). Significant correlarions were found between all the parameters. It is evident from results that wheat is a suitable host for mass multiplication of *A.laevis* when mustard seed waste is to be used as substrate.

For mass production of *G.mosseae*, it is perceptible from Table 2 that both the host plants (wheat and barley) showed highest degree of mycorrhization at maximum (180 gm) concentration of mustard seed waste. The intensity of mycorrhizal root infection (67.33±3.54, 54.21±2.56: wheat, barley) as well as spore density (83.33±3.51, 61.33±2.51: wheat, barley) were found more in wheat as compared to barley. But, the percent increment in both root colonization (200.45, 375.11: wheat, barley) and spore count (247.21, 252.01: wheat, barley) was found higher in barley than in wheat. At the same time, mycelium, vesicles and arbuscules of *G.mosseae* were recorded most abundant when wheat was grown in presence of 180 gm of substrate. Likewise, shoot biomass and root biomass for both the hosts were observed maximum at 180 gm concentration of substrate. On the whole experiment, results contemplate the most appropriate suitability of wheat as host with mustard seed waste as substrate. Spore density, root infection as well as plant biomass were found to be positively and significantly correlated to each other.

Positive influence of seed wastes on AM fungal growth might be attributed to the increased porosity. In the current examination, application of different concentrations of the substrate influenced the formation of vesicles and arbuscules. Also, a positive correlation was observed between root colonization and spore production in all the treatments that could be attributed to the soil nature and the amount of substrate mixed thereby affecting the root infection, number of vesicles per root and ultimately the spore population. Gupta *et al*. (2006) also reported number of vesicles to be correlated with spore number per root.

Table: 1. Inoculum production of *Acaulospora laevis* using different hosts and Mustard seed waste as substrate

Sr. No.	Treat-ments	Substrate Concen-tration (gm)	Sand:Soil 1 : 3 (gm)	Host Plant Used	Type of Infection			AM Root Colonization (%)	AM Spore Count	Shoot Weight (gm)		Root Weight (gm)	
					#M	#V	#A			Fresh	Dry	Fresh	dry
1.	**Control**	**0**	150:450	**Wheat**	+	-	-	*18.55±1.79^{e}	20.33±3.51^{f}	8.63±0.03^{f}	1.55±0.04^{f}	4.83±0.03^{d}	1.15±0.04^{f}
2.	**T_1**	**60**	135:405	**Wheat**	++	++	-	32.32±3.03^{c}	49.00±4.00^{c}	14.44±0.04^{c}	2.93±0.05^{c}	8.55±0.03^{c}	2.18±0.03^{c}
3.	**T_2**	**120**	120:360	**Wheat**	+++	++	+	43.07±2.52^{b}	56.66±4.04^{b}	18.52±0.02^{b}	3.97±0.04^{b}	10.75±0.04^{b}	2.98±0.02^{b}
4.	**T_3**	**180**	105:315	**Wheat**	+++	+++	+++	58.33±2.58^{a}	73.33±3.51^{a}	21.85±0.04^{a}	4.51±0.03^{a}	11.22±0.02^{a}	3.24±0.03^{a}
5.	**Control**	**0**	150:450	**Barley**	+	-	-	15.84±2.77^{e}	13.33±4.16^{g}	7.13±0.02^{g}	0.92±0.05^{h}	1.85±0.05^{h}	0.61±0.04^{h}
6.	**T_4**	**60**	135:405	**Barley**	++	+	-	23.89±3.66^{d}	28.33±3.51^{e}	9.87±0.03^{e}	1.37±0.03^{g}	2.52±0.03^{g}	1.03±0.03^{g}
7.	**T_5**	**120**	120:360	**Barley**	++	++	+	39.93±2.52^{b}	41.33±2.51^{d}	12.58±0.02^{d}	1.82±0.03^{e}	2.96±0.03^{f}	1.38±0.04^{e}
8.	**T_6**	**180**	105:315	**Barley**	+++	+++	++	56.03±2.52^{a}	53.00±3.00^{bc}	14.49±0.03^{c}	2.49±0.04^{d}	3.39±0.04^{e}	1.94±0.03^{d}
Annova (F)								106.230	95.640	84542.626	3154.286	25515.555	2429.761
LSD (P≤0.05)								4.7121	6.18055	0.0516	0.0684	0.0717	0.0574
F values			**Substrate (s)**					264.686	1289.286	1250656.0	1213488.0	1110916.0	158010.8
			Parameter (p)					3787.145	237.532	1055819.0	125090.2	21882.029	66086.750
			s x p					57.351	3.669	751555.0	6315.556	6416.651	5970.750

* Each value is mean of three replicates

M : Mycelium, **V** : Vesicles, **A** : Arbuscules

± : Standard Deviation

Mean values followed by different alphabet/s within the same column are significant over one another at *P*= 0.05.

Table: 2. Inoculum production of *Glomus mosseae* using different hosts and Mustard seed waste as substrate

Sr. No.	Treat-ments	Substrate Concen-tration (gm)	Sand:Soil 1 : 3 (gm)	Host Plant Used	Type of Infection			AM Root Colonization (%)	AM Spore Count	Shoot Weight (gm)		Root Weight (gm)	
					#M	#V	#A			Fresh	Dry	Fresh	dry
1.	**Control**	**0**	150:450	**Wheat**	+	-	-	*22.41±2.65[d]	24.00±4.00[g]	9.49±0.04[f]	2.42±0.02[e]	5.34±0.04[d]	2.00±0.03[d]
2.	**T_1**	**60**	135:405	**Wheat**	+++	++	+	36.36±3.03[c]	54.66±3.51[d]	15.23±0.03[d]	3.37±0.03[d]	10.35±0.05[c]	3.51±0.03[c]
3.	**T_2**	**120**	120:360	**Wheat**	+++	+++	++	65.60±3.03[a]	67.33±2.51[b]	24.41±0.02[b]	5.44±0.04[b]	14.57±0.02[b]	3.98±0.02[b]
4.	**T_3**	**180**	105:315	**Wheat**	+++	+++	+++	67.33±3.54[a]	83.33±3.51[a]	25.76±0.03[a]	5.60±0.03[a]	16.44±0.03[a]	5.47±0.04[a]
5.	**Control**	**0**	150:450	**Barley**	+	-	-	11.41±1.59[e]	15.66±3.51[h]	5.48±0.04[h]	0.63±0.04[g]	2.28±0.03[g]	0.66±0.03[gs]
6.	**T_4**	**60**	135:405	**Barley**	++	+	-	24.91±3.54[d]	38.00±3.00[f]	8.75±0.03[g]	1.57±0.02[f]	2.82±0.02[f]	0.89±0.02[f]
7.	**T_5**	**120**	120:360	**Barley**	++	++	+	39.39±3.03[c]	47.33±3.51[e]	11.46±0.02[e]	2.38±0.05[e]	3.18±0.03[e]	1.03±0.03[e]
8.	**T_6**	**180**	105:315	**Barley**	+++	+++	++	54.21±2.56[b]	61.33±2.51[c]	19.29±0.04[c]	3.96±0.03[c]	5.29±0.04[d]	1.97±0.04[d]
Annova (F)								148.062	140.256	163317.8	8168.977	80267.623	9325212
LSD (P≤0.05)								5.07675	5.70805	0.0558	0.0587	0.0587	0.0533
F values			**Substrate (s)**					4420.455	13467.00	2.7	20617.230	8231977.0	0.00
			Parameter (p)					3550.908	7849.00	1793474.0	44722.721	1106505.0	58646.375
			s x p					261.156	135.276	1759275.0	647.348	124690.6	37807.125

* Each value is mean of three replicates

[#] **M** : Mycelium, **V** : Vesicles, **A** : Arbuscules

± : Standard Deviation

Mean values followed by different alphabet/s within the same column are significant over one another at *P*= 0.05.

Higher colonization was reported in all the seedlings receiving different substrates when compared with control. Also, accretion in AM fungal spore density was observed with the addition of substrates. Variation in root colonization and sporulation could be ascribed to the soil mixture that affected number of vesicles per root as well as spores in the rhizosphere. Enhancement in the formation of vesicles and arbuscules as an effect of application of substrate has been reported by Baby and Manibushanrao (1996).

AM fungal colonization depends upon the type of host as well. Type of root system determines the extent of interaction between endophyte and host and the supply of carbohydrates to the fungal partner. The presence of more number and fine nature of fibrous roots facilitates the entry of AM fungus (Al-Raddad, 1995). As monocots have larger root system, a positive effect on root colonization was observed. In the present study, both wheat and barley proved to be suitable hosts for mass multiplication of *A.laevis* and *G.mosseae*, as these grew fast with extensive root system while providing favourable conditions for higher root colonization and sporulation. When spore producing capacity of two hosts was analyzed, both the hosts i.e. wheat and barley gave maximum spore number which could be because graminaceous hosts provide better root system for sporulation (Al-Raddad, 1995). In the present investigation, spore density differed with different plant species that could be ascribed to the characteristics of the plant hosts which vary in their ability to adapt to the growth conditions like soil temperature, soil pH, soil moisture, soil fertility, interaction of soil microorganisms, light conditions and others (Mukerji *et al*., 2002).

The stability of plant growth is considered as an important factor for AM fungal production. In the present study (with both host plants), intensity of mycorrhization showed significant positive correlations with plant biomass. The current findings are strongly validated by the results of Dabire *et al*. (2007) who found that density of AM inoculum was positively related to the plant growth. Spore numbers tend to increase with age of the crop. Chaurasia and Khare (2005) while mass producing AM fungi with four different host plants reported a gradual increase in root colonization and spore number with period of growth and increase in size of the plants.

Historically, several plant families including the Brassicaceae and Chenopodiaceae have been believed to be non-mycorrhizal. Although members of family Brassicaceae are considered to be inhibitive to arbuscular mycorrhizal fungi yet some positive results were observed in the present case while using mustard seed waste as substrate. Inhibition of AM fungal growth by the members of Brassicaceae could be due to the presence of glucosinolate (Schreiner and Koide, 1993) and isothiocyanates (Ghosh *et al*., 2004) in their oil. Glenn *et al*. (1985, 1988) examined the effects of *Brassica* species with varying glucosinolate levels on AM fungus interactions and found no clear relationship between glusinolate concentrations and the development of AM fungi. However, in the present study, waste left after the oil has been taken out was used which might be devoid of both these inhibitory compounds. Gray (2012) isolated sinapic acid after the mustard seeds were pressed for oil that showed antimicrobial properties against some pathogenic microbes, which could help in minimizing the competition of rhizospheric microorganisms for better growth of mycorrhizal fungi. Thus, the present study might be highly significant as the goal of our research was to make a species specific and highly effective inoculum that was very inexpensive. And as the substrate used was a waste, it was an eco-friendly step too.

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