Research Article

Effects of Arbuscular Mycorrhizal Fungi on Spores Density and Root Colonization of Four Hybrids of Sunflower (*Helianthus annuus* L.) at Different Rock Phosphate Levels

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Abstract | The present experiment was carried out in a net house at the University of Peshawar, Pakistan. The main purpose of this research was to find out the effects of arbuscular mycorrhizal fungal (AMF) inoculation (with and without Mycorrhiza) along with application of various levels (0%, 25%, 50% and 100%) of rock phosphate (RP) fertilizer on spores density and percent root colonization of selected sunflower (*Helianthus annuus* L.) hybrids in P-deficient soil. It was observed that spore density and AMF root colonization was higher in the soil of control (RP0) plants, which decreases progressively with increasing fertility level. Less number of spores and percent root colonization was found at high RP level (RP100) in all hybrids. Higher P doses declined the sporulation and colonization. There was total seven AMF species that were observed and recorded. The dominant genus was *Acaulospora* which was followed by *Glomus*, *Sclerocystis* and *Gigaspora*. The average AMF spore density ranged from 56-260 spores/100g soil while root colonization ranged from 32-100%. Mycorrhizal enhancement regarding AMF spores density and root colonization ranked as-RP0>RP1>RP2>RP3 in all hybrids i.e 0%>25%>50%>100%.

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Introduction

Mycorrhiza is universal mutualistic associations between soil fungi and vascular plants and is essential in improving plant fitness and soil quality. These fungi improve the resilience of plant communities against environmental, nutritional and drought stresses (Barea et al., 2011). The AM fungi are known to be of great importance due to their high capability to increase growth, yield, and quality of crops through efficient nutrient acquisition in infertile soils and consequently lessening the prerequisite for Phosphate-based fertilizers (Khalafallah and Abo-Ghalia, 2008; Roy-Bolduc and Hijri, 2011). In turn, the fungi get carbon from the host plant. AM fungi have the capability of absorbing all essential macro and micro nutrients which are required for plant growth (Lester, 2009).

Sunflower (*Helianthus annuus* L.) belonging to the family Asteraceae is one of the important oilseed crops in the world and ranks fourth in production of vegetable oil (Weiss, 2000). About 18% of edible vegetable oil of the world is obtained from sunflower (Hussain et al., 2010). Sunflower production was 194,544 tons in 1998-99 which increased to 407224 tons in 2006-07.

Like most of the arid and semiarid soils of world soils

of Pakistan are mostly Phosphorus (P) deficient due to their alkaline and calcareous nature, affecting plants adversely (Memon et al., 1992; NFDC, 2001). Total phosphorus contents of an average soil is about 0.05%, out of which only 0.1% of the total P is brought in use by plants because of its low solubility. To overcome this problem plants have adopted different strategies to acquire sufficient phosphorus (Sharma, 2004). Arbuscular mycorrhizal fungal association is one among these adaptations (Khade and Rodrigues, 2009; Coline et al., 2011). Due to scarcity of phosphorus reserves in the soil and their rapid utilization, efforts are being made to supplement plants with low grade rock phosphate. Mycorrhizal inoculation can help plants by solubilizing rock phosphate into available forms, which helps in plant growth (Sabanavar and Lakshman, 2009).

Throughout the world, scientists are focused on developing alternative technologies to minimize dependence on chemical fertilizers. Keeping the importance of AM fungi as bio-fertilizers, the present investigation was carried to find out the feasibility of inoculation of some high yielding sunflower hybrids with indigenous tropical AM fungi in low fertility soils, to achieve adequate production level.

Materials and Methods

Experimental Site

The present study was conducted at the Department of Botany, University of Peshawar, Pakistan.

Plant Material

Authentic seeds of four hybrids of sunflower i.e NKS-278, Hysun-33, SMH-0917 and SMH-0907 were obtained from Oil Seeds Research Program, NARC Islamabad, Pakistan.

Soil

The soil used was sandy loam with pH 7.8, electric conductivity 0.675dSm^{-1}, Nitrogen 0.032% and Phosphorus 0.8 mgkg^{-1} with low organic matter (0.6%). All 96 pots having 89 cm diameter and 48 cm depth were filled with 6 Kg of this nutrient deficient soil.

Experimental Design and Treatments

The experimental work was carried out in a randomized complete block design (RCB) along with eight treatments; each treatment was replicated three times with five plants in each pot.

Application of AMF Inoculums

In the experimental work, mixed constorium of different AMF species i.e. *Glomus fasciculatum, G.mosseae, G. aggregatum, Sclerocystis pakistanica*, *Gigaspora gigantea* along with roots of wheat and maize infected with arbuscular mycorrhiza were used as rhizobase inoculum. The roots were cut into 1 cm pieces, which along with soil base inoculum (rhizospheric soil) were spread uniformly in pots, at a depth of 3 cm and 6 cm in layers before sowing. Inoculum for each pot consisted of 160 g of mycorrhizal infected roots and adhering soil. Mycorrhizal inoculum preparation, placement and application were done by the method given by Brundrett et al. (1996).

Fertilizer Application

Fertilizers were applied by following Krishna and Bagyaraj (1982) method. Rock phosphate fertilizer was obtained from Hazara deposits. Four levels of P fertilizer treatments 0 mg P_2O_5/kg soil, 100 mg P_2O_5/kg soil, 200 mg P_2O_5/kg soil and 500 mg P_2O_5/kg soil in form of RP were applied. The recommended dose was 80 kg P_2O_5/ha. These treatments were applied in combination with or without AMF.

Extraction of Spores

The spores were isolated from the soil samples by wet-sieving and decanting technique (Gerdemann and Nicolson, 1963).

Spore Density Calculation

Density of spores in each soil sample was calculated by following Stahl and Christensen (1982) standard method. The observed spores were micro photographed at two magnifications (4x, 10x).

Spores Identification

Spores were identified with the help of keys following Hall and Fish (1978), Trappe (1982) and Schenck and Perez (1990).

Assessment of Root Colonization

Roots were carefully dug out and washed thoroughly with water and stored in FAA (Formalin:Acetic acid:Alcohol) solution. The roots were stained and processed following the procedure of Phillips and Hayman (1970). For the assessment of root colonization + slide method of Giovannetti and Mosse (1980) was followed. Total of 25 segments of roots of individual plant each approximately 1 cm long were randomly selected for microscopic study. Morphology of AM

entophyte was studied and expressed in percentage (%). The infection percentage was calculated by using the following formula (Giovannetti and Mosse, 1980):

$$\% \text{ age mycorrhizal infection} = \frac{\text{No. of infected segments}}{\text{Total No. of segments studied}} \times 100$$

Table 1: *Effect of mycorrhiza on AMF spores in the roots of sunflower hybrids at various levels of rock phosphate (RP)*

Rock Phosphate levels	Spores density / 100 g Soil			
	NKS-278	Hysun-33	SMH-0917	SMH-0907
RP0 (0%)	202	260	251	246
RP1 (25%)	171	202	182	171
RP2 (50%)	103	172	145	122
RP3 (100%)	56	102	88	79

Results and Discussion

AMF Spores Density

The results in Tables 1, 2 and Figure 1 shows the effect of various rock phosphate levels on the AMF spores density in the rhizosphere soil of selected sunflower hybrids. Mycorrhizal enhancement regarding AMF spores density followed RP0>RP1>RP2>RP3 trend in all hybrids (Figure 1). It has been observed that spores density was higher in the soil of control (RP0) plants, which decreased progressively with increasing fertility level. Less number of spores was found at RP3 level in all hybrids. Generally the population of AMF spores and soil phosphorus are inversely related to each other (Hao et al., 1991). Chandrasekara et al. (2005) and Panwar and Tarafdar (2006) also found that interaction of mycorrhiza and phosphorus fertilizer had no significant effect on AMF spores density. Guillemin et al. (1995), Antunes et al. (2007) and Arumugam et al. (2010) reported that the spores density got declined sharply at high P level but these results negate the findings of Sharathbabu and Manoharachary (2006) who reported that dual inoculation of AMF (*Glomus fasciculatum*) and rock-phosphate significantly enhanced percentage of mycorrhizal colonization than in single inoculation or in control *Tylophora indica* plants.

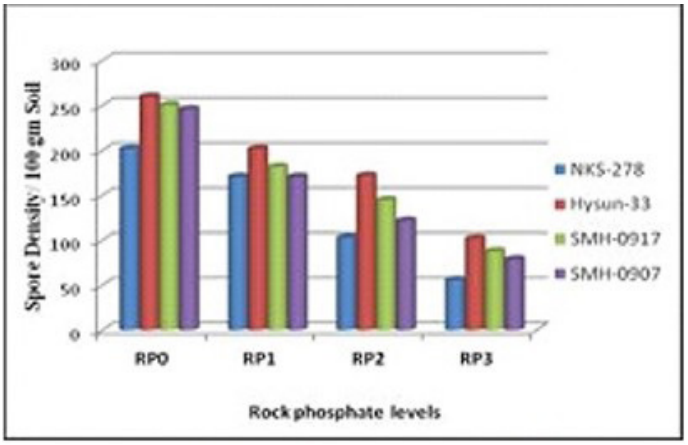

Figure 1:*Effects of rock phosphate (RP) fertilizers on spore density / 100 gm soil of four hybrids of Helianthus Annuas L.*

Table 2:*Effect of mycorrhiza on AMF spores species in sunflower hybrids*

Percent Rock phosphate levels	AMF spores	Spores density in NKS-278	Spores density in Hysun-33	Spores density in SMH-0917	Spores density in SMH-0907
RP0 (0%)	Acaulospora	+++	+++	+++	+++
	Sclerocystis	++	++	++	++
	Glomus	+++	+++	+++	+++
	Gigaspora	-	+	-	-
RP1 (25%)	Acaulospora	++	+++	+++	+++
	Sclerocystis	-	++	+	-
	Glomus	+	++	++	+
	Gigaspora	-	-	+	-
RP2 (50%)	Acaulospora	+	+++	++	+
	Sclerocystis	-	+	+	-
	Glomus	-	+	+	+
	Gigaspora	-	-	+	-
RP3 (100%)	Acaulospora	-	+	-	-
	Sclerocystis	-	-	-	-
	Glomus	-	+	-	-
	Gigaspora	-	-	-	-

+:Present; - :Absent; +++:100 %; ++:75 %; +:25 %

Average number of spores counted per 100 g of soil was different from hybrid to hybrid at various levels of treatments (Table 1 and Figure 1). The AMF spore densities ranged from 56-260 spores/ 100 g soil in selected sunflower hybrids. It was found that among control plants, the highest number of spores was recorded for Hysun-33 (260/100 g of soil) followed by SMH-0917 (251/100 g of soil), SMH-0907 (246/100 g of soil) and NKS-278 (202/100 g of soil). The combined effect of AMF+ RP results showed that AMF spores density followed Hysun-33>SMH-0917, SMH-0907> NKS-278 trend at all RP levels (Table 1).

AMF spores are ubiquitous in most ecosystems (Marleau et al., 2011), and are essential component of soil micro biota (Hindumathi and Reddy, 2011) AMF exists in soil as spores, hyphae, as vegetative propagules or infected root pieces for infecting plants, but mostly inoculation of plants is brought about by extraradical mycelium (ERM) (Sylvia and Jarstfer, 1992). Occurrence or distribution of AMF varies with host ranges (Sarwade et al., 2011).

AMF Species

Table 2 shows that the following species were identified in rhizosphere soil of selected sunflower hybrids at different rock phosphate levels (Figure 2).

- *Acalospora mellae* (Spain and N.C. Schenck)
- *Acaulospora laevis (*Gerd and Trappe, 1974)
- *Glomus fasiculatum* (Thaxt. Gerd. and Trappe)
- *Glomus mosseae* (T.H. Nicolson and Gerd)
- *Glomus aggregatum* (N.C. Schenck and G.S. Sm.)
- *Sclerocystis pakistanica* (S.H. Iqbal and Perveen)
- *Gigaspora gigantea* (Gerd. and Trappe)

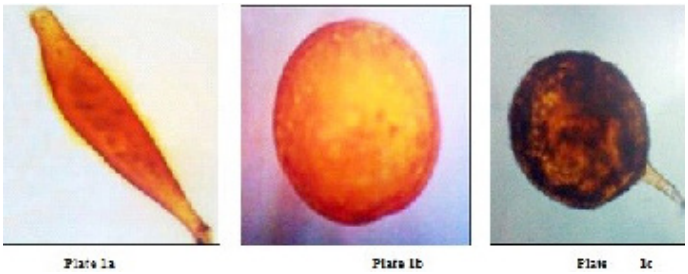

Figure 2: a*) Spores of Sclerocystis pakistanica;* **b***) Spores of Acalospora mellae;* **c***) Spores of Glomus mosseae*

Nasim et al. (1998) showed that spores are the means of identification of these fungi. In the present work soil was collected from different pots with plants at reproductive stages of growth. Four genera of endogonaceous spores were identified which were Acaulospora (2 spp. i.e. *A. mellae, A. laevis*), Glomus (3 species i.e. *G. mosseae*, *G. aggregatum and G. fasiculatum*), Sclerocystis 1 specie i.e. (*S. pakistanica)* and Gigaspora (1 spp. *G. gigantean)* (Figure 2a, b, c).

In this research we found that the species of *Acaulospora* were most common and predominant followed by *Glomus*, *Sclerocystis*, and Gigaspora. Our findings are further supported by the work of other researchers (Lovelock et al., 2003; Wongmo, 2008; Tchabi et al., 2008; Charoenpakdee et al., 2010; Gao and Guo, 2010; Songachan and Kayang, 2011) who investigated that there is higher number of *Acaulospora* in the soil followed by Glomus species. The predominance of Acaulospora species might be due to their adaptation to wide variety of soil types, host species and pH and nutrient availability etc (Jefwa et al., 2006; Straker et al., 2010). It suggests that AMF strains are biological specific for the host plant as reported by Bever et al. (1996). The large number of AMF spores may be attributed to the deficiency of low phosphorus in the soil. Generally, the population of VAM spores and soil phosphorus are inversely related to each other (Hao et al., 1991).

The presence of small number of Gigasporaceae might be due to the fact that they are usually found in sandy dunes (Lee and Koske, 1994) and are usually large sized, which requires long period for their development than the small sized spores species (Hepper, 1984). Moreover, *Gigaspora* are very common in wild plants than field crops (Gai et al., 2006).

Table 3*:Effects of various levels of RP fertilizers on REC index in the roots of sunflower hybrids*

Rock phosphate levels	NKS-278	Hysun-33	SMH-0917	SMH-0907
	% age infection	% age infection	% age infection	% age infection
RP0 (0%)	100%	100%	100%	100%
RP1 (25%)	72%	96%	91%	88%
RP2 (50%)	66%	88%	84%	72%
RP3 (100%)	32%	46%	42%	35%

AMF Colonization in Roots

The results given in Tables 3 and 4 and Figure 3 show the effect of various rock phosphate levels on the percent root colonization in the rhizospheric soil of selected sunflower hybrids. Mycorrhizal enhancement regarding percent root colonization ranked as RP0>RP1>RP2>RP3 in all hybrids (Figure 3).

Table 4: *Effects of various levels of RP fertilizers on AM infection Morphologies, external hyphae, internal hyphae, Arbuscules, vesicles, (%) in roots of mycorrhizal Sunflower hybrids*

Percent AMF infection (%)					
Hybrids	**Treatments**	**External Hyphae (%)**	**Internal hyphae (%)**	**Vesicles (%)**	**Arbuscules (%)**
NKS-278	RP0	24±5.29	24.33±6.65	24±5.29	7.33±6.42
	RP1	20±10	23.33±5.77	23.33±5.77	4±6.08
	RP2	6.66±5.77	13.33±5.77	13.33±5.77	*
	RP3	*	3.33±5.77	6.66±11.54	*
Hysun-33	RP0	40.66±9.01	44±6.92	50.66±1.15	14±6.37
	RP1	33.33±5.77	33.33±11.54	36.66±5.77	6.66±5.77
	RP2	13.33±11.54	20±0	20±0	3.33±0
	RP3	*	10±0	13.33±5.77	*
SMH-0917	RP1	40±10	43.33±5.77	50±0	7.33±6.42
	RP0	23.33±5.77	23.33±5.77	23.33±5.77	3.33±6.35
	RP2	10±10	16.66±5.77	20±0	*
	RP3	*	6.66±5.77	10±10	*
SMH-0907	RP0	36.66±1.54	37.33±5.77	40±0	13.33±5.94
	RP1	20.66±10.06	24±6.92	24±5.29	4±6.92
	RP2	3.33±5.77	10±0	10±0	*
	RP3	*	3.33±5.77	3.33±5.77	*

±*:Standard error;* **RP0***:0% rock phosphate;* **RP1***:25% rockphosphate;* **RP2***:50% rock phosphate;* **RP3***:100% rockphosphate*

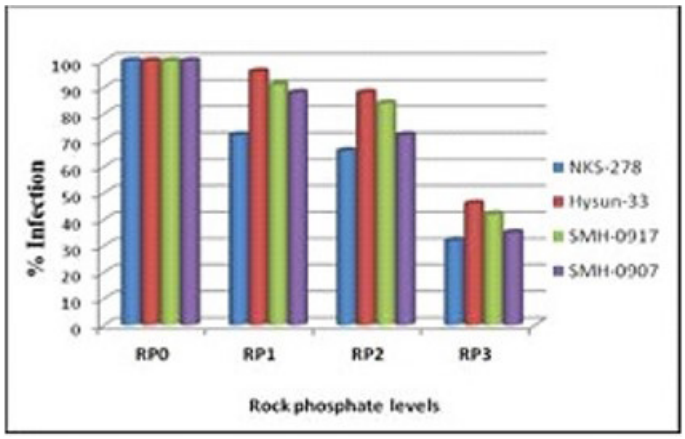

Figure 3*: Effects of rock phosphate (RP) fertilizers on % infection of 4 hybrids of sunflower*

AMF root colonization was determined by the presence of external hyphae, internal hyphae, vesicles and arbuscules. The general AMF infection in sunflower hybrids at various rock phosphate levels was low as compared to control (RP0) viz 100% (Table 3). Vesicular infection was common and maximum at all RP levels in all hybrids (Figure 4)as reported by Iqbal and Bareen (1986) in *Narcissus poeticus* and Burni et al. (1993) in *Targionia hypophylla*. Moreover, Al-Raddad (1995) observed that the type of crop and harvesting greatly affect the root colonization. The comparison revealed that highest number of vesicles was recorded in Hysun-33 followed by SMH-0917, SMH-0907 and NKS-278 at all RP levels shown in Table 4, as reported by Linderman and Davis (2004) in marigold, Janoušková et al. (2007) for tobacco and Sensoy et al. (2007) for *Capsicum annuum* L.

The results (Table 3 and 4) shows that AMF inoculated plants had significant positive effects on AMF root colonization. However, this positive effect of AMF inoculated plants decreased with increasing RP level; lowest root colonization was found at RP3 level in all hybrids. Redecker (2005) found that high concentration of phosphate seems to induce low fungal colonization level by the plants.

It was found that AMF colonization was higher in the control plants of all hybrids whereas it decreased to a minimum 32% in NKS-278, 35% in SMH-0907, 42% in SMH-0917 and 46% in Hysun-33 at high P levels (RP3) (Table 3, Figures 3 and 4). These results agreed the results of Soleimanzadeh (2012) who showed that positive effect of AMF colonization decreases with increasing P levels. Similar results were reported by Chandrashekara et al. (1995), Mohammad et al. (2003) and Pragatheswari et al. (2004). This might be attributed to the fact that low phosphorus

result in exudation of certain chemicals from the root which enhances AMF colonization and spore germination but such exudations does not take place when phosphorus level is high (Juniper and Abbott, 2006; Murkute et al., 2009).

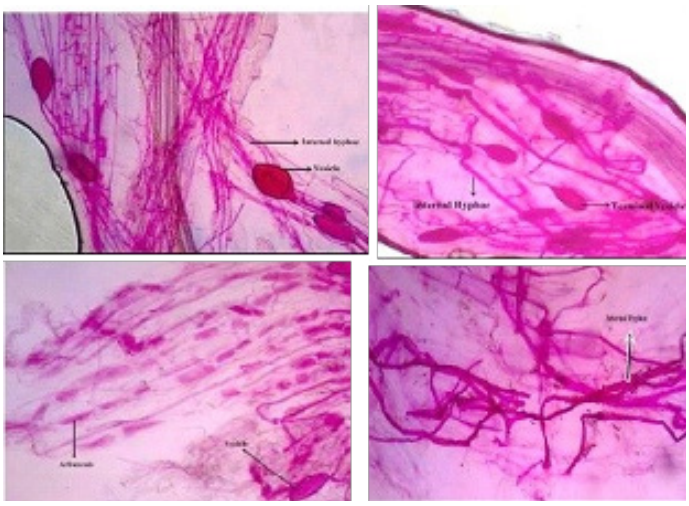

Figure 4: *AM infection in the roots of subflower hybrid*

Sharif et al. (2011) and Manske (1990) showed that low availability of soil phosphorus increases AMF colonization. But, results of Satpal and Kapoor (2000) showed that dual inoculation of *Vigna radiata* plants with rock phosphate and AMF stimulated root colonization as compared to those without rock phosphate. The results also shows that at control and low level of rock phosphate the internal hyphae and arbuscular infection were moderately frequent and scattered throughout the cortex which is actually the sites of nutrients exchange (Table 4) . The external hyphae and arbuscules were not seen in any of the studied root segments at high RP level (RP3) as shown in Table 4. High soil phosphate level has direct effect on reduction of hyphal growth and spore production.

Conclusion

This study clearly indicates the potential of using indigenous biofertilizer such as AMF for oil seed crops in low fertility soils, to achieve adequate production level with least utilization of synthetic fertilizers for sustainable agriculture practice. The use of biofertilizer is not only eco-friendly but also economical as it reduces our dependence on expensive chemical fertilizers.

Authors' Contribution

Prof. Dr. M. Ibrar has supervised Ms. Sayeda Sarah in her PhD program and this article is a portion of the research.

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