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Interaction of mycorrhiza, earthworm and rhizobium on growth of annual medic under light stress

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A major tenet of sustainable agriculture is to create and maintain diversity. The influence of arbuscular mycorrhizal fungi (*Glomus intraradices*, AMF), earthworm inoculation (*Pheretima* sp., EW) and rhizobium (*Rhizobium melilotus*, R) separately, and in combination, on growth of annual medic (*Medicago scutellata* (L.) Mill) were studied in potted culture at different planting density (6 and 20 plants pot^{-1}) with a 96 h light stress at seed formation. EW activity significantly increased mycorrhizal colonization rate. The population of low plant (6 plants pot^{-1}) produced higher mycorrhizal colonization rate. With a combined AM+EW+R inoculant, the greatest shoot biomass was observed. The maximum root nodule number obtained with a combined AMF+EW+R inoculant at the low planting density. At low population plant, EW inoculant caused annual medic to retain fresh green leaf and leaf chlorophyll content after 96 h light stress at seed formation.

Key words: Annual medic, mycorrhiza, earthworm, rhizobium, plant density, light stress

Introduction

The activities of earthworms benefit plant growth productivity, particularly in pasture (Stockdill, 1982) and agricultural production systems (Edwards and Bater, 1992) are commonly accepted. Shoot biomass of plant was found to increase significantly in 79% of published studies when earthworms were present (Scheu, 2004). The positive effects of earthworms can include improved soil structure (Shipitalo and Protz, 1988), increased macroporosity (Binet *et al.*, 1997), promoted microflora and fauna growth (Clapperton *et al*., 2001), improved soil physical and chemical conditions (Baker *et al*, 1999). Rhizobium

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(R) are ubiquitous symbionts of plants, including some agricultural crops. The bacteria in root nodules of legumes (*Rhizobium*) are important agents in N_2 fixation of atmospheric nitrogen. Many factors affect nodule formation, such as soil moisture, pH, and the availability or toxicity of minerals (Jardin, 1982), but the availability of R, carbohydrate serving as a source of electrons for nitrogenase activity, and an adequate plant water status can be singled out as the most limiting to nodule function in legumes (Phillips, 1980). Arbuscular mycorriza fungi (AMF) are a mutualistic symbiosis between AMF and the roots of terrestrial plants. The ancient fungi colonize approximately 90% of the earth's land plant species (Gadkar *et al*., 2001). The AMF symbiosis can also enhance uptake of other nutrients such as P, N, Cu and Zn (Clark and Zeto, 2000; Marschner and Dell, 1994). While positive effects of earthworm (EW) activity, AMF and rhizobium (R) separately have been demonstrated, the effects of them in combination, on different plan density are not well understood. Our research objective was to investigate the effects of AMF- EW –R on different plant density of annual medic (*Medicago scutellata* (L.) Mill.), leaf chlorophyll content under light stress, mycorrhizal colonization rate, root nodule formation and shoot biomass. Annual medics are native to the Mediterranean region, but are found in the major agricultural regions of world (Grawford, 1985). Annual medics are important winter annual pasture legumes in west of Iran provinces where they provide forage for livestock. The purpose of the experiment was to determine the effect of AMF+EW+R inoculant on growth annual medic at the different planting density, chlorophyll rate and resistance green leaf under light stress.

Materials and methods

Soil preparation

Clay soil used for the present experiment was collected from kordan grassland of Tehran province. It was air-dried, sieved (2 mm mesh). The soil was well-watered and stored at 20ºC for 48 h to cause micro organisms activate and then was sterilized (120 ºC for 2 h) by oven to eliminate native bacteria and other micro organisms, before it was divided into each 12 equal parts of 1.0 Kg and amended to contain 20 g organic matter. The soil had a pH (in water) of 7.1.

Host plant

The seed of annual medic (*Medicago scutellata*) were sterilized in a 10% V/V solution of hydrogen peroxide for 10 min. they were sown at a rate of 30 seeds in pot (20cm ×10cm × 20cm, L×W×H) and were thinned to 6 and 20 seedlings per pot once week after emergence.

Mycorrhiza, earthworm, rhizobium and plant density

Mycorrhizal fungus inoculums, consisting of spore, hypha and root fragment from a stock culture of *Glomus intraradices*. The inoculated dosage was 30 g of inoculums per pot (350 spores g^{-1} of inoculum). Mycorrhizal inoculums were placed at 2 cm blow annual medic seeds at sowing time.

Earthworms (EW) were washed free of surface soil with distilled water and kept in a sterilized glass vessel for 24 h to minimize the number of naturally occurring arbuscular mycorrhizal fungi (AMF) and bacteria associated with their surfaces or gut contents. Earthworms of similar fresh weight (0.61 g) and length (6.2 cm**)** were added to the plots. The population density of earthworms was similar to the natural populations near the field experiment.

Annual medic seeds inoculate by the R (*R. melilotus*) (purchased from water and soil institute research, Tehran, Iran). The population of plants was at 2 levels including 6 and 20 plants pot^{-1}.

Darkened shock

Light stress (10 µ mol m^{-2} s^{-1}) began after 40 days of acclimation (at seed formation) in growth chamber condition, at which time well-light pots were controlled with 250 µ mol m^{-2} s^{-1}.

Measurements

After 65 d, plants were harvested and shoot and root yield, root nodule number and pod number recorded. Leaf chlorophyll rate was assayed by chlorophyll meter (SPAD-502 Minota Co., Ltd. Japan) from 5 samples per plant pot^{-1} before and after light stress. Chlorophyll rate was non-destructively measured on leaves that were fully expanded. The mycorrhiza colonization assessment was carried out using the method described by Brundrett *et al*. (1996). Root were stained in trypanblue, and mycorrhiza colonization levels determined using the gridline intersect method of Giovanetti and Mosse (1980).

Experiment design

The experiment consists of two levels of plant density (6 and 20 plant plants pot^{-1}) and treatments: control (C), mycorrhiza inoculum (AMF), earthworm inoculum (EW), *rhizobium* inoculum (R), mycorrhiza+earthworm inoculum (AMF+EW), mycorrhiza + rhizobium inoculum (AMF+R), earthworm + rhizobium inoculum (EW+R) and mycorrhiza + earthworm + rhizobium inoculum (AMF+EW+R), giving 32 treatments each with three replicates, arranged under a

randomized block in a growth chamber, at the temperature control (25°C, 60% humidity), with illumination of 250 µ mol m^{-2} s^{-1}, under 14/10 h-light/dark cycle.

Statistical analysis

The experimental data were statistically analyzed by variance (ANOVA) with SAS 8.1 software. The significance of the differences between treatments was estimated using the Duncan range test, and a main effect or interaction was deemed significant at $P \leq 0.05$.

Results

Micorrhiza colonization

The mycorrhiza colonization rate was higher with a combined AMF+EW and AMF+EW+R inoculant treatments, although there was an obvious effect of planting density on mycorrhiza colonization rate. The population of 6 plants pot^{-1} produced higher mycorrhiza colonization (Table 1).

Shoot and root dry weight

The present experiments showed that inoculation of AMF, EW and R in combination resulted in enhanced annual medic plant dry weight at the various planting populations (Table 2). The combined AMF+EW+R inoculant achieved the highest shoot and dry yield at the rate of 20 plants pot^{-1}. The present experiment also showed that AMF, R and EW in combination resulted in enhanced annual medic shoot and root dry yield.

Pod number and root nodules

At the rate of 6 plants pot^{-1}, the combination of EW+R and AMF+EW+R resulted in enhancement annual medic root nodule (Table 3). The maximum pod number pot^{-1} was obtained by a combined AMF+EW+R inoculant (6 plants pot^{-1}) (Table 3). At the rate of 20 plants pot^{-1}, there was not significant difference between R, EW+R, AMF+R and AMF+EW+R on Root nodules. Moreover, there was not significant difference between AMF, EW and R, separately, and in combinations on pod number at the higher density (20 plants pot^{-1}).

Table 1. Mycorrhiza colonization rate of annual medic (*Medicago scutellata*) roots in response to plant densities, AMF, EW, and R inoculants (% of total length colonization).

Treatments	Planting densities (PD)	
	6(plants pot^{-1})	20(plants pot^{-1})
Control	0a	0a
R	0a	0a
EW	0a	0a
AMF	20.21b	18.1b
EW+R	0a	0a
AMF+R	21.1b	19.94b
AMF+EW	32.5c	24.01c
AMF+EW+R	33.7c	25.04c
Significance		**Mycorrhiza infection**
PD		*
AMF		**
EW		*
R		NS
AMF×PD		*
EW× PD		NS
R×PD		NS
AMF×EW		**
AMF×R		NS
EW× R		NS
AMF×EW×PD		**
AMF×R×PD		NS
EW×R×PD		NS
AMF×EW×R		**
AMF×EW×R×PD		**

Note: the same letter within each column indicates no significant difference among treatment (P<0.01).NS-not significant. *P<0.05, **P<0.01.

Leaf chlorophyll content

In this experiment the lowest leaf chlorophyll content in response to plant density, AMF, EW and R were observed in the control treatments (Table 4). Compared with the lower plant density, planting at the higher rate (20 plants pot^{-1}) resulted in reducing leaf chlorophyll content (Table 4). At the rate of 6 plants pot^{-1} earthworm addition increased chlorophyll content of leaf. Moreover, 96 h darkens also demonstrates that earthworm activity significantly sustain and retain green fresh leaf (Table 4).

Table 2. Effects of mycorrhiza (AMF), earthworm (EW), rhizobium (R) inoculants and planting density on annual medic (*Medicago scutellata* (L.) Mill.) shoot and root yield in response to plant densities (g dry matter basis pot^{-1}).

Treatments	Planting densities			
	6 (plants pot^{-1})		20(plants pot^{-1})	
	Root	Shoot	Root	Shoot
Control	4.6c	1.5c	12.24c	5.02c
R	5.6b	2.61b	15.6b	7.02b
EW	5.5b	2.71b	15.41b	8.21b
M	5.5b	2.7b	15.32b	7.9b
EW+R	5.6b	2.82b	15.2b	7.89b
AMF+R	5.0b	2.97b	15.42b	8.01b
AMF+EW	5.9b	2.4b	14.99b	8.2b
AMF+EW+R	6.5a	3.9a	16.0a	8.26a
Significance	**Root yield**		**Shoot yield**	
PD	*		**	
AMF	*		NS	
EW	**		NS	
R	**		NS	
AMF×PD	*		NS	
EW× PD	NS		NS	
R×PD	*		NS	
AMF×EW	*		NS	
AMF×R	*		NS	
EW× R	**		*	
AMF×EW×PD	*		*	
AMF×R×PD	*		*	
EW×R× PD	**		*	
AMF×EW×R	*		**	
AMF×EW×R×PD	*		**	

Note: the same letter within each column indicates no significant difference among treatment (P<0.01).NS-not significant. *P<0.05, **P<0.01.

Table 3. Effects of mycorrhiza (AMF), earthworm (EW), rhizobium (R) inoculants and planting density (PD) on annual medic pod number and root nodules number in response to plant densities(basis pot^{-1}).

Treatments	Planting densities (plants pot^{-1})			
	6		20	
	Root nodules	Pod no.	Root nodules	Pod no.
Control	0d	12b	0b	21.4b
R	12b	12b	5.2a	25.1a
EW	0d	12b	0b	24.2a
AMF	0d	12b	0b	26.4a
EW+R	16a	12b	6.21a	25.3a
AMF+R	11.2b	7.5c	4.01a	24.1a
AMF+EW	3c	8.5c	0b	23.5a
AMF+EW+R	14ab	18a	4.1a	25.3a
Significance	**Root nodules no.**		**Pod no.**	
PD	*		**	
AMF	*		NS	
EW	**		NS	
R	**		NS	
AMF×PD	*		NS	
EW× PD	NS		NS	
R×PD	*		NS	
AMF×EW	*		NS	
AMF×R	*		NS	
EW× R	**		*	
AMF×EW×PD	*		*	
AMF×R×PD	*		*	
EW×R× PD	**		*	
AMF×EW×R	*		**	
AMF×EW×R×PD	**		**	

Note: the same letter within each column indicates no significant difference among treatment (P<0.01).NS-not significant. *P<0.05, **P<0.01.

Discussion

Effect of earthworm and rhizobium on mycorrhiza mycorrhiza colonization and growth of annual medic

At harvest, soils were burrowed completely by the earthworms. All earthworms were alive at the 6 plants pot^{-1}. The present experiment shows that EW activity at the low plant population produced higher mycorrhizal colonization rate of root than high population plant (Table 1). The enhancement of root colonization rate by mycorrhizal inoculant in the presence

Table 4. Effects of mycorrhiza (AMF), earthworm (EW), rhizobium (R) inoclants and planting density (PD) on annual medic chlorophyll content before and after light stress in response to plant densities.

Treatments	Planting densities			
	6 (plants pot^{-1})		20 (plants pot^{-1})	
	Chlorophyll content			
	Before light stress	After light stress	Before light stress	After light stress
Control	58.5c	-	55.7c	-
R	60.4b	-	57.3b	-
EW	61.3a	58.1a	59.9ab	-
M	57.2c	-	56.3b	-
EW+R	61.0a	56.2a	60.2a	-
AMF+R	60.7b	-	60.3a	-
AMF+EW	63.1a	57.4a	60.1a	-
AMF+EW+R	64.7a	47.9b	58.6ab	-

Significance	Chlorophyll content	
	Before light stress	After light stress
PD	NS	*
AMF	*	NS
EW	*	**
R	*	NS
AMF×PD	*	NS
EW× PD	*	**
R×PD	NS	NS
AMF×EW	**	**
AMF×R	*	NS
EW×R	*	**
AMF×EW×PD	*	**
AMF×R×PD	*	NS
EW×R× PD	*	**
AMF×EW×R	*	**
AMF×EW×R×PD	*	**

Note: the same letter within each column indicates no significant difference among treatment (P<0.01).NS-not significant. *P<0.05, **P<0.01.

of EW may due to the production of phytohormones by earthworms and microorganism, which apparently stimulate mycorrhizal infection (Azcon *et al.*, 1978). Our results demonstrated that mycorrhiza colonization rate of annual medicago plant was enhanced by EW activity. Yu *et al.* (2005) reported that AMF infection rate of ryegrass root enhanced by earthworm activity. Increasing the plant-to-plant competition for available water, nutrient and light caused to decrease mycorrhiza colonization (Table 1). We conclude at higher planting density, each plant produces low assimilation and in return decrease

symbiosis of mycorrhizal fungi. The present result also indicated that inoculation of EW, AMF and R, in combination, increased shoot biomass, root nodule and pod number. The beneficial effect of inoculation of both EW and AMF has been well documented (Yu *et al.*, 2005). EW also produced humic substance that can influence plant growth via physiological effects (Hu *et al.*, 1998). AMF are capable of forming hyphal interconnections between mycorrhizal plants, through which nutrients can be transferred (Newman, 1988). The AMF is important in nutrient transfer in soil, but this process is affected by the activities of EW (Tuffen *et al.*, 2002). EW may graze preferentially on soil containing mycorrhizal fungal propagules and as a result, concentrate them in the casts (Gange, 1993). EW activities benefit plant growth, soil structure, fertility and productivity by their influence on organic matter breakdown and nutrient cycling (Lee, 1985). The present experiment also demonstrated that EW activity significantly decreased soil PH (data not shown), which confirmed results of Cheng and Wong (2002), Yu and Cheng (2003) and Yu *et al.* (2005), who indicated that EW activity decreased soil PH (by 0.2-0.5 units). The slight decrease in soil PH may in increase bioavailability of some nutrient and heavy metal in soils (Yu and Cheng, 2003). Many factors affect nodule formation, such as soil moisture, pH, and the availability or toxicity of minerals (Jardin, 1982), but the availability of R, carbohydrate serving as a source of electrons for nitrogenase activity, and an adequate plant water status can be singled out as the most limiting to nodule function in legumes (Phillips, 1980). The AMF symbiosis can enhance uptake of other nutrients such as P, N, Cu and Zn (Clark and Zeto, 2000; Marschner and Dell, 1994). Low plant population (plants pot^{-1}) had a greater number of branch pod per node that high population, while also producing a greater of branch reproductive nodes (data not shown). High plant density increased plant-to-plant completion for available water, nutrient and light and decrease biomass production and in return decrease symbiosis of R.

Effect of mycorrhiza, earthworm, and rhizobium on leaf chlorophyll content

This research demonstrated that EW addition increased chlorophyll content of leaf. Shaobing *et al.* (2002) reported a significant increase in single-leaf net photosynthetic rate by rhizobial inoculation on rice. Dejong and Phillips (1981) reported higher leaf apparent photosynthesis and increased leaf N content in Alaska pea (*Pisum sativum* L.) following rhizobial inoculation. A close relationship between photosynthetic rate and leaf N content was reported for rice plants (Yoshida and coronel, 1976; Peng *et al*., 1995; Peng *et al*., 2002).

The present experiment showed that mycorrhiza colonization rate was affected by the plant density and EW. A combined AMF+EW+R inoculant affected annual medic root and shoot yield. AMF+EW+R combination increased shoot and root yield. EW activity significantly increased leaf chlorophyll content while mycorrhiza inoculation and R inoculation alone or in combination had a little affect on leaf chlorophyll content. In conclusion, AMF, EW, R and their combination may have a potential role on plant growth and enhancement chlorophyll rate and retain green leaves. Complex interaction between roots, microorganisms and fauna in the rhizosphere have a fundamental effect on agricultural sustainable. More extensive research is needed to test the interactions between root, microorganisms and animals in rhizosphere.

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