International Journal of Agriculture Innovations and Research
Volume 4, Issue 6, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy) :
Received : 06/05/2016 | Accepted on : 15/05/2016 | Published : 25/05/2016

Effect of Arbuscular Mycorrhizal on Growth and Stress Eesistance of Alfalfa (Medicago Sativa. L) Plants in Different Depths of Soil Overlying Coal Fly Ash

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***Abstract* – The pressure from large area mining in China drives projects to rehabilitate such areas with restoration of vegetations, but efforts to establish vegetation in barren coal mining environments have not been successful due to the phytotoxic substrates in the mining area. It has been frequently reported that arbuscular mycorrhizal fungi (AMF) can promote plant growth, but the effects of AMF on plants to be grown in the mining area substrates have not been reported. We report a pot experiment in greenhouse that compared the effects of AMF including Glomus mosseae (G.m), Glomus versiforme (G.v), mixed fungi Glomus mosseae + Glomus versiforme (G.mv), and the non-mycorrhizal control treatment (CK) on the growth, chlorophyll content and antioxidant enzyme systems of alfalfa (Medicago sativa L.) grown with different depths of soil layer and overlying coal fly ash. The highest inoculation rates up to 45.9% after the plants were inoculated with the AMF. Inoculation of AMF increased plant growth and chlorophyll content compared with the CK treatment. Inoculation of G.mv produced higher yields of alfalfa than G.m and G. v at the same depths of soil. Inoculation of AMF aslo increased activity of SOD, POD, and CAT and decreased MDA content in leaf. Increasing soil depth led to increased plant yield and improved the antioxidant enzyme systems. These results indicated that inoculation of AMF can promote alfalfa plants growth in the substrates from the mining area, which is related to its stimulation effects on plant antioxidant enzyme systems, and soil depth is also important for plant performance.**

***Keywords* – Alfalfa, Coal Fly Ash, Mycorrhizal, Mine Subsidence, Phytoremediation.**

I. Introduction

The Shan xi province is a major coal mining area in China, and there are substantial impacts of coal mines exploitation on the environments. In china, most of coal mining were underground mining and lead to large areas of lands subsidence, which hinders the sustainable development of local social economy and ecosystem [1]. According to statistics, in china, the total area of coal mining lands increases up to 6,000,000 hm2, and that of destructed lands is 2,000,000 hm2 as estimated, and the subsidence lands are still increasing at the rate of 40,000 hm2/year [2]. Surface subsidence processes often cause serious environment problems, such as loss of surface water, destruction of soil texture and injury of plant roots [3]. To improve the ecological environments in the coal mining area, it is urgent to restore the subsidence land, establish vegetations and remediate and improve soil properties [4].

Coal fly ash (FA), a byproduct of coal combustion in thermal power plants, has been considered as a problematic solid waste and its safe disposal is a cause of great concern [5]. Global FA production may cover up to 3235 km2 of land area by 2015 [6]. Disposal of such huge amount of FA as a soil filling is unsustainable for the environments [7]. In china, one of the main ways about reclamation that used FA filled in the coal mining subsidence utilized as land reclamation at present [8]. Moreover, FA has been recognized as a soil ameliorant [9, 10] along with forestry and agricultural production [11]-[13] because of the presence of plant’s micro and macro-nutrients. But the hostile conditions (i.e., high pH, heavy metals toxicity, poor organic matter, lack of nitrogen and phosphorus) of FA inhibit the vegetation growth and speed on FA basins [14]; also FA have the characteristics of low water retentivity and low fertility, plants are difficult to grown on it [15]. Therefore, it is very important to increase the vegetations speed and efficiency on mining area recovery [16]. It has been proposed that FA with microbial inoculants could be used to formulate a soil benefaction strategy, which would help to improve the properties of the soil and enrich its nutrient status [17]-[19].

Due to the harsh environments in coal mining area, the role of microorganisms in promoting plant growth and the technique of applying microorganisms in vegetation establishment have been receiving increase [20]. Arbuscular mycorrhizal fungi (AMF) can form mutualistic symbiotic associations with the roots of 80% of terrestrial plant species [21]. AMF can improve soil microflora environments and enhance plants growth [22, 23]. In extreme environments, AMF would enhance the ability of plants to acquire nutrients and to tolerate heavy metal, drought and pest. These beneficial effects of AMF can be utilized to accelerate phytoremediation of degraded and contaminated ecosystems, particularly in metal mine tailing sites [24]. Preliminary studies have shown that inoculation with AMF greatly increased growth and nutrients content of Sainfoin (Onobrychis viciaefolia Scop.) under the drought resistance in mining area [25]. Studies also found inoculation with G. mosseae increased leaf chlorophyll content, nitrate reductase and SOD of tobacco (Nicotiana tabacum L.) seedlings [26]. When inoculated with AMF, the praline content, POD and CAT activities of maize (Zea mays L.) leaves under drought

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Volume 4, Issue 6, ISSN (Online) 2319-1473

condition were also increased [27]. In this paper, a pot experiment was conducted to test whether inoculation of AMF can enhance the plants growth in the overlying soil and the FA. The effects of different depths of soil and FA were also in vesitaged to determine whether plants can maintain growth when grown on the thin soil layer.

II. Materials and Methods

2.1 *Soil and Coal Fly Ash*

The raw soil was collected from Linfen City in Shanxi Province, China. The soil was air-dried at room temperature, sieved using a 2-mm diameter sieve before analysis. The FA was collected from the He xi thermal power plant. The FA was sieved using a 2-mm diameter sieve before the treatments. All the substrates were autoclave-sterilized (121℃, 2h) to eliminate indigenous AMF propagules and other microorganisms.

The raw soil and FA were analyzed for PH (soil: water ratio1:2.5), organic matter content, total nitrogen (TN) content, total phosphorus (TP), total potassium (TK) content, available nitrogen (AN) content and available potassium (AK) content. The physical and chemical properties of the soil and ash are listed in Table 1.

2.2 *AMF*

The AMF species were Glomus mosseae (G.m) and Glomus versiforme (G.v) provided by the institute of plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. The two AMF species were propagated in the rhizosphere of sorghum (Sorghum propinquum H.) plants in a soil-sand mixture (1:3, w/w) for three mouths prior to the experiment. The resulting inoculums consisted of a cultivation substrate containing spores, extraradical mycelium and colonized sorghum root fragments.

2.3 *Plants*

A perennial herb plant, alfalfa (Medicago sativa L.), was used as a target study in this study. It is one of most widely cultivated legume forages and preliminary tests showed that it can grow in the substrates from the coal mining area. The alfalfa seeds were purchased from Wheat Research Institute of Linfen, China. The seeds were surface-sterilized and pre-germinated in incubator at 25 ℃ for about 36 h until the radicles appeared and the seedlings with identical size were selected before planting.

2.4 Experiment Design

The pots used were 13.5 cm depth plastic pots. The FA with depth of 3, 5, and 7 cm, was placed in the bottom part of the pots. Afterwards, above the FA were covered with sterilized soil with depth of 9, 7, and 5 cm, respectivley. Therefore, the total depth of growth substrate remained as 12 cm for all the treatments. In all the mycorrhizal, 60 g of fresh inoculums (G.m, G.v, and G.m+G.v, a mixture of G.m, G.v, and abbreviated as G.mv) was added to each pot, while non-mycorrhizal inoculated plants received indentical amount of sterilized inoculums. Inoculums were added and mixed with the surface soil of each pot. Afterwards, 25 pre-germinated seeds were transplanted into the soil layer in each pot. The experiment followed a random blocks design with mycorrhiza inoculation and growth substrate as the main factors. Each combination of mycorrhiza inoculation and growth substrate treatments was replicated three times.

The pot experiment was performed in a greenhouse in Shanxi Normal University under natural light conditions. The treatments lasted for 80 days, from 1 August to 20 October 2015. During this period, the temperature ranged between 15-30 ℃. Deionized water was added as required to maintain the moisture content at 70%-80% of the soil water holding capacity as determined by regular weighing.

2.5 *Plant Sampling and Analysis*

When the plants were harvested, the shoots and roots were separated and then the root samples were carefully washed with tap water to remove adhering soil and sand particles. Plants heights were measured by ruler, the roots length were measured by root scanner (EPSON). The shoot and root fresh weights were direct weighed; the shoot and root dry weights were weighed after oven-drying to a constant weight at 70 ℃ for 48 h.

Some of the fresh roots from subsample were randomly selected and cut into approximated 1 cm pieces for determination of the root mycorrhizal colonization. The root sample were cleaned in 10% KOH (w/v) and stained with lactic acid fuchsin solution [28]. The root colonization rates were counted with the gridline-intersect method under a microscope [29]. Leaf chlorophyll content was measured by spectrophotometer. Leaf superoxide dismutase (SOD) activity was determined with nitroblue tetrazolium (NBT) colorimetry and peroxidase (POD) activity with guaiacol method; catalase (CAT) acticity using ultraviolet spectrophotometry [30]; Malondialdehyde (MDA) content was measured with the method of glucosinolates barbituric acid reaction [31].

2.6 *Statistical Analysis*

The effects of the AMF inoculation and different soil/FA depth combination treatments on all the root parameter (Length, Projected area, Surface area, Average diameter, and Volume) were analyzed with two-way ANOVA (SPSS 17.0). Prior to ANOVA, the data were checked for normality and homogeneity of variance. Between the treatments of AMF inoculation or the soil/FA combination treatments, differences of colonization rate, root parameters were analyzed by Duncan's multiple range tests. When p value is less than 0.05, the difference is considered significant. The figures were depicted by origin 8.5.

III. Results

3.1 *Arbuscular Mycorrhizal Colonization*

All the inoculation treatments successfully infected the plants roots, but there were significant differences among the AMF treatments or among the substrates treatments (Table 2, 3, $P < 0.05$). No arbuscular mycorrhizal colonization was observed in the roots of the non-inoculated alfalfa plants in different soil/fly ash depth

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Volume 4, Issue 6, ISSN (Online) 2319-1473

combinations (Table 3). The root colonization rates of inoculation of G.mv were significantly lower than that of G.m or G.v (Table 2, P < 0.05). The highest root colonization rates reached 45.9% in the treatment of G.mv growing in soil of 9 cm. The treatments of soil/fly ash depth combinations also significantly affected plants colonization rates which declined with the decreasing soil depth (Table 2, 3, P < 0.05). In the treatments with soil depth of 5cm, the colonization rate only reached 28.4%. There were also significant interaction effects between AMF and growth substrate (Table 2, P < 0.05).

Table 1. Physical and chemical properties of the soil and FA.

	PH	Organic matter g/kg	TN mg/kg	TP mg/kg	TK g/kg	AN mg/kg	AK mg/kg
soil	7.8	10.62	134.3	450.0	4.45	31.34	108.4
fly ash	8.1	23.87	104.0	631.0	2.63	15.86	50.0

Table 2. Two-way ANOVA for the effects and interactions of different treatments on alfalfa

Index	Substrate treatments		AMF treatments		Substrate*AMF	
	F	P	F	P	F	P
Colonization rate (%)	78.99	0.000***	436.92	0.000***	12.25	0.000***
Plant height (cm)	4.20	0.027*	103.13	0.000***	0.32	0.919 ^{NS}
Root length (cm)	5.72	0.009**	10.70	0.000***	1.14	0.317 ^{NS}
Shoot fresh weight (g)	4.54	0.021*	36.92	0.000***	0.54	0.777 ^{NS}
Root fresh weight (g)	8.64	0.001**	107.32	0.000***	0.892	0.516 ^{NS}
Shoot dry weight (g)	69.87	0.000***	285.63	0.000***	8.15	0.000***
Root dry weight (g)	47.02	0.000***	415.28	0.000***	8.15	0.000***
Chlorophyll a (mg)	1.858	0.178 ^{NS}	40.87	0.000***	0.171	0.982 ^{NS}
Chlorophyll b (mg)	2.13	0.14 ^{NS}	22.00	0.000***	0.152	0.987 ^{NS}
Total chlorophyll content (mg)	2.624	0.093 ^{NS}	44.73	0.000***	0.087	0.997 ^{NS}
Carotenoid content (mg/g)	1.27	0.299 ^{NS}	13.40	0.000***	0.116	0.994 ^{NS}
SOD (U/g)	158.19	0.000***	218.37	0.000***	3.15	0.020*
POD (U/g ·min)	132.55	0.000***	122.84	0.000***	9.04	0.000***
CAT (U/g· min)	59.56	0.000***	35.14	0.000***	2.08	0.093 ^{NS}
MDA (nmol/g)	18.66	0.000***	21.83	0.000***	0.79	0.585 ^{NS}

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. NS: non-significant.

Table 3. Effect of different AMF on alfalfa growth grown in in three different mediums

Soil depth (cm)	AMF fungus	Plant height (cm)	Root length (cm)	fresh mass (g)		dry mass (g)	
				Shoot	root	Shoot	root
9	CK	5.83c	8.92b	0.20b	0.27d	0.03d	0.04c
	G.m	14.33ab	15.80ab	0.56a	0.90b	0.13b	0.12b
	G.v	12.40b	12.01b	0.29b	0.57c	0.064c	0.072c
	G.mv	16.50a	24.82a	0.73a	1.35a	0.154a	0.168a
7	CK	5.50c	7.99b	0.16c	0.24c	0.028d	0.031d
	G.m	14.00a	12.54ab	0.46b	0.71b	0.096b	0.104b
	G.v	11.10b	10.69ab	0.27c	0.54b	0.054c	0.056c
	G.mv	15.33a	15.48a	0.63a	1.11a	0.14a	0.15a
5	CK	4.07d	6.87c	0.15c	0.17d	0.02d	0.03d
	G.m	13.47b	11.21ab	0.39ab	0.64a	0.07b	0.08b
	G.v	9.83c	9.38c	0.24bc	0.45b	0.04c	0.051c
	G.mv	15.23a	13.33a	0.51a	1.05c	0.11a	0.11a

Values within columns in the same substrate marked with the same letter are not significantly different according to Duncan's multiple range test at the p < 0.05 level.

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Table 4 Mycorrhizal colonization rate of alfalfa inoculated with AMF in three different mediums

Soil depth (cm)	FA Depth (cm)	AMF fungus	Substrate total weight /pot (g)	Mycorrhizal colonization rate (%)
9	3	CK	3400	0.00c
		G.m	3400	43.64a
		G.v	3400	28.14b
		G.mv	3400	45.89a
7	5	CK	3040	0.00c
		G.m	3040	31.44a
		G.v	3040	23.87b
		G.mv	3040	35.15a
5	7	CK	3120	0.00c
		G.m	3120	22.53b
		G.v	3120	18.73b
		G.mv	3120	28.40a

The colonization rate values marked with different letters are significantly different between the AMF treatments ($P < 0.05$).

3.2 *Plant Growth and Chlorophyll Content*

Inoculation of AMF significantly promoted the plants growth including shoot height, root length and total biomass (Table 2, 4, $p < 0.05$). AMF also significantly increased leaf chlorophyll a, chlorophyll b and the total chlorophyll content (Table 2, 5, $p < 0.05$). The mixed fungi G.mv imposed the strongest effects than G.m and G.v on plants growth and chlorophyll content, and G.m and G.v had stronger effects than unvaccinated treatment CK. The substrates also significantly affected plant growth (Table 2, 4, $p < 0.05$). The chlorophyll and carotenoid content decreased with decreasing soil depth, but the effects were not significant (Table 5). The interaction effects of AMF and substrate significantly affected plant growth (Table 2, $p < 0.05$). The highest plant growth was achieved with 9 cm of soil depths and inoculation with G.mv.

Table 5. Leaf chlorophyll content of alfalfa inoculated with AMF in three different mediums

Soil depth (cm)	AMF fungus	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll content (mg/g)	Carotenoid content (mg/g)
9	CK	0.47c	0.17c	0.64c	0.057b
	G.m	0.66b	0.29b	0.95b	0.097ab
	G.v	0.50c	0.25b	0.74c	0.068ab
	G.mv	0.83a	0.33a	1.17a	0.115a
7	CK	0.40b	0.16c	0.56b	0.056c
	G.m	0.65a	0.26ab	0.91a	0.086ab
	G.v	0.47b	0.22bc	0.69b	0.064bc
	G.mv	0.77a	0.32a	1.10a	0.107a
5	CK	0.39b	0.16b	0.55b	0.053b
	G.m	0.64a	0.24ab	0.88a	0.077ab
	G.v	0.43b	0.22ab	0.66b	0.060b
	G.mv	0.74a	0.29a	1.03a	0.097a

Values with different letters from the AMF treatments with the same soil depth are significantly different from each other ($p < 0.05$)

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International Journal of Agriculture Innovations and Research
Volume 4, Issue 6, ISSN (Online) 2319-1473

3.3 *Plant Stress Resistance Indexes*

All the AMF treatments significantly increased antioxidant enzymes (SOD, POD, and CAT) and MDA content and the strongest effects were found in the G.mv treatment (Table 2, Fig. 1, 2, 3, 4, $p < 0.05$). Generally, the effects of the mycorrhizal inoculation treatment showed a pattern of G.mv > G.m > G.v > CK. Substrates also significantly affected antioxidant enzyme activities and MDA content (Table 2, Fig. 1, 2, 3, 4, $p < 0.05$), but There were no significant interaction effects between AMF and soil depths on stress resistance indexes (Table 3, $p > 0.05$). The highest antioxidant enzyme activity values and the lowest MDA value were found in 9 cm of soil overlying 5 cm of FA.

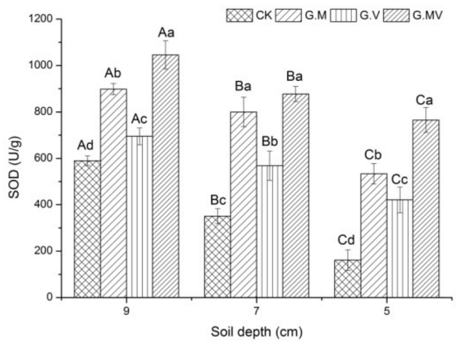

Fig. 1. Activity of SOD (U/g) of the alfalfa plants grown with different soil depth: 9 cm soil (overlying 3 cm of FA), 7 cm soil (overlying 5 cm of FA), 5 cm soil (overlying 7 cm of FA). Plants were either left non-inoculated, or inoculated with AMF (G.m, G.v, and G.mv). The data are means of three replicates ± SE. Columns marked by the different lowercase letters letter are significantly different within a particular substrate ($P < 0.05$); different capital letters represent the significantly difference among different substrates treatments ($P < 0.05$). The same as below.

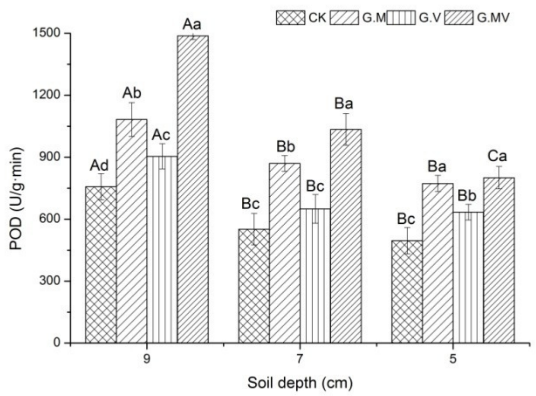

Fig. 2. POD activity (U/g •min) of alfalfa grown with different soil depth

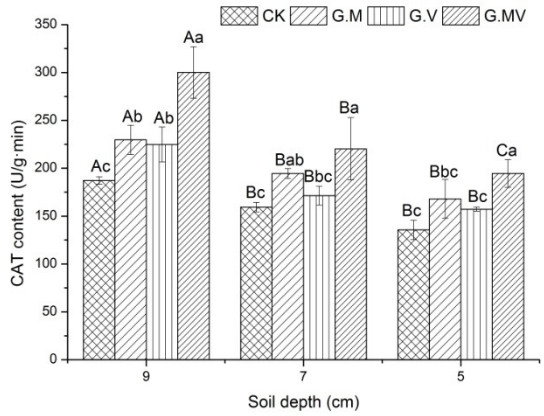

Fig. 3. CAT content (U/g• min) of alfalfa grown with different soil depth.

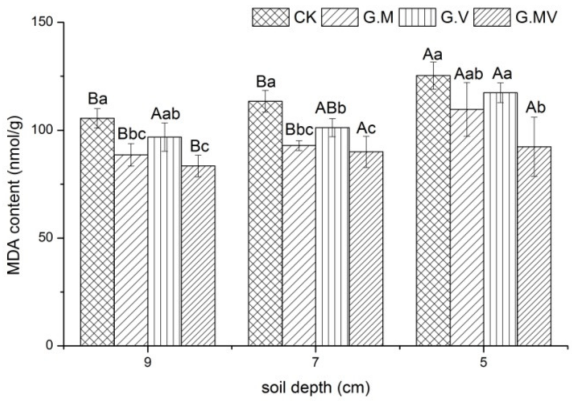

Fig. 4. MDA content (nmol/g) of alfalfa grown with different soil depth.

IV. Discussion

4.1 *Mycorrhizal Colonization Rate*

In this study mixed fungi G.mv inoculation led to higher colonization rate than the single fungi G.m and G.v. This is possibly because different AMF species may have positive interactive effects rather than competitive effects. The decreasing of AM colonization rate with increasing depth of FA under the soil may be attributed to changes in substrate conditions caused by FA. It is well known that FA application could change soil nutrient level and soil microbial activity. Change of these soil properties may affect mycorrhization of AM fungi [32]. On the whole, AMF had a crucial role and exhibited a very distinct tolerance for FA.

4.2 *Plant Growth and Chlorophyll Content*

Chlorophyll is the main pigment for photosynthesis of plants, it is an important material to capture light energy for photosynthesis [33]. Our study indicated that AMF significantly increased the plants growth and chlorophyll contents compared to non-inoculated plants, suggesting a beneficial effect from AMF inoculation. This is in accordance with many other studies. It is reported that AMF could promote plants photosynthesis growth and development for the plants [34]. Liu and Ma [35] found

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that AMF could significantly improve the caper (capparis spinosa L.) biomass, chlorophyll content, soluble sugar, and praline. The effects of mixed fungi were better than single fungi, similar results were obtained by Ren and Xia [36], who observed much more positive effects of mixed inoculation fungi than single inoculation treatment on soluble sugar content, chlorophyll content, and net photosynthetic rate of maize (Zea mays .L) plants. This is due to higher mycelium density in the mixed fungi treatments than single fungus treatments. The results indicate that mixed fungi may increase survival rate or growth of variety of plants that is unable to sucessfully establish on the FA when inoculated with only single fungus species.

The maximum soil depth studied (9 cm) is required for satisfactory plant growth (highest total biomass, plant height, chlorophyll content et al) under the conditions of experiment. On the contrary, when grown with the minimum soil depths, plants showed stunted growth (lower plant height and total biomass) and the lowest chlorophyll content. Bi and Li [37] also demonstrated that maize (Zea mays L.) plant inoculated with AMF had the lowest biomass in grown in minimum soil depth with overlying FA. This is probably due to the decrease of photosynthetic pigment content and low availability of soil nitrogen and phosphorus at higher FA doses [38]-[40]. Ma et al [41] reported that the weight of 20%-25% fly ash to substrates was optimal for plants growth. Overall, FA content directly affects plants growth and development. Therefore, deeper depth is advisable and further work is required to determine the optimum soil depth under natural conditions.

4.3 *Plant Stress Resistance*

There are several mechanisms that may explain the promoting effects of AMF on plant growth. First, plant stress resistance is increased when colonized with AMF. When plant growth are stressed, the activity of antioxidase in the plant, such as POD, SOD and CAT will continuously increase to protect cells against the damage of reactive oxygen species [42]; MDA could inhibition of protein synthesis so that made enzyme deactivation [43]. Therefore, MDA is a key indicator of the cell membrane damage level and membrane lipid peroxidantion [44, 45]. In our study, activity of SOD, POD, and CAT were significantly increased and MDA content decreased after AMF inoculation compared to unvaccinated treatments. Similar results were found in some other studies. He et al [46, 47] found that AMF can improve SOD, POD, and CAT activities under salt stress and drought stress plants and reduce MDA content. Han et al [48] also found that AMF could improve the cucumber (Cucumis sativus L.) antioxidant enzyme activities and root activity while decreased MDA and electrolyte leakage under the low temperature stress.

Second, when plants are inoculated AMF, more mycelium outside plant roots may be produced, which have larger root absorption area and higher water and nutrients uptake efficiency [49]. AMF can also provide other macro- and micro- nutrients such as N, K, Mg, Cu, and Zn, particularly in soils where they are present in less soluble forms [50]-[52]. The results form this study also indicated that the effects of AMF on plants antioxidant capacity depends on AMF species. Singh et al [53] confirmed that G.m was better than G.v to increased antioxidant enzyme activities of Chrysanthemum (Flos Chrysanthemi) plantlets. However, He et al [54] reported that that G.v had stronger promotive effects on SOD, POD, PAL, and PPO of tomato (Lycopersicon esculentum Mill) than G.m. Therefore it is necessary and possible to select the most effective AMF species.

V. Conclusion

Our study indicates that successful establishment of alfalfa is possible in soil with overlying FA and alfalfa plants growth can be improved by inoculation of AMF. Thus, remediation of coal subsidence areas infilled with FA may be possible using either undisturbed soil containing viable propagules of indigenous AM fungi or disturbed soil inoculated with effective strains of AMF. AMF can improve plants growth, increase chlorophyll content and antioxidant enzymes under the adverse conditions conferred by FA. Mixed fungi effects was obvious than the single fungi, indicated that the presence of a variety of AMF can compensate for the shortage of single AMF, and consequently had a positive improvement on alfalfa physiological effects. This study suggests that the application of AMF technology may be a sustainable method to recovery degrade and contaminated ecosystems, particularly in coal mine tailing sites. Further studies are required to determine the optimum depth of soil required.

V. Acknowledgments

We thank Dr. Ye, X.Q for the revision of an early version of the manuscript. Financially supported by the National Natural Science Foundation of China (NO.31270461); the School Fund of Shanxi Normal University (NO.ZR1211).

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