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Effects of Three Fungicides on Arbuscular Mycorrhizal Fungi and Transformed Carrot Hairy Roots/AM Fungus Association

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The effect of three fungicides (chlorothalonil, carbendazim, thiram, each fungicide set for three concentration: (0.1, 1, 10 mg/l) on spore germination, hyphal elongation, the biomass and malondialdehyde content of carrot hairy roots were investigated using a strict in vitro cultivation system associating the Ri T-DNA-transferred carrot hairy roots with *Glomus etunicatum*. The results show that three fungicides at all three concentration except for thiram at the 0.1 mg/l impacted significantly on spore germination and hyphal elongation. The impacts of the fungicides were more significant with the increasing concentration. Both of the biomasses of the fungus colonized carrot hairy roots and no fungus colonized roots treated with fungicides were decreased compared with the control. *G. etunicatum* root colonization and spore production as well as hyphal Succinate dehydrogenase and Alkaline phosphatase activity reduce with the concentration of fungicide increasing. These evidences suggest that the fungicides have adverse effects on both plant and arbuscular mycorrhizal fungus during the plant-fungus interactions. Both of the malondialdehyde of the fungus colonized and non-colonized roots were increased, but the contents of the fungus colonized hairy roots were lower than that of non-colonized roots, indicating that the arbuscular mycorrhizal fungus can protect plants to a certain degree in the adverse conditions. The results of the impacts of fungicides on carrot hairy roots/AM fungus association in this paper will be useful in instruction the use of fungicides with the consideration of ecological effects.

Key word: Fungicide Arbuscular mycorrhizal Hairy root Root colonization.

Plant diseases often cause significant damage to agricultural industry. It is estimated that the yields of the world's crops reduce approximately 500Mt a year caused by the diseases. Famine caused by plant diseases have happened several times, even starve tens of thousands of people to death in history. In order to maintain high and stable yields of the crops, many techniques, such as molecular breeding, fertilizers, antibiotics etc., for pest control were used in agricultural industry. Among those, application of fertilizer is an efficient method to maintain the high and stable yields of crops (Ding *et al*., 2010). Farmers often use excessive fertilizers to increase the yields. However, excessive use of chemical fertilizer will cause environmental pollution and lead to many health problems (Ongley, 2004). To avoid the adverse effects of synthetic fertilizer, investigators pay more attention to some new fertilizers such as controlled-release fertilizer, arbuscular mycorrhizal (AM) fungi.

AM fungi are believed to be a safe and no pollution fertilizer. These fungi are beneficial

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soil microorganisms that live symbiotically in association with the vast majority of plant species, including most agricultural and horticultural economically-important crops (Brundrett, 2002). A large number of studies have shown that AM fungi can enhance the uptake of nitrogen (Leigh *et al*., 2009), phosphorus (Weber *et al*., 1992; Jayachandran and Shetty, 2003), zinc (Al-Karaki and Clark, 1998; Ryan and Angus, 2003; Seres *et al*., 2006), copper (Marschner and Dell, 1994; Toler *et al*., 2005), iron (Kim *et al*., 2010) and others ions (Ryan *et al*., 2004). Furthermore, many researchers have proved that AM fungi can also improve host plant's resistance against drought stress (Fagbola *et al*., 2001; Zhu *et al*., 2011; Sohrabi *et al*., 2012), salt stress (Feng *et al*., 2002; Giri *et al*., 2007; Yu *et al*., 2012); low-temperature stress(Wu and Zou, 2010; Zhu *et al*., 2010; Zhou *et al*., 2012) etc. Some studies have also revealed that inoculation of AM fungi can improve the nutrient quality of crops (Hart and Forsythe, 2012). For the above reasons, AM fungi are believed to have bright future in sustainable agriculture.

Application of antibiotics, particularly fungicide(s) in crops prevention and control of plant diseases is another way to maintain stable yields of the crops besides using fertilizer. Although thousands of kinds of fungicides had been invented, the most common used chemicals are chlorothalonil, carbendazim, thiram etc. Chlorothalonil is a greatly used broad-spectrum organochlorine pesticide (Liang and Tang, 2010; Wu *et al*., 2012). This chemical reduces fungal intracellular glutathione molecules to alternate forms which cannot participate in essential enzymatic reactions, ultimately leading to cell death of fungi (Tillman *et al*., 1973). Carbendazim is a systemic broad-spectrum fungicide which belongs to benzimidazole compounds. The mode of action is that carbendazim can inhibition of nuclear division as well as binding to tubulin and inhibition of microtubule assembly which can lead to death of fungi (Davidse, 1986). Thiram pertains to the widely used protective dimethyldithiocarbamate pesticides utilized for decades. Several lines of evidence indicate that after administration to cells, thiram is rapidly reduced to its corresponding thiol, dimethyldithiocarbamic acid. The dithiocarbamate anion, which is used as a fungicide, is considered the active moiety because of its chelating properties (Elskens and Penninckx, 1997). The three fungicides are widely used in China and many other countries for their usefulness in the control of fungias plant pathogens (Xiao *et al*., 2013).

Although AM fungi and fungicides are both beneficial for crops when they are used separately, the outcome might be different while they are applied together. Most of fungicides usually disturb the function of fungi via respiration, lipid synthesis or cell division (Leroux, 2003). However, the results of fungicides on AM fungi were different in the field studies (Trappe *et al*., 1984; Kurle and Pfleger, 1994). The investigation by He *et al*. (1994) showed that *Glomus mosseae* generally maintain the normalfunction to promote the growth of host plant after the application of fungicides. The fungicide applied as soil drenches effects on AM fungi colonization were relatively minor in Burrows and Ahmed's study (2007). Similar results was also showed in the study of Chiocchio *et al*. (2010) only the high doses of fungicide can hinder the germination of AM fungi spores. While others studies showed that almost all the tested fungicides inhibited the AMF colonization and formation of spores significantly (Kjoller and Rosendahl, 2000; Assaf *et al*., 2009; Bharat, 2011). The effects of fungicides on AM fungi are different in different studies, which might due to that almost all the studies were implemented in pot or field experiment. Results in field experiments may be affected by factors such as soil particle size, temperature difference, rainfull, local microbial community etc. In order to precisely assess the effects of fungicides on AM fungi, studies with less influencing and uncertain factors should be adopted.

In this study, we used a strict in vitro cultivation system associating the Ri T-DNA transformed carrot hairy roots with *G. etunicatum* to assess the effects of three fungicides (chlorothalonil, carbendazim, thiram) on the AM fungus and transformed carrot hairy roots/AM fungus association. This *in vitro* culture system offers the advantages that the environmental factors can be restricted to a limited number of variables, allowing more precise investigation of the impact of fungicides on the plant-fungus association.

MATERIALS AND METHODS

Plant and fungal strains, media and growth conditions

The inoculum of *G. etunicatum* and carrot hairy roots were obtained and cultured as well as the dual cultivation system of *G. etunicatum* with Ri T-DNA transferred carrot hairy roots was established, as described previously (Li *et al*., 2013). Modified Strullu-Romand (MSR) medium (Declerck *et al*., 1998) solidified with 0.3% phytagel (Sigma), pH 5.8 was used to cultivate the plant and fungal strainsin aninverted position at 28° C in a growth chamber under dark conditions.

Fungicides

Standard sources of chlorothalonil, carbendazim and Thiram were purchased from Hefei Rongyu Biological Technology Co.Ltd. (Hefei, China). For each experiment, high temperature sterilization MSR medium solidified with 0.3% Phytagel (Sigma) was used. Three fungicides were dissolved in a solution of acetone, filter-sterilized with 0.22-μmfilterand added to MSR medium. Each fungicide prepared for three concentrations (0.1, 1 and 10 mg l^{-1}). A control treatment (MSR medium supplemented with acetone but without fungicides, 0 mg l^{-1}) was also prepared.

G. etunicatum spore germination analysis

Spore germination was investigated based on the method described by Cheng *et al*.(2012). The method of sterilized spore extracted from the culture medium was described previously (Li *et al*., 2013). Sterilized spores were inoculated to sterilized MSR medium supplemented with each of the three fungicides at three different concentrations. Each experiment treat with ten Petri dishes and each dish with 80 to 100 spores. All treatments were repeated three times. After inoculation, spores incubated in the dark at 28 °C. 15 days later, germinating spores were counted, and hyphal length were measured using an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan) with Image-Pro Plus 6.0 Image Analysis Software (Fig. 3).

Effects of fungicides on mycorrhizal development

Approximately 5 cm of Ri T-DNA transferred carrot hairy root was cut from cultured carrot hairy root and placed in the fresh sterilized MSR medium which supplemented with different concentrations of fungicides, respectively. Approximately 50 sterilized spores were transferred to the MSR medium adjacent to the hairy roots. The co-cultures were sealed and cultured at 28 °C under dark conditions. After 60 days, the fresh weight and MDA content of the carrot hairy roots (with or without AMF), mycorrhizal colonization, daughter spore production and hyphal SDH and ALP activity were analyzed. All of these were analysis by the methods described by Li and Miao *et al*. (2013).

Enzyme histochemical staining

Succinate dehydrogenase activity (SDH) and Alkaline phosphatase activity (ALP) were analyzed by enzyme histochemical staining (Li *et al*., 2013). The stained Ri T-DNA transferred carrot hairy root which infected by AMF was assessed by fluorescence microscopy (Olympus IX71; Olympus) and was expressed as the percentage of roots exhibiting SDH or ALP activity based on the total length of 30 root segments.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS software of least significant difference. Results with $p < 0.05$ were considered significant. All figures were drawn using Sigma Plot 10.0 statistical software (Li *et al*., 2013).

RESULTS

The effect of three fungicides on spore germination and hyphal elongation

In this study, all three fungicides at all concentrations tested (0.1, 1 and 10 mg/l) except for the thiram at 0.1 mg/l did significant effects on spore germination (Table 1), while all the treatments significantly inhibited hyphal growth of *G. etunicatum*. No significant difference on spore germination mediated by chlorothalonil and carbendazim were observed at 0.1 mg/l and 1 mg/l. When the concentrations of the fungicides are 10mg/l, thiram has the largest effect on spore germination (34.47%), carbendazim (46.62%) and chlorothalonil (45.19%) are in the second place. Thiram has the least effect on *G. etunicatum* hyphal elongation, secondly is carbendazim, chlorothalonil has the greatest influence on hyphal elongation. Compared with control group, the germination rate of spores decreases by an average of 33.10% (The sharpest decreases was the thiram

at 10 mg/l, down by 60.75%; and the minimum decreases was the thiram at 0.1 mg/l, down by 1.06%), while the hyphal elongation decreases by an average of 79.45% (The sharpest decreases was the chlorothalonil at 10 mg/l, down by 89.18%; and the minimum decreases was the thiram at 0.1 mg/l, down by 60.98%). And looking at the overall, the impacts of fungicides were more significant with the increasing concentration.

Effects of fungicides on the biomass of carrot hairy roots

All the biomass of the fungus-colonized and no fungus colonized carrot hairy roots that were treated with three fungicides declined significantly compared with each control. More significant decrease can be observed at the highest concentration (Table 2). The biomass of no fungus colonized carrot hairy roots decreases by an average of 44.08% compared with controls, while the biomass of the fungus-colonized roots decreases by an average of 37.69%. The biomass of the fungus-colonized roots was consistently higher than that of non-colonized roots with the same concentrations.

Effects of fungicides on malondialdehyde content of carrot hairy roots

It can be seen from Fig. 1 that all the malondialdehyde (MDA) content of the no fungus colonized roots increased after the fungicide treatments, but the MDA content the roots inoculated with AM fungus were much less than that of non-colonized roots at the same concentrations. In the non-colonized treatments, three fungicides obviously increased the MDA

Table 1. Effects of three fungicides on spore germination and hyphae length of *G.e*

Treatments	Concentrations(mg l^{-1})	Spore germination (%)	Average hyphae length(mm)
CK	0	87.83±2.16a	3.05±0.22a
	0.1	65.12±2.08b	0.55±0.03def
chlorothalonil	1	61.98±2.13bc	0.56±0.02def
	10	45.19±2.47d	0.33±0.03f
	0.1	64.34±2.26bc	0.60±0.04cde
carbendazim	1	65.38±1.18b	0.51±0.05def
	10	46.62±1.96d	0.38±0.02ef
	0.1	86.90±1.57a	1.19±0.08b
thiram	1	58.82±2.19c	0.83±0.08c
0	10	34.47±1.87e	0.69±0.06cd

Note: The data are obtained 15 days after the application of fungicides. Different letters in the same column indicate significant differences among treatments (n = 3, $p< 0.05$). Data are the mean ±SE.

Table 2. Effects of three fungicides on the biomass of carrot hairy roots

Treatments	Concentrations (mg l^{-1})	Biomass(g) (no colonized)	Biomass(g) (colonized)
CK	0	1.83±0.07a	2.65±0.11a
	0.1	1.24±0.03c	2.07±0.05bc
chlorothalonil	1	1.22±0.03c	2.12±0.07bc
	10	0.74±0.06e	1.27±0.03e
	0.1	1.21±0.04c	1.94±0.06cd
carbendazim	1	1.05±0.06d	1.83±0.03d
	10	0.34±0.03g	0.50±0.04g
	0.1	1.46±0.06b	2.21±0.11b
thiram	1	1.38±0.04b	2.16±0.11bc
0	10	0.57±0.05f	0.76±0.08f

Note: The data are obtained 60 days after the application of fungicides. Different letters in the same column indicate significant differences among treatments (n = 3, $p< 0.05$). Data are the mean ±SE.

content of the roots, furthermore, higher MDA content was observed with increasing concentrations of fungicides. There was no significant difference on the MDA content mediated by all three fungicides between 0.1 mg/l and 1 mg/l. When the concentration was 10 mg/l, carbendazim had the largest impact on the MDA content, with the MDA content found to be two-fold greater than that in the control. However, the impact was greatly reduced after inoculation with *G. etunicatum*.

Effects of three fungicides on indicators of AM fungus

When the roots were treated with 0.1 mg/l chlorothalonil and 0.1 mg/l thiram, there were no significant effects on mycorrhizal colonization compared to the control (76.6% vs. 82.9%, 76.8% vs. 82.9%)(p>0 .05) (Fig. 2a), while other treatments significantly reduced mycorrhizal colonization rate. Furthermore, no significant effects on mycorrhizal colonization were mediated by chlorothalonil and carbendazim between 0.1 mg/l and 1 mg/l. However, three fungicides at 10 mg/l, mycorrhizal colonization rate declined sharply to 46.0% (by chlorothalonil), 37.3% (by carbendazim) and 31.7% (by thiram).

Daughter spore production of the fungus was severely reduced by three fungicides. Daughter spore production in the control group was significantly higher than that in all treated groups (Fig. 2b). There was no significant difference on daughter spore production between chlorothalonil and thiram at 0.1 mg/l, although a reduction of 39.3%and 42.5% was observed compared with the control. At 1 mg/l, thiram had the largest effects and chlorothalonil had the smallest effects on daughter spore production. When the concentration was 10 mg/l, the spore production declined 73.6%, 86.5% and 89.2%

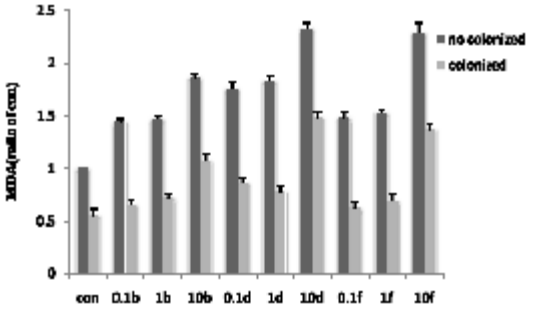

Fig. 1. Effects of fungicides on malondialdehyde of no colonized and colonized carrot hairy roots in different concentrations. The data were obtained 60 days after the application of fungicides. The test was performed three times. In x-axis, 'con' represents control, 'b' represents chlorothalonil, 'd' represents carbendazim and 'f' represents thiram.

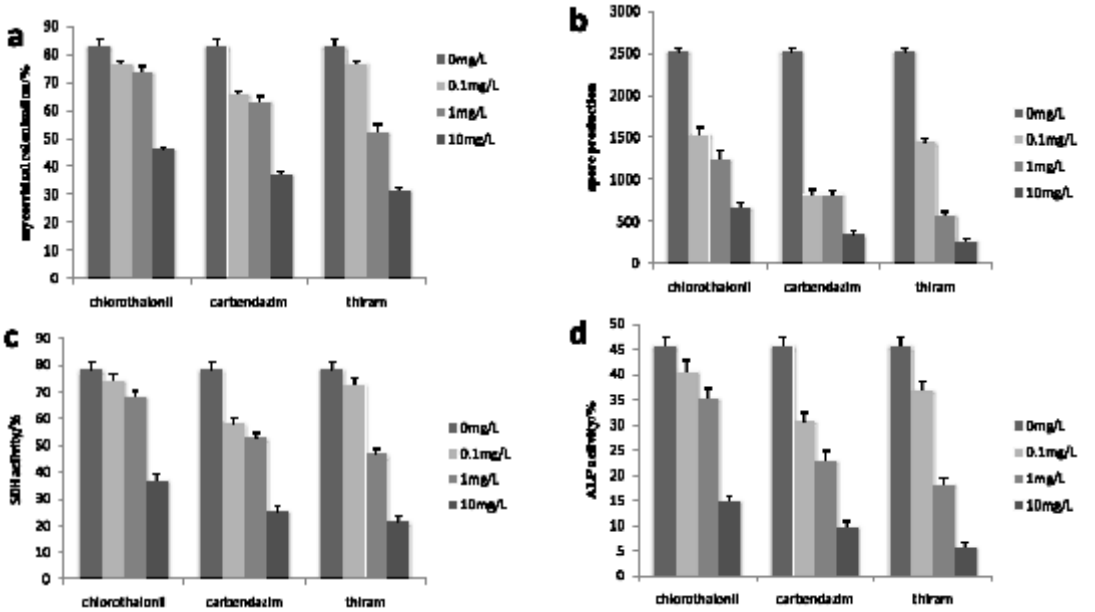

Fig. 2. Effects of fungicides on mycorrhizal colonization (a), spore production (b), the activity of hyphal succinate dehydrogenase (c) and Alkaline phosphatase (d) in different concentrations. The data were obtained 60 days after the application of herbicides. The test was performed three times

compared to the control. Generally, spore production decreased with increasing concentrations of fungicides. Among the three fungicides, chlorothalonil had the smallest effects on spore production.

Succinate dehydrogenase (SDH) and Alkaline phosphatase (ALP) activities which are regarded as indexes of vitality and activity of AM fungi mycelium were also measured in this investigation. There was no significant difference in the proportion of hyphae exhibiting SDH activity when treated with chlorothalonil and thiram respectively at 0.1 mg/l compared with the control (Fig. 2c), while other treatments significantly decreased the hyphal SDH activity. No significant effects on hyphal SDH activity were mediated by chlorothalonil and carbendazim at 0.1 mg/l and 1 mg/l. However, three fungicides at 10 mg/l, hyphal SDH activity was reduced by 52.9%, 67.6% and 72.6% respectively compared with the control. Moreover, SDH activity decreased with increasing fungicides concentrations and chlorothalonil had the smallest effects on the hyphal SDH activity.

All three fungicides greatly reduced the extracellular ALP activities released by AM fungus (Fig. 2d). No significant difference in ALP activity was observed in response to treatments with 0.1 mg/l chlorothalonil and 0.1 mg/l thiram. At 1 mg/l,

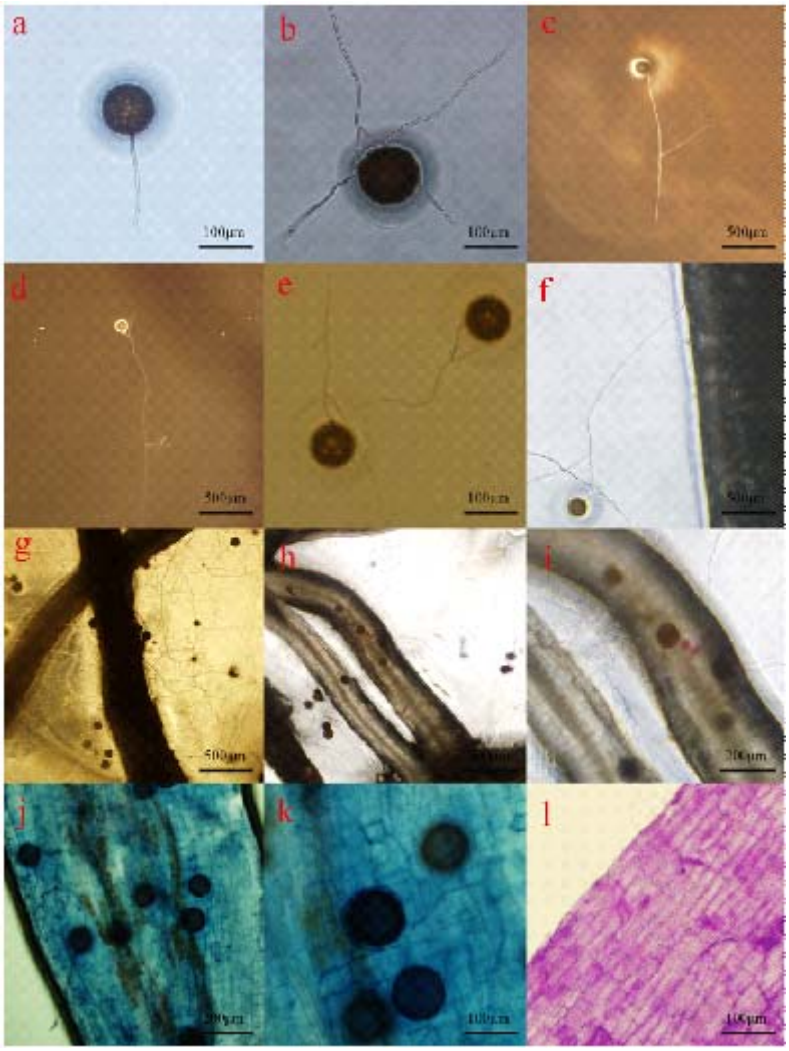

Fig. 3. Micrographs of *Glomus etunicatum* (a-b) spore germination, (c-e) growth and branch, (f) inoculation to carrot root, (g-h) extraradical spores, (i-k) intraradical spores, (l) vesicle

their ALP activity declined respectively from average 45.7% to 35.3%, 22.9% and 18.1%. However, at 10 mg/l, ALP activity was reduced by 67.4%, 78.6% and 87.7% respectively compared with the control. Furthermore, ALP activity decreased with increasing concentrations of fungicides. Chlorothalonil had the smallest effects on the hyphal ALP activity which is similar to SDH activity.

DISCUSSION

Uncertain factors often greatly impact the results of effects of fungicides on AM fungi in field experiments. The effects of fungicides on fungi will influenced by soil particle properties, heavy metal ions, rain, UV and microbes, since fungicides can be divided or converted to other chemicals in fields. The *in vitro* dual culture system of Ri T-DNA-transferred carrot hairy roots with *G. etunicatum* was often used to investigate the potentialities and/or mechanism of different plant species to tolerate, accumulate, and/or remove environmental pollutants, such as PCBs, pharmaceuticals, heavy metals etc (Agostini *et al*., 2013). Factors with the dual culture system are much fewer than that in field experiments, and thus application of the dual culture system to investigate the effects of fungicides on AM fungi will be more accurate. For this purpose, the effects of widely used fungicides (chlorothalonil, carbendazim, thiram) on AM fungus (*G. etunicatum*) were assessed with the dual system in our study.

The results of the fungicides on *G. etunicatum* spores germination and the fungus hypha in Table 1 showed that under the condition of low concentration of fungicides (only thiram at 0.1 mg l^{-1}), the spore germination rate of AM fungus were similar while the hyphal elongation were severely hindered. For higher concentration, all the tested fungicides significantly reduced *G. etunicatum* spores germination and inhibited the fungus hyphal elongation, and thus reduce fungal colonization of host plants (Fig. 2a) and the production of daughter spores (Fig. 2b). Abnormal germination hyphae often observed in the germination of *G. etunicatum* spores affected by fungicides. A spore often germinated more than one hypha (Fig. 3). Although the results of fungi influenced by many fungicides have been assessed before, the conclusions were controversy in different reports attributed to field experiments (Wang *et al*., 2005). The observations in this study suggested that three fungicides hinder the initial growth of *G. etunicatum* spores directly, which was different from some assessments of several fungicides in field studies (Busse *et al*., 2004; Stok L Osa *et al*., 2011). The result indicating that AM fungi are directly affected by fungicides, which was confirmed by the data of Succinate dehydrogenase and Alkaline phosphatase activities. One possible reason is that fungicides were often applied onto the surface of the soil and often hard to penetrate into the deep part of the soil which is usually inhabited by AM fungi. The other possible reason is that the presence of fungicides may also affect the production of cell wall–degrading enzymes by AM hyphae. These enzymes are essential for root infection by mycorrhizal fungi to occur (Abd-Alla *et al*., 2000). Another possible reason is that a chemical can be converted or divided into other chemicals by some soil prokaryotes. The toxicity of the converted chemicals can be stronger or weaker than the former chemicals, which were often observed in the studies related to degrade toxic waste (Maruyama *et al*., 2007; Saien and Khezrianjoo, 2008).

Fungicides were reported to affect the metabolism as well as physiological and biochemical functions of the mycelium, reduce the activity of enzymes related to growth, metabolism and differentiation, and thus ultimately produce toxic effects on the mycelium Diedhiou *et al*. (2004) reported that under greenhouse conditions, before AM fungus and host plant establish symbiosis, azoxystrobin and kresoxim-methyl application significant reduced the root colonization and the production of daughter spores; but after the symbiosis had been established, the influence of root colonization was not so obvious. Similar results was also observed in our study with the dual culture system, the fungicides not only inhibited the spores germination and hyphal elongation of fungus *G. etunicatum*, but also inhibited the growth of carrot hairy roots with/without fungus *G. etunicatum*to some extent. Nevertheless, the biomasses of the roots inoculated with fungus *G. etunicatum* were more than that of the roots without fungus inoculation as seen in Table 2.

MDA is one of the most important products of cell lipid peroxidation and normally used to show the degree of lipid peroxidation in the host plants. It could be seen from Fig. 1 that all the MDA contents of the roots treated by fungicides were higher than that without fungicides treatment, while the MDA contents of the roots with *G. etunicatum* inoculation were higher than that without fungus inoculation. The data indicated that the AM fungus in the symbiotic system reduces the toxic effects of fungicides on host plants to a certain extent by decreasing the accumulation of MDA and relieving lipid peroxidation in plants, and thus protecting the growth of the host plants in adversely environments. A possible explanation of protection mechanism is that AM fungus improves plant uptake of mineral nutrition by expanding the absorption area of the plant root and enhancing the transport rate, accompanied by secretion and activation of a variety of enzymes. The results of this study similar to the alleviation effects of AM fungus to plant in adversely environments such as herbicide, heavy metal and salt stress (Aroca *et al*., 2012; Forgy, 2012; Li and Miao *et al*., 2013; Mohammad and Mittra, 2013). Colonization of host plant roots is a prerequisite for growth, development, and production of daughter spores of AM fungi. Nevertheless, the application of fungicides often exerts detrimental effects on gemination of spores and mycelial elongation. This means that high dose and long time application of fungicides on plant fungal diseases might result reduction in the number and diversity of AM fungi in the agricultural ecosystems, which in turn adverse to the growth of host plants. Without the protection of probiotics in the fields like the AM fungus, more soil-borne diseases will take place. In the long run, the ecosystems will trap in vicious circles. Therefore, limitation of the toxic effects of agricultural chemicals such as fungicides, herbicides and pesticides on mycelial growth and metabolism, as well as the formation, function and colonization of arbuscular mycorrhiza, is a key factor in maximizing the positive effects of arbuscular mycorrhizal symbiosis.

In conclusion, the present study clarified that fungicides (chlorothalonil, carbendazim and thiram) had adverse influence on the AM fungus under strict *in vitro* culture conditions. Thiram was less toxic to the plant compared than the other two fungicides. The results of this study are useful in instruction the use of fungicides with the consideration of ecological effects.

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