Arbuscular mycorrhizal fungi can alleviate the adverse effects of chlorothalonil on *Oryza sativa* L.

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Abstract

A glasshouse pot experiment was conducted to investigate the effect of the fungicide chlorothalonil on the growth of upland rice, in the absence or presence of the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* (NM and GM treatments). The plants were grown with three concentrations of chlorothalonil (0, 50 and 100 mg kg\(^{-1}\) soil). Mycorrhizal colonization decreased significantly with increasing chlorothalonil concentrations. Plant biomass decreases were smaller in GM plants than in non-mycorrhizal (NM) plants. Mycorrhizal dependency was the highest with 50 mg kg\(^{-1}\) chlorothalonil. Chlorothalonil affected physiological processes in upland rice irrespective of inoculation. Chlorothalonil at 50 and 100 mg kg\(^{-1}\) increased ascorbate peroxidase (APX) activity and soluble protein concentrations in shoots and roots of NM upland rice. However, values of APX, catalase (CAT) and peroxidase (POD) were reduced more in GM plants than in NM plants. These results showed that chlorothalonil induced oxidative stress in upland rice and it is needed to evaluate the side effects of chlorothalonil on rice and AMF.

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1. Introduction

Application of the fungicide chlorothalonil to soil to control plant diseases has become a common practice in crop production in many parts of the world. Chlorothalonil is a chlorinated isophtalonitrile fungicide, used widely in agriculture, which reacts with functional cellular thiols and inhibits fungal respiration and energy metabolism (Godard et al., 1999). The fungicide chlorothalonil was considered to be non-selective and was used commonly to control a broad spectrum of plant diseases such as Brown Patch (*Rhizoctonia solani*), Dollar Spot (*Sclerotinia homoeocarpa*), Southern Blight (*Sclerotium rolfsii*) and Pythium Blight (*Pythium aphanidermatum*) (Sherrard et al., 2003). Chlorothalonil can be used in turfgrass as well as silviculture, particular in humid regions such as the Pacific Northwest and Georgia–Florida river basins. With a half-life of 45 days (USEPA, 1986), chlorothalonil can be translocated well beyond its point of application; it has been found in locations as remote as the surface microlayer and fog of the Chukchi Arctic ecosystem (Chernyak et al., 1996). Rice is one of the main crops in many regions of Southeast Asia. Rice blast (*Pyricularia oryzae* Cav) is the most harmful disease induced by fungi which occur widely in rice producing regions and can lead to severe yield loss. In many parts of China, chlorothalonil are being used to control this disease.
Because of non-systematic and the application characteristics of chlorothalonil, it is not yet clear whether its application can affect the growth of rice plants. It is well known that the application of this fungicide is primarily targeted to plant leaf surface. However, a thin plant canopy, over-application, or application followed by irrigation or rainfall may cause the accumulation of this fungicide in soils (Cisar and Snyder, 1991; Petrovic et al., 1996). Like other pesticide chlorothalonil has adverse effects on the non-target organisms such as soil microorganisms, plants and animals which not only interfere with the biochemical and physiological reactions but also influence the population of non-target organisms (Chen et al., 2001). As a non-selective fungicide chlorothalonil has the potential to cause changes to other soil microbes including arbuscular mycorrhizal fungi (AMF), a group of beneficial fungi in soils. Generally, there is a lack of understanding of the impacts of fungicides such as chlorothalonil on the AMF community in arable soils (Sigler et al., 2000). In previous research it had been proved that chlorothalonil can induce oxidative damage, one of the toxicity mechanisms, in isolated rat hepatocytes (Suzuki et al., 2004). The persistence of chlorothalonil, along with its frequency of use, warrants investigation of its short-term impacts on the soil bacterial and fungal populations, and hence the risk to soil ecosystems.

AM fungi are ubiquitous in terrestrial ecosystems, forming symbiotic associations with roots from the majority of plant species (Smith and Read, 1997). It has been shown that rice can support and benefit from AMF: Glomus mosseae (Secilia and Bagyaraj, 1992; Bilou et al., 2000; Zhang et al., 2005). In exchange for carbon from host plants, AMF colonization can facilitate plant uptake and transport of less mobile soil nutrients such as phosphorus (Bolan, 1991; Thingstrup et al., 2000; Jakobsen et al., 2001), enhance drought tolerance (Davies et al., 1993; Ruiz-Lozano et al., 2001; Kaya et al., 2003) and reduce pathogenic infections (Newsham et al., 1995; Abdalla and Abdel-Fattah, 2000). Previous studies have also shown that AMF can alleviate the toxicity of heavy metals to many plants, such as Trifolium subterraneum, Viola calaminaria and Oryza sativa L. (Tonin et al., 2000; Zhang et al., 2005). Nevertheless, there is little information available on the effects of chlorothalonil on AMF and plant growth. Due to the potential benefit of AMF-rice symbiotic relationship in upland rice production system, it is essential to understand the mechanisms by which chlorothalonil affects AMF-rice symbiosis. However, the effects of chlorothalonil on the mycorrhizal upland rice plants are unresolved now, especially whether the oxidative damage induced by this fungicide can occur in the plants is very interesting.

The aims of the present study were to examine the effects of chlorothalonil on the growth of upland rice, AMF-rice symbiosis, biochemical and physiological reactions especially the oxidative stress occurring in the upland rice with AMF.

2. Material and methods

2.1. Growth medium

The soil (paddy soil) was collected from Huzhou, Zhejiang province, China. Soil was sampled from the surface layer (0–20 cm) of cultivated fields which were free of contamination by fungicide. In Chinese Soil Taxonomy, the soil is defined as Stagnic Anthrosols (Cooperative Research Group On Chinese Soil Taxonom, 2001). The soil was sieved to pass a 2 mm mesh, autoclaved (121 °C, 2 h) to eliminate indigenous AMF, and then air-dried. Soil pH (soil:water 1:2.5) was 6.3 before sterilization. Olsen P was extracted by 0.5 mol l⁻¹ NaHCO₃ and determined colorimetrically by the vanadomolybdate method. The soil contained 11.75 mg Olsen P kg⁻¹ soil. In all treatments basal nutrients in solution were mixed in the soil at rates of 684 mg N (NH₄NO₃), 300 mg K (K₂SO₄), 180 mg Mg (MgSO₄) and 300 mg Ca (CaCl₂·2 H₂O) kg⁻¹ soil. The chlorothalonil concentrations of the soil were adjusted to 50 or 100 mg kg⁻¹ by adding chlorothalonil dissolved in 5 ml acetone, in order to maintain consistence with 50 and 100 mg kg⁻¹ chlorothalonil treatments, 5 ml acetone was added into the without chlorothalonil treatment. Each chlorothalonil level contained two treatments: with and without AMF. Each treatment had four replicates.

2.2. Plant growth conditions

Seeds of upland rice (Oryza sativa cv. 91 B 3) were surface-sterilized with 10% (v/v) peroxide (H₂O₂) for 10 min and immersed in deionized water for 24 h. They were then germinated on moist filter paper until radicals appeared and were selected for uniformity before sowing. The rice was inoculated with Glomus mosseae (BGC, XJ01) isolates (GM treatment) or left uninoculated control (NM treatment). Inoculum of Glomus mosseae from Professor YS Wang (Beijing Academy of Agriculture and Forestry, China) contained dried roots of grain sorghum, hyphae and spores. Plants were grown in round plastic pots (diameter: 4 cm; height: 8 cm) containing 460 g soil plus 40 g inoculum for the mycorrhizal treatments or 460 g soil plus 40 g inoculum which had been sterilized beforehand to eliminate the fungus for the NM treatments. The inoculum was mixed thoroughly with the soil. Three pre-germinated seeds were sown into each pot. Pots were regularly watered to weight with ddH₂O to maintain soil moisture at 70% of field water holding capacity. The pots were randomly arranged in an environment-controlled growth chamber for six weeks with 14 h photoperiod at 280 µmol m⁻² s⁻¹. Plants were harvested after eight weeks of growth. Shoots were firstly cut off, and roots were carefully washed free of soil with tap water.

2.3. Root colonization

The clean roots were cut into segments about 1 cm long. A randomly selected subsample of fresh root was taken for
assessment of root colonization. Roots were assessed the root colonization according to Phillips and Hayman (1970) with some modifications. Roots were cleared in 10% KOH at 90 °C for 30 min in a heat-water bath before KOH was removed from the root samples. The roots were then stained with acid fuchsins at 90 °C for 30 min in a heat-water bath before acid fuchsins was removed from root samples. Percent colonization determined by the grid intersection method (Giovannetti and Mosse, 1980).

2.4. Plant biomass

Shoots and roots of harvested plants were washed free of soil with tap water and dried at 70 °C for 72 h. The dry weight of shoots and roots was then determined and samples were ground to pass a 0.5-mm sieve.

2.5. Mycorrhizal dependency (MD)

MD was calculated as the ratio of dry weight of mycorrhizal upland rice shoots or roots to that of non-mycorrhizal upland rice with the same level of chlorothalonil (Mengen et al., 1978).

2.6. P analysis

Dried subsamples of shoots and roots were ground and submitted to double acid digestion (HNO₃–HClO₄) to analyze P concentrations by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Andrade et al., 2004).

2.7. Enzyme extractions and assays

Total enzyme activity was determined according to a modified method of Cord and Fridovich (1969). Fresh root/leaf samples were ground in liquid N₂ using a mortar and pestle. The ground samples were homogenized on ice in 10 ml homogenizing solution containing 50 mmol l⁻¹ potassium phosphate buffer, 1% (w/v) polyvinylpyrroli- done (pH 7.8) and extracted at 4 °C. The homogenate was filtered and centrifuged at 4000 g at 4 °C for 15 min. The supernatants were used for further analyses. POD activity was determined at 25 °C with guaiacol (Lagrimini, 1991). In the presence of H₂O₂, POD catalyzes the transformation of guaiacol to tetruguaiacol (brown product). This reaction was recorded at 470 nm with a U-3010 spectrophotometer (Hitachi, Co. Japan). The reaction mixture contained 100 mmol l⁻¹ potassium phosphate buffer (pH 6.0), 33 mmol l⁻¹ guaiacol and 0.3 mmol l⁻¹ H₂O₂. CAT activity was determined according to Chance and Maehly (1955). 0.2 ml enzyme supernatants were adding to 1 cm quartz cuvettes containing 3 ml CAT reagent (50 mmol l⁻¹ phosphate buffer, pH 7.0; 19 mmol l⁻¹ H₂O₂). The mixture was homogenized and the decomposition of H₂O₂ was followed at 240 nm (extinction coefficient of 0.036 mmol⁻¹ cm⁻¹) by decrease in absorbance. Enzyme specific activity was expressed as µmol of H₂O₂ oxidized min⁻¹ (g fresh weight (FW))⁻¹. Total APX activity was determined by measuring the oxidation rate of ascorbate at 290 nm (Asada, 1992).

2.8. Soluble protein concentration

After POD, CAT and APX activity were determined, the extractable enzyme solution was analyzed with an ultraviolet and visible spectrophotometer at 280 and 260 nm to determine the soluble protein concentration.

2.9. Data analysis

Results are presented as the mean ± se. Data were calculated and analyzed statistically using the analytical tools of Microsoft Excel 2003. Multiple comparisons between treatments were carried out using the SAS software.

3. Results

3.1. Mycorrhizal colonization

Root colonization was inhibited significantly with increasing chlorothalonil concentrations (Fig. 1). An 18.7% and 52.5% decrease was recorded respectively with 50 and 100 mg kg⁻¹ chlorothalonil compared with chlorothalonil-free soil.

3.2. Plant growth

Chlorothalonil significantly inhibited the growth of NM and GM upland rice (Fig. 2). A significant increase in shoot and root dry weights was observed in GM plants with 50 and 100 mg kg⁻¹ chlorothalonil treatments. Plants in
the 50 mg kg\(^{-1}\) chlorothalonil treatment showed the highest mycorrhizal dependency (data not shown).

### 3.3. Plant P concentrations

P concentration in shoots of upland rice was significantly decreased with increasing chlorothalonil concentrations (Table 1). With 50 mg kg\(^{-1}\) chlorothalonil, fungal colonization significantly decreased P concentration in the shoot, however, increased P concentration in the root compared with NM treatments. With 100 mg kg\(^{-1}\) chlorothalonil, AMF colonization significantly enhanced P concentration in shoots compared with NM plants.

### 3.4. Enzyme activities

Chlorothalonil at 50 and 100 mg kg\(^{-1}\) increased shoot and root APX activity of NM plants compared with chlorothalonil-free treatments (Table 2). However, this enzyme was most active in NM treatments at 50 mg kg\(^{-1}\) chlorothalonil and decreased significantly at 100 mg kg\(^{-1}\) chlorothalonil. Colonization by *Glomus mosseae* decreased APX activity significantly compared with NM plants with or without chlorothalonil addition.

AMF colonization significantly decreased shoot and root CAT activity compared with NM plants at the three chlorothalonil levels (Table 2). Application of 50 and 100 mg kg\(^{-1}\) chlorothalonil increased shoot CAT activity in NM plants. However, CAT activity in roots was much lower than in shoots with chlorothalonil-free treatments in NM plants.

With increasing chlorothalonil concentration, shoot POD activity in NM plants increased (Table 2). At the rate of 100 mg kg\(^{-1}\) chlorothalonil, this enzyme had the highest activity compared with chlorothalonil-free treatments in the absence of AMF. At three levels of chlorothalonil, inoculation by *Glomus mosseae* decreased shoot and root POD activity significantly compared with non-inoculation treatments.

### 3.5. Soluble protein concentration

Soluble protein concentrations of roots and shoots increased with increasing chlorothalonil concentrations (Table 2). However, values were lower in GM plants than in NM plants.

### 4. Discussion

It was demonstrated that chlorothalonil, even at the recommended application did (50 mg kg\(^{-1}\)) inhibit growth of NM plants, especially root growth. There was less effect of 50 mg kg\(^{-1}\) chlorothalonil on growth of GM plants than the effects on NM plant, which indicated that the rice plants benefit from the AMF colonization. However both NM and GM plants were severely affected by 100 mg kg\(^{-1}\) chlorothalonil which maybe lie in the fact that colonization by *Glomus mosseae* was negatively affected by chlorothalonil. Nevertheless, the benefit of AMF colonization was greater in the presence of fungicide than in the absence. This was shown especially by the 61% and 307% increase in shoot and root MD with 50 mg kg\(^{-1}\) chlorothalonil.
compared with NM plants. The benefits of AMF colonization to rice growth possible lied in the fact that the addition of chlorothalonil reduced the P concentration in plant tissues and AMF colonization improved P nutrition (higher P concentrations in plant tissues). In the previous studies it was reported that the application of fungicides inhibited the development of AMF symbioses. For example, Sreenivasa and Bagyaraj (1989) found a reduction in root colonization by AMF and spore production by copper oxychloride and Carbofuran. As a non-selective fungicide chlorothalonil has the potential to inhibit the growth of AMF indicating the percentage of colonization was significantly decreased with increasing chlorothalonil concentration. Most reports attributed the negative effects of fungicides on root colonization by AMF to their effects on spore germination at initial colonization stage (Nemec, 1980; Dodd and Jeffries, 1989). Fungicides may also affect root exudation (Ratnayake et al., 1978; Schwab et al., 1982) or the release of cell wall-degrading enzymes from AMF hyphae that are essential for AMF colonization in a manner similar to the case of root hair infection by rhizobia.

Chlorothalonil can induce biochemical and physiological reactions to non-target organisms such as oxidative stress. The induction of biochemical and physiological reactions is one of the toxicity mechanisms as indicated in isolated rat hepatocytes (Suzuki et al., 2004) and in plants (Chen et al., 2001). Plants cope with oxidative stress by the operation of antioxidative systems, comprising both enzymatic systems such as ascorbate peroxidase (APX), peroxidases (POD), catalase (CAT) and non-enzymic systems such as ascorbate peroxidase (APX), and catalase (CAT) and non-enzymic systems that act as free radical scavengers such as ascorbic acid, thiols, soluble protein and GSH (Foyer et al., 1994; Malecka et al., 2001; Sinha et al., 2005). The present study indicated that chlorothalonil produced higher enzyme activities and elevated the concentration of soluble protein in the shoots and roots of NM plants, showing that chlorothalonil induced oxidative stress. Colonization by *Glomus mosseae* led to lower enzyme activities and decreased the soluble protein concentration in the shoots and roots compared with NM treatments indicating that the oxidative stress was alleviated by AMF colonization. There are two possible hypotheses. On one hand, *Glomus mosseae* might make use of the chlorothalonil as carbon source and degrade the fungicide in the soil. More likely, nutritional improvements of AMF colonization might increase the resistance to chlorothalonil by different mechanisms. Further study will be needed to understand the molecular mechanisms underline chlorothalonil metabolism and plant responses.

In conclusion, the results of the present experiments reveal that chlorothalonil application, even at recommended application rates (50 mg kg\(^{-1}\)), caused reduction in plant growth. The plant growth reduction may be induced by the application of chlorothalonil directly or indirectly. This result emphasizes the need to evaluate the side effects of chlorothalonil on soil microbes, non-target organisms, which play a vital role in mineral nutrition of plants, before application in the field. However, inoculation with *Glomus mosseae* can alleviate the side effects induced by chlorothalonil.

**Acknowledgements**

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**References**


**Table 2**

Enzyme activities in shoots and roots of upland rice with three concentrations of chlorothalonil

<table>
<thead>
<tr>
<th>Chlorothalonil concentration (mg l(^{-1}))</th>
<th>Isolates</th>
<th>APX activity (μmol min(^{-1}) g(^{-1}) FW)</th>
<th>CAT activity (μmol min(^{-1}) g(^{-1}) FW)</th>
<th>POD activity (OD min(^{-1}) g(^{-1}) FW)</th>
<th>Soluble protein concentration (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>0</td>
<td>NM</td>
<td>1083 ± 51 c</td>
<td>78 ± 35 b</td>
<td>472 ± 18 b</td>
<td>14 ± 4 ab</td>
</tr>
<tr>
<td></td>
<td>GM</td>
<td>811 ± 122 d</td>
<td>51 ± 23 bc</td>
<td>370 ± 48 b</td>
<td>5 ± 1 abc</td>
</tr>
<tr>
<td>50</td>
<td>NM</td>
<td>1577 ± 243 a</td>
<td>178 ± 6 a</td>
<td>659 ± 36 a</td>
<td>19 ± 4 a</td>
</tr>
<tr>
<td></td>
<td>GM</td>
<td>1017 ± 175 c</td>
<td>64 ± 14 bc</td>
<td>482 ± 40 b</td>
<td>8 ± 0.4 bc</td>
</tr>
<tr>
<td>100</td>
<td>NM</td>
<td>1290 ± 41 b</td>
<td>129 ± 29 b</td>
<td>669 ± 43 a</td>
<td>15 ± 3 ab</td>
</tr>
<tr>
<td></td>
<td>GM</td>
<td>876 ± 47 d</td>
<td>47 ± 11 c</td>
<td>501 ± 77 b</td>
<td>5 ± 2 bc</td>
</tr>
</tbody>
</table>

Data in the table are expressed as mean ± se, n = 4. Different letters following means in columns indicate significant difference in the means of inoculation and non-inoculated treatments under levels of chlorothalonil by LSD at 5% level.


