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Influences of excessive Cu on photosynthesis and growth in ectomycorrhizal *Pinus sylvestris* seedlings

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Abstract: Growth and photosynthesis responses were measured for Scots pine(*Pinus sylvestris* L. cv.) inoculated with ectomycorrhizal fungi(*Suillus bovinus*) under 6.5 and 25 mg/L Cu treatments to evaluate ectomycorrhizal seedlings' tolerance to heavy metal stress. Results showed that excessive Cu can significantly impair the growth and photosynthesis of pine seedlings, but such impairment is much smaller to the ectomycorrhizal seedlings. Under 25 mg/L Cu treatment, the dry weight of ectomycorrhizal seedlings is 25% lower than the control in contrary to 53% of the non-mycorrhizal seedlings, and the fresh weight of ectomycorrhizal roots was significantly higher than those of non-mycorrhizal roots, about 25% and 42% higher at 6.5 and 25 mg/L Cu treatments respectively. Furthermore, ectomycorrhizal fungi induced remarkable difference in the growth rate and pigment content of seedlings under excessive Cu stress. At 25 mg/L Cu, the contents of total chlorophyll, chlorophyll-a and chlorophyll-b were 30% higher in ectomycorrhizal plants than those in non-mycorrhizal plants. O_2 evolution and electron transport of PSI and PSII were restrained by elevated Cu stress. However, no significant improvement was observed in reducing the physiological restraining in ectomycorrhizal seedlings over the non-mycorrhizal ones.

Keywords: copper; ectomycorrhizal *Pinus sylvestris*; photosynthesis

Introduction

It is well known that infection by mycorrhizal fungi can increase the tolerance of host plants to Cu(Bradley, 1982; Jones, 1986; Hartley, 1999; Karagiannidis, 2002), while the mechanism by which the fungi confer tolerance to the host plants is unclear. Lower concentration of Cu in stems and leaves, and higher amounts of the respective metals in roots were observed in tolerant ectomycorrhizal pine seedlings (Weissenhorn, 1995; Karagiannidis, 2002). This led to the hypothesis that the mycobiont may bind the metal to reduce the amount available for translocation to the shoots. However, not all fungi are equally effective in protecting the hosts. Furthermore, the relative tolerance of the fungus under axenic culture may not reflect its ability to enhance the tolerance of the phytobiont. Thus, there is a need to examine the relationship between mycorrhizal infection and the physiological aspects of metal toxicity in greater detail.

Copper, as an essential nutrient element for plants, is a component of various proteins and involved in photosynthetic and respiratory processes(Wilkins, 1985), but excessive Cu in environment is severe phytotoxic. While the overall inhibitory effects of Cu have long been recognized, and the specific physiological mechanisms through which it affects plants are poorly understood(Kabata-Pendias, 1985). Some studies have indicated that excessive Cu may alter membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthetic and respiratory processes, and even activate senescence(Sandmann, 1980; Van Assche, 1990; Fernandes, 1991). Thus exposure to Cu affects several essential metabolic processes in plants.

The objective of this study was to further investigate the methods by which mycorrhizal fungi alter Cu tolerance in pine. To facilitate this, an ectomycorrhizal fungus strain *Suillus bovinus* was chosen to associate with Scots pine seedlings(*Pinus sylvestris*), and two aspects of the effects of the fungi on Cu toxicity were compared: their effects on seedling growth and their effect on major physiological processes.

1 Materials and methods

1.1 Growth condition

1.1.1 Inoculums cultivation

Suillus bovinus mycelia were suspended in nutrition solution after Kottke *et al*.(Kottke, 1987) that contained in culture tubes(length 45 cm, ϕ 4 cm, previously sterilized for 4 h at 160℃) with gas inlet at the bottom. For an ample supply with oxygen for heterotrophic growth and to prevent the hyphae from settling down, the suspensions were continuously aerated with compressed air, purified by a passage through cotton filters. Cultivations incubated in water at 25℃ in the dark for 7 d. Then mycelia were harvested by filtering the suspension through a tea sieve. The separated mycelium was suspended in glucose-free nutrition solution and broken into smaller pieces by 3 min of gentle homogenization in a mixer. The resulting suspension used as the inoculums.

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1.1.2 Seedlings cultivation

Seeds of Scots pine were sterilized by shaking in 30% H_2O_2 for 10 min and placed on wet filter papers in Petri dishes, and then kept in a growth chamber (light intensity 160 μmol/(m^2 · s); 13 h light at 25℃ and 11 h dark at 18℃; 55% relative humidity). After one week when the primary roots were just visible, the seeds were transferred to Perlite (Agriperl, Perlite-Dämmstoffe GmbH & Co, Dortmund, Germany) soaked with diluted nutrition solution (10% full nutrition solution after Kottke *et al*. (Kottke, 1987)) and kept in the growth chamber for 4 weeks before they are treated for experiment.

1.1.3 Inoculation

4 weeks old Scots pine seedlings were moved out from the substrates carefully and the bare roots of seedlings were dipped into the above-described suspension of *Suillus bovinus*. Thereafter, the plants were transplanted into freshly prepared Perlite at a density of 25 seedlings per 0.1 m^2. When the seedlings had 35—45 needles and a well-developed root system with mycorrhiza on about 10%—20% of the short roots (Six weeks after application of *Suillus*), they were treated with different concentrations of excessive copper.

1.2 Treatment

According to the previous experiment (Huang, 2001), copper was added as $CuSO_4 \cdot 5H_2O$ to diluted nutrient to obtain 6.5 mg/L and 25 mg/L copper excessive nutrient solution before autoclaved. Afterward, the solution was used for watering the mycorrhizal and non-mycorrhizal plants. Control was watered by diluted nutrient solution. Four weeks later the seedlings were harvested for measurement and analysis.

1.3 Measurement

1.3.1 Biomass

After carefully removed from the substrates, the seedlings were washed with distilled water and either as a whole or separated into roots and shoots dried at 105℃ for 1 h, then 80℃ for other 10 h. They then were cooled in desiccators for 5 h and weighed on a semimicro-balance (Sartorius, Analytic AC 120s, Göttingen, Germany).

1.3.2 Pigments

0.25 g fresh leaves were thoroughly smashed in a mortar with 80% acetone (v/v) and kept in dark for 15 min for pigment extraction. Centrifugation at 5000 g for 10 min yielded a light to dark green supernatant. The sediment was washed with 80% acetone and centrifuged at 5000 g for 3 times and lasting 10 min each. The resulting supernatants were collected and filled up to 25 ml with 80% acetone. The extraction was measured and the amounts of total chlorophylls, chlorophyll-a, chlorophyll-b and total carotenoids were calculated follow Lichtenthaler (Lichtenthaler, 1987). To transform extracted chlorophylls into pheophytines one drop of 30% HCl was added to the above acetone extract.

1.3.3 Photosynthesis

Release of oxygen per minute was taken as a measure for photosynthesis rate with the Warburg technique (Umbreit, 1972). Two seedlings were placed in a Warburg vessel containing 0.1 mol/L Na_2CO_3/$NaHCO_3$-buffer at pH 9.0 librated in 25℃ water bath. Buffer in the bottom of the vessel supplied CO_2.

Photosynthetic electron transport was measured polarographically with isolated chloroplasts prepared after modified Walker, Oku and Tomita (Walker, 1971; Oku, 1976). Needles of 5 g fresh weights were ground in a mortar with 40—50 ml grinding medium in a Moulinex mixer for 30 s in dark. The resulting homogenate was filtered through 8 layers of gauze and the filtrate centrifuged at 2000 g (Labofuge III, Heraeus GmbH, Hamburg, Germany) for 10 min. The sediment was suspended in 40 ml washing medium, centrifuged at 500 g for 5 min and the chloroplasts located in the supernatant spun down at 2000 g for 5 min. The sediment-unbroken-chloroplasts were finally suspended in 1.0 ml storage medium and used for the experiments. The whole procedure was performed at 4℃.

Photosynthetic electron transport capacity of photosystem I (PS I) was determined after Han-Rho (Han-Rho, 1994) and Messdaghi (Messdaghi, 1995). Measurement of photosystem II (PS II) was according to Specht (Specht, 1987), Han-Rho (Han-Rho, 1994) and Messdaghi (Messdaghi, 1995) using a Clark-type electrode (Rank Brothers, Bottisham, Cambridge, England) (Kowallik, 1967). Measurements were performed at 25℃.

To break the chloroplasts, 1.5 ml of medium A (Han-Rho, 1994; Messdaghi, 1995) was added to 0.1 ml chloroplast suspension and filled in the electrode chamber. After 1 min, 0.5 ml of medium B (Specht, 1987; Han-Rho, 1994; Messdaghi, 1995) were added to this mixture and oxygen exchange was followed for 6 min in darkness and subsequently for 6 min in white light of 797 μmol/(m^2 · s).

1.3.4 Respiration

Consumption of oxygen was taken as a measure for respiration. It was determined manometrically with the Warburg technique. Two seedlings were placed in 0.1 mol/L phosphate buffer at pH 6.0 librated in 25℃ water bath. CO_2 release was absorbed by KOH. For details see Umbreit *et al*. (Umbreit, 1972).

1.4 Data analysis

Data were statistically analyzed using a one-way analysis of variance (ANVOVA), and when differences observed were significant, means were compared by the multiple range *t*-test at level of significance of 0.05 (Mirocsoft Excel).

2 Results

2.1 Growth analysis

After 4 weeks exposure to excessive copper, significant reductions of fresh weight were observed from 10 weeks old non-mycorrhizal seedlings, while no indication of impairing

influences by Cu^{2+} on ectomycorrhizal seedlings under both 6.5 and 25 mg/L Cu^{2+} treatments. As shown in Fig.1a, the non-mycorrhizal seedlings lost 40% to 50% fresh weight.

Comparing to the control, dry weight of both non-mycorrhizal and mycorrhizal seedlings were significant decreased under the two Cu-applications. The fresh weight of mycorrhizal seedlings was 25% and 42% higher than non-mycorrhizal seedlings at 6.5 and 25 mg/L Cu treatments respectively, while no significant differences of dry weight were observed between ectomycorrhizal and non-mycorrhizal seedlings.

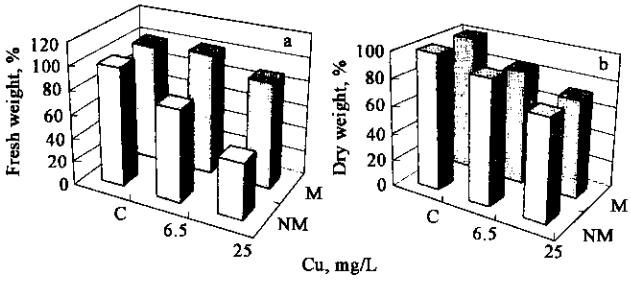

Fig.1 Influence of Cu on growth of *Pinus sylvestris* seedlings with/without association of ectomycorrhizal fungus *Suillus bovines*

a: fresh weight; b: dry weight (n = 6); C: control; M: mycorrhizal seedlings; NM: non-mycorrhizal seedlings

To understand the different responses of under-ground and aboveground components of plants to Cu stress, growth of roots and shoots was analyzed separately and the normalized results are presented in Fig.2. The measurements showed that the reduction of both fresh and dry weights of seedlings under excessive Cu was mainly due to decreased root weights. The fresh weight of Cu treated non-mycorrhizal shoots was only 2%—7% lower than the control, and the dry-weight was even higher at 6.5 mg/L Cu-application than that of the control, while the fresh weight and dry-weight of roots under Cu stress significantly decreased to 30%—50% of the control(Fig.2). Fresh and dry weights of mycorrhizal shoots, similar to non-mycorrhizal shoots, had no significant decrease under Cu-stress compared to the control (Fig. 2b, 2d). However, compared with the non-mycorrhizal roots, the mycorrhizal roots increased by 17%—20% in dry weight, and even by 100% in fresh weight. The biomass accumulation of mycorrhizal roots was significant higher(Fig. 2a, 2b). With the evidence that the fresh weights of the mycorrhizal roots gave no indications of impairing influence by excessive Cu, it led to a conclusion that a remarkable protective effect of the ectomycorrhizal fungi exists in ectomycorrhizal seedlings.

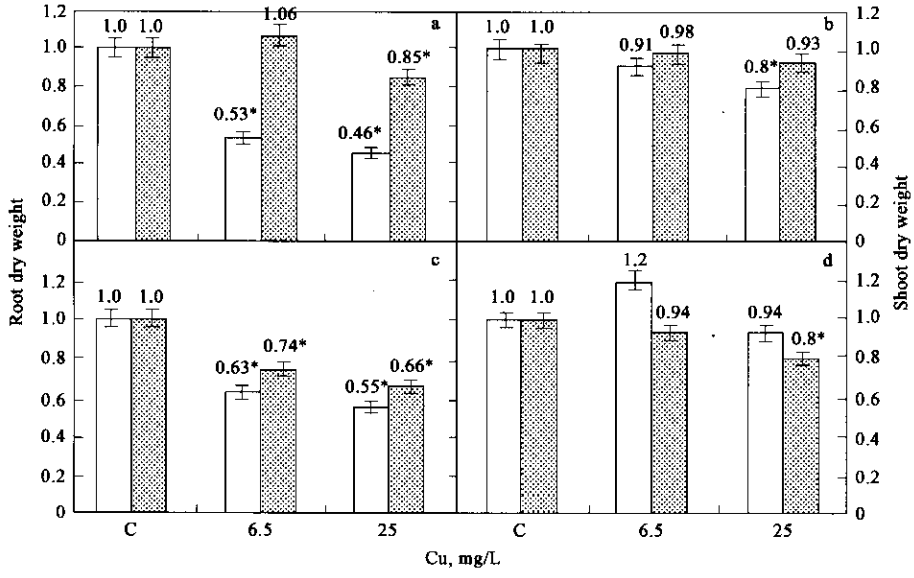

Fig.2 Comparison of biomass accumulation of Cu-treated mycorrhizal(▨) and non-mycorrhizal (□) seedlings of *Pinus sylvestris*

a: root fresh weight; b: shoot fresh weight; c: root dry weight; d: shoot dry weight(n = 6; data were normalized with control); * denotes significantly different from control at $p < 0.05$

2.2 Pigments

In non-mycorrhizal needles, the chlorophyll content had dropped significantly to about 70% of the control, but no significant difference was observed between both given Cu concentrations. The decrease was more pronounced in chlorophyll-a (25% to 35%) than in chlorophyll-b (about 12%), so chlorophyll-a/chlorophyll-b-ratios changed slightly from 2.7/L to 2.6/L and 2.3/L, respectively. The amounts of carotenoids were not significantly altered. The chlorophyll/carotenoid-ratio, therefore, was only about 4/L instead of 5/L in the control (Table 1).

The reddish-brown color of the needles which clearly observed in Cu treated seedlings' needles depended on anthocyanines. They could also be extracted from control plants, but in the seedlings exposed to 6.5 or 25 mg/L Cu treatment their amount was 2.7 and 6.4 times higher respectively(Table 1).

In ectomycorrhizal seedlings, the content of total chlorophyll dropped down to 84% of that of the control exposure to 6.5 mg/L and 68% under 25 mg/L Cu. This

resulted from a decrease in chlorophyll-a(Table 1). The ratio of chlorophyll-a/chlorophyll-b, therefore, dropped from 3.2 to 2.5 in the Cu-treated plants. This was different from the non-mycorrhizal plants in which chlorophyll-a and chlorophyll-b decreased at comparable rates under the influence of external Cu. Compared with non-mycorrhizal plants, content of the total chlorophyll, chlorophyll-a and chlorophyll-b were significantly 29%, 27% and 35% higher respectively in mycorrhizal plants under 25 mg/L Cu-treatment

Table 1 Influence of Cu on pigment contents in needles of mycorrhizal and non-mycorrizal *Pinus sylvestris* seedlings(n = 6)

	Control, μg/gDW		Cu treated (6.5 mg/L), μg/gDW		Cu treated(25 mg/L), μg/gDW	
Pigments	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza
Chlorophylls	1.75 ± 0.09	1.89 ± 0.06	*1.20 ± 0.05	*1.29 ± 0.03	*1.22 ± 0.05	*1.58 ± 0.04^x
Chlorophyll-a	1.28 ± 0.06	1.44 ± 0.06	*0.84 ± 0.04	*0.92 ± 0.02	*0.88 ± 0.02	*1.12 ± 0.03 ^x
Chlorophyll-b	0.47 ± 0.024	0.45 ± 0.010	0.36 ± 0.02	0.37 ± 0.006	0.34 ± 0.01	0.46 ± 0.006 ^x
Pheophytine	0.24 ± 0.01	0.25 ± 0.01	0.29 ± 0.015	0.28 ± 0.01	0.14 ± 0.007	0.30 ± 0.02 ^x
Carotenoids	0.35 ± 0.011	0.40 ± 0.004	0.31 ± 0.016	0.34 ± 0.010	0.33 ± 0.01	0.38 ± 0.004

Notes: *denotes significantly different from control at $p < 0.05$; ^denotes significantly different between mycorrhizal and non-mycorrhizal treatment

Although no statistically significant variations were observed in total carotenoids contents among all treatments and control, the mean carotenoids content of mycorrhizal seedlings was 5%—15% higher than non-mycorrhizal seedlings.

2.3 Photosynthesis

2.3.1 Photosynthesis rate

As expected from the lower chlorophyll content of the Cu-treated non-mycorrhizal seedlings, the photosynthetic oxygen production per unit fresh weight was lower than that of the control. It dropped down to about 60% at application of 6.5 mg/L Cu and only about 40% under the influence of 25 mg/L Cu. This impairment of photosynthesis did not only depend on the reduced amount of chlorophylls, but also the decreased efficiency of chlorophylls at high Cu stress(25 mg/L), at about 44% of that of the control plants(Table 2).

Table 2 Influence of excessive Cu on true photosynthetic oxygen production in mycorrhizal and non-mycorrizal *Pinus sylvestris* seedlings(n = 6)

	Control		Cu treated (6.5 mg/L)		Cu treated (25 mg/L)	
	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza
$\mu lO_2/(h \cdot mg\ FW)$	0.87 ± 0.01	0.83 ± 0.09	* 0.51 ± 0.03	* 0.38 ± 0.01	* 0.36 ± 0.012	* 0.28 ± 0.03
$\mu lO_2/(h \cdot mg\ Chl)$	1421.0 ± 4.26	1494.0 ± 36.76	1403.8 ± 7.2	1280.0 ± 230.72	' 619.8 ± 9.31	' 640.9 ± 72.96

Notes: * denotes significantly different from control at $p < 0.05$

Photosynthetic ratio of mycorrhizal plants showed the same impairment by excessive Cu. The O_2-production per total chlorophylls was decreased to 86% at 6.5 and only 43% at 25 mg/L Cu. There was no significant difference of photosynthetic oxygen production rate between mycorrhizal and non-mycorrhizal seedlings. This corresponded with the data of accumulated biomass of mycorrhizal and non-mycorrhizal seedlings under excessive Cu stress(Fig.1).

2.3.2 Activity of PS I and PS II

To narrow down the point of action of the extraneous Cu, the capacities of the two photo-systems were examined separately. Photo-system I activity was measured by oxygen consumption deriving from re-oxidation of methylviologen previously reduced by electrons from photo-system I in the light.

Both photo-systems exhibited lower activities in isolated chloroplasts from the Cu-treated than control seedlings with or without mycorrhiza, except mycorrhizal seedlings at 6.5 mg/L Cu that had a slightly higher activity than control. However, photo-system II was much more impaired than photo-system I. While non-mycorrhizal seedlings were not significantly affected at 6.5 mg/L Cu and still reached 80% of the control at 25 mg/L, photo-system II-activity was about 65% of the control at 6.5 mg/L and only 40% at 25 mg/L Cu (Fig.3). Whether inhibition of photosynthesis in presence of excessive Cu was additionally caused by negative effects on enzymes of the Calvin cycle shall be tested further.

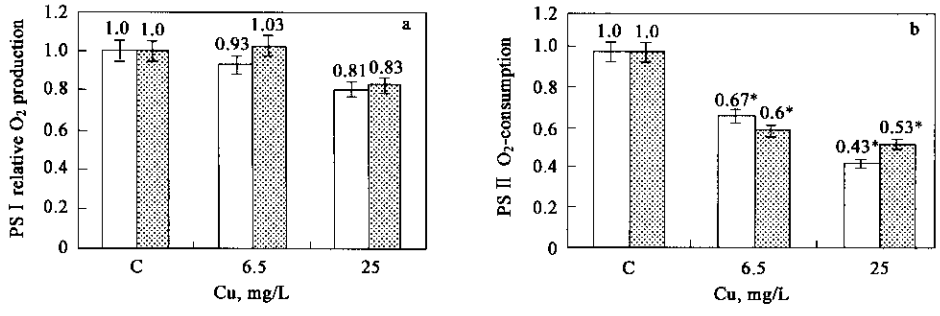

Fig.3 Normalized activities of photosynthetic systems(PS) I and II of mycorrhizal(▨) and non-mycorrhizal(□) seedlings under different Cu concerntrations
a: PS I; b: PS II (n = 6; data were normalized with control; * denotes significantly different from control at $p < 0.05$)

The mycorrhizal seedlings showed almost the same results and there is no significant difference in activity of photo systems between mycorrhizal and non-mycorrhizal seedlings under excessive Cu stress. Therefore, no protective effect of the fungi on photosynthesis of the seedlings was detected.

2.3.3 Respiration

Determined by O_2-uptake in the dark, respiration of Cu treated non-mycorrhizal seedlings was more than two times higher at exposure to 6.5 mg/L and about 15% higher at 25 mg/L Cu. The increase at 6.5 mg/L might generally be considered as a response to physiological stress situations. Considering this for the plants at 25 mg/L Cu-treatment, their lower O_2-uptake would indicate some other not identified damages (Table 3). Respiration of mycorrhizal seedlings showed the same response to Cu stress as non-mycorrhizal seedlings. Its respiration of Cu treated seedlings was 70% higher at 6.5 g/ml and 10% at 25 mg/L Cu than that of the control plants(Table 3).

Table 3 Influence of excessive Cu on respiratory O_2-uptake of mycorrhizal and non-mycorrhizal seedlings of *Pinus sylvestris* ($n = 6$)

	Control		Cu treated(6.5 mg/L)		Cu treated(25 mg/L)	
Pigments	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza	Non mycorrhiza	Mycorrhiza
$\mu lO_2/(h \cdot mg\ FW)$	0.12 ± 0.01	0.15 ± 0.03	0.17 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.01
$\mu lO_2/(h \cdot mg\ Chl)$	200.61 ± 8.2	264.68 ± 45.91	456.15 ± 17.7	450.40 ± 43.37	232.81 ± 10.4	292.95 ± 34.55

The ectomycorrhizal seedlings' respiratory O_2-uptake under high Cu-concentration was largely identical with that of non-mycorrhizal seedlings(Table 3). However, at the low Cu concentration(6.5 mg/L), the respiratory O_2-uptake rate of mycorrhizal seedlings was significantly 33% lower than that of non-mycorrhizal plants. Given the above result was derived from the reactions of seedlings and attached hyphae, it did not allow conclusion on specific mutual influences of the partners.

3 Discussion

Excessive Cu can significantly reduce the accumulation of biomass of seedlings, and under-ground components(root system) are much more sensitive to heavy Cu stress(Fig.1). This inhibiting effect of Cu on biomass accumulation in roots was observed in both mycorrzhial and non-mycorrhizal seedlings. However, the fresh weight of mycorrhizal roots was significant higher than that of non-mycorrhizal roots, showing improved ability of mycorrhizal seedlings to keep water in roots. This result also matches with Jones and Hutchinson's research result illustrating that Cu is one of the heavy metals that can easily be accumulated in roots(Jones, 1988), and lead to inhibition of root development through disturbing metabolisms of roots.

The increased water content in plants may lead to a lower concentration of heavy metal ions in cell and alleviate the photoxicity from metal ions. Therefore, keeping high water content in roots to dilute toxic ions in cell might be one of the ways in which mycorrhiza protect the host plant against excessive Cu stress. Furthermore, ectomycorrhizal root system is composed of mantle and bare roots. Mantle is a weft of loose mycelium over the surface of short roots. There are great amount of slim and air filled in the interspaces of mycelia(Jackson, 1984) that may also contribute to a higher fresh weight of mycorrhizal roots. According to Denny and Ridge's research on Zn, slim of ectomycorrhizal fungi could chelate with excessive heavy metal ions that might decrease ions bioavailability and phytoxicity of its host plant(Denny, 1995). The higher tolerance of mycorrhizal seedlings to excessive Cu may cause by exudates of mycelia and mycorrhizal roots that chelate free Cu and reduce bioavailability of Cu. Further research need to be done on relationship between exudates of mycorrhizal plant and its tolerance to excessive heavy metals.

Increases of respiration O_2 consumption were measured in both mycorrhizal and non-mycorrhizal seedlings exposed to Cu treatments compared to the control. Plants' respiration has two components, growth respiration and maintenance respiration (Amthor, 1984). Physiological adaptations to harsh environments can increase the need for maintenance respiration, and thus the overall respiration rates would be expected to increase. Jones and Hutchinson's research on Ni toxicity also obtained similar result (Jones, 1988). In comparison to non-mycorrhizal seedlings, mycorrhizal seedlings had a significantly lower respiration rate, indicating that mycorrhizal fungi might provide help to host plants and reduce the maintenance respiration. In this experiment, a decreasing trend of respiration O_2 consumption was monitored at a higher Cu stress. This might occur due to the severe damage to the plants from Cu, and the physiological processes became very weak under extreme harsh environment.

Cu is reported to change a number of physiological processes and reduce the content of chlorophyll to induce red leaf of seedlings(Prasad, 2001). Similar result also observed from this experiment, i.e. the chlorophyll content was reduced in needles of Cu treated mycorrhizal and non-mycorrhizal seedlings (Table 1). As a result of low chlorophyll content, the treated plants have lower photosynthesis, and this result match the result of biomass measurement described in above. This also illustrated that excessive Cu stress in environment can cause damage to normal physiological processes of plants.

The observed significant effect of Cu on chlorophyll

content indicated that it had been taken up into the photobiotic cells. The mycorrhizal seedlings, compared to non-mycorrhizal ones, had much higher content of chlorophyll-a, b and total chlorophyll, and this can be also observed from the healthier and greener look of the mycorrhizal plants. As the loss in chlorophyll content could be due to proxidation of chloroplast membranes mediated by Cu (Baszynski, 1988), and Cu induced damage to photoxidative mechanisms (Prasad, 1999; Chettri, 1998), mycorrhizal fungi may help the host plants to prevent the Cu induced detrimental changes to chlorophlast structure and functions, and alleviate the damage from excessive Cu stress.

However, there is no significant difference in activity of PS I and PS II between mycorrhizal and non-mycorrhizal seedlings (Fig.2). This result consents with Pankovica *et al*. (Pankovica, 2000) and Jones and Hutchinson's (Jones, 1988) work on Cd and Ni. They found that ectomycorrhizal fungi increased host plants' tolerance to heavy metals, but not by preventing metal-induced reductions in photosynthesis rates or by affecting shoot respiration rates. The incompatible result between growth rate and chlorophyll content can due to copper inhibits the process of cell division independently of any effect on the production of new cell material (Stauber, 1987). Further research on a Cu accumulation and distribution in different cell of mycorrhizal plant need be carried out to understand if ectomycorrhizal can prevent the Cu to transport into shoots cell of plant.

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