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Micronutrient deficiencies accelerate leaf senescence in *Amomum villosum*

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ABSTRACT. Fruit yield decreases gradually after several years of cultivation in *Amomum villosum*, an important Chinese medicinal herb, which is generally attributed to plant senescence. However, the reasons for plant senescence are still not clear until now. In this study, we investigated the effects of micronutrient deficiencies on leaf senescence. We hypothesized that micronutrient deficiencies may cause oxidative stress by disturbing cell metabolisms, which in turn leads to leaf senescence and decrease growth in *A. villosum* plants. Deficiencies of copper, zinc, and manganese decreased light-saturated photosynthetic rate and the contents of chlorophylls and soluble proteins but increased malondialdehyde content in *A. villosum* plants. The results indicated that oxidative stress and senescence occurred in leaves of *A. villosum* plants grown under micronutrient deficient conditions. *Amomum* plants were more susceptible to zinc deficiencies than to deficiencies of copper and manganese. The negative effects of micronutrient deficiencies on CO_2 assimilation in chloroplast stroma were more serious than on electron transfer in thylakoids, increasing excess excitation energy. In most cases, micronutrient deficiencies decreased thermal dissipation of excess excitation energy and carotenoid content, leading to oxidative stress although antioxidant enzyme activities increased. Our results indicated that the micronutrient deficiencies caused oxidative stress, exacerbating leaf senescence and inhibiting biomass accumulation in *Amomum* plants.

Keywords: Antioxidant enzymes; Chlorophyll *a* fluorescence; Copper; Growth; Malondialdehyde; Manganese; Oxidative stress; Photosynthesis; Zinc.

INTRODUCTION

Leaf senescence, as the final stage of leaf development, involves degradation of many cellular and sub-cellular structures and macromolecules, and mobilization of the products of the degradation to newly emerged leaves and other parts of the plant (Ohe et al., 2005). The decreases in photosynthesis and chlorophyll content are two of the most striking events during leaf senescence due to the degradation of both structural and functional components of chloroplasts (Yoshida, 2003; Xie et al., 2011). Many earlier studies showed that senescent leaves usually experienced a substantial decrease in photosynthetic capacity accompanied by only a slight decrease in photosystem II (PS II) photochemistry, which may potentially expose the leaves to excess excitation energy. Under such conditions, molecular oxygen operates as an alternative acceptor of non-utilized electrons and light energy, thus resulting in the generation of reactive oxygen species (Mittler et al., 2004). Among these species, superoxide anion and hydrogen peroxide (H_2O_2) are relatively unreactive when separate, but they can react with each other in a Haber-Weiss reaction to form highly reactive hydroxyl radical and other derivatives (Bowler et al., 1992). These reactive oxygen species can cause lipid peroxidation and therefore accumulation of malondialdehyde, protein denaturation and DNA mutation. To prevent oxidative damage, plant cells are equipped with an antioxidant system consisting of low-molecule-weight antioxidants and protective enzymes. Superoxide anion is scavenged by superoxide dismutase (SOD), while the product of this reaction, H_2O_2, can be detoxified in the ascorbate-glutathione cycle (Nakano and Asada, 1981).

Unfavorable environmental factors such as water stress, extreme temperatures, UV-B irradiation and nutrient deficiencies can result in induced premature senescence. Micronutrient deficiencies including zinc, copper and manganese can cause inhibition of growth and development of plants and eventually severe reductions in yield. Increasing evidence indicates that oxidative damage to critical cell compounds is the basis of disturbances in plant growth

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caused by micronutrient deficiencies (Mittler et al., 2004). Zinc deficiency is very common in different climatic regions of the world according to an extensive soil survey. Sillanpää (1990) found that approximately 30% of the soil samples collected from 25 countries contained a deficient level of plant-available zinc. In China about 60 million ha cropland is prone to be zinc deficient (Liu, 1996). Zinc plays a fundamental role in several critical cellular functions such as protein metabolism, gene expression, structural and functional integrity of biomembranes (Marschner et al., 1996; Woo et al., 2010; Breeze et al., 2011). Enhanced superoxide anion generation by NADPH oxidase and impaired detoxification of superoxide anion and H_2O_2 due to zinc-deficiency-related inhibition of antioxidant enzymes could lead to an elevated level of superoxide anion and its derivatives, thus creating oxidative stress (Wenzel and Mehlhorn, 1995). Copper deficiency can directly impact energy metabolisms because it affects the synthesis of plastocyanin and cytochrome oxidase, two copper-containing electron carriers. Under the conditions of copper deficiency, photosynthesis and respiration decrease and more electrons contribute to the formation of reactive oxygen species (Pilon et al., 2006). Manganese is essential for growth and survival of plants because of its function as a redox cofactor activating at least 35 enzymes (Allen et al., 2007). In photosynthetic organisms, manganese is also present as a polynuclear cluster in PS II, where it catalyzes the water-splitting reaction (Merchant and Sawaya, 2005). Manganese-SOD and PS II are expected to be the prime targets of the impact of manganese deficiency in plants (Yu and Rengel, 1999). Manganese deficiency has been found to decrease quantum yield efficiency and change fluorescence induction kinetics in *Hordeum vulgare* (Husted et al., 2009).

Amomum villosum Lour. (Zingeraceae), a traditional Chinese medicinal herb, has been cultivated in the understory of seasonal rainforest for more than 40 years in Xishuangbanna, Yunnan Province, southwest China. Fruits of this plant are widely used to cure gastritis, stomachache, and digestive troubles in East and Southeast Asia (Zou, 1993). The economic benefit from cultivating *A. villosum* in rainforest is very important for minorities in Xishuangbanna, which sometimes accounts for almost half of their total income (Liu et al., 2006). However, *A. villosum* fruit yield decreases obviously after about seven-year cultivation due to plant senescence, which is suspected to be partially associated with micronutrient deficiencies. Recently, Li et al. (2007) found that nutrient deficiencies including potassium, calcium, and magnesium were closely related to the incidence of leaf blight disease, which caused a 30-40% decrease in fruit yield of *A. villosum*. Until now no experimental study was conducted to explore the relationship between micronutrient deficiencies and leaf senescence in *A. villosum*, and a thorough understanding of the relationship is helpful to improve *A. villosum* cultivation practice. In this study, we investigated the effects of deficiencies of zinc, copper, and manganese on leaf senescence in *A. villosum* plants. We hypothesized that micronutrient deficiencies may cause oxidative stress by disturbing cell metabolisms, which in turn results in leaf senescence and decreased growth in *A. villosum* plants.

MATERIALS AND METHODS

Materials

This study was carried out at Xishuangbanna Tropical Botanical Garden (21°56′ N, 101°15′ E) of the Chinese Academy of Sciences, located in Mengla County, Yunnan Province, southwest China. The mean annual temperature and precipitation are 21.5°C and 1,557 mm, respectively (Feng et al., 2002). In May, similar-sized seedlings with one or two small leaves of *A. villosum* plants were collected from a 100-year-old seasonal rainforest, and were singly planted into 3 L plastic pots containing 2 L Hoagland's solution. The seedlings were grown in a shade house with 36% irradiance, on top of which a white plastic film was put to avoid the influence of rain on the concentration of nutrient solution and the solution was changed every two weeks. The amount of water evapotranspired from each pot was supplied daily at 17:00 to maintain the nutrient solution concentration. Three months later (in August), 60 similar-sized seedlings were chosen and randomly divided into four groups. The control group was cultivated with full Hoagland's solution during the experiment and for treatments designated as zinc, copper, and manganese deficiencies, the corresponding element was omitted from the solution. Photosynthesis and chlorophyll *a* fluorescence were measured on 10 plants per treatment at the 12th and 42th dates of treatments, respectively. Afterwards, the youngest fully expanded leaf (the fourth leaf from top) was sampled from each sample plant (three per treatment) for chemical determinations. After determination of the above variables, 10 plants per treatment were harvested by taking them (including roots) from cultural solution, dried at 80 °C for 48 h and weighed.

Measurements

Leaf gas exchange. Light-saturated photosynthetic rate (P_{max}) was measured at 1500 μmol m^{-2} s^{-1} irradiance (based on preliminary experiments) using a portable photosynthesis system (Li-6400, Li-Cor, Lincoln, NE) during 8:30 - 10:30 in a sunny day. During this period, no photoinhibition occurred and maximum photosynthetic rates could be measured for *A. villosum* plants. Leaf temperature, CO_2 concentration in reference chamber and air humidity in leaf cuvette were controlled at 25°C, 360 μmol mol^{-1} and 60%, respectively.

Chlorophyll a fluorescence. Chlorophyll *a* fluorescence emission was measured during 8:30 - 10:30 using a portable pulse-modulated fluorometer (FMS 2, Hansatech, UK). The sample leaf was dark-adapted for at least 15 min, and the minimum fluorescence yield (F_o) was measured under a weak modulating beam. Maximum fluorescence yield (F_m) was determined by irradiating the dark-adapted leaf with a saturated pulse (5,000 μmol

m^{-2} s^{-1}, 0.7 s). After the fluorescence yield dropped from F_m nearly to F_o, "actinic light" source (400 μmol m^{-2} s^{-1}) was switched on, and the fluorescence yield started to increase. Steady-state fluorescence yield (F_s) was recorded when the fluorescence yield becomes stable. Afterwards, the leaf was again irradiated with a pulse of the saturated beam and the maximum light-adapted fluorescence yield (F_m') was determined. Finally, the minimum light-adapted fluorescence yield (F_o') was detected by turning off the "actinic light" and 3 s later switching on the far-red radiation source for 5 s. Maximum photochemical efficiency of PS II was calculated as: $F_v/F_m = (F_m - F_o)/F_m$, quantum yield of PS II photochemistry as: $\Phi_{PSII} = (F_m' - F_s) / F_m'$, photochemical quenching coefficient as: $q_p = (F_m' - F_s) / (F_m' - F_o')$, and non-photochemical quenching coefficient as: NPQ = $(F_m/F_m' - 1)$ (Bilger and Björkman, 1990).

Malondialdehyde, soluble protein and chlorophyll pigments. About 0.5 g leaf was homogenized in 10 ml of 10% trichloroacetic acid, and the homogenate was centrifuged at 12,000 ×*g* and 4°C for 10 min. After that, 2 ml 0.6% thiobarbituric acid in 10% trichloroacetic acid was added to an aliquot of 2 ml from the supernatant. The mixture was incubated in boiling water for 30 min, and then quickly cooled in an ice bath. After centrifugation at 10,000 ×*g* for 10 min, the absorbance of the supernatant was determined at 450, 532, and 600 nm, respectively. Malondiadehyde content was calculated according to Hodges et al. (1996). Leaf soluble protein content was determined with the method of Bradford (1976), using bovine serum albumin as a calibration standard. Leaf chlorophyll and caroteniods were extracted with 80% acetone, and were measured with the method of Lichtenthaler and Wellburn (1983).

Antioxidant enzyme activities. Enzymes were extracted by homogenizing sample leaf in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 ×*g* and 4°C for 20 min and the supernatant was used for the following enzyme assays. Superoxide dismutase (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium at 560 nm (Giannopolitis and Ries, 1977). Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was analyzed by following the decrease in ascorbate at 290 nm (Nakano and Asada, 1981). Catalase (CAT) (EC 1.11.1.6) activity was measured as the decline of the extinction of H_2O_2 at 240 nm (Aebi, 1984).

Statistical analysis. A one-way ANOVA with a post-hoc test (Duncan test) was conducted to test the differences among treatments in each variable measured in this study. The analysis was carried out using SPPS 13.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Chloroplast pigments and soluble proteins

The contents of chlorophylls and carotenoids decreased significantly during the 42 days experiment in *A. villosum* plants grown in full nutrient solution (Figure 1a, b). Micronutrient deficiencies (zinc, copper, and manganese) intensified the decreases in chlorophylls and carotenoids. Micronutrient deficiencies also decreased soluble protein content in *A. villosum* plants (Figure 1c). Harmful effects of zinc deficiency on this plant were the most serious among the three micronutrients; visible symptoms such as pallid or chlorotic leaves appeared in zinc deficient plants after 40 days of treatment. However, no plant died during the experiment.

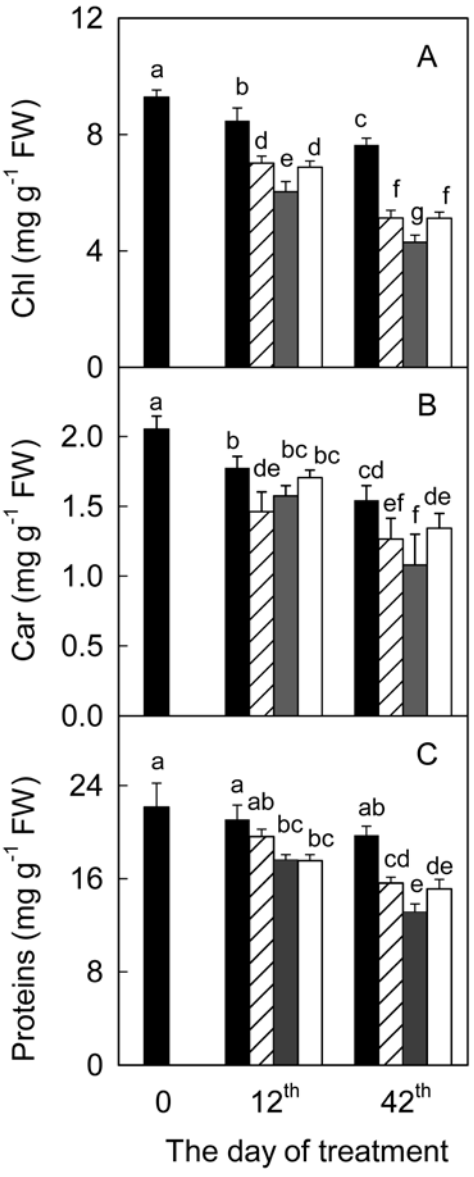

Figure 1. Effects of micronutrient deficiencies on the contents of chlorophylls (Chl; a), carotenoids (Car; b), and soluble proteins (c) in leaves of *Amomum villosum* plants grown at the 12th and 42th days of treatment. Means ± SE (n = 3). Different letters indicate significantly different at P = 0.05 level. Filled bar, control; hatched bar, copper deficiency; gray bar, zinc deficiency; open bar, manganese deficiency.

Photosynthesis and chlorophyll *a* fluorescence

Light-saturated photosynthetic rate decreased significantly during the 42 days experiment in full-nutrient-grown *A. villosum* plants, and micronutrient deficiencies significantly accelerated the decrease in P_{max} (Figure 2a). Maximum photochemical efficiency of PS II changed little with time in full-nutrient-grown plants, and the effects of micronutrient deficiencies on F_v/F_m were small. At the 12th day of treatment, zinc, copper, and manganese deficiencies did not significantly decrease F_v/F_m; at the 42th day of treatment, only zinc deficiency significantly decreased F_v/F_m (Figure 2b). Quantum yield of PS II photochemistry decreased significantly during the 42 days experiment in full-nutrient-grown *A. villosum* plants (Figure 2c). The effects of micronutrient deficiencies on Φ_{PSII} were not significant at the 12th day of treatment. However, the effects of copper and zinc deficiencies became significant at the 42th day of treatment. Micronutrient deficiencies decreased q_p significantly at the 42th day of treatment, while only manganese deficiency decreased q_p significantly at the 12th day of treatment (Figure 2d). For plants grown in full nutrient solution, NPQ increased at the 12th day of treatment, and then decreased significantly at the 42th day of treatment (Figure 2e). At the 42th day of treatment, micronutrient deficiencies decreased NPQ significantly. However, zinc deficiency increased NPQ at the 12th day of treatment.

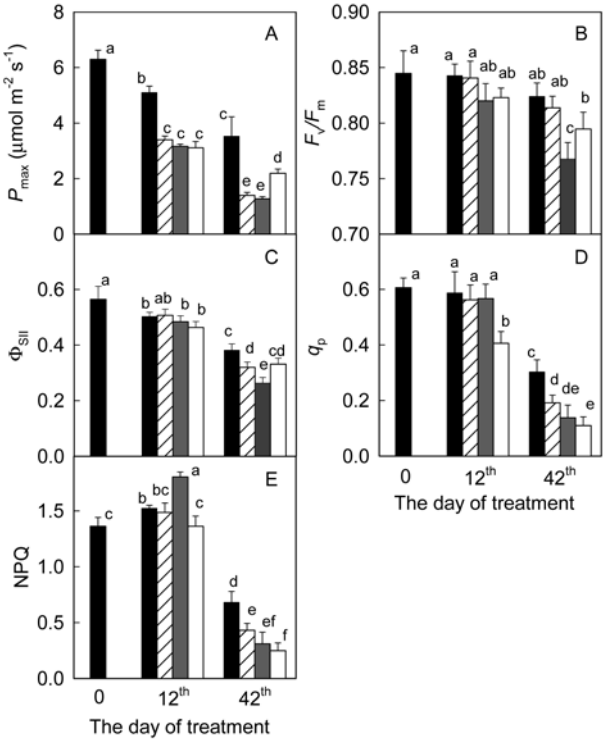

Figure 2. Effects of micronutrient deficiencies on light-saturated photosynthetic rate (P_{max}; a), maximum photochemical efficiency of photosystem II (F_v/F_m; b), quantum yield of photosystem II photochemistry (Φ_{PSII}; c), photochemical quenching coefficient (q_p; d), and non-photochemical quenching coefficient (NPQ; e) in leaves of *Amomum villosum* plants grown at the 12th and 42th days of treatment. Means ± SE (n = 10). Different letters indicate significantly different at P = 0.05 level. Filled bar, control; hatched bar, copper deficiency; gray bar, zinc deficiency; open bar, manganese deficiency.

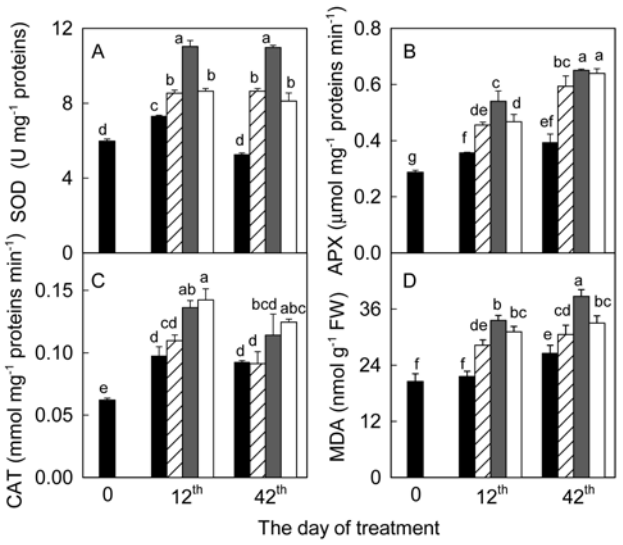

Figure 3. Effects of micronutrient deficiencies on the activities of superoxide dismutase (SOD; a), ascorbate peroxidase (APX; b), catalase (CAT; c), and the content of malondialdehyde (MDA; d) in leaves of *Amomum villosum* plants grown at the 12th and 42th days of treatment. Means ± SE (n = 3). Different letters indicate significantly different at P = 0.05 level. Filled bar, control; hatched bar, copper deficiency; gray bar, zinc deficiency; open bar, manganese deficiency.

Antioxidant enzyme activities and malondialdehyde content

For full-nutrient-grown plants, SOD increased significantly at the 12th day of treatment, then decreased sharply to the original level at the 42th day of treatment (Figure 3a). However, APX and CAT increased significantly at the 12th day of treatment and maintained at high level at the 42th day of treatment for the control plants (Figure 3b, c). Micronutrient deficiencies increased activities of SOD and APX. Activity of CAT was also increased by deficiencies of zinc and manganese but not by copper deficiency. Malondialdehyde content increased at the 42-day of treatment in full-nutrient-grown *A. villosum* plants, and micronutrient deficiencies increased malondialdehyde content significantly (Figure 3d).

Total biomass

After 42 days of treatments, zinc, copper, and manganese deficiencies decreased total biomss of *A. villosum* by 16.9%, 37.3%, and 20.4%, respectively. However, the effects of copper and manganese deficiencies were not significant (Figure 4).

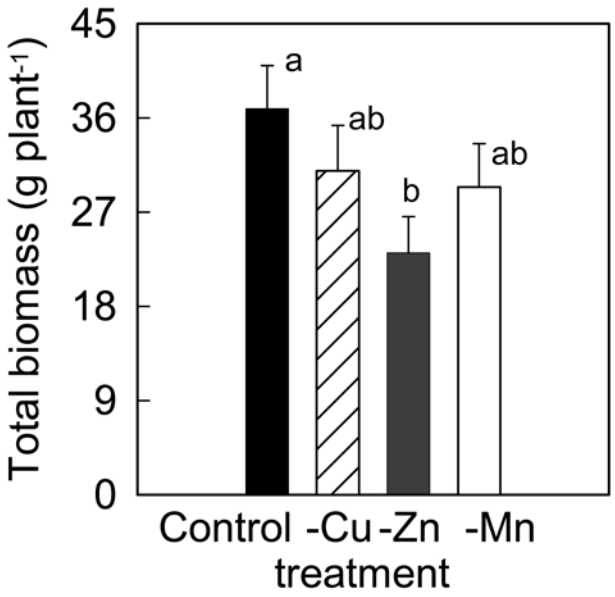

Figure 4. Effects of micronutrient deficiencies on total biomass in *Amomum villosum* plants grown at the 42th day of treatment. Means ± SE (n = 10). Different letters indicate significantly different at P = 0.05 level. Filled bar, control; hatched bar, copper deficiency; gray bar, zinc deficiency; open bar, manganese deficiency.

DISCUSSION

Leaves of *A. villosum* plants grown in full nutrient solution experienced significant senescence during the 42 days experiment (in August and September), as indicated by the decreased chlorophyll content and P_{max} (Figures 1a; 2a). Leaf chlorophyll content can be used as an indicator of leaf ontogeny and a rapid decrease in chlorophyll content is widely observed during leaf senescence in many plants (Hunter et al., 1999; Prochazkova et al., 2001; Yoo et al., 2003; Xie et al., 2011). A substantial decrease in photosynthesis is another striking change during leaf senescence (Wen et al., 2004), which is associated with the loss of Rubisco, a primary target of proteolysis (Yoo et al., 2003). Leaf senescence detected in plants grown in full nutrient solution was a normal developmental phenomenon; in fields *A. villosum* plants also senesce in August. Deficiencies of zinc, copper, and manganese accelerated the process of leaf senescence as judged by the reduced contents of chlorophylls and soluble proteins and the reduced P_{max} (Figures 1a, c; 2a). The harmful effects of zinc deficiency were more serious than those of copper and manganese deficiencies, consistent with the fact that zinc is more closely involved in synthesis of chlorophylls and proteins than copper and manganese (Yu et al., 1999).

Micronutrient deficiencies significantly reduced thermal dissipation at the 42th day of treatment in *A. villosum*, as judged by the decreased NPQ (Figure 2e). The reduced thermal dissipation, combined with the reduced photosynthetic light utilization (Figure 2a), may cause more serious photoinhibition in micronutrient deficient *A. villosum* plants than in full-nutrient-grown plants. This prediction was confirmed by the lower values of F_v/F_m, Φ_{PSII}, and q_p in the former (Figure 2b - d), which are widely used as indicators of photoinhibition (Feng et al., 2002; Ort and Baker, 2002). The decrease in Φ_{PSII} may be the result of a functional down-regulation of PS II, a mechanism to maintain the balance between electron supply by photoreaction and electron utilization by CO_2 assimilation (Lu and Zhang, 1998). The lower q_p indicated a higher proportion of Q_A in reduced state in micronutrient deficient *A. villosum* plants, imposing a higher excitation pressure on PS II (Lu et al., 2001). The excitation pressure has been shown to be a determinant of photodamage to photosynthesis (Wen et al., 2004). Thus a substantial decrease in P_{max} accompanied by only a slight decrease in PS II photochemistry in leaves of micronutrient deficient plants may potentially expose these leaves to excess excitation energy, which, if not safely dissipated, may result in accumulation of reactive oxygen species (Demmig-Adams and Adams, 1992). This was the case for micronutrient deficient *A. villosum* plants. Copper, zinc, and manganese deficiencies indeed caused oxidative stress in leaves of *A. villosum* plants as indicated by the increased malondialdehyde content (Figure 3d), decreasing plant biomass accumulation (Figure 4).

Increasing evidence indicates that oxidative damage to critical cellular metabolisms resulting from direct or indirect attacks by reactive oxygen species is the basis of disturbances in plant development and growth caused by micronutrient deficiencies. Enhanced generation due to increased NADPH oxidase and impaired detoxification due to reduced SOD may lead to an elevated level of superoxide anion and its derivatives in zinc deficient plants, creating oxidative stress (Wenzel and Mehlhorn, 1995; Cakmak and Marschner, 1988). The obvious decrease in carotenoid content may also contribute to oxidative stress in leaves of *A. villosum* plants grown in micronutrient deficient solutions (Figure 1b); carotenoids can effectively scavenge singlet oxygen and quench triplet state of chlorophyll (Bagci et al., 2007). Copper deficiency can decrease the level of plastocyanin, a copper-containing protein involved in transfer of electrons from PS II to PS I, and therefore leaves more electrons in PS II, contributing to the formation of reactive oxygen species (Pilon et al., 2006). Manganese is necessary for water-splitting in PS II and functions of a variety of enzymes such as manganese-SOD (Kabata-Pendias, 2004), and manganese deficiency can lead to oxidative stress in chloroplast (Yu and Rengel, 1999).

The production of reactive oxygen species is supposed to be a general alarm signal that serves to alert or notify metabolism and gene expression about possible modifications (Foyer et al., 1994). The activities of antioxidant enzymes usually increase in the initial stage of environmental stress, providing certain degree of protection against oxidative damage, and then decrease with the prolonged

duration of the stress due to either reduced synthesis or enhanced degradation or inactivation of the enzymes. An increase in the activities of SOD and CAT at the initial stage of drought or high irradiance stress followed by a decrease was founded in wheat (Zhang and Kirkham, 1994). Ascorbate peroxidase activity increased at the sixth day of magnesium deficient treatment and then decreased in leaves of *Mentha pulegium* plants (Candan and Tarhan, 2003). Micronutrient deficiencies did not reduce the activities of SOD, APX and CAT in *A. villosum* plants (Figure 3a-c), indicating that our treatments were not stressful enough to inhibit activities of these enzymes. In contrast, zinc, copper, and manganese deficiencies significantly increased the activities of SOD, APX, and CAT (in three cases) (Figure 3a-c). However, the increased activities of these enzymes did not prevent oxidative stress in leaves of micronutrient deficient *A. villosum* plants. Similar results was also found in rice (Xie et al., 2011)

In conclusion, deficiencies of zinc, copper, and manganese accelerated leaf senescence in *A. villosum* plants as indicated by the decreased light-saturated photosynthetic rate and the contents of chlorophylls and soluble proteins, and induced serious oxidative stress as judged by increased malondialdehyde content. The negative effects of zinc deficiency on *A. villosum* plants were the most serious among the three micronutrients. The harmful effects of micronutrient deficiencies on P_{max} were greater than on F_v/F_m, q_p, and Φ_{PSII}, disturbing the balance between electron supply by photoreaction in thylakoid and electron utilization by CO_2 assimilation in chloroplast stroma. The results contributed to the oxidative stress in leaves of micronutrient deficient *A. villosum* plants. Our results indicated that micronutrient deficiencies induced oxidative stress, exacerbating leaf senescence and inhibiting growth in *A. villosum* plants.

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微量元素缺乏加速砂仁葉片衰老

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栽培幾年後藥用植物砂仁（*Amomum villosum*）的果實產量逐漸降低，這常常被歸因於植株衰老。然而，至今砂仁衰老的原因還不清楚。本文研究了微量元素缺乏對砂仁葉片衰老的影響。我們假設微量元素缺乏可能通過干擾細胞代謝而導致氧化脅迫，進而導致砂仁葉片衰老以及生長減慢。銅、鋅和錳缺乏使砂仁葉片光飽和光合速率、葉綠素含量和可溶性蛋白質含量降低，丙二醛含量升高，表明發生了氧化脅迫和衰老。與缺銅和錳相比，缺鋅對砂仁的危害更大。缺少微量元素對葉綠體基質中 CO_2 同化的負面影響大於對類囊體膜上電子傳遞的負面影響，導致葉綠體中過剩激發能增加。在大多數情況下，微量元素缺乏使砂仁葉片熱耗散能力和類胡蘿蔔素含量降低，減少過剩激發能的耗散，導致氧化脅迫，儘管此時抗氧化酶活性升高。本研究表明，缺少微量元素導致了砂仁葉片氧化脅迫，加速了葉片衰老，抑制了砂仁生長。

關鍵詞：丙二醛；光合作用；抗氧化酶；錳；生長；銅；辛；氧化脅迫；葉綠素螢光。