Light Intensity Affects Growth, Photosynthetic Capability, and Total Flavonoid Accumulation of Anoectochilus Plants

Zengqiang Ma, Shishang Li, and Meijun Zhang
College of Life Science, Capital Normal University, Beijing 100048, China; and Yangtze Delta Region Institute of Tsinghua University, Zhejiang 314100, China

Shihao Jiang
Yangtze Delta Region Institute of Tsinghua University, Zhejiang 314100, China

Yulan Xiao
College of Life Science, Capital Normal University, No. 105 North Xisanhuan Road, Beijing 100048, China; and Yangtze Delta Region Institute of Tsinghua University, Zhejiang 314100, China

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Abstract. Anoectochilus formosanus, a medicinal plant used to treat hypertension, lung disease, and liver disease, was grown to maximize biomass and secondary metabolite production in a controlled environment under four levels of photosynthetic photon flux (PPF), namely, 10, 30, 60, or 90 μmol m⁻² s⁻¹, that is L₁₀, L₃₀, L₆₀, and L₉₀ treatments, respectively. On Day 45, all growth values were greatest for the L₃₀ plants. Dry weight was lowest for the L₁₄ plants. Leaf area, stem length, and fresh weight were lowest for the L₉₀ plants. The chlorophyll concentration was highest in the L₁₀ treatment and decreased with increasing PPF. Electron transport ratios of leaves were highest in the L₃₀ treatment and lowest in the L₉₀ for the second leaf (counted down from the apex) and in the L₁₀ for the third leaf. An increase in light intensity from 10 to 60 μmol m⁻² s⁻¹ increased the superoxide dismutase activity and was associated with an increase in the total flavonoid concentration. The total flavonoid concentration (mg g⁻¹ DW) was greatest in the L₆₀ and lowest in the L₉₀. However, the total flavonoid content (mg/plant) was highest in the L₁₀ plants as a result of great biomass. The results indicated that A. formosanus is a typical shade plant suitable to grow under low light intensity at PPF of 30 to 50 μmol m⁻² s⁻¹ for both growth and production of total flavonoid. A light intensity of 90 μmol m⁻² s⁻¹ induced stress on plant growth and reduced photosynthetic capability and the flavonoid accumulation.

Anoectochilus, a perennial herb, belonging to the Orchidaceae family, which comprises more than 35 species that are widespread in the tropical regions, from India through the Himalayas and Southeast Asia to Hawaii (Asahishinbun, 1997). Anoectochilus is a short (10 to 15 cm in length) and typical shade plant and mainly distributed at an altitude of 400 to 1200 m of the wet zone. Several species have been used in Chinese medicine. Among them, Anoectochilus formosanus, only found in Taiwan and Okinawa, has been used for hypertension, lung disease, and liver disease and underdeveloped children as a folk remedy (Kan, 1986). One of the most active components in Anoectochilus was flavonoid (Takatsuki et al., 1992). Many of flavonoid constituents have antioxidant properties (Shih et al., 2003).

The international trade of medicinal plants is becoming a major force in the global economy, and the demands of A. formosanus are rapidly increasing in Chinese medicine. However, overharvesting wild species is one of the serious issues for plant conservation. In addition, the explosive demand in plant materials for medicinal preparation has been accompanied by issues of quality and consistency, because the natural sources of A. formosanus are exhausted and growth of A. formosanus in the field has many problems, for instance, small seeds with slow germination rate, slow growth, and sensitive to stressful environments. To conserve wild species and to meet the demands for medicinal plant materials, new technologies for A. formosanus plant production with great biomass and high quality are required.

In 2000, a new concept for the production of transplants in a controlled environment was introduced (Kozai et al., 2000). In this system, artificial light is the sole light source for plant growth; photosynthetic photon flux (PPF), CO₂ concentration, air temperature, relative humidity, and air speed are well controlled for optimizing plant productivity and quality. The major advantages of growing plants under controlled environments are maximization of plant biomass, consistency, and quality. Environmental factors also have remarkable effects on secondary metabolite biosynthesis. The secondary metabolites are defined as bioactive molecules, which provide the plant with defense mechanisms to survive from herbivores, environmental stresses, disease, or competition and may affect the growth and development of other organisms (Seigler, 1996). Quality of medicinal plants is determined by their superior genetic characteristics and great biomass with high and consistent secondary metabolite content (Kozai, 2005). High-quality medicinal plants can be produced only under carefully controlled environments (Afreen et al., 2005). Therefore, growing plants under a controlled environment can be considered an alternative way for medicinal plant production to ensure safety and efficacy.

A number of earlier investigations have reported that environmental factors such as light intensity can significantly improve growth and alter the metabolite concentrations. For example, the increasing light intensity at a PPF of 100 μmol m⁻² s⁻¹ significantly improved the growth and photosynthetic capability of in vitro Momordica grosvenori plantlets (Zhang et al., 2009). Briskin and Gawienowski (2001) reported that growing St. John’s wort plants at a PPF of 400 μmol m⁻² s⁻¹ significantly increased the hypericin concentration with enhanced photosynthetic activity. Light is also involved in regulating antioxidant enzymes and secondary metabolites. Mohammad et al. (2005) found that micropropagated Phalae-nopsis plantlets had higher superoxide dismutase (SOD) content to adapt the increasing light intensity. Zhong et al. (1991) successfully increased anthocyanin production in cell culture of Perilla frutescens (shiso) by increasing light intensity.

However, there is little information about the effects of light intensity on growth and secondary metabolite of A. formosanus. Therefore, the objective of this study was to determine the effects of light intensity on the growth, photosynthetic capability, and total flavonoid accumulation of A. formosanus plants in a controlled environment.

Materials and Methods

Plant material, treatments, and culture conditions. Anoectochilus formosanus plantlets were cultured in vitro for 30 d and acclimated ex vitro for 10 d. The acclimated plantlets, each with four or five unfolded leaves, were used in this study. The average leaf area,
fresh and dry weight, and stem length per plant were 764 ± 16 mm², 821 ± 17.3 mg, 78 ± 2.6 mg, and 6.98 ± 0.09 cm, respectively. The plants were transplanted into a plastic tray (540 × 270 × 50 mm) containing vermiculite and peat moss (Growing Mix I; Fafard Co., Ltd., Inkerman, Canada) in a 1:2 ratio by volume and placed in a growth chamber (2.3 m in length, 1.8 m in width, 1.4 m in height, Zhejiang Qunxin Biological Industry Development Co., Ltd., Jiaxing, China). The experiment included four treatments, L10, L30, L60, and L90, in which L indicates light intensity and subscripts denote PPF. Light intensity was measured on the surface of the empty shelves with a light meter (Model LI-250A; LI-COR, Lincoln, NE).

The air temperature and relative humidity in the growth chamber were maintained at 25 ± 1 °C and 80% ± 5%, respectively. The photoperiod was 14 h per day supplied with cool-white fluorescent lamps. The CO2 concentration was maintained at 1000 µmol mol⁻¹ in the growth chamber. The CO2 concentration was measured and controlled by a CO2 controller with a nondispersive infrared detector CO2 sensor (T6004; Telaire Co., Ltd., Shoreview, MN). Seventy-two replications (72 plants) were used for each treatment.

The experiment was conducted twice.

Measurements, calculation, and statistical analysis. The plants were harvested on Day 45 for destructive measurements and analysis. Plant growth, chlorophyll concentration and fluorescence, SOD activity, and total flavonoid concentration were measured. Leaf area was determined using image and quantify analysis software (LIÅ for Windows 95; K. Yamamoto, Nagoya, Japan) with an image scanner. Dry weight of the plants was determined after oven-drying at 80 °C for 72 h.

For chlorophyll measurement, the second and third fully expanded leaves from the apex were cut into pieces, soaked in 5 mL N, N-dimethylformamide solution, and shaken once every 10 min for 2 h at 4 °C (Moran and Porath, 1980). Absorbance was measured with an ultraviolet-vis spectrophotometer (Helios Gamma; Thermo Spectronic Co., Ltd., Cambridge, UK) at wavelengths of 664 nm, 647 nm, and 603 nm. The chlorophyll concentration, including chlorophyll a and chlorophyll b, was determined on a fresh weight basis (µg/g⁻¹) and calculated using the formula of Moran (1982).

The ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) and electron transport rate (ETR) were measured and calculated with a Maxi-Imaging-PAM chlorophyll fluorescence measuring system (Walz, Effeltrich, Germany). After 43 d of culture, the second and third fully expanded leaves (counted down from apex) were used for the measurement. Six replications or six leaves were measured in each treatment. For chlorophyll fluorescence measurement, the plants were placed in a dark room to adapt for 15 min. Next, randomly chosen intact leaves from four treatments were placed in the measuring head (10 × 13 cm) to measure the Fv/Fm. The leaves without dark adaptation were used to evaluate ETRs. Each ETR value was obtained using actinic irradiance for 10 s. All relevant fluorescence parameters, including actinic irradiance and leaf temperature, were recorded and ETRs were calculated in the Maxi-Imaging-PAM system.

The extraction and activity analysis of SOD were performed by using the method described by Rao and Sresty (2000). In brief, the 0.5 g leaves were ground with 5 mL ice-cold 0.05 mol/L phosphate buffer (pH 7.8). The homogenates were centrifuged at 4 °C for 20 min at 12,000 × g and the resulting supernatants were used for determination of enzyme activity. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The 3.0 mL reaction mixture contained 1.5 mL phosphate buffer (pH 7.8), 13 µM methionine, 75 µM NBT, 2 µM riboflavin, 10 µM EDTA-Na₂, and 50 µL of enzyme extract. The reaction was initiated by exposing the tubes to 4000 lx light intensity for 20 min. A nonirradiated complete reaction mixture served as a blank and another complete reaction mixture without enzyme, which gave the maximal blue color, served as a control. The reaction was stopped by quenching the irradiation light, and then the tubes were covered with a black cloth waiting for testing. The absorbance was recorded at 560 nm by the ultraviolet-vis spectrophotometer and one unit of SOD was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction.

Total flavonoid concentration in the leaves of plants grown at different light intensities was measured following the method of Sakamura et al. (2005). The reaction contained 0.25 mL of the methanolic plant extract or (+)-catechin standard solution and 1.25 mL of distilled water followed by the addition of 0.75 mL 5% sodium nitrite solution. After 6 min, 0.15 mL of 10% aluminum chloride solution was added and the mixture was allowed to stand 5 min and then 0.5 mL of 1 M sodium hydroxide was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The data were calculated using (+)-catechin for total flavonoid concentration.

Statistical significance was determined by one-way analysis of variance using Sigma Stat (SigmaStatTM for Windows Version 2.03; SPSS Inc., Chicago, IL). Differences among means were assessed with the Holm-Sidak test and a significance level of P ≤ 0.05.

Results

Plant growth. The results of plant growth with different light intensities on Day 45 are summarized in Table 1 and Figure 1. Among all treatments, the plant growth was greatest in the L30 treatment followed by L60. Leaf area, stem length, and fresh weight were 1.4, 1.1, and 1.3 times greater, respectively, in the L30 than those in the L90. Dry weight was 1.4 times greater in the L30 than that in the L10. There was no significant difference in dry weight between L60 and L90 treatments. An increase

Table 1. Effects of four levels of light intensity on the growth of Anoectochilus formosanus per plant grown for 45 d under a controlled environment.

| Treatment | Leaf area (mm²) | Stem length (cm) | Fresh Plant weight (mg) | Dry Plant weight (mg) | DW/FW (%) |
|-----------|----------------|------------------|------------------------|-----------------------|-----------|
| L10       | 963 ± 16 b     | 8.26 ± 0.10 b    | 995 ± 13 c            | 103 ± 1.3 c           | 10.4      |
| L30       | 1167 ± 22 a    | 8.57 ± 0.06 a    | 1184 ± 22 a           | 140 ± 2.7 a           | 11.8      |
| L60       | 986 ± 15 b     | 7.97 ± 0.08 c    | 1043 ± 20 b           | 126 ± 2.4 b           | 12.1      |
| L90       | 850 ± 19 c     | 7.90 ± 0.07 c    | 936 ± 13 d            | 121 ± 1.5 b           | 12.9      |

aData are mean ± se, and means denoted by the same letter in a column were not significantly different (P ≤ 0.05).

DW/FW = dry weight/fresh weight.

Fig. 1. Anoectochilus formosanus grown under controlled environment with four levels of light intensity, 10, 30, 60, or 90 µmol mol⁻¹ s⁻¹. For 45 d, L10, L30, L60, and L90 were four light intensity treatments, in which L indicates light intensity and subscripts denote photosynthetic photon flux density.
in light intensity increased the ratio of dry weight to fresh weight of plant (DW/FW).

The plants cultured in the L10 treatment not only had lowest dry weight, but also had lowest ratio of DW/FW. The plants cultured in the L90 treatment had the highest ratio of DW/FW, but the thickest stem, and small, thick, and yellow leaves were observed in the L90 treatments (Fig. 1).

Chlorophyll concentration and chlorophyll a/b ratio. The chlorophyll concentration of plants was significantly influenced by the different light intensities. Among all treatments, the chlorophyll concentration was greatest in the L10 treatment and then decreased consistently as the light intensified, lowest in the L90 treatment (Fig. 2). In contrast, the ratio of chlorophyll a to chlorophyll b increased to a maximum (3.72) as the light intensified to a PPF of 60 µmol m⁻² s⁻¹ and then decreased to 3.67 at a PPF of 90 µmol m⁻² s⁻¹, creating a parabola.

Ratio of Variable to Maximum Chlorophyll Fluorescence and Electron Transport Rate. The Fv/Fm ratio, reflecting the maximal photochemical efficiency of photosystem II (PSII), is usually steady with fluctuations from the second leaf and a PPF of 30 µmol m⁻² s⁻¹ (Fig. 1). The Fv/Fm ratio of variable to maximum chlorophyll concentration of the plant increased to the highest in the L60 treatment, and then significantly decreased to the lowest in the L90 (Fig. 5). The total flavonoid concentration of the L60 plant was 1.3 times greater than that of the L90 plant.

Discussion

Plant growth and photosynthetic capability. Light intensity is one of the key environmental factors influencing plant growth. The results clearly showed that A. formosanus plants grown under L10 achieved a great biomass with largest leaf area and longest stem length among all treatments (Table 1; Fig. 5). Chen et al. (1994) reported that the light saturation point of Anoectochilus was ~60 µmol m⁻² s⁻¹, indicating Anoectochilus is a typical shade plant. Moreover, a light intensity either too low or too high would not be suitable to growth of A. formosanus plants. In the present study, the plant grown under L10 and L90 had lower biomass and smaller leaf area. The low biomass of plants has frequently been explained by low photosynthetic ability. The low photosynthesis can result in low growth rate. ETRs allow for the rapid and noninvasive assessment of light response of PSII and are used to study the photosynthetic performance and photoclimation of photosynthetic organisms. The maximum ETR was related to the maximum photosynthetic capability, which was obtained when the photosynthetic rate was limited by the activity of the electron transport chain or Calvin cycle enzymes (Behrnfeld et al., 2004; Ralph et al., 2005). As shown in Figure 3, the L30 plants had the highest ETRs, which contributed to a great dry weight. The L10 and L90 plants, however, had the lowest ETRs in the third and second leaves, respectively, which contributed to low dry weight in both treatments.

Photoinhibition may occur when light intensity exceeds what is required for the saturation of photosynthesis. The decline in Fv/Fm represents the accumulation of a photodamaged PSII center (Rosenqvist and Kooten, 2003). In our study, the decline in Fv/Fm was observed in the second leaves of the L90 plants (Fv/Fm = 0.36). The 90 µmol m⁻² s⁻¹ (L90) probably exceeded the photoprotective capacity of the plants and resulted in photodamage. This adverse phenomenon can manifest as a loss of PSII activity (Masuda et al., 2002). A loss in the reaction-center content of PSII was associated with a slower ETR (Pell et al., 1994). Thus, the photoinhibition could account for the lower capacity of ETR as shown in the leaf from the L90 treatment in our study. Vasilikiotis and Melis (1994) also reported that in photoinhibition, the chloroplasts contained much of the same amount of PSII as they did under low light intensity. However,
up to ∼80% of PSII were photochemically inactive because of photodamage. This is the reason why the L10 plant with low light intensity and the L90 plant with photoinhibition had low ETRs and resulted in low carbohydrate accumulation in both treatments. In the present study, we also found that the ETRs in the second leaves from the L90 treatment were lower than those from the L10. In contrast, the ETRs in the third leaves from the L90 were higher than those from the L10. The phenomenon can be explained by the fact that the upper leaves may create a dense canopy to protect lower ones from photooxidation during plant growth. Thus, the dry weight of plants from the L90 treatment was greater than that from L10.

Although the decline in Fv/Fm was not observed in the leaves of the L60 plants, it could be assumed that the L60 plants also probably had little light stress resulting from lower ETRs compared with the L90 plants. Moreover, as shown in Figure 3, the PPF for the maximum photosynthetic capability of *A. formosanus* plants in all the treatments was 35 μmol•m⁻²•s⁻¹ in the second leaf and 55 μmol•m⁻²•s⁻¹ in the third leaf. We therefore suggest that PPF of 30 to 50 μmol•m⁻²•s⁻¹ would be suitable for growth of *A. formosanus* plants.

**Chlorophyll concentration.** The chlorophyll concentration of plants plays an important role in the absorption of light during photosynthesis. In the present study, low light intensity significantly enhanced chlorophyll concentration of plants, and the chlorophyll concentration decreased as the light intensity increased. The greatest chlorophyll concentration was estimated at a lowest PPF of 10 μmol•m⁻²•s⁻¹. The results agree with the finding of Walters et al. (2003) that leaves of plants growing at low PPF have relatively higher contents of chloroplastic pigments, electron carriers, and increased number of chloroplast. The result also demonstrates that plants could balance light absorption and translation by regulating chlorophyll synthesis (Bailey et al., 2001). The change of chlorophyll concentration was possibly an acclimation of plants to different light intensities.

For many plants, changes in light intensity may elicit physiological responses at the level of leaf and chloroplast (Bailey et al., 2001). In our study, the leaf responded to the light intensity by adjusting or reducing the chlorophyll concentration. Chloroplast responded to light intensity by changing chlorophyll a/b and adjusting to the ETR. As shown in Figure 2, an increase in light intensity from L10 to L60 was associated with an increase in chlorophyll a/b and a decrease in the size of the PSII light-harvesting antenna (Bailey et al., 2001; Masuda et al., 2002). Thus, interconversion of chlorophyll a and chlorophyll b is significant for the establishment of required chlorophyll a/b ratio during the adaption of leaves to high and low light intensity (Ito et al., 1993).

**Superoxide dismutase activity.** SOD is an important enzyme, which contributed to clear up superoxide in plants in the case of light stress, and SOD activity can give us some valuable information whether the plant was stressed by high light. Plants can fix carbon and photosynthesize by absorbing light energy at a proper light intensity. Excess light absorption resulted in acidic chloroplast lumina, reduced electron transport chain, and excess excitation energy (EEE) within chloroplast (Powles, 1984). EEE is toxic for plants by producing singlet oxygen, which can cause photooxidation mainly resulting from oxidative damage to the PSII. Depletion of the NADP⁺ pool under EEE causes an increase in the rate of electron flow from the donor side of PSII to oxygen, generating reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Asada, 1999). If these ROS are not removed immediately, they can easily cause damage to the cellular and molecular machinery, protein modification, and lipid peroxidation. Plants have evolved various antioxidative enzymes to avoid damage. SOD is an important antioxidative enzyme, which contributes to clear up superoxide in plants. In the present study, increasing light intensity from 10 to 60 μmol•m⁻²•s⁻¹ increased SOD activity, which suggested SOD plays an important role against the ROS caused by light stress. However, when light intensity reached 90 μmol•m⁻²•s⁻¹, SOD activity was found inversely, which probably related to the susceptibility of the plant to photodamage or to a higher temperature around the plants under high light intensity. Similarly, Rabinowitch (1980) found that in scouring conditions, SOD activity decreased; without scouring, SOD activity should increase.

**Total flavonoid content.** The production of total flavonoid content can be explained by the interaction of oxidative stress and photosynthesis. It is widely believed that the synthesis of secondary metabolites in plants is part of the defense responses of plants to oxidative stress. A number of earlier investigations have suggested that oxidative stress plays an important role for the synthesis of secondary metabolite in plant growth (Wojtaszek, 2002). In our study, as the light intensity increased from 10 to 60 μmol•m⁻²•s⁻¹, the SOD activity increased along with an increase in the total flavonoid concentration. The greatest total flavonoid concentration found in the L60 plants may be the result of the increased ROS induced by oxidative stress. The secondary metabolite synthesis of plants requires endogenous signal components such as ROS (Menke et al., 1999). ROS can function as a signal for the induction of defense systems and could enhance secondary metabolite production (Berglund and Ohlsson, 1995). This enables the plants to protect them against oxidative stress (Scalet et al., 1995).

Meanwhile, for many carbon-based metabolites, several hypotheses have been developed to explain phenotypic and evolutionary patterns in the distribution of plant secondary metabolites (Heyworth et al., 1998). For example, according to the "overflow metabolism" concept, when carbon production exceeds the carbon demand associated with plant growth, the excess carbon is channeled into biosynthesis of secondary metabolites (Matsuki, 1996). In the present study, the total flavonoid concentration (mg•g⁻¹•DW) in the L30 plants was lower than that of the L60 plants. However, the total flavonoid content (mg•plant) in the L30 plants was higher than that of the L60 plants as a result of a higher dry mass or enhanced photosynthesis. The total flavonoid concentration significantly decreased in the L90 plants can be explained by low photosynthesis resulting from photooxidation and synthesis mechanism damage. Further study is required to better understand the relationship among photosynthesis, oxidative stress, and secondary metabolite contents in *A. formosanus* plants.

**Conclusion**

To summarize, *A. formosanus* is a typical shade plant suitable to grow under lower intensity of PPF ranging from 30 to 50 μmol•m⁻²•s⁻¹ for both growth and production of total flavonoid content. Higher light intensity such as a PPF of 90 μmol•m⁻²•s⁻¹ induced stress on plant growth and reduced photosynthetic capability and the flavonoid accumulation. The production of *A. formosanus* plants with high total flavonoid content under a controlled environment can be further increased by optimizing other environmental parameters such as light quality, photoperiod, temperature, and relative humidity and by optimizing the nutrient composition. Such studies are currently underway in our laboratory. Therefore, growing plants in a controlled environment can be considered a good alternative way of producing high-quality *A. formosanus* to meet the demand for its medicinal use. We are hopeful that application of a controlled environment with the optimization of environmental factors will be an efficient method for producing *A. formosanus* plants with great biomass and high secondary metabolite.

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