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Citation	Eurasian Journal of Forest Research, 10(2), 201-207
Issue Date	2007-12
Doc URL	<a href="http://hdl.handle.net/2115/30313">http://hdl.handle.net/2115/30313</a>
Type	bulletin (article)
File Information	10(2)_201-207.pdf



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## Photosynthetic Characteristics of Regenerated Plantlets of *Camptotheca acuminata* as a Diagnostic for Tissue Cultured Plantlets in Acclimatization in Field Growth

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### Abstract

To establish an efficient *in vitro* rooting protocol and assist the acclimatization of regenerated plantlets, we determined the gas exchange rates of regenerated plantlets and seedlings of *Camptotheca acuminata*, an endangered Chinese plant species. Between the regenerated plantlets and control seedlings, no clear differences were observed in the light compensation point, light saturation point, CO<sub>2</sub> compensation point and carboxylation efficiency, but the seedlings had 30% higher photosynthetic capacity. Low photosynthetic rate accompanied by low stomatal conductance may be due to poor root development of regenerated plantlets during *ex vitro* acclimatization, in contrast to the seedlings. The ratio between root tips and leaf area in regenerated plantlets was significantly lower than in the seedlings, and this ratio was positively correlated with stomatal conductance. These findings indicate that the balance between above- and below-ground parameters specifying the plant body should be taken into account during *in vitro* rooting, with the aim of improving performance during *ex vitro* acclimatization.

**Key words:** *Camptotheca acuminata*, photosynthesis, root development, stomatal conductance, *ex vitro* acclimatization

### Introduction

Development of mass-cultured plantlets is essential in tissue-culturing for the production of commercially important materials. Methods of establishing mass vegetation of endangered and medical plant species are needed in conservation ecology, the maintenance of natural resources and in commercial production. Although we have successfully established tissue culture methods (Wang *et al.* 2007), an acclimation method for new plantlets in field culture is still needed.

*Camptotheca acuminata* Decaisne (Nyssaceae) is an endangered tree species native to south China. It is a well-known natural source of the monoterpene-indole alkaloid camptothecin (CPT) (Wall *et al.* 1966). For commercial production, mass vegetation of *C. acuminata* must be established (Wang *et al.* 2007). The most important process in *ex vitro* acclimatization is the development of well expanded roots of plantlets. Root development including root tips, measured by the root projection area and leaf/root ratio during *ex vitro* acclimatization, is very important for water and mineral nutrient absorption, and may even affect gas exchange. Little is known about the effect of root development on leaf function (Lambers *et al.* 1998). An understanding of root development and its effect on leaf function for rooted plantlets of *C. acuminata* will suggest measures for improving the survival rate following transplantation and for accelerating *ex vitro* acclimatization.

Stomatal conductance of leaves should be a good

criterion for evaluating the development of root systems. If the root system develops poorly or an imbalance arises between the top and the root, the stomatal conductance of plantlets may be lower than that of seedlings. Roots of regenerated plantlets from tissue culture, and control seedlings, differed in root tip number and tap root status (Wang *et al.* 2007). We therefore used these two stages of *C. acuminata* to determine the relations between root development and leaf photosynthesis from the viewpoint of whole plant physiology. We accordingly learn how to stimulate root development in an *in vitro* culture.

### Materials and Methods

After culturing in the rooting medium for three weeks, regenerated plantlets with normal roots were transferred to pots (55cm in length, 38cm in width, 12cm in height) containing a mixture of sterilized sand and soil (1:1,v:v). The plantlets were covered with clear plastic film to maintain high humidity for about two weeks (Wang *et al.* 2007). The control seedlings were germinated from seed, and the seedlings were transferred to similar pots about 10 days after sprouting. The experiment began on June 11, 2005 and finished on August 1. The basal diameter and height were measured at the beginning and end of the experiment, to give the growth rate.

The leaf size and total leaf area per plant were measured by the leaf profile method, in which leaf profiles were drawn on paper and then cut by a pair of

scissors. The leaf area was measured on the paper by a projection area meter (Li-3000, LiCor, USA). Leaf thickness was measured by a spiral micrometer (Precision, 0.001mm). A total of 20 replications of regenerated plantlets and seedlings were measured in this experiment. The rate of survival of the rooted plantlets was recorded at the end of the experiment.

To find the relation between root development and leaf photosynthesis, we compared the regenerated plantlets and seedlings cultivated in a greenhouse. Root development was specified by the ratio of total root tip number to total leaf area. Leaf stomatal conductance and the leaf net photosynthetic rate were measured simultaneously by a Li-6400 (LiCor, USA). During measurement of photosynthesis of regenerated plantlets, the leaf chamber was tilted slightly to assist in leaf clamping, because the height of some plantlets was very low. CO<sub>2</sub> response curves (A/C<sub>i</sub> curves) were measured by adjusting the CO<sub>2</sub> concentration with LI-6400-CO<sub>2</sub> bomb, and the CO<sub>2</sub> concentration in the leaf chamber varied from near zero to ambient CO<sub>2</sub> concentration (400ppm). Light was kept at the saturation value of 1000μmol.m<sup>-2</sup>s<sup>-1</sup>. Light response curves were measured in total darkness (0μmol.m<sup>-2</sup>s<sup>-1</sup>), and at 25μmol.m<sup>-2</sup>s<sup>-1</sup>, 50μmol.m<sup>-2</sup>s<sup>-1</sup>, 100μmol.m<sup>-2</sup>s<sup>-1</sup>, 500μmol.m<sup>-2</sup>s<sup>-1</sup>, 1000μmol.m<sup>-2</sup>s<sup>-1</sup> and 1500μmol.m<sup>-2</sup>s<sup>-1</sup>. A hyperbolic curve was used to fit the light response curves:

$$P_n = \frac{b \times PAR}{1 + a \times PAR} - R_{day},$$

where  $P_n$  is the net photosynthetic rate,  $PAR$  is photosynthetic active radiation, and  $a$ ,  $b$  and  $R_{day}$  are parameters to be estimated by best-fitting. Here  $b$  indicates the gradient of the light response curve at zero light intensity and  $R_{day}$  denotes the dark respiration rate of a leaf. The compensation point  $I_c$  (the light intensity at which the net photosynthetic rate is zero) and saturation point  $I_s$  (the light intensity at which the photosynthetic rate is 95% of its theoretical maximum value of photosynthesis,  $A_{max}$ ) can be calculated as (Wang et al., 2001)

$$I_c = \frac{R_{day}}{b - a \times R_{day}},$$

$$A_{max} = \frac{b}{a},$$

$$I_s = \frac{0.95 \times A_{max}}{b - a \times 0.95 \times A_{max}} = \frac{19}{a}.$$

Stomatal limitation was calculated according to the method proposed by Farquhar and Sharkey (1982):

$$L_s = \frac{CO_2S - C_i}{CO_2S - C_c},$$

where  $CO_2S$  is the CO<sub>2</sub> concentration in the leaf

chamber and  $C_i$  denotes the intercellular CO<sub>2</sub> concentration.  $C_c$  is the CO<sub>2</sub> compensation point calculated from the A/C<sub>i</sub> curves. When the CO<sub>2</sub> concentration in the leaf chamber is lower than the ambient CO<sub>2</sub> concentration, the net photosynthetic rate ( $P_n$ ) varies linearly with the intercellular CO<sub>2</sub> concentration ( $C_i$ ),

$$P_n = B \times C_i + A,$$

where  $B$  and  $A$  are parameters to be estimated by best-fitting. The value of  $B$  measures the carboxylation efficiency (CE), and the CO<sub>2</sub> concentration point ( $C_c$ ) can be calculated as

$$C_c = -\frac{A}{B}.$$

## Results

### Growth of plantlets

The regenerated plantlets were cultured in WPM medium for root induction (Fig. 1 A). Three weeks later, the resulting well rooted plantlets (Fig. 1B) were transferred to soil for greenhouse acclimatization (Fig. 1C). Leaf photosynthesis was measured during acclimatization in the greenhouse. Following UGPase transfer to improve the wood quality, the plantlets were field grown in FuYang, Zhejiang Province, China. The three-year-old saplings from the regenerated plantlets were about 2.5m high, with basal diameter 3cm (Fig. 1D).

As shown in Fig. 2 A and B, the seedlings and regenerated plantlets differed greatly in height, but only slightly in basal diameter. In June the height of the seedlings was about 6cm, and in August was 10cm, whereas the height of the regenerated plantlets was 1cm in June and 4cm in August. In June the basal diameter of the seedlings was almost equal to those of regenerated plantlets, and the regenerated plantlets were 0.2 mm larger in diameter than the seedlings. During the experimental period, the growth rates of the seedlings and regenerated plantlets were quite similar; statistical analysis found no significant differences in diameter ( $p=0.114$ ) or height ( $p=0.60$ ) (Fig. 2C). In contrast to the 100% survival rate of seedlings, about 10% of the regenerated plantlets died during greenhouse acclimatization. The death of poorly developed plantlets increases the growth rate in Fig. 2C, since dead plants were excluded from the data analysis. Statistical analysis of leaf thickness data found no significant difference between the regenerated plantlets and seedlings (Fig. 3A). The leaf size of seedlings was significantly larger (1.8 times) than in the regenerated plantlets ( $p<0.01$ ) (Fig. 3B). Moreover, the total leaf area per seedling was 1.6 times higher in the regenerated plantlets, but statistical analysis showed no significant difference between the two types of plant ( $p=0.08$ ); see Fig. 3C. The total root tip number per plant was 3.0 times higher in seedlings than plantlets, and the root tip/leaf area was 3.8 times higher for seedlings than plantlets (Fig. 3D, E).

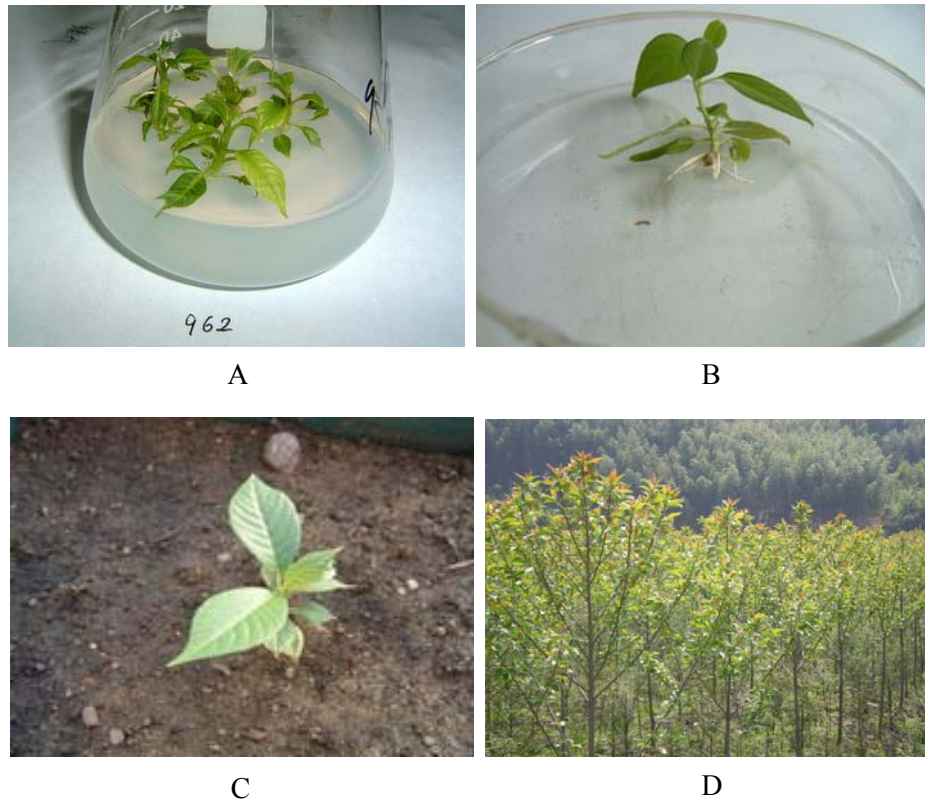


Fig. 1. Plantlets of *C. acuminata* from *in vitro* flask, greenhouse acclimatization to field cultivation.

A) the *in vitro* plantlets cultured in flask; B) Rooted plantlet for greenhouse acclimatization; C) Plantlets grown at greenhouse soil; D) 3-yr-old field grown individuals originated from *in vitro* plantlets.

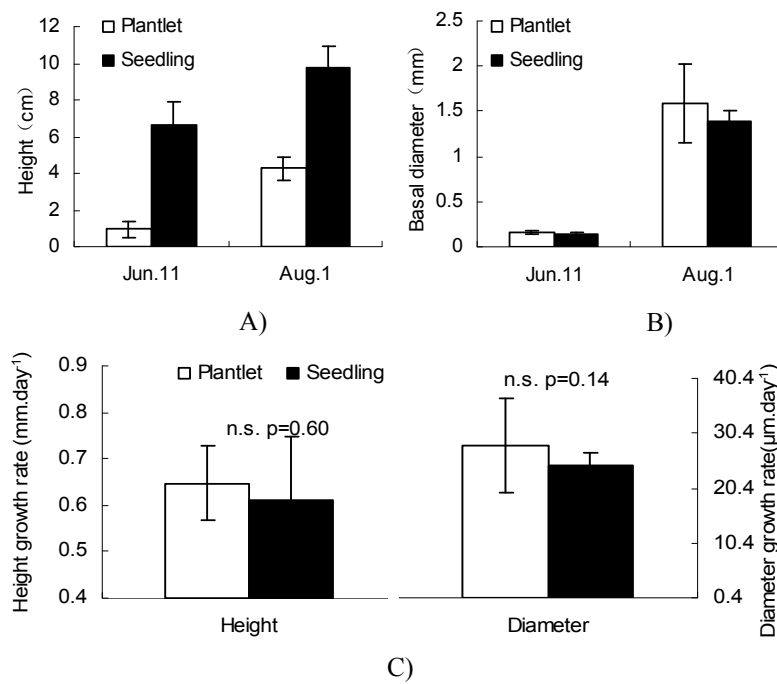


Fig. 2. Height, diameter and their growth rate of regenerated plantlets and seedlings as control from seed germination.

A) Height changes of plantlets and the seedlings during the experiment; B) Diameter changes of the plantlets and the seedlings during the experiment; C) Growth rate of height and diameter during the experiment. The vertical bar shows the standard deviation of the data.

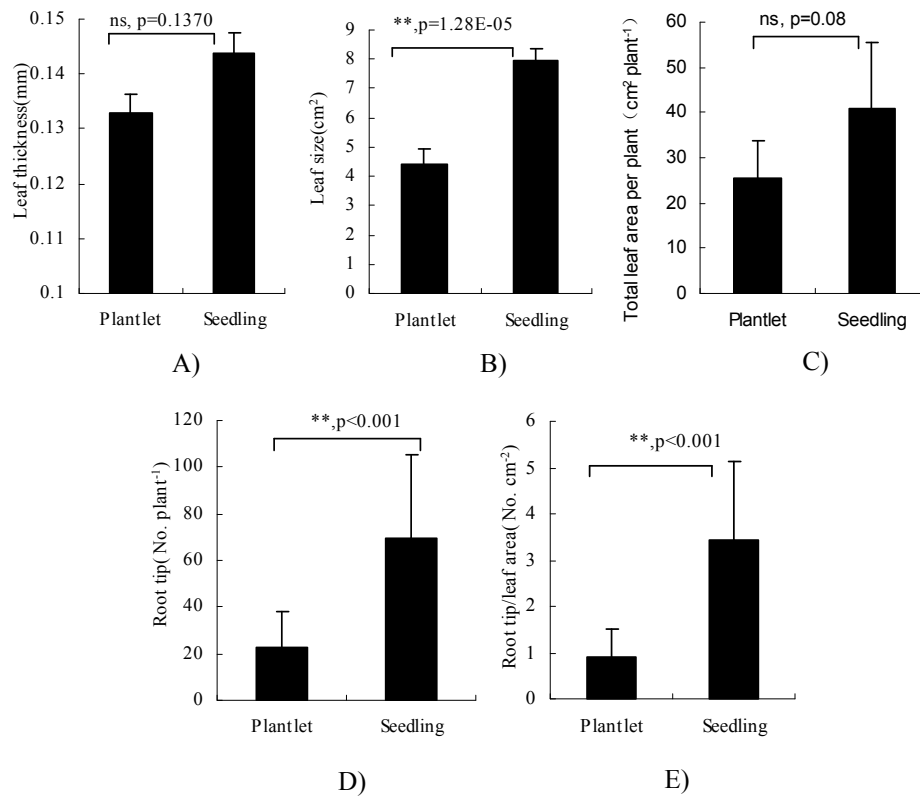


Fig. 3. Difference in leaf features and root development between regenerated plantlets and the seedling as control of *C. acuminata* during the experiment. The vertical bar shows the standard deviation of the data. A) Leaf thickness difference; B) Leaf size difference; C) total leaf area per plant; D) Root tips; E) Root tip/ leaf area.

### Gas exchange traits

Fig. 4 shows the differing light response curves for regenerated plantlets and seedlings. Upon estimating the parameters from curve fitting, the light compensation point for both plants ranged from 3.9 to 4.6  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , and the light saturation point varied from 350 to 390  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . These two parameters differed little between regenerated plantlets and the

seedlings (Fig. 4A). At the lower end of the light response curves there were no differences between the two types of plant, but in the light saturation region a clear difference was observed. Upon pooling data for the saturated photosynthetic rate ( $P_{\text{sat}}$ ), statistical analysis finds a significantly lower  $P_{\text{sat}}$  in the regenerated plantlets ( $2.0\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) than the seedlings ( $2.6\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) ( $p<0.001$ )(Fig. 4B).

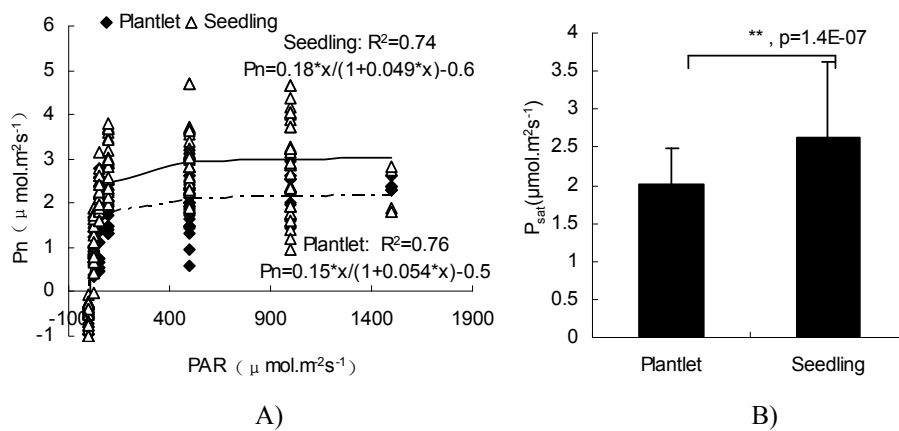


Fig. 4. Differences in light response curves of regenerated plantlets and the seedlings. (A) light response curve; (B) saturated net photosynthetic rate. The vertical bar shows the standard deviation of the data.

Fig. 5 shows A/C<sub>i</sub> curves for the regenerated plantlets and seedlings. Correlation analysis shows that the photosynthetic rates of both plant types increased linearly with CO<sub>2</sub> concentration ( $R^2 > 0.65$ ,  $p < 0.001$ ). No significant differences were found in carboxylation efficiency (CE) between seedlings (0.0188 mol.mol<sup>-1</sup>) and regenerated plantlets (0.0163 mol.mol<sup>-1</sup>) ( $p > 0.05$ ); see Fig. 5B. By linear regression analysis between photosynthesis and CO<sub>2</sub> concentration, the CO<sub>2</sub> compensation point of the seedlings and regenerated

plantlets were estimated at 74  $\mu\text{mol.mol}^{-1}$  and 82  $\mu\text{mol.mol}^{-1}$ , respectively (Fig. 5A).

The regenerated plantlets and the seedlings had a similar linear correlation between stomatal conductance and photosynthesis, but the stomatal conductance of the regenerated plantlets was much lower than that of seedlings (Fig. 6A). The intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) in leaves of the regenerated plantlets was substantially smaller than in the seedlings ( $p < 0.001$ ). Moreover, the stomatal limitation for the

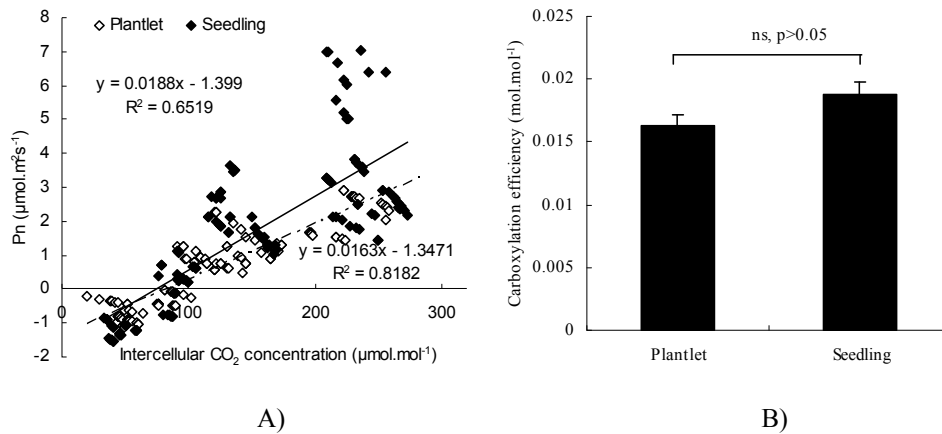


Fig. 5. Differences in CO<sub>2</sub> response (A/C<sub>i</sub>) curves.

(A) A/C<sub>i</sub> curves (B) Carboxylation efficiency. The vertical bar shows the standard deviation of the data.

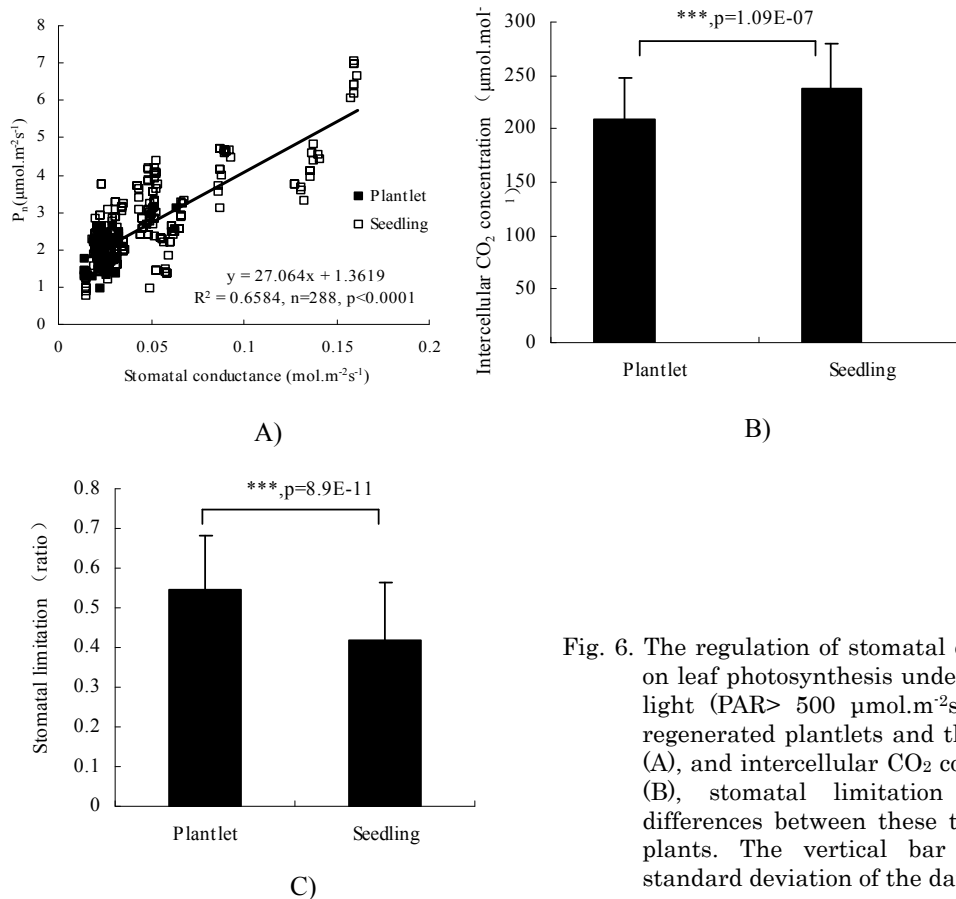


Fig. 6. The regulation of stomatal conductance on leaf photosynthesis under saturation light ( $\text{PAR} > 500 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) for both regenerated plantlets and the seedlings (A), and intercellular CO<sub>2</sub> concentration (B), stomatal limitation value (C) differences between these two types of plants. The vertical bar shows the standard deviation of the data.

regenerated plantlets was significantly greater than in seedlings ( $p < 0.001$ ); see Fig. 6 B, C. We used the ratio between root tip number and total leaf area to describe the root development. The stomatal conductance of both the regenerated plantlets and the seedlings increased as this ratio increased (Fig. 7).

## Discussion

### Gas exchange characteristics

*In vitro* plantlets are in general "photo-mixotrophism" plants. Acquisition of photosynthetic capability is the basis for survival of micro-propagated plantlets in open conditions. How plantlets adjust their photosynthetic apparatus during *ex vitro* acclimatization is of interest. According to the review of Pospíšilová *et al.* (1999), adjustment in photosynthesis, anatomical structure and water relations are commonly observed during *ex vitro* acclimatization. Wang *et al.* (2006) found that *in vitro* plantlets and *ex vitro* acclimated plantlets differ in their stomatal ( $g_s$ )-photosynthetic ( $P_n$ ) relations:  $P_n$  was almost independent of  $g_s$  during the first week of acclimatization, but was significantly correlated with  $g_s$  thereafter. We also found that the  $g_s$ - $P_n$  relations of the regenerated plantlets at 51 days after transplantation were the same as for the seedlings.

Some photosynthetic related parameters, such as the light compensation point, light saturation point,  $\text{CO}_2$  compensation point, and carboxylation efficiency were in fairly similar ranges for the regenerated plantlets and the seedlings (Figs. 4 and 5). The photosynthetic capacity of the regenerated plantlets was much lower than that of the seedlings, however (Fig. 4). The carboxylation efficiency of the regenerated plantlets did not differ significantly from that of seedlings (Fig. 5), indicating that carboxylation activity is not the reason for this difference. What, then, is the reason for this photosynthetic difference as seen in Fig. 4B?

A shortage of water supply could give rise to low stomatal conductance both for water and  $\text{CO}_2$ , and this shortage of  $\text{CO}_2$  might directly slow the photosynthetic rate (Jones 1992, Wang *et al.*, 2001). We found that the regenerated plantlets and seedlings had similar linear relations between their stomatal conductance and photosynthesis, but the stomatal conductance of the regenerated plantlets was much lower than that of the seedlings (Fig. 6A). This finding indicates that the function of stomata in regulating  $\text{CO}_2$  from the atmosphere to intercellular space is the same in the regenerated plantlets and seedlings. According to Farquhar and Sharkey (1982), it could be determined whether photosynthetic reduction is induced by stomatal limitation by examining the relative changes in intercellular  $\text{CO}_2$  concentration ( $C_i$ ) and also the stomatal limitation. When a decrease in  $C_i$  and increase in the stomatal limitation are jointly observed, the decrease in photosynthesis can be attributed to stomatal limitation.

The regenerated plantlets had a significantly lower  $C_i$  ( $p < 0.001$ ), and a significantly higher stomatal limitation value ( $p < 0.001$ ), than the seedlings (Fig. 6B, C). We therefore conclude that the much lower photosynthetic

capacity of the regenerated plantlets is due to their significantly greater stomatal limitation than in the seedlings. A protocol providing the establishment of *in vitro* regeneration of leaf explants and rooting of *C. acuminata* has been proposed, with success (Wang *et al.*, 2005 & 2007). A natural question is then how to increase growth vigor during *in vitro* culture. Our results for stomatal limitation indicate that improvement of stomatal function is involved in tackling this question.

### Evaluation of gas exchange traits in plantlets

A further issue is how to improve the openness of stomata. By using seedlings as controls, we found that the closure of stomata is due mainly to imbalance between root- and shoot-development, as measured by the ratio of root tips/leaf area (Fig. 7). The generation of balanced plantlets during *in vitro* culture is therefore important for the survival and growth of plantlets during *ex vitro* acclimatization. Researchers have found that high air humidity, low irradiance,  $\text{CO}_2$  shortage, and the presence of sugars and phyto-hormones in the medium during *in vitro* culture could result in plantlets with specific structure and functioning (Pospíšilová *et al.*, 1997). These tissue-cultured plants must overcome the sudden change in environmental conditions after transfer to open air and soil.

Hazarika (2003) has proposed several measures to improve the growth vigor of *in vitro* plantlets for better acclimatizing in *ex vitro* environments. These include changing the sucrose concentration, to improve the dry mass of plantlets, or improving the photosynthetic capacity; photoautotrophic culture to make persistent and productive *in vitro* leaves; application of growth retardant to shorten internodes and leaf size; reduction

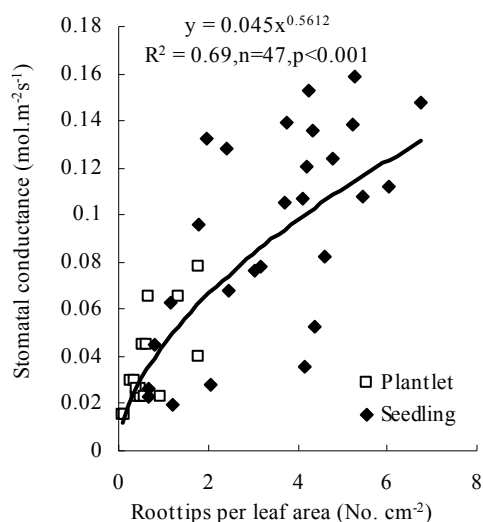


Fig. 7. Influences of root development on the stomatal conductance of seedlings and regenerated plantlets of *C. acuminata* under saturated light range ( $\text{PAR} > 500 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ).

of air humidity and application of anti-transpirant to reduce the water loss from leaves. Most of these techniques aim to change the leaf structure and function, but focus less on root development and even less on the balanced development of roots and shoots. Smith *et al.* (1990) reported that paclobutrazol (0.5-4mg l<sup>-1</sup>) in the rooting medium led to reduced stomatal apertures, increased epicuticular wax, shortened stems and thickened roots, and reduction in wilting after transfer to compost; it also increased the chlorophyll concentration per unit area of leaf.

In some species, such as tea (Sharma *et al.* 1999), walnut and peach (Sahay and Varma 2000), simultaneous rooting and acclimatization generally increase the survival rate of transplanting. However, many species do not root easily in *ex vitro* environment (Hazarika 2003). The present study indicates that, instead of separately considering the leaf structure and root development, the balance between aboveground (=leaves, branches and stem) and belowground (=root) components must be considered in the *in vitro* culture procedure. Comparison between seedlings and regenerated plantlets is a useful method in such research.

### Conclusion

By using the seedlings as control, we sought to understand the growth differences and photosynthetic performance of regenerated plantlets during *ex vitro* acclimatization. Both regenerated plantlets and controls grew at a similar rate, though the regenerated plantlets were much smaller than the seedlings. Many photosynthetic parameters of regenerated plantlets were quite similar to control values, but the photosynthetic capacity of the regenerated plantlets was significantly less than in controls. We believe the reason is the much higher stomatal limitation observed in the regenerated plantlets, which induces lower C<sub>i</sub> value. Stomatal conductance was positively correlated with the ratio of root tip number to total leaf area, indicating that the balance between root and shoots (or the top part of the plant) is important for improving the leaf photosynthesis. Our findings indicate that the balance between topside and root must be considered in any *in vitro* culture procedure as part of improving plant performance during *ex vitro* acclimatization.

### Acknowledgements

This work was financially supported by the Natural Science Fund for Youth of Heilongjiang Province, China (QC05C70 to W.H.), the State Key Basic Research and Development Plan of China (G19990160 to Z.Y.) and the Application Fund of Agricultural Research Production (03EFN216700297 to Z.Y.). Special thanks are due to Prof. Sasa Kaichiro at Hokkaido University Forests for providing the position of the Field Science Center of Northern Biosphere, Hokkaido University.

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