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Artemisinin accumulation and enhanced net photosynthetic rate in Qinghao (*Artemisia annua* L.) hardened *in vitro* in enriched-CO_2 photoautotrophic conditions

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Abstract

In vitro hardening or acclimatisation is one of the most practices in plant tissue culture, especially medicinal plant namely *Artemisia annua* (qinghao), which is very sensitive to *ex vitro* environments in transplanting process. The aim of this investigation was to enhance the production of artemisinin, sesquiterpene bioactive compound to cure parasite as a major antimalarial drug, and develop a high photosynthetic rate in qinghao plantlets, using photoautotrophic CO_2-enrichment. The artemisinin content in acclimatised plantlets grown in liquid MS medium with vermiculite supporting material was increased when compared to controlled plantlets. Net photosynthetic rate (P_n) and growth characters were greatest in the acclimatized plantlets grown in liquid medium with 65±5% relative humidity, leading to the highest survival percentage (81.7%) when transplanted to *ex-vitro* conditions. In addition, the P_n and growth performance of hardened plantlets in enriched CO_2 condition were better than those of hardened plantlets without CO_2 enrichment, leading to an improvement in the *ex-vitro* survival percentage (91.7%). Healthy plantlets of qinghao can be produced effectively *in vitro*, using a liquid medium with CO_2 enrichment in photoautotrophic conditions, resulting in plants to survive in *ex-vitro* environments, as well as having effective artemisinin accumulation.

Keywords: artemisinin, chlorophyll content, growth, net photosynthetic rate, survival percentage
Abbreviation: BA (N^6 benzyladenine), DMRT (Duncan's new multiple range test), HPLC (high performance liquid chromatography), NAA (α-naphthalene acetic acid), P_n (net photosynthetic rate), PPFD (photosynthetic photon flux density), RH (relative humidity)

Introduction

Artemisia annua L., belonging to the Asteraceae family, is a well known plant in China as the discovery of the antimalarial drug "artemisinin", which is an oxidized sesquiterpene lactone. Artemisinin accumulation in qinghao is dependent on internal factors, including genetics (de Jesus-Gonzalez and Weathers, 2003; Ferreira et al., 2005), plant organs (Tellez et al., 1999; Covello et al., 2007) and their developmental stages (Gupta et al., 2002; Zhang et al., 2006; Baraldi et al., 2008; Ma et al., 2008), and external factors, such as cultivation management (Kumar et al., 2004; Ferreira et al., 2005) and precursor or elicitor treatments (Lommen et al., 2007; Towler and Weathers, 2007; Wang and Weathers, 2007). *In vitro* culture is a practice procedure in medicinal plant species for rapid propagation, secondary metabolite production (cell suspension, hairy root culture and bioreactor) and genetic resource preservation (genebank). In the case of micropropagation, plant tissue culture is the most popular technique used to speed-up the production of high quality plantlets, in terms of genetic and physiological uniformities, which are pathogen free. However, low survival percentage of plantlets in *ex vitro* environments is a large barrier on final step of plant micropropagation, especially medicinal plant species. The environmental factors of *in vitro* cultivation are quite different from *ex vitro* and lead to physiological, morphological and anatomical disorders prior to plant death, due to environmental stresses following the direct transfer to soil (Chen, 2004; Chen, 2006; Hazarika, 2006). *In vitro* hardening is an important technique, used to improve the vigour of plantlets before transplantation to *ex vitro* conditions (Kozai et al., 1997; Kozai et al., 2005; Cha-um et al., 2010). There are many environmental factors which can be controlled *in vitro* in order to produce healthy plants. Low relative humidity, CO_2 enrichment and photoautotrophic culture (sugar free) in the acclimatisation process have been reported as the critical environmental factors for large scale micropropagation (Pospíšilová et al., 1999; Talbott et al., 2003; Cha-um et al., 2003; Chen, 2004; Kozai et al., 2005; Qu et al., 2009) which are mentioned in the present study. The aim of this investigation was to discover the environmental factors of *in vitro* hardening including supporting material, carbon sources and relative humidity enhancing healthy qinghao plantlets.

Table 1. Chlorophyll content (Chl), shoot height (SH), number of roots (NR), number of leaves (NL), leaf area (LA), fresh weight (FW) and dry weight (DW) of qinghao plantlets hardened in various substrates and relative humidity for 42 days.

Substrate	RH (%)	Chl (μg g^{-1} FW)	SH (cm)	NR	NL	LA (cm^2)	FW (g)	DW (mg)
Phytagel®	65±5	260.3c	10.8b	10.3c	23.6c	53.4b	2.8bc	264c
	95±5	256.4c	10.9b	10.9c	23.1c	40.6c	2.7c	260c
Vermiculite	65±5	290.2a	13.6a	14.0a	34.9a	59.2a	3.3a	298a
	95±5	274.2b	13.0a	12.9b	31.6b	56.0ab	3.1ab	279b

Different letters in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

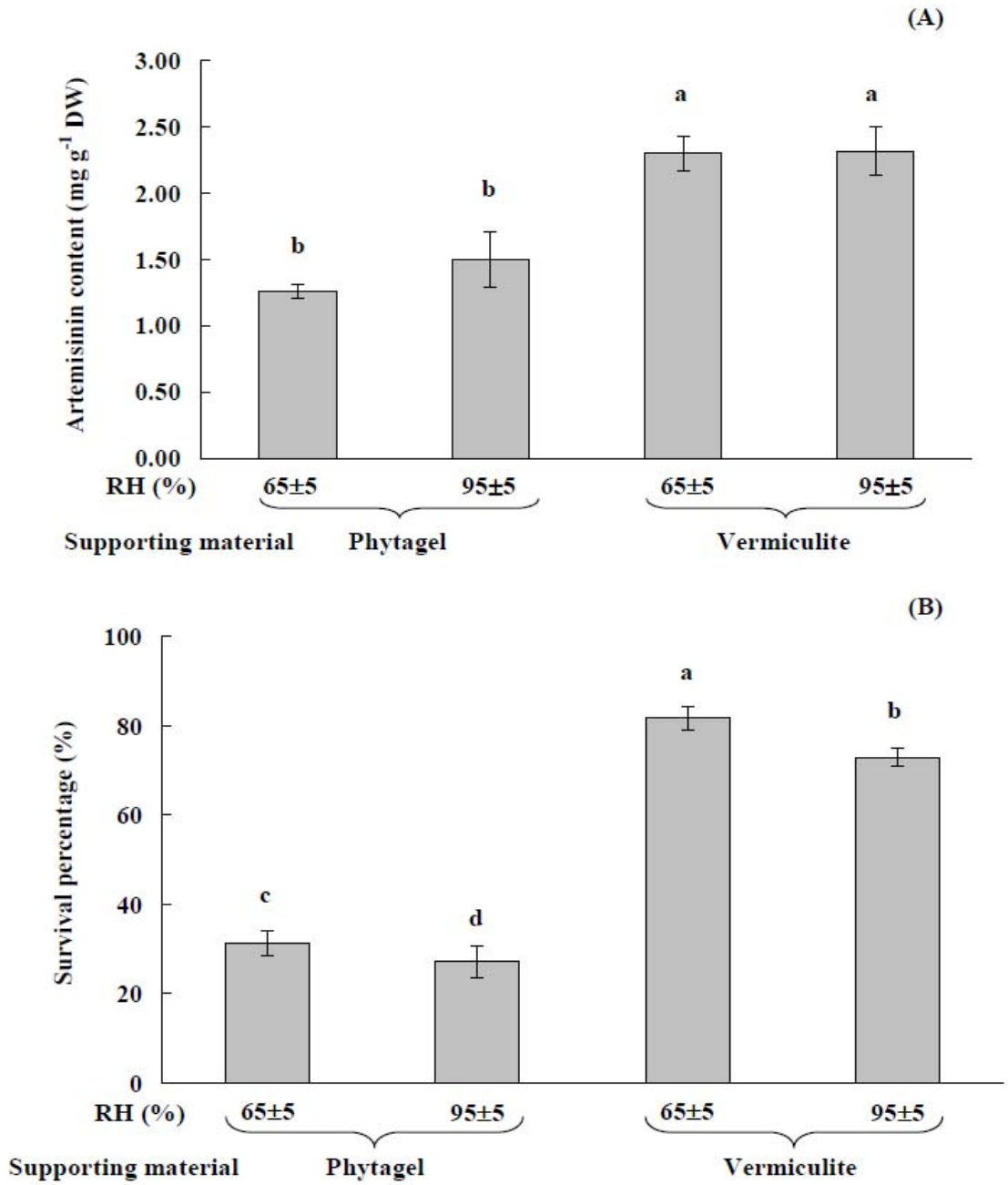

Fig 1. Artemisinin content (A) and survival percentage (B) of qinghao plantlets hardened in various substrates and relative humidity for 42 days. Error bars represented by ±SE. Different letters in each bar show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

Materials and methods

Plant materials

Seeds of qinghao (*Artemisia annua* L.), obtained from China were surface disinfected using 5% (v/v) Clorox® (5.25% active ingredient sodium hypochlorite, The Clorox, Loveland, USA) for 10 h and rinsed three times using sterilised distilled-water. Disinfected seeds were germinated on 0.25% (w/v) Phytagel® solidified-MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose in a 250 mL glass vessel. The media were adjusted to pH 5.7 before autoclaving. Seedlings were germinated *in vitro* and microshoots induced from node cuttings on MS medium containing 0.44 μM N^6 benzyladenine (BA) and 0.27 μM α-naphthalene acetic acid (NAA), 3% (w/v) sucrose and 0.25% (w/v) Phytagel®. The culture vessels containing microshoots were incubated in a culture room under 25±2°C ambient temperature, 60±5% relative humidity (RH) and 80±5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps with a 16 h d^{-1} photoperiod for 42 days.

Supporting material and relative humidity

Single shoots (3.0±0.2 cm in height) were excised and transferred directly to Phytagel® solidified MS or liquid MS medium using vermiculite as supporting material, for 7 days and then transferred aseptically to a culture box chamber (Carry Box Model P-850, size 26×36×19 cm, Japan) with controlled relative humidity (RH) at 65±5% by 1.5 L saturated NaCl solution or 95±5% by 1.5 L distilled water (Cha-um et al., 2003). The qinghao plantlets in the culture chambers were acclimatised for 42 days in a Plant Growth Incubator under a temperature shift of 28±2°C/25±2°C (light/dark), 500±100 μmol mol^{-1} CO_2 concentration, 60±5% RH, 120±5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps with a 16 h d^{-1} photoperiod. Morphological, physiological characteristics and artemisinin content of *in vitro* hardened plantlets were collected and the survival percentage was recorded after transplantation to plastic pots (10 × 15 cm) containing soil and vermiculite (1:1) for 15 days.

Carbon sources

Single shoots (3.0±0.2 cm in height) were excised and transferred directly to MS liquid medium using vermiculite as supporting material supplemented with 88.8 mM sucrose or without sucrose (0 mM sucrose) for 7 days and then transferred aseptically to a culture box chamber with RH controlled at 65±5% by 1.5 L saturated NaCl solution (Cha-um et al., 2003). The qinghao plantlets in the culture chambers were acclimatised in a Plant Growth Incubator under 500±100 or 1,500±100 μmol mol^{-1} CO_2 concentration with a temperature shift of 28±2°C/25±2°C (light/dark), 60±5% RH, 120±5 μmol m^{-2} s^{-1} PPFD with 16 h d^{-1} photoperiod for 42 days.

Data collection

Artemisinin measurement

The third leaf from shoot tip was quickly dripped into liquid nitrogen and keep at -80°C until extraction process. Crude artemisinin was extracted from leaf tissues following the modified method of van Nieuweburgh et al. (2006). One grams of leaf sample was ground in liquid nitrogen using cool mortar. Powder samples were incubated in 10 mL chloroform and shake for 1 min. The solvent was evaporated in a fume hood at room temperature until absolutely dry. The crude extract was dissolved in 1mL methanol and 200 μL of solution was collected into a new reaction tube. Eight-hundred microlitres 0.2% (w/v) of sodium hydroxide solution was added to the tube and the solution was mixed using a vortex mixture before incubating in a water bath at 50°C for 30 min. After cooling, the reaction mixture was added to 200 μL methanol and 800 μL 0.05M acetic acid. The mixture was filtrated through 0.45 μm Sartorious® membrane and then 10 μL of the filtrated solution was injected to HPLC (High Performance Liquid Chromatography; Water, USA). The filtrated solution was separated on a C_{18} Bondapak column (3.9×300 mm) with 1 mL min^{-1} flow rate. A mixture of 55% (v/v) acetonitrile in water containing 0.05% (v/v) formic acid was used as the mobile phase. The UV signal at 260 nm was monitored with a photodiode array detector (Vandenberghe et al., 1995). Authentic artemisinin (Khunming Phamaceutical Corp., China) was purified by recrystallised step in hexane and chloroform and was used as the standard chemical.

Chlorophyll detection

Chlorophyll content was analysed following the methods of Shabala et al. (1998). One hundred milligrams of leaf material was collected from the second and third nodes of the shoot tip. The leaf samples were placed in 25 mL glass vials, along with 10 mL 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation, and then stored at 4°C for 48 h. The chlorophyll contents were measured using a UV-visible spectrophotometer (HACH Model DR4000, HACH Co. Ltd., Nebraska, USA) at 662 nm and 644 nm wavelengths. A solution of 95.5% acetone was used as a blank.

Net photosynthetic rate measurement

Net photosynthetic rate (P_n) was calculated by comparing the different concentrations of CO_2 inside and outside the glass vessel containing with plantlets. The CO_2 concentrations inside (C_{in}) and outside (C_{out}) the glass vessel and at steady state were measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Tokyo, Japan). The GC capillary column and detector were a GS-Q (J&W Scientific®, Germany) and a thermal conductivity detector (TCD), respectively. The detector and injector were set to a temperature of 250°C. The temperature program of the column was set at 30°C for 1 min at initial state, then increased by 20°C per min to 100°C, and held for 1 min. The net photosynthetic rate (P_n) of *in vitro* cultivated plantlets was calculated according to the method of Fujiwara et al. (1987).

Plant growth measurement

Shoot height, number of lateral shoots, number of leaves, number of nodes, internode length, number of roots, fresh weight, dry weight and leaf area of *in vitro* hardened plantlets were measured. Hardened plantlets were dried at 80°C in a hot-air oven for 3 days, and then incubated in a desiccator before measurement of dry weight. The leaf area of plantlets

Table 2. Chlorophyll content (Chl), shoot height (SH), number of roots (NR), number of leaves (NL), leaf area (LA), fresh weight (FW) and dry weight (DW) of qinghao plantlets hardened in 88.8 mM sucrose or without sucrose and different CO_2 concentrations for 42 days.

Sucrose (mM)	CO_2 (μmol mol^{-1})	Chl (μg g^{-1} FW)	SH (cm)	NR	NL	LA (cm^2)	FW (g)	DW (mg)
0	500±100	250.3c	13.6b	13.8b	33.9b	60.5b	3.25b	299b
	1500±100	256.4c	14.2a	15.2a	37.1a	62.8a	3.46a	311a
88.8	500±100	274.2b	8.2c	7.3c	15.8c	31.6c	1.80c	181c
	1500±100	290.2a	8.1c	7.1c	15.2c	30.8c	1.58d	170c

Different letters in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

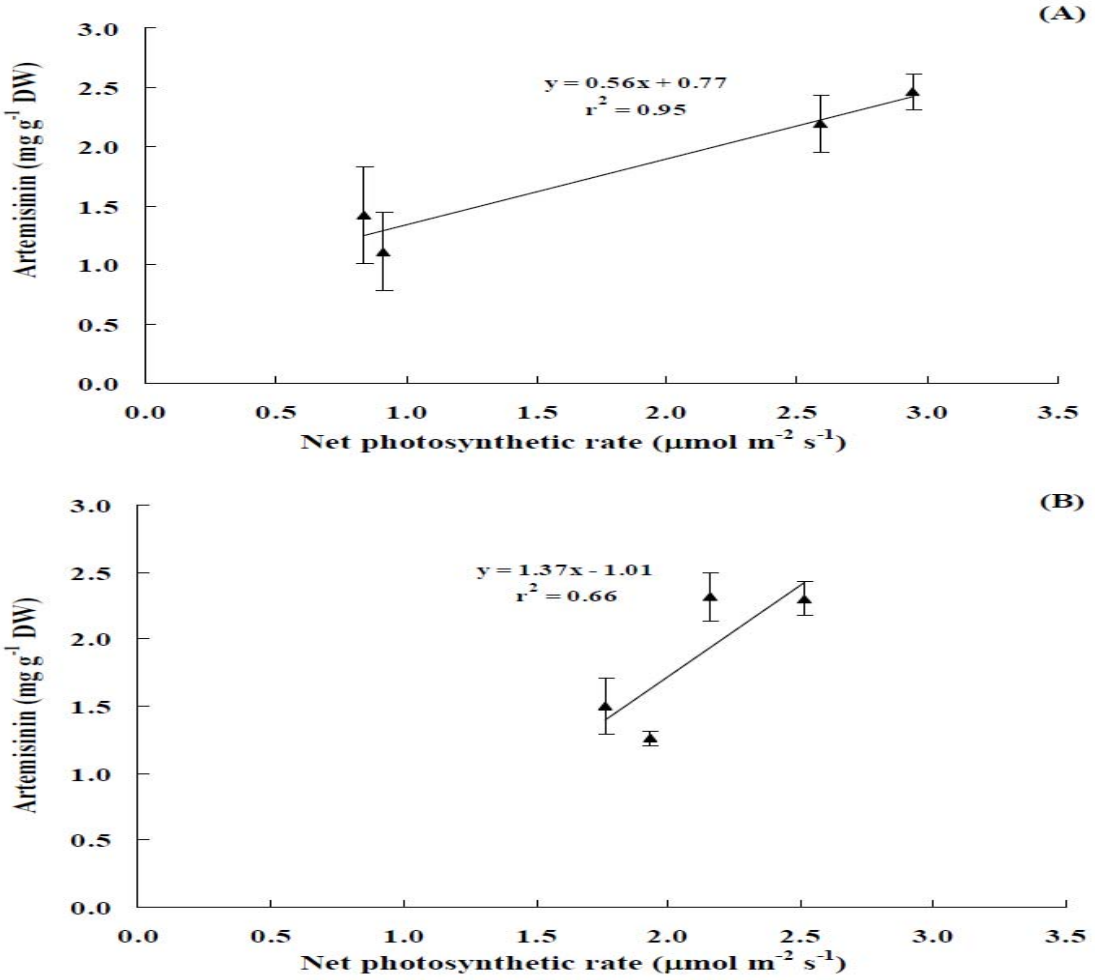

Fig 2. Relationship between net photosynthetic rate (P_n) and artemisinin content of qinghao plantlets hardened in various substrates and relative humidity (A) and plantlets in 88.8 mM sucrose or without sucrose and different CO_2 concentrations (B) for 42 days. Error bars represented by ±SE.

was measured using a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., UK).

Experimental design and analysis

The experiment was arranged as 2×2 factorials in completely randomized design (CRD) with six replications (n = 6). The mean value in each treatment was compared using Duncan's New Multiple Range Test (DMRT) and analysed by SPSS software (SPSS for Windows, SPSS Inc., Chicago, USA). The correlation between P_n and artemisinin was evaluated using Pearson's correlation coefficients.

Results and discussion

Supporting material and relative humidity

Artemisinin in the leaf tissues of plantlets, hardened in liquid medium using vermiculite as supporting material accumulated significantly and reached a higher level than in plantlets hardened in solidified-Phytagel® medium (Fig. 1A).

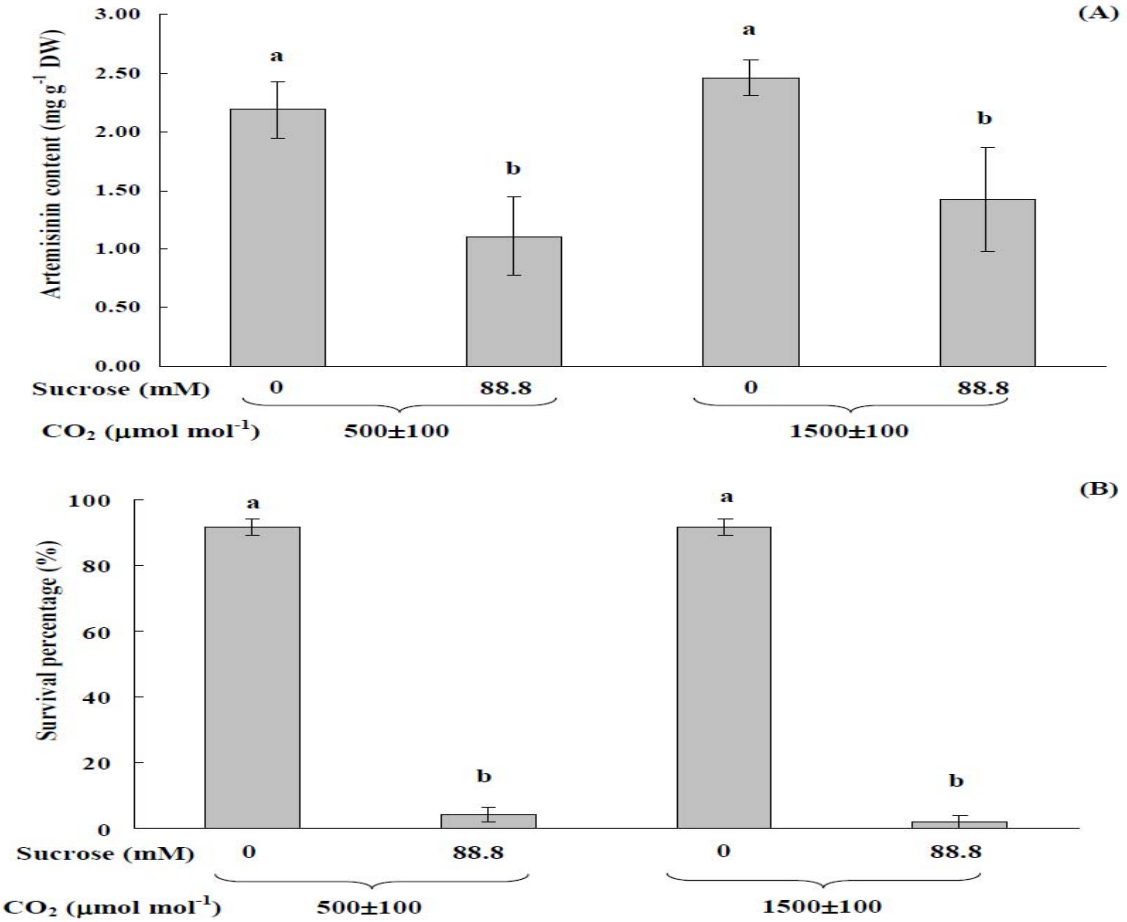

Fig 3. Artemisinin content (A) and survival percentage (B) of qinghao plantlets hardened in 88.8 mM sucrose or without sucrose and different CO_2 concentrations for 42 days. Error bars represented by ±SE. Different letters in each bar show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

Total chlorophyll content peaked in plantlets hardened in liquid MS medium with 65±5% RH, leading to enhanced net photosynthetic rate (P_n) and improved overall growth characters. Increasing P_n in qinghao was positively correlated to artemisinin accumulation in the leaf tissues ($r^2 = 0.95$) (Fig. 2A). Growth characters *i.e.* shoot height, number of roots, number of leaves, leaf area, fresh weight and dry weight of hardened plantlets in liquid medium with 65±5% RH were superior to those in solidified-Phytagel® with 95±5% RH by 1.25, 1.28, 1.51, 1.46, 1.22 and 1.15 times, respectively (Table 1). In addition, the survival percentage of plantlets acclimatised in vermiculite substrate liquid medium was 81.9%, which was higher than that of plantlets acclimatised in solidified Phytagel® medium (Fig.1B). In the present study, artemisinin compound was accumulated in the leaf tissues of plantlets hardened in a liquid medium using vermiculite supporting material. During the hardening process, the plantlets are defined as being in the vegetative stage, which is reported as having low artemisinin content when compared to the plants in the flowering stage in the field trial (Gupta et al., 2002; Zhang et al., 2006; Baraldi et al., 2008). In addition, the stervioside and benzopyrans content in micropropagated *Stevia rebaudiana* and *ex-vitro* acclimatised *Hypericum polyanthemum* amounted to 10.68 mg g^{-1} DW (Hwang, 2006) and 1.62 g % DW (Bernardi et al., 2008), respectively which was nearly the same as in the mother plant. The total chlorophyll content in qinghao plantlets hardened in liquid medium with low RH increased, leading to exhibition of P_n and growth characters, including shoot and root development. A healthy plantlet derived from *in vitro* hardening was successfully transplanted to *ex-vitro* conditions in order to ensure a high survival percentage. Liquid culture medium and low RH of *in vitro* acclimatized environments have been reported as effective protocol to promote healthy plantlets, helping them to adapt quickly to *ex-vitro* environments with a high survival rate in many plant species (Cha-um et al., 2003; Sánchez-Blanco et al., 2004; Bañon et al., 2006; Yang and Yeh, 2008).

Carbon sources

In this experiment, carbon sources, sucrose and CO_2, were applied for rapid *in vitro* hardening of qinghao plantlets. Artemisinin in the leaf tissues of plantlets hardened under CO_2-enriched photoautotrophic conditions was accumulated to a significantly higher level than in plantlets hardened in

photomixotrophic conditions (Fig. 3A). The total chlorophyll content was highest in plantlets hardened under enriched-CO_2 photoautotrophic conditions (Table 2). Shoot height, number of roots, number of leaves, leaf area, fresh weight and dry weight of plantlets hardened under CO_2-enriched photoautotrophic conditions were better than plantlets hardened in ambient CO_2 photomixotrophic conditions by 1.73, 2.08, 2.35, 1.99, 1.92 and 1.72 times, respectively (Table 2). Also, the survival percentage of photoautotrophic acclimatised plantlets in both enriched and ambient CO_2 was 91.7% and was superior to plantlets acclimatised in photomixotrophic conditions (Fig. 3B). Artemisinin antimalarial compound was accumulated in the leaf tissues of hardened qinghao plantlets, relating to high P_n ($r^2 = 0.66$) (Fig. 2B).Sugar classes play an important role as elicitors to enhance artemisinin accumulation in qinghao plants (Wang and Weathers, 2007). In this study, the artemisinin content peaked in qinghao plantlets acclimatised under a CO_2 carbon source, especially in CO_2 enrichment (2.46 mg g^{-1} DW). Artemisinin accumulation may be positively related to P_n and the gland density in the leaves (Tellez et al., 1999). Sugar added to the medium adversely affected photosynthesis by feedback inhibition and inhibitory effects on gland production in the leaf tissues. In contrast, the artemisinin content in qinghao seedlings treated with 3% (w/v) glucose in the culture medium is enriched and is twice as high as in seedlings grown with sucrose and fructose supplementation (Wang and Weathers, 2007). Moreover, the enhancement of P_n in St. John's wort plants, using environmental control systems, especially CO_2 enrichment, has been investigated and has been found to be closely related to hypericin content (Mosaleeyanon et al., 2005; Zobayed et al., 2006). The reduction of sugar, or absence from the culture media in enriched-CO_2 conditions, has been widely applied to promote P_n and is an effective strategy for the production of healthy plantlets before transplantation to *ex vitro* conditions (Le et al., 2001; Yoon et al., 2009; Xiao and Kozai, 2006; Martin, 2004; Khan et al., 2002; Mosaleeyanon et al., 2004). Sugar in the culture medium has been reported as having a negative effect on P_n (Mosaleeyanon et al., 2004; Xiao and Kozai, 2006; Yoon et al., 2009).

Conclusion

In vitro hardening of qinghao plantlets cultured in liquid sugar-free media with CO_2 enrichment, was an effective procedure to produce artemisinin, P_n exhibition and healthy plantlets, as identified by growth parameters and a high survival percentage in *ex-vitro* environments.

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