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A More Efficient Transplanting System for Thai Neem (*Azadirachta siamensis* Val.) by Reducing Relative Humidity

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ABSTRACT: The objective of this study was to develop an *in-vitro* acclimatization technique through relative humidity reduction for direct transplanting of woody species to *ex-vitro*. The survival percentage, growth efficiency and physiological characteristics of *in-vitro* acclimatized plantlets and *ex-vitro* adaptation were then evaluated. Thai neem (*Azadirachta siamensis* Val.) was selected for this research as it is a species with high potential for regenerating soils with high salt content. Twenty-eight day-old plantlets were transferred to environmental control chambers under controlled relative humidity (RH), which are 65±5% and 95±5% for low and high RH respectively, in order to acclimatize plantlets for 42 days. It was found that leaf water content, chlorophyll content and net photosynthetic rate (*NPR*) of plantlets acclimatized under low RH conditions were significantly higher than those acclimatized under high RH conditions (control). The relationship of leaf water content and chlorophyll content with *NPR* directly affects growth efficiency, leaf area and dry weight, of plantlets. After transplanting, the water relation system [water use efficiency (*WUE*), stomatal conductance (*G*) and transpiration rate (*E*)] and photosynthetic system [*NPR*, maximum quantum yield (F_v/F_m) and quantum efficiency of photosystem II (Φ_{PSII})] were measured at days 0, 1, 3 and 5. The plantlets acclimatized under low RH conditions showed higher physiological adaptation in *G*, *NPR*, F_v/F_m and Φ_{PSII}, except *E*, than those acclimatized under high RH conditions. The plantlets acclimatized under low RH conditions protected water loss through the leaf tissues by the function of stomata closure as indicated by low *E* values. Consequently, the survival percentage of plantlets acclimatized under low RH conditions was higher by a factor of 2.3 than those acclimatized under high RH conditions. *In-vitro* plantlets acclimatized by reducing RH produced vigorous plantlets and directly transplanted to *ex-vitro* with a high survival rate of 87.5%.

KEYWORDS: acclimatization, photoautotrophic condition, photosynthetic system, physiological adaptation, water relation.

INTRODUCTION

Plant micropropagation is widely used in horticulture and silviculture since it has many advantages over conventional propagation when rapid propagation, genetic and physiological uniformity, pathogen-free plantlets are considered. However, micropropagation is still limited in many species, especially woody species, because of the usually low survival percentage after transplanting to *ex-vitro*. The environmental conditions of plant micropropagation induce morphological disorders, including hyperhydricity, curled leaves, inhibited leaf expansion, incomplete rooting and few secondary roots, anatomical disorders such as poor vascular, spongy and palisade tissues, low stomatal density, and physiological disorders such as low net photosynthetic rate and high transpiration rate.[1-3] These abnormal characteristics directly contribute to the low survival percentage of plantlets after transplanting to *ex-vitro*. However, the main cause of plantlet death after transplanting to *ex-vitro* is still unclear.

The environmental factors, especially relative humidity (RH) of *in-vitro* and *ex-vitro* conditions play a critical role on transition from *in-vitro* to *ex-vitro*.[2,4,5] The RH plays an important role in both physiological and biochemical functions such as water relation, stomatal conductance, transpiration rate and water oxidation.[6-11] The reduction of water relation including relative water content, water potential and osmotic potential is due to water loss through the foliage. The *ex-vitro* plantlets normally show responsive damage

such as wilting, chlorosis, necrosis and senescence.[12,13] There are several reports on reducing RH during *in-vitro* acclimatization.[4,5,14-16] Reduction of RH *in-vitro* successfully improved the quality of plantlets as indicated by epicuticular wax deposition, restored stomatal function and photosynthetic ability, resulting in a high percentage of survival and a high growth rate after transplanting to *ex-vitro*.[17-19]

In these experiments, Thai neem was used as a model of woody species. The objective of this study is to develop the *in-vitro* acclimatization technique by RH reduction for direct transplanting to *ex-vitro*. The survival percentage, growth efficiency and physiological characteristics of *in-vitro* acclimatized plantlets and *ex-vitro* adaptation were evaluated.

Materials and Methods

Establishment of Plant Materials

In-vitro propagation

Shoots of a salt-tolerant clone of Thai neem were multiplied on 0.25% Phytagel®-solidified MS medium[20] supplemented with 8.88 μM 6-benzylaminopurine (BA). The multiple shoots (2.50±0.25 cm in length) were cultured on MS medium without plant growth regulator (PGR) and then roots were induced on MS medium supplemented with 2.46 μM indole-3-butyric acid (IBA). The culture medium was supplemented with 90 mM sucrose (photomixotrophic condition) and pH adjusted to 5.7 before autoclaving. The shoots were cultured in 240 ml glass vessels and incubated at 25±2 °C ambient temperature, 60±5% relative humidity (RH) and 60±5 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPF). The PPF was provided by fluorescent lamps (TLD 36W/84, Cool White, Philips, Thailand) with a 16 h d^{-1} photoperiod.

Photoautotrophic growth

The plantlets (2.50±0.25 cm in length) with 3-4 leaves were used as plant material and then transferred into 240 ml glass vessels containing 10 g vermiculite as a supporting material, and 30 ml sugar-free liquid MS medium (photoautotrophic condition). The air exchange rate in the culture vessel was adjusted to 2.3±0.2 h^{-1} by punching hole in the plastic cap (∅ 1 cm) and replacing this with a gas-permeable microporous polypropylene film (0.22 μm of pore size, Nihon Millipore Ltd., Japan). The plantlets were cultured under temperature shift of 28±2 °C /25±2 °C (16 h photoperiod/8 h dark period), 60±5% RH, 100±5 μmol $m^{-2}s^{-1}$ PPF and CO_2-enrichment (1000±100 μmol mol^{-1}) conditions in Plant Growth Incubator (EYELA, Model EYELATRON FLI-301LH, Japan) for 28 days.

In-vitro Acclimatization

The glass vessels containing plantlets were transferred into an aseptic culture chamber (Carry Box Model P-850, size 26×36×19 cm, Japan) in which RH condition controlled at 95±5% (high RH) by 1,500 ml distilled-water, and at 65±5% (low RH) by 1,500 ml saturated-NaCl solution (Fig 1). The air exchange rate in the culture chambers was increased to 5.1±0.3 h^{-1} by punching the side of the plastic chambers with 32 holes and replacing this with a gas-permeable microporous polypropylene film (0.22 μm of pore size) over each hole. These chambers were incubated under temperature shift of 28±2 °C / 25±2 °C (16 h pho-

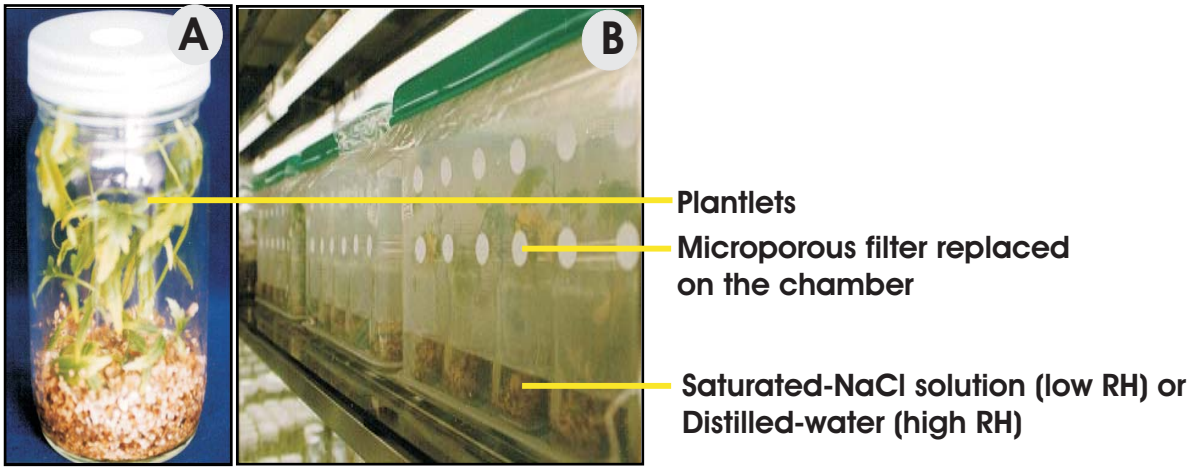

Fig 1. Culture of Thai neem plantlets. (a) The plantlets were grown under photoautotrophic conditions in glass vessels containing vermiculite as a supporting material. (b) The glass vessels (without the plastic caps) were placed in the culture chamber, in which the low (65±5%) and high (95±5%) RH conditions were controlled by saturated NaCl solution and distilled water, respectively, and the air-exchange rate by microporous filter.

toperiod/8 h dark period), 100±5 μmol $m^{-2}s^{-1}$ PPF, 60±5% RH and CO_2-enrichment (1,000±100 μmol mol^{-1}) conditions in Plant Growth Incubator (EYELA, Model EYELATRON FLI-301LH, Japan) for 42 days. Leaf water content, chlorophyll content, netphotosynthetic rate (*NPR*), stomata number, leaf area, fresh weight and dry weight were measured.

Ex-Vitro Adaptation

The plantlets acclimatized under high RH and low RH conditions for 42 days were transplanted into open plastic bags (size 10W×10L×20H cm), containing two parts of soil and one part of vermiculite (one plantlet per bag). Eighty plantlets in each treatment were planted out in a glass house at 30±2 °C ambient temperature, 75±5% RH and 700-800 μmol $m^{-2}s^{-1}$ PPF light intensity at plant level with 10 h d^{-1} photoperiod. All plants were watered twice a day and measured for *NPR*, maximum quantum yield (F_v/F_m), quantum efficiency of photosystem II (Φ_{PSII}), water use efficiency (*WUE*), stomatal conductance (*G*), transpiration rate (*E*) and survival percentage at days 0, 1, 3 and 5.

Measurement of Anatomical, Morphological and Physiological Characteristics

Anatomical and morphological characteristics

The stomata of the third leaf (abaxial part) from the shoot tip was immediately painted with nail-enamel solution, taped with transparent tape and then the printed-stomata was placed on a glass slide. The stomata were counted under a light microscope, 40× for 16 fields per treatment. Fresh weight, dry weight, leaf area and leaf area ratio (*LAR*) of plantlets were analyzed for growth efficiency by following the Lutt method.[21]

Physiological characteristics

Water relation, transpiration rate (*E*; mol $m^{-2}s^{-1}$) and stomata conductance (*G*; mol H_2O $m^{-2}s^{-1}$), were measured on the third leaf from the shoot tip by Infrared Gas Analyser (IRGA; Model Portable Photosynthesis System LI 6400, LI-COR® Inc, USA) and calculated by following Pan equations.[22] Water use efficiency (*WUE*) of the leaves was calculated by the ratio of *NPR* to *E* according to Estrada-Luna.[5] Leaf water content (*LWC*) was calculated from the ratio of leaf fresh weight (FW) and leaf dry weight (DW) by following the Bonnet method.[23]

The photosynthetic system such as chlorophyll content, chlorophyll *a* fluorescence and netphotosynthetic rate were measured. The chlorophyll content, chlorophyll *a* and chlorophyll *b*, in the leaf tissues was extracted with 95.5% (v/v) acetone and analyzed by UV-visible Spectrophotometer (HACH DR/4000; Model 48000, HACH Company, USA) using the methodology as described by Shabala.[24] The chlorophyll *a* fluorescence, maximum quantum yield (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}), of the adaxial surface of the third leaf from the shoot tip was monitored by Fluorescence Monitoring System (*FMS* 2; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggini.[25] Carbon dioxide concentration inside and outside the chamber was measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan) and the net photosynthetic rate (*NPR*) of *in-vitro* was calculated according to Fujiwara[26] and Kirdmanee methods,[4] while the *ex-vitro* plantlets were measured by Infrared Gas Analyser (IRGA; Model Portable Photosynthesis System LI 6400, LI-COR® Inc, USA) and calculated as described by Pan.[22]

Experimental Design

The experiment was designed as a Completely Randomized Design (CRD) with 4 replications and 20 plantlets per replication. The mean of treatments was compared by *t*-test ($P \leq 0.01$) and analyzed using SPSS software (SPSS for Windows, SPSS Inc., USA). The correlation of leaf water content and *NPR*, chlorophyll content and *NPR*, and leaf water content and survival percentage were evaluated by Pearson's correlation coefficients.

Results and Discussion

In-Vitro Acclimatization

In-vitro plantlets acclimatized under low RH conditions (Fig 2a) had significantly better growth than those acclimatized under high RH conditions (Fig 2b). Leaf area and leaf area ratio (*LAR*) of plantlets acclimatized under low RH conditions were markedly higher than those acclimatized under high RH conditions by 1.4 and 4.0 times, respectively. The fresh weight and dry weight of plantlets acclimatized under low RH conditions were higher than those acclimatized under higher RH conditions by 1.3 and 1.4 times, respectively (Table 1). The low RH conditions of *in-vitro* acclimatization promoted the leaf expansion as indicated by the leaf area and *LAR* parameters (Table 1). The growth efficiency of *in-vitro* plantlets can be predicted by leaf area, fresh and dry weights, as these are the best indicators of vigorous plantlets.[27] The leaf tissues are composed of mitochondrion and chloroplast organelles as a primary factory for energy production by respiration and carbon gain accumulation in storage organs, indicated by high value of fresh and dry weight (Table 1). The low RH conditions of *in-vitro* acclimatization strongly promoted the uniformity and higher density of stomata in the abaxial part of the leaf (Fig 2c) compared to those acclimatized under high RH conditions (Fig 2d). In addition, the plantlets acclima-

Table 1. Leaf water content, leaf area, *LAR*, fresh weight, dry weight and survival percentage of *in-vitro* acclimatized plantlets under high RH and low RH conditions for 42 days. In all catagories, values for high and low RH conditions are significantly different at $p \leq 0.01$ by *t*-test.

Treatment	Leaf water content (%)	Leaf area (cm^2)	*LAR*	Fresh weight (g)	Dry weight (g)	Survival (%)
High RH (95±5%)	36.4	92	0.5	2.43	0.49	37.5
Low RH (65±5%)	46.3	124	2.0	3.25	0.63	87.5

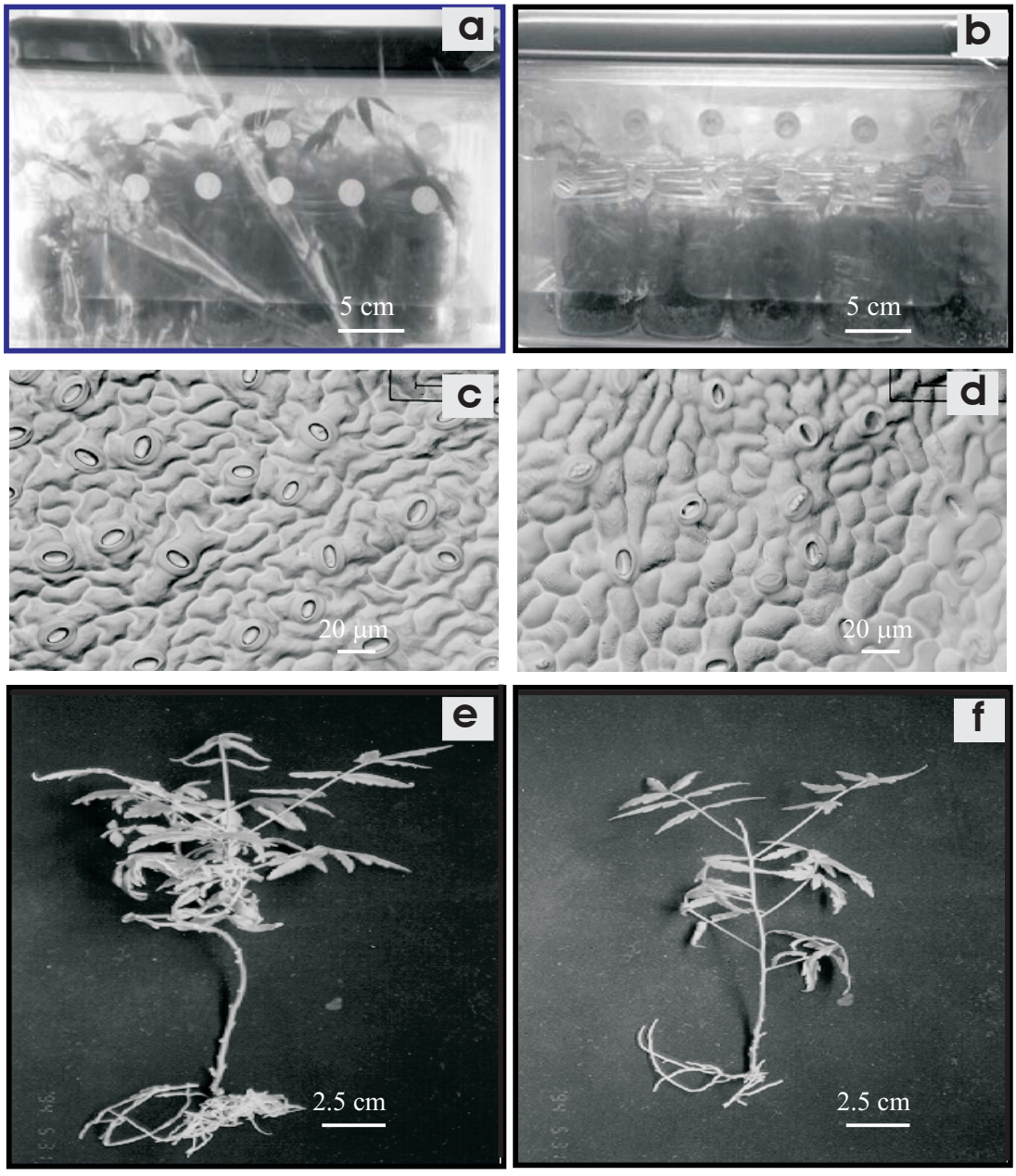

Fig 2. Comparisons of growth and development of *in-vitro* acclimatized plantlets under low RH condition (65±5%) and high RH condition (95±5%). The growth of plantlets acclimatized under low RH condition (a) and high RH condition (b), stomata of plantlets acclimatized under low RH condition (c) and high RH condition (d) and morphological characteristics of plantlets acclimatized under low RH condition (e) and high RH condition (f) for 42 days.

tized under low RH conditions were significantly different in their morphological characteristics, including plant height, leaf area, root number and root length, when compared to those acclimatized under high RH conditions (Figs 2e, 2f). The reduction of RH in the culture vessel using saturated-salt solutions has been used to produce vigorous plantlets in several species such as potato,[14,16] radiata pine[15] and *Eucalyptus*.[4]

Water content in the leaf tissues of plantlets acclimatized under low RH conditions was 46.3% higher than those acclimatized under high RH conditions (Table 1). The water content in the leaf tissues of plantlets plays an important role in water relation, causing the stomatal functions such as stomatal closure, gas-exchange and transpiration rate.[13,28] The leaf water content closely relates to net photosynthetic rate of the plantlets ($R^2 = 0.97$) (Fig 3). The leaf water content mainly affects water oxidation of photosystem II (PSII), as well as the stomata opening for carbon dioxide (CO_2) fixation.[13,29-30] In addition, there was a higher accumulation of chlorophyll *a* and chlorophyll *b* in the leaf tissues of plantlets acclimatized under low RH conditions. The chlorophyll content positively relates to net photosynthetic rate ($R^2 = 0.93$) (Fig 4). The chlorophyll pigments convert electronic energy into chemical energy by transferring electrons from reaction centers to acceptor molecules in photosystem II (PSII) of light reaction.[13,24]

Results from this study showed that the low RH conditions of *in-vitro* acclimatization promoted the growth and development of plantlets, as evaluated by morphological, anatomical and physiological characteristics.[14,16] These characteristics indicating vigorous plantlets, were directly responsible for the high survival percentage after transplanting to *ex-vitro* (Table 1). The high survival percentage of plantlets was strongly related to leaf water content ($R^2 = 0.85$) (Fig 5). Thus, appropriate RH conditions in the culture chamber are an important factor in producing vigorous plantlets before transplanting to *ex-vitro*.[31]

Ex-Vitro Adaptation

The water relation of *ex-vitro* plantlets was indi-

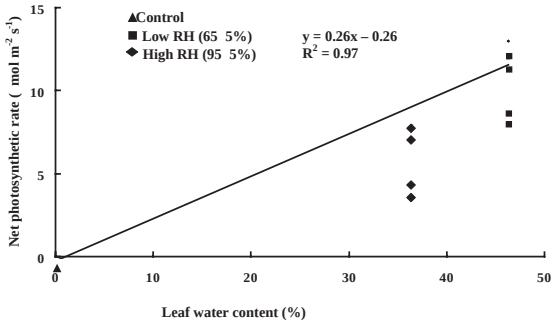

Fig 3. Correlation of leaf water content and net photosynthetic rate of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) for 42 days. Control (▲) represents the direct transplanting of *in-vitro* plantlets (photomixotrophic growth) to *ex-vitro*.

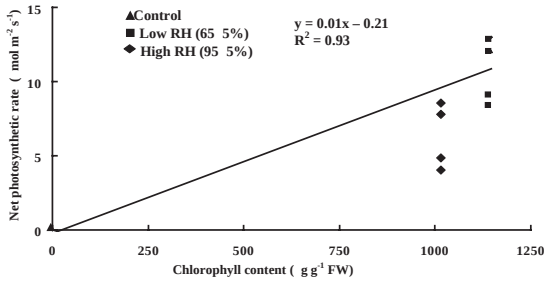

Fig 4. Correlation of chlorophyll content and net photosynthetic rate of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) for 42 days. Control (▲) represents the direct transplanting of *in-vitro* plantlets (photomixotrophic growth) to *ex-vitro*.

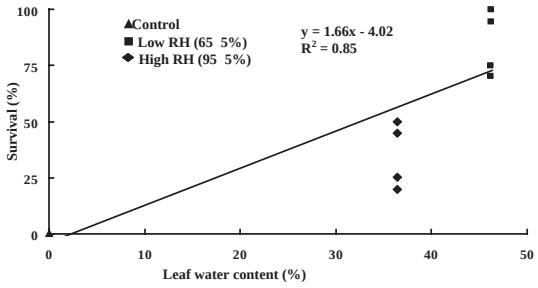

Fig 5. Correlation of leaf water content and survival percentage of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) for 42 days. Control (▲) represents the direct transplanting of *in-vitro* plantlets (photomixotrophic growth) to *ex-vitro*.

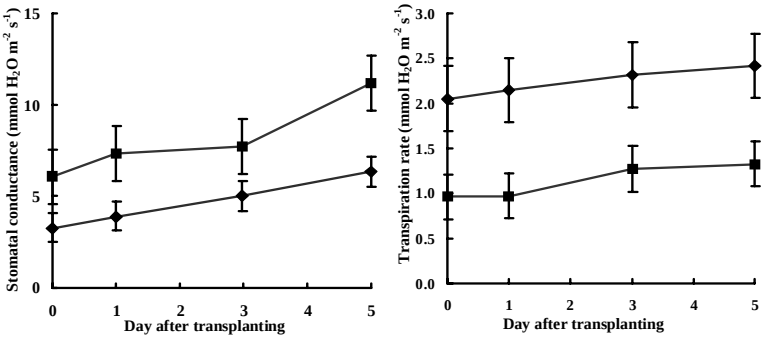

Fig 6. Stomatal conductance (a) and transpiration rate (b) of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) after transplanting to *ex-vitro*. Error bars represent ±SE.

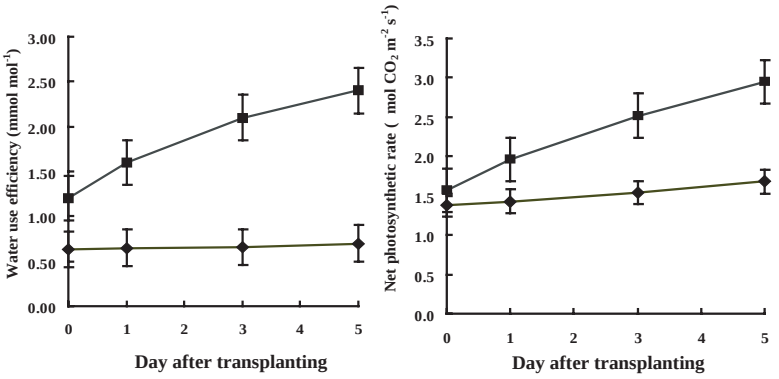

Fig 7. Water use efficiency (a) and net photosynthetic rate (b) of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) after transplanting to *ex-vitro*. Error bars represent ±SE.

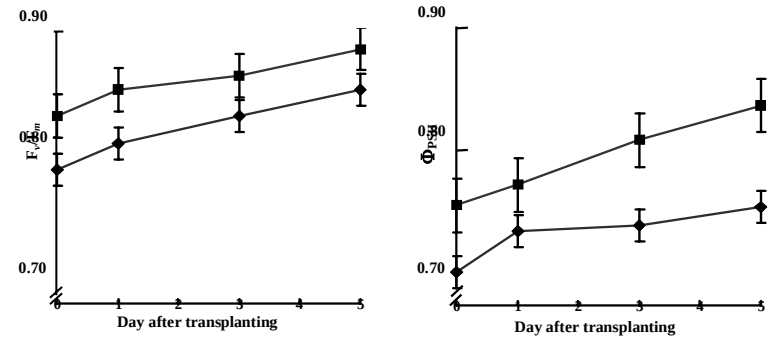

Fig 8. Maximum quantum yield of photosystem II photochemistry (F_v/F_m) (a) and quantum efficiency of photosystem II (Φ_{PSII}) (b) of *in-vitro* acclimatized plantlets under low RH condition (■) and high RH condition (◆) after transplanting to *ex-vitro*. Error bars represent ±SE.

cated by stomatal conductance (*G*), transpiration rate (*E*) and water use efficiency (*WUE*). The G values of plantlets acclimatized under low RH conditions was higher than those acclimatized under high conditions (Fig 6a). High *G* values retarded the E values of plantlets (Fig 6b). The *WUE* of plantlets acclimatized under low RH conditions slightly increased within the day after transplanting and were higher than those acclimatized under high RH conditions (Fig 7a). The net photosynthetic rate (*NPR*) of plantlets in both treatments responded to *ex-vitro* conditions as the *WUE* (Fig 7b). During the stomatal function monitoring, *G* of leaf tissues is a critical point for water loss and carbondioxide fixation.[5,13] The *E* value is initially high because of the low *G* from high rate of water loss, resulting in wilting, chlorosis, necrosis, and senescence.[32] The *G* value plays an important role in regulating water loss from plantlets, controlling the carbondioxide uptake

through stomata, as well as sustaining CO_2 concentration during photosynthesis.[4,13] The causes of a low survival rate are the low water use efficiency and insufficient CO_2 fixation through stomata.[12,13,33]

Chlorophyll *a* fluorescence represents maximum quantum yield of PSII photochemistry (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}). The plantlets acclimatized under low RH conditions had significantly higher chlorophyll *a* fluorescence after transplanting than those acclimatized under high RH conditions (Figs 8a, 8b). Reaction center of PSII (F_v/F_m and Φ_{PSII}) generated in the thylakoid membrane of the water-oxidizing step is more sensitive in its response to water deficiency.[34,35] The F_v/F_m and Φ_{PSII} parameters have been used as indices for water-deficit damaging the PSII reaction center that has been demonstrated in many plant species such as cedar, sorghum and wheat.[34,35] These parameters are indicative of physiological adaptation of *in-vitro* plantlets after transplanting to *ex-vitro*.

The role of RH reduction in *in-vitro* acclimatization is to improve the potential survival rate of vigorous plantlets before transplanting to *ex-vitro*. This potential can be determined by high chlorophyll content, *NPR*, leaf area, *LAR*, fresh weight and dry weight. Such plantlets could be directly transplanted and would be highly adapted to *ex-vitro*, as indicated by the high values of *G*, *NPR*, *WUE*, F_v/F_m and Φ_{PSII}.

In conclusion, our experiments determined that plantlets acclimatized under low RH conditions promote the growth and development of *in-vitro* plantlets and promote rapid adaptation after direct transplanting to *ex-vitro*, resulting in a higher survival percentage of 87.5%. It is proposed that in the future, the water-uptake and water translocation in the root system of plantlets should be further studied.

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