Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

Asian Journal of Plant Sciences 4 (2): 109-116, 2005
ISSN 1682-3974
© 2005 Asian Network for Scientific Information

The *ex vitro* Survival and Growth of Ginger (*Zingiber officinale* Rocs.) Influence by *in vitro* Acclimatization under High Relative Humidity and CO_2 Enrichment Conditions

[1]Suriyan Cha-um, [2]Nguyen Minh Tuan, [3]Kongchay Phimmakong and [1]Chalermpol Kirdmanee
[1]National Center for Genetic Engineering and Biotechnology,
National Science and Technology Development Agency,
113 Thailand Science Park, Paholyothin Rd., Klongluang, Pathumthani 12120, Thailand
[2]Plant Cell Technology Laboratory, Institute of Tropical Biology,
01 Mac Dinh Chi Street, District 1, Ho Chi Minh City, Vietnam
[3]The National University of Laos, Department of Biology, Faculty of Science, Dongdok, Laos

Abstract: The aim of this investigation was to acclimatize the ginger (*Zingiber officinale* Rosc.) plantlets cultured photoautotrophically *in vitro* under different RH and CO_2 concentrations and subsequent *ex vitro* adaptation. Plantlets acclimatized *in vitro* under medium or high RH (80±5 or 95±5% RH) with 1,000±100 μmol mol^{-1} (CO_2-enrichment) conditions possessed significantly higher Relative Water Content (RWC) than those acclimatized *in vitro* under low RH (65±5% RH) with 450±100 μmol mol^{-1} (CO_2 non-enrichment) conditions. The RWC of acclimated plantlets was positively related to maximum quantum yield of PSII photochemistry (F_v/F_m) (r = 0.77), quantum efficiency of PSII (Φ_{PSII}) (r = 0.89) and stomatal conductance (G) (r = 0.99), while negatively related to transpiration rate (r = 0.98). Moreover, the total chlorophyll concentration was closely related to Φ_{PSII} (r = 0.77), lead to increase net photosynthetic rate (NPR) (r = 0.87). As well as, the intracellular CO_2 concentration (C_i) and WUE of acclimatized plantlet positively related to NPR (r = 0.96 and r = 0.85, respectively), resulting in growth promotion (r = 0.99), as defined by the parameters of leaf area, fresh weight, dry weight, root number and root length. Five days after transplantation, the WUE, G, F_v/F_m and Φ_{PSII} of ginger plantlets acclimatized *in vitro* under high RH with CO_2-enriched conditions were significantly higher than those acclimatized *in vitro* under low RH and without CO_2 enrichment, while E and transpiration ratio (Tr) were significantly lower. The plantlets acclimatized under high RH with CO_2-enrichment conditions showed the highest adaptive abilities and WUE, resulting in the highest survival percentage (90-100%) after transplantation to *ex vitro*.

Key words: Chlorophyll concentration, net photosynthetic rate, relative water content, survival percentage, vigorous plantlet, water use efficiency

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is an annual crop belonging to the family *Zingiberaceae*. In addition to its common usage as a spice, ginger rhizomes have a long history of use as a medicine throughout parts of Asia for its purported antiemetic and anti-inflammatory properties. Unfortunately, ginger is susceptible to soil borne diseases, most notably soft rot (caused by *Pythium aphanidermatum*) and bacterial wilt (caused by *Ralstonia solanacearum*). One of the most practical and efficient ways to solve this problem is to produce disease-free ginger from meristem-tip culture[1,2]. Nevertheless, use of disease-free ginger from *in vitro* propagation is still limited due to the complicated transplantation process and low survival percentage after transplantation, resulting in a high cost of production[3].

Although, micropropagation has many advantages over the conventional propagation for large-scale production, the technique is limited by the low growth rate and low survival percentage of plantlets after being transferred to *ex vitro* conditions[4]. The artificial conditions during *in vitro* growth result in various morphological, anatomical and physiological disorders in plantlets, which may impair the ability of plantlets to adjust to sudden changes in environmental conditions,

Corresponding Author: Suriyan Cha-um, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Paholyothin Rd., Klongluang, Pathumthani 12120, Thailand
Tel: +662 5646700 Fax: +662 5646707 E-mail: suriyanc@biotec.or.th

there by leading to high rates of mortality following *ex vitro* transplantation[5]. The poor survival percentage of plantlets after transplantation to *ex vitro* is a critical topic of investigate in the area of plant micropropagation and thus many attempts have been reported on solving this problem[4,5]. The application of an *in vitro* acclimatization phase can increase the vigor of plantlets, resulting in a higher survival percentage after transfer to *ex vitro* conditions[6].

Relative Humidity (RH) is an important environmental factor of *in vitro* acclimatization[7-9]. RH influences the Relative Water Content (RWC) of whole plant. The RWC, in turn, affects a number of physiological characteristics, including stomatal conductance (G), CO_2 assimilation, water oxidation and Net Photosynthetic Rate (NPR). Decreasing the RWC of plantlets progressively decreased G and limited on CO_2 assimilation, thereby leading to a reduction in NPR[10,11]. The NPR of *in vitro* plantlets can also be restricted by the low CO_2 concentrations within culture vessels lacking adequate gas exchange[12]. The NPR of *in vitro* plantlets cultured under CO_2-enriched conditions was greater than those cultured on the sugar-containing media under CO_2 non-enrichment conditions[12,13]. CO_2-enrichment also promotes the growth of plantlets during the *in vitro* acclimatization stage[14,15]. The environmental factor in term of RH and CO_2 plays an important role on vigorous plantlet production with high survival percentage after transferred to *ex vitro* conditions[9,14,15]. The objectiveness of this investigation is to examine the acclimatization of ginger plantlets cultured photoautotrophically *in vitro* under different RH and CO_2 concentrations. The subsequence growth of plantlets after transplantation to *ex vitro* is evaluated.

MATERIALS AND METHODS

***In vitro* acclimatization:** Single shoots of disease-free ginger (*Zingiber officinale* Rosc.) with 2-3 fully-expanded leaves (fresh weight; 300±20 mg) were aseptically excised from micropropagated-plantlets. The shoots were cultured photoautotrophically on sugar-free MS media using vermiculite as a supporting material. All shoots were cultured for 7 day at 25±2°C ambient temperature, 65±5% RH and 100 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) with a 16 h d^{-1} photoperiod provided by fluorescence lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand). For each treatment, twenty glass vessels containing *in vitro* cultured plantlets were placed in a plastic chamber box (length×width×height; 32×24×18 cm). Each box contained either 1500 mL KCl, a NaCl saturated-salt solution or distilled water in order to maintain RH at 65±5, 80±5 or 95±5% RH, respectively. The number of air exchange in the chamber boxes was adjusted to 5.1±0.3 h^{-1} by punching the side of the plastic boxes with 32 holes and replacing with gas-permeable microporous polypropylene film (0.22 μm pore size) over each hole. The chamber boxes were placed into either a Plant Growth Incubator (EYELA, Model EYELATRON FLI-301LH, Japan) for CO_2 enrichment (1,000±100 μmol mol^{-1}) or a culture room for growth under the CO_2 non-enrichment (450±100 μmol mol^{-1}) condition (Table 1). All chamber boxes were incubated at 25±2°C ambient temperature and 100 μmol m^{-2} s^{-1} PPF with 16 h d^{-1} photoperiod for 35 days (Fig. 1). The pigment concentration, net-photosynthetic rate and growth characteristics of *in vitro* acclimatized plantlets were measured prior to *ex vitro* adaptation, as described below.

Table 1: Description of treatment codes in the experiment

Treatment codes	CO_2 concentration (μmol mol^{-1})	RH (%)
LL	450±100	65±5
LM		80±5
LH		95±5
HL	1,000±100	65±5
HM		80±5
HH		95±5

***Ex vitro* adaptation:** Thirty-five days acclimatization, the plantlets were transplanted into pots containing a mixture of two parts soil and one part vermiculite. Twenty plantlets in each treatment were planted out in a glass house, at 30±2°C air temperature, 75±5% RH and 300-400 μmol m^{-2} s^{-1} PPF of natural light intensity at plant level with 10 h d^{-1} photoperiod (Fig. 1). All plants were watered twice a day. Maximum quantum yield (F_v/F_m), quantum efficiency of photosystem II (Φ_{PSII}), stomatal conductance (G), transpiration rate (E), transpiration ratio (Tr) and Water Use Efficiency (WUE) were measured 5 days after transfer to *ex vitro* conditions.

Measurement of growth and physiological characteristics

Growth characteristics: Leaf area, fresh weight, dry weight, number of root and root length of plantlets were analyzed following by Lutt method[16].

Physiological characteristics: Transpiration rate (E, mol m^{-2} s^{-1}), stomatal conductance (G, mol H_2O m^{-2} s^{-1}) and transpiration ratio (Tr) were measured by the Infrared Gas Analyzer (IRGA; Model Portable Photosynthesis System LI 6400, LI-COR® Inc, USA) and calculated by the Pan equation[17]. Water Use Efficiency (WUE) of leaves was calculated by the ratio of NPR to E according to Estrada-Luna[18].

The photosynthetic systems, pigment concentration, chlorophyll a fluorescence and net photosynthetic rate, were measured. The pigment concentrations

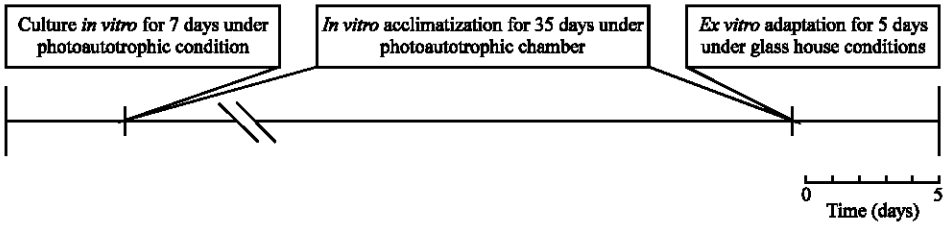

Fig. 1: Scheme of the experiment on *in vitro* acclimatization for 35 days and subsequently transferred to *ex vitro* under glass house conditions for 5 days

(chlorophyll a, chlorophyll b and total carotenoid) were analyzed by the methods of Shabala[19] and Lichtenthaler[20]. The maximum quantum yield of PSII photochemistry (F_v/F_m) and quantum efficiency of PS II (Φ_{PSII}) of the adaxial leaf surface were measured by Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggini[21]. Carbon dioxide concentration inside and outside the chamber was measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan) and the NPR of *in vitro* plantlets were calculated according to Fujiwara[22], while the NPR of *ex vitro* plantlets were measured by IRGA and then were calculated as described by Pan[17].

Experimental design: The experiment was designed as 2×3 factorials in a Completely Randomized Design with 4 replications and 5 plantlets per replication. Means of each treatment were compared by SPSS software (SPSS Inc., USA). The correlation between RWC and F_v/F_m, RWC and ϕ_{PSII}, RWC and G, RWC and E, chlorophyll concentration and ϕ_{PSII}, ϕ_{PSII} and NPR, C_i and NPR, WUE and NPR, NPR and dry weight, WUE and survival percentage, were evaluated by Pearson's correlation coefficients.

RESULTS AND DISCUSSION

***In vitro* acclimatization:** The growth characteristics (i.e. leaf area, fresh weight, dry weight, number of root and root length) of *in vitro* plantlets acclimatized under high RH with CO_2-enrichment conditions were significantly enhanced when compared with those acclimatized under low RH with CO_2 non-enrichment conditions (Table 2). In addition, high RH and CO_2-enrichment conditions significantly enhanced on the leaf area, fresh weight, dry weight, number of root and root length. The combination of these factors was also strongly affected the leaf area, fresh weight, dry weight, number of roots and root length of plantlets. Moreover, present results showed that both root length and number were greater under high RH and CO_2-enriched conditions than under low RH without CO_2 enrichment by the factors of 2.4 and 2.8 times, respectively. The root system of the plantlets plays a critical role in water uptake and the translocation of many metabolites. Plantlets acclimatized under low RH, with or without CO_2-enrichment, exhibited the symptoms of water-deficit (i.e. chlorosis, leaf burn and growth inhibition; data not shown).

The highest Relative Water Content (RWC), 94%, was obtained when ginger plantlets acclimatized under high RH (95±5% RH) with CO_2-enrichment (1000±100 μmol mol^{-1}). The RWC of acclimatized plantlets was directly reduced to 56% by the low percentage of

Table 2: Leaf area, fresh weight, dry weight, number of root and root length of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days

Treatment	Leaf area (mm^2)	Fresh weight (mg)	Dry weight (mg)	Number of root	Root length (cm)
LL	1280d	199e	48d	1.6b	3.1c
LM	1473d	306de	58d	2.8a	4.9bc
LH	1489c	424cd	68cd	2.8a	5.4b
HL	1789b	484c	73bc	2.8a	6.8bc
HM	2255b	1012b	87b	3.3a	8.8a
HH	2864a	1518a	91a	3.8a	8.6a
Significant level					
Relative humidity (RH)	**	**	**	**	*
CO_2 concentration (CO_2)	**	**	**	**	**
RH × CO_2	**	**	*	**	*

Table 3: Carotenoid, chlorophyll a, chlorophyll b and total chlorophyll concentrations of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days

Treatment	Carotenoid (μg^{-1}g FW)	Chlorophyll a (μg^{-1}g FW)	Chlorophyll b (μg^{-1}g FW)	Total chlorophyll (μg^{-1}g FW)
LL	128b	259c	103c	362c
LM	159b	444c	159c	603c
LH	209b	595bc	211bc	806bc
HL	289a	1030ab	342ab	1372ab
HM	361a	1280a	408a	1688a
HH	339a	1300a	441a	1741a
Significant level				
Relative humidity (RH)	**	*	NS	*
CO_2 concentration (CO_2)	**	**	**	**
RH×CO_2	*	*	*	*

**,* Significance at p≤0.01 or 0.05, respectively
Means within a row followed by the different letters in each column are significantly different at p≤0.01 by New Duncan's Multiple Range Test

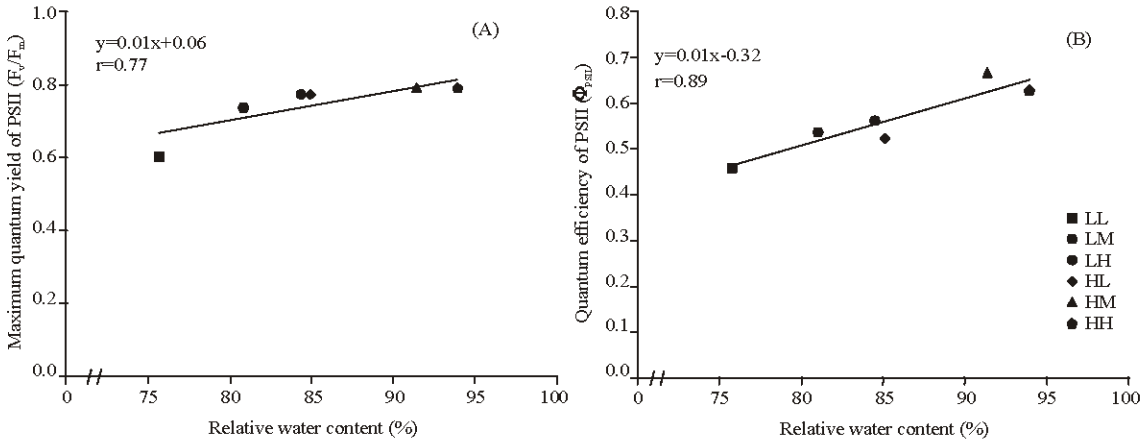

Fig. 2: Correlation between relative water content and maximum quantum yield of PSII photochemistry (A) as well as relative water content and quantum efficiency of PSII (B) of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

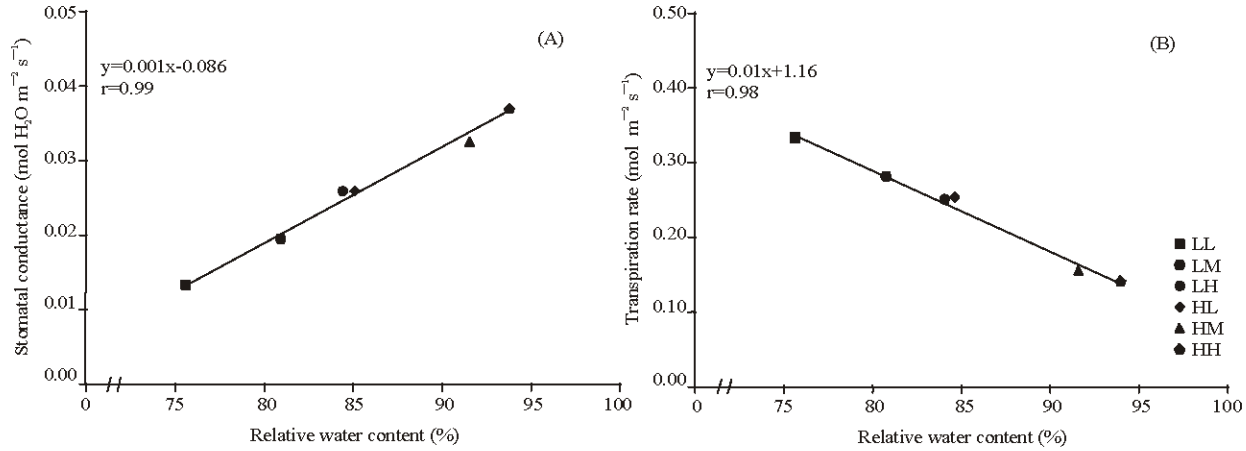

Fig. 3: Correlation between relative water content and stomatal conductance (A) as well as relative water content and transpiration rate (B) of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

RH (65±5%RH) and without CO_2 enrichment in the culture chamber. The RWC of acclimatized-plantlets was positively related to F_v/F_m (r = 0.77), ϕ_{PSII} (r = 0.89) and G, but negatively related to E (r = 0.98) (Fig. 2 and 3). The total carotenoid, chlorophyll a, chlorophyll b and total chlorophyll concentrations of *in vitro* plantlets acclimatized under high RH with CO_2-enrichment conditions were significantly higher than those acclimatized under low RH without CO_2 enrichment by factors of 2.65, 5.02, 4.28 and 4.81 times, respectively (Table 3). RH factors significantly affected on chlorophyll concentration but not carotenoid concentration, whereas CO_2-enrichment increased the concentration of caroteoid and both chlorophyll a and b. The total chlorophyll concentration of acclimatized-plantlets positively related to ϕ_{PSII} (r = 0.77) (Fig. 4A), which is consistent with the role of chlorophylls as the light harvesting complexes of the photosynthetic system. The ϕ_{PSII} of plantlets acclimatized under high RH with CO_2-enrichment conditions was 1.38 times higher than those acclimatized under low RH with CO_2 non-enrichment conditions. The decreasing of this parameter was associated with a reduction in NPR (r = 0.87) (Fig. 4B).

The stomata function (conductance) of acclimatized plantlets was strongly stimulated by the high RH and CO_2-enrichment conditions, defined by high G parameter (Fig. 3A). Normally, the stomata pores of plantlets under high RH and CO_2-enrichment are widely opened for CO_2-fixation. On the other hand, the stomata of plantlets under low RH or water-deficit condition are less opened, or even remain closed to prevent water loss (Fig. 3B). In addition, the intracellular CO_2 (C_i) and WUE of

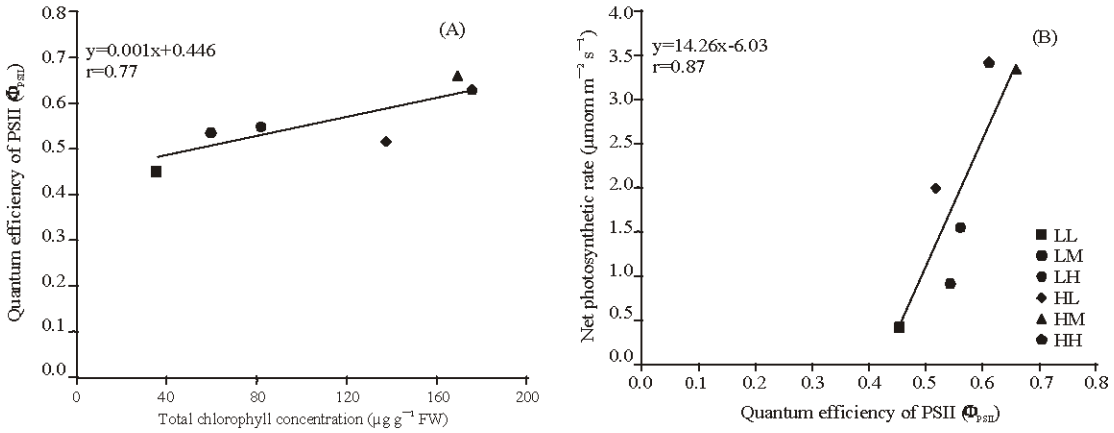

Fig. 4: Correlation between total chlorophyll concentration and quantum efficiency of PSII (A) as well as quantum efficiency of PSII and net photosynthetic rate (B) of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

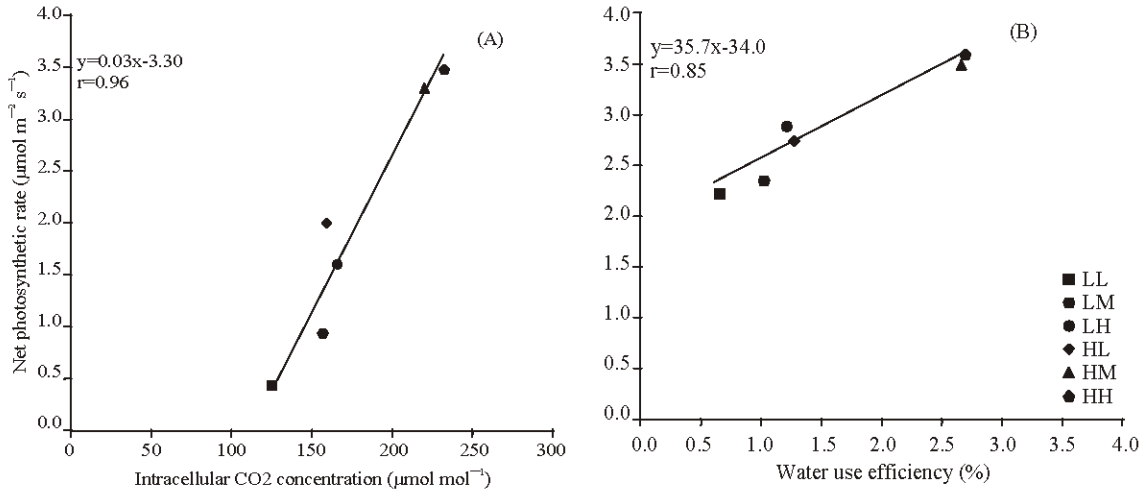

Fig. 5: Correlation between intracellular CO_2 concentration and net photosynthetic rate (A) as well as water use efficiency and net photosynthetic rate (B) of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

acclimatized-plantlets under high RH and CO_2-enrichment conditions were significantly higher than those acclimatized under low RH with CO_2 non-enrichment conditions. The C_i and WUE parameters of acclimatized-plantlets closely related to NPR (r = 0.96 and r = 0.85, respectively) (Fig. 5). The NPR of acclimatized-plantlets under high RH with CO_2-enrichment conditions was enhanced more than those acclimatized under low RH without CO_2 enrichment. This parameter positively related to dry weight or growth characteristics of acclimatized-plantlets (r = 0.99) (Fig. 6A).

The ideal RH for *in vitro* plantlets acclimatization is dependent upon the plant species and the transferring procedures used[23,24]. However, ginger plantlets acclimatized under low RH exhibited on low RWC or water deficit. Reducing RH conditions during acclimatization results in better adaptation to *ex vitro* conditions in the cases of potato[7,8], *Eucalyptus*[9] and Thai neem[25]. Low RWC in plantlets has a negative effect on both the dark reaction of photosynthesis [i.e. low stomatal conductance (G), low CO_2-assimilation and high transpiration rate (E)] and light reaction [*i.e.* low F_v/F_m and low ϕ_{PSII}], resulting in an overall reduction in NPR[10,11,25]. On the other hand, the high RWC of ginger plantlets acclimatized under high RH with CO_2-enrichment conditions prevented on pigment degradation (Table 3), resulting in stimulation of light harvesting for water oxidation or ATP production in the light reaction, as represented by the regulation of chlorophyll a fluorescence (*i.e.* F_v/F_m and ϕ_{PSII}), as well as regulation of stomata for CO_2-assimilation[26,27]. The photosystem II regulation and CO_2-assimilation of ginger plantlets acclimatized under high RH with CO_2-enrichment

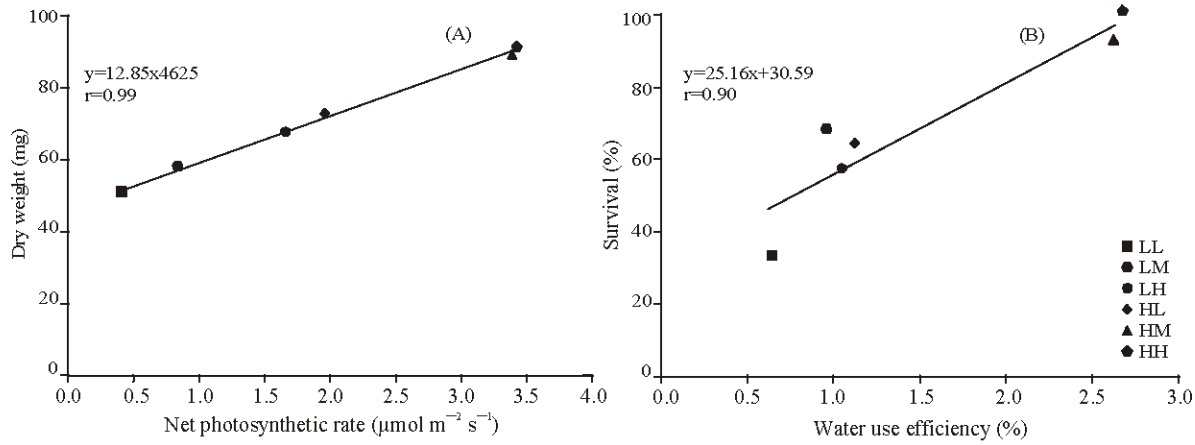

Fig. 6: Correlation between NPR and dry weight (A) as well as water use efficiency and survival percentage (B) of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

Table 4: Water use efficiency, stomatal conductance, transpiration rate, transpiration ratio, maximum quantum yield of PSII photochemistry and quantum efficiency of PSII of ginger plantlets acclimatized *in vitro* under different relative humidities and CO_2 concentrations for 35 days and subsequently transferred to *ex vitro* conditions for 5 days

Treatment	Water use efficiency (%)	Stomal conductance (μmol H_2O $m^{-2}s^{-1}$)	Transpiration rate (mmol $m^{-2}s^{-1}$)	Transpiration ratio	Maximum quantum yield of PSII	Quantum efficiency of PSII
LL	0.6c	13.2c	0.35a	6.72a	0.53c	0.42c
LM	1.1bc	26.4b	0.28b	3.26b	0.65b	0.47bc
LH	0.9bc	27.8b	0.25b	0.85cd	0.78a	0.57b
HL	1.1bc	26.8b	0.24b	1.85c	0.78a	0.62b
HM	2.6a	32.6ab	0.13c	0.52d	0.79a	0.73a
HH	2.7a	39.9a	0.14c	0.28d	0.80a	0.70a
Significant level						
Relative humidity (RH)	*	*	*	**	*	*
CO_2 concentration (CO_2)	**	**	**	**	**	**
RH×CO_2	*	*	*	**	*	*

**,* Significance at p≤0.01 or 0.05, respectively
Means within a row followed by the different letters in each column are significantly different at p≤0.01 by New Duncan's Multiple Range Test

directly enhanced NPR and growth. In photoautotrophic system, CO_2 in the culture vessel is the main carbon source for photosynthesis. Normally, the CO_2 inside of sealed culture vessels is quite low due to the limited rate of the gas exchange. Thus, the elevation of CO_2 in a photoautotrophic system is an alternative method for NPR promotion. CO_2-enrichment of *in vitro* plantlets directly enhanced CO_2-uptake by increasing stomatal conductance or stomata-opening for CO_2-assimilation, resulting in the high C_i[28] for photosynthesis system.

***Ex vitro* adaptation:** The WUE, G, E, Tr, F_v/F_m and ϕ_{PSII} were measured after *ex vitro* adaptation. Plantlets acclimatized under high RH with CO_2-enrichment conditions were better adapted to *ex vitro* conditions than those acclimatized under low RH with CO_2 non-enrichment conditions. The WUE and G of *ex vitro* plantlets were enhanced when acclimatized under high RH with CO_2-enrichment conditions, while E was decreased. The WUE of acclimatized-plantlets was positively related to survival percentage (r = 0.90) (Fig. 6B). In addition, chlorophyll a fluorescence as determined by F_v/F_m and ϕ_{PSII}, the energy production of photosystem II was highest for plantlets acclimatized under high RH with CO_2-enrichment conditions (Table 4). Present results indicate that plantlets acclimatized under high RH with CO_2-enrichment conditions possess vigorous root systems, a high efficiency of water relation and a high NPR consistent with vigorous plantlets. These plantlets displayed the highest survival percentage when transplanted to *ex vitro* conditions.

The physiological adaptations of vigorous plantlets with high survival percentage in *ex vitro* conditions have been widely investigated in many plant species i.e. pepper[18], tobacco[14], carnation[15], neem tree[25] and *Eucalyptus*[9]. Ginger plantlets acclimatized under high RH with CO_2-enrichment conditions grew vigorously *in vitro* and rapidly adapted to *ex vitro* conditions. These

plantlets increased CO_2 assimilation through higher WUE and G, while reducing water-loss through decreases in E and Tr, most likely through the regulation of stomata[14,24]. Furthermore, these plantlets possessed the highest water oxidation, F_v/F_m and ϕ_{PSII} after transplant to *ex vitro*, as well as the high survival percentage[24,25]. The F_v/F_m and ϕ_{PSII} parameters have been used as an indicator of water-deficit damages of PSII reaction center, as well as of physiological adaptation after transfer to *ex vitro* conditions[11,25].

In conclusion, *in vitro* acclimatization of ginger plantlets under high RH with CO_2-enrichment produced plantlets with both vigorous shoot and root systems of the various combinations of RH and CO_2 concentration tested, they exhibited the highest water use efficiency, pigment concentration and water oxidation in PSII, leading to the highest NPR. These plantlets rapidly adapted to *ex vitro* conditions and demonstrated by their enhanced growth and high survival percentage.

ACKNOWLEDGMENTS

We are grateful to Asean Foundation and National Center for Genetic Engineering and Biotechnology (BIOTEC) for funding source of Short-term Training in Biotechnology Program: Year 2003.

REFERENCES

1. Noguchi, Y. and O. Yamakawa, 1988. Rapid clonal propagation of ginger (*Zingiber officinale* Rosc.). Japan J. Breed., 38: 437- 442.
2. Sharma, T.R. and B.M. Singh, 1994. Production of disease-free encapsulated buds of *Zingiber officinale* Rosc. Plant Cell Rep., 13: 300-2.
3. Sharma, T.R. and B.M. Singh, 1995. *In vitro* micro-rhizome production in *Zingiber officinale* Rosc. Plant Cell Rep., 15: 274-277.
4. Kozai, T., C. Kubota and B.R. Jeong, 1997. Environmental control for large-scale production of plants through *in vitro* technique. Plant Cell Tiss. Org. Cult., 51: 144-151.
5. Seon, J-H., Y.Y. Cui, T. Kozai and K-Y. Paek, 2000. Influence of *in vitro* growth conditions on photosynthetic competence and survival rate of *Rehmannia glutinosa* plantlets during acclimatization period. Plant Cell Tiss. Org. Cult., 61: 135-142.
6. Pospisilova, J. and D. Haisel, 2000. Photosynthetic pigment and gas exchange during *ex vitro* acclimation of tobacco plants as affected by CO_2 supply and abscisic acid. Plant Cell Tiss. Org. Cult., 61: 125-133.
7. Kozai, T., K. Tanaka, B.R. Jeong and K. Fujiwara, 1993. Effect of relative humidity in the culture vessel on the growth and shoot elongation of potato (*Solanum tuberosum* L.) plantlets *in vitro*. J. Japan Soc. Hort. Sci., 62: 413-417.
8. Tanaka, K., K. Fujiwara and T. Kozai, 1992. Effect of relative humidity in the culture vessel on the transpiration and net photosynthetic rates of potato plantlets *in vitro*. Acta Hort., 319: 59-64.
9. Kirdmanee, C., T. Kozai and J. Adelberg, 1996. Rapid acclimatization of *in vitro Eucalyptus* plantlets by controlling relative humidity *ex vitro*. Acta Hort., 440: 616-21.
10. Lawlor, D.W., 2002. Limitation to photosynthesis in water-stressed leaves: Stomata *vs.* metabolism and the role of ATP. Ann. Bot., 89: 871-85.
11. Lu, C. and J. Zhang, 1999. Effects of water stress on photosystem II photochemistry and its thermostability in wheat plant. J. Exp. Bot., 50: 1199- 1206.
12. Desjardins, Y., 1995. Factors affecting CO_2 fixation in striving to optimize photoautotrophy in micropropagated plantlets. Plant Tiss. Cult. Biotech., 1: 13-25.
13. Capellades, M., L. Lemeur and P. Debergh, 1991. Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured *in vitro*. Plant Cell Tiss. Org. Cult., 25: 21-26.
14. Pospisilova, J., H. Synkova, D. Haisel, J. Catsky, N. Wilhelmova and F. Sramek, 1999. Effect of elevated CO_2 concentration on acclimatization of tobacco plantlets to *ex vitro* conditions. J. Exp. Bot., 330: 119-126.
15. Solarova, J. and J. Pospisilova, 1997. Effect of carbon dioxide enrichment during *in vitro* cultivation and acclimatization to *ex vitro* conditions. Biol. Plant, 39: 23-30.
16. Lutt, S., J.M. Kinet and J. Bouharmont, 1996. Effect of salt stress on growth, mineral nutrient and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Plant Growth Reg., 19: 207-218.
17. Pan, Q., Z. Wang and B. Quebedeaux, 1998. Response of the apple plant to CO_2 enrichment: Changes in photosynthesis, other soluble sugars and starch. Aust. J. Plant Physiol., 25: 293-297.
18. Estrada-Luna, A.A., F.T. Davies and J.N. Egilla, 2001. Physiological changes and growth of micropropagated chile ancho pepper plantlets during acclimatization and post-acclimatization. Plant Cell Tiss. Org. Cult., 66: 17-24.

19. Shabala, S.N., S.I. Shabala, A.I. Martynenko, O. Babourina and I.A. Newman, 1998. Salinity effect on bioelectric activity, growth, Na^+ accumulation and chlorophyll fluorescence of maize leaves: A comparative survey and prospects for screening. Aust. J. Plant Physiol., 125: 609-616.
20. Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol., 148: 350-380.
21. Loggini, B., A. Scartazza, E. Brugnoli and F. Navari-Izzo, 1999. Antioxidant defense system, pigment composition and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol., 119: 1091-1099.
22. Fujiwara, K., T. Kozai and I. Watanabe, 1987. Fundamental studies on environment in plant tissue culture vessels. (3) Measurements of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. J. Agric. Meteorol., 43: 21-30.
23. Siddique, M.R.B., A. Hamid and M.S. Islam, 2000. Drought stress effects on water relations of wheat. Bot. Bull. Acad. Sin., 41: 35-39.
24. Tezara, W., V. Mitchell, S.P. Driscoll and D.W. Lawlor, 2002. Effect of water deficit and its interaction with CO_2 supply on the biochemistry and physiology of photosynthesis in sunflower. J. Exp. Bot., 375: 1781-1791.
25. Cha-um, S., K. Mosaleeyanon, K. Supaibulwatana and C. Kirdmanee, 2003. A more efficient transplanting system for Thai neem (*Azadirachta siamensis* V.) by reducing relative humidity. Science Asia, 29: 189-196.
26. Flexas, J. and H. Medrano, 2002. Drought-inhibition of photosynthesis in C_3 plants: Stomatal and non-stomatal limitations revisited. Ann. Bot., 89: 183-189.
27. Medrano, H., J.M. Escalona, J. Bota, J. Gulias and J. Flexas, 2002. Regulation of photosynthesis of C_3 plants in response to progressive drought: Stomatal conductance as a reference parameter. Ann. Bot., 89: 895-905.
28. Wheeler, R., C.L. Mackowiak, N.C. Yorios and J.C. Sager, 1999. Effect of CO_2 on stomatal conductance: Do stomata open at vary high CO_2 concentrations? Ann. Bot., 83: 243-251.