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ORIGINAL ARTICLE

In vitro photoautotrophic acclimatization, direct transplantation and ex vitro adaptation of rubber tree (*Hevea brasiliensis*)

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Abstract

We investigated the effect of carbon dioxide (CO_2)-ambient (350 µmol CO_2 mol^{-1}) and CO_2-enriched (1500 µmol CO_2 mol^{-1}) conditions of in vitro photoautotrophic system on two cultivars, 'RRIM600' and 'RRIT413' of rubber tree (*Hevea brasiliensis*) in an acclimatization process of 45 days. Survival percentage of in vitro rubber tree plantlets derived from somatic embryos under ambient CO_2 was better than those under CO_2-enriched conditions, especially in cv. 'RRIT413'. Subsequently, the survival rate of ex vitro transplanted plantlets was similar to the in vitro plantlets and abnormal morphological characters such as light-green leaves (SPAD), small leaves in cv. 'RRIT413' acclimatized under CO_2-enriched conditions were demonstrated 30 days after the plantlets were transferred into the soil. Maximum quantum yield of PSII, photon yield of PSII, stomatal conductance and transpiration rate in cv. 'RRIT413' acclimatized under CO_2-enriched conditions were sharply declined by 39.0, 50.6, 47.1 and 45.8%, respectively as compared to those acclimatized under ambient CO_2 conditions. In contrast, the in vitro acclimatized plantlets of cv. 'RRIM600' were un-responsive under both ambient- and enriched-CO_2 conditions. In conclusion, genotypic dependent in response to CO_2 enriched conditions in in-vitro acclimatization of rubber tree plantlets was evidently demonstrated as a key result to regulate plant growth and development in ex vitro environments. Interestingly, soluble sugar contents (sucrose, glucose and fructose) were increased after transplanting the plantlets of cv. 'RRIM600' acclimatized under CO_2-enriched condition into the soil and thus, can be considered as an adaptive indicator of ex vitro adaptation.

Keywords Chlorophyll fluorescence · CO_2 enrichment · Net photosynthetic rate · SPAD · Survival percentage

Introduction

Plant micropropagation is a simple technique to produce a large number of uniform, genetic fidelity (true-to-type plants), and healthy plantlets. A large number of protocols for plant micropropagation i.e. organogenesis (direct or indirect organogenesis) and somatic embryogenesis are available in the literature (Rani and Raina 2000; Beruto and Debergh 2004; Haque and Ghosh 2013a, b, 2016). In vitro microclimates, i.e. enriched macro- and micro-mineral nutrients, closed vessel without ventilation, high relative humidity and low light intensity cause the morphological and physiological disorders such as hyperhydricity, stomatal disorder, loss of photosynthetic abilities and abnormal growth and development (Hazarika 2006; Kumar and Rao 2012). Subsequently, the low survival rate in transplanting process has been a major concern, especially in the woody plant species. Low survival rate (< 60%) in aquatic rotula (Martin 2003), strawberry tree (Mereti et al. 2002) and black berry (Liu and Pijut 2008), has led to the high costs required for plant micropropagation (Pence 2011). In vitro acclimatization is a procedure to train the plantlets grown in the closed vessels (Chandra et al. 2010). Photoautotrophic growth (sugar free medium) of in vitro plantlets, before transplanting it to the

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soil, results in a healthy plantlet production (Xiao et al. 2011; Hoang et al. 2017). However, the microenvironments such as enriched-CO_2, increased air-ventilation rate, porous supporting material, reduced macronutrient supply, high light intensity, controlled relative humidity and reduced ethylene gas in the culture vessel, in photoautotrophic culture system have been well established (Seon et al. 2000; Carvalho et al. 2001; Kadleček et al. 2001). Physiological and morphological adaptations, i.e. root development, prevention of water loss by stomatal function (low transpiration rate), improved CO_2 assimilation (high net photosynthetic rate), new leaf/root emerge, leaf expansion (leaf area), increased shoot and root biomass and greenish leaves of in vitro acclimatized plantlets are very important indices of the high survival and rapid growth rate of the plantlets prior to their ex vitro (Xiao et al. 2011; Hoang et al. 2017). Recently, CO_2-enriched in vitro acclimatization has been reported as an effective strategy to produce healthy plantlets by adapting them to the ex vitro conditions (Shin et al. 2014; Pérez-Jiménez et al. 2015). In contrast, some plant species are very sensitive to high CO_2 condition, leading to impaired physio-morphological performances of CO_2-enriched in vitro acclimatization (Carvalho et al. 2002; Osário et al. 2005).

In rubber tree, there are many evidences of in vitro micropropagation via nodal cutting, and somatic embryogenesis from inner integuments and roots. In vitro seedling production of rubber tree has been well investigated with high survival rate (80%) (Sanguansermsri et al. 2015). However, the information on survival percentage of transplanted plantlets in somatic embryos derived from inner integuments, root tissues and anther initial materials of rubber tree is still lacking and needs to be investigated for commercial production (Zhou et al. 2010; Karumamkandathil et al. 2015; Nor Mayati 2015). The aim of this investigation was to improve the survival percentage and physiological and morphological adaptations of in vitro acclimatized plantlets of rubber tree under CO_2-enriched conditions when transferred to greenhouse environments.

Materials and methods

Plant materials and in vitro culture

Somatic embryos derived from the inner integuments of *Hevea brasiliensis* cvs. 'RRIM600' and 'RRIT413' immature seeds via callus induction on MH-IN medium under darkness (Carron et al. 1989), somatic embryogenesis using MH-DEN medium under dark condition and embryos formation by MH-MAT and MH-GER under 60 ± 5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) for 16 h day^{-1} photoperiod provide by fluorescent lamps prior to plantlet regeneration were used for the investigation (Prommee et al. 2014; Fig. 1). Somatic embryos were selected and cultured on the modified MH-PL medium for plantlet conversion. Plantlets were grown under conditions of 25 ± 2 °C air temperature, 60 ± 5% relative humidity (RH), and 60 ± 5 µmol m^{-2} s^{-1} photosynthetic proton flux density (PPFD) provided by fluorescent lamps (TLD 36W/84 Cool White 3350 Im, Philips, Bangkok, Thailand) with a 16-h day^{-1} photoperiod. The entire scheme of the development of the plantlets by somatic embryogenesis from the inner integuments of immature seeds has been given in Fig. 1.

In vitro photoautotrophic acclimatization and ex vitro transplantation

Plantlets (5 ± 0.5 cm in shoot height) in the test tube (Fig. 1e) were chosen as the test plant material for in vitro photoautotrophic acclimatization. Healthy plantlets were transferred to MS (Murashige and Skoog 1962) sugar-free liquid medium (photoautotrophic conditions), using vermiculite as the supporting material in vented vessels. The culture vessels containing the plantlets were incubated in an EYELA Plant Growth Incubator (model FLI-301NH, Tokyo, Japan) in CO_2-enriched culture room (1500 ± 50 µmol CO_2 mol^{-1}) or in the culture room with ambient CO_2 conditions (350 ± 50 µmol CO_2 mol^{-1}) at a temperature shift of 28 ± 2 °C for 16 h in daytime and 25 ± 2 °C for 8 h in nighttime, 60 ± 5% RH, and 120 ± 5 µmol m^{-2} s^{-1} PPFD provided by fluorescent lamps with a 16-h day^{-1} photoperiod for 45 days. The survived plantlets were directly transplanted into the plastic bags (10 cm in diameter and 30 cm in length) containing 2 kg mixed soil (EC = 2.69 dS m^{-1}; pH 5.5; organic matter = 10.36%; total nitrogen = 0.17%; total phosphorus = 0.07%, and total potassium = 1.19%). The plantlets planted in the plastic bag containing soil were incubated in a greenhouse at 500–1000 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 10 h d^{-1} photoperiod, 28 ± 2 °C temperature, and 80 ± 5% relative humidity for 30 days.

Biochemical and physiological changes

Soluble sugars (sucrose, glucose and fructose) in the leaf tissues were assayed following the modified method of Karkacier et al. (2003). In brief, 50-mg of plant sample was ground in a mortar with liquid nitrogen. One millilitre of nanopure water was added and centrifuged at 12,000 rpm for 15 min. The supernatant was collected and filtered through a 0.45 µm membrane filter (VertiPure™, Vertical®). Twenty micro-litters of the filtrate was injected into a Waters HPLC equipped with a MetaCarb 87C column (300×7.8 mm) (Varian Inc., Palo Alto, USA) and an Agilent MetaCarb 87C guard column (Part No. A5201, Agilent Technologies, Inc., Santa Clara, CA, USA). Column was incubated in the heat

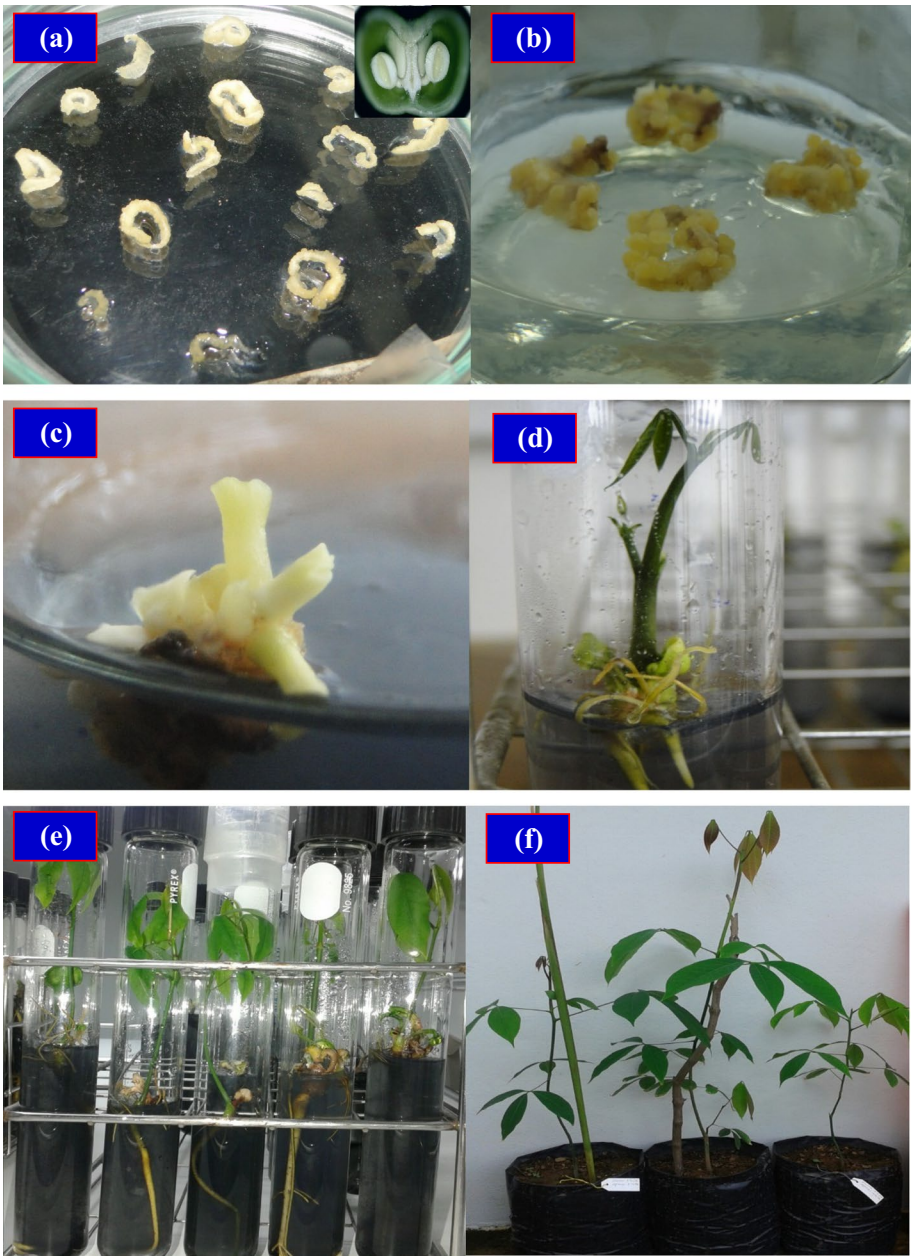

Fig. 1 A scheme of somatic embryos derived from inner integuments of rubber tree. Inner integument explants cultured on modified MH medium (**a**), callus induction (**b**), somatic embryos (**c**), plantlets derived from somatic embryogenesis (**d**), initial plantlets (**e**) and 3 month-old plant cv. 'RRIM600' in the plastic bag containing garden soil (**f**)

jacket controlled the temperature at 85 °C. Deionized water was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. The online detection was performed using a Waters 410 differential refractometer detector to control the temperature at 40 °C and the data was analysed by Empower® software. Sucrose, glucose and fructose (Fluka, USA) were used as the standards.

Chlorophyll content in the second fully developed leaf from the top was measured using Chlorophyll Meter (Model SPAD-520Plus, Konica Minolta, Osaka, Japan). Chlorophyll fluorescence emission was measured from the adaxial surface on the leaf using a fluorescence monitoring system (model FMS 2; Hansatech Instruments Ltd., Norfolk, UK) in the pulse amplitude modulation mode (Loggini et al. 1999; Maxwell and Johnson 2000). In brief, a leaf, kept in dark for 30 min was initially exposed to the modulated measuring beam of far-red light (LED source) with typical peak at 735 nm. Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (< 85 µmol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (> 1500 µmol m^{-2} s^{-1} PAR) and calculated using FMS software for Windows®. The variable fluorescence yield (F_v) was calculated using the equation: $F_v = F_m - F_0$. The ratio of variable to maximum fluorescence (F_v/F_m) was calculated as the maximum quantum yield of PSII photochemistry. The photon yield of PSII (Φ_{PSII}) in the light was calculated as:

$\Phi_{PSII} = (F_m' - F)/F_m'$ after 45 s of illumination, when steady state was achieved. Net photosynthetic (P_n; μmol m^{-2} s^{-1}) and transpiration rate (E; mmol m^{-2} s^{-1}) were measured using a Portable Photosynthesis System with an Infra-red Gas Analyzer (Model LI 6400, LI-COR® Inc., Lincoln, Nebraska, USA). The g_s and E were measured continuously by monitoring the content of the air entering and existing in the IRGA headspace chamber, according to Cha-um et al. (2007). The air-flow rate of IRGA chamber was fixed at 500 μmol s^{-1} and chamber temperature was set at 28 °C. The light intensity was adjusted to 1000 μmol m^{-2} s^{-1} PPFD of 6400-02B red-blue LED light source.

Survival percentage and morphological characters

Survival percentage of in vitro acclimatized plantlets under photoautotrophic growth conditions was recorded after incubation in the plant growth incubator at a temperature shift of 28 ± 2 °C for 16 h in daytime and 25 ± 2 °C for 8 h in nighttime, 60 ± 5% RH, and 120 ± 5 μmol m^{-2} s^{-1} PPFD provided by fluorescent lamps with a 16-h day^{-1} photoperiod for 45 days. Survival percentage of ex vitro transplanted plants was calculated 30 days after transplanting the plantlets in the green house. Plant height, number of leaves, and leaf length and width of ex vitro transplanted plants were measured.

Experiment design and statistical analysis

The experiment was arranged as 2 × 2 factorial in Completely Randomized Design (CRD) with five replicates ($n = 5$). Survival percentage was calculated in each treatment with five replicates (20 plantlets per replicate). The mean values obtained from four treatments were compared using Tukey's HSD and analyzed by SPSS software.

Results and discussion

In vitro acclimatization

Overall morphological characteristics of the survived plantlets were similar irrespective of the cultivars and acclimatization conditions. Acclimatized plantlets of cv. 'RRIT413' grown under CO_2 enriched conditions showed the toxic symptoms, i.e. leaf burn, stem die back, and death, leading to small leaves (leaf length and leaf width) after transplanting to ex vitro (Table 1). Survival percentage of in vitro acclimatized plantlets in the ambient CO_2 (350 ± 50 μmol CO_2 mol^{-1}) was greater than those acclimatized in the CO_2-enrichment, especially in cv. 'RRIT413' (Fig. 2a). Rubber tree plantlets of cv. 'RRIT413' was sensitive to high CO_2 condition (1500 ± 50 μmol CO_2 mol^{-1}), resulting in a low survival rate (only 20.0%). Therefore, it was improved using in vitro photoautotrophic acclimatization under ambient CO_2 conditions for both the cvs. 'RRIM600' (66.5%) and 'RRIT413' (75.0%).

Overall growth performance i.e. new shoot initiation, leaf expansion, number of leaves and leaf area of survived rubber tree plantlets under in vitro photoautotrophic acclimatization was a key index to regulate physiological adaptation in both in vitro and ex vitro conditions, leading to lift-up survival rate of acclimatized plantlets. In woody species, CO_2 enrichment of in vitro photoautotrophic acclimatization plays a critical role in overall growth elevation (Morini and Melai 2003; Vyas and Purohit 2003; Cha-um et al. 2011). Subsequently, survival percentage of the acclimatized plantlets under CO_2-enriched condition is high as compared to those under ambient CO_2 (Li et al. 2001; Pérez-Jiménez et al. 2015). In contrast, rubber tree plantlets were familiar to ambient CO_2 condition in the in vitro photoautotrophic acclimation, especially cv. 'RRIT413'. In carob tree also,

Table 1 Shoot height, number of leaves, leaf length and leaf width of rubber tree plantlets derived from somatic embryo of cvs. 'RRIM600' and 'RRIT413' acclimatized under ambient (350 μmol CO_2 mol^{-1})- or enriched (1500 μmol CO_2 mol^{-1})-CO_2 of photoautotrophic condition, subsequently transplanted into plastic bag containing mixed soil, measured after 30 days. Data presented as mean ± SE

Cultivar	CO_2 (μmol CO_2 mol^{-1})	Shoot height (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)
RRIM600	350	10.4 ± 0.8	5.4 ± 0.4a	5.9 ± 0.6ab	1.8 ± 0.3ab
	1500	11.7 ± 0.4	5.0 ± 0.8ab	5.2 ± 0.4b	1.3 ± 0.3b
RRIT413	350	9.6 ± 0.5	4.6 ± 0.7ab	7.8 ± 0.5a	2.6 ± 0.2a
	1500	10.7 ± 0.3	2.3 ± 0.3b	2.7 ± 0.2c	1.2 ± 0.1b
Significant level					
Cultivar		ns	*	ns	ns
CO_2		ns	ns	**	**
Cultivar × CO_2		ns	ns	**	ns

Different letters in each column show significant difference at $p \leq 0.05$ (*) and $p \leq 0.01$ (**) by Tukey's HSD

ns, * and ** represents non-significant, significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$, respectively

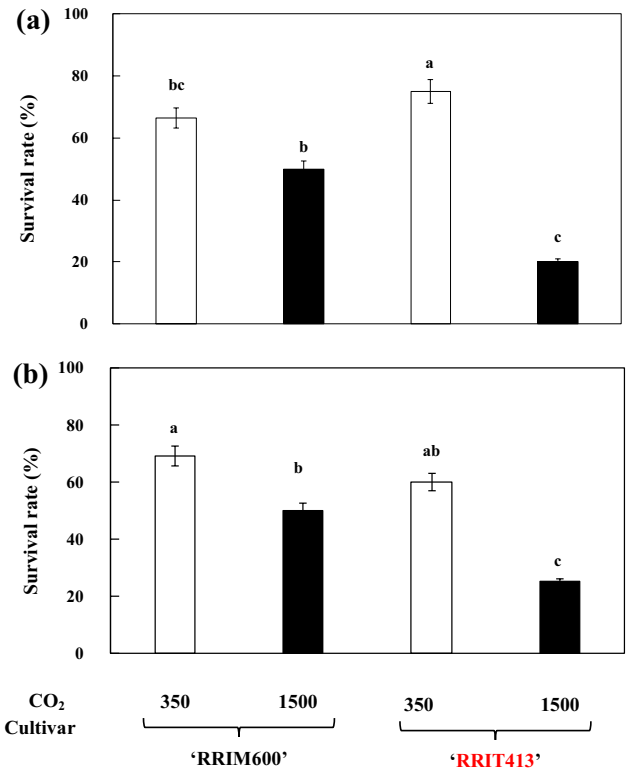

Fig. 2 Enriched (1500 µmol CO_2 mol^{-1})-CO_2 photoautotrophic condition for 45 days (**a**) and survival percentage of transplanted plants into plastic bag containing mixed soil for 30 days (**b**). Data presented as mean ± SE. Different letters in each column show significant difference at $p \leq 0.01$ by Tukey's HSD test

the responses of acclimatized plantlets to the CO_2 concentrations were cultivar dependent. It was found that carob tree cv. 'Mulata' was very sensitive to the CO_2 enrichment (810 µmol CO_2 mol^{-1}) when compared with cv. 'Galhosa', identified by a decline of the net photosynthetic rate (P_n) (Osório et al. 2005). It was possible that high amount of CO_2 (1500 ± 50 µmol CO_2 mol^{-1}) under in vitro conditions negatively affected the CO_2 assimilation, and consequently retarded the plant growth and development. In *Hosta* 'Blue Vision', growth and net photosynthetic rate (P_n) of in vitro acclimatized plantlets were evidently promoted when grown under 2800 µmol CO_2 mol^{-1} (Toler et al. 2003). The optimum intracellular CO_2 concentration (C_i) that promotes P_n in rubber tree cultivars grown in the field trial was identified as "1000–1200 µmol CO_2 mol^{-1}" (Kositsup et al. 2010; Nataraja and Jacob 1999). Alternatively, an increased light intensity during in vitro acclimatization under CO_2 enrichment was a minimal requirement for ex-vitro adaptation (Cavalho et al. 2002a, b; Shin et al. 2014; Toler et al. 2003).

Ex vitro adaptation

Plant morphological characters of ex vitro transplanted rubber tree cvs. 'RRIM600' and 'RRIT413' are given in Fig. 3. Rubber tree plantlets derived from in vitro acclimatization under ambient CO_2 condition were represented as vigorous donor plant, as indicated by elongated stem, fully expanded, dark-green leaves and continuous branching. In contrast, the short stem, tiny and light-green leaves and lesser branching were found in plantlets acclimatized under CO_2 enriched conditions, especially in cv. 'RRIT413', resulting in a low survival percentage (only 25.0%) (Fig. 2b). Survival percentage of plantlets derived from in vitro acclimatization under ambient CO_2 condition was significantly higher in both rubber tree cultivars, 'RRIM600' (69.2%) and 'RRIT413' (60.0%) (Fig. 4b). In rubber tree, the low survival rate of the transplanted plantlets in ex vitro conditions was a major concern, especially in case of the plantlets derived from somatic embryogenesis (Zhou et al. 2010; Karumamkandathil et al. 2015) and the anther culture process (Nor Mayati 2015).

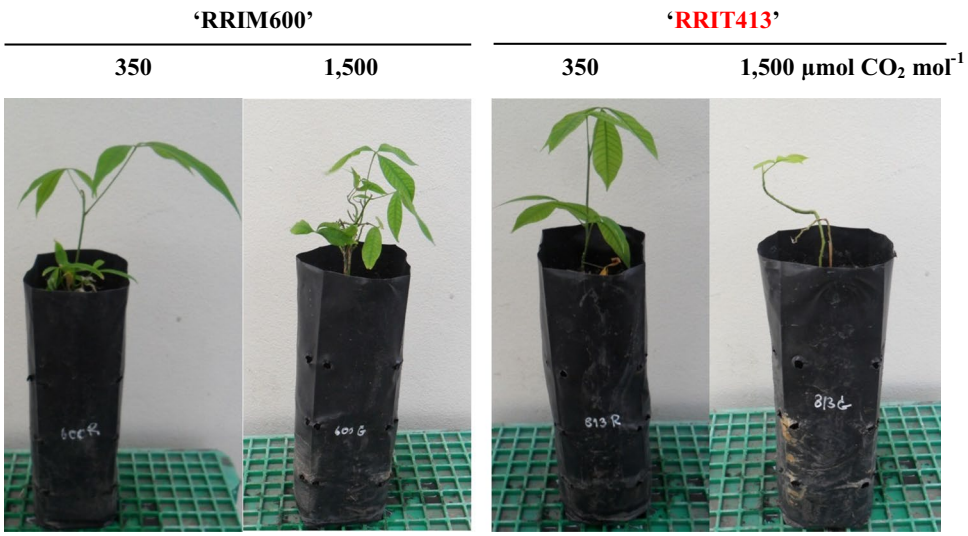

Fig. 3 Morphological characters of rubber tree derived from acclimatized plantlets under ambient (350 µmol CO_2 mol^{-1}) or enriched-(1500 µmol CO_2 mol^{-1}) CO_2 photoautotrophic condition, subsequently transplanted into plastic bag containing mixed soil for 30 days

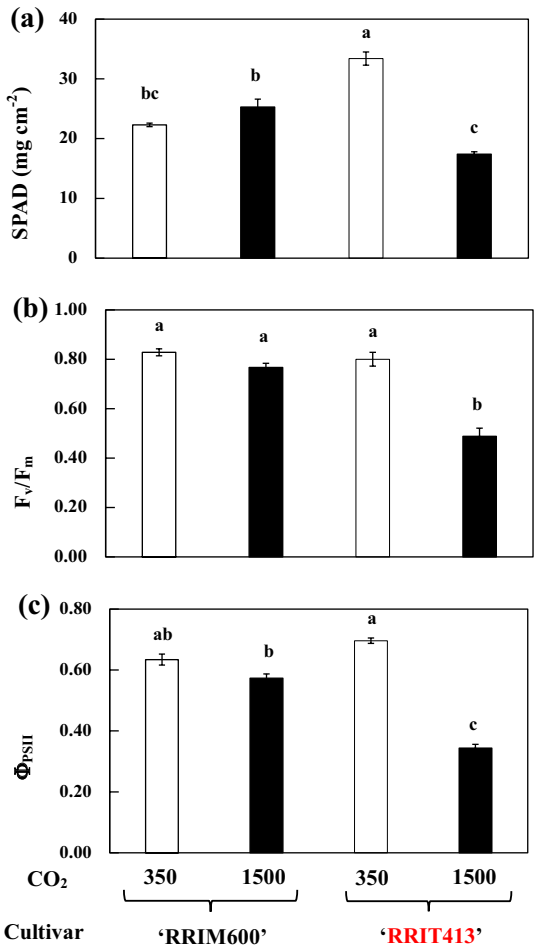

Fig. 4 SPAD (**a**), maximum quantum yield of PSII (F_v/F_m) (**b**) and quantum efficiency of PSII (Φ_{PSII}) (**c**) of rubber tree derived from somatic embryo cvs. 'RRIM600' and 'RRIT413' acclimatized plantlets under ambient (350 µmol CO_2 mol^{-1}) or enriched (1500 µmol CO_2 mol^{-1})-CO_2 photoautotrophic condition for 45 days, subsequently transplanted into plastic bag containing mixed soil for 30 days. Data presented as mean ± SE. Different letters in each column show significant difference at $p \leq 0.01$ by Tukey's HSD test

In general, the in vitro seedlings of rubber tree has been reported as "easy transplanted material" with 80.0% survival percentage in the greenhouse for a month (Sanguansermsri et al. 2015). In a previous report, only two plantlets derived from clone 'SH/RD1-B2' and one plantlet each from the clones 'SH/RD1-C1', 'SH/RD1-E2', 'B5/RD1-B1' and 'B5/RD1-E2' survived successfully in the greenhouse, but leading to the wilting and death when cultivated for long term (Nor Mayati 2015). In present study, survival percentage of rubber tree plantlets derived from in vitro acclimatization under ambient CO_2 condition was significantly greater than those under CO_2 enrichment condition. In chestnut hybrid, survival rate (> 82%) of acclimatized plantlets under ambient CO_2 (350 µL L^{-1}) and enriched-CO_2 (700 µL L^{-1}) with low light (150 µmol m^{-2} s^{-1} PPF) or high light intensities (300 µmol m^{-2} s^{-1} PPF) was indifferent (Carvalho and Amâncio 2002a). In contrast, survival percentage of in vitro acclimatized plantlets of apple under CO_2-enriched condition (1000 µmol CO_2 mol^{-1}) with 100 µmol m^{-2} s^{-1} PPF was 80%, which was better than those under ambient CO_2 (only 50% survival) (Li et al. 2001). Moreover, 100% active growth of acclimatized *Cynara scolymus* plantlets under CO_2-enriched conditions (800 µmol CO_2 mol^{-1}) was found, whereas only 42.3% of acclimatized plantlets under ambient CO_2 condition (380 ± 40 µmol CO_2 mol^{-1}) could survive (Pérez-Jiménez et al. 2015).

Plant height of ex vitro transplantions derived from different CO_2 concentrations was indifferent. In addition, leaf characteristics of rubber tree cv. 'RRIM600' acclimated in vitro under enriched CO_2 conditions were similar when compared to the ambient CO_2 conditions (Table 1). In contrast, number of leaves, leaf length and width of rubber tree cv. 'RRIT413' in ex vitro transplantation declined significantly by 2.00, 2.89, and 2.17 fold, respectively, when exposed to in vitro acclimatization under CO_2 enriched conditions (Table 1). Overall growth characters, number of leaves, leaf length and width of rubber tree cv. 'RRIM600' acclimated in vitro under CO_2 enriched conditions were unaffected while those in cv. 'RRIT413' were declined. Similarly, new leaves of acclimatized plantlets of carob tree cvs. 'Galhosa' and 'Mulata' under CO_2 enrichment emerged slowly when compared with those under ambient CO_2 (Osório et al. 2005). In contrast, leaf area (in case of *Cynara scolymus*) and specific leaf area (in case of chestnut hybrid) of acclimatized plantlets under CO_2 enrichment were improved (Carvalho and Amâncio 2002a; Pérez-Jiménez et al. 2015). The growth response of in vitro acclimatized plantlets under CO_2 enrichment depends on genetic factors (Cavalho et al. 2002a, b; Osório et al. 2005).

Chlorophyll content (SPAD) in the leaf tissues of in vitro acclimatized plantlets of rubber tree cv. 'RRIM600' under ambient CO_2 and enriched-CO_2 conditions was stabilized when transplanted to the greenhouse for 30 days (Fig. 4a), resulting in the maximum quantum yield of PSII (F_v/F_m, Fig. 4b) and photon yield of PSII (Φ_{PSII}; Fig. 4c). In contrast, chlorophyll degradation was evidently found in the in vitro acclimatized plantlets cv. 'RRIT413' under enriched-CO_2 conditions (47.9% degradation), causing on F_v/F_m and Φ_{PSII} to diminish by 39.0 and 50.6%, respectively (Fig. 4). Total chlorophyll pigment (SPAD),chlorophyll fluorescence (F_v/F_m and Φ_{PSII}) and P_n in the leaf tissues of in vitro acclimatized rubber tree cv. 'RRIT413' was sensitive to ex vitro environments, whereas those in cv. 'RRIM600' were maintained. Previously, chlorophyll degradation with subsequent reduction in F_v/F_m, Φ_{PSII} and P_n was evidently found in ex vitro transplantation of grapevine cv. 'Touriga' (Carvalho et al. 2002), chestnut hybrid (Carvalho and Amâncio 2002a) and carob tree cvs. 'Galhosa' and 'Mulata' (Osório et al.

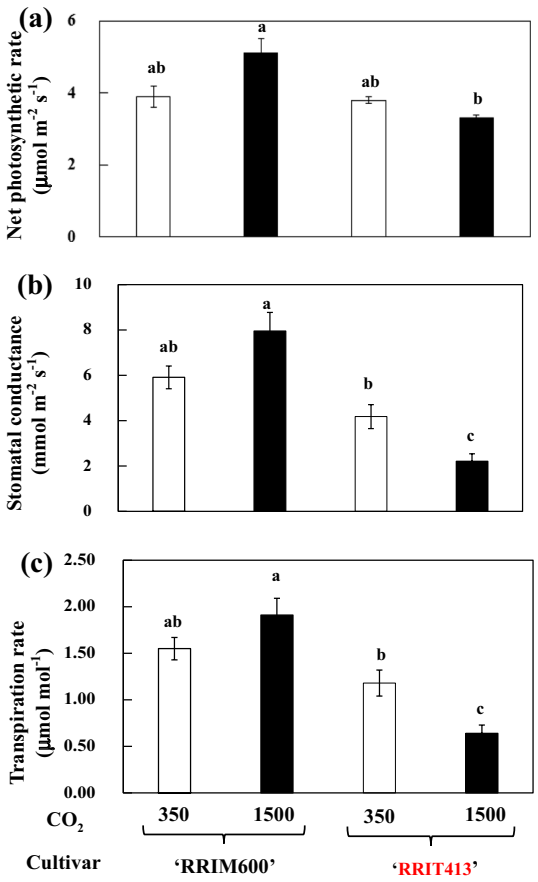

Fig. 5 Net photosynthetic rate **a**, stomatal conductance **b** and transpiration rate **c** of rubber tree derived from somatic embryo cvs. 'RRIM600' and 'RRIT413' acclimatized plantlets under ambient (350 μmol CO_2 mol^{-1}) or enriched (1500 μmol CO_2 mol^{-1})-CO_2 photoautotrophic condition for 45 days, subsequently transplanted into plastic bag containing mixed soil for 30 days. Data presented as mean ± SE. Different letters in each column show significant difference at $p \leq 0.01$ by Tukey's HSD test

2005). Photosynthetic pigments of ex vitro plantlets in tobacco acclimatization were unaffected by enriched-CO_2 supply (Pospíšilová et al. 2000). In contrast, chlorophyll a and chlorophyll b in *Cynara scolymus* acclimatized plantlets were enhanced by CO_2 enrichment (800 μmol CO_2 mol^{-1}) (Pérez-Jiménez et al. 2015).

Similarly, net photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration rate (E) of in vitro acclimatized plantlets cv. 'RRIM600' under ambient CO_2 and enriched-CO_2 conditions were maintained (Fig. 5). In cv. 'RRIT413', P_n was retained (Fig. 5a), whereas g_s (Fig. 5b) and E (Fig. 5c) were sharply decreased by 47.1 and 45.8%, respectively. The photosynthetic responses of in vitro acclimatization under CO_2 enrichment depend on the plant species and the degree of CO_2 concentrations (Zhou et al. 2005; Shin et al. 2014). The present study demonstrated that the physiological adaptation of rubber tree cv. 'RRIT413' acclimatized under CO_2-enriched condition was very poor, leading to low survival rate and abnormal growth characters in ex vitro environments. High CO_2 conditions during in vitro acclimatization might regulate ethylene production, leading to chlorophyll degradation, dark respiration, stomatal closure, photosynthesis inhibition and leaf senescence (Reuveni and Bugbee 1997). Soluble sugar enrichment in rubber tree cv. 'RRIM600' acclimatized plantlets under CO_2 enriched condition was a good indicator to identify "healthy plantlets", suitable for ex vitro environments (Carvalho and Amâncio 2002b; Carvalho et al. 2002; Shin et al. 2014).

Soluble sugars in the leaf tissues of in vitro photoautotrophically acclimatized plantlets of cvs. 'RRIM600' and 'RRIT413' was in the order sucrose > fructose > glucose (Table 2). Interestingly, sucrose, glucose, fructose and total soluble sugar in the leaf tissues of in vitro acclimatized plantlets of cv. 'RRIM600' under enriched-CO_2 conditions were increased by 3.6, 12.4, 6.3 and 4.9 fold, respectively (Table 1). In contrast, these sugar types were constant in the leaf tissues of in vitro acclimatized plantlets cv. 'RRIT413' under both ambient-CO_2 and enriched-CO_2 conditions. Soluble sugars such as glucose, fructose and sucrose are the primary products of photosynthesis that play a central role in providing energy, sensing/signaling networks in building blocks for plant growth and development and osmotic adjustment under fluctuation of microenvironments (Smeekens 2000; Rolland et al. 2002; Gupta and Kaur 2005; Smeekens et al. 2009).

In conclusion, survival percentage of in vitro photoautotrophic acclimatization under ambient CO_2 condition was better in rubber tree cvs. 'RRIM600' and 'RRIT413', subsequently leading to rapid physiological adaptation (photosynthetic abilities) and improved growth performance in ex vitro environments. Soluble sugar enrichment in the leaf tissues of transplanted plants can be considered a biochemical indicator for the rapid adaptation of acclimatized plantlets in ex vitro conditions, especially in case of rubber tree cv. 'RRIM600'.

Table 2 Sucrose, glucose, fructose and total soluble sugar (TSS) in the leaf tissues of rubber tree plantlets derived from somatic embryo of cvs. 'RRIM600' and 'RRIT413' acclimatized under ambient (350 µmol CO_2 mol^{-1})- or enriched (1500 µmol CO_2 mol^{-1})-CO_2 of photoautotrophic condition, subsequently transplanted into plastic bag containing mixed soil for 30 days. Data presented as mean ± SE

Cultivar	CO_2 (µmol CO_2 mol^{-1})	Sucrose (µg g^{-1} DW)	Glucose (µg g^{-1} DW)	Fructose (µg g^{-1} DW)	TSS (µg g^{-1} DW)
RRIM600	350	44.4 ± 0.7c	4.4 ± 1.0c	16.5 ± 2.6c	65.3 ± 4.2c
	1500	159.4 ± 7.1a	54.7 ± 3.8a	103.6 ± 4.1a	317.7 ± 13.6b
RRIT413	350	77.4 ± 2.5b	32.9 ± 4.4b	50.5 ± 6.6b	160.7 ± 11.0b
	1500	72.8 ± 0.5b	24.3 ± 0.9b	35.4 ± 0.3b	132.5 ± 0.8b
Significant level					
Cultivar		**	*	**	**
CO_2		**	**	**	**
Cultivar × CO_2		**	**	**	**

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Tukey's HSD

* and ** represents significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$, respectively

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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