

In-Vitro Acclimatization of *Curcuma Zedoaria* Under Iso-Osmotic Treatments, Ex-Vitro Adaptation and Agronomic Traits in Greenhouse Conditions

Rujira Tisarum (✉ rujira.tis@biotec.or.th)

NSTDA: National Science and Technology Development Agency

Unenzaya Bayaraa

Mongolian Academy of Sciences

Thanyaporn Sotesaritkul

NSTDA: National Science and Technology Development Agency

Thipmanee Hleepongpanich

NSTDA: National Science and Technology Development Agency

Thapanee Samphumphuang

NSTDA: National Science and Technology Development Agency

Suriyan Cha-um

NSTDA: National Science and Technology Development Agency <https://orcid.org/0000-0002-7662-363X>

Research Article

Keywords: Curcuma zedoaria, curcuminoids, iso-osmotic acclimatization, physiological adaptation, sugar alcohol, white turmeric

Posted Date: March 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-304281/v1>

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Abstract

Low survival rate, poor adaptation to *ex-vitro* environments, and time required for hardening the plants to cope with fluctuated environments of field trial are identified as major barriers in this technology. In present study, iso-osmotic adjustment in the culture medium using sucrose and/or mannitol was applied to the *in-vitro* cloning of *Curcuma zedoaria* (white turmeric) plants, which were transferred to *ex-vitro* conditions and subsequently cultivated in the greenhouse conditions prior to harvest after 9 months. During both *in-vitro* and *ex-vitro* development of plant, growth and physiological traits under 3% sucrose (Suc) + 2.5% mannitol (Man) were lower than those in control (3% Suc; conventional tissue culture). Interestingly, pseudostem height and root length in acclimatized plantlets under 3% Suc + 2.5% Man were sharply dropped by 60.13% and 92.37% over control, respectively, resulting in a decrease in the *ex-vitro* adaptation by 56.27% and 33.33% over the control. A positive relationship between reduction of net photosynthetic rate (P_n) and sucrose concentration in the leaf tissues was evidently observed. Remarkably, the morphological and physiological traits of aboveground and underground parameters of acclimatized plantlets under 3% Suc + 2.5% Man were maximized over control, leading to high yield of curcuminoids ($229.4 \text{ mg plant}^{-1}$) in the dry rhizome (31 g plant^{-1}) when cultivated under greenhouse microenvironments for 9 months. Based on this investigation, we propose that plantlets of *C. zedoaria* micropropagated using 3% Suc + 2.5% Man can readily acclimatize under *ex-vitro* conditions and subsequently develop as healthy plants with compact and uniform size.

Introduction

Curcuma zedoaria (Christm.) Roscoe (white turmeric; Zingiberaceae) is a perennial rhizomatous plant, commonly used as a food ingredient and traditional medicine. It is originated in Bangladesh, India and Sri Lanka and distributed throughout Asia including China, Japan, Nepal, Thailand and Vietnam (Lobo et al. 2009). Essential oil in several parts of the plant including mature rhizome, roots, pseudostem shoots and leaves have been used in ethnomedicinal practices (Ayati et al. 2019). Likewise, its leaf extracts are used for dropsy and leprosy, fresh roots cure leucorrhoeal discharge and powdered rhizome is antiallergic in nature (Lobo et al. 2009). Rhizome of *C. zedoaria* contains curcuminoids, which are specific to *Curcuma* species and widely used in pharmacology for anti-cancerous, anti-inflammatory and antimalarial treatments (Paramapojn and Gritsanapan 2009; Peng et al. 2010; Jena et al. 2020). Demethoxycurcumin (DEM) has been identified as the major compound in the rhizome of *C. zedoaria* along with curcumin (CUR) and bisdemethoxycurcumin (BIS) as the minor compounds (Syu et al. 1998; Burapan et al. 2020). Moreover, curcuminoid biosynthesis related genes, *i.e.*, diketide-CoA synthase (DCS), curcumin synthase 1 (CURS1), curcumin synthase 2 (CURS2), and curcumin synthase 3 (CURS3) are identified and characterized as major routes for their production in the rhizome of white turmeric (Lan et al. 2018). Seasonal variations are one of the most important environmental factors, which regulate fluctuations in the major compounds including curcumenol and dihydrocurdione (Pamplona et al. 2005). Mother rhizome propagation has been wildly practiced for cultivation of white turmeric but it results in slow growth rate, non-uniformity in the produce and the risk of soil-borne infections, which are major concerns in its production at commercial scale (Ajitomi et al. 2015; Prameela and Bhai 2020). Micropropagation in elite clones of *C. zedoaria* has been well established to overcome the barriers for a large-scale production (Bharalee et al. 2005; Loc et al. 2005; Stanly et al. 2010; Jena et al., 2020).

Plant micropropagation via *in-vitro* culture under aseptic conditions is a novel technology to produce elite clones as mother stock so as to facilitate the industrial scale production. Acclimatization of the *in-vitro* plantlets before transferring them to field conditions is a critical concern since sudden change from a delicate growth conditions with high nutrients, high relative humidity and low light intensity to harsh environmental conditions with resource competition, fluctuating climate and uneven light intensity (Hazarika 2006; Kumar and Rao 2012). Several factors affect the success of this process of hardening, *i.e.*, CO_2 concentration, relative humidity, suitable substrates, inoculated arbuscular mycorrhizal fungi and optimized carbon sources in the media (Loc et al 2005; Tisarum et al. 2018; de Souza Ferrari et al. 2020). Carbon source supplementation in the media is a major factor, which controls plant morphological characteristics and adaptive strategies (van Huylenbroeck et al. 1998; Seon et al. 2000; Kadlecak et al. 2001; Badr et al. 2015; Bohra et al. 2016). Increasing sucrose in the media by 6-8% has been reported to induce osmotic stress, which facilitates efficient acclimatization of *Zingiber* and *Curcuma* species (Nayak 2000; Shirgurkar et al. 2001; Chirangini and Sharma 2005; Diem 2019). Addition of sugar alcohols, including mannitol and sorbitol, in the medium might cause the osmotic effect, thereby, replacing the carbon source for *in-vitro* acclimatization (Custodio et al. 2004; Waman et al. 2014). Sugar alcohols are, therefore, successfully applied as osmopriming agent in several plant species (Kaur et al. 2005; Papastylianou and Karamanos 2012; Maiti and Pramanik 2013). Limited information is available in the literature regarding effect of the combined application of sucrose (carbon source) and mannitol (osmotic adjustment) in the culture medium for *in-vitro* hardening of plantlets. The aim of this study was to investigate the impact of iso-osmotic conditions of *C. zedoaria* on *in-vitro* acclimatization and *ex-vitro* adaptation and agronomic traits under greenhouse conditions.

Materials And Methods

***In-vitro* acclimatization**

Mother rhizomes of *C. zedoaria* were collected from Nakhon Nayok province, central region of Thailand (latitude 14.090956, longitude 100.932454) and the rhizomes were allowed to sprout to give rise to new shoot buds. The sprouted shoots with 1 cm length were dissected and surface-sterilized with 1% hypochlorite solution (Clorox[®], 6% sodium hypochlorite, v/v; ai, Clorox Company, Oakland, USA) for 15 min and washed thrice using 100 mL sterile distilled water. Leaf sheaths were removed, and apical shoot was inoculated onto 35 mL Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 1 mg L⁻¹ BA in the 125 mL glass vessel. Then, culture vessels containing plantlets were incubated under 60±5% relative humidity (RH), 25±2°C ambient temperature, and 60±5 µmol m⁻² s⁻¹ photosynthetic photon flux (PPF) intensity provided by fluorescent lamps (Cool white, Philips, Thailand) with a 16 h d⁻¹ photoperiod for 30 days. Single shoots were dissected and transferred to the fresh MS medium on monthly basis for shoot proliferation. The single plantlet (2.0±0.2 cm in length) was transferred to MS medium containing 3% sucrose (control; -1.0 MPa) and iso-osmotic conditions at -1.3 MPa using 6% sucrose (Suc), 4% mannitol (Man) and 3% sucrose + 2.5% mannitol with 1 plantlet per glass vessel. After 45 days of *in-vitro* acclimatization, pseudostem height, number of leaves, leaf area, pseudostem fresh and dry weight, leaf osmotic potential, root length, number of roots, root fresh and dry weight, and root osmotic potential of acclimatized plantlets under isosmotic conditions were collected.

***Ex-vitro* adaptation**

Plantlets derived from *in-vitro* acclimatization using isosmotic conditions were directly transplanted to peat moss substrate (K[®] 1913 Class man, K>Select Aquasave, Germany), irrigated regularly and incubated in a greenhouse under 28 ± 2 °C ambient temperature, 500–1000 µmol m⁻² s⁻¹ photosynthetic photon flux density with a 10 h d⁻¹ photoperiod, and 80 ± 5% relative humidity for 2 weeks, subsequently transferred to plastic bag (5'14 cm) containing 1 kg garden soil (EC = 2.687 dS m⁻¹; pH = 5.5; organic matter = 10.36%; total nitrogen = 0.17%; total phosphorus = 0.07%; total potassium = 1.19%) and daily irrigated in a greenhouse condition for 4 weeks. Thereafter, pseudostem height, number of leaves, leaf area, root length, number of roots, pseudostem fresh and dry weight, root fresh and dry weight, photosynthetic abilities, and soluble sugars in leaf tissues were measured.

Long-term cultivation and agronomic traits

Ex-vitro adapted plants in each treatment were subsequently transferred to plastic bags (14'30 cm) containing 5 kg garden soil in a greenhouse for 9 months. Daily irrigation and 10 g Osmocote[®] 13-13-13 slow-release fertilizer were supplied as per water and fertilizer application schedule. Pseudostem height, number of leaves, leaf length, leaf width, pseudostem fresh and dry weight, root length, number of roots, root fresh and dry weight, rhizome length, rhizome width, leaf greenness, maximum quantum yield of PSII, photon yield of PSII, net photosynthetic rate, transpiration rate, and stomatal conductance were measured after harvest. In addition, bisdemethoxycurcumin (BIS), dimethoxycurcumin (DEM), curcumin (CUR) and total curcuminoids in the rhizomes were also measured.

Morphological characteristics

Pseudostem height, number of leaves, leaf length, leaf width, leaf area, pseudostem fresh and dry weight, root length, number of roots, root fresh and dry weight, rhizome length, rhizome width, rhizome fresh and dry weight in white turmeric plants were measured as growth parameters. Leaf area was measured by Leaf Area Meter (Model CL-203, CID[®] Inc, WA, USA). Pseudostem, rhizome and root of white turmeric plants were dried at 80°C in a hot air oven for 48 h, and then placed in a desiccator before the measurement of dry weight.

Physiological characteristics

Osmotic potential in the root and leaf tissues of each treatment was measured according to Lanfermeijer et al. (1991). In brief, 10 mL cell sap was dropped directly onto a filter paper in an Osmometer chamber (5520 Vapro[®], Wescor, Utah, USA). Then, the osmolarity (mmol kg⁻¹) was converted to osmotic potential (MPa) using conversion factor of osmotic potential measurement according to Fu et al. (2010).

Leaf greenness (SPAD value) in the second fully expanded leaf from the shoot tip of each treatment was measured using Chlorophyll meter (Model SPAD-520Plus, Konica Minolta, Osaka, Japan) according to Hossain et al. (2015).

Chlorophyll fluorescence emission including maximum fluorescence (F_v/F_m) and photon yield of PSII (F_{PSII}) from the adaxial surface of second fully expanded leaf of the shoot tip was measured using a fluorescence monitoring system (model FMS 2; Hansatech Instruments Ltd., Norfolk, UK) (Loggini et al. 1999; Maxwell and Johnson 2000).

Net photosynthetic rate (P_n ; mmol m⁻² s⁻¹), stomatal conductance (g_s ; mmol CO₂ m⁻² s⁻¹), intracellular CO₂ concentration (C_i; mmol mol⁻¹) and transpiration rate (E; mmol m⁻² s⁻¹) of second fully expanded leaf of the shoot tip were measured by a portable photosynthesis system (LI 6400XT, LI-COR, Lincoln, NE, USA), following the method of Cha-um et al. (2007).

Biochemical assays

Curcuminoids assay, the dried rhizomes were ground into fine powder in the mortar with liquid nitrogen. Twenty milligrams of the powder was then taken into a glass vial and 5 mL of methanol was added. The mixture was mixed thoroughly by vortex followed by sonication for 30 min. Then, the solution was filtered (Whatman® #1, Maidstone, UK) and crude extract was dried by allowing the methanol to evaporate. Curcuminoid content in crude extract was analyzed using High Performance Liquid Chromatography (HPLC). Curcuminoids were dissolved in 1 mL methanol (HPLC grade) and then filtrated through 0.45 µm (Millipore™, Nihon Millipore Ltd., Japan) nylon filter. Ten microliters of sample were injected into injection loop and analyzed by HPLC (Waters Associates, Milford, MA, USA) equipped with photodiode array detector (Water 2998) at 425 nm. Bis-demethoxycurcumin (BIS), demethoxycurcumin (DEM) and curcumin (CUR) were separated using C₁₈ (Vertisep® UPS C₁₈ HPLC) column under 25°C room temperature. The mobile phase consisted of acetonitrile (100% HPLC grade) and acetic acid (0.25%, v/v). The elution was carried out with a gradient set at a flow rate of 0.8 mL min⁻¹. The solvent gradient was 50% acetonitrile for 0 to 8 min, then 50% to 40% acetonitrile from 8 to 10 min, 40% acetonitrile constant from 10 to 15 min, and 40 to 50% acetonitrile from 15 to 16 min (Pothitirat and Gritsanapan 2007).

Sucrose, glucose, and fructose in the leaf tissues of second fully expanded leaf from the shoot tip) were extracted using nanopure water and then the contents of soluble sugar were assayed by HPLC according to the method of Karkaci et al. (2003).

Experimental layout and statistical analysis

The experiment was arranged as Completely Randomized Design (CRD) with four replicates (n=4). The mean values obtained from all treatments were compared using Tukey's HSD test and analyzed by SPSS (Statistical Package for Social Science) software (version 11.5 for Window®, SPSS Inc., Chicago, USA). Relationships between physiological and morphological data of each treatment were validated using Pearson's correlation coefficient.

Results And Discussion

In-vitro acclimatization

Overall morphological characteristics of white turmeric plantlets grown under iso-osmotic conditions for 45 days were demonstrated (Fig. 1). Growth and development patterns of 6% Suc and 3% Suc + 2.5% Man (-1.3 MPa iso-osmotic potential) were similar to control (3% Suc; -1.0 MPa osmotic potential), whereas growth inhibition by 4% Man was observed (Fig. 1). Pseudostem height, number of leaves, leaf area and pseudostem fresh weight in plantlets propagated under 3% Suc (control) and 6% Suc were similar, while these parameters were significantly declined by 60.13%, 69.77%, 91.3% and 50.00% over control, respectively, when subjected to 3% Suc + 2.5% Man (Table 1). Interestingly, 100% inhibition of leaf emergence and leaf expansion in 4% Man were observed. Underground parameters, root length and root fresh weight in plantlets propagated under 3% Suc + 2.5% Man were significantly decreased by 92.37% and 59.18% over control and lacked microrhizome (Table 1). Number of roots were the maximal (2.24-folds over control) in plantlets propagated under 3% Suc + 2.5% Man (Table 1). Pseudostem- and root-dry weights in plantlets propagated under 3% Suc (control, -1.0 MPa) were significantly greater than those under iso-osmotic treatment (-1.3 MPa) (Fig. 2A and 2B). Interestingly, leaf osmotic potential (Fig. 2C) and root osmotic potential (Fig. 2D) in plantlets propagated under 3% Suc + 2.5% Man were significantly dropped compared to 3% Suc (control) and 6% Suc. Microrhizome fresh and dry weights in plantlets acclimatized under 3% Suc (control, -1.0 MPa) were increased to 0.44g and 35.7 mg, respectively, when compared with iso-osmotic treatments (Fig. 2E and 2F). A positive relation between decreasing root osmotic potential and root fresh weight (Fig. 3A; R² = 0.6718), leaf osmotic potential and pseudostem fresh weight (Fig. 3B; R² = 0.6102) in acclimatized plantlets was detected. Moreover, a close relation between pseudostem height and root length (Fig. 3C; R² = 0.8669) as well as number of leaves and leaf area (Fig. 3D; R² = 0.9446) in acclimatized plantlets was observed.

Growth inhibition in *C. zedoaria* plantlets grown under 4.5% Man was evidently observed. Mannitol is a sugar alcohol that induces osmotic stress in several plant species if supplied via culture medium (Watanabe et al. 2000; Molassiotis et al. 2006; Carloni et al. 2017; de Oliveira et al. 2018) and assist *in-vitro* acclimatization of banana (Waman et al. 2014). Poor growth responses such as low sprouting and multiplication rate of plantlets under 1%, 2% and 3% Man have been previously demonstrated (Waman et al. 2014). Shoot height of *Populus euphratica* *in-vitro* plantlets under 3.64% Man sharply declined, whereas number of leaves significantly decreased when subjected

to 7.28% Man for 2 weeks. In contrast, 100% death of plantlets of *P. alba* × *P. tomentosa* under 7.28% Man was observed and the shoot height and number of leaves in plantlets under 3.64% Man were decreased (Watanabe et al. 2000). In addition, chlorophyll pigments in the leaf tissues of apple rootstock MM106 shoot culture under 10.5% Man were significantly degraded by 18.34% over control (Molassiotis et al. 2006). One possible way of shoot and root initiation in Man culture media is to reduce IAA (indole-3-acetic acid) content to ensure down-regulation of expression levels of IAA-biosynthesis related gene(s) (Barbier et al. 2015). Moreover, root and shoot traits of plantlets acclimatized under 2-3% Suc plus different degrees of Man were sharply decreased in relation to their concentrations in the culture medium (Custódio et al. 2004; Madhulatha et al. 2006; Samarina et al. 2020). In *Ruta graveolens*, the growth performance and production of coumarins and rutin in the shoot cultures were regulated by 3% Suc + 1% Man in the culture medium (Mohamed and Ibrahim 2012). Leaf and root osmotic potential of plantlets acclimatized under 3% Suc + 2.5% Man were more negative than control and 6% Suc. Interestingly, leaf osmotic potential of *Arabidopsis* grown under mixtures of 50 mM Suc with different degrees of Man (50-250 mM) decreased in relation to the concentrations in culture medium (Déjardin et al. 1999). In addition, osmotic potential in leaf tissues was more negative than the root tissues, especially in plants grown under mannitol-induced osmotic stress (Darko et al. 2019). Increase in Suc content in the acclimatized plants was evidently observed when plants were exposed to Man-induced osmotic stress, which plays a key role in osmotic adjustment at the cellular levels (Déjardin et al. 1999; Darko et al. 2019). In present study, inhibition of pseudostem height and root length of plantlets propagated under 3% Suc + 2.5% Man was evidently observed. Root and shoot traits of plantlets propagated *in-vitro* are sensitive to declined osmotic potential in the culture medium, leading to short root and delayed shoot growth (Custódio et al. 2004; Madhulatha et al. 2006; Mohamed and Ibrahim 2012; de Olivera et al. 2018). Alternatively, some plant species including *Zingiber* and *Curcuma* requires 4.5-6.0% Suc in the culture medium for the development of healthy acclimatized plantlets with the maximal survival percentage and abilities to quickly adapt under *ex-vitro* environments (Fuentes et al. 2005; Jo et al. 2009; Sharmin et al. 2013; Kusumastuti et al. 2014; Matysiak and Gabryszewska 2016).

***Ex-vitro* adaptation**

Plantlets grown under 4% mannitol (-1.3 MPa) had a 0% survival rate after being transferred to peat moss substrate. In contrast, survival rate of plantlets acclimatized under iso-osmotic potential (-1.3 MPa; 6% Suc or 3% Suc + 2.5% Man) and control (-1.0 MPa; 3% Suc) was 100%. Overall growth performances in both aboveground and underground of adapted *ex-vitro* plants were observed (Fig. 4). Pseudostem height, number of leaves, leaf area, pseudostem fresh weight in the control plants (3% Suc) were highest at 31.1 cm, 16.3 leaves, 207.6 cm² and 11.0 g, respectively, whereas these parameters in plants propagated under 3% Suc + 2.5% Man were declined by 56.27%, 30.67%, 68.55% and 58.18% over the control (Table 2). Pseudostem height and root length were identified as the most sensitive parameters that significantly decreased by 26.69% and 31.97% over control when plantlets were acclimatized under 6% Suc (Table 2). Root length, root fresh weight and minirhizome width in control plants (3% Suc) were observed to be 14.7 cm, 6.1 g and 1 cm, respectively, but in 3% Suc + 2.5% Man treatment these were decreased by 33.33%, 52.46% and 40.00% over control (Table 2). Interestingly, number of roots and minirhizome length were unaffected in all the treatments. Moreover, pseudostem dry weight, root dry weight, minirhizome fresh and dry weights in acclimatized plants under 3% Suc + 2.5% Man were sharply declined by 62.50% (Fig. 5A), 66.67% (Fig. 5B), 73.44% (Fig. 5C) and 75.00% (Fig. 5D) over control, respectively.

In the present study, plantlets acclimatized under 4% Man were undeveloped and consequently died when transplanted in the peat moss substrate. On the other hand, the plantlets subjected to iso-osmotic treatments (6% Suc and 3% Suc + 2.5% Man; -1.3 MPa) and control (3% Suc; -1.0 MPa) did fairly well under *ex-vitro* conditions. In *C. zedoaria*, the survival percentage of plantlets transplanted into *ex-vitro* conditions was observed to be 82-100% using conventional plant tissue culture containing with 2.0-3.0% Suc in MS medium (Bharalee et al. 2005; Loc et al. 2005; Stanly et al. 2010; Jena et al. 2020). There are so many steps to acclimatize the plants in greenhouse conditions including *ex-vitro* hardening, evaporation prevention, mist spray to keep high relative humidity, and reduced transpiration rate from plantlets, etc. A novel protocol to harden plantlets is required to facilitate their direct transplantation to *ex-vitro* environments. Previously, survival percentage of carob tree (*Ceratonia siliqua*) was significantly improved by adding 58 mM glucose + 58 mM mannitol in the culture medium (Custódio et al. 2004). Similarly, shoot and root traits of bromeliad plants (*Vriesea inflata*) derived from *in-vitro* sucrose containing medium were decreased (Freitas et al. 2015), in relation to concentrations (4-6% Suc) and plant species (Wojtania et al. 2019). In addition, root traits in the plantlets of magnolia cv. 'Yellow Bird' (cross between *Magnolia acuminata* and *M. × brooklynensis*) acclimatized under 3-4% Suc were inhibited, resulting in more than 75% of leaf necrosis in the plants (Wojtania et al. 2019).

P_n of adapted plantlets derived from *in-vitro* acclimatization under 3% Suc + 2.5% Man was significantly declined by 22.81% over control (Fig. 5E), leading to decreased sucrose concentration in the leaf tissues (Fig 6A; $R^2 = 0.5636$). Sucrose concentration in the leaf tissues of *in-vitro* acclimatized plants under iso-osmotic potential (-1.3 MPa) was dropped by 43.65-47.09% over control (Fig. 5F). Therefore, glucose, fructose and total soluble sugar in the leaf tissues were unaffected. Physiological parameters such as leaf greenness (SPAD), F_v/F_m , F_{PSII} , E and g_s in the second fully expanded leaves of each treatment were found to be unchanged (Table 3). In addition, strong

relationships between leaf area and microrhizome dry weight (Fig. 6B; $R^2 = 0.9478$), pseudostem height and root length (Fig. 6C; $R^2 = 0.7555$), and number of leaves and leaf area (Fig. 6D; $R^2 = 0.8396$) were noticed.

Physiological strategies of plants derived from *in-vitro* acclimatization, such as water loss prevention by stomata and CO₂ assimilation to produce large amount of carbohydrate for plant growth and development have been validated in relation to field conditions (Seon et al. 2000). In general, maintained P_n along with limited transpiration (E) by stomata is a good indicator for rapid *ex-vitro* adaptation of acclimatized plantlets, which ensures a high survival rate of the plants under field conditions (van Huylenbroeck et al. 1998; Osório et al. 2012; Badr et al. 2015). Also, sucrose is a primary product of photosynthesis, which significantly increases in the healthy *ex-vitro* plants (van Huylenbroeck et al. 1998; Seon et al. 2000; Badr et al. 2015). Cuticular wax in the leaf surface of *ex-vitro* plants derived from *in-vitro* acclimatization play a key role in preventing water transpiration rate (Monja-Mio et al. 2015). In the present study, the *ex-vitro* adaptation of *in-vitro* acclimatized white turmeric plants under 3% Suc + 2.5% Man was longer (> 6 weeks) compared to previous reports (Kadleček et al. 2001; Valero-Aracama et al. 2006).

Long-term cultivation of white turmeric and agronomic traits

Morphological characteristics of white turmeric plants observed during its entire life cycle from *ex-vitro* adaptation to final harvest are presented for all the treatments in Fig. 7. Aboveground traits, *i.e.*, pseudostem height, number of leaves, leaf length and leaf width in all the treated plants were unaffected. It means that there is uniformity in the aboveground parameters of white turmeric plants irrespective of their *in-vitro* treatments. Remarkably, pseudostem fresh- and dry-weights were maximal in plants derived from 3% Suc + 2.5% Man acclimatization (1.53 and 1.31-folds over control, respectively) (Fig. 8A and 8B). Interestingly, the underground parameters including root length, number of roots, rhizome length and rhizome width of plants derived from 3% Suc + 2.5% Man treatment were significantly improved by 3.54, 1.5, 1.69 and 2.71-folds over control, respectively (Table 4). Root fresh weight, root dry weight, rhizome fresh weight and rhizome dry weight of plants derived from 3% Suc + 2.5% Man treatment were strongly enhanced upon -1.3 MPa iso-osmotic treatments and recorded to be 145.93 g (Fig. 8C), 10.7 g (Fig. 8D), 248.39 g (Fig. 8E) and 31.0 g (Fig. 8F), respectively. In white turmeric, DEM in the rhizome was identified as major compound, whereas BIS and CUR were the minor compounds (Fig. 9A-9C). The BIS, DEM, CUR, total curcuminoids and curcumin yield in plants derived from 3% Suc + 2.5% Man treatment were peaked at 0.7 mg, 5.9 mg, 0.8 mg, 7.4 mg and 229.4 mg plant⁻¹, respectively (Fig. 9A-9E). A positive relation between rhizome dry weight and total curcuminoids were observed (Fig. 9F; $R^2 = 0.9467$). In term of plant physiological responses, leaf greenness (SPAD) and P_n in plants derived from 3% Suc + 2.5% Man treatment were increased by 1.34 and 1.22-folds, respectively (Table 5). In addition, E and g_s in acclimatized plants under 6% Suc and 3% Suc + 2.5% were significantly improved over controlled plants (Table 5). In contrast, F_v/F_m and F_{PSII} in plants were unaffected irrespective of the treatment.

After 9 months, overall growth performances in both aboveground and underground traits of *in-vitro* plantlets acclimatized under 3% Suc + 2.5% Man were better than those in control, including total curcuminoids in the mature rhizome. Overall growth performances and yield traits of plants derived from Man osmoprimer were significantly better than non-priming control (Kaur et al. 2005; Papastylianou and Karamanos 2012). In *C. zedoaria*, the morphological parameters, *i.e.*, plant height, number of tillers, number of leaves, leaf length, leaf width, rhizome diameter, total rhizome weight and rhizome length as well as secondary metabolites in rhizome and leaf oil were nearly same in mother plant and *in-vitro* derived plants under field trial (Jena et al. 2020). In tea (*Camellia sinensis*), the major compounds, epigallocatechin and epigallocatechin gallate, in the fresh leaves of plantlets acclimated under 200 mM mannitol were enriched over control (Samarina et al. 2020). Fresh mass, dry mass, number of shoots and COX1-inhibition activity (%) in plants of *Eucomis autumnalis* acclimatized under 4% Suc were increased when compared with low sucrose treatment (2%; w/v) (Taylor and van Staden 2001). It was confirmed that the physiological and morphological traits as well as curcuminoids yield of *in-vitro* plantlets acclimatized using iso-osmotic treatment of 3% Suc + 2.5% Man were better compared to control during long-term cultivation. In *Ruta graveolens*, plant morphological characters in mannitol acclimatized plantlets were unaffected, whereas coumarins and rutin in terms of content and total yield were regulated in response to increase in Man concentration in the culture medium (Mohamed and Ibrahim 2012).

Conclusions

The present study demonstrated a successful hardening of *in-vitro* plantlets of white turmeric using 3% Suc + 2.5% Man (-1.3 MPa) resulting in compaction by ~50% in terms of aboveground and underground growth performances, thereby requiring small area for master stock production. Healthy plants (uniform-sized) derived from *in-vitro* acclimatization under 6% Suc and 3% Suc + 2.5% Man (-1.3 MPa) treatments were rapidly adapted to *ex-vitro* greenhouse conditions within 6 weeks, and subsequently adapted to long-term greenhouse

microclimates. Interestingly, physiological characters including photosynthetic abilities and underground rhizome traits in plants derived from 3% Suc + 2.5% Man *in-vitro* acclimatization were improved, leading to an increase in rhizome and curcuminoids yield.

Declarations

Acknowledgements

The authors would like to thank National Science and Technology Development Agency for funding support (Grant number P-18-529840).

Author contributions

Conceptualization, SC and RT; formal analysis, UB, ThS, TH; investigation, RT, TS; writing—original draft preparation, RT; writing— review and editing, RT, SC; supervision, SC; project administration, RT; funding acquisition, SC, RT, TS. All authors have read and approved the manuscript for submission.

Funding

Financial support provided by the National Science and Technology Development Agency for funding support (Grant number P-18-529840). The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Compliance with ethical standards

Conflict of interest

We declare no competing interest with respect to the current study.

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Tables

Table 1 Pseudostem height (Ps-H), number of leaves (NL), leaf area (LA), pseudostem fresh weight (Ps-FW), root length (RL), number of roots (NR), root fresh weight (RT-FW), microrhizome length (RhL) and microrhizome width (RhW) of acclimatized *C. zedoaria* under *in vitro* iso-osmotic treatments for 45 days. Data represented as mean ± standard error (SE) (n = 4).

Treatment	Aboveground				Underground				
	Ps-H (cm)	NL	LA (cm ²)	Ps-FW (g)	RL (cm)	NR	RT-FW (g)	RhL (cm)	RhW (cm)
3% Suc	14.3±1.0a	4.3±0.2a	16.1±2.3a	1.6±0.2a	11.8±1.2a 3.8±0.6bc		0.49±0.03a	0.50±0.03a	0.30±0.03a
6% Suc	11.4±0.8a	3.8±0.7a	11.5±2.2a 1.1±0.1ab		8.2±1.9a 6.3±1.0ab		0.41±0.02b	0.50±0.01a	0.40±0.01a
4% Man	2.4±0.1c	0.0±0.0b	0.0±0.0b	0.5±0.0b	0.9±0.4b	2.0±0.5c	0.20±0.01c	0.00±0.00b	0.00±0.00b
3% Suc + 2.5% Man	5.7±0.2b	1.3±0.2b	1.4±0.3b	0.8±0.1b	7.1±0.8a	8.5±1.0a	0.27±0.01c	0.50±0.05a	0.40±0.03a

Different letters in each column represented significant difference at p ≤ 0.05 by Tukey's HSD test.

Table 2 Pseudostem height (Ps-H), number of leaves (NL), leaf area (LA), pseudostem fresh weight (Ps-FW), root length (RL), number of roots (NR), root fresh weight (RT-FW), minirhizome length (RhL) and minirhizome width (RhW) of transplanted *C. zedoaria* plantlets from *in*

vitro iso-osmotic treatments to soil substrate under greenhouse conditions for 6 weeks. Data represented as mean ± standard error (SE) ($n = 4$).

Treatment	Aboveground				Underground				
	Ps-H (cm)	NL	LA (cm ²)	Ps-FW (g)	RL (cm)	NR	RT-FW (g)	RhL (cm)	RhW (cm)
3% Suc	31.1±1.5a	16.3±2.5a	207.6±14.7a	11.0±1.1a	14.7±1.5a	8.0±1.4 ^{ns}	6.1±1.3a	0.70±0.08 ^{ns}	1.00±0.04a
6% Suc	22.8±1.7b	17.5±1.8a	173.9±2.8a	10.4±0.8a	10.0±0.7b	4.3±0.6	7.1±1.2a	0.50±0.04	0.70±0.12ab
3% Suc + 2.5% Man	13.6±1.1c	11.3±1.6b	65.3±3.7b	4.6±1.3b	9.8±1.7b	7.8±0.9	2.9±1.1b	0.60±0.06	0.60±0.07b

Different letters in each column represented significant difference at $p \leq 0.05$ by Tukey's HSD test. ^{ns} represented as non-significant different in statistical analysis.

Table 3 Glucose, fructose, total soluble sugar (TSS), leaf greenness (SPAD), maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (F_{PSII}), transpiration rate (E), and stomatal conductance (g_s) in the leaf tissues of transplanted *C. zedoaria* plantlets from *in vitro* iso-osmotic treatments to soil substrate under greenhouse conditions for 6 weeks. Data represented as mean ± standard error (SE) ($n = 4$).

Treatment	Soluble sugar (mg g ⁻¹ DW)			Physiological parameters					
	Glucose	Fructose	TSS	SPAD	F_v/F_m	F_{PSII}	E (mol H ₂ O m ⁻² s ⁻¹)	g_s (mmol H ₂ O m ⁻² s ⁻¹)	
3% Suc	14.1±0.9 ^{ns}	11.0±1.4 ^{ns}	62.9±1.5 ^{ns}	26.8±1.2 ^{ns}	0.80±0.03 ^{ns}	0.60±0.03 ^{ns}	0.91±0.30 ^{ns}	0.030±0.008 ^{ns}	
6% Suc	22.1±0.4	19.2±2.9	61.3±5.6	23.9±2.3	0.80±0.02	0.60±0.04	0.77±0.26	0.030±0.009	
3% Suc + 2.5% Man	19.2±0.2	17.7±2.3	58.2±4.7	24.9±0.5	0.80±0.01	0.60±0.02	0.68±0.18	0.020±0.009	

^{ns} represented as non-significant different in statistical analysis.

Table 4 Pseudostem height (Ps-H), number of leaves (NL), leaf area (LA), leaf length (LL), leaf width (LW), root length (RL), number of roots (NR), rhizome length (RhL) and rhizome width (RhW) of *C. zedoaria* plants derived from *in vitro* iso-osmotic treatments, subsequently transplanted to soil substrate under greenhouse conditions for 9 months. Data represented as mean ± standard error (SE) ($n = 4$).

Treatment	Aboveground				Underground			
	Ps-H (cm)	NL (cm)	LL (cm)	LW (cm)	RL (cm)	NR (cm)	RhL (cm)	RhW (cm)
3% Suc	53.5±2.8 ^{ns}	5.8±0.8 ^{ns}	51.3±1.7 ^{ns}	13.5±0.5 ^{ns}	17.1±0.9c	6.0±1.0b	7.0±0.5b	7.0±1.0b
6% Suc	45.2±4.3	5.0±0.4	46.8±2.9	12.0±1.3	49.0±1.2b	7.0±1.0b	10.0±0.5a	13.0±3.0ab
3% Suc + 2.5% Man	53.3±1.0	5.0±0.4	52.3±1.6	11.8±0.3	60.5±0.5a	9.0±2.0a	11.8±1.3a	19.0±0.5a

Different letters in each column represented significant difference at $p \leq 0.05$ by Tukey's HSD test. ^{ns} represented as non-significant different in statistical analysis.

Table 5 Leaf greenness (SPAD), maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (F_{PSII}), net photosynthetic rate (P_n), transpiration rate (E) and sttmatal conductance (g_s) of *C. zedoaria* plants derived from *in vitro* iso-osmotic treatments, subsequently transplanted to soil substrate under greenhouse conditions for 9 months. Data represented as mean ± standard error (SE) ($n = 4$).

Treatment	SPAD	F_v/F_m	F_{PSII}	P_n (mmol m ⁻² s ⁻¹)	E (mol H ₂ O m ⁻² s ⁻¹)	g_s (mmol H ₂ O m ⁻² s ⁻¹)
3% Suc	23.7±1.6b	0.81±0.01 ^{ns}	0.59±0.02 ^{ns}	6.05±0.3b	0.37±0.05b	0.013±0.002b
6% Suc	27.7±1.7ab	0.81±0.01	0.55±0.04	6.30±1.1ab	0.63±0.15a	0.025±0.006a
3% Suc + 2.5% Man	31.7±3.7a	0.79±0.02	0.55±0.05	7.09±0.6a	0.83±0.09a	0.033±0.003a

Different letters in each column represented significant difference at $p \leq 0.05$ by Tukey's HSD test. ^{ns} represented as non-significant different in statistical analysis.

Figures



Figure 1

Morphological characteristics of acclimatized Curcuma zedoaria plantlets under in-vitro iso-osmotic treatments for 45 days.

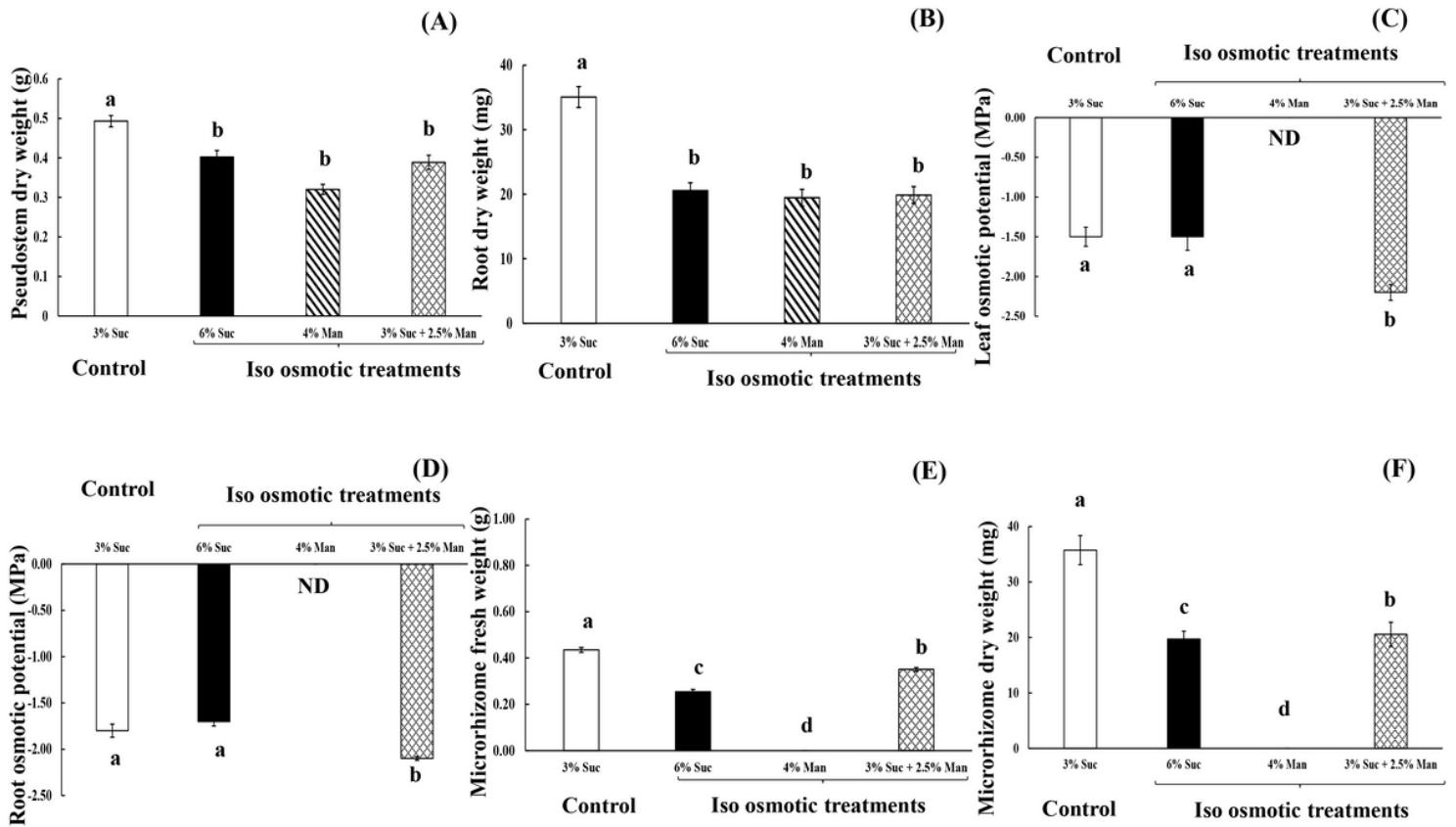


Figure 2

Pseudostem dry weight (A), root dry weight (B), leaf osmotic potential (C), root osmotic potential (D), microrhizome fresh weight (E) and microrhizome dry weight (F) of acclimatized *Curcuma zedoaria* plantlets under in-vitro iso-osmotic treatments for 45 days. Data represented as mean \pm standard error (SE) ($n = 4$). Different letters above bars represent significant difference at $p \leq 0.05$ according to Tukey's HSD test. NA = not available for detection

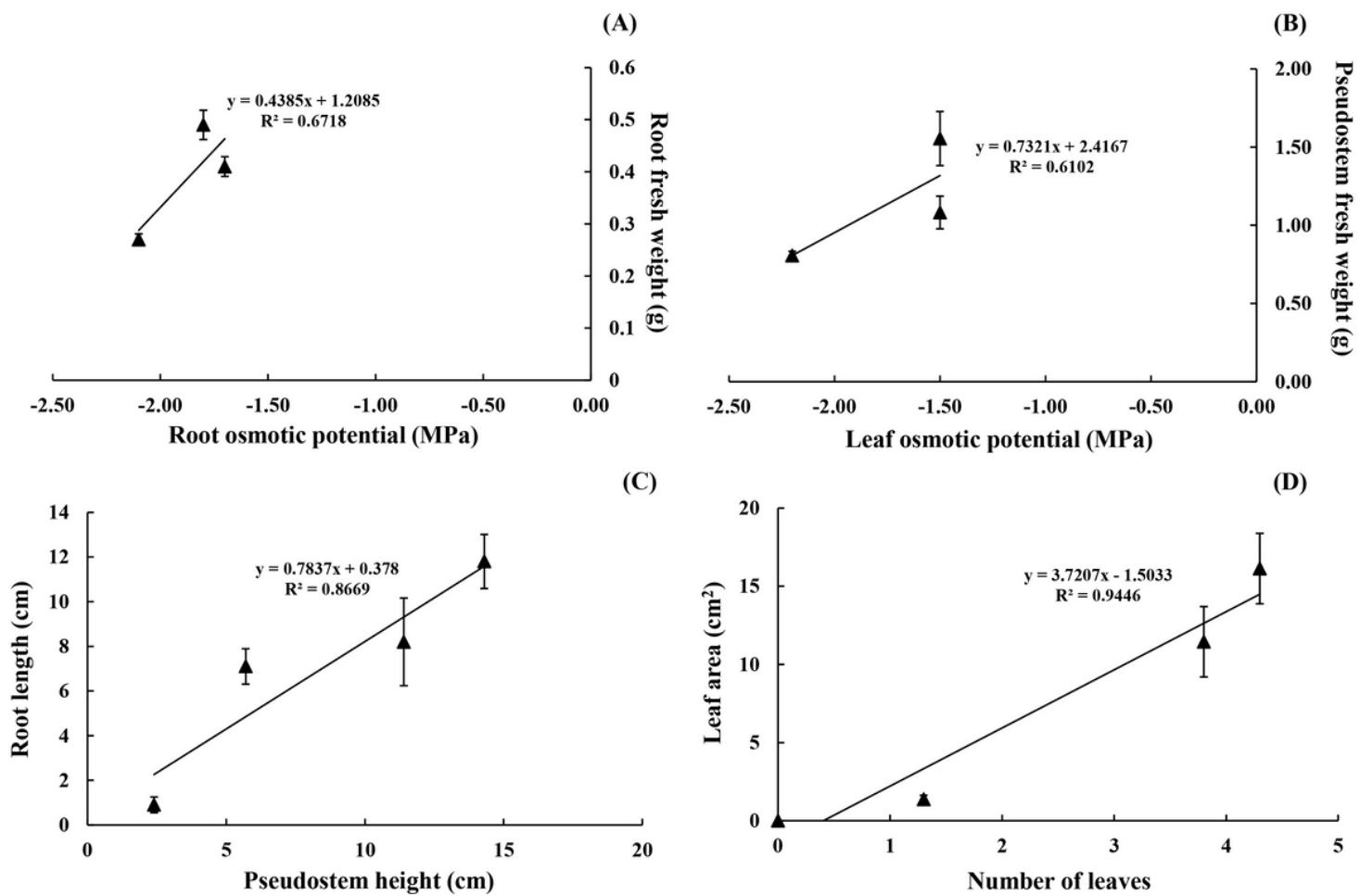


Figure 3

Relationships between root osmotic potential and root fresh weight (A), leaf osmotic potential and pseudostem fresh weight (B), pseudostem height and root length (C), number of leaves and leaf area (D) of acclimatized *Curcuma zedoaria* plantlets under in-vitro iso-osmotic treatments for 45 days. Data represented as mean \pm standard error (SE) ($n = 4$).



Figure 4

Morphological characteristics of transplanted *Curcuma zedoaria* plantlets from in-vitro iso-osmotic treatments to soil substrate under greenhouse conditions for 6 weeks (top panel: overall growth performance, middle panel: bareroot individual plant and bottom panel: minirhizome characteristics).

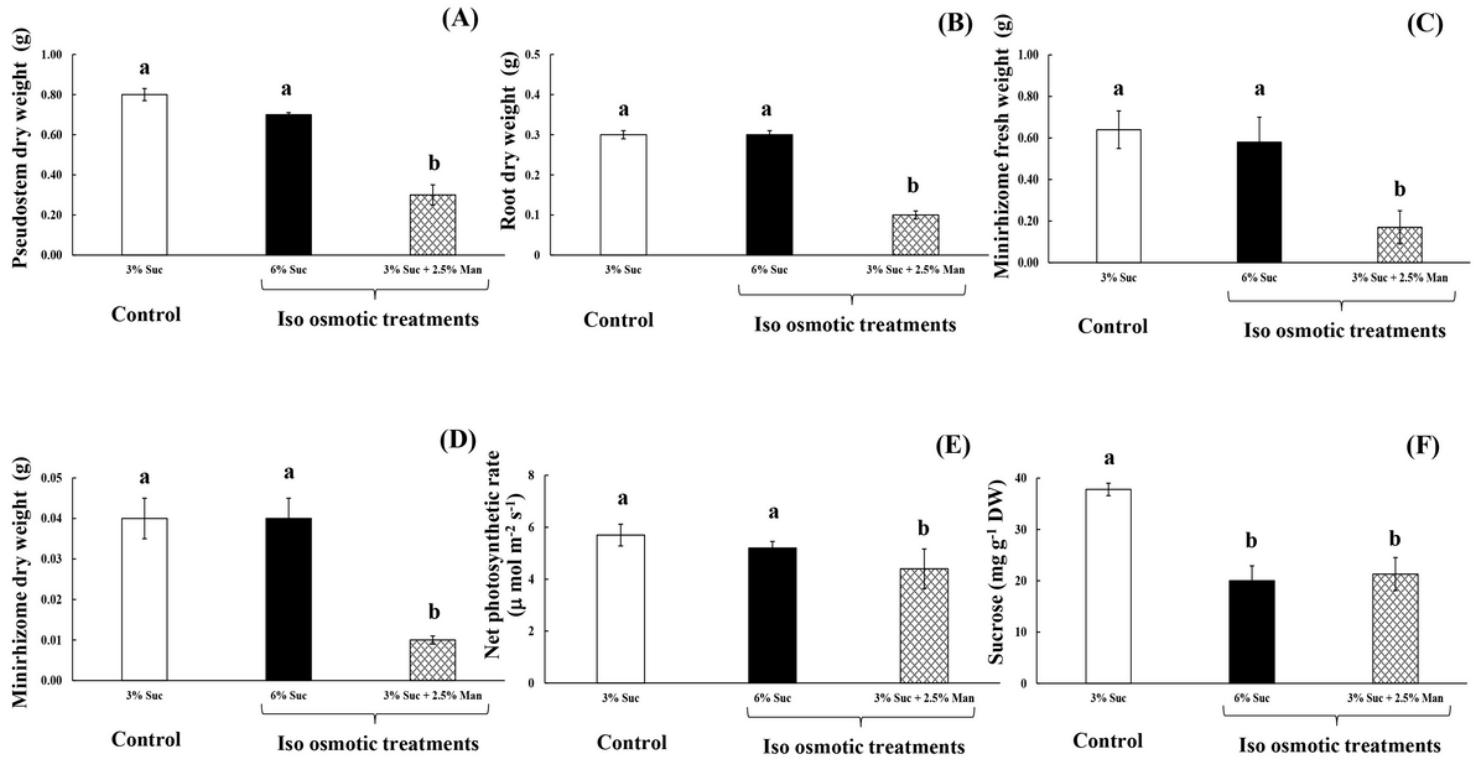


Figure 5

Pseudostem dry weight (A), root dry weight (B), minirhizome fresh weight (C), minirhizome dry weight (D), net photosynthetic rate (E) and sucrose concentration in the leaf tissues of transplanted *Curcuma zedoaria* plantlets from in-vitro iso-osmotic treatments to soil substrate under greenhouse conditions for 6 weeks. Data represented as mean \pm standard error (SE) ($n = 4$). Different letters in each bar represented significant difference at $p \leq 0.05$ according to Tukey's HSD test.

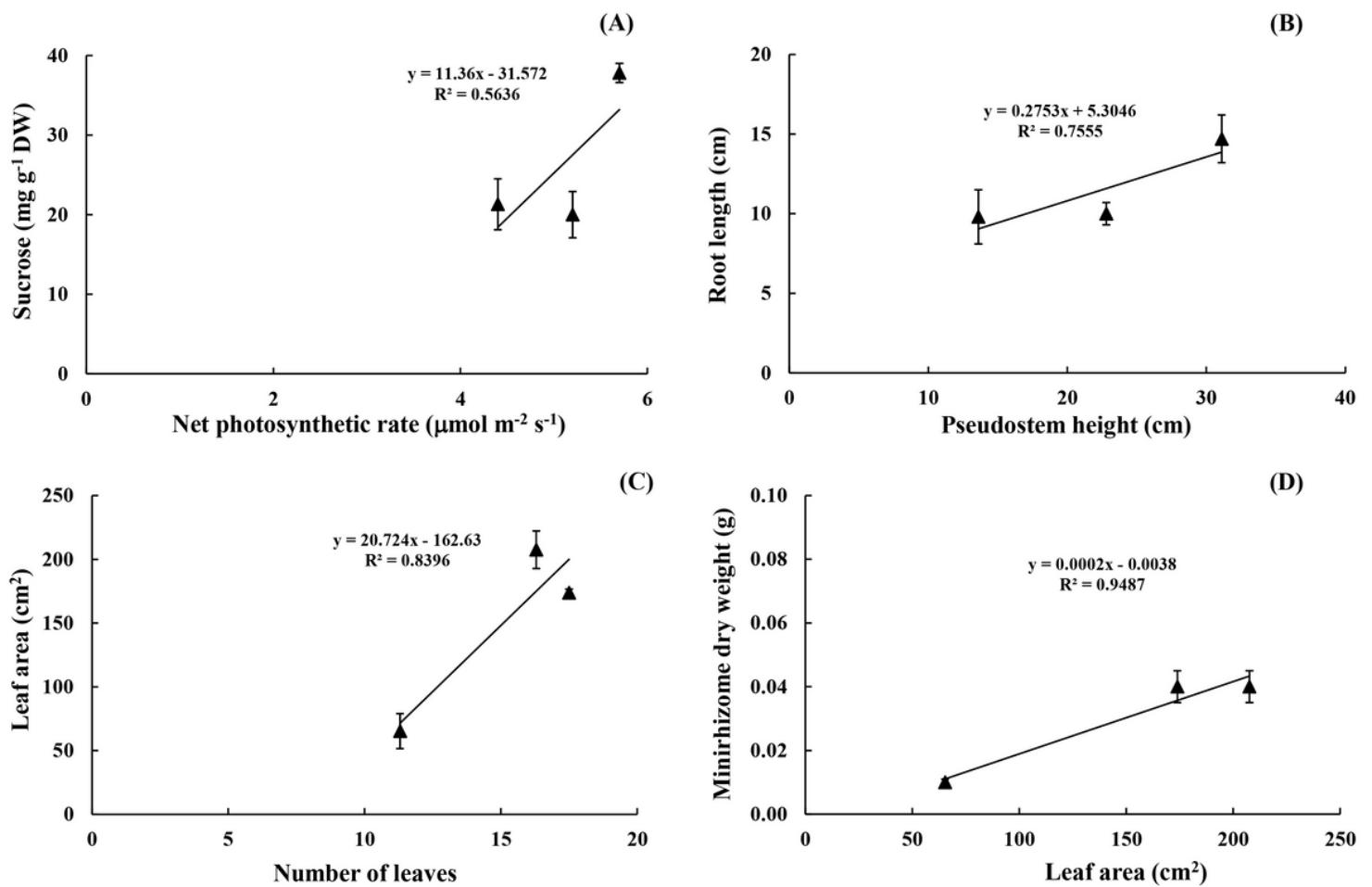


Figure 6

Relationships between net photosynthetic rate and sucrose concentration in the leaf tissues (A), pseudostem height and root length (B), number of leaves and leaf area (C), leaf area and minirhizome dry weight (D) of transplanted *Curcuma zedoaria* plantlets from in-vitro iso-osmotic treatments to soil substrate under greenhouse conditions for 6 weeks. Data represented as mean \pm standard error (SE) ($n = 4$).



Figure 7

Morphological characteristics on whole plant and rhizome of *Curcuma zedoaria* plants derived from in-vitro iso-osmotic treatments, subsequently transplanted to soil substrate under greenhouse conditions for 9 months.

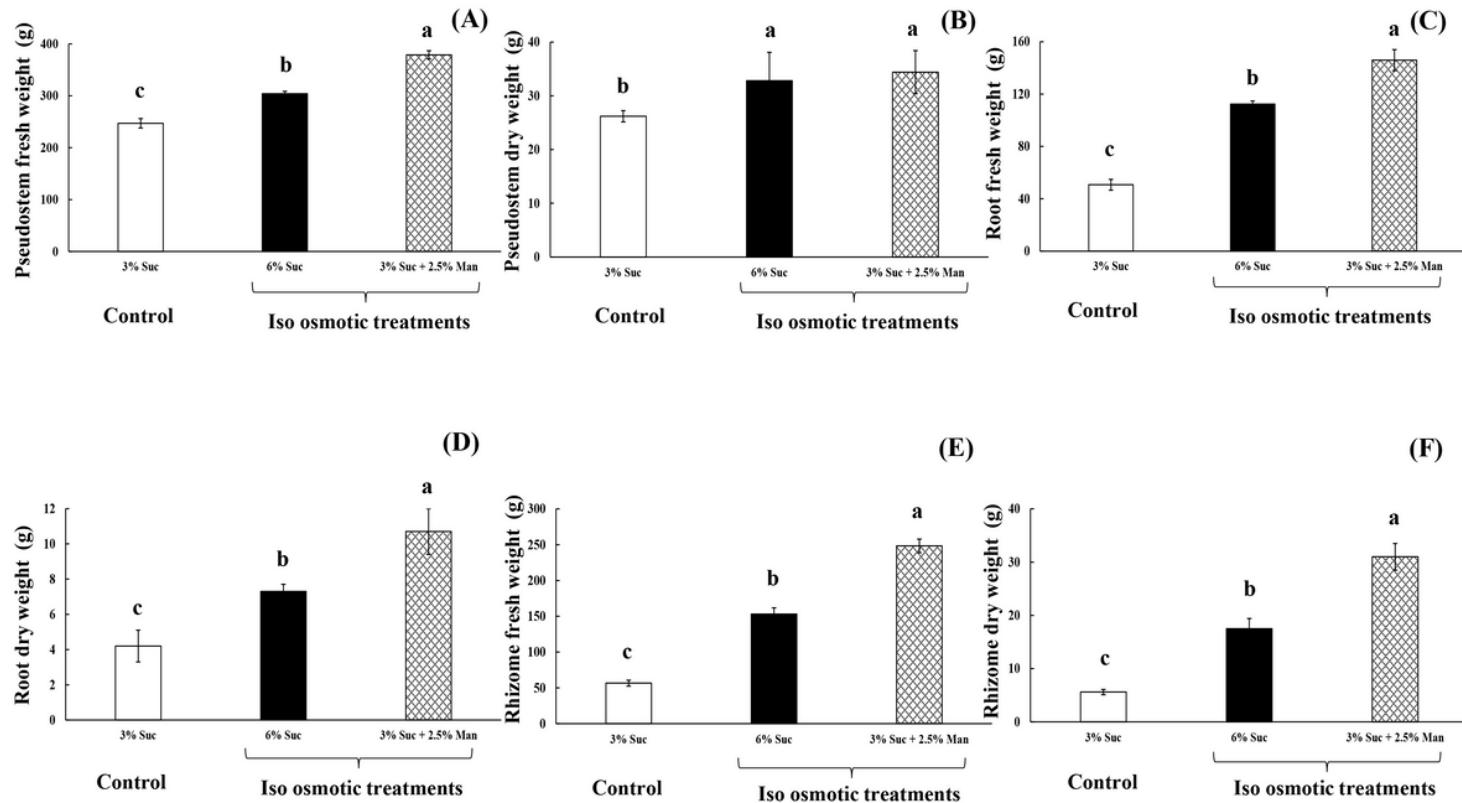


Figure 8

Pseudostem fresh weight (A), pseudostem dry weight (B), root fresh weight (C), root dry weight (D), rhizome fresh weight (E) and rhizome dry weight (F) of *Curcuma zedoaria* plants derived from in-vitro iso-osmotic treatments, subsequently transplanted to soil substrate under greenhouse conditions for 9 months. Data represented as mean \pm standard error (SE) ($n = 4$). Different letters in each bar represented significant difference at $p \leq 0.05$ according to Tukey's HSD.

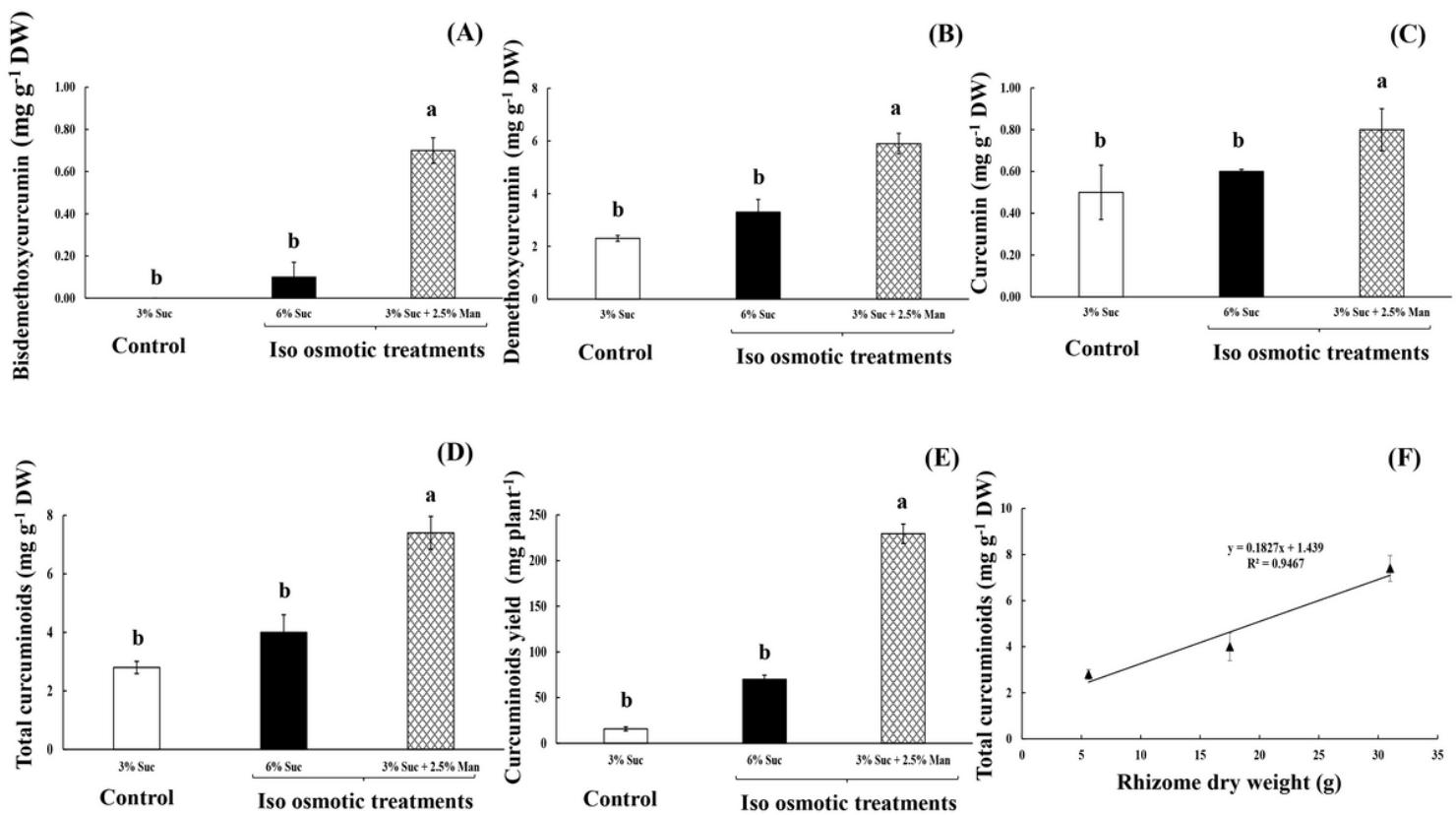


Figure 9

Bisdemethoxycurcumin (A), demethoxycurcumin (B), curcumin (C), total curcuminoids (D), curcuminoid yield (E) and relationship between rhizome dry weight and total curcuminoids (F) of *Curcuma zedoaria* plants derived from in-vitro iso-osmotic treatments, subsequently transplanted to soil substrate under greenhouse conditions for 9 months. Data represented as mean \pm standard error (SE) ($n = 4$). Different letters in each bar represented significant difference at $p \leq 0.05$ according to Tukey's HSD.