In vitro acclimatization of Curcuma longa under controlled iso-osmotic conditions

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Abstract In vitro acclimatization has been validated as the successful key to harden the plantlets before transplanting to ex vitro conditions. In the present study, we investigated the potential of different sugar types (glucose, fructose, galactose, sucrose) in regulating morphological, physiological and biochemical strategies, survival percentage and growth performance, and rhizome traits of turmeric under iso-osmotic potential. Leaf greenness (SPAD value) in acclimatized plantlets (4% glucose; −1.355 MPa osmotic potential) of ‘ST018’ was retained and greater than in ‘PB009’ by 1.69-fold, leading to maintain high Fv/Fm (maximum quantum yield of PSII), ΦPSII (photon yield of PSII) and Pn (net photosynthetic rate) levels, and retained shoot height, leaf length, leaf width, shoot fresh weight and shoot dry weight after one month upon transplanting to ex vitro conditions. In addition, Pn, Ci (intracellular CO₂), gs (stomatal conductance) and E (transpiration rate) in acclimatized plantlets (6% sucrose; −1.355 MPa osmotic potential) of ‘PB009’ were stabilized as physiological adapted strategies, regulating the shoot and root growth and fresh and dry weights of mini-rhizome. Interestingly, the accumulation of total curcuminoids in mini-rhizome derived from 6% sucrose acclimatized plantlets of ‘ST018’ was greater than in ‘PB009’ by 3.76-fold. The study concludes that in vitro acclimation of turmeric ‘PB009’ and ‘ST018’ using 6% sucrose and 4% glucose, respectively, promoted percent survival, physiological adaptations, and overall growth performances under greenhouse conditions.

Key words: acclimatization, curcuminoids, ex vitro conditions, hardening, iso-osmotic potential, total soluble sugar, turmeric plant.

Introduction

Turmeric (Curcuma longa L.; Zingiberaceae), a herbaceous perennial, is an important commercial spice crop that is generally propagated by rhizome (Yadav and Tarun 2017). India is the largest producer of turmeric and accounts for 78% of world’s turmeric production (with 173,055 ha area and 855,763 ton or 4.59 ton ha⁻¹), followed by P.R. China (8%) and Myanmar (4%) (Deepa 2010). Several bioactive compounds, i.e., curcumin (CUR), demethoxycurcumin (DEM) and bisdemethoxycurcumin (BIS), playing as anti-cancer, anti-inflammatory, anti-free radical, anti-microbial, and anti-mutagenic activities in turmeric rhizome have been well established (Amalraj et al. 2017; Kocaadam and Şanlier 2017; Nasri et al. 2014; Rauf et al. 2018). The increase in production and maintenance of good quality of turmeric are the major economic strategies to regulate the small farmers and industrial private sectors (Angles et al. 2011). Elite varieties of turmeric exhibiting high rhizome yield and enriched curcuminoids are required as master stock to make a good profit because seed stock and sowing consume 26.8% of production cost (Karthik and Amarnath 2014).

In agricultural practices, turmeric requires a long period (7–9 months) to grow from sprout to rhizome harvest. It is sensitive to soil-borne diseases including bacterial wilt caused by Ralstonia solanacearum (Ajitomi et al. 2015). Disease-free turmeric plants derived from meristem cutting are the minimal requirements for production.

Abbreviations: BAP, benzyl amino purine; BIS, bis-demethoxycurcumin; Ci, intracellular CO₂ concentration; CRD, Completely Randomized Design; CUR, curcumin; DEM, demethoxycurcumin; E, transpiration rate; EC, electro conductivity; Fruc, fructose; Fv/Fm, maximum quantum yield of PSII; Gluc, glucose; gs, stomatal conductance; HPLC, High Performance Liquid Chromatography; MS, Murashige and Skoog medium; Pn, net photosynthetic rate; ΦPSII, photon yield of PSII; PPF, photosynthetic photon flux; Suc, sucrose; TSS, total soluble sugar.

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for disease-elimination and to produce the healthy master stock (Sharma and Singh 1997). In vitro micropropagation of turmeric disease-free elite clone via clonal multiplication (Goyal et al. 2010; Naz et al. 2009), organogenesis (Roopadarshini 2010), somatic embryogenesis (Raju et al. 2015) and bioreactor (El-Hawaz et al. 2016) has been well established.

In vitro acclimatization is one of the most important processes to acclimatize in vitro plantlets before transplanting into soil in the greenhouse with high survival rate (>90%) and rapid growth performances (Thingbaijam et al. 2012). The key factors for acclimatization process of in vitro and ex vitro using physical and chemical treatments have been reported to regulate the physiological adaptations, i.e., water relation, photosynthetic abilities (chlorophyll pigment, chlorophyll fluorescence, photosynthesis) and stomatal function as well as overall growth promotion in the greenhouse and field conditions (Salvi et al. 2002; Thingbaijam et al. 2012). Previously, only 70–75% survival rate of acclimatized turmeric plantlets has been reported, and poorly acclimatized plantlets are not adapted to field conditions (only 45–50% survival rate, Perić et al. 2012). Alternatively, in vitro microrhizome induction in turmeric plants has been reported as one of the most important techniques to improve the survival rate in acclimatized greenhouse conditions (Hashemy et al. 2009) but it is low frequency, leading to consume the long-period for microrhizome production (>18 months). In the present study, we aimed to produce the healthy plantlets of two turmeric cultivars, ‘PB009’ and ‘ST018,’ using iso-osmotic adjustment in the culture medium by several sugar types before transplanting into soil and validating the physiological adaptations, biochemical changes (including curcuminoids in mini-rhizome), and growth characteristics.

Materials and methods

Plant materials and in vitro treatments

Disease-free turmeric plantlets of ‘PB009’ and ‘ST018,’ derived from meristem cutting were proliferated on MS medium (Murashige and Skoog 1962) with 3 mg l−1 BAP (benzylaminopurine). Individual plantlets were subsequently transferred to glass vessel (250 ml) containing 50 ml MS medium and using vermiculite (Propolimer, Proplast Kimyevi TIC. LTD., Turkey) as supporting material. The air exchange rate (2.13 µmol CO₂ h⁻¹) of culture vessel was increased by punching a hole in the plastic cap (ϕ=1 cm) and covering the hole with gas-permeable microporous polypropylene film (0.22 µm pore size, Nihon Millipore LTD., Japan). Glass vessels containing turmeric 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, Phillip, Thailand) with a 16 h d⁻¹ photoperiod. During in vitro acclimatization, the iso-osmotic potential (−1.355 MPa) of MS culture medium was adjusted with 6% sucrose, 4% glucose, 4% galactose and 4% fructose in parallel to 3% sucrose (−1.045 MPa) as control for 21 day.

Ex vitro transplantation and adaptation abilities

In vitro acclimatized plantlets were directly transferred to peat moss (K® 1913, Class man, K-Select Aquasave, Germany) under greenhouse conditions (32±2°C day/28±2°C night temperature and 85±5% RH) and daily irrigated as ex vitro acclimatization for 14 day. Subsequently, individual plants were transferred to plastic bag (4×6 cm) containing 0.5 kg of gardening soil (EC=2.687 dS m⁻¹; pH=5.5; organic matter=10.36%; total N=0.17%; total P=0.07%; total K=1.19%, Seda Mixed Soil, Thailand) under greenhouse conditions as ex vitro adaptation for 1 month.

Morphological characteristics

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

Physiological characters

Osmotic potential in the culture medium containing different sugar classes was measured according to Lanfermeijer et al. (1991). In brief, 20 µl of the medium solution 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 first 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 (Fᵥ/Fᵣᵥ) and photon yield of PSII (ΦᵥPSII) from the adaxial surface of first 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 (Fᵥ; µmol m⁻² s⁻¹), stomatal conductance (gₛ; mmol CO₂ m⁻² s⁻¹), intracellular CO₂ concentration (Cᵢ; µmol mol⁻¹) and transpiration rate (E; mmol m⁻² s⁻¹) of first 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 changes

For curcuminoids assay, the dried mini-rhizomes were grinded into fine powder in the mortar with liquid nitrogen. Twenty milligram 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® No. 1, Maidstone, UK) and crude extract was dried by allowing the methanol to evaporate. Curcuminoids 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 C 18 (Vertisep™ UPS C8 HPLC) column incubating 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 with 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 first 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 Karkacier et al. (2003).

Experimental layout and statistical analysis

The experiment was arranged as 2×5 factorial in Completely Randomized Design (CRD) with four replicates (n=4). The mean values obtained from eight treatments were compared using Tukey’s HSD 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 evaluated using Pearson’s correlation coefficient.

Results

Growth characteristics

In vitro turmeric plantlets of both ‘PB009’ and ‘ST018’ acclimatized using different sugar classes (including control), except 4% galactose, survived when directly transplanted into the greenhouse condition. However, under 4% galactose plantlets turned yellow and did not survive (100% mortality). In aerial zone, leaf width in ‘PB009’ plantlets acclimatized under 3% sucrose (control) were significantly larger than those in ‘ST018’ by 1.80 folds, (Table 1). In 4% fructose treatment, shoot height in ‘PB009’ was greater than in ‘ST018’ by 1.41-fold (Table 1). Shoot height and shoot fresh- and dry-weights in turmeric plants of ‘PB009’ derived from 6% sucrose acclimatization were significantly improved by 1.74, 2.26 and 2.82 folds, respectively, over the control (3% sucrose) (Table 1). Moreover, shoot height, leaf length and leaf width, shoot fresh weight and shoot dry weight in turmeric plants of ‘ST018’ derived from 4% glucose acclimatization were significantly increased by 1.74, 1.56, 1.82, 3.42 and 5.2 folds, respectively, over the control (Table 1). Shoot height, leaf length, leaf width, shoot fresh weight and shoot dry weight in acclimatized turmeric plants of ‘PB009’ under 6% sucrose were greater than those in ‘ST018’ by 1.46, 1.39, 1.46, 2.81 and 4.43 folds, respectively (Table 1). Overall shoot performances in ‘PB009’ plantlets acclimatized under 6% sucrose, and in ‘ST018’ plantlets acclimatized with 4% glucose were identified as healthy plants with rapid adaptation in ex vitro conditions (Table 1).

In the root zone, root length and number of roots in ‘PB009’ acclimatized plantlets under 3% sucrose (control) were significantly greater than those in ‘ST018’ by 1.74 and 1.79 folds, respectively (Table 2). Root fresh- and dry-weights in turmeric plants ‘PB009’ derived from 6% sucrose acclimatization were significantly increased by 3.10 and 3.60 folds, respectively, over the control (3% sucrose) (Table 2). Root length, number of roots, and root fresh- and dry-weights in acclimatized turmeric plants of ‘PB009’ under 6% sucrose were greater than

| Variety | Sugar class | Shoot height (cm) | Leaf length (cm) | Leaf width (cm) | Number of leaves | Shoot fresh weight (g) | Shoot dry weight (g) |
|---------|-------------|-------------------|------------------|-----------------|-------------------|------------------------|----------------------|
| ‘PB009’ | 3%Suc       | 13.15±0.79de      | 11.54±0.56abc    | 4.10±0.11a      | 1.00±0.16b       | 1.58±0.16cd           | 0.11±0.01bc          |
|         | 6%Suc       | 22.88±2.95a       | 13.20±0.27a      | 4.10±0.23a      | 1.50±0.29ab      | 3.57±0.67a            | 0.31±0.08a           |
|         | 4%Gluc      | 19.65±0.97abc     | 12.05±0.87ab     | 3.13±0.43bc     | 1.75±0.25ab      | 2.00±0.30bc           | 0.21±0.03ab          |
|         | 4%FruC      | 17.75±1.16bcd     | 10.30±0.81bcd    | 3.38±0.31b      | 1.25±0.25b       | 1.45±0.18c            | 0.20±0.03ab          |
| ‘ST018’ | 3%Suc       | 12.00±0.79e       | 8.25±0.41c       | 2.28±0.14c      | 2.25±0.25a       | 0.78±0.05d            | 0.05±0.02c           |
|         | 6%Suc       | 15.70±1.95cde     | 9.50±0.87cd      | 2.80±0.30bc     | 2.25±0.48a       | 1.27±0.10c            | 0.07±0.02c           |
|         | 4%Gluc      | 20.88±1.25ab      | 12.88±1.48a      | 4.15±0.51a      | 1.75±0.25ab      | 2.67±0.33ab           | 0.26±0.03a           |
|         | 4%FruC      | 12.63±0.97e       | 8.75±0.48d       | 2.75±0.14bc     | 1.50±0.12ab      | 1.01±0.22d            | 0.06±0.02c           |

Different letters in each column represent significant difference at p≤0.01 according to Tukey’s HSD test.
In vitro acclimatization of turmeric

Table 2. Root length, number of roots, root fresh weight and root dry weight of acclimatized turmeric plantlets of 'PB009' and 'ST018.' Data presented as mean ±SE (n=4).

| Variety | Sugar class | Root length (cm) | Number of roots (cm) | Root fresh weight (g) | Root dry weight (g) |
|---------|-------------|------------------|----------------------|-----------------------|---------------------|
| 'PB009' | 3%Suc       | 20.10±3.43abc    | 6.25±0.75ab          | 1.40±0.39bc           | 0.05±0.02bcd        |
|         | 6%Suc       | 25.75±1.83a      | 7.25±1.18a           | 4.34±0.82a            | 0.18±0.03a          |
|         | 4%Gluc      | 22.20±1.87ab     | 6.25±1.11ab          | 1.98±0.66b            | 0.09±0.02b          |
|         | 4%Fruc      | 17.35±2.2bcd     | 4.75±0.48bc          | 1.09±0.11bc           | 0.09±0.01b          |
| 'ST018' | 3%Suc       | 11.55±2.46de     | 3.50±0.65c           | 0.26±0.07c            | 0.02±0.01cd         |
|         | 6%Suc       | 13.65±2.60cde    | 3.75±0.25c           | 0.72±0.21bc           | 0.04±0.01bcd        |
|         | 4%Gluc      | 10.58±3.34de     | 4.50±0.29bc          | 1.11±0.51bc           | 0.07±0.03bc         |
|         | 4%Fruc      | 7.55±2.39cde     | 3.25±0.48c           | 0.19±0.10c            | 0.01±0.00d          |

Different letters in each column represent significant difference at p<0.01 according to Tukey's HSD test.

Figure 1. Physiological adaptation of turmeric plantlets 'PB009' and 'ST018' acclimatized in 3% sucrose (Suc; control −1.045 MPa), 6% Suc, 4% glucose (Gluc) and 4% fructose (Fruc) with iso-osmotic control at −1.355 MPa for 21 day, and subsequently transferred to plastic bag containing commercial soil for 1 month in the greenhouse conditions. A) Leaf greenness (SPAD value), B) maximum quantum yield of PSII (Fv/Fm), C) photon yield of PSII (ΦPSII) and D) net photosynthetic rate (Pn). Data presented as mean ±SE (n=4). Different letters in each column show significant difference at p<0.01, according to Tukey's HSD test.

those in 'ST018' by 1.89, 1.93, 6.03 and 4.50 folds, respectively (Table 2). Moreover, root length and root dry weight in 'PB009' acclimatized plantlets under 4% fructose were significantly greater than those in 'ST018' by 2.30 and 9.00 folds, respectively (Table 2). Overall root traits in 'PB009' plantlets acclimatized with 6% sucrose and in 'ST018' plantlets acclimatized with 4% glucose were maximized (Table 2).

Physiological adaptation

Leaf greenness (SPAD value) in acclimated turmeric plants of 'ST018' was greater than in 'PB009,' especially under 4% glucose (1.69-fold) and 4% fructose (1.71-fold) (Figure 1A). In 'PB009,' SPAD value in acclimatized plantlets under iso-osmotic potential (−1.355 MPa) using 6% sucrose and 4% fructose was significantly reduced by 37.33% and 31.10%, respectively, over the control (Figure 1A). Leaf greenness in acclimatized plantlets of 'ST018’ with 6% sucrose was decreased by 26.66% of the control, whereas it was maintained in other treatments (Figure 1A). The Fv/Fm in acclimatized plantlets of 'ST018' under 4% glucose and 4% fructose was retained and higher than that in 'PB009' by 1.25 and 1.20 folds, respectively (Figure 1B). Moreover, ΦPSII in 'ST018' acclimatized plantlets under 4% glucose was maintained better than that in 'PB009' by 1.55-fold (Figure 1C). In 'PB009,' ΦPSII in acclimatized plantlets under 6% sucrose, 4% glucose and 4% fructose was sensitive to ex vitro conditions, thereby diminishing ΦPSII by 22.54%, 39.13% and 31.88% of the control, respectively (Figure 1C).

Interestingly, Pn in acclimatized plantlets of 'ST018' under 6% sucrose and 4% glucose was promoted by 1.49 and 1.74 folds, respectively, over the control (Figure 1D). Pn in 'ST018' acclimatized plantlets under 4% glucose was retained better than that in 'PB009' by 2.11-fold (Figure 1D). In 'PB009,' Pn in acclimatized plantlets
under 6% sucrose was peaked at 3.4 µmol m⁻² s⁻¹, which was greater than in 4% glucose (40.88% reduction) and 4% fructose (42.35% reduction) (Figure 1D). The gₛ in acclimatized plantlets of ‘PB009’ under 4% glucose and 4% fructose was declined by 66.67% of the control (Figure 2A), whereas E was decreased by 58.09% and 61.77%, respectively, over the control (Figure 2B). Surprisingly, in acclimated plants of ‘PB009’ under 6% sucrose these parameters were retained, while these were unchanged in ‘ST018’. The Cᵢ in acclimatized plantlets of both ‘PB009’ and ‘ST018’ under 6% sucrose was significantly enriched over the control by 1.61 and 2.17 folds, respectively (Figure 2C). In addition, the Cᵢ in acclimatized plantlets of ‘ST018’ was lower than that in ‘PB009’ by 3% sucrose, 48.05%, 53.41% (4% glucose) and 36.67% (4% fructose) (Figure 2C). Positive relations between SPAD value and Fᵥ/Fₘ (Figure 3A; R²=0.6216), Fᵥ/Fₘ and Φₚₛᵢ (Figure 3B; R²=0.5044), Φₚₛᵢ and Pₙ (Figure 3C; R²=0.392), and Pₙ and mini-rhizome dry weight (Figure 3D; R²=0.348).
were demonstrated.

**Mini-rhizome yield and biochemical changes**

TSS in acclimated plants of 'ST018' under 4% glucose was significantly dropped by 18.27% of the control and was lower than in 'PB009' by 24.12% (Figure 2D). Sucrose level in the leaf tissues of acclimatized plants of 'PB009' (42.8 mg g\(^{-1}\) DW under 6% sucrose; 1.32-fold over control) was increased in relation to a degree of exogenous pretreated plants, whereas glucose was unchanged (Table 3). Fructose was maximized at 49.5 mg g\(^{-1}\) DW in acclimatized plantlets of 'ST018' under 4% fructose, which was higher than in 'PB009' by 1.37-fold (Table 3). Pseudostem and mini-rhizome characteristics of acclimated turmeric mini-rhizomes in both cultivars were better demonstrated than in the control (Figure 4A). Mini-rhizome fresh weight in acclimatized plantlets of 'PB009' under 6% sucrose was maximized (0.76 g plant\(^{-1}\)), which was larger than control by 2.71-fold and greater than in 'ST018' by 4.47-fold (Figure 4B). In addition, mini-rhizome fresh weight in acclimatized plantlets of 'ST018' under 4% glucose was maximized (0.43 g plant\(^{-1}\)), which was larger than of the control by 4.30-fold (Figure 4B). In parallel, mini-rhizome dry weight in 'PB009' and 'ST018' was strongly improved by acclimatized plantlets under 6% sucrose (2.5-fold over the control) and 4% glucose (5.0-fold over the control), respectively (Figure 4C). Total curcuminoids in mini-rhizome of 'ST018' under 3% sucrose (control; 118.2 µg g\(^{-1}\) DW) and 6% sucrose (89.4 µg g\(^{-1}\) DW) were higher than in 'PB009' by 2.01 and 3.76 folds, respectively (Figure 4D). Interestingly, BIS, DEM and CUR in acclimatized plantlets of 'ST018' under 6% sucrose were accumulated at 25.0, 26.2 and 38.1 µg g\(^{-1}\) DW, respectively.

### Table 3. Soluble sugars including sucrose, glucose and fructose in the leaf tissues, curcuminoids (bis-demethoxycurcumin, BIS), demethoxycurcumin, DEM, and curcumin, CUR) in the mini-rhizome of acclimatized turmeric plantlets of 'PB009' and 'ST018.' Data presented as mean ±SE (n=4).

| Variety | Sugar class | Soluble sugar (mg g\(^{-1}\) DW) | Curcuminoids (µg g\(^{-1}\) DW) |
|---------|-------------|----------------------------------|---------------------------------|
|         |             | Suc  | Gluc | Fruc | BIS  | DEM  | CUR  |
| 'PB009' | 3% Suc      | 32.5 ± 3.7cd | 29.0 ± 1.5b | 34.0 ± 2.8bc | 17.9 ± 2.5abc | 18.9 ± 2.5bc | 22.1 ± 2.9bc |
|         | 6% Suc      | 42.8 ± 4.4a | 25.2 ± 2.5bc | 29.5 ± 1.9bc | 7.1 ± 1.8c | 7.6 ± 1.8c | 9.1 ± 2.4c |
|         | 4% Gluc     | 34.1 ± 1.0bc | 29.1 ± 3.7b | 38.8 ± 5.8b | 15.0 ± 3.0bc | 15.4 ± 2.7bc | 18.2 ± 3.4bc |
|         | 4% Fruc     | 45.3 ± 1.9a | 26.6 ± 1.7bc | 36.1 ± 3.1bc | 18.0 ± 5.2abc | 25.5 ± 5.4abc | 25.2 ± 3.9bc |
| 'ST018' | 3% Suc      | 33.8 ± 0.8bc | 24.8 ± 0.5bc | 36.1 ± 2.3bc | 32.0 ± 2.7a | 35.4 ± 3.1a | 50.8 ± 3.5a |
|         | 6% Suc      | 39.9 ± 0.8ab | 19.3 ± 1.0c | 27.4 ± 0.5c | 25.0 ± 2.1ab | 26.2 ± 2.7ab | 38.1 ± 2.8ab |
|         | 4% Gluc     | 26.1 ± 0.7de | 23.4 ± 1.6bc | 27.9 ± 2.4c | 18.9 ± 4.2abc | 21.5 ± 4.5abc | 44.3 ± 3.5ab |
|         | 4% Fruc     | 21.5 ± 0.7e | 43.9 ± 3.5a | 49.5 ± 5.1a | 23.9 ± 5.9ab | 25.5 ± 6.7ab | 34.4 ± 4.3ab |

Different letters in each column represent significant difference at p≤0.01 according to Tukey’s HSD test.
DW, which were larger than in ‘PB009’ by 3.52, 3.45 and 4.19 folds, respectively (Table 3). In 3% sucrose acclimated plantlets, DEM and CUR in ‘ST018’ was significantly enriched by 1.87 and 2.30 folds, respectively, over ‘PB009’ (Table 3). The turmeric plant of ‘ST018’ was identified as high curcuminoids one (including DEM and CUR), whereas ‘PB009’ was classified as low curcuminoids, thus helping to rapidly detect at micro-rhizome developmental stage.

Discussion

Photomixotrophic in vitro culture with sucrose as carbon source in the culture medium has been well established to harden the plantlets before transplanting into soil with high survival percentage (>80% survival in banana; Emara et al. 2018), rapid ex vitro adaptation (Martins et al. 2020), and fast growth strategies (Fuentes et al. 2005). In the present study, 100% survival of acclimatized turmeric plantlets (−1.355 MPa osmotic potential) was evidently observed, except that turmeric plantlets acclimatized with 4% galactose did not survive. Previously, galactose has been reported to exhibit negative effects on higher plants, inhibiting auxin-induced growth (root inhibition) of wheat, maize, barley, azuki bean, mung bean, cucumber, and pea plants (Pritchard et al. 2004; Yamamoto et al. 1988). In Arabidopsis mutant (AtGALK; with galactokinase T-DNA insertion), three independent transformed lines contain low galactose in the leaf tissues and were insensitive to exogenous galactose application (Egert et al. 2012). Galactose has been reported to play an important role in the somatic embryogenesis of Citrus sinensis (‘Caipira’ and ‘Valencia’) and C. reticulata (‘Cleopatra I’ and ‘Cleopatra II’) (Tomaz et al. 2001). Sucrose in culture medium is a major carbohydrate source for plant growth and development. In elephant’s ear (Colocasia esculenta), acclimatized plantlets under 3% sucrose were optimized with 85% survival in ex vitro conditions, and promoting overall growth performances, i.e., shoot length, root length, number of leaves, and number of roots; however, fresh-, dry-weights and corn size were peaked in acclimatized plantlets under 9% sucrose (Jo et al. 2009). Previously, 3.4% sucrose in the acclimated plantlets of Rindera umbellata has been reported to promote survival rate in the greenhouse (71.43%) and field conditions (42.86%), leading to greater leaf length, fresh weight, dry weight, number of roots and root length than without hardening process (Perić et al. 2012). Shoot dry weight of bromeliad Guzmania ‘Hilda’ plants grown in 4.5% sucrose exhibited positive relation to sucrose uptake (Lembrchts et al. 2017). Moreover, survival percentage, number of shoots and vigorous growth with green plant of acclimatized pineapple plantlets under 3% table sugar and sucrose were validated as optimum dose when compared with glucose and fructose (Mengesha et al. 2020). In native bromeliad, survival percentage of acclimatized plantlets under 1–6% sucrose was 100% with normal growth pattern, as indicated by number of leaves, number of roots, leaf length, root length, fresh mass and dry mass after transplanting to Pinus bark substrate for 90 day (Freitas et al. 2015). In silk banana, survival rate in acclimatized plantlets under 3% glucose, fructose and sucrose was 100% with high growth performances, i.e., shoot length, shoot thickness and number of leaves, after transplanting for 45 day (secondary ex vitro hardening; Waman et al. 2014). Moreover, the number of roots, root length, shoot length, number of leaves, leaf length and leaf width of acclimatized Ney Poovan banana under 2% glucose were identified as healthy plant in ex vitro conditions when compared with sucrose and fructose and the other doses (Bohra et al. 2016).

In general, the reduction in osmotic potential in the culture medium using sugar supplementation (6% sucrose) negatively affected the leaf water potential (Jo et al. 2009). The optimum doses and type of sugars for photosynthetic pigment retention, and consequently high levels of F v/F m and Φ PSII (Jo et al. 2009; Rybczyński et al. 2007) during acclimatization process depends on plant species; for example, in coconut at 4.5% sucrose (Fuentes et al. 2005); in banana with 3% glucose or 3% fructose (El-Mahdy and Youssef 2019). In addition, total chlorophyll in Chinese foxglove (Rehmannia glutinosa) plantlets acclimatized using 3% sucrose (heterothophic growth) or without sucrose (photosautotrophic growth) was significantly lower than those in field plant (Seon et al. 2000). In tobacco, total chlorophyll level in the leaf tissues of acclimatized plantlets using 3% sucrose under high light intensity (200 µmol m−2 s−1) was maintained better than in photosautotrophic plantlets, leading to quickly adapt to greenhouse conditions (Kadleček et al. 2001). The P n and g s in 3.0–4.5% sucrose acclimatized plantlets of elephant’s ear (Jo et al. 2009) and coconut (Fuentes et al. 2005) were better than those in high degree of sucrose or without sucrose, thereby resulting in enrichment of total soluble sugar (TSS; sucrose, glucose and fructose) and starch in the leaves (Pospůšilová et al. 1999; van Huylenbroeck and de Riek 1995; van Huylenbroeck et al. 1998).

Increased sugar level in root and leaf tissues of acclimatized plantlets depends on the types of sugar and a degree of exogenous application. For example, sucrose was rapidly taken up by root organs and then translocated to leaf tissues of acclimatized bromeliad ‘Hilda’ plantlets in relation to an initial sucrose concentration in the medium (15–117 µmol g−1 medium) within 4 weeks (Lembrchts et al. 2017). Total soluble sugar (fructose, glucose and sucrose) in leaves of 17 day ex vitro adapted tobacco plants (acclimatized under
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3% sucrose) was peaked, whereas it was undetected in sugar-free plantlets (Haisel et al. 2001). In elephant’s ear plant, glucose, fructose and starch in the leaf tissues of plantlets acclimatized with 6% sucrose for 30 day were peaked (Jo et al. 2009). Based on sugar enrichment, it possible that there was source (leaves) to sink (micro-rhizome enriched with starch over control) regulation in 5% sucrose acclimatized plantlets (Lo-apirukkul et al. 2012). Micro-rhizome formation of Thai black ginger plantlets acclimatized under 6% sucrose were evidently observed (Zuraiida et al. 2015). Similarly, micro-rhizome in painted spiral ginger (Costus speciosus) was produced using 9% sucrose with 1.7 micro-rhizomes per explant (5.12 g micro-rhizome fresh weight) (Punyarani and Sharma 2010). Thus, the mini-rhizome and micro-rhizome formation in response to sugar classes and concentration in the culture medium depends on plant species. Tuber formation, size and fresh weight in acclimatized plantlets of yam under 3% sucrose was maximized after transplanting for 60–120 day (Ovono et al. 2009), whereas 6% sucrose or 8% maltose was required for cocoyam (Xanthosoma sagittifolium) for mini-tuberization (Djeuani et al. 2014). The secondary metabolites, BIS, DEM and CUR, in mini-rhizomes of wild turmeric plantlets acclimatized under 3% sucrose were highest, whereas micro-rhizome formation (>50%) and fresh weight (>0.025 g) were maximized in 5–9% sucrose (Wu et al. 2015). In cassumunar ginger (Zingiber montanum), phenolic, floravonoid, tannin, alkaloid, saponin and steroid in induced micro-rhizomes using 6% sucrose were enriched over the natural rhizomes, whereas curcuminoid content was equal with natural rhizomes (Rajkumari and Sanatombi 2020). In Brahmi (Bacopa monnieri), mixed sucrose/maltose or glucose/fructose was recommended for bacoside A production in the leaf tissues (Naik et al. 2017). It is possible that total curcuminoids in mini-rhizome of acclimatized turmeric needs further evaluation at the harvest stage (9 months-old).

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