

Thidiazuron induces high-frequency indirect shoot organogenesis of *Bienertia sinuspersici*: a single-cell C₄ species

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Abstract Induction of high-frequency shoot organogenesis using stem-derived callus of *Bienertia sinuspersici* was achieved on Murashige and Skoog basal medium containing 0.0045–9.0 μM thidiazuron (TDZ). Other cytokinins [kinetins, and 6-benzyladenine (BA)] were also tested for their shoot induction potential but only TDZ was effective at promoting shoot morphogenesis and development. The highest shoot induction efficiency (80 %) was obtained on a medium containing 0.45 μM TDZ. The TDZ-derived shoots were transferred to medium containing 8.8 μM BA for multiplication followed by gibberellic acid (1.45–14.5 μM) treatment to promote shoot elongation. The elongated shoots spontaneously developed adventitious roots during the elongation process. The rooted plantlets were successfully transplanted to soil, acclimated in the greenhouse and then transferred to a growth chamber under controlled conditions. Histological analysis of leaf anatomy of the regenerated plants showed that the distinctive cytoplasmic compartmentation feature of the single-cell C₄ photosynthetic system was re-established. Moreover, Western blots detected high levels of key C₄ enzymes in mature leaves of TDZ-derived shoots in vitro and the regenerated plants. Overall, these findings show that in vitro regenerated plants possessed the single-cell C₄ leaf anatomy and C₄ enzymes comparable to those of the seed-derived plants.

Keywords *Bienertia sinuspersici* · Indirect organogenesis · Single-cell C₄ photosynthesis · Regeneration · Thidiazuron

Introduction

Bienertia sinuspersici is one of four members of family Chenopodiaceae that possesses a novel form of C₄ photosynthesis known as single-cell C₄ where the entire C₄ cycle takes place within a single cell as opposed to the classical Kranz-type C₄ which requires two cell types: mesophyll and bundle sheath (Hatch and Slack 1970; Edwards et al. 2004; Akhani et al. 2005; 2012). These species achieved the single-cell C₄ pathway by spatially partitioning organelles such as chloroplasts, mitochondria and peroxisomes and key photosynthetic enzymes into two distinct intracellular cytoplasmic compartments within individual chlorenchyma cells (Voznesenskaya et al. 2001, 2002, 2005; Akhani et al. 2005; Chuong et al. 2006).

Since C₄ plants have higher water and nitrogen efficiency and increased yield compared to C₃ plants, it has been proposed that transforming C₃ crop plants with C₄ genes could increase their yield and carbon fixation efficiency (Hibberd and Covshoff 2010). The discovery of these single-cell C₄ photosynthetic systems has altered our understanding of the traditional C₄ dogma and offered new perspectives for introducing C₄ traits in C₃ crops without the development of Kranz anatomy. Such modifications to C₃ crop plants could revolutionize agriculture through increased yield. Although genetic transformation is a tool that can be applied to achieve this biotechnological effort, the recovery or production of transgenic plants is often limited by lack of rapid and efficient regeneration protocols. To the best of our knowledge, a genetic

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transformation protocol is lacking for *B. sinuspersici* thus far. Currently, only transient transformation protocols using polyethylene glycol (PEG)-mediated transformation of protoplasts and biolistic bombardment of leaves have been successfully used to introduce genes of interest and examine their expression in cells of *B. sinuspersici* (Chuong et al. 2006; Lung et al. 2011; Lung and Chuong 2012). Therefore, establishment of callus cultures that facilitate the regeneration of plants is essential for developing a stable genetic transformation protocol for *B. sinuspersici*. Furthermore, establishment of an efficient transformation system is important in dissecting the regulatory mechanisms underlying the development of single-cell C_4 photosynthesis and to provide novel solutions for enhancing carbon assimilation in C_3 crops.

In vitro regeneration has long been employed commercially for the propagation of plant material for agricultural, horticultural, pharmaceutical, or research purposes and for generating disease-free stock (Thorpe 2007). There are several paths for plant regeneration including vegetative propagation via cuttings and organogenesis directly from plant explants or indirectly from callus tissue. Dedifferentiation of plant material into callus tissue generally depends on the ratio of cytokinin to auxin in the plant medium (Murashige and Skoog 1962). Organogenesis may be induced indirectly from stem or leaf explants through the production of callus tissue by using a cytokinin, such as 6-benzyladenine (BA), thidiazuron (TDZ), or kinetin, to differentiate callus cells into new shoots (Thorpe 2007). Indirect organogenesis provides a means of dedifferentiating plant tissue into callus allowing for transformation and selection protocols, and regenerating whole plants in vitro from transformed callus (Robinson and Firoozabady 1993). Currently, there are only three reports documenting the in vitro propagation of *B. sinuspersici* using callus (Rosnow et al. 2011), bud explants (Northmore et al. 2012) and cuttings (Northmore et al. 2015). However, the in vitro regeneration success reported by Rosnow et al. (2011) only occurred in cultures grown under elevated CO_2 and saline conditions.

In this study, the effect of TDZ on shoot organogenesis of stem-derived callus of *B. sinuspersici* was examined. The ultimate goal of this study is to use the regeneration protocol in genetic transformation of *Bienertia*. Furthermore, the unique single-cell C_4 anatomical and biochemical features such as the intracellular compartmentalization of organelles and presence of key C_4 enzymes in the leaves of regenerated plants were examined by histological and immunoblot analyses.

Materials and methods

Plant material

Seeds of *B. sinuspersici* (courtesy of Mr. Abdulrahman Alsirhan, Kuwait) were germinated on moist filter paper in Petri dishes at room temperature. Seedlings were transplanted to 11 cm pots containing Sunshine Mix #4 potting soil (Jack Van Klavereen, Ontario, Canada) and grown for 3 weeks at room temperature under $60 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were then maintained in a growth chamber (model GCW-15H; Environmental Growth Chambers, Ohio, USA) under $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 14 h/10 h light/dark photoperiod and a 25/18 °C day/night temperature regime. Plants were regularly watered and fertilized with Miracle-Gro fertilizer (24-8-16; Scott's Miracle-Gro Company, Ontario, Canada) once a week. Stems from 6-month-old plants were used as explants. Explants were surface sterilized for 10 min in 1.0 % (v/v) sodium hypochlorite solution followed by three 5 min rinses in sterile distilled water prior to inoculation on the culture medium (Northmore et al. 2012).

Induction of callus on explants

Primary callus induction was initiated by culturing sterilized stem explants (~5 mm) on callus inducing medium (CIM) consisting of Murashige and Skoog (MS) basal medium (Sigma-Aldrich, Ontario, Canada; Murashige and Skoog 1962; Gamborg et al. 1968) that contained various combinations of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthalene acetic acid (NAA); Sigma-Aldrich, Ontario, Canada] and cytokinins (BA, kinetin; Sigma-Aldrich, Ontario, Canada). The following concentrations of plant growth regulators (PGRs) used were: 2.25, 4.5 and 15.0 μM 2,4-D; 2.7 and 5.4 μM NAA; 2.5 and 5.0 μM kinetin; 2.2 and 4.4 μM BA. For the above experiments, 20 explants were cultured in each petri dish (100 × 15 mm; Sarstedt Inc., Quebec, Canada) containing 20 ml medium. Petri dishes were sealed with Parafilm, wrapped in aluminum foil and maintained in a growth chamber at 22 °C. Each treatment consisted of three replicate plates and the experiments were repeated at least three times. Explants were examined at 2, 4, and 6 weeks intervals. The percentage of explants producing callus were recorded and imaged using a Zeiss stereomicroscope (Stemi SV11; Carl Zeiss Canada Ltd., Ontario, Canada). Images were captured using a cooled CCD camera (Retiga 1350 Exi Fast, Qimaging, British Columbia, Canada) and the OpenLab

(OpenLab, Ontario, Canada) imaging software. Healthy callus were continuously subcultured on CIM every 6 weeks and maintained in the dark.

Indirect organogenesis

Three cytokinins were used for shoot induction experiments: BA and kinetin were applied at 2.2, 4.4, 8.8, 22.0, and 44 μM whereas TDZ was used at 0.0045, 0.0225, 0.045, 0.45, 1.125, 2.25, 3.375, 4.5, and 9.0 μM . Callus were cultured on shoot induction medium (SIM) and maintained in a growth chamber with a 14 h/10 h light/dark photoperiod at 22 °C under 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 6 weeks, callus exhibiting the formation of pre-shoots (green, smooth clusters of cells) were observed and recorded. Callus with pre-shoots were subcultured every 3 weeks onto fresh SIM containing 0.45 μM TDZ until true shoots completed their development. Shoots were then isolated and multiplied by culturing on medium containing 8.8 μM BA (Northmore et al. 2012). Elongation of shoots was obtained on medium containing 0.88 μM BA or 1.45–14.5 μM gibberellic acid (GA_3 ; Sigma-Aldrich, Ontario, Canada; Northmore et al. 2012). Elongated shoots were cultured on medium containing 4.9 μM indole-3-butyric acid (IBA; Sigma-Aldrich, Ontario, Canada) for root induction (Northmore et al. 2015). For the above experiments, ten explants were cultured in each Magenta GA7 culturing vessel (77 × 77 × 97 mm; Phytotechnology Laboratories, Kansas, USA) containing 30 ml medium. Rooted plantlets were transplanted to Sunshine Mix #4 potting soil (Jack Van Klavereen, Ontario, Canada) and kept under a humidifying dome to maintain humidity and slowly acclimatized to greenhouse conditions.

Light microscopy

Ten leaf samples from five individual TDZ-induced shoots cultured on BA-containing medium, 4-month old regenerated plants or seed-derived plants in a growth chamber were randomly harvested and fixed in 2 % (v/v) paraformaldehyde and 2 % (v/v) glutaraldehyde [Electron Microscopy Sciences (EMS), Pennsylvania, USA] in 50 mM piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES) (Sigma-Aldrich, Ontario, Canada) buffer, pH 7.2 overnight at 4 °C. The samples were dehydrated with a graded ethanol series for 30 min each at room temperature, gradually infiltrated with increasing concentrations of London Resin White (LR white; EMS, Pennsylvania, USA) acrylic resin, and embedded in pure LR white overnight at 60 °C. Sections (1 μm) were prepared on a Reichert Ultracut E ultramicrotome (Reichert-Jung, Heidelberg, Germany), dried onto glass slides, and stained with 0.1 % (w/v)

Toluidine blue (TBO) (Sigma-Aldrich, Ontario, Canada). For freehand sections, ten fresh leaves were obtained from TDZ-induced shoots in vitro or from 4-month old regenerated plants in a growth chamber. Sections (~200 μm) were made using a double edge stainless steel PERSONNA razor blade (EMS, Pennsylvania, USA) and immediately fixed in 2 % (v/v) paraformaldehyde and 2 % (v/v) glutaraldehyde (EMS, Pennsylvania, USA) in 50 mM PIPES buffer, pH 7.2. Plastic or freehand sections were observed using a Zeiss Axiophot (Carl Zeiss Canada Ltd., Ontario, Canada) light microscope. Images were captured using a cooled CCD camera (Retiga 1350 Exi Fast, Qimaging, British Columbia, Canada) and OpenLab (OpenLab, Ontario, Canada) imaging software. Image processing was performed using Adobe Photoshop CS (Adobe, California, USA).

Western blot analysis

Leaf samples were collected, frozen in liquid nitrogen, and stored at –80 °C until use. Young (~5 mm) and mature (10 mm) leaves of TDZ-induced shoots maintained under in vitro conditions were sampled. Mature leaves from seed-derived *B. sinuspersici* plants grown under greenhouse conditions were used as positive controls. *Suaeda linifolia*, a C_3 species of the Chenopodiaceae family, was also used as a control to demonstrate the absence of key C_4 enzymes. Briefly, total proteins were extracted by homogenizing leaf tissues in liquid nitrogen to a fine powder with a mortar and pestle. The powdered samples were further ground in extraction buffer [100 mM Tris-HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 % (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 10 $\mu\text{l ml}^{-1}$ protease inhibitor cocktail (Sigma-Aldrich, Ontario, Canada), 1 mM phenylmethylsulfonyl fluoride (PMSF)] with a pinch of sea-washed sand (Fisher Scientific, Ontario, Canada). The homogenates were centrifuged at 14,000 rpm for 5 min at 4 °C and the supernatants were carefully transferred to fresh tubes on ice and used for quantification of protein concentrations according to the procedure described by Bradford (1976) using bovine serum albumin as a standard.

Discontinuous gels [for the stacking gel: 4 % (w/v) acrylamide/bisacrylamide 37.5:1, 125 mM Tris-HCl, pH 6.8; for the separating gel: 10 % (w/v) acrylamide/bisacrylamide 37.5:1, 375 mM Tris-HCl pH 8.8, 0.1 % (w/v) sodium dodecyl sulfate (SDS), 0.05 % (w/v) ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine (TEMED)] were used to separate proteins according to Laemmli (1970). Protein samples (10 μg) were mixed with 5 × sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [62.5 mM Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.05 %

(w/v) bromophenol blue, 5 % (v/v) β -mercaptoethanol] and heated at 95 °C for 5 min. The boiled samples were loaded into submerged lanes containing 1 \times SDS-PAGE electrophoresis buffer [25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3] and electrophoresed at a constant voltage of 120 V for 45 min or until the bromophenol blue dye front reached the bottom of the gels. The separated proteins were transferred to nitrocellulose (Bio-Rad, Ontario, Canada) membrane using the Bio-Rad semi-dry transblotter (Bio-Rad, Ontario, Canada). The membrane was briefly stained with Ponceau S stain [0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid] to determine whether proteins had been effectively transferred, rinsed with distilled water, and then incubated in blocking buffer [5 % (w/v) skim milk in TBS-T buffer: 25 mM Tris-HCl pH 7.4, 137 mM NaCl, 27 mM KCl, 0.1 % (v/v) Tween 20] for 1 h at room temperature. The blocked membrane was incubated with the appropriate primary antibody in blocking buffer overnight at 4 °C with gentle shaking. The primary antibodies used were: rabbit antiribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (1:10,000; Agrisera, Vannas, Sweden), rabbit antiphosphoenolpyruvate carboxylase (PEPC) (1:20,000; Chemicon, USA), rabbit antinicotinamide adenine dinucleotide malic enzyme (NAD-ME) α -subunit (1:4000; courtesy of James Berry, SUNY, New York, USA), and rabbit antipyruvate orthophosphate dikinase (PPDK) (1:10,000; courtesy of Chris Chastain, MNSU, Minnesota, USA). The membrane was then rinsed three times in TBS-T buffer for 15 min each and incubated with the secondary antibody goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated (1:10,000; Sigma-Aldrich, Ontario, Canada) in blocking buffer for 2 h at room temperature with gentle shaking, and then washed again three times with TBS-T for 15 min each. The membrane was incubated in ECL solution (1.25 mM Luminol, 2 mM *p*-coumaric acid, 5.3 mM H₂O₂ in 100 mM Tris-HCl, pH 8.8) in the dark for 5 min. Luminescence was detected by exposing the membrane to Amersham hypersensitive film (GE Health Care, Ontario, Canada) and developed using a CP1000 Agfa photodeveloper (AGFA, Ontario, Canada). The film was scanned and processed using Adobe Photoshop CS (Adobe, California, USA). Representative blots were presented after similar results were obtained from at least three independent experiments.

Statistical analysis

The experiments were performed in a complete randomized design. The experimental data were subjected to statistical analyses by one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences

(SPSS) version 18 (SPSS, IL, USA). Mean values of treatments were compared by the Tukey test with the level of significance set at 5 %.

Results

Callus induction

Basal MS medium containing various combinations and concentrations of auxins (2,4-D and NAA) and cytokinins (BA and kinetin) were used for the induction of primary callus from *B. sinuspersici* stem explants. Callus tissue was observed on stem explants after 2 weeks of culture in all treatments (Fig. 1a). The frequency of callus formation varied considerably with PGR combinations, but in majority of the experiments, 60–100 % of explants produced callus within 6 weeks of culture (Table 1; Fig. 1b). Callus were also observed qualitatively to determine which combination of PGRs produced the most prolific and healthiest callus. Generally, healthy callus should appear light yellow in colour and smooth in texture, and proliferate rapidly (Fig. 1c). Callus formed on explants cultured on medium containing NAA were less abundant and appeared less healthy than those from explants cultured on medium containing 2,4-D. In the present study, it was observed that the medium containing 15.0 μ M 2,4-D, 2.5 μ M kinetin consistently produced the highest quality callus (Fig. 1c).

Shoot induction from stem-derived callus

Healthy callus were cultured onto solid medium containing various cytokinins (kinetin, BA, or TDZ) for shoot induction. Callus that were cultured on medium containing kinetin turned grayish-brown, stopped proliferating, and did not produce any shoots (data not shown). Callus cultured on medium containing BA proliferated large quantities of healthy, green callus, but failed to differentiate shoots (data not shown). However, the addition of TDZ to the medium improved the shoot induction potential of the callus as indicated by the formation of many greenish meristemoids after 6 weeks of culture (Table 2; Fig. 1d). A low concentration of TDZ (0.0045 μ M) did not substantially increase the percentage of callus that developed shoot meristemoids, whereas concentrations of 0.0225 μ M or higher were more effective in promoting meristemoid formation (Table 2). The number of callus that differentiated shoots was significantly higher (80 %) with the 0.45 μ M TDZ treatment (Table 2). Furthermore, this treatment also induced many healthy meristemoids per callus, making it highly effective for shoot regeneration. Callus with morphogenic meristemoids subcultured on

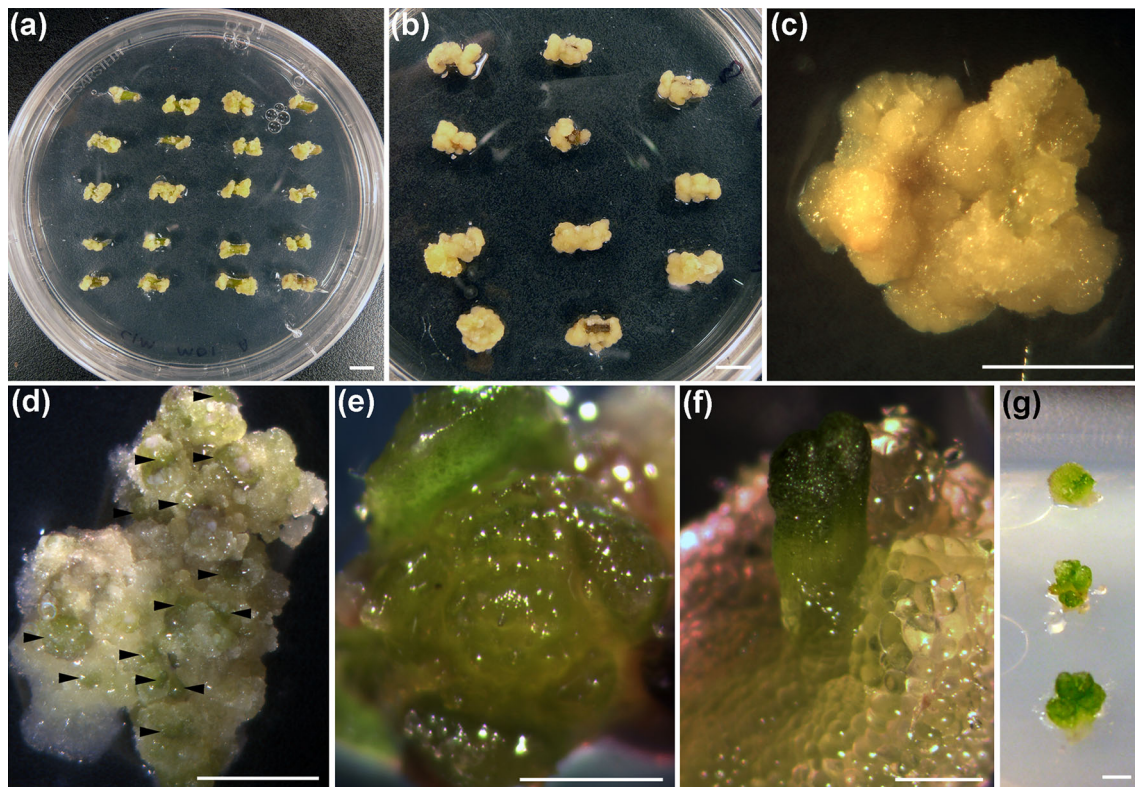


Fig. 1 Induction of callus tissue from *Bienertia sinuspersici* stem explants on medium containing 2,4-D and kinetin and effect of TDZ on shoot organogenesis from callus tissue. **a** Stem explants on callus induction medium (CIM) containing 15.0 μM 2,4-D, 2.5 μM kinetin after 2 weeks. **b** Stem explants with prolific callus after 6 weeks on CIM. **c** Healthy callus maintained on CIM. **d** Callus cultured on shoot

induction medium containing 0.45 μM TDZ showing numerous greenish meristemoids (*arrowheads*). **e** Top view of an organized shoot primordium. **f** Shoot primordium emerging from callus tissue. **g** Well-developed shoots on multiplication medium containing 8.8 μM BA. Scale bars 5 mm

fresh TDZ-containing medium subsequently developed into shoot primordia (Fig. 1e, f). Individual shoot primordia were isolated and cultured on TDZ-containing medium to further the shoot development process (Fig. 1g).

Multiplication, elongation and rooting of TDZ-induced shoots

Well-developed shoots were excised and transferred to medium containing 8.8 μM BA, which had previously been optimized for shoot multiplication in direct organogenesis experiments (Fig. 2a; Northmore et al. 2012). Repeated subcultures on BA-containing medium produced large quantities of TDZ-induced shoots (Fig. 2b). Occasionally during the shoot multiplication process, a few in vitro shoots displayed hyperhydricity rendering them incapable of regeneration (Fig. 2b inset). Healthy shoots were cultured on medium containing 0.88 μM BA or different GA₃ levels, previously determined to be effective in promoting shoot elongation in experiments involving direct organogenesis (Table 3; Fig. 2c; Northmore et al. 2012).

However, the rate of shoot elongation was not significantly affected by the concentrations of GA₃ tested (Table 3). To evaluate the root induction capacity, elongated TDZ-induced shoots were cultured on medium containing 4.9 μM IBA based a recent report which showed that IBA treatment was ideal for adventitious root induction (Northmore et al. 2015). Prolonged incubation of shoots up to 8 weeks resulted in callusing, necrosis of the cut ends and hyperhydricity (Fig. 2d, e). However, close inspection of the IBA-treated in vitro shoots after 2 weeks revealed root primordia formation (Fig. 2f, g). Consistently, it was observed that approximately 20–30 % of the TDZ-induced shoots spontaneously developed adventitious roots during subculture on elongation medium containing low levels of BA or GA₃ (Fig. 2h). These rooted shoots were transplanted to soil and successfully acclimated to greenhouse conditions (Fig. 2i–k). Cytological analysis of root tips of the regenerated plantlets derived through indirect organogenesis revealed chromosome number ($2n = 18$) (Fig. 2j inset) identical to that reported by Akhiani et al. (2005) from seed-derived plants.

Table 1 Effect of plant growth regulators on callus formation of stem explants of *Bienertia sinuspersici* after 6 weeks of culture

Growth regulator (μM)	% Explants showing callus
0	0 ^d
2.25 2,4-D + 2.5 kinetin	89.7 \pm 4.4 ^{ab}
2.25 2,4-D + 5.0 kinetin	100.0 \pm 0.0 ^a
4.5 2,4-D + 2.5 kinetin	94.5 \pm 3.9 ^{ab}
4.5 2,4-D + 5.0 kinetin	90.0 \pm 6.3 ^{ab}
15.0 2,4-D + 2.5 kinetin	94.8 \pm 3.0 ^{ab}
15.0 2,4-D + 5.0 kinetin	75.6 \pm 9.6 ^b
2.25 2,4-D + 2.2 BA	87.0 \pm 10.0 ^{ab}
2.25 2,4-D + 4.4 BA	80.0 \pm 10.7 ^{ab}
4.5 2,4-D + 2.2 BA	93.3 \pm 4.2 ^{ab}
4.5 2,4-D + 4.4 BA	91.0 \pm 3.2 ^{ab}
15.0 2,4-D + 2.2 BA	91.1 \pm 6.0 ^{ab}
15.0 2,4-D + 4.4 BA	96.1 \pm 1.6 ^{ab}
2.7 NAA + 2.5 kinetin	57.7 \pm 22.6 ^{bc}
2.7 NAA + 5.0 kinetin	78.7 \pm 19.7 ^{ab}
5.4 NAA + 2.5 kinetin	91.5 \pm 4.7 ^{ab}
5.4 NAA + 5.0 kinetin	93.3 \pm 2.8 ^{ab}
2.7 NAA + 2.2 BA	57.8 \pm 22.6 ^{bc}
2.7 NAA + 4.4 BA	10.0 \pm 6.3 ^c
5.4 NAA + 2.2 BA	92.3 \pm 4.9 ^{ab}
5.4 NAA + 4.4 BA	100.0 \pm 0.0 ^a

Values represent the mean \pm SE of three replicates, each with 20 explants. Means followed by same letters do not differ statistically at $P = 0.05$ according to the Tukey test

Table 2 Effect of thidiazuron (TDZ) on shoot organogenesis of stem-derived callus of *Bienertia sinuspersici* after 6 weeks of culture

TDZ (μM)	% Callus showing shoot primordia
0	0 ^d
0.0045	18.3 \pm 4.9 ^c
0.0225	57.5 \pm 19.8 ^{ab}
0.045	54.0 \pm 8.1 ^{ab}
0.45	80.0 \pm 10.9 ^a
1.125	48.0 \pm 16.5 ^{bc}
2.25	55.0 \pm 13.9 ^{ab}
3.375	66.0 \pm 11.7 ^{ab}
4.5	45.0 \pm 19.4 ^{bc}
9.0	50.0 \pm 25.8 ^{bc}

Values represent the mean \pm SE of three replicates, each with ten explants. Means followed by same letters do not differ statistically at $P = 0.05$ according to the Tukey test

Histological analysis of leaf anatomy of TDZ-induced shoots

Histological analysis of leaves of regenerated plants was performed to determine whether the *Bienertia's*

characteristic single-cell C_4 anatomy is maintained in the regenerated plants. Leaves of *Bienertia* species generally have chlorenchyma cells that display two distinct cytoplasmic domains: a thin peripheral cytoplasm (pc) and a ball-like central cytoplasm (ccc) suspended in the centre of the cell (Fig. 3a, b). Leaf cross sections prepared from regenerated plants via indirect organogenesis revealed the presence of a central cytoplasmic compartment, as indicated by a cluster of chloroplasts in most chlorenchyma cells (Fig. 3d–f). The development of this unique cellular organization was also observed in leaves of TDZ-induced shoots maintained under in vitro conditions. In the leaves of in vitro shoots, the central cytoplasmic compartment was not in the center in chlorenchyma cells, but appeared to be shifted to one side of the cell (Fig. 3c). However, leaf cross sections of a 4-month old regenerated plant that had been acclimatized and maintained under greenhouse conditions showed chlorenchyma cells with well-developed central cytoplasmic compartments similar to those found in the seed-derived plants (Fig. 4c, d).

Immunoblot analysis of TDZ-induced in vitro shoots and regenerated plants

Western blot analysis was performed to further determine whether key C_4 enzymes were present in leaves of regenerated plants. Figure 4 shows the results of the Western blot analysis of total proteins isolated from leaves of TDZ-induced shoots maintained under in vitro conditions, and leaves of 2-week and 4-month old regenerated plants transplanted to soil and maintained in a growth chamber under controlled conditions. Although the amounts varied somewhat between the different tissues and species, Rubisco large subunit polypeptides were detected in all extracts. As expected, Rubisco was present in higher quantities in *S. linifolia*, a C_3 plant of the Chenopodiaceae family that served as a control, than in all *B. sinuspersici* leaf tissues (Fig. 4, lane 1, lower panel). Key C_4 photosynthetic enzymes (PEPC, PPK, and NAD-ME) were present in all *B. sinuspersici* leaf extracts, with similar amounts accumulating in mature leaves of the regenerated plants, and those of the greenhouse-grown plants (Fig. 4 lanes 2–8). These C_4 enzymes were not detected in the C_3 plant, *S. linifolia* (Fig. 4, lane 1, top three panels).

Discussion

The establishment of regenerate plants via indirect organogenesis is an essential step in developing transgenic plants for *B. sinuspersici*. This study describes an optimized protocol for indirect organogenesis of *B. sinuspersici*, and will provide researchers with a foundation through

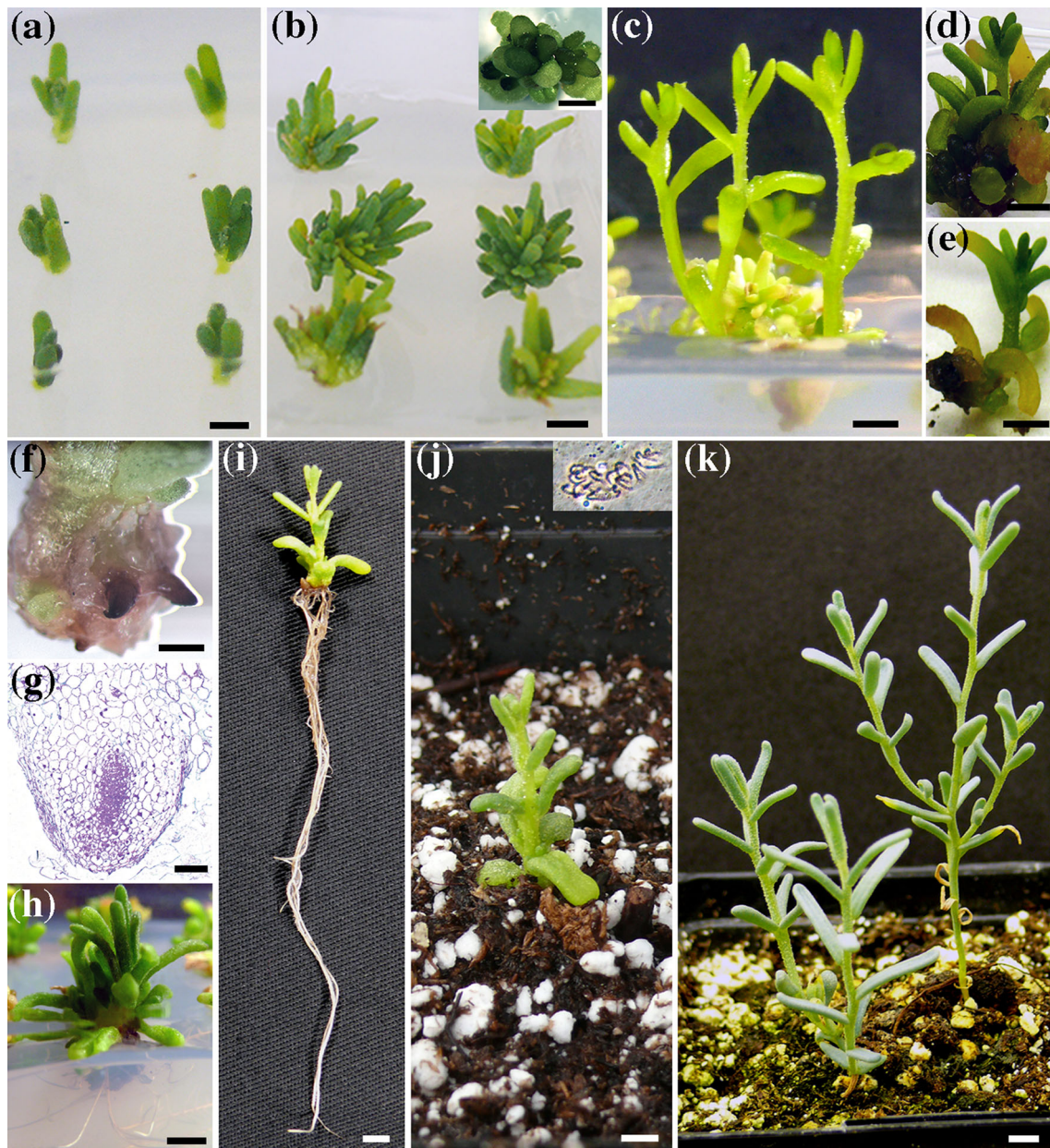


Fig. 2 Whole plant regeneration from TDZ-induced shoots in *Bienertia sinuspersici*. **a** In vitro TDZ-induced shoots cultured on shoot multiplication medium containing $8.8 \mu\text{M}$ BA. **b** Replicated shoots after 4 weeks on shoot multiplication medium. *Inset* to **(b)** in vitro shoots displaying hyperhydricity on multiplication medium. **c** In vitro shoots after 4 weeks on elongation medium containing $2.9 \mu\text{M}$ GA_3 . **d** and **e** In vitro shoots with prolific callus and necrosis on medium containing $4.9 \mu\text{M}$ IBA. **f** In vitro shoot

displaying root primordia after 2 weeks on IBA-containing medium. **g** Longitudinal section of a root primordium. **h** In vitro shoots spontaneously rooted on multiplication medium. **i** Elongated in vitro plantlet with long, healthy roots. **j** In vitro-derived plantlet transplanted to soil. *Inset* to **(j)** late mitotic prophase chromosomes from regenerated plants. **k** Regenerated plant after 4 months in greenhouse conditions. *Scale bars* 5 mm in **(a–e)** and **(h–k)**, 1 mm in **(f)** and 100 μm in **(g)**

which to transform and regenerate plant material. The optimum ratio of auxin:cytokinin varies between species based on their individual growth requirements. In *B. sinuspersici*, the best quality callus was obtained on medium containing $15.0 \mu\text{M}$ 2,4-D and $2.5 \mu\text{M}$ kinetin.

A study reported the regeneration of *B. sinuspersici* from callus on MS medium containing BA; however, the

callus were subjected to special treatment under CO_2 enrichment and high NaCl conditions (Rosnow et al. 2011). In our study, successful shoot induction in *B. sinuspersici* was only achieved from callus on medium containing different levels of TDZ, whereas other tested cytokinins including BA and kinetin were not effective under ambient CO_2 level. The highest rate of shoot induction (80 %) was

Table 3 Effect of GA₃ on the elongation of TDZ-derived shoots of *Bienertia sinuspersici* after 5 weeks of culture

GA ₃ (μM)	Average shoot elongation (mm)
0	1.2 ± 0.9 ^b
1.45	4.5 ± 1.5 ^a
2.9	4.8 ± 1.3 ^a
8.7	3.6 ± 1.3 ^a
14.5	4.2 ± 1.3 ^a

Values represent the mean ± SE of three replicates, each with ten explants. Means followed by same letters do not differ statistically at $P = 0.05$ according to the Tukey test

observed in callus cultured on medium containing 0.45 μM TDZ. The effect of TDZ on the induction of shoots has been observed in many species that are considered to be recalcitrant to shoot organogenesis, including bean *Phaseolus vulgaris* L. (Malik and Saxena 1992), the medicinal “Cancer bush” *Sutherlandia frutescens* L. (Dewir et al. 2010), and potatoes, *Solanum tuberosum* L. (Sajid and Aftab 2009). Although the exact mode of TDZ action has not been determined, evidence suggests that it regulates endogenous cytokinin biosynthesis and/or metabolism (Mok et al. 2000). Several studies have also attributed the effectiveness of TDZ in inducing shoot organogenesis to its ability to enhance cytokinin accumulation or enhance the accumulation and translocation of auxin within the tissue (Murthy et al. 1998; Victor et al. 1999).

Although shoot elongation can be achieved in medium with low levels BA, the in vitro shoots elongated optimally at a concentration of 2.9 μM GA₃. GA₃ induces the transcription of genes that are involved in loosening the polymers within cell walls, resulting in cell elongation (Sun 2010). It is recommended that shoot elongation be initiated as early as possible during plant regeneration, as repeated subcultures lead to hyperhydricity and stress that inhibits elongation and rooting. Hyperhydricity in shoot cultures of *B. sinuspersici* was reported in our previous study (Northmore et al. 2012). The lower rate of elongation may be caused by stress induced from the extended in vitro culturing conditions of the shoots. In addition, the in vitro environment may be very stressful to cultured explants due to high humidity, the presence of accumulated gases, unusual nutrient and hormone, and the lack codependent microorganisms (Kevers et al. 2004). During the elongation process, formation of adventitious roots of in vitro-derived shoots spontaneously occurred on medium containing low concentrations of BA or GA₃ (Fig. 2h, i). Spontaneous adventitious root development has been documented in species such as eucalyptus (Dibax et al. 2010), and bamboo (Jiménez et al. 2006). The ability of IBA as an effective growth regulator of root induction has been previously

demonstrated in *B. sinuspersici* cuttings (Northmore et al. 2015). Other studies also showed successful in vitro root induction using IBA and activated charcoal have been reported in many species including *Cannabis sativa* (Lata et al. 2009), wood-apple (Vyas et al. 2004) and an important Indian medicinal creeper plant (Manjula et al. 1997). However, in the present study in vitro rooting of elongated TDZ-induced shoots on medium containing IBA was not effective. This correlates with our previous observations which reported that IBA treatments of in vitro shoots of *B. sinuspersici* from direct organogenesis resulted in no root formation (Northmore et al. 2012). Similarly, auxins did not induce adventitious root formation in *Elegia capensis* (Verstraeten and Geelen 2015). Inhibition of adventitious root formation may be related to the impact of extended auxin exposure causing oxidative stress as indicated by the changes in explant stem morphology. The discoloration and necrosis of cultured explants caused by oxidation of phenolic compounds is a major concern in tissue culture and is considered as one of the main causes for rooting-recalcitrance (Ozyigit et al. 2007; Ozyigit 2008). The inhibitory effect of phenolic compounds on in vitro regeneration was also reported in difficult-to-propagate *Protea cynaroides* (Wu and Lin 2012). Alternatively, the rooting-recalcitrance in *B. sinuspersici* may be associated with high accumulation of BA conjugates from shoot multiplication in the base of in vitro-derived shoots. These BA metabolites have been proposed to inhibit root formation of in vitro shoots in many plant species (Werbrouck et al. 1995, 1996). It is also possible that the stress of hyperhydricity may inhibit root formation, but this could be corrected by improving ventilation of culture dishes (Lai et al. 2005).

Genetic instability is often a concern associated with materials derived from in vitro studies, particularly with plants indirectly derived from callus tissue. Therefore, cytological analysis was performed to determine the chromosome number in the regenerated plants. The diploid chromosome number of $2n = 18$ observed in root samples of the regenerated plants suggested cytological stability. This finding is in agreement with cytological analysis of vegetatively propagated plants (data not shown) as well as with previous analysis of seed-derived *B. sinuspersici* plants (Akhani et al. 2005). However, further biochemical studies are required to confirm clonal homogeneity.

Histological analysis of in vitro-derived shoots suggests that *B. sinuspersici*'s characteristic single-cell C₄ anatomy is present in the regenerated plants. During shoot induction and replication, both young and mature leaves display evidence of a central cytoplasmic compartment. Chlorenchyma cells of leaves from greenhouse-grown plants have the central cytoplasmic compartment localized in the center of the cell, whereas those of leaves maintained under

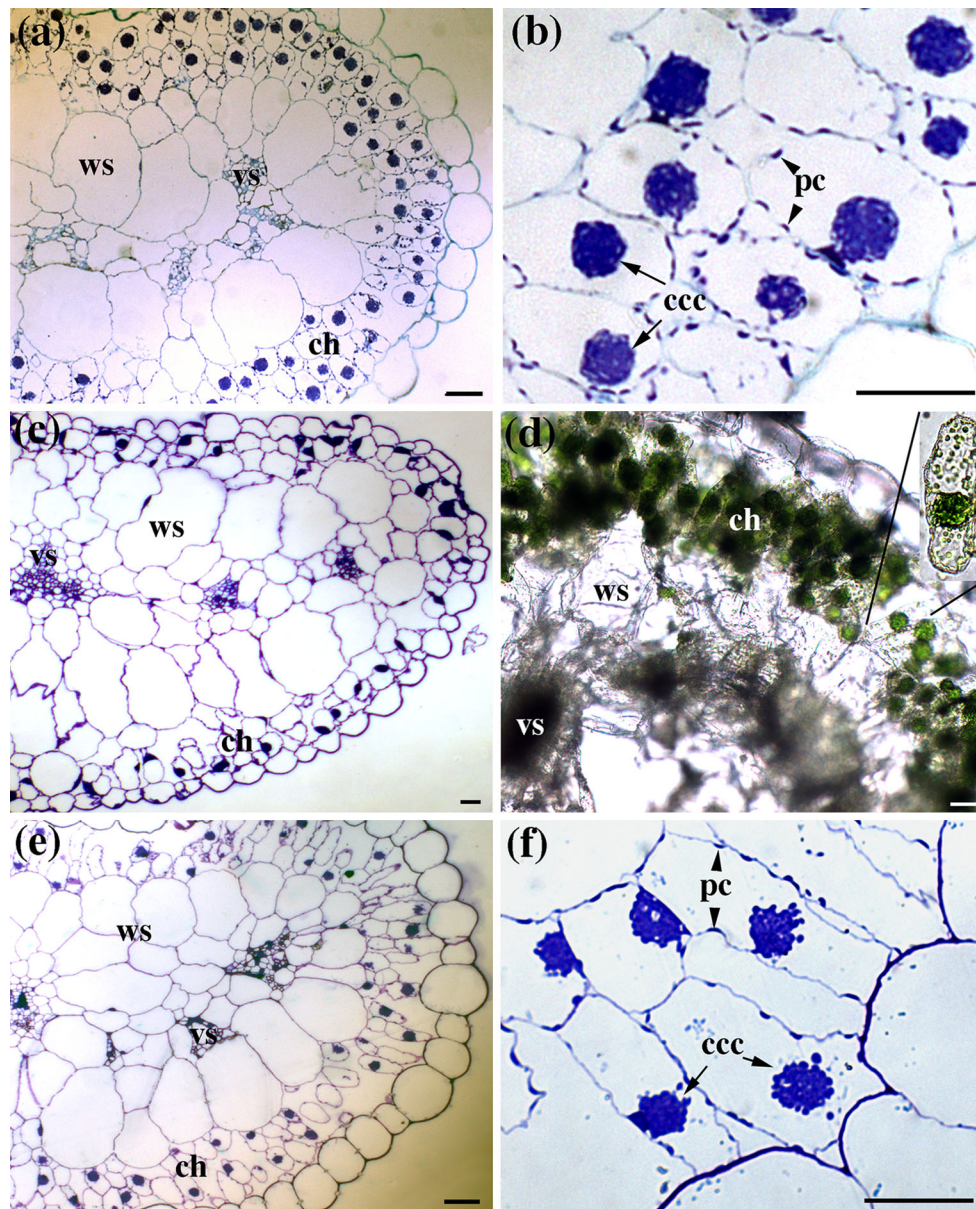


Fig. 3 Light micrographs of leaf cross sections comparing the anatomy of a regenerated *Bienertia sinuspersici* plant to that of a seed-derived plant. **a** and **b** Cross sections of leaves showing the general anatomy of seed-derived *B. sinuspersici* plants grown in the growth chamber or greenhouse. **c** Cross sections of leaves from regenerated shoots maintained under in vitro conditions. **d** Fresh free-

hand cross sections of leaves from 4-month old regenerated plant growing in soil. The *inset* shows an isolated chlorenchyma cell. **e** and **f** Cross sections of leaves from 4-month old regenerated plant (*ws* water storage cells, *vs* vascular tissues, *ch* chlorenchyma cells, *pc* peripheral cytoplasm, *ccc* central cytoplasm). Scale bars 50 μ m

in vitro conditions displayed less developed central cytoplasmic compartments that are shifted to one side of the cell (Fig. 3c). In a previous study, similar altered anatomy was observed in chlorenchyma cells of mature leaves from in vitro shoots that were obtained via direct organogenesis (Northmore et al. 2012). This altered anatomical development may be associated with in vitro confined conditions causing the plant to be less dependent on photosynthesis due to the exogenous supply of carbohydrates.

Abnormalities in the anatomy are generally found in in vitro-derived leaves may also be associated with environmental differences between tissue culture and greenhouse environments such as temperature, humidity, and light conditions. It has been shown that in vitro-derived plants often have altered anatomies such as large intercellular spaces, hypertrophy, less lignified vascular tissue, more spongy mesophyll cells, and reduced palisade layer (Kevers et al. 2004). Previous studies documenting altered

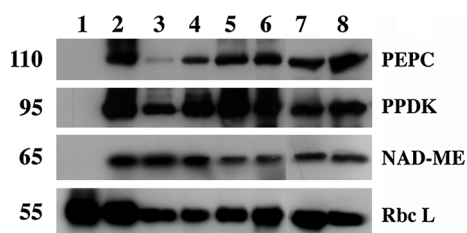


Fig. 4 Western blot analysis of Rubisco and key C_4 enzymes in leaves of in vitro TDZ-induced shoots and regenerated *Bienertia sinuspersici* plants. Ten μ g of total proteins from a C_3 plant, *Suaeda linifolia* (1), mature leaves of greenhouse-grown *B. sinuspersici* (2), young leaves of TDZ-induced in vitro shoots (3), mature leaves of TDZ-induced in vitro shoots (4), young leaves of 2-week old regenerated plant (5), mature leaves of 2-week old regenerated plant (6), young leaves of 4-month old regenerated plant (7), mature leaves of 4-month old regenerated *B. sinuspersici* plant (8). Numbers to the left indicate the molecular weight of marker proteins in kDa

leaf anatomy and physiology in vitro have been reported (Grout and Aston 1978; Wetzstein and Sommer 1982; Smith et al. 1986). However, leaf sections of regenerated plants grown in greenhouse conditions for 4 months clearly showed the development of a peripheral compartment and a central cytoplasmic compartment that is localized in the centre of most chlorenchyma cells, suggesting the unique single-cell C_4 photosynthetic anatomy can be re-established under proper growth conditions (Fig. 3d–f). Moreover, immunoblot analysis of key photosynthetic proteins in regenerated plants demonstrated that key C_4 enzymes (PEPC, PPKK, and NAD-ME) are present in high levels similar to those observed in parent stock plants maintained in the greenhouse (Fig. 4). These results provide further evidence that in addition to the inherited single-cell C_4 anatomy, the in vitro regenerated plants possess the biochemistry required for the C_4 pathway.

In conclusion, a reproducible protocol for whole plant regeneration from stem-derived callus in *B. sinuspersici* has been developed, allowing for the rapid propagation of this interesting single-cell C_4 plant. The in vitro technique will also be helpful for conservation of this genus and establishment of genetic transformation system aimed at understanding the mechanisms underlying the development of the single-cell C_4 pathway.

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References

Akhani H, Barroca J, Koteeva N, Voznesenskaya E, Franceschi V, Edwards G, Ghaffari SM, Ziegler H (2005) *Bienertia*

sinuspersici (Chenopodiaceae): a new species from Southwest Asia and discovery of a third terrestrial C_4 plant without Kranz anatomy. *Syst Bot* 30:290–301

Akhani H, Chatrevoor T, Dehghani M, Khoshravesh R, Mahdavi P, Matinzadeh Z (2012) A new species of *Bienertia* (Chenopodiaceae) from Iranian salt deserts: a third species of the genus and discovery of a fourth terrestrial C_4 plant without Kranz anatomy. *Plant Biosyst*. doi:10.1080/11263504.2012.662921

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 72:248–254

Chuong SDX, Franceschi VR, Edwards GE (2006) The cytoskeleton maintains organelle partitioning required for single-cell C_4 photosynthesis in Chenopodiaceae species. *Plant Cell* 18:2207–2223

Dewir YH, Singh N, Shaik S, Nicholas A (2010) Indirect regeneration of the Cancer bush (*Sutherlandia frutescens* L.) and detection of L-canavanine in in vitro plantlets using NMR. *In Vitro Cell Dev Biol Plant* 46:41–46

Dibax R, Quisen RC, Bona C, Quoirin M (2010) Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* dehn and histological study of organogenesis in vitro. *Braz Arch Biol Technol* 53:311–318

Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. *Annu Rev Plant Biol* 55:173–196

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. *Exp Cell Res* 50:151–158

Grout BWW, Aston MJ (1978) Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. *Ann Bot* 42:993–995

Hatch MD, Slack CR (1970) Photosynthetic CO_2 -fixation pathways. *Annu Rev Plant Physiol* 21:141–162

Hibberd JM, Covshoff S (2010) The regulation of gene expression required for C_4 Photosynthesis. *Annu Rev Plant Biol* 61:16.1–16.27

Jiménez VM, Castillo J, Tavares E, Guevara E, Montiel M (2006) In vitro propagation of the neotropical giant bamboo, *Guadua angustifolia* Kunth, through axillary shoot proliferation. *Plant Cell Tissue Organ Cult* 86:389–395

Kevers C, Franck T, Strasser RJ, Dommes J, Gaspar T (2004) Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. *Plant Cell Tissue Organ Cult* 77:181–191

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685

Lai CC, Lin HM, Nalawade SM, Fang W, Tsay HS (2005) Hyperhydricity in shoot cultures of *Scrophularia yoshimurae* can be effectively reduced by ventilation of culture vessels. *J Plant Physiol* 162:355–361

Lata H, Chandra S, Khan I, ElSohly MA (2009) Thidiazuron-induced high-frequency direct shoot organogenesis of *Cannabis sativa* L. *In Vitro Cell Dev Biol Plant* 45:12–19

Lung SC, Chuong SDX (2012) A transit peptide-like sorting signal at the C terminus directs the *Bienertia sinuspersici* preprotein receptor Toc159 to the chloroplast outer membrane. *Plant Cell* 24:1560–1578

Lung SC, Yanagisawa M, Chuong SDX (2011) Protoplast isolation and transient gene expression in the single-cell C_4 species, *Bienertia sinuspersici*. *Plant Cell Rep* 30:473–484

Malik KA, Saxena PK (1992) Regeneration in *Phaseolus vulgaris* L.: high-frequency induction of shoot formation in intact seedlings by N^6 -benzylaminopurine and thidiazuron. *Planta* 186:384–389

Manjula S, Thomas A, Daniel B, Nair GM (1997) In vitro plant regeneration of *Aristolochia indica* through axillary shoot

- multiplication and organogenesis. *Plant Cell Tissue Organ Cult* 51:145–148
- Mok MC, Martin RC, Mok DWS (2000) Cytokinins: biosynthesis metabolism and perception. *In Vitro Cell Dev Biol Plant* 36:102–107
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15:473–497
- Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent in vitro plant morphogenesis. *In Vitro Cell Dev Biol Plant* 34:267–275
- Northmore JA, Zhou V, Chuong SDX (2012) Multiple shoot induction and plant regeneration of the single-cell C₄ species *Bienertia sinuspersici*. *Plant Cell Tissue Organ Cult* 108:101–109
- Northmore JA, Leung M, Chuong SDX (2015) Effects of media composition and auxins on adventitious rooting of *Bienertia sinuspersici* cuttings. *Adv Biosci Biotechnol* 6:629–636
- Ozyigit II (2008) Phenolic changes during in vitro organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. *Afr J Biotechnol* 7:1145–1150
- Ozyigit II, Kahraman MV, Ercan O (2007) Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). *Afr J Biotechnol* 6:3–8
- Robinson KEP, Firoozabady E (1993) Transformation of floricultural crops. *Sci Hortic* 55:83–99
- Rosnow J, Offermann S, Park J, Okita TW, Tarlyn N, Dhingra A, Edwards GE (2011) In vitro culture and regeneration of *Bienertia sinuspersici* (Chenopodiaceae) under increasing concentrations of sodium chloride and carbon dioxide. *Plant Cell Rep*. doi:10.1007/s00299-011-1067-1
- Sajid ZA, Aftab F (2009) Effect of thidiazuron (TDZ) on in vitro micropropagation of *Solanum tuberosum* L. cvs. Desiree and Cardinal. *Pak J Bot* 41:1811–1815
- Smith MAL, Palta JP, McCown BH (1986) Comparative anatomy and physiology of microcultured, seedlings, and greenhouse-grown Asian white birch. *J Am Soc Hort Sci* 111:437–442
- Sun T (2010) Gibberelin signal transduction in cell elongation and leaf growth. *Plant hormones 3: biosynthesis, signal transduction, action!*. Springer, Netherlands, pp 308–328
- Thorpe TA (2007) History of plant tissue culture. *Mol Biotechnol* 37:169–180
- Verstraeten I, Geelen D (2015) Adventitious rooting and browning are differentially controlled by auxin in rooting-recalcitrant *Elegia capensis* (Burm. f.) Schelpe. *J Plant Growth Regul* 34:475–484
- Victor JMR, Murthy BNS, Murch SJ, Krishnaraj S, Saxena PK (1999) Role of endogenous purine metabolism in thidiazuron induced somatic embryogenesis of peanut (*Arachis hypogea* L.). *Plant Growth Regul* 28:41–47
- Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C₄ plant photosynthesis. *Nature* 414:543–546
- Voznesenskaya EV, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE (2002) Proof of C₄ photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). *Plant J* 31:649–662
- Voznesenskaya EV, Koteyeva NK, Chuong SDX, Akhani H, Edwards GE, Franceschi VR (2005) Differentiation of cellular and biochemical features of the single-cell C₄ syndrome during leaf development in *Bienertia cycloptera* (Chenopodiaceae). *Am J Bot* 92:1784–1795
- Vyas S, Joshi N, Tak K, Purohit SD (2004) In vitro adventitious shoot bud differentiation and plantlet regeneration in *Feronia limonia* L. (Swingle). *In Vitro Cell Dev Biol Plant* 41:296–302
- Werbrouck SPO, van der Jeugt B, Dewitte W, Prinsen E, VanOnckelen HA (1995) The metabolism of benzyladenine in *Spathiphyllum floribundum* Schott 'Petite' in relation to acclimatization problems. *Plant Cell Rep* 14:662–665
- Werbrouck SPO, Strnad M, Van Onckelen HA, Debergh PC (1996) Meta-topolin, an alternative to benzyladenine in tissue culture? *Physiol Plant* 98:291–298
- Wetzstein H, Sommer H (1982) Leaf anatomy of tissue cultured *Liquidambar styraciflua* (Hammamelidaceae) during acclimation. *Am J Bot* 69:1579–1586
- Wu HC, Lin CC (2012) Red light-emitting diode light irradiation improves root and leaf formation in difficult-to-propagate *Protea cyanroides* L. plantlets in vitro. *HortScience* 47:1490–1494