RESEARCH ARTICLE

Influence of polyamines on hyperhydricity reversion and its associated mechanism during micropropagation of China pink (*Dianthus chinensis* L.)

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Abstract Hyperhydricity (HH) is a physiological disorder that frequently occurs in plant tissue cultures, affecting healthy growth and development of clonal plants. The primary cultures raised in Murashige and Skoog (MS) medium supplemented with 2.5 μ M N⁶-benzyladenine (BA) produced normal microshoot (6.3 shoots/ culture) with least HH. However, the third subculture onwards, HH becomes a major problem. The role of ethylene on HH induction through stomatal closure mechanism were proved by the supplementation of ethephon (5 μ M) in the culture medium containing 2.5 μ M BA. In the present study, the application of polyamines (putrescine, spermidine, or spermine) to minimize the HH was examined. Supplementation of 5 μ M spermine in MS medium significantly reduced the percentage of HH to 0.33%, in contrast to control (100%), while a maximum number of healthy reverted shoots (11.0) were observed in 5 μ M spermidine treatment. The addition of polyamines effectively reduced H₂O₂ content (50%) characterized by increased chlorophyll content with proper stomatal morphology. The relative gene expression profile of ethylene biosynthesis genes, 1-Aminocyclopropane-1-carboxylase synthase (ACS1) and 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO1) at 5 μ M spermine added medium was 1.09 and 1.3 over normal (1) or HH cultures (1.93 and 2.53) respectively, and thus directed restoration of normal morphology of shoots. The present finding in brief, forward a novel method to regulate HH in terms of endogenous ethylene by adopting polyamines exposure and the

procedure can be applied to many other plants facing similar HH problems.

Keywords ACC synthase · Ethephon · Spermine · Stomatal bioassay · Vitrification

Introduction

Plant tissue culture is a technique widely employed in the field of agriculture, horticulture, plant breeding, and forestry due to its accepted advantages over conventional tools. The in vitro propagation systems can be of beneficial use in many plant species for rapid multiplication, germplasm conservation, and genetic manipulation. However, the in vitro propagation can sometimes be problematic due to the development of various tissue abnormalities. Hyperhydricity (HH) or vitrification is one of the significant problems that impose extreme oxidative stress to in vitro growing tissues (Saher et al. 2005). The hyperhydric (glassiness) plantlets are easily breakable, and shoots are translucent, thick, and pale green. These physiological disorders are characterized under extreme culture conditions viz., high relative humidity, weak gaseous exchange, and consequent ethylene accumulation in culture flask. The glassy nature of hyperhydric shoot is due to abnormal accumulation of water in the intercellular space of nascent in vitro formed tissues (Gao et al. 2017b) and can lead to irreversible damage to cultures, leading to various metabolic disorders (Lai et al. 2005). The hyperhydricity induction in cultures can affect the overall quality and consequent reduction of healthy plantlets. Although a fascinating fact is the possibility that HH shoot can revert to its normal state or morphology. In some cases, HH has been reported to reduce by changing water relation

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(Vanderschaege and Debergh 1987), increasing agar concentration (Saher et al. 2005), altering media components (Yadav et al. 2003; Ivanova and Van Staden 2011), enhancing vessel ventilation (Thomas et al. 2000), and by bottom cooling (Piqueras et al. 2002). Under a highly stressed condition, microshoots induce the production of reactive oxygen species (ROS) that may affect the photosynthetic pigment by reduced production of chlorophyll (Chakrabarty et al. 2006; Petruş Vancea 2018). Moreover, HH plantlets show reduced transpiration due to non-functional or closed stomata (Sreedhar et al. 2009; Gao et al. 2017b). In *D. caryophyllus*, oxidative injury of HH tissues was reported along with elevated activity of peroxidase, increased accumulation of malondialdehyde, or reduced lignification (Piqueras et al. 2002). These anomalies cause anatomical and physiological changes of microshoots that will significantly reduce the survival rate of plants during the hardening process (Gao et al. 2017a). Therefore, an efficient HH reversion step is indispensable for proper in vitro establishment of such plants.

In the past, several researchers focused on reducing HH syndromes in different plant species, such as *D. chinensis* (Kantia and Kothari 2002; Gao et al. 2017a, b), *D. caryophyllus* (Jain et al. 2001; Muneer et al. 2018), *Aloe vera* (Ivanova and Staden 2011), *Malus domestica* (Chakrabarty et al. 2006; Tabart et al. 2015) *Allium sativum* (Tian et al. 2015) and *Olea europaea* (Bayraktar et al. 2020). HH is first reported in the herbaceous ornamental plant species *D. chinensis*. Due to its high frequency of regeneration and high susceptibility to HH, *D. chinensis* is considered as the best model plant to study HH. In the past, researchers were concentrated on stress related plant hormone ethylene, their role on growth and development of in vitro shoots, and interactive mechanism towards HH (Turhan 2004; Lai et al. 2005). While addressing the problem, several researchers opine that ethylene biosynthesis is the prime reason or an independent signal accompanying on HH (Gao et al. 2017a; Hassanein et al. 2018). In support to this, the occurrence of HH syndrome in *Dianthus* species has been confirmed by the higher level of ethylene accumulation on tissues (Gao et al. 2017a). In methionine related ethylene biosynthesis, two key enzymes viz., 1-Aminocyclopropane-1-carboxylase synthase (ACS; S-adenosyl-L-methionine methyl thioadenosine-lyase, EC 4.4.1.14) and 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO; 1-aminocyclopropane-1-carboxylate oxygenase, EC 1.14.17.4) catalyzes the conversion of S-adenosyl methionine (SAM) to 1-Aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene respectively (Wang et al. 2002). The direct application of ethylene releasing hormone ethephon on in vitro cultures and recording its effect can serve as the stimulated system to elicit exact reason for HH. Anti-ethylene compounds AgNO₃ or aminoethoxy vinyl glycine (AVG) has been used as the inhibitors of ACC synthase

(Bernard et al. 2015; Hassanein et al. 2018). Sometimes, the ethylene biosynthetic pathway is modulated by the biosynthesis of polyamines (PAs) since SAM act as a common intermediate (Pandey 2000). In such a condition, the competitive inhibition may cause a reduction in ethylene production by the action of PAs. Li et al. (1992) suggested that PAs specifically regulate ethylene biosynthesis by inhibiting the ACC synthase expression in the tomato plant.

PAs act as a multifunctional regulator within the cell by its polycationic property, which could act as free radical scavengers by regulating membrane fluidity and control on many aspects of molecular turn over (Handa and Mattoo 2010; Parra-Lobato and Gomez-Jimenez 2011). This indicates the influence of PAs on anti-oxidant protection against the stressed HH condition (Hassannejad et al. 2012). The recent reports on HH reversion in *D. chinensis* elaborate ethylene gene expression level by AgNO₃ treatments (Gao et al. 2017a). Nevertheless, the ethylene gene expression pattern concerning PAs treatments in HH reversion experiments has never been documented. These facts inspired us to undertake a study on the effect of PAs viz., diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) supplementation to MS medium to avoid HH syndrome and to ascertain how ACS and ACO gene expression patterns will support the reversion in different types of PAs treated cultures.

Materials and methods

In vitro culture and hyperhydricity (HH)

Chinese pink (*Dianthus chinensis* L.) cultivar *Dianthus* telstar scarlet procured from District Horticultural Nursery, Thiruvananthapuram, Kerala state, India served as the explant source. It was maintained in the glasshouse of the Department of Botany, University of Kerala. Single node segments prepared from selected shoots. They were thoroughly washed under running tap water for 30 min, followed by treatment (2 min) in 2% polysorbitol detergent solution (Labolene Mfg. Fisher Fine Chemicals, Mumbai, India). These explants were then treated with 0.1% carbendazim fungicide (Bavistin, Mfg. BASF Mumbai, India) for 15 min and washed 3–4 times with distilled water. The explants then taken into a laminar air flow work station and surface sterilized with 1% chlorine containing sodium hypochlorite (5% NaOCl solution, Spectrum Reagents, and Chemicals, Ernakulum, Kerala) for 5 min and washed 4 times with sterile distilled water. The surface-sterilized explants were trimmed at the cut ends into ~ 1.5 cm nodal segments and cultured to test tube containing 15 ml Murashige and Skoog (MS) medium with 3% sucrose and 0.85% agar (Sisco Research Laboratories, Mumbai, India).

The pH of the medium was adjusted (5.80 ± 0.02) before autoclaving at 121°C for 20 min. To study the effect of cytokinins on shoot proliferation and hyperhydric induction, the nodal segments were inoculated on agar gelled MS medium fortified with varying concentrations (0.0, 2.5, or $5\ \mu\text{M}$) of N^6 -benzyladenine (BA; MW = 225.25 g/mol; Sigma–Aldrich, Bangalore, India) and subsequently subcultured at 30 days of culture interval. Each subculture treatment continued up to 4 times, and data on the number of shoots per explant and HH frequency (%) recorded. HH frequency (%) was estimated by using the formulae;

$$\text{HH frequency (\%)} = \frac{\text{No. of hyperhydric shoots}}{\text{total no. of shoots}} \times 100$$

Effect of ethephon on HH syndrome and stomatal bioassay

To examine the role of ethylene on HH induction, a synthetic compound ethephon (ethrel, 2-chloroethylphosphonic acid; MW = 144.5 g/mol; Sigma–Aldrich, Bangalore, India) was filter-sterilized ($0.22\ \mu\text{m}$ pore size) and added at different concentrations (0.0, 1, 2, 3, 4, or $5\ \mu\text{M}$) to pre autoclaved MS media containing $2.5\ \mu\text{M}$ BA, 3% sucrose, and 0.85% agar. Nodal segments derived from basal cultures transferred into the ethephon containing medium, after 30 days of culture, HH frequency (%), and relative water content (RWC) were estimated and compared with the control raised in $2.5\ \mu\text{M}$ BA. RWC of plantlets calculated by using the formula;

$$\text{RWC (\%)} \text{ w/w} = \frac{[\text{FW} - \text{DW}]}{(\text{TW} - \text{DW})} \times 100$$

where, FW, TW, and DW represent fresh weight, saturated weight, and dry weight respectively (Karimi et al. 2012).

To study the direct effect of ethephon on stomata morphology, the detached leaves from the cultures were floated on 2-(N-morpholino) ethanesulfonic acid (MES)-KCl buffer (10 mM MES/ 50 mM KCl/ 0.1 mM CaCl_2 , pH – 6.15) solution under 2 h treatments at room temperature for opening the stomata. Then the treated leaves were transferred to the same buffer with various concentrations of ethephon (25, 50, 100, or $150\ \mu\text{M}$) for another three hours. After the treatment, the abaxial epidermal portion peeled from the leaves, and stomatal apertures were observed (Ge et al. 2015) by light microscopy (Leica Model DM 2000 (BF/PH/DE)).

Effect of polyamines on HH reversion

The MS medium supplemented with 0.0, 2.5, or $5\ \mu\text{M}$ concentrations of PAs; Spm, Spd, or Put (Sigma–Aldrich, St. Louis, US) in full strength MS medium were tested for

the reversion of hyperhydric cultures. Before the reversion experiments, each hyperhydric cultures were subcultured for one month on MS basal medium containing 0.4% agar to induce uniform HH (100%). Nodal segments excised from hyperhydric cultures were cultured on PAs supplemented medium up to 30 days. The experiments were conducted by 4 replication blocks of 6 culture vessels each, and all the cultures were maintained in the culture room at $25 \pm 2^\circ\text{C}$ under a 16/ 8 h light/ dark cycle with $55 \pm 5\%$ relative humidity and $50\ \mu\text{mol}/\text{m}^2\ \text{s}$ irradiance provided by cool white fluorescent tubes (40 W, Philips, India). After 30 days of culturing, data on plantlet regeneration (shoot number and shoot length), as well as HH (% HH and RWC) were scored. The reverted plantlets from the best treatments (Spm, Spd, or Put) were randomly selected and subjected to stomatal bioassay, histological localization of H_2O_2 , estimation of total chlorophyll content, and ethylene gene expression to confirm HH reversion.

Stomatal bioassay, histological localization of H_2O_2 and estimation of total chlorophyll

To study the effect of PAs on related mechanism during HH reversion, the normal cultures in $2.5\ \mu\text{M}$ BA, hyperhydric (hormone-free control), or reverted ($5\ \mu\text{M}$ Put, $5\ \mu\text{M}$ Spd, or $5\ \mu\text{M}$ Spm) leaves samples were randomly collected from 30 days cultures. The stomatal bioassay of in vitro samples analyzed (He et al. 2013), and stomatal apertures were calculated as described previously (Ge et al. 2015). H_2O_2 content was quantified as per the standard protocol available (Tian et al. 2015), and absorbance was recorded at 390 nm. Histological staining of H_2O_2 was conducted by staining the plantlets with 3,3'-diamino benzidine (DAB) as proposed previously (Lee et al. 2012) with slight modification. The freshly collected plantlets were immersed in 1 mg/ml DAB staining solution (10 mM Na_2HPO_4 -DAB staining solution (pH 7.2) and vacuum infiltrated using needleless syringe for 2 min and incubated for 24 h in the dark at room temperature. Samples were then washed in 95% ethanol and incubated at 70°C for 10 min. The photographs of stained plantlets were then captured using a digital camera (Model: Nikon COOLPIX S3100 Mfg. Nikon CORP., Japan). The total chlorophyll content was estimated as per the standard procedure (Lichtenthaler 1987). The acetone (100%) extract was quantified by reading the absorbance at 652 nm in a spectrophotometer (Shimadzu, Japan, UV-1700, Pharma spec, Model No. 1700).

Analysis of expression of genes associated with ethylene biosynthesis

The expression of ethylene biosynthesis gene (ACS1 and ACO1) during HH reversion was studied by qRT-PCR (qTOWER 2.2, Analytic Jena, Germany) and analyzed by *aj*, qPCRsoft 3.0. The total RNA was extracted from 30 days old in vitro raised normal (2.5 μ M BA), hyperhydric (hormone-free medium) or reverted leaves from 5 μ M PAs (Spm, Spd, or Put) treated cultures, using trizol reagent (Ambion by Life Technologies, Invitrogen, Carlsbad, USA) method (Simms et al. 1993). The isolated RNA for qRT-PCR was quantified by Nanodrop Spectrophotometer (NANO DROP ONE, Thermo Fisher, Scientific, Wilmington, USA). Collected total RNA (500 ng) were converted into cDNA using Primer ScriptTM1st strand cDNA Synthesis Kit (Takara Bio India Pvt. Ltd, New Delhi, India). To confirm the expression of our gene of interest, actin (ACT) was used as the internal control. The primer sequences were designed for qRT-PCR (Table 1) and purchased (Geno Biosciences Pvt. Ltd, Noida, India). qRT-PCR analysis was performed with 2X Real Time PCR Master Mix with SYBR Green (Origin Diagnostics and Research, Karunagapally, India) all in the volume of 20 μ l. The thermal profile of the ACS1 and ACO1 polymerase reaction was 95 °C for 7 min, followed by 40 cycles of 95 °C denaturation for 20 s, 58.1 °C (ACS1) or 58.7 °C (ACO1) annealing for 15 s and 72 °C extension for 15 s. At the end of the program, the melting curve was activated by increasing the temperature from 60 to 95 °C. Each copy number of ACS1 and ACO1 were normalized against that of ACT, and the relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The RNA isolation was conducted with pooled replicas, and data represented the mean relative expression of three repeats with standard error bars.

Statistical analysis

All the experiments on HH reversion were conducted using a randomized complete block design (RCBD) method. Every in vitro experiment consisted of four replication blocks of 6 culture flask. The data subjected to analysis of

variance (ANOVA) and the differences among the mean values were compared with Tukey's—test ($p < 0.05$) using IBM SPSS Statistics (Ver. 22, SPSS Inc., Chicago, IL, US) and all results were expressed as mean $\pm$ SD.

Results and discussion

Shoot induction and hyperhydricity

The fresh nodal segments, after four days of culture showed signs of axillary bud break and resumed an active growth resulting in the initiation of adventitious microshoots on 2.5 μ M BA supplemented medium. The ANOVA revealed a significant ($p < 0.001$) effect of BA on shoot number and percentage of HH at different subcultures (Table 2). The best shoot proliferation (6.3 shoots per culture) with least HH (0.6%) was observed in 2.5 μ M BA added medium after 30 days of fresh inoculation. The number of shoot buds and the percentage of HH were increased with increasing concentration of BA as well as subsequent subcultures. At 5 μ M BA, the rate of HH was increased significantly, and third subculture (70% HH) onwards, it became a serious hindrance to produce normal shoots. HH symptoms such as thick watery shoots with leaf necrosis and stunted growth were noticed (Fig. 1a) compared to the healthy plantlets (Fig. 1b). Medium containing 2.5 μ M BA was optimized as the best treatment for shoot multiplication with minimum HH. BA is widely used, most effective, affordable cytokinin, both in experimental and commercial micropropagation including carnation and China pink (Kantia and Kothari 2002; Fu et al. 2008). However, continuous maintenance of cultures in BA medium, caused in vitro stress, which induces HH in pink cultures. Previous report found that a dose dependent use of cytokinins involved in the induction of HH (Ivanova and Van Staden 2011; Vasudevan and Van staden 2011). Recent study suggested that supply of high conc. cytokinin in the culture medium could enhance ethylene accumulation, and consequent induction of HH (Liu et al. 2017).

Table 1 Nucleotide sequences of primers used for quantitative real-time PCR analysis

Gene ID	NCBI ID	Primer sequence (5' → 3')
ACS1	AB605175.1	F: CGAGGGAGAGTTAAAGTTATGGAG R: ATTGAGTGCAACGCTCTAGGG
ACO1	AB042320	F: GACAAAGTGGAGAGGATGACAA R: GAGACTCAGCAGACACTAAACC
ACTIN	AY007315	F: TTTGGACTCTGGTGATGGTG R: CAGCCAAATCAAGACGCAAG

Table 2 Effect of subculture trail and BA concentrations on regeneration and hyperhydricity induction in *D. chinensis*

Subculture trail	Conc. of BA (μM)	No. of Shoots	% of HH
Fresh Inoculation	0	1.00 ± 0.0^f	0.00 ± 0.0^f
	2.5	6.33 ± 0.9^{ef}	0.66 ± 0.7^f
	5.0	7.33 ± 0.9^e	4.87 ± 0.4^{ef}
Subculture I	0	1.33 ± 0.3^f	0.00 ± 0.0^f
	2.5	9.00 ± 0.6^e	11.13 ± 0.6^e
	5.0	14.23 ± 1.3^d	20.17 ± 3.0^d
Subculture II	0	1.80 ± 0.2^f	0.00 ± 0.0^f
	2.5	15.00 ± 1.3^d	24.00 ± 1.5^d
	5.0	22.50 ± 1.4^{bc}	41.60 ± 2.3^c
Subculture III	0	2.13 ± 0.1^f	1.67 ± 1.7^f
	2.5	21.00 ± 2.6^c	49.67 ± 2.7^c
	5.0	26.53 ± 1.0^{ab}	70.00 ± 5.3^b
Subculture IV	0	2.23 ± 0.1^f	3.33 ± 1.7^{ef}
	2.5	28.00 ± 1.1^a	72.17 ± 0.7^b
	5.0	29.00 ± 1.7^a	87.67 ± 1.5^a
Main effect Df ($n-1$) = 14		84.76***	230.62***
Subculture(S) Df ($n-1$) = 4		83.60***	341.91***
BA conc. (C) Df ($n-1$) = 2		349.81***	620.42***

***Significant at $p < 0.001$ level; Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Tukey's test

Fig. 1 Culture establishment and hyperhydricity induction in *D. chinensis* L. **a** Hyperhydric plantlets in 4th subcultures of 5 μM BA (Scale bar 1.2 cm); and **b** normal plantlet on 2.5 μM BA (Scale bar 1.5 cm)

Effect of ethephon on hyperhydricity of shoots and stomata

Ethephon supplementation had a positive influence on HH induction. After 4 weeks of culture on medium containing 5 μM ethephon along with 2.5 μM BA, 100% of cultures

showed HH (Fig. 2a) characterized by glassy, brittle, and pale yellow coloured shoots. The hyperhydric plantlet grown in ethephon medium displayed a significant ($p < 0.001$) increase in the relative water content (93.8%) and in medium containing higher concentration (5 μM) of ethephon, maximum morphological abnormalities were

Fig. 2 Effect of ethephon on hyperhydricity and stomatal closure mechanism of in vitro raised normal leaves of *D. chinensis* L. **a** Hyperhydricity induction and relative water content; **b** stomatal aperture bioassay on in vitro raised normal leaves; **c** stomatal opening on normal control set; **d** stomatal aperture on 50 μM ethephon

treatment; **e** partial stomatal closure on 100 μM ethephon; and **f** complete stomatal closure on 150 μM ethephon. Vertical bars represent mean ± SEs, means denoted by different alphabets are significantly different ($p < 0.05$) as per Tukey's test. Least mean value representing alphabet 'a' next highest as 'b' and so on

noticed. Ethephon at 3 μM onwards creates a high risk of HH with a range greater than 90% RWC. The ethephon in the medium created a stressed condition to induce HH. In vitro application of ethephon stimulates peroxidase or catalase activity in a dose-dependent manner (Wang et al. 2002; De and De 2003), induce oxidative stress finally resulting in the development of HH symptoms.

The higher rate of stomatal opening and stomatal density were observed under MES-KCl buffer solution on normal in vitro raised leaves. The effect of ethephon on in vitro leaves revealed the reduction of stomatal aperture at

varying concentration (Fig. 2b). The control in vitro leaves showed normal stomatal opening (Fig. 2c) however, direct treatment of ethephon on in vitro raised leaves gradually reduced their stomatal aperture (Fig. 2d) with increasing the application of ethephon (25–150 μM) after 3 h. Irregular stomatal aperture was observed on 100 μM ethephon (Fig. 2e). A treatment using 150 μM ethephon displayed more than 50% stomatal closure (Fig. 2f) than control. Ge et al. (2015) reported both ethylene gas or ethephon treatment on *Arabidopsis* leaves induced stomatal closure mechanism in a dose-dependent manner. Gao et al. (2017a)

have reported partial or complete closure of stomata in ethylene accumulated HH state. Our study also showed functioning of partial to complete stomatal closure mechanism at different conc. of ethephon. These may hamper transpiration or evaporation of plant tissues and finally leads to cellular water accumulation. Hence the result can be summarized as; ethylene have an important role in the induction of HH through blocking stomatal movement by H_2O_2 like ROS production in guard cells.

Effect of polyamines on HH reversion

Hyperhydric nodal segments after one week of PAs treatment showed signs of axillary bud enlargement and associated initiation of adventitious microshoots from the basal region of explant. The ANOVA revealed significant ($p < 0.01$) effect of PAs on shoot number, shoot length (cm), and percentage of HH (Table 3). The hyperhydric nodal segments cultured on hormone-free medium treated as control demonstrated the development of HH cultures (Fig. 3a). After two weeks of culture in PAs containing medium, healthy shoots have emerged with reduced shoot transparency, and dark green well-expanded leaves indicate the reversion of HH. The effect of PAs treatment on the induction of normal shoot was characterized by the formation of broad and healthy leaves with normal intermodal elongation. The addition of Spm (5 μ M) in agar (0.85%) gelled medium displayed 0.3% HH frequency, which indicates a significant reduction in HH with a normal level of RWC (79.1%). Based on the observation, we can deduce that microshoots $< 85\%$ of RWC signify the healthy nature and free from HH (Fig. 3b). These characteristics are also supported by Gao et al. (2017a, b) through decreased water accumulation or increased water loss in reverted *D. chinensis* shoots by $AgNO_3$ treatment. The control set raised

from hyperhydric node on basal medium developed 100% HH with 94.3% RWC. A significant reduction (13.7%) of HH frequency over control (100%), coupled with the development of 11.0 healthy shoots was achieved when HH nodal segments were cultured in MS medium supplemented with 5 μ M Spd. The addition of Put (2.5 μ M) in the medium reduced HH to 17% with maximum shoot length (10.2 cm, Fig. 3c) compared to control. Tabart et al. (2015) report that Spd or polyamine precursors like ornithine or arginine reduced the frequency of HH in micropropagated shoots of apple. A similar signature on the efficiency of Spm was reported in HH reversion by salicylic acid in *Thymus daenensis* shoots, where free spermine could be involved in the reversion process (Hassannejad et al. 2012). However, Piqueras et al. (2002) state that the level of free, conjugated, and bounded PAs regulated HH in *D. caryophyllus*. Some reports on PAs indicated their physiological, developmental, and response to various stress conditions (Viu et al. 2009). The addition of Put may reduce the production of unwanted ethylene and can enhance the healthy shoot development through blocking HH. Kumar et al. (2009) demonstrated that an intermediate compound SAM works behind the interplay of polyamine and ethylene biosynthesis pathway. Here Put utilizes SAM for the conversion of Spd, which possibly helps to reduce the availability of SAM for ethylene production (Bais et al. 2000). Another reason for stress regulation is the conjugation effect of PAs with other phyto-compounds. Medium fortified with free polyamines enhanced the production of conjugated polyamines and forward antioxidant activity (Kuznetov and Shevyakova 2007). Bassard et al. (2010) postulated that endogenous application of PAs favored the production of a conjugated polyamine phenolamide, when phenolic acid combine with PAs. PAs alter the HH syndrome in several ways. For instance, during osmotic stress,

Table 3 Effect of different polyamines (PAs) on microshoot induction and HH reversion in *D. chinensis*

Polyamine type	Polyamine conc. (μ M)	Shoot number	Shoot length (cm)	HH frequency (%)	Relative water content (%)
Control	0	3.67 $\pm$ 0.33 ^c	4.40 $\pm$ 0.31 ^d	100.00 $\pm$ 0.00 ^c	94.33 $\pm$ 1.20 ^c
Spermidine	2.5	6.33 $\pm$ 0.88 ^{bc}	7.77 $\pm$ 0.15 ^{bc}	20.33 $\pm$ 5.75 ^{bc}	83.87 $\pm$ 0.47 ^{bc}
	5.0	11.00 $\pm$ 0.58 ^{ab}	8.97 $\pm$ 0.32 ^{abc}	13.67 $\pm$ 3.57 ^b	80.67 $\pm$ 1.20 ^b
Putrescine	2.5	6.00 $\pm$ 0.58 ^{bc}	10.20 $\pm$ 0.23 ^a	17.02 $\pm$ 3.41 ^{bc}	84.67 $\pm$ 0.88 ^{bc}
	5.0	8.00 $\pm$ 1.15 ^a	9.90 $\pm$ 0.78 ^a	13.77 $\pm$ 3.37 ^b	83.33 $\pm$ 0.67 ^{bc}
Spermine	2.5	4.67 $\pm$ 0.67 ^{bc}	9.40 $\pm$ 0.50 ^{ab}	0.33 $\pm$ 0.33 ^a	80.67 $\pm$ 0.88 ^b
	5.0	6.33 $\pm$ 0.88 ^{bc}	7.00 $\pm$ 0.64 ^c	0.33 $\pm$ 0.33 ^a	79.10 $\pm$ 0.67 ^a
Main effect Df (n–1) = 6		9.69***	18.85***	121.35***	32.30***
PAs type(T) Df (n–1) = 2		8.55**	9.49**	17.17***	10.78**
PAs conc.(C) Df (n–1) = 1		19.71**	1.70NS	1.66NS	7.82*

*Significant at $p < 0.05$ level, **Significant at $p < 0.01$ level, ***Significant at $p < 0.001$ level, NS Non-significant, Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Tukey's test

Fig. 3 Effect of polyamines on hyperhydricity reversion. **a** Hyperhydric plantlets on hormone free medium (Scale bar 3.6 cm); **b** reverted shoot developed from 5 μ M spermine (Scale bar 4.3 cm); **c** reverted shoot developed from 2.5 μ M putrescine (scale bar 4.3 cm); **d** and **e** chlorophyll and H₂O₂ content in reverted plantlets from 5 μ M putrescine, spermidine and spermine treatments; **f** localization of

H₂O₂ content by DAB staining; **g** microscopic image of stomatal opening of normal leaves sampled from 2.5 μ M BA; **h** reverted plants from 5 μ M spermine; and **i** stomatal closure of HH leaves. Vertical bars represent mean $\pm$ SEs, means denoted by different alphabets are significantly ($p < 0.05$) different as determined by Tukey's test. Least mean value representing alphabet 'a' next highest as 'b' and so on

the cell homeostasis is maintained by the biosynthesized putrescine in cells through regulation of proline content (Aziz et al. 1999). Polycationic nature of spermine or spermidine helps to contribute membrane stabilization (Cohen 1998).

Effect of PAs on total chlorophyll content, H₂O₂ level and stomatal bioassay

The loss of chlorophyll seems to be a pioneering trait to the formation of hyperhydric shoots. In vitro developed normal shoots showed 2.35 mg/ g total chlorophyll content. In HH shoots, chlorophyll content was less than normal shoots (Fig. 3d), and during the process of reversion by Put (5 μ M), 2.28 mg/ g chlorophyll was recorded after 30 days of culture. Present findings are in conformity to comparative studies of hyperhydric and non-hyperhydric *Vanilla*

planifolia shoots (Sreedhar et al. 2009) and *Beta vulgaris* (Petruș-Vancea 2018), where a significant increase in chlorophyll content was noticed in non-hyperhydric shoots. Likewise, Chakrabarty et al. (2006) point out the lesser number of chloroplasts in hyperhydric leaves of *Malus domestica*. The high rate of total chlorophyll in reverted plants indicated that the plants had normal metabolism and higher survival rate on outdoor conditions.

Reactive oxygen species (ROS) in HH cultures studied by determining H₂O₂ synthesis in microshoots. H₂O₂ production in hyperhydric cultures (4.9 μ M g/ FW) was significantly higher than normal shoots (2.1 μ M g/ FW) in 2.5 μ M BA (Fig. 3e). Spm or Put treated shoots sampled after 30 days of culture contain nearly 50 and 60% less H₂O₂ respectively than hyperhydric shoots (control). H₂O₂ accumulation of reverted plantlets could suppress by PAs, as evident by enhancing antioxidant activity. Franck et al.

(1995) reported in *Prunus avium* that in contrast to normal shoots, the hyperhydric shoots showed higher activity of H_2O_2 generating enzyme, superoxide dismutase and lower activity of H_2O_2 detoxifying enzymes viz., catalase, peroxidase, ascorbate peroxidase, glutathione reductase, and dehydro-ascorbate reductase. A recent report suggested that treatment of Spd alleviated accelerated ageing process of rice seedlings through enhanced scavenging activity of ROS (Hu et al. 2020). It suggests that the exogenous supply of PAs regulate abiotic stress condition of plants (Tabart et al. 2015). This stressed condition was also coupled with regulating ethylene biosynthesis in $AgNO_3$ induced reverted plants (Gao et al. 2017a). Like $AgNO_3$, the polyamines also act as anti-ethylene compounds to rescue HH. Reduction in H_2O_2 level in reverted microshoot was further confirmed by DAB staining (Fig. 3f). The image showed that hyperhydric shoot stained dark brown at the initial stage become light brown through the activity of PAs in the medium. Hence reduction in H_2O_2 could be linked to the avoidance of HH, and it also signifies the need of suitable culture environment and media components for normal development of shoots. The stomatal bioassay study revealed that reverted leaves have similar water mechanism like that of normal in vitro leaves. Here the maximum stomatal opening was found in normal leaf developed in vitro (Fig. 3g). The reduced H_2O_2 or ethylene accumulation in the reverted shoots showed increased stomatal apertures (Fig. 3h). But the stomata remain closed in complete HH leaves (Fig. 3i).

Previous result suggests that exogenous application of PAs enhance the abiotic stress tolerance in plant tissues (Kuznetov and Shevyakova 2007; Amri et al. 2011). Amooaghaie and Moghym (2011) suggested spermidine can stabilize cell membrane, protecting from cell damage under stress. These anti-oxidant properties of PAs depends on the conjugation nature and conjugated polyamines become a good precursor for peroxidase activity, lead to eliminate H_2O_2 and helps in cell wall strengthening in apoplast (Bassard et al. 2010). The reversion of HH helps to restore the phenotype of hyperhydric shoots, its growth and metabolism.

Expression pattern of ethylene biosynthesis genes in PAs treated cultures

The effect of PAs on the biosynthesis of ethylene was also investigated by measuring the expression levels of two ethylene biosynthesis associated genes, ACS1 and ACO1. Hyperhydric plantlets cultured in PAs supplemented medium displayed lower expression pattern for ACS1 and ACO1 compared to those cultured in medium without PAs (control). ACS1 transcript level in HH leaves was about twofold higher than that of normal in vitro leaves sampled

from 2.5 μM BA (Fig. 4a). The microshoots from 5 μM Spm treatment showed 50% decreased ACS1 expression than those from HH cultures. Similarly, Put and Spd (5 μM) treatment resulted in 1.8 and 1.3 times decreased ACS1 expression from HH cultures, respectively. This data indicate the expression pattern of ACS1 is decreased from HH to reverted shoots after PAs treatment. ACO1 transcript of hyperhydric leaves upregulated 1.9-fold in contrast to the normal leaf sample. Compared to the hyperhydric sample, the expression pattern of ACS1 and ACO1 was reduced by PAs supplementation. Among PAs, Spm showed the lowest expression (1.09) for ACO1 transcript, and fold change indicates ethylene transcript abundance of reverted plants are almost similar to normal ones. The amplification profile thus evident the effect of PAs to reduce ethylene biosynthesis at transcriptional level. Furthermore, both ACS1 and ACO1 gene expression forward the ethylene production and have an important role in HH reversion in *D. chinensis*. A negative correlation of endogenous PAs and ACS or ACO level has been reported in the stage of fruit ripening (Handa and Mattoo 2010; Parra-Lobato and Gomez-Jimenez 2011). In olive fruit, the endogenous application of Put down-regulated ACS while,

Fig. 4 Effect of polyamines on expression of genes associated with ethylene biosynthesis. **a** Changes in the expression level of ACS1; and **b** ACO1 gene in in vitro normal, hyperhydric and polyamines (putrescine, spermidine, and spermine) treated cultures. The expression levels of all treatments were normalized against the level of actin. Vertical bars represent mean $\pm$ SEs, means denoted by different alphabets are significantly ($p < 0.05$) different as determined by Tukey's test. Least mean value representing alphabet 'a' next highest as 'b' and so on

Spd down-regulated ACO expression (Parra-Lobato and Gomez-Jimenez 2011). The same pattern of expression was reported in the treatments of AgNO₃ in *D. chinensis*, where reverted shoot have displayed lower expression level of ethylene biosynthesis associated genes compared to those cultured in a medium not supplemented with AgNO₃ (Gao et al. 2017a). It could, therefore, be concluded that PAs suppressed ethylene production and effectively regulating the activity of ethylene on in vitro plantlets. During reversion, reduced gas accumulation in guard cell prevent H₂O₂ production, and stomata become open, the overall effect finally block water accumulation in plant tissue (Gao et al. 2017b).

Conclusion

In the present communication, we report for the first time the HH reversion property of polyamines concerning ethylene biosynthesis. Hyperhydric nodal segments cultured in medium fortified with Spd, Put, and Spm exhibited reduced level of HH and RWC, leading to green healthy plantlets. The HH reverted shoots produced through PAs treatment displayed low level ACS1 and ACO1 expression compared to HH shoots. The present study demonstrated that reversion is triggered by the reduced ethylene accumulation of nascent shoots characterized by significant reduction in H₂O₂ and enhancement in stomatal aperture, allowing normal functioning of the water loss mechanism. The results deduced that the excessive accumulation of ethylene in microshoots was a critical factor that is leading HH, and fixed the role of PAs in HH reversion of *D. chinensis* L.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Amooaghaie R, Moghym S (2011) Effect of polyamines on thermo tolerance and membrane stability of soybean seedling. *African J Biotechnol* 10:9673–9679. <https://doi.org/10.5897/AJB10.2446>

Amri E, Mirzaei M, Moradi M, Zare K (2011) The effects of spermidine and putrescine polyamines on growth of pomegranate (*Punica granatum* L. cv ‘Rabbab’) in salinity circumstance. *Int J Plant Physiol Biochem* 3:43–49

Aziz A, Martin-Tanguy J, Larher F (1999) Salt stress-induced proline accumulation and changes in tyramine and polyamine levels are linked to ionic adjustment in tomato leaf discs. *Plant Sci* 145:83–91. [https://doi.org/10.1016/S0168-9452\(99\)00071-0](https://doi.org/10.1016/S0168-9452(99)00071-0)

Bais HP, Sudha GS, Ravishankar GA (2000) Putrescine and silver nitrate influences shoot multiplication, in vitro flowering and endogenous titers of polyamines in *Cichorium intybus* L. cv. Lucknow Local *J Plant Growth Regul* 19:238–248. <https://doi.org/10.1007/s003440000012>

Bassard JE, Ullmann P, Bernier F, Werck-Reichhart D (2010) Phenolamides: bridging polyamines to the phenolic metabolism. *Phytochem* 71:1808–1824. <https://doi.org/10.1016/j.phytochem.2010.08.003>

Bayraktar M, Hayta-Smedley S, Unal S, Varol N, Gurel A (2020) Micropropagation and prevention of hyperhydricity in olive (*Olea europaea* L.) cultivar ‘Gemlik’ South African. *J Bot* 128:264–273. <https://doi.org/10.1016/j.sajb.2019.11.022>

Bernard F, Moghadam NN, Mirzajani F (2015) The effect of colloidal silver nanoparticles on the level of lignification and hyperhydricity syndrome in *Thymus daenensis* in vitro shoots: a possible involvement of bonded polyamines. *Vitro Cell Dev Biol Plant* 51:546–553. <https://doi.org/10.1007/s11627-015-9700-2>

Chakrabarty D, Park S, Ali M, Shin K, Paek K (2006) Hyperhydricity in apple: ultrastructural and physiological aspects. *Tree Physiol* 26:377–388. <https://doi.org/10.1093/treephys/26.3.377>

Cohen S (1998) A guide to the polyamines. Oxford University Press, Oxford, pp 396–442

De D, De B (2003) Effect of ethephon on antioxidant enzymes and diosgenin production in seedlings of *Trigonella foenum-graecum*. *Food Chem* 82:211–216. [https://doi.org/10.1016/S0308-8146\(02\)00514-9](https://doi.org/10.1016/S0308-8146(02)00514-9)

Franck T, Kevers C, Gaspar T (1995) Protective enzymatic system against activated oxygen species compared in normal and vitrified shoots of *Prunus avium* L. raised in vitro. *Plant Growth Regul* 16:253–256. <https://doi.org/10.1007/BF00024782>

Fu X, Yang S, Bao M (2008) Factors affecting somatic embryogenesis in anther cultures of Chinese pink (*Dianthus chinensis* L.). *Vitro Cell Dev Biol Plant* 44:194–202. <https://doi.org/10.1007/s11627-008-9107-4>

Gao H, Xia X, An L, Xin X, Liang Y (2017a) Reversion of hyperhydricity in pink (*Dianthus chinensis* L.) plantlets by AgNO₃ and its associated mechanism during in vitro culture. *Plant Sci* 254:1–11. <https://doi.org/10.1016/j.plantsci.2016.10.008>

Gao H, Xu P, Li J, Ji H, An L, Xia X (2017b) AgNO₃ prevents the occurrence of hyperhydricity in *Dianthus chinensis* L. by enhancing water loss and antioxidant capacity. *Vitro Cell Dev Biol Plant* 53:561–570. <https://doi.org/10.1007/s11627-017-9871-0>

Ge XM, Cai HL, Lei X, Zhou X, Yue M, He JM (2015) Heterotrimeric G protein mediates ethylene-induced stomatal closure via hydrogen peroxide synthesis in *Arabidopsis*. *Plant J* 82:138–150. <https://doi.org/10.1111/tpj.12799>

Handa AK, Mattoo AK (2010) Differential and functional interactions emphasize the multiple roles of polyamines in plants. *Plant Phys Biochem* 48:540–546. <https://doi.org/10.1016/j.plaphy.2010.02.009>

Hassanein AM, Salem JM, Faheed FA, El-nagish A (2018) Effect of anti-ethylene compounds on isoenzyme patterns and genome stability during long term culture of *Moringa oleifera*. *Plant Cell Tiss Organ Cult* 132:201–212. <https://doi.org/10.1007/s11240-017-1326-0>

Hassannejad S, Bernard F, Mirzajani F, Gholami M (2012) SA improvement of hyperhydricity reversion in *Thymus daenensis* shoots culture maybe associated with polyamines changes. *Plant*

- Physiol Biochem 51:40–46. <https://doi.org/10.1016/j.plaphy.2011.10.006>
- He JM, Ma XG, Zhang Y, Sun TF, Xu FF, Chen YP, Liu X, Yue M (2013) Role and interrelationship of Gα protein, hydrogen peroxide, and nitric oxide in ultraviolet B-induced stomatal closure in *Arabidopsis* leaves. *Plant Physiol* 161:1570–1583. <https://doi.org/10.1104/pp.112.211623>
- Hu Q, Chen M, Song T, Cheng C, Tian Y, Hu J, Zhang J (2020) Spermidine enhanced the antioxidant capacity of rice seeds during seed aging. *Plant Growth Regul* 91:397–406. <https://doi.org/10.1007/s10725-020-00613-4>
- Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. *Plant Cell Tiss Org Cult* 104:13–21. <https://doi.org/10.1007/s11240-010-9794-5>
- Jain A, Kantia A, Kothari SL (2001) De novo differentiation of shoot buds from leaf-callus of *Dianthus caryophyllus* L. and control of hyperhydricity. *Scient Horticult* 87:319–326. [https://doi.org/10.1016/S0304-4238\(00\)00185-0](https://doi.org/10.1016/S0304-4238(00)00185-0)
- Kantia A, Kothari SL (2002) High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. *Sci Hort* 96:205–212. [https://doi.org/10.1016/S0304-4238\(02\)00081-X](https://doi.org/10.1016/S0304-4238(02)00081-X)
- Karimi S, Yadollahi A, Nazari-Moghadam R, Imani A, Arzani K (2012) In vitro screening of almond (*Prunus dulcis* (Mill.)) genotypes for drought tolerance. *J Biol Environ Sci* 6:263–270
- Kumar V, Parvatam G, Ravishankar GA (2009) AgNO₃—a potential regulator of ethylene activity and plant growth modulator. *Elctron J Biotechnol* 12:2. <https://doi.org/10.2225/vol12-issue2-fulltext-1>
- Kuznetov VV, Shevyakova N (2007) Polyamines and stress tolerance of plants. *Plant Stress* 1:50–71
- 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. <https://doi.org/10.1016/j.jplph.2004.07.015>
- Lee S, Seo PJ, Lee HJ, Park C (2012) A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*. *Plant J* 70:831–844. <https://doi.org/10.1111/j.1365-313X.2012.04932.x>
- Li N, Parsons BL, Liu D, Mattoo AK (1992) Accumulation of wound inducible ACC synthase transcripts in tomato fruit is inhibited by salicylic acid and polyamines. *Plant Mol Biol* 18:477–487. <https://doi.org/10.1007/bf00040664>
- Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol* 148:350–382. [https://doi.org/10.1016/0076-6879\(87\)48036-1](https://doi.org/10.1016/0076-6879(87)48036-1)
- Liu M, Jiang F, Kong X, Tian J, Wu Z, Wu Z (2017) Effects of multiple factors on hyperhydricity of *Allium sativum* L. *Sci Horticult* 217:285–296. <https://doi.org/10.1016/j.scienta.2017.02.010>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>
- Muneer S, Park YG, Jeong BR (2018) Red and blue light emitting diodes (LEDs) participate in mitigation of hyperhydricity in in vitro-grown carnation genotypes (*Dianthus caryophyllus*). *J Plant Growth Regul* 37:370–379. <https://doi.org/10.1007/s00344-017-9733-3>
- Pandey S, Ranade SA, Nagar PK, Kumar N (2000) Role of polyamines and ethylene as modulators of plant senescence. *J Biosci* 25:291–299. <https://doi.org/10.1007/BF02703938>
- Parra-Lobato MC, Gomez-Jimenez MC (2011) Polyamine-induced modulation of genes involved in ethylene biosynthesis and signalling pathways and nitric oxide production during olive mature fruit abscission. *J Exp Bot* 62:4447–4465. <https://doi.org/10.1093/jxb/err124>
- Petrus-Vancea A (2018) Cell ultrastructure and chlorophyll pigments in hyperhydric and non-hyperhydric *Beta vulgaris* var *Conditiva* plantlets, treated with deuterium depleted water. *Plant Cell Tiss Org Cult*. <https://doi.org/10.1007/s11240-018-1439-0>
- Piqueras A, Cortina M, Serna MD, Casas JL (2002) Polyamines and hyperhydricity in micropropagated carnation plants. *Plant Sci* 162:671–678. [https://doi.org/10.1016/S0168-9452\(02\)00007-9](https://doi.org/10.1016/S0168-9452(02)00007-9)
- Saher S, Piqueras A, Hellin E, Olmos E (2005) Prevention of hyperhydricity in micropropagated carnation shoots by bottom cooling: implications of oxidative stress. *Plant Cell Tiss Org Cult* 81:149–158. <https://doi.org/10.1007/s11240-004-4043-4>
- Simms D, Cizdziel PE, Chomczynski P (1993) TRIzol: a new reagent for optimal single-step isolation of RNA. *Focus* 15:99–103
- Sreedhar RV, Venkatachalam L, Neelwarne B (2009) Hyperhydricity-related morphologic and biochemical changes in *Vanilla (Vanilla planifolia)*. *J Plant Growth Regul* 28:46–57. <https://doi.org/10.1007/s00344-008-9073-4>
- Tabart J, Franck T, Kevers C, Dommes J (2015) Effect of polyamines and polyamine precursors on hyperhydricity in micropropagated apple shoots. *Plant Cell Tiss Org Cult* 120:11–18. <https://doi.org/10.1007/s11240-014-0568-3>
- Thomas P, Mythili JB, Shivashankara KS (2000) Explant, medium and vessel aeration affect the incidence of hyperhydricity and recovery of normal plantlets in triploid watermelon. *J Hort Sci Biotechnol* 75:19–25. <https://doi.org/10.1080/14620316.2000.11511194>
- Tian J, Jiang F, Wu Z (2015) The apoplastic oxidative burst as a key factor of hyperhydricity in garlic plantlet in vitro. *Plant Cell Tiss Org Cult* 120:571–584. <https://doi.org/10.1007/s11240-014-0623-0>
- Turhan H (2004) The effect of silver nitrate (ethylene inhibitor) on in vitro shoot development in potato (*Solanum tuberosum* L.). *Biotechnol* 3:72–74. <https://doi.org/10.3923/biotech.2004.72.74>
- Vanderschaege AM, Debergh PC (1987) Technical aspects of the control of the relative humidity in tissue culture containers. In: Ducate G, Jacob M, Simeon A (eds) *Plant micropropagation in horticultural industries*. Presses Universitaires, Liege, pp 68–76
- Vasudevan R, Van Staden J (2011) Cytokinin and explant types influence in vitro plant regeneration of Leopard Orchid (*Ansellia africana* Lindl.). *Plant Cell Tissue Organ Cult* 107:123–129. <https://doi.org/10.1007/s11240-011-9964-0>
- Viu AFM, Viu MAO, Tavares AR, Vianello F, Lima GPP (2009) Endogenous and exogenous polyamines in the organogenesis in *Curcuma longa* L. *Scientia Horticult* 121:501–504
- Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. *Plant Cell* 14:s131–s151. <https://doi.org/10.1105/tpc.001768>
- Yadav MK, Gaur AK, Garg GK (2003) Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. *Plant Cell Tiss Org Cult* 72:153–156. <https://doi.org/10.1023/A:1022236920605>