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RESEARCH ARTICLE

Overcoming Hyperhydricity and Profiling the affected proteins in Micropropagated Carnation

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Abstract

Nodal cultures of carnation (*Dianthus caryophyllus* L.) showed hyperhydric growth when grown in standard *in vitro* conditions. This afflicts their survival rate *ex vitro*. When the concentration of agar in the medium was modified (ranging from 0.9 to 1.4%), the regenerated axillary shoots perturbed vitrification on increasing the agar concentration up to 1.2%, probably owing to the reduced water content available. Additionally, lower pigment concentration was observed in the hyperhydric shoots. The total soluble protein content decreased with increasing hyperhydric state of the shoots. Protein profiling of the isolated proteins from hyperhydric and normal shoots showed that several proteins existed distinctively in the normal shoots and were absent or present in lower amounts in hyperhydric shoots. However accumulation of 110 and 30 kDa proteins in regenerated hyperhydric shoots indicated that these polypeptides may play a role in regulating hyperhydricity in carnation.

Keywords

Carnation, Gelling agents, Hyperhydricity, Proteins, Vitrification

Introduction

Hyperhydricity (Vitrification) is a morphogenic response of *in vitro* grown cultures when subjected to various stressful culture conditions. The so-called vitrified, vitreous or hyperhydric shoots (HS) appear turgid, watery at their surface and hypolignified. Their organs are somehow translucent, in some cases green, as in carnation, and easily breakable (Kevers *et al*, 2004). These shoots root poorly, when they do and hence do not generally survive at the acclimatization step. This is a serious problem during *in vitro* culture of carnation, since it directly affects the production of this commercially important ornamental plant at the economic level. Poor survival rate of the regenerated plantlets in the soil leading to losses have been reported in HS of *Dianthus* (Piqueras *et al*, 2002) and *Tagetes* (Aguilar *et al*, 2000). Despite the meticulous efforts involved in growing plants *in vitro*, this physiological and developmental problem limits the potential of *in vitro* technique for mass propagation (Winarto *et al*, 2004; Ivanova and van Staden, 2008).

Extensive studies on hyperhydricity have been conducted on several plants such as *Dianthus caryophyllus* (Piqueras *et al*, 2002; Saher *et al*, 2004, 2005; Casanova *et al*, 2008; Hazarika and Bora, 2010; Kharrazil *et al*, 2011), *Tagetes* (Aguilar *et al*, 2000; Modi *et al*, 2009), *Malus* (Chakraborty *et al*, 2005; Lucyszyn *et al*, 2005), *Euphorbia* (Dewir *et al*, 2006), *Prunus* (Perez-Tornero *et al*, 2001), *Simmondsia chinensis* (Apostolo and Llorente, 2000; Mills *et al*, 2004) and *Arabidopsis* (Delarue *et al*, 1997). Several factors responsible for hyperhydricity have been investigated like the mechanical stresses that the explants are exposed to involving excision, wounding and possible air embolism due to dissection, the factors responsible include high osmoticity or high sucrose content (Hazarika and Bora, 2010), the osmotic shock due to infiltration of the culture medium in the intercellular spaces (Bottcher and Goring, 1987), abnormal mineral nutrition like high amounts of nitrogen (Brand, 1993; Tsay, 1998; Mohamed, 2011), high cytokinins application (Jain *et al*, 2001; Ivanova *et al*, 2006) and the gaseous environment in relation to the culture vessel used (Majada *et al*, 2000; Park *et al*, 2004).

Various approaches reported to overcome hyperhydricity comprise good gaseous exchange (Majada *et al*, 2000, 2002; *Casanova et al*, 2008, Modi *et al*, 2009), different concentrations of agar (Miller *et al*, 1991; Ivanova and van Staden, 2011; Zhao *et al*, 2011), type and concentration of PGRs used such as BA, ABA (Kim *et al*, 1988; de Oliveira *et al*, 1997), IAA (Li *et al*, 1997), GA_3 (Jain *et al*, 2001) and NAA (Kharrazil *et al*, 2011), the ratio of nitrate to ammonium ions (Tsay *et al*, 1998; Mohamed , 2011), bactopeptone (Sato *et al*, 1993; Jain *et al*, 2001) and changing levels of calcium chloride, ammonium nitrate, potassium nitrate, ferrous sulphate and magnesium sulphate (Choudhary *et al*, 1993; Yadav *et al*, 2003).

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Nonetheless, the physiological and molecular base for such *in vitro* induced problem is poorly understood. Therefore, carnation can apparently be an experimental model to better understand hyperhydricity-associated processes.

In the present study, an account of the effect of different gelling agents on controlling induction of hyperhydric shoot buds from nodal segments of carnation has been reported. Differences in amount of plant pigments in normal and hyperhydric shoots has also been studied. Further a quantitative and qualitative analyses of proteins was carried out based on differences in total protein pattern as determined by SDS-PAGE.

Materials and Method

Plant material and Culture Conditions

Seeds of *D. caryophyllus* (Namdhari Seeds Pvt. Ltd., Bengaluru, India) were surface sterilized with 0.1%(w/v) mercuric chloride for 3 minutes followed by four washes with sterile distilled water. MS (Murashige and Skoog, 1962) medium of half strength with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Qualigens, bacteriological grade), pH adjusted to 5.8 before autoclaving at 121°C and 1.2-1.3kg/cm^{2} pressure for 20 minutes was prepared for seedling formation. Seven seeds were kept in a flask (100ml Erlenmeyer with 40 ml medium in each). Cultures were incubated at a temperature of 26°C ± 1°C and 16 h photoperiod and light intensity of 25µmol/m^{2}/s provided by white fluorescent tubes. Nodal segments of 0.5mm were aseptically dissected out from 21 days old aseptically raised seedlings and cultured on MS medium supplemented with BAP (2.2 µM) and NAA (2.8 µM).

Effect of Gelling Agent

The media used in the present investigation was gelled with 1.0%-4.0% isabgol, 0.1-0.5% CleriGel (Merck, India) or 0.9-1.4% agar. The most suitable agent and its optimum concentration were assessed. The nodal explants were cultured in 100ml Erlenmeyer flasks containing 40 ml medium and three explants each.

Pigment Content Analysis

Pigment contents were quantified from samples of leaf from both normal and hyperhydric shoots according to the procedure by Arnon (1949). Pigments were extracted by homogenizing 1-2 g of leaves with 80% acetone; the extract was filtered through Whatman No. 1 filter paper. Carotenoid and chlorophyll a and b contents were estimated. Absorbances were determined at 451 and 503 nm for carotenoids, and 647 and 664.5nm for chlorophyll.

Protein Extraction, Quantification and SDS-PAGE Electrophoresis

Nodal explants, normal and hyperhydric frozen leaf samples were ground in liquid nitrogen with a pestle and mortar and homogenized at 25°C with extraction buffer (100 mM Tris-HCl, 50 mM NaCl and 1 mM PMSF, pH 7.5) at a ratio of 500 mg of tissue per 5 ml of extraction buffer. Cell debris was removed by centrifugation (14,000rpm, 15 mins.) and protein-containing supernatants retained. Protein quantification and gel electrophoresis were performed according to Bradford (1976) and Laemmli (1970), respectively. Protein extracts were incubated at 100°C, for 3 min, with sample buffer [10% (v/v) glycerol, 2.3% (v/v) SDS, 0.25% bromophenol blue, 5% β-mercaptoethanol, 0.0625 M Tris-HCl, pH 6.8], before loading the gels. Aliquots of protein extract (30 µl) were loaded onto 5% SDS-PAGE, electrophoresed for 16 h at 37 V in a running buffer [0.0025 M Tris-HCl. 0.2 M glycine, 1 mM EDTA and 3.5 mM SDS]. The gel was stained with 40% (v/v) methanol, 7.5% (v/v) acetic acid and 0.1% Comassie Brilliant Blue R-250 for 8 h and then destained in 10% (v/v) methanol and 7.5% (v/v) acetic acid.

Statistical Analysis

Three replicates per treatment were made. Mean of all repetitions and standard deviation of each observation from the mean was calculated from the data obtained.

Results and Discussion

Effect of Gelling Agent

Increase in the concentration of agar promoted healthy shoot proliferation. The highest organogenic response was observed on 1.2% agar with an average of 7.3 shoots (Fig. 1b). The number decreased to 2.0 shoots/explants when the agar concentration was increased to 1.4% (Table 1). The extent of medium solidification and the type of solidifying agent affect the water content of the shoots along with the shoot regeneration capacity and hence the increase in number of normal shoots with the increase in amount of agar has been observed in the present study consistent with previous findings in carnation (Casanova *et al*, 2008) and *Aloe* (Ivanova and van Staden, 2011). In contrast to such studies, Yadav *et al* (2003) had reported that decrease in the agar concentration from 0.8% to 0.6% resulted in overcoming hyperhydricity in carnation.

CleriGel too showed a positive response, with maximum number of healthy shoots obtained at a concentration of 0.2%. It has been reported with other genera that medium solidified with phytagel resulted in highest number of HS (Turner and Singha, 1990; Kadoka and Niimi, 2003; Modi *et al*, 2009). The medium solidified using 3% *Isabgol* also overcame vitrification i.e. all the shoots obtained were

Table 1. Effect of gelling agents on shoot bud induction from nodal explants of *Dianthus caryophyllus* cultured on MS + BAP (2.2 μM) and NAA (2.7 μM)

Gelling agent (%)	% Response	Mean ± S. D.	
		NS	HS
Agar			
0.9	100	2.3 ± 0.5	3.5 ± 1.2
1.0	60	4.5 ± 0.3	2.9 ± 0.2
1.2	40	7.3 ± 0.2	0
1.4	40	2.0 ± 1.2	0
CleriGel			
0.1	100	2.3 ± 0.1	4.6 ± 0.2
0.2	100	3.3 ± 1.2	0.2 ± 1.8
0.3	90	3.1 ± 1.5	2.5 ± 0.7
0.5	80	0	0
Isabgol			
1.0	90	0.4 ± 1.2	2.3 ± 0.8
2.0	100	2.2 ± 1.8	0.3 ± 0.1
3.0	100	6.2 ± 1.0	0
4.0	80	4.2 ± 0.8	0

NS- normal shoots, HS- hyperhydric shoots, S.D. - standard deviation

totally healthy and no signs of vitrification were observed (Fig. 1d). Though it overcame vitrification, the shoots were not completely unvitrified hence the subculturing was unworkable due to the loss of the mother tissue and so the shoots could not proliferate. Henceforth, 1.2% agar was used in subsequent experiments.

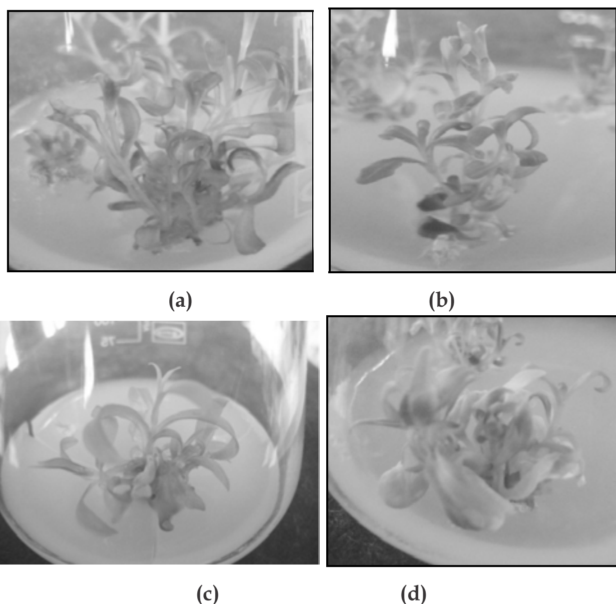

Fig.1. Shoot bud induction from nodal explants of *Dianthus caryophyllus* cultured on MS medium with BAP (2.2 μM) and NAA (2.7 μM) and (a) 0.9 % agar as gelling agent. (b) 1.2% agar as gelling agent. (c) 1.0% agar as gelling agent. (d) 3% isabgol as gelling agent

Pigment Content

The amount of chlorophyll reduced drastically in the HS contrary to carotenoid content (Fig.2). Deficiency of chlorophyll in HS can be attributed to the watery tissues of the vitrified shoots. Similar observations have been accorded in carnation (Winarto *et al.*, 2004), *Tagetes* Modi *et al*, 2009) and *Allium* (Wu *et al*, 2009).

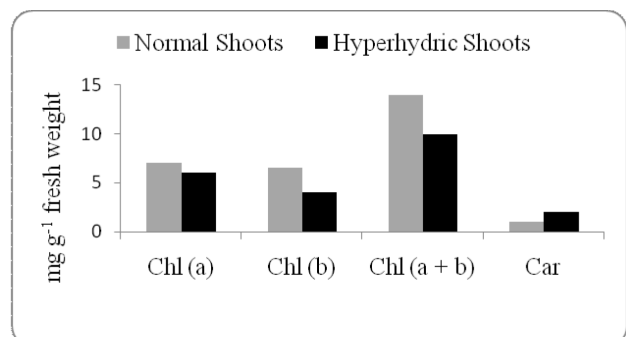

Fig.2. Pigment content in 4 weeks old normal and hyperhydric shoots of *in vitro Dianthus caryophyllus.* Chl (a)- chlorophyll a, Chl (b)- chlorophyll b, Chl (a+b)- chlorophyll a plus b, Car- carotenoids

Additionally, according to Chakraborty *et al*, (2005) chlorophylls a and b and carotenoid concentrations were significantly reduced as compared to the healthy leaves of apple.

Total Protein Content

The total amount of protein was found to be less in hyperhydric shoots (HS) as compared to the normal

shoots (NS). A remarkably discrete profile of proteins was obtained on performing the SDS-PAGE electrophoresis (Fig. 3). The analysis of protein accumulation in *in vitro* regenerated HS of carnation revealed the inhibited or under-expression of 80 kDa proteins and the distinct expression of 110 kDa protein. Secondly, it revealed that a 55- 60 kDa protein accumulates as a major protein in NS but evidently is subdued in HS. Referring to similar findings by Ziv and Ariel (1992) and Jones *et al* (1993), since the electrophoretic migration of this protein is consistent with the molecular weight of the large subunit of the enzyme 1,5-ribulose bisphosphate carboxylase/ oxygenase (Rubisco) and also because a low photosynthetic rate has been recorded owing to the decrease in plant pigments resulting from hyperhydricity, this down-regulated protein may be identified as Rubisco (Fontes *et al*, 1999). The up-regulation of this polypeptide as a major protein in NS substantiates the finding.

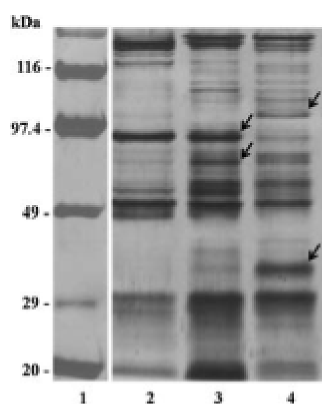

Fig.3. SDS-PAGE pattern of proteins during shoot bud induction of *Dianthus caryophyllus*: molecular mass marker (1), explant (2), normal shoots (3) and hyperhydric shoots (4). Equal amounts of protein (40 □g) were loaded in each lane. *Arrows* indicate the new and affected bands detailed in the text

Furthermore, a 30 kDa protein was found to be present in HS but not in NS (Ziv, 1991; Ziv and Ariel, 1992). This observation was also made earlier by Picoli *et al* (2008) stating that it may be a polypeptide regulating important aspects in the plant tissue. Peroxidases associated with lignin synthesis, related to lignifications were also detected in large amounts in HS and found to have a molecular weight of 30-32 kDa (Van Huystee, 1987). Peroxidases have also been detected in large amounts in HS in several other studies (Phan and Hegedus, 1986; Piqueras *et al*, 2002; Saher *et al*, 2004). Further several proteins expressed themselves distinctively in the NS and are absent in the HS, or their amounts are lower than in NS.

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