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EFFECT OF PERMEABLE VESSEL CLOSURE AND GELLING AGENT ON REDUCTION OF HYPERHYDRICITY IN *IN VITRO* CULTURE OF CARNATION

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ABSTRACT

Hyperhydricity, an abnormal morphological appearance and physiological function, is an important problem in carnation tissue culture. The problem causes premature flowering, high occurrence of abnormal shoots, difficulty in transferring hyperhydric plantlets to soil, and low survival rate of plantlets. High relative humidity and the water potential are considered as the key factors involved in the abnormality. Furthermore, permeable culture vessel and gelling agent were assured to be high potential treatment to eliminate it. Objective of this research was to reduce hyperhydricity in regenerants of carnation using different permeable vessel closures and gelling agents and to assess the multiplication and acclimatization abilities of recovered shoots. Experiment was arranged in randomized complete block design with four replications. First factor was different types of closure, i.e. cotton wool, plastic wrap, parafilm and aluminium foil, while second one was gelling agents, i.e. bacto agar, phytagel, swallow agar, and Type 900 agar. The recovered shoots were then multiplied, rooted, and acclimatized. The results showed that hyperhydricity was successfully reduced by applying permeable closure (cotton wool and plastic wrap) in combination with Type 900 agar. The combination of plastic wrap and Type 900 agar was the most appropriate treatment in reducing hyperhydricity and producing good quality shoots. The treatment reduced the problem down to 23% of total condition of hyperhydricity (100%) and increased leaf chlorophyll content from 0.0883 to 0.1288 mg mg^{-1}. The plastic wrap was easily applied and cheaper material compared to cotton wool. The recovered shoots were able to produce 1-3 healthy axillary shoots and easily rooted on half-strength MS. The recovered plantlets were simply acclimatized with survival rate up to 100% on kossas peat + soil (1:1, v/v) and flowered 4-5 months after acclimatization with decreasing in number and size of flower.

[*Keywords*: *Dianthus caryophyllus*, *in vitro* culture, *in vitro* regeneration, adaptation]

INTRODUCTION

Hyperhydricity is an abnormal condition morphologically and physiologically on herbaceous and woody plants during *in vitro* vegetative propagation (Debergh *et al*. 1981). High relative humidity and the water potential are the key factors involved in the abnormality (Debergh *in* Ziv 1991). Superfluous nutritional factors both minerals and carbohydrates, high levels of growth regulators, and low light intensity are also major causes of shoot malformation (Ziv 1986). The problem is commonly found in plant tissue culture especially in Caryophyllaceae (Mii *et al*. 1990). The hyperhydricity causes reduction of apical dominance, hypertrophy cells of plantlets, and defective cell walls (Werker and Leshem 1987). Debergh *in* Ziv (1991) reported that high relative humidity and water potential are the key factors inducing hyperhydricity in *in vitro* culture.

Hyperhydritised plants are characterized by low leaf chlorophyll content (less than 0.10 mg mg^{-1}) (Ziv *et al*. 1983; Olmos and Hellin 1998), higher fresh weight and low dry weight (Böttcher *et al*. 1988), and hypertrophy of cells with large vacuolated mesophyll cells and intercellular spaces (Olmos and Hellin 1998). The plants are more susceptible to desiccation when transferred to the *ex vitro* conditions (van Altvorst *et al*. 1996; Majada *et al*. 2001).

The undesirable effects of hyperhydricity due to high relative humidity and water potential can be lowered by increasing the agar concentration, applying slanted agar surface (Leshem 1983a; Ziv *et al*. 1987; Böttcher *et al*. 1988), and using different types and concentrations of gelling agent in the medium (Debergh *et al*. 1981; Leshem 1983a, 1983b). Other studies used looser than tighter closures or ventilated culture vessels (Hakkaart and Versluijs 1983; Majada *et al*. 2000; Dantas *et al*. 2001). Application of looser closures or permeable closure or ventilated culture vessels successfully overcame hyperhydricity as reported by Hakkaart and Versluijs (1983) on carnation, Dillen and Buysens (1989) on *Gypsophila paniculata*, and Majada *et al*. (1997, 2000, 2001) on several carnation cultivars. Application of permeable closures combined with gelling agents has high potential to be applied in reducing the problem.

The objective of this research was to transform hyperhydricity in regenerants of carnation into normal condition of *in vitro* explants and to assess

the multiplication of shoots and acclimatization abilities of recovered plantlets. One of combination treatments of vessel closure and gelling agent was expected to decrease hyperhydricity in regenerants of hyperhydritised explants. The recovered shoots would easily be multiplied and acclimatized as it happened on the normal ones.

MATERIALS AND METHODS

Preparation of Hyperhydritised Shoots

Dianthus caryophyllus L. cv. Maldives is a spray type of carnation, has red flower and was obtained from Cameron Highland farmers. Rooted shoot-cuttings were planted in combination media of paddy charcoal + kossas peat (1:1, v/v) in polyethylene bag of 15-cm diameter and put under glasshouse. Hyperhydritised shoots of this cultivar were prepared by culturing the nodal explants on Murashige and Skoog (MS) medium containing 2 mg l^{-1} 6-benzyladenine (BA) and 0.9 mg l^{-1} α-naphthalene acetic acid (NAA) using tighter closure (aluminium foil or seal tip). All cultures were maintained at 25 ± 2ºC with 16-hour photoperiod under cool white fluorescent lamps providing 25 µmol m^{-2} s^{-1} light intensity for 6 weeks, and 100% of hyperhydritised shoots were obtained after 6-week incubation. The first and the second nodes of hyperhydritised shoots were excised and cultured with the basal cut surface down on the medium. Hyperhydritised shoots that were almost similar in height and size were used in the experiment.

Assessment of Different Types of Closure and Gelling Agents to Minimize Hyperhydricity

The types of closure tested were cotton wool, plastic wrap, parafilm, and aluminium foil, while the gelling agents were bacto agar (Difco-USA, 10 g l^{-1}), phytagel (Sigma-USA, 3 g l^{-1}), swallow agar (Swallow Globe-INA, 8 g l^{-1}), and Type 900 agar (LCP-MAL, 7 g l^{-1}). The different types of closure were used as first factor and different gelling agents as second factor. MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA was used.

Parameters observed were percentage of hyperhydritised shoots, leaf chlorophyll content, and number of shoots produced per explant. Relative water loss, increase in agar concentration, and evaporation rate as described by Dillen and Buysens (1989) were measured for supporting information on discussion.

Water loss of cultures for any specific treatment was approximated by averaging the weight loss of medium. The increase in agar concentration (IAC) due to evaporation could be calculated from the relative water loss (RWL) described by Dillen and Buysens (1989) as follows: RWL (%) = weight loss x (initial weight of medium)$^{-1}$x 100; IAC (%) = {100 x (100 – RWL) $^{-1}$ - 1} x 100. The evaporation rate was measured by calculating weight loss per day.

Leaf chlorophyll content was measured before culture and at 6 weeks after culture initiation using direct (Minolta ™ SPAD chlorophyll meter model 502) and indirect (Scanning Spectrophotometer type UV 3101PC) measurement methods as described by Coombs and Hall (1987). Leaf chlorophyll was first measured by SPAD, followed by Spectrophotometer at 664 and 647 nm. Two hundred and fifty milligrams of leaves (normally using 3 cm^2 of leaves) were used. The concentration of leaf chlorophyll content was calculated using the formula described by Coombs and Hall (1987) with minor modification as follows: chlorophyll a (mg cm^{-2} modified to mg mg^{-1}) = {3.5/250 x (13.19 A_{664}-2.57 A_{647})}; chlorophyll b (mg cm^{-2} modified to mg mg^{-1}) = {3.5/250 x (22.10 A_{664}-5.26 A_{647})}; and total chlorophyll = chlorophyll a + b. Other parameters were observed at 6 weeks after culture initiation. Transferable shoot is a shoot that had more than 1 cm in length and could be cut and subcultured easily on the fresh medium after 6 weeks of culture initiation.

Percentage of hyperhydritised shoots was calculated using the formula described by Pathak (1972), while level of leaf hyperhydricity was scored from 0 to 5 (Jain *et al.* 1997) as follows: 0 = no hyperhydricity, 1 = 1-5% of leaf hyperhydricity, 2 = 6-25% of leaf hyperhydricity, 3 = 26-50% of leaf hyperhydricity, 4 = 51-75% of leaf hyperhydricity, 5 = 76-100% of leaf hyperhydricity.

The factorial experiment was arranged in a randomized complete block design with four replications. Each treatment consisted of 12 explants per replication. The data were analyzed by analysis of variance (ANOVA) using SAS for Window Release 6.12. In cases where significant differences were obtained ($p = 0.05$), Duncan's Multiple Range Test (DMRT) was used for comparison between means.

Multiplication of Recovered Hyperhydritised Shoots

Upper nodes of recovered shoots obtained from the previous experiment were cultured on two selected concentrations (SC) of BA and NAA, i.e. MS medium containing 0.1 mg l^{-1} BA + 0.02 mg l^{-1} NAA (SC-3) and 0.1 mg l^{-1} BA + 0.01 mg l^{-1} NAA (SC4). Forty-eight explants were cultured into 12 flasks. Subculture of

shoots and collection of data were carried out after most of the shoots reached the 100-ml flask closure (6-7 weeks after culture initiation). Parameter observed was total shoots produced at 6 weeks after culture initiation.

Acclimatization of Recovered Plantlets

Well-rooted recovered shoots were prepared on half-strength MS medium with 6 g l^{-1} of Type 900 agar, slanted agar surface, and carton paper as vessel closure for rooting. After 1-month incubation, rooted shoots were taken out from the culture vessels and then placed under running tap water to remove the agar. Plantlets were immersed in 1% benomyl solution for 1 minute, then planted in potting media covered with polyethylene plastic for 7 days. Gradual steps of plantlet establishment were started from incubation of plantlets under room condition for 1 month, followed by potting plantlets in small plastic polybags and placed in the glasshouse.

Media for plantlet acclimatization were paddy charcoal and kossas peat + soil in the ratio of 1:1 (v/v). Due to limitation in acclimatized-plantlet material, the media were used in different times. Fifteen plantlets were planted in the media and replicated six times. Percentage of survival, leaf chlorophyll content, plant height, and number of leaves per plant were observed after 1 month in incubation room and 1.5 months in glasshouse.

RESULTS AND DISCUSSION

Effect of Different Types of Closure and Gelling Agents on Hyperhydricity

Recovered shoots were monitored for 7-9 days after culture initiation. The shoots generally had greener and thinner leaves than hyperhydritised ones, and found in flask with bacto agar and Type 900 agar as gelling agents with cotton wool and plastic wrap as the closure. However, inhibited growth of shoots was generally observed on MS medium with bacto agar. The recovered shoots maintained their normal and healthy growth until the end of observation (Fig. 1).

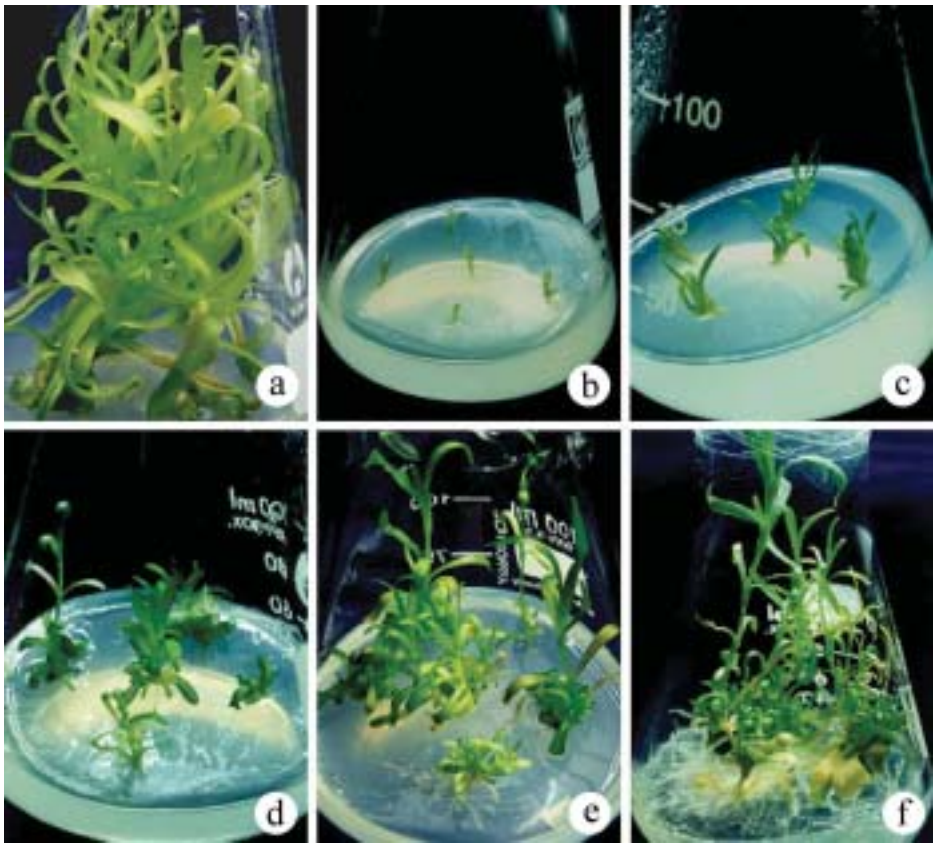

Fig. 1. Development of hyperhydritised explants of carnation cultured on MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA with Type 900 agar as gelling agent and plastic wrap as vessel closure; a = hyperhydritised shoots as explant source, b = first and second nodes at culture initiation, c, d, e, f = explant growth at 1, 2, 3-4, and 5-6 weeks after culture initiation, respectively.

Different growth performance of hyperhydritised shoots in different gelling agents was presumably affected by gel strength, mineral composition, inhibitory compounds, water availability, and pH of the gelling agents (Scholten and Pierik 1998). From the four gelling agents tested, at least three different characteristics of shoot growth were observed (Table 1).

Inhibited growth of explant indicated by shorter internodes and smaller leaves were showed by bacto agar. Its characters were usually accompanied by high pH and low moisture content which inhibited the growth of explants (Pierik 1991; Scholten and Pierik 1998). Faster elongation and improvement in shoot height with high hyperhydricity on phytagel were supported by high gel strength, low concentration of salt, suitable pH, and low Cl^- content that indicated high purity of the gelling agent (Scholten and Pierik 1998). Nairn *et al.* (1995) reported that high purity of gelling agent indicated non-toxic, but resulted hyperhydric tissues. Normal growth with longer internodes and greener leaves was indicated by Type 900 agar. According to Nairn *et al*. (1995), the agar contains components that control hyperhydricity. The gelling agent also contains carbohydrates, traces of amino acids, and vitamins (Torres 1989).

Combination of plastic wrap and Type 900 agar showed highest reduction (23%) on hyperhydricity (Table 2) and highest leaf chlorophyll content (0.1288 mg mg^{-1}) (Table 3). However, the combination did not significantly differ with that of cotton wool and Type 900 agar. The first combination improved percentage of leaf chlorophyll content up to 62% and produced 4.2 shoots per explant (Table 4). The second best ones improved percentage of leaf chlorophyll content till 57.7%. The combination yielded the highest number of shoots per explant (9.2) and was significantly different with those other ones.

High ability of cotton wool and plastic wrap in reducing hyperhydricity was also indicated by high capacity of both closures in increasing relative water loss and agar concentration of the medium (data not shown). The highest relative water loss (0.13% day^{-1}) was noted by cotton wool, followed by plastic wrap (0.027% day^{-1}). The cotton wool and plastic wrap increased agar concentration up to 1.17 and 0.24 mg l^{-1} day^{-1}, respectively, with higher evapotranspiration rate (130 and 27 mg day^{-1} for cotton and plastic wrap, respectively) compared to aluminium foil and parafilm (15 and 4 mg day^{-1}, respectively). The high capacity of plastic wrap in lowering hyperhydricity was also

Table 1. Characteristics of gelling agents on growth of carnation explants.

Characteristics	Bacto agar (10 g l^{-1})	Phytagel (3 g l^{-1})	Swallow agar (8 g l^{-1})	Type 900 agar (7 g l^{-1})
Gel strength	Low	High	Moderate	Moderate
Salt concentration	High	Low	High	Moderate
Chloride content	High	Low	High	Low
Color	Yellow-brown	Clean	Light yellow	Light yellow
Growth response	Inhibited	Fast elongation	Light inhibited	Normal

Table 2. Effect of closures and gelling agents on percentage of hyperhydritised shoots of carnation at 5-6 weeks after culture initiation.

Closure	Hyperhydritised shoots (%)				CV (%)
	Bacto agar	Phytagel	Swallow agar	Type 900 agar	
Cotton wool	47.6a	64.3a	50.9a	26.2c	12.38
	A	A	A	B	
Plastic wrap	35.1a	61.9a	64.0a	22.7c	6.04
	B	A	A	C	
Parafilm	46.5a	64.9a	56.4a	70.7a	9.46
	B	A	A	A	
Aluminium foil	46.0a	64.1a	63.9a	42.5b	7.04
	B	A	A	B	
CV (%)	7.20	6.39	10.69	11.35	

Means followed by the same letter in the same column are not significantly different based on DMRT (p = 0.05).

Table 3. Effect of closures and gelling agents on leaf chlorophyll content of carnation shoots.

Closure	Leaf chlorophyll content (mg mg^{-1})				CV (%)
	Bacto agar	Phytagel	Swallow agar	Type 900 agar	
Cotton wool	0.0943a	0.0894a	0.1006a	0.1252a	10.70
	B	B	B	A	
Plastic wrap	0.0905a	0.0966a	0.0894b	0.1288a	12.10
	B	B	B	A	
Parafilm	0.0860a	0.0884ab	0.0917ab	0.0840c	12.08
	A	A	A	A	
Aluminium foil	0.0893a	0.0816b	0.0846b	0,0977b	9.69
	B	C	CB	A	
CV (%)	9.71	10.82	11.24	12.25	

Means followed by the same letter in the same column are not significantly different based on DMRT (p = 0.05).

Table 4. Effect of closures and gelling agents on number of shoot per explant of carnation at 5-6 weeks after culture initiation.

Closures	Number of shoots per explant				CV (%)
	Bacto agar	Phytagel	Swallow agar	Type 900 agar	
Cotton wool	3.4a	6.7a	4.3a	9.2a	12.07
	C	B	C	A	
Plastic wrap	2.4a	4.2b	5.1a	4.2bc	11.40
	B	A	A	A	
Parafilm	2.1a	5.4ab	4.2a	3.3c	15.73
	B	A	A	AB	
Aluminium foil	3.3a	4.5ab	4.2a	5.3b	12.84
	B	A	A	A	
CV (%)	19.12	11.93	9.44	12.18	

Means followed by the same letter in the same column are not significantly different based on DMRT (p = 0.05).

affected by no shading effect of the treatment compared to others.

Application of permeable closures (cotton wool and plastic wrap) successfully reduced hyperhydricity, so improved explants recovered from hyperhydricity. Cotton wool and plastic wrap gave possibility in increasing gas exchange potential that occurred between inside and outside of culture vessel, reducing relative humidity and water potential (Debergh *et al*. 1981; Hakkaart and Versluijs 1983; Ziv *et al*. 1983; Ziv 1986, 1991; Majada *et al*. 1997). The permeable closure reduced the risk of water logging and ethylene accumulation, which caused enzymatic inhibition and poor development of the plantlets (Kevers *et al*. 1984; Rossetto *et al*. 1992). In carnation, a higher amount of ethylene was produced during the first, second, and fourth weeks of culture (Fal *et al*. 1999). Utilization of parafilm and aluminium foil as closure was not able to reduce hyperhydricity due to less and/or the absence of gas exchange potential occurred. The condition was indicated by low evapotranspiration rate of parafilm (4 mg day^{-1}) and aluminium foil (15 mg day^{-1}) compared to cotton wool (130 mg day^{-1}) and plastic wrap (27 mg day^{-1}).

Increasing the potential of gas exchange between the external and internal atmospheric condition of culture vessel and lowering the high humidity lead to improve CO_2 concentration in culture vessel (Capellades-Queralt *et al*. 1991). Furthermore, higher concentration of CO_2 stimulated high photosynthetic ability of *in vitro* plants and reduced chlorophyllous deficiency (Kozai 1991). Less chlorophyllous deficiency was also recorded in this study due to the application of permeable closures. The treatments had successfully improved leaf chlorophyll content of shoots raised from hyperhydritised plants and induced the stomata to develop and conduct the function normally (Ziv *et al*. 1987; Majada *et al*. 2001).

Although cotton wool was the best closure to obtain healthy axillary proliferated shoots with low

hyperhydricity, in commercial scale, application of the closure was not practical due to difficulty in preparing the same compactness. Moreover, the material is more expensive compared to plastic wrap. Type 900 agar was the most suitable gelling agent to produce good quality axillary shoots and in combination with plastic wrap exhibited good results in reducing percentage of hyperhydritised shoots and improving leaf chlorophyll content and growth of shoots. The plastic wrap is a cheaper material and easier in application compared to cotton wool. From another study, it was also informed that normal condition of leaf chlorophyll content of carnation field plants was 0.3198 mg mg^{-1} in average, 0.2471 mg mg^{-1} for healthy *in vitro* plantlet, and less than 0.0883 mg mg^{-1} for severe hyperhydricity.

Multiplication of Recovered Hyperhydritised Shoots

Both selected concentration of BA and NAA did not show differences in all parameters observed. Although shoot height and percentage of transferable shoots tended to increase respectively from 1.96 to 3.61 cm and from 75 to 90%, production of axillary shoot decreased in each subculture. The highest shoot production was 130 shoots on SC-3 at the first subculture and 106 shoots on SC-4 after the first culture. From this study, one node produced 3.3 shoots on SC-3 and 2.5 shoots on SC-4. The total shoot production tended to reduce in the next subculture (Fig. 2). In each subculture, 2-3 shoots were obtained at the end of the observation.

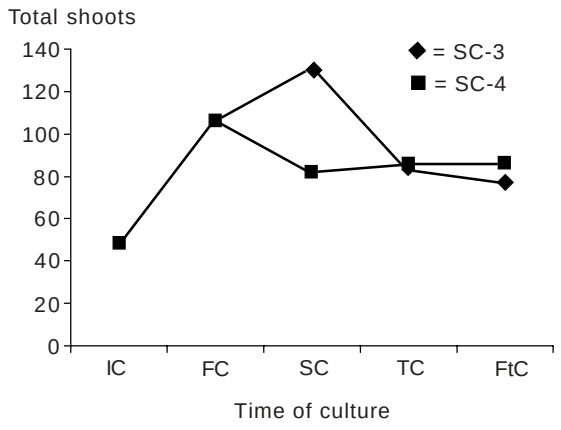

Fig. 2. Effect of selected concentrations of BA and NAA on total shoot production from node explants of recovered carnation shoots; SC-3 = MS + 0.1 mg l^{-1} BA + 0.02 mg l^{-1} NAA; SC-4 = MS + 0.1 mg l^{-1} BA + 0.01 mg l^{-1} NAA, IC = initial culture, FC = first culture, SC = second culture, TC = third culture, FtC = fourth culture.

SC-3 and SC-4 usually produced less axillary proliferated shoots. The axillary shoots produced in each subculture had greener and broader leaves, longer stem internodes. The shoots were good for preparing rooted shoots (Fig. 3).

The low ability of recovered shoots to produce axillary shoots was influenced by the hyperhydricity condition of explants as reported by Werker and Leshem (1987). The recovered shoots grew as normal shoots. Node explants of recovered shoots harvested from the first culture and placed on selected medium with slanted agar surface in ventilated culture vessel could maintain a normal growth of shoots. Some of them were able to continue their growth in the absence of roots (Leshem and Shachs 1985). The recovered shoots were easily rooted on half-strength MS medium.

Acclimatization of Recovered Plantlets

Gradual steps of acclimatization improved the ability of recovered plantlets to adapt to the *ex vitro* environment. After opening the transparent plastic cover, plantlets underwent a shock for 2-3 days. The plantlets generally appeared wilted, especially in young leaves. Five days later, plantlets began to grow and develop. New leaves were usually produced 10-13 days after opening the plastic.

Leaves from tissue culture condition easily turned brown and dried up during acclimatization especially in recovered plantlets cultured in paddy charcoal, but did not occur in recovered plantlets planted in soil + kossas peat (1:1, v/v). The leaf browning was most probably influenced by changing environmental growth (temperature and humidity) and nutrient availability in the acclimatization media.

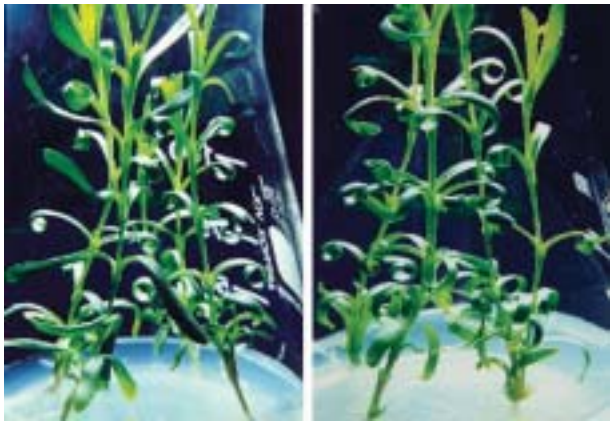

Fig. 3. Similar performance of carnation shoots on MS + 0.1 mg l^{-1} BA + 0.02 mg l^{-1} NAA (left) and MS + 0.1 mg l^{-1} BA + 0.01 mg l^{-1} NAA (right).

Recovered shoots exhibited high survivability in both media tested. Quite similar results were observed in all parameters measured during *ex vitro* plantlet establishment in the incubation room. Recovered plantlets cultured in paddy charcoal medium indicated high percentage of survival. However, the survival rate was not as high as in kossas peat + soil (Table 5).

All acclimatized plantlets in kossas peat + soil medium from incubation room were transferred to small polybags containing the same medium and acclimatized in the glasshouse. The plantlets still exhibited high survivability upon transfer to the glasshouse. The percentage of plantlet survival was 89-100% with 94.4% on the average (Table 6). All plantlets showed normal growth (Fig. 4). It showed that plantlets recovered from hyperhydricity had high survivability as normal plants during glasshouse acclimatization.

Recovered plantlets flowered 4-5 months after acclimatization. Flower color was red, similar to those produced from normal plantlets. However, the flowers were reduced in size and number (Fig. 5).

High survivability of recovered plantlets both in paddy charcoal and combination of kossas peat + soil (1:1, v/v) were 97% in the incubation room and 94% in the glasshouse. The results showed that plantlets recovered from hyperhydricity were still able to survive in *ex vitro* environment. The high percentage of survival was affected by all treatments previously applied with the major factor due to the application of ventilated culture vessel during preparation of rooted shoots.

Well preparing rooted shoots under ventilated culture vessel followed by gradual steps of acclimatization greatly influenced plantlet ability to adapt with *ex vitro* environment. The method promoted *in vitro* hardening of micropropagated plantlets as reported by Dillen and Buysens (1989) in *Gypsophila paniculata*, Dantas de Oliveira *et al*. (1997) in carnation, and Majada *et al*. (1997; 2000; 2001) in several carnation cultivars, reduced degree and duration of wilting after plantlet transfer to acclimatized media (Hofman *et al*. 2002) and produced healthy plantlets with good quality shoots and roots.

Table 5. Acclimatization of recovered plantlets of carnation in incubation room.

Parameters	Media	
	Paddy charcoal	Kossas peat + soil (1:1, v/v)
Survivability(%)	93.3 ± 8.16	100.0 ± 0.00
Leaf chlorophyll content (mg mg^{-1})	21.6 ± 2.36	22.5 ± 3.95
Plant height (cm)	7.57 ± 0.81	7.69 ± 0.91
Number of leaf per plant	15.0 ± 2.45	16.6 ± 2.37

Data ± standard deviation (SD) were mean values from measurement of 15 plantlets replicated six times.

Table 6. Acclimatization of recovered plantlets of carnation in glasshouse.

Parameters observed	Kossas peat + soil (1:1, v/v)
Survivability (%)	94.4 ± 4.60
Leaf chlorophyll content (mg mg^{-1})	26.1 ± 3.76
Plant height (cm)	13.4 ± 2.52
Number of leaf per plant	26.4 ± 6.01

Data ± standard deviation (SD) was mean values from measurement of 15 plantlets replicated six times.

Fig. 4. Similar growth performance of recovered plantlets (a) and normal plantlets (b) of carnation during acclimatization in the glasshouse.

Fig. 5. Different performance of carnation flowers produced from recovered (left) and normal plants (right).

CONCLUSION

Plastic wrap in combination with Type 900 agar was the most appropriate treatment in reducing hyperhydricity and producing good quality shoots of carnation. The combination reduced the problem down to 23% of total condition of hyperhydricity (100%) and gave higher leaf chlorophyll content (0.1288 mg mg^{-1}) compared to that of severe hyperhydricity (less than 0.0883 mg mg^{-1}). The plastic wrap was easily applied and cheaper material compared to cotton wool. The recovered shoots were able to produce 1-3 healthy axillary shoots and easily rooted on half-strength MS.

The recovered plantlets were simply acclimatized with high survivability (up to 100%) on kossas peat + soil (1:1, v/v). Acclimatized plants flowered 4-5 months after acclimatization with reducing in number and size of flower. The suitable combination treatment of the study has high potential to be applied in reverting and overcoming hyperhydritised explants into their normal condition.

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