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Reduction of Hyperhydricity in Micropropagated French Marigold (*Tagetes patula* L.) Plants by Modified Medium Parameters

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ABSTRACT

Adventitious shoot bud regeneration was obtained on Murashige and Skoog (MS) medium supplemented with 4.4 µM 6-benzylaminopurine (BA) and 2.8 µM indole-3-acetic acid (IAA) from cotyledon explants of 12 *Tagetes patula* varieties. In order to reduce the occurrence of hyperhydrated shoots (HS), the effect of various gelling agents, culture vessels and ammonium ions in the medium was investigated in var. 'French Marigold Safari Red'. When the induction medium contained agar (0.9-1.5%) as the gelling agent, the highest organogenic response was observed on 0.9% agar with 11.2 normal shoots (NS) per explant. Among different culture vessels, Petri dishes were found to be most suitable for induction of NS. Vigorous shoots were produced on optimized medium [MS medium containing 10.3 mM NH_4^+ (half the standard MS level of 20.6 mM), BA (4.4 µM) and IAA (2.8 µM)] yielding 25.4 NS per explant. Both chlorophyll content and peroxidase activity were lower in HS than in NS. Full-strength MS medium without any plant growth regulator was effective for elongation and rooting. Plantlets with a well developed shoot and root system obtained on optimized medium were acclimatized with a maximum survival rate of 90% on soil and organic manure in a 1: 1 ratio.

Keywords: ammonium ions, chlorophyll, isabgol, peroxidase, phytagel
Abbreviations: BA, 6-benzylaminopurine; **CT**, culture tube; **EF**, Erlenmeyer flask; **GA_3**, gibberellic acid; **HS**, hyperhydric shoot; **IAA**, indole-3-acetic acid; **MS**, Murashige and Skoog; **NS**, normal shoot; **PD**, Petri dish

INTRODUCTION

Tagetes patula L. (Family: Asteraceae), commonly called French marigold, is used for ornamental, medicinal and industrial purposes. It is native to Mexico and South America. The plant contains bioactive compounds that are used in traditional medicine and employed as insecticides, fungicides and nematicides (Vasudevan *et al*. 1997). Lutein is the main carotenoid in the flowers of marigold (Delgado Vargas *et al*. 2000); it is an antioxidant and possesses pharmacological importance (Tsao and Akhtar 2005; Bashir and Gilani 2008). The essential oils present in the plant are also used in medicine (Pérez Gutiérrez *et al*. 2006; Reichling *et al*. 2009).

Clonal propagation of an elite genotype helps to develop a regeneration protocol for genetic manipulation which would provide a promising approach by facilitating the transfer of valuable genes. Adventitious shoot regeneration using floret (Kothari and Chandra 1984), hypocotyl (Belarmino *et al*. 1992), cotyledon (Mohamed *et al*. 1999), shoot tip (Misra and Datta 2001) and leaf (Vanegas *et al*. 2002) explants has been established for several *Tagetes* species. Qi *et al.* (2005) developed a regeneration protocol for *T. patula* using leaves, cotyledons and hypocotyls. Hairy root cultures of *T. patula* have been established for thiophene production by various groups (Arroo *et al.* 1995; Bais *et al*. 2000; Suresh *et al*. 2001). Szarka *et al.* (2007) investigated the occurrence of volatile compounds in flowers and roots of intact plants and *in vitro* hairy root cultures of *T. patula*.

The growth and development rates of plant cultures *in vitro* are genetically determined (Casanova *et al.* 2008; Kakani *et al*. 2009) although they are limited by the physical and chemical microenvironment in the cultures. Hyperhydricity is a morphological and physiological disorder that has been commonly experienced in tissue culture. Losses have been recorded in *Tagetes* (Aguilar *et al.* 2000), *Dianthus* (Piqueras *et al*. 2002) and *Thapsia* (Makunga *et al*. 2006). These losses together with poor survival rate of hyperhydric shoots (HSs) when transferred to *ex vitro* condition limit the potential of *in vitro* technique for mass propagation (Kevers *et al*. 2004; Winarto *et al.* 2004; Ivanova and van Staden 2008).

Several factors are responsible for hyperhydricity (Debergh *et al*. 1992). A number of investigations have reported that high level of exogenous cytokinins, usually in a concentration-dependent manner (Ivanova *et al*. 2006), large quantities of ammonium ions (Brand 1993), the gelling agent in the culture medium (Tsay 1998) and the composition of gaseous environment (Park *et al*. 2004) significantly influence hyperhydricity in different species (reviewed by Olmos 2006).

Hyperhydric malformation has been associated with biochemical deviations in the synthesis of tetrapyrrole-containing compounds like chlorophyll (Franck *et al*. 1995), the activity of different enzymes such as peroxidase (POD) (Letouze and Daguin 1987), and ethylene and auxin metabolism (Phan 1991). Hyperhydricity leads to a deficiency of chlorophyll accumulation resulting in low photosynthetic capacity of the leaves. In HSs, due to lower activity of antioxidative enzymes, there is accumulation of undetoxified H_2O_2 (Wang *et al*. 2007). The phenomenon influences photosynthesis, transpiration, CO_2 and O_2 gas exchange, the inhibition of which could be detrimental for the survival of the plant. The lower activity of POD could result in the decrease of lignin synthesis and cell wall ligination, which might be the key factor leading to the increase in water con-

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tent, as observed in *Populus suaveolens* (Lin *et al.* 2004). The deficiency of detoxification capacity caused by the decreased activity of the protective enzymatic system might lead to a large accumulation of H_2O_2 and the enhancement of membrane lipid peroxidation, which might be the main cause leading to the occurrence of HSs (Lin *et al*. 2004).

The present work focuses on quantitative and qualitative physiological analyses carried out on normal shoots (NSs) and HSs based on differences in chlorophyll content and POD activity by defining the optimal composition of culture media under constant environmental condition to overcome hyperhydricity and to develop a highly efficient regeneration protocol.

MATERIALS AND METHODS

Plant material

Seeds of 12 varieties of *Tagetes patula* were procured from Namdhari Seeds Pvt. Ltd., Bangalore, India. Screening was done on the basis of germination response of different varieties on half-strength MS medium to select the best responding variety (**Table 1**). The seeds were washed in tap water and rinsed with 20% (v/v) detergent 'Extran' (Merck, India) and then surface sterilized with 0.1% mercuric chloride (w/v) for 3 min followed by three rinses with sterile distilled water. The seeds were soaked overnight in sterile distilled water and then placed on half MS (Murashige and Skoog 1962) medium for germination. The cotyledons from *in vitro*-germinated seedlings (5-7 days) were used as explants. Out of 12 varieties, 'French Marigold Safari Red' responded best, so it was selected for further studies.

Shoot regeneration medium and culture conditions

Various concentrations of 6-benzylaminopurine (BA; 2.2-22.2 µM) in combination with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and phenyl acetic acid (PAA), all at 2.8-17.1 µM, were tested for the induction of shoot buds. MS medium was supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (Merck, bacteriological grade). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 1.2-1.3 kg cm^{-2} for 20 min. All the cultures were incubated in a growth room at 26 ± 2°C and a 16-h photoperiod with 30 µmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps (Philips, India, 36W). The cotyledons cut from the petiolar end were placed with their dorsal surface in contact with the sterile medium. Weekly observations were recorded. After 4 weeks of culture, the shoot buds induced on cotyledon explants were excised from the base along with some mother tissue and placed on proliferation medium supplemented with BA (4.4 µM), IAA (2.8 µM) and gibberellic acid (GA_3; 0.5 µM).

Effect of gelling agent

The media used in the present investigation was gelled with 1.0-4.0% isabgol, 0.1-0.5% phytagel (Sigma, USA) or 0.9-1.5% agar (Qualigens, bacteriological grade). The most suitable quantity or concentration of these gelling agents was assessed. The cotyledon explants were cultured in 100 ml Erlenmeyer flasks (EFs) containing 40 ml of medium and three explants each.

Effect of culture vessel

The internal volume of the three culture vessels used viz. EF, plastic Petri dishes (Tarson, India, 90 mm diameter)(PDs) and culture tubes (Borosil, India, 25 × 150 mm) (CTs) were 135, 70 and 60 ml, respectively. Each EF, PD and CT contained 40, 30, and 20 ml MS medium, respectively. In the present study, we did not find any effect of explant density on regeneration in our initial experiments, so we accommodated maximum number of explants in the culture vessel that could grow easily and distinctly visualised for taking observations. There was no effect of explant density in the investigation. Hence, we cultured three explants in EF, two in CT and five in PD.

Table 1 Germination response of different varieties of *Tagetes patula* on half-strength MS medium.

Name of variety	% Germination
French marigold Boy Gold	60 d
French marigold Boy Orange	43.3 e
French marigold Boy Spry	23.3 g
French marigold Boy Yellow	30 f
French marigold Hero Spry	63.3 d
French marigold Little Hero Flame	90 b
French marigold Safari Bolero	70 c
French marigold Safari Queen	33.3 f
French marigold Safari Red	96.6 a
French marigold Safari Scarlet	90 b
French marigold Sparky Mix	70 c
French marigold Gold Safari	73.3 c

Means with the same letter(s) within the same column are not significantly different at the 5% level according to Duncan's Multiple Range Test (n = 30).

Effect of ammonium ions

Five concentrations of NH_4^+ ions were supplied in the form of NH_4NO_3 (0.0-41.2 µM) in MS medium containing BA (4.4 µM) and IAA (2.8 µM).

Chlorophyll estimation

Chlorophyll (Chl) was estimated in 4-week-old shoot buds (both NS and HS) by extraction of the pigment with 80% acetone (Arnon 1949). Fresh mass of the shoot buds (1 g) was ground in 10 ml acetone and centrifuged. Absorbance at 663 and 645 nm was measured with UV-Vis spectrophotometer (Shimadzu, UV-1700).

POD estimation

1 g fresh leaves of 4 week-old shoot buds was separated, chilled in a deep freeze (-20°C) and then homogenized in a mortar in Na-phosphate buffer (0.2 M, pH 7.0) and centrifuged at 10,000 × g for 15 min. The supernatant was used for estimating POD activity (Chanda and Singh 1997). POD (E.C. 1.11.1.7) activity was measured by recording the changes in absorbance at 470 nm (ΔA_{470}) for guaiacol due to the oxidation of hydrogen donors in the presence of H_2O_2 (Racusen and Foote 1965). The assay mixture (4 ml) consisted of 20 mM Na-phosphate buffer (pH 7.0), 50 mM hydrogen donor, enzyme and 10 mM H_2O_2. The activity was expressed as ΔA_{470} min^{-1} g fresh $weight^{-1}$.

Histological studies

Shoot buds (2 weeks-old) were fixed by immersion in formalin: acetic acid: ethanol (FAA, 5: 5: 90) for 48 hrs. After dehydration in a tertiary butyl alcohol (TBA) series, the material was infiltrated with liquid paraffin followed by three changes of paraffin wax with ceresin (Merck). Sections 10 µm thick were cut with a rotary microtome (WESWOX DPTIK, MT-1090 A 11101). Sections were kept for 2 days in xylene to remove the wax, passed through a decreasing alcohol series, stained with safranine (Himedia, India) (1%) and fast green (Merck) (0.5%), and again passed through an increasing alcohol series. The slides were mounted in DPX mountant (Merck), analyzed under a light microscope (Olympus, MAGNUS MLM 70702076, India) (Johansen 1940).

Experimental design and statistical analyses

The experiments were conducted in a complete randomized block design. The treatment for EFs consisted of 10 replicates, each with 3 explants. For PDs, it consisted of 6 replicates each with 5 explants and for CTs, 15 replicates of 2 explants each. The experiment was repeated in triplicate under identical conditions and values are expressed as the mean ± SD. Analysis of variance (ANOVA) appropriate for the design was carried out to detect the significant differences among the treatment means, which were compared using Duncan's multiple range test at the 5% probability level according to Gomez and Gomez (1984).

RESULTS

Adventitious shoot regeneration

Among various auxins (IAA, IBA, NAA, PAA) tried in combination with BA, IAA was found to be best for producing adventitious shoot buds (**Table 2**). Medium enriched with IBA or NAA or PAA in combination with BA could induce mainly pale, friable, rhizogenic callus which was non-morphogenic. The highest mean number of shoots (18.1) with a 100% regeneration rate was observed when cotyledons were cultured in EFs containing MS medium supplemented with 0.9% agar, BA (4.4 µM) and IAA (2.8 µM), but almost 35% shoots were hyperhydric (**Table 2**). This culture condition was considered as the 'control'. Further experiments were designed to reduce the problem of hyperhydricity by modifying different parameters in cultures.

Table 2 Effect of different concentrations of BA and auxin on shoot bud induction from cotyledon explants of *Tagetes patula*.

Plant growth regulators (µM)		Mean ± S.D		Callus/ Rhizogenesis
BA	**IAA**	**NS**	**HS**	
2.2	2.8	10.5 ± 0.5 b	4.4 ± 0.5 ef	-
4.4	2.8	11.7 ± 0.5 a	6.4 ± 0.4 b	-
8.8	2.8	8.6 ± 0.5 c	7.2 ± 0.5 a	-
13.3	2.8	6.8 ± 0.8 d	4.2 ± 0.5 f	-
22.2	2.8	5.0 ± 0.7 h	2.8 ± 0.4 jk	-
4.4	5.7	8.8 ± 0.8 c	6.2 ± 0.5 b	-
8.8	5.7	5.8 ± 0.5 e	2.4 ± 0.5 l	-
13.3	5.7	5.6 ± 0.8 ef	2.6 ± 0.5 kl	-
22.2	5.7	5.2 ± 1.3 gh	3.2 ± 0.5 hi	-
8.8	11.4	5.4 ± 1.1 fg	3.6 ± 0.3 g	-
13.3	11.4	7.0 ± 1.0 d	5.0 ± 0.7 d	-
22.2	11.4	5.2 ± 0.8 gh	5.6 ± 0.8 c	-
13.3	17.1	3.8 ± 0.4 j	2.0 ± 0.7 m	-
BA	**IBA**			
2.2	2.8	0 q	0 p	C
4.4	2.8	0 q	0 p	C+
8.8	2.8	1.8 ± 0.8 n	3.0 ± 1.2 ij	C
13.3	2.8	1.4 ± 0.5 o	4.2 ± 0.8 f	C
22.2	2.8	0 q	4.6 ± 0.5 e	C
4.4	5.7	0 q	0 p	C+
8.8	5.7	2.2 ± 0.8 m	3.4 ± 0.5 gh	C+
13.3	5.7	0 q	0 p	C+
22.2	5.7	0 q	0 p	C++
8.8	11.4	0 q	0 p	C++
13.3	11.4	0 q	0 p	C++
22.2	11.4	0 q	0 p	C++
13.3	17.1	0 q	0 p	C++
BA	**NAA**			
2.2	2.8	2.6 ± 0.5 l	0 p	CR
4.4	2.8	4.2 ± 0.4 i	0 p	CR
8.8	2.8	1.6 ± 0.5 no	0 p	CR
13.3	2.8	0.6 ± 0.5 p	0 p	C+R
22.2	2.8	0 q	2.4 ± 0.8 l	C+R
4.4	5.7	0 q	1.4 ± 0.5 n	C+R
8.8	5.7	0 q	1.2 ± 0.4 n	C++R
13.3	5.7	0 q	0.8 ± 0.4 o	C++R
22.2	5.7	0 q	0 p	C++R
8.8	11.4	0 q	0 p	C+++R
13.3	11.4	0 q	0 p	C+++R
22.2	11.4	0 q	0 p	C+++R
13.3	17.1	0 q	0 p	C+++R
BA	**PAA**			
2.2	2.8	0 q	0 p	C+
4.4	2.8	0 q	0 p	C+
8.8	2.8	3.0 ± 1 k	0 p	C+
13.3	2.8	1.4 ± 0.5 o	2.6 ± 0.5 kl	C+
22.2	2.8	0 q	3.6 ± 1.3 g	C+
4.4	5.7	0 q	4.2 ± 0.8 f	C+
8.8	5.7	0 q	2.6 ± 0.5 kl	C+R
13.3	5.7	0 q	1.4 ± 0.5 n	C+R
22.2	5.7	0 q	1.2 ± 0.4 n	C+R
8.8	11.4	0 q	0 p	C++R
13.3	11.4	0 q	0 p	C++R
22.2	11.4	0 q	0 p	C++R
13.3	17.1	0 q	0 p	C++R

NS = normal shoots, HS = hyperhydric shoots, S.D. = standard deviation, C = callus, R = rhizogenesis
C = 0.1- 0.25 g, C+ = 0.25-0.5 g, C++ = 0.6-1 g, C+++ = 1-3 g,
Each treatment consisted of ten replicates, each with three explants.
Means with the same letter(s) within the same column are not significantly different at the 5% level according to Duncan's Mutiple Range Test (n = 30).

Effect of gelling agent on shoot bud induction

In the case of induction medium containing agar (0.9-1.5%) as gelling agent, the highest organogenic response was observed on 0.9% agar with 11.2 NSs (**Fig. 1A**). This value decreased to 5.0 shoots/explant when the agar concentration was increased to 1.5% which amounted to a 2.2-fold decrease in shoot regeneration capacity. But with increasing agar concentration the number of HSs also decreased and at 1.25%, hyperhydricity was totally overcome. The maximum number of shoot buds was obtained on induction medium gelled with 0.2% phytagel (**Table 3**), but severely abnormal shoots (highly hyperhydric; 78%) were observed in the cultures (**Fig. 1B**). The addition of 3% isabgol as gelling agent in the induction medium produced many healthy shoot buds (15.4) and no morphological characters related to hyperhydricity; however, these shoot buds got entangled in the medium during subculture, so most of the mother tissue was lost and thus the shoots failed to proliferate (**Fig. 1C**). So, 0.9% agar was used in subsequent experiments.

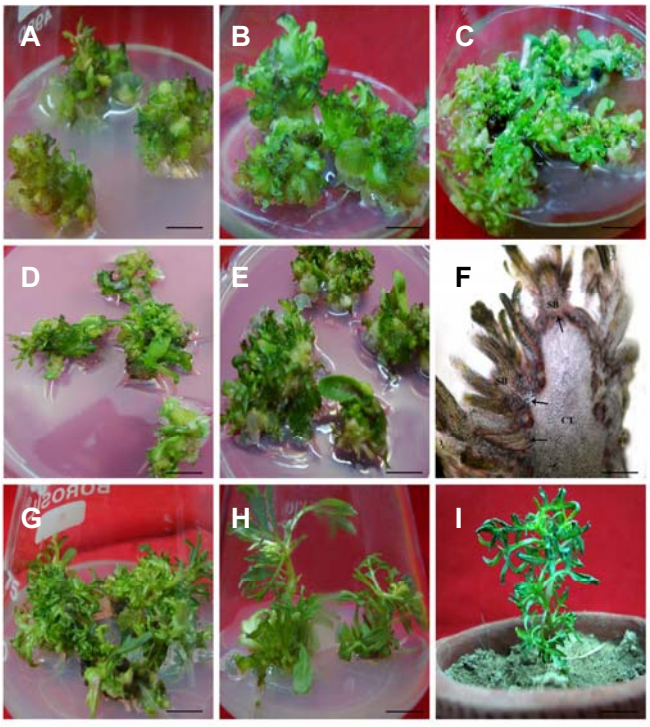

Fig. 1 Shoot bud induction from cotyledon explants of *Tagetes patula*. (**A**) Induction of shoot buds on MS + BA (4.4 µM) + IAA (2.8 µM) in EF using agar as gelling agent (bar = 1 cm). (**B**) Induction of shoot buds on MS+BA (4.4 µM) + IAA (2.8 µM) in EF using phytagel as gelling agent (bar = 1 cm). (**C**) Induction of shoot buds on MS+BA (4.4 µM) + IAA (2.8 µM) in EF using isabgol as gelling agent (bar = 1 cm). (**D**) Induction of shoot buds on MS+BA (4.4 µM) + IAA (2.8 µM) in PD using agar as gelling agent (bar = 1 cm). (**E**) Induction of shoot buds on optimized medium MS containing 10.3 mM NH_4NO_3+ BA (4.4 µM) + IAA (2.8 µM) in PD using agar as gelling agent (bar = 1 cm). (**F**) Histological details of adventitious shoot bud induction (2 weeks-old) (bar = 100 µm). Arrows indicate direct shoot bud induction from cotyledon, SB = shoot bud, CT = cotyledon. (**G**) Proliferation of shoot buds upon subculture on MS+ BA (4.4 µM) + IAA (2.8 µM) + GA_3 (0.5 µM) (bar = 1 cm). (**H**) Elongation and rooting of shoot buds on MS medium (bar = 1 cm). (**I**) Field transferred plant (bar = 1 cm).

Table 3 Effect of gelling agent on shoot bud induction from cotyledon explants of *Tagetes patula* cultured on MS + BA (4.4 µM) and IAA (2.8 µM).

Gelling agent (%)	% Response	Mean ± S.D.	
		NS	HS
Agar			
0.9*	100 a	11.2 ± 0.8 c	6.2 ± 0.8 e
1	60 d	9.6 ± 1.1 d	5.2 ± 0.8 f
1.25	40 e	8.8 ± 0.8 e	0 i
1.5	40 e	5.0 ± 0.7 h	0 i
Phytagel			
0.1	100 a	2.5 ± 0.5 j	13.2 ± 0.5 c
0.2	100 a	4.2 ± 0.4 i	15.6 ± 0.8 a
0.3	90 b	4.5 ± 0.5 i	13.8 ± 0.8 b
0.5	80 c	5.8 ± 0.8 g	8.0 ± 1.0 d
Isabgol			
1	90 b	8.0 ± 1.0 f	3.4 ± 0.5 g
2	100 a	9.4 ± 0.5 d	2.8 ± 0.8 h
3	100 a	15.4 ± 1.1 a	0 i
4	80 c	13.8 ± 0.8 b	0 i

* Control, NS = normal shoots, HS = hyperhydric shoots, S.D. = standard deviation
Each treatment consisted of 10 replicates, each with three explants.
Means with the same letter(s) within the same column are not significantly different at the 5% level according to Duncan's Multiple Range Test (n = 30).

Table 4 Effect of culture vessel on shoot bud induction from cotyledon explants of *Tagetes patula* cultured on MS + BA (4.4 µM) and IAA (2.8 µM).

Culture vessel	% Response	Mean ± S.D	
		NS	HS
Erlenmeyer flask*	100 a	12.0 ± 0.7 b	5.8 ± 0.8 a
Petri dish	100 a	16.2 ± 0.9 a	3.8 ± 0.6 c
Culture tube	100 a	12.2 ± 0.8 b	4.5 ± 0.8 b

* Control, NS = normal shoots, HS = hyperhydric shoots, S.D. = standard deviation
The treatment in Erlenmeyer Flask consisted of 10 replicates each with three explants; in Petri dish six replicates each with five explants and in culture tubes fifteen replicates with two explants each.
Means with the same letter(s) within the same column are not significantly different at the 5% level according to Duncan's Multiple Range Test (n = 30).

Table 5 Effect of various concentrations of ammonium nitrate (NH_4NO_3) on shoot bud induction from cotyledon explants of *Tagetes patula* cultured on MS + BA (4.4 µM) + IAA (2.8 µM).

μNO_3 (mM) in MS medium	Mean ± S.D	
	NS	HS
0	0 d	0 e
5.1	17.5 ± 0.5 b	2.6 ± 0.4 c
10.3	25.4 ± 1.1 a	1.7 ± 0.9 d
20.6*	17.0 ± 0.8 c	3.8 ± 0.8 b
41.2	0 d	12.0 ± 0.8 a

* Level of NH_4NO_3 in standard MS medium
NS = normal shoots, HS = hyperhydric shoots, S.D. = standard deviation
Each treatment in Petri dish consisted of six replicates, each with five explants.
Means with the same letter(s) within the same column are not significantly different at the 5% level according to Duncan's Multiple Range Test (n = 30).

Effect of culture vessel on shoot bud induction

The number of shoots regenerated from cotyledon explants was significantly higher in PDs (16.2 NS) than in EFs (12.0 NS) and CTs (12.2 NS). In PDs the shoots appeared healthy with least percentage of hyperhydricity (**Fig. 1D; Table 4**). So, PDs were selected as the best culture vessel and used in subsequent experiments.

Effect of ammonium ions on shoot bud induction

Vigorous shoots were produced on PDs containing MS medium with 0.9% agar, 10.3 mM NH_4^+ (half the standard MS level), BA (4.4 µM) and IAA (2.8 µM) yielding an average of 25 shoots/explant (**Fig. 1E; Table 5**). This was considered as the optimal induction medium. By increasing the NH_4NO_3 concentration to 41.2 mM (twice the standard MS level) produced a much softer gel and 100% HSs.

The shoot buds from cotyledon explants differentiated directly without any intervening callus phase (**Fig. 1F**). Proliferation of shoot buds was observed on MS medium fortified with 0.9% agar, BA (4.4 µM), IAA (2.8 µM) and GA_3 (0.5 µM) in EF (**Fig. 1G**). Full-strength MS medium without any growth regulator worked well for the purpose of elongation and rooting (**Fig. 1H, 1I**).

Chlorophyll content

The amount of chlorophyll was found to be maximum (0.489 mg.g fresh wt.$^{-1}$) in the shoots developed under optimized medium conditions (PD, 10.3 mM NH_4^+, 4.4 µM BA and 2.8 µM IAA, **Fig. 2**). Shoots induced in control medium showed lower (0.356 mg. g fresh wt.$^{-1}$) chlorophyll content.

POD activity

POD enzyme activity was half the level in the HSs as it was in the NSs (**Fig. 3**).

DISCUSSION

Hyperhydricity is considered as a physiological response that can be induced by different stresses. A pathway of biochemical events leading to hyperhydricity has been proposed by Kevers *et al*. (2004). This pathway was considered as an adaptive response of tissue submitted to several stresses simultaneously. Among the different stress inducers, high amounts of ammonium ions and cytokinins, different concentrations of gelling agents and type of culture vessel played a major role in hyperhydricity. These different stresses can cause sustained a build-up of reactive oxygen species (ROS) which can cause lipid peroxidation, DNA mutation and plant cell death (Cassells and Curry 2001). Plants have an efficient antioxidant system which scavenges ROS like POD, but POD activity was found to be low in HS of *Beta vulgaris* (Hagege *et al*. 1990). It has been hypothesized that the accumulation of undetoxified H_2O_2 due to the absence of sufficient anti-oxidative enzymes leads to

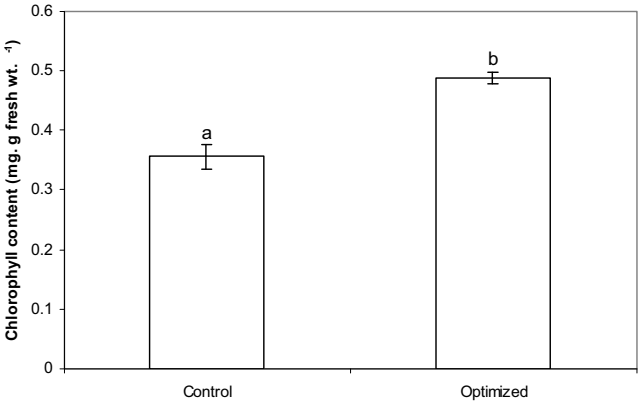

Fig. 2 Chlorophyll content in 4 week old shoot buds of *Tagetes patula*.

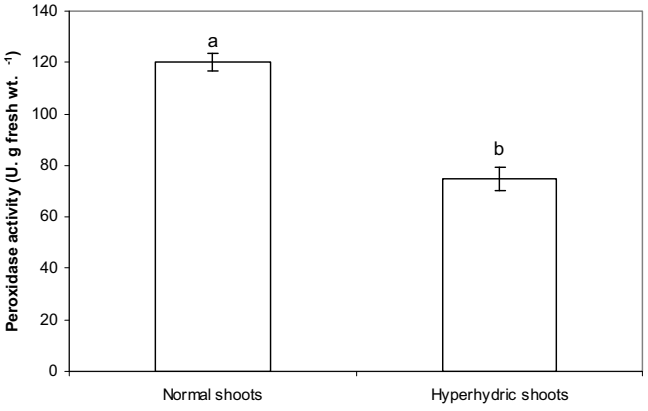

Fig. 3 POD activity in 4-weeks-old shoot buds of *Tagetes patula*.

hyperhydricity (Dily *et al*. 1993). Many studies support this hypothesis: Franck *et al*. (1995) in *Prunus* and Olmos *et al*. (1997) in *Dianthus* found that POD activity in hyperhydric leaves of regenerated shoots was significantly reduced compared to normal leaves. Similar results were obtained in *Tagetes* also, where the POD activity in HSs was almost half than that of NSs (**Fig. 3**). These results lead to the conclusion that the malformation during hyperhydricity resulted from the inability of the shoots to defend themselves against the toxic forms of oxygen.

The watery tissue leads to deficiency of chlorophyll accumulation. In our study chlorophyll content in HSs was lower than in NSs (**Fig. 2**). The result is supported by similar reports characterized by low leaf chlorophyll content in carnation (Winarto *et al*. 2004).

The degree of medium solidification, as well as type of gelling agent strongly affect not only the adventitious shoot regeneration capacity in *Tagetes* explants but also the water content of the shoots (Jain *et al*. 2001). In the present investigation it was observed that as the agar concentration increased the number of HS decreased. This observation is consistent with findings of micropropagated plantlets of several species, including *Dianthus* (Casanova *et al*. 2008). In addition to a reduction in hyperhydricity, increased agar concentration can drastically reduce the multiplication rate (George 1996). In the case of phytagel-solidified medium the highest number of HSs was found in various species viz., *Malus* (Turner and Singha 1990), *Pyrus* (Kadoka and Niimi 2003) and *Tagetes* (78%, this study).

Culture vessel had pronounced effect on HS in *Tagetes*. The head space of vessel accumulates components like ethylene, CO_2, acetyldehyde and ethanol. The differential effect of culture vessels on regeneration and hyperhydricity can be attributed to different amount of gases that accumulated in the head space which might have influenced morphogenic response. The effect of culture vessel on shoot bud induction and proliferation has been reported in *Magnolia* (De Proft *et al.* 1985), *Carica* (Magdalita *et al.* 1997) and *Gossypium* (Agrawal *et al.* 1997). Hazra *et al.* (2000) also found a marked difference in the number of multiple shoots produced from *Gossypium* explants when cultured in two different vessels, keeping the media and incubation conditions unaltered. The differences in two vessels were in the shape, volume and quantity of medium. Contrary to our findings, they concluded that a larger culture vessel with more medium favors the growth of shoots. Jain *et al.* (2008) also observed pronounced effect of culture vessel on regeneration in *Stevia.*

In the *Tagetes* cultures, increasing ammonium ion concentration above the standard MS level produced a softer gel thus providing substances responsible for the induction of hyperhydricity such as water, cytokinin, NH_4^+ readily available to the plant. Reducing the concentration of ammonium ion to half the normal MS (20.6 to 10.3 mM) reduced hyperhydricity in *Tagetes*, supported by a similar result in *Castanea sative* (Vieitez *et al*. 1985) and *Amelanchier arborea* (Brand 1993). Ivanova and van Staden (2008) studied the effect of NH_4^+ concentration, cytokinin type and cytokinin concentration on hyperhydricity. They found that increasing the concentration of zeatin or BA to 15 µM, resulted in an increase of the multiplication rate but only on media with 10.3 mM NH_4^+. With elevating the amount of NH_4^+ in the medium, an inverse correlation between cytokinin concentration and multiplication rate was observed.

CONCLUSION

In *T. patula* HSs, a low amount of chlorophyll and lower POD activity indicated a condition of stress leading to hyperhydricity. A high multiplication rate and reduced hyperhydricity were obtained on MS medium containing half the normal [NH_4^+] (10.3 mM) and PD as the culture vessel.

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