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ORIGINAL PAPER

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EFFICIENT *in vitro* PROPAGATION OF *Amaranthus viridis* L. USING NODE EXPLANTS

Tour Jan[1]✉, Ikram Ullah[1], Bilal Muhammad[1], Tariq[1], Ali Mansoor[1], Zaheer Ullah[1], Muhammad Asif Nawaz[2]

[1] Plant Tissue Culture Laboratory Department of Botany, University of Malakand, Dir Lower, KP, Pakistan

[2] Department of Biotechnology, SBBU, Sheringal, Dir Upper, KP, Pakistan

ABSTRACT

Hyperhydricity is a frequently problem in plants during *in vitro* culture and affected micropropagation of plants. To develop an efficient *in vitro* regenerated system without hyperdydricity, we demonstrated the effect of different disinfected agents (mercuric chlorite and hypochlorite), growth regulators, their concentrations and combinations, Agar, pH, ammonium nitrate (NH_4NO_3) and number of subcultures. Mercuric chlorite at 0.07% and exposing time (9–10 min) was appropriate for hygienic culture. The shoots induced by Benzyladnine (BA) alone or in combination with α-Naphthaleneacetic acid (NAA) exhibited maximum multiplication with symptoms of hyperhydricity than those induced by Kinetin alone or in combination with NAA. Hyperhydricity was also reduced by increasing the concentration of agar, pH and elimination of NH_4NO_3 from the macroelements of Murashig and Skoog (MS) medium. Repeated subcultures affected both multiplication and hyperhydricity. The multiplication of shoots increased from parental culture up to 5th subculture and thereafter declined in 6th subculture. Although shoot hyperhydricity were observed from 1st subculture (19%) and then increased up to 85% in 6th subculture. This increased in hyperhydricity could be due to the remaining influence of hormones. In shoots of 5th subculture the content of chlorophyll (dark green) were higher than shoots of 6th subculture.

Key words: agar, *Amarinthus viridis*, $HgCl_2$, hyperhydricity, pH, subcultures

INTRODUCTION

Amaranthus viridis belong to family *Amaranthaceae*, is a fast growing herb, locally known as 'Chalwai' which has been used as vegetable by indigenous people. It is mostly cultivated in Asia, Africa and Latin America [Amin et al. 2006]. This species resist to drought, hot climate, pest and little requirement of cultivation, this pseudocereal has attracted much attention as an important food commodity [Saxena et al. 2007]. The increased anthropogenic activities limited the size of the population of *Amarinthus viridis*. Therefore, developing of an effective system of regeneration would be a useful tool to active protection of this species. The technique of *in vitro* culture supports conservation of plant genetic resources without depleting their natural position, because it requires small fragment of plants to initiate the regeneration process [Mikula and Rybczynski 2006]. There are extensive literatures on the tissue culture. In vitro culture techniques are now being widely applied for improvement of field crops, forest, horticultural and agricultural crops, e.g. *Amaranthus cruentus* [Yaacob et al. 2012], *Amaranthus* species [Bennici et al. 1992]. The main recognized problem that influence *in vitro* propagation of plant is hyperhydricity [Kevers et al. 2004, Ivanova and

✉ tour_jan@yahoo.com

Van-Staden 2008]. Hyperhydricity obstruct mass multiplication of plants, therefore the prevention of this problem is very important. Several studies have been conducted to stunned hyperhydricity i.e., Masanori and Yoshiji [2003] studied the effect of plant growth regulators, Ivanova and Van-Staden [2008] ammonium nitrate and Casanova et al. [2008] agar concentrations. In order to study the influencing factors on hyperhydricity, we investigated the effects of BA, Kinetin, NAA, Agar, pH, NH_4NO_3 and repeated subcultures on shoots hyperhydricity and multiplication. The present study contribute to our understanding of effect of factors on hyperhydricity in *Amarinthus viridis*. This study deliver a reference for prevention of hyperhydricity in other plants. Developing an effective system of regeneration would be a useful method for conservation of this species.

MATERIALS AND METHODS

Plant material and sterilization of explants. The plant material free from any external injury of pest and insect attack was collected from the premises of the Botanical Garden and Herbarium, University of Malakand. Excised axillary branches were placed under running tap water for 30 min to wash dust and other unwanted materials and then sterilized with mercuric chloride ($HgCl_2$) solution containing few drops of Tween-20 and Sodium hypochlorite (NaClO) (Tab. 1). Sterilized explants were rinsed 3–4 times with sterile distilled water prior culture initiation.

Basal medum and culture conditions. MS [Murashige and Skoog 1962] medium supplemented with 3% sucrose was used throughout this investigation. Growth hormones, benzyladenine (BA), kinetin (Kin) and α-Naphthaleneacetic (NAA) were always added before sterilizing the medium. In all experiments, the chemicals used were from Sigma-Aldrich, USA. After addition of all constituents of media, pH was adjusted to 5.5 and 0.6% agar (agar-agar Microbiologie, Merck, USA) was used as solidifying agent. All cultures were maintained at 26 ±2°C, under a 16/8 h day/night photoperiod. In the growth chamber light was provided from cool white fluorescent tubes having intensity 2000 flux.

Initiation of shoot culture. For initiation of shoot culture, nodal explants were cut (3–4 cm) and placed horizontally on Murashige and Skoog [1962] medium supplemented with BA (0.5, 1.0, 1.5, 2.0, 2.5 mg·dm^3), Kin (0.5, 1.0, 1.5, 2.0, 2.5 mg·dm^3), NAA (0.5 and 1.0 mg·dm^3) added singly or in combination: BA/NAA (1.0/0.5 and 1.5/0.5 mg·dm^3) and Kin/NAA (1.0/0.5 and 1.5/0.5 mg·dm^3). Each treatment had seven replicates with two explants per replicate. Percentage response of explants, number and length of shoots and roots were recorded after four weeks of culture.

Shoot multiplication. Vigorous shoots of the previous culture were selected and subcultured on media supplemented with BA/NAA (1.0/0.05, 1.0/0.1, 1.0/0.5, 1.5/0.05, 1.5/0.1, and 1.5/0.5 mg ·dm^3) (Experiment 1). In order to evaluated the effect of subculture on multiplication and hyperhydricity of shoots, after 30 days, vigorous shoots of the first subculture were selected and successively subcultured up to six cultures on media augmented with BA (1.5 mg·dm^3), Kin (1.5 mg·dm^3) or BA/NAA (1.5/0.1 and 1.5/0.5 mg·dm^3) and Kin/NAA (1.5/0.1 and 1.5/0.5 mg·dm^3) (Experiment 2).

Experiment 3. In order to evaluated the effect of agar concentration on hyperhydricity, normal shoots of the 3rd subculture were selected and subcultured on the media augmented with BA/NAA (1.5/0.1 mg/l) and solidified with different agar concentrations (0.5%, 0.6%, 0.7%, 0.8% and 0.9%).

Experiment 4. In order to test the effect of media pH values on hyperhydricity, normal shoots of the 3rd subculture were selected and subcultured on media containing BA/NAA (1.5/0.1 mg/l) with pH values (4.6, 5.0, 5.8, 6.0 and 6.5).

In vitro rooting. For roots induction individual shoots were transferred on rooting media supplemented with NAA (1.0, 1.5 and 2.0 mg·dm^3), IAA (1.0, 1.5 and 2.0 mg·dm^3 and sucrose 3%. After 20–25 days of subculture, the percentage of rooted shoots, number of roots per rooted shoot and length of the roots were recorded.

Statistical analysis. Standard deviation was evaluated of the collected data using the SPSS statistical package software version 16.0.

RESULTS

Establishment of *in vitro* culture. The nodal explants of *Amarinthus viridis* was successfully decon-

taminated by exposing to $HgCl_2$ and NaClO in variable sterilization time and concentrations (Tab. 1). On average, 95.25% of the explants treated with $HgCl_2$ (0.07%) in sterilization time of 9–10 min and 94.65 with NaClO (9%) in sterilization time 14-15 min survived and did not manifest symptoms of fungal and bacterial contamination (Tab. 1). When the sterilization time was reduced to 7–8 min and $HgCl_2$ was kept the same (0.07%), 83.52 percent explants were found contaminated whereas exposure time of 12–13 min showed no contaminated explants but caused explants browned that proved to be lethal. Concentrations of $HgCl_2$ above 0.07 percent was also completely eliminated microorganisms with sterilization time of 9–10 min but caused explants browned that led to dead of explants. Similar results was also observed with NaClO (Tab. 1). Brown dead tissues present on the edges of the decontaminated explants were trimmed before initiation of culture.

Multiple shoot regeneration. Direct axillary shoot formation and morphogenesis *in vitro* were achieved from nodal explants on MS medium containing different concentrations/combinations of plant growth regulators. Nodal explants initiated axillary shoots after two week of setting up the culture with different concentrations of BA and Kinetin or in combination with NAA. Explants on MS medium supplemented with BA maintained only less than one shoots at 2.0 and 2.5 mg/l and less than two shoots at 0.5, 1.0 and 1.5 mg·dm^3. Length of the regenerated shoots were lower than 2 cm at all concentrations of BA (Tab. 2). In these cultures leaves of the *in vitro* shoots were broad and petiole was normal as in the wild plants (Fig. 1a). Similar results were found on media containing Kinetin (Tab. 2). The regeneration frequency of shoots in the presence of both type of cytokinins (BA and Kinetin) was not more than 40%. When 1.5 mg·dm^3 BA was used in combination with NAA, the regeneration frequency increased to 53%, with an average two shoots per explant. At both cut end of node, small amount of callus has been observed. In the presence of BA/NAA 1.0/0.5 mg·dm^3, shoots were able to elongate reaching on average 5.81 cm in height (Tab. 2). The shoots formed on medium supplemented with cytokinin and auxin were vigorous and the regeneration frequency ranged from 38 to 53%.

Shoot multiplication and appearance of hyperhydricity. The regenerated shoots were subcultured on medium containing different concentrations of BAP (1.0 and 1.5 mg·dm^3) added sole or in combination with NAA (1.0/0.05, 1.0/0.1 and 1.0/0.5 mg·dm^3 and 1.5/0.05, 1.5/0.1 and 1.5/0.5 mg·dm^3). Number of shoots induction increased as the BA concentrations increases. When the BA concentration was 1.0 mg/l the shoot induction number was 5.64 ±0.83 per explant while at 1.5 mg/l, 7.76 ±1.034 shoot per explant were formed (Tab. 3). However, the symptoms of hyperhydricity (curling of leaf, narrow leaf lamina and pale green shoots) were observed (Fig. 1c) (Tab. 3). When sole NAA was used at 0.1 mg·dm^3, the frequency of number of shoots formation per explants decreased to 3.58 ±0.62 but morphologically the shoots formed were normal as the wild plants (Tab. 3). Combinations of plant growth regulators (BA and NAA) were found essential for maximum axillary shoots formation with normal shoots morphology (Tab. 3). When the concentration of BA was kept constant (1.0 mg·dm^3) with variable concentrations of NAA (0.05, 0.1 and 0.5 mg·dm^3) less shoots formation per explants occurred (Tab. 3). When the concentrations of NAA was kept the same (0.05, 0.1 and 0.5 mg·dm^3), shoots regeneration per explants increased as the concentration of BA (1.5 mg·dm^3) increased in the medium (Tab. 3). Therefore the combination of BA/NAA (1.5/0.1 mg·dm^3) was found the most suitable for maximum shoots proliferation (17.62 ±7.62). In combination BA/NAA (1.5/0.05 mg·dm^3) only 6% shoots shown hyperhydricity (Tab. 3).

Successive subculture and hyperhydricity. Regenerated shoots from 1st subcultured were subcultured on media supplemented with cytokinin and auxin (Tab. 4). The data presented in Table 4 revealed that sole BA produced less number of shoot per explant and more percentage hyperhydricity in all 6th subcultures against combination (BA/NAA). Further it was distinguished that the percentage response and number of shoots enhanced progressively with each subculture on either media containing single hormone (BA) or combination (BA/NAA). During sequential subcultures the highest number of shoots achieved in the 1st subculture was (17.62 ±7.62), in 2nd subculture (17.56 ±2.06), in 3rd subculture (17.85 ±1.97), in 4th subculture (19.48 ±2.69), in 5th subculture (22.59 ±1.89) and

Table 1. Effect of sterilizing agents and exposing time on the control of microbial contamination

Explant	Sterilization agents (%)		Exposure time (min)	Explants contaminated (%)	Explant survived (%)
	$HgCl_2$	NaClO			
Nodes	0.07	–	12–13	0.0	0.0
		–	9–10	4.75	95.25
		–	7–8	83.52	16.48
	0.2	–	9–10	0.0	8.34
	0.5	–		0.0	3.62
	1.0	–		0.0	0.0
	–	9.0	17–18	0.0	0.0
	–		14–15	5.35	94.65
	–		11–12	87.24	12.76
	–	12.0	14–15	0.0	0.0
	–	15.0		0.0	0.0

Table 2. Direct shoots initiation from nodal explants under the influence of cytokinins and auxin

Growth regulators	Conc. ($mg \cdot dm^3$)	Shoots formation (%)	Number of shoots ±SE	Height of shoots
BA	0.5	26	1.06 ±0.4	Less than 2 cm
	1.0	37	1.86 ±1.02	
	1.5	40	1.49 ±0.51	
	2.0	38	0.92 ±0.13	
	2.5	39	0.78 ±0.61	
Kin	0.5	33	0.58 ±0.73	Less than 2 cm
	1.0	29	0.82 ±0.34	
	1.5	40	1.03 ±0.86	
	2.0	37	0.59 ±0.25	
	2.5	40	0.84 ±0.71	
NAA	0.5	20	0.32 ±0.13	Less than 2 cm
	1.0	24	0.45 ±0.35	
BA/NAA	1.0/0.5	46	2.07 ±0.96	5.81 ±1.54
	1.5/0.5	53	2.27 ±1.04	5.09 ±0.94
Kin/NAA	1.0/0.5	41	1.06 ±0.8	3.64 ±1.02
	1.5/0.5	38	1.00 ±0.63	4.72 ±1.32

Conc. = Concentration

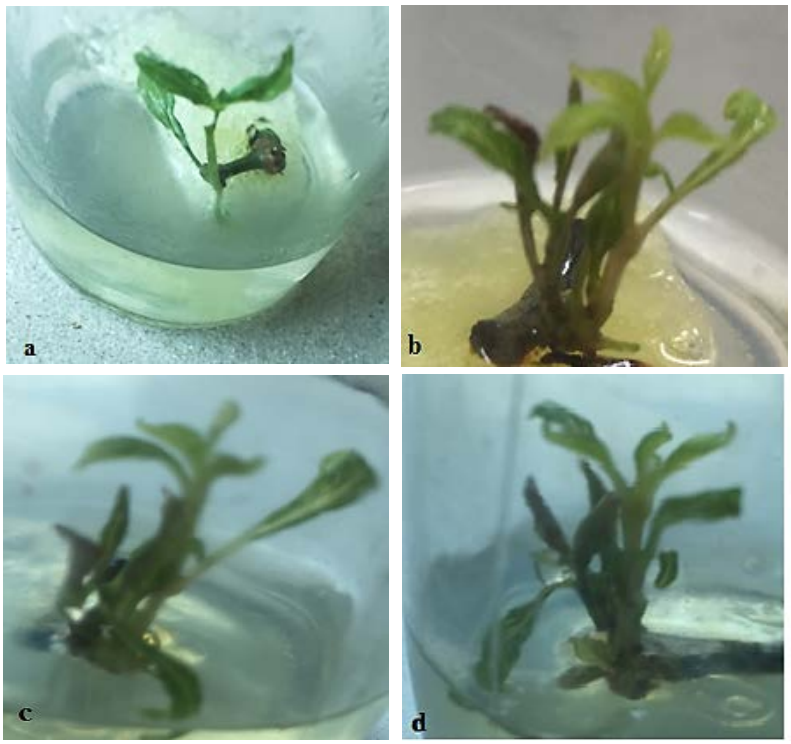

Fig. 1. Shoot regeneration in *Amarinthus viridus*; a) regenerated shoot in parental culture on medium with BA, b) shoot multiplication in subculture on medium containing BA/NAA (1.5/0.5 mg/l), c) abnormal shoots on medium containing BA (1.5 mg/l), d) abnormal shoots on medium containing BA/NAA (1.5/0.05 mg/l)

Table 3. Effect of plant growth regulators on multiplication and morphology of shoot in subculture

Plant growth regulators ($mg \cdot dm^3$)		Number of shoots ±ES	Length of shoots (cm) ±ES	Hyperhydricity (%)	Symptoms of hyperhydricity
BA	NAA				
1.0	–	5.64 ±0.83	3.56 ±0.34	14	Pale green shoots with curled leaves and narrow lamina
1.5	–	7.76 ±1.03	3.82 ±0.19	19	
–	0.1	3.58 ±0.62	2.79 ±1.02	–	–
1.0	0.05	11.85 ±1.32	4.33 ±0.63	–	–
1.0	0.1	13.37 ±0.93	5.09 ±0.58	–	–
1.0	0.5	13.92 ±1.03	5.94 ±0.79	–	–
1.5	0.05	15.45 ±1.62	3.39 ±0.08	6	Pale green shoots with curled leaves and narrow lamina
1.5	0.1	17.62 ±1.66	7.28 ±1.48	–	–
1.5	0.5	15.68±1.85	6.59±0.69	–	–

Table 4. Effect of successive subculture on regeneration efficacy and hyperhydricity of shoot. Subculture were performed every 30 days

PGR ($mg \cdot dm^3$)		Subcultures									
		2^{nd}		3^{rd}		4^{th}		5^{th}		6^{th}	
BA	NAA	Hy (%)	TNS	Hy (%)	TNS	Hy (%)	TNS	Hy (%)	TNS	Hy (%)	TNS
1.5	–	27	8.37 ±0.21	47	10.62 ±1.27	62	10.84 ±1.64	78	12.78 ±1.23	85	12.49 ±1.78
1.5	0.1	15	17.56 ±2.06	32	17.85 ±1.97	37	19.48 ±2.69	51	21.72 ±2.42	68	21.33 ±2.58
1.5	0.5	19	16.97 ±1.68	45	17.69 ±1.89	46	17.85 ±2.27	49	22.59 ±1.89	61	19.49 ±2.86
Kin											
1.5	–	21	6.58 ±0.37	35	8.25 ±0.63	54	8.53 ±0.69	58	11.08 ±1.07	49	9.29 ±1.38
1.5	0.1	14	8.74 ±1.61	18	11.19 ±1.57	25	11.34 ±1.39	28	13.48 ±1.49	35	13.72 ±1.59
1.5	0.5	15	5.27 ±0.57	25	7.73 ±0.85	29	7.88 ±0.48	31	11.24 ±1.62	31	10.52±1.48

Hy – hyperhydricity, TNS – total number of shoots

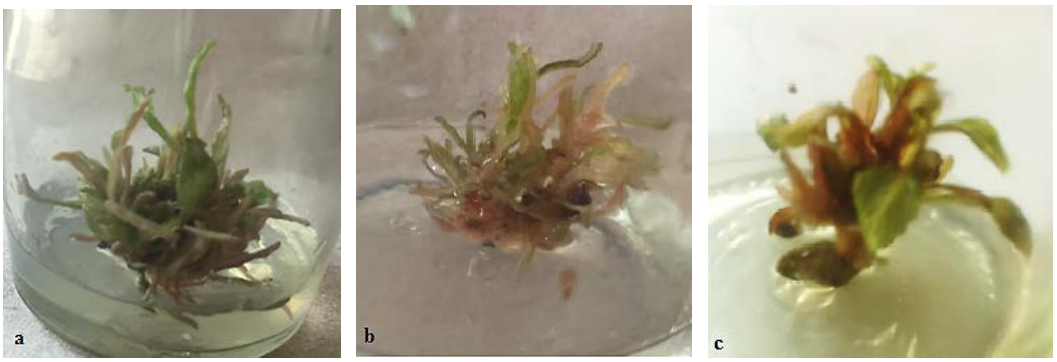

Fig. 2. Hyperhydric (abnormal) and normal shoots regenerated in successive subculture; a) hyperhydric (abnormal) shoots of 5^{th} subculture, b) hyperhydric shoots of 6^{th} subculture, c) normal shoots

Table 5. Effect of agar concentrations on hyperhydricity and multiplication of shoots in media containing BA/NAA (1.5/0.1 mg/l)

Treatment	Concentration (%)	% rate of hyperhydricity	% rate of normal shoots	Total number of shoot per explant
Agar	0.5	84.71	16.51	21.05 ±2.61
	0.6	56.95	44.75	16.37 ±1.85
	0.7	39.83	61.36	13.72 ±0.92
	0.8	25.16	73.19	7.28 ±0.68
	0.9	0.0	8.28	3.27 ±0.09

in 6th subculture (21.33 ±2.58) on combination (BA/NAA 1.5/0.1 mg·dm^3) (Tabs. 3, 4). It was noted in consecutive subculture that symptoms of hyperhydricity (Pale green, glassiness of shoots, curled leaves with narrow lamina and elongated petiole) increased with increasing the numbers of subculture (Figs. 1c–d, 2a–b). Furthermore, shoots of the 5th subculture were dark green (higher chlorophyll content) while shoots of the 6th subculture were light green (low chlorophyll content) (Fig. 2 a–b). In these subcultures, medium containing (BA) hyperhydricity ranged 27 to 85% while on medium containing combination (BA/NAA) hyperhydricity ranged 15 to 68% (Tab. 4). Replacement of cytokinin (BA) by Kin in combination with auxin (NAA), hyperhydricity ranged 14 to 31%. When single Kin was used hyperhydricity ranged 21 to 49%. However, shoots regeneration frequency per explant dropped with supplementation of Kin in the medium (Tab. 4).

Effect of agar and NH_4NO_3 on hyperhydricity. When concentration of agar was raised to 0.6% or above, both shoot multiplication and shoot hyperhydricity decreased. Agar at the range of 0.6 to 0.7% in the medium resulted vigorous shoots formation 16.37 ±1.85 to 13.72 ±0.92 respectively with limited symptom of hyperhydricity (39.83 to 25.16). Maximum multiplication and hyperhydricity of shoots occurred at 0.5% agar (Tab. 5). A concentration above 0.8% was deleterious which reduced shoots multiplication and only 8% explants formed shoots without any symptom of hyperhydricity (Tab. 5). The number of hyperhydric shoots decreased with half and complete elimination of NH_4NO_3 from culture MS medium (data not shown).

Effect of media pH on hyperhydricity. The highest percentage of hyperhydricity (89.52) (curled, wrinkled and needle like leaves) were found on medium whose pH was 4.6 and no any symptoms hyperhydricity were found on medium whose pH valve was 6.5 (Tab. 6). Minimum hyperhydricity and maximum multiplication of shoots were recorded on medium whose pH valve was 5.8.

In vitro rooting. For the induction of roots individual microshoots were transferred to hormone free and hormones supplemented MS medium. Two weeks after inoculation, root formation occurred from the cut end of microshoots without callusing on media comprising different concentrations of NAA and IAA (Tab. 7). Of the two auxins used, NAA was found best hormones for the induction of roots. The highest (4.27 ±1.02) number of root per explant were managed on medium having 1.0 mg·dm^3 of NAA.

Acclimatization of plantlets. After 5 weeks, the rooted plantlet were gradually subjected to non-sterile condition by step wise loosening the screw of culture containers and finally removed the cape within two days. Plantlet were gently isolated from the culture media and washed with sterilized distilled water to remove the adhering media. These plantlets were transferred to plastic cups that contained sterile garden soil and sand (2 : 1). Initially plantlet were covered with plastic cup and irrigated with half MS medium without sugar. The survival potential of the *in vitro* regenerated plants into *ex vitro* condition was 65%. There was no detectable variation among the acclimatized plants with respect to morphological characteristic. After 45 days, plantlets were then transferred to soil in the experimental garden of the department. Thus the successful in vitro protocol and acclimatization of *Amarinthus viridis* was achieved.

DISCUSSION

Explants sterilization is an important stage in establishing tissue culture of any plants; all upcoming steps really dependent on it. In our experiment with *Amarinthus viridis*, the concentration and exposure time to mercuric chlorite and sodium hypochlorite greatly affected the survival frequency of the explants. High concentration and period of exposure to mercuric chlorite and sodium hypochlorite leads the explants to browning and death. The surface sterilization of *Alternanthera sessilis* [Wesely et al. 2011] and *Rhinacanthus nasutus* [Johnson et al. 2005] above 5 min was lethal to explants whereas, the present study showed that above 9 to 10 min with mercuric chlorite and 14 to 15 min with sodium hypochlorite of surface sterilization was lethal to nodal segments of *Amarinthus viridis*.

Sole BA and Kin or Kin in combination with NAA was less effective compared to combination of BA/NAA because shoots from any sole BA or Kin concentration in the initial culture were always less than two per explants, confirming study where combination (BA/NAA) promoted maximum shoots formation [Faisal et al. 2018]. BA at 1.5 mg·dm^3 added together

Fig. 3. Multiple roots formation with NAA (1.5 mg·dm^3)

Table 6. Effect of pH concentrations on hyperhydricity and multiplication of shoots in media containing BA/NAA (1.5/0.1 mg/l)

Treatment	Concentration (%)	% rate of hyperhydricity	% rate of normal shoots	Total number of shoot per explant
pH	4.6	89.52	11.79	9.68 ±1.36
	5.0	58.43	42.14	17.59 ±2.59
	5.8	21.47	79.59	18.93 ±2.63
	6.0	19.21	53.38	18.04 ±1.97
	6.5	0.0	17.25	5.45 ±0.29

Table 7. Multiple root regeneration in the *in vitro* produced shoots

Hormones (mg·dm^3)		Rooting %	Number of root per explants	Length of roots (cm)
NAA	IAA			
0.5	–	47	2.31 ±0.31	3.26 ±0.82
1.0	–	53	3.25 ±0.63	4.85 ±0.18
1.5	–	51	4.27 ±1.02	3.69 ±0.52
2.0	–	39	3.51 ±0.72	4.69 ±0.38
–	0.5	36	1.65 ±0.09	2.4 ±0.33
–	1.0	47	2.71 ±0.46	5.48 ±0.41
–	1.5	58	3.19 ±0.84	5.37 ±1.03
–	2.0	34	2.36 ±0.28	2.7 ±0.27
Control		–	–	–

with NAA induced no hyperhydricity in the 1st subculture. Sole BA at high concentration (1.5 mg·dm^3) induced 19% and low (1.0 mg·dm^3) 14% hyperhydricity in the 1st subculture. In the initial culture of *Allium sativum* 94.17% hyperhydricity was observed with Kinetin [Liu et al. 2017]. Ivanova et al. [2006] reported that high level of exogenous cytokinin in concentration dependent manner influence hyperhidricity. A similar results reported by Martin et al. [2006].

Increasing trend of morphological abnormalities (hyperhydricity) were observed with repeated subculture, regardless of their hormones supplementation to the medium in this study. We noticed hyperhydricity in the first subculture and increased progressively in successive subcultures whereas [Piatczak and Wysokinska 2003] observed hyperhydricity after 5th subculture in *Centaurium erythraea*. In our study, variety of cytokinins (BA & Kin) and cytokinins and auxin combination play an essential role in formation of hyperhydric shoots in the successive subcultures. BA promoted additional hyperhydricity than Kinetin. This phenomenon Kataeva et al. [1991] observed when excess amount of single BA was used in the medium. All the explants in the presence of cytokinin became hyperhydric [Ivanova and Johannes 2011]. However, when BA or Kin was added together with auxin (NAA), the intensity of hyperhydricity reduced. The mechanism of cytokinins-to-auxin interaction is not clear for hyperhydricity but this interaction may be allowed the synthesis of certain substances that inhibited hyperhydricity. In our experiments, the consecutive subculturing positively affected the multiplication of shoots up to 5th subculture and then declined (Tab. 4). Similarly, shoots multiplication increased up to 6th subculture in *Bacopa monnieri* and then declined [Naik et al. 2013]. Likewise, in *Potentilla fruticosa* shoot multiplication was at its maximum at the beginning of the experiment and then declined [Remphrey et al. 1993]. A sharp decline in shoots formation of *Sterculia urens* may be overcome by the reduction of thidiazuron concentration which favour the continuous production of shoots formation [Hussain et al. 2007].

Increasing agar concentration from 0.5 to 0.8% decreased hyperhydricity in our study. With low agar concentration, more hyperhydricity occurred. We discussed that low concentration of agar increases the chances of availability of water and moisture in the culture container, permitted more uptake of water, resulting hyperhydricity. It has been reported that hyperhydricity can be alleviated by increasing the concentration of agar [Casanova et al. 2008]. In the *in vitro* culture the common pH for most of the plants species is 5.5 to 5.7. The lower level of pH, the more serious of hyperhydricity. The increasing level of pH up to 5.8 was found suitable for normal shoots regeneration. A similar tendency was found in *Allium sativa* [Liu et al. 2017].

The highest number of roots (4.27 ±1.02) were observed on medium containing NAA (1.5 mg/l) whereas uppermost length of roots (5.48 ±0.41) was noticed on medium containing IAA (1.0 mg/l). In *amaranthus* species rooting was stimulated by NAA [Tisserat and Galleta 1988]. The best rooting rate (58%) was achieved on IAA at 1.5 mg/l in our study. IAA was reported as a potential auxin for rooting of *Arachis stenosperma* and *Arachis villosa* [Vijayalakshmi and Giri 2003]. Similar results were obtained with *Sesbania drummondii* [Cheepala et al. 2004].

CONCLUSIONS

In conclusion, the factors (BA, Kin, NAA, Agar, pH and number of subcultures) effected hyperhydricity and multiplication efficacy of cultured *Amarinthus viridis*. Shoot proliferation in initial culture was little and subsequently increased in repeated subcultures. Our results suggested the combination of cytokinis and auxin, media pH values 5.8 to 6.0 and solidifying agent 0.6% to 0.7% essential for normal shoots formation. The regenerated shoots then need a rooting stage for roots formation which was obtained on MS medium added with 1.0 mg/l NAA. Plantlets thus obtained was successfully acclimatized into natural environment. This is the first detail study on hyperhydricity in *Amarinthus viridus*. We hope this study will provides detail information for understanding the prevention and controlling of hyperhydricity in other plants species. This protocol will be applied to other species of *Amarenthus*.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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