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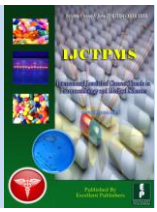

Original Research Article

Hyperhydricity (Vitrification) of Micropropagated Shoots of Two Grapevine (*Vitis vinifera* L.) Cultivars

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Abstract

Hyperhydricity of micropropagated shoots also known as vitrification is unconditionally results from growth and culture condition, which affects the survival and quality of micro-propagated plants. The occurrence of *in vitro* shoot vitrification in grapevine was examined. MS medium supplemented with 5 concentrations of BAP and agar were used. From the tested five different concentrations of BAP and agar, the best mean number of normal shoots were obtained at 0.5 mg/l BAP in 7.5g/l agar for both Chenin Blanc (6.0 ± 0.1) and Canonannon (5.0 ± 0.2) cultivars. The maximum mean numbers of normal shoot roots/explant (5.8 ± 0.3) were obtained when gelling agent was 7.5g/l in 4mg/l IAA for both cultivars. In contrast, maximum vitrified shoot roots of Canonannon variety were occurred in medium gelled at 7.5g/l in 2mg/l IAA. For both varieties, the maximum mean length of normal shoot roots were observed at medium supplemented with 4mg/l IAA and 7.5g/l gelling agent. Of the normal transferred shoots, the survival percentages were 94% and 75% for Chenin Blanc and Canonannon varieties, respectively. The vitrified plantlets of both varieties were not survived. In general, frequencies of *in vitro* normal shoots regeneration were greatly influenced by the concentrations of BAP and agar.

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Hyperhydricity
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Introduction

Hyperhydricity of micropropagated shoots also known as vitrification, unquestionably results from growth and culture conditions, exposed to stressing factors, wounding, and using of soft culture media. Generally, of high ionic strength, rich in nitrogen and growth regulators in special balance in a humid and gaseous confined atmosphere can create vitrification (Kevers et al., 2004). Hyperhydricity affecting herbaceous as well as woody shoots during their *in-vitro* vegetative propagations. The vitrified shoots appear turgid, watery at their surface, sometimes less green and easily breakable. Vitrified shoots root poorly when they do. They fit a problem of survive at acclimatization steps. Stems of vitrified plantlets are broad and thick in diameter. Nodes are short. Leaves are thick and elongated (Kevers et al., 2004). The hyperhydricity is also characterized by an excessive accumulation of water which is apparently associated to cellular oxidative stress. It also, gives place to a number of morphological, physiological and anatomical abnormalities. This condition is most likely to develop in vegetative materials grown *in*

vitro (Osuna et al., 2011). In most micropropagation laboratories, vitrification is very serious problem most face of their cultures and many tissue cultures have focused their efforts on practical means of avoiding vitrification (Kevers et al., 2004).

Vitrification affects the survival and quality of micro propagated plants. It highly affects the leaf. The thoughtful of fundamental mechanism of *in vitro* controlling vitrification can produce more efficient micropropagation (Rasco and Patena, 1997). As, vitrification of shoots appear during the multiplication stages, Reductions of vitrification *in vitro* result in increment of shoot numbers. There are a number of mechanisms used to reduce vitrification: can be reduced by aeration of culture volume and changing of the concentration of growth regulators (Sharma and Mohan, 2006). In another way, an effective procedure for obtaining healthy shoots from *in vitro* culture of propagates was ventilating the culture vessels (Laia et al., 2005).

Liquid and low agar media also one causing agent of vitrification as it induced cellulose formation along with induced and disoriented cellulose biosynthesis which is manifested in non-functional guard cells. Mal-functioning stomata in addition affect the cuticle contributed to increased transpiration and desiccation of *in vitro* formed leaves. Thus, agar should not be considered simply as a means of solidifying culture media: In general, the concentrations of agar affect the chemical and physical characteristics of a culture medium (Ziv, 1991). Though it is known that, grapevine (*Vitis vinifera* L.) is one of the most widely distributed fruit crop of the world, today the need for grapevine fruit is increasing (Richard et al., 2010). This happened because of increase in the number of wine industries and more demand for fresh and dried fruits (Fayek et al., 20090. So to fit the demand for grape a healthy micro-propagation is too much needed (Patrice et al., 2006). A vitrification is a serious problem during the *in vitro* propagation of grapevine. The problem of vitrification on micropropagation of the grapevine has been reported (Alizadeha et al., 2010; Beza Kinfe, 2010) but there are no reports which mention decreases of vitrification. This study was not covering effect of all types of phyto-hormones on vitrification. The aim of this work was to assess the effects of agar and BAP concentrations in the reduction of vitrification in shoots of grapevine under tissue culture conditions.

Materials and methods

Explant source and maintenance in the laboratory

In vitro cultivated of two grapevines (Chenin Blanc and Canonannon*)* varieties were obtained from Holeta Agricultural Research Center and used as plant material for this study. The study was conducted in Addis Ababa University at plant propagation and tissue culture laboratory. Shoot induction of *in vitro* cultivated stock plant varieties were maintained by sub culturing shoots and nodes at one-month intervals on induction medium (MS medium supplemented with 1mg/l BAP in combination with 0.1mg/l IBA and 30 g/l sucrose). Magenta GA-7 box were used as vessels and sealed with Para-film. The medium was gelled with 7.5 g/l agar and the pH was adjusted to 5.8 prior to autoclaving at 121^0C for 15 min.

Media stock solution and determination of the optimum BAP and agar concentration for better shoots multiplication

The MS (Murashige and Skoog, 1962) nutrient medium with its full macro, micro and vitamin compositions was used. The plantlets induced from the induction medium were cultured in the medium supplemented with five different concentration of BAP (0.5, 1.0, 1.5, 2 and 2.5 mg/l) and agar (6, 6.5, 7, 7.5 and 8 g/l). Then the cultures were incubated in a growth room at 27^0C. The length and number of shoots were recorded after culture at day intervals (0, 5, 10, 15, 20, 25, 30, 35, days).

In vitro rooting of shoots

Three weeks sub-cultured shoots of Canonannon and Chenin Blanc were rooted on full strength 20 ml MS medium supplemented with 3% (w/v) sucrose, four different concentrations of IAA (1mg/l, 2mg/l, 3mg/l and 4mg/l) and five different concentrations of agar (6, 6.5, 7, 7.5, 8 g/l). The length and number of main roots were recorded after culture, in day intervals (0, 5, 10, 15, 20, 25, 30, 35, days).

Acclimatization

Both (normal and vitrified) plantlets, having better roots and shoots systems were taken out from the culture vessels and washed under running tap water to remove the agar and sucrose. The plantlets were then transferred to 12cm diameter plastic bag containing sterilized red soil, sand and cow dung manure at the ratio of 1:2:1,

respectively. The plantlets were covered with transparent plastic bag to maintain moisture and watered within one day interval. The plastic cover was gradually removed after the plantlets successfully established in insect proof glasshouse for one month.

Experimental design, data collection and analyses

The one-way analysis of variance (ANOVA) was used to compute the mean number and length of normal and vitrified shoots and roots. Complete Randomized Design (CRD) was used. All data were analyzed at p ($\alpha<0.05$) using SPSS 16 version statistical software.

Results

Effect of agar and BAP on normal and vitrified shoots of grapevine at 3 weeks after culturing

Hyperhydricity (vitrification) was a serious problem observed in this study. During the conduction of this research, there was an observation of maximum number of normal and vitrified shoots at different concentrations of BAP. In both cultivars, 0.5mg/l BAP and 7.5g/l agar concentrations were contributed in production of maximum number of normal shoots/explant (Table 1).

Table 1. Effect of agar and BAP on normal and vitrified shoots of grapevine at 3 weeks after culturing.

Agar (g/l)	BAP (mg/l)	'Canonannon'		'Chenin Blanc'	
		Mean no. of normal shoots/explant	Mean no. of vitrified shoots/explant	Mean no. of normal shoots/explant	Mean no. of vitrified shoots/explant
0.0	0.0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
6.0	0.5	2.5± 0.8^{b}	2.5± 0.8^{b}	2.0± 0.1^{c}	2.0± 0.1^{c}
6.0	1.0	1.0± 0.6^{c}	1.0± 0.6^{c}	0.7± 0.2^{c}	0.7± 0.2^{c}
6.0	1.5	2.2± 0.3^{c}	2.2± 0.3^{c}	2.1± 0.8^{c}	2.1± 0.8^{c}
6.0	2.0	1.5± 0.2^{c}	1.5± 0.2^{c}	1.3± 0.6^{c}	1.3± 0.6^{c}
6.0	2.5	1.0± 0.1^{c}	1.0± 0.1^{c}	0.8± 0.5^{c}	0.8± 0.5^{c}
6.5	0.5	2.9± 0.5^{b}	2.5± 0.5^{b}	2.3± 0.4^{c}	2.3± 0.4^{c}
6.5	1.0	2.0± 0.8^{c}	2.0± 0.8^{c}	1.6± 0.3^{c}	1.6± 0.3^{c}
6.5	1.5	2.2± 0.2^{c}	2.2± 0.2^{c}	2.1± 0.1^{c}	2.1± 0.1^{c}
6.5	2.0	1.2± 0.1^{c}	1.2± 0.1^{c}	1.2± 0.8^{c}	1.2± 0.8^{c}
6.5	2.5	2.8± 0.7^{b}	2.8± 0.7^{b}	2.4± 0.9^{c}	2.5± 0.9^{b}
7.0	0.5	3.0± 0.9^{ab}	0.5 ± 0.9^{c}	2.5.± 0.5^{b}	0.0 ± 0.0^{d}
7.0	1.0	2.7± 0.5^{b}	1.5± 0.5^{c}	2.2± 0.8^{c}	1.0 ± 0.8^{c}
7.0	1.5	2.1± 0.3^{c}	1.1± 0.3^{c}	1.1± 0.4^{c}	0.0 ± 0.0^{d}
7.0	2.0	2.3± 0.5^{c}	0.3± 0.5^{c}	2.0± 0.3^{c}	1.0± 0.3^{c}
7.0	2.5	1.9± 0.2^{c}	0.9± 0.2^{c}	2.5± 0.4^{b}	0.0± 0.0^{d}
7.5	0.5	6.0± 0.1^{a}	0.0 ± 0.0^{d}	5.0± 0.2^{a}	0.0± 0.0^{d}
7.5	1.0	2.8±0.1^{b}	0.8± 0.1^{c}	2.2± 0.8^{c}	0.0 ± 0.0^{d}
7.5	1.5	3.0 ± 0.3^{ab}	0.0 ± 0.0^{d}	2.5± 0.1^{b}	1.0 ± 0.1^{c}
7.5	2.0	3.0 ± 0.3^{ab}	1.0 ± 0.3^{c}	2.8± 0.2^{b}	1.0 ± 0.2^{c}
7.5	2.5	2.9± 0.3^{b}	0.9± 0.3^{c}	2.5± 0.1^{b}	0.8 ± 0.1^{c}
8.0	0.5	2.0± 0.2^{c}	0.0± 0.0^{d}	1.8± 0.6^{c}	1.4± 0.6^{c}
8.0	1.0	2.2± 0.2^{c}	0.2± 0.2^{c}	2.0± 0.3^{c}	1.0± 0.3^{c}
8.0	1.5	1.2± 0.8^{c}	0.2± 0.8^{c}	1.1± 0.1^{c}	0.1± 0.1^{c}
8.0	2.0	1.8± 0.6^{c}	0.8± 0.6^{c}	1.0± 0.7^{c}	0.2± 0.1^{c}
8.0	2.5	1.0± 0.8^{c}	0.0± 0.0^{d}	0.9± 0.2^{c}	0.1± 0.2^{c}

Means followed by the same letters in the same column are not significantly different at 5% level of probability.

Effect of time intervals on number of normal and vitrified shoots /explant

Culturing time intervals had a great effect on production of normal and vitrified shoots. Thus, as the time of culture increased, there was an increment of vitrified shoots of both varieties of grapevine This result, suggested that the time interval for obtaining maximum mean number of normal shoots was 21 days after culture (Fig. 1).

At the day intervals after culture were 0 to 20; numbers of vitrified shoots were low. But, when the days after culture were increased to 20 to 40, normally produced shoots were changed to vitrified shoots in both varieties (Fig. 2).

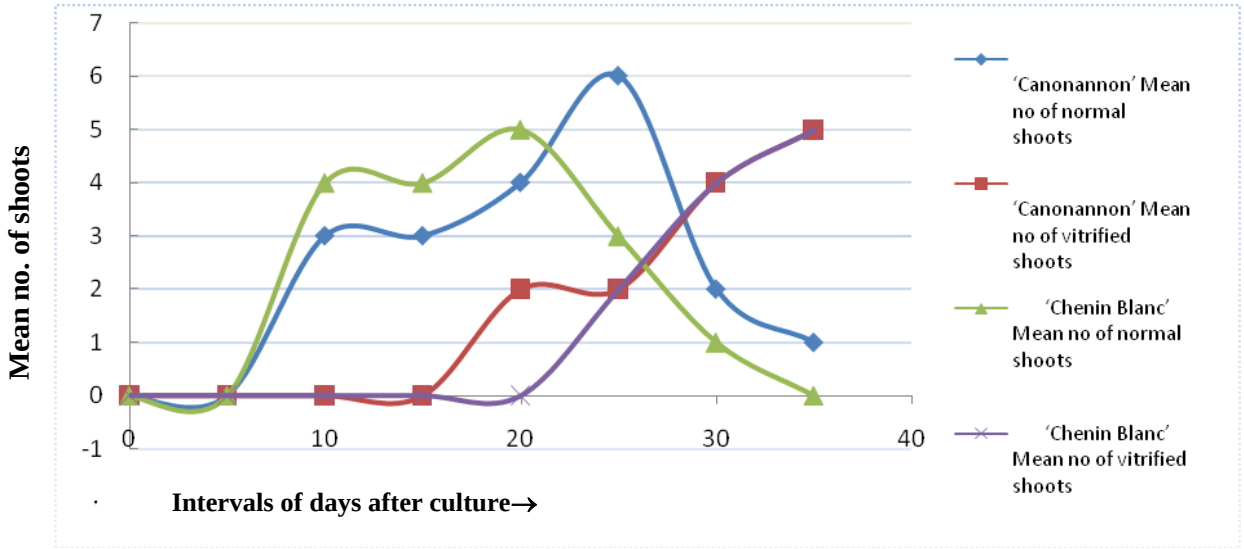

Fig. 1: Effect of time on number of normal and vitrified shoots/explant on MS medium supplemented with 0.5mg/l BAP and 7.5g/l agar.

Fig. 2: A= Chenin Blanc at 21 days, B= Chenin Blanc after 30 days, C= Chenin Blanc after 4 weeks, D= Canonannon at 21 days, E= Canonannon after 30 days, F= Canonannon after 4 weeks).

Effect of agar and IAA on differentiation of roots /explant

The maximum mean numbers of normal shoot roots/explant (5.8 ± 0.3) were obtained when gelling agent was 7.5g/l in 4mg/l IAA for both varieties. In contrast maximum vitrified shoot roots of Canonannon variety were occurred in medium gelled at 7.5g/l in 2mg/l IAA. The maximum mean length of normal shoot roots were observed at medium supplemented with 4mg/l IAA and 7.5g/l gelling agent for both cultivars (Table 2).

Even though there were a maximum number of normal shoot roots at 21days after culture, gradually, it started to retard the growth of root number and length after 21 days after culture. The culturing time interval had a great effect on production of roots number and length. When the time of culture increased, there is an increment of vitrified shoots/explants which, resulted in mal-growth of plant roots/explant in both cultivars. These results suggests that, at 4mg/l IAA and 7.5g/l agar, the time interval for obtaining maximum mean number of normal shoots roots was at 21 days after culture for both varieties (Fig. 3).

Discussion

The present study, deals with improvement of protocol by manipulating agar and BAP concentrations in the medium. The maximum vitrified numbers of shoots were observed at low concentrations of agar (6g/l, 6.5g/l and 7g/l) for both varieties of grapevine. Thus, during the *in vitro* propagation of grapevine it is important to consider the concentrations of agar as it were resulted in vitrified plant when its concentration was lowered. It was reported that agar should not be considered simply as a means of solidifying culture media: its concentrations affected the chemical and physical characteristics of a culture medium (Ziv, 1991). In both varieties, an explant's shoot numbers and length were increased when 7.5g/l agar was supplemented with 0.5mg/l BAP. However, vitrified shoots numbers of both cultivars, were not observed at 0.5g/l agar supplemented with 0.5mg/ BAP, where maximum mean numbers of normal shoots were observed. Related findings had been reported when *in vitro* propagated of *Boswellia serrata* Roxb. cultivars (Suther et al., 2010).

Table 2: Effect of agar and IAA concentrations on differentiation of roots/explant at 3 weeks after culturing.

Agar (g/l)	IAA (mg/l)	'Canonannon'			'Chenin Blanc'				
		Mean no. of normal shoot roots/ explant	Mean length of normal shoot roots /explant	Mean no. of vitrified shoot roots /explant	Mean Length of vitrified shoot roots/explant	Mean no. of normal roots /explant	Mean length of normal shoot roots /explant	Mean no. of vitrified shoot roots/explant	Mean length of vitrified shoot roots/explant
0.0	0.0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
6.0	1.0	1.0± 0.2^{c}	2.0± 0.8^{c}	0.5 ± 0.1^{c}	0.5± 0.1^{c}	1.5± 0.8^{c}	1.5± 0.8^{c}	1.0± 0.1^{c}	0.5± 0.1^{c}
6.0	2.0	1.0± 0.6^{c}	1.0± 0.6^{c}	0.7± 0.2^{c}	0.7± 0.2^{c}	1.0± 0.6^{c}	1.0± 0.6^{c}	0.7± 0.2^{c}	0.7± 0.2^{c}
6.0	3.0	1.2± 0.3^{c}	2.2± 0.3^{c}	1.0± 0.8^{c}	1.1± 0.3^{c}	2.0± 0.3^{c}	2.8± 0.3^{b}	1.0± 0.8^{c}	1.8 ± 0.8^{c}
6.0	4.0	2.5± 0.2^{b}	2.5± 0.2^{b}	2.1± 0.6^{c}	2.3± 0.3^{c}	1.5± 0.2^{c}	3.5± 0.2^{ab}	1.0± 0.6^{c}	2.5± 0.6^{b}
6.5	1.0	2.8± 0.5^{b}	2.5± 0.5^{b}	2.3± 0.4^{c}	2.3± 0.1^{c}	2.9± 0.5^{b}	2.5± 0.5^{b}	2.3± 0.4^{c}	2.3± 0.4^{c}
6.5	2.0	2.9± 0.8^{b}	2.0± 0.8^{c}	2.4± 0.3^{c}	1.6± 0.3^{c}	2.0± 0.8^{c}	2.0± 0.8^{c}	1.6± 0.3^{c}	1.5± 0.2^{c}
6.5	3.0	2. 8± 0.2^{b}	2.3± 0.2^{c}	2.5± 0.1^{b}	2.1± 0.1^{c}	2.2± 0.2^{c}	2.2± 0.2^{c}	2.1± 0.1^{c}	1.1± 0.1^{c}
6.5	4.0	3.0 ± 0.1^{ab}	2.5± 0.1^{b}	2.8± 0.8^{b}	1.2± 0.8^{c}	2.8 ± 0.1^{b}	1.2± 0.1^{c}	2.2± 0.8^{c}	1.2± 0.8^{c}
7.0	1.0	3. 0± 0.9^{ab}	2.8 ± 0.9^{b}	2.9.± 0.5^{b}	2.0 ± 0.5^{c}	3.0± 0.9^{ab}	1.0 ± 0.9^{c}	2.5.± 0.5^{b}	0.5 ± 0.5^{c}
7.0	2.0	3.1± 0.5^{ab}	2.9± 0.5^{b}	2.5± 0.8^{b}	2.0 ± 0.8^{c}	2.7± 0.5^{b}	1.5± 0.5^{c}	2.2± 0.8^{c}	0.5 ± 0.8^{c}
7.0	30	3.1± 0.3^{ab}	3.1± 0.3^{ab}	2.9± 0.4^{b}	2.0 ± 0.4^{c}	2.1± 0.3^{c}	1.1± 0.3^{c}	2.1± 0.4^{c}	0.1 ± 0.4^{c}
7.0	4.0	3.3± 0.5^{ab}	3.3± 0.5^{ab}	2.8± 0.3^{b}	2.1± 0.3^{c}	2.3± 0.5^{c}	0.3± 0.5^{c}	2.0± 0.3^{c}	1.0± 0.3^{c}
7.5	1.0	3.2 ± 0.2^{ab}	6.6 ± 3.0^{a}	2.6± 0.1^{b}	2.8 ± 0.1^{c}	3.0 ± 0.2^{ab}	6.1 ± 4.1^{a}	2.1± 0.1^{c}	2.8 ± 0.1^{b}
7.5	2.0	4.1 ± 0.1^{ab}	8.0 ± 4.2^{a}	3.0± 0.8^{ab}	3.2± 0.8^{ab}	3.2 ± 0.2^{ab}	7.0 ± 3.2^{a}	2.2± 0.8^{c}	3.2± 0.8^{ab}
7.5	3.0	4.5 ± 0.3^{ab}	7.0 ± 0.3^{a}	2.8.± 0.5^{b}	3.5.± 0.5^{ab}	3.8 ± 0.1^{ab}	7.0 .± 0.2^{a}	2.5.± 0.5^{b}	3.5.± 0.5^{ab}
7.5	4.0	5.8 ± 0.3^{a}	8.5 ± 1.4^{a}	2.1± 0.8^{c}	3.2± 0.8^{ab}	4.0 ± 0.5^{ab}	7.2 ± 0.3^{a}	2.2± 0.8^{c}	3.0± 0.8^{ab}
8.0	1.0	5.0± 0.2^{a}	5.2± 0.2^{a}	2.2± 0.4^{c}	2.1± 0.4^{c}	2.5.± 0.5^{b}	2.5.± 0.5^{b}	2.1± 0.4^{c}	1.1± 0.4^{c}
8.0	2.0	3.2± 0.2^{ab}	4.2± 0.2^{ab}	2.0± 0.3^{c}	2.0± 0.3^{c}	2.2± 0.8^{c}	2.2± 0.8^{c}	2.0± 0.3^{c}	2.0± 0.3^{c}
8.0	3.0	2.2± 0.8^{c}	3.2± 0.8^{ab}	1.1± 0.1^{c}	2.1± 0.1^{c}	1.1± 0.4^{c}	1.1± 0.4^{c}	1.1± 0.1^{c}	1.1± 0.4^{c}
8.0	4.0	1.8± 0.6^{c}	2.8± 0.6^{b}	1.0± 0.7^{c}	2.0± 0.1^{c}	2.0± 0.3^{c}	2.0± 0.3^{c}	1.1± 0.2^{c}	1.1± 0.4^{c}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability

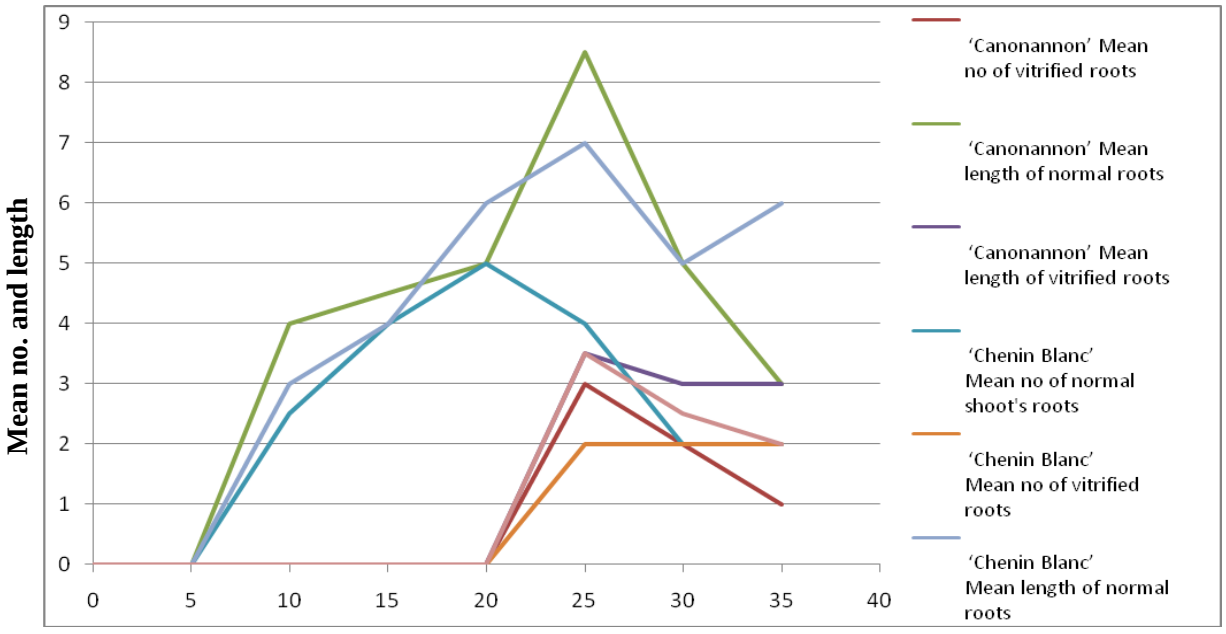

Fig. 3: Mean number and length of normal and vitrified roots on MS medium supplemented with 4mg/l IAA at day intervals after culture.

At a constant concentration of agar treatment, the different BAP concentration had also shown different number of normal and vitrified shoots (Table 1). Any way the problem of vitrification were fixed when the

agar concentrations were increased to 7.5g/l, which resulted in recovering the maximum number of shoots 6.0±0.1 and 5.0± 0.2 for Canonannon' and Chenin Blanc cultivars, respectively. This is in reliable with the initiated better shoot number and length after *in vitro* controlling of vitrification of Shallot plant (Rasco and Patena, 1997).

In the current investigation it's observed that, at a normal shoots induction medium (a medium supplemented with 0.5mg/l PAP and 7.5g/l agar) , an increment of culture time intervals (days between 21 to 40) affect the normal shoots which, had been resulted in changing the normal shoots to losing leaves from shoot tips and formed vitrified shoots. Such condition was highly observed on Chenin Blanc cultivar. But, on similar medium, the better time to recover maximum number and length of shoots/explant was at 21 days after culture. About 6.0± 0.1 and 5.0± 0.2 mean numbers of normal shoots/explants were obtained from Canonannon and Chenin Blanc cultivars, respectively. This similar day culture time was recorded to obtain maximum number (5.8 ± 0.3) and length (8.5 ± 1.4) of normal shoot roots/explants for Canonannon variety.

The normal transferred shoots were survived when brought to *ex-vitro* conditions. The survival percentages were 94% and 75% for Chenin Blanc and Canonannon, respectively. But, the acclimatization of vitrified plantlets of both varieties was not successful. This was due to the developed roots number and length was lower in comparing to the normal plantlets. Correspondent findings were also reported for other cultivars (Kevers et al., 2004).

Conclusion and recommendations

The incidence of *in vitro* shoot vitrification /hyperhydricity/ in grapevine was examined under different concentrations of BAP and agar. From the tested five different concentrations of BAP and agar, the best mean number of normal shoots was obtained at 0.5 mg/l BAP in 7.5g/l gelling agent for both cultivars. Similarly, maximum mean numbers of normal shoot roots/explant were obtained at 7.5g/l gelling agent and 4mg/l IAA for both varieties. Acclimatized normal shoots/explant were survived while all vitrified shoots/explant were not survived. Testing all phyto-hormones for reduction of grapevine vitrification is recommended. In general, frequency of *in vitro* normal shoot regeneration was greatly influenced by the concentrations of BAP, gelling agent and culture time intervals.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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