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Effects of Ultrasound, Tryptophan and Proline on embryogenesis and regeneration of grape (*Vitis vinifera* L.)

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ABSTRACT

Genetic improvement of Grape is limited by traditional methods. An effective regeneration system for tissues culture of transgenic adult plants could facilitate genetic modification of them. So it is necessary to develop and improve embryogenesis and regeneration systems in plants. Accordingly the aim of the present study was to evaluate the effects of ultrasound (0 (as control), 60, 120 and 240 second), tryptophan (0 (as control), 50,100, 200 µM) and proline content (0 (as control), 50, 100 and 200 µM) on grape stem internodes explants in Kodori cultivar. This project was performed in factorial experiment (two factors) in the basis of completely randomized design with three replications at tissue culture laboratory of Shahed University of Tehran. Results showed that both ultrasound and two explained amino acids had significant effects on studied characteristics such as callus frequency, callus length and width, fresh weight, embryo numbers in each callus and their germination percentage. Generally, using 100 µM tryptophan and proline coincide with 120 second ultrasound had highest positive effects on the most studied characteristics.

INTRODUCTION

Grape is cultured worldwide, making it one of the most important commercial fruit crops in terms of economic value (Kurmi et al. 2011). Although it has been relatively easy to achieve somatic embryogenesis from grapevine, the frequency of plant regeneration from these embryos usually is low (Rajasekaran and Mullins 1979; Martinelli et al. 1993).

Several *Vitis vinifera* L. genotypes have been regenerated by somatic embryogenesis from explants derived from different plant tissues (Martinelli and Gribaudo 2001). In grapes somatic embryogenesis and regeneration of complete plants was first described by Mullins and Srinivasan (1976), they used unfertilized ovules of the *Vitis vinifera* cv 'Cabernet Sauvignon.' Subsequently, regeneration of *V. vinifera* somatic embryos has been reported from anthers (Salunkhe et al.1999; Bouamama et al. 2007; Cutanda et al. 2008) immature zygotic embryos (Stamp and Meredith 1988a), leaves (Martinelli et al. 1993; Das et al. 2002), pistals and stamens (Cutanda et al. 2008), tendrils (Salunkhe et al. 1997), ovaries (Motoike et al. 2001), as well as from Styles and stigmas (Nakajima and Matsuta 2003; Morgana et al. 2004). The limitation of the explants is that anthers, styles and stigmas and zygotic embryos are available for experimentation for only a brief period. High regeneration frequency is important, because stable transformation of grapevine cells occurs at relatively low frequency (Iocco et al. 2001). The induction of somatic embryogenesis is not yet routine in most laboratories and the percentage of embryogenesis differs among grapevine genotypes. The explant type and induction medium have been reported to be critical for establishment of embryogenic cultures (Martinelli and Gribaudo 2001). Factors that influence somatic embryogenesis, including explant type and developmental stage, macro- and microelement composition of the culture medium and growth regulator concentration warrant examination (Thorpe and Stasolla 2001).

Ultrasound is the field of science dealing with the application of sound frequencies in the inaudible range, generally from 20-100 kHz, although special applications occur outside that range (Gaba et al. 2008). At the organism (plant) level ultrasound enhances the germination of

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various seeds and the subsequent growth of the seedling (Gaba et al. 2008).

Low-frequency ultrasound, as an abiotic stress with thermal and chemical effects in living organism, has several biological effects (Jaime and Judit 2014). The growth and development of several plant species in vitro has been stimulated by ultrasound, although the exact response depends on the frequency and exposure period. To date, sound has shown positive effects on carrot (*Daucus carota* L.) (Wang et al. 1998), rice (*Oryza sativa* L.) (Liu et al. 2003), aloe (*Aloe arborescens* Mill.) (Liu et al. 2003), hazelnut (*Corylus avellana* L.) (Safari et al. 2013). It was recently reported direct regeneration from in vitro seedling-derived cotyledons of squash in vitro (Ananthakrishnan et al. 2007).

Exogenously added amino acids play an important role in plant tissue culture but culture media of existing regeneration protocols are scarcely supplemented with amino acids (Asad et al. 2009). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon 2007). In this study, we describe the use of nodal segments as valuable explants excised from mature somatic tissue and available throughout the year for the induction of somatic embryogenesis by evaluate the effect of ultrasound and amino acids (proline and tryptophan).

MATERIALS AND METHODS

Explants obtained by stem internodes of grape (Kodori cv) collected in May 2015 from research garden of faculty of agriculture, Shahed University. After sampling, the explants were transmitted to laboratory and each sample was washed three times for 15 minutes with running water and suitable detergent. The next step was followed by separating the leaves from stem and washing with sodium hypochlorite (0.1%). In order to remove sodium hypochlorite from sample surfaces, the explants were washed three times for 5 minutes with distilled water and placed in ethanol 70% for 1 minute and washed three times with distilled water. After that, cultured dishes were kept in complete darkness condition for callus induction. Aseptic explants were cultured in MS medium containing 0.1 Mg/ L 2,4-D, 1 $Mg.L^{-1}$ BAP and 30 g sucrose. Obtained calluses were transferred to MS embryogenesis medium containing 1µM 2,4-D, 0.1 µM BAP supplemented with proline and tryptophan (0 (as control), 50, 100 and 200 µM). After a week- long, ultra sound treatments were applied with the power of 2 W and a frequency on 20 kHz in four times (0 (as control), 60, 120 and 240). Spending two months, embryonic cotyledons were observed and cultural dishes were maintained in chamber room with 16h light, 8h darkness and controlled temperature of 25 ^{o}C. In order to germination, embryonic cotyledons were cultured in MS medium with 1 $Mg.L^{-1}$ BAP without auxin and ultrasound treatment. Finally callus frequency, length, height, fresh and dry weight, number of embryos per callus and embryo germination were evaluated. This project was performed in factorial experiment in the basis of completely randomized design with three replications. Data analysis was performed by SPSS software and means comparisons was done using Duncan's multiple range test.

RESULTS

Callusing frequency

According to variance analysis in the first experiment (Table 1), ultrasound, tryptophan and interaction of these two factors had significant ($P\leq0.01$) effects on callusing frequency. The most callusing frequency was in 50µM tryptophan and 240 second ultrasound treatment and 100 µM with 60 ultrasound waves which the means was 3.66. It was in same group with 100µM tryptophan in 120 and 240 second ultrasound treatment and 200µM tryptophan in 60 and 120 second ultrasound (table 2).

Second experiment variance analysis (Table 3) showed that the effect of ultrasound waves and interaction of ultrasound and proline was significant ($P\leq0.01$) on callusing frequency. The most callusing frequency was obtained in 100µM proline and applying 60 and 120 second ultrasound. It was in same group with 50µM proline and 60

Table 1. Analysis of variance of the data for the effect of tryptophan and ultrasound in studied traits of grape

S.O.V	df	MS						
		Callusing frequency	Callus length	Callus weight	Callus fresh weight	Callus dry weight	Embryo count in each callus	Embryo's germination percentage
Ultrasonic	3	0.916*	120.96**	82.47**	19.06**	0.012ns	61.39**	10820.3**
Tryptophan	3	2.08**	20.52**	6.02**	2.82**	0.021ns	5.83**	1330.9**
US×T	9	1.95**	18.63**	10.71**	6.62**	0.021ns	16.29**	1763.8**
Error	32	0.27	0.791	0.416	0.070	0.014	0.044	13.14
CV (%)	-	19.82	10.59	10.98	5.99	25.37	7.82	11.24

ns: non-significant; * and ** are significant at the level of 0.05 and 0.01

second ultrasound treatment (Table 4).

Callus length

The results showed that the effect of ultrasound, tryptophan and interaction of ultrasound wave and tryptophan had significant (P≤0.01) effect on callus length statistically (Table 1). Mean comparison of interaction effects indicated that maximum callus length is related to 100 µM tryptophan in combination with 120s ultrasound waves with the average of 66.14 mm and the lowest one was observed in combination treatment of 50 µM tryptophan without ultrasound (Table 2). Callus cells elongation in different stages, embryogenic callus and embryos derived from stem explants was presented in Figure 3 A, B and C. Results of second experiment showed that (Table 3) the effect of ultrasound wave and proline and interaction of them was significant (P≤0.01) on callus length. The highest and lowest callus length in this experiment was observed in applying 100 µM proline with 120s ultrasound wave (12.66 mm) and 50 µM proline without ultrasound (4 mm), respectively (Table 4).

Callus latitude

Variance analysis of data obtained by first experiment showed that the main effect (tryptophan and ultrasound) and combination effects of tryptophan and ultrasound had significant (P≤0.01) effects on callus width. According to mean comparisons, the maximum callus width obtained in 60s and 120s applying ultrasound wave in three levels of tryptophan 50, 100 and 200 µM and minimum values was in 50 µM tryptophan without ultrasound wave (Table 2).

Table 3, showed the significant effects of ultrasound, proline and combination effects of these two factors on callus width (P≤0.01). According to mean comparisons of interactions maximum callus width was obtained in applying 100 µM proline in 120s ultrasound wave (9 mm). However, statistically, applying 50 µM proline with 60s and 120s ultrasound was in same group with 200 µM proline in 60s ultrasound having high callus width.

Callus fresh weight

Variance analysis (Table 1) showed that ultrasound, tryptophan and interaction of them had significant effects on callus fresh weight statistically (P≤0.01). Interaction effects means comparisons showed that applying 100 µM tryptophan with 120s ultrasound yield maximum callus weight (6.89g) and the lowest values was obtained by applying 50 µM tryptophan without ultrasound wave (0.3g averagely) (Table 2).

According to variance analysis of second experiment (Table 3) the effect of ultrasound, proline and interaction of them on callus fresh weight was significant. The most callus fresh weight was in combination treatment of applying 50 and 100 µM proline with 120s ultrasound wave (respectively with 6.13 and 6.04 means of callus fresh weight) (Table 4). The lowest callus fresh weight was observed in highest applying levels of proline and ultrasound (200 µM proline and 240 ultrasound wave).

Callus dry weight

According to Table 1 in first experiment no significant results were seen in callus dry weight but Table 3 showed that in second experiment proline, ultrasound and combination of them had significant effect on callus dry weight statistically (P≤0.01). Means comparison of combination effects revealed that maximum callus dry weight

Table2. Mean comparisons of different sonication and tryptophan levels

Sonication (s)	Tryptophan (µM)	Callusing frequency (number)	Callus length (mm)	Callus high (mm)	Callus fresh weight (g)	Callus dry weight (g)	Embryo count in each callus	Embryo's germination percentage
0	0	2.66^{bc}	3^{g}	2g^{h}	3.73^{fg}	0.10^{b}	0^{h}	0^{f}
	50	2.66^{bc}	8^{e}	4.66^{de}	4.1^{et}	0.110^{b}	0.7^{g}	0^{f}
	100	2.33^{cd}	9.66^{cd}	7^{b}	4.66^{d}	0.130^{b}	7.36^{a}	63.33^{c}
	200	2.33^{cd}	8^{e}	5.66^{cd}	4.38^{de}	0.271^{a}	0^{h}	0^{f}
60	0	1^{e}	1^{h}	0^{h}	0.3^{j}	0.13^{b}	0^{h}	0^{f}
	50	1.33^{e}	11^{bc}	8.33^{a}	3.43^{gh}	0.16^{b}	2.76^{f}	34.33^{e}
	100	2.66^{bc}	9.66^{cd}	8.66^{a}	5.91^{b}	0.21^{ab}	5.93^{b}	76.33^{ab}
	200	3.66^{a}	8^{e}	8.33^{bc}	6.24^{b}	0.22^{ab}	5.74^{bc}	45.66^{d}
120	0	2.66^{bc}	8.66^{de}	5.33^{cd}	4.15^{et}	0.18^{b}	0^{h}	0^{f}
	50	3.66^{a}	11.33^{b}	8.32^{a}	5.43^{c}	0.22^{ab}	0^{h}	0^{f}
	100	3.33^{ab}	14.66^{a}	9^{a}	6.89^{a}	0.28^{a}	5.49^{c}	81^{a}
	200	3a^{bc}	4.66^{f}	2.66^{fg}	3.94^{et}	0.24^{ab}	5.96^{b}	61.66^{c}
240	0	2.66^{bc}	7.33^{e}	3.66^{ef}	3.06^{h}	0.15^{b}	0.86^{g}	0^{f}
	50	3a^{bc}	12.33^{b}	9.33^{a}	5.99^{b}	0.21^{ab}	4.66^{d}	73.33^{b}
	100	3.33^{ab}	12^{b}	9^{a}	5.95^{b}	0.18^{b}	3.8^{e}	72.33^{b}
	200	1.66^{de}	5^{f}	2.66^{fg}	2.55^{i}	0.08^{c}	0^{h}	0^{f}

Different letters in each column indicate significant difference at p≤0.05

Figure 3. callus cells at different stages of growth and division (A), Embryogenic callus (B), Embryos derived from stem explants (C).

obtained when using 100 µM proline with 120s ultrasound (0.23 g) that it was in same group with 200 µM proline with 60s ultrasound wave (Table 4).

Number of embryo in each callus

According data obtained from Table 1 the effect of tryptophan, ultrasound wave and their combination effects was significant (P≤0.01) on average of embryo number in each callus. Maximum embryo number in each callus in mean comparisons of combination effects was in applying no tryptophan and 120s ultrasound (Table 2). Embryo number of callus in second experiment is affected by ultrasound wave, proline and contraction effects of ultrasound×proline significantly (Table 3). As it shown, in combination treatment of 50µM proline with exposing 60s ultra-wave maximum means of embryo number (4.43) was observed (Table 4).

Embryo germination percent

Results obtained by first and second experiment showed that in both of them the main interactions effects were significant in embryo germination percentage (P≤0.01) (Table 1 and 3). Mean comparison of interaction effects of ultrasound and tryptophan showed that maximum embryo germination was in 100 µM tryptophan with 120s ultrasound waves (81% averagely) (Table 2). Also interaction of ultrasound and tryptophan revealed that in 100 µM proline with 120s ultrasound (82% averagely) maximum germination percent was obtained (Table 4).

DISCUSSION

Callus production take place in the grapes with different objectives. The aim of callus production can be regeneration and embryogenesis. Embryogenesis considered a variety of purposes such as plant micro propagation, genetic changes, somatic hybridization and soma clonal variation. Another callus applications (in addition to embryogenesis) is using in gene transfer programs and secondary metabolites production. Results obtained by Liu et al. (2006) showed that exposure callus of *Aloe arborescens* to ultrasound were helpful and increase their adaptation to environmental stresses by increasing Ca-ATPase activity in solid media. Activity of this pump to maintain calcium (Ca+) homeostasis is necessary for plant cell growth and development. Results of this study showed that using ultrasound wave increase callus frequency which is corresponded with other results. In another study the use of ultrasound in *chrysanthemums* increased callus cell division (Hassanien et al. 2014). Review of various researches showed that the effect of ultrasound on frequency depends on intensity and duration of exposure (Rokhina et al. 2009). In vitro callus and somatic embryogenesis of grape leaves (c.v Crimson Seedless) changes by different plant growth regulators and amino acids used in callusing environment (Nookaraju and Agrawal 2015). Chrysanthemums affected by ultrasound waves decrease cell namber in G0/G1 stage but increase in S stage. Mentioned changes show that

Table 3. Analysis of variance of the data for the effect of Proline and ultrasound on studied traits of grape

S.O.V	df	MS						
		Callusing frequency	Callus length	Callus weight	Callus fresh weight	Callus dry weight	Embryo count in each callus	Embryo's germination percentage
Ultrasonic	3	3.50**	60.72**	44.08**	10.40**	0.0085**	16.56**	4573.1**
Proline	3	3.72**	15.83**	10.36**	3.90**	0.0131**	17.35**	3540.9**
US×P	9	1.11**	7.77**	6.36**	1.68**	0.0069**	5.77**	1703.1**
Error	32	0.31	0.56	0.52	0.05	0.00008	0.032	3.39
CV (%)	-	18.13	8.73	12.28	5.54	6.20	12.48	8.22

ns: non-significant; * and ** are significant at the level of 0.05 and 0.01

Table 4. Mean comparisons of different sonication and Proline levels

Sonication (s)	Proline (µM)	Callusing frequency (number)	Callus length (mm)	Callus high (mm)	Callus fresh weight (g)	Callus dry weight (g)	Embryo count in each callus	Embryo's germination percentage
0	0	2.66^{cd}	5.33^{h}	$2g^{j}$	3.43^{ef}	0.109^{g}	0^{e}	0^{f}
	50	2.66^{cd}	10^{cd}	6.66^{det}	3.67^{de}	0.105^{gh}	0^{e}	0^{t}
	100	2.66^{cd}	8^{et}	4.33^{h1}	4.23^{c}	0.102^{gh}	0^{e}	0^{t}
	200	2.33^{de}	7^{tg}	6.33^{etg}	3.23^{t}	0.091^{h}	0^{e}	0^{t}
60	0	1.66^{e}	4^{i}	2.66^{j}	2.61^{g}	0.091^{h}	0^{e}	0^{f}
	50	4^{ab}	11.33^{b}	8.33^{ab}	4.71^{b}	0.197^{cd}	4.43^{a}	53.33^{c}
	100	3.33^{bc}	11^{bc}	8.33^{ab}	6.13^{a}	0.211^{bc}	3.46^{c}	61^{b}
	200	3.66^{b}	10.33^{bc}	6^{efg}	4.16^{c}	0.190^{de}	3.36^{c}	40.66^{e}
120	0	2.66^{cd}	9^{de}	5.33^{gh}	4.24^{c}	0.177^{ef}	0^{e}	0^{f}
	50	4.66^{a}	11.33^{b}	7.66^{bcd}	4.67^{b}	0.188^{de}	3.6^{c}	48.66^{d}
	100	4.66^{a}	12.66^{a}	9^{a}	6.04^{a}	0.230^{a}	4.03^{b}	82^{a}
	200	3.33^{bc}	6.66^{g}	5.66^{etg}	3.52^{et}	0.092^{h}	0^{e}	0^{t}
240	0	2.66^{cd}	4.66^{h1}	4^{i}	2.76^{g}	0.169^{t}	0.76^{d}	0^{t}
	50	$3.33a^{bc}$	10^{cd}	8^{abc}	4.99^{b}	0.217^{ab}	3.43^{c}	62.66^{b}
	100	2.66^{cd}	8.66^{e}	7^{cde}	4.01^{cd}	0.105^{gh}	0^{e}	0^{f}
	200	2.33^{de}	7.33^{fg}	2.66^{j}	2.04^{h}	0.094^{gh}	0^{e}	0^{r}

Different letters in each column indicate significant difference at $p \leq 0.05$

the radiation hastened callus growth in Chrysanthemums (Wang et al. 2003). Results obtained by this experiment are in compliance with all the results. Compare the above mentioned about working with ultrasound and its effect on callus growth implies that appropriate intensity and optimal duration of ultrasound is different in plant species. As it shown in this experiment using 240s ultrasound decrease studied characteristics and decrease means as compared to 120s. It seems that using longer time of ultrasound cause destroyed and damage to samples. On the other hand is considered as a stress induction factor and ultimately reduce means.

Ananthakrishnan et al. (2007) reported that ultrasound treatment between 0.5-2 minutes increase stem regeneration of *Cucurbita pepo* L. (c.v Ma'yan and Bareqet) cotyledon explants in high levels (five times more than control) in in vitro condition. Regeneration was occurred in supplementary culture medium with 4.4µM Adenin Banzil while in a controlled environment without ultrasound treatment regeneration only was limited to a few small branches and bud like structures. Ultrasound treatment also stimulated the subsequent growth of seedlings. This the first study that demonstrated ultrasound stimulates regeneration. Hassanien et al. (2014) explain that in chrysanthemums callus treated with ultrasound due to increase activity of H^{+}-ATPase pomp, total sugar content, soluble protein and amylase activity increase in addition to RNA content and transcription levels. Increase these materials is suitable for production embryonic structures. The results of this study are corresponded with other results and indicate that proper duration and range would help embryogenesis. Callus tissue growth stimulate by amino acids significantly. This suggested that organic nitrogen is the limiting factor plant tissue culture. Cells needs nitrogen atoms to construct molecules such as nucleotides, amino acids, sugars and vitamins. When glucose levels are low and energy demand is high, cell can be use amino acids for metabolism (Elmeer 2013). Nitrogen sources often have a beneficial effect on callusing. Tyrosine and phenylalanine increase organ formation in tobacco callus (Siriwardana and Nabors 1983). It is reported that tryptophan increase rice cultivars Calrose 76, IR 36 and Pokkali callusing significantly compare to control treatment (without amino acid application) (Siriwardana and Nabors 1983). Nieves et al. (2008) in their experiments on sugarcane reported that amino acid enhance callus growth and embryogenic tissues. Also, it has positive effects on conversion non-embryonic callus to embryonic callus. Asad et al. (2009) demonstrated that various amino acids are beneficial for plant cells processing and reproduction. When amino acids are added to culture mediums as combination have a better effect than added them to the medium separately. Elmeer (2013) suggested that amino acids help to growth cells which require a large amount of energy for nucleic acid and protein synthesis. Furthermore organic sources of nitrogen used as a supplementary source of energy for cells which are inefficient to use glucose. So it is appearing that tryptophan as a source of nitrogen that is available for plant simply, improve callus cell growth characteristics.

Addition 12.5- 50 MM proline to the medium culture with basic salt of MS medium in *Miscanthus ogiformis* for callus induction showed that embryonic callus increase significantly.

Healing effect of proline is depending on proline concentration and medium culture salts. A proper concentration of proline for plant regeneration was reported 5.12 MM (Holme et al. 1997). In recent study using 200 µM proline had less positive effects than 100 µM and in some cases decease means compare with using 100 µM. Studies on shoot regeneration of sugar cane was reported that glycine (0.75 MM), arginine (0.75 MM) and cysteine (0.25 MM) had significant effect (94%) on shoot regeneration compared to medium cultures without amino acid (Asad et al. 2009). When tryptophan was added to culture medium of rice variety in appropriate level (100 µM), regeneration increase significantly that exactly supported this research results. Embryogenesis and regeneration increased when proline and tryptophan was added to rice culture medium in combination with 2, 4-D (Zafar et al. 1992). Obtained results showed that adding 100 µM tryptophan or proline to each experiment had positive and multiplier effects on embryogenesis and regeneration. Amino acids regulate cell growth and differentiation and influence cell metabolism and morphogenesis positively (Vasanth et al. 2006). Adding proline affects embryogenic callus formation and growth. Proline effectiveness is depending on proline and base salts of medium culture. Proline also increase suspended culture growth (Holme et al. 1997). Furthermore proline promoted embryonic callus formation of maize and sugar cane in solid medium and suspensions (Holme et al. 1997). Vasanth et al (2006) demonstrated that amino acids play a vital role in branch induction and development of cotyledon explants and embryonic axis of peanut (*Arachis hypogaea* L.). Adding various concentration of proline to the anther medium culture had bear successful results and increase embryogenesis (Özkum and Tipirdamaz 2011).

CONCLUSION

The results showed that ultrasound, tryptophan and proline had a significant effect on grape embryogenesis and regeneration. Proline and tryptophan application in 100 µM and ultrasound wave in 120s had the most favorable results and increase embryogenic characteristics (callusing frequency, callus width and length, fresh and dry weight of callus and germination percentage).

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