The First Successful Report: Control of Browning Problem in in vitro *Culture of Iranian Seedless Barberry, a Medicinally Important Species*

Rahim Nazary Moghaddam Aghayeh, Bahram Abedy, Ahmad Balandari, Leila Samiei & Ali Tehranifar

Erwerbs-Obstbau

ISSN 0014-0309

Erwerbs-Obstbau
DOI 10.1007/s10341-021-00574-6

Springer

Erwerbs-Obstbau
https://doi.org/10.1007/s10341-021-00574-6

ORIGINAL ARTICLE / ORIGINALBEITRAG

The First Successful Report: Control of Browning Problem in *in vitro* Culture of Iranian Seedless Barberry, a Medicinally Important Species

Rahim Nazary Moghaddam Aghayeh[1] · Bahram Abedy[1] · Ahmad Balandari[2] · Leila Samiei[3] · Ali Tehranifar[1]

Received: 28 March 2019 / Accepted: 26 March 2021

Abstract

The current study was aimed to solve the problem of browning in Iranian seedless barberry *in vitro* culture by using a variety of different approaches. As such, several experiments were carried out. The results revealed that addition of 225 mg/l citric acid + 50 mg/l ascorbic acid could alleviate phenol browning compared to control. Presoaking of explants in 300 mg/l citric acid solution for 30 min and culturing them in media containing 225 mg/l citric acid could further reduce browning rate. Results related to the effect of sampling time indicated that explants cut off in April showed the lowest browning rate and those cut off in January, February and March showed the highest browning rate. Effects of conditions related to stock plant were also investigated, and the results displayed that explants harvested from upper position of current-year shoot and sucker showed the lowest browning rates, respectively, while those collected from lower position of one- and two-year old shoots exhibited the highest browning rate, respectively. Data obtained from shoot color and diameter also demonstrated that explants taken from pinkish-colored shoots with small diameter showed the lowest browning rate and those collected from dark brown-colored shoots with large and very large diameter contained the highest browning rates. Use of 8-hydroxyquinoline and Fe-EDDHA for reduction of browning was investigated for the first time, and interestingly the results showed that both compounds, in particular 8-hydroxyquinoline, caused a significant reduction in phenol exudation. More interestingly, they could cause a significant increase in bud break rate. Furthermore, phenol exudation and peroxidase activity in treatments supplemented with 8-hydroxyquinoline and Fe-EDDHA were lower than those in treatments containing antioxidants.

Keywords Iranian seedless barberry · Tissue culture · Browning · Explant · Antioxidant

Ein erster Erfahrungsbericht: Die Regulierung des Verbräunungsproblems bei der In-Vitro-Kultur der iranischen kernlosen Berberitze, einer für die Medizin wichtigen Species

Schlüsselwörter Iranische kernlose Berberitze · Gewebekultur · Verbräunung · Explantat · Antioxidans

Introduction

Seedless barberry (*Berberis vulgaris* L. var. asperma), a highly valued member in the family of *Berberidaceae*, is a drought-tolerant species which is mainly grown in dry regions of Iran. This economically important crop is the only source of income in some parts of the country. The economic importance of this multipurpose shrub is due to its fruits, which are used as raw or cooked (Arayne et al. 2007). Due to containing high contents of phenolic compounds (anthocyanins) and ascorbic acid, the fruits are also regarded as healthy additives that are used in many foods.

✉ Bahram Abedy
Abedy@um.ac.ir

[1] Department of Horticultural Science and Landscape, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

[2] Khorasan Science and Technology Park (KSTP), Mashhad, Iran

[3] Department of Ornamental Plants, Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

Published online: 22 July 2021

With high medicinal value, which is mainly due to the presence of alkaloid berberine in bark of root, stem and unripe fruits, this species has been used for curing diseases such as infectious fevers, typhus, arterial blood pressure, diabetes, different types of cancer, Alzheimer and human immunodeficiency virus (HIV) (Fatehi et al. 2005; Arayne et al. 2007; Asai et al. 2007; Ebrahimi-Mamaghani et al. 2009; Bodiwala et al. 2011; Yin et al. 2012).

Moreover, beautiful yellow flowers of this crop in the early spring which later turn into red berries in summer and early autumn create an attractive view in the landscape. The shrub also can be used as border wall around orchards and lands owing to having thorn and compacted growth habit. There is a growing desire in the country to cultivate this valuable crop in many parts of the country, especially in regions that impending changes of climate threaten the cultivation of high-water demanding crops and thus cultivation of low water demanding plants is inevitable, but it is difficult to find suitable clone of this shrub due to its recalcitrant propagation. Finding a way to propagate this species vegetatively, which cannot be propagated sexually since it is seedless, has for long time been the subject of many studies but the results obtained so far have not been fruitful. Sucker is the only means of propagation of this multipurpose crop but it has its own limitations. Not only suckers should be divided from mother plants at specific time of the year but also they should be in suitable size (60–70 cm in length) and age (2–3 years old) with adequate roots (Balandary and Kafi 2001). Besides, damages occur during division and transportation cause a high percentage of the plants to fail to grow after cultivation, making the cultivation very time-consuming, laborious and expensive process. In addition, provision of high-quality and uniform suckers on a large scale is not easily possible. As an efficient technique, micropropagation has been successfully used for propagation of many plants. Unlike many fruit trees, whose tissue culture have been successfully optimized, *in vitro* culture of Iranian seedless barberry has not been successful thus far, as disappointingly reported by Azizi et al. (2007) and Mohammadi et al. (2011). One of the most serious obstacles in the way of successful micropropagation of Iranian seedless barberry is the exudation of phenolic compound. Browning posed serious problems for successful micropropagation of Iranian seedless barberry as shown in the preliminary tests conducted for 1 year, and the many unpublished attempts made to tackle this problem proved futile up to now. Control of phenol-induced browning of explants in *in vitro* culture is an important factor determining the success rate of establishment phase of all fruit trees (Leal et al. 2007; Pérez-Tornero and Burgos 2007). Since this crop has been found as a species with high degree of browning and there is no background about the problem in this crop, doing some researches to overcome this obstacle can lay the foundations for improvement of Iranian seedless barberry *in vitro* culture. The present investigation thus tried for the first time to assess the influence of a variety of factors including compounds controlling phenol-induced browning, sampling time and conditions related to explants on browning problem in barberry *in vitro* culture.

Materials and Methods

Plant Material

The current research was performed in tissue culture laboratory of Horticultural Sciences Department, Ferdowsi University of Mashhad, Iran, in a 3-year period from 2014 to 2017. After being collected from barberry shrubs located in research orchard of Agricultural Faculty, 25–30 cm shoots were transferred to the laboratory. Shoots were then stripped off leaves and thorns, cut into 2–3 cm pieces and subjected to different treatments designed for controlling phenol-induced browning.

For designing of the treatments, four issues were hypothesized to play a role in reducing phenol-induced browning and accordingly were taken into consideration: 1) compounds controlling phenol exudation, 2) time of sampling, 3) conditions of explants on stock plant, and 4) subculture.

Experiments Related to Phenol Controlling Agents

The part related to the compounds controlling phenolic browning was divided into four experiments. In the first experiment, the combined effects of addition of citric acid (0, 75, 150 and 225 mg/l) and ascorbic acid (0, 50, 100) to culture medium were investigated. In the second experiment, citric acid was used in sequential manner, so that explants were first soaked in solutions containing 150 and 300 mg/l citric acid for 15 and 30 min, and then cultured in media containing the same antioxidant at three levels of 0, 150 and 225 mg/l. These two mentioned experiments were carried out in factorial based on completely randomized design with 5 replications. The third experiment was aimed at studying the effect of 8-hydroxyquinoline at concentrations of 0, 50, 100, 150 and 200 mg/l on browning rate. Effect of adding Fe-EDDHA at concentrations of 0, 50, 100, 150 and 200 mg/l to the media on browning rate was evaluated in the fourth experiment. The third and fourth experiments were performed based on completely randomized design with 5 replications. At the end of the experiments, parameters of browning rate and bud break percentage were recorded.

Experiments Related to Explants

Two experiments were designed to study the effects of some characteristics related to the explants on browning. In the first experiment, the effects of shoot types including sucker, current-year shoot, one- and two-year old shoots, and explant positions including lower, middle and upper positions on the browning rate were explored. The second experiment was performed to assess the effects of shoot color and diameter on browning rate. Explants were chosen from dark and light brown-, pinkish- and green-colored shoots with small, medium, large and very large diameters. In both experiments, explants were treated with the best citric acid treatment determined in the previous experiment (soaking explants in solution containing 300 mg/l citric acid for 30 min and culturing them in medium containing 225 mg/l citric acid). Both experiments were laid out in factorial based on completely randomized design with 5 replications. At the end of the experiments, parameters of browning rate and bud break percentage were recorded.

Experiments Related to Sampling Time and Subculture

One experiment was also designed for studying the impact of sampling time on browning rate. Explants were collected each month and were subjected to the best treatment of citric acid selected in the previous experiment. Besides, effects of different periods of subculture (7, 14, 21 and 28 days) on browning rate were investigated in a separate experiment. Explants in this experiment were treated with the same treatment of citric acid as the previous experiment. The experiments were arranged based on completely randomized design with 5 replications. At the end of the experiments, parameters of browning rate and bud break percentage were recorded.

Comparison of the Best Treatments for Biochemical Analyses

In a separate experiment, the best treatment of the experiments examining the effect of chemical compounds on browning (part one of the experiments) was selected and compared in terms of phenol exudation, peroxidase activity, browning and bud break rates. Phenol exudation was measured at the end of 28 days based on the method described by Krishna et al. (2008). The peroxidase activity was measured according to the method described by Sadasivam and Manickam (1996).

Experimental Conditions

In all the experiments mentioned above, MS medium containing 0.5 mg/l benzyl adenine, 8 mg/l agar and 30 g/l sucrose was used as the basal medium, and pH of the media was adjusted to 5.8 with 0.1 N NaOH and/or 0.1 N HCl before autoclaving. All the cultures were maintained at 23 ± 2 C under 8/16 h photoperiod and PPFD of 40 µmol $m^{-2} s^{-1}$ provided by cool white fluorescent light.

Data was subjected to an analysis of variance by JAMP software and means comparison was performed using LSD at 5% probability level.

Results

Effect of Citric Acid and Ascorbic Acid

Table of analysis of variance (ANOVA) showed that the effect of citric acid and ascorbic acid on browning and bud break rates was significant at 1% level, but their interaction effect was not significant (Table 1).

Results obtained from addition of citric acid and ascorbic acid, alone or together, to media showed that degree of browning varied significantly based on the concentrations of the two antioxidants. Treatments containing no citric acid + 0 and 50 mg/l ascorbic acid exhibited the highest rate of phenol-induced browning. By contrast, treatment supplemented with 225 mg/l citric acid + 100 mg/l ascorbic acid showed the lowest browning rate (63%), however it did not show significant differences from most of the treatments. For bud break, significant differences were found among the different treatments. Explants showed no bud break when medium was devoid of both citric acid and ascorbic acid, whereas they showed the highest bud break rate when cultured in the treatment containing 225 mg/l citric acid + 100 mg/l ascorbic acid; however, this treatment was not significantly different from most treatments containing higher concentrations of citric acid and ascorbic acid (Table 2).

Table 1 ANOVA results of experimental factors on different factors

Source		Mean Square	
	DF	Browning rate	Bud break
Citric acid	3	2207.08^{**}	1654.2^{**}
Ascorbic acid	2	473.7^{ns}	716.5^{**}
Citric acid * Ascorbic acid	6	48.7^{ns}	42.4^{ns}
Error	48	167.2	57.5

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 2 Effects of citric acid + ascorbic acid on browning and bud break rates

Treatments (Citric + Ascorbic acids [mg/l])	Browning rate (%)	Bud break rate (%)
0 + 0	100 a	0 f
0 + 50	100 a	14 e
0 + 100	87 ab	19.6 de
75 + 0	85 ab	22.6 cde
75 + 50	80 bc	28.6 abcd
75 + 100	77 bcd	32.2 abc
150 + 50	77 bcd	34.6 ab
225 + 0	74 bcd	30.6 abc
150 + 0	73 bcd	25.6 bcd
150 + 100	68 cd	35.6 a
225 + 50	67 cd	35.6 a
225 + 100	63 d	37.6 a

Levels not connected by same letter are significantly different according to LSD at 5% level

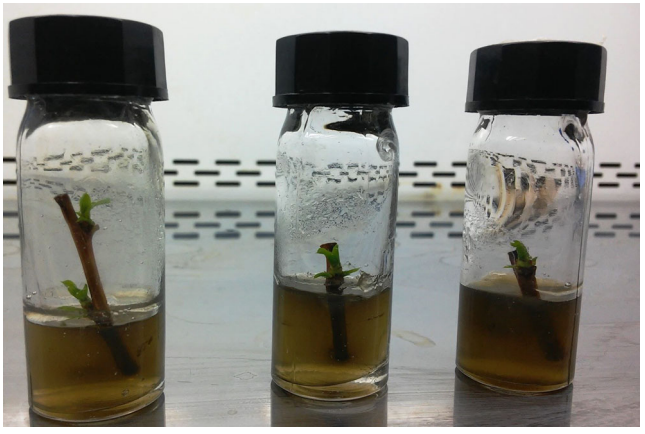

Fig. 1 Explants cultured in media containing 150 mg/l 8-hydroxyquinoline

Effect of Citric Acid Presoaking, Time and Citric Acid Addition

ANOVA results in Table 3 showed that interaction of citric acid presoaking, presoaking time and addition of citric acid to the media did not cause significant effects on the parameters of browning and bud break rates, but the simple effects of citric acid presoaking and citric acid addition to the media on the two parameters were significant at 1% level.

According to means comparison of the treatments, significant differences were observed among the different treatments (Table 4). Explants presoaked in 150 mg/l citric acid for 15 and 30 min, respectively, and cultured in media lacking citric acid showed the highest browning rates, while explants presoaked with 300 mg/l citric acid for 15 and 30 min and cultured in media containing 225 mg/l citric acid showed the lowest rate of browning.

On the matter of bud break, the highest rate was found in the treatments of presoaking explants in 300 mg/l citric acid for 30 min and addition of 225 and 150 mg/l citric acid to the media, respectively, while the lowest rate of bud break was recorded in the treatment of presoaking in 150 mg/l citric acid for 15 min + no citric acid in the media. Generally, agitation of explants in citric acid solution and their culture in media supplemented with the same antioxidant resulted in the highest bud break and lowest browning rate.

Effect of 8-hydroxyquinoline

As seen in ANOVA Table 5, use of 8-hydroxyquinoline caused significant impact on browning and bud break rates at 1% probability level.

Results related to the means comparison of the effect of 8-hydroxyquinoline on browning and bud break rates are presented in Table 6. Lower browning rate was observed in treatments containing 8-hydroxyquinoline at different concentrations compared to control. Interestingly, bud sprouting rates were significantly higher in treatments supplemented with different concentrations of 8-hydroxyquinoline when compared with the treatment lacking 8-hydroxyquinoline (Fig. 1).

Table 3 ANOVA results of experimental factors on different factors

Source		Mean Square	
	DF	Browning rate	Bud break
Presoaking	1	3285.6^{**}	1490.01^{**}
Time	1	147.26^{ns}	673.35^{**}
Citric acid addition	2	2067.8^{**}	3091.25^{**}
Presoaking*Time	1	11.267^{ns}	126.15^{ns}
Presoaking*Citric acid addition	2	26.6^{ns}	6.11^{ns}
Time*Citric acid addition	2	199.26^{ns}	27.15^{ns}
Presoaking*Time*Citric acid addition	2	50.86^{ns}	52.35^{ns}
Error	48	96.967	80.342

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 4 Effects of presoaking explant in citric acid solution at different times + citric acid addition to media on browning and bud break rates

Treatments (Pre * Time * Addition)	Browning rate (%)	Bud break rate (%)
150 * 15 * 0	88 a	13 g
150 * 30 * 0	82 ab	18.6 fg
300 * 15 * 0	75 bc	24.6 ef
150 * 15 * 150	68 cd	29.6 def
150 * 30 * 150	67.2 cd	37.6 bcd
150 * 15 * 225	64 cde	32.6 cde
150 * 30 * 225	64 cde	47.8 ab
300 * 30 * 0	60.2 def	27.2 def
300 * 30 * 150	56 def	48 ab
300 * 15 * 150	53.2 ef	41.2 abc
300 * 15 * 225	50 f	48 ab
300 * 30 * 225	50 f	50 a

Levels not connected by same letter are significantly different according to LSD at 5% level

Table 5 ANOVA results of 8-hydroxyquinoline on different parameters

Source	DF	Mean Square	
		Browning rate	Bud break rate
8-hydroxyquinoline	4	1806**	2746**
Error	20	54.4	82

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 6 Effects of 8-hydroxyquinoline on browning and bud break rates

8-hydroxy levels (mg/l)	Browning rate (%)	Bud break rate (%)
0	64 a	40 c
50	25 b	87 b
100	22 b	100 a
150	20 b	92 ab
200	20 b	82 b

Levels not connected by same letter are significantly different according to LSD at 5% level

Effect of Fe-EDDHA

Use of Fe-EDDHA resulted in significant effect on browning and bud break rates at 1% probability level, as shown in ANOVA table (Table 7).

Results of means comparison revealed that there were significant differences among the different treatments. Treatments fortified with different concentrations of Fe-EDDHA displayed lower browning and higher bud break rates in comparison with Fe-EDDHA-free treatment. Fe-EDDHA at concentration of 200 mg/l caused the greatest effect in terms of both browning rate and bud break percentage compared to other concentrations, though the difference was not statistically significant (Table 8).

Table 7 ANOVA results of Fe-EDDHA on different parameters

Source	DF	Mean Square	
		Browning rate	Bud break rate
Fe-EDDHA	4	963.50**	2784.94**
Error	20	70.20	109.14

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 8 Effects of Fe-EDDHA on browning and bud break rates

Fe-EDDHA levels (mg/l)	Browning rate (%)	Bud break rate (%)
0	64 a	30.2 c
50	40 b	59 b
100	35 b	74 a
150	33 b	86 a
200	29 b	87 a

Levels not connected by same letter are significantly different according to LSD at 5% level

Table 9 ANOVA results of sampling time on different parameters

Source	DF	Mean Square	
		Browning rate	Bud break rate
Month	11	1386.309**	296.21**
Error	48	194	100.8

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 10 Effects of sampling time on browning and bud break rates

Months	Browning rate (%)	Bud break rate (%)
August	82 a	36.2 bc
September	82 a	37.6 abc
July	79 a	46 ab
June	77 ab	44.6 ab
May	61 bc	44.6 ab
October	61 bc	49.6 a
November	56 cd	48.6 ab
December	52 cd	38.2 abc
April	50 cd	28.2 c
January	40.6 d	31.2 c
February	39.2 d	30.6 c
March	39 d	30.2 c

Levels not connected by same letter are significantly different according to LSD at 5% level

Effect of Sampling Time (Month)

Results presented in ANOVA table indicated significant effect of sampling time on the parameters of browning and bud break rates at 1% probability level (Table 9).

Values of means given in Table 10 are clear indication that there are significant differences among various treatments. Browning of explants cut off in wintering months of January, February and March were lower than other months, while browning in those collected in August, September and

Table 11 ANOVA results of experimental factors on different factors

Source	DF	Mean Square	
		Browning rate	Bud break
Shoot type	3	8534.106**	7968.461**
Explant position	2	8744.017**	5504.450**
Shoot type * Explant position	6	235.106*	644.561**
Error	48	100.25	104.46

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 12 Combined effects of shoot type and explant position on browning and bud break rates

Treatments (Shoot type * Explant position)	Browning rate (%)	Bud break rate (%)
2-year old, Lower	90 a	0 e
1-year old, Lower	79 ab	15 d
2-year old, Middle	79 ab	15 d
1-year old, Middle	70 bc	20 cd
Sucker, Lower	65 c	23.2 cd
2-year old, Upper	63 c	19 cd
Current-year, Lower	48 d	45 b
1-year old, Upper	37 de	31 c
Sucker, Middle	33.2 e	48.6 b
Current-year, Middle	24.6 ef	52 b
Sucker, Upper	15 f	86 a
Current-year, Upper	0 g	79 a

Levels not connected by same letter are significantly different according to LSD at 5% level

Table 13 ANOVA results of shoot color and diameter on different factors

Source	DF	Mean Square	
		Browning rate	Bud break
Shoot color	3	4193.55**	4846.48**
Shoot diameter	3	24,108.05**	28,826.75**
Shoot color * Shoot diameter	9	1892.113*	330.36*
Error	64	79.41	92.09

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

July months showed the highest browning rates, respectively. Furthermore, explants displayed the best response when taken in October and November, with 49.6 and 48.6% of them exhibiting bud break, respectively. By contrast, explants showed the lowest bud break rates of 28.2, 30.2 and 30.6% when cut off in April, February and March months, respectively.

Effect of Shoot Type and Position

According to ANOVA results presented in Table 11, interaction of shoot type and shoot position had significant influence on browning and bud break rates.

Table 14 Combined effects of shoot color and diameter on browning and bud break rates

Treatments (Color*Diameter)	Browning rate (%)	Bud break rate (%)
Dark brown, Very large	100 a	0 h
Dark brown, Large	100 a	0 h
Green, Very large	100 a	0 h
Light brown, Very large	100 a	0 h
Light brown, Large	86 b	0 h
Pinkish, Very large	82 b	15.6 g
Green, Large	69 c	27.2 fg
Dark brown, Medium	62 cd	44.6 e
Pinkish, Large	54 d	34.2 ef
Light brown, Medium	42.6 e	59 d
Dark brown, Small	34.2 ef	66 cd
Green, Medium	34.2 ef	73 bc
Light brown, Small	26 fg	71 bcd
Pinkish, Medium	23.6 fg	100 a
Green, Small	15 g	82 b
Pinkish, Small	0 h	100 a

Levels not connected by same letter are significantly different according to LSD at 5% level

Table 15 ANOVA results of subculture on different parameters

Source	DF	Mean Square	
		Browning rate	Bud break rate
Subculture	3	246.18ns	1528.98**
Error	16	82.65	86.60

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 16 Effects of subculture on browning and bud break rates

Subculture (Day)	Browning rate (%)	Bud break rate (%)
28	54 a	31.2 c
21	52 a	39.2 c
14	43 ab	57 b
7	39 b	70 a

Levels not connected by same letter are significantly different according to LSD at 5% level

Results of means comparison for browning rate parameter showed that there were significant differences among the different treatments (Table 12). Based on the results, browning rate was the highest in the explants taken from lower position of the two-year-old shoots, while it was the lowest in the explants prepared from upper position of both current-year shoot (0) and sucker (15%). Moreover, explants of upper position taken from both sucker (86%) and current-year shoot (79%) had the highest bud break rates compared to those of lower position taken from two-year-old shoots with no bud break.

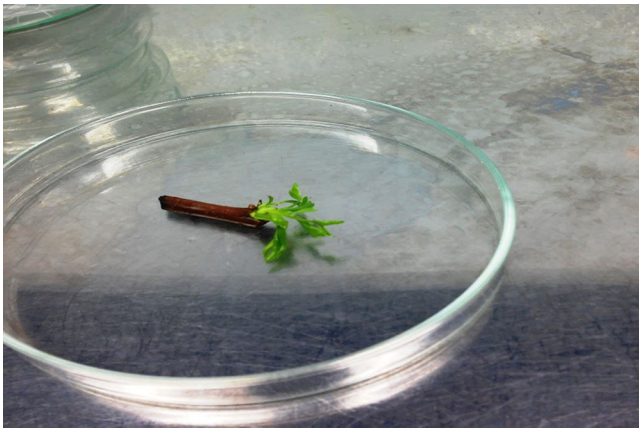

Fig. 2 Explant collected from dark brown-colored shoot

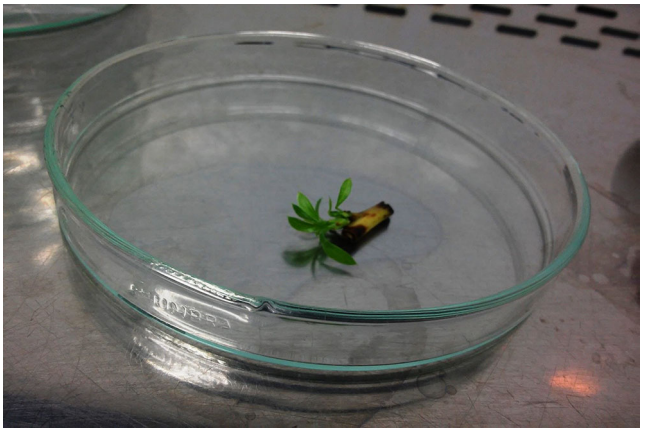

Fig. 3 Explant collected from green-colored shoot

Effect of Shoot Color and Shoot Diameter

It is evident from ANOVA table that interaction effects of shoot color and diameter on browning and bud break rates were significant at 5% probability level, and their simple effects were significant at 1% level (Table 13).

As can be seen in Table 14 and Fig. 2 and 3, browning and bud break rates were significantly different among the treatments. Explants collected from shoots with large diameter, irrespective of shoot color, recorded the highest phenol rate. Explants taken from pinkish-colored shoots with small diameter showed the lowest rate of phenol exudation (0%), followed by the explants taken from green-colored shoots with the same diameter as the pinkish ones, with just 15% of them showing phenol-induced browning. Referring to bud sprouting rate, explants provided from shoots with pinkish color and with small and medium diameter showed 100% bud break. Dark and light brown-colored shoots with large and very large diameter plus green-colored shoots with very large diameter showed no bud sprouting.

Effect of Subculture

Table 15 presents the results of ANOVA related to the effect of different periods of subculture on browning and bud break rates. As it is clearly evident, different periods of subculture brought about significant influences on both parameters of browning and bud break at 1% probability level.

According to Table 16, there were significant differences among different periods of subculture. Explants subcultured after 7 days showed significantly lower browning rate and higher bud break rate than those subcultured after longer periods. Explants transferred to new culture after 28 days showed the highest browning rate and the lowest bud break rate, but they did not show significant differences from those subcultured after 21 days with respect to both parameters.

Table 17 ANOVA results of the best treatment of each experiment on different parameters

Source			Mean Square		
	DF	Phenol exudation	Peroxidase activity	Browning rate	Bud break rate
Treatment	4	14,796.14 **	5.800**	4976.50**	6297.56**
Error	20	77.7	0.053	70.00	77.76

Ns non-significant, *significant at $p \leq 0.05$, **significant at $p \leq 0.01$

Table 18 Effects of the best treatment of each experiment on phenol exudation, peroxidase activity, browning rate and bud break rate

Treatments	Phenol exudation (mg/ml)b	Peroxidase activity (ΔA 470 of 1.0 for 1 mg of protein in 10 min)	Browning rate (%)	Bud break rate (%)
A	187 a	3.91 a	100 a	0 d
B	126.2 b	2.93 b	63 b	37.6 c
C	100.8 c	2.13 c	50 c	50 b
D	75.2 d	1.60 d	29 d	87 a
E	43.6 e	1.22 e	20 d	82 a

Levels not connected by same letter are significantly different according to LSD at 5% level
A control, *B* 225 mg/l citric acid + 100 mg/l ascorbic acid, *C* Presoaking in 300 mg/l citric acid for 30 min + culturing in media containing 225 mg/l citric acid, *D* 200 mg/l Fe-EDDHA, *E* 200 mg/l 8-hydroxyquinoline

Biochemical Analyses

As ANOVA results in Table 17 showed, the best treatment selected from the experiments of part one of the present study significantly affected phenol exudation and peroxidase activity at 1% probability level.

Means values presented in Table 18 indicated that there were significant differences among the different treatments. In vitro phenol exudation was lower in explants cultured in media supplemented with 8-hydroxyquinoline and Fe-EDDHA than antioxidant-treated and control explants. Peroxidase activity also followed the same pattern, so that treatments containing 8-hydroxyquinoline and Fe-EDDHA showed lower peroxidase activity than other treatments.

Discussion

The present study concentrated on the possibility of reducing browning in *in vitro* culture of barberry, a species with high degree of browning in tissue culture. Based on the preliminary experiments conducted for 1 year, browning was determined as a serious problem limiting the success of barberry *in vitro* culture. So, according to the information obtained from preliminary experiments, different experiments were designed for solving the mentioned problem.

Phenol browning is a very common problem in tissue culture which can be eliminated by using different approaches including addition of antioxidants and activated charcoal to medium, presoaking of explants in antioxidants solution (Raghuvanshi and Srivastava 1995), and frequent subculture and selection of proper explants (Chandra et al. 2004), but the degree of success varies based on the plant species.

In the present study, the explants showed browning within 48 h after the culture. To reduce the harmful effect of phenolic compounds on explants, two antioxidants of citric acid and ascorbic acid were tried either as single or in combination, among which mixture of 225 mg/l citric acid + 100 mg/l ascorbic acid was found significantly better compared to control, but still rate of explant browning was relatively high. Similar results have been reported by Tao et al. (2007), who noted that using citric acid at 2–4 g/l did not result in any change in browning rate of *Platanus occidentalis* L., and Desai et al. (2018), who found that supplementing of media with ascorbic acid was not successful in controlling of phenol exudation in pomegranate cv. Bhagwa. Interestingly, Sharma and Ramamurthy (2000) reported that explants of *Eucalyptus tereticornis* exhibited somehow more browning in the presence of antioxidants than in their absence. Contrary to Desai et al. (2018), Patil et al. (2011) reported successful alleviation of phenol browning in pomegranate by using 150 mg/l ascorbic acid and 100 mg/l citric acid. These conflicting results can be attributed to the type of plant species. Since single or combined use of citric acid and ascorbic acid did not yield promising results in the present study, the mentioned antioxidants were employed in two steps. To do so, explants were first dipped in solutions containing antioxidants at different times and then cultured in media containing the same antioxidants. This approach also failed to cause a significant reduction in browning rate, with low rate of the explants being able to successfully establish. Unlike to our results, Krishna et al. (2008) in mango reported lower browning rate by agitating explants in 100 mg ascorbic acid + 50 mg citric acid antioxidant solution and culturing them in medium supplemented with the same antioxidant level. Alleviation of phenol browning as a result of presoaking the explants in antioxidants solution has been also reported in other plants (Chavan et al. 2000; Huang et al. 2002; Tabiyeh et al. 2005; Phulwaria et al. 2012).

In addition to antioxidants, the effect of explant collection season on the reduction of browning was also investigated. The influences of season on browning of explants in *in vitro* culture have been reported in many studies (Christiansen and Fonnesbech 1975; Biedermann 1985; Prasad and Chaturvedi 1988; Wang et al. 1994; Bhatt and Dhar 2004; Martin et al. 2006; Arora et al. 2010; Phulwaria et al. 2011, 2012), but there are conflicting reports regarding the most suitable time for collection of explants. In this context, Wang et al. (1994) in their study on 'Fuji' apple and 'Jinhua' pear reported that explants collected from November to February produced low rates of browning and those collected in April and August showed maximum browning rate, while Sharma and Ramamurthy (2000), working on 4-year-old elite *Eucalyptus tereticornis* trees, found 100% browning in explants cut off in February, and no browning in those harvested in March–April and August–January. It was also reported in mango that explants collected in March-April and September-October were more responsive and showed lower phenol exudation (Chandra et al. 2004). Similarly, Tao et al. (2007) reported that browning rates of the explants collected in January and March were lower than those in July, and Dhavala and Rathore (2010) in a study on micropropagation of *Embelia ribes* Burm f. found that explants collected during March to May contained lower rate of browning. In the present study, explants showed higher browning rate in spring and summer seasons compared to wintering months. These seasons correspond with the timing of the most active growth phases. Fresh sprouts appear vigorously in spring and summer, and new shoots are seen emerging and growing rapidly during spring and summer (June through August). Possible reason for high phenol rate in spring and summer might be related to the new growth of shoots, which provides the possibility of phenol exudation.

Dalal and Rai (2001) and Bhatt and Dhar (2004) noted that explants taken during the winter season showed maximum bud break, which is contrary to the present report where the lowest rate of bud break was seen in winter season. Chaturvedi et al. (2004) and Arora et al. (2010) in neem plant, and Phulwaria et al. (2012) in *Terminalia catappa* reported that explants taken in March and April showed maximum bud break which is not in line with the results of the present study, where the highest bud break rates were seen in October and November. This time is known to coincide with maximum new growth in trees; that is the reason why the explants showed the highest bud break rate. This may be related to the active growth phase of barberry during the period with high temperature and low humidity, as shown by Dhavala and Rathore (2010) in *Embelia ribes* Burm f. Generally, bud break rate in all months of the year was low and differences among them were not high.

In the current study, for better controlling of phenol exudation, effects of physiological conditions of explants were also studied. The results showed that shoot type and explant positon on shoot can have significant effect on browning and bud break rates. Explants collected from upper position of both current-year shoot and sucker displayed the lowest rate of browning, respectively, when compared with older shoots. Arora et al. (2010) pointed out that not only the survival of explants but also their later performance are determined by their size, thickness and maturity. Rai et al. (2010) and Ram and Shekhawat (2011) also revealed that woody nodal explants exuded high rate of phenol and were not thus good for culture establishment. In pomegranate, explants collected from the older shoots showed higher rate of browning compared to those collected from younger shoots (Naik et al. 1999), which is in line with the results reported by the current study. By contrast and similar to our results, Krishna et al. (2008) in an investigation reported that younger explants responded better compared to explants taken from older shoots. This may be due to this issue that young shoots synthesize lower phenolic substances (Rai et al. 2009). In neem plant, explants collected from lower position which are older in age showed the highest bud break rate (Arora et al. 2010), which is not in agreement with the results of the present study. Our results also displayed that explants taken from dark and light brown-colored shoots with very large diameter showed 100% browning rate, and those taken from pinkish-colored shoots with small diameter were devoid of browning. Contrary to our results, Hsia and Korban (1996) in case of Rosa sp reported that explants with larger diameters developed larger buds due to better nutrient translocation from the donor mother plant, hence showed sooner bud break when cultured in media. Furthermore, 4–6 week old shoots with dark green stem displayed higher bud break rate and lower phenol exudation compared to 1–2 week old shoots with reddish brown stem (Chandra et al. 2004).

The interesting part of this study was related to the experiments investigating the effects of 8-hydroxyquinoline and Fe-EDDHA for the first time on browning and bud break rates. Surprisingly, the results showed that use of these compounds could significantly reduce browning rate and increase bud break rate. To the best of our knowledge, there is no report about the use of these compounds for controlling phenol-induced browning in other fruit trees, and this is the first study reporting the beneficial effect of these compounds on alleviation of browning and improvement of bud break rate. 8-hydroxyquinoline is an antioxidant (Zhong et al. 2002) which has been mainly used in forcing solution for bud break and also as an antimicrobial agent for preservation of cut flowers (van Doorn et al. 1989; Verma et al. 2012). Based on the results of the present study, use of 8-hydroxyquinoline could cause a high reduction in browning rate compared to control. It also significantly increased bud break rate so that over 82% bud break was observed in media supplemented with different concentrations of 8-hydroxyquinoline. Another interesting observation in media containing 8-hydroxyquinoline was the reduction of contamination. It seems that reduction of browning as a result of using 8-hydroxyquinoline might be due to the blackening of media. However, the reasons for this observation are not clear at this time.

Our results regarding the effect of Fe-EDDHA on browning and bud break rates showed that both parameters were improved as a result of supplementation of media with Fe-EDDHA. No report has been published so far about the use of Fe-EDDHA for control of browning and increase of bud break in plants. Reports on Fe-EDDHA just show its superiority over Fe-EDTA with respect to the production of higher leaf chlorophyll content, shoot length and shoot dry weight. It was also reported that EDDHA has higher photostability than EDTA that becomes unavailable due to its degradation by light (Stasinopoulos and Hangarter 1990; Van der Salm et al. 1994). The higher stability of Fe-EDDHA was also noted in bitter almond, where higher acquisition of iron was reported in shoots cultured in media containing Fe-EDDHA in comparison to the shoots cultured in Fe-EDTA-supplemented media (Shibli et al. 2002). Shibli et al. (2002) also found out that increase in iron concentration led to the enhancement in iron acquisition, and it was also observed in the present study that bud break rate, as an index of growth, increased in Iranian seedless barberry when Fe-EDDHA concentration increased.

Furthermore, in the present study, the treatment showing the best response in terms of phenol browning rate was chosen from the experiments examining phenol controlling materials and was subjected to a comparison in terms of phenol exudation and peroxidase activity. The results demonstrated

that the rates of phenol exudation and peroxidase activity in treatments supplemented with 8-hydroxyquinoline and Fe-EDDHA were significantly lower than those containing antioxidants. The activity of oxidative enzymes, which depends on phenols oxidation, can be aggravated by light (Crippen and Morrison 1986; Mole and Waterman 1994). Therefore, exclusion of light from culture media helps reducing the explants browning (George 1996). Reduction of browning exudation and peroxidase activity in media containing 8-hydroxyquinoline may be due to the darkening of media as a result of using this compound. Besides, higher photostability of Fe-EDDHA may play a role in the reduction of peroxidase activity, which can be increased by light.

Conclusion

The present study explored the control of browning in Iranian seedless barberry by taking several factors into consideration. It seems that manipulation of several factors can be a possible approach for obtaining the desired results. Use of suitable chemical compound, proper source of explant and the best time of sampling can successfully reduce phenol-induced browning. Considering the findings of the current research, it is obvious that 8-hydroxyquinoline and Fe-EDDHA led to a significant reduction in phenol browning compared to the common antioxidants of citric acid and ascorbic acid. Furthermore, explants cut off in April showed lower browning rate than other months of the year. Finally, explants taken from upper position of current-year shoot and sucker as well as those obtained from pinkish-colored shoots with small diameter showed the lowest browning rate.

Funding This work was financially supported by Ferdowsi University of Mashhad.

Conflict of interest R.N.M. Aghayeh, B. Abedy, A. Balandari, L. Samiei and A. Tehranifar declare that they have no competing interests.

References

Arayne MS, Sultana N, Bahadur SS (2007) The berberis story: Berberis vulgaris in therapeutics. Pak J Pharm Sci 20:83–92

Arora K, Sharma M, Srivastava J, Ranade SA, Sharma AK (2010) Rapid in vitro cloning of a 40-year-old tree of Azadirachta indica A. Juss.(Neem) employing nodal stem segments. Agrofor Syst 78:53

Asai M, Iwata N, Yoshikawa A, Aizaki Y, Ishiura S, Saido TC, Maruyama K (2007) Berberine alters the processing of Alzheimer's amyloid precursor protein to decrease Aβ secretion. Biochem Biophys Res Commun 352:498–502

Azizi M, Aghabozorgi M, Farsi M, Tehranifar A, ZolAli J, Ghabooli M (2007) Study of rooting some horticultural plants inoculated with Agrobacterium rhizogenes. J Agric Sci Technol 21:79–87

Balandary A, Kafi M (2001) Berberis production and processing. Zaban va Adab Press, Mashhad

Bhatt ID, Dhar U (2004) Factors controlling micropropagation of Myrica esculenta buch.–Ham. ex D. Don: a high value wild edible of Kumaun Himalaya. Afr J Biotechnol 3:534–540

Biedermann IEG (1985) Factors affecting establishment and development of Magnolia hybrids in vitro. In: Symposium on In Vitro Problems Related to Mass Propagation of Horticultural Plants, vol 212, pp 625–630

Bodiwala HS, Sabde S, Mitra D, Bhutani KK, Singh IP (2011) Synthesis of 9-substituted derivatives of berberine as anti-HIV agents. Eur J Med Chem 46:1045–1049

Chandra R, Padaria JC, Srivastava S (2004) Factors influencing in vitro establishment of mango shoot buds. Indian J Plant Physiol 9:136–144

Chaturvedi R, Razdan MK, Bhojwani SS (2004) In vitro clonal propagation of an adult tree of neem (Azadirachta indica A. Juss.) by forced axillary branching. Plant Sci 166:501–506

Chavan SS, Ranade SS, Deore AC, Deshpande RS, Dhonukshe BL (2000) Cloning of Alphonso mango through vegetative explants. Ann Plant Physiol 14:178–181

Christiansen J, Fonnesbech M (1975) Prevention by polyvinylpyrrolidone of growth inhibition of Hamamelis shoot tips grown in vitro and of browning of the agar medium. In: Symposium on Propagation in Arboriculture, vol 54, pp 101–104

Crippen DD, Morrison JC (1986) The effects of sun exposure on the compositional development of Cabernet Sauvignon berries. Am J Enol Vitic 37:235–242

Dalal NV, Rai RV (2001) In vitro propagation of Ochreinauclea missionis (Wall. EX G. Don), an ethnomedicinal endemic and threatened tree. In Vitro Cell Dev Biol 37:820–823

Desai P, Patil G, Dholiya B, Desai S, Patel F, Narayanan S (2018) Development of an efficient micropropagation protocol through axillary shoot proliferation for pomegranate variety 'Bhagwa. Ann Agrar Sci 16:444–450

Dhavala A, Rathore TS (2010) Micropropagation of Embelia ribes Burm f. through proliferation of adult plant axillary shoots. In Vitro Cell Dev Biol 46:180–191

van Doorn WG, Schurer K, de Witte Y (1989) Role of endogenous bacteria in vascular blockage of cut rose flowers. J Plant Physiol 134:375–381

Ebrahimi-Mamaghani M, Arefhosseini SR, Golzarand M, Aliasgarzadeh A, Vahed-Jabbary M (2009) Long-term effects of processed Berberis vulgaris on some metabolic syndrome components. Iran J Endocrinol Metab 11:41–47

Fatehi M, Saleh TM, Fatehi-Hassanabad Z, Farrokhfal K, Jafarzadeh M, Davodi S (2005) A pharmacological study on Berberis vulgaris fruit extract. J Ethnopharmacol 102:46–52

George EF (1996) Plant propagation by tissue culture. Part 2: in practice. Springer, Dordrecht

Hsia C-N, Korban SS (1996) Factors affecting in vitro establishment and shoot proliferation of Rosa hybrida L. and Rosa chinensis minima. In Vitro Cell Dev Biol 32:217–222

Huang L-C, Lee Y-L, Huang B-L, Kuo CI, Shaw JF (2002) High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture. In Vitro Cell Dev Biol 38:358

Krishna H, Sairam RK, Singh SK, Patel VB, Sharma RR, Grover M, Nain L, Sachdev A (2008) Mango explant browning: effect of ontogenic age, mycorrhization and pre-treatments. Sci Hortic 118:132–138

Leal DR, Sánchez-Olate M, Avilés F, Materan ME, Uribe M, Hasbún R, Rodríguez R (2007) Micropropagation of Juglans regia L. In: Protocols for micropropagation of woody trees and fruits. Springer, Dordrecht, pp 381–390

Martin G, Geetha SP, Raja SS, Raghu AV, Balachandran I, Ravindran PN (2006) An efficient micropropagation system for Celastrus paniculatus Willd.: a vulnerable medicinal plant. J For Res 11:461–465

Mohammadi M, Bagheri A, Marashi H, Moshtaghi N, Balandari A (2011) Investigation into seasonal effect and browning inhibitor on callus regeneration of seedless barberry (Berberis vulgaris var. asperma). Plant Tissue Cult Biotechnol 21:161–168

Mole S, Waterman S (1994) Analysis of phenolic plant metabolites. Blackwell Scientific, Oxford, Boston

Naik SK, Pattnaik S, Chand PK (1999) In vitro propagation of pomegranate (Punica granatum L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Sci Hortic 79:175–183

Patil VM, Dhande GA, Thigale DM, Rajput JC (2011) Micropropagation of pomegranate (Punica granatum L.) 'Bhagava'cultivar from nodal explant. Afr J Biotechnol 10:18130–18136

Pérez-Tornero O, Burgos L (2007) Apricot micropropagation. In: Protocols for micropropagation of woody trees and fruits. Springer, Dordrecht, pp 267–278

Phulwaria M, Ram K, Gahlot P, Shekhawat NS (2011) Micropropagation of Salvadora persica—a tree of arid horticulture and forestry. New For 42:317–327

Phulwaria M, Ram K, Gupta AK, Shekhawat NS (2012) Micropropagation of mature Terminalia catappa (Indian Almond), a medicinally important forest tree. J For Res 17:202–207

Prasad RN, Chaturvedi HC (1988) Effect of season of collection of explants on micropropagation of Chrysanthemum morifolium. Biol Plant 30:20–24

Raghuvanshi SS, Srivastava A (1995) Plant regeneration of Mangifera indica using liquid shaker culture to reduce phenolic exudation. Plant Cell Tissue Organ Cult 41:83–85

Rai MK, Asthana P, Jaiswal VS, Jaiswal U (2010) Biotechnological advances in guava (Psidium guajava L.): recent developments and prospects for further research. Trees 24:1–12

Rai MK, Jaiswal VS, Jaiswal U (2009) Shoot multiplication and plant regeneration of guava (Psidium guajava L.) from nodal explants of in vitro raised plantlets. J Fruit Ornam Plant Res 17:29–38

Ram K, Shekhawat NS (2011) Micropropagation of commercially cultivated Henna (Lawsonia inermis) using nodal explants. Physiol Mol Biol Plants 17:281–289

Sadasivam S, Manickam A (1996) Biochemical methods. New Age International, New Delhi

Sharma SK, Ramamurthy V (2000) Micropropagation of 4-year-old elite Eucalyptus tereticornis trees. Plant Cell Rep 19:511–518

Shibli RA, Mohammad MJ, Ajlouni ZI (2002) Growth and micronutrient acquisition of in vitro grown bitter almond and sour orange in response to iron concentration from different iron chelates. J Plant Nutr 25:1599–1606

Stasinopoulos TC, Hangarter RP (1990) Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. Plant Physiol 93:1365–1369

Tabiyeh DT, Bernard F, Shacker H (2005) Investigation of glutathione, salicylic acid and GA3 effects on browning in Pistacia vera shoot tips culture. In: IV International Symposium on Pistachios and Almonds, pp 201–204

Tao F, Zhang Z, Zhou J, Yao N, Wang DM (2007) Contamination and browning in tissue culture of Platanus occidentalis L. For Stud China 9:279–282

Van der Salm TPM, Van der Toorn CJG, ten Cate CHH et al (1994) Importance of the iron chelate formula for micropropagation of Rosa hybrida L. 'Moneyway'. Plant Cell Tissue Organ Cult 37:73–77

Verma AK, Prasad KV, Singh SK, Kumar S (2012) In vitro isolation of red coloured mutant from chimeric ray florets of chrysanthemum induced by gamma-ray. Indian J Hort 69:562–567

Wang Q, Tang H, Quan Y, Tang YQ (1994) Phenol induced browning and establishment of shoot-tip expiants of 'Fuji' apple and 'Jinhua' pear cultured in vitro. J Hortic Sci 69:833–839

Yin J, Ye J, Jia W (2012) Effects and mechanisms of berberine in diabetes treatment. Acta Pharm Sin B 2:327–334

Zhong MQ, Lou CF, Tan JZ (2002) Function of $AgNO_3$ during mulberry genetic translation. J Trop Subtrop Plant 10(1):74–76 (in Chinese with an English abstract)