Prevention of Browning during *in vitro* Cultures of *Verbascum scamandri*

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Abstract

Browning, a common problem in plant tissue cultures, can hinder callus production and the synthesis of secondary metabolites. This study investigates browning in the in vitro callus culture of Verbascum scamandri, a plant species with significant medicinal potential. This study aims to evaluate the efficacy of various browning inhibitors. In the study, leaf explants from 15-week-old plants grown in vitro were used to investigate browning in callus cultures. Explants were cultured in MS basal medium supplemented with 3% sucrose, 0.7% phyto agar and 2 mg/L Kinetin, which was chosen for its high callus proliferation. Treatments included various concentrations (50, 100, 200 and 300 mg/L) and combinations of browning inhibitors (PEG, PVP, AA, CA, AC and AA+CA), as well as dark culture conditions. The density of browning and callus was recorded and calculated after 28 days including texture and color data of induced calli.

According to the results, it was found that the most effective treatment for the prevention of browning is culturing in dark conditions. Additionally, it was observed that in this treatment, the density of callus induction was higher and the induced calli did not brown and had a compact texture.

Keywords: Ascorbic Acid, Browning, Callus, Citric Acid, Explant, Mullein.

Introduction

In tissue culture, the successful initiation of cultures without the loss of explants is the initial step towards maximizing culture efficiency and the production of plant-derived compounds such as secondary metabolites. However, one of the most significant problems frequently encountered in plant tissue cultures is browning[1]. Browning refers to the secretion of brown compounds or phenolics into the culture medium because of oxidation during tissue or redifferentiation stages of explants[26]. Moreover, browning is a problem that impedes the growth and cell proliferation of cultures and can even lead to cell death[43].

Particularly, in callus cultures of plants rich in secondary metabolites, browning is commonly observed[17]. Browning processes can be classified as enzymatic or non-enzymatic depending on the presence of enzymes[41]. In plant tissue culture, explant browning generally arises from enzymatic browning[45]. It is suggested that two key enzymes, polyphenol oxidase (PPO) and peroxidase (POD), are the main causes of callus browning[11]. PPOs are copper-containing enzymes that catalyse the oxidation of quinones to polymerize phenols, resulting in browning and the production of toxic substances[8,31].

PODs, on the other hand, catalyse the reduction of hydrogen peroxide to protect tissues and cells from oxidative damage by utilizing phenolic compounds as hydrogen donors[44]. Drought, salinity, UV, temperature and other abiotic stress conditions can lead to an increase in reactive oxygen species (ROS) such as free radicals resulting in lipid peroxidation, plasmalemma disintegration and organelle breakdown.

Consequently, PPO release and initiation of oxidative reactions may occur[12]. In normal plant cells, phenols are in vacuoles and enzymes in plastids and their simultaneous presence prevents substrates from contacting phenolics, thereby preventing browning. Phenols act as respiratory intermediates in normal plant tissues[15]. However, mechanical damage caused by cutting explants in tissue culture leads to the release of phenols, resulting in increased ROS accumulation and subsequent browning[42].

Browning is influenced by various factors such as plant species, explant size, genotype, age, density of damage, physiological condition, medium composition, culture conditions and culture period[1]. Additionally, it has been noted that the addition of plant growth regulators, adsorbents, or antioxidants (such as polyvinylpyrrolidone, ascorbic acid, activated carbon and citric acid) at different levels and concentrations in culture media reduces browning in explants or callus[27,35]. However, determining suitable culture conditions for the problem of browning is essential due to the different responses of each plant species to culture media and components.

The genus *Verbascum* (mullein) has a significant endemism rate (80%) in Türkiye and species belonging to this genus have medical potential due to their various secondary metabolites such as iridoids, phenylethanoids, flavonoids, neolignan glycosides, saponins and spermine alkaloids[2-4,10,13,22]. Consequently, they are widely used in folk medicine for the treatment of various ailments[28,32]. *Verbascum scamandri* Murb. is biennial and endemic species. In a previous study where we conducted *in vitro* callus culture of *V. scamandri* species, the major issue encountered was browning[5]. Browning constitutes one of the most significant problems in the production of callus and secondary metabolites of this valuable plant. Therefore, this

study aimed to investigate the effect of different browning inhibitors on *in vitro* callus culture of *V. scamandri* to find an effective solution to the browning problem.

Material and Methods

Plant Material: The plants of *V. scamandri* germinated *in vitro* in the previous study[5] were used as plant materials. The plants were grown under controlled conditions in a plant growth chamber with 55-60% humidity, 16-hour light/8-hour dark photoperiod, temperature of 25±2°C and light intensity of 72 µmol $m^{-2}s^{-1}$. Leaves from 15-week-old plants were used as explant for callus culture.

Effect of Different Browning Inhibitors on Browning: Murashige and Skoog basal medium[20] (MS, Duchefa Biochemie-M0222) supplemented with 2 mg/L Kinetin (Duchefa Biochemie-K0905), 3% (w/v) sucrose (Duchefa Biochemie-S0809) and 0.7% (w/v) phyto agar (Duchefa Biochemie-P1003) were used as the callus induction medium. To inhibit browning, the addition of 0, 50, 100, 200 and 300 mg/L polyethylene glycol (PEG, Merck-8.07490), polyvinylpyrrolidone (PVP, Sigma Aldrich-856452), ascorbic acid (AA, AFG Bioscience-180476), citric acid (CA, Isolab Chemicals-910.046), activated carbon (AC, Merck-102183), as well as combinations of ascorbic acid and citric acid, in the callus induction medium were evaluated.

These experiments were conducted under 55-60% humidity, 16-hour light/8-hour dark photoperiod, temperature of 25±2°C and light intensity of 72 µmol $m^{-2}s^{-1}$. In addition to introducing chemicals, the effectiveness of incubating the samples *in vitro* under dark photoperiod for 28 days was also assessed. Each treatment was set up with 3 replicates and 5 explants (5x5 mm). The morphological changes in the explants were observed and photographed daily using a stereomicroscope. On the 28th day of the culture period, the density of browning, the density of callus induction, the color and the texture data were recorded.

Results and Discussion

One of the most significant issues in *in vitro* cultures is browning which can not only inhibit the growth and development of plant cells but also lead to cell or explant death, thereby reducing product yield[21]. Phenolics, which are released from the cut surfaces of explants, oxidize and have a toxic effect on plant tissue, causing browning of the medium[24]. Many researchers have used various methods to prevent browning such as adding antioxidants[19] or adsorbents[38], frequent subcultures[34] and dark culture conditions[16]. However, browning also depends on factors such as plant species, tissue type and age and optimizing *in vitro* conditions is necessary for species that exhibit browning[30].

In our study, leaf explants of *V. scamandri* were cultured in MS basal medium containing 2 mg/L Kinetin (Figure 1a). Thirty-seven different treatments were attempted, where chemicals were added to the medium and culture conditions were altered to prevent browning. According to the results obtained, browning could not be prevented on the 7th and 14th days of culture in media containing PEG (50, 100, 200 and 300 mg/L), PVP (50, 100, 200 and 300 mg/L), AC (100, 200 and 300 mg/L), AA (50, 100 and 300 mg/L), CA (50, 100, 200 and 300 mg/L) and the combination of AA with CA (50+50, 50+100, 50+200, 50+300, 100+50, 100+100, 100+300, 200+200, 200+300, 300+100 and 300+200 mg/L). Consequently, no morphological differences or callus induction were observed on the 28th day of these culture (Figure 1b-d).

Additionally, based on observations from the treatments, browning was observed on the entire surface of the explants on the 7th day with 100 mg/L AS and in the 100+50, 100+100 and 200+300 mg/L combinations of AS+SA, while in the medium containing only 300 mg/L AS, browning started from the middle of the explants on the 7th day. Browning was determined to start from the cut areas of the explants on the 7th day in all remaining treatments. On the 14th day, while the density of browning was generally moderate, it was observed to accelerate in the control group, 200 mg/L PEG, 50 and 200 mg/L PVP, 100 and 300 mg/L AC, 100 mg/L AS, 50 and 100 mg/L SA and some combinations of AC+CA (100+50, 100+100, 200+300, 300+100 and 300+200 mg/L). In the medium containing 200+200 mg/L AA and CA, browning was observed to progress slowly.

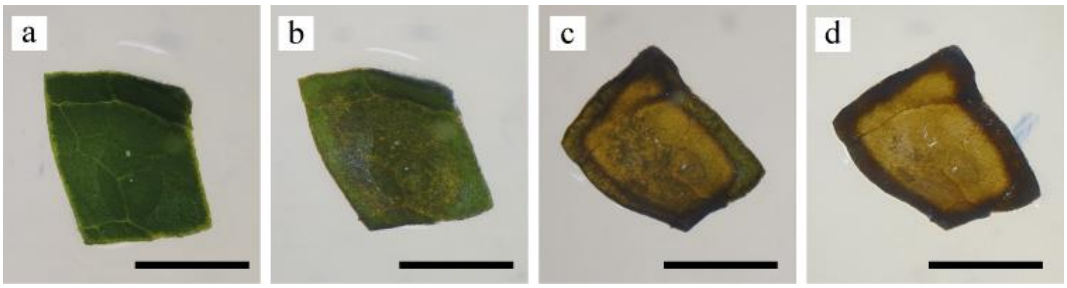

Figure 1: Leaf explant of *V. scamandri* were cultured on MS medium containing 2 mg/L Kinetin with PEG (50, 100, 200 and 300 mg/L), PVP (50, 100, 200 and 300 mg/L), AC (100, 200 and 300 mg/L), AA (50, 100 and 300 mg/L), CA (50, 100, 200 and 300 mg/L) and the combination of AA with CA (50+50, 50+100, 50+200, 50+300, 100+50, 100+100, 100+300, 200+200, 200+300, 300+100 and 300+200 mg/L) at the (a) 0th, (b) 7th, (c)14th, and (d) 28th day of culture (bar = 1 cm).

Table 1
The data obtained from treatments with browning inhibitors regarding callus browning density, callus induction density, callus color and texture.

Chemical Treatments (mg/L)						
AC	**AA**	**CA**	**Browning Density**	**Callus Color**	**Callus Texture**	**Callus Density**
50	-	-	+	Yellowish-Greenish	Compact	+
-	200	-	++++	Brown	Compact	+++
-	100	200	+	Greenish-Brown	Compact	++
-	100	300	+	Greenish-Brown	Compact	++
-	200	50	++++	Greenish-Brown	Compact	+
-	200	100	+++	Brown	Compact	+
-	300	50	++	Greenish	Compact	+
-	300	300	+++	Greenish-Brown	Compact	+
Culture Condition Treatment						
Dark Culture Condition			-	Yellowish	Compact-Friable	++++

Density Scale= Dense (100-90%, ++++), High (80-60%, +++), Moderate (50-40%, ++), Low (30-10%, +) and None (%0, -).

By the 21st day, in most treatments, the entire explants were observed as brown, while only in the medium containing 200+200 mg/L AA and CA, browning was determined to continue at a moderate level. In addition, cultures showing differentiation and callus induction were further examined from the 14th day onwards and data regarding the density of browning, callus induction, callus color and texture were recorded on the 28th day of culture in these media (Table 1). The scale for browning and callus density was determined by calculating the browning rate and callus induction rate as well as through observations.

Adsorbents such as activated carbon, as well as antioxidants like ascorbic acid and citric acid, are known to inhibit the activity of PPO and POD enzymes, thereby preventing tissue browning, protecting plant cells and tissues from oxidative damage[25, 46]. In our study, in the MS nutrient medium supplemented with 50 mg/L AC as an adsorbent, it was observed that browning was more effectively inhibited compared to other chemical inhibitors on the 28th day of culture (Figure 2a). However, callus induction was observed to be at a low level in this treatment. Similarly, in a previous study, the use of 200 mg/L AC in nodal explants of *Punica granatum* L. has been reported to reduce browning[33]. However, it is known that AC adsorbs nutrients and plant growth regulators in the medium[9].

Therefore, in our study, it is considered that AC affects callus formation by reducing it. When 200 mg/L AA was added to the nutrient medium as an antioxidant, browning was not inhibited. On the contrary, it occurred intensively and the callus induction that developed to a moderate level underwent oxidation and turned brown (Figure 2b). Another study on *P. granatum* found that the addition of 50, 100 and 150 mg/L AA to the culture media was not effective in preventing browning[34]. When media containing combinations of AA and CA were examined, the lowest browning of explants was observed in media containing 100+200 mg/L and 100+300 mg/L AA with CA, with browning of induced calli from the excised regions of these explants starting on the 28th day (Figure 2c-d). Additionally, it was determined that the browning density did not change in media containing increasing concentrations of CA (200 and 300 mg/L) with 100 mg/L AA. The highest browning density was observed in media containing 200 mg/L AA with combinations of 50 and 100 mg/L CA. While it was determined that the combination of 200 mg/L AA with a low CA concentration (50 mg/L) did not prevent or did not sufficiently reduce browning, it was also found that the combination of 200+100 mg/L AA with CA did not prevent browning but did result in a slight decrease in its density compared to 200+50 mg/L AA with CA (Figure 2e-f).

In explants containing 300 mg/L AA with combinations of 50 and 300 mg/L CA, the browning density decreased compared to media containing 200 mg/L AA with 50 and 100 mg/L CA, but browning was still not prevented in these media (Figure 2g-h). Furthermore, in terms of callus induction density, the highest density was observed in media containing 100+200 and 100+300 mg/L AA with CA, while lower densities were found in other media. In media containing combinations of AA and CA, an increase in browning of explants and a corresponding decrease in callus induction density were observed.

Although some studies suggest that combinations of AA and CA can reduce browning[6,39], this study demonstrates the ineffectiveness of AA and CA in *V. scamandri*. This finding may be attributed to the ineffectiveness of AA and CA in scavenging oxygen radicals generated by tissue damage and in preventing the release of phenols into the medium in the plant species used in our study. In media containing chemical inhibitors, browning was observed to commence slowly from the 7th day of culture at the cut areas of the explants.

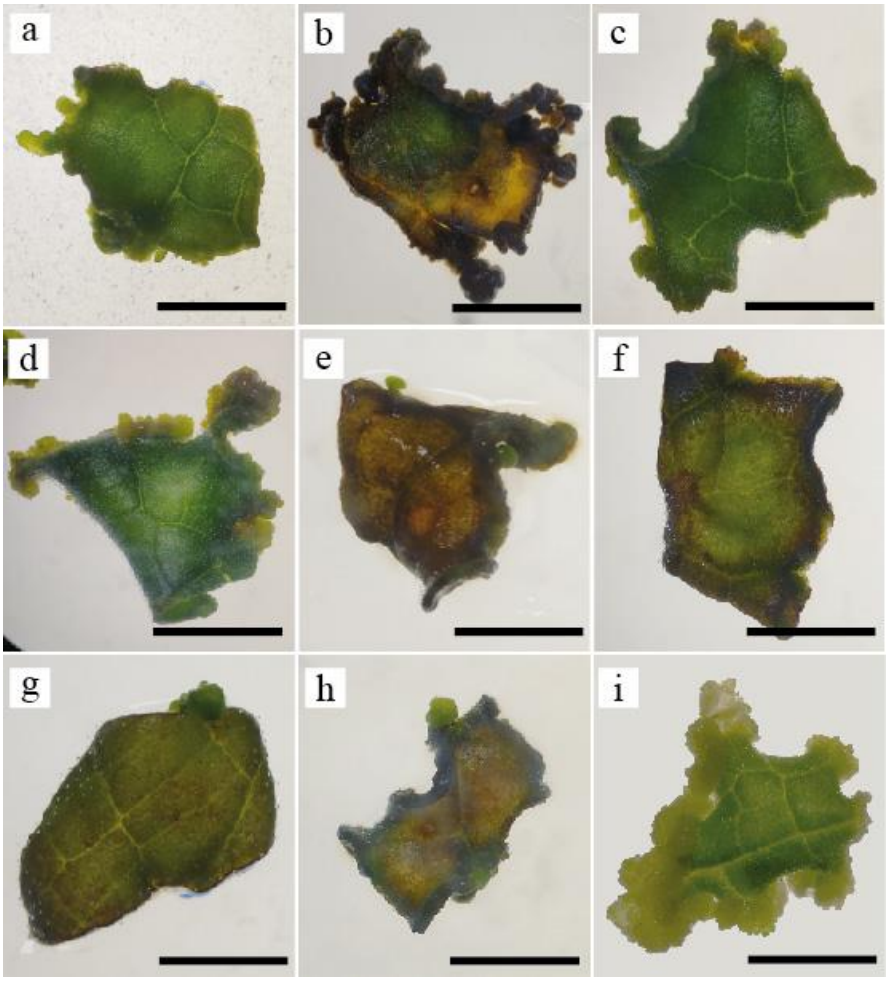

Figure 2: Leaf explants of *V. scamandri* cultured on MS medium containing 2 mg/L Kinetin with (a) 50 mg/L AC, (b) 200 mg/L AA, (c) 100 mg/L AA + 200 mg/L CA, (d) 100 mg/L AA + 300 mg/L CA, (e) 200 mg/L AA + 50 mg/L CA, (f) 200 mg/L AA + 100 mg/L CA, (g) 300 mg/L AA + 50 mg/L CA, (h) 300 mg/L AA + 300 mg/L CA, and (i) in dark culture conditions on the 28th day (bar = 1 cm).

However, in media containing 50 mg/L AC and 200+100 mg/L AA+CA, browning began to develop slowly from the 14th day onward. In media with 200 mg/L AA, the extent of browning increased further by the 21st day of culture. Light is an abiotic factor that accelerates browning by enhancing enzyme activity[7]. Studies across different plant species have noted that tissues cultured in dark culture conditions show lower levels of browning compared to those cultured under light conditions[16, 23]. Additionally, culturing explants in dark culture conditions have been suggested to slow down the degradation of plant growth regulators, thereby increasing culture efficiency[41]. Therefore, in our study, the explants were cultured in dark culture conditions. In this treatment, it was determined that neither the explants nor the calli exhibited browning on the 28th day of culture (Figure 2i). Consequently, the callus induction density was found to be highest in this treatment.

Furthermore, when comparing the effects of adding chemical inhibitors to the nutrient medium with those of culturing under dark conditions, it was observed that dark culture conditions completely prevented browning in *V. scamandri*. Likewise, Taghizadeh and Dastjerdi[37] reported in their study on *Spartium junceum* L. that the lowest level of explant browning and the highest callus induction were achieved under dark conditions. In a study, it has been reported that in chlorophyll-free habituated sugar beet callus, the dark culture conditions increased callus growth and prevented necrosis[14].

Another study on *Actaea racemosa* L. indicated that *in vitro* culture in the dark culture conditions reduced explants loss from 63.9% to 2.8%[29]. In a study on *Musa ssp.*, it was found that the dark culture conditions reduced browning compared to the light photoperiod in the French Clair and Batard varieties[18]. Furthermore, a study on Srikandi putih corn (*Zea mays* L.) reported that root and stem explants induced 49.3% more callus under dark culture conditions[36]. It has been determined that our results are consistent with previous studies.

Conclusion

The browning of explants is significantly influenced by the addition of chemicals to the culture medium and by incubating *in vitro* cultures under different environmental conditions. In this study, the effectiveness of different

chemicals (PEG, AC, PVP, AA, CA and AA+CA) added to the nutrient medium and the efficacy of dark culture conditions, were evaluated to prevent browning in the *in vitro* culture of *V. scamandri.*

According to our findings, dark culture conditions were found to be the most effective method for preventing browning in *V. scamandri* leaf explants. For future *in vitro* cultures of this species, especially when establishing callus cultures intended for secondary metabolite production, it would be beneficial to initially culture under dark conditions to establish healthy and productive growth.

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