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Control of browning in Plant Tissue Culture: A Review

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Abstract

Browning is one of the severe problems in plant tissue culture that hampers successful *in vitro* propagation of plants especially woody and perennial plants. In order to control the browning problem, different attempts have been made *in vitro* such as presoaking of explants in antioxidant solution, incorporation of antioxidants in to medium, culturing in the dark period and frequent subculturing of explants. Presoaking of explants in antioxidant solution like polyvinylpyrrolidone (PvP) and ascorbic acid (AC) is one of the most frequently used. Incorporation of antioxidants such as 0.2-0.5g/l PvP and 15-250mg/l ascorbic acid in to MS medium are commonly used to control browning in different plants and explants followed by activated charcoal, citrc acid, MES, and AIP. Moreover, frequent sub culturing and incubation of explants in the dark period is the other alternative. This review attempts to explore and provide comprehensive idea on the different methods used to control browning problem in plant tissue culture which need to be further optimized for successful control of browning when using the same or different crops as well as explants.

Keywords: Activated charcoal, Antioxidants, Browning, Phenolic compounds, Polyvinylpyrrolidone.

INTRODUCTION

Browning in plant tissue culture refers to a phenomenon in which the explants release brown substances or phenolics to the medium from its own tissues in the course of dedifferentiation and/or re-differentiation [1, 2]. Phenols are chemical compounds that embraces a wide range of plant substances which possess in common, an aromatic ring bearing one or more hydroxyl constituents [3].

Phenolic compounds are secreted from wounded regions of explants as a defense response [4], and oxidation of these compounds results in browning of culture media and plant tissues [5]. Phenolic compounds are oxidized by PPOs to their quinine derivatives and further oxidized to form the pigment melanin, which is found in organisms and is responsible for browning reactions [6]. Besides PPO, phenylalanine ammonia lyase (PAL) and peroxidase (POD) are also responsible for browning arising from wound as a catalyser of polyphenol biosynthesis [7].

While phenolic compounds are generally present in healthy plant tissues and can accumulate in specialized cell types [8], they are produced in greater abundance and/or released as a defense response, especially following tissue wounding or stress [8, 9]. The majority of tissue culture protocols involve wounding the material in order to remove explants and culturing them in potentially stressful environments; often eliciting the production and release of phenolic compounds. As a result, this natural defense response can lead to the accumulation of toxic compounds that ultimately damage or kill plant cells and tissues.

In addition, accumulation of ethylene in the culture medium as a result of low gas exchange is another cause of browning of explants during in vitro culture [11]. Other types of phenolic exudates appear at the end of incubation period and are apparently products of dying cells [12]. The phenolic exudation is aided by light and is autocatalytic. For example, tissues cultured in the dark often display lower levels of browning than those grown in the light [7, 9, 13].

The prevalence of browning varies among species, cultivars, the physiological state of the plant/tissue, and size of explants and age of explants [14, 15, 16, 17]. Oxidative browning is a common problem in plant tissue culture; resulting in reduced growth [7, 18], lower rates of regeneration or recalcitrance [19, 20, 21], and can ultimately lead to cell/tissue/plant death [7, 20, 22, 23, 24]. Different attempts has been made to eliminate browning problem in woody plant species like pre-socking of explants in antioxidants solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent sub culturing

of explants. However, the effectiveness of these methods varies from species to species and physiological conditions of plant.

Therefore, this study provides comprehensive ideas on the type, effect of different browning agents on avoiding of explants with particular regards to effectiveness of the agent, concentrations as well as the type of plant/variety used and how it is applied to the explant.

Pre-soaking of explants in antioxidant solution

The successful use of antioxidant applied during explants preparation to prevent lethal browning is reported by several authors. An antioxidant wash of 0.125% potassium citrate: citrate (K-C: C in a ratio of 4:1 w/w) solution was useful for explants preparation of *Musa* spp.cv [26]. Similarly**,** treating the explants with 1.2 g/l of ascorbic acid during explants preparation controlled the extent of lethal browning of local *Musa* spp.cv. Mzuzu [27]. Pre-socking of apical and axillary buds in 0.5% polyvinylpyrrolidone (PVP) in combination with 3% sucrose for 30 min was found effective for browning control in mango [28]. pre-soaking of nodal explant in 1 g/l activated charcoal for 5 hours significantly reduced media browning in micro propagation of Guava (*Psidium guajava* L.) [29]. Treating seeds of faba bean with 1000 mg/l PVP solution for 1 h, followed by culturing in Murashige and Skoog medium (MS medium) supplemented with ascorbic acid (1 mg/l) or activated charcoal (10 g/l), greatly reduced lethal browning in explants and improved shoot regeneration [30]. Pre-treating shoot tip of Curculigo *latifolia* with PVP, ascorbic acid and citric acid (0.1%) for 9 hr was the best technique for reducing browning [31]. Dipping the explants in 0.5 g·L-1 NaCl solu tion was effective in suppressing browning of the petal explants of herbaceous *Paeonia Lactiflora* Pall [32]. Presoaking/ pretreatment of aux bud of plantain (*Musa paradisiaca*) in 0.1-0.5 mg mL-1 of potassium citrate and citrate (K-C: C) for 2 hr prevented browning [3].

Table 1: Antioxidants used in presoaking of explants

Antioxidant	**Concentration**	**Variety**	**Explants used**	**Reference**
PVP +Sucrose	0.5%+3%	Mango	Apical and axillary buds	Chavanetal.,2000
PVP+ AA[b] +CA	**0.1**%	Curculigo latifolia	Shoot tip	Babaei *et al*. (2013
AA[b]	0.125	Musa spp.	Shoot tip	Titov *et al*. (2006)
AA[b]	1.2g/l	Musa spp.	Shoot tip	Ngomuo *et al*. (2014 (2014)
PVP	1000 mg/l	faba bean	cotyledon	Abdelwahd *et al*. (2008)
NaCl	0.5 g·L-1	*Paeonia Lactiflora* Pall	Petal	Cai *et al*. (2020)

PVP= polyvinylpyrolidone, AA[b] =ascorbic acid, CA= citric acid

Incorporation of antioxidants into medium

The addition of 0.3 g·L-1 polyvinylpyrrolidone (PVP) to the medium can effectively inhibit browning followed by 0.2 mg·L-1 ascorbic acid in stem segments of 'Hongyang' kiwifruit [33]. The addition of aminoindane-2-phosphonic acid (AIP) up to 10 µM into culture media resulted in significant reductions in visual tissue browning of *Artemisia annua* [5]. Similarly, AgNO3 is a potent ethylene inhibitor and its presence in culture medium has been reported to inhibit browning in vitro shoot production in many plant species [34, 35, 36]. In addition to its role as an antibrowning agent, several reports indicate that it is effective in regulating morphogenesis and induces multiple shoot production [37, 38]. AC adsorbs the free phenolic compounds secreted by explants into the culture medium [39] and prevents tissue browning.

Supplementation of 15 mg·LL1 ascorbic acid to basal media minimized the phenolic secretion, improved culture quality, and survival from cotyldonary node explant of Okra (Abelmoschus esculentus L.) [40]. AA has been shown to inhibit the browning of cultured tissues and improve morphogenesis in Cavendish banana and Brachylaena huillensis [41, 42]. In addition, this compound has an essential role during plant morphogenesis [43] and is involved in cell division, cell differentiation, and cell elongation of apical meristems of Aloe barbadensis Mill [44] and cotyledonary nodes of Vicia faba [30]. Ascorbic acid contains ascorbate that has a direct inactivating effect on PPO [42]. In addition, AA converts colorless o-quinones resulting from PPO action back to diphenols and prevents browning [45]. According to Titvo *et al*. (2006), AA scavenges oxygen radicals to prevent the oxidation of phenolic compounds in wounded tissues, thereby reducing tissue browning [26].

The addition of 1.0 g/L morpholine ethane sulfonic acid (MES) into MS medium significantly reduced browning in *Sideritis trojana* [46]. Murashige and Skoog medium supplemented with 0.2 gL-1and 0.3 gL-1 of Polyvinylpyrrolidone has gave 100% and 80% survived explants of C86-56 and C86-12 sugarcane genotypes respectively after 30 days of culturing [47]. Dipping excised explants in a 0.5 g·L-1 NaCl solution, adding 0.5 g·L-1 PVP to the medium, storing planted explants at 4 ◦C for 24 h, and transferring planted explants to the same fresh medium after 24 h could effectively inhibit browning in Petal explants of *Paeonia Lactiflora* Pall [32]. Peach can be successfully propagate in media supplemented with 50m/l ascorbic acid, 20 mg/l stabs vitamin mixture [48].

It has been found 60% and 40% browning free explants for two different sugarcane genotypes at a PVP concentration of (0.5-1) gL-1[49]. This could be due to genotypic differences among the materials used. MS medium supplemented with 0.5 *g*L-1 PVP resulted in successful initiation of large embryogenic callus ranging from 80 to 90% which were free of browning [50]. This difference may be happened due to the difference in genotypes and the type of *in vitro* regeneration path used.

Incorporation of 200-250 mg/litre of ascorbic acid into the medium significantly controlled lethal browning in nodal culture of *Brahylaena huillensis* [42]. Supplementation of 0.5% PVP into culture medium prevented explants browning in callus culture of nodal explant of *Spartium junceum L.* [51]. Adding a combination of 100 mg/l ascorbic acid and 50 mg/l citric acid to the murashige and skoog (MS) medium was found as the most effective treatment during micro propagation of Sideritis trojana bornm, an endemic medicinal herb of Turkey [46]. Addition of 200 mg/L activated charcoal into the medium was found quite effective to minimize browning problem in nodal segment of mature explant *Punica granatum* L [52].

Supplementation of 300 mg L PVP in conjunction with 2 g L-1 activated charcoal ascorbic acid, is recommended for minimizing the effects of phenol oxidation in nodal segments of *E. pyriformis* [53]. Pre-socking of apical and axillary buds in 0.5% polyvinylpyrrolidone (PVP) + 3% sucrose for 30 min was found effective for browning control in mango [54]. Best results found in browning control with 150 mg/L ascorbic acid and 100 mg/L citric acid in pomegranate [55].

The addition of 15 mL L-1 ascorbic acid to the MS culture medium was efficient in preventing oxidation in banana tree explants *Musa* spp. [56]. Adding the antioxidants cysteine and silver nitrate improved the maximum recovery of chickpea plantlets *in vitro* after agro-inoculation [57]. Similarly, the addition of cysteine to the growth media reduced explant blackening in banana tissue culture [58]. Incorporation of activated charcoal to the culture medium prevented the effect of leached phenolics that hindered regeneration of *Celastrus paniculatus* and *C. orchioides* respectively [59, 60].

Table 2: Incorporation of antioxidants into medium

Antioxidants	Concentration	Variety /plant name	Explants used	Reference
Pvp	0.3g/l	Hongyang kiwifriut	Stem segments	Jiufeng *et al.*,2018
AA[b]	0.2g/l	Hongyang kiwifriut	Stem segments	Jiufeng *et al.*,2018
AIP	10M	Artmesia annua	-	Jones and Saxena (2013)
AA[b]	15mg/l	Okra	Node	Mohammed [et al.]., (2018)
MES	1g/l	*Sideritis trojana*	-	Corduk and Aki (2011)
PVP	0.2g/l	*C86-56*		Shimelis *et al.*(2015)
PVP	0.3g/l	*C86-12*		Shimelis *et al.*(2015)
AA[b]	15mg/l	Okra (Abelmoschus esculentus L.)	cotyldonary node	Muhammad *et al.* (2028
PVP	0.5 g·L-1	*Paeonia Lactiflora* Pall	Petal explants	Xuan *et al.* (2019)
AA[b]	200-250 mg/litre	*Brahylaena huillensis.*	Node	Ndakidemi *et al.* (2014
AA[c]	200 mg/l	*Punica granatum* L	Node	Singh and Patel (2016)
AA[b] + CA	100 mg/l + 50 mg/l	Sideritis trojana bornm	Leaf	Corduk and Aki (2011)

AA[b=] ascorbic acid, AA[c]=activated charcoal, CA= citric acid, MES= morpholine ethane sulfonic acid, AIP=aminoindane-2-phosphonic acid, PVP= polyvinylpyrolidone

Frequent sub culturing

Quick transfer of explants within the same spell or to fresh medium 2 or 3 times, at short intervals, is the simplest and fairy successful method to protect the explants from the detrimental effect of oxidative browning [61]. Frequent transfer of explants within the same medium or into fresh medium fairly prevents *in vitro* browning of explants [61, 62]. During this period the cut ends of explant may become sealed up and the leaching of phenolics stops. Sub culturing of explant consecutively thrice at an interval of 24 hours controlled browning completely in pomegranate [63]. Subsequent transfer of explants on fresh medium resulted in complete disappearance of browning in nodal segment explants of mature plants in pomegranate [64, 65, 66]. Sub culturing of nodal explants of *Punica granatum* L.twice, at the first day and third day of inoculation was effective in browning control [52]. Pushpraj and Patel (2016) result revealed, that the most effective browning control was observed in sub culturing of nodal explants twice, at the first day and third day of inoculation, which also found better in establishment of explants of pomegranate [67].

Incubation of culture into dark period

Presence of light and high temperature raise browning rate by increasing the enzyme activity [68]. For example, tissues cultured in the dark often display lower levels of browning than those grown in the light [7, 11, 12]. MS media supplemented with 1.6 mgl-1 IAA and 4.0 mgl-1 BAP without ascorbic acid and activated charcoal in darkness for 4 weeks was the most suitable media for shoot regeneration [69] (Nisyawati & Kusuma, 2013**).** Keeping the cultures initially in the dark may also help to reduce browning problem [70] by preventing or reducing the activity of the enzymes concerned with both biosynthesis and oxidation of phenols [26].

CONCLUSION

The purpose of this review was to systematically analysis the previous research done to control browning problem in plant tissue culture. It is normal that the practice of plant tissue culture involves cutting/wounding of explants in a way that is able to grow in a defined nutrient medium. While cutting of explants from stock plant as well as reducing the size of explant in vitro is the normal practice/procedure to remove the residual effect of surface steriliants leftover during the process of surface sterilization/disinfection which is phytotoxic and to the enable explant take up nutrients through the wounded surface from the medium, on the other side the explant secrete phenolic compounds as defense mechanism into the nutrient medium which hinders nutrient uptake by the explants, making the medium brown and eventually kill the explants which is more severe in woody and medicinal plant species. To overcome the problem, different attempts have been in vitro such as presoaking of explants in antioxidant solution, incorporation of antioxidants in to MS medium, frequent subcultuirng of explant and incubation in to dark period. Moreover, it is essential that the mother plant be grown in the greenhouse or lath house than field grown so that the browning intensity than can be minimized.

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