Lethal Effects of Secondary Metabolites on Plant Tissue Culture

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Abstract: Development of a reliable in vitro regeneration protocol is necessary for researchers as well as for commercial tissue culture plant producers. However, leaching of phenolics from the explants of most woody and some herbaceous plants, to the culture medium causes browning, proven lethal to explants, hampering in vitro regeneration that leads to death of explants. Pre-treated with polyvinylpyrrolidone (PVP), then cultured different types of explants on tissue culture media supplemented with an adsorbent (activated charcoal) and antioxidants (ascorbic acid, cysteine and silver nitrate). As major problem during the micropropagation is lethal browning caused by the exudation of phenolic compounds (secondary metabolites) from excised portion of explants. Overall, the main purpose of this review is to highlight the lethal effect of phenolic compounds and provide methods to solve oxidative browning problem, especially in woody plants.

Key words: Phenolic compounds • Oxidative Browning and media darkening

INTRODUCTION

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. More than 8,000 phenolic structures are currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. The oxidation of the hydroxycinnamoyl residues results to the formation of phenolic bridges between polysaccharide chains, polysaccharide and lignins and between polysaccharide and structural proteins [1]. Micropropagation of fruit trees is an important tool through which we can produce genetically identical plants at rapid pace [2]. However very low frequency of organogenesis has been induced due to inhibitors [3]. Woody plants such as mango high phenolic exudation cause activation of oxidative enzyme when explants are cut, which causes failure of mango tissue culture. It is due to the activity of polyphenol oxidase (PPO) leads to the death of tissue [4]. Including PPO, some other oxidative enzymes like phenylalanine ammonia lyase (PAL) and peroxidase (POD), a catalyzer of polyphenol bio-synthesis also take part in browning at wounded region of explants [5, 6]. In addition, POD and PPO may act collectively in oxidative browning, as PPO may promote POD in oxidation of phenolic compounds. Biotic or abiotic stress stimulates oxidative browning of explants which involves severing from mother plant and wounding of tissue during culture preparation [7].

A blackening or browning of tissues excised from many woody species of the tropics can be observed. This process is called phenolics oxidation, inactivates the growth of the tissues in culture. For instance, coffee contains a high concentration of phenols which exude when any organ is existed and their subsequent oxidation in the presence of copper-containing oxidase enzymes such polyphenoxidase and tyrosinase causes the blackening or browning. The medium in which explants are grown become colored within an hour or two of planting the material as is observed in tissue culture of many tropical and sub-tropical woody species. The browning and black colour developing in callus cell cultures is due to the formation of quinines. Also, possibly as a result of binding between phenols and proteins and its subsequent oxidation to quinines a loss of enzyme activity might result [8, 9]. One of the most common problems associated with the in vitro establishment is the deleterious effects of oxidized...
phenols [10]. Phenolic exudation during the excision of plants of several woody tree species including guava cause browning of media occurred [11]. Browning of tissue is caused by the oxidation of tannin and polyphenols and the formation of quinones which are highly reactive and toxic to the plant tissues plant tissues contain these substances in separate pools or compartments. During tissue wounding or senescence these pools are integrated and the oxidation process is initiated [12] which is enormous problem for Establishment of in vitro cultures of woody plants. The cinnamic acid is believed to be the first step in the biosynthesis of phenolic compounds. It is synthesized from amino acid-phenylalanine ammonia lyase (PAL) [13]. Phenolics are inhibitory to cellular growth [14, 15] Quinoline substance which are produced as a result of oxidation of phenolic compounds will gradually enter the tissues cultured on the medium and further represses the activities of other enzymes and as a result, poison other contents of the medium [16].

As production of phenolic compounds indirectly stimulated by several factors like age of the plant, size of plant, biotic and abiotic stresses, probably young seedlings do not synthesize higher quantities of phenolics when grown in a shoot chamber [17]. Increase in age of mother plant will directly affect in vitro browning as there is positive correlation between age of explant and phenolic exudation in tissue culture of cotton [18]. The qualitative and quantitative estimates of phenolic compounds in different explant types of jamun (Syzygium cumini L.) were found different [19]. Young tissue and organs of pistachio contained less phenolic compounds, while these were biosynthesized and accumulated more in older cells. Browning and total phenolic content of explants increased gradually throughout a proliferation cycle. During later phases of sub culturing explants browned heavily and started shrinking. It is suggested that explants be taken from young shoots of pistachio and that regular transfer of subcultures occur to control browning by reducing polymerized phenols in the media, Media browning that resulted in the death of explants of Pistachio (Pistacia vera L.) in a few days after culturing [6, 20, 21]. Cultures initiated from immature embryos show low browning as compared to the older explants of pines [22, 23]. However severity of browning varies between species and varieties, tissues and development phase of organ. Vigorous browning results in tissue degeneration and stops development embryogenesis in Hevea brasiliensis [24]. Browning of media occurred as a result of oxidation of polyphenols exuded from explants [11]. Tissue browning seriously decreases in vitro regeneration from callus cultures, especially plant regeneration. Tissue browning is associated with both accumulation of polyphenol oxidase and decrease of putrescine, spermidine and spermine in browning calluses, which inhibited callus growth, shoot differentiation and rooting of shoots derived from browning tissues in Virginia pine. However, more research is necessary, In callus derived from mature trees activity of oxidative enzymes increased during culture of. These cultures browned early and after 6 weeks of culture had significantly higher enzyme activities compared to the cultures derived from immature embryos. It is considered that browning seemed to be related to the culture time moreover Plastids Differentiation rate varies in embryos and those embryos contain both protochlorophyll and chlorophyll. The variations in the plastid differentiation rate, PPO concentration in the plastids and the onset of tannin synthesis explain the differences in browning at the maturation stage in cultures from immature seeds [25-27]. In case of bamboo three species gave varied responses to the traditional substances. Nutrient medium addenda of some PPO inhibitors, named ascorbic acid, cysteine, kojic acid and thiourea, mainly enhanced browning. However, ferulic acid at 3 mM and lower concentrations reduced the number of brown shoots per culture, but not the percentage of cultures that browned [28]. As browning is dependent of species A comparison was made between European and Canadian yew (Taxus baccata L. and T. canadensis Marsh.) from results it was suggested that a high level of phenolic compounds in yew calli was high and cause death of explants [29]. In micropropagation of guava (Psidium guajava L.) and olive (Olea europaea L.) lethal browning inhabits the morphogenetic activity [30]. Browning and subsequent death of the explants is usually depended on the phenolic compounds and the quantity of total phenols. Different studies, due to exudation of phenolics various problems such as media discoloration, rooting deficiencies and explant browning and death. In addition, amount of phenolics can be more or less in different stages of organogenesis due to metabolic actions. Therefore determination of phenolics and calculating the amounts of phenols may be another research area for many tissue culture studies [31]. Guava is a recalcitrant species and in vitro oxidative browning, which restrict efficient micropropagation protocol [32]. It has been suggested that high POD and PPO activities may minimize growth and stop embryonic development in non-differentiating
callus was characterized by a high phenolic polymers, pine callus having high-tannin, the amount of declining and dying cells has also been found to increase in parallel with browning [23, 33]. Although some technical approaches can occasionally circumvent immediate micropropagation limitations, general solutions await the development of a deeper understanding of physiological bases of such genetically predetermined phenomena; Browning can be caused by phenolic compounds at the chemical level. The phenolic compounds could activate polyphenoloxidase (PPO) of explants and change the metabolism of tissue cells and form brown quinine substances through its oxidation [34, 35]. To determine if lethal browning is caused by deficiency or toxicity of mineral nutrients, the mineral contents of leaves and corns of diseased and healthy plantlets of Cavendish banana cv. Nitrogen and phosphate were analyzed calorimetrically with an UV-1 spectrophotometer while potassium was analyzed by Eppendorf flame photometer [36]. Development of Shoot was completely inhibited and the explants died within a week. The browning problem was more rapid with explants collected from the older shoots than those from the younger shoots. This problem was overcome by sub-culturing the nodal segments to a fresh medium of the same composition at 24 h interval. The browning exudation was stopped after two consecutive transfers and the explants exhibited normal shoot development [37]. In case of mango there is difference in phenolic exudation from different explants source, minimum browning was observed when immature embryos were cultured and high regeneration rate was obtained [38-42]. Banana tissues are known to contain large amount of latex and phenolic compounds and hence micropropagation of banana has a serious problem caused by lethal browning of plantlets [43]. Actually these antioxidants donate hydrogen and can also react with compounds that are delivered from mechanical injury and environmental stress from stable complex compounds [44, 45]. However, very little is known about the precise mechanism by which antioxidants reduce browning effectively in many horticulture crops and would make interesting field of research. It has been reported that peroxidase catalyzes the reduction of hydrogen peroxide and protects tissues and cells from oxidative damage [46-48]. In damaged cells the contents of cytoplasm and vacuoles are mixed and phenolic compounds can readily become oxidized by air, PODs or PPOs. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal for explants [49]. As browning of media prevents further progress in biotechnology of woody plants especially, therefore it is necessary to explore a suitable protocol for successful micropropagation [50].

How to Control: Different attempts have been made to alleviate this problem, including pretreatment of explants with antioxidants, incorporation of antioxidants into the culture medium, incubation of cultures in the dark and frequent subculture to fresh medium, etc. These manipulations have proved to be either successful or not depending on the species and type of explant propagated [51].

Growth medium provided with different concentrations of ascorbic acid, citric acid, activated charcoal and polyvinylpyrrolidone had no conclusive ameliorative effect on the browning of pine callus [52]. Special attention is drawn to the optimization of the salt content of the nutrient media for getting overcome the browning of the shoot tip, the existence of red pigmentation on the leaves and improvement of the vitality of the plants [53].

The effects of ascorbic acid and 4-hexylresorcinol on pear polyphenoloxidase (PPO) activity and stability have been investigated in vitro. Ascorbic acid does not interact directly with PPO but prevents browning by reducing oxidized substrates. The 4-hexylresorcinol exerts a dual role on PPO. If no substrates are present, it interacts preferably with the deoxy form of PPO to inactivating it. If substrates and 4-hexylresorcinol are both present they compete for the catalytic site. The 4-hexylresorcinol behaves then as a canonical enzyme inhibitor, binding to the net form of PPO. Simultaneous addition of 4-hexylresorcinol and AA has synergistic inhibition effects depending on the presence or the absence of PPO substrates [54].

Quick transfer of explants within the same spell or to fresh medium 2 or 3 times, at short intervals, is the simplest and fairest successful method to protect the explants from the detrimental effect of oxidative browning (Kotomari and Murashige, 1965; Morel, 1972). During this period the cut end of the explant may become sealed up and the leaching of phenolics stop. Keeping the cultures initially in the dark may also help to reduce the browning problem [55] by preventing or reducing the activity of the enzymes concerned with both biosynthesis and oxidation of phenols [56]. Phenolic compounds secretion was successfully stopped by pre-soaking them in an antioxidant solution of 0.125% potassium citrate: citrate [57]. Through sub-culturing lethal browning can be controlled in many crops [58, 59] find in his investigation
that *strelitzia reginae* can be propagate successfully through controlling oxidative browning using 1% activated charcoal, 0.05% polyvinylpyrrolidone (PVP), 0.04% dihydroxytol (EDT). Browning was also controlled cultured after agitation pretreatment of 100 mg/l and 150 mg/l ascorbic acid and citric acid respectively, [9], silver nitrate is also helpful in elimination of browning in micropropagation of *taxus mairei* [60]. Basal medium provided with AC (activated charcoal) has great capability to absorb phenolics [61]. Oxidative stress can be overcome with the help of mycorrhization [3, 62] significant reduction in phenolic exudation was observed in PVP treated explants. Effectiveness of ascorbic acid in resolving the lethal browning problem of banana cv. It is believed that ascorbic acid may have been absorbed by the plantlets transplanted to leaves and prevented the oxidation of phenolic compounds on the target site [63]. The explants of Sharixi walnut were pretreated with 20% sodium hyposulphite to decrease the browning rate of various pretreatments of Sharixi walnut, it was found that the effects of AC would last only for a short time, the effects of PVP remained over a longer period and AC made the plantlets bloom [64, 65]. This problem would probably be controlled by suspending the explants in a sterile solution and by adding PVP 40 to the media [66]. Shoots tips of strawberry were cultured under dim light to develop into plantlets on a hormone-free MS medium, The survival rate of three genotypes was between 89.2 - 100% when cultured under dim light (500 lux). It is resulted that light intensity significantly affected survival rate of explants by induction of the phenolic [51]. In case of banana to prevent phenolic oxidation it was suggested that use of filter sterilized ascorbic acid 2.5 mg per ml. Maximum number of healthy and contaminant free cultures obtained [67]. Partial etiolating of stock plant of guava before explant excision resulted in early bud sprouting and significant increase in explant survival due lower degree of browning of culture medium [68].

Soaking the explants in the solution of citric acid and ascorbic acid for 12 hours decreases browning slightly, while 24-hour immersion reduced browning considerably [69]. Soaking wounded portion of explants in the solution of low pH solution reduced the discoloration of media [70]. Satisfying results were obtained when explants were placed in running tape water for at least 1 h before sterilization, as suggested [71, 72]. The reduction of oxidative browning was best achieved by stirring the explants for 1 h in an antioxidant solution mixture containing 100 mg/l ascorbic acid and 1500 mg/l citric acid before inserting them into the medium [43]. Shoot tips of guava cv. Sañeda were washed by running tape water for 30 minutes [73, 74] found that browning significantly controlled by addition of activated charcoal into basal MS media in case of *Sequoiadendron giganteum*. [75] reported that micropropagation of apple can is problematic as high phenolic exudation at initial stage, however can be controlled by combination of ascorbic and citric acid. Similar effect was observed in *Caesalpinia pulcherrima*. Activated charcoal adsorbs the phenolics which leach into the medium from the cut ends of the explants [76]. Peach (Prunus persica L.) can be successfully propagate in media supplemented with supplemented with 50 mg/l ascorbic acid, 20 mg/l stabs vitamin mixture [77]. Media browning was higher in MS medium not supplemented with antioxidants or adsorbents [78]. Charcoal, PVP, citric acid and ascorbic acid beneficial to control the phenolic oxidation and all suppressed browning of callus tissues. Among them, supplementation of PVP (15 mg dm-3) was superior [79].

Stem node explants excised from seedlings produced dark brown exudates at the stem base. Guava stems and leaves are rich in phenolic compounds [80]. Higher browning in shoot tips than nodal segments may be due to higher phenolics as the shoot tips are actively growing and herbaceous in nature [81]. Explants obtained from grafted seedlings. However, they compared performance of shoots from grafted seedlings to those obtained from greenhouse grown seedlings show minimum blackening [82]. Utilization of immature inflorescence tissue as explant material can favours minimal exudation of phenolic compounds [83]. Sever pruning of grape vine is helpful in Control of oxidative browning resulted increase in multiple shoot formation per initiating culture [84]. The seasonal influence on browning of explants derived from mature trees has been reported previously [85]. Percent establishment, percent mortality and degree of browning as influenced by pretreatment of antioxidants for different duration hours [69]. There is limitation of season because the strawberry only produces runners during the vegetative development phase. If we can obtain explain materials from off shoot, this problem will be overcome. But the off shoot larger than runner size is also more difficult for disinfection. In addition the browning at initial establishing stage of in vitro culture is the main cause leading to explant death [86, 87]. The highest explant establishment (77.88%) was observed during spring, which was on a par with explant establishment frequency during winter season (76.21%) [88]. The relatively poorer effectiveness of antibrowning agents during summer, rainy and autumn seasons in
comparison to spring and winter seasons may be due to accumulation higher in vivo phenolic content in the stock plant [89]. Low browning in spring and winter seasons may be due to low in vivo phenolic content as the short day length prevailing during this period has been reported to reduce the in vivo phenol ics [90]. The present study also showed that seasonal changes greatly influenced the browning of shoots. Browning was maximum (70-85%) in explants obtained during the months of May to August and was relatively low (35-40%) for explants collected in December to March [91]. In vitro propagation protocol has been developed using nodal explants from a mature 'elite' tree of Acacia siuata. Maximum shoot proliferation (75.2%) was achieved from nodal explants collected during the December to March season [92].

Shoot tip and nodal segment explants were cultured on MS medium modified with different growth regulators for mass propagation of 'Hybrid Tea' roses evs. 'Rosy Cheek' and 'Whisk Mac'. The Murashige and Skoog medium modified with BAP (3 mg/l) + IAA (3 mg/l) + activated charcoal (5 mg/l) effectively minimized production of phenolic compounds and gave early shoot proliferation, maximum shoot length and number of leaves per shoot [93]. Establishment of in vitro cultures of several plant species, especially woody plants, is greatly hampered by the lethal browning of the explant and culture medium. Browning is generally considered to result from the oxidation of phenolic compounds, released from the cut ends of the explants, by polyphenoloxidases [94]. The basic medium was MS, with a test playing temperature of 25±2°C, light for 12 h and light intensity of 1000-1500 lx results lower down the browning rate up to 48.0%, while the control reached 100% [95]. Buds of x Malosorus florentina (Zucc.) Browicz (Rosaceae) were collected in March, micro shoots from buds collected in March were subculture five times on MS media give butter result against darkening [96]. Adsorptive materials on the incidence of lethal browning of banana tissue culture plantlets, the SM medium was amended with 10% each of Dowex anion exchange resins, Lowatit cation exchange resins (Bayer Corporation, Pitts- burgh), polyvinylpyrrolidone or activated charcoal (Taipei Chemical Industry Co., Hsinchu, Taiwan). To remove possible inhibitory substances, 100 g each of this compound was soaked in 1,000 ml distilled water. The pH of mixture was adjusted to 5.7 and filtered through Whiteman no. 1 filter paper after shaking for 30 min [97]. In case of P. graminifolia dark treatment, a countermeasure, reduced exudation of the browning compounds during floret culture [98].

CONCLUSION

Phenolic compounds or secondary metabolites in plants are produced in response of biotic and abiotic stress. Actually these are involved in defense mechanism of plants. In tissue culture when explants are excised during preparation for culturing this stimulate phenolic exudation. The oxidation of exuded phenolics cause darkening or browning of media which blocks the uptake of nutrients ultimately leads to death of explants. Their exudation is minimized by use of different absorbents and antioxidants.

REFERENCES


