Influence of media supplements on inhibition of oxidative browning and bacterial endophytes of *Camellia sinensis* var. *sinensis*

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Abstract

Explant oxidative browning and necrosis of *Camellia sinensis* var. *sinensis* is a severe problem in tissue culture, often associated with the exuded phenolic compounds and microbial contamination from the explants. In this study, 2-aminoindane-2-phosphonic acid (AIP), an inhibitor of the polyphenol production-required enzyme phenylalanine ammonia lyase (PAL), and different antibiotics were tested to control tea explant necrosis and browning. These compounds were supplemented in the regular plant growth medium together with 6-benzylaminopurine and thidiazuron at different concentrations. Our data indicated that application of 2 μ M of AIP was able to effectively inhibit callus browning, significantly reduce EGC abundance, and greatly improve callus induction and growth. Moreover, the use of 150 mg/L of timentin and 30 mg/L gentamycin resulted in an effective elimination of the surface and endophytic microbes associated with explants of *C. sinensis* var. *sinensis*. Our study revealed that the inhibition of PAL using AIP combined with the two tested antibiotics could open up new doors to control oxidative tissue browning and endophyte contamination in tissue culture for tea genetic manipulation.

Keywords 2-Aminoindane-2-phosphonic acid (AIP) · Bacterial endophytes · Phenolic compounds · Phenylalanine ammonia lyase (PAL)

Introduction

Camellia sinensis (L.) belongs to the family Theaceae. It originated from southern China and the Chinese were the first to consume tea as medicinal drink later as beverage (Eden 1976). The cultivated taxa consist of several variants such as *C. assamica* (Masters) or Assam type, *C. assamica* sub spp. *lasiocalyx* (Planchon ex. Watt.) or Cambod or Southern type and *C. sinensis* (L.) O. Kuntze or China type (Costa et al. 2007). Compared to Assam type cultivars, those of China type have smaller leaves and are more resistant to drought and cold stress conditions (Harler 1964). They generally contain extraordinarily high levels of polyphenols (16–30% of dry weight) in leaves (Graham 1992; Almajano et al. 2008). Very often, attempts of in vitro manipulations and genetic improvements of Chinese tea cultivars fail, not only because of phenol oxidative browning of the explant, but also likely due to the contamination of the endophytic

microbes associated with explants of the field-grown tea plants (Wang et al. 2016). Effective inhibition of explant browning/necrosis and eradication of microbial contaminants are prerequisites for successful *Agrobacterium*-mediated genetic transformation in tea plants.

In plants, due to the action of polyphenol oxidase (EC 1.14.18.1) (PPO), phenolic compounds may form *o*-diphenols, which can be further oxidized to form highly colored *o*-quinones (Sapers and Hicks 1989; Janovitz-Klapp et al. 1990). The *o*-quinones condense spontaneously with other *o*-quinones and proteins, reducing sugars, etc. to result in the precipitated polymers in browning plant tissues (McEvily et al. 1992). Silencing of PPO expression has successfully resulted in non-browning Arctic® apples (Xu 2013). Moreover, phenylalanine ammonia lyase (EC 4.3.1.24) (PAL) is the first committed enzyme in the phenylpropanoid pathway, converting L-phenylalanine to *trans*-cinnamic acid (Hahlbrock and Scheel 1989). Subsequent reactions result in the production of some cinnamates (caffeoyltartaric acid and dicaffeoyltartaric acid) and quinic acid derivatives (5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid), which are associated with increased browning in lettuce (Tomás-Barberán et al. 1997). Some chemicals such as calcium

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chloride and 2,4-dichlorophenoxyacetic acid (2,4-D) have been studied for reduction of biosynthesis of phenolic compounds by inhibiting PAL activity to control tissue browning in lettuce (Tomás-Barberán et al. 1997). Application of some reductants such as L-cysteine or ascorbic acid can lead to tissue necrosis reduction (Enriquez-Obregon et al. 1999; Olhoft and Somers 2001). Though different adsorbents and antioxidants have been used in the culture media to mitigate tissue browning (necrosis), they may have significant negative effect on callus induction, *Agrobacterium*-mediated transformation and *vir* gene expression (Rana et al. 2016). The effective inhibition of PAL activity and phenolic compound production using the phenylalanine structural analog 2-aminoindane-2-phosphonic acid (AIP) is documented in a variety of plant systems (Teklemariam and Blake 2004; Nybakken et al. 2007; Oh et al. 2009). However, effects of AIP application on tea leaf explant browning is quite uncertain because of the high levels of polyphenols in tea leaves, which suggest active phenylpropanoid pathways in tea. It is interesting to find out whether AIP application can efficiently inhibit PAL-related tissue browning in tea tissue culture.

In the establishment of plant cells or tissues on artificial media using in vitro technique, fungal and bacterial, and endophyte contamination of explants can be a major barrier. The presence of bacterial endophytes in the pith, within sclerenchyma, in xylem lumen and parenchyma tissues causes plant culture losses and affects the micropropagation efficiency. In strawberry, multiple microorganisms such as *Pseudomonads*, *Xanthomonads*, enterobacteria and some other unidentified bacteria are present in plant tissues as endophytes and combination of antibiotics timentin, streptomycin sulfate, and gentamycin can effectively eliminate them (Tanprasert and Reed 1997). Wang et al. (2016) reported recently a group of 24 endophytic bacteria belongs to six groups such as *Acinetobacter*, *Burkholderia*, *Geobacillus*, *Herbaspirillum*, *Microbacterium*, and *Staphylococcus* are present in *C. sinensis* cv. “Zijuan” and “Yunkang 10”. It would be interesting to try to eliminate endophytes specifically associated with tea plants using antibiotics. Thus, in the present study an attempt has been made to evaluate role of antibiotics in controlling bacterial endophytes and AIP in reduction of tissue browning in callus cultures of *C. sinensis* var. *sinensis*.

Materials and methods

Plant material

The experiment was carried out using seeds of 8-year-old healthy tea plants (*Camellia sinensis* var. *sinensis*) cv. “Nong

Kangzao” obtained from the experimental tea farm of Anhui Agricultural University, Hefei, China.

Preparation of explants

Compared to leaf and stem explants excised from the field-grown plants of Chinese tea varieties, seeds can be easily sterilized without significant browning using the following surface sterilization protocol. Fresh seeds were washed with tap water; the outer coat was removed and soaked overnight. Using a strainer, the “floaters” and “sinkers” were separated. “Sinkers” were used as primary batch for the study. The seeds were transferred into a sterile 500-mL conical flask and washed twice with water and soaked in 4% bavistin overnight. The bavistin-treated seeds were rinsed with sterile distilled water and surface sterilized using 70% ethanol for 3 min followed by 0.1% mercuric chloride for 4 min and thrice rinsed with sterile distilled water. Surface sterilized seeds were blot-dried with Whatman No. 1 filter paper and the seed shells were removed under aseptic condition using sterile knife and pincette. The seeds were then placed on half strength MS medium (Murashige and Skoog 1962) to raise in vitro seedlings. The shoots generated from the seedlings were multiplied on half strength MS medium with 2 mg/L of 6-benzylaminopurine (BAP) and 0.1 mg/L of indole-3-butyric acid (IBA) according to Sarathchandra et al. (1988) (Fig. 1a, b).

In vitro callus induction

The young leaves were collected from the in vitro shoots and transferred on Gamborg B5 (B5) medium (Gamborg et al. 1968) with cytokinins for callus induction. Different concentrations of BAP (1, 1.5, 2.0, 2.5 mg/L) and thidiazuron (TDZ) 0.1, 0.5, 1.0, 2.5 mg/L, 10% coconut water, 300 mg/L L-glutamine were supplemented in B5 medium with a pH of 5.8 to study their role in controlling tissue browning. In addition, 2 µM of AIP was supplemented in the medium based on a thorough standardization of various concentrations of AIP (0.5, 1.0, 1.5, 2, 2.5 and 3.0 µM) for in vitro tea culture. Different concentrations of timentin (50, 100, 150 mg/L) and gentamycin (10, 20, 30 mg/L) were used to evaluate their effect on bacterial endophytes with a duration of 12 and 24 days. The hormones, coconut water, and antibiotics were filter sterilized and added after autoclaving the media components at 121 °C, 15 lbs for 20 min. After 3 months the actively growing callus tissues were sub-cultured and maintained in the dark to carry out further studies.

HPLC analysis

The polyphenols and caffeine were extracted from tea calli using a published protocol (Ashihara et al. 2010).

Fig. 1 Callus induction of *C. sinensis* var. *sinensis* grown on B5 medium supplemented with cytokinins, antibiotics and AIP. **a** In vitro-grown seedlings on $\frac{1}{2}$ MS medium after 6–8 weeks. **b** Shoot multiplication on $\frac{1}{2}$ MS medium with 2 mg/L BAP and 0.1 mg/L IBA after 5 weeks. **c, d** Leaf explants on antibiotic-free medium showing different types of bacterial endophytes after 6–10 days of culture initiation. **e** Leaf explants showing absence of bacterial endophytes on

B5 medium with 2 mg/L of BAP, 1 mg/L TDZ, 10% coconut water, 300 mg/L L-glutamine, 150 mg/L of timentin and 30 mg/L gentamycin after 12 days. **f** Callus showing oxidative browning on B5 medium supplemented with 2 mg/L of BAP, 1 mg/L TDZ, 10% coconut water and 300 mg/L L-glutamine after 12 weeks. **g** Creamy white callus cultured on B5 medium supplemented with 2 μ M AIP, 2 mg/L of BAP, 1 mg/L TDZ, 10% coconut water and 300 mg/L L-glutamine

Briefly, about 200 mg of calli were milled into fine powder in liquid nitrogen and extracted with 10 mL of 80% (v/v) aqueous MeOH containing 20 mM sodium diethyldithiocarbamate. Supernatants from two-round extraction after centrifuge (12,000g for 20 min) were pooled together and reduced to dryness in vacuo, before being dissolved in 2 mL of distilled water. Chemical quantification was performed as outlined by Kerio et al. (2013) using an HPLC (600 Controller, 2489 UV/Visible Detector; Waters, Milford, MA) equipped with a C18 column (5 μ m $\times$ 4.6 mm $\times$ 250 mm; Phenomenex). Solvents A (ethanoic acid:water, 49.9:0.1) and B (acetonitrile) were run in linear gradients and eluted compounds were identified by comparing their retention times and absorption spectra with those of authentic standards, including caffeine from Winherb Medical Technology Co. (Shanghai, China), catechins (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-gallate (EGCG), all from Ruifensi Biotechnol. (Chengdu, China). The identified compounds were quantified using calibration curves determined from the standards according to Han et al. (2016). All the experiments were carried out in triplicate and the data were subjected to analysis using SPSS 17.0 statistical package.

Results and discussion

In the present study, the effects of timentin and gentamycin at different concentrations on bacterial endophytes were assessed at different time intervals (12 and 24 days). Our results indicated that 150 mg/L of timentin and 30 mg/L of gentamycin was found effective to eliminate bacterial endophytes up to 24 days of observation. After 24 days, antibiotics were removed and no bacterial endophytes were observed in the culture medium. In general, the leaf explants and callus tissues grown on B5 medium with lower concentrations of antibiotic still had bacterial endophytic contamination up to 24 days. As indicated in Table 1, the percentage of callus contaminated with bacterial endophytes were 24.4 and 8.3% when the concentration of timentin and gentamycin in the B5 medium was at 50, 10 mg/L and 100, 20 mg/L, respectively (Fig. 1c–e).

Moreover, based on our results for endophyte eliminations, different combinations of cytokinin in the B5 medium were further tested. Among the different concentrations of cytokinins, the percentage of callus induction on B5 media supplemented with 2 mg/L BAP, 1 mg/L TDZ, 10% coconut water (Sigma, USA), and 300 mg/L

Table 1 Elimination of bacterial endophytes from the callus cultures of *C. sinensis* var. *sinensis* by different concentrations of gentamycin and timentin

Antibiotics (mg/L)		Percentage (%) of callus contaminated with bacterial endophytes	
Timentin	Gentamycin	12-days*	24-days*
50	10	20.6a	24.4a
100	20	14.1a	8.3a
150	30	0b	0b

Each treatment was replicated three times and consisted of 30–90 friable calli

*Data separated by different letters represents statistical difference (at 0.05) within the same column using Duncan's multiple range test

L-glutamine was 1.2-fold higher than that in the medium containing 1.5 mg/L BAP and 0.5 mg/L TDZ (Table 2). For further multiplication of friable calli, the combination of 2 mg/L BAP and 1 mg/L TDZ was found to be optimum for callus growth compared to other cytokinin treatments (Table 2).

The effect of 2 μ M of AIP on callus polyphenol production was evaluated by HPLC. The abundances of four components of polyphenols (EGCG, epigallocatechin gallate; EC, epicatechin; C, catechin; EGC, epigallocatechin) and caffeine (CAF) in the treated samples were found to be varied when compared to control (with coconut water, L-glutamine and without AIP; Fig. 1f). A significant decline of EGC (28.6–4.42 mg/g) was observed in the AIP-treated samples, whereas the abundances of C, EGCG, EC and CAF were not significantly reduced (Figs. 1g, 2). HPLC chromatograms of the extracts from the calli treated and non-treated with AIP exhibited differential changes in the tested metabolites (Fig. 3b, c).

In this study, callus induction from tea leaf explants was studied to evaluate the effect of different medium components on tissue browning. In plant tissue culture oxidative browning is a common problem leading to reduced growth (Krishna et al. 2008), genetic transformation recalcitrance (Parthasarathy et al. 2007), and even plant death (Panaia et al. 2000). Tissue browning varies among

Fig. 2 HPLC analysis of AIP-treated callus of *C. sinensis* var. *sinensis* showing significant reduction in EGC as compared to other polyphenols and caffeine. EGCG epigallocatechin gallate, EC epicatechin, C catechin, EGC epigallocatechin, CAF caffeine

different species and cultivars. The cause of oxidative tissue browning has been extensively studied in plant tissue culture. Beckman (2000) reported that the accumulation of phenolic compounds in culture media and specialized tissues is the cause of tissue browning. Ke and Saltveit (1989) and Tomás-Barberán et al. (1997) observed that the phenols from the cut surface or wounding of explants and its altered metabolism can result in the increased synthesis of major phenolic compounds in green tea, which includes epigallocatechin gallate, epigallocatechin, epicatechin, and epicatechin gallate. In order to control browning, manipulation of tissue culture medium components with different cytokinins and antioxidants were found to be effective. In the current study the maximum callus induction rate (72.5%) was obtained from the leaf explants by supplementing B5 medium with 2 mg/L BAP, 1 mg/L TDZ. The stimulatory effect of TDZ at 5, 10 and 15 μ M on callus induction has been achieved from all the explants of tea resulting to 90–99% in combination of different concentrations (5, 10 and 15 μ M) of 2,4-D or IBA (Mondal et al. 1998). Similarly, a combination of BAP and TDZ (0.1–2.0 mg/L) was found effective in callus induction of *Lycopersicon esculentum* Mill, cv. Omdurman (Osman

Table 2 Effect of BAP and TDZ on in vitro callus induction from leaf explants of *C. sinensis* var. *sinensis* on Gamborg B5 medium after 12 weeks

Growth regulators	Conc. of cytokinin (mg/L)	% of callus induction	Fresh weight of callus mean $\pm$ SE (in mg)
BAP+TDZ	1.0+0.1	45.6 $\pm$ 0.32	32.5 $\pm$ 0.83
	1.5+0.5	58.1 $\pm$ 0.46	41.3 $\pm$ 0.01
	2.0+1.0	72.5 $\pm$ 0.29*	67.6 $\pm$ 0.40*
	2.5+2.5	45.1 $\pm$ 0.80	35.6 $\pm$ 0.73

*Values in bold are significantly different from other cytokinin treatments ($P < 0.05$)

Fig. 3 HPLC chromatograms of a mixture of authentic compounds (**a**), extracts of the calli treated (**b**) and not treated with AIP (**c**). Peak 1, EGC; peak 2, caffeine; peak 3, catechins; peak 4, EC; peak 5, EGCG; peak 6, gallic acid (GC); peak 7, gallic acid gallate (GCG)

et al. 2010). Forrest (1969) reported that the slow growth of tea callus might be due to the deficiency of nitrogen and found that callus growth can be promoted by supplementing coconut water in medium. Further studies showed that addition of coconut water to the co-cultivation medium improves *Indica* rice callus survival rate (Karthikeyan et al. 2011). The findings of our present study are in harmony with earlier reports on the role of coconut water on callus growth. The role of L-glutamine in controlling tissue browning is well documented. Kumar et al. (2013) proposed that amino acids can react with quinones to form amino catechins, consequently inactivating the quinones

and oxidized products of polyphenols. They also observed that 0.5 mg/L of L-glutamine was effective in reducing tissue browning (Kumar et al. 2013). Thus, in our present study, 300 mg/L of L-glutamine was supplemented in callus generating medium and was found suitable in inhibiting tissue browning of calli.

The efficiency of micropropagation in plant tissue culture can be reduced by endophytic contamination. In general, antibiotics can be used to control bacterial endophytes. According to Tanprasert and Reed (1997), combination of timentin (1000 µg/mL), streptomycin sulfate (250 µg/mL) and gentamycin (25 µg/mL) was found to

be most effective against endophytes in strawberry. Our results revealed that timentin (150 mg/L) and gentamycin (30 mg/L) were able collectively to eliminate bacterial endophytes in tea leaves.

A novel approach to control tissue browning in tea using AIP has been achieved in this study. Jones and Saxena (2013) demonstrated that the supplementation of AIP in tissue culture medium prevented browning in *Artemisia annua*, sugar maple and American elm. Similarly, the abundance reduction for dicaffeoyltartaric and 5-caffeoylquinic acids, respectively, by 98 and 92%, in the cut midribs of iceberg lettuce has been reported due to the application of 50 μ M AIP (Peiser et al. 1998). Phenolic compounds are known being involved in the programmed cell death in plants responded to pathogen stress (Tamagnone et al. 1998). In addition, PAL is also involved in the production of salicylic acid (SA) (Verberne et al. 1999), which plays crucial roles in the activation of various plant defense responses such as host cell death and the containment of pathogen spread (Durner et al. 1997). To reduce synthesis of phenolic compounds in a variety of systems, AIP can be used both in vitro and in vivo as a competitive inhibitor of PAL (Appert et al. 2003; Bagheri et al. 2011). In this study, 2 μ M of AIP was found able to effectively inhibit callus browning, significantly reduce EGC abundance in the treated plant materials, and greatly improve callus induction and growth. But it was not able to reduce the abundances of other polyphenols, probably because the applied AIP might not be optimal to inhibit PAL activity. In addition, high levels of AIP were found not beneficial for callus growth.

Conclusions

In the present study, application of AIP and other medium supplements effectively inhibited tissue browning of leaf explants and significantly improved their callus induction and growth of *C. sinensis* var. *sinensis*. Our HPLC data indicate a significant reduction in EGC in the AIP-treated tea calli as compared to non-treated ones. Our study has demonstrated that incorporation of AIP and other necessary supplements in a regular plant growth medium allows practical in vitro manipulations of recalcitrant tea plants.

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Author contributions KA and LFS conducted the experiments and prepared the manuscript; SW provided critical suggestions over the study and finalized the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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