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Investigation of the interaction between abscisic acid (ABA) and excess benzyladenine (BA) on the formation of shoot in tissue culture of tea (*Camellia sinensis* L.)

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Abstract

The effect of abscisic acid (ABA) and excess benzyladenine (BA) on the formation of shoot from tea (*Camellia sinensis* L. *assamica* × *sinensis*) leaf was investigated in this research. Callus was formed and grew well when explants were cultured on LS basal medium supplemented with (in mg/L) thiamine-HCl, 1.25; pyridoxine-HCl, 0.625; nicotinic acid, 0.625; indole-3-acetic acid (IAA), 30; naphthaleneacetic acid (NAA), 30; kinetin, 0.1; myoinositol, 100; as well as 3% (w/v) sucrose. After two months, the calli were transferred to a modified B5 medium in order to regenerate plant. As a result, we observed root formation in the transferred calli to B5 medium after one month. Subsequently, the calli were transferred to the aforesaid B5 medium supplemented with ABA (2 mg/L) and excess BA (400 mg/L) to form shoot. The calli turned green and showed differentiation of globular and heart embryos when transferred to the modified B5 medium, without formation of shoot. These findings showed that the applied concentration of ABA may cause inhibition of conversion of globular and heart embryos to shoot. The increased level of BA, however, was not able to ameliorate the effect of ABA.

Keywords: Abscisic acid (ABA); Benzyladenine (BA); B5 medium; Shoot formation; Tea plant

Introduction

Conventional tea breeding is well established, though time-consuming and labors-intensive due to its perennial nature and long gestation period (4-5 years). Additionally, tea breeding has been slowed by lack of reliable selection criteria. Vegetative propagation is standard, yet limited by slow multiplication rate, poor survivability of some clones, and need for copious initial planting material (Tahardi et al., 2003). Seed-borne plants are heterogeneous due to their highly allogamous nature; consequently, it is difficult to maintain their superior character. Research on tea micro-propagation has recently focused on exploring the potential of somatic embryogenesis as a more efficient means of plant manipulation and regeneration (Tahardi et al., 2003). Somatic embryos have been used as a

model system to understand the mechanisms regulating plant embryogenesis, being an alternative for the propagation of plants with high rates of multiplication, with relevance in tree improvement programmes (Sghaier et al., 2009). To induce calli from tea explants and to cultivate the calli to produce plants, various kinds of media have been designed. Generally, it would be better to use only one or two kinds of basal media in combination of different kinds and concentrations of phytohormones (Misawa, 1994). The most suitable medium composition should be optimized afterwards in order to obtain higher level of products as well as higher growth rate. Plant hormones play essential roles in plant metabolism and can influence cell cycle proteins (Sánchez et al., 2005).

Tea (*Camellia sinensis* L.) is the oldest non-alcoholic caffeine-containing beverage crop in the world and health benefits attributed to tea consumption are well proven (Mondal, 2004). Tea is one of the most important plantation crops in the world. At present, the most parts of tea fields are planted with various clonal propagated cultivars. Fields performance of micro-propagated tea plants and the impact of cultural operations on growing tea plants have been reported previously (Marimuthu and Kumar, 2001). For these reasons, tea plants were introduced into tissue culture for plant regeneration and genetic manipulation. Unlike other crops, reports are not available on the basic physiology of micro-propagated tea plants (Marimuthu and Kumar, 2001).

Abscisic acid (ABA) is one of the five classical plant hormones that have been studied for its application for callus induction and regeneration. Because of its preventive effects on cell cycle, ABA causes decrease in callus induction and percentage of regeneration, as well as decrease of callus volume and callus fresh weight (Fazelienasab et al., 2004). Maturation of somatic embryos may be induced by the application of exogenous ABA (Corredoira et al., 2003). This hormone plays an important role in both somatic and zygotic embryo maturation (Label and Lelu, 2000). Thus, it promotes embryo maturation, and supports the accumulation of storage proteins, lipids and starch, suppresses the formation of embryo structures, and prevents the precocious germination of mature embryos (Preeti et al., 2004).

On the contrary, benzyladenine (BA), a synthetic cytokinins, may regulate cell cycle and cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, and inhibit root formation (Taiz and Zeiger, 2003). The objective of the present research was to enhance the development of a culture method for improving the conversion of calli to tea plant via combination of both ABA and excess BA.

Materials and Methods

Tea (*Camellia sinensis* L. *assamica* × *sinensis*) leaves were obtained from 2-years-old rooted cuttings of tea plants grown in a green house, with a 16 h photoperiod and photosynthetic photon flux of 101.5 μmol m^{-2} S^{-1} (400-700 nm) at the plant level. Leaf samples were washed with tap water and surface sterilized in a drop of liquid detergent for 1 min, followed by three rinses in sterile distilled water. Then, they were re-sterilized with 70% ethanol for 30s and with 20% sodium hypochlorite for 20 min, followed by three rinses in sterile distilled water, all under laminar flow. Discs of ca. 0.5 cm^2 diameter were cut from the leaves and were cultured on LS (Linsmaier and Skoog, 1965) basal medium supplemented with (in mg/L) thiamine-HCl, 1.25; pyridoxine-HCl, 0.625; nicotinic acid, 0.625; indole-3-acetic acid (IAA), 30; naphthaleneacetic acid (NAA), 30; kinetin, 0.1;

myoinositol, 100; as well as 3% (w/v) sucrose. PH was adjusted to 5.8 before adding 0.8% (w/v) agar. Autoclaving was done for 20 min at 120 ˚C and 150 KPa (Fazelienasab et al., 2004). The cultures were maintained in dark at 27 ± 3 ˚C.

After approximately two weeks the calli were emerged on the aforesaid modified LS media and after 2 or 3 subcultures (with 3 weeks intervals), they were transferred to a B5 medium, here in after referred as "modified B5-1". This medium was B5 basal medium (Gamborg et al., 1968) supplemented with (in mg/L) thiamine-HCl, 10; pyridoxine-HCl, 1; nicotinic acid, 1; benzyl adenine (BA), 2.253; naphthaleneacetic acid (NAA), 18.621; myoinositol, 100 as well as 2% (w/v) sucrose. PH was adjusted to 5.5 before adding 0.8% (w/v) agar. Autoclaving was done for 20 min at 120 ˚C and 150 KPa. The viability of the cells was examined using Evans blue (1% w/v aqueous solution) (Morita et al., 2006) in different media and different stages. The cells were observed with an Olympus BH-2 light microscope (Olympus, Tokyo, Japan) equipped with a camera. After two months growing on aforesaid media (2 or 3 subcultures with 20 days intervals), the calli were transferred to another modified medium, which herein after is referred as "modified B5-2". This medium was B5 basal medium in which 2 mg/L ABA and 400 mg/L excess BA were added. Other than ABA which was added to the media after filter sterilization under laminar air flow, other hormones were added to the media before autoclaving them. The concentrations of ABA and BA were decided based on our preliminary studies as well as on previous studies (Sghaier et al., 2009; Xing et al., 2008; Preeti et al., 2004; Corredoira et al., 2003; Label and Lelu, 2000; Saito and Suzuki, 1999). PH was adjusted to 5.5 before adding 0.8% (w/v) agar. Subsequently, the cultures were maintained at 27 ± 3 ˚C with the light intensity of 17 µmol m^{-2} S^{-1} (400-700 nm) at the flask level. Histological studies were conducted on samples fixed with FAA (17.5 ml of EtOH 80%, 2 ml of formaldehyde 37%, and 0.5-1 ml acetic acid) for 18h, washed with water and kept in EtOH 70%. Thin (14-15 µM in diameter by microtome) or free hand sections were stained by nuclear Feulgen reaction and Brachet test (Clark, 1981), localizing DNA and RNA, respectively.

Results

As shown in Figure 1, initiation of callus occurred two weeks after culture on a modified LS medium. Initiation of callus was remarkable in 70% of explants. After 2 or 3 subcultures (with 3 weeks intervals), the viability of the cells was measured (91%) and they were transferred to modified B5-1 media for pushing the calli to differentiation. After about 10 days, the roots were regenerated and emerged in 20% of the calli (Figure 2). In this stage, the viability of the cells was the same as initial stage (Figure 3). As mentioned, the calli were then transferred to modified B5-2 media containing 2 mg/L ABA and 400 mg/L excess BA and were grown at 27 ± 3 ˚C with the light intensity of 17 µmol m^{-2} S^{-1} (400-700 nm) at the flask level. In this media, the calli were turned green (Figure 4. a) and further analysis of these calli confirmed that somatic embryogenesis (globular and heart) was induced (Figures 4. b and 4. c, Table 1). However, theses embryos did not differentiate to shoot; however, was not occurred. The observation of the stained sections of globular and heart embryos showed that they have precious potent for cell differentiation because of both longed nuclei and high concentrations of RNA (Figure 5).

Table 1. Effects of phytohormones on callus formation and somatic embryogenesis.

	Phytohormones (mg/L)		Callus formation rate (% ± S.E)	Embryo formation rate (% ± S.E)
	ABA	BA		
Modified LS	0	0	70 ± 1.0	6 ± 0.5
Modified B5-1	0	2.25	65 ± 0.5	8 ± 1.0
Modified B5-2	2	400.00	30 ± 0.5	20 ± 2.4

Rates were calculated from 30 explants (tea leaves derived calli) cultured on LS, B5 and renewed B5 media. This experiment was performed with 30 explants.

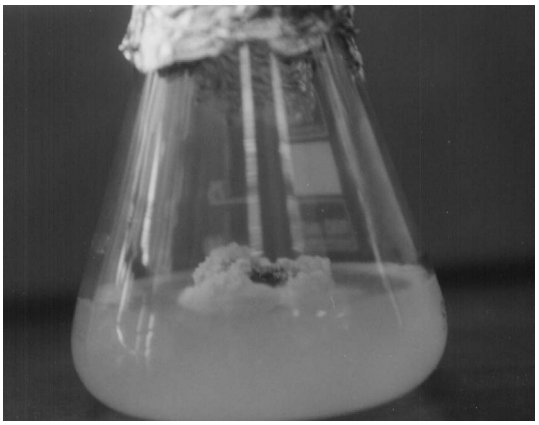

Figure 1. Initiation of callus on tea leaf explants after 2 weeks in a modified LS medium.

Figure 2. Formation of root after culture on a modified B5 medium. The roots are pointed by arrows.

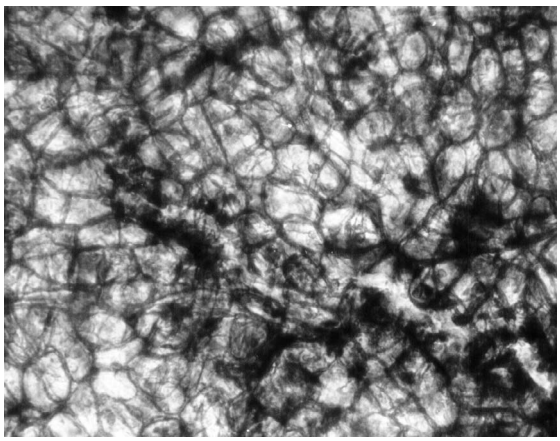

Figure 3. The viability of the cells using Evans blue (1% w/v aqueous solution) after culture on a modified B5 medium.

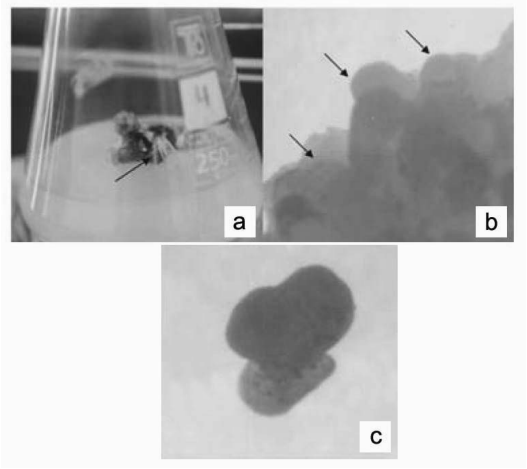

Figure 4. Differentiation of tea callus in a modified B5 medium containing 2 mg/L ABA and 400 mg/L BA. a: Greening of the calli in the presence of light intensity of 17 µmol m^{-2} S^{-1} (400-700 nm) at the flask level. The roots are pointed by arrows, b: globular embryos, c: heart embryos.

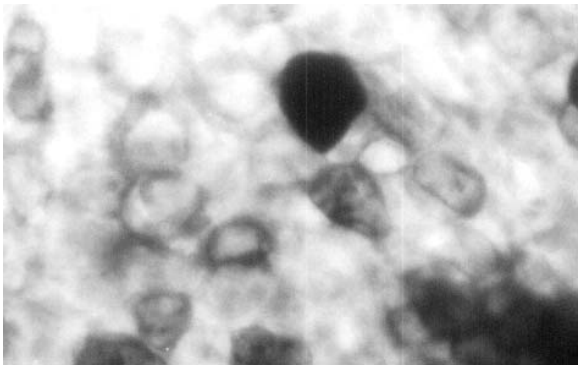

Figure 5. Stained sections of globular and heart embryos with Brachet test. Pink colored cells represent accumulation of RNA.

Discussion

The results of the present research confirmed that the formulation of basal LS (or MS) medium is likely to be the most suitable media for induction of callus in tea plant. The significant feature of this medium can be attributed to its very high concentration of nitrate, potassium, ammonia as well as sugar. Although inorganic nutrients of basal B5 is lower than that of LS (Gamborg et al., 1968), in the present study, regeneration of roots was occurred in modified B5 media.

Tahardi and his colleagues reported that a major problem for in vitro propagation of tea (*Camellia sinensis* L.) was poor conversion of somatic embryos into plantlets as they failed to complete normal stages of embryogenesis, generally common to zygotic embryos (Tahardi et al., 2003). They also mentioned that while endogenous level of free ABA was highest during maturation stage of zygotic embryos, treatment of somatic embryos, with exogenous ABA (0.5 mg/L for 14 days) could alleviate the problem of lack of reserve accumulation during maturation of somatic embryos, the major cause of poor and abnormal somatic embryo germination (Tahardi et al., 2003).

In the present study, growth on B5 medium containing ABA and excess BA was accompanied by greening the calli and differentiation of globular and heart embryos.

ABA is one of the five classical plant hormones that has shown to prevent callus induction phase in tissue culture of different plant species via inhibition of cell cycle in different stages i.e., G_1 to S and G_2 to M (Nadina et al., 2001). When it was used in tissue culture, ABA inhibits the growth of shoots and the germination of embryos. Since ABA can induce the expression of cyclin-dependent kinase inhibitors (ICKs) that bind and inhibit cyclin-dependent kinase A (Cdk-A) activity (Sánchez et al., 2005), perhaps it inhibits germination through inhibition of cell cycle process in G_1. Conversely, exposure to a high level of ABA (0.5 mg/L) during embryos developmental phase appeared to be beneficial in accelerating the development of torpedo and cotyledonary embryos in liquid culture and, subsequently in enhancing their plant conversion efficiency (Tahardi et al., 2003). It also acts as an inducer of somatic embryogenesis and maturation of these embryos in some plants such as carrot, coconut and date (Nadina et al., 2001; Fernando and Gamage, 2000).

Similarly, in our research, ABA acted as inducer of somatic embryogenesis (globular and heart), but without maturation of these embryos to shoot. It is in coincident with other literature (Kumari et al., 1998).

Progress through the cell cycle is regulated by distinct families of cyclin-dependent kinases (Cdks) whose mechanisms of function are conserved in plant cells (Sánchez et al., 2005). Cdks are the chief regulators in cell cycle and may be the target of ABA. Abscisic acid induces Cyclin-dependent kinase inhibitory protein (ICK) which inhibits Cdk-A, leading to a reduced rate of cell cycle progression. Abscisic acid also interacts with other classes of hormones such as auxin, cytokinin and gibberllin. The location of this interaction is in G2-M transition with induction of encoding of ICK proteins or prevention of expression of genes that are responsible for Cdk (Fazelienasab et al., 2004). Exogenous application of ABA that up-regulates ICK1 expression may lead to blockage of G1-S transition (Wang et al., 1998; Wang et al., 2000; Świstek et al., 2002).

In plant tissue cultures, cytokinin is required for callus growth (an undifferentiated, tumor-like mass of cells) and ratio of cytokinin to auxin is important to determine the fate of the callus. Moreover, cytokinin is known to promote the light-induced formation of chlorophyll and conversion of etioplasts to chloroplasts (Smart, 2008). This greening process was also observed in our experiments.

Benzyladenine (BA) as a cytokinin is generally used in plant cell culture at a concentration range of 0.1-10 mg/L. Cytokinins stimulate Cdk-A activity in the G_1-S transition and in the G_2-M phases, regulate cell cycle progression partly by inducing CycD3/1 transcription. When BA is added in appropriate concentrations, it may regulate cell cycle and cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA and protein synthesis and enzyme activity, delay senescence and promote nutrient uptake and cause greening process (Liu et al., 1997; Smart, 2008).

Interference between ABA and BA may be because of association and commonality of molecules and elements in their signaling pathways. In the present research, increased level of BA was not able to overcome the effect of ABA so that the conversion of globular and heart embryos to plants was arrested in these stages. So, further studies are required to elucidate the mechanism of interaction of these hormones leading to enhanced frequency of conversion of embryos to plants. Since optimization of media is essential for promotion of differentiation of embryo to plantlet, sequential manipulations of culture conditions should be achieved.

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