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Original Paper

Efficient embryogenesis in the callus of tea (*Camellia sinensis*) enhanced by the osmotic stress or antibiotics treatment

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Abstract In developing a technique for plantlet regeneration *in vitro* by somatic embryogenesis and adventitious bud formation, callus formation must be avoided to prevent somaclonal variation. However the somaclonal variation caused by callus formation is considered to be important because it potentially promotes diversity in the breeding material. In the present study, we developed an efficient method for somatic embryogenesis from callus derived from shoot apex of tea *Camellia sinensis* (L.) O. Kuntze. The addition of mannitol at 0.31 M improved somatic embryogenesis relative to that on medium without mannitol (25% and 7%, respectively). When the callus was cultured on medium with small amounts of hygromycin ($5-10\,mg\,l^{-1}$), the differentiation rate increased up to 43% that of control. Results suggested that culturing with osmotica or antibiotics was quite effective to induce somatic embryogenesis from vegetative-derived callus tissue of tea plants.

Key words: *Camellia sinensis*, osmotic pressure, phytohormones, somatic embryogenesis.

Tissue culture techniques for plant regeneration *in vitro* by somatic embryogenesis or adventitious bud formation have been improved for potential application to plant breeding. It is desirable to regenerate plants without formation of callus in order to avoid somaclonal variation. There are many reports on the successful induction of somatic embryogenesis and adventitious bud differentiation without callus formation, from nodal segment (Akula and Dodd 1998), mature seed (Akula et al. 2000b), cotyledons (Bano et al. 1991; Jha et al. 1992; Furukawa and Tanaka 2004; Kato 1986; Mondal et al. 2001a; Nakamura 1988; Ponsamuel et al. 1996; Wachira and Ogada 1995) or immature leaves of *in vitro* grown shoots (Kato 1996) of tea plants. From a different point of view, the somaclonal variation induced during callus formation is important because it is expected to promote diversity in the breeding material (Adkins et al. 1995; Arnold et al. 1995; Seliskar and Gallagher 2000). Although there have been several reports on the plantlet regeneration via bud formation from callus tissues (Kato 1985; Nakamura 1989; Nakamura 1990; Phukan and Mitra 1984; Sarwar 1985), the callus was not used as initial explants and the callus was formed adventitiously on the explant in these explanations. Besides, there has been no report on the induction of somatic embryogenesis from vegetative tissues via callus formation or from vegetative-tissue-derived callus in tea plants.

Recently several reports have been described the morphogenetic efficiency of osmoticum treatment (Alula et al. 2000b; Biahoua and Bonneau 1999; Kamada et al. 1993; Satoh et al. 2000; Tremblay and Tremblay 1995), or the addition of antibiotics to the medium in inducing plant regeneration *in vitro* (Nakano and Mii 1993; Rao et al. 1995; Torregrosa et al. 2000; Yepes and Aldwinckle 1994; Zhang et al. 2001).

I report here on the efficiency upon somatic embryogenesis from shoot apex-derived callus tissue of tea by addition of carbohydrates and antibiotics in preference to phytohormones.

Callus were derived from the shoot apex of "Surugawase cultivar" of *Camellia sinensis* on MS medium (pH 5.8) (Murashige and Skoog 1962), containing $3\,mg\,l^{-1}$ BA, 0.09 M sucrose and 0.3% gellan gum in 9 cm Petri dishes at 27°C for a 16-h photoperiod (3,000–4,000 lx. white light) for about 30 days. The proliferated callus was characterized as milk-white-colored and compact. They were then cultured on MS hormone-free medium for about eight weeks, and used for the experiments as shoot apex-derived callus.

Each callus was divided into about 5 mm in size and transferred as explant on MS medium containing 0.09 M sucrose with or without $0.5\,mg\,l^{-1}$ IBA and 0, 1, 5, or $10\,mg\,l^{-1}$ BA. These conditions were decided based on

Abbreviations: BA, benzyladenine; IBA, indolebutyric acid; MS, Murashige and Skoog; PEG, polyethylene glycol.

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

Phytohormones ($mg\,l^{-1}$)		Callus formation rate[a] (%±S.E.)	Embryo formation rate (%±S.E.)
IBA	BA		
0	0	75±4.5	6±0.5
0	1	80±5.6	11±1.1
0	5	60±4.7	8±0.7
0	10	55±7.2	0±0.0
0.5	0	50±3.7	8±0.7
0.5	1	35±8.6	5±0.5
0.5	5	25±1.8	0±0.0
0.5	10	25±2.5	3±0.3

Rates were calculated from 20 explants (shoot apex−derived callus) cultured on MS medium (pH 5.8) with IBA and BA, 0.09 M sucrose and 0.3% gellan gum in the dark at 27°C for 60 days. Each experiment was performed with 20 explants and repeated four times. Callus formation rate[a]: Rate of explant on which new callus secondarilly proliferated.

Table 2. Effects of mannitol on somatic embryogenesis.

Mannitol (M)	Survival rate (%±S.E.)	Callus formation rate[a] (%±S.E.)	Embryo formation rate (%±S.E.)
0	80±6.7	62±7.2	7±0.5
0.11	93±12.4	79±9.7	5±0.3
0.31	77±4.8	19±2.4	25±3.2
0.51	67±7.4	18±1.3	1±0.1
0.71	60±5.1	10±1.1	0±0.0

Rates were calculated from 20 explants (shoot apex−derived callus) cultured on MS medium (pH 5.8) with mannitol at various concentrations and 0.3% gellan gum in the dark at 27°C for 60 days. Each experiment was performed with 20 explants and repeated four times. Callus formation rate[a]: Rate of explant on which new callus secondarilly proliferated.

the previous reports (Kato 1986; Nakamura 1988). All plates were incubated at 27°C in the dark for 60 days. After incubation, the generation rate of somatic embryogenesis from callus was 11% at most (Table 1).

Each shoot-apex-derived callus was divided and incubated on phytohormone-free MS medium containing 0.09 M sucrose with mannitol at various concentrations of 0–0.71 M in the dark for 60 days to apply osmotic stress. An addition of mannitol at 0.31 M improved somatic embryogenesis predominantly relative to that on medium without mannitol (25% and 7%, respectively) (Table 2). Conventionally mannitol, sucrose, or polyethylene glycol (PEG) have been used as the osmotica for some plants (Biahoua and Bonneau 1999; Satoh et al. 2000). Recently, the effects of osmotica such as mannitol, sucrose, PEG, and betaine on plantlet regeneration have been reported for some plants. Betaine, in particular, is remarkably effective in inducing somatic embryogenesis from tea seeds (Akula et al. 2000b). In the present study using mannitol, a high level of osmoticum was effective in inducing somatic embryogenesis from the shoot apex-derived callus. I have yet to examine in detail the effects of the concentration and type of carbohydrate osmoticum upon somatic embryo differentiation from the tissue-derived callus.

Table 3. Effects of antibiotics on embryogenesis.

Antibiotics	($mg\,l^{-1}$)	Survival rate (%±S.E.)	Callus formation rate[a] (%±S.E.)	Embryo formation rate (%±S.E.)
Kanamycin	0	88±5.2	65±5.1	7±0.6
	25	85±9.1	80±6.8	11±0.9
	50	60±4.7	40±6.1	8±0.9
	125	75±11.3	50±5.9	12±1.1
	250	50±10.4	35±2.7	5±0.9
Hygromycin	0	85±7.2	78±8.1	4±0.3
	5	50±4.8	35±4.2	43±5.7
	10	44±7.1	18±2.7	22±1.8
	20	28±4.7	8±1.7	11±1.0
	40	11±1.7	0±0.0	0±0.0

Rates were calculated from 20 explants (shoot apex−derived callus) cultured on MS medium (pH 5.8) with kanamycin or hygromycin, 0.09 M sucrose and 0.3% gellan gum in the dark at 27°C for 60 days. Each experiment was performed with 20 explants and repeated four times. Callus formation rate[a]: Rate of explant on which new callus secondarilly proliferated.

Each shoot-apex-derived callus was divided and incubated on MS medium containing 0.09 M sucrose with 0–250 $mg\,l^{-1}$ kanamycin or 0–40 $mg\,l^{-1}$ hygromycin at 27°C in the dark for 60 days.

The effects of antibiotics on somatic embryogenesis from callus tissue are shown in Table 3 and Figure 1. When the callus was cultured on medium with small amounts of hygromycin (5–10 $mg\,l^{-1}$), the differentiation rate of somatic embryogenesis increased up to 43%. Recently there have been some reports describing the effect of antibiotics such as kanamycin, cefotaxime, or carbenicillin upon embryo differentiation from callus or adventitious roots (Nakano and Mii 1993; Yu et al. 2001; Zhang et al. 2001), and upon bud differentiation from callus (Rao et al. 1995) in cotton, sorghum, and woody species like apple, grapevine and papaya. In this study, two kinds of antibiotics i.e. kanamycin and hygromycin, generally used as selectable markers for gene transformation of tea (Aoshima et al. 2001; Matsumoto and Fukui 1998; Mondal et al. 2001b), were investigated. The addition of hygromycin showed remarkable effects on somatic embryogenesis from shoot apex-derived callus tissue. Generally hygromycin is considered to be more toxic to plants than kanamycin. Therefore, kanamycin has been used for plants rather than hygromycin, to minimize damage to the explants (Torregrosa et al. 2000). Also in tea, kanamycin has been generally used and the optimal dose for selecting transformed plant cells was elucidated in detail (Tosca et al. 1996; Matsumoto and Fukui 1998; Mondal et al. 2001b). In the present investigation, hygromycin was more effective in inducing somatic embryogenesis from

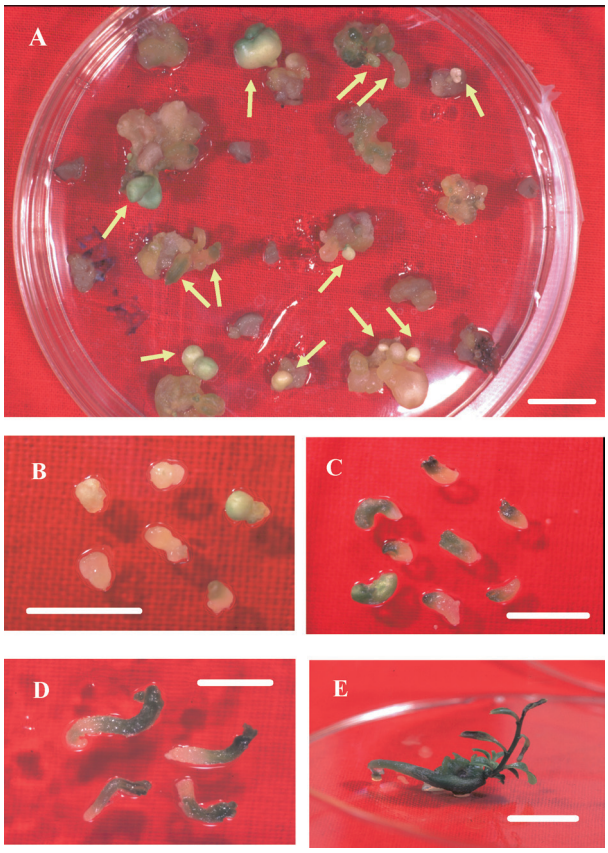

Figure 1. Plant regeneration from embryogenic cultures of tea (*Camellia sinensis*). Somatic embryos (arrows) were differentiated from callus on the medium with 5 mg l^{-1} hygromycin (A). Embryos were cultured on the MS medium with 0.01 mg l^{-1} IBA, 1 mg l^{-1} BA and 10 mg l^{-1} GA3 after 0 days (B), 8 days (C), 14 days (D), and 20 days (E), respectively. Bar = 1 cm.

shoot apex-derived callus. So in the physiological viewpoint, the influence of hygromycin should be evaluated on plant in detail. The somatic embryos formed in this experiment grew to plantlets with normal appearances by culture on the medium that has already been proved optimal for the growth of the segments of tea shoot *in vitro*: MS + 0.01 mg l^{-1} IBA + 1.0 mg l^{-1} BA + 10 mg l^{-1} gibberelline (pH 5.8) without antibiotics (Figure 1) (Kuranuki and Aono-Shibata 1993).

As mentioned above, addition of high osmotic stress or treatment with antibiotics was effective on somatic embryogenesis from shoot apex-derived callus of tea.

The system established here will be hopefully useful for tea breeding system involved in the somaclonal variation. The system would be applied more practically by optimizing the condition for maturation (Tremblay et al. 1995), germination (Mondal et al. 2002) of somatic embryo and secondary embryogenesis (Mondal et al. 2001a; Akula et al. 2000a) in tea.

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