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COMPARATIVE STUDIES ON SOMATIC EMBRYOGENESIS IN *CAMELLIA SINENSIS* VAR. *SINENSIS* AND *C. SINENSIS* VAR. *ASSAMICA* (MAST.) PIERRE.

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ABSTRACT

Excised cotyledons of China tea (Camellia sinensis *var.* sinensis) *and Assam tea* (C. sinensis *var.* assamica)*were cultured on modified MS basal medium containing 0.1-4 mg/l IBA and 0-10 mg/l BA and B_5 basal medium supplemented with 0.1-0.3 mg/l NAA and 1-5 mg/l 2iP. Somatic embryos were formed directly on the surface of the explants of both varieties without callus formation within 3 months after culturing. Excised cotyledons of China tea responded to all media tested but those of Assam tea did not. The percentage of cultured explants producing somatic embryos were higher but the average number of embryos produced per culture was lower for China tea than for Assam tea. The best medium to produce the highest percentages of cultures with somatic embryos and the largest number of somatic embryos produced per culture in both tea varieties was B_5 containing 0.1 mg/l NAA and 2 mg/l 2iP in which plantlet regeneration of China tea was observed. Somatic embryos of Assam tea did not develop to the plantlet stage upon successive subculturings but produced numerous secondary adventitious embryos under the same cultural conditions.*

(Abbreviations IBA-indolebutyric acid, NAA = naphthaleneacetic acid, BA = 6-benzyladenine, 2iP = 2-isopentenyladenine)

INTRODUCTION

Among the three economic beverage crops (tea, cocoa and coffee), only coffee has been progressively improved qualitatively and quantitatively through the application of tissue culture techniques.[1] Of tea only a few reports on tissue culture have been published. Multicelled pollen embryiod and callus formation have been produced from tea anther cultures.[2, 3] Roots have also been regenerated from anther calli but not buds.[4] The possibility of shoot regeneration from nodal explants and shoot tips has also been reported.[3, 5, 6] Many workers have induced callus formation from stem segments[3, 7, 8] and eventually root formation from such calli.[3] Buds and plantlets were also reported to regenerate from calli originating from the epidermal layer of stem segments.[7] Again, tea plantlets were found to differentiate from cotyledon-derived callus cultures[9] and proved to be new clones.[10] In cotyledon culture, Kato[11] obtained somatic embryos and plantlet regeneration induced from excised cotyledons of tea after 2 and 10 months in culture respectively. In these earlier reports of cotyledon culture, only cotyledons of Chinese jats were used in experiments. Although various botanical varieties of tea have been given from time to time as they all

intercross with ease, the cultivars may therefore be considered[12] under 2 main groups, *namely, China tea (Camellia sinensis* var. *sinensis)* and Assam tea *(C. sinensis* var. *assamica* (Mast.) Pierre). Genotypes differ in the ease with which somatic tissues can be regenerated *in vitro,*[10, 13-15] and this paper therefore deals with somatic embryogenesis in culture of cotyledons of Assam tea compared with that of China tea under the same culture conditions.

MATERIALS AND METHODS

The seeds of each tree of Assam tea and China tea were collected from a 40-year-old tea plantation at the Fang Horticultural Research Station, Chiang Mai. Healthy mature seeds were surface sterilized in 20% commercial bleach solution for 20 minutes and then washed thoroughly with ample sterile distilled water. Before the seed coats were removed, the seeds were dipped in 95% ethanol and flamed for 10 seconds. Two cotyledons of a seed were then cut into 4-8 pieces after excision of zygotic embryo and were used as the experimental explants.

The basal media used were modified MS (as described elsewhere[11]) and B_5.[16] The two media were supplemented with the same combinations of 0-5 mg/l NAA or IBA and 0-10 mg/l 2iP or BA respectively. Media were adjusted to pH 5.7-5.8 and solidified by 0.7% Difo bacto agar before autoclaving.

The cultures were incubated under a 12-hour photoperiod regime at 28° ± 1°C. Standard paraffin methods were employed for histological observation.

RESULTS

Among those combinations of plant growth regulators experimented, only 5 combinations of NAA and 2iP supplemented in B_5 and 4 combinations of IBA and BA in modified MS (Table 1) showed positive results. Most of the cultured cotyledonary explants gradually turned from white to dark green in colour within 2 weeks and gradually swelled up. Somatic embryos appeared directly on the surface of such cultured explants of both China tea (Fig. 1a) and Assam tea (Fig. 1b) after 3 months in culture bypassing callus formation. Histological observations of these somatic embryos (Fig. 2a-c) showed that they probably originated from the epidermal and sub-epidermal layers of the cultured explants as earlier described by Kato[11] and in other *Camellia* spp.[17, 18] The explant responses to the media in term of the ability to produce somatic embryo were recorded as the percentage of cultured explants producing somatic embryos and the average number of somatic embryos produced per cultured explant (as shown in Table 1). The regenerability of tea cotyledon was low[10, 11] and independent of the levels of growth regulator.[11] It was quite clear that the cotyledonary explants of both tea varieties responded better to B_5 containing NAA and 2iP than to modified MS supplemented with IBA and BA. The best medium for both China and Assam teas giving the highest responses of cultured explants was B_5 containing 0.1 mg/l NAA and 2 mg/l 2iP. B_5 with 0.1 mg/l NAA and 1 mg/l 2iP showed the greatest difference in the percentage of cultures producing somatic embryos between China tea (28%) and Assam tea 2%.

Comparing between the 2 varieties of tea, it was obvious that cotyledonary explants of var. *sinensis* responded to wider range of media tested (Table 1) than those of var. *assamica*. Although there were more explants of var. *sinensis* responding to each medium, less somatic embryos generally formed from each responsive explant than those of var. *assamica*. The highest number of somatic embryos per culture, 3 in var. *sinensis* and 13 in var. *assamica*, was observed again in the medium supplemented with 0.1 mg/l NAA and 2 mg/l 2iP. The growth of these embryos was slow similar to those previously described.[10, 11] About 2-3 months after initation, most of Assam somatic embryos swelled up around their cotyledonary portion assuming a trumpet-like structure (Fig.3a) while those of China tea generally showed normal development, many of which regenerated well-developed roots (Fig. 1a) which could be observed in the medium containing 0.1 mg/l NAA and 2 mg/l 2iP. Upon 2-3 monthly subculturings to the same freshly-prepared medium, only secondary adventitious embryos formed from the original embryos of Assam tea without differentiation of shoot or root. The colour of these embryos gradually turned from green to yellow or white after successive subculturings. In China tea, on the other hand, only a few of its embryos produced adventitious embryos but most of them increased in size. Many of these *sinensis* somatic embryos developed shoots without roots (Fig. 3b) and many developed shoots with roots (Fig. 3c) from which finally full plantlets were regenerated (Fig. 4) after 1 year of successive subculturings. Attempts to induce plantlet regeneration of Assam tea was performed by using various differentiating media including those described by Kato[11] with only limited success.

DISCUSSION

Cotyledon culture has been used as a means of micropropagation in many plant species.[19] Calli have been formed from cotyledonary explants and shoot buds regenerated for *Brassica* spp.[14], cucumber[15] and many tropical fruits.[20] Somatic embryos have also been reported to form directly on the surface of the explants without callus formation in the case of Douglas fir[21] and many of the *Camellia* spp. such as *C. reticulata*[17] and *C. chrysantha*.[18] In *C. japonica* and *C. sinensis* both callus formation and somatic embryogenesis were observed.[10, 11, 22]

Among Chinese jats, Wu *et al.*[10] obtained callus tissues from cultured cotyledons of var. Chyi-Men and Pyng-Shoei in MS medium containing 2-8 mg/l 2, 4-D, the growth regulator which was claimed to be responsible for callus formation. Ten tea plantlets were regenerated from such calli upon subculturing to differentiating medium. Kato,[11] in contrast, reported the production of somatic embryos from cotyledons of cv. Yabukita when cultured in modified MS medium supplemented with 0-4 mg/l IBA and 0-2 mg/l BA. In the present study, both var. *sinensis* and var. *assamica* also produced somatic embryos from cotyedon culture employing modified MS medium with 0.1-4 mg/l IBA and 0-10 mg/l BA or B_5 medium with 0.1-0.3 mg/l NAA and 1-5 mg/l 2iP. These results indicated that cotyledons of *C. sinensis* were capable of regeneration, depending on the environmental influences represented by growth media and hormonal interactions as described in *Brassica*.[14] The

genotypic determination also seemed to work as a control of regeneration to a certain extent when the environmental factors were appropriate. In B_5 medium containing 0.1 mg/l NAA and 1-5 mg/l 2iP, for examples, the responses of the 2 varieties differed greatly both in the percentage of responsive explants and the number of embryos per explant. The regenerability of *C. sinensis* was reported to be much lower than other *Camellia*spp.[8, 11] Kato[11] obtained only the average of 12% of responsive explants of cv. Yabukita and 17% was the highest when the culture medium involved was without growth hormone. In the same basal medium, var. *sinensis* produced only 6% of the average response and 10% was the highest and showed no response at all in the medium without growth hormone (unpublished data).

Intraspecific genotypic variations for the rates of somatic embryogenesis and differentiation were observed between China and Assam teas in this work. *Assamica* produced more embryos than *sinensis* did, especially in B_5 containing 0.1 mg/l NAA and 2 mg/l 2iP. But they did not differentiate to form shoots and roots while *sinensis* embryos did under the same cultural conditions. China tea was therefore more amenable to *in vitro* culture than Assam tea.

The results indicated that in cotyledon culture of *C. sinensis,* genotypic determination seemed to work under environmental influences. Since most tea has been planted from seed and, as the flowers are cross-pollinated, it is very heterogeneous. The variations of culture media and combination of growth regulators should be considered if the highest yield is to be obtained for any applicable use of cotyledon culture. The production of plantlets from such culture of tea was described[11] as a method for rapid, disease-free, clonal propagation of selected hybrid clones and some were proved to be new clones.[10] Further studies are required to define the cultural conditions for plantlet regeneration of Assam tea and a higher rate of plantlet production for both varieties of *C. sinensis.*

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บทคัดย่อ

เมื่อนำชิ้นส่วนใบเลี้ยงของชาจีน *(Camellia sinensis* var. *sinensis)* และชาอัสสัม *(C. sinensis* var. *assamica)* ไปเลี้ยงในอาหารพื้นฐาน MS สูตรปรับปรุง ผสม IBA0. 1-4 มก./ลิตร และ BA 0-10 มก./ลิตร และ B_5 ผสม NAA 0.1-0.3 มก./ลิตร และ 2iP 1-5 มก./ลิตร พบว่าหลังจากเพาะเลี้ยงไปได้ 3 เดือน เกิด somatic embryo ขึ้นบนผิวของใบเลี้ยงโดยตรงโดยไม่ผ่านแคลลัส เนื้อเยื่อใบเลี้ยงของชาจีนแสดงการตอบโต้ต่ออาหารทุกชนิดที่ศึกษา ส่วนของชาอัสสัมไม่ทุกชนิด จำนวนร้อยละของเนื้อเยื่อที่เพาะเลี้ยงของชาจีนที่ผลิต somatic embryo มีค่าสูงกว่าของชาอัสสัม แต่จำนวน somatic embryo โดยเฉลี่ยที่เกิดขึ้นบนผิวเนื้อเยื่อแต่ละชิ้นมีค่าต่ำกว่า อาหารที่ดีที่สุดที่สามารถผลิตเนื้อเยื่อเพาะเลี้ยงที่มี somatic embryo ได้ในจำนวนร้อยละที่สูงที่สุด และจำนวน somatic embryo ต่อเนื้อเยื่อแต่ละชิ้นมีค่ามากที่สุด ทั้งในชาจีนและชาอัสสัมคือ B_5 ผสม NAA 0.1 มก./ลิตร และ 2iP 2 มก./ลิตร ซึ่งเป็นอาหารที่ชักนำให้เกิดการพัฒนาของ somatic embryo ของชาจีนไปเป็นต้นชาที่สมบูรณ์ด้วย แต่ภายใต้สภาพการเพาะเลี้ยงเดียวกันนี้ somatic embryo ของชาอัสสัมกลับผลิต adventitious embryo จำนวนมากมายแทนที่จะพัฒนาไปเป็นต้นชาที่สมบูรณ์

TABLE 1 Formation of somatic embryos on cotyledonary explants of China tea *(C. sinensis* var. *sinensis)* and Assam tea *(C. sinensis* var, *assamica)* after 6 months in culture.

Basal medium	Growth regulators (mg/l)		Total explants cultured		% Cultures with embryos		av. no. embryos/ culture	
	NAA	2iP	*sinensis*	*assamica*	*sinensis*	*assamica*	*sinensis*	*assamica*
	0.1	1.0	32	40	28.1	2.5	1.4	3.3
	0.1	2.0	35	33	34.2	27.2	3.4	13.6
B_5	0.1	5.0	28	28	14.2	10.7	0.85	1.07
	0.2	2.0	25	25	4.0	0	0.24	0
	0.3	2.0	20	30	5.0	6.6	0.25	0.4
	IBA	BA						
	0.1	0	30	28	3.3	0	0.2	0
modified	1.0	2.0	20	25	10.0	8.0	0.6	1.28
MS	4.0	2.0	28	30	7.7	6.6	0.2	0.3
	5.0	10.0	20	30	5.0	6.6	0.1	0.2

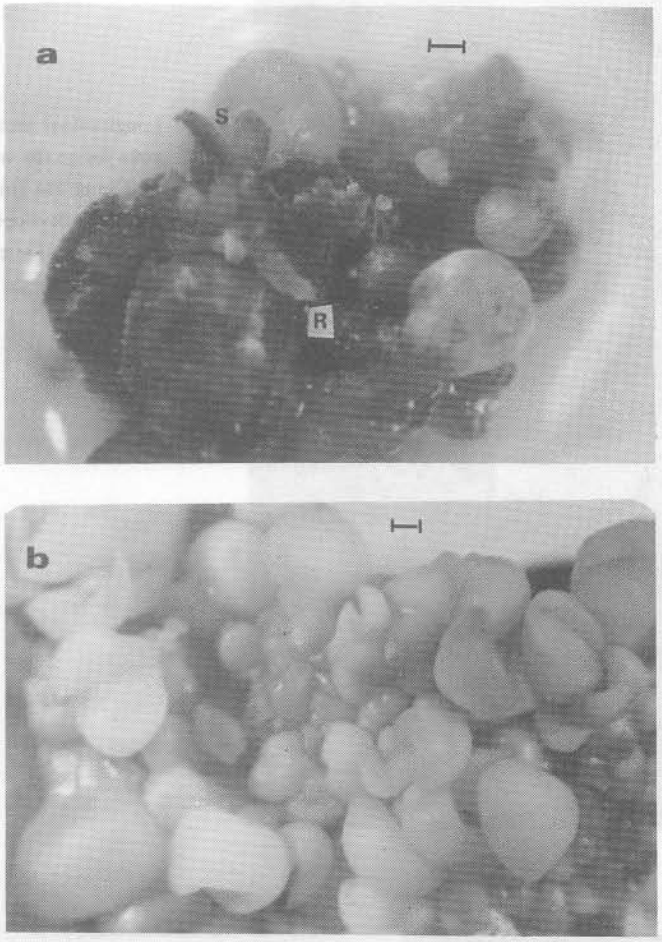

Fig. 1 Development of somatic embryos on excised cotyledons of China tea (a) and Assam tea (b) cultured on B_5 containing 0.1 mg/l NAA and 2 mg/l 2iP (S=shoot, R=root) (bar=1 mm)

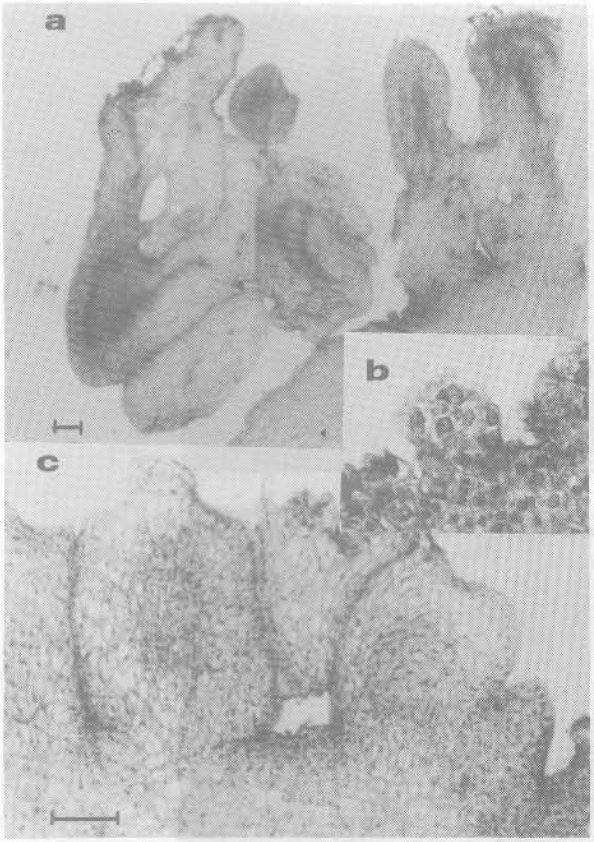

Fig. 2 Longitudinal sections of regenerating embryos on the surface of the explants of China tea (a) showing a primordium of a developing embryo (b) and of Assam tea (c) after 5 months in culture (bar = .1 mm)

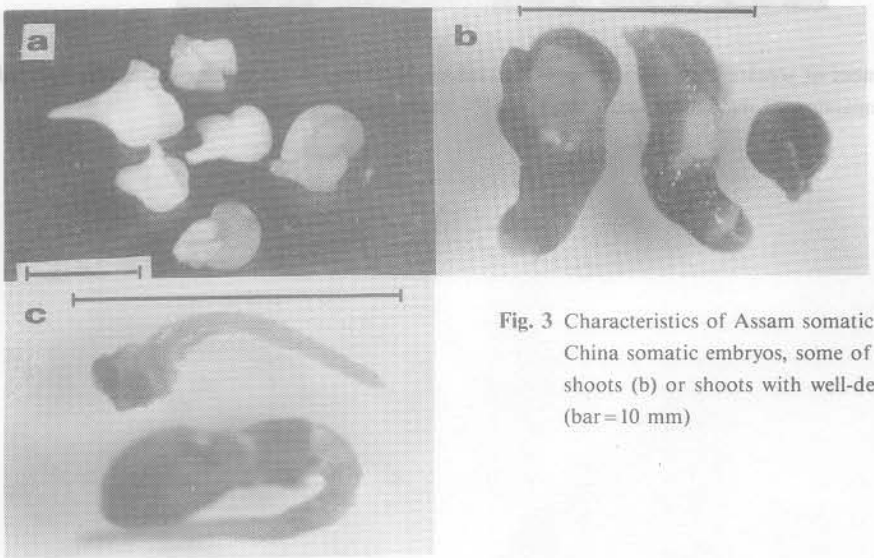

Fig. 3 Characteristics of Assam somatic embryos (a) and China somatic embryos, some of which developed shoots (b) or shoots with well-developed roots (c) (bar = 10 mm)

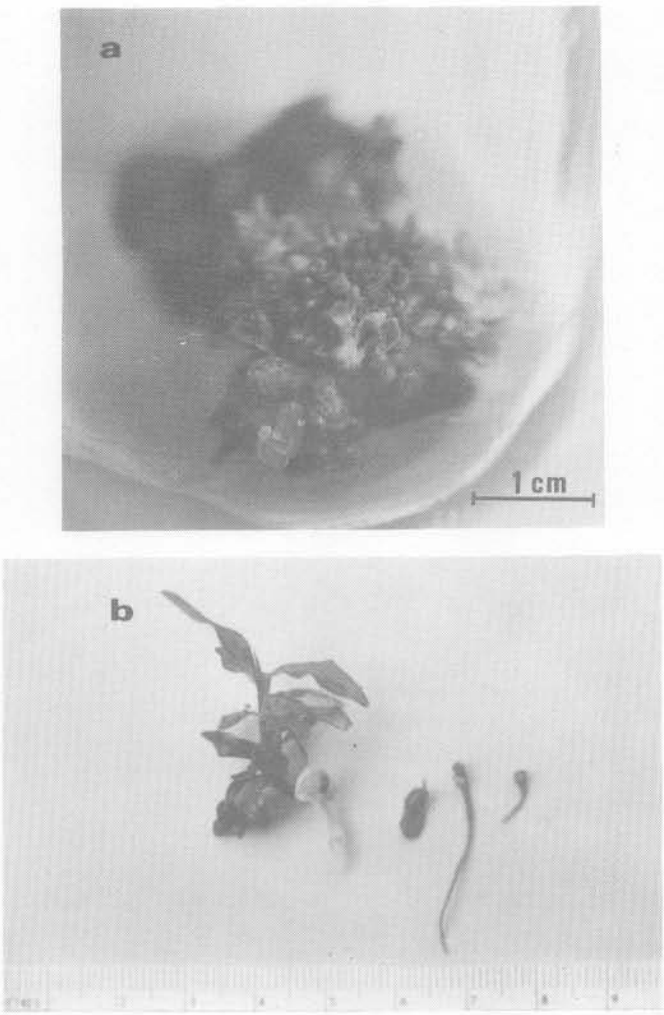

Fig. 4 Plantlet regeneration from a cotyledon of *C. sinensis* var. *sinensis*. (a) Shoots in 7-month culture. (b) Stages of development of somatic embryos to full plantlet during 1-year's culturing.