Bot. Bull. Academia Sinica **26**: 1-12 (1985)

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION ON *SASSAFRAS RANDAIENSE* (HAY.) REHD[1,2]

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(Received May 5, 1984; Accepted December 6, 1984)

Abstract

Callus was induced and plantlets were regenerated by culturing excised seed embryos of *Sassafras randaiense* (Hay.) Rehd on 1/2 strength of Murashige and Skoog's (MS) liquid media containing 2,4-dichlorophenoxy acetic acid (2,4-D) or α-naphthalene acetic acid (NAA). The highest percentage of embryoid formation was obtained after 8 to 12 weeks on 5×10^{-5}M 2,4-D via calli derived from seed embryos. Multiforms of somatic embryos were observed. 6-Benzyladenine (6-BA) did not stimulate rates of callus induction and embryogenesis. The somatic embryos developed into rosette form, after being transferred from liquid to solid medium. The rosette would develop elongated weak terminal bud when gibberellic acid (GA_3) was added to the medium. Eight percentage of the somatic embryos rooted and developed into normal plantlets after being transferred to liquid medium having no growth regulator. The rooted plantlets could not survive after being transplanted to soil containing pots because of unknown reasons. The results revealed that embryogenesis of seed embryo calli of *Sassafras randaiense* would provide a feasible way for propagation *in vitro* because the *Sassafras randaiense* is difficult to propagate by seed and asexual propagation.

Key words: *Sassafras randaiense* (Hay.) Rehd; tissue culture; embryo culture; embryogenesis; somatic embryos.

Introduction

Sassafras randaiense (Hay.) Rehd (ie. Taiwan sassafras), of Lauraceae family, is a tree angiosperm. It spreads around 1100–2000 m elevation of northern Taiwan and the Central Mountains (Hsien and Yang, 1969). *Sassafras* has recently become an important native forest tree in Taiwan because of heavy logging (Ku, 1977 a). However, owing to a number of reasons (Ku, 1979 a), it is difficult to obtain a

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[1] Paper No. 283 of the Scientific Journal Series, Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China.

[2] This Work was supported by the National Science Council Grant NSC-72-0204-B001-01, Republic of China.

satisfied demand of seeds (Ku, 1977 a). Besides, mass *Sassafras* propagation from seeds becomes more difficult because of low germination and asexual propagation rate (Ku, 1977 a, b). Therefore, a more feasible way is *in vitro* propagation via embryo culture for *Sassafras* breeding.

During the last decade, many attempts (Banga, 1978) have been made to improve the breeding of forest trees with tissue or organ culture *in vitro*. Excised embryos have frequently been used as the explant source for adventitious bud production for several species of gymnosperms (Sommer *et al.*, 1975; Cheng, 1975; Webb and Street, 1977; Winton and Verhagen, 1977; Arnold and Eriksson, 1978, 1980; Arnold, 1982). Comparing with organogenesis, embryogenesis can provide a rapid propagation especially for woody species which need long breeding period. The first report about the induction of embryoid formation from embryo culture of woody gymnosperm was *Biota orientalis* (Konar and Obero, 1965), and that of angiosperm was *Santalum album* L. (Rao, 1965). References of embryo culture on angiosperm were rather few. Two reports on the embryoid induction of woody angiosperm by embryo culture were found on *Santalum album* L. (Rao, 1965) and *Ilex aquifolium* (Hu and Sussex, 1971). This paper reports the success of embryogenesis case via embryo culture on *Sassafras randaiense* (Hay.).

Materials and Methods

Seeds of *Sassafras randaiense* were collected from the trees planted in the Taiping mountain of Taiwan in 1981. The seeds were cold stored (4°C) in a low humidity (45%) chamber. After being surface sterilized in 75% ethyl alcohol for 30 seconds and 0.5% sodium hypochlorite solution containing 1% (V/V) Tween-20 for 20 min, the seeds were washed with three changes of sterile distilled water and soaked overnight in sterile water at room temperature. Brown seed coats were removed and embryos (ca. 1 mm in length) excised and immediately inoculated into the liquid embryo culture medium under sterile conditions.

A modified MS (Murashige and Skoog, 1962) medium (half-strength of macroelements and microelements) was used as basal medium (Table 1). Factors tested in various experiments were concentrations of 6-Benzyladenine (0, 10^{-7} and 10^{-5} M) and types of auxins (2,4-dichlorophenoxy acetic acid (2,4-D) and α-naphthalene acetic acid (NAA)) at different concentrations (2,4-D (5×10^{-7}, 5×10^{-6} and 5×10^{-5} M) and NAA (5×10^{-6}, 5×10^{-5} and 5×10^{-4} M)). One isolated embryo was placed in each test tube (1.7×17 cm, containing 5 ml liquid medium). Cultures were incubated at 25°C with a 16 hrs photoperiod of 2,000 lux under fluorescent light on a rotary shaker at 150 rpm.

After two-month embryo culturing, the regenerated embryoids were transferred to solid and liquid modified MS medium (Table 1) supplemented with different

Table 1. *Composition of the basal medium for* ***Sassafras randaiense*** *embryo culture*

Constituent	Concentration	
	g/l	mM
Inorganic macroelements		
NH_4NO_3	0.83	20.6
KNO_3	0.95	9.4
KH_2PO_4	0.09	0.63
$M_gSO_4 \cdot 7H_2O$	0.19	0.8
$C_aCl_2 \cdot 2H_2O$	0.22	1.5
Inorganic microelements		
$M_nSO \cdot 4H_2O$	11.2	50.0
H_3BO_3	3.1	50.0
$Z_nSO_4 \cdot 7H_2O$	5.3	15.0
KI	0.42	2.5
$N_{a2}M_0O_4 \cdot 2H_2O$	0.13	0.5
$C_uSO_4 \cdot 5H_2O$	0.13	0.05
$C_oCl_2 \cdot 6H_2O$	0.013	0.05
N_{a2}-EDTA	37.3	200.0
$F_eSO_4 \cdot 7H_2O$	27.8	100.0
Vitamins		
Thiamine•HCl	0.1	0.3
myo-inositol	100.0	555.0
Sucrose	30 g/l	
PH	5.8	

concentrations of gibberellic acid (GA_3) (0, 2, 5 and 10 ppm). The GA_3 was added to the medium by millipore filter when the media were autoclaved and cooled to 50°C. Solid medium was added to half of the 100-ml Erlenmeyer flasks in each treatment. The conditions of temperature and light were the same as mentioned above. After another one month of culture, the effect of GA_3 on the germination of regenerated embryoids derived from embryo culture was examined.

The rooted plantlets were washed with tap water and transplanted in 50% vermiculite and 50% sand mixture at 25°C. The planted embryos were grown under fluorescent lamp of 4,000 lux, and 12 hrs photoperiod in a growth chamber. The humidity was kept high with plastic bags on the pots and watering rather regularly.

Result and Discussion

Our earlier investigations on *in vitro* propagation of *Sassafras*, only a low

percentage shoots harvested from multiple-bud-masses could rooted and successfully developed into whole plants from 5 year-old grafted Taiwan sassafras (Wang, 1980; Wang, 1984; Hu and Wang, 1984). In the present paper we now report the success of embryogenesis of the *Sassafras randaiense* (Hay.).

As it was identified (Wang, unpublished data) that some inhibitors existed in the seed coat and cotyledons of *Sassafras* seed. The chemical inhibited seed germination. So the embryo isolated from cotyledons was cultured. Normally germination of *Sassafras* embryos was observed in hormone-free medium after one month culturing (Fig. 1). The result could be explained as the removing of inhibitory substances in cotyledons.

Embryogenic Callus Induction and Somatic Embryogenesis of Sassafras

The effects of 2,4-D, NAA and 6-BA on embryogenesis of embryo culture were observed. Most embryo culture (Arnold, 1978; Arnold *et al.*, 1980; Winton *et al.*, 1976), the formation of multiple shoots and embryoids of woody plants have been induced through embryos on cytokinin media. We found that auxin was essential for the callus induction and embryogenesis of *Sassafras*. At low auxin concentration, 5×10^{-7} M 2,4-D or 5×10^{-6} M NAA, embryo root development was inhibited. The radicle axis swelled or elongated and might form calli (Figs. 2 and 3). Calli were derived from embryonal axis and embryoid derived from callus on 2,4-D and NAA media. Medium with 2,4-D induced more calli and regenerated more embryos than that with NAA (Tables 2 and 3). For embryogenic callus induction, 2,4-D is more effective than NAA. The embryoids developed directly from the cotyledons of cultured embryos in *Ilex* (Hu *et al.*, 1971, 1978) and *Biota orientalis* (Konar and Oberoi, 1965). But the embryoids of *Sassafras* and *Santalum album* (Rao, 1965) were not regenerated directly from cultured embryos. They were found to regenerate from the calli induced from embryos.

The highest frequency of somatic embryo regeneration (30% on the average) was obtained at 1/2 strength MS medium containing 5×10^{-5} M 2,4-D (Table 2). Embryogenic callus appeared hard yellowish initiated after 6–8 weeks on embryo culture (Figs. 4 and 5). The compact embryo-like structure (small globular protuberance) was visible on the embryogenic callus (Figs. 6 and 7). These induced embryoids were snow-white, hard and easily seperated from the parent callus (Fig. 8). Approximately 12 weeks on 2,4-D 5×10^{-5} M medium, several accessory embryos redifferentiated from the callus. The number of somatic embryos on each embryo-derived callus varied from 2 to 35, with an average of 10 per embryo.

Multiforms of somatic embryos were observed. Some somatic embryos were produced in tight clusters arising from the embryogenic callus (Fig. 9). Some have suspensor-like structure (Figs. 10 and 11), and the others (Fig. 12) develop directly on the callus without a constricted rootpole as in *Ilex* (Hu, 1971). Some embryoids

have cup-like cotyledons (Fig. 13), and some with various shapes of fused cotyledons (Figs. 11, 14 and 15) or multiple cotyledons (Fig. 16). Similar response of multi-cotyledons were also found in *Ilex* somatic embryos (Hu, 1971).

6-BA was reported to induce embryos of *Picea sitchensis* (Webb and Street, 1977) and Douglas-fir (Winton and Verhagen, 1977) to produce multiple shoots. However, in *Sassafras,* no multiple shoots was formed in 6-BA medium. At high concentration of 6-BA, with or without 2,4-D and NAA, the stem and leaves swelled and turned red. The lateral buds grew and some leaves became rosette (Fig. 17). The callus-induction and embryogenesis could not be induced by 6-BA, even at high concentration (10^{-5} M). It seemed that 6-BA retarded auxins induced callus formation and embryogenesis (Tables 2 and 3).

Table 2. *Morphogenetic response of embryos of* ***Sassafras*** *to various concentrations of 2,4-D and 6-BA*

2,4-D	6-BA	Callus formation	Embryoid formation
5×10^{-7}M	0	+	−
	10^{-7}M	+	−
	10^{-5}M	−	−
5×10^{-6}M	0	++	+
	10^{-7}M	+	+
	10^{-5}M	+	−
5×10^{-5}M	0	+++	+++
	10^{-7}M	++	+
	10^{-5}M	+	−

Table 3. *Morphogenetic response of embryos of* ***Sassafras*** *to various concentrations of NAA and 6-BA*

NAA	6-BA	Callus formation	Embryoid regeneration
5×10^{-6}M	0	−	−
	10^{-7}M	−	−
	10^{-5}M	−	−
5×10^{-5}M	0	+	−
	10^{-7}M	+	+
	10^{-5}M	+	−
5×10^{-4}M	0	+	+
	10^{-7}M	+	+
	10^{-5}M	−	−

Germination of Somatic Embryos

No normal plantlets was found from *Sassafras* somatic embryos on any solid medium. The embryos grew into rosette-like on solid media after one month in culture (Fig. 18). The cotyledons of somatic embryos would also form rosette after being continuously kept on callus inducing liquid medium for two months or longer. The morphological change of rosette has been observed during the germination of immature *Sassafras* seeds (Wang, unpublished data). In the center of a rosette, a small dormant apical bud could be found (Fig. 19). The bud was germinated from rosette somatic embryos. However, the older tissue of the shoots remained rosette after being transferred to GA_3-agar medium (Fig. 20). Some of them could grow roots as normal somatic embryos in GA_3-liquid medium (Fig. 21). Since NAA and 2,4-D were found to inhibit the rooting of normal embryos (Tables 2 and 3), only GA_3 was added into media to induce the germination of somatic embryos.

Somatic embryos developed roots well in liquid medium, while only few embryos roots on solid media. Some somatic embryos developed shoot apex and root (Fig. 22); some developed abnormal shoots and roots (Fig. 23) and some only developed roots (Fig. 24) in liquid media. The best medium for somatic embryo development was hormone-free 1/2 strength MS liquid medium. About 8% of the somatic embryos formed vigorous shoots and roots (Figs. 25, 26, 27 and 28), 32% of embryos formed vigorous roots with or without shoot development (Table 4). The rooted embryos usually have one to two roots (Fig. 27). As reported by Ku (1977 a, b), the rooting of *Sassafras* was very weak; only 1.3% cuttings might develop roots. In this experiment, the rooting of somatic embryos (32%) was improved by the use of liquid culture. It is likely that the liquid medium dilutes the unknown inhibitor present in somatic embryos during embryo development, while the solid medium could not.

After being transferred to unsterile condition, the rooted plantlets continued to grow for 3 weeks. Later on, their roots and stems became brown and dry, while the leaves and shoots were still vigorous. Similar phenomenon was often observed

Table 4. *Development of somatic embryos after transferred to 1/2 MS liquid medium*

Organ formed	Total number	Percentage
Shoot (with or without root)	45/324	14%
Root (with or without shoot)	103/324	32%
Shoot + Root	26/324	8%
Dormant	183/324	56%
Rosette*	19/324	6%

* Swelling of cotyledons and no vegetative leaves expanded.

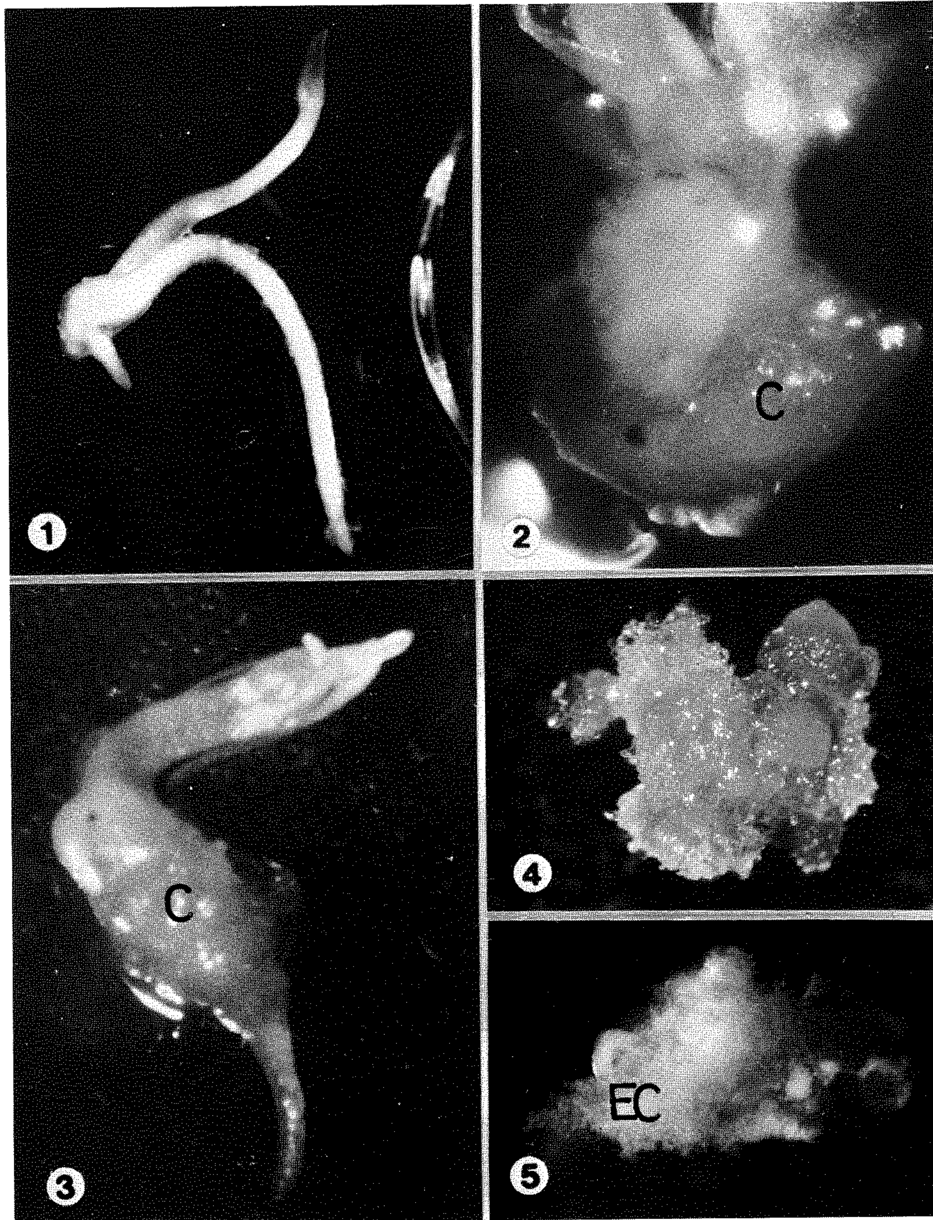

Fig. 1. *Sassafras* embryo germinated into seedling on hormone-free medium.

Fig. 2. Callus was induced on root pole of *Sassafras* embryo on 5×10^{-7}M 2,4-D liquid medium. (C: callus).

Fig. 3. The radicle axis of swollen *Sassafras* embryo and formed a callus on 5×10^{-6}M NAA medium.

Fig. 4, 5. Embryogenic calli initiated from *Sassafras* embryo callus. (EC: embryogenic callus).

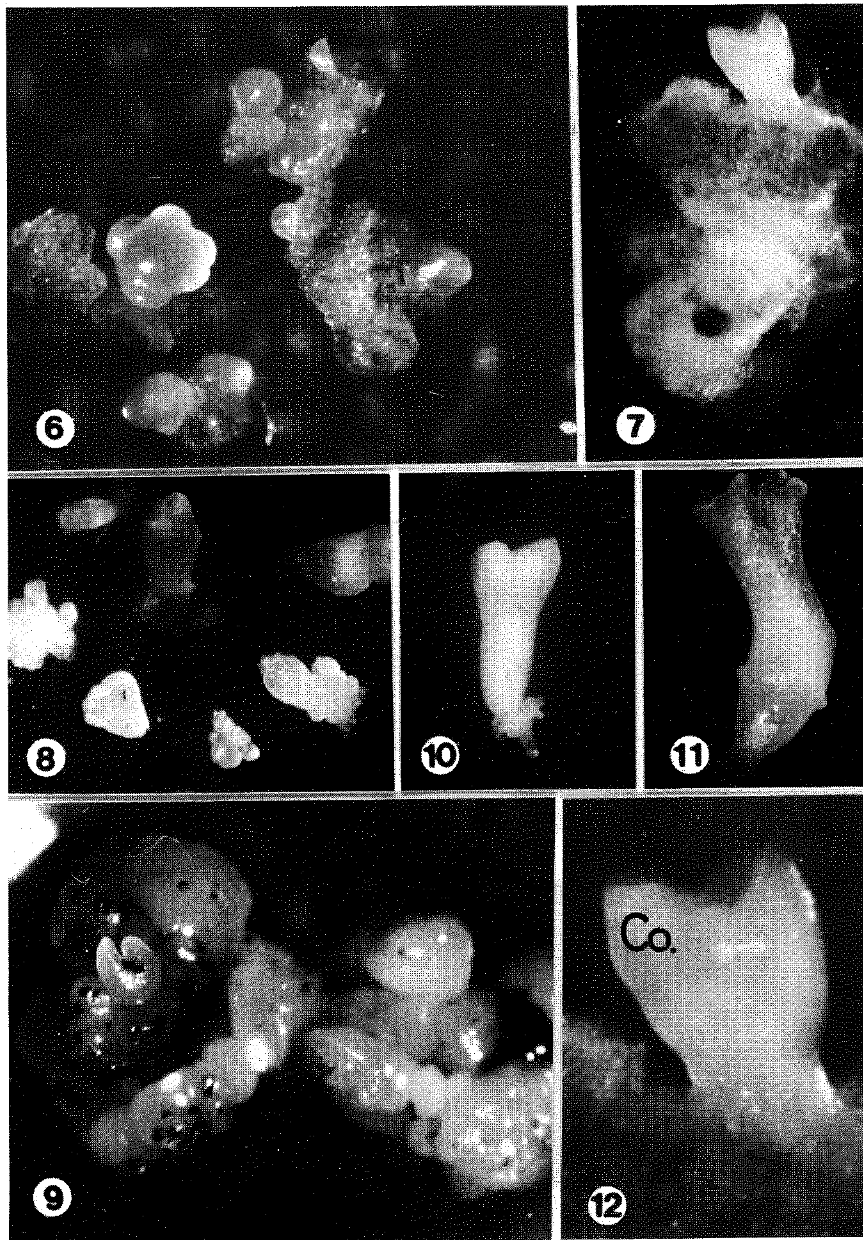

Fig. 6. The globular structure regenerated from embryo-derived calli after 2 month.
Fig. 7. Somatic embryo regenerated from callus.
Fig. 8. Individual embryoids separated from calli in liquid medium.
Fig. 9. Somatic embryo in tight clusters.
Fig. 10, 11. Suspensor-like structure of *Sassafras* somatic embryo.
Fig. 12. Close-up of regenerated somatic embryo without suspensor-like structure. (Co.: cotyledon).

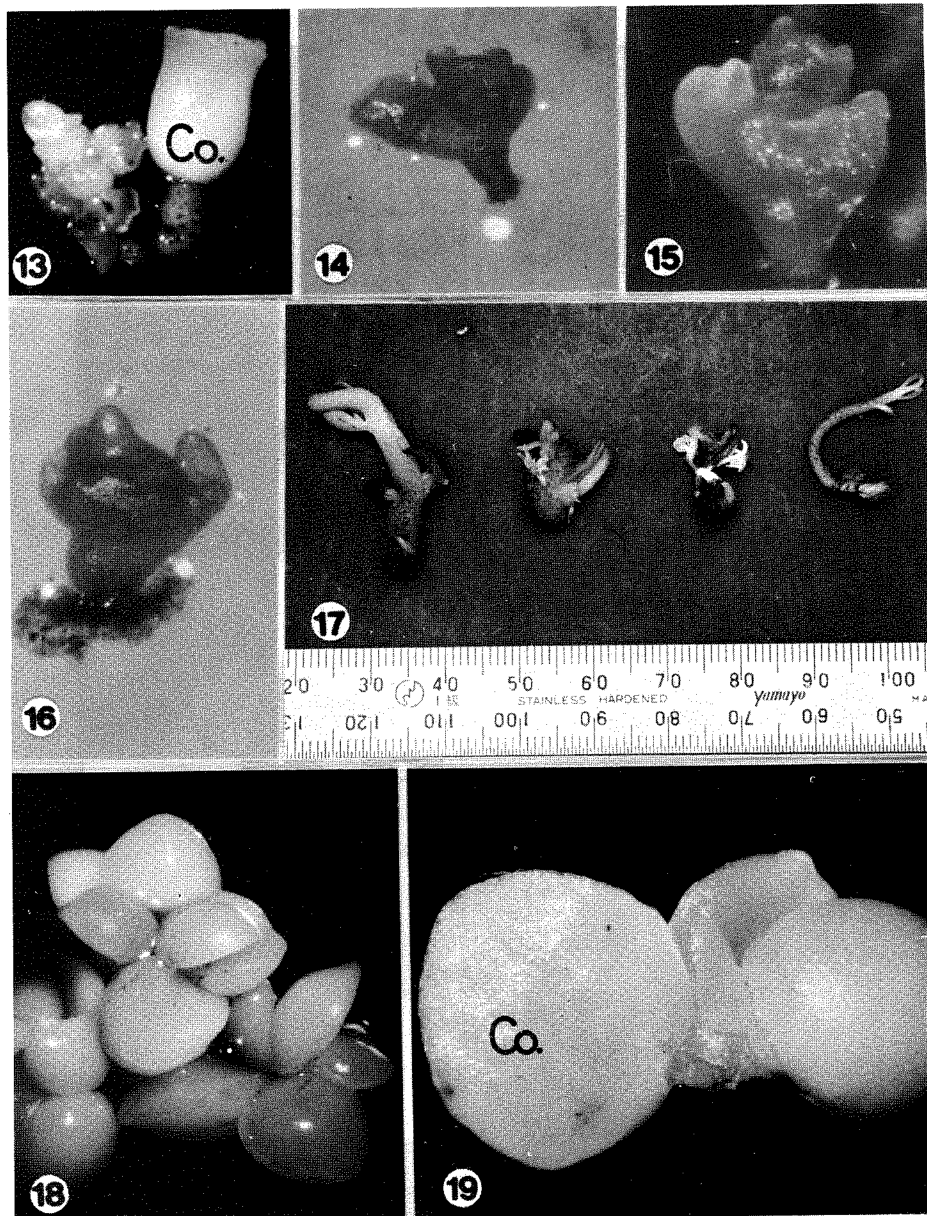

Fig. 13. Cup-like cotyledon of *Sassafras* somatic embryo.
Fig. 14, 15. Fused cotyledon of *Sassafras* somatic embryo.
Fig. 16. Multiple cotyledons of *Sassafras* somatic embryos.
Fig. 17. The morphological changes of *Sassafras* seedlings cultured on 10^{-5}M BA media.
Fig. 18. Rosette of *Sassafras* somatic embryos after 6 weeks culture on solid medium.
Fig. 19. The terminal bud in the center of rosette-like *Sassafras* somatic embryos.

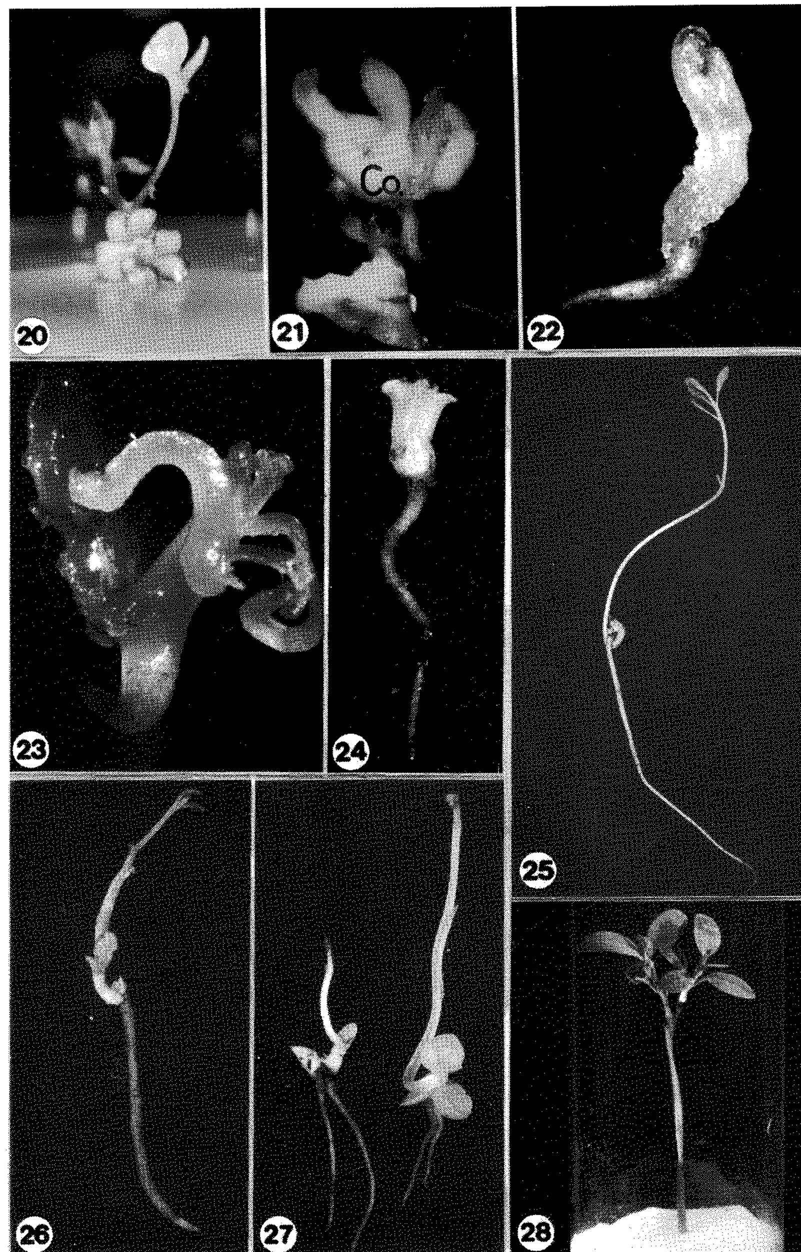

Fig. 20. Shoot from rosette on GA_3- solid medium.
Fig. 21. Shooting and rooting of rosette on GA_3- liquid medium.
Fig. 22. Apical shoot and root from somatic embryos on hormone-free liquid medium.
Fig. 23. Abnormal germination of *Sassafras* somatic embryo on liquid medium.
Fig. 24. Rooting of *Sassafras* somatic embryo on liquid medium.
Fig. 25, 26. Plantlets germinated from *Sassafras* somatic embryos on liquid medium.
Fig. 27. Plantlets with two roots developed from somatic embryos on liquid medium.
Fig. 28. Normal leaves expanded from somatic embryos.

in planting *Sassafras* wild seedlings (Ku, 1977 a). However, a suitable culture condition has not yet been found which can simutaneously support a good shoot and root growth. A complete experiment from the induction of embryoid to the development of a whole plant of *Sassafras* embryo culture takes 5 to 8 months.

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檫樹的胚培養及體胚分化

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將臺灣檫樹 (*Sassafras randaiense* (Hay.) Rehd) 的胚由種子挑出來單獨培養時，在含NAA 或 2,4-D 的液體培養基下形成癒傷組織 (Callus)，並獲得擬胚及植株的再分化。本實驗將MS 培養基的無機鹽濃度稀釋½倍做爲基本培養基。由實驗結果得知植物生長素 NAA 及 2,4-D皆可誘導癒傷組織及體胚形成 (Somatic embryogenesis)，且 2,4-D 的誘導效果較 NAA大。細胞分裂激素 BA 的添加對癒傷組織的形成及體胚的誘導沒有促進作用。體胚的分化以含 5×10^{-5}M 2,4-D 的培養基分化最多，所分化的體胚在形態上呈現多樣變異。將這些體胚移入不含任何植物荷爾蒙的液體培養基中，32%可發根，14%抽芽，8%證實可生長發育成完整的植株。經由體胚再分化獲得的小苗，移到砫石中培養，最初三個星期生長良好，嗣後原因不明，根及莖相繼死亡。因爲檫樹種子萌芽及扦插發根都相當困難，所以利用胚培養誘導癒傷組織並獲得體胚再分化的方法，可能應用於檫樹之無性繁殖。