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Shoot Formation in *Populus* Callus Tissues precultured in vitro on Some Different Media

By

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Summary : The effect of 4 kinds of synthetic media containing various inorganic and organic compounds, and auxins subjected to an earlier subculture of callus tissues upon the frequency of shoot regeneration in callus tissues derived from juvenile cambial parts was investigated. Sixty-eight days after the callus tissues were subcultured to these media, they were transferred to a medium already found to be effective for shoot regeneration from *Populus* callus tissues, and incubated : (1) under a dark condition for 30 days and subsequently under a fluorescent illumination of about 5,000 lx for 16 hr daily for 57 days, and (2) under only the above mentioned illumination for 16 hr daily for 87 days without a dark treatment. The frequency of shoot regeneration varied among test media subjected to subculturing callus tissues and between illumination conditions of the medium. Certain media (Medium-3 and -2) subjected to a subculture of callus tissues were found to be effective for shoot formation. Moreover, the shoot formation frequency was increase by dark treatment of the callus tissues. The pattern of analytical thin layer gel electrofocusing of peroxidase extracted from callus tissue grown on synthetic media subjected to an ealier subculture showed some quantitative and qualitative iso-enzyme differences with the media.

I Introduction

The induction of shoots from somatic callus tissue has been attempted for a large number of woody plants[1]. It has been demonstrated by numerous researchers[1)2)4)5)] that shoot formation from the callus tissues of various higher plants is controlled initially by the concentrations of chemical factors, especially those of phytohormones in the medium applied to induce shoots and by the variations of physical factors surrounding callus tissues. In the tissue culture of higher plants, the cultural conditions and other nongenetical factors are obviously also important because they facilitate or prevent the manifestation of genetical information. The understanding of the physiological basis for successful regeneration from callus tissues is now well known in this regard. However, some woody plants, except *Populus*, are extremely difficult to regenerate from somatic callus tissues in spite of much effort.

In this paper, we report evidence that the frequency of shoot formation from callus tissues of *Populus deltoides* grown on a synthetic medium found to be effective for shoot formation varies among test media used for earlier subcultures, and then the pattern of thin layer gel electrofocusing of peroxidase extracted from callus tissues subcultured on the test media varies in the same manner.

II Materials and methods

1) Procedure of tissue culture

Pieces 10 cm in length were cut from newly elongated branches of 3 year old cuttings

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Table 1. Composition of nutrient media used for subculture of callus (mg/*l*)

Components	Medium-1	Medium-2	Medium-3	Medium-4
KCl	130	130	130	130
$NaH_2PO_4 \cdot 2H_2O$	40	40	40	40
$MgSO_4 \cdot 7H_2O$	1,230	1,230	1,230	1,230
KI	0.8	0.8	0.8	0.8
$Ca(NO_3)_2 \cdot 4H_2O$	710	710	710	710
KNO_3	1,750	1,870	1,870	1,750
Na_2SO_4	426	426	426	426
ethylenediamine tetraacetic acid, iron salt (Fe·EDTA)	5.6	5.6	5.6	5.6
$NiCl_2 \cdot 6H_2O$	0.03	0.03	0.03	0.03
NH_4NO_3	400	220	220	400
$ZnSO_4 \cdot 7H_2O$	6	6	6	
H_3BO_3	3.2	3.2	3.2	
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.025	0.025	
$CuSO_4 \cdot 5H_2O$	0.03	0.03	0.03	
$MnSO_4 \cdot 4$–$6H_2O$	18	18		
$NaNO_3$	1,800		670	1,800
urea		200		
L-arginine	50	50	50	50
L-glutamic acid	10	10	10	
L-phenylalanine	2	10		
L-glycine	3	3		3
sodium citrate ($3H_2O$)	4	4	4	4
adenine sulfate	1	1	1	
adenosine-5-triphosphate, disodium salt (ATP)	1			1
guanosine	10		10	
succinic acid				11
maleic acid hydrazide	2			2
niacin	0.8	0.8	0.8	0.8
inositol	100	100	100	100
thiamine HCl	0.6	0.6	0.6	0.6
choline chloride	1	1	1	1
vitamin B_{12} (VB_{12})	0.0015	0.0015	0.0015	0.0015
calcium pantothenate	0.1	0.1	0.1	0.1
biotin	0.1	0.1	0.1	0.1
riboflavin	0.1			
pyridoxine HCl	0.1	0.1		0.1
L-ascorbic acid (vitamin C, VC)				1
DL-α-tocopherol (vitamin E, VE)	1			1
gibberellin (GA_3)	0.5	0.5	0.5	
3-indolebutyric acid (IBA)	0.5	0.5	0.5	0.5
2, 4-dichlorophenoxy acetic acid (2, 4-D)	0.009	0.009	0.009	0.009
α-naphthylacetic acid (NAA)		0.16	0.09	
3-indoleacetic acid (IAA)	0.088			0.088
β-naphthoxyacetic acid (NOAA)	0.02	0.02	0.02	0.02
6-benzylaminopurine (BAP)	0.40	0.40	0.40	0.40
sucrose	20,000	20,000	20,000	20,000
agar	8,000	8,000	8,000	8,000

Notes : pH was adjusted to 5.8 with HCl or NaOH before adding the agar.

of *Populus deltoides* MARSH. in June, 1977. The cut ends of these pieces were sealed tightly with paraffin, surface-sterilized with 70% ethyl alcohol for 5 min and then rinsed twice with sterile distilled water. These sterilized branch pieces were peeled aseptically with a surgical knife which had been rinsed in 2% liquid sodium carbonate. The cambial part of the peeled sections was cut into pieces about 5 mm square and incubated aseptically in 18 mm × 180 mm test tubes containing 20 m*l* of the solid nutrient medium for inducing and subculturing callus tissues as shown in Table 1 of the previous paper[8] and which was developed to encourage the induction and growth of *Populus* callus. The test tubes were subjected continually to a temperature of 25°C and a fluorescent illumination of about 5,000 lx for 16 hr daily.

The callus tissue derived from the explant after 12 weeks was cut into roughly rectangular pieces each having an average weight of 40～50 mg. Ten pieces were placed in each test tube containing fresh medium. The tissue obtained by subculturing in this manner for 10～12 weeks showed active growth. This subculturing was repeated numerous times.

In order to investigate the frequencies of shoot regeneration from somatic callus cultured in vitro, the callus tissues were transferred to 4 kinds of media, as shown in Table 1, in September 1980. Sixty-eight days after the inoculation, the callus tissues from each subcultured medium were transferred to another nutrient medium for inducing shoots as shown in a previous paper[8], and incubated (1) under dark conditions for 30 days and subsequently a fluorescent illumination of about 5,000 lx for 16 hr daily for 57 days, and (2) under a fluorescent illumination of about 5,000 lx for 16 hr daily for 87 days throughout the culture period.

2) **Procedure of analytical thin layer isoelectric focusing**

Callus tissues obtained 82 days after inoculation with 4 kinds of media, as shown in Table 1, were utilized. The callus tissues corresponding to 0.5 g were weighed and added to 5 m*l* of Tris buffer (1 M, pH 7.5) including 12% sucrose, 1% Triton X-100, 0.58% ethylenediamine tetraacetic acid (EDTA) and 10% insoluble polyvinyl pyrrolidone (PVP) and then homogenized with a moter at high speed. The supernatants obtained after the homogenization were absorbed by filter paper cut into 5 mm × 10 mm pieces and subjected for electrophoretic separations. Analytical thin layer gel electrofocusing was performed on prepared polyacrylamide gel plates containing Ampholine carrier ampholytes in the pH range of 4.0～6.5 (L. K. B., Sweden). An Model 2117 Multiphor (L. K. B., Sweden) apparatus was used for the isoelectric focusing. The electrode buffer was as follows :

(−) 1 M NaOH　　(+) 1 M H_3PO_4

The isoelectric runs were made at 50 V, 10 mA for 30 min and then at 200 V, 15 mA for 210 min just after the filter papers impregnated with the liquid extracts were plated.

In order to detect isoenzyme of peroxidase, the gels were stained with 2 mM 3-amino-9-ethylcarbazole in 0.1 M Tris buffer pH 4.0 (mixed solution consists of 2 mM β-naphthol, 20% acetone and 3% H_2O_2) for 40 min. The gels were washed and fixed repeatedly with 7% acetic acid solution.

The diagrammatic banding patterns consisting of broad dense (A), narrow dense (B), linear (C) and dotted (D) bands were used to indicate various standards of the staining portions of the gels. The A, B and C bands indicate visible bands on the gels, although the D band is a less-dense band. The last D band indicates a non-visible, very fine band.

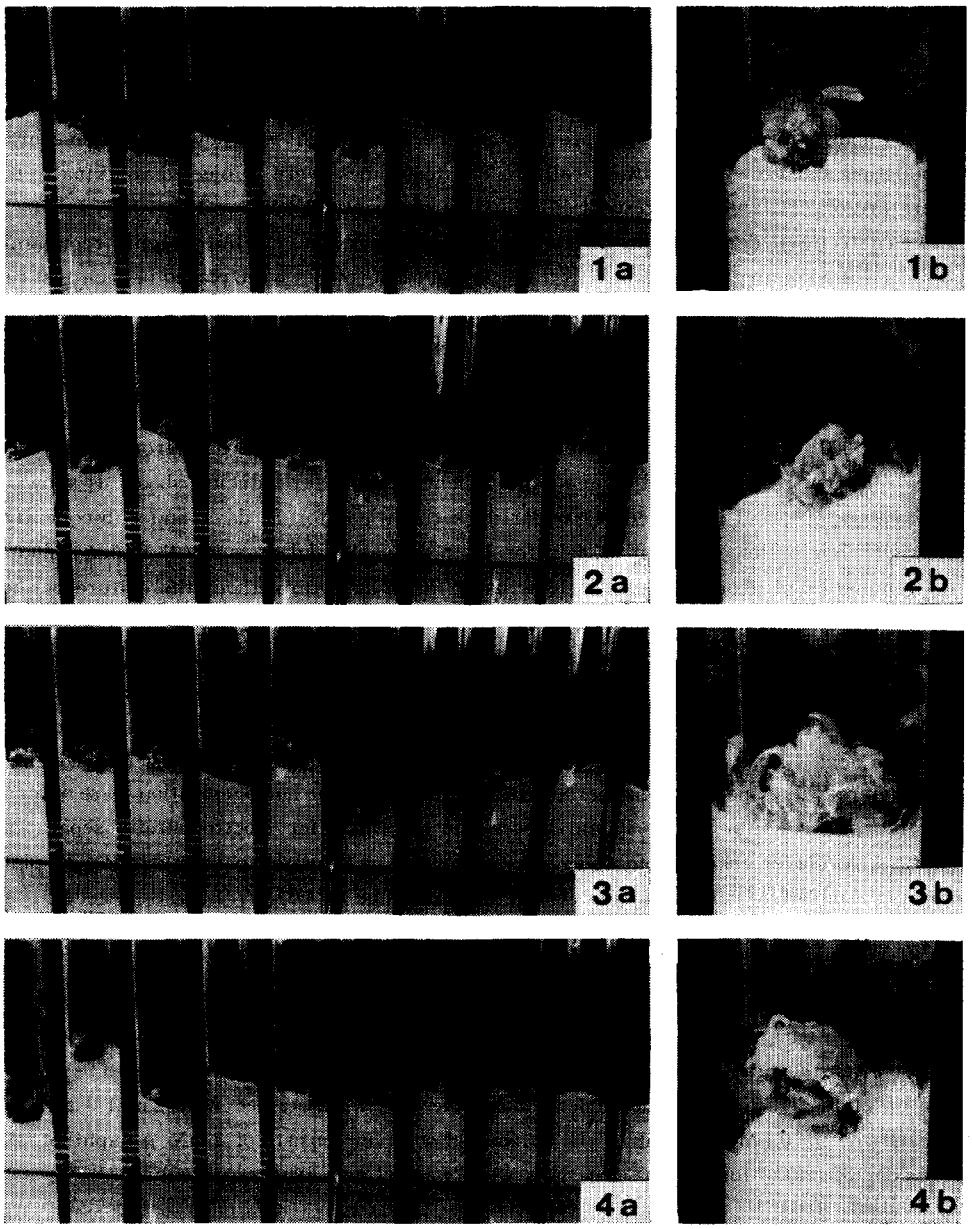

Fig. 1 Juvenile shoot formations from callus tissues of *Populus* subcultured to medium-1 (1 a, b), -2 (2 a, b), -3 (3 a, b) and -4 (4 a, b) under dark condition for 30 days, and subsequently under a fluorescent illumination of about 5,000 lx for 16 hr daily for 57 days. b; Close up view of shoots. Test tube diameter; 18 mm.

III Results and discussions

1) Frequency of shoot regeneration

All callus tissues transferred to 4 kinds of media, shown in Table 1, showed active growth. When the callus tissues were transferred to another nutrient medium developed for successive shoot regenenration, as described in a previous paper[8], 68 days after the inoculation, greenish shoots appeared on the slowly growing brown callus in about 40 days (Fig. 1, 2). The frequencies of shoot regeneration from the somatic callus cultured in vitro are shown in Table 2. The variations among media subjected to an earlier subculture were distinct. A greater amount of shoot regeneration was induced in callus tissues subcultured with medium -3 and -2 in which the elements of IAA, VE, ATP and maleic hydrazide were cut, while under a fluorescent illumination of about 5,000 lx for 16 hr daily for 30 days after being cultured under the dark condition.

These results suggest that in order to induce an embryogenic response of *Populus* callus, the selection of a medium to be subjected to an earlier subculture is a most important problem, although such physical conditions as the illumination factor mentioned above are necessary.

2) Pattern by isoelectric focusing

The diagrams of iso enzymes by analytical thin layer isoelectric focusing of peroxidase carried out on callus tissues subcultured on 4 kinds of media (shown in Table 1) are shown in Fig. 3. A comparison of the peroxidase patterns revealed that the number of A, B, C

Fig. 2 Juvenile shoot formations from callus tissues of *Populus* subcultured to medium-3 under a fluorescent illumination of about 5,000 lx for 16 hr daily for 87 days. b; Close up view of shoots. Test tube diameter; 18 mm.

Table 2. The frequency of shoot regenerations in callus tissues of *Populus* transferred on a medium for shoot establishment for 68 days after an earlier subculture to test media shown in Table 1. The frequency of shoot regeneration was presented by number of test tubes with shoot differentiation among 10 test tubes

Optical condition	Medium			
	1	2	3	4
Dark treatment for 30 days and then illumination for 16 hr daily for 57 days	6	8	10	5
Illumination for 16 hr daily for 87 days			8	

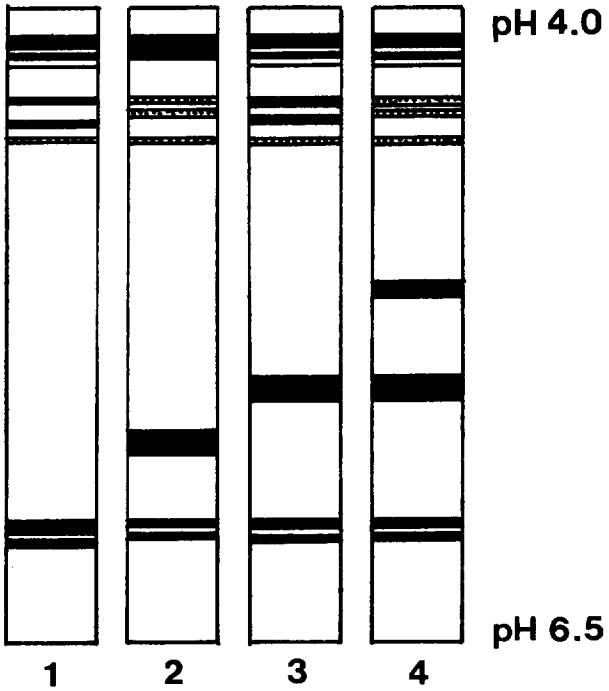

Fig. 3 Applications of isoelectric focusing (IF) in polyacrylamide gel. Electrophoretic runs; 50 V, 10 mA, 30 min, 200 V, 15 mA, 180 min. Electrode buffer; (−) 1 M NaOH, (−) 1 M H_3PO_4 1～4; medium.

tissues derived from cambial tissues of woody plants.

visible and D faint bands varied between callus tissues of each medium. The presence of only one A band found in the center area of the gel was observed with callus tissues subcultured on medium -3 and -2 in which the higher frequencies of shoot regeneration were obtained. This A band occured twice in medium-4 whereas it was absent in medium-1.

Little attention has been paid to the components of mediums used in earlier subcultures in relation to shoot regeneration from callus. The results of the present paper support the possibility that embryogenesis in callus tissues is controlled by encouraging the growth of a few proembryos carried over from earlier subcultures. The electrophoretic patterns of peroxidase by isoelectric focusing may be useful as indicators of shoot regenerative ability in callus

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各種培地で継代培養されたポプラカルスにおける茎葉分化のちがい

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摘　要

いろいろな無機成分，有機成分ならびにオーキシンを含む4種類の合成培地を用意し，これに枝の若いじん皮部から誘導されたポプラカルスを継代培養すると，その後の茎葉分化能力にちがいが生じるかどうかをしらべた。これらの培地に継代培養してから68日目のポプラのカルスを，カルスからの茎葉分化に効果的であることがすでにしられている分化用培地に移し，2つの培養条件すなわち，（1）30日間の暗処理ののちに57日間毎日16時間蛍光灯（5,000ルックス）で照明した条件と，（2）暗処理をしないで87日間毎日16時間照明の条件で培養した。その結果，茎葉分化の程度は，カルスを継代培養するために用いられた供試培地間で，また茎葉分化用培地に対する光条件間でちがいがみとめられた。カルスの継代培養のために用いられた4種の培地のうち，2種の培地がそのあとの茎葉分化に効果をあらわすことがわかった。さらに，茎葉分化の程度はカルスの暗処理によって高められた。

また，継代培養のために用いられた合成培地で培養されたカルスから抽出したパーオキシダーゼの薄層ゲル等電点電気泳動パターンは，培地によって質的ならびに量的にイソ酵素の変化を示した。このイソ酵素の変化は，カルス細胞の培養過程での遺伝的安定性との関連で興味ある問題点を提起する。この遺伝的安定性は組織培養の分野ではもつとも重要な課題であり，その辺の事情をさらに詳しく解明する必要があると考えられる。

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