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ORGANIZATION AND ALKALOID PRODUCTION IN TISSUE CULTURES OF *SCOPOLIA PARVIFLORA*

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Abstract—Callus cultures have been established from stem and rhizome segments of *Scopolia parviflora*, a tropane alkaloid-producing plant. These cultures initiate organs, especially large numbers of roots in spite of the presence of auxin in the culture medium. This root-forming capacity of the callus tissues has been maintained through serial subcultures for more than 2 years. The alkaloid content of the cultures grown on the basal medium is much less than that of the intact rhizome, although it can be increased to 0·12% by a sufficient supply of tropic acid, the precursor of the acidic moiety of the ester alkaloids hyoscyamine and scopolamine. In contrast to the unorganized tissues, the roots initiated from callus cultures tend to produce the normal pattern of alkaloids, suggesting the operation of self-regulation for alkaloid synthesis as part of the organization. Alkaloid content can be increased to 0·08% by the transfer of root-initiated tissues to an appropriate liquid medium for the development of roots in suspension culture.

INTRODUCTION

CALLUS and cell suspension cultures from certain species of *Atropa*, *Hyoscyamus* and *Datura* (Solanaceae) have been examined for the ability to synthesize tropane alkaloids contained in the original plants.[1-10] All these studies indicate that 'dedifferentiated' tissues or cells propagated in synthetic culture media produce little or no alkaloids. We detected only small amounts of alkaloids (average 0·006%) also in the cultures of *Scopolia japonica* Maxim., a species producing hyoscyamine and scopolamine as the main tropane alkaloids.[11] Recently, we have established tissue cultures from *Scopolia parviflora* Nakai, a native Korean species closely resembling *S. japonica* not only in morphology but in alkaloid composition and pharmacological activity.[12,13] Thus the rhizome extracts of both species are being used medicinally in Japan as antispasmodics. In spite of these similarities, the callus cultures of *S. parviflora* are clearly distinguished from those of *S. japonica* by their

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[13] M. KONOSHIMA, M. TABATA, Y. KANO and S. TANAKA, *Jap. J. Pharmacog.* **24**, 105 (1970).

remarkable ability to exhibit organogenesis, particularly vigorous root formation. Examination of *S. parviflora* cultures has indicated an intimate relation between organization and alkaloid formation and furthermore suggested a possible utilization of the morphogenetic ability of cultured tissues for efficient production of the useful alkaloids. In the present paper, the characteristic behaviors of *S. parviflora* cultures and some factors that affect root development and alkaloid production are described.

RESULTS

Callus Growth and Organization

Callus tissues were initiated from excised segments of the rhizome or stem of *Scopolia parviflora* cultured in the Linsmaier–Skoog medium[14] containing 10^{-7}–10^{-5} M of 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-acetic acid (IAA) and 9 g/l. of agar. The supply of 10^{-6} M 2,4-D induced the most vigorous growth of callus, whereas the effect of kinetin was quite insignificant (Table 1). As the undifferentiated mass proliferated on the

TABLE 1. EFFECTS OF GROWTH REGULATORS ON CALLUS FORMATION AND ORGANIZATION IN EXCISED RHIZOME SEGMENTS OF *S. parviflora* CULTURED ON THE BASAL MEDIUM IN LIGHT AT 25°

2,4-D concentration (M)	Kinetin concentration (M) 0	10^{-6}	10^{-5}
0	−	−	−
10^{-7}	++ *R*	++ *R*	++ *R*
10^{-6}	+++ *R*	+++ *R*	+++ *R*
10^{-5}	+	+	++

−, +, ++, +++ = degree of callus proliferation.
R = root formation.

culture media containing either 10^{-6} or 10^{-7} M 2,4-D, many roots differentiated on the surfaces of callus tissues. As compared with 2,4-D, IAA was less effective for callus induction, unless the medium was fortified by the addition of kinetin; but it was more favorable for root formation, which occurred in most of the calluses supplied with 10^{-7}–10^{-5} M IAA. In addition to root formation, some of the cultures grown on media containing IAA (10^{-5} M) and kinetin (10^{-7}–10^{-5} M) under fluorescent light formed shoots or occasionally plantlets, which developed into apparently normal plants when transplanted to the soil in pots.

Calluses induced from the rhizome or stem segments were successfully subcultured on a basal medium containing either 10^{-5} M IAA or 10^{-6} M 2,4-D. Inoculated tissue pieces showed an average 20-fold increase in fresh weight during 6 weeks of incubation. The occurrence of root primordia on the surface of callus was already visible 1 week after the initiation of subculturing, and they continued to grow outward to become fibrous roots of various lengths. Although the shoot-forming capacity of calluses was hardly expressed after a few culture passages, their root-forming capacity has been retained through successive passages carried out over 2 years. As seen in Table 2, the growth of subcultured callus was dependent on the concentration of auxin in the culture medium, but was little affected by

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TABLE 2. EFFECTS OF 2,4-D AND KINETIN ON GROWTH, ROOT FORMATION, AND ALKALOID PRODUCTION IN THE CALLUS CULTURES OF RHIZOME ORIGIN (6-MONTH-OLD) GROWN IN LIGHT FOR 6 WEEKS AT 25°

Concentrations of 2,4-D (M)	Concentrations of Kinetin (M)	Fresh wt. (g/flask)	No. of roots per piece	Length of roots (mm)	Alkaloid content (% of dry wt., $\times 10^3$)
0	0	0·80	5–10	5–40	10
0	5×10^{-6}	1·28	10–20	5–40	14
10^{-7}	0	2·36	10–20	5–40	9
10^{-7}	5×10^{-6}	2·32	10–20	5–40	2
10^{-6}	0	5·75	20–30	*ca.* 5	6
10^{-6}	5×10^{-6}	4·15	20–30	5–10	4
10^{-5}	0	3·55	0	0	9
10^{-5}	5×10^{-6}	2·98	0	0	11
10^{-4}	0	5·02	20–30	5–10	8
10^{-4}	5×10^{-6}	4·96	30–40	*ca.* 5	12

kinetin. Roots formed on calluses regardless of the concentrations of growth regulators, except when 10^{-5} M 2,4-D was added. The absence of organogenesis at 10^{-5} M 2,4-D was ascertained by a repeated experiment, but the reason for this peculiar effect has not been clarified. With the increase of 2,4-D concentration from 0 to 10^{-6} M, the number of roots per callus piece increased, whereas the length of roots became shorter. Apparently 2-,4-D tends to stimulate the initiation of root primordia from undifferentiated tissues, but it is rather inhibitory to the elongation of initiated roots at higher concentrations.

Alkaloid Formation

The formation of alkaloids was detected by TLC in all the cultures of different passage generations, but the average contents of total alkaloids produced were only 0·010% for the cultures of stem origin and 0·009% for those of rhizome origin. It is interesting in connection with the 'dedifferentiation' that there is no difference in alkaloid-producing ability between the cultures of different origin in spite of a wide difference in alkaloid content between the stem (0·043%) and the rhizome (0·340%) in the mature plant.

Experiments were carried out to examine the effects of environmental factors on alkaloid production in cultured tissues. As seen in Table 2, no definite correlation between auxin concentration and alkaloid content has been established in the cultures of *S. parviflora*. As to the effect of the light, the cultures grown under continuous light (7000 lx) showed a 50–75% reduction in alkaloid content, as compared with those grown in the dark. On the other hand, both alkaloid production and tissue growth were promoted to an appreciable extent when the cultures grown in the dark were supplied with 2 g/l. of casamino acids (Difco), peptone (Kyokuto), or yeast extract (Difco); the respective alkaloid contents being 0·012, 0·016 and 0·017% in comparison with 0·008% in the control cultures. The effects of possible precursors of tropane alkaloids,[15–17] i.e. phenylpyruvate, L-phenylalanine, tropic acid, L-ornithine, L-proline, and tropine were tested individually in the concentration range between 50 and 1000 μM. Of these, only tropic acid was found to be effective in increasing the alkaloid content, which reached 0·12% as the concentration of tropic acid was raised to

[15] E. LEETE and M. L. LOUDEN, *Chem. & Ind.* 1405 (1961).
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2×10^{-3} M, although the growth of tissues was reduced to about 60% of the control (Table 3).

TABLE 3. EFFECTS OF TROPIC ACID ON GROWTH AND ALKALOID PRODUCTION IN *S. parviflora* CALLUS CULTURES. THE CALLUS TISSUES OF RHIZOME ORIGIN (15-MONTH-OLD) WERE GROWN ON BASAL MEDIA CONTAINING 10^{-6} M 2,4-D, 10^{-6} M KINETIN, AND DL-TROPIC ACID IN THE DARK FOR 6 WEEKS AT 25°

DL-Tropic acid concentration (μM)	Relative growth*	Alkaloid content (%, $\times 10^3$)
0	100	7
250	108	16
1000	74	91
2000	58	120

* 100 corresponds to 4·7 g fresh wt./flask.

The alkaloids produced by *S. parviflora* tissue cultures were compared with the alkaloids of the intact plant by TLC. Callus tissues, whether they had been derived from stem or rhizome, gave seven Dragendorff-positive spots, most of which were present in the intact plant and included hyoscyamine, scopolamine, apoatropine and tropine. However, the relative proportions of hyoscyamine and scopolamine were remarkably low as compared with those of other alkaloids detected. This alkaloid pattern in the unorganized tissue was hardly changed even when the alkaloid production was promoted by the addition of tropic acid to the culture medium.

In striking contrast to the unorganized portion, the roots that formed on the callus showed a pattern of alkaloids similar to that of the normal plant; two major alkaloids corresponding to hyoscyamine and scopolamine and six minor alkaloids including apoatropine and tropine. The two main alkaloids in the root clusters, obtained in a large amount by a suspension culture of callus tissues, were identified as hyoscyamine and scopolamine (see Experimental). Apoatropine was also isolated from the same material.

Root Development and Alkaloid Production in Suspension Cultures

Although the roots initiated from callus cultures grown on agar media resumed a normal pattern of alkaloid synthesis, their alkaloid contents were as low as those of the callus tissues. However, alkaloid content of the regenerated roots increased from a low level of 0·003–0·05% within 6 weeks if the root-bearing callus was transferred to a liquid medium containing 10^{-5} M IAA. In this suspension culture, large numbers of roots developed from the aggregates, resulting in clusters or balls of fibrous or somewhat thickened roots with rudimentary pieces of callus.

Based on this finding, a series of experiments were carried out in an attempt to improve the chemical environment of the culture medium. For this purpose, the stem callus (3-month-old) was propagated in the liquid medium with 10^{-5} M IAA to obtain numerous root-bearing aggregates, which were then inoculated in various test media and cultured for 5–6 weeks in the dark at 25°. The results obtained from a comparative test against various basal media containing 10^{-6} M 1-naphthaleneacetic acid indicated that the solutions of

White[18] and Wood-Braun[19] compare favorably with the Linsmaier–Skoog solution[14] in supporting the root growth; but the average alkaloid contents of the roots harvested after a 40-day culture were 0·074, 0·048 and 0·082%, respectively. The optimal concentration of sucrose in the medium for root development was determined to be 3 g/l., and the optimal pH of the medium 5·8 (before autoclaving). Moreover, vitamins played an important role in the elongation of roots; the addition of a vitamin mixture consisting of 0·1 ppm thiamine-HCl, 0·3 ppm pyridoxine-HCl, 0·5 ppm nicotinic acid, and 100 ppm *myo*-inositol stimulates the root elongation much more than the addition of a mixture lacking any one of them.

TABLE 4. EFFECTS OF VARIOUS AUXINS ON GROWTH AND ALKALOID PRODUCTION IN THE ROOT CLUSTERS DEVELOPED FROM THE STEM CALLUS. ROOT-INITIATED CALLUS PIECES (75–90 mg DRY WT.) WERE INOCULATED INTO EACH FLASK CONTAINING THE BASAL LIQUID MEDIUM SUPPLEMENTED WITH AUXIN AND CULTURED IN THE DARK FOR 7 WEEKS AT 25°

Concentration (M)	IAA		NAA		2,4-D	
	Dry wt.*	Alkaloid content†	Dry wt.	Alkaloid content	Dry wt.	Alkaloid content
10^{-9}	315	35	290	63	253	60
10^{-8}	331	45	341	45	359	38
10^{-7}	423	36	432	29	451	31
10^{-6}	502	42	512	30	393	27
10^{-5}	381	57	356	36	290	55

* mg/flask.
† % of dry wt., × 10^3.

Table 4 shows the effects of different auxins on growth and alkaloid production in suspension cultures. The best growth was obtained when 10^{-6} M of IAA or NAA was added to the medium. The elongation of roots was slightly inhibited at higher concentrations of auxins, and many of the cultured roots formed callus tissues in the presence of 10^{-6} or 10^{-5} M 2,4-D. The alkaloid content of the roots varied from 0·027 to 0·063% with the

TABLE 5. EFFECTS OF YEAST EXTRACT ON GROWTH AND ALKALOID PRODUCTION IN THE ROOTS INDUCED FROM THE STEM CALLUS. ROOT-INITIATED CALLUS PIECES WERE CULTURED IN THE LIQUID MEDIUM SUPPLEMENTED WITH 10^{-6} M IAA AND YEAST EXTRACT IN THE DARK FOR 48 DAYS AT 25°

Yeast extract concentration (g/l.)	Fresh wt. (g/flask*)	Alkaloid content (% of dry wt., × 10^3)
0	7·5	33
0·1	7·9	43
0·5	7·6	53
1·0	8·2	72

* 500-ml Sakaguchi flask containing 250 ml of the medium.

[18] P. R. WHITE, *The Cultivation of Animal and Plant Cells*, p. 74, Ronald Press, New York (1954).
[19] H. N. WOOD and A. C. BRAUN, *Proc. Nat. Acad. Sci. U.S.A.* **47**, 1907 (1961).

culture, but it does not seem to be correlated directly with auxin concentration, as has been found in the static cultures mentioned earlier. Although the various hormonal treatments of cultured roots failed to stimulate alkaloid production significantly, the supply of a complex nutrient like yeast extract is effective to a certain extent in increasing the alkaloid content of roots, as shown in Table 5. These results together suggest that both root development and alkaloid production in submerged cultures are influenced by a number of exogenous substances and that their yields may be increased through further improvements in the cultural conditions.

DISCUSSION

Callus cultures derived from the stem and rhizome of *Scopolia parviflora* exhibit a remarkable morphogenetic capacity; although both roots and shoots arise on calluses during the first few passages after callus induction, only roots regenerate during the later subcultures. In this respect, *S. parviflora* cultures form a striking contrast in morphogenetic expression to the callus strain of *Nicotiana tabacum* that forms only shoots after a series of subculturing,[20] but they are rather similar to the suspension culture of *Atropa belladonna*, in which large numbers of roots arise on the aggregates.[9] In both the tobacco and belladonna cultures, however, organogenesis was induced only when calluses were transferred to an auxin-omitted medium. Although the tendency for callus cultures to form organized structures when cultured in a medium without auxin has been reported in many other plants, *S. parviflora* cultures are capable of forming roots persistently in the media that do contain IAA or 2,4-D. Another feature of *S. parviflora* cultures is that their morphogenetic potential is retained without any noticeable reduction after serial culture passages. In the aggregates from belladonna calluses, however, the frequency of morphogenesis became extremely low with the increasing number of previous culture passages.[9]

It has been demonstrated that *S. parviflora* cultures usually contain much less alkaloid than the rhizome or root of the intact plant. The results obtained from precursor experiments seem to suggest that the metabolic activity pertaining to the biosynthesis of tropic acid from phenylalanine or phenylpyruvic acid might be lowered in the 'dedifferentiated' tissues. Although Thomas and Street[9] found that the addition of tropic acid enhanced root development in belladonna cultures, such an 'antiauxin'-like action of tropic acid was not observed in either *Scopolia* or *Datura* cultures. However, tropic acid may play an important role not only in alkaloid synthesis but also in growth regulation, as the growth of *Scopolia* or *Datura* cultures are strongly suppressed in the presence of this compound even at a concentration of hormonal level.

That the cultured cells of *S. parviflora* maintain the inherent potentiality to synthesize specific alkaloids has been indicated by the nearly complete restoration of the normal alkaloid pattern in the roots initiated from subcultured calluses. In belladonna cultures, Bhandary *et al.*[8] pointed out that the alkaloid formation is associated with organogenesis, since the tropane alkaloids, which are absent from cultured callus, are present in the excised roots of callus origin. The contents of total alkaloids from their excised root cultures were 0·048% and from var. *lutea* 0·096%. It is of interest that similar values have been obtained with *S. parviflora* suspension cultures. In *Datura stramonium*, Steinstra[21] reported that the total alkaloids in the cultures of roots excised from normal plants were much less (0·06%) than in

[20] M. TABATA, H. YAMAMOTO, N. HIRAOKA, Y. MARUMOTO and M. KONOSHIMA, *Phytochem.* **10**, 723 (1971).
[21] T. M. STEINSTRA, *Proc. Koniki. Ned. Aka. Wetenschap.* **57C**, 584 (1954).

normal plants (0·21–0·25%), suggesting that this difference must be caused by differences in environmental factors, nutritional supply, and the age or stage of development between the cultured roots and the normal roots. This concept may hold good for *S. parviflora* cultures, where the alkaloid content is significantly increased through the development of roots in an appropriate culture medium.

Although our studies with *Scopolia japonica* and several species of *Datura* have indicated that small amounts of tropane alkaloids (0·002–0·020%) are synthesized by the callus or suspension cultures showing no microscopical organization, our experience with *S. pariflora* cultures suggests[22] that it might be more desirable to grow specific organized structures in submerged cultures that have the proper self-regulation necessary for the production of useful metabolic products than to grow rapidly dividing but undifferentiated cell suspensions. However, it is still possible that normal synthesis of tropane alkaloids might be induced artificially in unorganized tissues cultured under the right conditions.

EXPERIMENTAL

Plant material and culture method. Callus tissues were derived from the stem and rhizome of a mature plant of *Scopolia parviflora* Nakai that was originally collected in Chung-choung Buck Province, Korea. For callus induction, a cylinder of tissue was aseptically excised from each organ with a cork borer, cut into disks (5 mm in diameter, 3 mm in thickness), and each disk was then placed on the Linsmaier–Skoog basal medium[14] in test tube. The basal medium was supplemented with various concentrations of auxin and kinetin and solidified with 9 g/l. of agar. The pH of the medium was adjusted to 6·0 before autoclaving. The callus tissues obtained were maintained through successive transfers of small pieces of callus to the same basal medium containing either 10^{-5} M IAA or 10^{-6} M 2,4-D carried out at 30–40 day intervals. In culture experiments, 100-ml Erlenmeyer flasks containing 50 ml of the solid medium were used for static cultures. Suspension cultures were done in 500-ml Sakaguchi flasks containing 250 ml of the liquid medium, which were shaken at a speed of 100 rev/min by a reciprocating shaker. The cultures were grown at 25°, usually in the dark, with 5 replicates for each treatment.

Extraction and analysis of alkaloids from the tissue cultures. Tissues harvested from culture flasks were dried at 50° for 2 days and then kept in a desiccator. The dry powdered sample was macerated overnight with a mixture of EtOH and 28% NH_4OH (9:1) and then Soxhlet-extracted with $CHCl_3$ for 6 hr. After evaporating the extract to dryness under reduced pressure, the residue was dissolved in 0·1 N HCl (20 ml) and extracted with $CHCl_3$ thrice (30 ml each). The aqueous layer was made alkaline (pH 9·5) with 1 N NaOH and extracted with $CHCl_3$ thrice. After drying with K_2CO_3 the $CHCl_3$ was evaporated, and the residue was dissolved in 0·05 N HCl (20 ml) to provide a sample for the quantitative estimation of alkaloids, which was done spectrophotometrically by the modified Vitali-Morin method.[23] The content of total alkaloids was calculated as the percent of atropine per dry weight of tissue.

The sample of alkaloids for chromatographic analysis was prepared through the procedure mentioned above and finally dissolved in $CHCl_3$. The alkaloids were developed on the Silica gel G (Merck) plate with $CHCl_3$–EtOH–28% NH_4OH (85:14:1) or with 70% EtOH–25% NH_4OH (99:1). The alkaloidal spots were detected by spraying with Dragendorff's reagent.

The isolation and identification of the two main alkaloids produced by the regenerated roots were done as follows: the dried sample (63 g) of the root clusters, which were harvested from a 7-week suspension culture of calluses in the basal medium containing 10^{-6} M IAA and 0·1% yeast extract, was Soxhlet-extracted with MeOH. The alkaloids obtained were separated by preparative TLC on Silica gel G with $CHCl_3$–EtOH–28% NH_4OH (85:14:1), and one alkaloid (I) corresponding to hyoscyamine in R_f (0·27) and another (II) corresponding to scopolamine (R_f 0·64) were extracted individually with acetone. The picrate of I (9·5 mg, m.p. 170–172°) and that of II (14·3 mg, m.p. 186–188°) were identified as the picrates of atropine and scopolamine, respectively, by direct comparison with authentic materials by mixed m.p., co-chromatography (two solvents, TLC) and IR analysis. In addition to these alkaloids, apoatropine (7·2 mg, m.p. 168–169·5° as picrate) was also isolated from the same material.

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[22] E. J. Staba, P. Laursen and S. Buchner, *Proc. Intern. Conf. Pl. Tissue Culture*, p. 191, McCutchan, California (1965).
[23] F. M. Freeman, *Analyst* **80**, 520 (1955).

Key Word Index—Scopolia parviflora; Solanaceae; tropane alkaloids; tissue culture; synthesis.