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STUDIES ON THE FORMATION AND GROWTH OF *UNCARIA ELLIPTICA* TISSUE CULTURE

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ABSTRACT.—The immature young leaves of *Uncaria elliptica* were found to be effective explants for the formation of callus culture. The initiation, growth, and viability of the calli were better when grown in one-fifth strength of Murashige and Skoog Medium. The order of effectiveness of the growth regulators for callus initiation was: 2,4-dichlorophenoxyacetic acid > α-naphthaleneacetic acid > indole-3-butyric acid (IBA) for auxins, and 6-γ,γ-dimethylallylamino purine > kinetin > N6-benzyladenine for cytokinins. Different combinations of kinetin and IBA showed synergistic effect on callus initiation. A mixture of 5 or 10 μM IBA with 10 μM of kinetin was optimal for callus maintenance. (−)-Epicatechin was found to be present in these cultures at a level comparable to that of the source plant.

Uncaria elliptica R. Br. exG. Don is a tropical woody climber belonging to the family Rubiaceae. It is reported to have been used in folk medicine and as a source of tannins (1). The presence of a large amount of the pharmacologically active flavonoid glycoside rutin and moderate amount of (−)-epicatechin in the various parts of the plant has earlier been reported (2–4). Rutin, a flavonol-3-glycoside, and its hydroxyethyl derivatives have been marketed as drugs such as "Venoruton" and "Paroven" for the treatment of vascular diseases in man (5). (−)-Epicatechin is a diastereoisomer of (+)-catechin, the later being marketed as drugs called "Catergen" and "Cianidanol-3" (6).

In view of the pharmacological importance of these flavonoids and the potential biotechnological application of plant tissue culture for the production of useful biochemicals (7), we have carried out empirical investigations with the long-term objective of establishing a plant tissue culture model or system whereby controlled production of the useful flavonoids could be achieved. Callus induction and maintenance are prerequisite for successful in vitro culture (8). Several important considerations such as the selection of an explant, medium composition, and culture environment can be different even for closely related species (9). Thus, in our present report, the selection of explant source and the effects of several auxins, cytokinins, and the use of several dilutions of Murashige and Skoog (MS) medium (10) on the initiation and growth of *U. elliptica* callus culture are described. The calli were also analyzed for their flavonoid content. This is the first report on the callus culture of the *Uncaria* genus of the Rubiaceae family.

MATERIALS AND METHODS

EXPLANT SOURCES.—Plant materials were collected from a 3-year-old field grown *U. elliptica* plant. Several plant parts were examined as potential sources for further studies: (i) apical buds, (ii) below apical, young leaves, (iii) intermediate, mature leaves, (iv) basal, old leaves, (v) below apical, young twigs, (vi) intermediate, mature stems, and (vii) basal, old stems.

TISSUE CULTURE.—The respective plant organs were excised and surface-sterilized by soaking in 70% EtOH for 2 min, washed with sterile deionized H_2O followed by 15 min incubation with occasional agitation in 6% calcium hypochlorite solution, and finally rinsed thoroughly with sterile deionized H_2O. The leaves were cut into sections of an area of about 0.5 cm^2 each. The stems were cut into segments of length 1 cm each, and the buds were either halved or cut into thin slices. These were then used as explants to initiate callus formation. Tissue culture was performed in 90-mm plastic sterile Petri dishes (Sterilin, Feltham, England) containing 25 ml of the nutrient medium solidified with 0.8% Phytagar (Gibco, Grand Island, NY). The Petri dishes were sealed with parafilm to avoid desiccation and kept in a growth chamber in the dark at 25 ± 1°. The calli formed after 30 days of culture were subcultured every 5 weeks, and the conditions for callus initiation were maintained.

NUTRIENT MEDIA AND GROWTH REGULATORS.—The growth regulators, namely 2,4-dichlorophenoxyacetic acid (2,4D), α-napthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 6-γ,γ-dimethylallylamino purine (2-IP), kinetin, and N6-benzyladenine (BA) were pure crystalline samples purchased from Gibco. Preliminary studies were carried out whereby several diluted concentrations of the basal medium of Murashige and Skoog (MS) (10) were used. The dilutions studied were half-strength (0.5 MS), one-fifth strength (0.2 MS), and one-tenth strength (0.1 MS) in the presence of 1% sucrose. Investigations based on explant and callus viability, rate of callus initiation, and growth, using one concentration for each growth regulator, indicated that the 0.2 MS dilution was the best among the four media tested. Thus, this dilution of the MS basal medium was used for the subsequent experiments.

The 0.2 MS medium was supplemented separately with individual auxins (2,4D, NAA, and IBA) and cytokinins (kinetin, BA, 2-IP) at a range of six different concentrations (0, 0.1, 1, 5, 10, 20 M) in order to study the effect of each growth regulator on callus initiation and maintenance. The pH of the media was adjusted to 5.7 prior to autoclaving at 1.10 Kgf/cm^2 for 15 min at 120°.

The effect of the combined use of an auxin (IBA) and a cytokinin (kinetin) on the callus initiation was examined. Different concentrations of both regulators were employed to form a grid of 30 ratios, and data were based on fresh wt measurement after 30 days of culture. The maintenance of culture using different IBA and kinetin combinations was also studied.

FLAVONOID ANALYSIS.—The calli were harvested and freeze-dried, and the flavonoids were extracted by MeOH as described earlier (2). The extracts were then analyzed by tlc, hplc, and nmr as described earlier (4, 22, 25).

RESULTS AND DISCUSSION

Buds and young twigs of *U. elliptica* did not provide a good explant source because they turned brown and eventually died after isolation and culture. Soaking the explants in sterile deioinized H_2O (11) and 1% ascorbic acid (12) or transferring the explants to a fresh medium (13) after a short time interval (2 days) did not increase tissue viability or help prevent tissue blackening.

Browning was more pronounced in the undiluted high salt MS media as previously noted in apple plant culture (14). In agreement with the general observation that stem sections of woody species often make very poor explants and that young tissues are more suitable than mature ones (15), the slow-to-zero rate of callus formation and higher rate of contamination ruled out mature stems, mature leaves, old stems, and old leaves as the explant of choice. The most appropriate explant was therefore the immature young leaves which were used effectively throughout the subsequent experiments in this study.

Seasons of the year are known to affect the efficiency of callus initiation and production in some plant species such as tea (16). However, there was no significant variation in the rate of callus formation from the young leaves of *U. elliptica* throughout the year. This may be due to the fact that this plant is found in the tropics where there are only wet and dry seasons. We found that there was higher rate of contamination during the rainy period than the hot, dry season, which is in agreement with observations for the tea calli (16). Although the high salt MS medium has been used with great success for many plant species (8,15), we found that the initiation, growth, and viability of the calli were better with 0.2 MS medium. Similar achievements of better growth of culture by salt dilution of culture media have been reported in a number of studies by other workers (15, 18–20). There is no apparent polarity involved in callus formation, and explant orientation is not important, as opposed to *Gasteria verrucosa* (17), *Haworthia faciata* (17), and *Camellia sinesis* (16).

2,4D was the most effective callus inducer, followed by 2-IP. Callus production was observed in all the concentrations of 2,4D used (Table 1). NAA and IBA were less effective. Kinetin and BA were ineffective in initiating callus. The results showed that the order of effectiveness was 2,4D > NAA > IBA for auxins and 2-IP > kinetin > BA for cytokinins. In the absence of growth regulator, there was no formation of callus. The use of single auxins, expecially NAA and IBA, resulted in frequent rhizogenesis of

TABLE 1. Effects of Different Concentrations of 2,4-Dichlorophenoxyacetic acid (2,4D), α-Napthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), and 6-γ-γ-Dimethylallylamino purine (2-IP) on the Amount of Callus Formation (mg wet wt per Petri dish ± SD, $n = 5$) after 35 Days of Culture.

Concentration (μM)	Growth (mg wet wt/Petri dish)			
	Growth regulator			
	2,4D	NAA	IBA	2-IP
0	—	—	—	—
0.1	4 ± 3	—	—	—
1.0	17 ± 8	5 ± 2	—	—
5.0	60 ± 22	19 ± 10	3 ± 1	4 ± 1
10.0	57 ± 10	17 ± 11	5 ± 3	22 ± 3
20.0	80 ± 18	8 ± 2	4 ± 3	44 ± 8

negatively geotropic adventitious root structures. Rhizogenesis was not desirable as it somehow decreased and/or arrested further growth and development of callus in the subsequent subculture. Subculturing in 0.2 MS medium supplemented with either a single auxin or cytokinin led to a decreased and/or cessation of callus growth. For the case of 2,4D, a similar phenomenon has been observed and was attributed to the plant cell's "decreased responsiveness" to the auxin (21). Newly mature stems did respond to the effect of 2,4D. However, studies using our established tlc (22) and hplc (4) procedures indicated that 2,4D strongly suppressed flavonoid formation; therefore the maintenance of culture in the presence of 2,4D was not further pursued.

Kinetin has earlier been reported to stimulate the biosynthesis of flavonoids (23). In view of this, a combination of auxin (IBA) and cytokinin (kinetin) was used to study their interaction. The result suggested some kind of synergistic effect of kinetin and

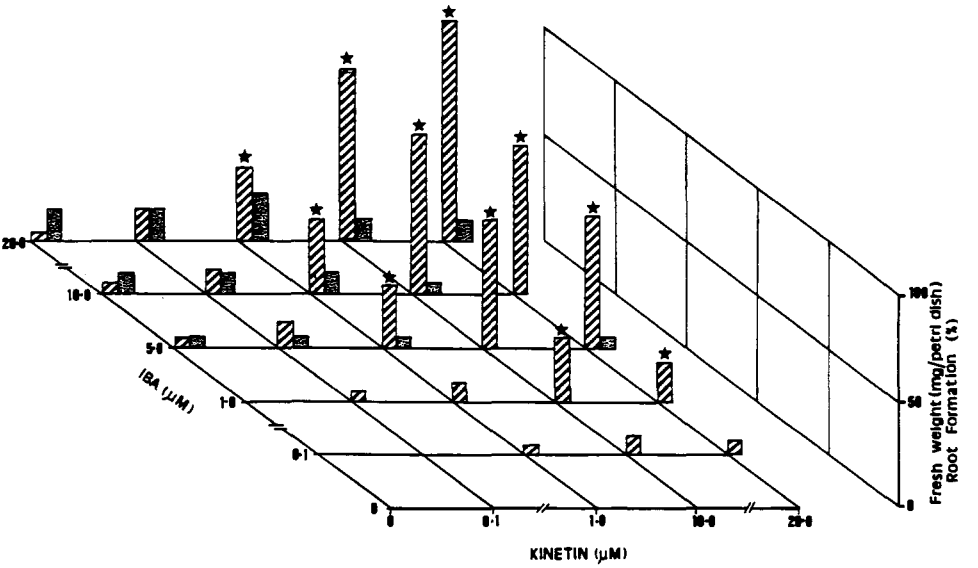

FIGURE 1. Effect of different concentrations of kinetin and indole-3-butyric acid (IBA) combination on the induction of callus formation (mg fresh wt per Petri dish, ▨) and percentage of calli with root formation (%, ▩) after 30 days of culture of young leaf of *Uncaria elliptica*. The combinations marked (★) were selected for maintenance studies.

IBA on callus initiation. When IBA was used alone, callus induction was poor (Table 1) and kinetin was ineffective (data not shown). However, when they were used in combination, a much more effective initiation of the callus was obtained (Figure 1). Therefore, we have found that the effectiveness of auxin and cytokinin could be very different depending upon whether it is used singly or in combination. The calli induced were pale yellow and nodular, with a significant part of the tissue growing downward into the media. IBA appeared to stimulate rhizogenesis, while kinetin depressed it (Figure 1).

Figures 2 and 3 show the pattern and rate of growth of the explant-free calli for the

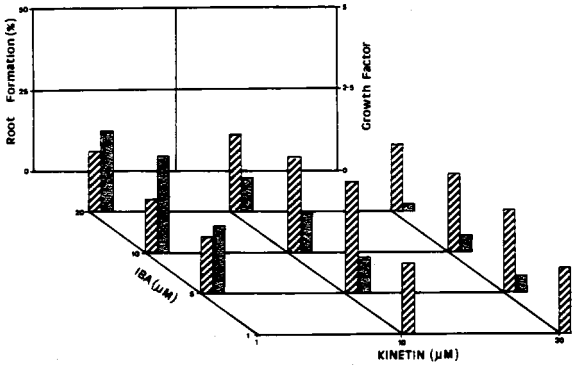

FIGURE 2. Effect of different concentrations of kinetin and idole-3-butyric acid (IBA) combination on the growth (growth factor, ▨) and percentage of calli with root formation (%, ▩) of explant-free calli of young leaves of *Uncaria elliptica* measured at the end (35th day) of second subculture. Growth was expressed as an increase factor defined as the g fresh wt of tissue at harvest per g fresh wt of the inoculum.

second and seventh subculture, respectively. The selection of callus for subculturing was based on the rate and uniformity of growth and the qualitative presence of flavonoids screened by tlc methods (22). The pattern and the rate of growth of the culture were more or less established by the fifth subculture. Although a relatively high level of auxin and cytokinin was essential for the initiation of calli (Figure 1), lower auxin levels could sustain good growth during subculture (Figures 2 and 3), and similar

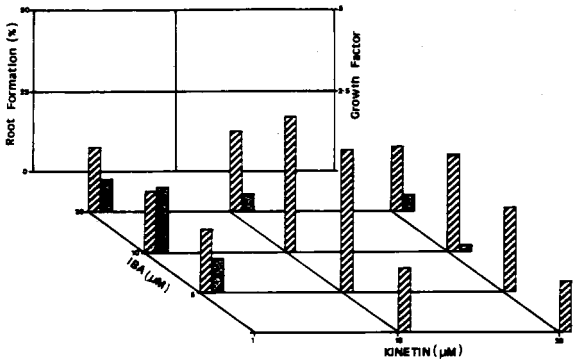

FIGURE 3. Effect of different concentrations of kinetin and indole-3-butyric acid (IBA) combination on the growth (growth factor, ▨) and percentage of calli with root formation (%, ▩) of young leaves of *Uncaria elliptica* callus culture, measured at the 35th day of the seventh subculture. Growth definition was expressed as an increase factor defined as the g fresh wt of tissue at harvest per g fresh wt of the inoculum.

reports have been given by other workers (24). The concentration of growth regulator for the optimum maintenance of callus was 5 or 10 μM of IBA combined with 10 μM of kinetin. The percentage of callus forming root structure decreased as the number of passage increased.

Hplc analysis (4) of the flavonoid content at the end of the seventh subculture showed the presence of (−)-epicatechin at a level (Table 2) approaching that of the

TABLE 2. (−)-Epicatechin Content (g % dry wt ± SE, $n = 5$) in the Calli Grown in 0.2 MS Medium Supplemented with Various Kinetin and Indole-3-butyric Acid (IBA) Combinations at the End of Seventh Subculture.

Kinetin (μM)	IBA (μM)			
	1.0	5.0	10.0	20.0
	(g % dry wt)			
1.0	—	1.8 ± 0.2	0.9 ± 0.2	0.4 ± 0.1
10.0	1.8 ± 0.3	1.9 ± 0.2	1.3 ± 0.2	0.8 ± 0.1
20.0	2.4 ± 0.1	2.0 ± 0.2	1.5 ± 0.3	1.1 ± 0.2

source plant (2–4). Increasing concentrations of kinetin appeared to result in a moderate increase in the amount of (−)-epicatechin. There was no significant fluctuation in the level of (−)-epicatechin in the subsequent analysis of the calli from the next two subcultures, indicating that the culture has reached a "stable" state for the flavonoid biosynthesis. However, rutin, the major flavonoid found in the source plant, was not detected under the present culture conditions. Nevertheless, our tissue culture has shown the potential of flavonoid biosynthesis. Further studies in order to obtain enhancement of flavonoid production are currently being carried out.

ACKNOWLEDGMENTS

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