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The Effects of Some Additives on Growth and Solasodine Production by Callus Cultures of *Solanum* spps.

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ABSTRACT

The established pharmaceutical steroid products derived from plants, mainly include the corticosteroids, contraceptives, sex hormones and anabolic agents. The majority of the steroid drugs are made by partial synthesis. The commercially valuable precursors are diosgenin, stigmasterol, cholesterol, and solasodine, Solasodine is obtained from some *Solanum* spps. which are potential raw materials for steroid drug manufacturing. In order to study the production of solasodine by callus cultures of *Solanum,* some species including (1) *S. nigrum* (native in Indonesia), (2) *S. nigrum* (native in Thailand), (3) *S. verbascifolium* (native in Thailand), (4) *S. khasianum* (native in India) were cultivated *in vitro* on RT medium containing 2, 4-D 1 mg/l, IBA 1 mg/l and BAP 0.6 mg/l in dark and light conditions. The additives were cholesterol 50 mg/l, cholic acid 10 mg/l, phloroglucinol 50 mg/l or coumarin 50 mg/l. These chemical compounds exerted certain effects on the growth and solasodine production of the tested *Solanum* cultures.

INTRODUCTION

Solanum species are known to contain a steroidal alkaloid, solasodine, which is used as the starting material for the production of steroid drugs (Mann, 1978). Several glycosides of solasodine, complexed with different sugar moities, occurred in *Solanum* species (Prelog and Jeger, 1960). Solasodine is a nitrogen analogue of diosgenin (Figure 1) and like diosgenin, can easily be converted to 16-dehydropregnenolone. It is currently promising steroid precursor, substituting for *Dioscorea* species. Uncertainty of the availability of *Dioscorea* tubers caused shortage of diosgenin (Asolkar and Rawat, 1979).

In plant cell cultures of various *Solanum* species, glycoalkaloids and alkamines belonging to the spirosolane- or solanidane- type alkaloids have been detected (Heble *et al.*, 1968). Production of solasodine in *in vitro* cultures have been reported from callus cultures of *Solanum acculeatissimum* (Kadkade and Madrid, 1977), undifferentiated callus tissue of *S. verbascifolium* L. (Jain and Sahoo, 1981), callus and suspension cultures of *S. laciniatum* Ait. (Chandler and Dodds, 1983), differentiated structures (shoots and/or roots) and callus of *S. nigrum* L. (Bhatt *et al.*, 1983).

The callus cultures of *Solanum* spps. were initiated and treated with some additives. The effects of these additives on growth of *Solanum* callus cultures and solasodine production were determined for the possibility of producing solasodine by *in vitro* cultures.

MATERIALS AND METHODS

Tissue culture

Seeds of *S. nigrum* native in Indonesia, *S. nigrum* native in Thailand, *S. verbascifolium* native in Thailand and *S. khasianum* native in India (Figure 2) were aseptically germinated on filter paper bridges soaked with water. Seven

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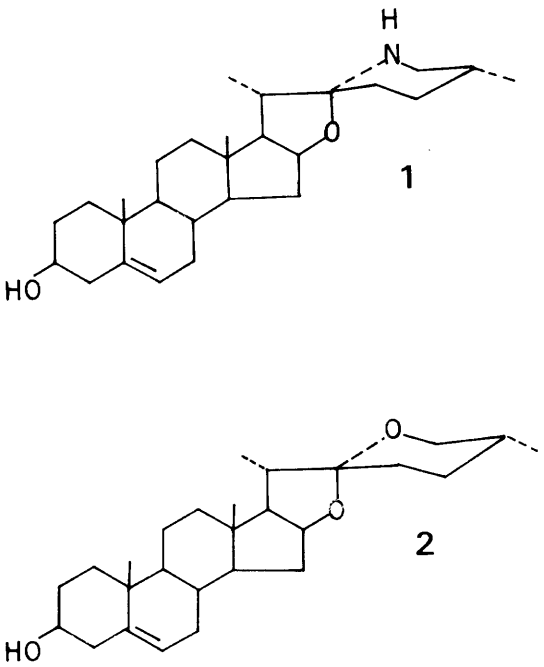

Figure 1 Structure of : (1) Solasodine, (2) Diosgenin

to fifteen-day old plantlets were cultivated *in vitro* on modified solid Revised Tobacco (RT) medium (Staba, 1969) containing 1 mg/l 2, 4-dichlorophenoxyacetic acid and 1 mg/l indolebutyric acid plus 0.6 mg/l benzylaminopurine in dark and light conditions. Calli, obtained within two to three weeks, were continuously cultivated for few passages until homogeneous cultures were obtained as shown in Figure 3. These callus cultures were then treated with the following additives: (1) 50 mg/l cholesterol (2) 10 mg/l cholic acid (3) 50 mg/l phloroglucinol (4) 50 mg/l coumarin. Growth rate was measured each week for a period of ten weeks. Weights of ten replicates of callus cultures were measured for each experiment. Comparison study on the growth of these *Solanum* cultures was done and the maximum growth index of each *Solanum* was also determined. The air-dried callus tissues were collected for analysis of solasodine.

Determination of solasodine

The quantity of solasodine was determined by using the modified method of Crabbe and Fryer, 1982. The sample of calli cultivated in different media for determination of their growth indices were collected, dried at 60-70°C, and ground into pieces by crushing and passing through a 60-mesh sieve. Three samples were pooled randomly from each test callus culture. Sample weighing 0.600 g was refluxed with 70 ml of methanol for one hour and then filtered. The filtrate and residue washings were made up to 100 ml with methanol and evaporated to dryness. The residue was hydrolyzed with 7.5 ml of 1 N HCl on a steam bath at 80°C for 4 hours. The solution was cooled and neutralized with 1 N NaOH and 5 ml of glacial acetic acid was added. The hydrolysate was transferred into a 25-ml volumetric flask and adjusted to the mark with water.

Eight milliliters of hydrolysate was transferred to a separatory funnel and 5 ml of sodium acetate acetic acid buffer pH 4.7 plus 1.0 ml of methyl orange was added. Five ml of chloroform was added and the resulting mixture shaken for 4 minutes. The chloroform layer was separated and dried over anhydrous sodium sulfate. The same method was carried out with the reference solution but without methyl orange. The solasodine content was determined colorimetrically and the maximum absorbance scanned and read in a Hitachi, model U-3200 spectrophotometer at 424 nm.

Calibration curve was prepared from 2.0 to 16.0 μg of solasodine per 5 ml of chloroform. The regression equation was as follows : $y = 0.0118 + 0.0024x$ ($r = 0.9752$).

RESULTS AND DISCUSSION

Phytohormones and additives affecting growth index

The phytohormonal concentration of auxins and cytokinins affect the development of *in vitro* tissue morphology. In media containing 0.5 to 2 mg/l of naphtholeneacetic acid or indolebutyric acid plus benzylaminopurine 0.01 to 2 mg/l, both shoots and roots were formed. Increasing the concentration of naphthaleneacetic

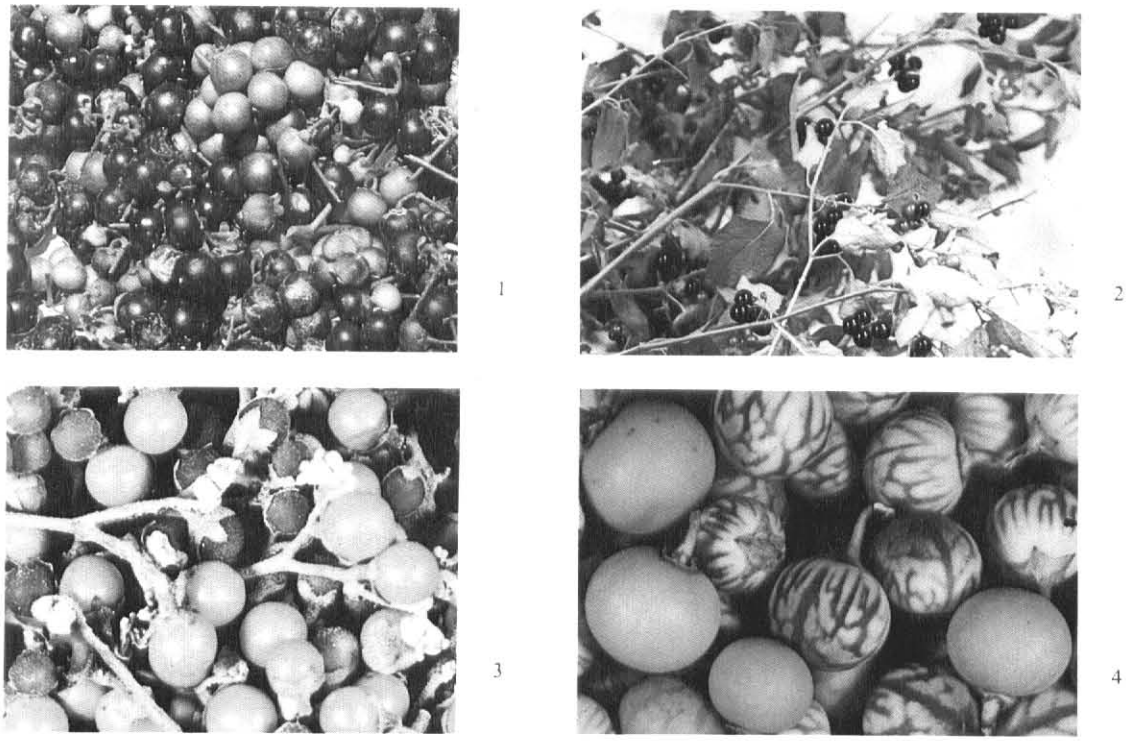

Figure 2 Fruits of *solanum* spps., (1) *S. nigrum* (native in Indonesia), (2) *S. nigrum* (native in Thailand), (3) *S. verbascifolium* (native in Thailand), (4) *S. khasianum* (native in India)

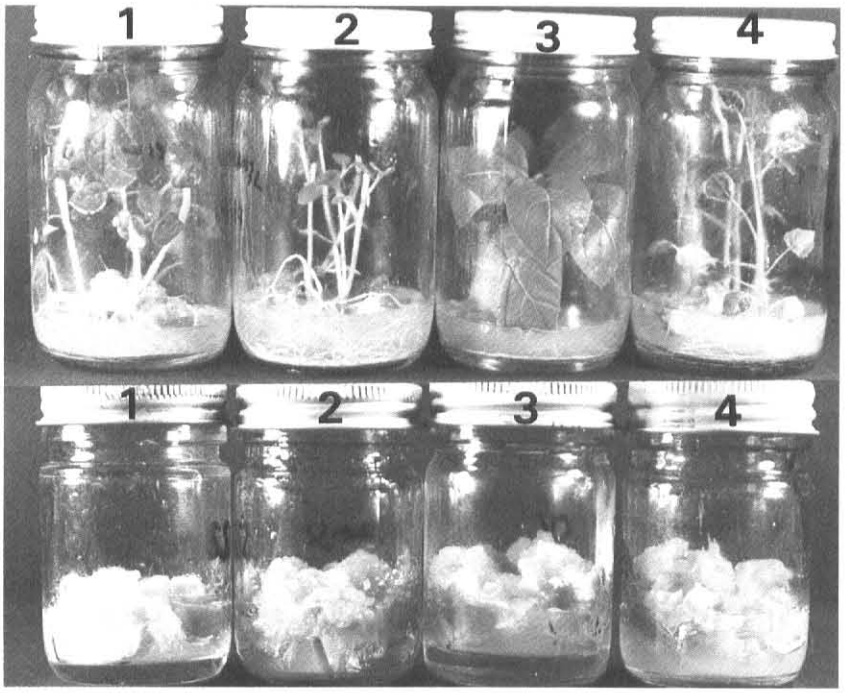

Figure 3 *In vitro* plantets and homogeneous callus cultures of *Solanum spps.*, (1) *S. nigrum* (native in Indonesia), (2) *S. nigrum* (native in Thailand), (3) *S. verbascifolium* (4) *S. khasianum*

acid or indolebutyric acid resulted in the proportional increase of root formation, and the combination of naphthaleneacetic acid or indolebutyric acid with benzylaminopurine at concentrations of 2 mg/l to 5 mg/l produced the fast proliferating callus cultures. In media containing 2, 4-dichlorophenoxyacetic acid in the range of 0.5 to 2 mg/l plus suitable cytokinins, homogeneous callus cultures which grow fast in both dark and light condition were obtained under 2,000 lux light condition, slightly green calli were observed.

The rate of growth of the *Solanum* callus cultures was measured in terms of growth index (GI = final fresh weight of callus - initial fresh weight of callus / initial fresh weight of callus). The results of their growth in folds per week (10 replicates per experiment) for the period of ten weeks are given in figure 4. The growth index for each week for the period of 10 weeks of *S. nigrum* (native in Indonesia), *S. nigrum* (native in Thailand), *S. verbascifolium* and *S. khasianum* cultivated on media containing no additive or either one of the additives, 50 mg/l cholesterol, 10 mg/l cholic acid, 50 mg/l phloroglucinol, 50 mg/l coumarin, respectively, are shown in figure 4. Mostly, the callus cultures grew faster to their maximum weight in the dark than in the light.

In the dark, the tested additives, 50 mg/l cholesterol, 10 mg/l cholic acid or 50 mg/l phloroglucinol accelerated growth of most of the *Solanum* species. In light condition, cholic acid accelerated growth of *S. nigrum* (native in Indonesia) and *S. verbascifolium* to their maximum growth index within 7 and 6 weeks, respectively. Other *Solanum* spp. proliferated in media containing only combination of phytohormones better than in media with additives. Addition of the growth inhibitor coumarin to the medium, decreased the proliferation rate of callus tissues in both dark and light conditions.

Table 1 Comparisons of maximum growth index for ten weeks and production of Solasodine of three *Solanum* species; *S. nigrum*, native in Indonesia (In), *S. nigrum*, native in Thailand (Th), *S. verbascifolium*, *S. khasianum*, cultured on RT solid medium containing 1 mg/l 2, 4-D and 1 mg/l IBA plus 0.6 mg/l BAP, or plus the following additives, 50 mg/l cholesterol, 10 mg/l cholic acid, 50 mg/l phloroglucinol or 50 mg/l coumarin.

Solanum spps.	No Additives		+ Cholesterol		+ Cholic acid		+ Phloroglucinol		+ Coumarin	
	Max GI folds/wk	Solasodine μg/g dw	Max GI folds/wk	Solasodine μg/g dw	Max GI folds/wk	Solasodine μg/g dw	Max GI folds/wk	Solasodine μg/g dw	Max GI folds/wh	Solasodine μg/g dw
In dark condition										
S. nigrum (In)	8.8/6	254 ± 7.5	15.3/6	376 ± 72.5	13.0/6	221 ± 45.2	8.1/6	443 ± 20.7	3.4/6	148 ± 10.1
S. nigrum (Th)	7.4/4	411 ± 24.8	6.9/7	258 ± 3.2	7.3/3	200 ± 7.5	11.4/3	433 ± 46.3	6.4/5	370 ± 14.0
S., verbascifolium	4.6/9	469 ± 85.1	7.4/8	659± 18.0	8.8/9	699 ± 17.5	8.6/4	691 ± 3.9	5.5/8	686 ± 1.7
S. khasianum	10.3/7	405 ± 61.4	10.1/10	899 ± 30.4	12.5/8	729 ± 28.7	15.3/10	801± 15.2	5.1/10	547 ± 1.1
In light condition										
S. nigrum (In)	7.5/7	266 ± 8.6	9.7/7	245 ± 20.8	13.1/7	220 ± 28.7	8.5/8	200 ± 9.3	5.5/7	479 ± 1.9
S. nigrum (Th)	11.9/7	350 ± 23.7	6.1/3	495 ± 31.2	4.9/7	280 ± 16.2	8.2/4	613 ± 15.2	7.2/4	542 ± 0.6
S. verbascifolium	5.7/6	433 ± 9.7	4.6/10	669 ± 1.1	8.1/6	689 ± 0.0	7.7/4	699 ± 3.9	5.7/7	643 ± 21.4
S. khasianum	17.3/10	438 ± 62.5	14.6/10	768 ± 41.7	11.6/8	762 ± 38.9	12.3/9	884 ± 72.1	6.8/10	691 ± 3.9

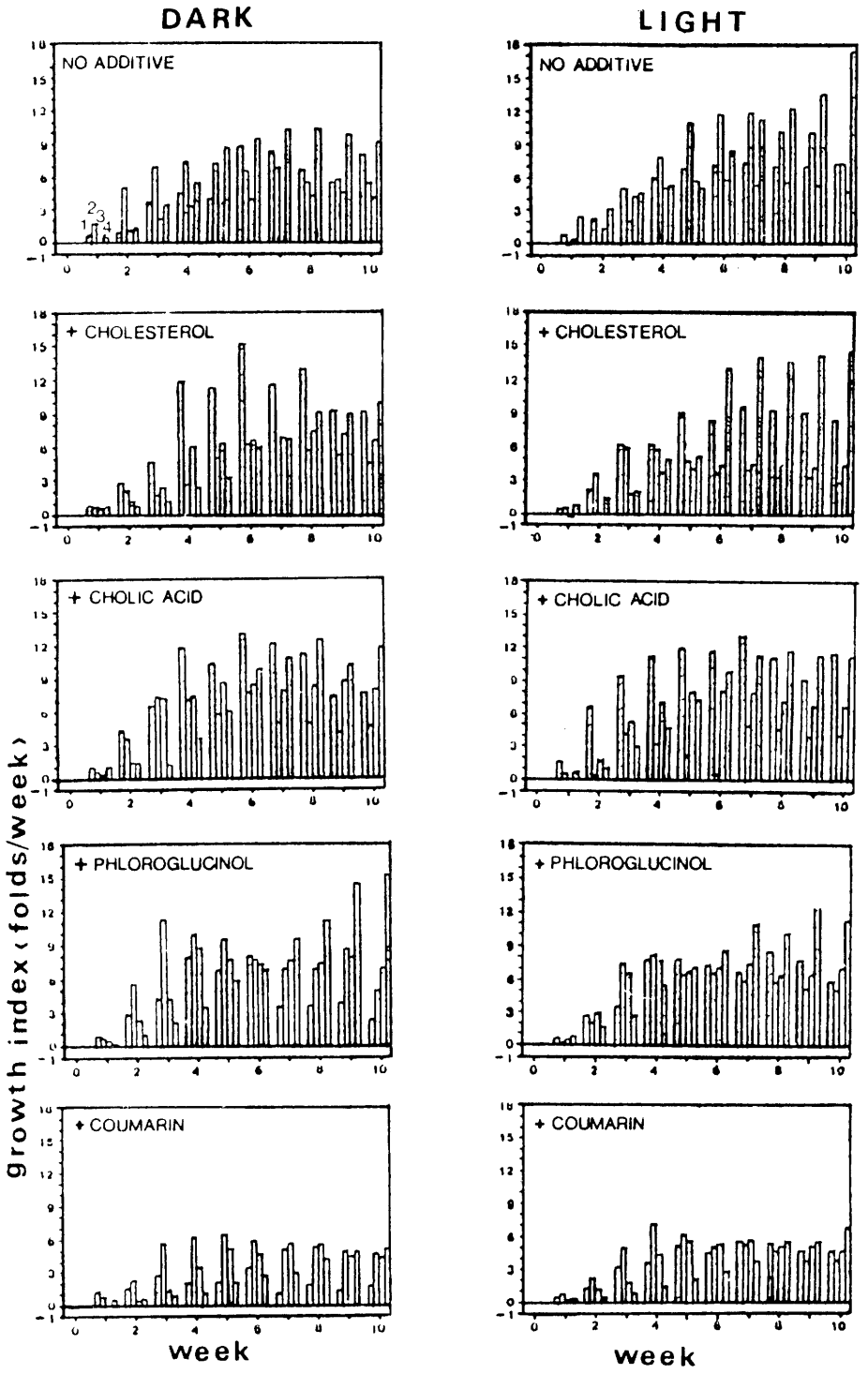

Figure 4 Growth indices of *Solanum* species cultivated on media with none and plus additives, column 1 = *S. nigrum* (native in Indonesia), column 2 = *S. nigrum* (native in Thailand), column 3 = *S. verbascifolium* and column 4 = *S. khasianum* were compaired in folds of initial subculturing weight weekly for the period of ten weeks in dark and light condition.

Both varieties of *S. nigrum* proliferated to maximum weight within 3 to 8 weeks. *S. verbascifolium* proliferated to maximum weight within of 4 to 10 weeks and *S. khasianum* proliferated to maximum weight within 7 to 10 weeks. *S. khasianum* cultivated on medium without additive in light condition gave the highest maximum growth index.

Additives affecting solasodine content:

The additives in the media affected the production of solasodine but not the morphogenetic of the homogeneous callus cultures which are controlled by the phytohormone combination of 1 mg/l 2, 4-dichlorophenoxyacetic acid and 1 mg/l indolebutyric acid plus 0.6 mg/l benzylaminopurine. Solasodine production by *Solanum* species cultivated on media with or without additives are shown in table 1. In medium with no additive and cultivated in dark condition, *S. verbascifolium* produced the highest concentration of solasodine (469 + 85.1 μg/g), however in light condition, *S. khasianum* produced the highest concentration (438 + 62.5 μg/g). Addition of cholesterol or phloroglucinol accelerated solasodine production of *S. verbascifolium* and *S. khasianum* better than both varieties of *S. nigrum*.

CONCLUSION

The effect of additives on the growth of callus cultures of *Solanum* varied according to the species and varieties of *Solanum*, combination of phytohormones, type of additives and period of subculturing. The *Solanum* callus cultures proliferated well in media with and without additives, with the exception if the medium plus coumarin. The callus cultures of *S. khasianum* has lower rate of proliferation than the other species, but the highest growth indices were obtained in both dark (medium plus phloroglucinol, 15.3 folds in 10 weeks) and light (medium without additive, 17.3 folds in 10 weeks) condition. Also, *S. khasianum* produced the highest amount of solasodine in both dark and light conditions. In medium containing cholesterol 899 + 30.4 μg/g of solasodine was produced and in medium with phloroglucinol 884 + 72.1 μg/g solasodine was produced. The results obtained are fundamental data for further investigation of the possibility to produce steroid precursors by *in vitro* cultures.

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