CONTROL OF ECDYSTERONE BIOGENESIS IN TISSUE CULTURES OF *TRIANTHEMA PORTULACASTRUM*

G. A. RAVISHANKAR and A. R. MEHTA

Department of Botany, The M. S. University of Baroda, Baroda-390 002. India.

ABSTRACT.—Seedling callus of *Trianthema portulacastrum* was established and screened for phytoecdysone by a bioassay on the larvae of house-fly *Musca domestica*. Methods for detection, extraction, separation, and estimation of ecdysterone are described. Effects of various phytohormones and different sucrose levels on growth and ecdysterone production by callus tissues were examined. Naphthaleneacetic acid supported maximum growth of callus and 2,4-dichlorophenoxyacetic acid (2,4-D) yielded maximum ecdysterone. Increasing kinetin concentrations were inhibitory to ecdysterone biogenesis. A combination of 2,4-D with kinetin enhanced growth and ecdysterone production. Gibberellic acid was antagonistic to callus growth enhanced by 2,4-D and kinetin; however, it stimulated ecdysterone biogenesis. The present study indicates the feasibility for increased ecdysterone production in callus cultures as compared to the intact plant.

The possibility of utilizing insect moulting hormone as third generation insecticide arose from the studies of Williams (15, 16). He demonstrated pathological effects of excessive ecdysone on insects which resulted in enhanced metamorphosis in larvae leading to the formation of abnormal adults. Though plants are known to produce more insect moulting hormone than the larvae themselves (2, 6, 13, 14), their utility as insecticides is yet to be demonstrated.

Ecdysterone, a widely occurring phytoecdysone, has been reported in a variety of plants which include *Polypodium vulgare* (6), *Sida carpinifolia* (9), *Achyranthes aspera, Trianthema portulacastrum, Sesuvium portulacastrum, Gomphrene celosioides* (1), and *Polypodium virginianum* (3). Ecdysterone has also been detected in tissue cultures of *Achyranthes* (4, 5, 12).

Ecdysterone and its analogues are used as chemosterilants, and are known to stimulate protein synthesis in insects and mammals (1). Ecdysterone is also reponsible for an increase in the number of lysosomes in the midgut epithelial cells of flesh-fly larvae (10). Though many functions are attributed to this compound in animals, its role in plants is still obscure. A rich source of phytoecdysone would facilitate studies on its physiological role in plants. With this view, tissue cultures of *Trianthema portulacastrum* L. were established and assayed for ecdysterone. The present communication deals with detection, extraction, isolation, and estimation of ecdysterone and effects of various phytohormones and sucrose levels on ecdysterone biogenesis in callus cultures of *T. portulacastrum.*

EXPERIMENTAL

INITIATION AND ESTABLISHMENT OF CALLUS CULTURES.—Seeds of *Trianthema*, surface sterilized with 0.1% (w/v) mercuric chloride, were germinated aseptically in petri dishes. Segments measuring 2–4 cm in length were excised from the seedlings and inoculated on Murashige and Skoog's (MS) medium (8) containing 2% sucrose and supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (K). The callus, which developed within two weeks, has been maintained on the same medium by regular transfers every 4 weeks for the last two years (see fig. 1a).

EXTRACTION OF ECDYSTERONE.—Dry callus tissue weighing 1.0 g was Soxhlet-extracted with methanol for 24 hr. The extract was evaporated and the residue redissolved in 1–5 ml of ethanol. The ethanolic extract was used for both the bioassay and the isolation of the compound.

BIOASSAY.—Bioassay was conducted on the larvae of the common housefly, *Musca domestica*, as described by Banerji *et al.* (1).

ISOLATION AND ESTIMATION OF ECDYSTERONE.—Aliquots of crude extract and the authentic sample of ecdysterone were spotted on tlc plates (20 x 20 cm) coated with 0.25 mm silica gel G (E. Merk). Chromatograms were run at 25±2° in four different solvent systems—1. EtoAc-MeOH (100:15) R_f X 100=30.3; 2. $CHCl_3$-MeOH (9:1) R_f X 100=20.2; 3. $CHCl_3$-MeOH (1:9) R_f X 100=90.0; 4. $CHCl_3$-MeOH (1:5) R_f X 100=36.0. The plates were air dried and sprayed with 1N HCl. They were then heated in a hot air oven at 60° for 6–8 hr and viewed under shortwave uv light. The spots corresponding to authentic ecdysterone were marked and eluted in 3 ml methanol. The absorption spectrum of the isolated compound was compared with that of authentic ecdysterone. The standard curve of authentic ecdysterone was prepared from its eluted spots on tlc and their absorption at 241 nm. Quantitative estimation of the isolated compound was made from the standard curve.

STUDIES ON GROWTH AND ECDYSTERONE BIOGENESIS.—Stock cultures were kept on hormone-free MS basal medium containing 2% sucrose for 15 days prior to inoculation so as to minimize any hormonal carry-over. Callus tissues weighing 300±10 mg were inoculated in 150 ml Erlenmeyer flasks containing 40 ml agar medium. Cultures were incubated for 30 days in light (3000 Lx) at 25±2°. The effects of various phytohormones and sucrose levels on growth and ecdysterone biogenesis in callus tissues were examined. The growth of the tissue was expressed in terms of fresh and dry weights, the latter being determined after the fresh tissue was dried in a hot air oven at 60° to constant weight.

RESULTS AND DISCUSSION

DETECTION AND ISOLATION OF ECDYSTERONE.—Three procedures were used to determine the presence of the active principle, ecdysterone. The first was bioassay. The second was a comparison of R_f of the isolated compound with an authentic sample of ecdysterone by use of tlc techniques. The final procedure involved the spectral analysis of the isolated compound.

The R_f's obtained for the isolated compound in the various solvent systems tested were identical to those of authentic samples in corresponding solvent systems. Of the four solvent systems tested, the second ($CHCl_3$-MeOH, 9:1) and third ($CHCl_3$-MeOH, 1:9) gave good resolution and were, therefore, adopted for isolation and estimation of the compound. Both the isolated compound and the authentic ecdysterone revealed a similar pattern of absorption with their absorption maxima at 241 nm.

EFFECT OF DIFFERENT AUXINS ON GROWTH AND ECDYSTERONE PRODUCTION.—Naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and 2,4-D were the three auxins tested for their effect on callus growth and ecdysterone production. Results presented in table 1 show that, of the three auxins, 0.2 ppm NAA supported

TABLE 1. *Effect of auxin and kinetin on growth of* Trianthema *callus and ecdysterone production.*[a]

Medium[b]	Fresh weight g/culture	Dry weight g/culture	% Ecdysterone	Ecdysterone μg/culture
A MS Basal medium (Control)	0.80134	0.0376	0.0039	1.5
B MS+0.2 ppm 2,4-D	1.5350	0.05405	0.01189	6.428
C MS+2.0 ppm 2,4-D	2.5635	0.09441	0.0349	33.0
D MS+10.0 ppm 2,4-D	0.75829	0.03577	0.0256	9.14
E MS+0.2 ppm NAA	3.7711	0 140762	0.01067	15.0
F MS+2.0 ppm NAA	3.3937	0.113420	0.00806	9.14
G MS+10.0 ppm NAA	2.5950	0.10270	0.00681	6.816
H MS+0.2 ppm IAA	0.97155	0.0590	0.0055	3.2
I MS+2.0 ppm IAA	0.82597	0.04295	0.00853	1.6
J MS+10.0 ppm IAA	0.69362	0.03970	0.00426	1.69
K MS+0.001 ppm K	2.7225	0.09572	0.0217	20.8
L MS+0.04 ppm K	2.8780	0.0841	0.009	7.5
M MS+0.4 ppm K	2.5308	0.10974	0.0047	5.2
N MS+2.0 ppm K	1.3822	0.05637	0.00369	2.07
O MS+2.0 ppm 2,4-D+0.4 ppm K	10.3873	0.3009	0.0085	42.85

[a]Data represent an average of 6 replicates.
[b]All media contain 2% sucrose.

maximum growth of callus. Increasing levels of NAA were inhibitory to growth as well as ecdysterone biogenesis. Though 2,4-D was less effective than NAA in supporting callus growth, it caused a marked increase in ecdysterone production (33 μg culture). NAA (0.2 ppm), which supported maximum growth of callus, yielded only 15 μg of ecdysterone per culture. IAA was least effective in supporting growth and ecdysterone biogenesis in *Trianthema* callus cultures.

EFFECT OF KINETIN ON GROWTH AND ECDYSTERONE PRODUCTION.—Of four concentrations of kinetin used, 0.4 ppm level supported maximum growth of the tissue. However, the ecdysterone production was maximum at low kinetin concentration and decreased with increasing doses of kinetin. It is important to mention that 0.001 ppm kinetin, which favored maximum ecdysterone production, also induced profuse rooting (see fig. 1b). Banerji *et al.*, (1) have shown that *Achyranthes aspera* roots contain more ecdysterone than stem and leaves. The increased level of ecdysterone obtained at 0.001 ppm kinetin level may be due to organized development of roots from the callus. Further studies are needed to establish any correlation between morphogenesis and ecdysterone biogenesis as has been suggested with certain other secondary metabolites (11).

In order to further increase biosynthetic potentiality of *Trianthema* callus tissues, a combination of 2 ppm 2,4-D and 0.4 ppm kinetin was tried. This boosted the ecdysterone level to 42.85 μg per culture. Subsequently the cultures were, therefore, grown on MS medium containing 2 ppm 2,4-D and 0.4 ppm kinetin (standard medium).

EFFECT OF GIBBERELLIC ACID (GA_3) ON GROWTH AND ECDYSTERONE PRODUCTION.—In comparison with the control, the medium containing 100 ppm GA_3 supported better growth and ecdysterone production (table 2). Though less effective for ecdysterone production, GA_3 at 50 ppm level supported better growth of the callus over the control as well as over that in 100 ppm GA_3 medium. GA_3 at 100 ppm level induced rooting in callus tissues (see fig. 1C). Similar higher ecdysterone production associated with root differentiation was observed earlier in low kinetin medium. Standard medium into which 100 ppm GA_3 had been incorporated enhanced the ecdysterone production more than GA_3 alone (50 or 100 ppm), both in terms of total yield and on a percent basis. The presence of GA_3 in the auxin-kinetin-containing medium reduced the growth markedly. Clearly, GA_3 stimulated ecdysterone biogenesis, but antagonized growth promotion by auxin and kinetin.

EXPLANATION OF FIGURE 1A, 1B AND 1C

FIGURE 1a. *Seedling callus of* T. portulacastrum *grown on MS medium containing 2% sucrose and supplemented with 2,4-D and K.*

b. *Root differentiation in callus of* T. portulacastrum *on MS medium containing 2% sucrose and supplemented with 0.001 ppm K.*

c. *Root differentiation in the callus of* T. portulacastrum *grown on MS medium containing 2% sucrose and supplemented with 100 ppm* GA_3.

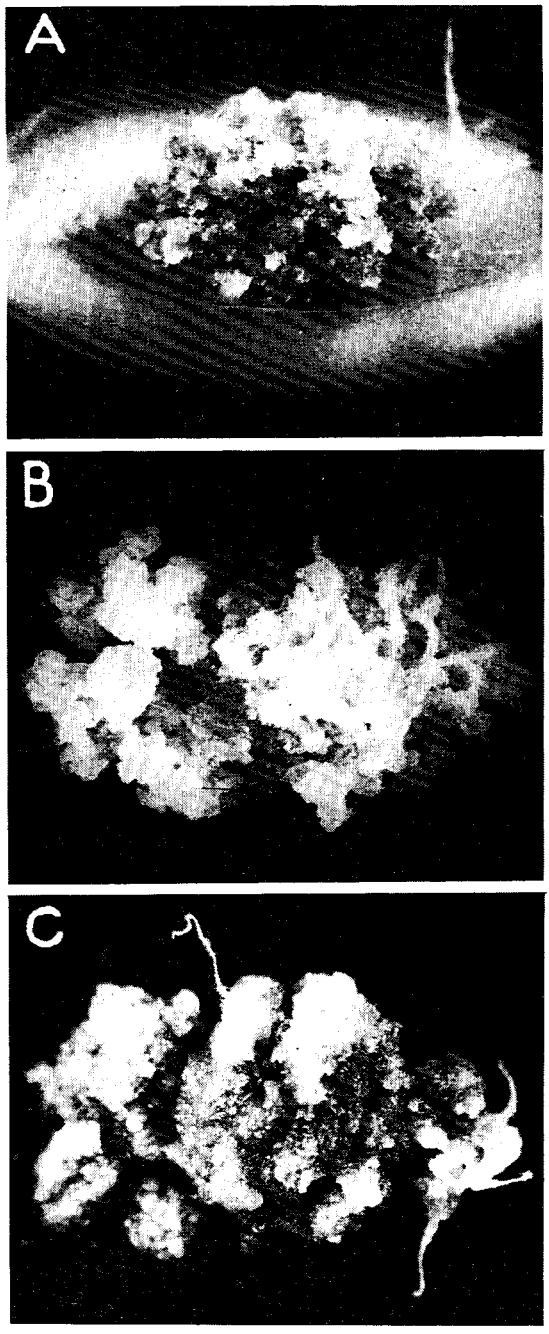

FIGURE 1A, 1B AND 1C

TABLE 2. *Effect of GA_3 and sucrose on growth of* Trianthema *callus and ecdysterone production.*[a]

Medium[b]	Fresh weight g/culture	Dry weight g/culture	% Ecdysterone	Ecdysterone μg/culture
P MS Basal medium (Control)	0.80134	0.0376	0.0039	1.50
Q MS+50 ppm GA_3	1.3448	0.06101	0.0040	2.44
R MS+100 ppm GA_3	0.9385	0.0506	0.0117	7.81
S MS+50 ppm GA_3+2 ppm 2,4-D+0.4 ppm K	1.2344	0.0662	0.0086	5.69
T MS+100 ppm GA_3+2 ppm 2,4-D+0.4 ppm K	4.4503	0.1481	0.0360	41.11
U MS+2 ppm 2,4-D+0.4 ppm K (standard medium)	10.3873	0.3009	0.0085	42.85
V MS standard medium with 1% sucrose	2.9991	0.0860	0.0097	8.4
W MS standard medium with 4% sucrose	4.7565	0.15275	0.00523	8.0

[a]Data represent an average of 6 replicates
[b]All media contain 2% sucrose except V and W.

EFFECT OF VARIOUS SUCROSE CONCENTRATIONS ON GROWTH AND ECDYSTERONE PRODUCTION.—This study was undertaken after the optimum hormonal requirement for growth and ecdysterone biogenesis was determined. As shown in table 2, sucrose at 2% level was most effective for increased growth and ecdysterone biogenesis. At 2% sucrose level, growth of the callus was 3.5 and 2.1 times higher than at 1% and 4% sucrose levels, respectively. Similarly ecdysterone content per culture at 2% sucrose level was 5.0 and 5.3 folds higher than at 1% and 4% sucrose concentrations, respectively.

PROGRESSIVE CHANGES IN GROWTH AND ECDYSTERONE CONTENT IN *Trianthema* CALLUS CULTURES.—It is clear from fig. 2 that when growth was measured in terms of dry weight, there was an initial lag phase till day 10; whereas a linear increase in growth took place from days 5 to 15, as measured in terms of fresh weight. The increase in fresh weight till day 10 was due to water accumulation rather than to an increase in dry mass. The lag phase was followed by a rapid increase in fresh and dry weights of callus from day 10 to 20. Thereafter, growth slowed down. A similar pattern of ecdysterone accumulation was noticed wherein the ecdysterone level tended to remain low during the lag phase. Concurrent with a spurt in growth during days 10 to 20, there was a rapid increase in the production of ecdysterone, *i.e.* from 3.5 μg per culture (day 10) to 48 μg per culture (day 20). Subsequently, the ecdysterone level declined. This suggested that ecdysterone production was restricted to the exponential growth phase in *Trianthema* callus cultures grown on standard medium.

Changes in the percentage of ecdysterone followed a pattern similar to that of changes in ecdysterone per culture (fig. 3).

It is claimed that plant cell cultures produce little, if any, of the particular secondary metabolites that are elaborated by the differentiated plant (7). However, Zenk *et al.* (17) showed that more anthraquinones could be produced by cell suspensions than by the differentiated plants by a factor of 9. In this communication, we have demonstrated that callus cultures produce more ecdysterone than the differentiated plants by a factor of almost 4. Such a system with enhanced

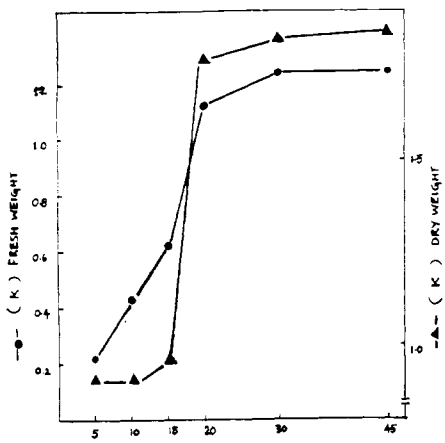

FIG. 2

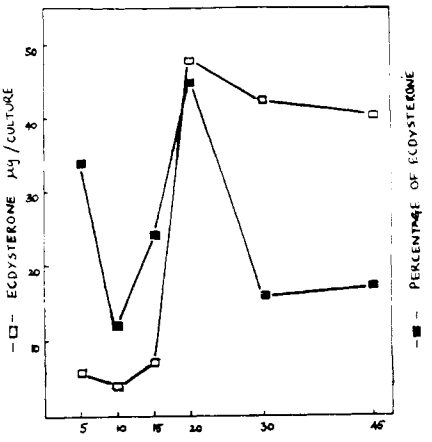

FIG. 3

FIGURE 2. *Progressive changes in growth of* T. portulacastrum *callus grown on standard medium.* — ● — (K) Fresh weight; — ▲ — (K) Dry weight.

FIGURE 3. *Changes in Ecdysterone content in* T. portulacastrum *callus grown on standard* medium. — □ — Ecdysterone µg/culture; — ■ — Percentage of ecdysterone.

biosynthetic potential could be utilized for the extraction of ecdysterone and to examine its effect on tissues which are unable to synthesize the compound. This might unravel the physiological and biochemical roles, if any, of this compound.

SUMMARY

Seedling callus of *Trianthema portulacastrum* was established and screened for phytoecdysone by a bioassay on the larvae of the housefly, *Musca domestica.* Methods for detection, extraction, separation, and estimation of ecdysterone are described. Effects of various phytohormones and different sucrose levels on growth and ecdysterone production by callus tissues are examined. Naphthaleneacetic acid supported maximum growth of callus, and 2,4-dichlorophenoxyacetic acid (2,4-D) yielded maximum ecdysterone. Increasing kinetin concentrations were inhibitory to ecdysterone biogenesis. A combination of 2,4-D with kinetin enhanced growth and ecdysterone production. Gibberellic acid was antagonistic to the growth enhanced by 2,4-D and kinetin; however, it stimulated ecdysterone biogenesis. The present study indicates the feasibility for increased ecdysterone production in callus cultures as compared to the intact plant.

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