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STUDIES ON GROWTH AND STEROID FORMATION IN TISSUE CULTURES OF *HOLARRHENA ANTIDYSENTERICA*

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Abstract—Callus cultures derived from the hypocotyl of germinated seedlings of the plant, *Holarrhena antidysenterica* showed an inherent lack of organ-forming ability when grown under the influence of a wide range of exogenous growth factors. Several sterols were isolated from the callus, of which the predominant ones were identified as cholesterol, 24-methylenecholesterol, 28-isofucosterol, sitosterol and stigmasterol.

INTRODUCTION

The metabolic events in plant tissues cultured *in vitro* are somewhat modified compared to the intact plant organs from which they are derived [1]. Such tissues are amenable to morphogenetic and biochemical manipulations and are therefore useful in studying the production of secondary products such as steroids and alkaloids and their physiological role. The function of steroids in animals has been extensively studied but their role in plants is still not fully elucidated owing to some limitations [2]. Plant tissue culture systems could be utilised to advantage to overcome some of the difficulties and studies were therefore initiated using the callus tissues derived from the plant *Holarrhena antidysenterica*, known to be a rich source of steroids [3].

RESULTS

Establishment of tissue culture. Callus induction occurred first at the cut ends of the hypocotyl segments and subsequently over the entire explant during four weeks of culture. The callus growth was enhanced by supplementing the medium with coconut milk, casein hydrolysate and meso-inositol. The tissue was non-pigmented, friable and could easily be grown as suspension culture.

Influence of growth factors on callus growth. Different concentrations of 2,4-D, IAA, NAA, NOA, OCPAA or PCPAA used singly and in combination with Kn (0.2 mg/l) in the medium did not result in the formation of either roots or shoots in the callus. Lower concentrations of 2,4-D (0.1 and 0.5 mg/l) were not favourable to proliferation of the tissue, whereas concentrations ranging from 1–2 mg/l were conducive for active growth of callus. Replacement of 2,4-D by IAA, NAA or NOA (0.1–2 mg/l) resulted in the inhibition of callus growth. Auxins like OCPAA or PCPAA were active in tissue growth comparable to that grown on 2,4-D medium. Addition of Kn (0.2–2 mg/l) or N^6-benzyladenine (0.2–2 mg/l) to the medium in conjunction with low levels of auxin did not result in organ initiation. Of the several combinations tested, low levels of cytokinins plus 2,4-D were conducive for growth of the callus. Incorporation of adenine (5–25 mg/l) or yeast extract (200–1000 mg/l) to the medium also failed to induce organogenesis. The data suggested that the callus lacked the potential for organ induction.

Isolation and identification of steroids. Column chromatography of the neutral fraction obtained by methanol extraction of the tissue gave a mixture of sterols. When this was subjected to TLC, three major spots (designated S, S_1 and S_2) were observed. These products were isolated by PLC and crystallised in methanol. Compound S_2 was identified as 24-methylenecholesterol on the basis of physical constants and spectral data [4]. Product S_1 (mp 133–134°) showed IR absorptions at ν_{max}^{KBr} 3450, 1060 (CHOH), and 800, 812, 840 cm^{-1} (tri-substituted double bonds). MS analysis of the compound showed a molecular ion peak at *m/e* 412 and fragments at *m/e* 314 and *m/e* 271, which are characteristic of sterols with a $\Delta^{24(28)}$ double bond in the side chain. The data indicated the constituent to be 28-isofucosterol [5], which was further confirmed through comparison of its acetyl derivative with authentic 28-isofucosterol acetate: mp 132–133°, MS base peak at *m/e* 296 [6], superimposable IR spectra.

GLC analysis of the product S showed it to be a mixture of cholesterol, stigmasterol ans sitosterol, which was further confirmed through the TLC comparison of the acetylated mixture with authentic sterol acetates. Quantitative estimation of the sterols by GLC showed that sitosterol was the major constituent (Table 1).

Isolation, identification and estimation of water-soluble sterols. GLC analysis of the sterol mixture isolated from water extract of the tissue showed the presence of sitosterol, 24-methylenecholesterol, cholesterol and stigmasterol and the absence of 28-isofucosterol (Table 1).

Identification of steroidal alkaloids. TLC separation of the basic fraction obtained from the methanol extract using an alkaline silica gel G plate showed six Dragendorff-positive spots of which one corresponded to the R_f value of the alkaloid conessine. Isolation of the compound by PLC gave less than 1 mg of chromatographically homogeneous product, which showed the same

Table 1. Composition of sterols in methanol and water extracts of callus tissue

Sterols	Sterol content in methanol extract (% dry wt)	Sterol content in water extract (% dry wt)
Total sterol	0 40	0 01
% total sterol		
Cholesterol	9 3	22 8
24-Methylenecholesterol	27.2	24.0
28-Isofucosterol	20 2	Nil
Sitosterol	30 2	41.0
Stigmasterol	12.4	11 5

mobility as authentic conessine in three different solvent systems.

DISCUSSION

Changes in basic metabolism of the cultured tissue were reflected in its failure to form organised structures and differences in the end products. The major secondary metabolites of the callus were cholesterol, 24-methylenecholesterol, 28-isofucosterol, sitosterol and stigmasterol unlike in the intact plant where sitosterol, stigmasterol, stigmastadienol and ergosterol have been identified [7]. The differences in the composition and concentrations of sterols in the water-extract of the callus as compared to the sterols from the methanol-extract, indicated the existence of structural specificity for their water solubility as observed in *Kalanchöe* [8]. The sterol spectrum in the callus cultures indicated a possible involvement of cholesterol in the biosynthesis of phytosterols, since 24-methylenecholesterol and 28-isofucosterol could function as intermediates in such a transformation. Conessine, the major alkaloid of the plant was present only in trace amounts in the callus. These observations indicated the occurrence of a modified steroid metabolism in the unorganised tissue as compared to the intact plant. Whether these changes in the metabolic events have a role in organogenetic response of the tissue, remains to be elucidated.

EXPERIMENTAL

Seeds of *Holarrhena antidysenterica* Wall. collected from Trombay hills, Bombay, India, were sterilized with chlorine water and germinated. Hypcotyl segments of 20-day-old seedlings were used for callus initiation.

Culture media. Explanted tissues were cultured on a modified Lin and Staba medium [9] containing 2,4-dichlorophenoxyacetic acid (2,4-D, 6 mg/l) and kinetin (Kn, 0.2 mg/l) for callus initiation. For subsequent growth of the tissue the auxin conc was lowered to 1 mg/l and the medium supplemented with meso-inositol (100 mg/l), casein hydrolysate (200 mg/l) and coconut milk (10% v/v). Growth hormones were added to the medium in combinations and conc as warranted: Auxins (0.1–2 mg/l)-2,4-D, indole-acetic acid (IAA), naphthaleneacetic acid (NAA), naphthoxyacetic acid (NOA), *o*-chlorophenoxyacetic acid (OCPAA), *p*-chlorophenoxyacetic acid (PCPAA); Cytokinins (0.2–2 mg/l)-Kinetin (Kn), N^6-benzyladenine (BA) and adenine (5–25 mg/l).

Extraction procedures. The callus was dried at 60°, powdered and extracted with MeOH and MeOH–C_6H_6(1:1). The organic extracts were pooled and separated into acidic, basic and neutral components by the method of Bennett and Heftmann [10]. The residual callus mass was extracted repeatedly with boiling H_2O and the extract was evaporated to dryness, refluxed with an alkaline methanolic pyrogallol soln [11], extracted with petrol and separated into basic and neutral components. The neutral fraction was subjected to chromatography on Si gel, eluting with mixtures of EtOAc in C_6H_6 to give a sterol mixture which was further chromatographed on Si gel (0.2–0.05 mm) impregnated with 10% $AgNO_3$ to separate the sterols differing in the number of double bonds. The sterols were purified by PLC on 10% $AgNO_3$–Kieselgel-G using $CHCl_3$. Steryl acetates were separated by TLC on 1% Rhodamine-6G-Anasil B using petrol–Et_2O (99:1) as the developing solvent system [4]. The initial separation of alkaloids was carried out on 0.1N NaOH–Kieselgel G using $CHCl_3$–*n*-PrOH–MeOH (75:15:10) as the solvent system. Subsequent purification and identification were carried out using Si gel G and the following solvent systems saturated with NH_3: $CHCl_3$–EtOAc (1:1), C_6H_6, C_6H_6–EtOAc (1:1). The alkaloids were made visible by spraying with Dragendorff's reagent [12]. Sterol mixtures were analysed by GLC using 2% OV-17. Authentic sterols were used for comparing retention times.

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REFERENCES

1. Krikorian, A. D. and Steward, F. C. (1969) *Plant Physiology* (Steward, F. C. ed.), Vol. VB, pp. 227–326, Academic Press, New York.
2. Heftmann, E. (1971) *Lipids* **6**, 128.
3. Tschesche, R. (1966) *Progress in the Chemistry of Organic Natural Products* (Zechmeister, L. ed.), Vol. xxiv, pp. 100–148, Springer-Verlag, New York.
4. Heble, M. R., Narayanaswamy, S. and Chadha, M. S. (1971) *Z. Naturforsch.* **26**, 1382.
5. Gibbons, G. F., Goad, L. J. and Goodwin, T. W. (1968) *Phytochemistry* **7**, 983.
6. Knights B. A. and Brooks, C. J. W. (1969) *Phytochemistry* **8**, 463.
7. Tschesche, R., Morner, I. and Snatzke, G. (1963) *Liebigs Ann. Chem.* **670**, 103.
8. Pryce, R. J. (1971) *Phytochemistry* **10**, 1303.
9. Lin, M. and Staba, E. J. (1961) *Lloydia* **24**, 139.
10. Bennett, R. D. and Heftmann, E. (1965) *Arch. Biochem. Biophys.* **112**, 616.
11. Adams, B. G. and Parks, L. W. (1967) *Biochem. Biophys. Res. Commun.* **28**, 490.
12. Cerny, V., Joska, J. and Labler, L. (1961) *Coll. Czech. Chem. Commun.* **26**, 1658.