BIOSYNTHETIC POTENTIAL OF CULTURED TISSUES AND REGENERATED PLANTS OF *PHYSALIS MINIMA*

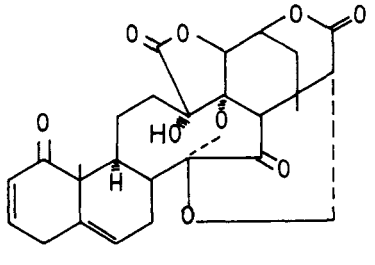
A. T. SIPAHIMALANI, V. A. BAPAT, P. S. RAO and M. S. CHADHA

Bio-Organic Division, Bhabha Atomic Research Centre, Bombay 400 085, India

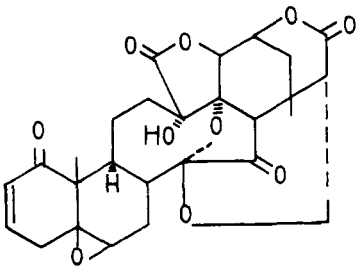
ABSTRACT.—Differentiated tissue cultures were established from the stem segments of diploid plants of *Physalis minima*, as well as from the triploid plants (obtained through anther culture). Plants regenerated from the two cultures were raised to maturity in the field. The two types of cultures showed striking differences in their biosynthetic abilities. Differentiated callus derived from diploid plants showed poor capacity to form physalins (459 μg/g) as compared to the callus established from triploid plantlets (912 μg/g). Physalin D (430 μg/g) was predominant in the diploid callus, whereas in the callus raised from triploid plantlets, the three physalins viz. physalin B (260 μg/g) epoxyphysalin B (200 μg/g) and physalin D (452 μg/g) were detected in significant amounts. The physalin profile of the regenerated plants of diploid and triploid origin was similar to and comparable to that of the field of grown plants.

Plant tissue and cell cultures, like microbial cultures, offer possibilities in studies on biosynthesis and biotransformation of commercially important products. In recent years a wide range of medicinal plant tissue cultures have been established, and valuable studies have been made with regard to the biosynthesis of secondary metabolites (1). Plants belonging to the family Solanaceae, such as *Atropa* (2), *Solanum xanthocarpum* (3), *Hyoscyamus* (4), *Scopolia* (5), and *Nicotiana* (6), have been extensively investigated since many of these contain products of pharmaceutical interest.

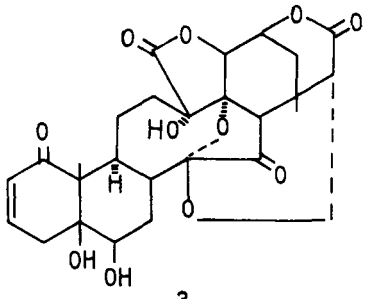
The present investigation was undertaken on *Physalis minima* L. (Solanaceae),



1



2



3

which is of medicinal value (7). Isolation and characterization of physalin B (**1**), 5,6-epoxyphysalin B (**2**), and physalin D (**3**) from the plants grown *in vivo* have been reported (8, 9, 10). These are C_{28} 13,14-seco steroids. Studies on *P. minima* dealing with experimental control of growth and differentiation in organ cultures, androgenesis through culture of anthers (11, 12), and isolation of Physalin D from its tissue cultures have been reported (13). It was, therefore, considered worthwhile to investigate the biosynthetic abilities of tissue cultures of *P. minima* and to explore the feasibility of adapting this system for extensive studies on bio-transformations.

EXPERIMENTAL[1, 2]

PLANT SOURCE MATERIAL.—The *Physalis minima* L. plants were grown at the Field Experimental Station, Bombay. The specimen sample of the plant is deposited in the National Botanical Gardens at Bhabha Atomic Research Centre, Bombay. Triploid strains of the plant were derived through culture of anthers (12). Stem segments excised from aseptically raised plants of *Physalis minima* were utilized as the experimental source material. Actively growing tissue cultures (diploid) were established on a basal medium supplemented with 2,4-D (1 mg/liter) + BA (1 mg/liter) (11). Callus tissues were also established from triploid plantlets (developed through anther culture) on the same medium.

TISSUE CULTURE CONDITIONS.—Tissues were periodically subcultured at monthly intervals on the fresh medium of the same composition. The cultures were maintained under continuous light at 25±2° and a relative humidity of 50 to 60%. At the end of the growth period, tissues were carefully freed from agar, weighed and dried in the oven for 24–30 hours at 60°. Oven-dried tissues were weighed and pooled separately according to their type. To obtain plantlets, shoot buds were isolated from the differentiated cultures and were transferred on BM+ NAA (1 mg/liter). In 5 weeks, robust plantlets with well organized root systems were developed. One batch of young plantlets was oven dried for analysis, whereas another batch was transferred to plastic vials containing vermiculite and were irrigated with nutrient solution as required. After a period of 4 to 5 weeks, the individual plants which had reached a height of over 20 cm, were transferred to soil in pots where they flowered and set fruit. Mature plants were then collected and were oven dried for chemical analysis.

CHEMICAL ANALYSIS.—Chemical analysis of the following types of tissues was carried out (i) Control plant (ii) differentiated diploid callus (iii) young plantlets from diploid callus (iv) mature plants from diploid callus (v) differentiated triploid callus[3] and (vi) mature plants from triploid callus.

EXTRACTION, CHROMATOGRAPHY AND ANALYSIS.—One gram of dry powdered tissue was extracted continuously with methanol (20 hrs). The volume was reduced to about 10 ml, diluted with water, and extracted first with petroleum ether and then with chloroform. The chloroform extracts, which contained crude physalins, were initially checked by tlc and later were put on small silica gel (<0.08 mm) columns (260 mm x 10 mm, 15 gm SiO_2). These were eluted with benzene, benzene-ethyl acetate (1:1) and ethyl acetate. Each fraction eluted from the

[1]Thin layer chromatography was done on glass plates coated with Silica gel C (ACME, India) and with chloroform-acetone (4:1) as an eluent. High performance liquid chromatographic (hplc) separation was carried out on a Waters Associates model ALC/GPC-244 liquid chromatograph with model 6000A solvent delivery system. The detector was Waters Associates model 440 absorbance detector set at 254 nm. Samples were injected via a model U6K injection system. A 30 cm x 3.9 mm I.D. μ-Bondapak C_{18} column (10 μ, Waters Associates, U.S.A.) was used. Methanol (UVasol, spectroscopy grade, E. Merck) and double glass distilled water were the two eluents. A flow rate of 1.2 ml/min was maintained with a mixture of methanol-water (60:40). Chart speed was maintained at 5 mm/min. Prior to injection, all sample solutions were filtered through a Millipore Fluoropore (PTFE) membrane filter of 0.5 μm pore size held in a stainless swinny fitting. Mass spectral analysis was carried out on Micromass mass spectrometer model 707OF. Physalin B, epoxyphysalin B and physalin D isolated from the control plant showed molecular ion fragments at 510, 526 and 544, respectively. Their identities were further confirmed by comparison of ir spectra with those of authentic samples.

[2]Abbreviations used: BA, 6-benzyladenine; BM, basal medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthalene acetic acid.

[3]Differentiated diploid callus applies to the callus (with shoot buds) obtained from diploid plants. Differentiated triploid callus applies to the callus (with shoot buds) obtained from triploid plantlets (through anther culture).

column was spotted on tlc; fractions of the same R_f were pooled. These pooled fractions were dissolved in a known volume of methanol and subjected to quantitative hplc analysis. The hplc profile of physalins, as observed in the case of three such fractions of the control plant, is shown in fig. 1. For calculation purposes, peak heights were taken into consideration for sharp peaks (physalin D and epoxyphysalin B). Peak areas (height x width at half-height) were taken into consideration for broad peaks (physalin B). For each treatment, the peaks with similar retention times (from different chromatographic fractions) were pooled. The concentrations of three physalins in various tissues were calculated from the respective standard graphs by plotting known concentrations versus peak areas.

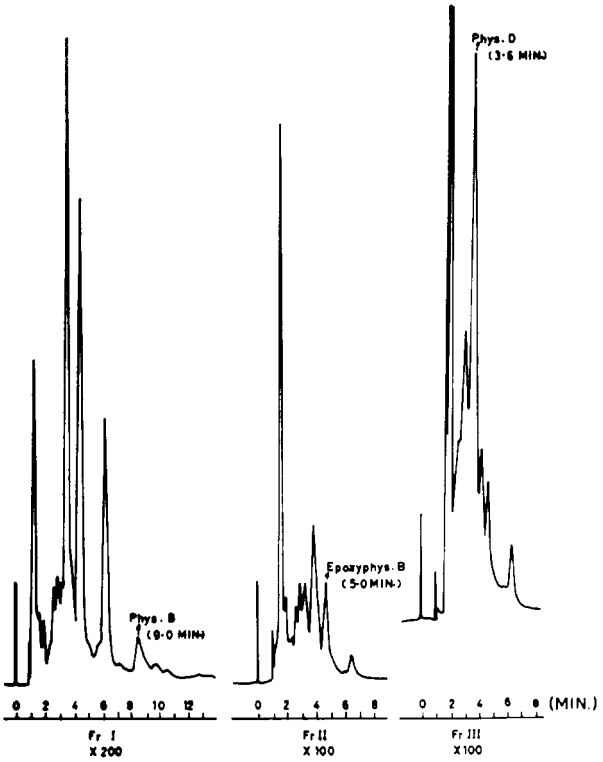


FIG. High performance liquid chromatography of 3 column chromatographic fractions of chloroform extract of *Physalis minima* plant. 100 and 200 represent the dilution factors of the fractions. Hplc conditions are as given under Experimental. Retention times of 3 physalins are given within brackets.

RESULTS AND DISCUSSION

(a) *Control plants.*—The chloroform extract showed the presence of a number of spots on tlc. These included physalin B, epoxyphysalin B and physalin D. Quantitative hplc analysis showed that these 3 physalins were present in the ratio of 2:1:5 (table 1).

(b) *Differentiated diploid callus.*—The chloroform extracts of the callus tissues showed the presence of a number of spots on tlc, but it was possible to identify only physalin D. However, the hplc analysis showed the presence of physalin B and epoxyphysalin B as well, though in negligible amounts (table 1). The overall yield of 3 physalins, as calculated by hplc, amounted to only 12% as compared

to the control plant. Physalin D contributed 95% to the yield, while the combined contribution of physalin B and epoxyphysalin B was only 5%.

(c) *Differentiated callus established from triploid plantlets.*—Physalin B, epoxyphysalin B and physalin D were detected on tlc. The overall profile of physalins as observed on tlc was similar to that of the control plant. The hplc analysis showed the sum formation of three physalins in differentiated callus established from triploid plantlets to be twice as much as compared to the diploid callus. Physalin D, though still a major component, was not as predominant as in the diploid differentiated callus. Its contribution to the total yield was 50%. Physalin B and epoxyphysalin B contributed 28% and 22%, respectively, to the yield.

TABLE 1. Physalin B (**1**), epoxyphysalin B (**2**) and physalin D (**3**) in different tissues of *Physalis minima* plant.

Material	Physalins analyzed (μg/g of dry tissue)			Total physalins (μg/g)
	1	**2**	**3**	
Control Plant	880	440	2380	3700
Differentiated Diploid Callus	12	17	430	459
Young Plantlets from Diploid Callus	270	165	1657	2092
Mature Plants from Diploid Callus	430	540	1074	2044
Differentiated Callus from Triploid Plantlets	260	200	452	912
Plants from Callus raised Through Triploid Plantlets	658	332	1005	1995

(d) *Young plantlets and mature plants regenerated from diploid callus.*—The tlc profile of both young plantlets and mature plants regenerated from diploid cultures was similar. The combined yield of the three physalins remained the same in both the young and the mature plants. However, the physalin D content decreased as the plants progressed to maturity. At the same time, the proportions of physalin B and epoxyphysalin B increased with the maturity of the plant. There was a significant increase in the content of epoxyphysalin B in mature plants as compared with the young plants.

(e) *Plants regenerated from callus raised through triploid plantlets.*—The plants regenerated from triploid cultures also maintained approximately the same proportions of the three physalins as in the triploid cultures except that the sum production of the three physalins doubled in the case of regenerated plants.

Thus cultures raised from triploid plantlets showed a better capacity to form physalins as compared to the cultures obtained from diploid plants. The formation of the three physalins was twice as much in the triploid cultures as compared to the diploid cultures. Another factor which distinguished the two cultures was their physalins composition. Whereas only physalin D was the most abundant in diploid cultures, all three physalins contributed significantly to the total physalins content in triploid cultures. The morphogenetic responses of the two cultures were similar. Thus, to what extent the formation of physalins is linked to the ploidy of the tissue is difficult to explain at this stage. On the other hand, the mature plants regenerated from the two cultures showed more or less

similar physalin contents. These did not show the differences which were exhibited by the diploid and triploid cultures.

The study of the physalin proportions in the diploid cultures, young plantlets and mature plants obtained from the cultures, indicated that the biosynthesis and accumulation of physalins is related to the development of the plant.

Received 7 April 1980

LITERATURE CITED

1. D. N. Butcher, Secondary products in tissue cultures. In: J. Reinert and Y. P. S. Bajaj, "Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture," 1977, pp. 668–693.
2. S. Eapen, T. S. Rangan, M. S. Chadha and M. R. Heble, *Pl. Sci. Lett.*, **13**, 83 (1978).
3. M. R. Heble, S. Narayanaswamy and M. S. Chadha, *Naturwissenschaften*, **55**, 350 (1968).
4. G. K. Dhoot and G. G. Henshaw, *Ann. Bot.*, **41**, 948 (1977).
5. M. Tabata, H. Yamamoto, N. Hiraoka and M. Koni Shima, *Phytochemistry*, **11**, 949 (1972).
6. T. Furuya, H. Kojima and K. Syono, *Chem. and Pharm. Bull.* (Tokyo), **15**, 901 (1967).
7. P. D. Sethi, Anti-inflammatory and anti-arthritic activity of some C_{28} steroidal lactones (withanolides and physalins). In: Second Common Wealth Pharmaceutical Association Conference. Indian Pharmaceutical Association, Bombay, India. Abstract C 18 (1977).
8. R. R. Alluri, R. J. Miller, W. H. Shelver and S. K. Wehbakhalil, *Lloydia*, **39**, 405 (1976).
9. E. Glotter, I. Kirson, A. Abraham, P. C. Sethi and S. S. Subramaniam, *J. Chem. Soc. Perkin-Trans.* I., 1370 (1975).
10. N. B. Mulchandani, S. S. Iyer and L. P. Badheka, *Planta Medica.*, **37**, 268 (1979).
11. V. A. Bapat and P. S. Rao, *Z. Pflanzenphysiol.*, **85**, 403 (1977).
12. L. George and P. S. Rao, *Protoplasma*, **100**, 13 (1979).
13. B. D. Benjamin and N. B. Mulchandani, *Planta Medica*, **37**, 88 (1979).