BOT. GAZ. 141(4):435–439. 1980.

COMPARATIVE PHYTOCHEMISTRY OF PARTHENIUM HYSTEROPHORUS L. (COMPOSITAE) TISSUE CULTURES[1]

KAREN WICKHAM,[2] ELOY RODRIGUEZ,[3] AND JOSEPH ARDITTI

Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Roots, stems, petioles, and leaf blades of mature plants of *Parthenium hysterophorus* and callus cultures derived from explants of these organs from aseptically grown seedlings were screened for the presence of sesquiterpene lactones. Parthenin was not detected in roots but was present in callus cultures from all sources. Coronopilin was detected only in petioles and leaf blades and callus cultures derived from these organs. These findings suggest that synthesis of parthenin and coronopilin is not limited to the glandular trichomes.

Introduction

Parthenium hysterophorus L. (Compositae), a plant indigenous to the Americas, has spread to Australia and India, becoming an aggressive weed. It contains the sesquiterpene lactone, parthenin (fig. 1), a constituent which causes allergic contact dermatitis in humans and is toxic to livestock (TOWERS et al. 1977). Because of the wide spectrum of biological activity exhibited by parthenin and related sesquiterpene lactones, we developed a tissue culture method for studying its biosynthesis and localization.

Material and methods

SEED GERMINATION.—Seeds of *Parthenium hysterophorus* were sterilized with saturated calcium hypochlorite solution (WILSON 1915) containing 2–5 drops of Tween 80 by shaking vigorously for 5 min and rinsing three times with sterile distilled water.

Hoagland's solution (HOAGLAND and ARNON 1938), solidified with 13 g agar/liter and autoclaved, was used as the germination medium. Erlenmeyer flasks (125 ml), each containing 50 ml medium and three or four seeds, were employed as culture vessels. The cultures were maintained under 22 ± 2 C, illuminated with 3 mW/cm², and 18-h photoperiods were provided by a combination of Gro Lux 40-W fluorescent tubes and 25-W incandescent bulbs.

TISSUE CULTURE.—Two media, Heller's (HELLER 1953) and MS (MURASHIGE and SKOOG 1962) were modified (table 1) by the addition of GA_3, two auxins (NAA and 2,4-D), and three cytokinins (BAP, kinetin, and 6-DMAP). The minerals, vitamins, sugars, and agar were autoclaved. Hormones (stock solutions in 95% ethanol) were added to the hot and still liquid medium. Test tubes (25 × 200 mm) containing 20 ml medium were used as culture vessels. Each tissue-medium combination was replicated 15 times.

EXCISION OF EXPLANTS.—Cotyledons were excised from newly germinated seedlings. Leaf blades, roots, stems, and petioles were removed from 30–150-day-old plants. The leaf blades were cultured intact. After removal of petiole stubs, stems were sectioned into ca. 5-mm sections. Roots were cut into ca. 2.5-cm lengths.

EXTRACTION.—Callus, roots, stems, leaf blades, or petioles were ground in chloroform and filtered (RODRIGUEZ 1976). If the filtrate contained water as well as $CHCl_3$, the aqueous fraction was evaporated in vacuo to an oil. Components of the oil were monitored by TLC (silica gel G developed with benzene:acetone, 4:1, vol/vol). Individual components were visualized with iodine vapor and/or concentrated $H_2SO_4/CeSO_4$ (50:5, vol/wt) and 0.5 g vanillin dissolved in a solution of 9 ml EtOH and 0.5 m H_2SO_4 (PICMAN, RANIERI, and TOWERS 1980). Authentic samples of parthenin and coronopilin were cochromatographed with extracts (fig. 1).

PREPARATORY CHROMATOGRAPHY FOR HPLC.—

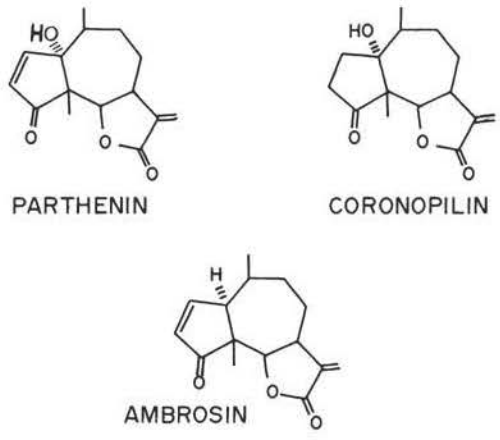

FIG. 1.—Sesquiterpene lactones of *Parthenium hysterophorus* and related species.

[1] Abbreviations used: BAP = benzylaminopurine; 2,4-D = dichlorophenoxyacetic acid; 6-DMAP = 6-dimethylaminopurine; GA_3 = gibberellic acid; HPLC = high performance liquid chromatography; MS = Murashige-Skoog medium; NAA = naphthalene acetic acid; TLC = thin layer chromatography.

[2] Undergraduate research project in phytochemistry and plant physiology.

[3] Address for reprint requests.

Manuscript received November 1979; revised manuscript received April 1980.

The crude oil was dissolved in $CHCl_3$, combined with 230–400 mesh silica gel 60 (E. Merck 5765), dried, and applied to the top of a microcolumn. Elution was with hexane, benzene, and increasing amounts of ethyl acetate and methanol. Four fractions were collected for analysis by TLC and HPLC.

TLC.—Two-dimensional chromatography of leaf, petiole, and callus extract, following preparatory chromatography, established the presence of small amounts of coronopilin and parthenin (PICMAN et al. 1980).

HPLC.—The yellow oil from the ethyl acetate fraction was dissolved in nanograde acetonitrile, filtered through a FH Millipore filter (0.5 μm pore size), and injected in a Waters Associates liquid chromatograph with a Model 440 absorbance detector set at 254 nm. The mobile phase was acetonitrile:water (30:70, vol/vol) using a C_{18} Bondapak Waters Associates column. The retention time was compared with standard solutions of authentic samples of sesquiterpene lactones, parthenin, coronopilin, and ambrosin (fig. 1, table 2).

TABLE 1

EFFECTS OF DIFFERENT ADDITIVES AND CONCENTRATIONS ON INITIATION AND GROWTH OF CALLUS ON MS MEDIUM

CULTURE	ADDITIVE (mg/liter)	TISSUE 1. Root	2. Petiole	3. Leaf blade	4. Stem	COMMENTS[a]
A.....	.1 2,4-D .1 kinetin	+++[b] ++[c]	+++ ++	+ −	++ +	1: Friable; root and shoot primordia 4: Not friable
B.....	.1 2,4-D .1 BAP	+ +	++++ +++	+++ +++	+++ +	1: Friable; rapid root growth, shoot primordia 2, 3: Friable 4: Not friable
C.....	.1 2,4-D .05 kinetin .05 BAP	++ +	++++ +++	+++ +++	+++ ++	1: Friable; roots on callus 2, 3: Friable
D.....	1.0 NAA .05 kinetin .05 BAP	+ −	+ −	− −	+ −	1: Thick, long roots develop
E.....	.1 2,4-D .05 kinetin .1 BAP	++++ ++++	+++ ++++	+++ +++	+++ +++	1: Friable; roots develop 2, 3: Friable
F.....	.1 2,4-D.... .1 6-DMAP	+ −	+ −	+ −	+ −	1: Thick, long roots; a few shoot primordia developed 2, 3, 4: Thick, long roots
G.....	.1 2,4-D .5 kinetin	++++ ++++	++ +	+ −	+++ +	1: Friable, roots develop
H.....	.1 2,4-D .5 BAP	++++ ++++	++ +	+ −	+++ +	1: Friable; roots develop 2: Friable
I.....	.1 2,4-D .1 BAP .1 kinetin 5.0 GA_3	+ −	+ −	+ −	+ −	1, 4: Vigorous, thickened roots develop
J.....	.5 2,4-D .05 BAP .05 kinetin	++ +	++ +	++ +	++ +	1: Friable; roots on callus 4: Friable
K.....	.5 2,4-D .1 BAP	+++ ++	++++ ++++	++++ ++++	++++ ++++	1: Friable; shoot primordia on a few callus masses 2, 3, 4: Friable
L.....	.5 2,4-D .5 kinetin	+++ +++	+++ +	+++ +	++ +	1, 2, 3, 4: Friable
M.....	.5 2,4-D .5 BAP	+++ ++	++ +	+++ +	+++ +++	1, 2, 3, 4: Friable
N.....	.5 2,4-D .5 kinetin .5 BAP	+++ +	++ +	++ +	++++ +++	1, 3, 4: Friable 2: Not always friable
O.....	.5 2,4-D .1 BAP .1 kinetin	+++ +	+++ +++	+++ +++	+++ +++	1, 2, 3, 4: Friable
P.....	.1 2,4-D .5 BAP .5 kinetin	++++ +	++++ +	++++ ++	+++ +	1, 3: Friable 2, 4: Not always friable
Q[d].....	.5 2,4-D .1 kinetin	++++ ++	++++ ++	++++ ++	++++ ++	1, 2, 3, 4: Friable

[a] Nos. correspond to tissue.

[b] Callus initiation on freshly excised material, described in subjective terms: − = none, + = poor, ++ = moderate, +++ = good, ++++ = very good.

[c] Growth of tissue with time: − = very slow, + = slow, ++ = moderate, +++ = rapid, ++++ = very rapid.

[d] Compare with media nos. K1–K4.

TABLE 2

HPLC RETENTION VALUES FOR SECONDARY CONSTITUENTS PRESENT IN CALLUS FROM DIFFERENT EXPLANTS OF PARTHENIUM HYSTEROPHORUS L.

		Retention time for secondary constituents[b,c]					
Culture medium[a]	Tissue source	Ua[d] 6.0	Ub[e] 6.3	Parthenin 6.8	Coronopilin 7.2	Uc[e] 8.3	Ud[e] 10.8
Mature plant	Root	+	+	−	−	−	−
G1	Root	+	+	+	−	+	+
E1	Root	+	+	+	−	+?	+
B1	Root	+	−	−	−	−	−
Mature plant	Petiole	+	−	+	+	−	−
H2	Petiole	+	−	+	+	−	−
K2	Petiole	+	−	+	+	−	+
E2	Petiole	+	−	+	+	−	+
Mature plant	Leaf blade	+	−	+	+	−	−
M3	Leaf blade	+	−	+	+	−	−
K3	Leaf blade	+	−	+	+	−	+
O3	Leaf blade	+	−	+	+	−	+
L3	Leaf blade	+	−	+	+	−	+
Mature plant	Stem	+	−	+	−	−	−
C4	Stem	+	−	+	−	+?	−
H4	Stem	+	−	+?	−	+?	−
B4	Stem	+	−	−	−	−	−
M4	Stem	+	−	+	−	+?	−

[a] For composition of media see table 1.

[b] Retention time shown as, e.g., 6.0 = 6 min after injection.

[c] Explanation of symbols: + = compound present, +? = presence of compound detectable with H_2SO_4 but not with HPLC, − = compound absent.

[d] Unidentified polar compound.

[e] Unidentified compounds.

Results

SEED GERMINATION.—Approximately 50% of the seeds germinated after 4 days. This increased to 75% within 1 wk and 95% in 10 days.

TISSUE CULTURE.—None of the explants produced calli on Heller's medium. Callus formation did occur on MS. Hormone combinations influenced the nature and growth of these calli (table 1).

Callus formed more readily from mature plant tissues than from those of young seedlings and cotyledons (table 1). Roots formed on callus derived from 1–2-mo-old seedlings. Shoots and roots were produced on callus derived from 5-mo-old plants. After 5 wk of culture, rapidly growing calli started to turn brown. Transfer to a new medium generally accelerated the browning and frequently caused death. Growth was slow following subculture of older calli.

Friable callus was always formed from root explants. At 0.1 mg/liter 2,4-D callus initiation, root development, and the appearance of shoots were enhanced by cytokinin concentrations of 0.1 or 0.5 mg/liter (table 1: media nos. A1, B1, G1, and H1).[4] A combination of kinetin and BAP, each at 0.1 mg/liter, increased callus formation and growth (table 1: medium no. E1). Increasing the 2,4-D concentration to 0.5 mg/liter did not promote callus growth (i.e., increase in volume), even at cytokinin levels that appeared optimal (table 1: media nos. J1, K1, L1, M1, N1, O1, Q1).

When the concentration of 2,4-D was 0.1 mg/liter, some cultures tended to form roots (table 1: media nos. A1, B1, C1, E1, G1, H1). Higher concentrations of the auxin suppressed this tendency (table 1: media nos. L1, M1, N1, O1, Q1). Roots produced by callus masses were very short and covered with hairs. They usually reverted back to callus (table 1: media nos. A1, B1, C1, E1, G1, H1, J1). On media containing NAA, 6-DMAP, and/or GA_3, the roots became long, thickened, and did not revert (table 1: media nos. D1, F1–F4, I1, I4). Root production on all tissues was enhanced by 6-DMAP.

Callus from stem explants developed most rapidly when auxin levels exceeded the concentration of either BAP or kinetin (table 1: media nos. K4, Q4). The addition of equal amounts of BAP and kinetin also promoted callus development in some instances (table 1: media nos. C4, E4, N4, O4). At higher levels of 2,4-D, the combination of auxin and BAP stimulated growth of callus (table 1: media nos. K4, M4). When the concentration of 2,4-D was equal to that of the cytokinin, callus was initiated but failed to grow (table 1: medium no. B4).

Leaf blade callus developed most rapidly when the auxin level equaled or exceeded that of BAP (table 1: media nos. B3, C3, K3, O3). The same was true for callus derived from petioles (table 1: media nos. B2, C2, E2, G2, K2, M2, N2, O2, Q2). Callus

[4] Numbers correspond to tissues.

initiated from both leaf and petiole explants failed to grow rapidly when the cytokinin concentration was greater than that of the auxin (table 1: media nos. P2, P3). Addition of equal amounts of BAP and kinetin promoted development of callus from both explant sources if auxin concentration equaled or exceeded that of either cytokinin (table 1: media nos. C2, C3, E2, E3, O2, O3).

Explants from leaf, petiole, and stem tissues responded to hormone combinations similarly, but their calli differed in appearance. Leaf callus was always friable and uniformly pale green. Stem callus was frequently firmer, nonuniform in color, and spread in concentric circles. Friability increased in direct proportion to increasing hormone levels. Petiole callus appeared more like that derived from stems when it developed on a cut end. When it emerged from the side of the petiole, it resembled leaf-derived callus.

Stems, petioles, and leaf blades contained substantial amounts of parthenin. Only petioles and leaf blades contained coronopilin (table 2). The chromatographic evidence (table 2) suggested the presence of parthenin (fig. 1) in nearly all calli regardless of explant origin or culture medium except for media nos. B1 and B4. An unknown polar compound, *Ua* (table 2), was detected in extracts of all calli and plant tissues. Compound *Ua* was always present in the callus in greater quantities than parthenin. The reverse was true for all plant tissues from which calli were derived, but parthenin was usually absent in roots (table 2). Peak heights suggested that the quantities produced were different. Generally the calli produced a larger array of compounds than plant tissues.

Two additional unknown compounds (*Ub* and *Uc*) were detected in callus tissues. Only *Ub* was found in roots of 116-day-old plants. The *Ub* and *Uc* were not detected with certainty in any other tissues or organs; *Ub* was more polar than parthenin, whereas *Uc* was less so. The polarity of *Uc* was intermediate between coronopilin and ambrosin.

A fourth unknown, *Ud*, was detected in 75% of the callus tissues derived from leaf blades, 67% of those obtained from petioles, and 75% of those cultured from root explants (table 2). Ambrosin was not detected in any tissue.

Discussion

Callus cultures derived from young *Parthenium hysterophorus* tissues tend to produce roots (table 1); those derived from older tissues produce roots and shoots. In contrast, explants from young leaves of *Phalaenopsis* (Orchidaceae) produce a larger number of protocorm-like bodies (i.e., shoots) than those from older ones (TANAKA, HASEGAWA, and GOI 1975). Young shoots of *Sequoia sempervirens* (Taxodiaceae) produce callus more readily than older ones (E. A. BALL and D. M. MORRIS, personal communication). Callus of *Salpiglossis sinuata* (Solanaceae) derived from older leaves undergoes organogenesis more readily than that from younger ones (LEE et al. 1977). These reports suggest that the tendency to produce roots or shoots from callus may not depend on the age of the tissue used as a source of explants.

Callus initiation from *P. hysterophorus* stem explants is better with 2,4-D than with NAA (table 1). The reverse is true for *Carica papaya* (Caricaceae) (ARORA and SINGH 1978) and *Araucaria araucana* (Araucariaceae) (BENIEST and DEBERGH 1976). Orchid cultures respond differently to various auxins (ARDITTI 1977), as do tobacco, peas, rice, and barley (SEKIYA, YASUDA, and YAMADA 1977). The reasons for these differences are not entirely clear, and that specificity of auxin acceptors, degradation, and molecular structure may be involved (SEKIYA et al. 1977).

High auxin/cytokinin ratios favor callus induction from stem, leaf blade, and petiole explants of *P. hysterophorus* (table 1). Similar responses have been obtained with stem explants of *Dioscorea alata* (Dioscoreaceae) (MANTELL, HAGUE, and WHITEHALL 1978), *Manihot esculenta* (Euphorbiceae) (PARKE 1978), and *A. araucana* (BENIEST and DEBERGH 1976), as well as with leaf tip cultures of *Laeliocattleya* and *Epidendrum* (Orchidaceae) (CHURCHILL, BALL, and ARDITTI 1973). On the other hand, low auxin/cytokinin ratios stimulate callus initiation from leaf blade explants of *Phalaenopsis* (TANAKA et al. 1975), *Peperomia* 'Red Ripple' (Piperaceae) (HENNY 1978), *Anthurium andraenum* (Araceae) (PIERIK 1976), and *Ficus lyrata* (Moraceae) (DEBERGH and DE WAEL 1977), as well as from petioles of *Phoenix dactylifera* (Palmae) (EEUWENS 1978). Initiation and growth of callus derived from *P. hysterophorus* root explants is enhanced by a low (1:5) auxin/cytokinin ratio. Roots of *Bryophyllum calycinum* (Crassulaceae) require somewhat higher (1:1 or 1:2) ratios (ROBBINS and HERVEY 1978). Hence, with the possible exception of stems, there seems to be no clear pattern regarding the effects of auxin/cytokinin ratios on root and stem formation in callus cultures or even a requirement for the latter. An interesting point in connection with cytokinins is the contrast between the effects of 6-DMAP and BAP or kinetin on root growth: 6-DMAP induces long thick roots; the others do not.

Phytochemical investigations of economically important plants in tissue culture have provided a wealth of information relating to biosynthesis, metabolism, and compartmentalization of natural products, indicating that they produce a greater diversity of constituents than intact plants (REINERT and BAJAJ 1977). In our investigation, *P. hysterophorus* callus cultures and mature plants differ in their terpenoid chemistry. Roots of mature plants do not accumulate the pseudoguaianolide sesquiterpene lac-

tones (table 2). Calli derived from stems, roots, petioles, and leaves all produced parthenin, while petioles and leaves also contained coronopilin and a series of unknown constituents.

Differences in chemical content of calli derived from the same explant were brought about by media composition. The greatest number of unidentified metabolites is produced by the root callus, which also contains parthenin. This is of interest since previous reports have implied that sesquiterpene lactones are synthesized in glandular trichomes. Our findings suggest that these compounds may be produced elsewhere (e.g., chloroplasts [GOODWIN 1967]) and possibly transported into trichomes. However, the possibility that sesquiterpene lactones are produced in trichomes cannot be ruled out because volatile monoterpenes are synthesized in these structures (LOOMIS and CROTEAU 1966).

Acknowledgments

This work was supported in part by NSF grant PSR78-25162 and NIH grant AI14646-02 to E. RODRIGUEZ. We thank Dr. M. S. STRAUSS for his help and Mr. JAN E. WEST for seeds of *Parthenium hysterophorus*. We also thank G. H. N. TOWERS and ANA PICMAN (Department of Botany, University of British Columbia) for TLC analysis of the extracts and helpful comments on the manuscript.

LITERATURE CITED

ARDITTI, J. 1977. Orchid biology: reviews and perspectives. Vol. 1. Cornell University Press, Ithaca, N.Y.

ARORA, I. K., and R. N. SINGH. 1978. Growth hormones and in vitro callus formation of papaya. Sci. Hort. **8**:357–361.

BENIEST, J., and P. DEBERGH. 1976. Nutritional and hormonal requirements for the growth of *Araucaria araucana* callus in vitro. Meded. Fac. Landbouww. Rijksuniv. Gent. **41**:1599–1610.

CHURCHILL, M. E., E. A. BALL, and J. ARDITTI. 1973. Tissue culture of orchids. I. Methods for leaf tips. New Phytol. **72**:161–166.

DEBERGH, P., and J. DE WAEL. 1977. Mass propagation of *Ficus lyrata*. Acta Hort. **78**:361–364.

EEUWENS, C. J. 1978. Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured in vitro. Physiol. Plantarum **42**:173–178.

GOODWIN, T. W. 1967. Regulation of terpenoid synthesis in higher plants. Pages 57–72 *in* T. B. PRIDHAM, ed. Terpenoids in plants. Academic Press, New York.

HELLER, R. 1953. Recherches sur la nutrition minérale des tissus végétaux cultivés in vitro. Ann. Sci. Natur. Bot. Biol. Vegetale **14**:1–22.

HENNY, R. J. 1978. In vitro propagation of *Peperomia* 'Red Ripple' from leaf discs. Hort. Sci. **13**(2):150–151.

HOAGLAND, D. R., and D. I. ARNON. 1938. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Circ. 347.

LEE, C. W., R. M. SKIRVIN, A. I. SOLTERO, and J. JANICK. 1977. Tissue culture of *Salpiglossis sinuata* L. from leaf discs. Hort. Sci. **12**(6):547–549.

LOOMIS, W. D., and R. CROTEAU. 1966. Biochemistry and physiology of lower terpenoids. Pages 147–186 *in* V. C. RUNECKLES and T. J. MABRY, eds. Recent advances in phytochemistry. Vol. 6. Academic Press, New York.

MANTELL, S. H., S. Q. HAGUE, and A. P. WHITEHALL. 1978. Clonal multiplication of *Dioscorea alata* L. and *Dioscorea rotunda* Doir yams by tissue culture. J. Hort. Sci. **53**:95–98.

MURASHIGE, T., and F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum **15**:473–497.

PARKE, D. 1978. Tissue culture of cassava on chemically defined media. Physiol. Plantarum **42**:195–201.

PICMAN, A., R. L. RANIERI, and G. H. N. TOWERS. 1980. Visualization reagents for sesquiterpene lactones and polyacetylenes on thin layer chromatograms. J. Chromatogr. **189**:187–198.

PIERIK, R. L. M. 1976. *Anthurium andraeanum* plantlets produced from callus tissues cultivated in vitro. Physiol. Plantarum **37**:80–82.

REINERT, J., and Y. P. S. BAJAJ. 1977. Applied and fundamental aspects of plant cell, tissue and organ culture. Springer-Verlag, New York.

ROBBINS, W. J., and A. HERVEY. 1978. Auxin, cytokinin and growth of excised roots of *Bryophyllum calycinum*. Amer. J. Bot. **65**:1132–1134.

RODRIGUEZ, E. 1976. Sesquiterpene lactones: chemotaxonomy, biological activity and isolation. Rev. Latinoamer. Quimica **8**:56–62.

SEKIYA, J., T. YASUDA, and Y. YAMADA. 1977. Callus induction in tobacco, pea, rice and barley plants by auxins and their analogues. Plant Cell Physiol. **18**:1155–1157.

TANAKA, M., A. HASEGAWA, and M. GOI. 1975. Studies on the clonal propagation of monopodial orchids by tissue culture. I. Formation of protocorm-like bodies from leaf tissues in *Phalaenopsis* and *Vanda*. J. Jap. Soc. Hort. Sci. **44**:47–58.

TOWERS, G. H. N., T. C. MITCHELL, E. RODRIGUEZ, F. D. BENNET, and P. V. SUBBA RAO. 1977. Biology and chemistry of *Parthenium hysterophorus* L: a problem weed in India. J. Sci. Ind. Res. **36**:672–684.

WILSON, J. K. 1915. Calcium hypochlorite as a seed sterilizer. Amer. J. Bot. **2**(8):420–427.