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Growth Hormone-Like Activities of Macrocyclic Trichothecenes in In Vitro Callus Induction and Growth of Four *Baccharis* Species

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Abstract. The ability of two plant-produced macrocyclic trichothecenes (baccharinoid B4 and roridin E) to induce callus growth of two trichothecene-producing *Baccharis* species (*B. coridifolia* and *B. megapotamica*) and two nontrichothecene-producing species (*B. halimifolia* and *B. neglecta*) was investigated. Roridin E had no effect in the induction of callus of *B. coridifolia*, a roridin-producing plant, but induced callus of nonroridin-producing plants (*B. megapotamica*, *B. halimifolia*, and *B. neglecta*). Baccharinoid B4 stimulated callus growth of *B. megapotamica*, a baccharinoid-producing plant, and inhibited growth of *B. coridifolia*, *B. halimifolia*, and *B. neglecta* callus tissues. The ability of roridin E to induce callus was most effective at concentrations of 10^{-8} and 10^{-6} M and when synergistically coupled with auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). The ability of baccharinoid B4 to stimulate callus growth appeared to increase with increased concentration in the culture medium. Analysis of callus cultures grown in medium amended with roridin E showed that B4, roridin E, and 8β-hydroxyroridin E and verrucarols were formed in the tissues but not in the medium. The results of this study indicated that while the callus-inducing ability of roridin E seemed to be nonspecies-specific in nature, the ability of B4 to stimulate callus was a highly species-specific phenomena. Callus-inducing activity of roridin E may depend on the capacity of plant species to transform exogenous roridin E into baccharinoids or other macrocyclic trichothecene derivatives.

Plant tissue cultures can be useful in selecting cell strains which accumulate valuable pharmaceutical compounds normally obtained from whole plants (Fujita and Tabata 1987). Two Brazilian shrubs, *Baccharis megapotamica* Sprengel and *B. coridifolia* APDC accumulate substantial amounts of closely related macrocyclic trichothecenes. These are baccharinoids (Jarvis et al. 1987a,c, Kupchan et al. 1977) and roridins (Habermehl et al. 1989, Jarvis et al. 1987c) which have antineoplastic (Kupchan et al. 1977), antimicrobial (Freeman 1955), and insecticidal (Kishaba et al. 1962) properties. The plant-produced trichothecenes belong to the same class of terpenoids that are normally found associated with fungal mycotoxins (Ueno 1983).

In order to produce these useful trichothecene compounds in large scale, we have been working on establishing a viable tissue culture system for in vitro production of trichothecenes from the *Baccharis* plants. Our initial efforts have met with little success. We have used several plant parts or explants of the *Baccharis* species to initiate callus and cell suspension cultures. In our experiments, the callus tissues obtained from *Baccharis* plants were extremely slow-growing and the cell populations in suspension cultures were too low for appreciable accumulation of trichothecene compounds (Kuti et al. unpublished results). Misawa et al. (1983) obtained appreciable callus biomass of *B. megapotamica* using a culture medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and no kinetin. They also isolated an unidentified cytotoxic product from the callus tissues which they thought was a trichothecene compound (Misawa et al. 1983). Recently, Kobayashi et al. (1989) reported that fungus-produced trichothecenes are capable of inducing callus formation from alfalfa (*Medicago sativa*) cotyledons. Their report indicated that roridins and verrucarins produced callus with twice the fresh weight of callus obtained under their optimal 2,4-D treatment. Therefore, the present study was initiated to determine the effect of the two plant-produced trichothecenes (baccharinoids and rori-

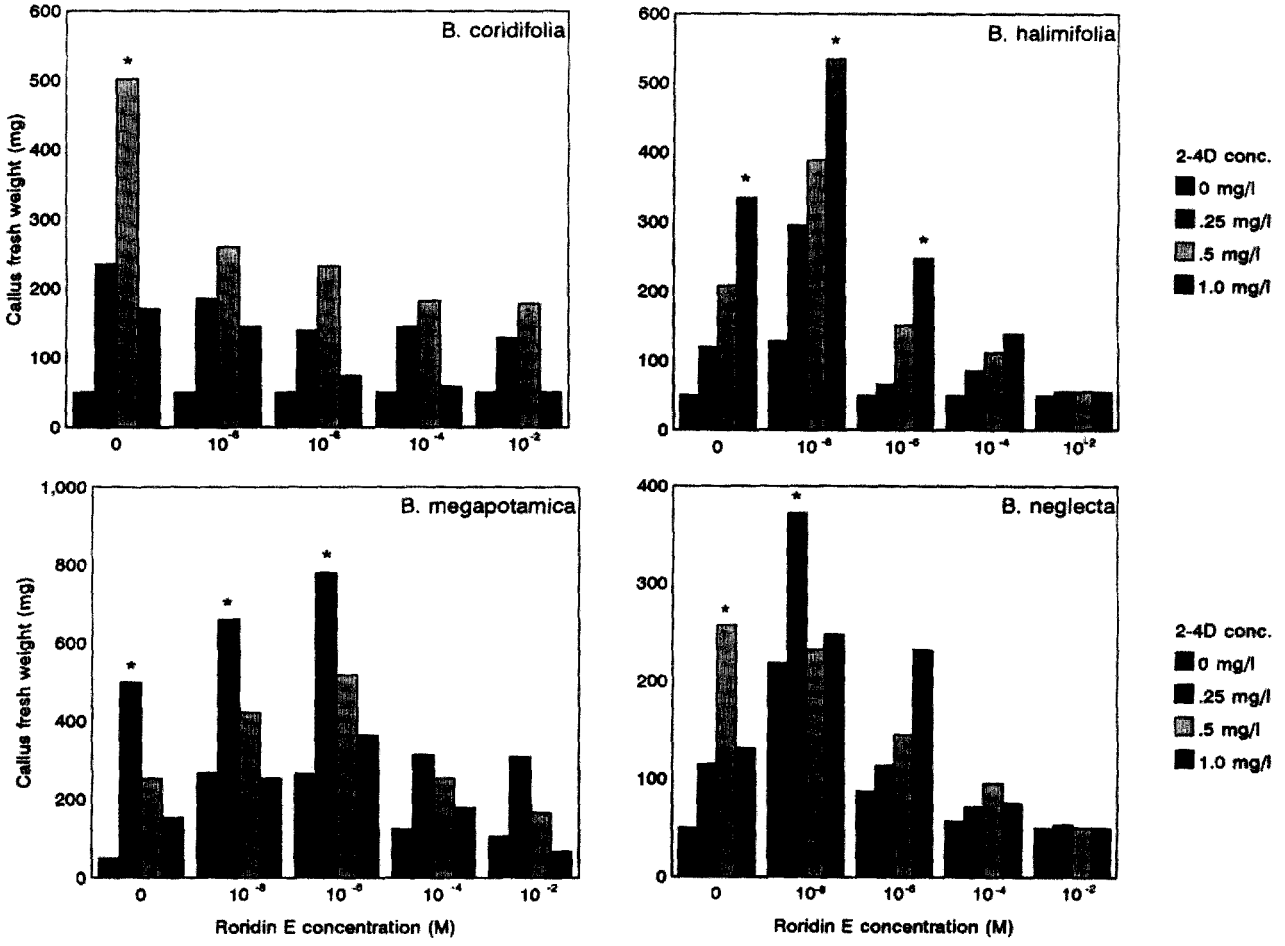


Fig. 1. Fresh weight of callus tissues of four *Baccharis* species produced on modified MS medium containing various concentrations of roridin E and 2,4-D after 4 weeks. An asterisk associated with a treatment mean indicates a significant ($p < 0.05$) difference from the control (i.e., no 2,4-D treatment).

dins) on induction and growth of callus tissues of the two trichothecene-producing *Baccharis* species (*B. coridifolia* and *B. megapotamica*) and two non-trichothecene producing species (*B. halimifolia* and *B. neglecta*).

Materials and Methods

Plant Material

Seeds of *B. coridifolia* and *B. megapotamica*, trichothecene-producing plants, were collected form various sites in Brazil, while seeds of *B. halimifolia* and *B. neglecta*, the nontrichothecene-producing plants, were collected from Maryland and Texas, respectively. All seeds were surface sterilized by immersion in 6% sodium hypochlorite solution for 30 min followed by three rinses with sterile-distilled water and were sown aseptically on solid agar medium without growth regulators. The cultured seeds were incubated in a 16-h photoperiod at 24 ± 2°C temperatures.

Callus Induction

Cotyledons from 10- to 14-day-old seedlings were used as the explant source. Cotyledons were cut into small portions (2 mm in width), weighed, and surface sterilized with 6% sodium hypochlorite solution containing 0.05% Tween 80 for 10 min and rinsed with sterile distilled water before they were placed on the culture medium.

The basal medium used in all experiments consisted of modified Murashige's (MS) macro- and micronutrients (Murashige and Skoog 1962) supplemented with 3% sucrose, 100 mg ml^{-1} *myo*-inositol, 1 mg L^{-1} thiamine-HCl, and 0.8% Bacto agar. The auxin used was 2,4-D. The trichothecenes used were greater than 95% pure as shown by HPLC analysis of roridin E (Jarvis et al. 1982) and baccharinoid B4 (Jarvis et al. 1987b). Each of the trichothecenes was initially dissolved in 250 µl acetone and made up to 5 ml to give a stock solution of 10^{-2} M. Volumes were adjusted with distilled water to obtain the appropriate concentrations (0, 10^{-8}, 10^{-6}, 10^{-4}, and 10^{-2} M). A factorial design was performed to provide levels of 0, 0.25, 0.5, and 1.0 mg L^{-1} for 2,4-D and 0, 10^{-8}, 10^{-6}, 10^{-4}, and 10^{-2} M for each trichothecene. The basal medium was supplemented with specific con-

centrations of 2,4-D and trichothecenes, the pH was adjusted to 5.7, and the medium was autoclaved at 20 psi (1.05 kg/cm^2) and 121°C for 15 min. Explants were placed on the medium and incubated in a 16-h photoperiod (60–85 μEinsteins m^{-2} s^{-1}, PAR) at 24°C. The explants were scored visually for callus initiation every 3 days, and callus production was measured as final fresh weight. There were five replicates per treatment and the experiment was repeated twice. Data obtained from the three experiments were combined and analyzed using analysis of variance.

Extraction of Trichothecenes from the Callus Tissues

Callus was removed from the agar slants and extracted three times with 3 ml of 10% MeOH in $CHCl_3$ under sonication for 30 min. The extracts were concentrated to dryness under nitrogen, divided into two equal portions and subjected to (a) high-performance liquid chromatography (HPLC) analyses and (b) hydrolysis followed by gas chromatography (GC) analyses.

Determination of Trichothecenes in the Callus Tissues

HPLC Analysis. The extracts were subjected to HPLC analyses using a Zorbax 5 μ silica column, 4.6 mm × 250 mm, with a gradient solvent system of hexane (solvent A) and 10% i-PrOH/EtOAc (solvent B). Solvent conditions were: flow rate of 1.5 ml min^{-1}, 50% solvent A, 50% solvent B at t = 0 with the gradient increasing to 100% solvent B over a 10-min period. The ultraviolet detector set at 260 nm was used to monitor eluted fractions. Retention times of standards on the column were roridin E (4.1 min), 8β-hydroxyroridin E (6.2 min), and baccharinoid B4 (13.6 min).

GC Analysis. The extracts were concentrated to dryness under nitrogen and taken up in 0.5 ml of MeOH. A 0.5-ml aliquot of 1 N NaOH in MeOH was added and the solution maintained at ambient temperature (22 ± 2°C) for 24 h. The solution was then passed through a short column of Dowex-50 resin (first washed with 2 N HCl followed by distilled H_2O, and then with MeOH), and the filtrate concentrated to dryness. The residue was dissolved in 100 μl of 0.05 M Et_3N in CH_2Cl_2, and the mixture treated with 10 μl of heptafluorobutyric anhydride. The mixture was concentrated to dryness under nitrogen and analyzed by GC as described by Barel et al. (1990).

Results

Modified MS media containing the lower concentrations of roridin E increased callus growth of *B. halimifolia, B. megapotamica,* and *B. neglecta* but not *B. coridifolia* (Fig. 1). The ability of roridin E to induce callus was most effective at concentrations of 10^{-8} and 10^{-6} M. Auxin (2,4-D) alone induced small amounts of callus but 2,4-D combined with roridin E significantly enhanced callus induction.

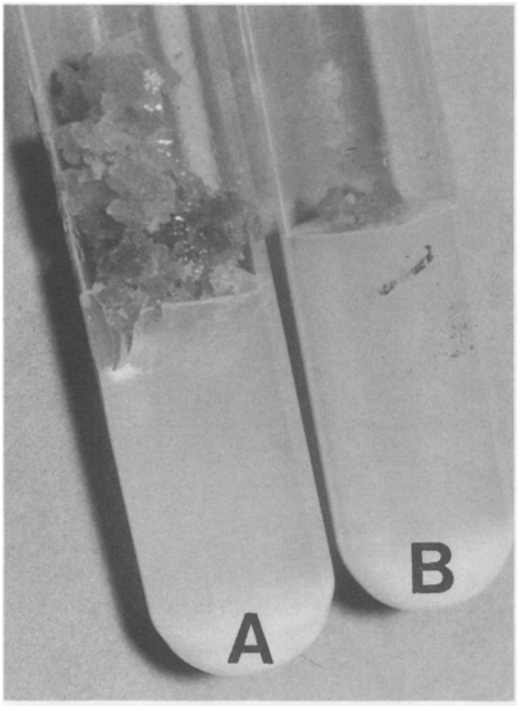


Fig. 2. Friable callus cultures of *Baccharis megapotamica* obtained on **(A)** modified MS medium containing 10^{-6} M roridin E supplemented with 2,4-D and **(B)** modified MS medium containing only 2,4-D.

Optimum callus production was obtained for *B. coridifolia* in medium containing 0.5 mg L^{-1} 2,4-D alone, for *B. halimifolia* in medium containing 1.0 mg L^{-1} 2,4-D and 10^{-8} M roridin E, for *B. megapotamica* in medium containing 0.25 mg L^{-1} 2,4-D and 10^{-6} M roridin E, and for *B. neglecta* in medium containing 0.25 mg L^{-1} and 10^{-8} M roridin E. Callus tissues formed in all treatments were light in color and highly friable (Fig. 2).

While modified MS media containing baccharinoid B4 stimulated callus growth of *B. megapotamica,* it strongly inhibited callus growth of *B. coridifolia, B. halimifolia,* and *B. neglecta.* A combination of 2,4-D and baccharinoid B4 in the media caused proliferation of *B. megapotamica* callus tissues and callus fresh weight increased with increased B4 concentrations. All *Baccharis* species produced callus when grown on media without trichothecenes (Fig. 3).

HPLC analysis of the callus tissues revealed the presence of a number of macrocyclic trichothecenes and their metabolic derivatives (Fig. 4) only in callus tissues grown on media containing roridin E. The results of HPLC analysis of callus tissues grown on medium containing 10^{-8} M roridin E are presented in Table 1. *B. megapotamica* callus

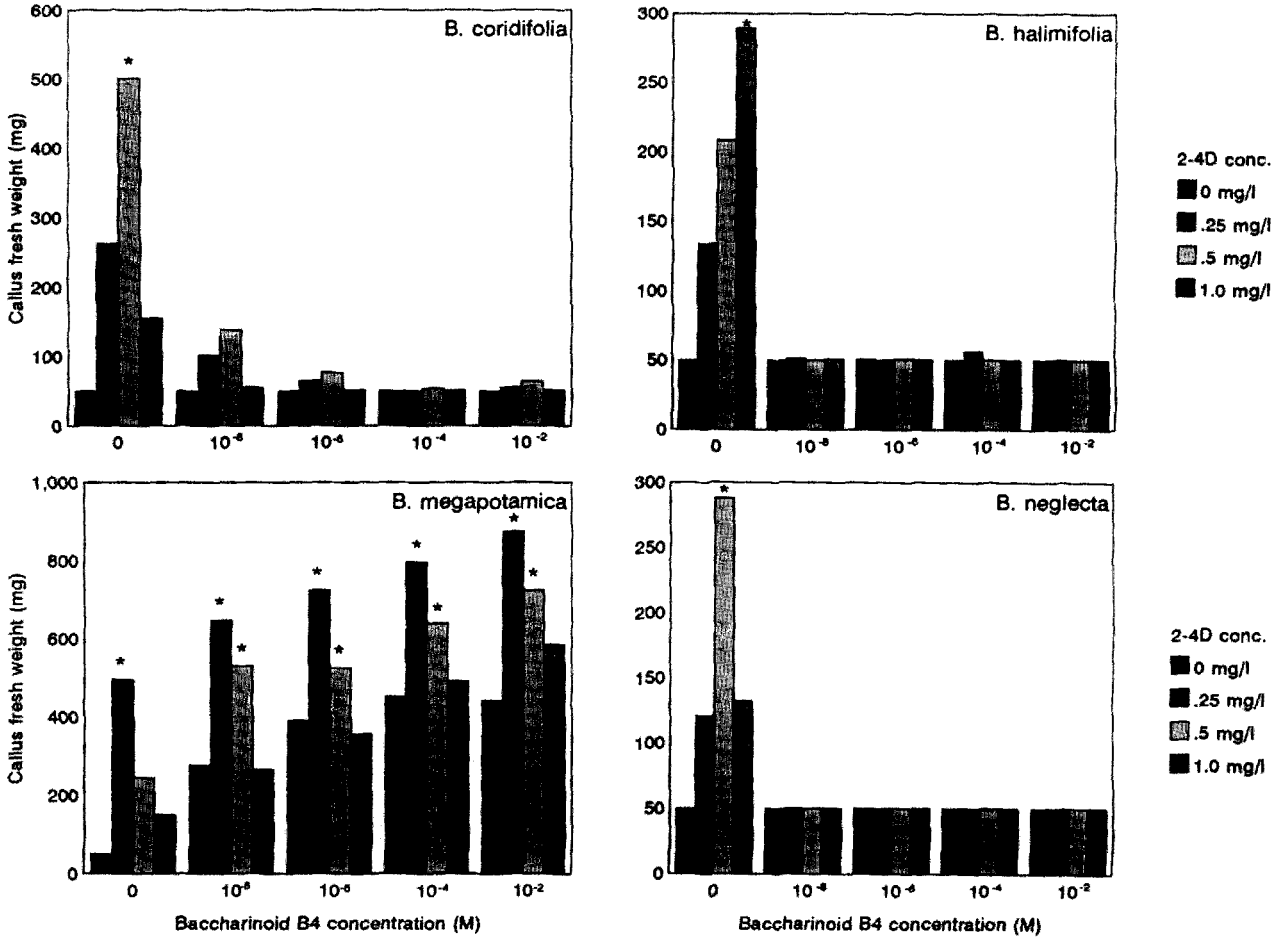
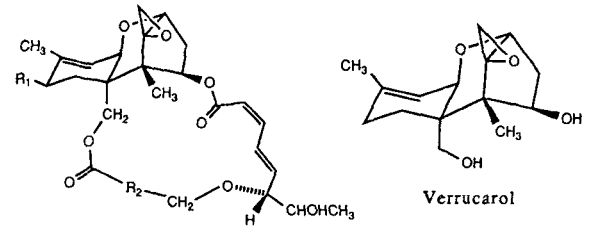


Fig. 3. Fresh weight of callus tissues of four *Baccharis* species grown on modified MS medium containing various concentrations of baccharinoid B4 and 2,4-D at the stationary growth phase. An asterisk associated with a treatment mean indicates a significant ($p < 0.05$) difference from the control.

tissues contained substantial amounts of B4, and smaller amounts of 8β-hydroxyroridin E and roridin E. Hydrolysis of the extract gave verrucarol as confirmed by GC analysis. Callus tissues of *B. halimifolia* and *B. neglecta* contained trace amounts of roridin E and B4 and upon hydrolysis, the extracts of these callus tissues gave trace amounts of verrucarol. The callus tissues of *B. coridifolia* contained traces of roridin E, and verrucarol was detected in the extract hydrolysate. No macrocyclic trichothecenes of substantial amount were detected in the culture medium.



Roridin E: $R_1 = H$, $R_2 = CH{=}CCH_3CH_2$
8β-Hydroxyroridin E: $R_1 = OH$, $R_2 = CH{=}CCH_3CH_2$
Baccharinoid B4: $R_1 = OH$, $R_2 = CH\text{-}CCH_3CHOH$ (epoxide)

Fig. 4. Structures of macrocyclic trichothecene metabolites isolated from callus tissues of four *Baccharis* species grown on modified MS medium containing roridin E.

Discussion

Plant cells normally require auxin for cell division and elongation during growth (Elliot et al. 1977). Interaction between exogenous and endogenous auxins are necessary in the regulation of plant growth in tissue culture. According to Moloney et al. (1983), plant cells are capable of rapid uptake of exogenous auxins, such as 2,4-D from the culture medium, especially during the lag phase of cell

Table 1. Macrocyclic trichothecenes extracted from callus tissues of four *Baccharis* species grown on medium containing 10^{-8} M roridin E.

Macrocyclic trichothecene[b]	*Baccharis* species[a]			
	BC	BH	BM	BN
Roridin E	+[c]	+	++	+
Baccharinoid B4	−	+	+++	+
8β-hydroxyroridin E	−	−	+	−
Verrucarol[d]	+	+	++	+

[a] BC, *B. coridifolia;* BH, *B. halimifolia;* BM, *B. megapotamica;* BN, *B. neglecta.*
[b] Trichothecenes were identified by GC and HPLC analyses.
[c] +++, indication of relative amount of trichothecenes in HPLC peaks; −, no trichothecene detected.
[d] Detected by GC analysis of the hydrolyzed extracts.

growth, converting it into biologically inactive 2,4-D glucosides. The plant cells consequently use endogenous auxins, such as indoleacetic acid (IAA), during the logarithmic growth phase up until the stationary growth phase.

Macrocyclic trichothecenes are generally known to inhibit plant growth (Cutler and Jarvis 1985). They act as potent inhibitors of protein synthesis by binding to susceptible (not resistant) eukaryotic 60S ribosomes thereby inhibiting peptidyltransferase activity during protein synthesis (Cundliffe et al. 1974). Recently, we reported that macrocyclic trichothecenes, such as roridins and baccharinoids, enhance seed germination and may play a vital regulatory role in reproduction of the *Baccharis* species that produce them (Kuti et al. 1990). While the precise role of macrocyclic trichothecenes in the induction of cellular growth is not known, Kobayashi et al. (1989) have suggested that macrocyclic trichothecenes may be stimulating the de novo synthesis of endogenous IAA or suppressing degradation of IAA in plant tissue culture. The exact mechanism of interactions between macrocyclic trichothecenes and exogenous or endogenous auxins deserves further investigation to further our knowledge of subcellular events that occur during *Baccharis* plant growth in tissue culture.

The results obtained with B4 (Fig. 3) indicated a growth hormone-like activity on callus growth of *B. megapotamica* with a linear relationship between B4 concentration and callus growth. A similar linear relationship was found between concentration of B4 with exogenous 2,4-D and callus growth. The stimulatory effect of B4 on callus growth was specific for *B. megapotamica,* and the inhibitory effect on the other three *Baccharis* species correlated with B4 phytotoxicity to cells of the other *Baccharis* species [i.e., *B. megapotamica* plants are more tolerant to B4 than other *Baccharis* species (Jarvis et al. 1988)]. A similar correlation cannot be inferred in the case of roridin E on callus growth of *B. coridifolia,* even though *B. coridifolia* plants were more tolerant to roridin E than other *Baccharis* species (Jarvis et al. 1988). The stimulatory effect of roridin E on callus induction seemed to be nonspecies-specific and occurred only in culture media containing low concentrations of roridin E and in the presence of 2,4-D. A similar finding was reported by Kobayashi et al. (1989) on induction of callus tissues of alfalfa by fungi-produced roridins.

Production of B4 by callus cultures of *B. megapotamica, B. halimifolia,* and *B. neglecta* grown on roridin E-containing medium may be due to the ability of these *Baccharis* species to metabolize exogenous roridins into baccharinoids (Jarvis et al. 1981). The absence of B4 in callus tissues of *B. coridifolia* indicates the inability of the plant to metabolize or transform roridin E. Cellular mechanisms involved in biotransformation of exogenous substances administered to cultured cells are complex and may be affected by various factors including the nature of the exogenous substance, its products, type of reaction required, and plant species used (Suga and Hirata 1990).

The unique observations reported in this study are that callus of *B. megapotamica* accumulates substantial amounts of B4 in the presence of a low concentration of exogenous roridin E, and that B4 and other roridin E metabolic derivatives may play a role in the induction and growth of *Baccharis* callus tissues. However, more fundamental information on the role of macrocyclic trichothecenes in growth and development of higher plant cells is needed before utilization of tissue culture methods to produce in vitro antineoplastic compounds, such as baccharinoids.

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