Callus induction in \textit{Phyllanthus} species and inhibition of viral DNA polymerase and reverse transcriptase by callus extracts

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Summary. Studies on callus induction and growth in \textit{Phyllanthus amarus} Schum. & Thonn. (Euphorbiaceae) and some related species are described, as well as the inhibition of enzymes of hepatitis B and related viruses by callus extracts. Callus was induced from stem or branch pieces of \textit{P. amarus} placed on several media combinations. Optimum induction and growth of friable, undifferentiated calli occurred on Murashige and Skoog medium supplemented with either 0.5 mg or 1 mg of BA/liter and 1 mg/liter of either 2,4-D or IBA, but not IAA. Callus induction using the same media was also attempted with other \textit{Phyllanthus} spp. The best success was with \textit{P. abnormis}. Aqueous extracts from field-grown plants were more active in vitro against viral DNA polymerase and reverse transcriptase than extracts of calli.

Key words: Antivirals - Hepatitis B - DNA polymerase - Reverse transcriptase - \textit{Phyllanthus}

Abbreviations: DNA\textsubscript{P}: DNA polymerase, RT: reverse transcriptase, MS: Murashige and Skoog salts medium, B5: Gamborg's B5 salts medium, 2,4-D: (2,4-dichlorophenoxy)acetic acid, BA: 6-benzylaminopurine, IBA: indole-3-butyric acid, IAA: indole-3-acetic acid, kinetin: 6-furfurylaminopurine, NAA: \(\alpha\)-naphthaleneacetic acid.

Introduction

\textit{Phyllanthus amarus} Schum. & Thonn. (Euphorbiaceae) is a small herb which has had many traditional and folk medicine uses (Unander et al. 1991). Recent interest in this plant was stimulated by reports that it possesses activity in vitro and possibly in vivo against hepatitis B virus and related animal viruses (Blumberg et al. 1989; Shead et al. 1990; Thyagarajan et al. 1988; Venkateswaran et al. 1987; Yanagi et al. 1989). \textit{Phyllanthus amarus} also has in vitro activity against the RT of retroviruses (Yanagi et al. 1989; PS Venkateswaran, personal communication) and possibly against retroviruses in vivo (I. Millman and A. Pangilinan, personal communication). Similar in vitro activity was found in several related species (Shead et al. 1990; Unander et al. 1990; Unander and Blumberg 1991). Results in vivo, however, have not always been reproducible (Berk et al. 1991; Leelarasamee et al. 1990; Niu et al. 1990; Shead et al. 1990).

Depending on the chemical structure responsible for the putative antiviral activity, a future therapeutic drug might be produced by synthesis de novo or might require extraction from whole plants. An intermediate alternative to these two possibilities would be the production of the active principle via culture of plant cells or organs (Flores 1987). Antiviral activity, expressed as the inhibition in vitro of viral DNA\textsubscript{P}, was present in extracts of both roots and shoots, as well as in extracts from plants ranging in age from a month to about six months old (Unander et al. in press). These results suggested the inherent presence of the active compound(s) and raised the possibility that such compounds would be produced in a callus culture.

Callus cultures were successfully established from young plants of the tree \textit{P. emblica} L. of the subgenus \textit{Emblica} Webster (Khanna and Staba 1968). \textit{Phyllanthus} may contain as many as 700 species in at least 10 subgenera, many of which contain predominately woody, perennial species (Holm-Nielsen 1979; Webster 1956; 1957; 1958). Herbaceous species of the subgenus \textit{Phyllanthus}, including \textit{P. amarus}, predominate among records of usage in systems of traditional medicine as
thoroughly rinsed with tap water and presoaked for 30-60 min in young, potted plants or from the new growth of older plants were first procedure: small sections of stems and phyllanthoid branches from Blumberg 1991). In preliminary attempts at surface sterilization, the Surface sterilization and in vitro culture conditions. Materials and methods

Results with extracts of whole plants.

Successful sterilization was accomplished using the following procedure: small sections of stems and phyllanthoid branches from young, potted plants or from the new growth of older plants were first washed with soap and water to remove any superficial dirt, then thoroughly rinsed with tap water and presoaked for 30-60 min in distilled water with Tween 20 added at 1 drop/100 ml. Tissue sections with sodium hypochlorite with the same amount of Tween 20, and rinsed (X3) in sterile, distilled water. Whole leaves, flowers and seed capsules, and sections of stems and phyllanthoid branches 5-10 mm long were then cut and placed on culture media in sterile petri dishes. Phyllanthoid branches, commonly found in many species of this genus, superficially appear to be compound leaves, but the presence of flowers and fruit at the base of each leaf shows them to be true branches (Webster 1956). Petri dishes were sealed with Parafilm M (American National Can, Greenwich, CN) and kept in the growth chamber under continuous fluorescent light (20 W Cool White tubes, Sylvania Co., Seneca, NY) at an average light intensity of 30 μE/m²/s. Temperature was maintained at an average of 28°C.

Callus induction was compared among media consisting of either MS salts (Murashige and Skoog 1962) with 100 mg/l myo-inositol and 0.4 mg/l thiamine HCl or B5 salts (Gamborg et al. 1968) with 100 mg/l myo-inositol, 1 mg/l nicotinic acid, 1 mg/l pyridoxine HCl and 10 mg/l thiamine HCl. All media also had sucrose (30 g/l) and Difco-Bacto agar (5 g/l). The plant growth regulators tested were 2,4-D alone (1 mg/l) in MS or B5 (referred to hereafter as MDB media, respectively), or with BA (0.5 mg/l) in MS and B5 (MDB0.5 and MDB1.5, respectively).

Comparison of callus growth among media. After successful callus initiation, subcultures were made with ca. 5 mm calli pieces taken from MDB0.5 or subsequent cultures (i.e., MDB1 and MBB1). These were transferred to various permutations of media to determine which supported maximum growth. Preliminary experiments (observations only, taken at 1 mon), compared 2 or 4 times the amount of BA as the original induction medium (i.e., MDB0.5 vs. MDB1 vs. MDB2). Fresh and dry weights of calli were then compared at 21 da among MDB1, MDB2, BDB1 and BDB2. This experiment was analyzed as a 2 X 2 factorial (media salts and auxin:cytokinin ratio) in a reps-in-blocks design of four calli in each of four petri plates. Dry weights were obtained after lyophilizing calli overnight. When Bartlett's test for the homogeneity of variance was significant (Snedecor and Cochran 1967), analyses of variance were compared with analyses done on the normalized ranks of the data (Conover 1980). If there were differences between the analyses, the results from the analysis of the ranks were used. This procedure was used throughout.

Since P. amarus is often found in calcareous sites (Webster 1957; personal observations), a medium was made to test the effect of twice the level of calcium of the MS salt formulation (MDB2 plus an additional 332 mg/l anhydrous CaCl₂). Fresh weights of calli at 18 da were analyzed as a reps-in-blocks design with four calli in each of five plates. Media incorporating the auxins IBA and IAA at 1 mg/l in place of 2,4-D (referred to hereafter as MBB and MAB media, respectively) were also tested. The first such experiment compared callus weight at 5 wk among MDB1, MDB2, MBB1, MBB2 and MBB3. A subsequent experiment measured callus weight at 6 wk in media using the cytokinins IBA and IAA and three cytokinin:auxin ratios closer to 1:1 among the media MAB0.5, MAB1, MAB1.5, MBB0.5, MBB1 and MBB1.5. Both of these experiments were analyzed as a 2 X 3 factorial (cytokinin type and auxin:cytokinin ratio) in a reps-in-blocks design with four calli in each of three plates.

Callus initiation in other species. Callus induction was also attempted with other species representing various degrees of taxonomic distance from P. amarus. The closest related species which were tested were P. abnormis Baill. and P. debilis Klein ex Willd., both occurring in the subgenus Swartzian Webster of the section Phyllanthus of the subgenus Phyllanthus (Webster 1957). Additional members of this subgenus which were tested were P. mimicus Webster of the subsection Phyllanthus of section Phyllanthus and P. urinaria of the section Urinaria Webster (Webster 1957). Phyllanthus debilis and P. urinaria are Asian species, whereas P. abnormis and P. mimicus are native to the Americas (Webster 1957). Phyllanthus abnormis is believed to be the closest relative to P. amarus (Webster 1957). In the subgenus Isocladus Webster, P. caroliniensis Walt of section Loxopodium Webster is a small New World herb (Webster 1956), whereas P. myrtifolius Moon of section Macres (Wight) Baill. (personal communication with G.L. Webster, Univ. of California, Davis), is a small shrub from Sri Lanka. Phyllanthus arbuscula (Sw.) Gmel. is a shrub of section Xylophylla (L.) Baill. in the New World subgenus Xylophylla (L.) Pers. (Webster 1958).

The plants of P. arbuscula and P. myrtifolius were obtained from the University of Pennsylvania, Philadelphia, PA, and from the Lyon Arboretum, Honolulu, HI, respectively. The plants of P. abnormis came from seeds collected from plants growing on Sanibel Island, FL. Details concerning the accessions of P. debilis, from India, P. mimicus and P. urinaria, from Venezuela, and P. caroliniensis, from the United States, are given in Unander and Blumberg (1991).

Leaves, seed capsules, stem, and phyllanthoid branch pieces of P. debilis were placed on MD, BDB, MBB1 and MBB2. Stem