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Bud Formation and Shoot Development *In Vitro:* Observations on Stem and Bud Explants of *Psychotria punctata* (Rubiaceae)

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Protuberances tentatively identified as buds were initiated on callus proliferating from the bases of excised terminal buds of *Psychotria punctata* (Rubiaceae) cultured on media containing 10^{-4} and 10^{-5}M zeatin. Only those cultures maintained in a greenhouse during mid-summer formed such buds. A high cytokinin concentration together with one or more specific environmental conditions appear to be required for their formation. In contrast, large nodule-bearing shoots developed directly from terminal buds when these were excised during the fall and cultured on the same medium in controlled environment chambers providing temperatures and light intensities lower than in the greenhouse. Zeatin was required for shoot development on this medium; little development occurred in its absence. Shoot development occurred in both 8- and 16-hr days in the chambers; it did not occur in darkness, in continuous, predominantly incandescent light, or in the greenhouse.

This investigation continues the earlier attempts by LaMotte and Lersten (1972) to induce bud formation in callus cultures derived from stem segments of the tropical, woody shrub, *Psychotria punctata*. The goal of these attempts is to obtain the bacteria-free plants needed for further critical studies of the leaf nodule symbiosis. Stem segments were used as starting material for cultures because they appear not to be inhabited by the endosymbiont (Lersten and Horner, 1967) and they readily form callus in culture. Callus from this source forms roots in culture (LaMotte and Lersten, 1972) but, until the work reported here, attempts by us and by J. E. Lombard (personal communication) of the University of Pretoria to induce bud formation had been without success. In this paper we report the formation of buds on callus proliferating from bases of excised terminal buds cultured *in vitro*. Although this was observed only once, and the buds were inadvertently killed before they could be unambiguously characterized, we feel this is important to report as it constitutes the first hint of success at inducing *Psychotria* bud formation *in vitro*, a step we consider essential to any strategy for complete analysis of the symbiosis.

MATERIALS AND METHODS

Main and branch shoots were excised from greenhouse-grown plants of *Psychotria punctata* Vatke (= *P. bacteriophila* Val.), washed with 95% (v/v) ethanol, and immersed in half-strength commercial bleach [Miracle Bleach; 5.25% (w/v) sodium hypochlorite] for 10 min. After rinsing twice in sterile water, terminal buds were excised. Two buds, each ca 3 X 3 mm and bearing 4-6 pairs of young leaves and leaf primordia, were placed on an agar medium of 50 ml volume. Sections ca. 1 cm long were cut from internodes of young shoots and prepared and cultured in similar manner. The basal medium was identical to the "stock callus medium" of LaMotte and Lersten (1972) except that KH_2PO_4 replaced K_2HPO_4 and IAA and kinetin were deleted.

Five environments were tested in one experiment, fewer in others. These environments were 1) a greenhouse, 2) a controlled environment chamber with 16 hr/day of light, 3) another chamber with 8 hr/day of light, 4) a laboratory cabinet illuminated predominantly by continuous incandescent light, and 5) a similar, dark cabinet in the same room. In the greenhouse, culture flasks were enclosed in transparent plastic containers and these were sealed in polyethylene bags. Temperatures as high as 45°C were measured in the containers on clear days. In the controlled environment chambers temperatures inside the containers were 27 ± 1°C in darkness and 32 ± 1°C in the mixed fluorescent and incandescent light of 1800-1900 ft-c. The laboratory cabinet was 24 ± 1°C and illuminated by one 40 watt incandescent bulb in a reflector hood 40-50 cm from the culture flasks and by fluorescent ceiling lights 2 m from them. This environment will be designated as "incandescent light." Darkness was maintained in another cabinet at 24 ± 1°C; cultures in it were examined briefly in light about twice weekly.

RESULTS

Because callus from mature stems had failed to form buds on media containing synthetic cytokinins (LaMotte and Lersten, 1972), young formative parts and a naturally occurring cytokinin were tried. Terminal buds were cultured on basal medium to which zeatin had been added in concentrations ranging from 10^{-7} to 10^{-4}M. Two cultures (2 buds/culture) on each medium were kept under mid-summer greenhouse conditions; one of each was kept in incandescent light and another in darkness. Callus proliferated from cut surfaces at the bases of terminal buds on all 4 media in all 3 environments but no roots were formed (Fig. 1). After 4 weeks in culture, callus growth was greatest on the 10^{-4}M zeatin medium and was similar in all 3 environments. At this time buds were evident on callus of some of the greenhouse cultures. Buds were identified by their shape and green color; small leaves were

Fig. 1-7. In vitro cultures of terminal buds excised from plants of *Psychotria punctata*. 1. Cultures after 4 weeks under mid-summer greenhouse conditions on basal medium plus zeatin at concentrations (left to right) of 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. × 0.5. 2. Newly formed buds (arrows) on callus proliferating from base of terminal bud after 4 weeks under mid-summer greenhouse conditions on basal medium plus 10^{-4}M zeatin. × 2. 3. As in Fig. 2 except medium contained 10^{-5}M zeatin. Arrows indicate buds on callus. 4. Shoots arising directly from terminal buds after 10 weeks of culture on basal medium plus 10^{-4}M zeatin in a growth chamber providing 16-hr daylength. × 1. 5. As in Fig. 4. Arrows indicate leaf nodules. 6. As in Fig. 4 except cultured on medium without cytokinin. × 1.5. 7. Effects of gentamicin at concentrations (left to right) of 10, 50, 100, and 200 mg/l after 10 weeks. Left flask contains a nodulated shoot. Medium and environment as in Fig. 4. × 0.5.

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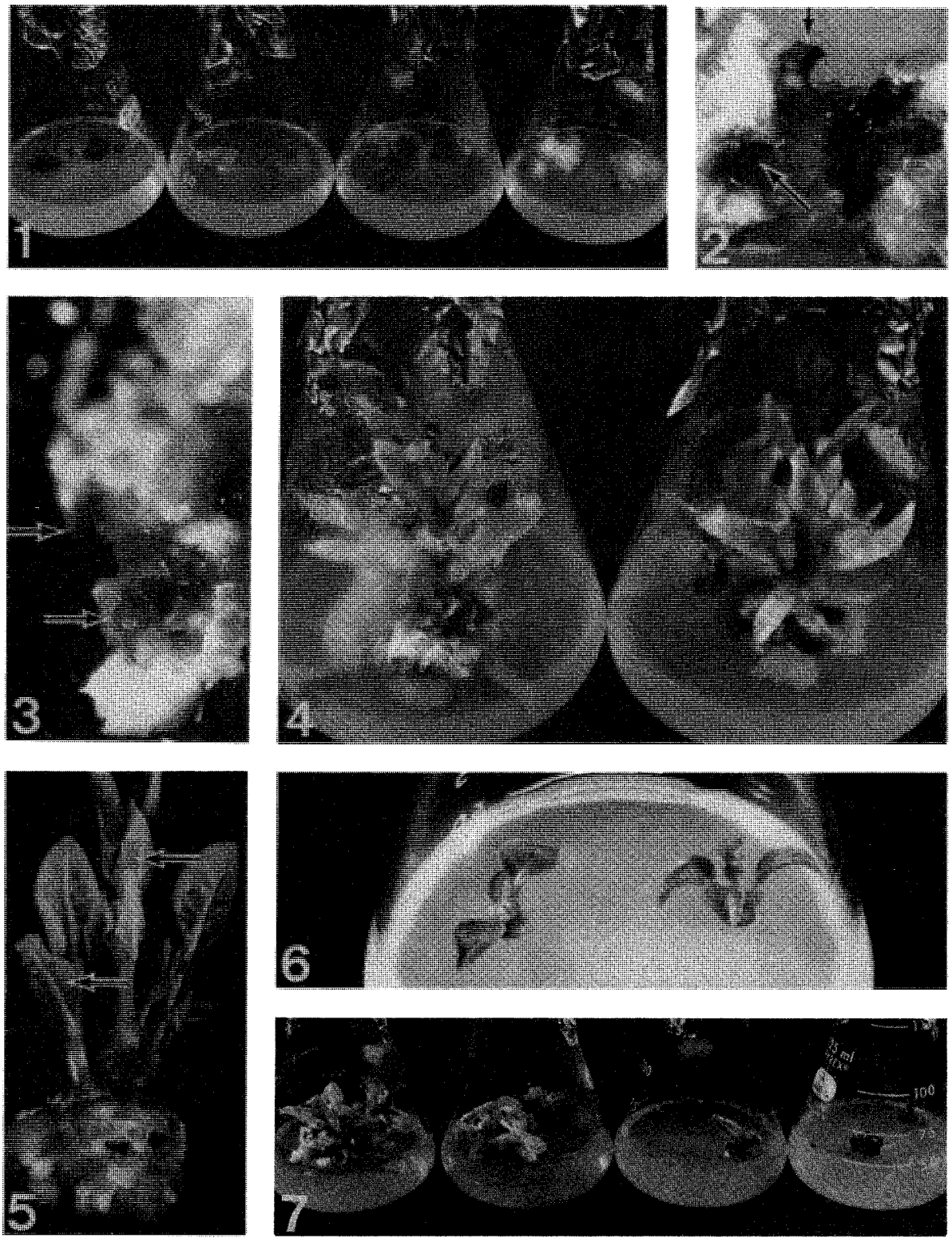

their most conspicuous parts (Fig. 2, 3). No buds had formed in darkness or in incandescent light. Six buds were formed by callus on 10^{-4}M zeatin (Fig. 2), one by callus on 10^{-5}M zeatin (Fig. 3), and none on the lower zeatin concentrations. All buds were formed on the callus undersides in direct contact with the agar medium.

In attempting to reproduce the above results, terminal buds were cultured under greenhouse conditions in early fall on the medium containing 10^{-4}M zeatin. No buds or roots and very little callus was

formed. Further tests were made in controlled environment chambers providing 8- and 16-hr days. Buds in both chambers produced results very different from those in the mid-summer greenhouse. One or more robust, leafy shoots (Fig. 4, 5) developed directly from each terminal bud on the zeatin medium, and to an equal extent in both photoperiods. Leaves of these shoots were nodulated (Fig. 5). Callus proliferated from the basal portions of the buds as in the greenhouse but did not initiate buds or roots. Callus grew more in the 8-hr than in the 16-hr photoperiod. In the absence of zeatin, terminal buds produced only small shoots consisting of expanded parts of the original terminal bud (Fig. 6). Little or no basal callus was formed.

Attempts were also made to induce bud formation on stem explants in early fall using the media with and without 10^{-4}M zeatin. Equal numbers of cultures were maintained in each of the 5 environments described. Callus was formed by those stem explants supplied with zeatin but buds were not formed.

In attempting to evoke development of bacteria-free shoots from excised terminal buds the antibiotic gentamicin, effective against a wide range of gram-negative and gram-positive bacteria, autoclavable, and stable over a wide pH range[3], was tested in the zeatin-containing medium on which buds were cultured. These cultures were all maintained in controlled environment chambers under those conditions shown to elicit shoot development (Fig. 4, 5). The recommended[3] bactericidal concentrations of 50-100 mg/l proved to be completely inhibitory to *Psychotria* shoot development (Fig. 7). The lowest tested concentration (10 mg/l) permitted shoot development from terminal buds but appears not to have eliminated the bacteria. These shoots developed leaves with conspicuous nodules.

DISCUSSION

Bud formation by callus tissue of *Psychotria punctata* appears to require a high concentration of exogenous cytokinin together with one or more conditions such as found in a greenhouse in mid-summer. Bud formation (Fig. 2, 3) was observed only once and only under these particular conditions. Repeated attempts to obtain it under controlled conditions were without success. However, conditions approaching those of the greenhouse in summer were never fully attained in these later experiments and we have not yet repeated the greenhouse test. Few instances of bud initiation by woody plant tissue cultures have been reported (Gautheret, 1955; Haissig, 1965; Jacquiot, 1966; Nitsch *et al.*, 1967; Staritsky, 1970; Winton, 1970, 1972a, b; and Venverloo, 1973) and the effects of photoperiod or temperature on bud formation by these has not been reported. Only Gautheret (1940a, b) has studied the effects of light intensity on bud formation in these. He used elm explants of predominantly cambial origin and found more buds formed in light than in darkness. Stone (1951) observed a large increase in bud formation by tobacco stem segments when these were cultured in sunlight instead of low intensity fluorescent illumination.

The finding of buds on callus growing on a medium containing a high concentration of a naturally occurring cytokinin (Fig. 2, 3) suggests that the callus may require this for bud formation. This report and that of Venverloo (1973) seem to be the only ones on bud formation by callus cultures of woody species supplied with a naturally occurring cytokinin. It is clear from this study that cytokinin will not suffice to elicit bud formation in *Psychotria* callus if yet unknown environmental conditions are not also provided.

Terminal buds cultured in the controlled environment chambers developed into normal shoots replete with nodules when cytokinin was supplied (Fig. 4, 5); those cultured in the other environments, or without cytokinin (Fig. 6), did not. Elliott (1970) found that excised shoot apices of *Rosa multiflora* bearing 1-3 leaf primordia required cytokinin for development into shoots or plantlets. A concentration of zeatin as low as 10^{-8}M sufficed for this. *Psychotria* buds, unlike *Rosa* apices, formed no roots at their bases. Callus which developed there in response to 10^{-4}M zeatin (Fig. 5) probably enhanced contact with the medium, but seems inadequate as sole explanation for the striking difference in shoot development in the presence and absence of cytokinin (Fig. 4, 5 vs. 6).

The physiological state of buds excised during mid-summer may be quite different from that of buds excised during fall and such state may be paramount in determining their development in culture, as shown for excised *Citrus* buds by Altman and Goren (1974). In our work, such differences in initial state are confounded with treatments when experiment to experiment comparisons are made, but are not confounded in the individual experiments.

Callus growth (Fig. 1; LaMotte and Lersten, 1972), bud formation (Fig. 2, 3), and shoot development (Fig. 4, 5) *in vitro* all appear to require exogenous cytokinin. These findings lend support to the idea that the leaf nodule symbiosis involves production of cytokinin by nodule bacteria which is required for normal development by the host plant. This is further supported by the demonstrated occurrence of high cytokinin concentration in nodule-bearing leaf discs compared with its low concentration in discs not bearing nodules (Edwards and LaMotte, 1975).

[3]Informational brochure from the Schering Corporation.

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