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DEVELOPING AN IMPROVED *IN VITRO* PROPAGATION SYSTEM FOR SLOW-GROWING SPECIES USING *GARCINIA MANGOSTANA* L. (MANGOSTEEN)

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Summary

This investigation disclosed that evaluation of tissue culture parameters of slowly developing species (e.g. *Garcinia mangostana*) requires monitoring of treatments through two or more successive, relatively long passages. Two 8-wk passages were necessary to observe differences in phytohormone effects. Photoperiod and temperature effects were not clearly evident until tissues had been cultured through three passages; the optimal photoperiod and temperature for shoot proliferation could not be established until after the fifth passage. Our investigation revealed that no auxin supplementation was necessary for bud primordium differentiation in cotyledon explants or proliferation of regenerated shoots. The optimum N^6-benzyladenine concentration for primordium differentiation was 13.3 μ*M*, and for shoot proliferation ranged from 4.4 to 13.3 μ*M*. Continuous culturing in an 8-h photoperiod at 30°C resulted in progressively intensified degeneration of shoots after three passages. In contrast, successive passages in a 16-h photoperiod/26°C regimen enabled sustained regeneration of shoots. The shoots rooted at a rate of 85% when precultured for 3 d in a medium containing 4921.3 μ*M* indole-3-butyric acid, or 10 d at 492.1 μ*M*, then cultured for two 8-wk passages in phytohormone-free medium. Following acclimatization by gradually lowering the relative humidity in the growth chamber, rooted shoots survived transfer to the greenhouse at a rate of 95%.

Key words: mangosteen; micropropagation; plant regeneration; two-step rooting; acclimatization.

Introduction

This investigation emerged from an attempt to obtain large quantities of mangosteen propagules for trial plantings. As acclaimed by earlier researchers (Goh et al., 1988; Normah et al., 1992, 1995), this tropical tree produces a most delicious 'queen of fruits'. But mangosteens are not widely available, even in the tropics, because of limited plantings. Mangosteen is an obligate apomict (Horn, 1940); thus all seedlings should be genetically identical. But seeds are scarce, short-lived and difficult to germinate, and cuttings are not readily rooted (Goh et al., 1988). We tried to obtain propagules rapidly by following the micropropagation protocol published by Normah et al. (1995). Rapid shoot proliferation and satisfactory rooting did not occur. Hence we were compelled to reassess specific aspects of the protocol, namely the phytohormone, photoperiod and temperature provisions for shoot regeneration and the inductive phytohormone treatment for rooting the regenerated shoots.

Materials and Methods

Tissue culture followed the procedure of Normah et al. (1995), and experiments focused on their key recommendations. Seeds were removed from freshly purchased ripe fruits and their densely pubescent outer coats were pared away. They were then immersed in sodium hypochlorite solution (1% active ingredient plus few drops Tween 20 surfactant) for 30 min and rinsed three times with autoclaved water. The cotyledons were divided into four segments by cutting once each, longitudinally and transversely. One segment was planted per tube to initiate cultures.

MS medium (Murashige and Skoog, 1962) with 3% sucrose was used as base for cotyledon cultures and shoot proliferation experiments. The medium was solidified with 0.25% Gelrite (gift from Kelco Division of Monsanto, San Diego, CA). The auxin α-naphthaleneacetic acid (NAA), and the cytokinins N^6-benzyladenine (BA) and N^6-isopentenyladenine (2iP) were examined for bud primordium differentiation in cotyledon explants and subsequent multiplication of emerged shoots. NAA was tested at levels of 0.5 and 2.7 μ*M*, and BA and 2iP were examined in the range of 1.3 to 49.2 μ*M*.

Lloyd and McCown's (1980) woody plant medium served as the base for rooting experiments. The two-step method of Goh et al. (1988), which is also the standard procedure in our laboratory for rooting woody genera, was evaluated. The medium for the induction step contained 1.5% sucrose, MS vitamins, 0.25% gelrite, and either 492.1 or 4921.3 μ*M* indole-3-butyric acid (IBA); no mineral salts were included. Shoots to be rooted were placed in inductive media for 3–14 d, then transferred to woody plant medium and further cultured for two passages, each 8 wk long.

The pH of all media was adjusted to 5.7 prior to adding gelrite. All media were sterilized by autoclaving at 121°C (1.05 kg cm^{-2}) for 10 min.

Culture vessels for cotyledon cultures and for the first passage of shoot multiplication experiments were 25 × 150 mm glass tubes, each containing 20 ml medium. Polypropylene closures (Kaputs from Bellco, Vineland, NJ) were used for these tubes. Cultures of the second and third passages of shoot proliferation experiments were conducted in 30 × 150 mm tubes, each containing 25 ml medium. Rooting experiments were also performed in the larger tubes. The larger tubes were closed with gas-permeable MEICO plugs (Microwave Enterprise, Taipei, Taiwan). For the fourth and fifth passages,

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culturing was done in 300 ml Erlenmyer flasks containing 100 ml medium to accommodate the larger and more numerous shoots. These were also capped with gas-permeable MEICO plugs.

We discovered at the outset that mangosteen tissue cultures developed extremely slowly. Single 4- to 6-wk passages, that are adequate for experimenting with most other species, enabled too few shoots and too low rooting frequencies for accurate determination of treatment responses. Thus all treatments were monitored through at least two passages, each 8 wk long. The critical experiment on photoperiod and temperature spanned five passages (40 wk). Data were recorded after each passage, and tissues were transferred to succeeding passages without dividing.

Except for evaluating photoperiod and temperature effects and for acclimatization of rooted shoots, all cultures were maintained in a large growth room under 16-h daily exposure to 25 $\mu mol\ m^{-2}\ s^{-1}$ cool white fluorescent light and at a constant 26° ± 2°C. The photoperiod and temperature experiments and acclimatization of rooted shoots were conducted in controlled environment chambers.

At least 10 cultures were employed per treatment in shoot multiplication experiments. Rooting experiments used 15 shoots per treatment. Inocula for shoot multiplication consisted of clusters of three 0.5–1.5 cm tall shoots with adjoining buds and primordia. Rooting observations were based on one 2–3 cm tall shoot per culture. All experiments were repeated at least once.

Rooted shoots were washed in running water to remove media that adhered to the roots, then transplanted in pots containing an equal-volume mixture of vermiculite, peat and perlite. They were acclimatized by lowering the relative humidity in the controlled environment chamber from 95 to 85% in 5% increments at weekly intervals, then transferred to a shaded bench (under a saran cloth cover) in the greenhouse.

Data were analyzed by calculating standard errors of means (Snedecor, 1957) or obtaining 95% confidence limits of binomials from published tables (Diem and Seldrup, 1982). Other than the final photoperiod and temperature study, only data from the second passage are presented.

Results

Cotyledon culture. At least 8 wk passed before primordia, or forerunners of buds and shoots, became clearly visible on cotyledon segments. Most differentiating cotyledons were found in the medium supplemented solely with 13.3 μ*M* BA. No differentiation was observed in the 44.4 μ*M* BA medium, with or without NAA. Both NAA concentrations, alone or in combination with varying levels of BA, inhibited tissue development. Thus 13.3 μ*M* BA was selected as the sole phytohormonal supplement for further culturing of cotyledons. The bud primordia differentiated in clusters directly from cotyledons; no intermediary callus developed. Cotyledon segments varied considerably in primordium differentiation. A few displayed prominent clusters after one 8-wk passage; others showed the first primordia after two passages, and still other segments failed to differentiate. All cotyledon segments browned intensely, but those failing to differentiate eventually blackened and died. Only a small proportion of the segments, 20% in one trial and 30% in another, differentiated. Bud and shoot emergence followed after several more weeks. We did not obtain quantitative data from these cultures. Instead, we allowed primordial differentiation and shoot emergence to progress continuously by transferring whole cultures to fresh medium every 8 wk until sufficient numbers of shoots became available for shoot-proliferation experiments.

Shoot proliferation: phytohormone requirement. Shoots excised from cotyledon segments also showed no auxin requirement for proliferation. Both levels of NAA repressed the BA-promoted shoot proliferation. The optimum BA concentration for shoot multiplication, without NAA addendum, ranged from 4.4 to 13.3 μ*M* (Fig. 1). An average of 13 shoots, or 10 new shoots, was obtained per three-shoot cluster after two passages in media containing 4.4 or 13.3 μ*M* BA. A lower BA concentration (1.3 μ*M*) gave substantially fewer shoots, although still more than the BA-free medium. The number of new shoots in the 44.4 μ*M* medium was no higher than in the control. All concentrations of 2iP resulted in significantly fewer shoots than the control (Fig. 1).

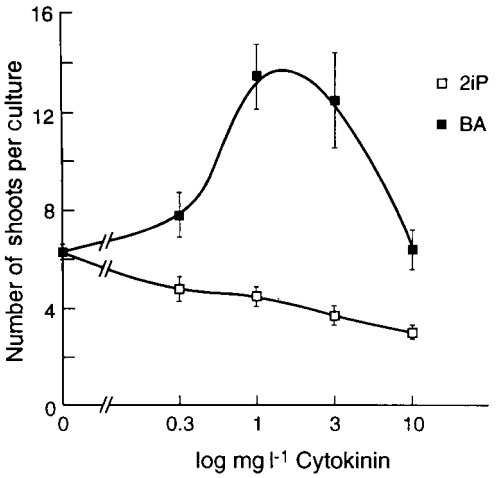

FIG. 1. Effects of N^6-benzyladenine and N^6-isopentenyladenine on proliferation of mangosteen shoots.

Shoot proliferation: photoperiod and temperature requirements. Initially, the shoot proliferation rates under the 8-h photoperiod recommended by Normah et al. (1995) was compared with the 16-h photoperiod that we routinely employ for tissue cultures of diverse species. The temperature was kept at 26°C. After two passages, mangosteen cultures under the 16-h photoperiod displayed slightly more shoots than the shorter photoperiod. Thus the investigation was repeated, but with temperature as an added variable and the treatments being monitored through five passages. The variables were 8- and 16-h photoperiods, and 26 and 30°C temperatures. The culture medium contained 4.4 μ*M* BA. Successive culturing under all combinations of 8-h photoperiod and 30°C temperature resulted in severe browning and death of shoots, even though the medium had been refreshed for each passage (Fig. 2). Thus shoot counts (Fig. 3) were confined to reculturable shoots.

The photoperiod × temperature effects first became evident after three passages (Fig. 3). Observations indicated that all combinations involving an 8-h photoperiod or a 30°C temperature produced significantly fewer shoots than the 16-h photoperiod × 26°C temperature regimen. The decrease in reculturable shoots became progressively more noticeable in succeeding passages. In the 16-h × 30°C combination (30C/16h in Fig. 3), the number of reculturable shoots actually decreased after five passages. Cultures in the 8-h photoperiod × 30°C (30C/8h) environment showed no further increase in reculturable shoots after three passages. In the 8-h photoperiod × 26°C treatment (26C/8h), reculturable shoots continued to increase, but at a distinctly slower pace than with the 16-h × 26°C. The latter enabled regeneration of more than 20 reculturable shoots per inoculum, or a six- to sevenfold shoot multiplication, after five passages (40 wk).

Rooting the regenerated shoots. A rooting rate of 85% was attained when individually severed shoots were first placed in an inductive medium containing 4921.3 μ*M* IBA for 3 d, or 492.1 μ*M*

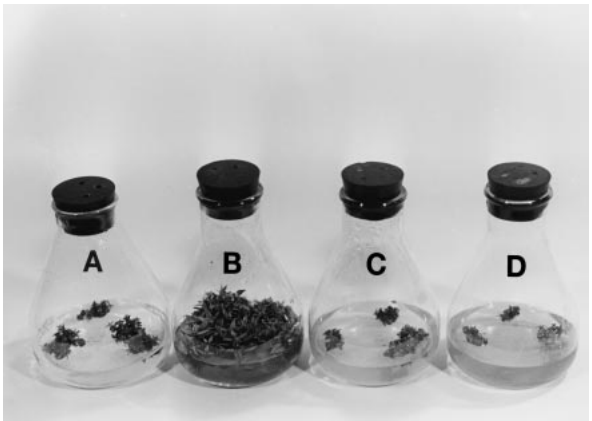

FIG. 2. Representative cultures showing effects of photoperiod and temperature. Left to right, A = 8 h/26°C; B = 16 h/26°C; C = 8 h/30°C; D = 16 h/30°C. Photographed after five passages of 8 wk each. Note severely darkened tissue, repressed growth and degenerated shoots of cultures maintained for prolonged periods under unfavorable photoperiod and/or temperature.

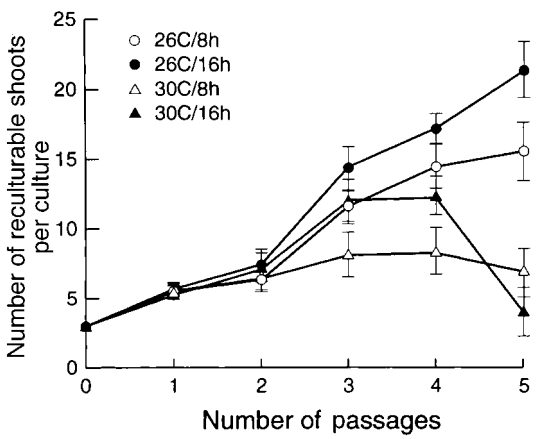

FIG. 3. Photoperiod and temperature effects on proliferation of mangosteen shoots. Only data for reculturable shoots are shown.

IBA for 10 d, then transferred to a second medium without auxin and further cultured for two passages (Fig. 4). No rooting occurred without the high-auxin induction treatment. More roots per shoot, three as opposed to two, resulted in the lower auxin concentration.

Acclimatization. Rooted shoots that were acclimatized in the growth chamber survived transfer to the greenhouse at a rate >95%.

DISCUSSION

Of foremost importance, this investigation of *Garcinia mangostana* demonstrated that accurate assessment of tissue culture variables for recalcitrant species requires monitoring of treatments through a number of relatively long, successive passages. The reports of Goh et al. (1988) and Normah et al. (1992, 1995) do not specify the passage lengths or the number of passages employed per experiment. Goh et al. (1988) present data which show that the

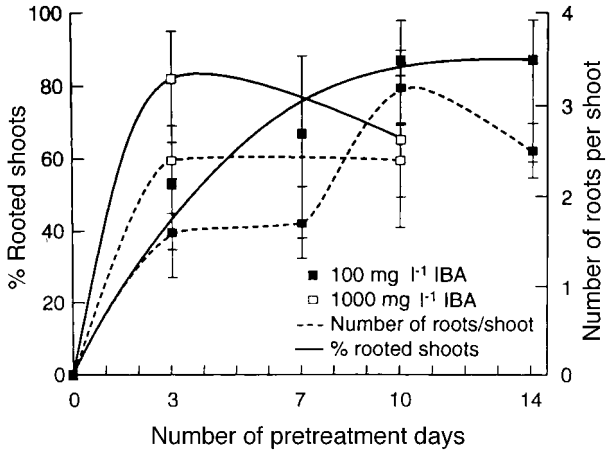

FIG. 4. Two-step rooting of regenerated mangosteen shoots. Shoots were precultured for varying periods in induction media containing 492.1 or 4921.3 μ*M* indole-3-butyric acid, then transferred to a medium without phytohormone for two 8-wk passages.

maximum differentiation of cotyledon segments was attained after 7 wk, and note that further proliferation by axillary branching occurred when shoots were subcultured. Normah et al. (1992, 1995) state that the maximum numbers of shoots were achieved after 14 wk; their regenerated shoots were rooted directly and transferred to soil.

In our investigation, the data recorded after two passages show that no auxin supplementation was needed for differentiation of bud primordia in cotyledon explants or proliferation of regenerated shoots. In fact, NAA consistently repressed shoot proliferation. However, the cytokinin BA was critical. Moreover, the 40 μ*M* concentration (ca. 9 mg l^{-1}) recommended by Normah et al. (1995) was far beyond the optimum. The comparable concentration in our investigation, 44.4 μ*M or*10 mg l^{-1}, produced a shoot number that was no better than the control. The more favorable BA concentration was in the range 4.4 to 13.3 μ*M*. Our findings with respect to the phytohormone requirement are thus in concurrence with those of Goh et al. (1988), but not Normah et al. (1995). 2iP, a very active cytokinin for some tissue cultures, was not effective for mangosteen, as it suppressed shoot number at all concentrations.

The principal focus of virtually all micropropagation investigations has been the requirements with respect to phytohormones and other nutrient medium constituents. This investigation demonstrated that systematic assessment of the light and temperature provisions can be equally important. Simply adopting the *in vitro* environment employed for other species, or for the same species by other investigators, may have unfavorable consequences. Thus we discovered that, when employed continuously through successive passages, the 8-h photoperiod × 30°C environment specified by Normah et al. (1995) caused degeneration and death of shoots. The more optimal regimen for mangosteen tissue culture was a combination of 16-h photoperiod and 26° temperature. The *in vitro* environment employed by Goh et al. (1988) for their mangosteen cultures consisted of a 12-h photoperiod and a 26°C temperature, which was probably less unfavorable. Systematic investigations of other species have resulted in similarly significant findings. For example, Paterson and Rost (1979) found that the maximum rate of plant regeneration in *Crassula argentea* leaf

segment cultures occurred under an 8-h photoperiod; much longer daylengths resulted in poor regeneration. Highest shoot differentiation in tobacco callus occurred under 16-h daylength; no shoots formed under an 8-h daylength or in continuous darkness, and caulogenesis was markedly repressed by continuous illumination (Murashige, 1977). Pellegrineschi and Tepfer (1993) revealed a higher plant regeneration rate of *Sesbania rostrata* callus under a 12-h than under a 16-h photoperiod. The temperature optimum established experimentally by Kato and Ozawa (1979) for shoot bud differentiation in tissue cultures of *Heloniopsis orientalis* was 21–25°C. Although not studied with aseptic cultures, Kefford and Caso (1972) found the optimum day temperatures for shoot regeneration in *Chondrilla juncea* roots to be 25–27°C, and the night temperatures, 16–22°C. More recently, Pestana et al. (1999) reported that the highest shoot regeneration rate of peanut cotyledon and leaf section cultures resulted at a relatively high 35°C, and not the more commonly employed 25–28°C.

The mangosteen shoots regenerated in this investigation have rooted at a rate of 85%, substantially higher than that reported by Goh et al. (1988). Goh et al. (1988), also employing the two-step procedure, obtained a rooting rate of 24% by preculturing for 7 d in an inductive medium supplemented with 98.4 μ*M* IBA. The two-step method may be the preferred alternative when the single-step method, widely employed for herbaceous species, fails to induce rooting of recalcitrant tree genera. The rationale is derived from the long-established concept (Thimann, 1977) that root initiation occurs in response to high concentrations of auxin, whereas root elongation is inhibited by the same concentrations. The two-step method allows the use of high doses of auxin for root initiation and avoidance of its inhibitory action during emergence.

In spite of our findings, the tissue culture method remains relatively slow for reproducing mangosteen plants. But improvements, particularly to eliminate factors that negatively affect shoot proliferation, can be proposed in modifying the protocol of Normah et al. (1995). First, the NAA supplement should be excluded. Second, the BA concentration should be reduced to ca. 4.4 μ*M* BA. Third, the culture environment should be modified by increasing the daylength to 16-h and lowering the temperature to 26°C. It is probable that when the new information is put into practice, the numbers of multiplied shoots can be increased substantially by dividing during subculturing, instead of simply reculturing them as was done in this investigation. Finally, the induction step for the two-step method of rooting regenerated shoots should employ a higher dosage of IBA than employed by Goh et al. (1988) for the induction medium, either 4921.3 μ*M* IBA for 3 d or 492.1 μ*M* for 10 d, instead of 98.4 μ*M* for 7 d. The acclimatization procedure of progressively lowering the ambient relative humidity in environment-controlled growth chambers has worked well in our hands for most species, woody as well as herbaceous.

Differences between our findings and those of earlier investigators (especially of Normah et al., 1992, 1995) may not be genetically based, inasmuch as the cultivated *G. mangostana* is an obligate apomict (Horn, 1940). Although the existence of somaclonal variants cannot be discounted, we believe the differences between this study and other reports can be attributed to experimental procedures, namely passage lengths and number of passages employed in evaluating treatments.

Acknowledgments

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