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Medium and calcium nitrate influence on shoot regeneration from leaf explants of guayule☆

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ABSTRACT

Basal media including Murashige and Skoog (MS), Woody Plant Medium (WPM), and Driver and Kuniyuki (DKW) media were compared for use in guayule regeneration and propagation. Explants grown on MS basal medium showed large size calli with multiple shoots, but the calli produced on DKW medium were of higher quality, and longer true shoots were produced. Adventitious root formation was best in WPM basal medium.

A comparison of macro and micro nutrient levels in MS, DKW, and WPM media revealed significant differences in the calcium levels. Under controlled conditions, guayule plants *in vitro* incorporated calcium into plant tissues in proportion to the amount available. Moreover, the addition of calcium nitrate tetrahydrate at moderate levels into MS medium significantly improved the emergence of single, elongated true shoots, suggesting a requirement for calcium in guayule tissue culture. We have further explored selection agent (antibiotic) concentration for the identification of transgenic plants using a spectinomycin resistance gene (aadA). Based on the results, we have improved tissue culture protocols for guayule including spectinomycin as the selection agent.

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1. Introduction

Natural rubber, *cis*-1, 4-polyisoprene, is a critical raw material derived from plants used in many industrial applications (Cornish and Backhaus, 1990). Guayule (*Parthenium argentatum* Gray), a perennial woody shrub native to the southwestern United States and northern Mexico desert (Bonner, 1943; Backhaus, 1985; Dhar et al., 1989; Madhavan et al., 1989) accumulates and stores high molecular mass natural rubber within the bark parenchyma cells that can be harvested for industrial uses (Mooibroek and Cornish, 2000). In addition, guayule rubber has similar molecular weight to *Hevea brasiliensis* rubber but does not contain the proteins that can cause Type I allergic reactions (Cornish, 1996). Although the plant is drought-tolerant and easily cultivated in semi-arid regions, guayule can be very recalcitrant to regeneration and transformation with *in vitro* plant tissue culture.

A guayule *Agrobacterium*-mediated transformation system developed by Pan et al. (1996) was used to introduce the kanamycin resistance *npt*II gene into guayule. Dong et al. (2006) improved guayule transformation using leaf tissue and controlled light intensity. However, both procedures suffer because of several limitations, including low efficiency of genetic transformation and time-consuming *in vitro* regeneration. Despite advances in the field, the efficiency of genetic transformation rates are generally low and *in vitro* propagation and regeneration very time-consuming. The reported methods (Pan et al., 1996; Dong et al., 2006) usually required 7–9 months or longer to complete. Another three months are required for growing small plantlets to a suitable size for potting in soil. Overall this limits the progress for transgenic analysis, functional genomics, and crop improvement studies.

The culture medium used is a critical component of plant cell and tissue culture. Also, addition of plant growth regulators (PGRs) into the culture medium is used to induce morphogenetic responses in plant tissue culture. In the previous studies (Pan et al., 1986; Dong et al., 2006), Murashige and Skoog (1962) medium was used, as commonly employed in many plant systems. We have evaluated various basal media including Murashige and Skoog (MS), Woody Plant Medium (WPM) (Lloyd and McCown, 1981), and Driver and

Abbreviations: ANOVA, analysis of variance; BA, 6-benzyladenine; $CaN_2O{\cdot}4H_2O$, calcium nitrate tetrahydrate; CRD, Completely Randomized Design; DKW, Driver and Kuniyuki; Guayule, *Parthenium argentatum* Gray; H_2O_2, hydrogen peroxide; HCl, hydrochloric acid; ICP-OES, inductively coupled plasma optical emission spectrometry; MS, Murashige and Skoog; NAA, naphthalene acetic acid; PGRs, plant growth regulators; WPM, Woody Plant Medium.

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Kuniyuki (DKW) (Driver and Kuniyuki, 1984) medium for guayule regeneration and propagation.

The objective of this research was the development of an improved, highly efficient propagation and regeneration protocol for guayule transformation. The focus of this report is optimization of the culture media. We have further explored selection agent (antibiotic) concentration for identification of transgenic plants using a spectinomycin resistance gene (aadA). Based on the results, we have improved tissue culture protocols for guayule including spectinomycin as the selection agent.

2. Materials and methods

2.1. Plant material

In vitro cultures of guayule were established from seeds of cultivar AZ-2 (Ray et al., 1999) obtained from the USDA-ARS US-Arid Lands Research Center (Maricopa, AZ). Seeds were excised from achenes, then soaked sequentially in a 2% Tween-20 solution for 5 min, 70% ethanol for 1 min and in a 0.525% sodium hypochlorite (Clorox®) solution for 15 min. Seeds were triple rinsed with sterile water for 5 min. After germination, 3 cm of shoot tip was placed into a magenta box containing 88 mL of Murashige and Skoog (MS) (1962) basal medium supplemented with MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS), 3% (w/v) sucrose and 0.3% (w/v) phytagel (Cat. No. p8169, Sigma). The solution was adjusted to pH 5.7–5.8 prior to autoclaving at 121 °C and 103.5 kPa (15 psi for 30 min. This was the established medium used to maintain *in vitro* guayule cultures in our laboratory (Dong et al., 2006) at the start of this study. The cultures were maintained in a growth chamber at 25 °C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 μmol m^{-2} s^{-1}, held at the top of the culture vessels. All of the stock cultures in the following experiments were maintained under these conditions.

2.2. Shoot regeneration media: plant growth hormones

All *in vitro* explants were aseptically grown on Woody Plant Medium (PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with 0.1% MS vitamins, 3% (w/v) sucrose and 0.3% (w/v) phytagel (Sigma). They were transferred to fresh medium (hormone free WPM) every 6–7 weeks as leaves were used as a source of explants for shoot regeneration. Thirty shoot induction media, differencing in nutrient formulation (MS, DKW) were employed. The effect of naphthalene acetic acid (NAA) (Sigma) and 6-benzyladenine (BA) (Sigma) addition on shoot generation of guayule explants was examined in combinations of six levels of NAA (0, 0.1, 0.25, 0.50, 0.75, and 1.0 mg L^{-1}) and five levels of BA (0, 0.25, 0.50, 0.75, and 1.0 mg L^{-1}). For regeneration, three replicate plates were used, each with five 0.5 cm^2 leaf segments. Each experiment was repeated two times. Petri-dishes (100 × 20 mm) were cultured and kept under a 16 h light period and 25 °C temperature. After 3 weeks the calli with explants were transferred to fresh plates of the same media types and maintained for an additional 3 weeks. Data was collected at 3 and 6 week time intervals. The explants were evaluated for both shoot regeneration and callus growth.

2.3. Shoot regeneration media: calcium nitrate tetrahydrate ($CaN_2O_6{\cdot}4H_2O$)

To evaluate the effect of calcium nitrate on guayule regeneration, guayule explants were cultured on MS basal medium with plant growth regulators (PGRs) (1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA) and calcium nitrate tetrahydrate ($CaN_2O_6{\cdot}4H_2O$) (Acros Organics, New Jersey, USA). Seven calcium nitrate concentrations, 0, 100, 250, 500, 750, 1000, and 1500 mg L^{-1}, were tested. Calcium nitrate was added to the culture medium before adjusting the pH to 5.7, prior to autoclaving. The effect of calcium nitrate addition was similarly studied in DKW basal medium (0.75 mg L^{-1} BA and 0.25 mg L^{-1} NAA). Explants were sub-cultured every two weeks and plant performance was evaluated at six weeks for callus appearance according to the following numeric criteria: (5-points) actively growing and callus size over 2 cm; (4-points) actively growing and callus size over 1.5 cm; (3-points) callus size over 1.0 cm; (2-points) callus size less than 1.0 cm; and (1-point) no callus.

2.4. Shoot regeneration media: spectinomycin resistance

Leaf segments from control plants were cultured on regeneration DKW (PhytoTechnology Laboratories, Shawnee Mission, KS) media that contained 0.75 mg L^{-1} BA and 0.125 mg L^{-1} NAA at various concentrations of spectinomycin antibiotic (PhytoTechnology Laboratories, Shawnee Mission, KS) (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mg L^{-1}). Plant survival, callus formation, and shoot formation from leaf segments was assessed following 4 weeks in culture.

2.5. Propagation media

To evaluate the effect of basal medium salt on plant propagation, guayule shoots were cultured aseptically on MS, WPM, or DKW medium. Vigorously growing 3-cm shoot tips were excised from shoot regeneration media (Table 1) for guayule propagation. All media were supplemented with 0.1% MS vitamins, 3% (w/v) sucrose and 0.3% (w/v) Phytagel (Sigma). To evaluate propagation efficiency, 10 shoots (one shoot per magenta box) generated from each type of shoot regeneration media were sub-cultured in the propagation media. The experiments were repeated three times. The length of each plant was measured every 2 weeks for 6 weeks.

2.6. Calcium content

Whole plant tissues (minus roots) were removed from magenta boxes, freeze-dried and finely cryo-milled (60 s) using a Retsch MM-200 stainless steel mixer mill (25 mm ball). Tissues were accurately weighed in triplicate to ~500 mg directly into 50 mL SCP digestion tubes (including a blank and standards). One perforated boiling bead was added to each digestion tube, then tubes placed into a SCP Science Digiprep graphite digestion block under a fume hood. Then 100 μL of 0.5 M ceric ammonium nitrate (CAN 99.5%, Fisher Sci) solution was added to each digestion tube followed by 3.0 mL concentrated nitric acid (HNO_3 trace metal grade, Fisher Sci). Tubes were covered with a SCP disposable watch glass and allowed to predigest for 3 h under the fume hood. Digestion proceeded at 110 °C for 1 h. Then 2 mL 30% hydrogen peroxide (H_2O_2 certified ACS grade, Fisher Sci) was added to each digestion tube 4 times, at 5 min intervals (total of 8 mL) and the digestion continued for 30 min at 91 °C. Then 0.5 mL concentrated formic acid (certified ACS grade, Fisher Sci) was added to each tube 2 times, at 5 min intervals, followed by careful digestion at 125 °C for 20 min to avoid drying. Digestion tubes were removed from the block and cooled to room temperature for at least 20 min. Then 20 mL concentrated hydrochloric acid (HCl trace metal grade, Fisher Sci) was added to each tube, tubes placed on the block, and digestion continued for 90 min at 100 °C. Tubes were again removed from the block and allowed to cool to room temperature. They were then brought up to volume with DI water, stopped and shaken, then placed in a refrigerator until analysis. Inductively coupled plasma optical emission spectrometry (ICP-OES) was performed on all samples in duplicate using a Varian Vista Pro-CCD Simultaneous ICP-OES (Varian Inc, Palo Alto, CA).