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A SIMPLIFIED PROTOCOL FOR MICROPROPAGATION OF GUAYULE (*PARTHENIUM ARGENTATUM* GRAY)[†]

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Summary

A simple, efficient protocol for *in vitro* micropropagation of guayule is reported. Shoot cultures were maintained on MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg l^{-1} (4.4 μM) 6-benzylaminopurine and 0.025 mg l^{-1} (0.13 μM) α-naphthaleneacetic acid. Excised shoots were treated for 14–18 h with 100 mg l^{-1} (492.1 μM) indole-3-butyric acid in 0.5 × MS salts to induce rooting. The shoots were subsequently inserted into cellulose plugs which were packed in sterile, ventilated plastic culture vessels and moistened with 0.5 × MS medium without growth regulators. Use of cellulose plugs, liquid medium and ventilated culture vessels facilitated acclimation. Rooted shoots were transplanted into potting medium and acclimated to greenhouse conditions by covering with a cloche for 2 d, followed by daily watering for the first week.

Key words: shoot cultures; acclimation; Sorbarod; cellulose plugs.

Introduction

Guayule (*Parthenium argentatum* Gray) is a woody desert shrub which is under development as a domestic source of hypoallergenic natural rubber (Siler and Cornish, 1994; Nakayama et al., 1996; Siler et al., 1996; Cornish, 1996, 1998). As part of this development process, existing and newly developed lines (developed either through plant breeding or genetic engineering) must be evaluated for field performance and yield. Guayule is a prolific seed producer. However, its apomictic nature and problems associated with direct seeding present obstacles for breeding, producing and evaluating large numbers of genetically identical plants (Thompson and Ray, 1989; Fangmeier et al., 1984). Micropropagation through tissue culture offers an alternative method for production of clonal material from improved or genetically engineered plants. There have been several reports of *in vitro* organogenesis and propagation of guayule that involved formation of callus on explants as an initial step (Dhar et al., 1989; Finnie et al., 1989; Zavala et al., 1982). Although these procedures did lead to regeneration of shoots and, in some cases, whole plants, induction of callus tissue often requires use of high growth regulator concentrations and can increase the occurrence of somaclonal variation (Phillips et al., 1994). Procedures for micropropagation of guayule, through *in vitro* multiplication of shoots followed by rooting and subsequent acclimation of plantlets, have been reported by only a few researchers (Pan et al., 1996; Trautmann and Visser, 1990; Smith, 1983). Although these methods have commonalities, they vary in terms of reported success rates as well as in terms of the time and labor that would be involved in applying these protocols to large scale micropropagation of plants. Here we report modifications to these methods that improve shoot proliferation and simplify protocols for both rooting and acclimation of plantlets to greenhouse conditions.

Materials and Methods

Establishment and maintenance of shoot cultures. Shoot cultures of guayule lines N6-5 and hybrid line AZ101 (*P. argentatum* × *P. tomentosum* var. *stramonium*; Thompson and Ray, 1989) were initiated from aseptically germinated seedlings by the method of Pan et al. (1996). After 2–3 wk, shoots with three or more nodes were excised from the rest of the seedling (above the primary leaves) and transferred to shoot multiplication medium consisting of MS basal medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 1.0 mg l^{-1} (4.4 μM) 6-benzylaminopurine (BAP), 0.025 mg l^{-1} (0.13 μM) α-naphthaleneacetic acid (NAA) and 0.8% TC agar (Carolina Biological Supply Co., Burlington, NC) in Magenta GA-7 boxes (Sigma Chemical Co., St Louis, MO). Individual shoots produced multiple shoots (~5–10) both from axillary buds and from callus-like tissue that grew from the cut end of the stem (Fig. 1). Shoot cultures were maintained on this medium and were subcultured every 4–6 wk by excising individual shoots and transferring these to fresh medium. Cultures were maintained in a growth chamber at 24°C under cool-white fluorescent light (~80 μmol m^{-2} s^{-1}; 16-h photoperiod).

Root induction on excised shoots. Individual shoots, 10–30 mm in length and having at least two nodes, were excised from 4–6-wk-old cultures and treated with indole-3-butyric acid (IBA) to induce root formation. The IBA treatment consisted of incubating excised shoots overnight (14–18 h) at room temperature in liquid medium consisting of 0.5 × MS salts (no vitamins or sucrose) and 100 mg l^{-1} (492.1 μM) IBA (Fig. 2*a*). This incubation was carried out in an open container in a transfer hood to maintain sterility while

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Fig. 1. Six-week-old shoot cultures of guayule hybrid line AZ101 on shoot multiplication medium exhibiting normal morphology. The numerous shoots visible originated from four individual shoots initially placed in the container.

allowing for adequate gas exchange. Shoots were subsequently removed and rinsed three times with sterile distilled water prior to insertion into individual cellulose plugs in sterile, ventilated plastic trays (Sorbarod® Micropropagation Systems, Sigma Chemical Co.) containing 250 ml of 0.5× MS medium (liquid) without growth regulators (Fig. 2b and c). Trays were returned to the growth chamber under the same conditions as given above for maintenance of shoot cultures.

Experiments were also conducted to compare the effects of IBA, indole-3-acetic acid (IAA) and NAA on root induction. These comparisons were conducted by following the same procedure given above for IBA treatment, but substituting IBA with water (control) or either of the other two auxins. After approximately 4 wk, the percentage of shoots producing roots was determined for each treatment (Fig. 4).

Transplanting and acclimation of rooted shoots. After 3–4 wk, rooted shoots were transplanted by removing plantlets from the Sorbarod® tray along with the cellulose plugs which had been penetrated by roots (Fig. 2d). The plugs were inserted into individual Rootrainer® pots (Spencer-Lemaire Industries, Ltd., Edmonton, Alb., Canada) filled with potting medium (Uni-Gro Cactus Mix, L&L Nursery Supply, Chino, CA). Plugs were placed in the pots ensuring that the top of the plug was even with the level of the potting medium (Fig. 3a). These were then covered with a layer of fine vermiculite so that the cellulose plug was no longer exposed, thereby reducing moisture loss from evaporation (Fig. 3b). The trays were then placed in the greenhouse, watered and covered with a cloche for 2–3 d (Fig. 3b). After the plastic tent was removed, plants were watered daily for 1 wk and three times a week thereafter. Greenhouse temperature was maintained between 14°C and 30°C, and natural lighting was supplemented with high intensity, metal halide lamps when skies were overcast. Humidity was not controlled and varied between 10% and 60%.

Results and Discussion

Shoot cultures were initially established following the protocol of Pan et al. (1996). Although adequate shoot proliferation occurred, shoots were rather short (5–10 mm) and sometimes hyperhydric (tissues had swollen, translucent appearance). The medium was

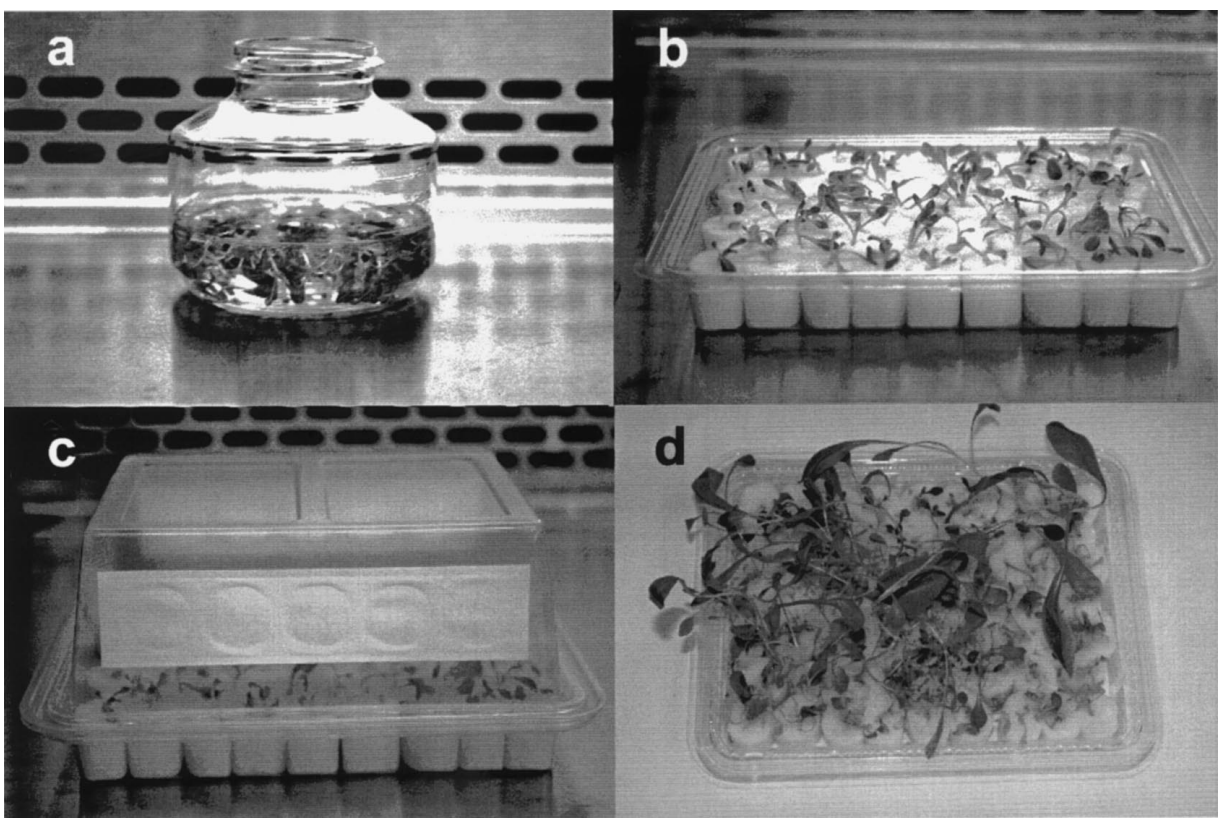

Fig. 2. Treatment of shoots with IBA medium and subsequent rooting in cellulose plugs. Excised shoots were first incubated overnight in IBA medium (100 mg l^{-1} IBA [492.1 μM] in 0.5× MS salts) (a), shoots were then inserted into sterile cellulose plugs contained in a plastic tray (b), covered with the ventilated lid (c) and placed in a growth chamber. Three weeks after IBA treatment rooted shoots were ready for transplanting (d).

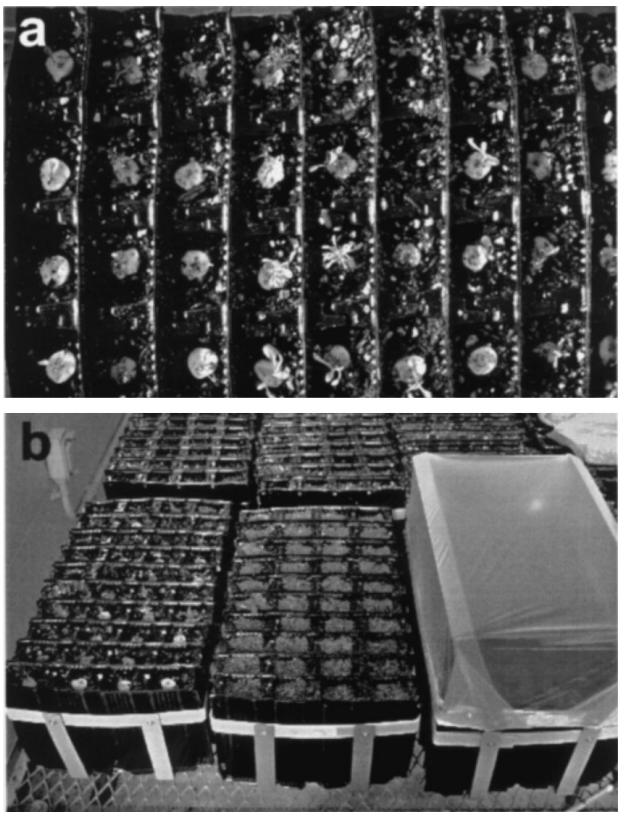

Fig. 3. Transfer of rooted shoots into pots in the greenhouse consisted of inserting plantlets (with cellulose plugs) into potting medium (a), covering the cellulose plugs with a layer of fine vermiculite (b) and then covering the plantlets with a cloche (b). The cloche was subsequently removed after 2 d.

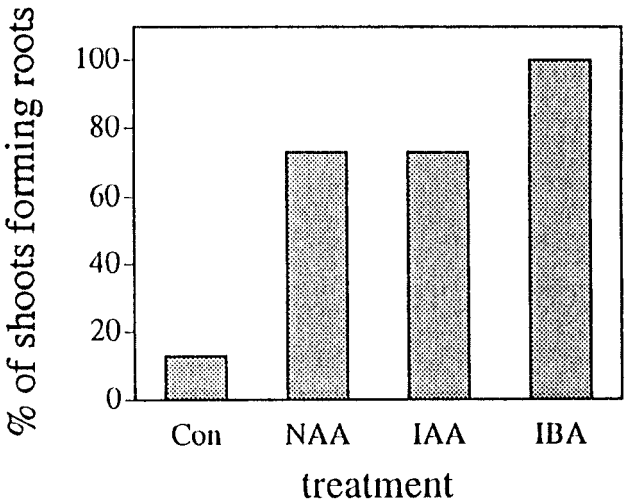

Fig. 4. Percentage of excised shoots which produced roots in cellulose plugs after an overnight treatment with either no auxin (Con) or 100 mg l^{-1} of NAA, IAA or IBA in 0.5 × MS salts.

subsequently modified by reducing the concentration of BA from 2 to 1 mg l^{-1} (from 8.8 to 4.4 μ*M*). This modification reduced the multiplication rate of the shoots, but increased shoot length. Hyperhydricity was also reduced after this modification and was only occasionally seen. Using this medium, 5–10 shoots were obtained from a single shoot after 4 wk and shoot length ranged from 10 to 40 mm (Fig. 1). This inverse relationship between shoot multiplication rates and shoot length in response to concentration of BA in the medium was also observed by Smith (1983). While several studies report the use of BA for shoot multiplication, optimal concentrations varied between 0.1 and 2.0 mg l^{-1} (0.44–8.8 μ*M*; Pan et al., 1996; Trautmann and Visser, 1990; Smith, 1983; Staba and Nygaard, 1983). This indicates that BA concentration adjustments may be necessary to obtain the desired multiplication rate and shoot length for specific guayule lines or for differences in culture conditions. In this study, 1 mg l^{-1} (4.4 μ*M*) BA produced good results for shoot cultures from both lines N6-5 and AZ101.

While treatment with auxin was not a prerequisite for adventitious root formation, it did increase the percentage of shoots producing roots. Comparison of optimal rooting percentages obtained with IBA, IAA, NAA and a control treatment (no auxin) revealed that IBA was most effective for induction of roots (Fig. 4). With IBA, 100% rooting of shoots was attainable (mean value: 90 ± 10.0%). Treatment with either IAA or NAA resulted in a maximum of 73% rooting (mean values: 71.5 ± 1.5% [IAA]; 61.5 ± 11.5% [NAA]), and shoots from the control treatment exhibited only 13% rooting (mean value: 11.5 ± 1.5%). Pan et al. (1996) achieved 80% rooting of cultured shoots without auxin treatment, but their protocol required adherence to strict criteria in order to obtain high rooting percentages. These criteria included careful selection (15–30 mm stem length, at least three nodes and 1–2 mm stem diameter), excision (2–4 mm below a node) and placement (5 mm into rooting medium) of shoots. Smith (1983) reported up to 90% rooting of shoots by including 0.5 mg l^{-1} (2.46 μ*M*) IBA in the rooting medium. Trautmann and Visser (1990) also reported up to 90% rooting by using 0.3 mg l^{-1} (1.48 μ*M*) IBA in the rooting medium and observed decreasing root length in response to increasing IBA concentration. In this study, an overnight treatment with a high concentration of IBA (100 mg l^{-1}; 492.1 μ*M*) was used (Fig. 2a), rather than inclusion of IBA in the rooting medium, to avoid effects of prolonged exposure to IBA on root development. Studies with other species have reported that auxin treatments to induce root formation are most effective when applied between 0 and 48 h after excision (de Klerk et al., 1995) and also tend to cause callus formation and suppressed root development when continuously present in the rooting medium (Mathur et al., 1995; de Klerk et al., 1990)

Another modification of previously reported *in vitro* propagation protocols for guayule was the use of cellulose plugs and liquid medium in a ventilated container, rather than agar-solidified medium, to root excised shoots (Fig. 2b and d). This eliminated the tedious process of removing agar from roots before transplanting to potting soil. This process is not only time-consuming, but often results in damage to the newly developed root system. Roberts and Smith (1990) reported the effectiveness of cellulose plugs in protecting roots of chrysanthemum plantlets from damage during potting. Also, these plantlets had reduced wilting and increased transpiration rates compared to plantlets that had been rooted in agar. Alderson and McKinless (1988) reported that rose shoots rooted in cellulose plugs developed root hairs, while those rooted in

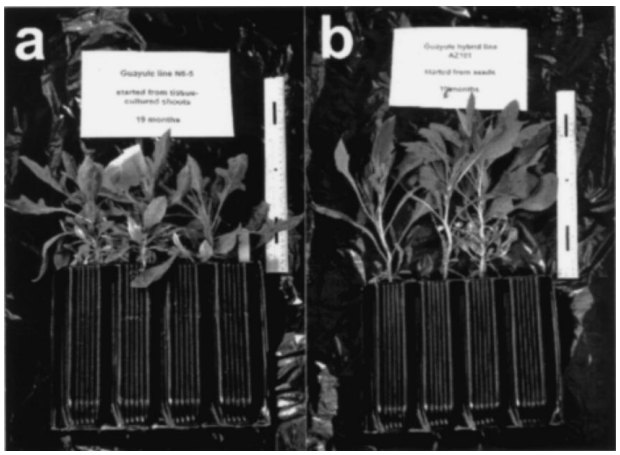

Fig. 5. Seedlings of (a) guayule line N6-5 and (b) guayule hybrid line AZ101 derived from micropropagated tissues exhibited normal morphology and were still growing well after 19 mo.

agar did not. Furthermore, use of liquid medium and a ventilated culture vessel allows the humidity level to decrease gradually over the course of the rooting period. This occurs as water from the liquid medium is used by plants for growth and is also lost through evaporation. This decrease in humidity allows acclimation of plantlets to begin before potting so that they are not as sensitive to changes in humidity levels when transferred to a greenhouse or growth chamber. Several other studies have also demonstrated that lowering humidity in the culture vessel prior to transferring plantlets to greenhouse conditions greatly reduces post-transfer acclimation times and increases the percentage of plants that survive (Smith et al., 1990, 1992; Short et al., 1987). The protocol reported here only requires that humidity be maintained at relatively high levels for 2–3 d after potting. This is easily achieved by covering plantlets with a cloche (Fig. 3b). Afterwards, the cloche can be removed provided that plantlets are watered daily for the first week and approximately every other day thereafter. In our experiments the percentage of plantlets which survived acclimation in the greenhouse generally ranged between 50% and 80%. However, we believe some of this variation is due to fluctuations in humidity levels which were not controlled in the greenhouse. The protocol employed by Pan et al. (1996) was much more labor-intensive and required covering potted plantlets with beakers for 3–5 wk and then removing the beakers every day for increasing periods of time until no wilting could be observed. While Trautmann and Visser (1990) and Smith (1983) do not give detailed descriptions of protocols employed for acclimation of micropropagated guayule plantlets, they acknowledge that this is a critical step in the *in vitro* propagation of guayule.

The authors would like to record that Sorbarod® Micropropagation Systems are no longer available from Sigma Chemical Co. However, Sorbarod® cellulose plugs can be purchased from Ilacon Limited (Churchdown, Tonbridge, UK), who are a distributor for the manufacturer, Baumgartner Papiers SA (Switzerland). Use of the cellulose plugs in Magenta GA-7 boxes with vented closures (Sigma Chemical Co.) has been tried in our laboratory and was also found to work, although the percentage of acclimated plantlets was slightly lower (~40%).

Conclusion

The procedures described here provide a simplified protocol for micropropagation and acclimation of guayule which can be effectively used to produce large numbers of morphologically normal plants (Fig. 5). Furthermore, this modified protocol can be employed for production of genetically identical plants and is easily adaptable for *in vitro* propagation of other woody species.

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