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In vitro multiplication of cashew (*Anacardium occidentale* L.) using shoot node explants of glasshouse-raised plants

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Abstract Using glasshouse-raised plants (1 month, 1 year and 5 years old), factors affecting shoot development from shoot nodes of two Brazilian and one Tanzanian elite selections of cashew (*Anacardium occidentale* L.) were assessed. Sprouting of buds decreased strongly with increasing age of mother plants. Solidified media, mainly when purified agar was used, gave better results than liquid medium. Murashige and Skoog salts containing 1/2-strength macroelements were the most suitable for bud sprouting and shoot elongation. Vitamins and sucrose concentration did not have a significant effect but by replacing 20 g/l sucrose with glucose or maltose all estimated parameters were improved. Gibberellins supported bud sprouting and shoot elongation but blocked rooting. Shoots developed in the presence of cytokinins were short and produced axillary branches. Activated charcoal, cultivation of explants in darkness for the first 7 days and superoptimal temperature (35 °C) decreased bud sprouting and supported shoot elongation. Microshoots rooted in vitro at a frequency of 42% when cultured for 5 days with 100 μM indole-3-butyric acid. Over 40% of rooted microshoots survived weaning.

Key words *Anacardium occidentale* · Bud sprouting · Cashew · Shoot nodes · Shoot elongation

Abbreviations *BAP* Benzyl-aminopurine · *GA* Gibberellic acid · *IBA* Indole-3-butyric acid · *2-iP* (2-Isopentenyl)adenine · *Kin* Kinetin · *MS* Murashige and Skoog · *TDZ* Thidiazuron · *WPM* Woody plant medium · *Zea* Zeatin

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Introduction

Cashew (*Anacardium occidentale* L.) is an important crop of many tropical countries through its production of nuts and apples. It is propagated mainly by seeds and this often results in high levels of variability (Philip and Unni 1984). Conventional vegetative propagation methods, e.g. airlayering, grafting or cuttings, are not sufficiently rapid, and techniques like micropropagation via multiple axillary branching and in vitro organogenesis or embryogenesis offer prospects of faster multiplication of elite genotypes. But cashew, like other Anacardiaceae, is strongly recalcitrant to in vitro culture and only limited successes have been achieved as yet: direct shoot and root regeneration was obtained from proximal ends of cotyledons (Philip 1984; Philip and Unni 1984) and calli of different explant origins have been reported to produce roots (Leva and Falcone 1990; Sy et al. 1991) or globular protuberances which developed into embryo-like structures but with many aberrations (Jha 1988). A protocol for large-scale somatic embryo production for this tree crop has not yet been established. Progress with application of micropropagation has been achieved using microshoots (Lievens et al. 1989; Leva and Falcone 1990) and cotyledonary nodes (D'Silva and D'Souza 1992; Das et al. 1996) but many problems still persist with in vitro explant viability, bud sprouting and shoot elongation. Additionally, in vitro rooting and survival of in vitro-produced plants remain constraints to more widespread application of micropropagation techniques to cashew (Leva and Falcone 1990; D'Silva and D'Souza 1992; Das et al. 1996). In the present study, shoot nodes derived from greenhouse-grown material of different age including relatively old (5 years) plants were used as standard explants in studies on cultural factors which influence cashew shoot production in vitro.

Materials and methods

Shoot nodes were collected from 1-month-, 1-and 5-year-old cashew (*A. occidentale* L.) grown in the glasshouse under natural daylight and daylength conditions, and temperature in the range 20–28 °C. The mother stock material used in the tissue culture experiments consisted of two Brazilian selections (cp09, cp1001), both of which were 1 month old, and one Tanzanian (AC4) selection (either 1 month, 1 year or 5 year old). Explants were surface sterilised in 70% ethanol for 1 min followed by 1% sodium hypochlorite for 30 min and placed singly on 10 ml culture medium in glass test tubes (2.5 cm diameter) which were covered by polypropylene film (Cannings, UK). Generally, the explants were incubated on media consisting of either woody plant medium (WPM) (Lloyd and McCown 1981) or Murashige and Skoog (1962) (MS) salts with 1/2-strength macroelements supplemented with 5 mM (2-isopentenyl)adenine (2-iP), 0.1 mg/l thiamine-HCl, 100 mg/l inositol, 40 g/l sucrose and solidified by 7 g/l technical agar (Oxoid), and the final pH adjusted to 5.8. Explants were kept at 25 °C under 16-h photoperiods delivered at 63 μmol m^{-2} s^{-1} by Philips TLD 50 W/84HF fluorescent lamps. Modifications of medium composition and experimental conditions used are as stated in the Results. The percentage of bud sprouting, lengths of new shoots developed from axils and numbers of nodes per extended axillary shoot were scored after 4 weeks culture. For rooting, shoots were cultured on medium (pH 5.5) with WPM salts, 0.1 mg/l thiamine-HCl, 100 mg/l inositol, 30 g/l sucrose, 7 g/l technical agar and different levels of indole-3-butyric acid (IBA) (0–300 mM) for 5 days and then on the same medium lacking this auxin. Growth regulators used in all experiments were added after autoclaving as ethanol solutions which did not exceed 0.02% of total medium volume. Agars tested were those supplied by Oxoid, while Phytagel, chemicals and salts were obtained from Sigma. The youngest branches with about six nodes were collected from 10 (5-year-old) and 50 (1-year-old) mother plants and used as explant sources for each experiment. For 1-month-old material, nodes from the whole seedlings were collected. A total of 40 explants were used for each treatment. Data were analysed by analysis of variance or by means of a chi-squared contingency test where appropriate.

Results

Effects of genotype

Explants from the two Brazilian selections showed poorer sprouting and elongation than those from the Tanzanian source (Fig. 1a, b). Furthermore, in the latter genotype, bud sprouting decreased with plant age (Fig. 1c) while shoot elongation was decreased only slightly (Fig. 1d). Axillary branching was achieved only with the Tanzanian material. In the following experiments we therefore focused on this genotype.

Effects of solidifying agent and activated charcoal

Media solidified by agar were more suitable for shoot multiplication than liquid medium (Fig. 2a–c). Purified agar gave the highest bud sprouting, shoot elongation and node formation; moreover, shoots were more vigorous and produced more leaves than those grown on other media. Explants on Phytagel medium tended to callus and produce hyperhydric shoots. Activated charcoal suppressed bud sprouting significantly, particularly in the case of the Tanzanian selection (Fig. 1a, c), while shoot elongation was improved in all three selections evaluated (Fig. 1b, d). Shoots on activated charcoal media were more vigorous than those without charcoal.

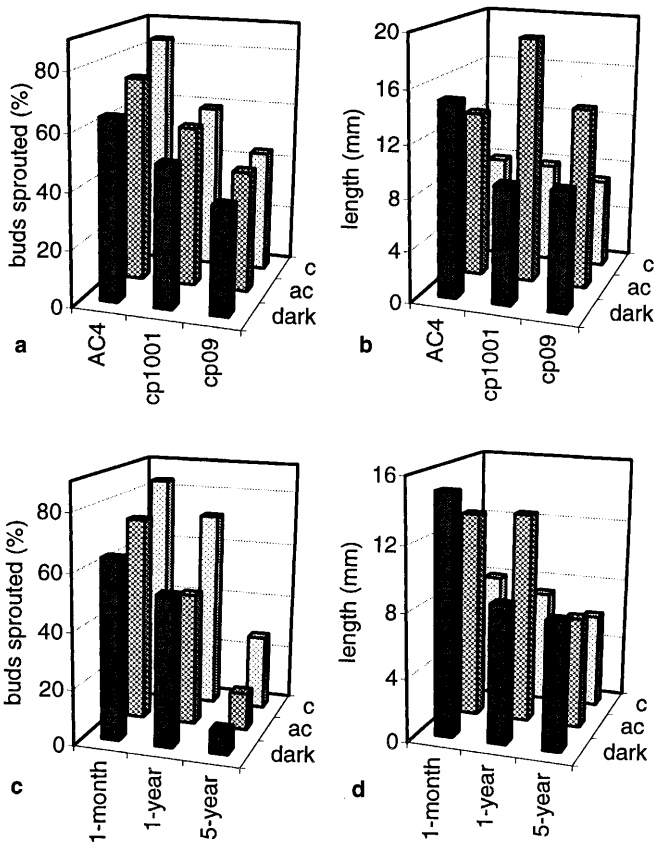

Fig. 1 Effects of genotype (*AC4* Tanzanian, *cp1001* and *cp09* Brazilian, all at 1 month old) (**a**, **b**), age (AC4 mother stock) (**c**, **d**), activated charcoal and 7 days treatment (for different ages and genotypes) (**a–d**), on shoot development [*dark* first days in darkness, *ac* medium with 0.5% (wt/vol) activated charcoal (explants kept at 16 h light/8 h dark photoperiod), *c* control (medium without activated charcoal; explants kept at 16 h light/8 h dark photoperiod)].Effects of activated charcoal and darkness were significant at $P \leq 0.05$ for **b–d**. Differences between genotypes and ages were significant at $P \leq 0.01$ for **c**, **d**, at $P \leq 0.05$ for **a**, **b**. Other effects were not significant

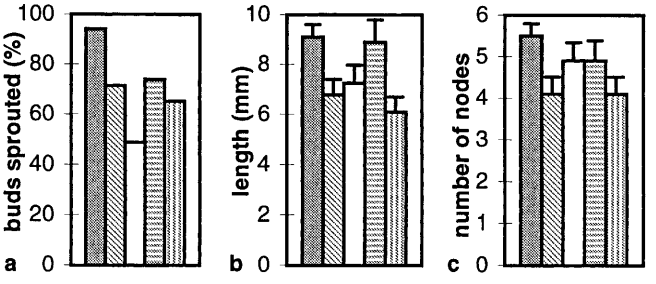

Fig. 2a–c Effects of gelling agents on shoot development (1-year-old plants) [gelling agents from left to right: 6.5 g/l purified agar, 7 g/l technical agar, 2.2 g/l Phytagel, 3.5 g/l technical agar + 1.25 g/l Phytagel, liquid medium wuth cellulose plugs (Sorbarods)]. Differences between treatments were significant at $P \leq 0.01$ for **a**, **b**, at $P \leq 0.05$ for **c** (*bars* SE of the mean)

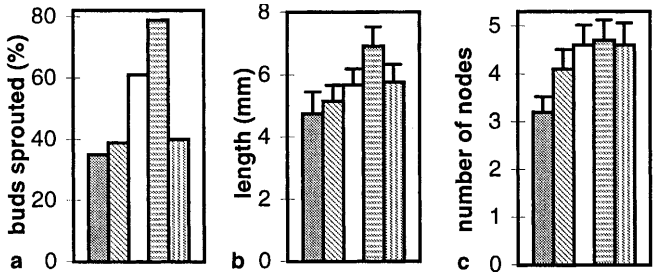

Fig. 3a–c Effects of salts on shoot development (1-year-old plant) [from left to right: White's (White 1942), *B*5 (Gamborg et al. 1968), WPM, MS with 1/2-strength macroelements, MS]. Differences between treatments were significant at $P \leq 0.01$ for **c**, at $P \leq 0.05$ for **a** and not significant for **b** (*bars* SE of the mean)

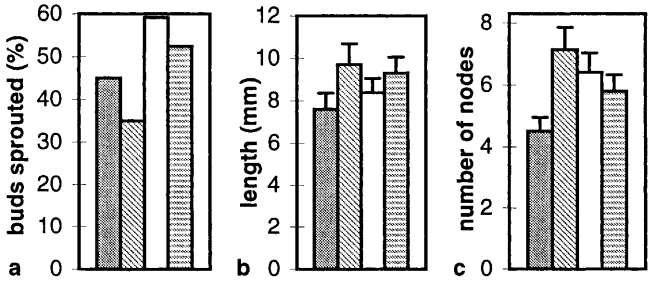

Fig. 5a–c Effects of saccharides on shoot develpment (1-year-old plant) (from left to right: 40 g/l sucrose, 20 g/l sucrose + 10 g/l fructose, 20 g/l sucrose + 10 g/l glucose, 20 g/l sucrose + 20 g/l maltose). Differences between treatments were significant at $P \leq 0.01$ for **a**, at $P \leq 0.05$ for **c** and not significant for **b** (*bars* SE of the mean)

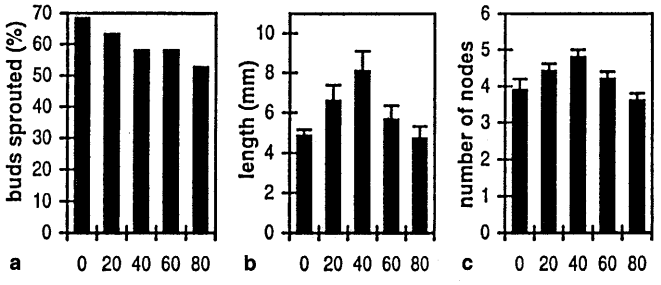

Fig. 4a–c Effects of sucrose concentration (g/l) on shoot development (1-year-old plants). Differences between concentrations were significant at $P \leq 0.01$ for **b** and not significant for **a**, **c** (*bars* SE of the mean)

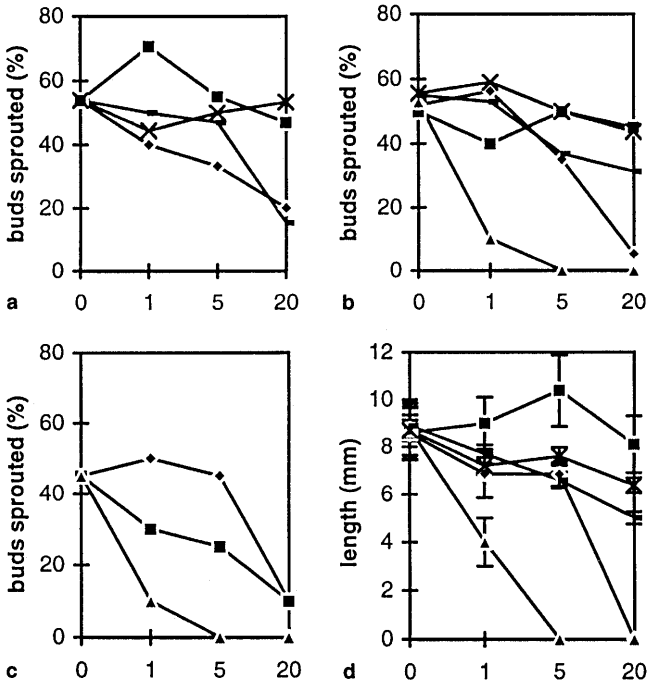

Fig. 6 Effects of cytokinins (μM) on bud sprouting and shoot elongation in 1-month-old glasshouse-grown stock (**a**), 1-year-old glasshouse-grown stock (**b**, **d**), and in vitro stock (**c**). Differences between single cytokinins were significant at $P \leq 0.01$ (*bars* SE of the mean; *diamonds* BAP, *squares* 2-iP, *rectangles* Kin, *crosses* Zea, *triangles* TDZ)

Influence of basal salts

Of the five salt compositions tested (see legend to Fig. 3), MS with 1/2-strength macroelements proved the most suitable for shoot multiplication. WPM gave satisfactory results with regards to bud sprouting while White's medium gave the poorest results for all parameters evaluated (Fig. 3a–c). No significant differences were found between the lengths of shoots on the five different salt combinations tested.

Effects of sugars and vitamins

The presence of sucrose in media was not essential for bud sprouting; in fact, when its concentration was increased, bud sprouting was slightly decreased (Fig. 4a). A significant effect of sucrose concentration on shoot elongation was found with an optimum at 40 g/l (Fig. 4b). The number of nodes per microshoot was also slightly affected (Fig. 4c). When 20 g/l sucrose was substituted by either equimolar levels of glucose or maltose (but not fructose), significantly more explants formed shoots (Fig. 5a). The number of nodes per shoot was significantly higher after application of each saccharide (Fig. 5c), while shoot elongation was only slightly improved (Fig. 5b). The addition of either usual levels of MS vitamins, or 100 μg/l thiamine with 100 mg/l inositol, did not improve any shoot growth parameter.

Influence of plant growth regulators

Bud sprouting was significantly influenced by cytokinins. In the case of explants obtained from glasshouse-raised stocks, the cytokinins benzylaminopurine (BAP), kinetin (Kin) and thidiazuron (TDZ) suppressed bud sprouting significantly while zeatin (Zea) and 2-iP had no suppressive effects (Fig. 6a, b). Callus formation was promoted by the highest level (20 μM) of BAP, Zea and Kin whereas callus was induced by TDZ at all concentrations tested (1, 5 and 20 μM). Shoots sprouted on medium with TDZ were hyperhydric and stunted in their growth. Bud sprouting from explants of in-vitro-produced stock was inhibited by all of

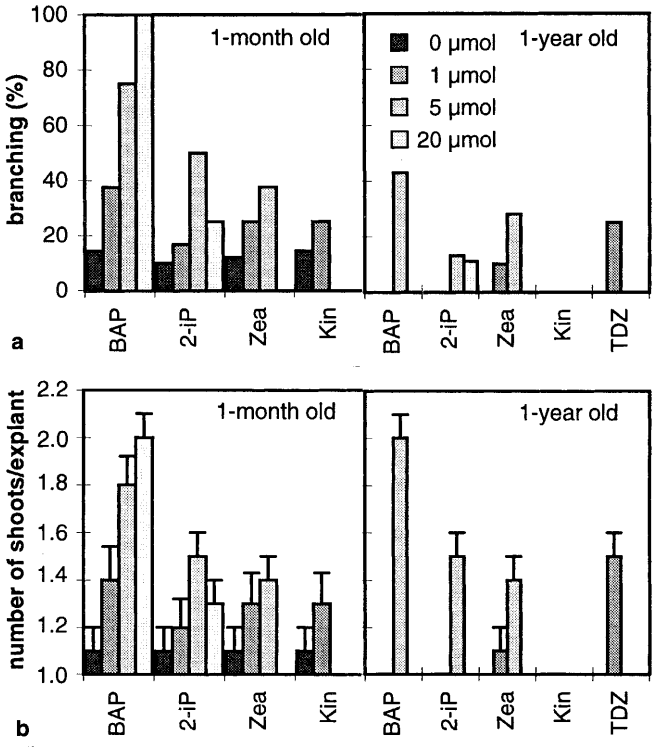

Fig. 7a, b Effects of cytokinins on axillary branching in 1-month- and 1-year-old AC4-stock. Differences between single cytokinins and between ages were significant at $P \leq 0.005$ (*bars* SE of the mean)

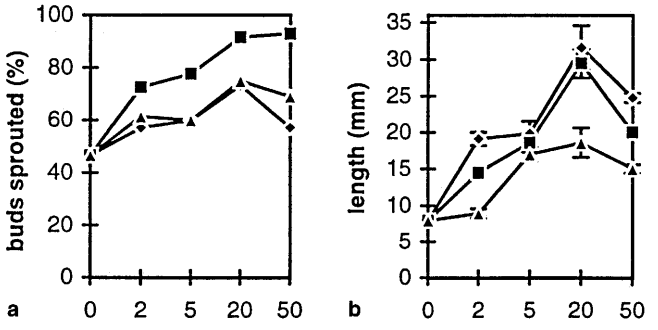

Fig. 8a, b Effects of gibberellerines (µM) on bud sprouting and shoot development (1-year-old plants). Differences between single gibberellins were significant at $P \leq 0.01$ (*bars* SE of the mean; *diamond* GA_3, *squares* GA_4, *triangles* Ga_7)

the cytokinins tested (Fig. 6c). Again, the cytokinins BAP, Kin and TDZ reduced shoot elongation significantly in sprouted buds from 1-year-old stock (Fig. 6d). By using the cytokinins Kin and Zea at 20 µM, it was possible to produce 5–6.5 and 6 nodes per main shoot, respectively. When used at appropriate concentrations, cytokinins were able to induce multiple axillary branching (Fig. 7a–d). Branches grew to lengths of 0.5 cm in 4 weeks and after cutting and further culture of these in the presence of a cytokinin (usually BAP in the range 5–20 µM) they elongated and produced new laterals. One-year-old material exhibited poorer branching ability than that originating from 1-month-old stock (Fig. 7a–d). Gibberellic acid (GA) supported bud sprouting and shoot elongation (Fig. 8a, b); however, the number of nodes per shoot was not affected. Of the three gibberellins tested (GA_3, GA_4 and GA_7), GA_4 was the most promotive for bud sprouting and GA_3 for shoot elongation. In vitro microshoots of cashew produced on high concentrations of GA, however, were weak and hyperhydric and their leaves were much reduced in size. In addition, the microshoots were unable to respond to rooting treatments (see below).

Temperature and light regime

Incubation temperatures in the range 19–29 °C did not affect bud sprouting but when they were raised to 35 °C, bud sprouting was drastically reduced and profuse callus occurred at the bases of microshoots (Fig. 9a). When temperatures were increased over the range 19–35 °C, shoot elongation increased (Fig. 9b). The number of nodes per shoot was significantly lower at 19 °C than at higher temperatures (Fig. 9c). At 35 °C, intact 0.5-mm-long microshoots derived from in vitro stock were able to elongate fourfold (Fig. 9d). An initial incubation of nodes for 7 days at 25 °C in darkness decreased bud sprouting while shoot elongation was significantly increased (Fig. 1a–d).

Rooting of microshoots

Microshoots formed roots after 5 days IBA treatment on root induction medium with the highest response being obtained at 100 µM IBA (Figs. 10a, b, 11). Rooted plantlets were weaned and hardened in 1:1 vermiculite/peat mixtures and the mean survival obtained was in the range

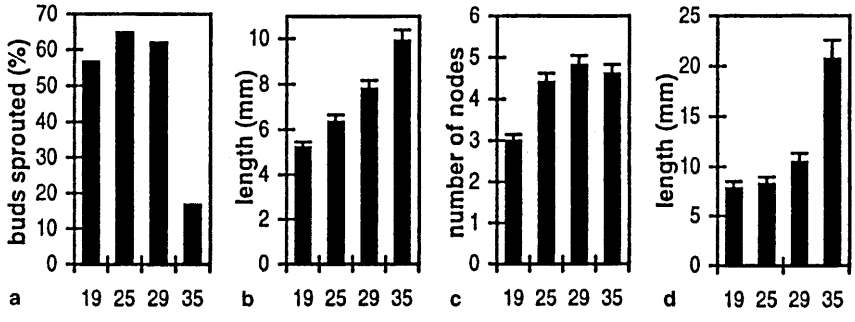

Fig. 9 Effects of temperature (°C) on shoot development in 1-year-old glasshouse-grown stock (**a–c**) and in vitro stock (**d**). Differences between treatments were significant at $P \leq 0.01$ for **a**, **b**, **d**, at $P \leq 0.05$ for **c** (*bars* SE of the mean)

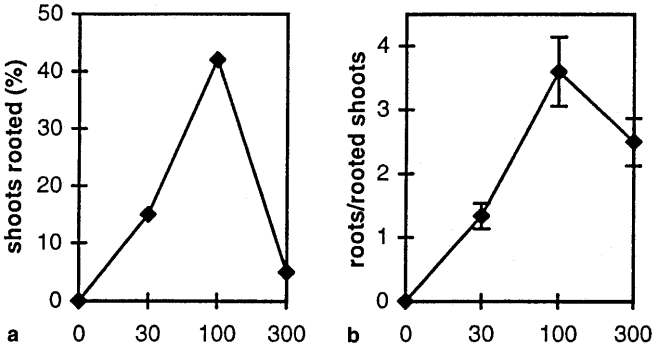

Fig. 10a,b Effects of IBA (μM) on the root induction. Differences between concentrations were significant at $P \leq 0.01$ (*bars* SE of the mean)

Fig. 11 Rooted cashew microshoots in vitro (*left*) and weaned plantlet after 6 months in the glasshouse (*right*)

40–50%. By 2 months after weaning, plantlets had reached 100–120 mm in height and carried approximately ten leaves ranging from 50–120 mm in length (Fig. 11).

Discussion

To reduce the risks of somaclonal variability during the multiplication of material, preformed apical and axillary meristems as sources of new plants are usually preferable to indirect organogenesis (George 1993). In cashew, attention has been paid previously to cotyledonary nodes which produced multiple shoots per node under appropriate conditions (D'Silva and D'Souza 1992; Das et al. 1996). However, seedlings are normally extremely heterozygous due to outbreeding (Philip and Unni 1984). The advantage of shoot nodes is that they are available in large numbers from single elite adult plants. Lievens et al. (1989) and Leva and Falcone (1990) successfully cultured microshoots derived from 6-/15-month- and 3-year-old plants, respectively, while Das et al. (1996) reported that shoot explants derived from adult field-grown stock plants failed to survive, browning and dying after surface sterilisation. In cashew, browning has been ascribed to the presence of secondary metabolites which oxidised after being released from the explants (Jha 1988; Das et al. 1996). Antioxidants or activated charcoal have been added routinely to the culture media to prevent this problem (Jha 1988; Lievens et al. 1989; Sy et al. 1991). Das et al. (1996) could increase explant viability in cotyledonary nodes from 15 to 90% only with cultivation in darkness for 1 week on media supplemented with activated charcoal. In our study, browning never inhibited the survival of more than 20% of explants. Moreover, either charcoal or cultivation in darkness for the initial 7 days had positive effects on shoot elongation but suppressed bud sprouting.

Several other factors affected axillary bud sprouting and shoot development in the current work with cashew. Increasing age of stock plants resulted in drastic decreases in the abilities of axillary buds on nodal explants to sprout. However, even from the 5-year-old mother stock plants it was possible to obtain over 20% explants which sprouted and went on to produce elongating shoot cultures. In vitro responses of explants from adult trees are usually enhanced once some type of partial rejuvenation in the shoots has been attained (Pierik 1990). We are now making efforts to increase the responses of adult-phase nodes by first micrografting adult-phase meristems onto seedling rootstocks to obtain partial rejuvenation of elite vegetative buds before these are subsequently used for micropropagation.

Generally, solidified media have been used in previous studies for culture of different cashew explants. However, Lievens et al. (1989) found that a short induction period on liquid medium with BAP improved axillary bud development, but that elongation of shoots was better carried out on solidified medium because liquid medium induced vitrification. In routine in vitro work, little attention is paid to agar quality, but in recalcitrant species like cashew, agar impurities may cause problems in explant responses. In the present work, different basal salt requirement were found for shoot nodes compared to cotyledonary nodes. According to Das et al. (1996), the MS formulation was the most efficient for bud development in cotyledonary nodes, but in our experiments, MS salts with reduced macrosalts were the most suitable. The sucrose concentration which has affected previously the numbers of buds developing from cashew cotyledonary nodes (D'Silva and D'Souza 1992) did not show any effect on bud sprouting. However, saccharides such as glucose or maltose added to 20 g/l sucrose appeared to enhance both bud sprouting and the numbers of nodes per explant; maltose also dramatically improved bud formation in cotyledonary nodes (D'Silva and D'Souza 1992).

Application of cytokinins at relatively high doses has been recommended for in vitro cashew bud development in microshoots and cotyledonary nodes (Lievens et al. 1989; Leva and Falcone 1990; D'Silva and D'Souza 1992; Das et al. 1996). In the present study, cytokinins never induced the multiple bud formation typical for cotyledonary nodes and only one bud developed in each axil. The continuous presence of strong cytokinins like TDZ, BAP or

Kin suppressed bud sprouting and inhibited shoot elongation – factors which are unsuitable for achieving shoot multiplication. The decrease in bud sprouting was partially compensated for by an increase in the numbers of side branches per microshoot, which after cutting could either give new lateral shoots or be used for rooting.

As small cashew shoots are difficult to root (D'Silva and D'Souza 1992), we attempted to elongate them first by application of three types of GA. GA is known to support bud sprouting and shoot elongation in many woody plants including cashew (Lievens et al. 1989; Leva and Falcone 1990). However, in our experiments, the positive effects of GA on bud sprouting and shoot elongation were accompanied by later problems with rooting of microshoots. Also, preculture or culture of cashew leaf explants on rooting medium supplemented with GA significantly reduced their root formation abilities (Boggetti 1997). Inhibitory effects of GA on root induction have been documented also for other woody plants, e.g. apple (Pawlicki and Welander 1992) and *Solanum aviculare* (Jasik et al. 1997).

Rooting of microshoots derived from nodal explants would appear to be more problematic than for those of cotyledonary node origin. D'Silva and D'Souza (1992), using appropriate combinations of auxins, induced 80.3% rooting in cotyledonary node-derived shoots. By contrast, in our experiments, only about 42% of microshoots derived from nodal explants of 1-year-old stock were able to root following exposure to an IBA concentration of 100 μM for 5 days. This may be simply due to the fact that the first were more juvenile in character.

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