In vitro root formation in *Anacardium occidentale* microshoots

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Abstract

Cultural conditions affecting the induction of rhizogenesis *in vitro* were evaluated in cashew (*Anacardium occidentale* L.) shoot-node-derived microshoots. The application of auxins was essential for the formation of adventitious roots. A 5-d indole-3-butyric acid (IBA) induction period was more suitable than continuous IBA treatment or a shorter induction period. N^6-[2-Isopentenyl]adenine in low concentrations (0.3 - 1 μM) in the root induction medium supported root formation. Precultivation of microshoots with gibberellic acid (GA_3) suppressed the subsequent rhizogenesis. Activated charcoal did not affect rooting. No significant differences in rooting abilities of cashew shoots were observed between 25, 29 and 35 °C and roots did not develop at 19 °C. Salts of low osmotic composition were more suitable than richer media. Microshoots originated from cotyledonary nodes showed higher rooting when compared to standard microshoots.

Additional key words: cashew, auxins, root induction, rhizogenesis.

Introduction

Cashew (*Anacardium occidentale* L.) is a tropical tree of high economic importance for the production of nuts and apples. In previous studies it has been shown to be strongly recalcitrant to *in vitro* culture. Only cotyledonary nodes (D'Silva and D'Souza 1992, Das *et al.* 1996), shoot nodes (Boggetti *et al.* 1999) and microshoots (Leva and Falcone 1990) have been found suitable explants for *in vitro* shoot multiplication. Limited success has been achieved in *de novo* organogenesis (Leva and Falcone 1990, Sy *et al.* 1991) and embryogenesis (Jha 1988) using cotyledon explants. Rooting, which in woody plants is still one of the major constraints for their *in vitro* propagation, is also a serious problem in cashew. In experiments by Das *et al.* (1996) and Leva and Falcone (1990), 40 and 25 %, respectively, of cotyledonary node derived shoots were able to produce adventitious roots. Moreover, percentage survival of *in vitro* produced microplants following acclimatisation was relatively low (between 30 and 68 % of rooted shoots, according to the soil mixtures used, D'Silva and D'Souza 1992). A poorly developed root system may contribute significantly to the low conversion rate. We recently established the protocol for *in vitro* microshoots production from shoot nodes (Boggetti *et al.* 1999). In the present study, conditions for *in vitro* rooting of these microshoots are studied.

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Abbreviations: B5 - medium according to Gamborg *et al.* (1968); BAP - 6-benzylaminopurin; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - medium according to Murashige and Skoog (1962); NAA - naphthalene acetic acid; SE - standard error of the mean; W - medium according to White (1942); WPM - woody plant medium according to Lloyd and McCown (1981); 2-iP - N^6-[2-isopentenyl]adenine.

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Materials and methods

Plants: Mature cashew (*Anacardium occidentale* L.) seeds collected in Recife, East Brazil (cv. cp 1001) and Tanzania (cv. AC4) were germinated in a 1:1 mixture of compost and perlite and grown in the glasshouse. Microcuttings about 1 cm long and 4 weeks old were obtained directly from shoot nodes (Boggetti *et al.* 1999) of 1-year-old plants (about 50) on MS medium with half-strength macroelements containing 40 g dm^{-3} sucrose, 1 μM 2-iP and 7 g dm^{-3} agar bacteriological (*Oxoid Ltd.*, Basingstoke, UK) and maintained at 25°C and a 16-h photoperiod (cool white fluorescent tubes, irradiance of 63 μmol m^{-2} s^{-1}). In one experiment (see Results) microshoots, which sprouted from cotyledonary node axils of decapitated seedlings germinated *in vitro*, were used.

Rooting studies: As a standard protocol, WPM (unless otherwise stated) supplemented with 0.4 mg dm^{-3} thiamine-HCl, 100 mg dm^{-3} myo-inositol and 30 g dm^{-3} sucrose was used. The pH of the media was adjusted to 5.5 before autoclaving. The medium was solidified with 2.5 g dm^{-3} *Phytagel*. The growth regulators were added to the autoclaved medium in an ethanol solution, amounting to a final concentration of no more than 0.02 %, because it was found by de Klerk *et al.* (1997a) that higher ethanol concentrations inhibited rooting *in vitro*. Auxins and other compounds were applied for the whole 30-d-culture period unless otherwise stated. The explants were kept at the same light regime as mentioned above unless otherwise stated. All chemicals were obtained from *Sigma-Aldrich Company Ltd.* (Gillingham, UK). At least thirty microcuttings were used for each treatment and they were placed singly on 10 cm^3 culture medium in 2.5 cm diameter glass test tubes that were covered by polypropylene film (*Cannings Packaging, Blackrock Co.*, Dublin, Ireland). The percentages of rooted microshoots and the mean numbers of roots per rooted microshoot were scored after 30 d of culture.

Statistical analysis: Data for number of roots were analysed by analysis of variance and for rooting percentage by χ^2 contingency test.

Results

The application of auxins (either IBA, NAA or IAA) was essential for the formation of adventitious roots in microshoots with the greatest induction effect when either IBA or IAA was used (Fig. 1*A*,*B*). A significant difference in rooting responses was observed for the three periods of IBA application (24 h, 5 d and continuous treatment) (Fig. 2*A*,*B*) with the best results when 100 μM IBA for 5 d was used. The cytokinin 2-iP when supplied at low concentrations significantly increased root formation induced by continuous 10 μM IBA application (Fig. 3*A*,*B*). When microshoots were previously cultured for 4 weeks on medium containing either 2, 5, 20 or 50 μM GA_3, no rooting occurred despite the continuous application of IBA at either 3, 10, 30 or 100 μM (results not shown). Moreover profuse callus arose at the cutting base of shoots, in greater quantity when 100 μM IBA was in the medium. Out of the different salt formulations

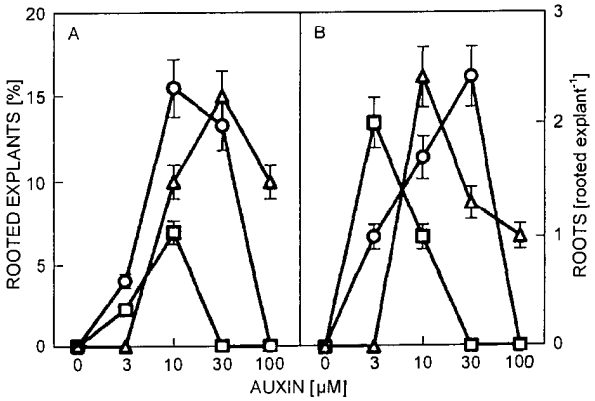

Fig. 1. Effects of continuous application of different auxins, IAA (*triangle*), IBA (*circle*) and NAA (*square*) on rooting (percentage of rooted explants and number of roots per explant). Effects were significant at $P \leq 0.05$ ($n = 30$). *Vertical bars* represent SE of means.

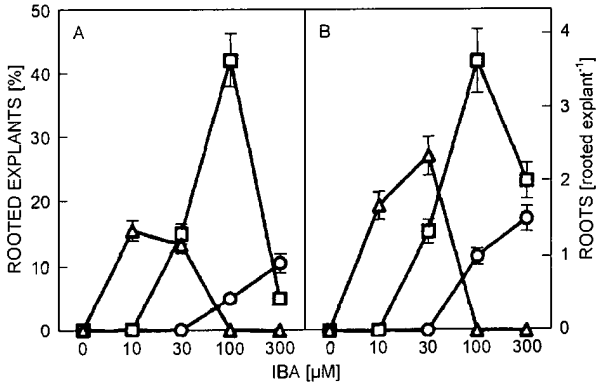

Fig. 2. Effects of 24 h (*circle*), 5 d (*square*) and continuous (*triangle*) IBA treatment on rooting. Effects were significant at $P \leq 0.05$ ($n = 40$). *Vertical bars* represent SE of means.

tested (W, WPM, B5, MS, MS at half strength macroelements), WPM was shown to be optimal for root formation (Fig. 4*A,B*). Activated charcoal did not affect rooting in microshoots when supplied at either 0.5, 1 or 5 g dm^{-3} during or after a 5-d root induction period by 100 µM IBA (results not shown). A 5-d darkness treatment decreased rooting induced by IBA (Fig. 5*A*) while it did not affect rooting induced by NAA (Fig. 5*B*). Callus developed at the cutting base in microshoots only when these were kept in darkness and to a greater extent in NAA media. Generally shoots under dark condition showed pronounced leaf abscission and browning of both stems and medium. No significant differences in rooting percentages of cashew microshoots induced by 30 µM

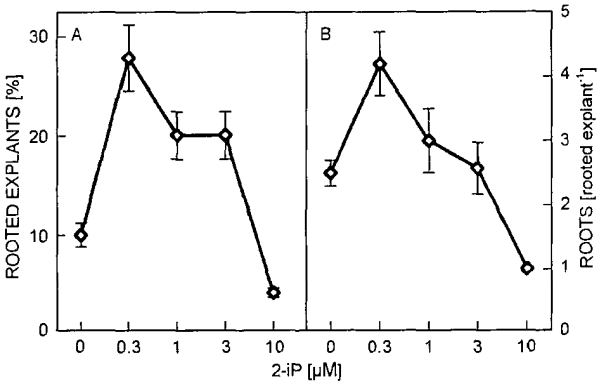

Fig. 3. Effects of 2-iP on rooting induced by continuous 10 µM IBA application. Effects were significant at $P \leq 0.05$ ($n = 30$). *Vertical bars* represent SE of means.

IBA were observed between 25 °C (about 15 %), 29 °C and 35 °C (both about 10 %) and roots did not develop at 19 °C. From two cashew selections, the Tanzanian material showed a slightly higher rooting percentage (Fig. 6*A*) and number of roots developed (Fig. 6*B*) compared to the Brazilian. A significant difference in the percentage of

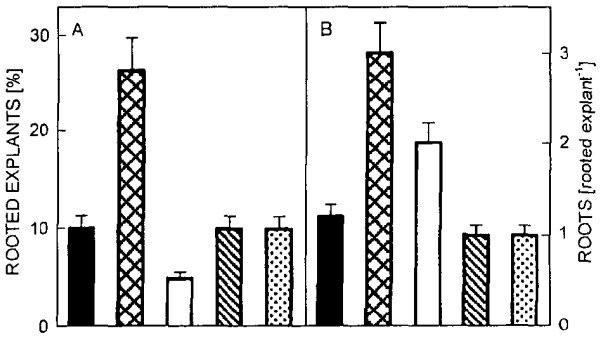

Fig. 4. Effects of salt formulation (W - *filled columns*, WPM - *crossed columns*, B5 - *open columns*, MS half macroelements - *hatched columns*, MS - *tinted columns*) on rooting of microshoots. Media were supplemented by 10 µM IBA and 0.3 µM 2-iP. Effects were significant at $P \leq 0.05$ ($n = 30$). *Vertical bars* represent SE of means.

rooting between shoots obtained from glasshouse-grown plants and from cotyledonary nodes of *in vitro*-raised seedlings was observed. Microshoots originated from cotyledonary nodes showed higher rooting percentage (64.5 %) when compared to standard shoots (30 %) under the same rooting treatment (Fig. 6*A*). No difference in the number of roots per rooted shoots was recorded (Fig. 6*B*).

Discussion

The correct morphological formation of the root system without callus proliferation is a necessary requirement to sustain the proper supply of solutes to the upper parts of the plant. Many factors, mainly growth regulators, affect adventitious root differentiation *in vitro* (de Klerk 1995, Jasik *et al.* 1997, De Klerk *et al.* 2001) and between them, auxins play substantial role in the induction phase (de Klerk 1995, de Klerk *et al.* 1997b). Differences between single auxins and their combinations in root induction have been previously found in cashew cotyledonary-nodes-derived shoots (D'Silva and D'Souza 1992). In both cotyledon and shoot-node-derived microshoots, which is so far the only material available for micropropagation in cashew, IBA seems to be the most effective auxin for root induction (D'Silva and D'Souza 1992, Das *et al.* 1996, Leva and Falcone 1990, and the present study). Moreover, NAA induces a massive production of callus that may cause problems with the acclimatisation of microplants.

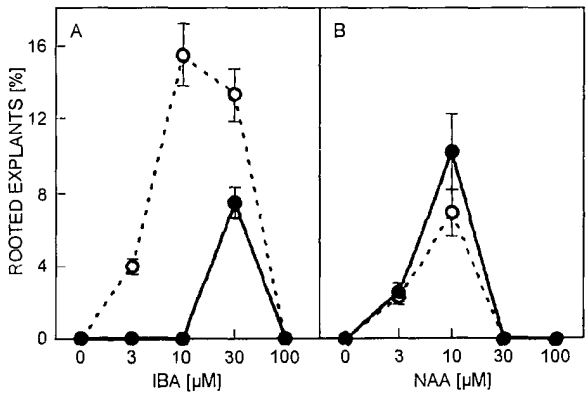

Fig. 5. Effects of a 5-day dark period (*filled circles*) and continuous light (*open circles*) on rooting induced by IBA and NAA. Effects were significant at $P \leq 0.05$ for IBA and not significant for NAA ($n = 30$). *Vertical bars* represent SE of means.

Adventitious root development consists of physiologically, biochemically and anatomically well defined phases (de Klerk 1995, Jásik and de Klerk 1997). Auxins promote root induction but they inhibit root primordia emergence (de Klerk 1995, de Klerk *et al.* 1997b). Thus the use of auxins in the medium for only the first hours or days is a frequently applied procedure. The current study shows that for node-derived microshoots a 5-d IBA treatment is more suitable than either its continuous presence or a 24 h pulse. In cashew cotyledonary-node-derived microshoots Das *et al.* (1996) obtained the highest rooting percentage (40 %) when treated them with 2.5 mM IBA for 2 h. Lievens *et al.* (1989) reached 30 % rooting with 2-3 roots per rooted explant in cashew microshoots after treating them with a solution of 4 mg dm^{-3} IBA for 16 h followed by transferring to 2 mg dm^{-3} IBA. However, dipping shoots in either 0.2 or 1 mM NAA for 20 s followed by transfer to auxin-free medium did not induce any rooting (Falcone and Leva 1987).

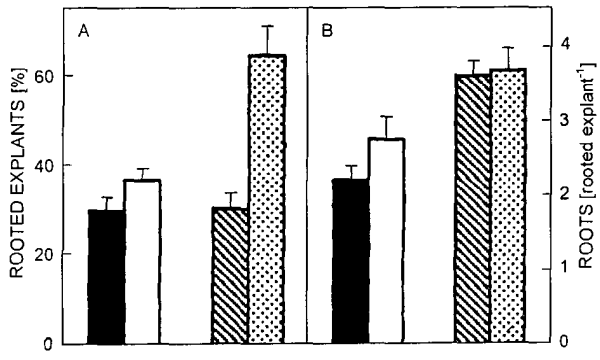

Fig. 6. Effects of genotype, (Brazilian - *filled columns*, Tanzanian - *open columns*) and explant source (microshoots from leaf axillary buds - *hatched columns*, microshoots from cotyledon axillary buds - *tinted columns*) on rooting. Medium was supplemented with 30 μM IBA and 0.3 μM 2-iP. Effects were significant at $P \leq 0.05$ ($n = 40$) for explant source (% rooted explant) and not significant for others. *Vertical bars* represent SE of means.

The role of cytokinins in root formation is still a matter of dispute. Cytokinins are generally known to suppress root formation, being effective mainly during the induction phase (de Klerk 1995, de Klerk *et al.* 2001). However, some cytokinins in low concentrations may even favour root formation (Jarvis 1986, van Staden and Harty 1988, de Klerk *et al.* 2001). 2-iP significantly increased root formation in cashew microshoots explants. Also in our other experiments with cashew explants (leaf fragments, cotyledons; unpublished results), even strong cytokinins like BAP or zeatin have not been shown to be so detrimental for rooting as they have been for other plant species. Leva and Falcone (1990) and Sy *et al.* (1991) working with leaf fragments and cotyledon explants, respectively, were also able to induce adventitious rooting on media with relatively high BAP levels. For practical micropropagation, the supplement of cytokinins and particularly 2-iP at low concentrations seems to be reasonable to reach a better developed root system in microshoots.

High concentrations of GA_3 were shown to inhibit root differentiation in apple microshoots (Caboni *et al.* 1992) and in *Solanum aviculare* leaf fragments (Jasik *et al.* 1997). In woody plants, gibberellins are generally used to elongate shoots *in vitro* (Junttila 1991). In cashew very short microshoots failed to root (D'Silva and D'Souza 1992) and gibberellins have been used successfully to support elongation in shoot node derived microshoots (Boggetti *et al.* 1999). However, the present study showed that such precultivation may result in the inhibition of rooting. GA_3 strongly suppressed root formation in cashew leaf explants (unpublished results).

Among the physical factors, irradiance has shown to be important during the rooting process. It has been reported by Epstein and Ludwig-Müller (1993) that a 95 % and 80 % reduction in IAA and IBA concentrations, respectively, occurred after only 3 d in the light. On the other hand, van der Krieken *et al.* (1992) concluded that the mode of action of light on root formation may be explained also in changes in the efficiency of perception of auxins by the plant tissues or in changes in the competence of the tissue to regenerate roots. In cashew secondary metabolites may also play an important role. They are released from the explants in great quantity and their oxidation in the light may give raise to various products that could affect rhizogenesis. However, surprisingly, in cashew darkness had a negative effect on microshoot healthiness (pronounced leaf abscission, browning of stem tissues, callus development on the cut surface) which probably caused their low rooting ability.

In the present study we tried to optimise the rooting of node-derived microshoots in the strongly recalcitrant cashew. Microshoots derived from axillary meristems could be a suitable material for micropropagation of cashew (Boggetti *et al.* 1999). After several approaches we have reached more than 40 % rooting of these microshoots. Despite this, in the present study microshoots derived from shoot nodes exhibited lower rooting abilities than microshoots originating from cotyledonary nodes as was found by D'Silva and D'Souza (1992) and in the current study. The better rooting responses observed in microshoots derived from cotyledonary nodes when compared to those of shoot origin may be caused by the more juvenile material source in the first case.

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