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HORMONAL CONTROL OF PROLIFERATION IN MERISTEMATIC AGGLOMERATES OF *EUCALYPTUS CAMALDULENSIS* DEHN

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Summary

Eucalyptus camaldulensis can be micropropagated through so-called meristematic agglomerates (MAs). MAs (4–6 mm diameter) are dense shoot clusters initiated by the outgrowth of numerous successive buds. Their reddish nature is associated with an increase in their endogenous cytokinin level during the exponential phase of growth. A simultaneous decrease in the auxin level favors a high cytokinin/auxin ratio. A low level of polyamines occurs at the time of the lowest level of auxins. Slow hormone release by activated charcoal plays a role in this very prolific organogenesis.

Key words: meristematic agglomerates; auxins; cytokinins; *Eucalyptus*; polyamines; micropropagation.

Introduction

Meristematic agglomerates (MAs) represent a powerful micropropagation method for *Eucalyptus camaldulensis* Dehn. (Arezki et al., 1998). MAs are clusters of small, reddish buds initiated *in vitro* in petri dishes from the culture of nodes in total darkness and in the presence of an auxin (indole-3-butyric acid; IBA) on Murashige and Skoog's (1962) medium. Each MA contains a large number of buds which can be divided up to 11 morphogenic explants for the subsequent monthly subcultures, which do not require any special care. Viable plantlets can be raised from such MAs. This MA technique can be related to the nodule systems developed by Aitken-Christie et al. (1988) on radiata pine; by McCown et al. (1988) on poplar; by Ito et al. (1996) on eucalypts; and to the cormlets propagation system proposed by Ziv (1989) on *Gladiolus*. However, for *E. camaldulensis* the multiplication and proliferation phases are obtained in total darkness in a confined environment and in the presence of IBA and activated charcoal. Moreover, MAs differ morphologically and histologically from nodules, which generally have a spherical form, and are vascularized tissues surrounded by an epidermic layer (McCown et al., 1988); MAs more closely resemble the clusters observed by Aitken-Christie et al. (1988) and Ziv (1989). In order to improve this typical organogenic process and to make it applicable to other species, the present approach aims at correlating the increase in the proliferating mass and number of buds in the MAs with their changing endogenous levels in auxins, cytokinins and polyamines. The control of organogenesis by the external application of a suitable auxin/cytokinin ratio is classical (George, 1993). However, there are far fewer studies dealing with the roles of endogenous auxins and cytokinins, and these indicate that the ratio of endogenous auxins/endogenous cytokinins (at some phases of their developmental processes at least) may be more important than the individual roles of each (Gaspar et al., 1996a, b). Polyamines are also involved in the control of organogenic processes (for reviews see Martin-Tanguy, 1997; Walden et al., 1997). Their metabolism, rather than their content *per se*, may be the determining factor in bud formation (Aribaud et al., 1999).

Materials and Methods

Plant material. The explants introduced *in vitro* were green nodes taken from young, soft shoots of 2-yr-old mother plants resulting from seeds (from Lake Albacutya, Victoria, Australia) cultivated in the greenhouse on peat substrate at 22 ± 2°C and a 16 h photoperiod. After preprocessing by steeping explants for 3 h in a solution of 4 g l^{-1} dithiocarbamate (TMTD, Ortocid 83, Protex, Leusden, The Netherlands) and some drops of Tween 20, the explants (1.5–2 cm long, 1–3 mm diameter) were disinfected with calcium hypochlorite (9% over 20 min), then placed horizontally in 9-cm-diameter petri dishes (six explants per dish) containing 25 ml fresh medium.

Culture medium and conditions. The initiation and proliferation medium for MAs consisted of Murashige and Skoog (1962) medium for the macro- and microelements and vitamins, to which were added 3% sucrose (BDH, Laboratory Supplies, Poole, U.K.); 0.5% agar (Pastagar B, Merck, Darmstadt, Germany); and 0.2% activated charcoal (No. 2186, Merck). This basic medium was supplemented by 1 mg l^{-1} (5 μM) IBA (Sigma, St. Louis, U.S.A.). The pH was adjusted to 5.6 before autoclaving.

The MA initiation and proliferation phases were obtained in total darkness (in a Conviron culture room) at a temperature of 27°C (±1°C). Transfers on fresh medium were made every 30 (±2) d. The MAs were subdivided into small clumps containing three or four buds each, and subcultured.

Biochemical analysis. The fresh plant material for analysis was sampled on days 0 (30th day of preceding subculture), 7, 15 and 30 of the 13th *in vitro* subculture. The explants were plunged into liquid nitrogen and stored at −70°C until analysis.

Extraction and determination of free and conjugated auxins. The methods used were as detailed previously (Nordström and Eliasson, 1991).

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Frozen samples (500 mg) were homogenized in liquid nitrogen. The powder was extracted with 5 mmol l^{-1} phosphate buffer (pH 6.5) containing [l-^{14}C]indole-3-acetic acid (IAA) as the internal standard, and butylated hydroxytoluene as the antioxidant. After centrifugation and filtration the samples were successively passed through Bond-Elut C18 columns, activated and conditioned to pH 6.5 and 2.5. Auxins were eluted with methanol (80%). Methanolic extracts (50 µl) were injected into a fully automated Merck–Hitachi (Darmstadt, Germany) HPLC system. HPLC analysis was performed on a 100-RP18, 5 µ*M* column (Merck Lichrocart 125-4). Detection was done by fluorescence (excitation 292 nm; emission 360 nm), and the solvent flow was 1 ml min^{-1}. Peak identification was done by injection of standards, IAA, IAA aspartate (IAAsp) and IBA, under the same conditions.

Cytokinin extraction and purification, HPLC separation and radioimmunoassay. The method used was as reported by Jemmali et al. (1995). A 1 g FW frozen sample was homogenized at 4°C in methanol (80%) containing butylhydroxytoluene (BHT, 40 mg l^{-1}); 100 Bq of [^{14}C]IAA was added as the internal standard. After centrifugation (10 000 rpm, 10 min, 4°C), the supernatants were purified using C18 cartridges. The cytokinins were recovered with 5 ml methanol (80%) and evaporated under vacuum. The residues were dissolved in 250 µl ultrapure water. Separation was performed on a 100-RP18, 5 µ*M* HPLC column (Merck Lichrocart 125-4). Fractions (0.5 ml) were collected, reduced under vacuum, and redissolved in buffer with sodium azide (200 mg l^{-1}) before assaying by radioimmunoassay. Recovery was estimated from the label found in the fractions corresponding to IAA (around 60%). Assays were performed using anti-CK serum from Phytoscience (Lyon). Bound radioactivity was measured by scintillation counting in 1 ml Optiphase (LKB, Bromma, Sweden) over 5 min.

Extraction and determination of free polyamines. The extraction, separation, identification and measurement of free polyamines by direct dansylation and HPLC have been described elsewhere (Walter and Geuns, 1987). 150 mg of frozen samples was homogenized in 2 ml 4% $HClO_4$ containing diaminoheptane–2HCl (5 mg l^{-1}) as the internal standard. The homogenate was centrifuged and dansylated. The dansylated polyamines were extracted with toluene and purified on 50 mg silica gel columns (MM Kieselgel 60), and finally eluted with ethyl acetate. The HPLC analysis was performed on a 100-RP18 5 µ*M* column (Merck Lichrocart 125–4). Detection was done by fluorescence (excitation 340 nm, emission 510 nm); the solvent flow was 1 ml min^{-1}.

The results are the means of at least three separate replications (± standard error).

Results

Structure and growth of the meristematic agglomerates. The final structure of an MA, as shown in Fig. 1, is initiated from the outgrowth of the axillary bud of the node explant. This axillary bud develops an average of three secondary axillary buds which soon stop growing. The secondary buds do the same. The MA at the end of the subculture period may contain 28–36 buds (each no longer than 5 mm) for a total fresh weight of ±60 mg. Its dissection provides an average of 11 explants, each consisting of three or four buds, which will continue to develop along the same pathway at subculture.

Fig. 2 shows the growth curve in terms of fresh weight and the increase in the number of buds inside the MA. Both are typical sigmoidal growth curves, with an exponential phase between the fifth and fifteenth days.

Endogenous levels of auxins, cytokinins and polyamines. Three auxins were identified in the extracts of *Eucalyptus* MAs: IAA, IAAsp and IBA. Table 1 shows the levels measured at days 0, 7, 15 and 30, and the variation in the total amount of auxins. A decrease occurs from the beginning of subculture to day 15, before an increase to a level approximating the initial value. Six cytokinins (zeatin, zeatin riboside, benzyladenine, benzyladenosine, 2-isopentenyladenine and 2-isopentenyladenosine) were measured in extracts of the same MAs on the same dates. The variation during a subculture of the total amount of cytokinins is shown in Table 2. An increase occurs from the beginning up to day 15, before a return to the initial value. There is thus an inverse variation in the levels of both types of hormones, resulting in a dramatic increase in the cytokinin/auxin ratio (Table 3) during the growth phase of the MAs. It is interesting to note that generally, as a cytokinin increases its corresponding riboside decreases, and *vice versa.* The amount and variation of the three classical polyamines (putrescine, spermidine and spermine) and their total are shown in Fig. 3. In our study, the

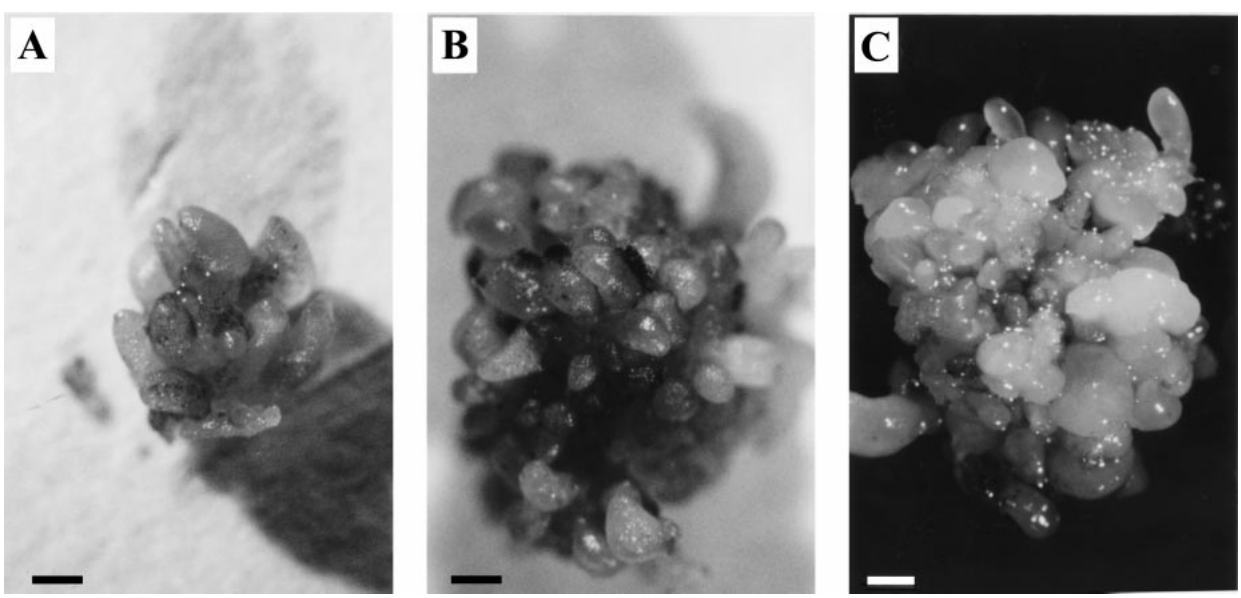

Fig. 1. Meristematic agglomerates (MAs) of *Eucalyptus camaldulensis* at days 5 (A), 15 (B) and 30 (C) in the *in vitro* culture cycle. Scale bars, 0.5 mm.

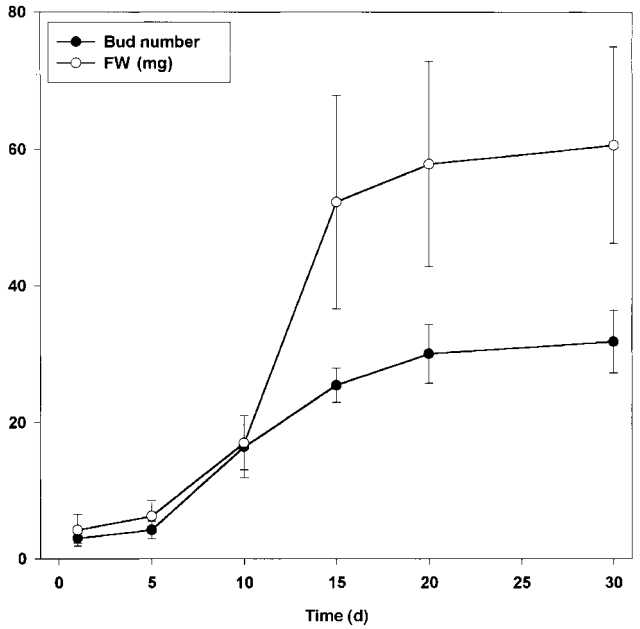

FIG. 2. Fresh weight (open circles) and bud number (closed circles) increases of *Eucalyptus camaldulensis* MAs (±SE) during the 30 d culture cycle in the dark.

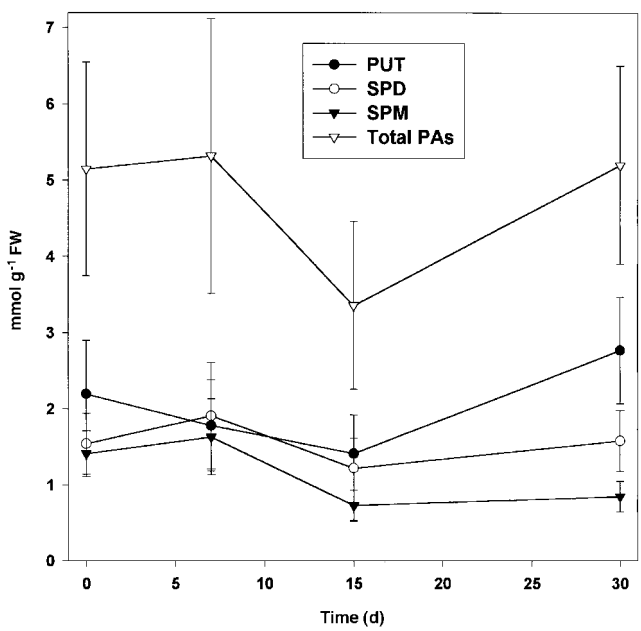

FIG. 3. Free polyamine (PA) content of *Eucalyptus camaldulensis* meristematic agglomerates during the culture cycle. PUT, putrescine; SPD, spermidine; SPM, spermine.

analysis of endogenous polyamines does not highlight a significant physiological action of these substances during the cycle of culture.

DISCUSSION

Dissection of the MAs has shown that they were true axillary shoot clusters where internodal outgrowth of successive buds was limited. The reddish color of the MAs might be the result of an accumulation of cytokinins during their culture, as it is well known that cytokinins favor anthocyanin accumulation (Mori et al., 1994; Deikman and Hammer, 1995). Indeed, the amount of endogenous cytokinins increased during the exponential phase of growth, corresponding to the development of additional axillary buds. This result was expected from the classical effect of the application of exogenous cytokinins, but might not be expected here as MA growth is controlled exclusively by an exogenous auxin. This is in agreement with the more accepted view that the determining organogenic factors are the endogenous growth hormones themselves, under the control of the exogenous growth regulators not necessarily of the same categories (Centeno et al., 1996; Gaspar et al., 1996b). That a cytokinin/auxin ratio increased in parallel with axillary proliferation was also an expected result, although the decrease in auxin level during the cytokinin increase has not generally been shown. These results thus confirm the relationship

TABLE 1

INDOLE ACETIC ACID (IAA), IAA ASPARTATE (IAASP), INDOLEBUTYRIC ACID (IBA) AND TOTAL ENDOGENOUS AUXINS (mmol g^{-1} FW) IN *EUCALYPTUS CAMALDULENSIS* MERISTEMATIC AGGLOMERATES DURING CULTURE

	Day of culture cycle			
Auxin	0	7	15	30
IAAsp	217.8 ± 15.1	49.8 ± 6.5	123.6 ± 7.5	335.8 ± 30.5
IAA	23.1 ± 3.1	106.5 ± 11.2	0	0
IBA	52.8 ± 1.5	119.4 ± 6.1	0	0
Total auxins	293.7 ± 14.7	275.7 ± 21.2	123.6 ± 7.5	335.8 ± 30.5

Content ± SE from three replicates.

TABLE 2

ENDOGENOUS LEVELS OF CYTOKININS (pmol g^{-1} FW) OF *EUCALYPTUS CAMALDULENSIS* MERISTEMATIC AGGLOMERATES AT POINTS IN THE CULTURE CYCLE

	Day of culture cycle			
Cytokinin	0	7	15	30
Zeatin	0.07 ± 0.03	2.2 ± 0.6	11.1 ± 1.0	0.5 ± 0.3
Zeatin riboside	14.9 ± 1.5	1.7 ± 0.3	0.8 ± 0.2	2.1 ± 1.1
Benzyladenine	1.6 ± 0.8	3.0 ± 1.4	44.4 ± 3.1	39.4 ± 5.2
Benzyladenosine	28.0 ± 4.9	40.7 ± 3.0	2.7 ± 0.9	2.4 ± 1.1
2-isopentenyladenine	2.1 ± 1.1	38.7 ± 4.5	157.1 ± 14.8	12.6 ± 6.3
2-isopentenyladenosine	44.3 ± 2.5	25.8 ± 3.6	16.6 ± 4.2	35.3 ± 3.5
Total cytokinins	91.0 ± 3.9	112.2 ± 2.2	232.6 ± 14.9	92.3 ± 6.1

TABLE 3

VARIATION OF THE CYTOKININ/AUXIN RATIO IN *EUCALYPTUS CAMALDULENSIS* MERISTEMATIC AGGLOMERATES CULTIVATED IN THE DARK DURING THE CULTURE CYCLE

	Day of culture cycle			
	0	7	15	30
Cytokinin/auxin ratio (10^{-3})	310	407	1882	275

between the elevation of the endogenous cytokinin/auxin ratio and an organogenic budding program, on the one hand, and the mutual control of endogenous auxins and cytokinins through the exogenous one (Smigocki and Owens, 1989; Eklôf et al., 1997), on the other hand.

Few papers have dealt with the relationships of polyamines with auxins and cytokinins in organogenic processes. Polyamines, close to these hormones, have been implicated in the control of cell division cycles (Del Duca and Serafini-Fracassini, 1993; Coenen and Lomax, 1997). The decrease in their level together with the auxins has been observed in the course of the rooting and flowering processes (Gaspar et al., 1996a).

MAs are obtained on a medium containing 0.2% activated charcoal (AC). Our tests showed that AC was absolutely necessary for the initiation and proliferation of these MAs. The actions of AC on plant tissue culture have been reported in many studies (Misson et al., 1983; Pan and Van Staden, 1998). Despite these various studies, the exact mechanisms of AC action on plant tissue physiology have not yet been elucidated. In our study, AC probably acts by adsorbing IBA and releasing it gradually in the culture medium. MAs are clumps of meristematic buds poor in vascular tissues (O. Arezki, unpublished results). The conjugated forms of the endogenous hormone could act while passing from one cell to another by simple diffusion much more slowly. The active forms would be released inside the cell. This slow but progressive release would have a very efficient morphogenetic effect.

Under our experimental conditions, the increase in the growth of MAs would be due to a double hormonal regulation: an internal action in the cells, and an external one due to AC action. This study clearly supports the concept that plant organogenesis is mediated by a balance of phytohormones.

More than 100 plantlets obtained by the MA system were adapted and are growing in the greenhouse. To the present, 3 yr after acclimatization, no phenotypical variation has been noted (compared with controls issued from seeds), and the shrubs show a great morphological uniformity.

However, we have not yet tested the MA system with mature trees, but we are sure that after rejuvenation it would be possible to apply it to 'elite' trees. From a practical point of view, the regulation system for MAs described here allows fast mass multiplication, a great reduction in propagation costs, and simplification of handling. This technique could also be used in genetic transformation programs, the MA structure facilitating gene transfers by biolistic transformation.

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