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Research note

BA enhances the germination of oil palm somatic embryos derived from embryogenic suspension cultures

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Abstract

Embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq.) allow mass propagation of somatic embryos; however regeneration rates are low. Histological observations have revealed that shoot development might be limited by the absence of a caulinary meristem. The addition of 6-benzyladenine during development was found to induce shoot apex differentiation and thus increased germination rates, by up to 70%. However, multiple shoot formation was a consequence of a longer period of cytokinin supply during the development of the embryo. In contrast, a short period of culture on medium with 6-benzyladenine at the begining of embryo development was found to result in single shoot production.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – 6-benzyladenine

Processes for the vegetative multiplication of oil palm through somatic embryogenesis on calluses have enabled the mass propagation of more than 1 million clonal plantlets to date (Duval et al., 1995). Liquid medium processes have also been investigated, with the aim of obtaining synthetic seeds on an industrial scale. Since 1991, two protocols involving embryogenic suspension cultures have been reported for the production of single somatic embryos (de Touchet et al., 1991; Teixeira et al., 1995), but regeneration rates are limited by poor shoot elongation.

Our purpose in this study was to use cytokinins to improve shoot development and subsequent germination rates. Indeed, since Skoog and Miller's work (1957), cytokinins have been frequently used *in vitro* to stimulate shoot induction. An exogenous supply of BA has also been found to improve somatic embryo development and germination in banana (Dhed'a et al., 1991) and rubber tree (Montoro et al., 1992). In coconut palm somatic embryogenesis, the lowering of the 2,4-D concentration in the medium followed by the addition of BA, was found to be essential for the complete bipolar differentiation of the embryo (Verdeil et al., 1994). We report here results on the effect of varying BA supply during the histodifferentiation phase of oil palm somatic embryos and its influence on their development.

Embryogenic suspensions were initiated as described by de Touchet et al. (1991). The basal medium contained Murashige and Skoog's macroelements as modified by Rabéchault and Martin (1976), Nitsch's microelements (1969), Morel and Wetmore's vitamins (1951) and 100 mg l^{-1} sodium ascorbate. Suspensions were transferred monthly on the basal medium supplemented with 20 g l^{-1} glucose, 30 mg l^{-1} adenine sulfate, 4.44 μM BA, 450 μM 2,4-D and 1 g l^{-1} activated charcoal. Embryogenic development was achieved following the transfer of cell clusters onto a plant growth regulator-free liquid medium containing the basal medium supplemented with 30 g l^{-1} sucrose and 0.5 g l^{-1} casein hydrolysate. The development of embryos occurred after sieving (mesh size=1 mm) and plating onto an 8 g l^{-1} agar (Agar-Agar, Sigma USA) gelled medium of the same organomineral com-

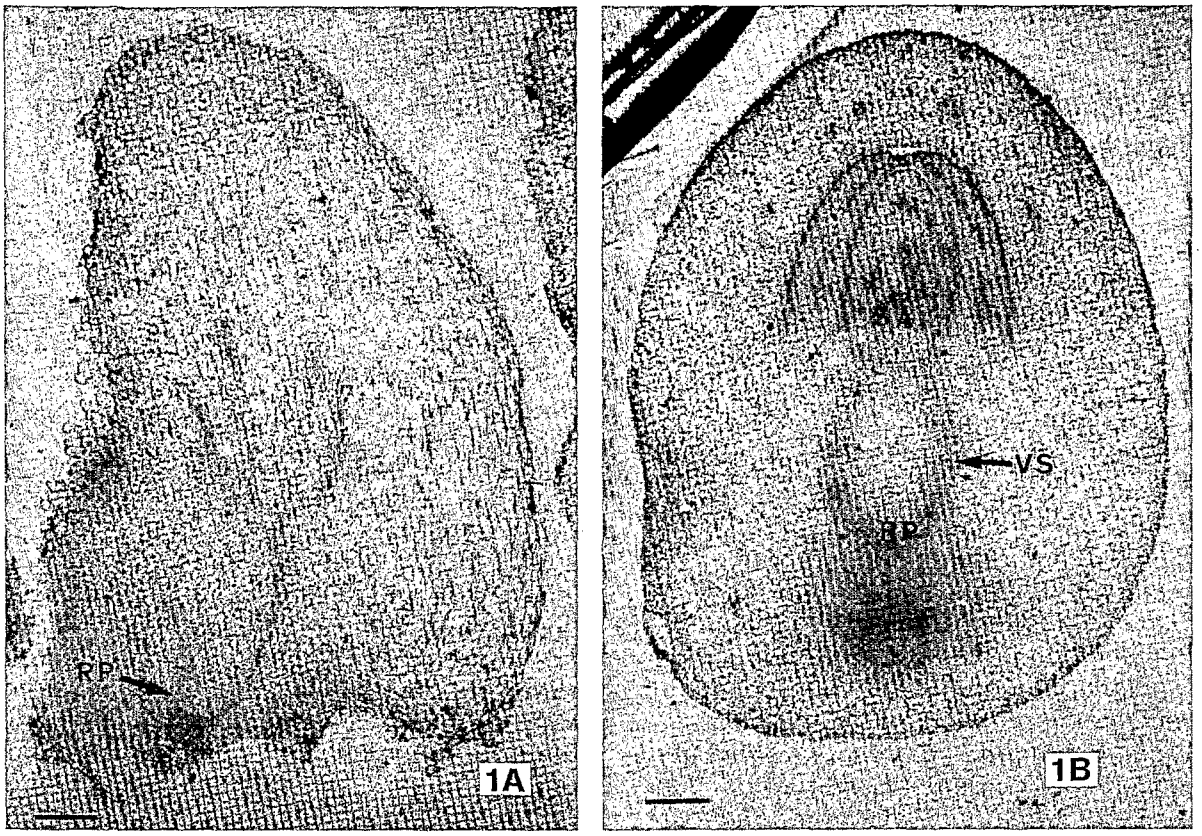

Figure 1. Oil palm somatic embryos cultured on hormone-free medium (*A*) (R.P: root pole; Bar=250 μm) and on BA containing medium (*B*) (S.A: shoot apex, V.S: vascular strand; Bar=300 μm).

position. Cultures were grown under 12 h light at 45 μmol m^{-2} s^{-1}, at 27 °C.

In a first experiment, 0, 1, 5, or 10 μM BA concentrations were tested on 4 clones (87, 121, 123 and 221) over a 4 week period. In a second experiment, 4 time periods on medium with 5 μM BA (1, 2, 3, or 4 weeks) were tested on clones 121 and 221. In both experiments, embryos were transferred weekly. The germination of somatic embryos was then performed on the same medium without growth regulators and containing 2 g l^{-1} phytagel. Embryos were transferred monthly.

Qualitative effects of BA treatments on embryo differentiation were investigated using various histological techniques (Schwendiman et al., 1990). Sections (3.5 μm thickness) of 3 mm long embryos were double-stained using the Schiff-periodic acid reaction and naphtol blue black. In the control cultures, most of the embryos had an unipolar axis composed of a single root apex (Figure 1A). In contrast, after BA treatment, embryos frequently exhibited a complete embryonic axis; i.e. a shoot apex surrounded by young leaves and a root pole (Figure 1B). Procambial strands connecting the two poles were also observed. The low germination rates previously recorded could be related to a poor differentiation of the shoot apices. This inhibition may be a consequence of prolonged subcultures on an embryogenic medium containing auxins (Verdeil et al., 1994). Moreover, according to Merkle (1995), the supplementation of cytokinins during the histodifferentiation phase can compensate for the detrimental effects of auxins on meristem development.

Quantitative effects of BA treatments were evaluated after a 2 month germination period by counting the numbers of embryos with single shoot and/or roots, and proliferating structures termed embryoids. A two-way ANOVA with fixed effects (4 clones, 4 treatments) was performed on triplicates of 15 embryos in order to test for factorial and interaction. In case of interaction, a multiple comparison of the

Table 1. (A & B). Effects of culture on media with various concentrations of BA on the percentage of single shoot (A) and roots (B) produced by somatic embryos after 2 months germination. Four clones were cultured over a period of 4 weeks.

1A

BA	Single shoot (%)			
(μmol)	clone 121	clone 123	clone 221	clone 87
0	22 a	7 a	47 a	22 a
1	60 b	67 b	73 b	33 a
5	29 a	56 b	64 ab	29 a
10	33 a	42 b	56 ab	20 a

BA effect: F (3,32)=27.13; p<0.000; ***;
Clone effect: F (3,32)=27.72; p<0.000; ***
Interaction: F (9,32)=3.73; p=0.0027; **.

1B

BA	Roots (%)			
(μmol)	clone 121	clone 123	clone 221	clone 87
0	89 a	33 a	93 b	78 b
1	67 a	47 a	82 ab	56 ab
5	71 a	62 a	60 ab	49 ab
10	78 a	42 a	51 a	33 a

BA effect: F (3,32)=5.59; p<0.0034; **;
Clone effect: F (3,32)=13.67; p<0.000; ***
Interaction: F (9,32)=3.51; p=0.004; **.

Means were calculated from triplicates of 15 embryos; ***, **, * and NS indicate the results of the two-ways ANOVA, respectively significant at p< 0.001, 0.01, 0.05 and non significant. Index letters indicate homogeneous groups in the same column according to the Newman and Keuls Test (p <0.05).

16 means was realised using the Newman and Keuls test (Newman, 1939; Keuls, 1952) and results were presented for each clone within separate column.

The effect of the concentration of BA on the percentage of embryos with single shoot was found to depend on the clone (Table 1A). For 3 out of the 4 clones (121, 123 and 221), the percentage of single shoot increased by up to 60 to 73% in the presence of BA, and was maximum with a BA concentration of 1 μM. The percentage of embryos with roots was also affected by BA, the effect again varying between clones (Table 1B). This percentage decreased as BA concentration increased for clones 87 and 221 and was unchanged for clones 121 and 123. Nevertheless, from a practical point of view, an auxin treatment performed after shoot emission allowed the development of a root system (Duval et al., 1988). Neither clone nor BA treatment produced significant effect on embryoids proliferation but an interaction was observed ($F_{9,32}$=2.62; p=0.0022), suggesting different effects of BA among clone. Nevertheless, the multiple comparison did not permit to show significant difference between clones due to the lack of capacity of the test in this case.

For the second experiment, a two-way ANOVA was applied again on triplicates of 20 embryos. The effect of different durations of culture on medium containing BA on the percentage of embryos with single shoot varied between clones. For clone 121, this percentage decreased as the duration of the treatment increased, whereas it was not affected for clone 221 (Table 2). In all cases, maximum was reached in the first week. Our results suggest that the induction of a unique shoot apex occurred early in differentiation of somatic embryos.

The clone x treatment interaction was also significant for the percentage of embryos with roots (Table 2) which decreased with the duration of the culture on medium with BA in clone 121. As in the first experiment, root development was inhibited by BA. In contrast, the effect of the duration of BA treatment on the number of embryoids did not depend on the clone (Table 2). The Newman and Keuls test was thus applied on the means of percentages of embryoids of the two clones. This percentage increased with the duration of culture on medium containing BA. Merkle (1995) observed that the presence of cytokinins in the medium may result in the formation of multiple apices in somatic embryos, resulting in structures that are difficult to characterize as embryogenic or organogenic, on the basis of their appearance alone. The increase of the number of embryoids may be the consequence of the stimulation of multiple shoot formation in oil palm somatic embryos by long term culture on medium with cytokinin.

Our result shows that for oil palm, as for some other tropical monocotyledoneous species, culture on medium with cytokinin may be used to achieve complete differentiation of the somatic embryo. The addition of BA to the culture medium resulted in a significant increase in the number of somatic embryos with single shoot which constitutes an improvement of the process for oil palm regeneration from embryogenic suspension cultures. Given that several papers report that cytokinins could be linked to a flower malformation induced during the oil palm regeneration process (Jones et al., 1990; Besse et al., 1992; Jones et al., 1995). It is necessary to examine the effect of

Table 2. Effect of various times *in vitro* on medium containing 5 μM BA on the percentage of single shoot, roots and embryoids obtained after 2 months germination of somatic embryos from 2 clones.

Number of week on BA medium		Single shoot (%)		Roots (%)		Embryoids (%)
		clone 121	clone 221	clone 121	clone 221	clone 121 and 221
1		48 b	40 a	23 b	20 a	28 a
2		42 ab	28 a	20 a	18 a	36 b
3		27 ab	30 a	25 b	10 a	38 b
4		20 a	43 a	10 a	15 a	41 b
Duration effect	$F_{3,16}$	3,769		3,753		7,42
	p	0.032		0.0324		0.0025
		*		*		**
Clone effect	$F_{1,16}$	0,127		3,52		1,88
	p	0.726		0.0789		0.188
		NS		NS		NS
Interaction	$F_{3,16}$	5,38		4,33		2,81
	p	0.0098		0.02		0.073
		**		*		NS

Means were calculated from triplicates of 20 embryos; ***, **, * and NS indicate the results of the two-ways ANOVA, respectively significant at $p< 0.001$, 0.01, 0.05 and non significant. Index letters indicate homogeneous groups in the same column according to the Newman and Keuls Test ($p<0.05$).

cytokinin supply during embryo development on the behavior of regenerated plants in the field.

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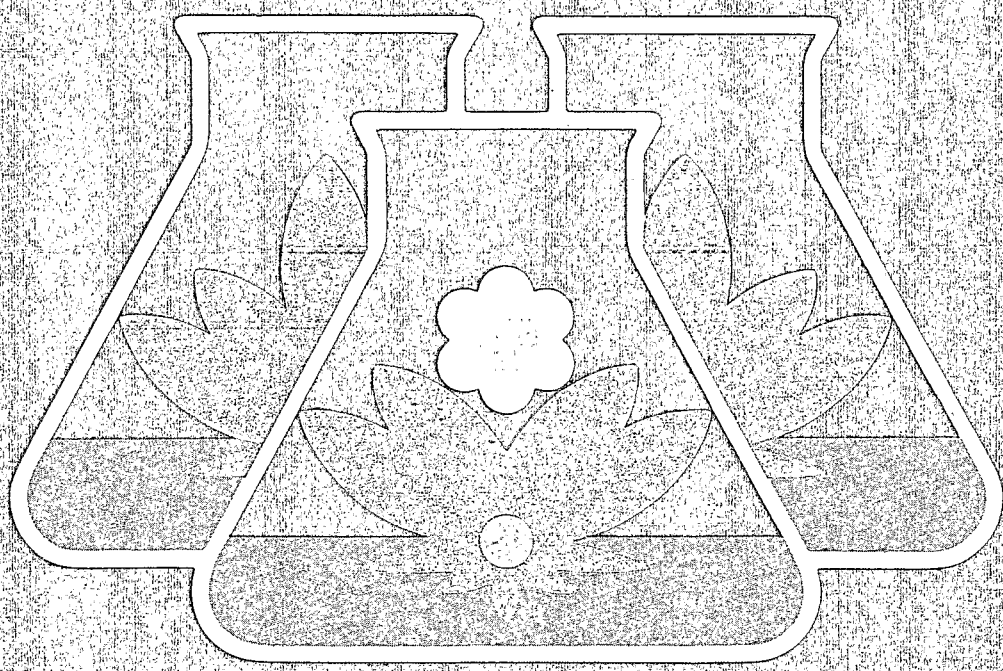

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