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Plant regeneration from embryo-derived callus of oil palm – the effect of exogenous polyamines

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Abstract

Regeneration in oil palm was achieved through somatic embryogenesis/organogenesis from embryo-derived callus. Callus was induced from mature embryos of the cross 281 (D)×18 (P) on modified MS medium supplemented with 2,4-D (113.12 μM) and 2-iP (14.76 μM). The embryogenic calluses obtained were transferred to Blaydes medium supplemented with 2,4-D (0.045 μM) and one of the following growth regulators: TDZ (4.54 μM), zeatin riboside (2.85 μM), putrescine (1 mM) and spermine (100 μM). Secondary somatic embryogenesis was found to occur in media supplemented with polyamines. The efficiency of formation of somatic embryos, secondary somatic embryos and shoot meristemoids were significantly higher in putrescine containing medium. Histological studies were also undertaken.

Abbreviations: BA – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; 2-iP – 6-(γ,γ,dimethylallyamino)-purine; IBA – indole-3-butryic acid; MS – Murashige and Skoog medium; TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (Thidiazuron); t – tonne

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a crop species producing high quality oil, which can be obtained from the mesocarp of the fruit (palm oil) and the kernel of the nut (palm kernel oil). Palm oil is used mainly for cooking, preparation of margarine, shortening and also for non-food applications (soap, detergent, cosmetics, etc.). Oil palm is the most productive oil crop with yields of up to 5–7 t of palm oil/ha/year under optimum conditions. Also, the production costs for palm oil in its ecosystem are the lowest among all the crops (Graille and Pina, 1999). All commercial palms are F1 hybrids between selections with small kernels (*dura*) and large kernels (*pisifera*), but the hybrids (*tenera*) show very high variation in oil yield with the best plants yielding 40% more than average. As a monocotyledonous species with a single growing apex, the plant cannot be multiplied vegetatively.

A reliable and efficient procedure for *in vitro* propagation of elite highest yielding palms will increase yields in a significant way. Extensive research from the 1980s has been successful in regenerating plants from culture (Duval et al., 1995; Parthasarathy et al., 2001). Many of the regenerants show flowering abnormalities ('mantling') that lead to fruit abortion and no yield due to a form of somaclonal variation. The fruiting abnormalities (mantleness) in field planted ramets, together with mediocre yield advantage of less than 20% over control *dura*×*pisifera* (D×P) seedling materials, created a loss of confidence in clonal oil palm. Unfortunately, many details regarding tissue culture on oil palm are not available because the research was generally carried out by commercial firms. Several papers have reported that long-term culture in the presence of cytokinins could be linked to the flower malformation induced during oil palm regeneration process (Jones, 1990; Besse et al., 1992; Jones et al., 1995).

Earlier studies from our laboratory were based on regeneration from leaf explants using BA and zeatin riboside (Raju et al., 1989; Anitha and Sajini, 1996). Polyamines function in a similar manner to some plant growth regulators with high levels of free polyamines promoting cell growth and DNA stability (Galston and Kaur-Sawhney, 1990). Exogenously supplied polyamines have been known to induce somatic embryogenesis and plant regeneration in many plant species (Galston and Flores, 1991; Martin-Tanguy and Carre, 1993; Adkins et al., 1998; Sargent et al., 1998). However, no work on the use of polyamines has been reported in oil palm. The objective of the present study was to substitute cytokinins with polyamines for improving the formation of somatic embryos and subsequent germination rates from embryo-derived callus of the cross 281 (D)×18 (P).

Materials and methods

Plant material

Mature oil palm nuts of the cross 281 (D)×18 (P) were obtained from Regional Station, National Research Centre for Oil Palm, Palode, Kerala, India. Embryos were extracted from the nuts and sterilized in 0.1% (w/v) mercuric chloride with two to three drops of Tween for 15 min. The embryos were then thoroughly washed in sterile water four to five times and inoculated onto the media.

Culture media

Modified Murashige and Skoog (1962) medium (Anitha and Sajini, 1996) and Blaydes (1966) medium were used for the experiments. The media were solidified with 0.55% (w/v) agar. The pH was adjusted to 5.7 prior to autoclaving at 1.06 kg cm^{-2} for 20 min.

Callus induction and maintenance

Sterilized embryos were inoculated into culture tubes containing 10–15 ml of modified MS medium (Anitha and Sajini, 1996) supplemented with 2,4-D (113.12 μM) and 2-iP (14.76 μM) for callus induction. The medium was solidified with 0.55% (w/v) agar. The cultures were incubated in the dark at 27±2 °C for 12 weeks until sufficient callus was obtained. They were subcultured at monthly intervals in the same medium during this period.

Plant regeneration

The calluses obtained were transferred to Blaydes medium (Blaydes, 1966) supplemented with 2,4-D (0.045 μM) and one of the following growth regulators: TDZ (4.54 μM), zeatin riboside (2.85 μM), putrescine (1 mM) and spermine (100 μM). The concentrations of these growth regulators were decided after initial optimization trials. Fifteen explants were used for each experiment and the experiments were repeated twice. Cultures were maintained under a 16-h light intensity. The somatic embryo production and plant regeneration rates were recorded at fortnightly intervals. The germinated plantlets were transferred into plain Blaydes medium solidified with 0.55% (w/v) agar in culture bottles for further growth. Plantlets with 4–5 leaves and with sufficient roots were transferred to a liquid medium (plain Blaydes medium) in big culture tubes (38×200 mm). When the plantlets were obtained from shoot meristemoids, they were transferred to Blaydes liquid medium supplemented with IBA (4.9 mM) for rooting when they reached 4–5 leaf stage.

Plant acclimatization

Healthy plants were removed individually from the culture tubes and washed with sterile water. Before transferring to pots, the plantlets were treated with Bavistin (1%) and thereafter with IBA solution (4 mM) for 1 h. The potting mixture used was sterilized soil, sand and coir dust in equal portions. Initially, the plantlets were covered with polythene bag. Gradually the bags were perforated and later the bags were removed during the night. After 4 weeks, the bags were removed completely.

Histological studies

Specimens for histological studies were fixed in Carnoy's B fixative (60% absolute alcohol, 30% chloroform, 10% acetic acid) for 24 h and were dehydrated in alcohol–butanol series before embedding in paraffin wax. Serial sections of 10 μM were taken using microtome. After deparaffinization, they were stained with 0.1% toluidine blue.

Statistical analysis

The experiments were replicated twice. Analysis of variances (one-way ANOVA) was used to test if there are significant differences between means obtained with different treatments at the 5% level of significance (Snedecor and Cochran, 1975). Means followed by the same letter are not significantly different from each other.

Results

Callus initiation was noticed within 4–5 weeks of inoculation of the embryos into the callus induction medium (Figure 1a). Callus proliferation occurred when subcultured in the same medium. The calluses were white in colour and compact in appearance.

The calluses were then subcultured into Blaydes medium supplemented with 2,4-D (0.045 μM) and other growth regulators. Within 2 weeks of subculture, two types of calluses could be distinguished: embryogenic calluses, which were friable, whitish yellow and fast growing, and non-embryogenic calluses, which were translucent and slimy.

The maximum percentage of embryogenic calluses formation occurred in putrescine-supplemented medium (70%), whereas the maximum percentage of non-embryogenic calluses formation occurred in TDZ-supplemented medium (80%). Within 3–4 weeks, globular structures were produced on the surface of embryogenic callus (Figure 2a) and these

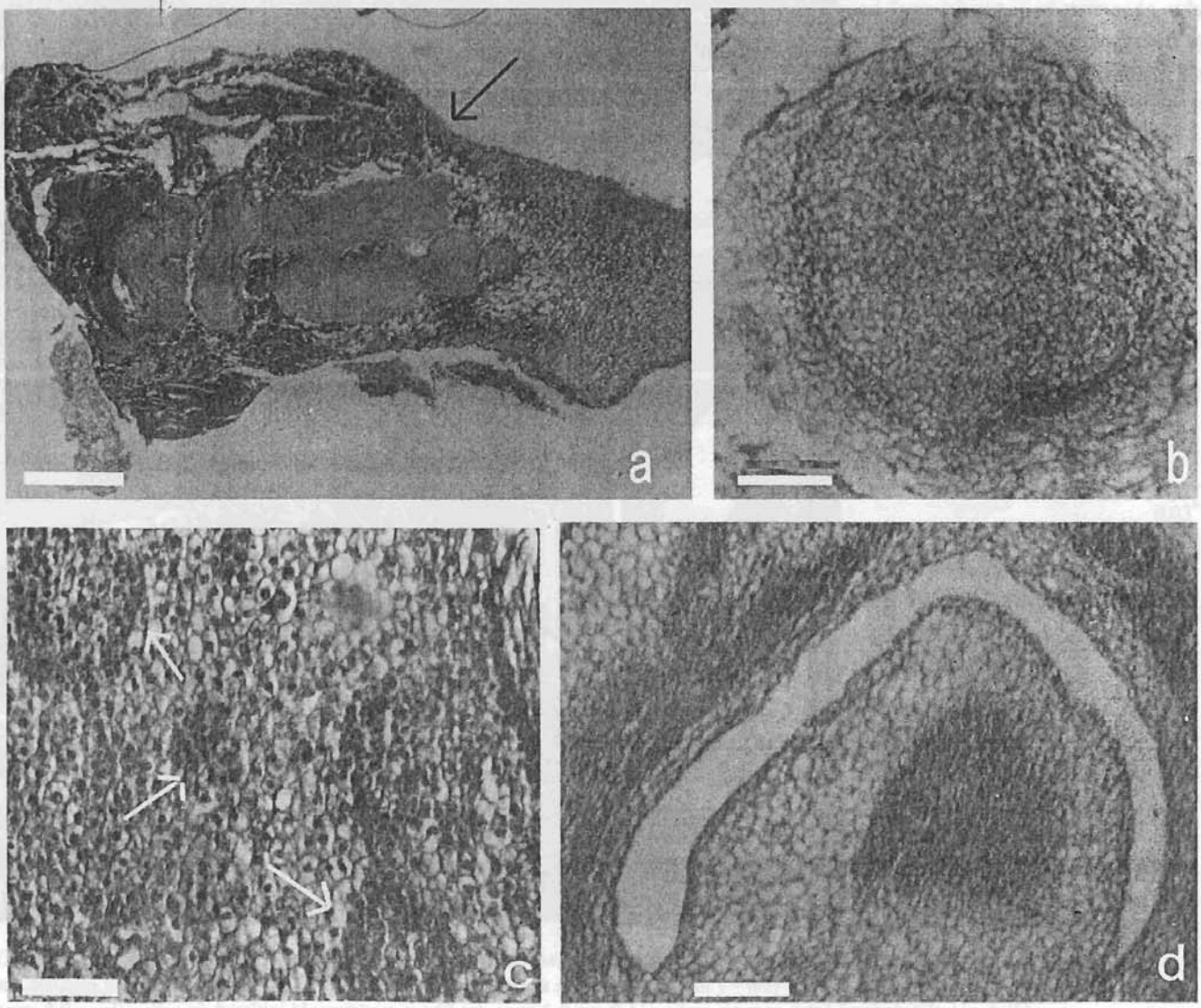

Figure 1. Histological studies of different stages of oil palm regeneration. (a) Initiation of callus from embryo explant (bar=500 μm). (b) Globular embryoid (bar=400 μm). (c) Initiation of meristematic centres inside the callus (bar=250 μm). (d) Initial stage of meristemoid development (bar=400 μm).

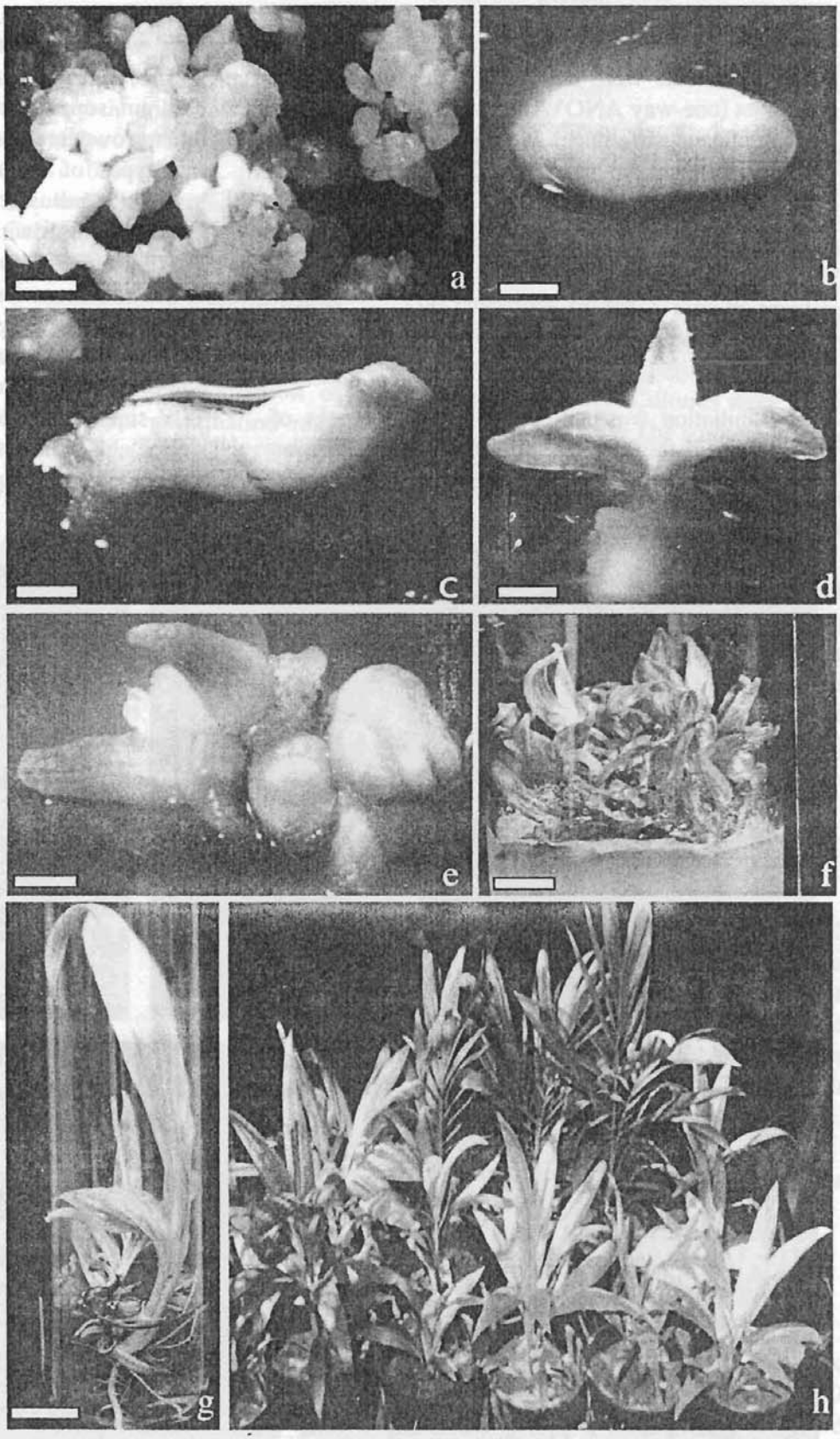

Figure 2. Regeneration through somatic embryogenesis/organogenesis from embryo-derived calluses of oil palm. (a) Globular structure on the friable callus surface (bar=1 mm). (b) A single somatic embryoid (bar=0.05 m). (c) A germinating somatic embryoid (bar=0.1 (d) A plantlet derived from somatic embryoid (bar=0.2 mm). (e) Shoot meristemoid initiated on callus (bar=0.2 mm). (f) Developing meristemoids (bar=0.8 mm). (g) An embryoid-derived plantlet with balanced shoot and root (bar=1 cm). (h) Established plantlets i

structures could be easily detached from the callus. When subcultured in the same medium, these structures (Figure 1b) enlarged and developed into embryoids (Figure 2b). When the primary embryoids were subcultured in the same medium, secondary somatic embryoids (2–12 embryoids per callus) were formed. Secondary embryoid formation could be achieved when the primary embryoids were transferred to a fresh medium. The efficiency of formation of somatic embryos and secondary somatic embryos were significantly higher in putrescine-supplemented medium (Table 1). The same results were obtained for germinating somatic embryoids giving rise to plantlets with both root and shoot poles. An average of 5.6 primary somatic embryos and 2.9 secondary embryos were produced in the putrescine-supplemented medium. Also, the formation of secondary somatic embryos was found only in medium supplemented with polyamines. The germinating embryoids (Figure 2c) were found to possess a bipolar organization with a shoot and root axis (Figure 2d).

Shoot/root primordia were also found to be initiated *de novo* from the callus tissue (Figure 2e,f). More than one meristemoid was generally seen to develop in a culture (Figure 1c). The meristemoid regions were found embedded in the callus unlike somatic embryos, which developed on the callus surface. A lateral prominence was formed on the side of the shoot apex (Figure 1d). Rhizoids were also initiated separately. The development of shoots and roots (organogenesis) was found to be monopolar, originating independently.

The formation of shoot meristemoids was found to be significantly higher ($p=0.05$) in putrescine and spermine-supplemented medium than in other media combinations (Table 1).

Embryoid-derived plantlets with balanced roots and shoots were transferred to plain Blaydes liquid medium in big culture tubes (Figure 2g). If the plantlets were derived from shoot meristemoids, when they reached 3–4 leaf stage, they were transferred to a liquid rhizogenesis medium (Blaydes medium supplemented with IBA). Plantlets were later transferred to pots (Figure 2h).

Table 1. The effect of different growth regulators on the formation of embryogenic callus, somatic embryos, secondary somatic embryos, shoot meristemoids and rhizoids in oil palm

Treatment	Average no. of embryogenic calluses	Average no. of somatic embryos	Average no. of somatic embryos germinating	Average no. of secondary somatic embryogenesis	Average no. of shoot meristemoids	Average no. of rhizoids
2,4-D (0.045 μM) alone	0.23	0.60[a]*	0.07[a]	0.00[a]	0.00[a]	0.40[a]
2,4-D (0.045 μM) +zeatin riboside (2.85 μM)	0.40	0.87[a]	0.47[a,b]	0.00[a]	0.57[a,b]	0.03[b]
2,4-D (0.045 μM) +TDZ (4.54 μM)	0.10	0.23[a]	0.23[a,b]	0.00[a]	0.33[a,b]	0.00[b]
2,4-D (0.045 μM) +spermine (100 μM)	0.47	2.13[b]	0.93[b]	0.50[b]	0.80[b,c]	0.10[b]
2,4-D (0.045 μM) +putrescine (1 mM)	0.70	5.60[c]	5.50[c]	2.97[b]	1.27[b]	0.37[a]

*Means followed by the same letter do not differ significantly ($p=0.05$).

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2,4-D (0.045 μM) +zeatin riboside (2.85 μM)	0.40	0.87^{a}	$0.47^{a,b}$	0.00^{a}	$0.57^{a,b}$	0.03^{b}
2,4-D (0.045 μM) +TDZ (4.54 μM)	0.10	0.23^{a}	$0.23^{a,b}$	0.00^{a}	$0.33^{a,b}$	0.00^{b}
2,4-D (0.045 μM) +spermine (100 μM)	0.47	2.13^{b}	0.93^{b}	0.50^{b}	$0.80^{b,c}$	0.10^{b}
2,4-D (0.045 μM) +putrescine (1 mM)	0.70	5.60^{c}	5.50^{c}	2.97^{b}	1.27^{b}	0.37^{a}

*Means followed by the same letter do not differ significantly ($p=0.05$).

Discussion

In the previous studies, embryogenic calluses in oil palm were induced and maintained in a medium containing growth regulators, especially auxins and cytokinins. They then had to be transferred to a medium with reduced growth regulator concentration to express their embryogenic potential, allowing embryo development and plant conversion (Parthasarathy et al., 2001). 2,4-D was the most commonly used auxin for the induction of somatic embryos in culture. BA and zeatin riboside are the cytokinins that have been used for regeneration in oil palm (Raju et al., 1989; Anitha and Sajini, 1996; Aberlenc-Bertossi et al., 1999). Prolonged culture in a cytokinin-supplemented media could be linked to the flower malformation induced during the regeneration process (Jones, 1990; Besse et al., 1992; Jones et al., 1995). In this study, the extent to which polyamines can replace cytokinins in the induction of embryogenesis/organogenesis and plant regeneration in oil palm has been presented. Most of the reported embryo induction media used for oil palm was MS based. However, in the present study, Blaydes medium was found to be the best based on our earlier experience.

Polyamines form a class of aliphatic amines that are ubiquitous in living organisms and have been implicated in a wide range of biological processes (Smith, 1985). Increased putrescine, spermine and spermidine levels have been observed during somatic embryogenesis of various plant species. Muhitch et al. (1983) reported that polyamines inhibited the senescence of non-photosynthetic Paul's Scarlet rose cells. Fienberg et al. (1984) reported that increased polyamine levels are required for cellular differentiation and development occurring during somatic embryogenesis in carrot cell cultures. Feray et al. (1993) demonstrated the effect of exogenously supplied polyamines on morphogenesis of *in vitro* potato plants. Chi et al. (1994) showed that exogenous application of polyamines enhanced plant regeneration in *Brassica* ssp. An intricate regulatory role of polyamines, specifically putrescine, was suggested by Yadav and Rajam (1997) in eggplant. Kevers et al. (2000) found that incorporation of polyamines or their precursors in the induction or regeneration media increased the number of somatic embryos produced in liquid cultures of *Panax ginseng* by up to four times. Martinez et al. (2000) reported the successful use of polyamines for the induction of gynogenic embryos and regeneration of onion plantlets.

The exogenous application of the two polyamii putrescine and spermine, significantly increased rate of somatic embryogenesis from the embryo-rived callus of the oil palm cross 281 (D)×18 (P) could be possible that the application of free pc amines may have provided a stimulus for som embryogenesis/organogenesis and subsequent pl regeneration. Similar results were reported by Sarg et al. (1998) in sorghum and sugarcane. Adkins et (1998) reported that polyamines, when applied coconut during the callus induction stage, enhan the somatic embryogenesis. It was suggested that role of polyamines might have been by a dir stimulation of somatic embryogenesis. TDZ is a su stituted phenyl urea that has been reported to exer high cytokinin activity and aid regeneration in ma plant species (Huetteman and Preece, 1993; Murthy al., 1998). But in oil palm, when the medium w supplemented with TDZ, the calluses had a tenden to turn non-embryogenic. The formation of embryoi in zeatin riboside containing medium was less co pared to the polyamine-supplemented media. This the first report of the use of polyamines for inducti of somatic embryogenesis/organogenesis and su sequent plant regeneration in oil palm.

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