

Optimizing rooting and survival of oil palm (*Elaeis guineensis*) plantlets derived from somatic embryos

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Abstract In species of the Arecaceae, initial conversion of somatic embryos to non-rooted plantlets (those with only shoot development and no roots) is common. A consistent methodology for improving rooting and survival of oil palm plantlets derived from somatic embryos was developed. Two experiments were carried out. In the first, non-rooted shoots regenerated *via* somatic embryogenesis were inoculated in nutrient medium supplemented with 0, 53.7, or 107.4 μM indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA). In the second, the optimum treatment as determined in the first experiment was evaluated in three culture systems: semi-solid medium (SS), stationary liquid (SL), and double-phase system (DP), with and without the addition of activated charcoal. Next, the plantlets were acclimatized in a greenhouse, where the influence of the presence or absence of roots, the number of roots, the length of the main root, and the height of the shoot on survival were assessed. Supplementation with 53.7 μM IBA and the DP system without activated charcoal provided the highest percentage of rooted plantlets (92.9%) and number of roots (6.3) and improved the length of the aerial parts (11.0 cm) and the survival of the plantlets during the acclimatization (82.1%). The best survival of plantlets was obtained when they were acclimatized with at least one root and with a height greater than 7.5 cm.

Keywords Arecaceae · Somatic embryogenesis · Liquid medium · Double-phase system · *In vitro* rooting · Acclimatization

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Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocotyledonous tropical tree that in adult age produces between 12 and 14 bunches of vegetable oil-rich fruits per year. The importance of this crop worldwide is due to its utility in the energy and ecological sectors, its high yield of oil from its fruits, and the high heating value of the oil, as well as its ability to be grown commercially for at least a 25-yr period (Adam *et al.* 2011; Jaligot *et al.* 2011).

In Brazil, oil palm cultivars used for commercial planting are mainly thin-shelled *tenera* genotypes and are obtained from crosses between *Dura* palm—the fruit of which has a thick shell surrounding the seeds—and *pisifera* palm—the fruit of which lacks a shell. The hybrids exhibit very high variability in oil yield, with the best plants yielding 20–30% more than the average. However, genetic improvement of oil palm through conventional breeding is extremely slow and costly as the breeding cycle can take up to 10 yr (Bakoum and Louise 2007; Low *et al.* 2008).

Micropropagation *via* somatic embryogenesis is one of the only forms of clonal propagation of elite plants (Duval *et al.* 1988). Despite the potential of this technique, it is difficult to control all the stages of somatic embryogenesis. One of the main problems is a high production of regenerated plants devoid of roots (Karun and Sajini 1996; Zamzuri 1998; Gallo-Meagher and Green 2002; Fki *et al.* 2003; Rajesh *et al.* 2003; Saénz *et al.* 2006; Konan *et al.* 2007; Nizam and Te-Chato 2009; Sumaryono and Riyadi 2011). This characteristic heavily impacts one of the final stages of the process—the acclimatization of cloned plants—making it a stumbling block for the success of the technique. Although *in vitro* rooting of oil palm plants derived from somatic embryos has been reported, plants with good shoot development, but no roots, can occur at frequencies of >50% of the regenerated plants. Therefore, a subsequent stage to *in vitro* rooting of the shoots,

supplementing the nutritional media with auxins, is generally necessary (Rival *et al.* 1997; Rival and Parveez 2004; Konan *et al.* 2007).

Other factors influencing plant recovery include the genotype, the physiological state of the explants, and the culture conditions. Furthermore, the use of an appropriate culture medium for each stage is a basic condition for the success of the process and is responsible for providing the nutrients necessary for the metabolism of the developing plant cells (Scherwinski-Pereira *et al.* 2012).

The majority of work on plant micropropagation is based on the use of semi-solid culture media. However, the use of liquid, double-phase nutrient media has provided equal or even greater efficiency for many plant species (Zamzuri 1998; Tarmizi *et al.* 2008; Steinmacher *et al.* 2011; Scherwinski-Pereira *et al.* 2012; Oliveira *et al.* 2013). Furthermore, this system features ease of the preparation and handling of the media, the reduced production costs provided by the elimination or reduction of gelling agent, and the possibility of using a small quantities of culture medium.

Activated charcoal can adsorb phenolic compounds that inhibit growth and development and stimulate *in vitro* rooting (Thomas 2008). Because roots of plants produced *in vitro* are very fragile, absorption of water and nutrients from the soil can be inadequate, which can lead to significant reductions in survival rates (Vidal *et al.* 2003; Sumaryono and Riyadi 2011).

The objectives of this work were to optimize rooting and survival efficiency of oil palm plantlets derived from somatic embryos and to evaluate the relationship between rooting and survival of the plantlets during acclimatization.

Materials and Methods

Plantlets from somatic embryos. Plantlets were obtained via somatic embryogenesis of the *tenera* oil palm FACM 589 hybrid variety, according to Balzon *et al.* (2013). Zygotic embryos were initially excised from mature seeds. The seeds were surface sterilized by immersion in 70% alcohol for 3 min, followed by immersion in 2.5% (*w/v*) sodium hypochlorite containing 50 μL Tween-20 (Sigma, St. Louis, MO) per 100 mL for 40 min, and then air-dried without rinsing. The mesocarp was then removed. The nuts were surface sterilized by immersion in 70% alcohol for 3 min, followed by immersion in 1.25% (*w/v*) sodium hypochlorite for 20 min, and subsequently washed in three changes of sterile distilled water. To facilitate embryo excision, nuts were immersed in sterilized water for 24 h. The embryos were then excised under aseptic conditions with a scalpel, rehydrated in sterile water for 1–2 h, and placed on culture medium.

The callus induction phase was performed in 9-cm diameter Petri dishes sealed with transparent plastic film containing

20 mL callus induction medium (CIM). CIM consisted of MS medium (Murashige and Skoog 1962) supplemented with 450 μM picloram, 2.5 g L^{-1} activated charcoal, 30 g L^{-1} sucrose, and solidified with 2.5 g L^{-1} Phytigel (Sigma). All culture media used in this study were adjusted to pH 5.7 and sterilized by autoclaving. During callus induction, the cultures were incubated in darkness at $25\pm 2^\circ\text{C}$ and subcultured every 4 wk on the same medium.

For callus proliferation and maintenance, embryogenic cultures induced on CIM were transferred onto MS medium supplemented with 40 μM picloram and 10 μM 2-isopentenyladenine (2iP), without charcoal. The cultures were maintained in darkness at $25\pm 2^\circ\text{C}$ for up to 12 wk. To induce embryo differentiation and obtain mature somatic embryos, embryogenic cultures from the proliferation and maintenance phase were transferred onto MS medium supplemented with 12.3 μM 2iP and 0.54 μM naphthaleneacetic acid (NAA), without charcoal. For plantlet development, calli containing mainly differentiating embryos were transferred to 250-mL glass bottles containing the medium used for differentiation, but with half-strength salt concentration, no plant growth regulators and 2.5 g L^{-1} activated charcoal. Somatic embryos with elongated shoots forming on the embryo clumps were isolated and transferred into test tubes (25 \times 150 mm) containing fresh medium to promote their adequate development before being used in the *in vitro* rooting experiments.

***In vitro* rooting.** Two experiments were performed to optimize *in vitro* rooting of non-rooted aerial parts. First, the auxins indole-3-butyric acid (IBA) and NAA in the concentrations of 0, 53.7, and 107.4 μM were evaluated in order to determine their effects on root formation from, and growth of, aerial parts. The optimal treatment as shown by this experiment was then used to evaluate the effects of three different culture systems: semi-solid (SS), stationary liquid (SL), and double-phase (DP) (semi-solid medium with a layer of liquid medium on the top). Each system was evaluated with and without the addition of activated charcoal. In these tests, plantlets with a height of between 4 and 5 cm, with 2–3 leaves, were used. Cultivation was carried out in test tubes (25 \times 150 mm), closed with plastic covers and sealed with transparent plastic film.

In both experiments, the plantlets were kept in light provided by cool white lamps with a photosynthetic photon flux of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a 16-h d^{-1} photoperiod and a temperature of $25\pm 2^\circ\text{C}$. The percentage of rooted plantlets, the number of roots, the length of the longest root, and the height of the aerial part (cm) were evaluated at 30, 90, and 150 d.

Effect of the type of auxin and concentration. To analyze the effects of auxins on *in vitro* rhizogenesis, non-rooted aerial parts were separated and then inoculated into test tubes (25 \times 150 mm) containing 10 mL of MS medium supplemented

with 0, 53.7, and 107.4 μM IBA or NAA, 30 g L^{-1} sucrose, and 2.5 g L^{-1} Phytigel, closed with plastic covers and sealed with transparent plastic film.

Effect of culture system and activated charcoal. To evaluate the effects of the different culture systems and activated charcoal on the induction of *in vitro* rhizogenesis, non-rooted aerial parts were separated and inoculated into SS, SL, or DP systems. The experiments used MS medium supplemented with 53.7 μM IBA and 30 g L^{-1} sucrose, with or without the addition of activated charcoal. At this stage, the concentration of activated charcoal was reduced from 2.5 to 1.5 g L^{-1} after preliminary experiments showed that higher concentrations were ineffective for inducing roots. Plantlets were cultivated in test tubes (25×150 mm) containing, for the first 30 d of growth, 10 mL of medium with semi-solid consistency (2.5 g L^{-1} Phytigel) (for SS and DP systems) or 3 mL of liquid medium (for SL system). After 30 and 90 d, 3 mL aliquots of liquid medium were added (for DP and SL systems).

Influence of the presence or absence of roots, the number of roots, length of the main root, and the height of the aerial part on the percentage of survival during acclimatization. Plantlets obtained during the rooting phase were cultivated for 45 d in 300-mL plastic cups filled with a mixture of commercial substrate (Bioplant[®], Nova Ponte, Brazil) and washed sand, at a proportion of 3:1 (v/v), respectively. For the first 3 wk, plantlets were kept covered with 400-mL transparent plastic cups with holes (approximately 5 mm diameter) at the top. After 45 d, plantlets were transplanted to black plastic bags ($20 \times 20 \times 20$ cm) in a greenhouse, where they were cultivated for 225 d to complete their development.

At greenhouse, plantlets were irrigated manually every 24 h in the first week of growth and every 48 h thereafter. Greenhouse conditions were $75 \pm 5\%$ relative humidity, $30 \pm 3^\circ\text{C}$, with photosynthetic photon flux density of 450–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 12-h photoperiod. After 60 d, plantlet survival was evaluated. The relationship between the presence, number, and length of roots and height of the aerial part with percentage of survival of the plantlets during acclimatization was also evaluated.

Statistical analysis. All the experiments used a completely randomized design with 20 replicates per treatment. Each replicate was composed of one non-rooted aerial part per test tube. The data obtained were examined *via* analysis of variance, and the average values were compared by the Scott-Knott test at a 5% significance level. Data obtained through counting (number of roots) were transformed according to $(x+1)^{0.5}$, while data obtained by calculating percentage were transformed by $\arcsin(x/100)^{0.5}$. All experiments were repeated at least once.

Results and Discussion

Effect of the type of auxin and concentration. The best results for rooting percentage (81.3%) and number of roots formed (7.1 per shoot) were obtained when the plantlets were treated with 53.7 μM IBA. NAA did not promote good root formation at any concentration used, with values consistently lower than those observed in the control treatment (Table 1). This is contrary to results obtained by Nizam and Te-chato (2009), who reported that NAA promoted better *in vitro* rooting of aerial parts of oil palm derived from somatic embryos. According to Zamzuri (1998) and Abahmane (2011), the quality of *in vitro* rooting is influenced by the genotype and the type and concentration of auxin added to the culture medium, thus explaining differential physiological responses in plantlets of the same species. On the other hand, the addition of auxins during *in vitro* rooting may stimulate ethylene synthesis, which in turn regulates plant growth and development (Vidal

Table 1 Effect of auxin type and concentration during the rooting of oil palm plantlets

Treatments		Period (d)		
Auxin	μM	30	90	150
Root induction (%)				
–	0.0	20.0 aB	20.0 bB	58.3 bA
IBA	53.7	9.3 aB	64.0 aA	81.3 aA
	107.4	7.7 aB	23.0 bB	53.7 bA
NAA	53.7	0.0 aA	7.3 bA	27.3 cA
	107.4	0.0 aB	0.0 bB	25.0 cA
Number of roots per shoot				
–	0.0	4.0 bB	6.5 aA	6.5 bA
IBA	53.7	3.0 bB	5.7 aA	7.1 aA
	107.4	6.0 aA	5.6 aA	5.6 bA
NAA	53.7	0.0 cC	1.0 bB	4.3 bA
	107.4	0.0 cB	0.0 cB	4.3 bA
Root length (cm)				
–	0.0	0.6 aB	0.8 bA	0.8 bA
IBA	53.7	0.3 bC	0.6 cB	1.2 aA
	107.4	0.4 bA	0.6 cA	0.5 cA
NAA	53.7	0.0 cC	1.0 aA	0.8 bB
	107.4	0.0 cB	0.0 dB	0.6 cA
Shoot height (cm)				
–	0.0	6.7 aB	9.2 aA	10.9 aA
IBA	53.7	6.5 aB	9.4 aA	11.0 aA
	107.4	7.7 aB	10.6 aA	11.8 aA
NAA	53.7	6.3 aB	8.8 aA	9.2 bA
	107.4	6.1 aA	7.3 aA	8.1 bA

Upper case letters represent significant differences among the periods at each treatment, and lower case letters represent differences among the treatments at each period

et al. 2003; Konan *et al.* 2007; Schaller 2012). However, in the present experiment, the growth of roots was only affected by auxin levels higher than 53.7 μM . Only NAA hindered shoot elongation, and NAA treatment on average provided shoots which were 2 cm shorter than the other treatments.

In the absence of auxins, 58% of rooting occurred after 150 d of cultivation. In *E. guineensis* and *Phoenix dactylifera*, shoots derived from embryo multiplication also showed development of roots in auxin-free media, although auxin supplementation favored rooting (Nizam and Te-Chato 2009; Sumaryono and Riyadi; 2011; Sané *et al.* 2012). These results likely can be explained by the existence of natural auxins, such as indole-3-acetic acid (IAA), in the tissues of the explants, or by the residual effects of the auxins used in the induction and multiplication phases of the embryogenic cultures (Klerk *et al.* 1999).

Rooting occurred after the first month of culture. However, the best rooting was obtained after the third month, reaching maximum values at 150 d. Thus, a minimum period of at least 90 d was needed for optimum rooting of the non-rooted aerial parts of oil palm (Table 1).

The use of exogenous auxins was even more important for *in vitro* rooting. In the absence of auxins, only 39.2% of the plantlets survived after 60 d (Table 2), whereas 85.7% survived when IBA was used in the rooting phase. Al-Salih *et al.* (1987) also found that culture media without auxins was associated with higher mortality rates during the acclimatization process of *P. dactylifera*.

Effect of culture system and activated charcoal. The best results for the percentage of rooting (92.9%) and the number of roots formed (6.3 per shoot) were obtained when the non-rooted aerial parts were grown in the DP system without activated charcoal (Table 3, Fig. 1). *In vitro* rooting of *E. guineensis* was also increased by about 20% with the DP system (Zamzuri 1998). These results likely are associated with increased availability of both water and nutrients

Table 2 Effect of auxin type and concentration on survival of oil palm plantlets during acclimatization

Treatments		Period (d)	
Auxin	μM	30	60
Survival (%)			
–	0.0	85.7 aA	39.2 bB
IBA	53.7	96.4 aA	78.5 aB
	107.4	96.4 aA	85.7 aA
NAA	53.7	40.0 bA	30.0 bA
	107.4	42.9 bA	0.0 cB

Upper case letters represent significant differences between the periods at each treatment, and lower case letters represent differences among the treatments at each period

Table 3 Effect of activated charcoal (AC) and *in vitro* culture system during the rooting of oil palm plants

Treatments		Period (d)		
Systems	AC	30	90	150
Root induction (%)				
Semi-solid	(–)	10.7 aC	57.3 aB	74.9 bA
	(+)	11.9 aA	12.0 bA	15.9 cA
Liquid	(–)	7.4 aB	22.1 bA	33.2 cA
	(+)	10.7 aB	28.5 bA	39.2 cA
Double-phase	(–)	10.0 aC	67.9 aB	92.9 aA
	(+)	3.9 aB	28.0 bA	27.9 cA
Number of roots per shoot				
Semi-solid	(–)	1.6 bC	2.5 aB	3.6 bA
	(+)	1.5 bA	1.5 cA	1.5 cA
Liquid	(–)	1.9 bB	1.9 bB	2.8 bA
	(+)	3.2 aA	2.2 aB	2.4 bB
Double-phase	(–)	0.0 dC	3.2 aB	6.3 aA
	(+)	0.5 cB	1.9 bA	1.9 cA
Root length (cm)				
Semi-solid	(–)	0.2 bC	0.7 cB	1.1 dA
	(+)	0.7 aC	1.7 aB	1.9 aA
Liquid	(–)	0.3 bB	0.4 dA	0.5 fA
	(+)	0.6 aC	0.8 cB	1.2 cA
Double-phase	(–)	0.0 cB	0.9 cA	1.0 eA
	(+)	0.2 bC	1.1 bB	1.7 bA
Shoot height (cm)				
Semi-solid	(–)	4.0 bB	7.4 bA	9.2 bA
	(+)	4.0 bA	5.2 bA	6.1 cA
Liquid	(–)	6.7 aC	9.5 aB	12.2 aA
	(+)	6.7 aB	9.2 aA	11.3 aA
Double-phase	(–)	4.9 bC	8.5 aB	11.0 aA
	(+)	4.2 bB	7.3 bA	8.8 bA

Upper case letters represent significant differences among the periods at each treatment, and lower case letters represent differences among the treatments at each period

provided by the liquid portion of nutrient medium (in which there is no physical resistance to the diffusion of these compounds) and the reduction of hyperhydricity of the tissues provided by part semi-solid culture medium (Scherwinski-Pereira *et al.* 2012; Oliveira *et al.* 2013).

Use of activated charcoal resulted in a reduction in the rooting percentage and number of roots formed per shoot. These results are probably due to the adsorption by the activated charcoal of the growth regulators and the consequent reduction or neutralization of their physiological activities (Thomas 2008). Pullman *et al.* (2005) also observed inhibition of the physiological effects of growth regulators in the presence of activated charcoal during *in vitro* culture of *Picea abies*. To obtain the benefits both from growth regulators

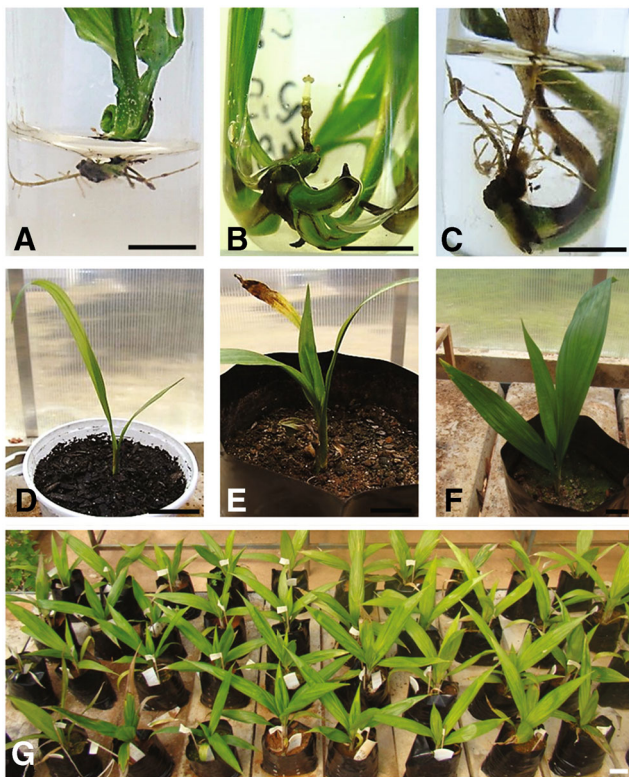


Figure 1 Oil palm plantlets regenerated via somatic embryogenesis during *in vitro* rooting and acclimatization. *A, B, C* *in vitro* rooting on semi-solid (SS), stationary liquid, and double-phase culture system, respectively. *D, E, F* oil palm plantlets acclimatized in greenhouse after 0, 60, and 90 d, respectively. *G* oil palm plantlets acclimatized in greenhouse after 180 d. Bars: *A–C* 1 cm; *D–F* 2.5 cm; *G* 5 cm.

and activated charcoal, it may be necessary to increase the levels of growth regulators.

Despite the reduction in the percentage of rooting and the number of roots formed, supplementation with activated charcoal, regardless of the culture system used, favored root elongation. This fact can also be explained by adsorption of auxins by the activated charcoal. Additional exogenous auxin may suppress shoot growth (Vidal *et al.* 2003; Konan *et al.* 2007; Thomas 2008) through the production of ethylene.

Although root development had already occurred by the third month, the best results for almost all the parameters were obtained only in the fifth month of growth. Thus, the importance was seen of allowing a minimum period of at least 150 d to optimize rooting of the non-rooted aerial parts of oil palm.

Although the use of the DP system without activated charcoal showed the best results for the rooting of plantlets, no statistical differences were observed in the process of acclimatization, between the charcoal-free rooting treatments. On average, survival was 78% after 60 d of acclimatization (Table 4).

Influence of the presence or absence of roots, the number of roots, the length of the main root, and the height of the aerial part on the

Table 4 Effect of *in vitro* culture system and activated charcoal (AC) on survival of oil palm plantlets during acclimatization

Treatments		Period (d)	
Systems	AC	30	60
Survival (%)			
Semi-solid	(–)	96.4 aA	78.5 aA
	(+)	57.1 bA	21.4 cB
Liquid	(–)	92.8 aA	85.7 aA
	(+)	96.4 aA	82.1 aA
Double-phase	(–)	89.2 aA	82.1 aA
	(+)	82.1 aA	53.5 bB

Upper case letters represent significant differences between the periods at each treatment, and lower case letters represent differences among the treatments at each period

percentage of survival in the acclimatization. Acclimatization involves transfer of plantlets to a transition environment, such as a greenhouse, before being transferred to the natural environment (Chandra *et al.* 2010). In this phase, stress caused by the reduced relative humidity, the increased light, the pathogens, and the transition from heterotrophic to

Table 5 Influence of the presence or absence of roots, the number of roots, the length of the main root, and the height of the aerial part on the percentage of survival of oil palm plantlets during acclimatization

	Period (d)	
	30	60
Survival (%)		
Roots	Survival (%)	
Present	90.3 aA	74.6 aB
Absent	77.7 bA	47.2 bB
Number of roots per shoot	Survival (%)	
0	77.7 aA	47.2 bB
1–2	92.3 aA	73.8 aB
3–4	89.6 aA	72.4 aA
≥5	87.1 aA	79.4 aA
Root length (cm)	Survival (%)	
≤0.5	86.8 aA	71.0 aA
0.6–1	89.1 aA	84.7 aA
1.1–1.5	92.8 aA	71.4 aA
>1.5	94.2 aA	68.5 aB
Shoot height (cm)	Survival (%)	
≤2.5	22.2 dA	22.2 bA
2.6–5	54.7 cA	21.4 bB
5.1–7.5	74.1 bA	37.6 bB
7.6–10	89.3 aA	62.7 aB
>10	94.1 aA	74.1 aB

Upper case letters represent significant differences between the periods at each treatment, and lower case letters represent differences among the treatments at each period

autotrophic metabolism, may be a limiting factor for the establishment of commercial plantations (Chandra *et al.* 2010; Fki *et al.* 2011; Kumar and Rao 2012).

Factors related to the rooting phase, such as the presence of roots, the total number of roots, the length of the main root, and the height of the aerial part of the plantlet, seem to be associated with the survival of the plantlets during the acclimatization process. Table 5 shows the effects of the presence of roots, the number of roots, the length of the main root, and the height of the aerial part, on the percentage of survival of oil palms during the acclimatization process.

It was found that 74.6% of rooted plantlets survived the acclimatization process, significantly higher than observed for plantlets devoid of roots (47.2%). Similar results were found by Steinmacher *et al.* (2007) and Othmani *et al.* (2009) for *Bactris gasipaes* and *P. dactylifera*, who reported survival rates of approximately 70% for complete plants. According to Steinmacher *et al.* (2007), the existence of roots in acclimatized plantlets was essential for survival.

Although in several plant species the number and length of roots are directly related to acclimatization success, in oil palm, no interaction was observed between the quantity and length of the roots produced *in vitro* and the percentage of survival of the plantlets during acclimatization. The treatments that gave enhanced rooting percentage, rather than the number and length of roots, were better predictors of plant survival.

The height of the acclimatized plantlets also significantly influences survival. According to Zamzuri (1998) and Abahmane (2011), shoots with heights above 12–15 cm are suitable for the process of acclimatization. In the present study, it was found that plantlets with heights less than 7.6 cm showed survival rates ranging from 22 to 37%, significantly lower than those with heights greater than 7.5 cm, for which survival rates were as high as 62–74%.

In conclusion, the present study describes a consistent methodology for improving levels of rooting of somatic embryo-derived oil palm plantlets which were devoid of roots after *in vitro* regeneration. In addition to determining the best auxin type and concentration, and culture system, we also determined the optimal time necessary for rooting and showed that the number and length of roots and height of aerial part affected acclimatization. It is important to highlight that this index refers to plantlets that were initially completely devoid of roots and which, therefore, could not be re-incorporated into the production process. An important observation that is not described in other similar works is that during acclimatization, it may be necessary to monitor the survival of the plantlets, beyond the usual 30 d in the greenhouse, as significant losses due to death occur after this period. Finally, we highlight the DP culture system, which enabled us to obtain better rooting. The DP system offers a simple, economical system, as it allows

liquid media to be used and reduces the need for direct handling of the crop.

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