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Changes in peroxidase activity during in vitro rooting of oil palm (*Elaeis guineensis* Jacq.)

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Abstract

Following induction by auxin treatment, the rooting performance of *Elaeis guineensis* Jacq. shoots obtained through in vitro clonal propagation by somatic embryogenesis varies considerably. This study involved a preliminary evaluation of guaiacol-peroxidase activity as a marker of in vitro rooting. It was carried out on 17 different clones obtained through a large-scale micropropagation procedure. No rooting occurred in the absence of exogenous auxins. Peroxidase activity evolved as previously described on other species, peaking between 10 and 14 days under optimum initiation conditions. The peak was found to shift in time when the rooting success rate was low. Peroxidase activity measurements, carried out prior to induction on 24 production batches belonging to 13 different clonal lines, revealed a significantly greater heterogeneity in batches with a low rooting success rate (< 50%). Several improved protocols aimed at optimising the rooting phase under large scale propagation conditions are proposed. © 1997 Elsevier Science B.V.

Keywords: Auxin; NAA; Rooting; Biochemical marker; Peroxidases

1. Introduction

A large scale oil palm clonal micropropagation process through somatic embryogenesis has been developed by ORSTOM and CIRAD as from the early eighties (Pannetier et al., 1981). To date, it has been used to produce more than a million clonal plantlets (Duval et al., 1988, 1995). In its final phase, this process involves a stage of shoot

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development in bunches, followed by isolation prior to rooting induction by an auxinic treatment (NAA). Scaling-up of this process in pilot production units on a large number of clones (more than 1000 to date) led to the discovery of substantial differences in clone performance during the rooting phase. Heterogeneity was seen between different clones, whose rooting abilities can vary—attributable to genetic variability from clone to clone, but also between different production batches of the same clone—which would rather seem to suggest physiological variations occurring on genetically homogeneous material. If the rooting phase on this plant material is to be optimised so as to limit losses, which are always costly at a finishing stage of production, it is important to identify a reliable biochemical marker for rooting (Gaspar et al., 1992).

Various studies on other species (*Sequoiadendron*, *Cynara*, *Asparagus*, *Populus*, *Prunus*, etc.) have shown the fundamental role played by peroxidases in controlling rooting initiation and expression (Quoirin et al., 1974; Van Hoof and Gaspar, 1976; Moncousin and Gaspar, 1983; Berthon et al., 1987; Hausman, 1993). Indeed, basic isoperoxidases are considered as IAA-oxidases, and are thus capable of modulating the endogenous auxin content (Berthon et al., 1989). Recent techniques for endogenous phytohormone titration (Hausman, 1993) have been used to validate the model proposed by Gaspar et al. (1977, 1992). The root neoformation process is now conventionally described in three phases: (a) an initial induction phase, characterised by a sharp drop in peroxidase activity, which is very rapid and fleeting and therefore very rarely described (Jouve et al., 1994), (b) a second phase of root initiation, corresponding to an increase in peroxidase activity, and (c) an expression phase, characterised by a gradual drop in peroxidase activity, which is followed by the first histologically visible signs (development of root primordia).

These three stages, during which endogenous auxin rates follow a reverse trend to that of enzymatic activity, are reported to be essential for root development in the in vitro plantlets studied (Moncousin et al., 1988; Gaspar et al., 1992). It has been shown that any disruption in one of these stages leads to defective in vitro rooting (Berthon et al., 1990).

The purpose of the work described in this article was to test the value of a peroxidase predictive marker for the first time on *Elaeis guineensis* Jacq. clonal plantlets obtained through somatic embryogenesis.

2. Material and Methods

2.1. Plant material

The oil palm (*E. guineensis* Jacq) in vitro grown plantlets analysed during this work were obtained by cloning elite individuals of *dura* × *pisifera* origin bred at the IDEFOR-DPO station in La Mé (Ivory Coast). Plant material isolation and maintenance conditions during the various stages of in vitro culture have already been described (Duval et al., 1995). Shootlets were obtained from polyembryonic cultures resulting from adventive secondary somatic embryogenesis. Somatic embryos from various development stage could be observed growing simultaneously on the same culture. For

rooting, vigorous 5–7 cm sized shootlets (with 2–3 leaves) were selected and manually isolated from bunches. This time is referred to as 'Day −1' throughout the experiment.

The process studied here involved a two-phase rooting stage in a liquid medium. The induction treatment was carried out on NAA at 1 mg l^{-1} for 24 h (standard conditions). The medium was then replaced by an expression medium of identical organomineral composition without growth regulators, in which root growth took place for 1 month. The time of this transfer to the expression medium is referred to as 'Day 0' throughout the experiment. The first roots visible to the naked eye appeared around 20 days after the auxin treatment.

When the induction is performed on a 'NAA0' medium, shootlets are soaked during 24 h in the expression medium (identical to the induction medium but deprived of growth regulators).

2.2. Experimental design

For the kinetic study of changes in peroxidase activity, the plantlets (sometimes with their roots removed) were frozen in liquid nitrogen (−196°C) at the time of sampling and stored at −80°C until enzyme extraction. Samples were taken daily in the first week, then every 3 days in subsequent weeks. Monitoring was continued for 3 weeks following shoot isolation and rooting induction. All the samples from the same clone were thawed and analysed at the same time. The rooting percentage was assessed 8 weeks after induction. In all, 250 shootlets from the same production batch were cultured: 117 plantlets (9 plantlets × 13 samples) were used for activity measurements. The success rate was estimated after 8 weeks on the remaining plantlets, approximately 133.

For the study of peroxidase activity prior to induction treatment (Day −1), the shoots obtained in bunches during the caulogenesis stage were isolated and frozen at −196°C immediately after isolation, then stored at −80°C until analysis. A sample of nine shootlets was taken at random from 23 different batches belonging to 14 clones.

2.3. Extraction and measurement of peroxidase activity

The plant material (0.5 to 1.0 g_{FW}) was thawed, then crushed in melting ice using an Ultra-Turrax apparatus (24 000 rpm for 30 seconds) in the presence of 20 ml g_{FW}^{-1} of K_2HPO_4/KH_2PO_4 buffer (50 mM), pH 6.0. The homogenate obtained was centrifuged at 18 000 × g for 30 min. The supernatant was kept at 0°C; this was the crude enzyme extract (CEE). Total guaiacol-peroxidase activity was estimated by adapting Darimont and Gaspar (1972) method. This measurement was chosen due to similarities between guaiacol-oxidase and IAA-oxidase, as demonstrated by Pythoud and Buchala (1989). The incubation medium comprised: 50 μl of CEE, 450 μl of extraction buffer and 500 μl of the guaiacol-H_2O_2 mixture (17 g of guaiacol + 11.2 ml of 30% hydrogen peroxide for 1 l of buffer). Optimum incubation conditions were fixed by a prior study (results not shown). The optimum incubation pH was 6.0 (in a range of pH 4.5 to pH 8.5). Activity measurement was linear up to 20 mg ml_{CEE}^{-1} of total soluble proteins in the tube and for up to 10 min incubation at 30°C. The reaction was stopped by adding 500 μl of NaOH (1N) after 5 min. The optical density of the reaction medium was measured at 400 nm after performing an absorption spectrum. The total soluble proteins in the

CEE were measured by Bradford (1976) method modified by Sedmark and Grossberg (1977).

2.4. Expression of results

A standard calibration curve was obtained by incubating known quantities of purified horseradish peroxidases under the aforementioned conditions (Horseradish Peroxidase (HRP) Boehringer, Ref. No. 108090). Specific peroxidase activity was expressed in μg of HRP equivalent mg^{-1} of total soluble proteins, according to Moncousin and Gaspar (1983). Each value shown on graphs is the mean of three different measurements taken in triplicate.

3. Results

3.1. Effect of NAA level in the induction medium

Three exogenous auxin levels were tested on a clone revealing satisfactory (80–90%) rooting (clone TRL44): 0 mg l^{-1} (control), 1 and 2 mg l^{-1} of NAA for 24 h. The trend for specific peroxidase activity is shown in Fig. 1. It can clearly be seen that auxin treatments specifically caused a sharp increase in peroxidase activity in the oil palm plantlet. In fact, in the control plants (NAA = 0) there was no change in activity during the first 3 weeks following isolation (Fig. 1).

When induction took place with 1 mg l^{-1} of NAA (Fig. 1), specific peroxidase activity increased from 19 μg_{HRP} mg^{-1}_{prot} (on Day 0) to 103 μg_{HRP} mg^{-1}_{prot} (on Day +12), meaning that activity was multiplied by a factor of ca. 5. When induction was carried out with an NAA concentration of 2 mg l^{-1} (Fig. 1), the specific activity induction peak was also substantial (×13) for an equivalent activity level on Day 0, but it occurred later (Day +18).

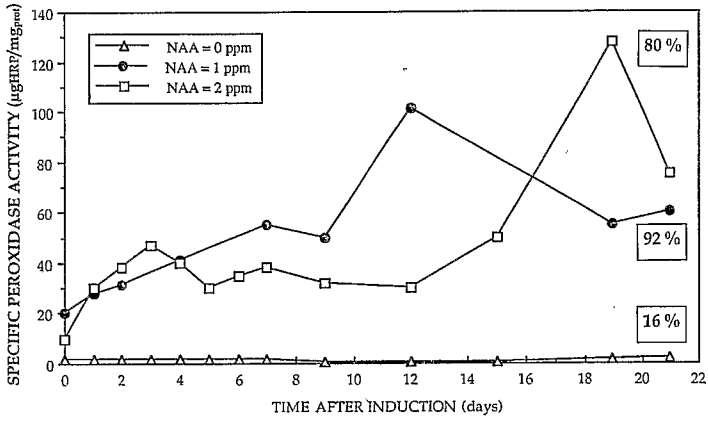

Fig. 1. Changes in peroxidase activity in oil palm shootlets in the presence of various concentrations of NAA (0, 1 or 2 mg l^{-1}) for clone TRL44. The 'time after induction' began when shootlets were transferred onto the induction medium. Incubation time in the presence of NAA was 24 h. The rooting percentage is given for each condition.

The rooting success rate measured after 8 weeks was 16% for the control, 92% for the induction treatment with 1 mg l^{-1} of NAA and 80% for the treatment with 2 mg l^{-1} of NAA.

3.2. Performance of two clones known for their opposite rooting responses

We studied two clones with very different responses to rooting treatment: clone TRL29 (little rooting, 20–30%) and clone TRL01, for which rooting was considerable (90–95%) in some batches under pilot-scale production conditions. The experimental protocol was identical to that described above. The induction treatment involved 1 mg l^{-1} of NAA for 24 h. Fig. 2 shows the results obtained during this experiment.

For clone TRL01 (Fig. 2), the same peroxidase activity trend was found as for TRL44 under the same conditions (Fig. 1); there was an activity peak after 12 days corresponding to around a three times increase compared to activity on Day 0. TRL01 also had an excellent rooting rate: 90%. As regards clone TRL29 (Fig. 2), the change in activity was very different. Activity on Day 0 was very high (200 μg_{HRP} mg^{-1}_{prot}). Moreover, the induction peak occurred earlier (Day +5) and showed a lower amplitude ($\times 1.5$ only). The peak activity was higher than for the other clones. A slight rise in activity seemed to occur on Day +19. For clone TRL29, the rooting success rate was mediocre: 29% after 8 weeks.

3.3. Relation between peroxidase activity prior to induction and rooting ability

We measured peroxidase activity prior to induction (Day −1) on 24 batches from 13 different clones. The rooting success rates were measured after 4 weeks of culture on an auxin-free expression medium.

The results are given in Table 1. These data revealed a substantial variability in peroxidase activity on Day −1 between the clones (from 30.6 to 250.0 μg_{HRP} mg^{-1}_{prot}),

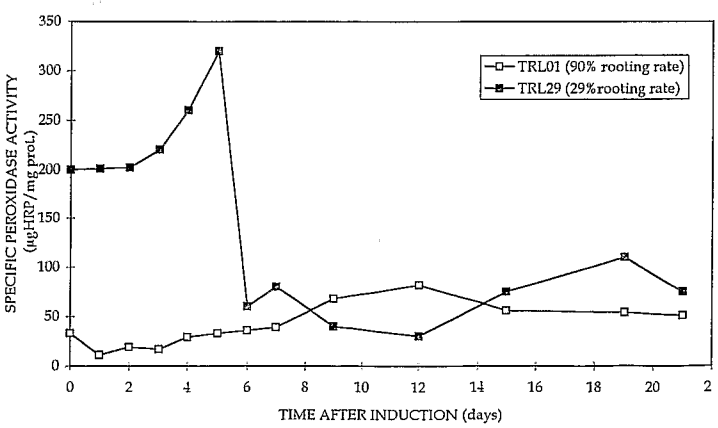

Fig. 2. Changes in peroxidase activity in oil palm shootlets in the presence of NAA (1 mg l^{-1}) for clones TRL01 (90% rooting rate) and TRL29 (29% rooting rate). Incubation time in the presence of NAA was 24 h.

Table 1
Peroxidase activity on Day −1 and rooting success rates measured on 24 oil palm in vitro production batches belonging to 13 different clones

Clone (TRL no.)	Production batch no.	Peroxidase activity[a]	Rooting %
1	158	53.4	45
6	97	75.6	56
6	98	89.5	66
14	188	68.1	6
16	160	250	27
16	161	179	3
16	162	51.6	85
22	305	59	90
46	72	91.1	80
52	224	51.3	70
60	14	100	80
68	142	44.7	30
68	143	155	36
68	154	61.8	60
69	289	88.9	43
69	294	120	13
69	296	68.4	6
70	44	69	66
70	45	51.9	76
70	48	103	80
70	54	147	40
73	48	74.1	58
73	50	38.6	73
80	107	30.6	73

[a]Specific activity: $\mu g_{HRP}\ mg^{-1}_{prot}$.

but also between different batches of the same clone (e.g., $160.2 \pm 82.0\ \mu g_{HRP}\ mg^{-1}_{prot}$ for clone TRL16). When an attempt was made to correlate peroxidase activity on Day 0

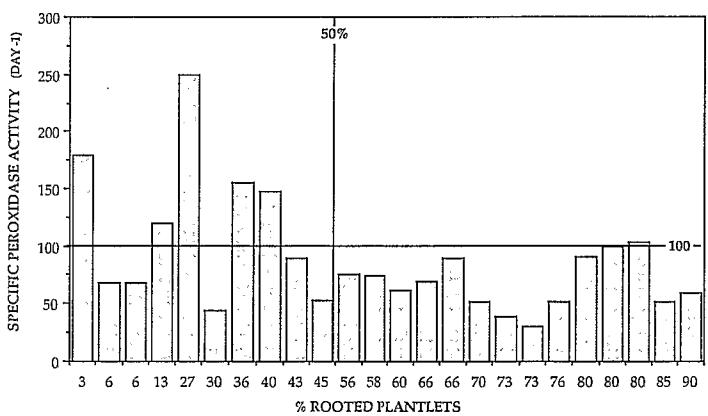

Fig. 3. Relation between specific peroxidase activity at the time of induction and in vitro rooting rates of oil palm shootlets (each bar is a single production batch).

Table 2
Contingency table showing the distribution of in vitro production batches of oil palm following rooting rate and peroxydase activity at the time of root induction

	Rooting rate < 50%	Rooting rate > 50%
Peroxidase activity > 100 μg_{HRP} mg_{prot}^{-1}	5	1
Peroxidase activity < 100 μg_{HRP} mg_{prot}^{-1}	5	13

Table 3
Relation between peroxidase activity on Day −1 and rooting performance for two different clones

Clone	Rooting rate	Peroxidase activity[a]
TRL44	87%	57.5 ± 10.8
TRL21	23%	82.4 ± 23.7

[a]Specific activity: μg_{HRP} mg_{prot}^{-1}.

to the rooting success rate for the 24 batches measured, a mediocre coefficient of correlation was obtained ($R^2 = 0.204$).

The graphs of the results (Fig. 3) revealed two distinct performances within the batches analysed. In fact, for batches with more than 50% rooting success, the peroxidase activities measured revealed values that were more clustered and virtually always lower than 100 μg_{HRP} mg_{prot}^{-1}. We drew up Table 2 to test the hypothesis that the different proportions were randomly distributed. A test of exact equality between two proportions (Dagnelie, 1975) revealed a highly significant difference between the two populations ($p = 0.026$). The distribution of the 24 samples between the four populations determined in this way was therefore not random.

3.4. Study of within-clone variability

In order to quantify the variability observed, 10 samples of 10 plantlets were taken on Day −1 (prior to induction) in two clones revealing different performances: TRL44 (satisfactory rooting) and TRL21 (mediocre rooting). Table 3 shows the results obtained. It thus appeared that the hypothesis put forward at the end of the previous experiment was not without its merits. Indeed, clone TRL44, which was characterized by uniform rooting, had a smaller standard deviation (10.8) than that measured for clone TRL21 (23.7). An analysis of variance revealed a significant difference ($\alpha = 0.010$) between the two clones studied.

4. Discussion

Changes in peroxidase activity measured after in vitro rooting induction in oil palm are consistent with the results previously described for various other species (see Gaspar et al., 1992, for a review).

An 'ideal' evolution curve could be drawn for specific peroxidase activity during root induction, as it reflects the behaviour of 'easy-to-root' clones. The curve, such as it was found for clones TRL44 and TRL01, showed an activity peak around the twelfth day after the auxin treatment, thus suggesting a 12-day initiation phase.

The induction intensity, for clones with satisfactory rooting, was 3 to 5 times the initial level of activity. The maximum specific activity value measured reached 300 $\mu g_{HRP}\ mg^{-1}_{prot}$, a comparable level to that encountered in various other species (Moncousin and Gaspar, 1983; Hausman, 1993; Jouve et al., 1994). It is interesting to note that, for a given clone, when the induction peak occurred earlier (Fig. 2) or later (Fig. 1) than the optimum (round Day +12), the rooting success rate was lower. Nevertheless, this response appeared to be clone-dependent, as a low rooting rate may be the consequence of an inadequate inductive treatment (clone TRL44) or of the recalcitrant nature of the considered clone (TRL29).

When the peak in activity occurs earlier than Day +12, root induction might have already occurred during the multiplication phase preceding isolation of single shootlets and rooting. If this hypothesis becomes justified after testing, any treatment aimed at decreasing peroxidase activity (transfer on an auxin-free or rutine-enriched medium) could probably improve the rooting rates, as it has been demonstrated for other species (Gaspar et al., 1992).

In a second set of experiments, peroxidase activity was studied just before root induction, to test its worth as an early marker of rooting ability. In clones recalcitrant to rooting, we discovered a significantly greater heterogeneity in activity on Day −1 than in clones with satisfactory rooting (Table 3). The small size of the sample (nine shootlets) may largely explain the heterogeneity found in our measurements. The recalcitrant clones contained probably a few individuals perfectly capable of rhizogenesis and with substantial activity on Day −1, alongside inapt populations within the same production batch. The size of the sample will have certainly to be re-estimated for future studies, to improve the accuracy of these measurements.

We observed substantial variability in peroxidase activity for induction-ready material, on three levels: between different clones, between production batches of the same clone, and between individuals within the same batch. This high heterogeneity must be linked to the very nature of the in vitro production process used (Duval et al., 1995). In fact, this batch process involves root induction on individuals with a different physiological background. The shoots were produced in bunches, in discontinuous flushes from polyembryonic cultures (Pannetier et al., 1981). In these cultures, somatic embryos developed asynchronously, all in a different nutritional, hormonal and gaseous environment. It is therefore clear that root initiation was applied on material with a highly variable physiological status. On clones with a low rooting success rate, our results suggested that rooting induction may have occurred naturally at the end of multiplication. In this case, the auxinic treatment would merely inhibit or delay rooting.

A novel process for oil palm micropropagation through embryogenic suspension has been set up in our group (de Touchet et al., 1991) which enables the synchronous production of single embryos at a large scale. This homogeneous plant material will be highly suitable for further studies on in vitro rooting of somatic-embryo derived oil palm plantlets.

The results obtained during this study suggest that the current rooting procedure should be modified for *E. guineensis* clonal plantlets.

Any improvement of the current protocol should be aimed at reducing the heterogeneity at the time of induction (Day −1), probably by introducing a 'buffer stage' between the isolation of shootlets and the inductive treatment, in order to better level and lower the peroxidase activity through the use of an auxin-free or rutine-enriched medium. In this case, the induction treatment (NAA 1 mg l^{-1}, 24 h) would remain the same.

One other proposal could be a procedure closer to the physiological events recorded: initiation over 10 to 15 days (using an NAA concentration lower than 1 mg l^{-1}), followed by an expression phase on a medium containing peroxidase inhibitors such as polyphenols (rutin) (Gaspar et al., 1977; Moncousin and Gaspar, 1983), or substances known to promote adventitious rooting, such as polyamines (Berthon et al., 1990; Hausman et al., 1994, 1995) and group D vitamins (Buchala and Schmidt, 1979).

5. Conclusion

This study is a preliminary step along the road to peroxidase predictive markers for use on *E. guineensis* Jacq. We have demonstrated that, for clones with high rooting rates, changes in peroxidase activity during in vitro rooting followed patterns similar to those already described for many other species. Furthermore, it appeared from this study that the current protocol used for shootlet production leads to very heterogeneous plant material, in terms of peroxidase activity at the time of induction. The highest was this heterogeneity, the lowest was the rooting rate. The in vitro rooting protocol thus needs to be amended, and various strategies have been proposed.

The present study needs to be completed by a full histological study on our plant material describing the chronology of anatomical events during the initiation/expression phases (Mitsuashi-Kato et al., 1978; Hicks, 1987).

Our preliminary results on the peroxidase marker will have to be tested on a wider range of clones in order to estimate and monitor its variability, and then assess its reliability under large scale production conditions.

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