Advances in tissue culture propagation of compact oil palm clones in Costa Rica

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After 20 years of research, ASD has developed a reliable protocol for cloning oil palm from inflorescence tissue, which has led to the planting of around 1,200 ha with compact clones since 2004, mainly in Central America.

Although the frequency of floral abnormalities observed in the field is very low (< 1%), a variable proportion of off-type plants showing erect leaves (juvenility character) and rather small bunches have shown up in some clones. However, it has been observed that any abnormal growth observed in vitro can be correlated with an undesirable behavior in the field, and these *ramets* can be discarded before leaving the laboratory. Shoot/root ratio can be used as an indicator of *ramet* performance during hardening, prenursery and nursery stages.

A morphometric characterization showed differences among six commercial compact clones (free of abnormalities), which can be grouped according to their vegetative traits and growth rate, both, in vitro and ex vitro. It was shown that *rametss* within a particular morphological group also had particular requirements of growth regulators and nutrients; particularly during the rooting stage, which resulted in high quality vitro-plants (pre-hardened ramets).

Key words: oil palm, clonal planting materials, morphological methods, in vitro off-type detection, micronutrients, growth regulators, auxins, interactions

Introduction

ASD´s research on oil palm cloning using inflorescences began in the early 1990s. In general, the cloning process using inflorescences or leaf tissue is about the same: formation of somatic embryos and its micro-propagation (proliferation including shoot differentiation) and rooting (Guzman 1995, Escobar et al. 2006). Nevertheless, there are some differences when using inflorescence tissue. Firstly, the *ortets* (tissue donor palms) do not suffer as much damage during sampling because inflorescence tissue is taken from fronds located quite distant from the growing point (apical meristem), making repeated sampling possible (every 6 to 8 months). Secondly, very little callus is produced from inflorescence tissue and direct embryogenesis is very common. Finally, low levels of growth regulators are required for induction of embryogenesis and *rhizogenesis*.

Nevertheless, some problems have been encountered in some clones in the field, such as a relatively high proportion of plants with an abnormal vegetative growth (erect habit) and rather small bunches. On the other hand, the rate of floral abnormalities, particularly mantled fruits, is very low and some clones seem to be free from this problem. So far, six compact clones with excellent agronomic characteristics (short leaves and stems, precocious and high yielding) have been selected for commercial production.

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The appearance of abnormalities in plantlets produced *in vitro* has been associated with stress-causing factors (Joyce et al. 2003) and morphological considerations for detecting of off-types *in vitro* have been used in some plants like banana (Grillo et al. 1998). Hence identifying stress causing factors and detecting off-types *in vitro* can be used for eliminating vegetative variations in the ex *vitro* phases in oil palm clonal material (nursery and field).

Our objective at ASD is to produce and commercialize uniform *ramets* with a low rate of abnormalities, which could also be managed ex vitro with a high rate of survival. This paper describes the work carried out for in vitro detection of vegetative off-types, the characterization of morphological differences between commercial clones and managing these differences through modifications in culture conditions in order to produce high quality, pre-hardened *ramets*.

Methodology

The studies were carried out at ASD's Tissue Culture in Coto, Costa Rica, where superior compact palms are routinely cloned from both male and female inflorescences. Shoots from stock cultures were rooted by modifying the standard protocol. Measurements on rooted shoots were taken two months after root induction.

Study of vegetative off-types: clones that had shown abnormal vegetative phenotypes in the nursery and the field were chosen for studying their behavior in vitro conditions (shoots and roots characteristics) (Table 1).

Characterization of commercial clones: plant height (from base to tip of longest leaf), number of leaves and roots, maximum root length and shoot, and root dry weight were recorderd. Leaves were also dissected to observe the shape of successive leaves in a shoot.

Study of culture conditions for production of pre-hardened ramets: a series of trials were carried out testing different levels of macro and micronutrients and growth regulators to observe the effect on root development.

Results and discussion

Detection of off-type *ramets* in vitro

Those clones that showed an abnormal vegetative growth (at the nursery or in the field), also showed distinctive characteristics in vitro, which separated them from those *ramets* that were normal *in vitro* and ex vitro (Table 1 and Figure 1).

Table 1. Morphological comparison between normal and abnormal *ramets*

	Normal *ramets*	Abnormal *ramets*
Shoot	-Successive leaves in a shoot differ in shape (gradual change of shape, hetero-phylly) -Lower (older) leaves are smaller than newly formed, expanded leaves -Soft and fibrous texture of leaves -Newly, expanded leaves with a prominent angle with re-spect to the vertical -Pale green new leaves	-Successive leaves in a shoot are very similar in shape (no heterophylly) -Older and new leaves attain similar size -Hard and plastic-like texture of leaves (sclerophylly) -Newly expanded leaves with little or no angle with respect the vertical -Dark green new leaves
Root	-4-6 roots, with a conspicuous inward curvature and long laterals	-4-10 erect, somewhat ligni-fied roots with a few short laterals

Fig. 1. Left. *Ramet* showing normal leaf development at the nursery stage. Right. Off-type *ra-met:* these plants also showed an abnormal growth in the field.

Normal *ramets* followed a typical development pattern, similar to that of seedlings at the nursery stage, by forming bifurcated leaves followed by pinnate ones, with newer leaves always longer than the older ones. On the contrary, the new leaves in off-type *ramets* had a low elongation rate and the production of bifurcated leaves was delayed. These growth patterns permit the early rejection of clones that will do poorly in the field.

<u>Characterization of commercial clones based on morphology</u>

A sample of six clones showing good agronomic behavior in the field was characterized according to several vegetative traits when still at the laboratory. These clones were separated into two groups based on internode length (estimated by the ratio between height increment and leaf production rate) and root growth rate (Table 2):

Type R+: clones with short internodes and vigorous root growth
Type S+: clones with long internodes and slow root growth

Values of some morphological ratios for the above clone types are given in Table 2.

Table 2. Separation of clones in two types according to some ratios in morphological characteristics

Ratios	Type S+	Type R+
Height/leaf number	50.0	38.6
Root dry weight/shoot dry weight (mg)	0.74	1.04
Root dry weight/total dry weight (mg)	0.40	0.54

Data are means of several trials with different clones for each type

Pre-hardened *ramets* presenting a good balance between root and shoot development are a viable option for transferring to ex vitro conditions or shipping abroad. The culling criteria differ according to the clone type as shown in Table 3.

Table 3. Culling criteria according to the clone type

	Type S+	Type R+
Shoot	-New leaves very elongated (partly folded in, cylinder-like), not fully expanded, showing etiolating-type symptoms	-Very small shoots with long roots
Root	-Poor root development	-High root/shoot ratio (>1.0)

The two clone types also showed different responses to environmental conditions in the laboratory. High light intensity favored the development of S+ type *ramets*, which under shade conditions (high density of flasks on the shelves for instance) suffered a further reduction in root development. On the contrary, high light conditions increased the root/shoot ratio of R+ type clones.

Several modifications of in-culture conditions were tried to improve the shoot and root development, and hence improve *ramet* quality.

Ramet quality of type S+ clones

Sugar deficiency could be involved in the formation of S+ type *ramets* since they tend to produce an etiolated leaf type and low root/shoot ratio, and increasing sugar level in the medium led to a rise in the root/shoot ratio, regardless of the clone type (Fig. 2). Increasing the sugar level from 60 to 75 g/l produced a higher proportion of optimum quality *ramets* from S+ type clones (Table 4).

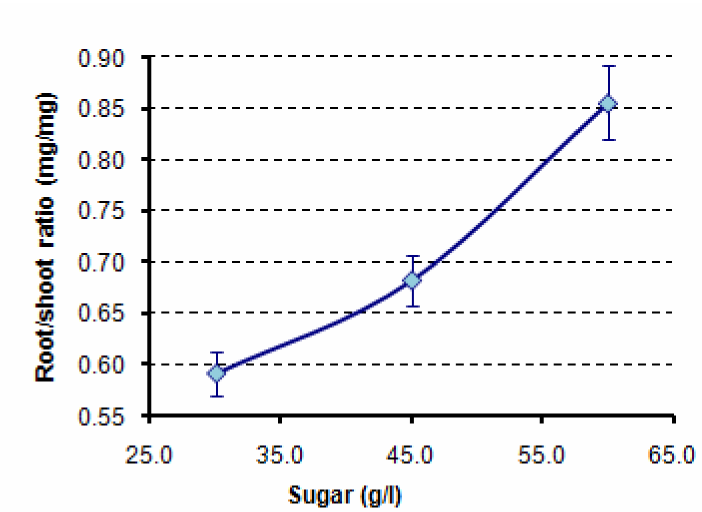

Fig. 2. Root/shoot ratio and sugar levels in the media

-24 replications with 6 *ramets* each
-Vertical bars represent mean standard error

Table 4. Percentage of optimum pre-hardened plantlets of Type S+ clones in media with two sugar levels

Trial	Sugar (g/l)	Pre-hardened plantlets (%)
1	60.0	66.7±11.1
	75.0	25.9±5.6
2	60.0	62.2 ±5.2
	75.0	82.2±6.2

Furthermore, an interaction between sugar and auxin levels was found in S+ type clones. The best quality *ramets* were obtained by increasing the sugar level and reducing auxin (naphthalene-acetic acid or NAA) (Fig. 3).

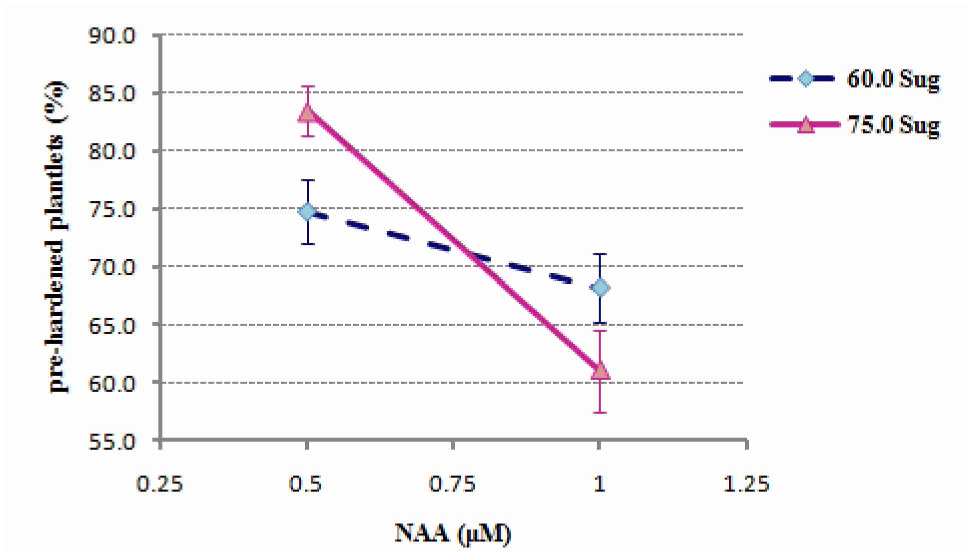

Fig. 3. Optimum quality pre-hardened plantlets of S+ type clones rooted in media with two levels of sugar and auxin (NAA).

The results indicate that increases in sugar level can, at least, partially replace light and auxin effects during development of *in vitro* plants, particularly for clones with higher shoot growth rate (S+ type clones). Similar results were observed by Ashburner et al. (1993) who also associated increases in sugar level in the culture medium with better root development in other palm species.

Ramet quality of R+ type clones

The quality, morphology and root/shoot balance of R+ type clones could be modified by changing the mineral and hormonal composition of the media. Auxin reduction to very low levels increased the quality of these *ramets*, but the effect also depended on the type and level of some micronutrients, as noted for in vitro plants by Kothari-Chajer et al. 2008. Although new media formulations used in oil palm have low copper and boron amounts (Nas and Read, 2004), these two elements are involved with the homeostasis of different hormones (Rodríguez et al. 1999; Goldbach and Wimmer 2007). Furthermore, the response to stress and the presence of abnormal morphologies have been associated with the hormonal homeostasis (King et al. 1999, Park et al. 2007).

Ramet quality improved as copper sulfate concentration increased at high auxin (NAA) levels, but low concentrations seemed more appropriate at low auxin levels (Fig. 4).

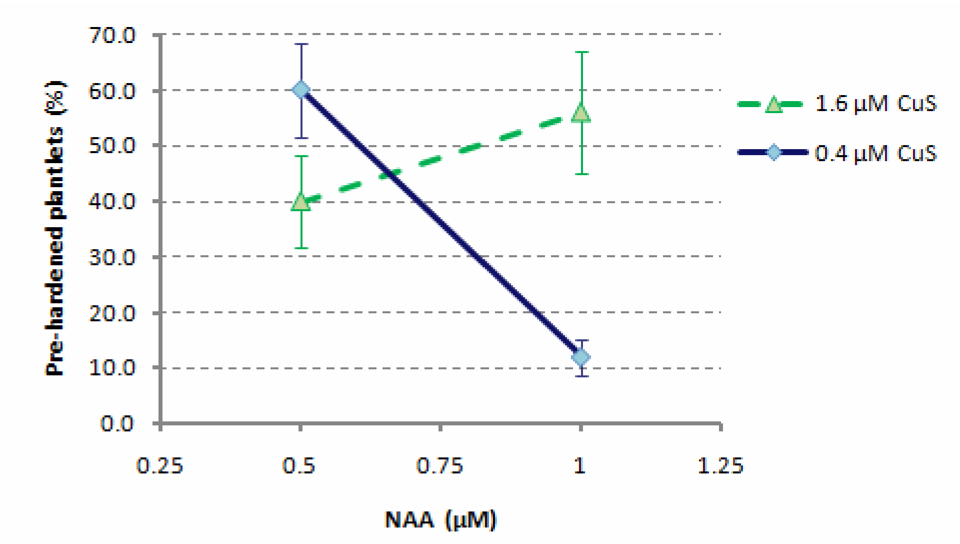

Fig. 4. Good quality pre-hardened plantlets (%) of a R+ clone rooted at two levels of naphthalene-acetic acid (NAA) and copper sulfate (CuS)

-Vertical bars represent standard error of the mean of 10 replicates (flasks), with 5 *ramets* each

A similar trend was observed between auxin and boron levels. With low levels of NAA, increasing boron levels had no effect on *ramet* quality. However, a boron increase had a positive effect on *ramet* quantity at high NAA levels (Fig. 5).

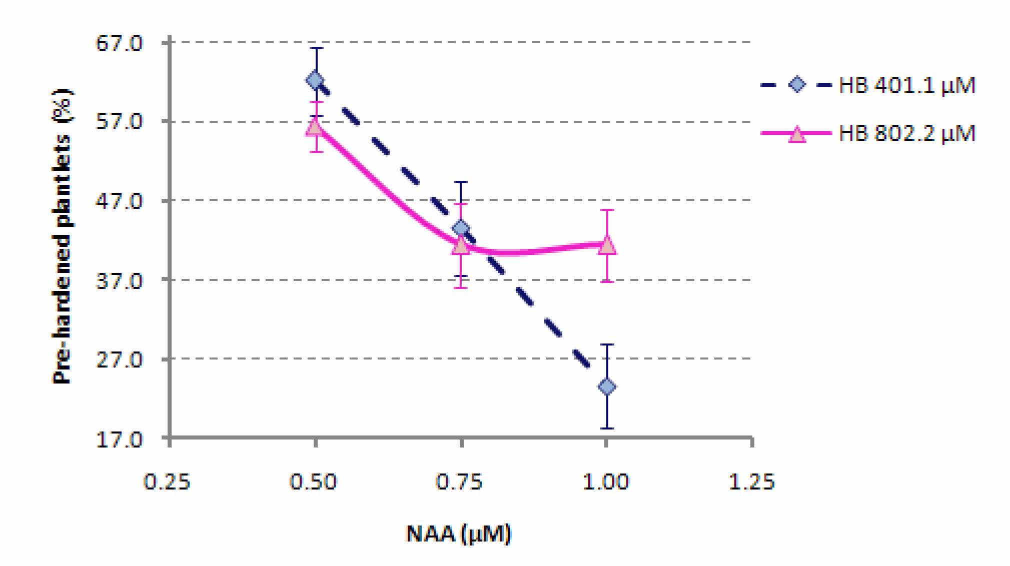

Fig. 5. Pre-hardened plantlets (%) of a R+ clone rooted at three levels of naphthalene-acetic acid (NAA) and two levels of boric acid (HB)
-Vertical bars represent standard error of the mean of 10 replicates (flasks), with 5 *ramets* each

Conclusions

Changes in morphology, particularly in leaf shape, permitted the early identification of vegetative off-types clones; hence, this parameter can well be used for culling abnormal *ramets* in vitro.

The compact clones studied could be grouped according to the balance between root and shoot growth (S+: higher shoot growth rate and R+: higher root growth rate).

Clones S+ and R+ showed opposite response to light intensity. High light intensity favored the development of S+ type *ramets*.

The best quality S+ type *ramets* were obtained by increasing sugar levels and reducing auxin (naphthalene-acetic acid) in the culture media.

Although very low levels of growth regulators are routinely used during clone production at ASD's laboratory, the quality of R+ clones can be increased by further reducing auxins or by changing the micronutrient composition in the rooting medium. A higher copper concentration was required when the auxin concentration was increased, and a similar trend was observed in the case of boron and auxin.

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