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Leaf Maturity, a Critical Factor in Embryogenesis

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Summary

The embryogenic capacity of leaf explants was found to be related to their physiological maturity in young palms of *Cocos nucifera*. Leaf tissues from 12- to 24-month-old palms were embryogenic but the potential was quickly lost with the onset of juvenility. Even in young palms, explants of tender leaves responded differently according to their maturity. Only a particular leaf in a particular state produces embryogenic cells: the leaf whose growth was observed to increase by about 13 times its initial size in 1 month. It may be concluded that a crucial factor(s) determining embryogenesis *in vitro* is elaborated at a certain physiological stage of maturity of one particular leaf in the crown of a young palm. Furthermore, only a portion of this leaf yields embryogenic explants and its competence is of short duration.

Key words: Cocos nucifera, leaf explants, embryogenic potential, growth rate, juvenility, leaf maturity.

Introduction

The vegetative propagation of coconut has been attempted using leaf, flower and zygotic embryo explants (Raju et al., 1984; Verdeil et al., 1989; Branton and Blake, 1983; Karunaratne and Periyapperuma, 1989). Leaf explants from young palms have responded to *in vitro* techniques better than those from older plants (Verdeil et al., 1989); leaves from adult palms have never responded. The immature embryo has proven to be better than the mature zygotic embryo (Karunaratne and Periyapperuma, 1989). However, in all these cases consistent plant regeneration is still awaited.

In many perennial plants, the capacity to propagate through tissue culture is shown to be closely associated with explant juvenility (Bonga, 1982). In coconut, in addition to palm maturity, the physiological maturity of the explant was also held to be critical (Verdeil et al., 1989; Pannetier and Morel, 1982; Raju et al., 1984). However, an experimental analysis of the concepts of palm and explant maturity is not an easy task since the coconut leaf and the inflorescence yield a variety of explants and the palm itself, at any given period, possesses a number of tender leaves and inflorescences in different stages of development.

In the present study we related leaf maturity to embryogenic capacity, using materials from young coconut palms to determine the most suitable developmental stage for explanting.

Materials

Plants of *Cocos nucifera* L. of the variety known as Sri Lanka tall were used.

Foliar development in the crown of the coconut palm originates in the apical bud and the tender leaves derived from it (Fig. 1 C). On an average, one leaf emerges every calendar month. At any time, therefore, the crown will exhibit a number of these emerging leaves in a succession of developmental stages. The emerging leaves are called spear leaves because of their characteristically furled appearance; they unfurl progressively to become the adult fronds of the palm. The youngest spear leaf just emerging from inside the crown is called S (Fig. 1 A, B), the next oldest S+1 etc. Similarly, the leaf immediately younger than S, within the cabbage, is S−1; it becomes S the next month.

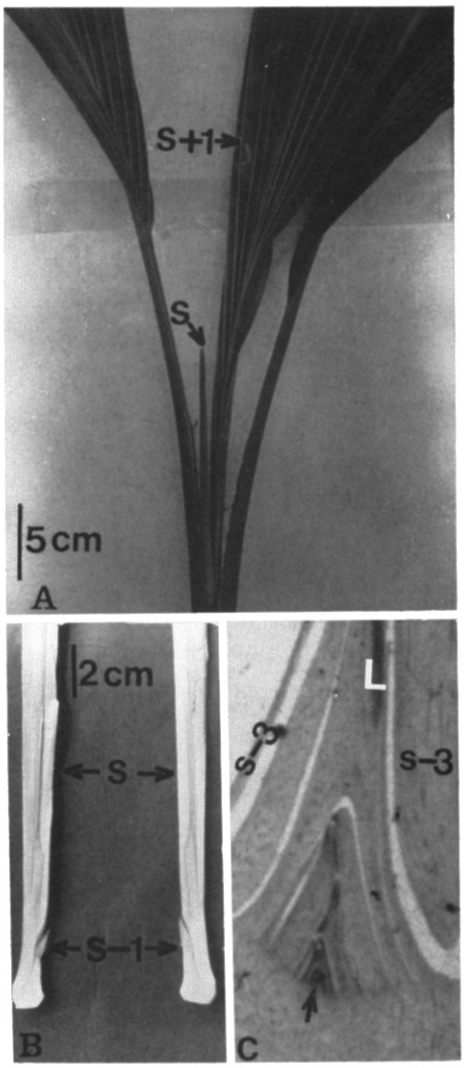

Fig. 1: Leaf emergence in coconut. A) Part of a 12-month-old coconut seedling showing the emergence of an S leaf. B) The apical bud and S leaf of (A) split and laid open to show the minute S–1 leaf halves. C) Vertical section of a cabbage showing S–3 and younger leaves. Each leaf appears as a cone. The central arrow indicates a structure consisting of the S–9 cone covering smaller leaf primordia and the apical dome (×7). S, just emerging spear leaf; S+1, leaf older than S; S–1, S–2, S–3 etc., leaves younger than S; L, developing leaflets.

Methods

Physiological stage, as denoted in the present report, covers two different concepts: the maturity of the whole palm, usually corresponding to its age, and that of the individual tissue or organ, indicated in the case of a leaf by its position or order of emergence.

Growth rate of emerging leaves

Physiological maturity was presumed to be reflected in the growth rate of leaves, whose lengths were measured. The sampling was thus destructive since most leaves of interest were buried in the cabbage. Therefore, from each of the age groups chosen (sprouting «beak» stage, 6, 12, 24, 36 and 60 months), 12–16 crowns were dissected and the lengths of leaves from S–3 to S+1 were measured. Growth rates were then calculated from the average lengths of leaves of a particular stage (e.g. S–2) and those of the next one (e.g. S–1) and expressed as cm/month.

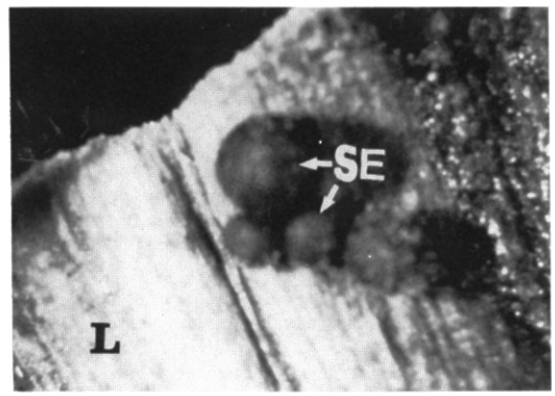

Fig. 2: Somatic embryogenesis from coconut leaf tissue. L, leaf explant; SE, somatic embryos.

Assay of embryogenesis

The cabbage was disinfected by washing in 2% calcium hypochlorite for 10 min and rinsing 3 times in sterile water. About 2 mm wide leaf segments were excised from the leaflets and placed in the culture medium (Karunaratne and Periyapperuma, 1989) solidified with 0.7% agar, which has been found to induce embryogenesis in leaf explants upon incubation in the dark at 25–27 °C (Fig. 2). The minimum number of replicates per treatment was five and each experiment was repeated three times. The number of leaf explants producing somatic embryoids was counted and expressed as a percent of the total number of explants per treatment.

Results

Embryogenic response of leaf explants from palms of different ages

Tender leaf tissues within a length of about 20 cm from above the shoot apex (Fig. 1 B, C) were excised from plants of different ages and cultured as described. Explants producing somatic embryoids were recorded after 4–6 months in culture (Table 1). Coconut sprouts (at the beak stage) possess only the coleoptile, a few sheaths and the first two emerging leaves; explants from them quickly turned brown in culture. Embryogenesis was low and inconsistent from 6-month-old material, which was also a poor source of explants. Leaves from 12- to 24-month-old plants were most embryogenic when they were about 10–20 cm long. The embryogenic potential diminished as the palm matured and it was about 30% from 36-month-old plants. Explants from 60-month-old palms were totally unresponsive *in vitro*.

Table 1: Embryogenesis from leaves of different stages of physiological maturity.

Leaf length* (cm)	Embryogenesis (%)				
Plant age (months)	<5.0	5–8	10–20	25–35	40
<6	No leaf explants				
6	0	Sporadic	Sporadic	No leaf explants	
12	0	Sporadic	59	33	0
24	0	Sporadic	55	30	0
36	0	Sporadic	30	0	0
60	0	0	0	0	0

* S–1 leaf in transition to S leaf. Material: Sri Lanka tall coconut. Only basal portions were cultured from leaves longer than 15 cm. Embryogenesis is expressed as a percent of the total explants cultured. Results are averages of three experiments.

Table 2: Embryogenic response from leaf segments of excisable leaves of Sri Lanka tall coconut.

Mean leaf length* (cm)	Embryogenesis (%)					
Distance from leaf base (cm)	3	6.5	10	18	30	40
1 (basal)	0	0	100	87	33	0
2	0	SP	78	90	60	0
3	NA	SP	60	33	57	0
4	NA	SP	66	50	50	0
5	NA	0	50	20	0	0
6 (apical)	NA	NA	0	33	0	0

* S–1 leaf in transition to S leaf. Material: 12-month old Sri Lanka tall coconut. Embryogenesis is expressed as percent of the total explants cultured. Results are averages of three experiments. SP = sporadic embryogenesis. NA = leaf explants not available.

Embryogenic response from leaves of different stages of development

The yellowish white leaves of the cabbage are structurally developed in that they possess a leaf base, rachis and leaflets, although fused and compactly packed. Leaf explants from S–6 to the S-stage and transitional stages from S–1 to S were cultured. It was not possible to obtain all the transitional stages from a single plant of a given age and leaves were thus removed from a group of plants of approximately the same age. The stage of development of the leaf was denoted by its length (Table 1) since the gradation could be indicated more precisely than by the series of S stages.

Irrespective of plant age, all explants derived from the rudimentary leaves expanded in culture with no differentiation. Later they turned brown and died. Explants from the yellowish white leaves were embryogenic but this response varied with individual leaf maturity, regardless of the age of the palm tested (Table 1). Generally, embryogenesis was sporadic from very young leaves up to 8 cm in length. It rose to about 50% in explants from 10- to 20-cm long leaves of 12- and 24-month-old seedlings and declined, as the leaf matured, to 30% when leaves were 25–35 cm long, terminating in no embryogenic response at all from leaves of 40 cm. The same morphogenetic pattern was observed in leaves derived from 36-month-old plants but the potential was comparatively low even from 10 to 20-cm long leaves and it was quickly lost as the leaf aged. None of the leaves tested had any embryogenic potential in 60-month-old palms.

A finer localization showed that embryogenic activity was highest at the leaf base, diminishing towards the apex (Table 2). Thus, explants from the basal two centimeters of 10 to 18-cm long leaves gave as much as 80 to 100% embryogenesis.

Growth rate of emerging leaves

In all four age groups of plants tested the S–1 leaf grows about 13 times its original length in about 28 days, enabling it to emerge the following month (Fig. 3). The S–2 leaf, which is still smaller, grows only 3–4 times its original length in 1 month to qualify as the next S–1 leaf and then it goes through a month's rapid growth phase in order to emerge from the crown as a S leaf. The same developmental trend may be expected from all leaves in their course from S–2 to S. In other words, each developing leaf remains dormant until its turn comes for emergence and out of some 24 developing leaves and leaf primordia in the bud, only one leaf evolving from S–1 to S singularizes itself by growing fast at a certain period of time within a given month. This event is repeated monthly by every subsequent young S leaf but restricted to the time when it is S–1.

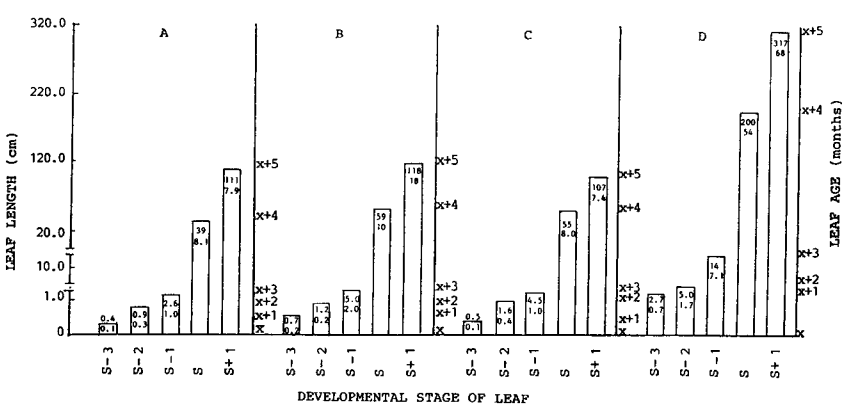

Fig. 3: Growth rate of explantable leaves. A–D, young coconut palms of ages 12, 24, 36 and 60 months, respectively. S, just emerging spear leaf; S–1 to S–4, successively younger leaves; S+1, leaf immediately older than the S leaf (please see materials). The age of the S–4 leaf is set as X (months). The upper figure on each block is the average leaf length and the lower figure the standard deviation.

Discussion

It is clear from our results, which agree with those of previous authors (Pannetier and Morel, 1982; Raju et al., 1984), that leaf explants from young palms are the most embryogenic. With adult coconut tissues Verdeil et al. (1989) obtained only callus and no apparent embryogenesis. In general, adult palm tissues are difficult to cultivate (Reynolds and Murashige, 1979) while older tissues and organs of the date palm, although yielding callus, have limited morphogenetic potential (Tisserat, 1984).

The loss of response that we have noted in old palms (36–60 months) may coincide with the transition from juvenility to flowering. In the variety of coconut we used, the inflorescence emerges 60–72 months after germination, about 24 months from flower inception. Juvenility, as commonly defined (Salisbury and Ross, 1978; Schwabe, 1971), could therefore last about 36 months before the physiological changes that govern flowering occur and that may be responsible for the decline in *in vitro* performance of leaf explants, dropping to a total absence of embryogenesis by 60 months. Indeed many dicotyledonous perennials can be propagated from juvenile material in contrast to tissues of mature donors that have to be rejuvenated prior to explanting by techniques inapplicable to coconut, like girdling, pruning and grafting (Bonga, 1982; David, 1982).

Table 1 further shows that in addition to palm maturity, leaf maturity also determines morphogenetic potential in all age groups of young plants tested. The most embryogenic material was explanted from 10 to 20-cm long leaves of 12- to 24-month-old plants. Rudimentary leaves and a few inner, yellowish-white ones, which immediately derive from the coconut shoot-tip meristem, were embryogenically inactive, both in seedlings and young palms. This situation is difficult to understand. Lu and Vasil (1981) reported that in panicum, the 3rd and 4th leaves from the shoot apex were the most morphogenetic while very young incompletely differentiated leaves did not respond. In coconut, however, some of the structurally developed leaves also did not respond. In those that did respond, morphogenetic capacity was highest at the leaf base (Table 2). This is expected as the leaf grows from the base, creating a gradient in tissue maturation from base to apex. A similar localization has been reported in panicum and sorghum leaf cultures (Lu and Vasil, 1981; Wernicke and Brettell, 1980).

Our experiments show that the ideal leaf to be explanted for optimum embryogenesis has an average length of 15 cm (Tables 1 and 2). Fig. 3 shows this leaf to be in transition from S–1 to S. The just emerging S leaf (the spear) is too mature and the S–1 leaf is too small to be excised. Furthermore, because of the arrested growth of young leaves (S–2 and younger), a continuous gradient in the developmental maturity of leaves does not exist in a given plant and there are no leaves of intermediate embryogenic competence to be excised. In other words, at certain times of the month (as when S–1 is too young and S too mature), although several tender «leaves» of various developmental stages are buried in the cabbage, none will be embryogenic. Thus, the only stage at which an embryogenically active leaf could be excised is during the course of development of an S–1 leaf into S, but before it reaches the latter.

The rapid growth from S–1 to S, while the leaf is still within the palm-heart, may be considered to reflect a profound physiological transition involving active cell division rather than elongation because the length increases to about 13 times that of the original in 1 month (Fig. 3). In contrast, the growth of the next older leaf developing from S to S+1, although by more than 100 cm in 1 month, amounts to only a doubling of its original length and appears to result mainly from cell elongation.

Leaf excision at a chosen developmental stage is difficult unless the crown is dissected. Growth of the S–1 leaf, totally buried until the S stage, cannot be monitored by direct methods. Thus, a number of plants may have to be sacrificed before one comes across an optimally morphogenetic leaf for explanting.

It should be noted that in all plants, the S–1 leaf, the lower half of the S leaf and the bottom leaflets of S+1 (depending on palm age) are so tender in comparion to other species, one is inclined to culture them. However, they are not truly embryogenic and that explains the frequent failure of coconut leaf culture. Besides, when attempting to reproduce results, it is essential to correctly assess the developmental stage of the leaf used in previous work. Misjudgement is all the more likely when leaves are removed without dissecting the crown in order to save the palm (Raju et al., 1984; Verdeil et al., 1989).

Our findings show that, in addition to juvenility of the palm, the developmental stage of a leaf strongly determines the embryogenic capacity of the explant. They provide a basis for selecting the morphogenetic window of leaf tissues opening out to consistent plant regeneration of coconut *in vitro*.

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