Micropropagation of *Cocos nucifera* L. from plumular tissue excised from mature zygotic embryos

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Abstract

Micropropagation of coconut depends initially on the induction of embryogenic calloid. This paper describes an improved procedure for obtaining such calloid using plumular tissue excised from mature zygotic embryos of Cocos nucifera *L. Embryogenic calloid production was assessed after pulse and continuous exposure of the tissue to varying amounts of 2,4-D. Plumular tissue gave calloid consistently in over 75% of the cultures which were exposed to media containing 4 x 10^{4} M 2,4-D. None of the remaining parts of the embryo produced calloid after removal of the plumule. After 12 months the embryogenic calloid produced clonal plantlets.*

Résumé

La micropropagation du cocotier dépend tout d'abord de l'induction de «calloïdes» embryogènes. Cet article présente un protocole d'obtention de ce type de calloïdes à partir de tissus plumulaires provenant d'embryons zygotiques matures de Cocos nucifera *L. La formation de calloïdes embryogènes est évaluée après l'exposition ponctuelle ou continue des tissus à des quantités variables de 2,4-D. Sur des milieux ayant une teneur en 2,4-D de 4 x 10^{4} M, plus de 75% des cultures de tissus plumulaires donnent systématiquement des calloïdes. A l'inverse aucune partie du tissu embryonnaire subsistant après prélèvement de la plumule, ne produit ce type de structure. Au bout de 12 mois, les calloïdes embryogènes produisent des vitroplants.*

Resumen

La micropropagación del cocotero depende en primer lugar de la inducción de «calloides» embriógenos. Este articulo presenta un protocolo de oblención de este tipo de calloides a partir de tejidos plumulares procedentes de embriones zigóticos maduros de Cocos nucifera *L. Se estima la formación de calloides embriógenos después de la exposición puntual o continua de los tejidos a cantidades variables de 2,4-D. En medios que tenien un contenido de 2,4-D igual a 4 x 10^{4} M, más del 75% de los cultivos de tejidos plumulares dan sistemáticamente calloides. A la inversa ninguna parte del tejido embrionar que quede después de la eliminación de la plúmula, produce este tipo de estructura. Al cabo de 12 meses, los calloides embriógenos producen plántulas clonales.*

The coconut palm is widely grown in many tropical areas. Propagation is currently from seed, producing a population of heterogenous plants whose yield is unknown until maturity is reached (5-7 years). There would be considerable value in establishing a system for clonal propagation to ensure that only elite palms are cultivated.

Tissues of coconut are recalcitrant *in vitro* and only limited success has been reported in inducing somatic embryogenesis in cultures which have been derived from a range of tissues including immature inflorescences, young leaf bases, immature and mature zygotic embryos (Verdeil *et al.*, 1993). Development of the resulting calloid to form somatic embryos and their subsequent germination to produce clonal plantlets has been erratic (Blake, 1990). The term calloid is used throughout this paper and is defined as a partly dedifferentiated callus (Brackpool *et al.*, 1986). There is no random cell proliferation as in most dicotyledons, but considerable organisation is retained which eventually leads to the production of somatic embryos (see figures 1B,C in Blake and Hornung, 1994).

Plumular tissue excised from mature zygotic embryos has been investigated as an alternative source of embryogenic calloid, from which clonal plantlets have been cultured.

Materials and methods

Nuts containing embryos, 9-11 months after pollination, were collected from palms of *Cocos nucifera* L. in the Philippines and in Jamaica and Mexico.

Cores of endosperm, containing mature zygotic embryos were extracted in the country of origin and transported by air to the U.K., where the following procedures were carried out aseptically. The cores were sterilised in 37.5% commercial bleach (Brobat, Jeyes U.K., 4.6% NaClO) for 10 min followed by three rinses with sterile water. Embryos were removed from the endosperm and sterilised in 10% commercial bleach for 5 min, followed by three rinses with sterile water. The embryos were then pretreated for 7 days in liquid MS-medium (Murashige and Skoog, 1962) supplemented with 2.5 g l^{-1} AC (Sigma) and 20 g l^{-1} sucrose. Using a dissecting microscope, the plumules were excised (plate, 1, 2) and cultured in glass tubes (7.5 x 2.5 cm) containing 10 ml of gelled MS-medium with 40 g l^{-1} sucrose, 10^{-6} M BAP and 2,4-D as specified for each experiment. The pH was adjusted to 5.8 before 2.5 g l^{-1} AC and 3 g l^{-1} Gelrite (Schweizerhall, U.K.) were added. The media were dispensed into the culture tubes and autoclaved for 20 min Unless otherwise stated, the cultures were maintained at 29°±1°C in darkness, and

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Abbreviations:
BAP = 6-benzylaminopurine;
AC = activated charcoal, acid-washed;
2,4-D = 2,4-dichlorophenoxyacetic acid.

the explants were transferred onto fresh medium after 6 weeks and then at 4-weekly intervals.

Calloid was isolated from the plumular explants after 10 weeks of incubation, and cultured separately on fresh medium.

Results

Early experiments in which mature zygotic embryos were cultured on medium with 2,4-D demonstrated the production of a compact, yellowish nodular calloid which appeared to arise from the region of the root pole. The yield of calloid was low, not exceeding 5%. Tests were therefore carried out in which the various parts of the embryo were isolated and cultured separately. It was shown that only the tissue of the plumule was able to produce embryogenic calloid. The plumule cultured *in situ* after the embryo was split open became necrotic, whereas fragments from the excised plumule gave rise to embryogenic calloid. When the outer leaves were dissected from a plumule they could be successfully cultured to give embryogenic calloid, while the remaining apical structure yielded a plant following culture on plant growth regulator-free medium.

Pulse treatment of plumular explants with 2,4-D

Plumular tissue of the cv. Laguna Tall was placed onto gelled media containing 2,4-D at 5 x or 8 x 10^{-4} M and 1 x, 2 x or 5 x 10^{-3} M for 48 or 96 hours. Each treatment consisted of 15 replicate tubes each containing plumular explants from a complete plumule excised from a mature zygotic embryo. After further culturing on media without auxin or cytokinin, the production of embryogenic calloid was assessed.

Table 1 shows that after a 48h-treatment only 2 x 10^{-3} M resulted in calloid formation. With the treatments lasting for 96h, the 2,4-D concentrations of 10^{-3} M and higher gave some calloid, and yields of 13% were recorded. There was considerable expansion of tissues and shoot development of some embryos in all treatments. Some plumules died at the higher levels of 2,4-D, particularly in the 96h treatment.

A disadvantage of this pulse procedure was that calloid usually became brown after approximately 15 weeks and ceased growth. An alternative technique was therefore adopted to provide a continuous 2,4-D supply.

Treatment of plumular tissue with various concentrations of 2,4-D

Forty replicate tubes were used for each treatment, with each tube containing explants from a complete plumule excised from a mature zygotic embryo cv. Laguna Tall. After 3 weeks of incubation on a range of 2,4-D concentrations from 2 x 10^{-4} M to 2 x 10^{-3} M, the first calloid was noted and after 6 weeks (plate, 3), 86% of the final yield of calloid had already been initiated. The frequency of embryogenic calloid production was assessed after 8 weeks and is shown in table 2. In addition to the induction of embryogenic calloid, there was also expansion and in some cases extension of the plumular leaves to produce a shoot.

As the 2,4-D concentration was increased, shoot production from plumules was suppressed in reverse proportion to the rate of calloid production. Over 70 % of cultures produced calloid at 4 x and 6 x 10^{-4} M 2,4-D. These concentrations were optimal as additional increments resulted in decreased calloid formation.

The results in table 2 were confirmed in three further experiments using plumules from the cv. Malayan Dwarf from Jamaica and Mexico. The first experiment was carried out using the cytokinin BAP at 10^{-6} M, which is the normal procedure with *in vitro* culture of coconut, but the work was also repeated omitting this regulator. The presence or absence of BAP failed to produce recognizable differences during the 15 months duration of the investigation.

Further development of plumular calloid into plantlets

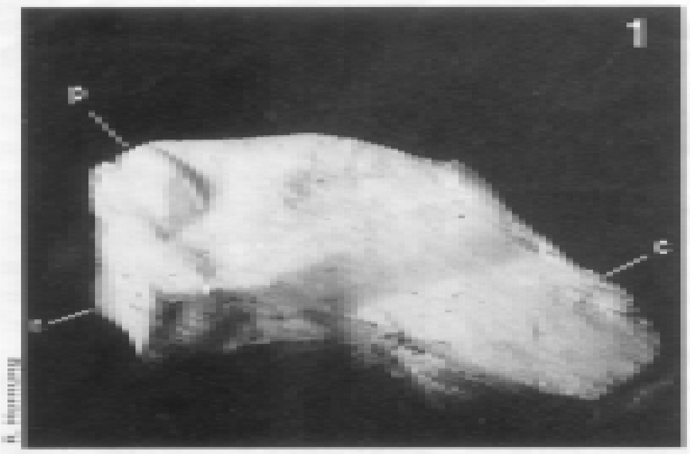
Calloid, formed on plumular tissue which had been subjected to continuous treatments of 4 x and 6 x 10^{-4} M 2,4-D, was separated from the original explant after 10 weeks and cultured for an additional 6 weeks on fresh medium. When embryo-like structures developed (plate, 4), the explants were transferred for 6 weeks to a 3 g l^{-1} Gelrite medium containing 2 x 10^{-4} M 2,4-D, 10^{-6} M BAP, 15 g l^{-1} mannitol and 2.5 g l^{-1} AC. At the end of this 6-week period the tissue was

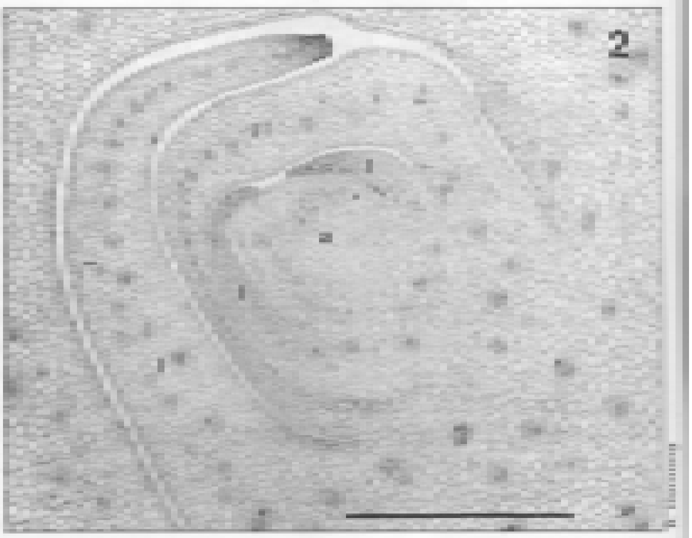
Table 1. Effect of 2,4-D pulse-treatment for 48 and 96 h on plumular explants (cv Laguna Tall). Percentages after 8 weeks of culture on plant growth regulator-free medium (n=15). / *Effet du 2,4-D sur les explants plumulaires (cv Grand Laguna) lors des traitements ponctuels de 48 et de 96 h. Pourcentages après 8 semaines de culture sur un milieu sans régulateurs de croissance (n=15).*

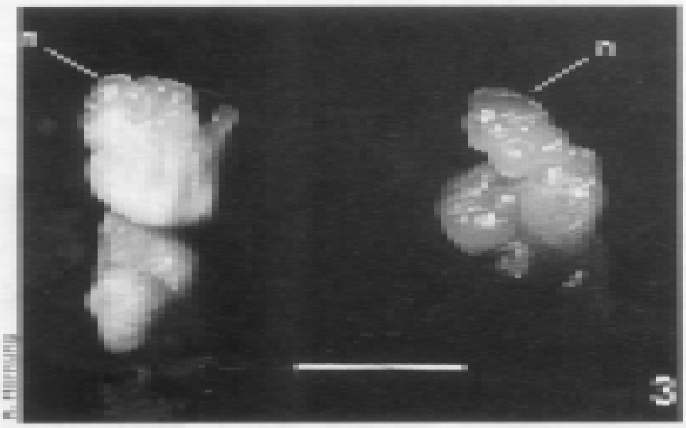
	48 h pulse-treatment / *Traitement ponctuel de 48 h*					96 h pulse-treatment / *Traitement ponctuel de 96 h*				
	10^{-4} M 2,4-D		10^{-3} M 2,4-D			10^{-4} M 2,4-D		10^{-3} M 2,4-D		
	5 x	8 x	1 x	2 x	5 x	5 x	8 x	1 x	2 x	5 x
Calloid / *Calloïde*	0	0	0	13	0	0	0	13	13	7
Shoot / *Pousse feuillée*	20	20	13	7	13	20	7	13	0	0
Expansion / *Gonflement*	80	80	87	67	54	80	66	61	67	66
Dead / *Mort*	0	0	0	13	13	0	27	13	20	27

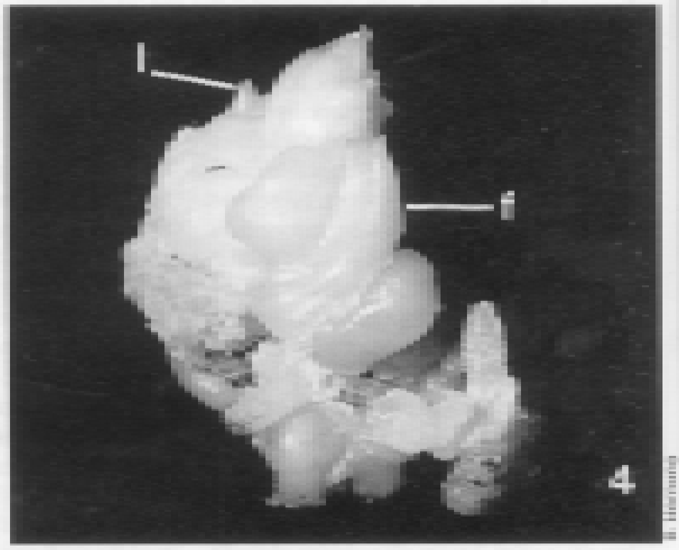
Table 2. Effect of 2,4-D treatments on plumular explants (cv Laguna Tall). Percentages after 8 weeks of culture (n=40). / *Effet des traitements au 2,4-D sur les explants plumulaires (cv Grand Laguna). Pourcentages après 8 semaines de culture (n=40).*

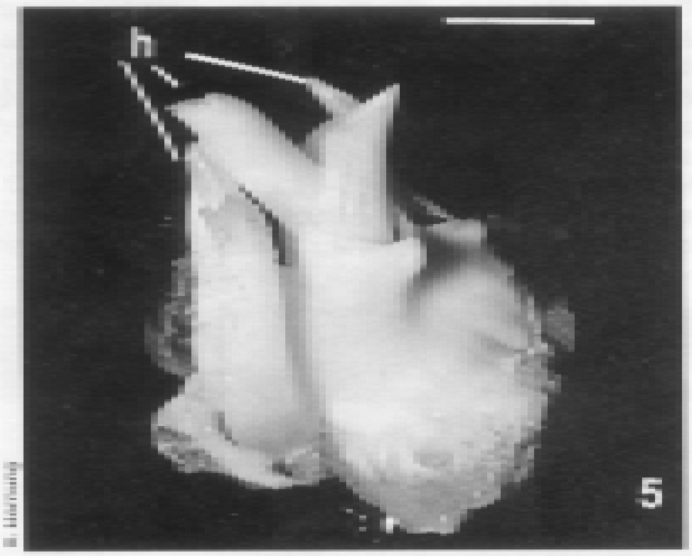
	10^{-4} M 2,4-D					10^{-3} M 2,4-D	
	2 x	3 x	4 x	6 x	8 x	1 x	2 x
Calloid / *Calloïde*	0	15	77.5	75	27.5	7.5	2.5
Shoot / *Pousse feuillée*	65	45	5	0	0	2.5	0
Expansion / *Gonflement*	35	40	17.5	25	25	17.5	22.5
Dead / *Mort*	0	0	0	0	47.5	72.5	75

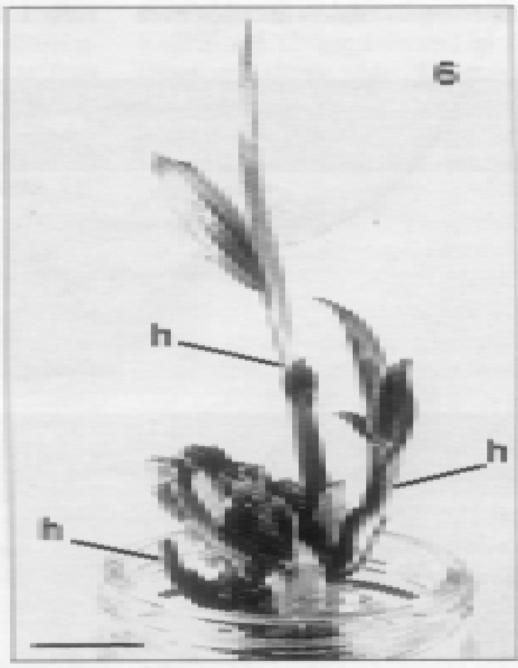












1. Mature zygotic embryo split open after pre-treatment, c = cotyledon; p = plumule; r = position of root. / *Embryon zygotique mature ouvert après prétraitement, c = cotylédon, p = plumule, r = emplacement de la racine.*
2. Section of plumule at excision, a = apex; l = plumular leaves. / *Coupe transversale de la plumule au moment de l'excision, a = apex, l = feuilles plumulaires.*
3. Nodular calloid (n) initiated on plumular tissue after 6 weeks. / *Calloïde nodulaire (n) sur tissu plumulaire après 6 semaines.*
4. Leafy structures (l) and fused somatic embryos (f). / *Structures feuillées (l) et embryons somatiques fusionnés (f).*
5. Germination of somatic embryos, h = shoot. / *Germination d'embryons somatiques, h = pousse.*
6. Some of 7 clonal plants derived from one plumule, h = shoot. Bar represents 5 mm except for plate 2, Bar = 0.25 mm. / *Quelques-uns des 7 vitroplants obtenus à partir d'une seule plumule, h = pousse. La barre représente 5 mm sauf pour la planche 2, barre = 0,25 mm.*

transferred to half strength MS-medium supplemented with 20 g l^{-1} mannitol, 20 g l^{-1} sucrose and 2.5 g l^{-1} AC in glass tubes (20 x 3 cm) containing 40 ml of medium for 12 weeks. Several shoots developed (plate, 5) and 8 weeks later the first roots emerged without any further treatment. The re-sulting clonal plantlets (plate, 6) are the first to be described which have been produced *in vitro* from coconut plumular tissue.

Discussion

Calloid production from immature inflorescence has not been reliable (Blake and Hornung, 1994) for reasons which have begun to be understood but not yet resolved. Progress with an alternative approach for producing embryogenic calloid using a different tissue, plumules, which is both high yielding and reproducible, has been developed here and is reported.

Early experiments in these laboratories with immature zygotic embryos were partially successful, but unsuccessful with mature embryos (unpubd. data). How-ever, separation of the mature zygotic embryos into their different components revealed that the plumular tissue, cultured in isolation, yielded embryogenic calloid. Tissue taken from a range of embryos of both Laguna Tall and Malayan Dwarf behaved uniformly and the data in table 2 illustrates the sensitivity of the system to the various levels of 2,4-D. Calloid production in excess of 75% was obtained from plumules excised from Laguna Tall zygotic embryos (table 2) which is higher than any previous reports in the literature with other coconut tissues and contrasts particularly with inflorescence explants (Blake, 1990 and Verdeil *et al.*, 1994) which have on rare occasions produced up to 30% calloid in our laboratories, but this appears to be an infrequent and random occurrence.

Fragments of plumules treated with 2,4-D-containing media have produced calloid (unpublished data). The meristem, removed and cultured separa-tely on an auxin-free medium, gave rise to a plantlet. When a half embryo con-taining the plumule was cultured on medium supple-mented with 2,4-D, no calloid was produced. This result is in agreement with the low calloid initiation rates with entire mature embryos. The production of embryogenic calloid from immature zygotic embryos (Karunaratne and Periyapperuma, 1989) together with results obtained in our laboratory, suggests that during the maturation of the embryos an inhibitory material accumulates, perhaps in the developing haustorium, and that this suppresses the growth of the plumule *in situ*; such an explanation fits with the observed data. As the plumules of the immature zygotic embryos are too small to excise it is advantageous to use the larger mature embryos and to remove the remaining tissues.

During the pulse application it must be concluded that either insufficient 2,4-D was being taken up or that it was being metabolised and required to be given over a longer period to be effective. Longer pulse treatments might have been beneficial in preventing browning and early loss of calloid.

Much variability in calloid production from inflorescence may be attributed to the genetic differences between palms. With zygotic embryos, differences between cultivars and individual nuts might be expected to result in similar variability patterns, but this is not the case in the present experiments. Here repetition revealed considerable uniformity.

In offering a method for the large scale production of calloid, the culture of plumular material has a considerable potential for research on embryogenesis in coconut. Like the procedures involving the use of entire zygotic embryos, the plumules can only produce clones of palms with unknown performance. If, however, zygotic embryos are produced from elite parents, then clones from these embryos are likely to show many of the desired characteristics of the parents.

The stages of development between the first appearance of embryo-like structures and the formation of plantlets needs further attention. With the availability of a plentiful supply of calloid from plumules it is now possible to carry out well-planned and replicated investigations. Plumular leaves from hybrid embryos could be used for producing clonal plants either by the process of somatic embryogenesis or organogenesis. Simultaneously, a palm could be established from the remaining apical structure. Thus the characteristics of the clonal plants could be pre-determined through the performance of the plant regenerated from the excised apex. ■

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