^{14}C-2,4-DICHLOROPHENOXYACETIC ACID UPTAKE AND FORMATION OF EMBRYOGENIC CALLI IN COCONUT PLUMULAR EXPLANTS CULTURED ON ACTIVATED CHARCOAL-FREE MEDIA

ABSORCIÓN DEL ÁCIDO ^{14}C-2,4-DICLOROFENOXIACÉTICO Y FORMACIÓN DE CALLOS EMBRIOGÉNICOS EN EXPLANTES DE PLÚMULA DE COCOTERO CULTIVADOS EN MEDIO SIN CARBÓN ACTIVADO

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SUMMARY

Research for the development of protocols for the micropropagation of coconut plants (*Cocos nucifera* L.) has encountered several difficulties, such as explant browning. Although browning could be overcome by adding activated charcoal, this additive can also bind components of the culture medium required for the tissue morphogenic responses; this adsorption can be up to 99 % of the growth regulators added to the medium. In this work we developed a protocol without the use of activated charcoal which is able to produce embryogenic calli from coconut palm plumules as explants. This protocol also allowed us to study the kinetic absorption of 2,4-dichlorophenoxyacetic acid (2,4-D) by the explant, otherwise impossible in medium with activated charcoal. After evaluating different concentrations of 2,4-D in culture media without charcoal or polyvinylpolypirrolydone (PVPP) instead, we found that about 43 % of coconut explants cultured in media containing 1 μM 2,4-D without activated charcoal (with or without PVPP) formed embryogenic calli after four months. Yields were reproducible and similar to those obtained with medium containing activated charcoal and responses occurred more rapidly. Morphological observations of calli obtained in activated charcoal-free media were similar to those obtained in activated charcoal-containing medium. However there were differences in the histological observations, mainly in the shape of the nuclei of the proembryos of both types of callus. Uptake of ^{14}C-2,4-D was studied in plumule explants cultured in activated charcoal-free medium. After 120 d of culture about 25 % of the original radioactivity originally in the medium was extracted from explants and their ^{14}C-2,4-D concentration peaked within the first 20 d, before appearance of any morphogenic response.

Index words: *Cocos nucifera*, plumule, embryogenic calli, activated charcoal, ^{14}C-2,4-D uptake.

RESUMEN

El desarrollo de un protocolo para la propagación del cocotero (*Cocos nucifera* L.) ha encontrado muchas dificultades, como el necrosamiento de los explantes. Aunque el necrosamiento puede evitarse con carbón activado agregado al medio de cultivo, este compuesto también adsorbe componentes del medio de cultivo requeridos para producir la respuesta morfogénica. Tal adsorción puede ser del 99 % en reguladores del crecimiento. En el presente trabajo se desarrolló un protocolo sin carbón activado capaz de formar callos embriogénicos en explantes de plúmula de cocotero, el cual también permitió estudiar la cinética de absorción del ácido 2,4-diclorofenoxiacético (2,4-D) en el explante, lo cual sería imposible en medio con carbón activado. Para ello se evaluó un medio con diferentes concentraciones de 2,4-D y otro sin carbón o con polivinilpolipirrolidona (PVPP) en lugar del carbón. Se encontró que aproximadamente 43 % de los explantes de cocotero cultivados en medio de cultivo con 1 μM de 2,4-D y sin carbón activado (con o sin PVPP) formaron callos embriogénicos después de cuatro meses. Los rendimientos fueron reproducibles y similares a los obtenidos con medio de cultivo con carbón activado, y la respuesta ocurrió más rápidamente. Las observaciones morfológicas de los callos formados en los medios de cultivo libres de carbón activado fueron similares a los obtenidos en medio con carbón activado, pero hubo diferencias histológicas en la forma de los núcleos de los proembriones en ambos tipos de callos. La absorción del ^{14}C-2,4-D en el explante se estudió en medio sin carbón activado, y a los 120 d de cultivo, aproximadamente 25 % de la radiactividad original en el medio fue extraída por los explantes y el máximo de la concentración de ^{14}C-2,4-D se alcanzó en los primeros 20 d, antes de la observación de alguna respuesta morfogénica.

Palabras clave: *Cocos nucifera*, plúmula, callo embriogénico, carbón activado, absorción de ^{14}C-2,4-D.

INTRODUCTION

Research for the development of coconut plants micropropagation protocols started in the seventies

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(Eeuwens, 1976, 1978; Eeuwens and Blake, 1977), and has encountered several difficulties (Verdeil and Buffard-Morel, 1995). One of them is that coconut explants are prone to browning. However, browning and the associated death of the of inflorescence explants cultures could be overcome by the addition activated charcoal (Blake and Eeuwens, 1980). Presently, important advances have been reported for the regeneration of coconut from inflorescences (Blake and Hornung, 1995; Verdeil *et al.,* 1994) and plumular (Chan *et al.,* 1998; Hornung, 1995) explants cultured in media containing activated charcoal. These reports varied on the optimal concentration of 2,4-D to produce embryogenic calli, in the case of Hornung (1995) it was 400 μM, while for Chan *et al*. (1998) it was 100 μM. This difference could be caused by the type of activated charcoal. In our case a Mexican brand was used, although currently we have changed to activated charcoal from Sigma and the optimal concentration of 2,4-D to produce embryogenic callus was 650 μM.

The beneficial action of activated charcoal in *in vitro* culture results from its powerful adsorption properties. It binds the growth inhibitors secreted by tissues, such as phenolic compounds (George and Sherrington, 1984) and ethylene (Mensuali-Sodi *et al.*, 1993). However, it can also bind culture medium components required for morphogenic responses and tissue growth such as vitamins (Weatherhead *et al.,* 1979), and plant growth regulators like auxins (Ebert and Taylor, 1990) and cytokinins (Ebert *et al.,* 1993). Adsorption can result very extensive. In the case of growth regulators, over 99 % of the initial amount added to the medium, can be adsorbed by activated charcoal (Ebert and Taylor, 1990; Ebert *et al.,* 1993). There is also evidence that binding can be affected by pH and other medium concentration component (Ebert and Taylor, 1990; Ebert *et al.,* 1993), and since pH (Oropeza and Taylor, 1994) and medium component concentration of (Dussert *et al.,* 1995; Magnaval *et al.,* 1995) change with time during coconut explants culture, the free/activated charcoal bound ratio of medium components may also change the initially well defined medium formulation into an unknown one. This can get even worse if we consider that there are differences in the adsorption capacity (as defined for 2,4-D) for different types and sources of activated charcoal (Pan and van Staden, 1998). In the particular case of the media used for regeneration from plumular explants (Chan *et al.,* 1998), activated charcoal was included based on previous reports for inflorescence culture.

The objective of the present work was to develop a protocol without activated charcoal or with polyvinyl-polypirrolydone (PVPP) being able to produce calli and embryogenic calli from coconut plumular explants. The histological studies helped us to determine more precisely if the cellular events were present in a similar way as in the calli developed in medium with activated charcoal. PVPP was chosen because it has been shown to be useful to avoid tissue browning of different species cultured *in vitro* (Abdullah *et al.,* 1987; Cervelli, 1987; Perl *et al.,* 1996). The activated charcoal free medium allowed to carry out studies on 2,4-D uptake that are practically impossible if an undefined interaction with activated charcoal takes place during culture.

These studies are important in order to understand how this growth regulator promotes morphogenic responses in coconut explants (Blake and Hornung, 1995; Verdeil *et al.*, 1994; Chan *et al.,* 1998). In inflorescence explants most of the auxins are uptaken within the first 3 d of preculture (Oropeza and Taylor, 1994) in an activated charcoal-free medium (Blake, 1990), but these studies have not been extended to the rest of the process because it requires activated charcoal-containing media. A few studies of 2,4-D uptake by plumular explants have been reported (López-Villalobos, Com. personal)[1]. The present study also reports the uptake time course of 2,4-D by plumular explants cultured in an activated charcoal-free medium.

MATERIALS AND METHODS

Plant material

The fruits were harvested 12-14 months after pollination from 15 year old Malayan Green Dwarf coconut palms at Dzidzantun, Yucatán, México. They were cut transversely to expose the embryos surrounded by solid endosperm. Mature embryos were excised from the open nuts using a cork borer (1.6 cm diameter) and placed in distilled water. Under aseptic conditions, the endosperm enclosing the embryo was washed in 70 % ethanol 3 min and rinsed three times with sterile water. A wash in a 6 % NaClO solution was followed for 20 min and rinsed three times with distilled sterile water. The embryos were excised from the endosperm and washed in a 0.6 % NaClO solution for 10 min and rinsed with sterile distilled water three times. Embryos were 5-7 mm long and weighed approximately 100 mg each. Plumules were excised from them using a stereoscopic microscope and placed directly in nutrient medium. All chemicals were supplied by Sigma (USA), except the activated charcoal, which was supplied by Reactivos y Productos Químicos Finos (RPQF, México).

[1] López-Villalobos A (2002) Roles of lipids in coconut (*Cocos nucifera* L.) embryogenesis. Ph. D. thesis. Department of Agricultural Sciences, Imperial College, University of London, United Kingdom.

Culture media and conditions

Medium preparation for the induction of callus and callus with embryogenic structures was based on the Y3 medium (Eeuwens, 1976), with additions according to Chan *et al.* (1998) as follow: 3 g·L^{-1} gelrite, 2.5 g·L^{-1} activated charcoal and 100 μM 2,4-D (medium I). Two modified media I were also prepared: one without activated charcoal and another with 2.5 g·L^{-1} PVPP instead of activated charcoal. In both cases the 2,4-D concentration was 1 μM (unless otherwise noted). Media pH was adjusted to 5.75 before autoclaving for 20 min at 120 ºC. Each explant was cultured in 35 mL glass vessels of 4.0 cm diameter and 5.5 cm height, containing 10 mL of medium and incubated in the dark for six months (or as indicated in the text) at 27 $\pm$ 2 ºC without subculturing (conditions I) and 1 μM 2,4-D and 50 μM 6-benzylaminopurine (6-BAP) for medium II (medium Y3, concentration of gelrite and activated charcoal were the same as in medium I) in conditions II [16 h illumination (45-60 μmol m^{-2} s^{-1} PPFD) / 8 h darkness photoperiod, at 27 ºC $\pm$ 2] and subculturing every two months.

Histology

The histological procedures were done according to Buffard-Morel *et al.* (1992), with slight modifications. Tissue samples were fixed in paraformaldehyde 4 % in phosphate buffer (pH 7.2) for 24 h under negative pressure. Samples were dehydrated in a stepwise manner from 30 % to 100 % aqueous ethanol. This was followed by impregnation with JB-4 resin (Polyscience, USA). 3 μm thick sections, were prepared from the resin-impregnated tissues and stained with the PeriodicAcid-Schiff (Sigma 395-2-016) reagent, and they were combined with protein-specific naphthol blue-black (Fisher, 1968).

Assessment of ^{14}C-2,4-D adsorption

Adsorption of ^{14}C-2,4-D by activated charcoal was estimated according to Ebert and Taylor (1990). Vials containing each 10 mL of 100 μM 2,4-D medium and spiked approximately with 0.045 μCi of 1-[^{14}C]-2',4'-D (specific activity 13.2 μCi·μmoL^{-1}, 0.0035 μMol of ^{14}C-2,4-D/vial), were added with activated charcoal or PVPP to a final concentration of 2.5 gL^{-1}. After a 1, 2, 3, 5 or 10 d incubation, supernatant aliquots from centrifuged medium were taken for measurements of radioactivity by scintillation counting. Four vials with culture medium were used for each incubation time and analysed independently.

Assessment of ^{14}C-2,4-D uptake

Tissue samples (one per vial) were cultured in 1-[^{14}C]-2',4'-D containing medium prepared as above. After different incubation times, each sample was extracted and radioactivity assessed according to Ebert and Taylor (1990). The tissue was rinsed with fresh medium, frozen with liquid nitrogen prior to homogenisation in 80 % acetone and extracted overnight. Aliquots, from both, the acetone extracts and the used medium, were counted after the addition of liquid scintillant. Three vials were used for each incubation time and analysed independently.

Fresh weigh and pmol of 2,4-D per fresh weight

In vitro cultured explants were weigh at different times utilising a Sartorius balance. The pmols of 2,4-D/fresh weight were calculated multiplying the percentage of ^{14}C-2,4-D absorbed by the explants by the concentration of 2,4-D in 10 mL of culture medium (1 μM) and divided by the fresh weight.

Statistical analysis

Each plumule was cultivated in a single vial. Each treatment consisted of 20-40 individual plumules as defined in each Table. Yields are expressed as percentage of explants forming calli or embryogenic calli and they were recorded monthly. Statistical analysis was performed on the binomial data using ANOVA on Ranks and Student-Newman- Keuls Test for multiple comparisons of means (Rickmers and Todd, 1967).

RESULTS

Use of activated charcoal-free media

Activated charcoal has been included by Chan et al. (1998) in their media for somatic embryogenesis from plumular explants. In the present study, formation of callus and embryogenic callus in coconut plumular cultures in the presence of 2,4-D (1 μM) was tested in activated charcoal free medium I, either containing PVPP or no additive at all, and compared to medium I containing activated charcoal and 100 μM 2,4-D, the optimal formulation for callus initiation from plumular explants, according to Chan et al. (1998).

In order to define a range of auxin concentration to be tested it was necessary to evaluate whether PVPP was able to adsorb any 2,4-D. Therefore ^{14}C-2,4-D was used to evaluate the adsorption capacity of PVPP. We found that PVPP did

not adsorb the auxin, since most of the radioactivity was found in the culture medium, contrasting with the strong 2,4-D adsorption shown by activated charcoal (Figure 1). Therefore the auxin concentration range included concentrations lower than those chosen for activated charcoal–containing media (Chan *et al.,* 1998). In an initial evaluation, medium I with PVPP or no activated charcoal included 2,4-D at 1, 10, 100 and 1000 μM. With both media the explants showed browning (100 %) at the three highest auxin concentrations. With 1 μM of 2,4-D (a concentration 100-fold lower than that used when activated charcoal was included in the medium), calli formed after two months (Figure 2A) and embryogenic calli after four months of culture (Figure 2B). Histological observations showed the occurrence of meristematic centers (MC) in calli (Figure 2C) and pro-embryos in embryogenic calli (Figure 2D). Similar morphological observations were made in explants cultured in activated charcoal containing medium (Figure 2E and 2F, respectively). The presence of meristematic centers in embryogenic calli was observed in Figure 2G. However, there were some differences in the histological observations, mainly in the shape of the nuclei of the proembryos of both types of callus (inserts Figure 2D and 2H).

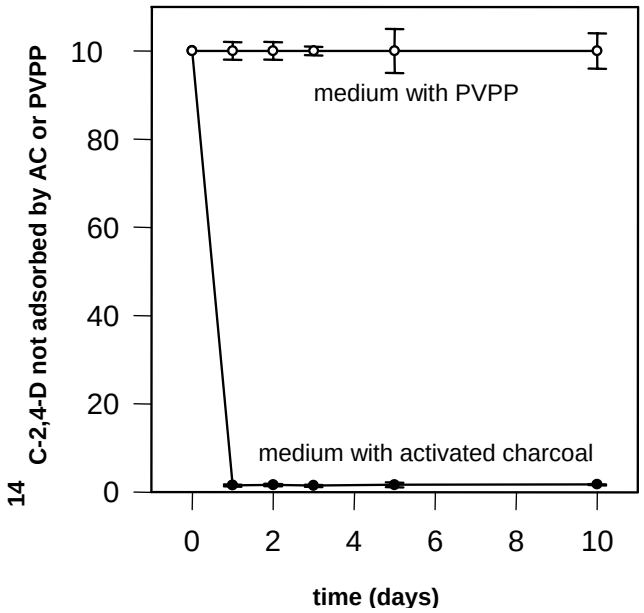

Figure 1. Time course of the adsorption of ^{14}C-2,4-D by activated charcoal (AC; 2.5 g L^{-1}) and PVPP (2.5 g L^{-1}) in liquid medium. Concentration of 2,4-D: 100 μM. Data presented are means (n = 4) and bars denote ± SD larger than symbols.

The responses in activated charcoal-free media appeared 1-2 months earlier than in medium containing activated charcoal and 100 μM 2,4-D (Table 1). In medium containing PVPP, callus was formed in 55 % of the explants and embryogenic callus bearing embryogenic structures in 42.5 % of the explants (Table 1). Similar responses and periods were observed using additive-free medium. These percentages of explants forming calli and embryogenic calli, were significantly different to those obtained with medium containing activated charcoal at two and four months respectively (Table 1).

Table 1. Formation of callus and embryogenic callus in plumular explants cultured in medium containing 2.5 g L^{-1} activated charcoal and in activated charcoal-free media with or without PVPP during six months of culture. Concentration of 2,4-D: 100 μM for medium with activated charcoal and 1 μM for activated charcoal-free media with or without PVPP 2.5 g L^{-1}, n = 40. Values followed by different letters vary significantly (P < 0.05).

Culture medium with	Percentage of explants forming callus after being cultured for		Percentage of explants forming embryogenic callus after being cultured for	
	2 months	3 months	4 months	6 months
2.5 g L^{-1} activated charcoal	15 a	62.5 a	17.5 a	47.5 a
activated charcoal-free 2.5 g L^{-1} PVPP	55 b	55 a	42.5 b	42.5 a
activated charcoal-free / without PVPP	50 b	60 a	47.5 b	47.5 a

In order to assess the optimal concentrations of 2,4-D for medium containing PVPP and additive-free medium, a range of concentrations was tested (Table 2). The results expressed on percentage of explants forming embryogenic calli showed that the optimal auxin concentrations were basically the same for activated charcoal-free medium (1 μM) and medium containing PVPP (0.95 μM). With lower auxin concentrations grew haustorium-like tissue, this is a spongy growth resembling the haustorium or cotyledon, which normally enlarges inside the nut supplying the developing zygotic embryo (see Branton and Blake, 1983), also germination of the plumules was observed. On the other hand, with higher concentrations there was callus formation although with slight growth (Table 2). Quantitatively, the results with both media were not significantly different to the response obtained with medium containing activated charcoal (55 %).

Results from five batches of plumules cultured in additive free media (1 μM 2,4-D) showed that the formation of embryogenic calli was reproducible, 43 % ± 9 (Table 3). The browning and necrosis in calli and embryogenic calli were rarely observed.

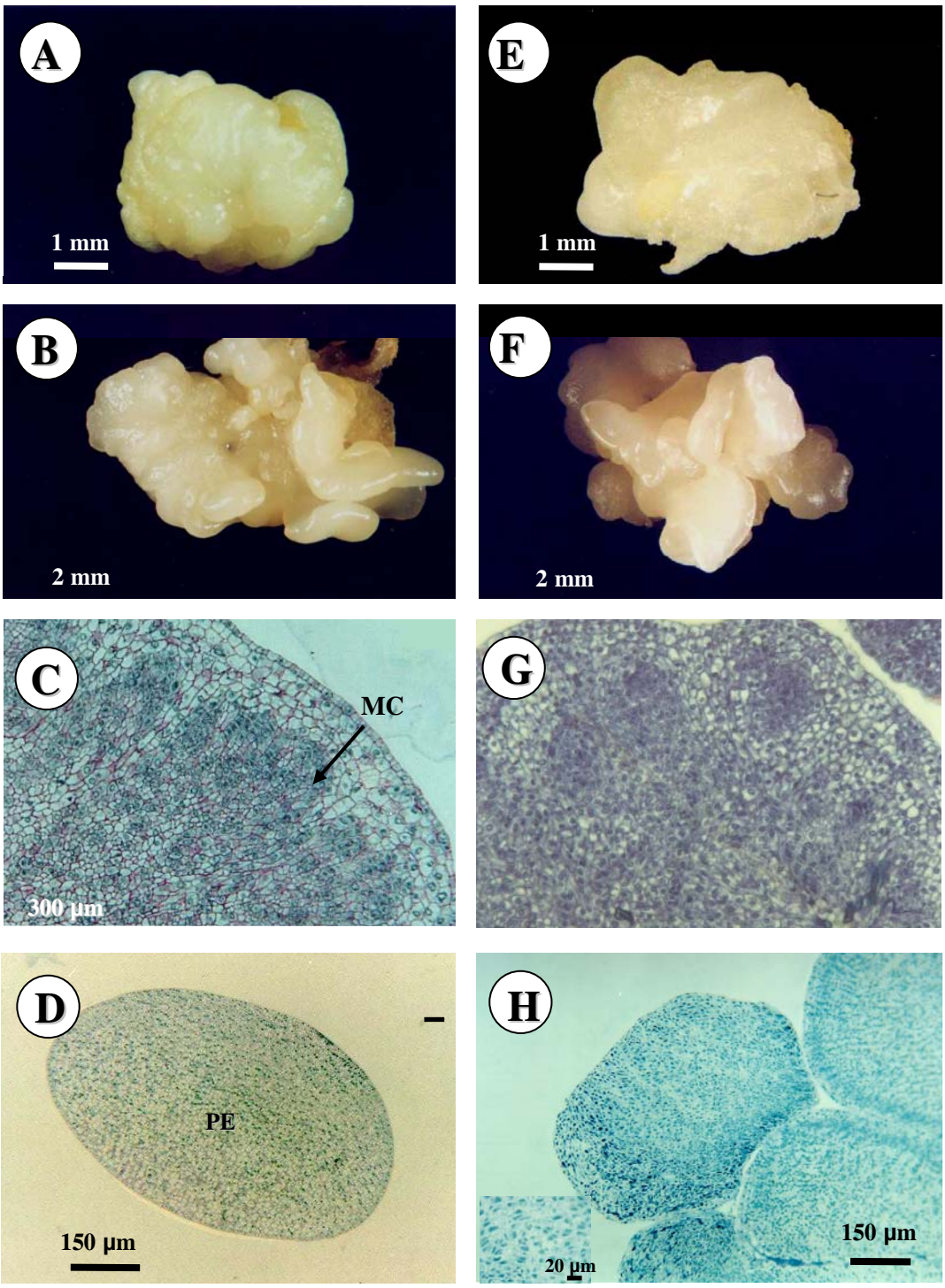

Figure 2. Plumular explants in gelled medium I without activated charcoal or PVPP (1 µM 2,4-D) or in medium with activated charcoal (100 µM) developed callus tissue after two or three months of culture (A y E correspondingly). Calli bearing embryogenic structures (ES) developed during the following two months of culture in medium without activated charcoal or PVPP (B) or three months with activated charcoal (F). Histological cross sections of this callus showed the presence of meristematic centers (MC) in medium without activated charcoal or PVPP (C) or with activated charcoal (G). Pro-embryos (PE) were present in histological sections of embryogenic calli in medium without activated charcoal or PVPP (D) or with activated charcoal (H). Inset: cells and nuclei from the proembryos.

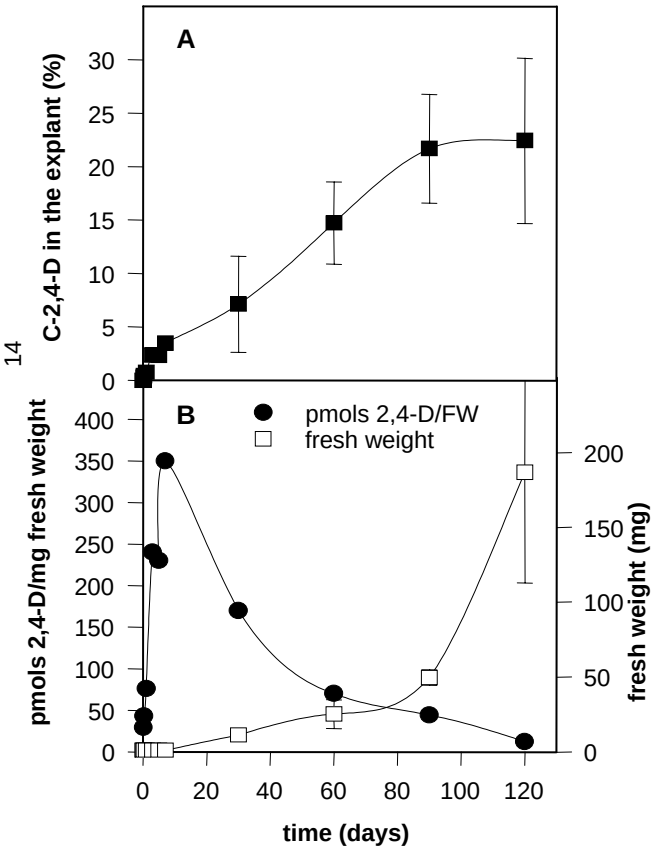

Figure 3. Time course of ^{14}C-2,4-D uptake by coconut plumular explants cultured in activated charcoal-free gelled medium (A). Time course of fresh weight gain by the plumular explants and of their 2,4-D content expressed on a fresh weight basis (B). Concentration of 2,4-D: 1 μM. Data presented are means (n = 4) and bars denote ± SD larger than symbols.

Uptake of ^{14}C-2,4-D

The time course of the uptake of ^{14}C-2,4-D by plumule explants cultured in activated charcoal free medium without additive, showed that the rate of uptake was fast during the first week of culture and thereafter the rate decreased, but total uptake continued increasing steadily, and by day 90 the rate stabilised (Figure 3A). Total uptake was about 22 % of the radioactivity originally in the medium (Figure 3A). Simultaneously, the pH of the medium decreased from 5.3 at the beginning to 4.3 by day 30 and to 4 by day 90 of culture without changing significantly afterwards (data not shown). The fresh weight of the explants increased steadily during the first 80 d of culture (Figure 3B), and afterwards it increased several fold faster. When ^{14}C-2,4-D taken up by the explants was expressed in terms of fresh weight, a peak was observed during the first 20 d of culture (Figure 3B). This experiment was repeated and the results were reproducible (not shown).

Table 2. Effect of different concentrations of 2,4-D on the formation of embryogenic calli in plumular explants after four months of culture in activated charcoal-free media with or without PVPP and after six months in medium containing 2.5 g L^{-1} activated charcoal (n = 20). Values within treatments followed by different letters vary significantly (P>0.05).

2,4-D (μM)	Percentage of explants forming embryogenic calli	Other responses in explants
(a) Activated charcoal-free / without PVPP medium		
3	0	Callus slight growth
2	0	Callus slight growth
1.5	25 b	
1	55 a	
0.95	50 a	
0.8	0	Haustorium-like tissue growth Some germinating
0.7	0	Haustorium-like tissue growth ome germinating
(b) Activated charcoal-free medium with 2.5 g L^{-1} PVPP		
3	0	Callus slight growth
2	0	Callus slight growth
1.5	45 b	
1	45 b	
0.95	60 a	
0.8	0	Haustorium-like tissue growth Some germinating
0.7	0	Haustorium-like tissue growth Some germinating
(c) Medium with 2.5 g L^{-1} activated charcoal		
100	55 a	

DISCUSSION

Activated charcoal is any form of carbon characterized by high adsorptive capacity for gases, vapors and colloidal solids. The charcoal is produced by destructive distillation of woods, peat, nut shells, vegetables and other carbonaceous matter (Pan and van Staden, 1998). The beneficial action of activated charcoal in *in vitro* culture results from its powerful adsorption properties. It binds the growth inhibitors secreted by tissues, such as phenolic compounds (George and Sherrington, 1984) and ethylene (Mensuali-Sodi *et al.*, 1993). However, it can also bind

components of the culture medium required for morphogenic responses and tissue growth, such as vitamins (Weatherhead *et al*., 1979) and growth regulators like auxins (Ebert and Taylor, 1990), cytokinins (Ebert *et al*., 1993) and gibberellins (Mohamed-Yasseen, 2001).

Table 3. Formation of embryogenic callus in different batches of plumule explants cultures in additive-free medium for four months. Concentration of 2,4-D 1 μM.

Batch	Number of explants	Percentage of explants forming embryogenic calli
1	20	40
2	20	55
3	20	50
4	60	33
5	100	38
Average	44	43.2±9.04

Activated charcoal is often added to plant tissue culture media, since it triggers beneficial effects in embryogenesis of *Cucumis sativus* (Chee and Tricoli, 1988), seed germination of orchids (Van Waes, 1987), rooting by providing a dark environment in *Pinus pinaster* (Dumas and Monteuuis, 1995), bud proliferation of *Lavandula officinalis,* and growth of *Anemona coronaria* seedlings (Mensuali-Sodi *et al*., 1993).

Although the use of activated charcoal for *in vitro* culture of coconut explants is beneficial to overcome tissue necrosis, there are associated disadvantages that may affect protocols performance in terms of efficiency and reproducibility. The use of activated charcoal for plumular explants reported by Chan *et al*. (1998) was based on formulations previously reported for inflorescence culture (Verdeil *et al*., 1994), but it was not known whether it was essential to obtain morphogenic responses from plumular explants. The present results show that it is possible to obtain calli (two months) and embryogenic calli (four months) from coconut plumular explants cultured in media devoid of activated charcoal at 0.95 - 1 μM of 2,4-D concentrations, 100-fold lower than those used when activated charcoal was included in the medium (100 μM). This is understandable since activated charcoal is known to bind most of 2,4-D present in the medium (Ebert and Taylor, 1990). Furthermore, both responses occurred quicker in activated charcoal-free media, than in activated charcoal containing medium. In quantitative terms, the responses in activated charcoal-free media were very similar to those obtained in activated charcoal-containing media, and were reproducible as well.

Morphological observations for calli and embryogenic calli obtained in activated charcoal-free media did not show differences compared to those obtained in activated charcoal-containing medium in this study and those reported previously by Chan *et al*. (1998). However, proembryos developed in activated charcoal free-medium showed cells with nuclei with a round shape, whereas those in proembryos forming in activated charcoal-containing medium had an irregular shape. There is evidence that the form of the nuclei of the cells of coconut *in vitro* cultures could change depending on the auxin medium concentration. Verdeil *et al*. (2001) reported that the nuclei of embryogenic cells in coconut calli changed from a round shape to an irregular shape (with invaginations), when the calli were transferred to a medium containing a higher (two-fold) concentration of 2,4-D. This could mean that changes in nucleus appearance may be merely associated to 2,4-D concentrations in the medium. Verdeil *et al*. (1999) showed a 1.25 to 5 fold increase in 2,4-D in coconut calli grown in activated charcoal-containing medium, depending on the state of the calli. Therefore, a difference in 2,4-D availability in the medium with or without activated-charcoal presence may explain the differences in nuclei appearance.

On the other hand, the present activated charcoal-free culture system offers the opportunity to carry out studies otherwise impossible when activated charcoal is included in the formulation, for instance, studies on the uptake of medium components such as growth regulators. As previously reported for inflorescence coconut explants (Oropeza and Taylor, 1994), ^{14}C-2,4-D was taken up by plumular explants. The rate was faster during the first week of culture, then reduced until reaching a plateau at day 90. It is interesting to note that when radioactivity was steadily taken up, calli were formed and once the calli started to form embryogenic structures, uptake practically stopped. This could be a proper time to transfer them to medium II with a 100-fold reduced 2,4-D concentration, instead of keeping them for a longer time, as reported by Chan *et al*. (1998). The explants fresh weight increased steadily during the first 80 d of culture, the period required for callus tissue development. Afterwards, fresh weight increased several fold faster, when embryogenic structures developed. It is interesting to note that this change in rate starts when 2,4-D uptake reached a plateau. The low uptake of ^{14}C 2,4-D could be due to ^{14}C2,4-D depletion in the vicinity of the explant. The ^{14}C-2,4-D concentration in the explants reached its maximum values within the first 20 d of culture, prior to the appearance of any morphogenic response. This result suggests that the uptake of 2,4-D may be related to the induction of these morphogenic responses. Actually, tyrosine kinase activity has been measured in this plumular system (although using medium containing

activated charcoal) and it also peaks at this time (Islas-Flores *et al.,* 2000). The tyrosine kinase activity catalyses the phosphorylation of protein tyrosine residues, a process that has been suggested to be mediating the control of cell differentiation responses from extracellular signals, including growth regulators, in animal cells (Hunter and Cooper, 1985; Alfaro-López *et al.,* 1998; Hubbard, *et al.*, 1998) and plant cells (Guo and Roux, 1995; Trojanek *et al.,* 1996).

The present results show that morphological responses in plumular explant can be obtained in activated charcoal-free media with a performance similar to that reported for activated charcoal-containing medium (Chan *et al.,* 1998). However, further research has to be carried out to determine whether further development of the cultures into somatic embryo formation and conversion, as reported previously (Chan *et al.,* 1998) can also be achieved in activated charcoal-free media. Furthermore, this activated charcoal-free system can be useful to carry out studies on the interaction between growth regulators and explants, as it is presently shown for 2,4-D uptake.

CONCLUSIONS

It was possible to obtain embryogenic calli in plumule explants cultured *in vitro* in activated charcoal free medium. The yields were reproducible and similar to those obtained with medium containing activated charcoal. Morphological observations were similar to those obtained in activated charcoal-containing medium, however the histological observations had slight differences. Only 25 % of ^{14}C-2,4-D was absorbed by the plumule explants cultured *in vitro*.

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