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ORIGINAL PAPER

Influence of form of activated charcoal on embryogenic callus formation in coconut (*Cocos nucifera*)

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Abstract Development of micropropagation protocols for *Cocos nucifera* has progressed slowly. Activated charcoal is included in the culture medium of each protocol, mainly to prevent tissue browning. Charcoal production procedures can affect the properties of different brands. In this study, eight types of activated charcoal were evaluated for their effects on free 2,4-dichlorophenoxyacetic acid level, pH, conductivity, and osmolarity of the culture medium and on the frequency of embryogenic callus induction. Moreover, the effect of particle size of the optimum charcoal type on embryogenic callus development was also studied. Charcoal type had a significant effect on (Y3) culture medium properties. Free 2,4-D was highest in Reactivos y Productos Químicos Finos-containing medium and pH was lowest in MERCK-containing medium. Charcoal type also influenced embryogenic callus induction, with acid washed for plant cell and tissue culture-, DARCO- and United States Pharmacopeia-containing media promoting ~60% embryogenic callus, but with different optimal 2,4-D concentrations. Particle size profiles varied among all charcoal types, although small particle fraction (<38 μm) was abundant in all. Use of small particle fractions produced higher frequencies of embryogenic callus (70%) than either large particle or whole charcoal fractions.

Keywords Somatic embryogenesis · 2,4-D adsorption · Conductivity · Osmolarity

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AC	Activated charcoal
AW	Acid-washed
EC	Embryogenic callus
NEUT	Neutralized
PAW	Acid-washed with phosphoric acid
PCCT	Acid washed for plant cell and tissue culture
PGRs	Plant growth regulators
RPQF	Reactivos y productos químicos finos
USP	United States Pharmacopeia testing specifications

Introduction

Induction of cellular differentiation in vitro depends on genetic totipotency, culture medium formulation, and incubation conditions. Phytohormones, most commonly auxins and cytokinins, are key medium components (Gaspar et al. 1996). During the in vitro culture process, undesirable or inhibitory compounds such as excess phenolic metabolites (Tisserat 1979; Carlberg et al. 1983; Mensuali-Sodi et al. 1993) and ethylene (Mensuali-Sodi et al. 1993) can be produced. Other deleterious compounds including 5-hydroxymethyl furfural, an inhibitory by-product of autoclaving sucrose can be produced during medium preparation (Weatherhead et al. 1978).

Activated charcoal is frequently added to culture medium formulations to either reduce or eliminate undesirable compounds and thus improve anticipated morphogenic responses of explants. AC provides many advantages in vitro, including promoting embryogenesis (Chee and Tricoli 1988) and enhancing rooting (Dumas and Monteuuis 1995).

However, it can also bind culture medium components such as vitamins (Weatherhead et al. 1978), copper and zinc (Van Winkle et al. 2003), and various PGRs such as auxins (Ebert and Taylor 1990), cytokinins (Ebert et al. 1993), and gibberellins (Mohamed-Yasseen 2001) required for explant growth and morphogenic responses. This adsorption can be very extensive and as much as 99% of the initial amount of PGRs added to a medium can be bound by AC (Ebert and Taylor 1990; Ebert et al. 1993) and therefore, the addition of AC into a well defined culture medium can therefore transform it into an undefined medium.

AC is produced from various carbonaceous materials following different methods (Pan and van Staden 1998). Several AC types and brands are used in plant tissue culture, and some are acid-washed, or meet particular chromatographic requirements. Differences between brands and types can confer different characteristics to AC thus influencing growth and morphogenic responses of in vitro cultured explants. For example, different types of ACs are reported to influence culture medium pH by altering mineral availability in a medium to induce embryogenesis in conifer explants (Van Winkle and Pullman 2003; Van Winkle et al. 2003). Moreover, use of non acid-washed AC can introduce impurities, such as Ca, Mg, and Si, into a culture medium (Van Winkle and Pullman 2003).

Developing micropropagation protocols for coconut (*Cocos nucifera* L.) has progressed slowly as this monocot species has proven to be difficult to regenerate (see Oropeza et al. 2005). This has been achieved through somatic embryogenesis (Blake 1990). Then in order to develop an improved protocol, different explants have been tested, including: inflorescences (Blake 1990; Verdeil and Buffard-Morel 1995), leaves (Verdeil and Buffard-Morel 1995), unfertilized ovaries (Perera et al. 2007), anthers (Perera et al. 2009) and plumules (Hornung 1995), the last one being the most responsive in terms of the formation of embryogenic callus, somatic embryos and their convertion to plantlets (Chan et al. 1998). Anatomical and histological characterization of these morphogenic responses was described, showing that an initial callus develops from the cotyledonary leaves, followed by growth of well defined translucid structures (TS), rich in meristematic cells (Sáenz et al. 2006). These TS formed a second set of well defined structures the embryogenic structures (ES) since from them somatic embryos formed (Sáenz et al. 2006). Further improvements in plumule based protocols, showed yields of nearly 100,000 somatic embryos from one plumule explant in a process based on a series of embryogenic callus multiplication, using the embryogenic structures as explants (Pérez-Nuñez et al. 2006).

In all these reports AC was included as a culture medium component to prevent high incidence of tissue browning that leads to loss of explants, and it was shown that its omission compromised importantly the efficiency of the process (Sáenz et al. 1999). Therefore, the objective of this study was to determine the optimal type of AC for in vitro culture of coconut by documenting the properties, including adsorption of 2,4-D, of different AC types, and evaluating their effects on embryogenic callus formation.

Materials and methods

Studies with activated charcoal of different sources in tissue-free medium

Eight types of charcoal from four suppliers (listed in Table 1) were characterized in their particle size profile. Also we studied the effect of each of the activated charcoals on the medium properties without cultivating any explant tissues. In each case the concentration was 2.5 g l^{-1} and charcoal was added to the medium before autoclaving. The parameters evaluated were: pH, conductivity, osmolarity and free 2,4-D in medium. The first set of measurements (day 0) was obtained immediately after autoclaving the medium.

Medium pH, conductivity and osmolarity determinations

Culture medium was prepared as described above. Two liters of culture medium were dispensed into vials and 20 vials were randomly taken for the measurements. Medium pH, conductivity and osmolarity were measured at 0, 1, 3 and 8 days after autoclaving. The pH was measured using a Beckman electrode (Fullerton, California, USA. Part number 511052) and conductivity with a Cole Parmer (Vernon Hills, Illinois, USA) electrode. Osmolarity was assessed using an osmometer (Advanced Instruments 3W2, Norwood, Massachusetts, USA) calibrated with standard NaCl solutions (100 mOsm and 500 mOsm).

Free ^{14}C-2,4-D in medium

Adsorption of ^{14}C-2,4-D by the ACs was measured indirectly according to Ebert and Taylor (1990). Vials were loaded with 10 ml of liquid Medium I supplemented with 0.65 mM 2,4-D, one type of AC (2.5 g l^{-1}) and an aliquot of 1–[^{14}C]–2,4-D (American Radiolabeled Chemicals, USA, specific activity 13.2 µCi·µmol^{-1}). After 1, 3 and 8 days incubation, supernatant aliquots from centrifuged media were used to take measurements by scintillation counting of residual medium radioactivity. Three vials from each culture medium and incubation time were analyzed independently in a scintillation counter (Beckman 3801, USA).

Table 1 Types of activated charcoal used

Brand (catalog)	Country	Relevant features[a]	Abbreviation
Sigma (C-6289)	USA	Acid washed for plant cell and tissue culture	PCCT
Aldrich (24 227-6)	USA	None described	DARCO
Sigma (C-4386)	USA	Acid-washed	AW
Sigma (C-5510)	USA	Acid-washed with phosphoric acid	PAW
Sigma (discontinued)	USA	Neutralized charcoal	NEUT
Sigma (C-7606)	USA	With *USP testing specifications	USP
Merck (002184.1000)	Germany	None described	MERCK
Reactivos y Productos Químicos Finos (C.N.)	México	None described	RPQF
None	None	Control: medium without AC added	-AC

[a] Features described by suppliers. * United States Pharmacopeia

Activated charcoal particle size

A particle size profile was generated for each AC type with eight inch sieves of different meshes (100, 90, 53, 45, and 38 μm) (W.S. Tyler, Inc. Mentor, Ohio, USA). For each type, 50 g sample was processed in a sieve shaker (Rotap, W.S. Tyler, Inc., USA) for 30 min. Each fraction was weighed to calculate the percentage of the total weight.

Gravimetric measurement of charcoal

As described above, 1 liter of liquid culture medium was prepared and dispensed into vials (10 ml per vial), and 55 vials chosen randomly for analysis. These were autoclaved and filtered using a 0.45 μm filter (Millipore, Bedford, Massachusetts, USA) with the aid of a Büchner funnel with a fritted disc in a vacuum. Filters were previously weighed. After filtering, those filters containing charcoal were dried at 80°C for 24 h in an oven, allowed to cool at room temperature in a vacuum desiccator, weights of dried filters were measured with an analytical balance (Ohaus, Pine Brook, New Jersey, USA), and charcoal weight was then calculated.

Studies with explants and the formation of embryogenic callus

Plumule explants

Plumules extracted from nuts (12–14 months after pollination), harvested from 15-year-old Malayan Green Dwarf palms were used as explants. All nuts were collected at San Crisanto, Yucatan, Mexico (l21°20′N, 89°09′W). Nuts were transversely cut with a machete to extract embryos surrounded by solid endosperm. These were excised from the open nuts with 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 for 3 min, rinsed three times with sterile water, washed again in 6% NaClO solution for 20 min, and rinsed three times with sterile distilled water. Embryos were excised from the endosperm, and washed in 0.6% NaClO solution for 10 min, and then rinsed three times with sterile distilled water. These embryos, 5–7 mm in length and ~100 mg in fresh weight, were used to extract plumules using a stereoscopic microscope (STEMI SV 11, Carl Zeiss, Jena, Germany).

Plumule explants were incubated on Medium I that consisted of Y3 medium (Eeuwens 1976) added with different concentration of 2,4-D, 3 g l^{-1} Gelrite (Sigma, St. Louis, Missouri, USA) and 2.5 g l^{-1} AC (for description see below). The addition of these components and the medium pH adjustment to 5.75 were carried out before autoclaving for 20 min at 120°C. Each explant was incubated in 35 ml vessels containing 10 ml medium, and incubated in darkness for three months at 27 ± 2°C without subculturing. After four weeks (initial) embryogenic calluses were formed and were morphologically consistent with the report by Sáenz et al. (2006) presented embryogenic structures (see Fig. 5). In this system we studied the effect of each of the different activated charcoals on the formation of embryogenic callus.

Embryogenic structure explants

Embryogenic structures were excised from embryogenic calluses and subcultured in Medium I to induce new embryogenic callus. This was repeated twice so three cycles of embryogenic callus multiplication were carried out. The embryogenic structures obtained from the calluses of the third cycle were used then on as explants for the experiments reported here. The medium and conditions used were the same as described above. The concentration of 2,4-D was 0.5 mM. With this system was studied the effect of activated charcoal (Sigma, PCCT) comparing the effect of the original product with its full complement of different particle sizes with fractions of the same charcoal of different particle sizes, on the formation of embryogenic callus.

Statistical analysis

Physicochemical parameters (pH, conductivity, and osmolarity) were determined for 20 randomly selected vials, and 2,4-D adsorption was determined in three replicates per each treatment. Morphogenetic responses were determined in three replicates per treatment using either 15 plumules or 30 embryogenic structures. Differences between the means were calculated with an analysis of variance (ANOVA) and a Student–Newman–Keuls test was performed to test the differences between the means.

Results

Here we present the results of two different types of evaluations. First the evaluations of different parameters of charcoal in tissue free medium to determine interaction of the ACs with the culture medium. Then evaluations with explants cultured in AC containing medium to determine the effect of the different ACs tested here and particle size on the formation of embryogenic callus.

Evaluations of ACs parameters in tissue free-medium

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

The level of adsorption of 2,4-D was calculated for each AC without tissue to determine if any adjustments to standardize the 2,4-D concentration were required. This was carried out indirectly, measuring the free ^{14}C-2,4-D level in the media with the different ACs. With RPQF AC the results showed that the level decreased from 100% to about 30% within 1 day and to 19% after 8 days (Fig. 1c). In contrast, with PCCT AC free ^{14}C-2,4-D was less than 1.5% after 1day, and remained unchanged until 8 days (Fig. 1b). With the rest of the ACs similar results were obtained but only measurements after 8 days are presented. The free auxin levels varied from as low as 0.72% (MERCK) to higher levels of 1.27% (PCCT) and 1.30% (NEUT) (Table 2).

Table 2 Percentage of free ^{14}C-2,4-D levels in culture media prepared with different types of activated charcoal (AC)

AC	% free ^{14}C-2,4-D
PCCT	1.27 ± 0.05 b
DARCO	1.00 ± 0.06 cd
AW	1.08 ± 0.06 cd
PAW	1.18 ± 0.05 c
NEUT	1.30 ± 0.03 b
USP	1.08 ± 0.12 cd
MERCK	0.72 ± 0.12 d
RPQF	19.00 ± 0.33 a
Control (-AC)	100.00 ± 0.00

Initial 2,4-D concentration was 0.65 mM

Data correspond to means ($n = 3$) of samples 8 d after preparation. Means with different letters are significantly different at $p < 0.05$

Medium pH

Responses in medium pH over 8 days fell into three groups with different patterns. The control medium without AC had the lowest overall pH and exhibited an increase in pH from 5.75 to about 6.0 at 3 days followed by a decrease to 5.6 at 8 days (Fig. 2a; Table 3). The medium containing PAW AC had the highest pH, and increased from 5.75 to 6.34 within 3 d and did not decrease thereafter (Fig. 2b; Table 3). All the remaining media manifested patterns similar to PCCT, in which pH increased from 5.75 to about 6.2 by 3 days and then remained constant at 6.12 (Fig. 2c; Table 2). In the media with the other ACs, the final pH ranged from 5.99 to 6.16 (Table 3). Each group was significantly different ($p < 0.05$) from the others, although within the third group, the medium containing MERCK AC had a lower pH (pH 5.99; $p < 0.05$) than the other media.

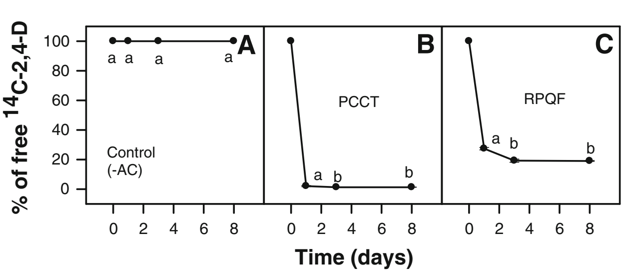

Fig. 1 Changes in free ^{14}C-2,4-D during an 8-day time course in media **a** without activated charcoal (AC); **b** containing PCCT; and **c** containing RPQF. Means ($n = 3$) with different letters are significantly different ($p < 0.05$)

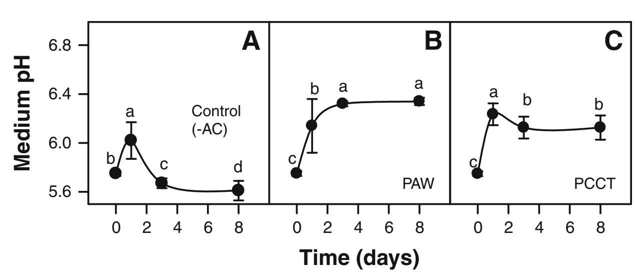

Fig. 2 Changes in pH during an 8-day time course in medium **a** without activated charcoal (AC); **b** with AC-PAW; and **c** with PCCT. Means ($n = 20$) with different letters are significantly different ($p < 0.05$)

Table 3 pH, conductivity and osmolarity of culture media prepared with different activated charcoal types

AC	pH	Conductivity (mS)	Osmolarity (mOsm/Kg H_2O)
PCCT	6.12 ± 0.05 b	6.02 ± 0.08 bc	300.90 ± 06.46 a
DARCO	6.13 ± 0.10 b	6.19 ± 0.08 ab	288.90 ± 04.56 ab
AW	6.12 ± 0.09 b	6.21 ± 0.17 ab	283.20 ± 07.44 ab
PAW	6.34 ± 0.03 a	5.96 ± 0.15 c	283.90 ± 10.75 ab
NEUT	6.16 ± 0.09 b	6.24 ± 0.16 ab	292.20 ± 05.14 a
USP	6.16 ± 0.08 b	6.21 ± 0.18 ab	295.20 ± 04.64 a
MERCK	5.99 ± 0.08 c	6.35 ± 0.09 a	272.40 ± 23.63 b
RPQF	6.09 ± 0.18 b	6.35 ± 0.18 a	282.60 ± 20.00 ab
Control (-AC) d 8	5.61 ± 0.08 e	6.06 ± 0.16 bc	284.50 ± 12.70 ab
Control (-AC) d 0	5.75 ± 0.01 d	6.14 ± 0.02 b	287.40 ± 15.48 ab
Mean (all treatments) ± SD	6.14 ± 0.10	6.19 ± 0.14	287.41 ± 08.83

Means ($n = 20$) of measurements taken 8 d after preparation. Mean values with different letters in the same column are significantly different at $p < 0.05$

Medium conductivity and osmolarity

Immediately after the preparation of all media with or without AC, measurements of conductivity and osmolarity were 6.14 mS and 287.40 m Osm/Kg H_2O, respectively. Then during the following 8 days although minor changes occurred, significant differences were observed among the media with the different ACs. In the case of conductivity, the lowest value was 5.96 mS in PAW AC medium and the highest 6.35 mS in RFQP and MERCK media (Table 3). For osmolarity, the lowest value was 272.40 m Osm/Kg H_2O in MERCK medium and the highest 300 m Osm/Kg H_2O in PCCT medium (Table 3).

Activated charcoal particle size

The profile of particle size was determined in the ACs by using sieves of different mesh sizes. The results showed a clear variation on the profiles in the different types of AC (Fig. 3). In all cases the majority of the particles were smaller than 38 µm and the less abundant particles were larger than 100 µm, however some charcoals as PAW, NEUT and USP presented higher amounts of particles larger than 100 µm particle than the other ACs (Fig. 3).

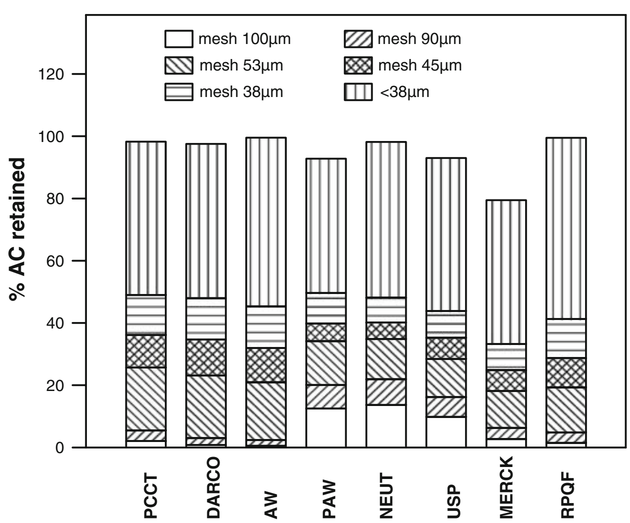

Fig. 3 Particle size profiles of eight tested activated charcoals (ACs)

Amount of AC delivered to tissue culture vial by medium with different particle fractions

During dispensing AC containing culture medium to the vials, the medium was agitated with a magnetic stirrer. In this process larger size particles sediment more rapidly than smaller particles. This sedimentation could generate variation in the amount of charcoal delivered to each vial with greater differences when dispensing large volumes of medium. An analysis was carried out to determine the amount of PCCT AC delivered to vials along the dispensing procedure when using media prepared with whole charcoal, small particle (≤38 µm) charcoal and large particle (>38 µm) charcoal. This AC was used for this particular evaluation because it is the one we had been using before and its performance was among the three best according to results presented above, and for embryogenic callus formation as shown below. The results revealed that there was a greater variation in the amount of charcoal delivered to the vials when whole charcoal or large particles were used, compared with small particles (Fig. 4).

Evaluation with explants

Media with different AC and EC formation

Plumule explants cultured in medium with any of the ACs presented a series of developmental changes during the time period studied (90 days). In responding explants a callus formed after 45 days of culture showing the formation of ear-shaped, translucent structures (Fig. 5a) as is referred to as initial callus. After 90 days, calluses were compact, smooth and easily distinguishable with small structures that

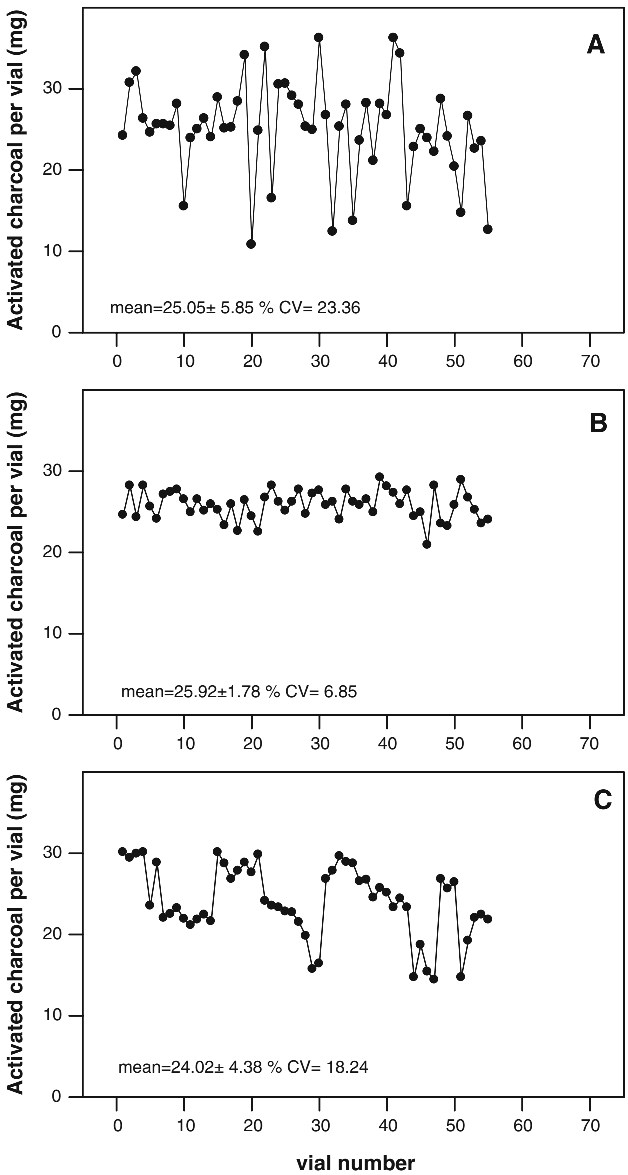

Fig. 4 Charcoal (PCCT, Sigma) content per vial of culture medium after dosing with different particle size fractions: **a** whole charcoal; **b** small particle fraction; and **c** large particle fraction

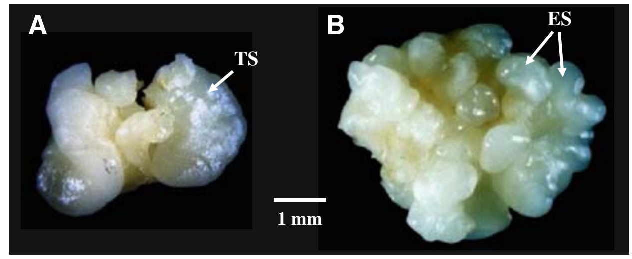

Fig. 5 Callus formation from explants. **a** Callus at 45 d, exhibiting translucid structures [TS]. **b** Fully developed embryogenic callus at 90 d, with embryogenic structures [ES]

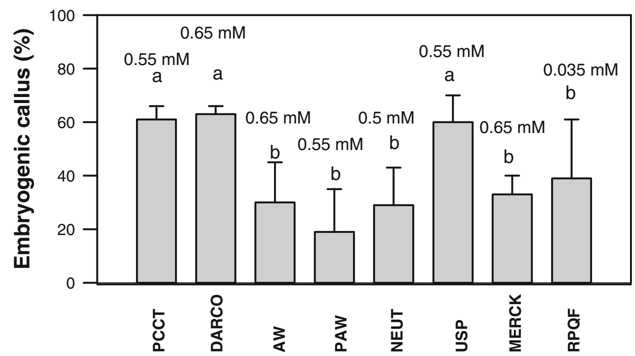

Fig. 6 Embryogenic callus formation rate (%) in culture media containing different activated charcoal types, showing optimum auxin concentration (mM). Means ($n = 3$; 15 plumule explants per replicate) with different letters are significantly different ($p < 0.05$)

appeared on the surface of the translucent structures. Initially these structures were globular and then they elongated and are referred to as embryogenic structures since somatic embryos formed later on from them (Fig. 5b). The callus at this stage is referred to as embryogenic callus (Fig. 5b).

The frequency of EC formation in explants cultured in media with different ACs was determined using 2,4-D at concentration ranges defined according to measured the medium free auxin level for each ACs (Table 2). Optimum auxin concentration for EC formation is presented in Fig. 6. Embryogenic callus formation ranged from 20% in medium with PAW AC to 60% or above in media with USP AC, DARCO AC or PCCT AC. Optimum auxin concentration was 0.035 mM for RPQF AC medium, contrasting with the 0.50–0.65 mM concentrations for the rest of the ACs; a greater than ten-fold difference generally consistent with the differences in the auxin adsorption capacity of ACs (Table 2).

AC particle size and EC formation

For this evaluation, we did not use plumule but embryogenic structures derived from embryogenic callus multiplication. The reason for this is that the when the present study started we were working with the formation of embryogenic callus from plumule explant only. Then by the end of the study the multiplication of embryogenic callus using embryogenic structures as explants was already established (Pérez-Nuñez et al. 2006) so there was particular interest in carrying out the last evaluation using this more advanced system for the micropropagation of coconut. Embryogenic structure explants presented an identical development of embryogenic callus to the one observed for plumule explants as previously reported by (Pérez-Nuñez et al. 2006).

Then embryogenic structures derived from the process of embryogenic callus multiplication were cultured 90 d as

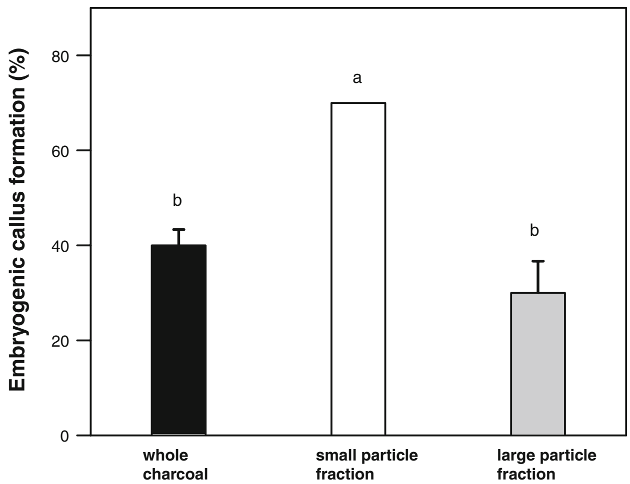

Fig. 7 Effect of activated charcoal (PCCT, Sigma) particle size fraction on embryogenic callus formation rate (%) with 0.5 mM 2,4-D. Means ($n = 3$; 30 embryogenic structures explants per replicate) with different letters are significantly different ($p < 0.05$)

explants in media containing small particle, large particle and whole PCCT AC added with 0.5 mM 2,4-D to test EC formation. The results showed that when small particle AC was used 70% ± 0 of the explants formed EC, whereas with large particle AC and whole AC 30% ± 7 and 40% ± 4 of the explants formed EC, respectively (Fig. 7). Differences were significant at $p < 0.05$.

Discussion

Activated charcoal has been included in the formulation of in vitro culture protocols for several plant species (Pan and van Staden 1998), primarily to avoid or retard browning (Verdeil and Buffard-Morel 1995). This is the case of protocols for coconut regeneration through somatic embryogenesis using plumule explants (Chan et al. 1998) and embryogenic structure explants (Pérez-Nuñez et al. 2006). AC used for in vitro culture is produced using different carbonaceous materials, such as wood, wood waste, paper mill waste liquors and peat (Pan and van Staden 1998) and processed in different ways (e.g. acid-washing) to meet certain requirements (e.g. chromatography), which confers different characteristics to the AC that could affect the medium culture properties and the growth and morphogenic response of in vitro cultured explants. Both types of effects are characterized here for eight different types of AC used for in vitro culture of coconut explants.

The present results demonstrate that the eight tested ACs modified medium pH and conductivity when added to culture medium. Individually, some ACs had a greater effect on these parameters than other. For instance the greatest change in pH was observed with PAW and the greatest change in conductivity with MERCK and RPQF. Therefore that we can conclude that the addition of AC to the culture medium do change some of its properties, in this case most particularly the pH. This is interesting since previous studies have revealed that precipitation of irons was pronounced in liquid medium when pH was higher than 5.8 resulting in 50% reduction of Mn and Fe irons, 20% reduction of Ca irons and 15% reduction of P irons (Van Winkle and Pullman 2003). In addition, the profile of particle size of each of the ACs was determined and there was a clear variation, but in all cases the majority of the particles were smaller than 38 µm and the less abundant particles were larger than 100 µm, however some charcoals as PAW, NEUT and USP presented higher amounts of particles larger than 100 µm particle than the other ACs. Considering these differences in profile, the adsorption capacity of the ACs was also studied by measuring free-^{14}C-2,4-D in the culture media prepared with each of the ACs. This is based in previous study demonstrating that 2,4-D remains stable after autoclaving (Berthon et al. 1991), and therefore measured radioactivity is equivalent to 2,4-D content and not to degradation products. The results showed differences in the 2,4-D adsorption capacity among the different ACs, this is particularly relevant since one of the most important factors affecting morphogenetic response in in vitro cultured coconut explants is the presence of this auxin in an optimum concentration (Ebert and Taylor 1990; Verdeil and Buffard-Morel 1995).

Therefore, it was expected that the differences in the way each AC changed the parameters studied could lead to differences in the way they could affect a morphogenic response of tissues cultured in media prepared with them. When media prepared with the different ACs was tested with plumule explants, indeed differences were observed in the formation of EC, some doing better than other: AC-USP, AC-DARCO and AC-PCCT were best. The last has been used routinely in our laboratory for the past five years in the development of coconut micropropagation protocols, however, these results also indicate that the DARCO and USP ACs are could be convenient alternative sources. Previous unpublished experiences in our laboratory using AC-PCCT have shown that differences in the morphogenic response can be found from batch to batch, but these can be overcome by defining optimum concentration of 2,4-D for each batch. Therefore this practice is recommended whenever a new batch is used and also when changing to a new source.

Finally, at the end of the present study another aspect of AC properties was studied: a relationship between particle size and embryogenic callus formation. When medium containing charcoal is dispensed to a batch of vials, the AC-medium suspension has to be agitated because AC particles are constantly sedimenting. We were interesting in testing if the amount delivered to each vial is the same or not. This evaluation was carried out with only one AC, PCCT for the reason mentioned above. AC delivered to vials along the dispensing procedure when using media prepared with whole charcoal, small particle charcoal and large particle charcoal. There was a variation in the amount of charcoal delivered to the vials that was larger when whole charcoal or large particles were used, and smaller with small particles. This finding is important because a the greater the variation in the amount of AC delivered to a vial, the greater the variation in the amount of 2,4-D and in the morphogenic response expected for explants cultured in the vials of the batch. Analysis of the EC formation showed a higher percentage of EC was obtained with small particle AC than with whole AC, a no variation between replicates with small particle AC, whereas as large variation was observed with whole AC. Therefore the more homogeneous delivery of the amount of AC (and assumed 2,4-D) per vial in a batch dispensed with medium prepared with small particle AC, seems to translate in improved performance in the morphogenic response of explants. The findings reported here can be directly incorporated into the propagation scheme proposed by Pérez-Nuñez et al. (2006) for coconut, which includes a series of EC multiplication steps (combined with secondary somatic embryogenesis). Incorporation of smaller AC particles into the culture medium would make this protocol much more efficient due to its cumulative nature.

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