Effect of 2,4-Dichlorophenoxyacetic Acid and Activated Charcoal on Somatic Embryogenesis of *Bactris gasipaes* H.B.K[1]

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ABSTRACT

Somatic embryogenesis of the pejibaye or peach palm (*Bactris gasipaes* H.B.K.) was induced by 2,4-dichlorophenoxyacetic acid (2,4-D) *in vitro* in a modified Murashige and Skoog (MS) medium. Callus cultures derived from shoot tips of greenhouse-grown seedlings produced the most somatic embryos in medium with 100 mg/l 2,4-D, 2.5 g/l activated charcoal, and 10% (v/v) coconut water. Maturation and germination of somatic embryos occurred when embryogenic calli were transferred to a modified MS medium without 2,4-D. The most prolific seedling explant produced 93 plantlets, mostly from easily separable somatic embryos after three months of callus induction under 24 h of darkness and four additional months of embryo maturation and germination in a 16 h-lighted growth chamber.

COMPENDIO

El ácido 2,4-diclorofenoxiacético (2,4-D) indujo embriogénesis somática en pejibaye (*Bactris gasipaes* H.B.K) *in vitro*, sobre un medio Murashige y Skoog (MS) modificado. Los cultivos de callo fueron derivados de ápices caulinares de plantas germinadas en invernadero. La mayoría de los embriones somáticos se generaron sobre un medio con 100 mg/l de 2,4-D, 2.5 g/l de carbón activado y 100 ml/l de agua de coco. La maduración y germinación de los embriones somáticos se dieron cuando los cultivos fueron transferidos a un medio MS modificado sin fitohormonas, carbón activado o agua de coco. Del cultivo más productivo se obtuvieron 93 plántulas, la mayoría de ellas provenientes de embriones somáticos fácilmente separables, generados tras un período de inducción de callo de tres meses a la oscuridad, seguido de un período de maduración y germinación de cuatro meses adicionales, a 16 h diarias de luz artificial en una cámara de crecimiento.

INTRODUCTION

The pejibaye or peach palm (*Bactris gasipaes* H.B.K.) is an important crop in limited areas of Central and South America and is used especially for fruit and heart of palm production (8). Pejibaye has many uses, both human and animal, but is a crop for which the full potential has yet to be realized (5). One way of increasing its use is to grow superior genotypes with uniformly high yields. At present, yields are very variable because the palm has a strong mechanism of self-incompatibility, and plants are grown primarily from heterozygous seedlings (6). Clonal propagation of superior genotypes by rooting basal offshoots has yielded discouraging results (1, 7), whereas *in vitro* clonal propagation is a promising alternative that has recently been demonstrated experimentally (2, 3, 10, 11, 14, 15).

Previous research indicated that the best embryogenic responses in pejibaye callus cultures could only be obtained with picloram (4-amino, 3,5,6-trichloropicolinic acid), a stronger auxin than the commonly used 2,4-dichlorophenoxyacetic acid (2,4-D) (14). Previous experiments with 2,4-D have yielded only mixed results, although sporadic cultures with very low embryogenic regeneration rates have been observed (3, 10, 11). Because of the widespread effectiveness of 2,4-D for inducing somatic embryogenesis in crop plants, including palms (13), the ease with which it can be obtained for laboratory use, and prospects that somatic embryogenesis could

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generate large numbers of propagules, the researchers wanted to persist in testing the effectiveness of 2,4-D at high concentrations, a condition that apparently needs to be met in this species, to maximize possibilities of obtaining somatic embryogenesis (2, 10, 11). The effectiveness of activated charcoal (AC) and coconut water (CW) in the callus-induction medium was also tested, as previous research indicated beneficial effects of AC in preventing browning of tissue cultures (16), and CW has been reported to enhance culture growth (4). Both additives have previously been used in pejibaye tissue culture research (10, 11, 14, 15).

MATERIALS AND METHODS

Seedlings were imported from Costa Rica when they had just emerged from the seeds, and were grown in individual pots in a greenhouse at 27°C. No artificial light was used. The seedlings were watered every other day and fertilized every other week with a soluble greenhouse fertilizer containing 200 ppm N.

Explants were obtained by cutting off the roots, foliage, and part of the stem of the palm seedlings until 9 cm stem segments were left, which included the broad base of the seedling. These segments were rinsed several times to remove the soil. The outermost tissue layer, i.e. the base of a leaf sheath, of each stem segment was peeled off, and the stem segments were subsequently surface-sterilized by immersing them in 70% ethyl alcohol for 1 minute. This step was followed by a transfer of the stem segments into a 30% (V/V) chlorine bleach solution (1.5% NaOCl), which also contained two drops of the surfactant "Tween 80". Immersed explants were shaken continuously for 15 min, after which they were rinsed four times in sterile deionized water under aseptic conditions. All further dissection steps were carried out aseptically. Two more tissue layers or leaf sheath bases were then removed from the segments, which left stem sections formed by the inner two or three layers of leaf-sheath tissue enclosing the leaf primordia, with the broad stem base still attached at the proximal end, where the roots had been attached.

To obtain the shoot tips, stem segments were held by hand at their distal end, where the foliage was originally attached, and thin slices were cut from the broad stem base. Contamination was avoided by holding the distal end outside the Petri dish while resting the proximal end inside the Petri dish in which the dissection was carried out. After a few slices had been removed from the broad stem base, several concentric "rings" became visible in the stem tissue. This indicated that the cuts had reached an area very close to the shoot tip. At this point a circle was cut into the stem tissue following the contour of the innermost "ring" visible in the center of the cut stem surface. By squeezing the base of the stem section gently between two fingers, the shoot tip, with a thin layer (1 - 2 mm) of stem tissue still attached at its base, was ejected from the stem section without suffering any visible damage. The shoot tips, which were 2 - 3 mm long, were placed vertically on semi-solid medium, analogous to their natural orientation in the seedlings. With this method of dissection, 90% of initiated cultures remained uncontaminated by bacterial or fungal growth during the experiment.

The basal culture medium was composed of MS salts (7), to which were added (in mg/l): myo-inositol (100), thiamine-HCl (0.1), pyridoxine-HCl (0.5), nicotinic acid (0.5), benzyladenine (5), and sucrose (30 g/l). To this basal medium was added 0, 25, 50, 75, or 100 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and either 0 or 2.5 g/l activated charcoal (AC), and 0 or 10% (v/v) coconut water (CW). There were 18 treatments (medium combinations poured into individual Petri dishes), with nine replications (shoot tips) per treatment and one shoot tip per Petri dish. Of the nine shoot tips per treatment, 1/3 came from 4-month-old seedlings, and 2/3 from 18-month-old seedlings. The pH of each of the 18 media was adjusted to 5.8, and 2 g/l Gelrite was added, after which the media were autoclaved 15 min at 1.1 kg/cm^2 and 121°C. Subsequently, 15-ml aliquots of each medium were dispensed aseptically into sterile, disposable 6 cm Petri dishes.

The cultures were placed in a dark growth chamber at 24°C - 27°C. Explants were kept on this callus-induction medium with each respective 2,4-D, AC, and CW concentration for three months, after which all explants, whether callused or not, were transferred to a regeneration medium, consisting of the basal MS medium without the addition of phytohormones, AC, or CW. All cultures were incubated on this medium in a growth chamber at 24°C - 27°C under a 16 h photoperiod at 45 μmol/m^2/s for nine months, after which final data were taken. All media were pH-adjusted, solidified, and autoclaved as described above. Cultures were

transferred to fresh media every six weeks throughout the experiment.

RESULTS AND DISCUSSION

Multiple plantlets were produced at a 2,4-D concentration of not less than 75 mg/l, despite the fact that in the induction phase of the experiment, good callusing had been observed at 2,4-D levels as low as 25 mg/l without AC in the medium. The effective concentration of 2,4-D may have been much less than 75 mg/l or 100 mg/l in the cultures that produced multiple shoots and plantlets, because of the presence of AC. Weatherhead *et al.* (17) found that AC can adsorb auxins from the culture medium, thus lowering their effectiveness for callus initiation, and in some cases even making them completely unavailable. This might explain why cultures with 25 mg/l and 50 mg/l 2,4-D and 2.5 g/l AC in the medium produced no calli at all, whereas the same 2,4-D concentrations without AC did produce calli. At 75 mg/l 2,4-D, none of the cultures produced calli, whether AC had been added to the medium or not. Only the medium with AC and CW produced multiple plantlets.

On the medium with AC but without CW only three shoot tips developed into single-shoot plantlets. All others failed to respond. Multiple shoots and plantlets were produced in medium with 100 mg/l 2,4-D plus AC, whereas without AC, no callus was formed and no plantlets were regenerated (Table 1). The explants on 75 mg/l and 100 mg/l 2,4-D without AC discolored and died, suggesting that 2,4-D was toxic at those concentrations and AC was perhaps necessary to effectively lower such high 2,4-D concentrations. Another possibility is that 2,4-D interacts negatively with substances secreted into the medium by the explant, and the addition of AC prevents this. Minor browning or oxidation of the callus tissue was observed in some of the cultures after transferring them to light, but we did not

Table 1. Effect of 2,4-D, activated charcoal, and coconut water in the callus-inducing medium on subsequent plantlet production (on medium without 2,4-D, charcoal or coconut water). All media treatments in the callus-induction phase contained 5 mg/l BA. There were nine replications per treatment.

2,4-D concentration mg/l	Activated charcoal 2.5 g/l	Coconut water 10% (v/v)	Callus produced	Number of cultures that regenerated shoots and plantlets	Number of cultures with more than 10 shoots and plantlets	Total number of shoots/plantlets regenerated
0	no	yes	no	0	0	0
	no	no	no	0	0	0
25	yes	yes	no	0	0	0
	yes	no	no	0	0	0
	no	yes	yes	0	0	0
	no	no	yes	0	0	0
50	yes	yes	no	0	0	0
	yes	no	no	0	0	0
	no	yes	yes	0	0	0
	no	no	yes	0	0	0
75	yes	yes	no	2	0	6
	yes	no	no	3	0	3
	no	yes	no	0	0	0
	no	no	no	0	0	0
100	yes	yes	yes	5	2	123*
	yes	no	yes	5	2	57**
	no	yes	no	0	0	0
	no	no	no	0	0	0

* One genotype alone (seedling 266Y, see Table 2) accounted for 76% of all plantlets produced

** Seedling 192Y (see Table 2) accounted for 54% of all plantlets produced

consider the browning to be as severe as have previous researchers (2, 12, 14). AC had no discernable effect on prevention of browning of explants in our study.

An equal number of 4-month-old and 18-month-old explants produced multiple (two or more) shoots and plantlets, but of the four cultures from which more than 10 plantlets were regenerated, three were derived from shoot tips of four-month-old seedlings. The most productive culture, which produced 93 plantlets, was not only derived from a four-month-old seedling, but was also grown on a medium containing CW (Table 2). Although too few cultures produced multiple shoots or plantlets to carry out an analysis of variance, it appears that young age and the addition of CW to the medium have a positive effect on embryogenesis. In a related experiment it was found that there is a statistically significant positive effect of young age on the production of callus in pejibaye, and that CW enhances callusing in young tissue (12).

Table 2. Itemization of individual shoots or plantlets produced by genotypes (explants) which showed regeneration *in vitro* after at least seven months in culture: three months in the dark on a given induction medium, and at least four months under 16 h light on a basal maturation/germination medium devoid of growth hormones, activated charcoal (AC), and coconut water (CW).

Medium*	Genotype**	Age (in months)	Number of shoots/ plantlets produced
A	177SP	18	1
	179SP	18	1
	224SM	18	1
B	273Y	4	4
	275Y	4	2
C	192Y	4	31
	193Y	4	19
	194Y	4	2
	225SM	18	2
	226SM	18	3
D	247SP	18	4
	248SP	18	18
	266Y	4	93
	288SM	18	3
	290SM	18	5

* The media specified here are the induction media on which the explants were originally grown. All were modified MS media with 5 mg/l BA.
Medium A contained 75 mg/l 2,4-D, 2.5 g/l AC
Medium B contained 75 mg/l 2,4-D, 2.5 g/l AC and 10% (v/v) CW.
Medium C contained 100 mg/l 2,4-D, 2.5 g/l AC
Medium D contained 100 mg/l 2,4-D, 2.5 g/l AC and 10% (v/v) CW

** The genotype number is an arbitrary number assigned by the researchers to each explant for record-keeping purposes. Explants were usually numbered in the order in which they were dissected and plated onto the medium. Each genotype number was a single shoot tip extracted form a single seedling.

The study suggests that a callus induction period of only three months in the dark suffices to induce embryogenesis. Valverde *et al.* (14) incubated pejibaye cultures for a total of six months in the dark and did not obtain higher embryogenesis rates. In fact, the variation in numbers of plantlets from different genotypes produced, under the conditions of this experiment, and by Valverde *et al.* (14), suggests that genotype is an important variable in number of plantlets regenerated. This should not be surprising, given the reproductive biology and resultant heterozygous nature of this crop (8).

Regeneration of pejibaye *in vitro* via somatic embryogenesis was induced by 2,4-D in at least four genotypes out of the 18 cultured on 100 mg/l 2,4-D and 2.5 g/l AC. After seven months of growth *in vitro* from the time of initiation, most regenerants had grown into individual plantlets, which could be separated easily from one another (Fig. 1). These somatic embryos had a continuous vascular system extending from shoot to root apex (Fig. 2), demonstrating a common feature of somatic embryos. Some of the regenerants grew together in clumps (Fig. 3). Many of these resulted from embryogenic structures, but some may have developed through organogenesis. The development of multiple shoots and plantlets through direct organogenesis is a phenomenon which has been previously documented

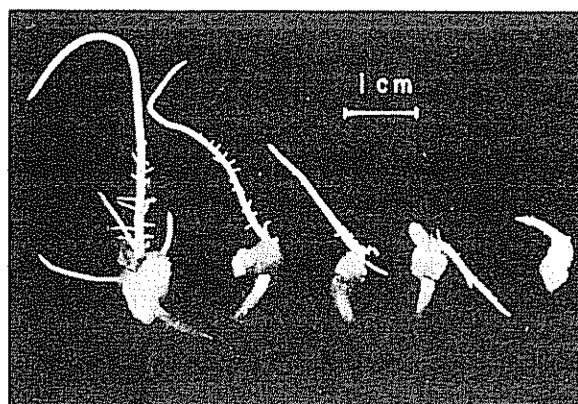

Fig. 1. Somatic embryos produced from callus culture of genotype 266Y four months after removal of 2,4-D from the culture medium.

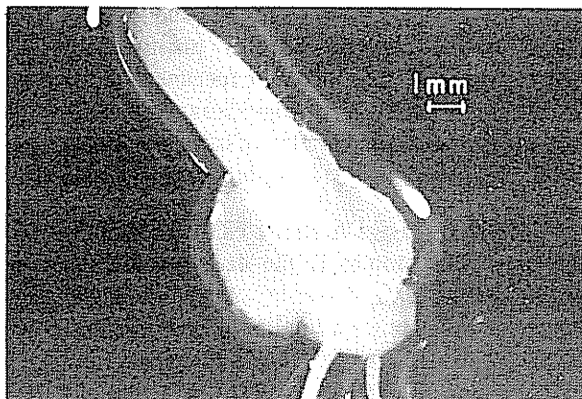

Fig. 2. Longitudinal section through one of the somatic embryos shown in Fig. 1, arising from a callus culture of genotype 266Y, showing a continuous vascular system from shoot apex (upper left) to root apex (lower right), with no attached callus.

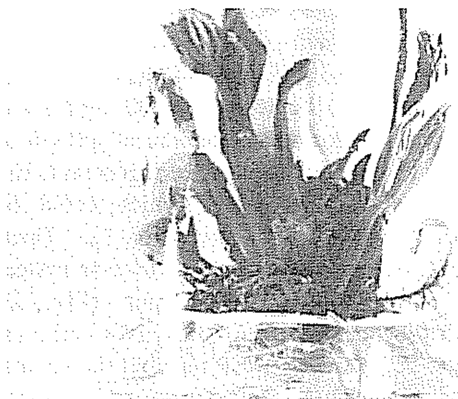

Fig 3. Pejibaye plantlets four months after removal from callus inducing medium.

in pejibaye tissue cultures (2, 10, 11, 14). Pinedo Panduro (10) proposed that multiple shoots may arise through organogenesis from pre-existing axillary buds, or from a *de novo* formation of such buds.

In our study, easily removable somatic embryos were found growing among such plantlet clumps in three cultures. This indicates two possibilities: either the development of somatic embryos in our cultures was asynchronous and embryos continued to develop among older somatic embryos, or somatic embryogenesis and direct organogenesis occurred simultaneously in a given culture. No embryogenic structures were observed in any of the cultures which produced fewer than 10 regenerants, so it is therefore assumed that they went through a strictly organogenic regeneration process.

CONCLUSION

The use of 2,4-D to stimulate embryogenic callus, followed by the maturation and germination of somatic embryos has the potential for use as a clonal propagation method. The main obstacle still remaining is the random rate at which embryogenesis occurs. Somaclonal variation cannot be ignored where a callus phase is involved, but the natural seedling variation is so great that, comparatively, somaclonal variation may not be important. It has been shown here that somatic embryogenesis may be induced by 2,4-D in callus from seedling explants. Recently, Salazar (11) showed that 2,4-D may also induce somatic embryogenesis on explants derived from offshoots of mature pejibaye palms, indicating that, in the future, superior genotypes may be clonally propagated in large numbers from mature plants despite the apparent advantage of seedling tissue to produce regenerants. If tissue culture techniques are improved so that shoot tips from older plants propagate as well *in vitro* as those from very young plants, adult pejibaye palms with good agronomic traits and a genetic propensity for somatic embryogenesis could be reproduced in very large numbers. This would be facilitated through a combination of two factors: the ability of some plants to form somatic embryos and the pejibaye palm's natural production of numerous basal offshoots in the field. The latter are essentially clones and can yield many shoot tips without sacrificing the mother plant.

LITERATURE CITED

1. ARIAS, O. 1979. Efecto del flurenol en la producción de hijos laterales de pejibaye (*Bactris gasipaes* H.B.K.). Agronomía Costarricense 3:47-52.

2. ARIAS, O.M.; HUETE, F.V. 1983. Propagación vegetativa *in vitro* de pejibaye (*Bactris gasipaes* H.B.K.). Turrialba 33(2):103-108.

3. CHAVERRI JIMENEZ, Y. 1984. Efecto de diferentes fuentes y niveles de nitrógeno en el medio nutritivo para el cultivo *in vitro* de ápices de pejibaye (*Bactris gasipaes* H.B.K.). Tesis M.S. San José, C.R., Universidad de Costa Rica. 63 p.

4. ERDELSKY, K.; CHOLVADTOVA, Z. 1970. Wirkung von Kokosmilch und Hefeextrakt auf das Wachstum von Gwebekulturen der Tabakpflanze *Nicotiana tabacum* L. Acta Fac. Rerum. Nat. Comenianae Physiologia Plantarum. 1:99-116.

5. HUNTER, R.J. 1969. The lack of acceptance of the pejibaye palm and a relative comparison of its productivity to that of maize. Economic Botany 23:237-244.

6. MORA-URPI, J. 1983. El pejibaye (*Bactris gasipaes* H.B.K.): Origen, biología flora y manejo agronómico. **In** Reunión de Consulta "Palmeras poco Utilizadas de América Tropical" (1983, Turrialba, C.R.). San José, C.R., FAO/CATIE. v.43, p.118-160.

7. MORA-URPI, J.; SOLIS, E. 1980. Polinización en *Bactris gasipaes* H.B.K. (Palmae). Revista de Biología Tropical 28:153-174.

8. MORA-URPI, J.; VARGAS, E.; LOPEZ, C.A.; VILLAPLANA, M.; ALLON, G.; BLANCO, C. 1984. The pejibaye palm (*Bactris gasipaes* H.B.K.). San José, C.R., FAO. 16 p.

9. MURASHIGE, T.; SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

10. PINEDO PANDURO, M.H. 1987. Organogénesis directa en apices caulinares de pejibaye (*Bactris gasipaes* H.B.K.). Tesis M.S. Turrialba, C.R., Centro Agrónomico Tropical de Investigación y Enseñanza. 110 p.

11. SALAZAR, S.S. 1986. The effect of 2,4-D, light and activated charcoal on offshoot-tip culture of the pejibaye palm (*Bactris gasipaes* H.B.K.). M.S. Thesis. Birmingham, England, University of Birmingham. 70 p.

12. STEIN, K.M. 1988. *In vitro* cultures of the pejibaye palm (*Bactris gasipaes* H.B.K.). M.S. Thesis. Iowa State University, Ames, Iowa, USA. 187 p.

13. TISSERAT, B. 1984. Clonal propagation: Palms. **In** Cell Culture and Somatic Cell Genetics of Plants. K. Indra Vasil. (Ed.). New York, Academic Press. v. 1, p.743-781.

14. VALVERDE, R.; ARIAS, O.; THORPE, T.A. 1987. Picloram-induced somatic embryogenesis in pejibaye palm (*Bactris gasipaes* H.B.K.). Plant Cell Tiss. Organ Cult. 10:149-156.

15. VALVERDE, R.; GOMEZ, L.; ARIAS, O.; THORPE, T. 1987. Respuesta morfogenética de los ápices de pejibaye (*Bactris gasipaes* H.B.K.) cultivado *in vitro* en condiciones de luz y oscuridad. Agronomía Costarricense 11(1):97-102.

16. WANG, P.J.; HUANG, L.C. 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. *In Vitro* 12:260-262.

17. WEATHERHEAD, M.A.; BURDON, J.; HENSHAW, G.G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. A. Pflanzenphysiol. 89:141-147.