FINAL REPORT

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IN VITRO PROPAGATION AND IMPROVEMENT OF FRUIT SPECIES WITH ECONOMIC POTENTIAL FOR SEMI-ARID ZONES

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3) EXECUTIVE SUMMARY

Within the project framework, the morphogenic potential of several types of explants of economically important and promising fruit species for semi-arid regions, Cherimoya (Annona cherimola), Lucumo (Pouteria lucuma), highland Papaya (Carica pubescens) including the Babaco (C. pentagona), was researched.

Briefly, new developments are: for A. cherimola (cherimoya), several morphogenic events could improve mass propagation prospects, plantlet recovery was possible; morphogenesis in vitro occured in hypocotyls, internodes, nodal sections and zygotic embryos, especially by multiple shoot formation in these explants. Responses were enhanced by control of browning of tissues, using combinations of antioxidants. Phenolic content found in tissues and culture media was related to antioxidants and morphogenesis. Seasonal variations of phenolics were also studied in cuttings, to determine the best propagation timing.

In Caricas, several types of explants, including leaf sections, meristems, axillary nodes, and cuttings led to plant regeneration. Somatic embryogenesis and adventive embryogenesis were also achieved in cell suspensions, as well as androgenesis from anthers. Rooting of cuttings was successful in Papaya and Babaco. Preliminary characterization of Papaya germplasm by means of isozymes was initiated.

Morphogenesis and regeneration were also achieved in several P. lucuma explants, including shoot tips, meristems, epicotyls, leaf sections and zygotic embryos. First stages of androgenesis were observed. Control of browning in vitro was achieved using several types of antioxidants; cell suspension protocols were developped for Lucumo, up to the green calli and proembryo stage. New protocols have been established to scale up production or perform in vitro selection work.

This body of valuable preliminary information will be of interest to scale up methods for future plantations, selection of germplasm and determination of elite cultivar parameters. Modern equipment was brought in for the project; young scientists were trained to use it for potential applications in future work.

The interaction of several scientists within the chilean and american Universities during meetings and training periods was of significant help in linking interests, sharing information, exchanging plant material and publishing several papers on the work performed.

Contacts with individual fruit growers were established and showed that there is a need to receive relevant information for the propagation of these species. Besides scientific information obtained throughout the project, their suggestion to convert data into application guidelines in the form of a booklet would favour propagation scale-up and production of these species.

4) RESEARCH OBJECTIVES

Great interest in little known crops of andean origin has been evidenced lately. Several species are cultivated marginally at present, whilst having a great potential. This point has been extensively illustrated in the book "Lost Crops of the Incas" (National Research Council, 1989).

As an example that could be applied to other sub-tropical countries of the world, Chilean exports of traditional fruit species increased continuously and production has almost quadrupled since the 80's. Future exports are predicated on the exploitation of non-traditional species, cultivated in semi-arid zones, so new or little known species will be brought into new markets.

The initial objectives of this project were to start propagation systems for three semi-arid zone fruit species: Carica pubescens (a higland papaya), Annona cherimola (cherimoya), and Pouteria lucuma. The specific objectives were: development of morphogenic responses in vitro (organogenesis, rhizogenesis, cell and callus embryogenesis, androgenesis) leading to plant regeneration; reduction of in vitro browning problems due to phenolic compounds oxidation; selection and characterization systems to be applied to elite clones.

The selected species are examples of potentially important export products; fruits and fruit-derived products, such as proteolytic enzymes found in papaya are beginning to enter new markets; the availability of plants is critical to meet future demand. Since cultivation is restricted to localized microclimes, information regarding physiology is very scanty. Little basic reseach has been carried out on these species and understanding basic points is necessary and useful to scale up production of plantlets for massive plantations.

Several basic research problems concerning the morphogenic potential exhibited in different explants, useful for further micropropagation and the control of browning, were solved for these three species. Electrophoretic studies of Papaya accessions from a North-South transect of Chile were planned, using material collected during the previous season.

Emphasis was to be placed on the development of plants from cell suspensions, in collaboration with Professor Don Durzan, University of California, Davis. Part of the experiments were started by a postgraduate student, Ms. Maritza Obando, who was trained in Dr. Durzan's laboratory, using plant material brought from Chile. Several metabolical and in vitro culture problems gave the opportunity to train research workers, carrying out pre- and post-graduate work in Santiago.

Several related species are cultivated in tropical or sub-tropical environments and research work is carried out at present in several Carica spp. Interaction with other researchers in this field has been established with Dr. Richard Litz in Florida, in tropical fruit species, chiefly C. papaya; Dr. Manshard in Hawaii working on breeding programs and transformation in this species; Babaco work carried out by Vega de Rojas (Ecuador); Chilean researchers (Universidad de Santiago, and Universidad de La Serena) working on papain, and related enzymes, with a view to export concentrated proteolytic enzymes. Work is done on Babaco and Papaya at the Catholic University of Valparaíso, and the Ministry of Agriculture Experimental Station at La Platina is evaluating the behaviour and physiology of the Babaco. Among the Annonas, morphogenic potential of different Annona squamosa explants was also studied by Nair et al (1983, 1984, 1986) in India. A. cherimola was researched in Spain by Martinez Cayuela et al, 1988 and Pliego-Alfaro (personal comm.). Less work has been carried out on Pouteria spp. Interest in this species provided support from the National Science Committee (CONICYT) to work on several aspects of these and other fruit species, including Solanum muricatum. A list of publications and presentations to meetings is attached.

5) METHODS AND RESULTS.

The morphogenic potential of the species studied, was evaluated testing different explants as listed in Table 5.1. on different basic media (Nitsch (1969), NN; Murashige & Skoog (1962) MS; McCown & Lloyd, Woody Plant Medium (1981) WPM; Gamborg et al, (1968) B6, supplemented with several growth regulator combinations including naphthaleneacetic acid (NAA); indoleacetic acid (IAA); 3-indolebutyric acid (IBA); 2,4-dichlorophenoxyacetic acid (2,4-D); 6-benzyladenine (BA); isopentyladenine (IPA); zeatin (Z); thidiazuron (TDZ) and gibberellic acid (GA3).

Table 5.1.
Morphogenic events obtained in Lucumo, Cherimoya, Papaya and Babaco explants.

Explant	Lucumo	Cherimoya	Papaya/Babaco
Anthers (microspores)	Androgenic embryos, calli	Somatic cells proliferation	Androgenic embryos
Leaves	Shoots, calli	Calli	Calli; shoots in Babaco
Petiole	Shoots and Roots	Multiple shoots.	Calli
Nodal sections	Rhizogenesis	Multiple shoots	Bud sprouting Calli
Internodes	Shoots	Multiple shoots, calli	Calli & somatic embryos
Meristem/axil. buds/shoot-tip	Development and rooting	Development and rooting	Multiple buds and rooting
Pericarp	Callus	not tested	Calli and embryos
Zygotic embryos	Adventitious embryos	Shoots, Calli	Calli & somatic embryos
Hipocotyls/ Epicotyls	Multiple shoots	Multiple shoots	Calli & somatic embryos
Cell suspensions	Cells, calli, proembryoids?	No growth	Calli & somatic embryos in papaya
Cuttings	Only bud development	Only bud development	Roots in both spp. profuse sprouting.

It is assumed that species with an initial low phenols level or an endogenous content reduced during culture, have a good morphogenic potential due to lower phenols oxidation. Browning usually occurred in most explants of A. cherimola and P. lucuma. When comparing leaf phenol content prior and after in vitro culture, changes are related to the metabolism of each species, affecting in vivo or in vitro morphogenic responses. In browning species such as cherimoya, phenol content increases during in vitro culture (as shown in Table 5.2), while other species normally remain green. Yearly variations of phenols affecting explants were observed for the several species studied; a tendency to an increase during the growth seasons is observed. (Table 5.3).

Table 5.2.
Level of Phenols in leaves of some fruit species grown in the field and after tissue culture. (Folin-Ciocalteau reagent; Abs at 730 nm/100 mg of fresh tissue).

Species	Leaf prior to culture*	Cultivated leaf plus calli**	Remarks
A. cherimola	0.1	0.93	Leaf brown, few calli.
P.lucuma	2.0	0.17	Leaf green, calli yellowish.
C. pubescens	0.18	0.02	Leaf/calli yellow, In babaco shoots.
S.muricatum(1)	0.21	0.38	Leaf green, calli, shoots.
A.chinensis(2)	1.00	0.06	Leaf and calli dark green, shoots.
C. pentagona	1.28	0.59	Shoots green, rest of the leaf brown.
C.betacea(3)	0.85	0.25	Shoots and little browning.

(1) Pepino, (2) Kiwi and (3) Tamarillo.* Average of two measurements. ** Measurements after 40 days in culture

Table 5.3.
Seasonal estimation of phenols in cuttings of Lucumo, Papaya and Cherimoya greenhouse plants.

Months 1992	Lucumo	Papaya	Cherimoya	Twig portion
January	1.39	0.90	1.41	apical
January	1.31	0.83	1.02	median
January	—	0.56	0.82	basal
April	—	0.29	1.26	apical
April	—	0.15	0.45	basal
July	0.30	0.13	0.65	apical
July	0.26	0.09	0.90	median
July	0.41	0.07	0.48	basal
October	0.81	0.06	0.47	apical
October	0.38	0.07	0.42	median
December	0.90	—	1.14	apical
December	0.48	—	0.58	median
December	0.40	—	0.51	basal

(Folin-Ciocalteau/ Abs/100 mg of fresh tissue; aver. of three measurements).

A. CHERIMOLA As phenols and browning affect A. cherimola regeneration responses, the effect of different antioxidant compounds to reduce browning in vitro was tested. NN basal medium was supplemented with 0.5 mg/l NAA and 2.0 mg/l BA and various compounds including PVP, ascorbic acid and glutathione which were very effective in reducing browning; DIECA and aminooxyacetic acid were less useful while cysteine and citric acid were not beneficial. Replacing sucrose by sorbitol did not prevent browning, but shoot formation improved. The results are shown in Table 2, Jordan et al, 1991.

Specific hormonal conditions regulating morphogenic responses of A. cherimola were determined in different types of explants, as shown in Table 5.4. Shoot initiation was the most common and abundant response, but rooting was difficult, differing from results reported for other Annona species.

Table 5.4.
Summary of morphogenic responses shown by different types of explants of A. cherimola, according to different growth conditions.

Cherimoya explants	Morphogenic responses	Growth Conditions mg/l
Leaves	Calli, browning	1.0-5.0 Z, 1.0-5.0 IPA 0.1 TDZ.
Petioles *	Multiple shoots.	0.5 NAA, 2.0 BA.
Nodes	Multiple shoots, calli.	0.1-5.0 NAA 0.1 1.0 BA.
Internodes	Multiple shoots calli.	0.5 NAA, 2.0 BA.
Axillary buds	Development	Several hormones.
Hypocotyls	Multiple shoots	0.5 NAA, 2.0 BA.
Shoots (subculture)	Roots	10.0 NAA.
Anthers	Somatic cell proliferation, browning.	1.0 NAA, 1.0 BA.
Zygotic embryos	Shoots, calli.	0.1, 2,4-D, 1.0 BA or 1.0 Z.

Hypocotyls: several treatments induced a significant morphogenic effect leading preferently to multiple shoot formation and development of the new outgrowths, but root formation was scarce (Figs 1,2, and 4). Polarity was a clear effect observed during morphogenesis in explants (Table 5.5). In hypocotyls normally oriented, most shoots formed directly on the distal region; the few roots formed on the proximal end. When placed horizontally, multiple shoots appeared over the whole segment; calli were formed only at the proximal end. Calli also formed at the proximal end of explants cultured in inverted position; this tissue was green and compact developed multiple shoots (Figs.5, a-d). Also, when subculturing shoots in darkness – in a higher concentration of NAA (results not shown) – roots formed only occasionally. Paclobutrazol has been used for rooting in several species, and was tested in shoots formed on hypocotyls. Although rooting was still unsuccessful, the use of paclobutrazol had three important effects on growth response: favored bud development, leaf expansion and reduced browning . All treatments maintained tissue green and healthy for five weeks. Shoot growth was affected by a level of paclobutrazol above 1.5 mg/l, as seen in Table 5.6. Apparently dishes sealed with Paraplast, having less oxygen available, limited polyphenoloxidase action.

Table 5.5.
Morphogenic responses of A. cherimola hypocotyl sections according to explant position.

Morphogenic responses	Horizontal %	Vertical Normal %	Vertical Inverted %
Multiple small shoots over the whole explant	50	20	0
Shoots formed only on proximal region	4	0	40
Calli formation on proximal region	32	40	80

Results after 40 days in culture as average of 40 hypocotyl/treatment (Petri dishes).

Table 5.6.
Morphogenic responses of A. cherimola shoots supplemented with paclobutrazol.

Paclobu-trazol (mg/l)	% Brown Tissue	% Callus formation (proximal)	% Bud elongation	% Leaf elongation	X Leaf length (cm.)
0.1	25	0	0	100	2.1
0.5	0	25	50	100	1.6
1	0	50	50	100	2.1
1.5	0	50	0	100	1.45

10 Petri dishes/treatment, 35 days in culture. Aver. of 40 shoots/treatment.

Axillary buds (undevelopped 2 mm long buds) excised in April (Fall) were tested versus different combinations of Z, BA, K, or TDZ/IAA on NN medium, and evaluated up to July. Buds developped in presence of NAA/TDZ initiating new axillary buds and a small callus at the base, without roots; finally browning occured (Fig. 6 and 7, Table 5.7).

Table 5.7.
Morphogenic responses of cherimoya undeveloped axillary buds in different growth regulator combinations.

Treatments		Results					
		Callus		Axillary Growth			
Growth.reg. (mg/l)		%	Average diameter (cm)	%	Average number shoots	Average length (cm)	Brown %
NAA	TDZ						
1.0	0.1	62.5	0.60	–	–	–	56.3
0.5	0.1	41.2	0.20	5.9	1	0.20	88.23
0.1	0.1	47.1	0.40	5.9	1	0.30	76.47
0.05	0.1	57.1	0.30	–	–	–	100
0.01	0.1	47.1	0.20	–	–	–	88.23

Average of 20 axillary buds

Nodal sections from one year old greenhouse plants were cultured on MS medium including several growth regulators (Table 5.8). Shoot formation occurs along the explant in presence of IBA, but no rooting was found after 40 days. Without subculture of excised shoots, new shoots turn brown after 60 days.

Table 5.8.
Effect of IBA and IAA on Cherimoya nodal sections grown in vitro.

Hormone mg/l	% Proximal Calli	% Browning	% Bud Development	% New Shoots
IBA				
1	3	22.5	58	9.6
2.5	25	33.3	–	4.1
5	50	54	–	–
IAA				
1	20	15	–	–
2.5	4.7	71	–	–
5	–	56.5	–	–
NAA/K				
3.0	0.01	87.5	12.5	100
1	0	100	80	80
0	1	100	0	75

Petioles. In this type of explants, as in hypocotyls, direct (no callus involved) shoot initiation with further development of the new shoots, was achieved (Figs. 8 and 9). In regions nearer the leaf lamina, embryogenic callus were more frequent, also deriving into shoots. As indicated above, paclobutrazol favored leaf expansion (Fig. 10).

Shoot-tips were also tested in combination of 0.6 mg/l IBA and 1 mg/l paclobutrazol and subcultivated on MS jelled with agarose and supplemented with IBA 0.3 and 15 mg/l calcium pantothenate. This treatment promoted elongation but had no effect on root formation. Isolated tips turned brown but after a time new shoots were formed on this tissue (Fig.11).

Zygotic embryos. To obtain adventitious somatic embryos from zygotic embryos, flowers excised 30, 40 and 60 days after anthesis were stored during 5 or 15 days at 4ºC, then cultured in darkness for 3 weeks on MS (macro half-strengh) or White-media, later in light. The whole seed was used for 30 and 40 days stages; and the half-seed for embryos at 60 days development. Embryos incubated 5 days (30 days) showed callus formation derived from endosperm; the best callus initiation occurred in MS-medium with 2,4-D 2.5 mg/l plus BA 0.1 mg/l, but browning was frequent. In assays with 15 day pretreatment at 4ºC for stages 30 and 60, a similar tendency was observed as regards browning, without calli development (Table 5.9, Figs. 12 and 13).

Table 5.9.
Response of immature A. cherimola zygotic embryos cultivated at three developing stages (30, 40 and 60 days after anthesis).

	Days after anthesis 30		40			60
Medium	Brown	Callus	Brown	Swell.	Callus	Brown
WH						
2.4D+BA 2.5+ 0.1	76.5	3.5	100	–	—	100
MS						
2.4D 0.1	87.5	12.5	94.1	–	5.9	100
2.4D 2.5	87.5	12.5	64.1	–	17.6	100
2.4D 5.0	73.3	26.6	87.5	6.3	6.3	100
2.4D+BA 2.5+ 0.1	64.7	35.3	94.1	–	—	100

Pretreatment 5 days 4ºC. Aver. of 20 zygotic embryos.

Germplasm collection: Seeds from different varieties grown at the Botanical Garden of the Univ. de La Serena were collected. These correspond to old clones or selections not used commercially now but represent useful material for future breeding programs. Fruit shapes of some selections are shown in Fig. 14.

P. LUCUMA. Positive responses of different Lucumo explants tested have been reported (Jordan and Oyanedel, 1982, Table 1 of reprint attached). Several morphogenic responses were found in most explants tested.

Leaf sections of Lucumo were cultivated on a modified WPM medium in different treatments with TDZ, or in combination with IAA. Explants were kept in the dark during 4 weeks, and then in 500 lux lighting. Shoot formation was induced; best treatments appear to be at the lowest TDZ concentration tested in combination with IAA (Table 5.10). However, unless the petiole is attached, leaf section responses are poor (Figs.15-17).

Table 5.10.
Lucumo leaf section responses.

Growth Regulator (mg/l)		Responses IAA 0.0				IAA 0.1		
TDZ	n	%Callus	%Brown	%Shoot	n	%Callus	%Brown	%Shoot
0.1	15	86.6	40.0	—	7	71.4	42.9	*
0.5	12	75.0	0.0	—	7	71.4	57.1	—
2.5	13	84.6	15.3	—	6	100	0.0	—
5.0	14	92.9	42.9	—	8	62.5	0.0	—
7.5	14	64.3	0.0	—	7	100	57.1	—
10.0	14	71.4	0.0	—	6	100	50.0	—

* Occasional events.

Petiole explants. A higher shoot formation percentage was achieved when petioles attached to leaf portions were used. Triggering conditions are given in Jordan and Oyanedel, 1982; Table 1. This effect ressembles responses of A. cherimola petioles. Plantlets were obtained from a petiole explant, forming shoot and roots in presence of TDZ and naphtalenacetic acid (Fig.18). Besides, other axillary buds develop at the base of the new shoot (Fig. 19).

Shoot-tips perform rhizogenesis when excised from 2 or 3-year old greenhouse trees and placed on Petri dishes with MS, WPM or NN media and growth regulators (Fig.20). However similar conditions tested adding paclobutrazol (Table 5.11) promoted bud and leaf elongation only.

Table 5.11.
Morphogenic responses of P. lucuma shoots in media supplemented with Paclobutrazol

Paclobu-trazol (mg/l)	% Brown Tissue	% Callus Formation Elongation	% Bud Elongation	% Leaf (cm.)	X Leaf length
0.1	0	0	50	80	0.8
1.0	0	0	100	50	0.5
1.5	0	0	0	100	1.2

40 days in culture (darkness). Aver. of 10 shoot-tips/treatment.

Axillary buds. Effect of branching and rooting affected by hormonal control was tested; different combinations of Z, BA, K, or TDZ/IAA, IBA were evaluated. The explants used were undeveloped axillary buds (2 mm) excised in March (fall) and evaluated up to June. The general response of this material entering dormancy or in recess was mainly callus development over the whole explant, the biggest being 0.9 cm in diameter. Only three combinations of K and IAA promoted growth without rooting, the percentage of browning was high, sometimes involving 100% of the sample (results not in Table). Some cells turned completely red, and by isolation, formed a callus line with intense anthocianin pigmentation.

Epicotyls and Nodal Sections. Multiple shoots can arise from an epicotyl section, as shown in cross section (Fig.21); polynodal sections, when cultivated in liquid medium, initiate axillary bud sprouting (Fig.22).

Micrografting. Seeds were sliced and the embryo cultured in nutrient media. When the apical bud reached 1 cm and had developed leaves (about 30 days), it was cut, and an inverted T cut was made. For the graft itself, buds from 2-3 year old greenhouse trees were excised and cultivated separately in a MS-medium without growth regulators following the method of Navarro (1992) and set in vitro for five days, to avoid infection. Then, they were placed in a BA 0,6 mg/l solution for 30 minutes, so that the scion should hold fast to the rootstock. Two treatments were assayed; basal medium plus 100 mg activated charcoal and 1g/l PVP 360 to prevent browning, or plus 500 mg/l hydrolized casein (acid). No browning was apparent in the first treatment, although a slower development was observed as compared to the second medium; the addition of casein allowed a larger development, but browning occurred. In time, grafting showed not to be useful: although growth of both explants was evident in vitro (Fig.23), wound tissue turned brown and vascular connections did not develop properly.

Anther culture. Androgenic proembryoids were obtained cultivating anthers after a cold temperature pretreatment and appropiate growth regulators. Table 5.12 summarizes results for the three species studied in this project. For lucumo, the cold pre-treatment consisted of 8 days at 8ºC, and is needed to start differentiation. Figs 24-30 show a sequence of androgenic events leading to pollen-derived lucumo proembryos.

Table 5.12
Growth conditions for androgenesis in anthers of Papaya, Lucumo and Cherimoya.

Responses/Conditions	Papaya	Lucumo	Cherimoya
Androgenesis (androgenic embryos)	6.5%	3.7%	No response
Calli	91.1%	80.0%	Occasionally
Browning	5.7%	51.9%	Over 90%
Culture media	MS-mod	NN	MS,NN,B6.
Sucrose conc.	60 gr/l	40 gr/l	Different conc.
Phytohormones	IAA 1.0	NAA 1.0	Different
hormones	BA 1.0	BA 1.0	combinations
Low Temperature Requirements	none	8 days/ at 8ºC	——

Zygotic embryos: Complete seeds with developing ovules were cultured (Fig.31). Three types of material: 30, 40 and 60 days after anthesis were tested. Assays made with immature embryos showed that the 30 days stage responded better to the culture media treatment, observing development of callus from carpelar tissue and partial development of the ovules (Fig.32). The best response was found with the MS medium plus 2,4-D 0.1 mg/l. Besides, less browning than in the 40 and 60 days stages was observed. There was callus development in the MS treatment plus 2,4-D 2.5 mg/l, in 60% of explants. Lucumo ovules are usually five; only one develops and the rest disintegrate. However, as can be seen in Fig.33 it was possible to obtain partial development of ovules.

Table 5.14
Immature Lucumo embryos cultivated at two developing stages, pretreated during 6 days at 4ºC.

Medium & Regulat. (mg/l)	Responses 30-day ovules				40-day ovules		
	%B	%S	%Cc	%G	%B	%S	%Cc
WH 2.4-D+BA 2.5+0.1	37.5	–	50.0	12.5	92.3	—	7.7
MS 2.4-D0.1	11.1	–	77.8	11.1	92.3	—	7.7
MS 2.4-D2.5	33.3	–	66.6	—	71.4	—	28.6
MS 2.4-D5.0	40.0	18	60.0	—	90.0	—	10.0
MS 2.4-D+BA 2.5+0.1	33.3	14	66.6	—	66.6	—	33.3

Cc: callus in carpel; B: Brown; S: Swelling; G: ovular growth, average of 10 immature Lucumo embryos

Cuttings Propagation from cuttings was attempted, with different hormones, in sand or liquid media (as for papaya) but no positive results were achieved.

Cell Suspensions. Lucumo cell suspensions were studied to determine best growth media, i.e. NN vs WPM, effect of reducing some N sources (NO3), replacing mineral sources with organic ones (putrescine, casein hydrolysate, glutamine), testing different hormones (TDZ, Z, BA, K) and the effect of 2,4-D during the suspension phase (softening) to obtain cells. Different explant sources were used including callus from axillary buds of 2-3 year old plants, callus from leaves and callus from plantlet nodes or internodes. Cell suspensions plated for fifteen days (although not fixed to the substrate – agarose) divide to form microcalli (Fig.34 and 35), and after 4-5 months, globular structures, ressembling proembryos (Fig 36) and profuse callus (Fig.37). Cells normally darken but remain alive, staining with Evans' blue and acetocarmin. Cell suspension work by Dr. Durzan in California helped to detect early stages of embryogenic cells and microcalli; a method to obtain green friable callus has been developed in Santiago and tested at Davis.

C. PUBESCENS and C.PENTAGONA

Leaves. Culture of Papaya and Babaco (Fig.38) leaf sections was carried out in a modified WPM medium (including casein hydrolysate 250, glutamine 300, cystein 20 mg/l respectively, besides sucrose 3%) in different treatments with TDZ, or in combination with IAA. In all treatments, explants were kept in the dark during 4 weeks, and then in 500 lux. New shoots were formed in

babaco, but the same conditions only formed nodular calli in papaya. Figs. 39-41 show several new shoots arising from previous callus formation on babaco leaf explants; initiation of new growth as seen by SEM is shown on Fig. 42. However, it is not clear yet if these are new shoots or somatic embryos. In papaya, only roots were formed from leaves in presence of 0.02 mg/l NAA and Z ranging 0.5-5.0 mg/l (Fig.48).

Table 5. 15.
C. pentagona leaf sections responses

TDZ	IAA 0.1 mg/l		
mg/l	% Callus	% Browning	% Shoots
0.1	33.3	66.6	—
0.5	57.1	42.8	—
2.5	66.6	33.3	—
5.0	80.0	0.0	20
7.5	80.0	0.0	—
10.0	90.9	0.0	—

Average of 15 leaf sections

Table 5.16
C. pubescens leaf sections responses

TDZ (mg/l)	Responses IAA 0.0 mg/l			IAA 0.1 mg/l		
	%C	%Chl	%NC	%C	%Chl	%NC
0.1	45.5	45.5	—	69.2	15.4	—
0.5	90.9	27.3	—	100	—	7.7
2.5	66.6	75.0	—	100	7.7	38.5
5.0	60.0	20.0	—	100	7.1	35.7
7.5	66.6	66.6	11	100	—	43.8
10.0	—	—	—	100	—	25.0

C: callus formation; Chl: leaf chlorosis; NC: nodular callus; average of 15 leaf sections.

Axillary buds were grown in NN-medium supplemented by different growth regulators (Table 5.16). Several combinations of Z, BA, K, or TDZ/IAA, IBA and paclobutrazol were evaluated. The explants used were undevelopped axillary buds (2 mm) excised in March (Fall) and evaluated up to June. Three main responses were found: elongation of numerous preformed axillary buds (best axillary development was found in combinations of IAA plus Z); bud branching into multiple shoots (Fig.44-45) and formation of proembryos on callus associated to the base of the explant (Fig.46). The highest percentage of proembryo development, 72.2%, was obtained using NAA 0.05 mg/l and BA 0.1 mg/l. NAA/TDZ and IAA/TDZ, and IAA/K in the range of 0.01-1/0.1 did not form proembryoids. Some embryos did not mature in culture; structures showing as green spots lost color in time and finally turned brown.

Table 5.17
Best conditions for papaya axillary buds morphogenic responses.

Treatments		Callus		Axillary growth			Proembryoids	
Growth.reg. (mg/l)	%	diam. cm	Aver. %	number expl.	Aver. length cm	Aver. %	E	
NAA –	BA							
1	0.1	33.3	1,50	–	–	–	33.3	++
0.5	0.1	62.5	1.30	–	–	–	43.7	++
0.1	0.1	53.3	1.50	–	–	–	46.6	+++
0.05	0.1	100	1.30	–	–	–	72.2	+++
0.01	0.1	53.0	1.50	–	–	–	35.3	+
IAA –	BA							
1	0.1	55.5	120	–	–	–	55.5	+
0.1	0.1	100	0.70	64.7	1.0	0.50	23.5	+
0.01	0.1	100	1.00	26.6	1.0	0.90	20.0	+
NAA –	Z							
0.5	0.1	100	1.30	6.3	1.0	0.60	18.8	+
0.1	0.1	100	1.60	14.3	2.0	0.80	28.6	+
0.05	0.1	100	1.00	8.3	1.0	1.50	41.6	++
0.01	0.1	100	1.00	15.4	1.0	1.00	23.0	+
IAA –	Z							
1	0.1	100	0.94	–	–	–	40.0	+
0.5	0.1	100	1.36	50.0	1.0	0.60	60.0	+
0.1	0.1	100	1.30	27.3	1.0	0.54	36.4	+
0.05	0.1	100	1.09	61.5	1.0	0.50	46.2	+
NAA –	K							
0.05	0.1	100	1.10	42.9	1.0	0.50	42.9	+
0.01	0.1	100	1.20	44.4	1.0	0.80	33.3	+

E: relative abundance of proembryoids. Aver. of 20 axillary buds

Shoots were treated with paclobutrazol; they were excised from 2 or 3-year old greenhouse trees, placed on Petri dishes with NN medium and kept constantly in the dark; first results were observed after 40 days. Fig. 47 shows extensive elongation of petioles and leaf lamina expansion using 0.1 mg/l paclobutrazol.

Table 5.18
Morphogenic responses of Papaya shoots supplemented with paclobutrazol.

Paclobu-trazol (mg/l)	% Brown Tissue	% Callus formation	% Bud elongation	% Leaf elongation	X Leaf length (cm.)
0.1	0	100	80	100	1.7
1.0	0	100	100	100	2.0
1.5	0	50	50	50	1.0

Cuttings. Rooting of Papaya cuttings in water was achieved using IBA (50 mg/l), in three different types of cuttings (apical, median and basal part of the twigs) and soaking times: 3, 7, 14 and 30 min. This was done at the beginning of summer. Fig. 50 shows rooting of apical cuttings after 30 days, soaking time 7 min. Rooting of Babaco cuttings (Fig. 43) occurred in the same conditions. When cuttings were treated with cytokinins, several "bruises", then growths and buds appeared along the whole segment (Fig. 50).

Zygotic embryos. Seeds from plants growing in Santiago and collected during winter were cultured to evaluate development of embryonary structures. The MS medium favored the development of callus over the whole surface of the seed, but there was no external evidence of embryonary structures (Table 5.19).

Table 5.19 Growth responses of papaya zygotic embryos.

Growth Regulat Medium (mg/l)	Responses			
	n	Browing %	Callus %	Average diameter (cm)
WH 2.4-D+BA 2.5+0.1	13	15.4	84.6	2.0
MS 2.4-D0.1	14	—	100	2.5
MS 2.4-D2.5	10	20.0	80.0	1.0
MS 2.4-D5.0	12	—	100	0.4
WH 2.4-D+BA 2.5+0.1	13	—	100	0.7

Anthers cultivated on MS with IAA 1 mg/l and 2.5 BA 1 mg/l supplemented with sucrose 6% formed androgenic embryos. Pollen derived embryos were observed in 6.5% of the surviving (green-yellow) anthers within 2 months. Calli formation occured in 91.1% of the anthers; some outgrowths were recognized as of androgenic origin(Figs. 51-54)

Cell suspensions

Callus derived from axillary buds, leaves and hypocotyls was obtained in NN medium supplemented with 0.1 mg/l TDZ in combination with 0.5 mg/l IAA after 2 months. Callus (0.72 cm diam.) was suspended in an orbital shaker at 100 rpm during 6 days in WPM-medium with 3% sucrose and 3% manitol, in presence of 1.1 mg/l 2,4-D and cytokinin combinations (TDZ; BA and/or Z), glutamine and casein hydrolysate. Later, cells were plated (Table 5.21) or resuspended in the same medium without hormones and maintained under agitation at 50 rpm during 5 months at 22ºC, fluorescent light 18 hrs. at 500 lux. Growth of plated cells was slow and only microcolonies and microcalli were obtained (Table 5.21). In resuspended cell cultures, cell density increased and single globular structures developped after 5 months (Fig.57); also proembryoids (heart and torpedo stages) were found (Table 5.20). These embryogenic suspensions were plated in NN medium without hormones but including 1% PVP (1 ml/plate) leading to the development of more embryoids and the formation of multiple adventive embryoids, turning green after 30 days (Fig. 56). This response was only found in cells derived from axillary buds. It was also possible to discriminate between embryonic and non embryonic cells (Fig. 55), with a stain reaction developed by Dr. Durzan. Fig. 58 show multiple stages of embryoid development what may allow to scale up multiplication by this procedure in the future.

Table 5.20
Best conditions to trigger somatic embriogenesis. Axillary bud-derived callus.

Growth regulators mg/l				Callus	Embryoids
2,4-D	Z	TDZ	BA		
1.1	0.4	0	0	+++ (16 embryoids/ml suspension)	+++
1.1	0	0.4	0	++	++
1.1	0	0	0.4	+	+

Table 5.21
Results of Papaya cell suspension treatments (Petri dishes).

Mother explant and media with Growth reg.mg/l	Obtention of cell suspension (shaking)	Plating	Micro-colonies %	Micro-calli %
Hypocot.NN NAA & BA 1.0	WPM 2,4-D 1.0 BA 0.4	WPM K 0.1	60%	0%
Embryo+Cotyled. NAA & BA 1.0	idem	idem	40%	0%
Leaves WPM NAA 0,1 TDZ 1.0	idem	idem	60%	0%
Leaves WPM NAA 0,1 TDZ 0,1	idem	idem	30%	60%
Leaves WPM NAA 0,1 TDZ 0,05	idem	idem	30%	50%

Other tests of organic and inorganic nitrogen compounds were done using callus from axillary buds and hypocotyls, MS-medium with 2,4-D 0.1 mg/l or 1 mg/l for 4 days. Calli were vortexed gently for plating. Growth conditions assayed were: NN medium reducing nitrogen 50% (KNO_3 or NH_4NO_3) as indicated in Table 5.22. Medium gellified with Agarose 0.6% was autoclaved or filter sterilized using 10 petri dishes/treatment, with 3 increasing levels of cells (10.000 cells/ml plating 0.5, 1.0 and 1.5 ml/plate)

Table 5.22
Conditions tested to grow Papaya cells in vitro.

1 Control, full medium, no growth regulators.					
2 Full strengh		K 0.1			
3 Full strengh			TDZ 0.01		
4 KNO3	50%				
5 KNO3	50%	K 0.1			
6 KNO3	50%		TDZ 0.01		
7 KNO3	50%	K 0.01	TDZ 0.01	CH 500	Gl 30uM
8 NH4NO3	50%	K 0.1		CH 500	
9 NH4NO3	50%		TDZ 0.01	CH 500	

CH, Casein hydrolysate, 500 mg/l; Gl, Glutamine, 30 uM; CM, Coconut milk (Sigma) 10%v/v).

Cell suspensions derived from hypocotyl calli grown in NN, NAA and BA 1 mg/l respectively using filter-sterilized media and kept in light (treatment 7) gave the best results, forming a green microcallus after 1 month, but not embryogenesis. Other good treatments were Nºs 5,6 and 9; many microcalli were formed although not green. Summarizing: suspending cells for 4 days in 0.1 mg/l 2,4-D gave the best response, lowering NO3 to 50% and enriching with glutamine and casein hydrolysate was beneficial, indicating a shift in N preference toward more reduced forms. K in conc. of 0.01-0.1 mg/l is beneficial but the addition of TDZ 0.01 mg/l works better. Embryogenesis mainly obtained in suspensed cells, not attached to agarose in plates.

Results on cell suspension work done within the training program of Ms. Maritza Obando, at Davis University, California showed that it was possible to follow cell divisions of discrete cell types on a daily basis and follow-up the validity of the morphogenic patterns using citochemical stains to characterize early zigotic embryo development. In papaya the recovery of cells from calli reveals a wide range of cells types and differentiation. Many cells are already independent of and dependent on previous light media effects, several cells specialize in producing anthocyanins. Cells can be distinguished by shape and color green clear, brown, black, red yellow either singly or in combination in clumps and could be separated mechanically and plated out to recover homogeneous cell lines. Somatic embryoids (white clusters) are visible. Cell division patterns varied with a few indications of internal divisions as expected in early stages of embryogenesis. Of special interest were the black cells in Papaya; daughter cells were not black and may be embryogenic.

Results indicate that good cells suspensions should be obtainable from Papaya calli even on a NN medium, but other changes may increase the frequency of embryogenesis. The induction process probably involves specific cell types and may require plating out the cell suspensions in Gelrite or agar for further observations. Early preliminar results on plating out generated good calli. Some Papaya cells appeared to contain 2 nuclei and this is not described in the literature.

The exudates from papaya were acetocarmin reactive and this is encouraging in term of a parallel with acetocarmin reactive exudates from conifers. The cytoplasm of Papaya cells was not strongly reactive and the acetocarmin-reactive material seems to be on the wall surface. For this reason, and to discern daugther cell divisions and growth patterns, it was considered better to use DAPI.

Characterization of Papaya germplasm along Chile.

C. pubescens is an introduced plant to Chile, where it is distributed in the central coastal region; important plantations exist between 29-35ºSL. It is not known if this material is related or may behave differently according to stress, showing traits such as cold tolerance. Characterization of this material using in vitro responses of seed explants collected at different localities, greenhouse growth and electrophoresis, was initiated.

Table 5.23

Growth differences observed in one-year old Papaya plants from four different localities between La Serena (30º S) and La Ligua (33º S), grown in the greenhouse.

LOCALITY	La Serena	Las Juntas	Huente-lauquen	La Ligua
Height X cm	19.0 +/-2.2	33.5 +/-3.33	22.8 +/-3.5	23.35+/-4.1
Stem diameter X mm*	0.69+/-0.1	0.96+/-0.06	0.94+/-0.09	0.79+/-0.14
Nºleaves X	12.9 +/-2.2	16.9 +/-2.3	13.3 +/-4.24	14.6+/-5.52
Leaf area X cm2	29.8 +/-10	57+/-9.4	51.4 +/-15.1	38.0+/-9.64

* Stem diameter was measured at a height of 10 cm. n = 20

Table 5.24
Pathogen incidence in Papaya plantlets from four different localities raised in the greenhouse during 1 year.

LOCALITY	La Serena	Las Juntas	Huente-lauquen	La Ligua
INFECTION				
Aphids	–	–	–	–
Scale	–	–	+++	+
Root-rot	++	–	+	+
Virosis	+	–	+++	++++

n = 20, (-)= No symptoms, (+)= Symptoms or pathogens, 30% or less of cases.

Isozyme variations in Papaya accessions.

Exploration of differences in catalase, lactate dehydrogenase, diaphorase, peroxydase, and aspartate amino transferase (AAT) bands was effected in papaya plantlets ranging from 20ª to 36ª S. The last two enzymes showed good differentiation. Technically, it was possible to avoid previous contamination with chlorophyll, and the use of PMSF (Phenylmethylsulfonyl fluoride) reduced proteolysis. Buffers were improved, enhancing extraction; and consisted of the following two solutions, mixed in equal parts.

a)	TRIS-HCl 0.05 M pH 8.3.	
b)	TRIS-HCl 0.05 M pH 6.8	12.5 ml
	PVP 40 (Sigma)	100 mg
	Glycerol (Sigma)	10.0 ml
	B-mercaptoethanol (Sigma)	5.0 ml
	PMSF	1.0 uM
	Distilled Water	45.0 ml

Peroxidase determination: When using peroxidase, variations appear as an extra band in localities Se 29º, Hu 31º, Co 35º2, Ta 36º but not in Li 32º and Ch 35º. (Fig.61). AAT determination: While there is little difference between samples from 20º to 36º (Fig. 60), it is possible to observe variations between close accesions, for example, between Co 35º and Ch 35º, it seems that when comparing plantlets within a group, the bands intensity also changes. Diaphorases did not show variations between accessions.

In vitro growth patterns were compared between accessions (Seeds gathered in 8 localities between the IV and VIIIth Regions, over 1.300 Km approx.) The seeds were germinated in the greenhouse and hypocotyls tested for embryogenesis. Data was insufficient to test differences on growth patterns, but it appears that the morphogenic potential is basically the same.

Table 5.25: Morphogenic responses of Papaya hypocotyls.

Locality	Results			
Nº tubes at 30 days		% Callus	% Embryogenesis	% Roots
Talcahuano	20	65	10	5
Talcahuano*	12	15	10	0
Constitucion	17	70	10	0
Llico	6	35	5	0
Chanco	20	85	5	0
Huentelauquen	20	80	0	5
V. Huasco	19	85	15	0
La Ligua	18	50	0	5

* Same locality. NN medium, NAA and BA 1mg/l. Callus after 30 days; embryogenesis after 60 days.

6) IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Many of the results of this project can already be used, it is possible to find relevant data on Papaya, as protocols, in the invited chapter: Micropropagation of papaya, (Carica sp). In: Biotechnology in Agriculture and Forestry (Ed. Y.P.S. Bajaj), Springer-Verlag, Vol.18, 418-459. (1992). C. pubescens propagation is based on induction of sprouting of axillary buds to be rooted; anther culture; induction of somatic embryogenesis from calli, and regeneration from cell suspensions. This is also important for the establishment of new Papaya and Babaco plantations; the latter requires asexual propagation since it is a sterile hybrid. Lucumo regeneration is presented in the paper: In vitro plant regeneration of Pouteria lucuma (R. et Pav.) O. Kze. (Sapotaceae). Plant Cell, Tissue and Organ Culture 31: 249-252, 1992. Choosing fast growing and producing lines of Pouteria and multiplying them is essential as this tree has a very long juvenile period. Recovery of plants obtained by cell suspensions has to be optimized, as the process is very slow.

For A. cherimola (cherimoya), several morphogenic events would improve prospects for mass propagation; especially multiple shoot formation and reduction of browning (several types of antioxidants) are important topics. The invited Chapter on Tropical and Subtropical Small Fruits, in: Biotechnology of Perennial Fruit Crops, Biotechnology of Agriculture Series, Nº 8. (eds. F. Hammerschlag and R. Litz). C.A.B. International. 570 pp., deals with Annonas, and gives detailed data on their morphogenetic potential expressed in tissue culture. It also deals with other promising subtropical fruits such as Solanum muricatum (pepino), and Solanum quitoense (naranjilla), as well as kiwi.

Micropropagation permits selection against pathogens in all species studied. Isozyme characterization may be helpful in detecting useful traits and also, the development of different expertises as electrophoresis, phenols determination, single cell culture and the beginning of transformation work will be helpful to improve and select more valuable germplasm of these species.

Use of the results presupposes the presentation of the protocols and techniques for in vitro propagation of these species in documents aimed to a specific public, i.e. fruit growers, who would undertake their own multiplication programs. The time of application depends largely upon the needs of these same growers.
Larger scale trials and development under in vivo conditions in the field are the necessary next steps to bring the results to practical use. In papaya, results are ready to be scaled-up to obtain material and establish new plantations to improve existing stands.

It is intended to prepare a booklet with the most relevant information obtained during the project to be used by growers to multiply these species; the booklet would be publicized in farmer's and farmer's unions specialized publications. This could be possible if supplementary funds were available for this purpose, a rough estimate is about US$ 4500.-

The species studied are very important for Chile, and there has been interaction of the scientists who work on them: Universidad de la Serena, concerning the industrialization of latex and papain;. Universidad de Santiago, characterization of the properties of the proteolytic enzyme. INIA and U.C. Valparaíso are interested in widening the range of fruit crops in Chile.

Internationally, the mountain papaya is important as regards studies of PRV resistance, since C. pubescens is one of the few Caricas with this trait. Seeds were sent to Hawaii to be evaluated for trials on interspecific crossing with C. papaya strains, looking for resistance.

7) PROJECT ACTIVITIES, OUTPUTS

Research results were communicated in several international meetings attended; interest was shown among scientists in sharing information, germplasm exchange and perform future work, specially during The Pacific Rim Food & Agricultural Biotechnology Conferences on Molecular Crop Agriculture, held at Davis and Sacramento and the meeting on In Vitro Culture and Horticultural Breeding, USDA/ARS Beltsville Agricultural Research Center, International Society for Horticultural Science held in Baltimore, Maryland, during 1992. The continous contact with Prof. Durzan and Prof. Litz also helped to improve this work.

Meetings attended (related).

1989 I Congreso Nacional de Biotecnología. 11-13 de Enero, Universidad de Talca. (Simposio Nº3: "Cultivos de Tejidos Vegetales").

Jordan, M. "Regeneración in vitro y aplicaciones a través del cultivo de células y tejidos vegetales".

Arce, P., Budge, C., Del Río, A., Garrido, J., Jordan, M., Leighton, F., Peralta, F., y Pichard, G. "Biotecnología Vegetal en la Universidad Católica de Chile".

1989 CTA/FAO Symposium: "Plant Biotechnologies for Developing Countries". 26-30 de Junio, Luxemburgo.

XL Congreso Sociedad Iberoamericana de Biotecnología Celular y XXXII Reunión Anual de la Sociedad de Biología de Chile, Valparaíso, Noviembre.

Jordan, M. "Regulación de respuestas morfogénicas de plantas in vitro". (Simposio Nº 7: Diferenciación celular en plantas). Biol. Med. Exp. 22(3) R 199.

1990 Tissue Culture Networking/Workshop Meeting. National Research Council, Davis, California, July 16-20.

Jordan, M. In vitro propagation and improvement of fruit species with economic potential for semi-arid zones.

Encuentro de Biotecnología. Pontificia Universidad Católica de Chile.

Jordan, M.; González, M.L.; Goreux, A.; Iturriaga, L.; Montenegro, G.; Mujica, A.M.; Roveraro, C.; Tesser, B.; Valenzuela, M.P.; Velozo, J. Panel: "Multiplicación in vitro de especies frutales de importancia económica para zonas semi-áridas". 8 de Agosto. Biotecnología en la Pontificia Universidad Católica de Chile: 85.

Celebración 20 años Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. Depto. de Ecología.

Panel: "Bases Biológicas del uso y Manejo de Recursos Bióticos y de la Preservación y Conservación de la Naturaleza – Micropropagación y Selección de Plantas in vitro". 7 de Agosto.

1990 XXXIII Reunión de la Sociedad de Biología de Chile. Punta de Tralca, Noviembre 21-23.

González, M.L., Iturriaga, L., Mujica. A. M., Oyanedel, E. Valenzuela, M.P., Velozo, J. Multiplicación in vitro de Especies Frutales de Importancia Económica para Zonas Semi-Aridas. (M. Jordan, Coordinador). Arch. Biol. Med. Exp. 23, R 230, 1990.

1991 Fourth Conference of the International Plant Biotechnology Network (IPBNet). San José, Costa Rica, January 14-18.

Jordan, M., Montenegro, G., González, M.L., Goreux, A., Iturriaga, L., and Valenzuela, M.P. In Vitro Propagation and Improvement of Fruit Species with Economic Potential for Semi-Arid Zones. p. 91.

PSTC Plant Biotechnology Networking Meeting. San José, Costa Rica, January 19.

Jordan, M., Biotechnological Potentials to Improve Some Underexploited Sub-Tropical Fruit Species.

Segundo Congreso Nacional de Biotecnología. 18-20 de Abril. Viña del Mar.

Jordan, M., Valenzuela, M.P., Velozo, J., Oyanedel, E., González, M.L., Sánchez, P., y Montenegro, G. Respuestas androgénicas en anteras de lúcuma (Pouteria lucuma) y papaya (Carica pubescens).

Simposio Argentino de Biotecnología Vegetal, Vaquerías, Córdoba, Junio 11 y 12.

Jordan, M., Valenzuela, M.P., Velozo, J., Oyanedel, E., Sánchez, P., y Montenegro, G. Potencialidades morfogénicas y multiplicación in vitro de tres especies frutales de importancia económica para zonas semi-áridas en Chile. Resúmenes: 67.

VIII Reunión Anual de Botánica. Santiago, 16-19 de Octubre, Universidad de Santiago.

Jordan, M., González, M.L., Goreux, A., Iturriaga, L., Montenegro, G., Mujica, A.M., Oyanedel, E., Roveraro, C., Tesser, B., Valenzuela, M.P. y Velozo, J. Regeneración in vitro y respuestas morfogénicas en algunas especies de importancia económica en Chile (Simposio). Resúmenes: 45.

42 Congreso Anual de la Sociedad Agronómica de Chile. Chillán, 11-15 de Noviembre.

Iturriaga, L., Goreux, A. y Jordan, M., Influencia de Antioxidantes en la Promoción de Brotes de Annona cherimola cultivados in vitro. Simiente 61: 123.

XXXIV Reunión de la Sociedad de Biología de Chile Puyehue, 27-30 de Noviembre.

Jordan, M., Valenzuela, M.P., Oyanedel, E. Respuestas androgénicas en anteras de lúcuma (Pouteria lucuma), papaya (Carica pubescens) y chirimoya (Annona cherimola). Arch.Biol. Med. Exp.24, R-191.

1992 PSTC/CDR Networking Meeting on Tree Crops. E.A.R.T.H., Costa Rica, April 2-8.

Jordan, M. In Vitro Propagation of Fruit Trees.

Molecular Crop Agriculture for the Pacific Rim. Pacific Rim Food & Agricultural Biotechnology Conferences. University of California, Davis. Sacramento. June 20-24.

Jordan, M. Micropropagation of some sub-tropical species. Abstracts. p. 35.

In Vitro Culture and Horticultural Breeding. USDA/ARS Beltsville Agricultural Research Center. International Society for Horticultural Science. Baltimore, Maryland. June 28 – July 2, 1992.

Jordan, M. Obando, M., Iturriaga, L., Goreux, A., and J. Velozo. Organogenesis and regeneration of some Andean fruit species.

XXXV Reunión de la Sociedad de Biología de Chile Puyehue, 25-28 de Noviembre.

Jordan, M., Obando, M., Goreux, A. y Velozo, J. Regeneración in vitro de algunas especies frutales andinas.Arch.Biol. Med. Exp.25, R-70

1993 XIV Jornadas de Investigación de la Universidad Nacional de Cuyo. II Jornadas Regionales y I Trasandinas de Investigación. 10-14 de Mayo 1993.

Jordan,. M., Velozo, J., Goreux A., y Mujica A.M. Organogénesis y Regeneración de algunas Caricaceas in vitro.

The following training seminars and theses were developped within the scope of the project:

Soledad Rivas: Cell suspensions.
Pablo Sánchez: Cell suspensions.
Ernesto Lay: Micrografting
Maria de la Luz Gonzales: Morphogenesis
Maria Paz Valenzuela: Anther culture
Eduardo Oyanedel: Tissue culture

Postgraduate training was done by Mrs. M. Obando, Magister of Science candidate, at Dr. Durzan's laboratory, Davis California, during 1992.

Related Publications

Jordan, M. (1989). Cell and tissue culture potentialities to regenerate three papaya species (Carica candamarcensis, C. papaya and C. pentagona. Erwerbobstbau 31:90-94.

Jordan, M. (1989). Estado actual de la Biotecnología Vegetal en Chile. P. Universidad Católica de Chile; Facultad de Ciencias Biológicas; Laboratorio de Botánica, Santiago de Chile. (Document prepared for FAO).

Jordan, M. (1990). Regeneración in vitro y aplicaciones a través del cultivo de células y tejidos vegetales. Arch. Biol. Med. Exp. 23:113-117.

Jordan, M. y Goreux, A. (1990). Perspectivas del cultivo in vitro de células y tejidos vegetales. Panorama Económico de la Agricultura. 69:16-22.

Jordan, M., Arce, P., Gutiérrez, A., y Roveraro, C. (1990). Inducción de respuestas morfogénicas in vitro mediante co-cultivo de diferentes especies frutales. Cien. Inv. Agr. 17 (1-2) 13-17.

Jordan, M., Arce, P., y Roveraro, C. (1990). Micropropagación in vitro de algunas especies frutícolas en Chile. Cienc. Inv. Agr. 17 (3) 111-116.

Jordan, M., Iturriaga, L., Roveraro, C., and Goreux, A. (1991). Promotion of Annona cherimola in vitro shoot morphogenesis as influenced by antioxidants. Gartenbauwissenschaft 56: 224-227, 1991

Jordan, M., Roveraro, C., Stipo, A., Aguirre, J., Velásquez, L., y Tesser, B. (1991). Inducción de variantes somaclonales y transformación en papa (Solanum tuberosum) empleando Agrobacterium tumefaciens. Cienc. Inv. Agraria 18: 129-136.

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Jordan, M. and Oyanedel, E. (1992). Regeneration of Pouteria lucuma (Sapotaceae) Plants in vitro. Plant Cell Tissue & Organ Culture 31 (3),249-252.

Jordan, M. and C. Botti (1992). Tropical and Subtropical Small Fruits. (Invited Chapter). In: Biotechnology of Perennial Fruit Crops, Biotechnology of Agriculture Series, Nº 8. (eds. F. Hammerschlag and R. Litz). C.A.B. International. 570 pp.

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8) PROJECT PRODUCTIVITY

We believe that the project fulfilled the proposed goals. We have now a better understanding of the physiology of the plants, morphogenic potential at the cellular and tisular level and regulation due to distinct types and concentration of phytohormones. Browning problems have also been solved to some extent. The results of androgenesis to obtain haploid plants in the future are also encouraging, as well the potential to scale up multiplication through somatic embryos derived from cell suspensions. Several articles and chapters in books were published and we believe this information will be useful for scientists working with related species.

9) FUTURE WORK

Research on morphogenic events should be continued via histology, to complete the sequences already begun in more detail. Electron microscopy will also help to detect viruses or pathogens in explants of the three species under study.

The group is interested in the continuation of the study of Andean subtropical fruit species. Work on cell suspensions has begun to be explored during this last period, and shows great promise as a valuable tool, for scaling-up and transformation work on disease resistance that could be performed later on.

Since progress has been achieved in the regeneration from leaf explants and cell suspensions, transformation work can now be envisaged. It was possible to start work with the plasmid pGV 2260 (having the gene GUS as marker) kindly provided by Dr. Luis Herrera Estrella, Mexico, for potato (Jordan et al, 1991). Having established this technique it would be possible to follow-up current research using a construction with a useful gene in collaboration with other scientists in the USA. This would mean to link our work with current research in the U.S., and eventually to prepare a joint project.

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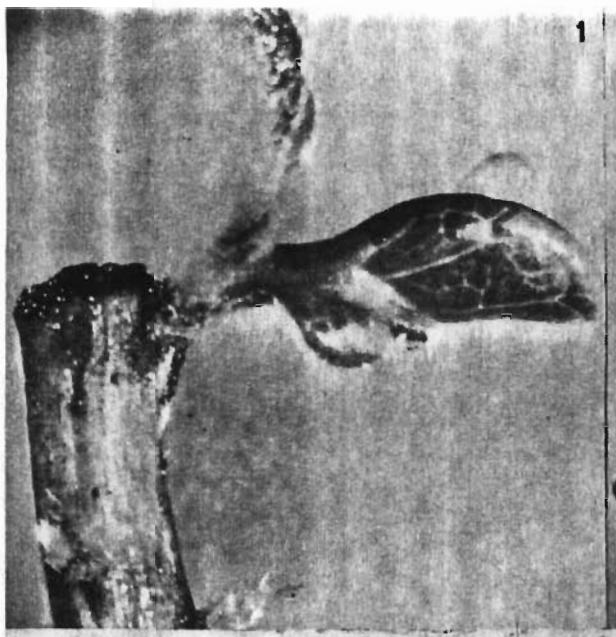
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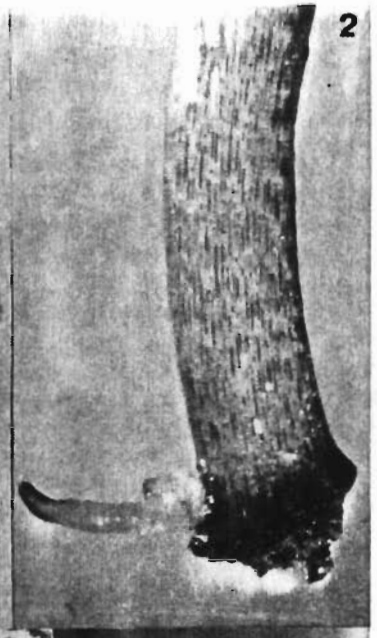
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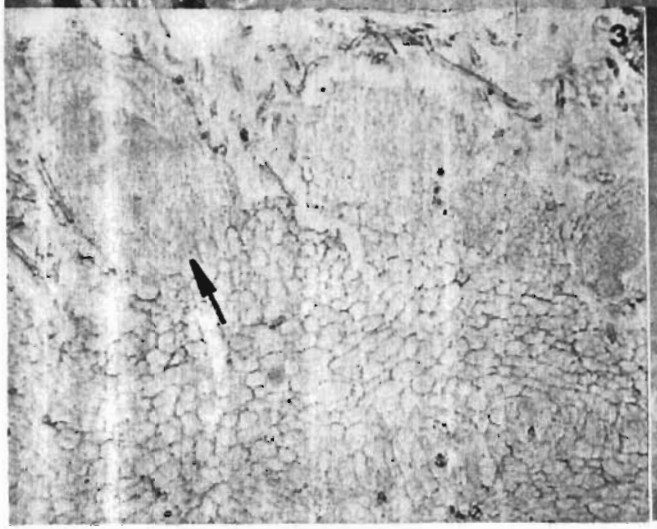
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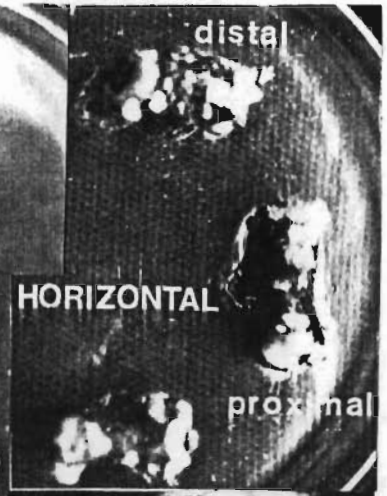
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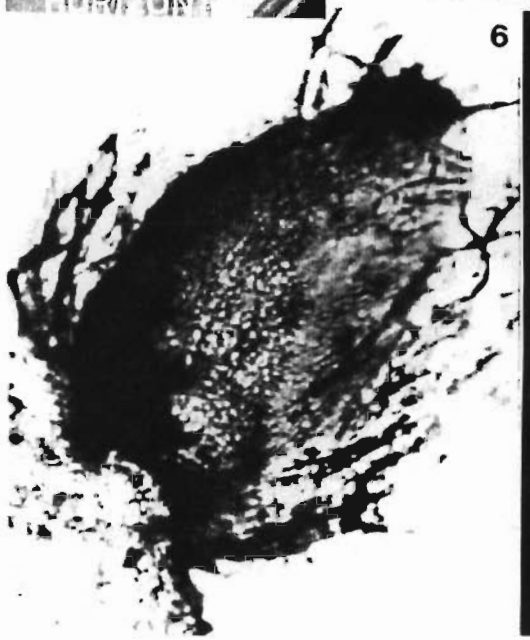
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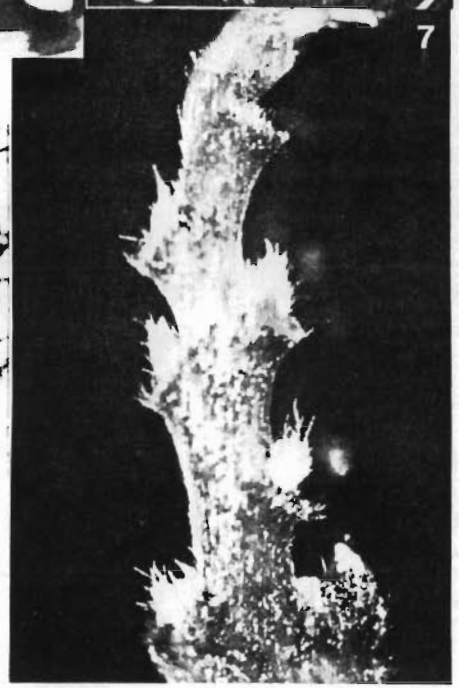
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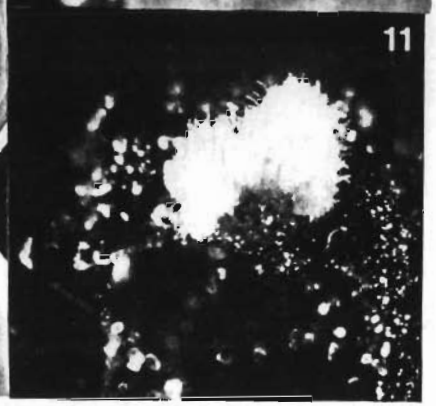
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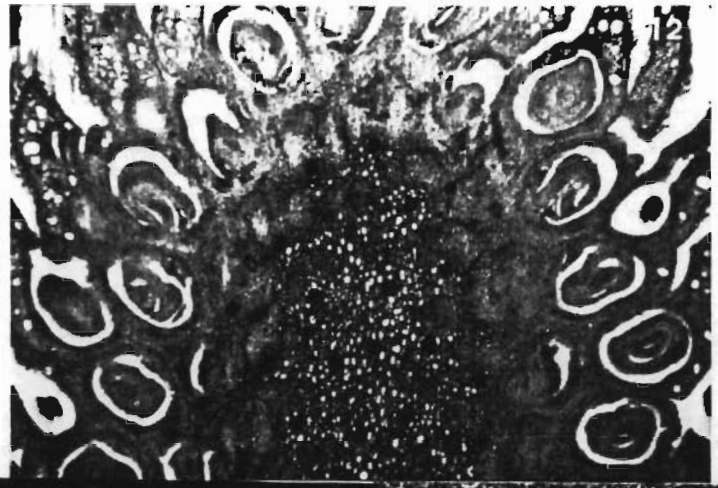
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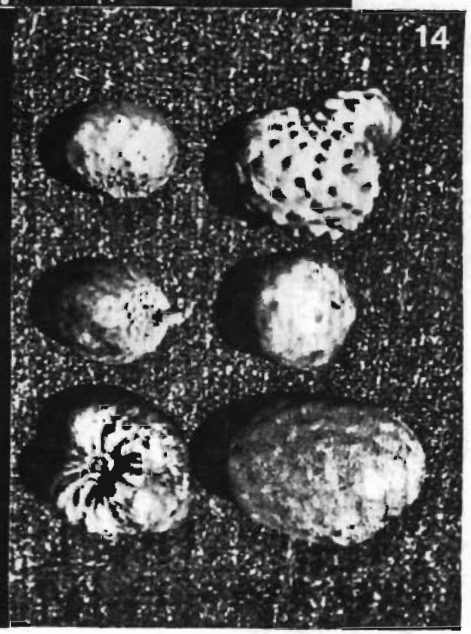
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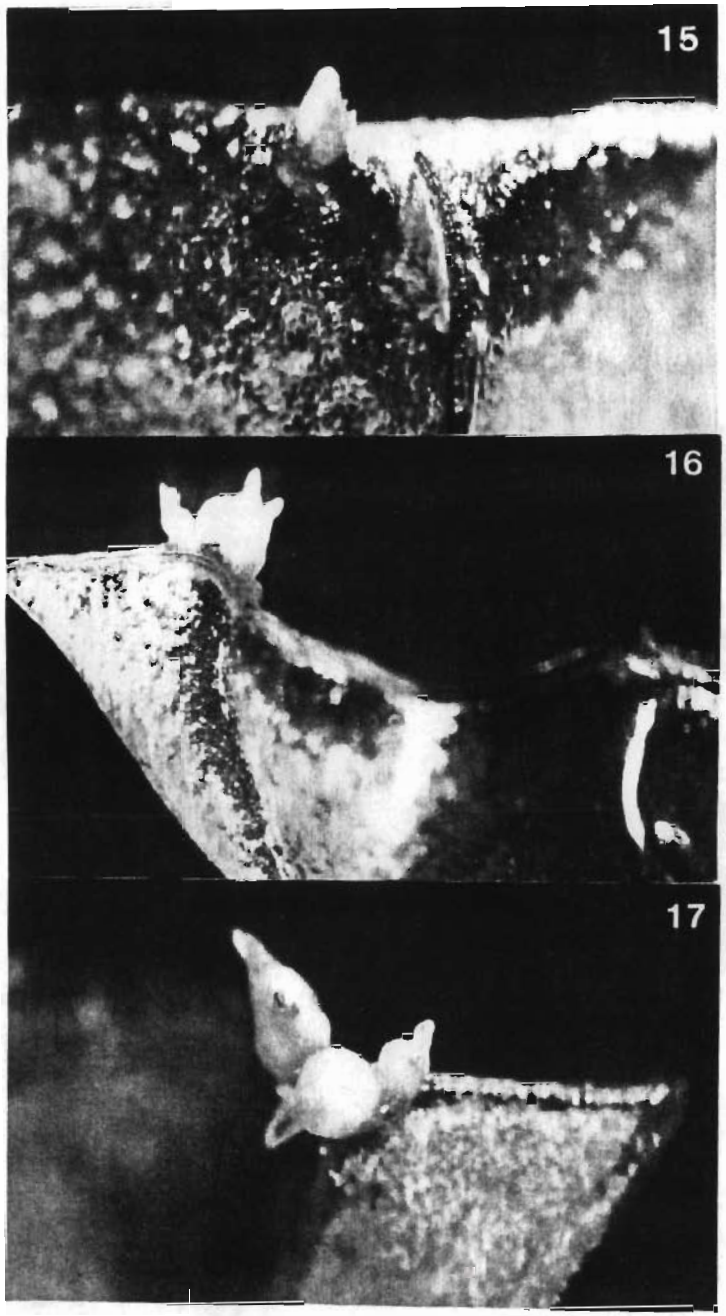
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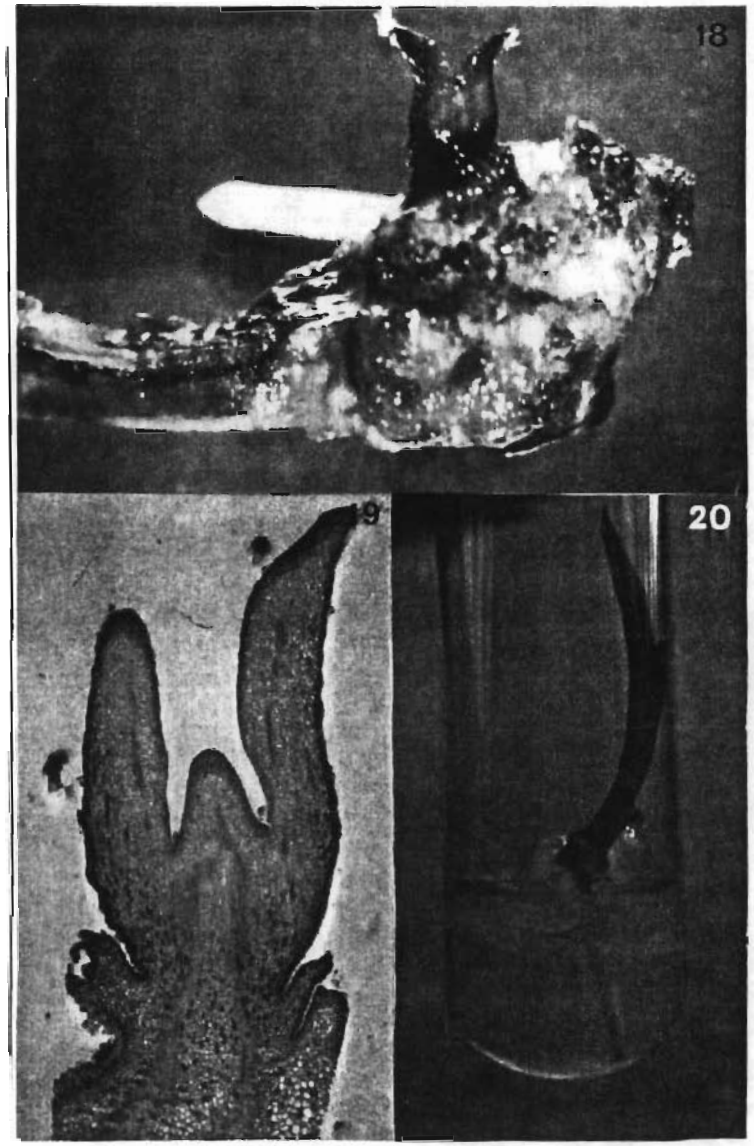
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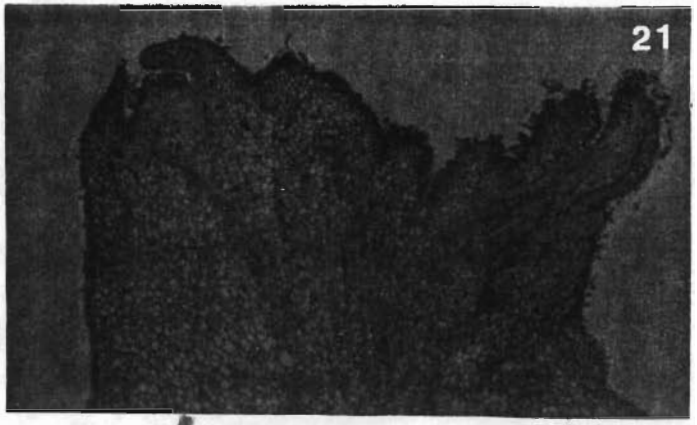
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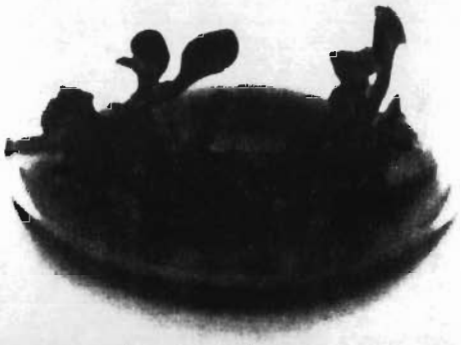
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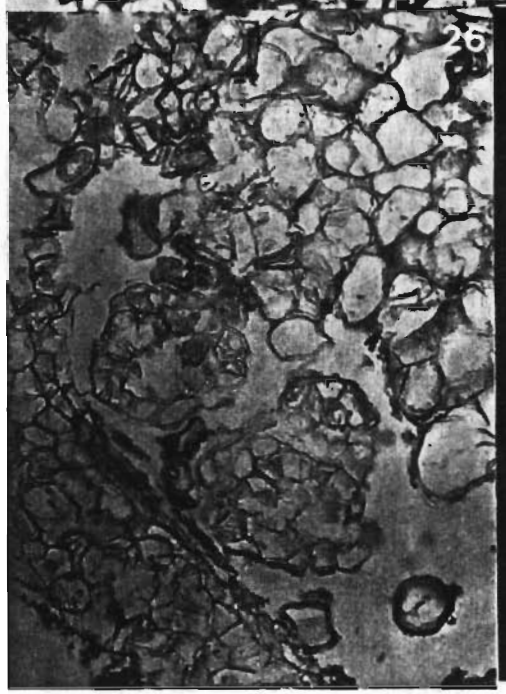
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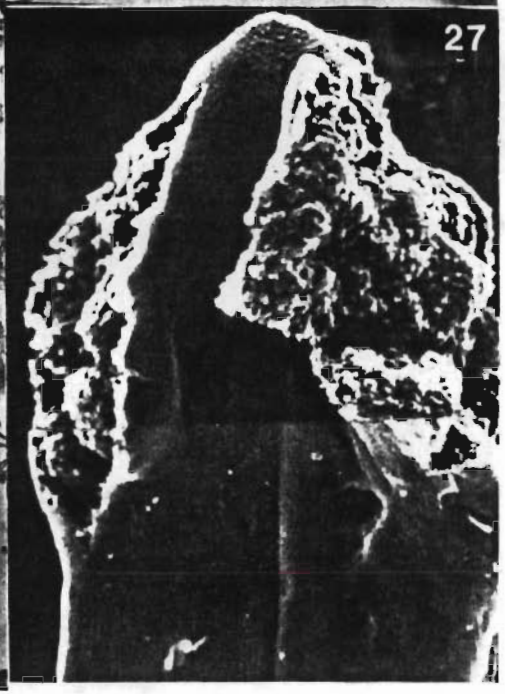
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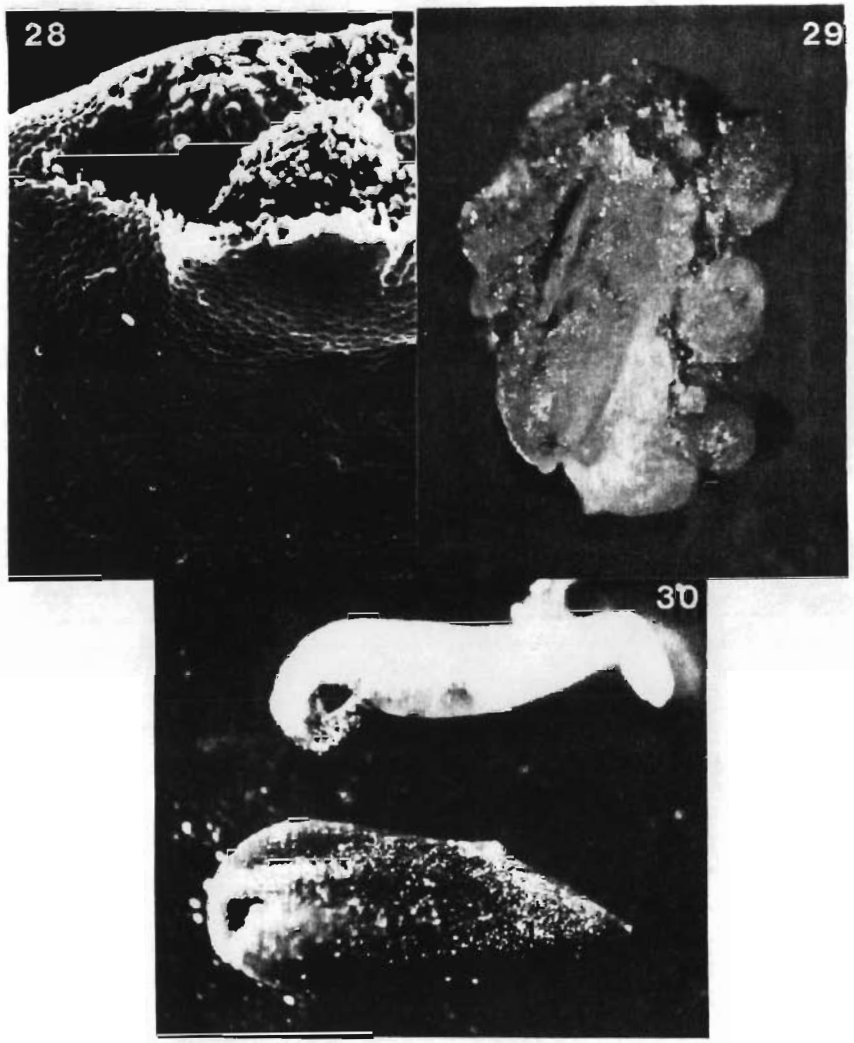

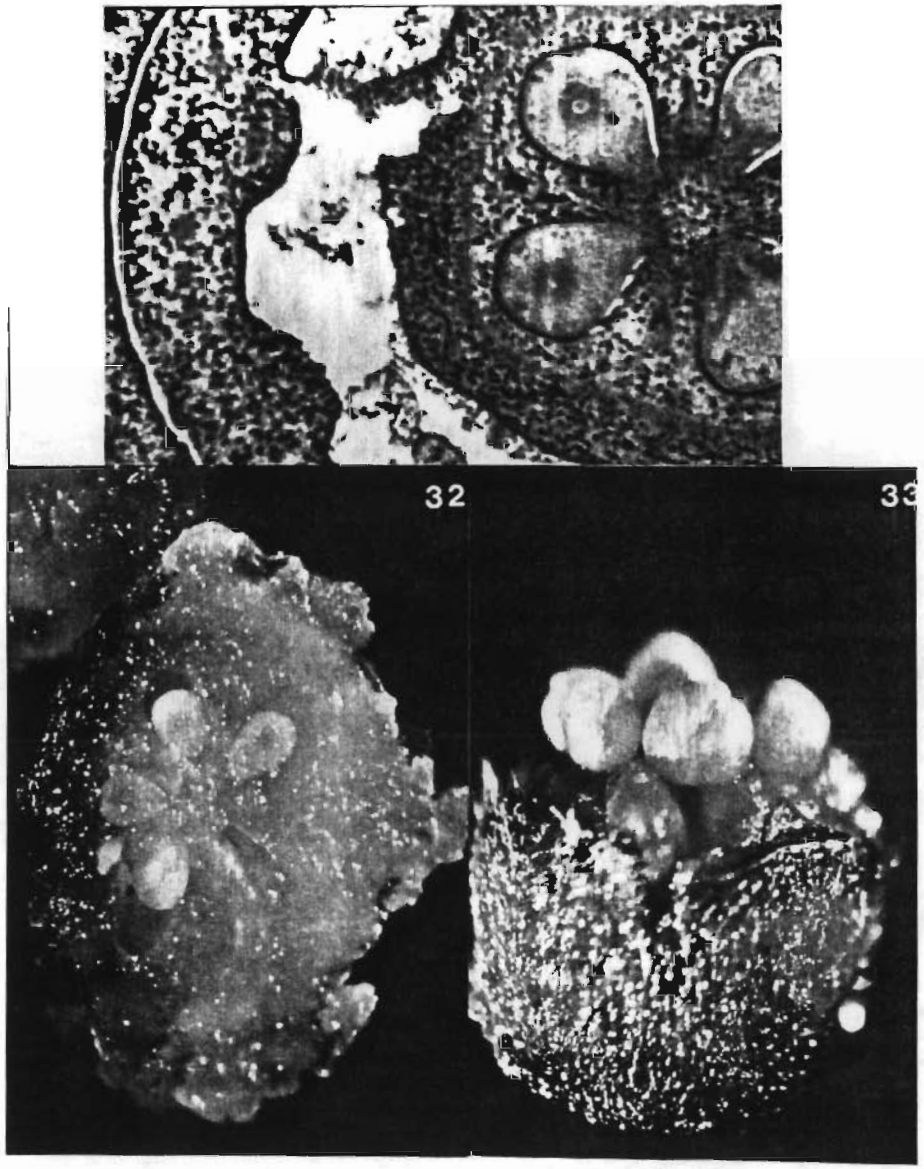

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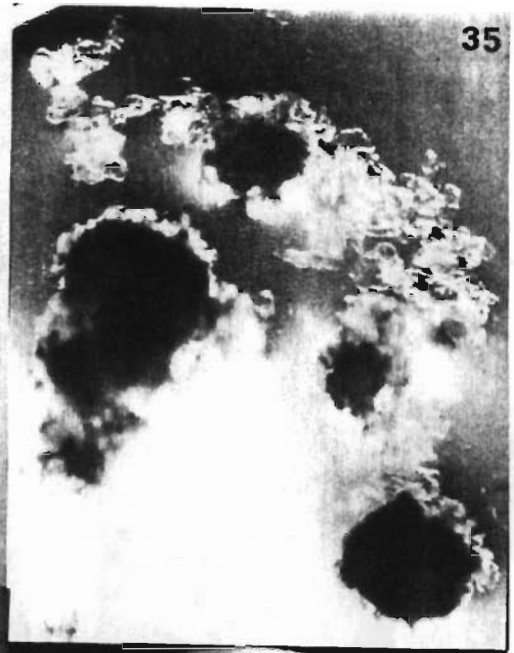
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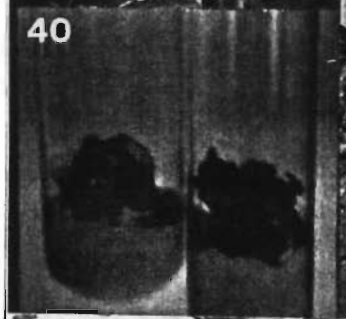
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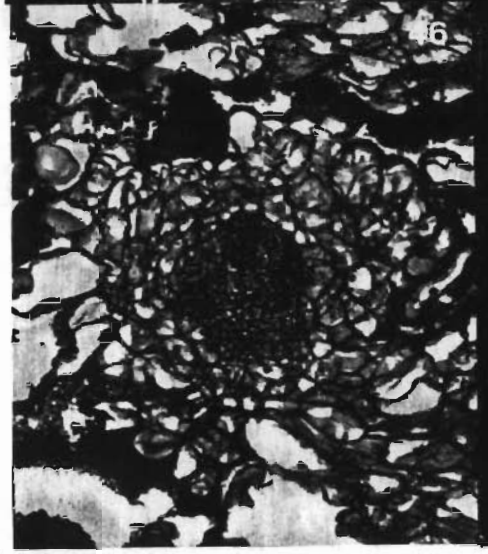
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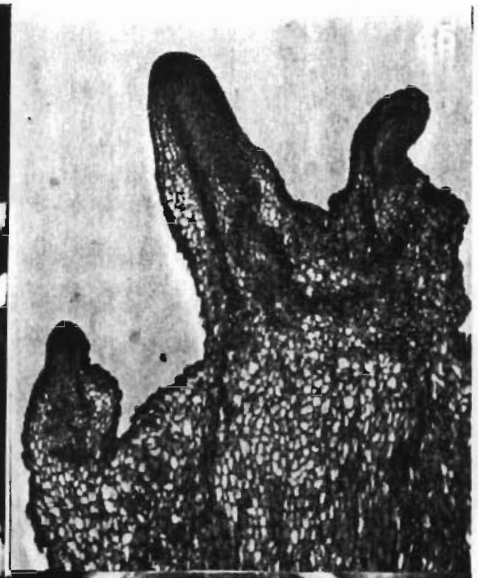
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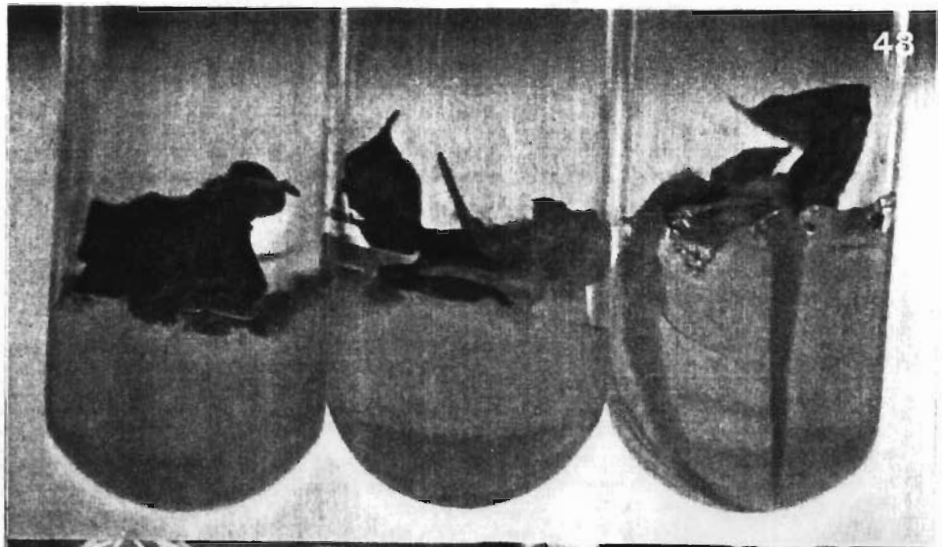
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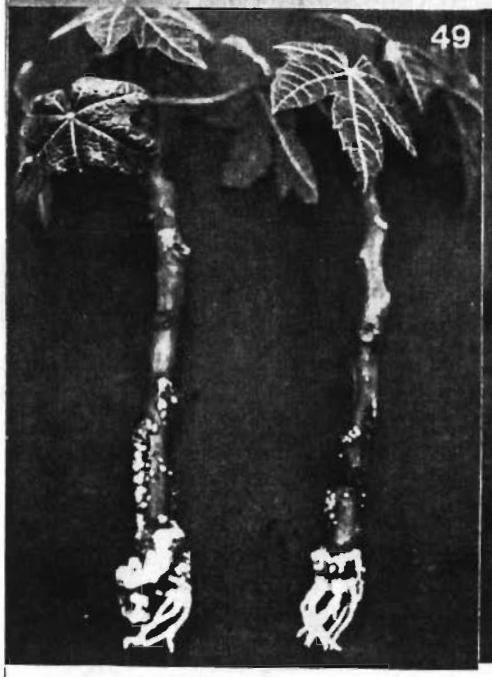
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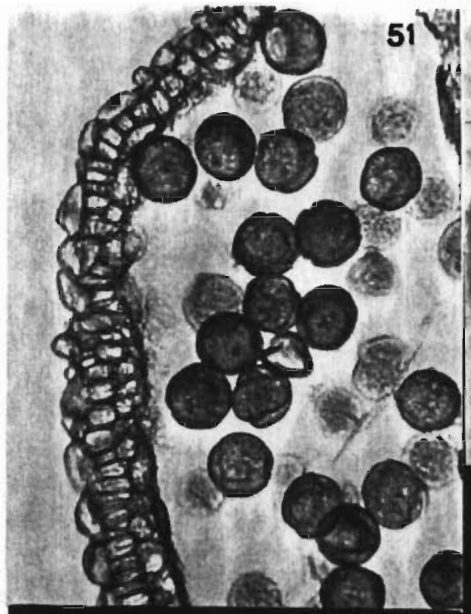
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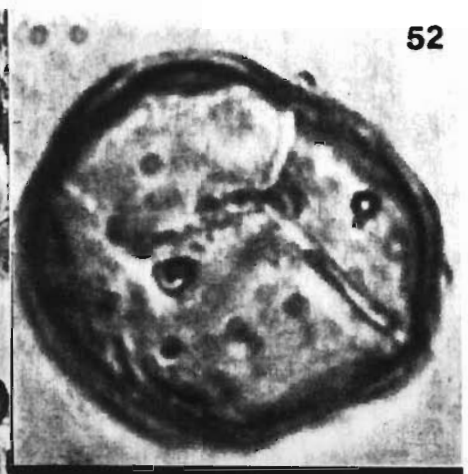
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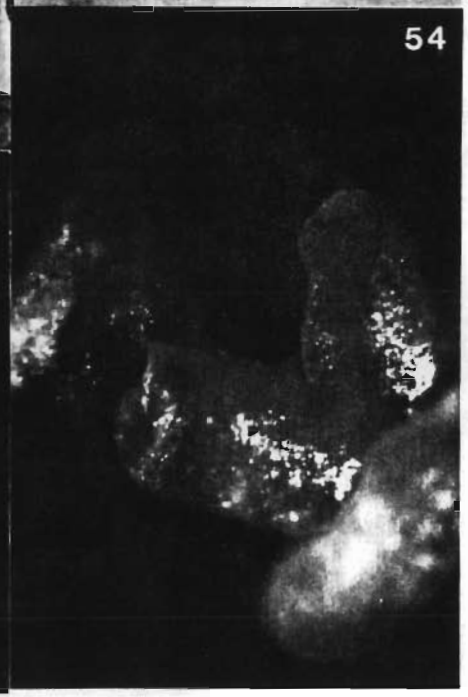
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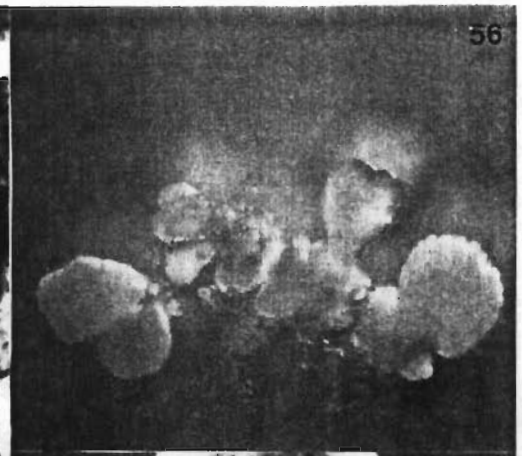
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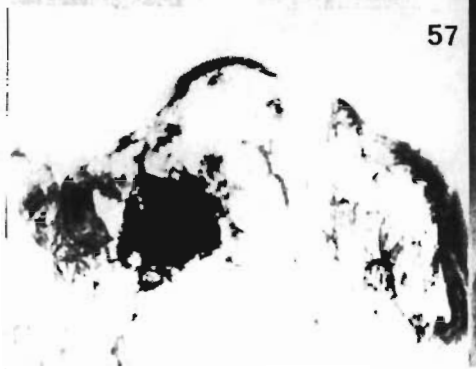
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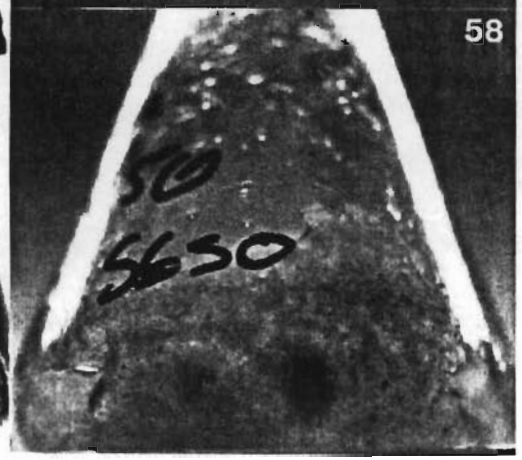
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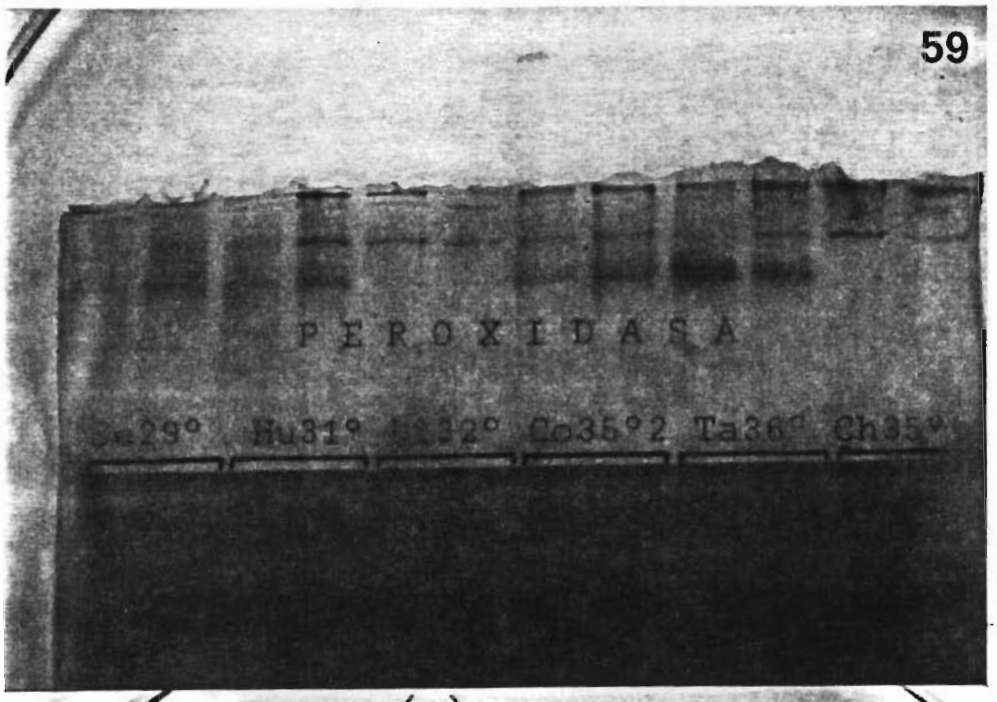
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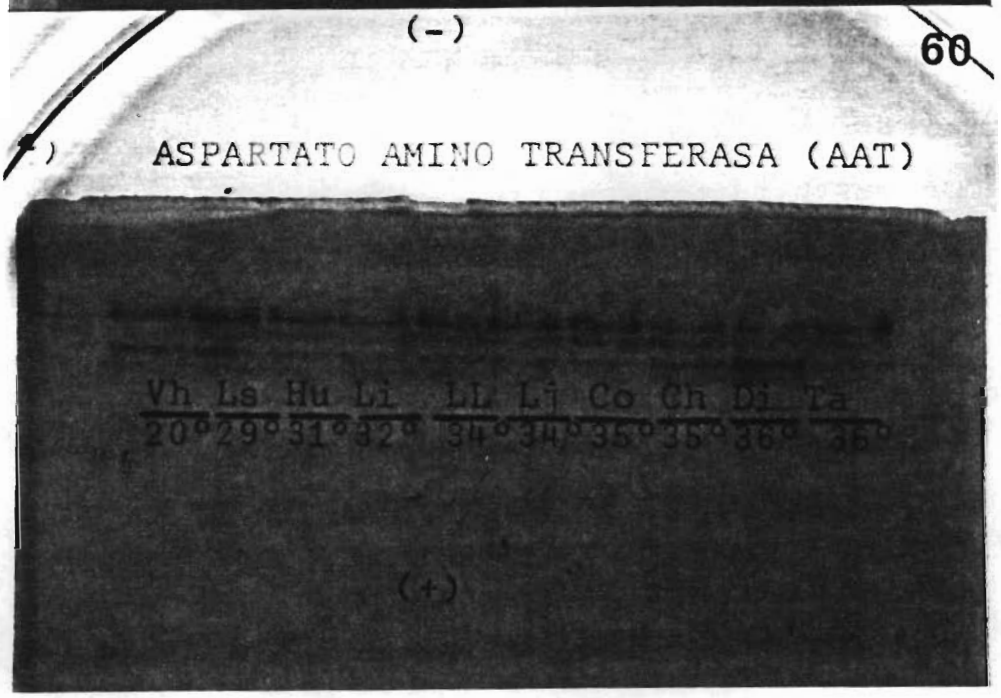
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II.12 Micropropagation of Papaya (*Carica* spp.)

M. Jordan[1]

1 General Account

Future policies linked to the exploitation of fruit species originating from regions where water resources are scarce, as is the case of the Central Coastal area of Chile (lat. 28° to 36° S), must consider species showing rapid growth, good production rates, and economic returns justifying the investment in controlled irrigation equipment and labor costs. Selection of germplasm and ecotypes showing special characteristics must be backed by quick and massive clonal multiplication systems, as a requisite to increase productivity. Within the Caricaceae species growing in Chile, some lines can be pre-selected as very useful as regards productivity or as enzyme sources, and little-known fruits of new hybrids or introduced material can become important. In Chile, three species of papaya are found; of these *Carica pubescens*, Lenné and Koch (*C. candamarcensis*, Hook f.) "mountain papaya" (Uphof 1968) and the recently introduced *C. x heilbornii*, Badillo n.m. pentagona "babaco" are the only edible fruit crops. Both have great potentialities for culture in mediterranean coastal regions, given their early yield, fruit quality, productivity, and, in the case of *C. pubescens*, a source of the proteolytic enzyme papain, required by the food, pharmaceutical, and leather industries. The third species corresponds to the endemic *C. chilensis* (Planch. ex. A.B.C.) Solms-Laub, "palo gordo" used as forage in some locations between lat. 30°–33 °S.

1.1 Botany

The genus *Carica* involves 22 species mainly found in the West Andean foothills (Upper Amazonian Basin; Muñoz 1988), only one native species is described for Chile, *Carica chilensis*, growing between 29° and 30 °S.

Carica pubescens (Fig. 1 A–D) "mountain papaya" is an arborescent herb-like species, 2–3 m high, growing mostly in temperate highlands (1500–3000 m.a.s.l.) from Panama to Bolivia; it is monoecious-dioecious (Muñoz 1965, 1988; Badillo 1971), and has 2n chromosomes = 18. According to Muñoz (1988), *C. pubescens* has numerous subterminal leaves, with five to seven lanceolated segments, and cylindrical petioles, 15–45 cm in length, distributed horizontally around the stem, and different flower forms: plants with female flowers only, set in a short inflorescence;

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Fig. 1. A Adult papaya in the field (*Carica pubescens*) B Flowers. C Fruits. D Stem

male flowers and incomplete hermaphroditic flowers; other mainly male. Fruits are fragrant, acid, 7–10 cm long, 4–6 cm wide, oblong-ovoid, spiculated, five furrows, thin pericarp green or yellow at maturity, brown apex. Fruit shape varies depending on flower type and season.

Morphologies of *C. heilbornii* and *C. chilensis* were fully described by Badillo (1971).

1.2 Distribution and Importance

Due to climatic conditions, only *C. pubescens* grows in the temperate coastal valleys of Chile, between 28° and 36 °S. Two crops a year are harvested in regions with most suitable climate (lat. 29° S). Although there is a relatively high demand in the local

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market, it is not very well known abroad, so extensive plantations were not made, as acreage depends on price fluctuations. Not more than 200 ha were planted between 1970 and 1980 (Lagos and Hederra 1979). Attention has been paid to this species lately, because: (1) the fruit is a quality material for the canning industry as it maintains its consistency and fragrance; (2) the fruit is used to flavor ice creams, desserts, preserves, juices, pastries, and as candied fruit (3) the plant is also important due to its papain content; this proteolytic enzyme is used in the brewery, food, and leather industries, and in pharmacy. Baeza et al. (1988) postulate that papain activity in *C. pubescens* is seven times higher than in *C. papaya*; perhaps this is why *C. pubescens* is not edible when raw (Fig. 2b).

The latex of plant tissues and fruits contains approximately 90% papain (Hermosilla 1979, 1984). A vertical incision is made in the fruit to extract latex, which flows into polyethylene bags. Surprisingly, the wounded papaya fruits do not rot, but heal perfectly. The scar is similar to a thrips attack, but is easily removed by peeling. It is necessary to do more research to implement dual or single utilization of fruits and latex.

Carica × *heilbornii* has been assayed in the field in areas where *C. pubescens* grows. Fruits develop normally, but work on phenology, fruit set, growth, and development of the whole plant must be done.

1.3 Limitations

Productivity is hampered by the polygamic conditions: identification of the most productive sexual forms, their selection and multiplication, and crosses can probably help. Selection is also important to obtain cold-tolerant lines and extend the cultivation area to the cooler central valley of Chile, as yield varies seasonally, assuming influence of photoperiod, and temperature on kind and placement of productive flowers (Moreno et al. 1979).

Increases in plantations of *C. pubescens* (and *C. heilbornii*) can be expected so far, in terms of resistance to viruses (affecting *C. papaya* crops in the tropics, i.e., PRV – papaya ringspot virus); but other pathogenic pressures may severely affect mountain papaya. Plants 6 to 7 years old are normally removed, because of lower yields probably due to viruses. Poor regeneration and diseased plants result from apparently healthy cuttings obviously affected by several hidden pathologies of unknown etiology.

As discussed by Litz (1986), ringspot virus is the most serious problem for papayas, but others, such as mosaic virus, apical necrosis virus, yellow crinkle virus, tobacco ringspot, and papaya bunchy top, also cause severe losses. Systematic assessment of *C. pubescens* resistance to these is then needed.

While the Chilean climate is not favorable to *C. papaya*, *C. pubescens* shows limitations due to polygamy and seasonal fluctuations limiting productivity (Kiger 1979; Moreno et al. 1979). The sexual forms, not recognizable in the juvenile stage, and differences due to seasonal variations are aggrevated by the traditional use of seeds, and in the field the number of "male" plants can be as high as 30%. If selection of the best specimens is accompanied by vegetative propagation systems, it will be possible to reduce variability in this species; however, other improvement alternatives, through the expression of new genotypes, must be explored.

C. pubescens is the species most cultivated in Chile, due to its good adaptation to local conditions; however, other members of the *Carica* genus are widely distributed in the subtropical and tropical areas of the world (Litz 1986). Species, and cultivars within species, have been chosen for their suitability to specific climatic conditions, but all are limited by temperature; here again, the cold tolerance of *C. pubescens* could be an advantage, and the need for controlled interspecific protoplast fusion becomes evident. From this point of view, the possibility to obtain somatic hybrids between species of this family, in vitro regeneration, and the evaluation of some criteria employed to characterize the plant material used, are most relevant and discussed here.

1.4 Conventional Propagation

Conventional propagation of *C. pubescens* is done by setting three or four seeds in clusters and cultivating plants until flower characteristics appear, and/or fruit yield is evident; later, male or less productive plants are eliminated, with additional work and costs for growers.

Attempts to propagate the most productive types asexually have been made. The results have been poor: although roots and plants can be recovered, they are stunted, chlorotic, and show severe disease symptoms not present in the parents (mostly viruses). This is a severe limitation to propagation in the field, and makes implementation of in vitro culture techniques necessary.

1.5 Need for in Vitro Culture of *Carica* spp.

In vitro culture of *C. pubescens* species is important to achieve the following goals:

- Obtaining virus-free plants.
- Multiplication of selected material, i.e., high yield floral forms (female clones?), highly productive lines.
- Multiplication of high papain level clones, cold-tolerant clones, or clones showing other useful traits. A useful approach could be to obtain homozygous material derived from haploids regenerated through androgenesis.
- Obtaining and regenerating protoplast fusion products, or embryos, from crosses with other *Carica* species. This can lead to new recombination products.
- Conservation, for *C. chilensis* (palo gordo). This species is considered in danger of extinction at present, and in vitro multiplication could rapidly rescue and increase material of existing genotypes or germplasms.
- In the case of Babaco this hybrid between *C. stipulata* and *C. pubescens* is propagated asexually only since no seeds are produced.
- Selection and propagation of ecotypes or clones with a wider adaptation range also require massive multiplication systems.

2 Brief Review of Tissue Culture Work on Papaya

Most work and attention have been devoted to *Carica papaya*. Several authors have reported intensively and defined protocols to regenerate this species through different in vitro propagation cell and tissue systems (see Litz and Conover 1979, 1980; Litz 1984; and George and Sherrington 1984 cited therein).

Since *C. papaya* plantlets were used in our work with the sole purpose of obtaining protoplasts for fusion experiments with *C. pubescens*, our protocols were envisaged mostly towards callus proliferation, as the resulting yellowish tissues and their cells were easy to distinguish from the green chloroplast-rich mesophyll cell used as protoplast source in *C. pubescens*.

In the case of *C.* × *heilbornii*, most work has been carried out by Cohen and Cooper (1982); also Cossio and Torelli (unpubl.) indicated methods to produce high numbers of plants, for commercial application, using micropropagation.

In our laboratory somatic embryogenesis from calli has been achieved in *C. papaya* and C. heilbornii. In this last species, isolated subcultured embryoids de-differentiated later into calli. The use of axillary buds was more successful, and, as reported, needs supplements (Cohen and Cooper 1982) and two-step cultures (see Protocol). Information on previous work done on *C. pubescens* is scanty (Jordan et al. 1983, 1986; Mosella and Iligaray 1985; Jordan 1986).

3 Micropropagation

3.1 Axillary Buds

Work has been done on propagation of *C. pubescens* via regeneration of plants starting from shoot tips and petioles (Mosella and Iligaray 1985). The shoot tips and petioles were obtained from growing adult plants, both from the field and greenhouse. The shoot tips were prepared and cut in the laboratory to obtain suitable explants. The size of shoots tips used as explants was from 0.4 to 0.5 mm, consisting of the meristem and one or two leaf primordia. These explants were sterilized in 5% calcium hypochlorite for 10 min and rinsed several times in sterile water. DIECA was added (0.15%) to avoid tissue browning.

Regeneration of plantlets from meristematic shoot tips was attempted in media containing 0.1 mg/l benzyl amino purine (BAP); 0.1 mg gibberellic acid (GA_3) and a phenolic compound, floroglucinol, 126 mg/l. Growth was carried out under controlled conditions (temperature 24 to 26 °C, 1600 1 × and 16 h/day photoperiod). Under these conditions explants grew rapidly, to an average size of 5.5 mm in 35 days. These results were apparent only when GA_3 was present in the media, and floroglucinol added.

In order to trigger root formation in developed apices, it was necessary to transfer them to fresh media, now containing indol butyric acid (IBA) 5 to 6 mg/l. Explants did not show responses to other standard auxins. Rooted plantlets did not exceed 10%, and responses were clearly dependent upon the season when in vitro

culture starts and the sex of the mother plant. There is evidence indicating that the best season is from November to March (summer) for explants obtained from female plants; plantlets then show good equilibrium between leaves and roots.

Transplant and autotrophic conditions are quickly reached under artificial mist. Transplanting can be done either to pots containing a peat moss-sand-earth mix or using Jiffy tablets.

3.2 Somatic Embryogenesis

3.2.1 Somatic Embryogenesis from Callus (Jordan et al. 1983)

Callus tissues were cultured starting from hypocotyls of 2-month-old plantlets from the greenhouse; explants were also isolated from the pericarp or the peduncle of immature fruits (80% of final size). Hypocotyl explants were about 3 mm long and weighed 4.5 mg on the average; pericarp and peduncle sections weighed 62 mg (average). All explants were sterilized in 2% commercial sodium hypochlorite for 3 min, and then cultivated in Pyrex tubes (25 × 125 mm) containing 13 ml of autoclaved nutrient media each (pH 5.5), on Whatman No. 1 paper bridges. Tubes sealed with aluminum foil were placed in a culture chamber at 25 °C, 18 h/day 56 $\mu E \cdot m^{-2} \cdot s$. Explants were fixed in formalin-acetic acid-alcohol (FAA), dehydrated in butyric alcohol series, embedded in Paraplast, sectioned at 10 µm and stained with safranin-fast green, for anatomical observations.

Three nutrient solutions with increasing salt levels were tested. Either Nitsch and Nitsch (NN) 1969, Murashige and Skoog (MS) 1962, or Jordan et al. (J) 1978 basal media consisting of MS micronutrients, iron, and NN vitamins combined with different concentrations of α-naphthaleneacetic acid (NAA) and 6-furfurylaminopurine (K) were used.

Subculture of calli was done on half-strength MS medium, solidified with 0.8% agar and supplemented with casein hydrolysate and IAA as a single hormone.

It was seen that hypocotyl and pericarp explants reacted quickly, initiating callus growth after 7 or 8 days in culture, in the different nutrient media and various hormone combinations tested. NAA was essential to start cell proliferation.

Increasing NAA concentrations, combined with K, enhanced growth noticeably with the sole exception of K 5 mg/l among those tested (Table 1). An average 1500 mg fresh weight/explant was reached after 70 days of culture for hypocotyl explant-derived tissue on MS medium (NAA 5; K 1 mg/l). However, there was considerable variation for growth among individual explants within the same treatments; some sections whitened and died after 1 or 2 days only.

The cells of hypocotyl-derived callus tissue proliferated, showing a typically parenchymatic structure, their size increased mainly at the periphery. A typical yellow color appeared, due to reduced chlorophyll content in the rapidly proliferating tissue, and this condition could not be overcome. However, the initial events leading to embryoid organization within callus tissue were evident (Fig. 2c,d) and after 2 months, outgrowths showing a green color and corresponding to different stages of developing embryoids (up to ten per callus) appeared (Fig. 2e, f, g).

At this stage no subculture was necessary. Somatic embryoids arising from tissue originally grown in relatively high levels of both phytohormones (NAA

Table 1. Effect of growth regulators and nutrient media on growth and regeneration of hypocotyl explants after 70 days. (After Jordan et al. 1983)

NAA (mg/l)	K (mg/l)	Nitsch and Nitsch		Murashige and Skoog		Jordan et al.	
		% Callus initiation	Relative growth	% Callus initiation	Relative growth	% Callus initiation	Relative growth
0.0	0.0	0.0	—	0.0	—	0.0	—
0.0	0.1	0.0	—	0.0	—	0.0	—
0.1	0.0	55.0	+	44.4	+	75.0	+
0.1	0.1	64.3	+	76.5	+	75.0	+
0.1	1.0	40.0	+	56.3	+ +	58.8	+
1.0	0.1	75.0	+ +	38.9	+ +	72.2	+ +
1.0	1.0	42.9	+ + + E	16.7	+ + + E	53.3	+ + E
5.0	1.0	41.2	+ E	12.5	+ + +	26.3	+ +
1.0	5.0	0.0	—	0.0	—	50.0	+

Each treatment consisted of 20 replicates. E = embryoids; callus growth:- no callus formation; + up to 500 mg/explant; + + 500–1500 mg; + + + over 1500 mg.

1–5 mg/l; K 1 mg/l) were not affected by the salt/sugar concentration or qualitative composition of the three nutrient media tested.

Finally, when single embryoids or callus portions with several embryoids were transferred to half-strength MS medium, supplemented with 0.5 mg/l IAA and 200 mg/l casein hydrolysate, normal plantlets were recovered.

Using the same medium, other subcultured callus portions could form roots and/or shoots, depending on the level of both growth regulators initially used. Additionally, induction was sustained after a long period, since yellow callus still gave rise to new embryoids.

On the other hand, pericarp sections showed only initial de-differentiation events, under the same conditions as described above, and peduncles did not respond at all.

Our experience leads us to say that *C. pubescens* has a reasonably good potential for in vitro regeneration using juvenile tissues. Transfer to the greenhouse followed as described in Section 3.1.

3.2.2 Somatic Embryogenesis from Cell Suspension Cultures (Jordan 1986)

We tested the in vitro embryogenic capacity of cell suspension cultures of *C. pubescens* using cells derived from hypocotyl calli, and obtained somatic embryos derived from cells in suspension or from microcalli. We saw that responses were affected by media and phytohormone levels. The best results were obtained when using Nitsch and Nitsch medium (NN) supplemented with NAA to grow the cells in suspension and then plating them on the same medium, but containing benzyladenine, or using combinations of both hormones.

As for callus, we used hypocotyl sections 3 mm long, from greenhouse plants, and cultured them on NN medium plus NAA and 6-BA 1 mg/l. After 30 days, there was no evidence of embryoids or organogenesis in calli examined under the

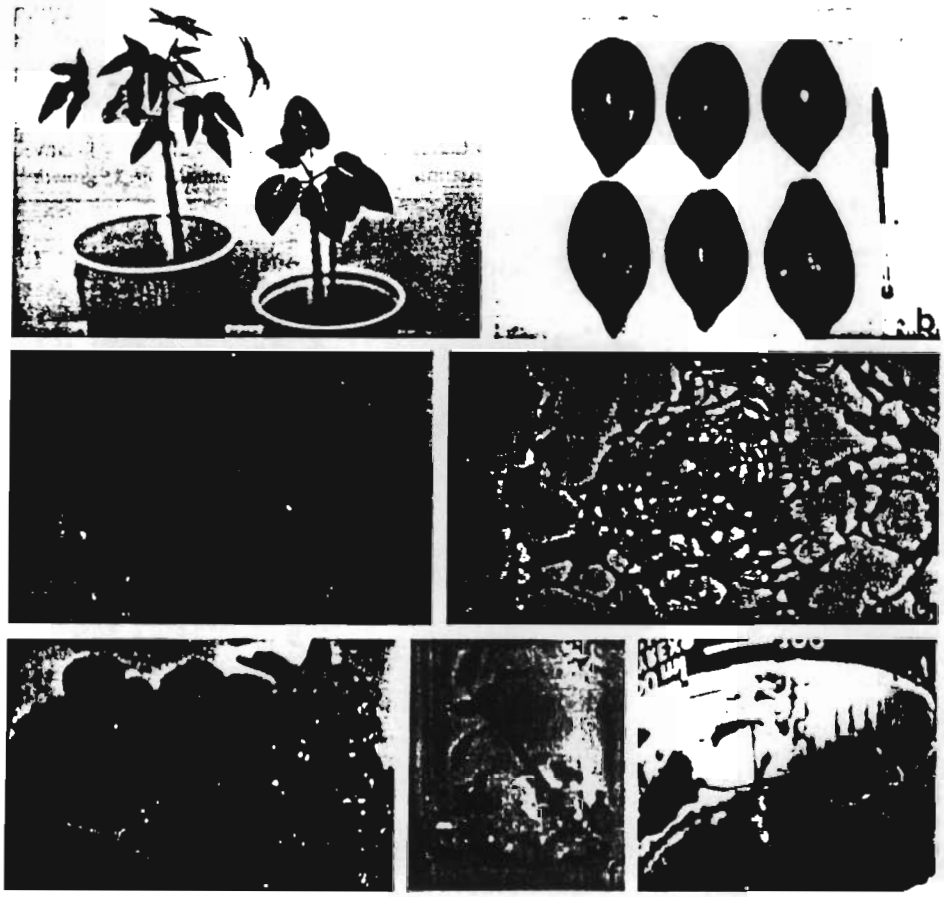


Fig. 2. **a** Plants of *C. papaya* (*left*) and *C. pubescens* (*right*) grown in the greenhouse. **b** Green fruits of *C. pubescens*, av. size 12 cm, used as pericarp explants. **c** Hypocotyl-derived callus of *C. pubescens* after 80 days in culture. Two green somatic embryoids rise at the surface. **d** Re-embryonalization in older callus tissue leading to embryoids. **e** Embryogenetic callus with several developing embryoids; the whole tissue gradually undergoes embryogenesis. **f** Complete plantlet and development of new embryoids after subculture in a solidified medium. **g** Plantlet transferred to soil (greenhouse)

microscope. Fresh weight of calli averaged over 2000 mg at the time. Cell suspensions were prepared using five sections of 5 mm diameter, average 60 mg each, of nonembryo forming unorganized calli, which were transferred to 50-ml Erlenmeyer flasks each containing 4 ml of NN or modified B5 medium, with combinations of NAA and BA at zero or 1 mg/l. The flasks were shaken at 120 strokes/min. Cultures were maintained at 28 °C and 50 $\mu E/m^2/s$, 18-h photoperiod. After 10 days, suspensions were examined to check for the presence of embryoids and viability. No embryoids were detected, only single cells, viability 90% and over. Then 2 ml of each suspension examined were used to plate four Petri dishes 5 cm in diameter. The dishes contained NN or B5 media with 0.5% agarose and NAA/BA combinations (either zero or 1 mg/l). Dishes were kept at 28 °C in the dark for 4 days, and then under 18-h photoperiod (100 $\mu E/m^2/s$).

Table 2. Morphogenic responses of *Carica candamarcensis* cell suspensions started from nonembryo forming callus[a] to phytohormones and nutrient media after 8 weeks in culture[b]. (After Jordan 1986)

Plating conditions[d]	No hormones	Suspension conditions[c] NAA(1 mg/l)	BA(1 mg/l)	NAA + BA(1 mg/l)
No hormone	N[e] n.r.[e] Ñ[d]	N clusters Ñ N calli G̃ G m-calli fibrilar G̃ shape	N m-calli fibrilar Ñ shape, *proembryoids* + N colonies, m-calli G̃ G n.r. G̃	N m-calli fibrilar Ñ shape N n.r. G̃ G calli G̃
NAA (1 mg/l)	N clusters and Ñ calli G n.r. G̃ G m-calli[f] Ñ	N m-calli Ñ N colonies G̃ m-calli G m-calli G̃	N m-calli Ñ N n.r. G̃ G m-calli G̃	N calli Ñ N m-calli G̃ G n.r. G̃
BA (1 mg/l)	N *proembryoids*[g] + Ñ and calli G m-calli Ñ G colonies G̃	N *proembryoids* + + + Ñ m-calli N *proembryoids* + G̃ G callus G̃	N n.r. Ñ N m-calli G̃ G m-calli G̃	N n.r. Ñ N m-calli G̃ *proembryoid* + G n.r. G̃
NAA + BA (1 mg/l)	N clusters and Ñ m-calli G m-calli G̃ G calli and Ñ *proembryoids* + G *proembryoid* + G̃	N clusters and Ñ *proembryoids* + + N n.r. G̃ G m-calli G̃	N n.r. Ñ N n.r. G̃ G fibrilar colonies G̃ and clusters	N n.r. Ñ N m-calli G̃ G n.r. G̃

[a] Calli were raised from hypocotyl sections in Nitsch and Nitsch (1969) medium supplemented with NAA + BA (1 mg/l) over 30 days. [b] Values represent observations averaged from four dishes/treatments. [c] suspension media or conditions. [d] Culture media (plating). [e] No response. [f] m-calli = microcalli. [g] Proembryoid formation (indicates treatments where at least this stage was observed) + + +, up to 30 proembryoids/dish; + +, up to 10 proembryoids/dish; +, only few proembryoids.

Several combinations of nutrients and hormones gave rise to somatic embryogenesis in suspension cultures, as summarized in Table 2. The best response was obtained when cells were suspended in NN medium containing NAA 1 mg/l and then plated in NN medium plus BA. After 8 weeks more than 20 yellowish green embryoids were counted in each Petri dish. Additionally, several developing

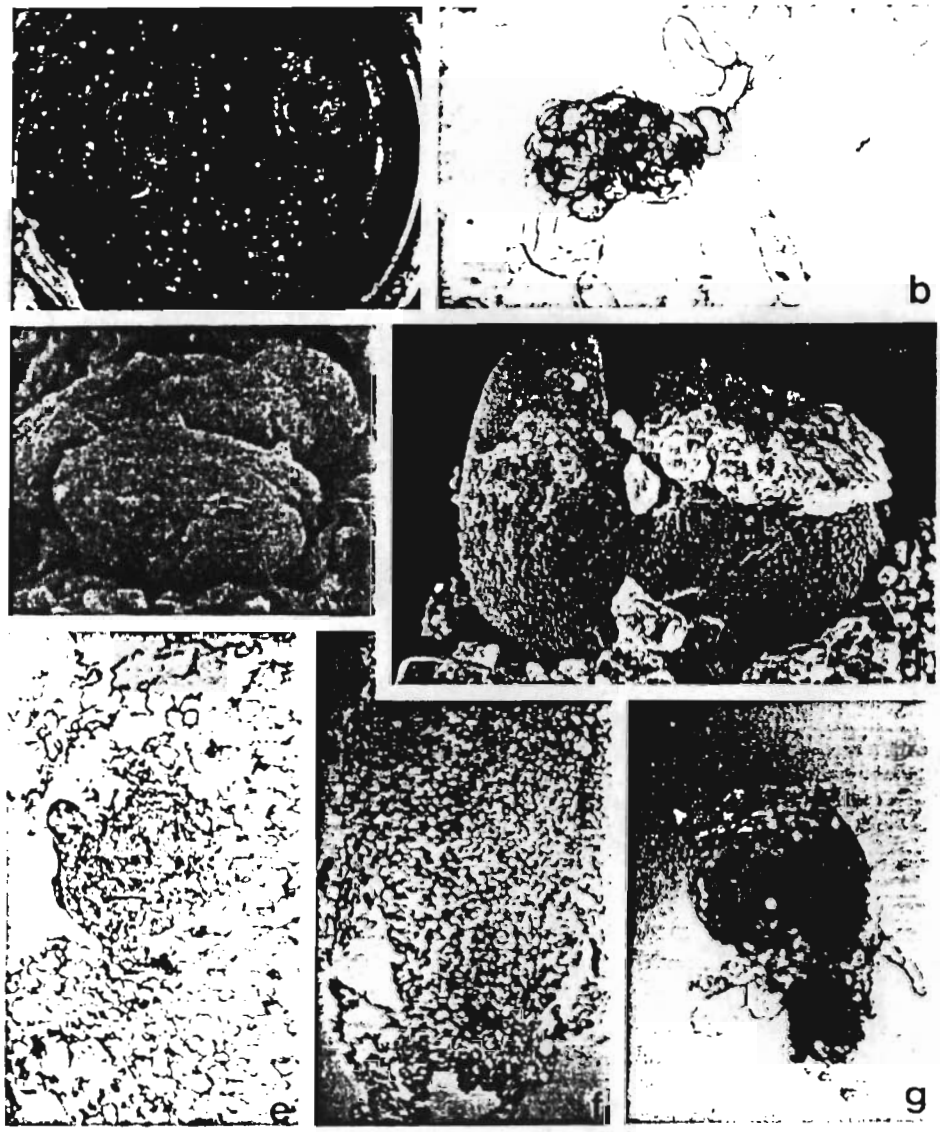


Fig. 3a–g (After Jordan 1986). **a** Embryogenic calli and embryoids developed from suspended cells after 8 weeks. Several other embryoids originating throughout the calli tissue are also visible. **b** A pro-embryo-like structure derived from cell suspension after 2–3 weeks in culture. **c, d** SEM photographs: embryoids protroduing from callus tissue. **e** Cross-section of an embryoid developing on the surface of calli. **f, g** Developed embryoids in a cross-section and isolated from the maternal callus tissue

proembryoids were observed under the microscope. Other treatments also led to embryoids, but at a reduced frequency.

The presence of BA (1 mg/l) either at the suspension or plating phases, was always necessary to obtain embryoids. Most embryos were obtained using NN medium only, but when using B5 medium for suspension and NN medium for plating, or vice versa, this was also possible. When using G medium only, microcalli did proliferate, but fewer embryoids were observed, even after 3 months under culture. Figure 3a–g represents some regeneration events (indirect embryogenesis) from cell suspensions through microcalli.

From our work, it can be seen that the composition of the NN medium and the presence of BA in the plating phase are favorable conditions to induce embryogenesis in *C. pubescens*, which seems more recalcitrant then *C. papaya* and *C. stipulata*; these species readily express somatic embryogenesis from cell suspensions (Moore and Litz 1984).

3.3 Androgenesis

Recently, direct embryoid formation from microspores of *C. pubescens* anthers was obtained (Jordan et al. 1991). Induction followed use of an MS-modified medium with 6% sucrose and including auxin and BA 1 mg/l each. Haploid or doubled homozygous material can be used for sex determination studies and breeding purposes.

Table 3. Preplasmolysis responses of juvenile leaves of *Carica candamarcensis* to different mannitol and sucrose concentrations. (After Jordan et al. 1986)

Osmoticum[a]	% approx.	Molarity	Time (min)	Preplasmolyzed cells %
Mannitol	11	0.60	30	30
			60	20
			90	2
	13	0.71	30	35
			60	90
			90	60
	15	0.82	30	15
			60	8
			90	0
Sucrose	20	0.58	30	40
			60	35
			90	20
	22	0.64	30	30
			60	85
			90	35
	24	0.70	30	35
			60	20
			90	8

[a]Osmoticum in inorganic CPW salts.

3.4 Protoplast Fusion

Considering that breeding work within the Caricaceae is hampered by sexual incompatibility, transfer of disease resistance and other desirable selected traits can be handled by means of interspecific protoplast fusion.

Methods are described by Jordan et al. (1986). As a first step to obtain somatic hybrids, special requirements to isolate, fuse, and culture protoplasts of *C. pubescens* and *C. papaya* must be defined. We worked with these two species, isolated protoplasts, fused them, and arrived at the first stages of cell cluster formation.

Preplasmolysis conditions, protoplast yield responses of different explants to enzyme concentrations, temperature, nutrient media, tissue sources, and manipulation were established for protoplast experiments (Table 3, Figs. 4, 5).

To obtain protoplasts, were used *C. pubescens* young leaves from greenhouse plantlets (4 months old) as well as adult leaves from 2-year-old plants in the field. Protoplasts of *C. papaya* cv. Pauna were isolated using calli arising from cultured hypocotyl sections. First, the material was disinfected with Captan 0.2% 10 min rinsed in sterile distilled water, immersed in 70% ethanol for 60 s. Leaves were then soaked in Na-hypochlorite 10% for 7 min and finally rinsed in distilled water.

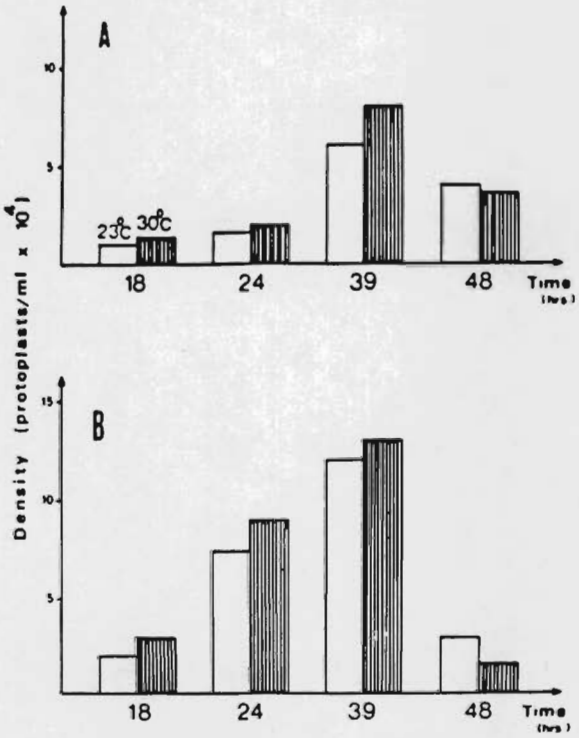


Fig. 4A, B. Effects of temperature, time, and enzyme concentration on protoplast yield of *C. candamarcensis* juvenile leaves. (A) Cellulase 0.5%, Macerozyme R-100. 1%, Mannitol 13%. B Cellulase 2% Macerozyme 0.5%, Mannitol 13%. (After Jordán et al. 1986)

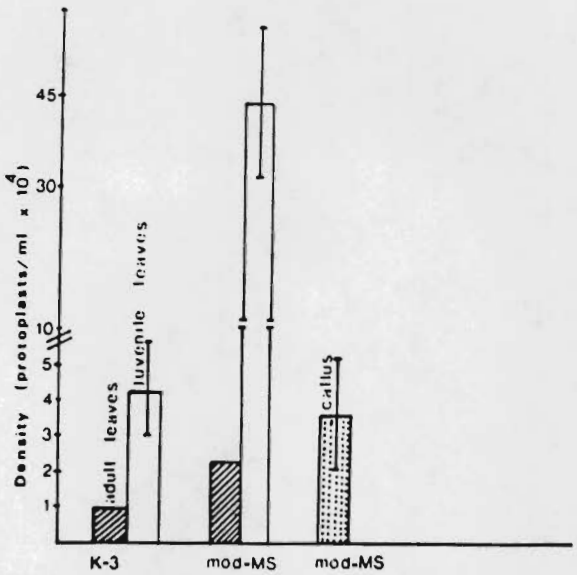


Fig. 5. Protoplast yield from juvenile leaves, adult leaves and calli of *C. candamarcensis* in two digesting media after 24 h at 30 °C.

For preplasmolysis, leaves were set in mannitol osmoticum (11–15%) or sucrose (20–24%) plus CPW inorganic salts (Reinert and Yeoman 1982) for different periods of time. Leaves were sliced and left to digest in the dark, in the same media containing Cellulase and Macerozyme in several concentrations, combined with mannitol, methane-ethane sulfonate, $CaCl_2$ in Kao et al. (1974) protoplast culture medium or modified MS nutrients.

Enzymes were filtered and media autoclaved. Protoplasts were centrifuged at 100 g, pellets suspended in CPW or NN media, filtered, centrifuged, washed three times in 13% Mannitol plus CPW salts, and then plated on modified NN medium or B5 medium, gelled using 0.5% agarose. The Petri dishes were kept in the dark for 48 h, at 22 °C, then drops of fresh NN medium were added to the dishes, lowering the concentration to 8% to allow wall regeneration and cell division.

For fusion, protoplasts from both sources were resuspended as above, and drops containing each kind of protoplast were mixed in a concave slide; drops of PEG 20 000 MW were added before plating. The sequences of protoplast isolation, fusion, and culture were followed under the microscope. Samples were stained with 0.1% bromphenol blue to check viability, and density was checked with a Reichert Bright-Line hematocymeter.

Best preplasmolysis response (85–90% average), occurred after 60 min when treated with 13% Mannitol or 22% sucrose; the highest yields of viable protoplasts were obtained with 2% Cellulase and 0.5% Macerozyme 0.5% at 30 °C during 39 h. Better yields were obtained from juvenile leaves, the mod-MS medium was better than the K_3. Figure 6a–e shows protoplasts and microcalli derived from fusion experiments of both *Carica* species.

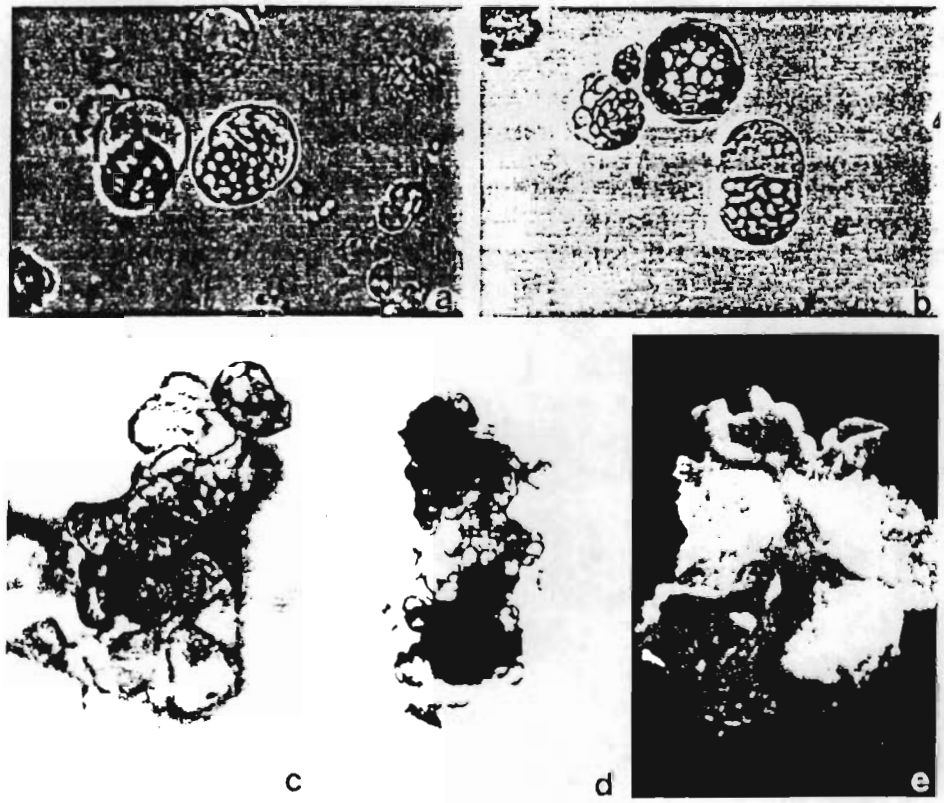


Fig. 6. **a, b** Isolated and fused protoplasts of *C. pubescens* (from green mesophyll parenchyma) and *C. papaya* (from yellow calli cells) **c, d** Cell clusters (microcallus) and organogenic callus with greenish outgrowths **e** Organogenic callus with abnormal leaves after 3 months of culture. Growth is stunted.

3.5 Biochemical Markers as a Selection Method

An important goal related to selection of better or more productive plants in the mountain papaya, following micropropagation, is the possibility to characterize patterns in adult plants related to productivity and eventually, to try to identify them, if expressed, in juvenile stages. Since *C. pubescens* shows great flower variability and "female types" of plants are most productive, attempts to recover plants belonging to this floral form would be desirable. On the other hand, biochemical markers related to the expression of those characters would be very helpful. Biochemical markers have been used before in papaya to characterize somatic embryos derived from incompatible hybrids of *C. cauliflora* and *C. papaya* (Moore and Litz 1984). It is easy to visualize the importance of these markers for other incompatible cross-products that may introduce resistance for papaya ringspot virus (PRV) in *C. papaya*. Since *C. cauliflora*, *C. stipulata* Badillo, and *C. pubescens* have been reported as resistant (Jiménez and Horowitz 1958; Horowitz and Jiménez 1967), fusion products, including protoplast fusion, if viable or organogenic, can be examined in early growth/development stages for their

characteristics if patterns of the parent are known. Enzyme activity can also be an interesting marker in crosses or selection programs since *C. pubescens* shows about seven times more papain enzyme activity than papainases found in *C. papaya*; differences also exist in the electrophoretic behavior of proteinases between both species (Baeza et al. 1988) and other changes in chemical composition are detectable within cvs. of *C. papaya* (Selvaraj et al. 1982).

In order to initiate studies to visualize differences found in plants with special floral forms in the mountain papaya, "female" and "male" plants were collected from the field. Leaves and flowers of three plants of each sex form were compared. Material was freeze-dried and tested for proteins by SDS-polyacrylamide gel electrophoresis. TLC of amino acids, HPLC of phenolic compounds, and isoelectrofocusing for phosphatases and peroxidases were performed. Results showed some electrophoretic but nonconclusive differences between the bands present in female flowers compared to male flowers (Fig.7) as also for amino acids (not shown); on the other hand, only small quantitative differences and no qualitative differences were observed comparing flower types; the same happened between leaves of both types of plants in the HPLC results. As expected, clear differences in the amount of some phenolics were found between leaves and flowers in each type of plants (Fig. 8). Regarding peroxidases and phosphatases, no differences were found either. These preliminary results (Jordan and Treutter unpubl.) found for mountain papaya must be confirmed in further work although it is doubtful if clearcut differences will appear when comparing floral forms in this species.

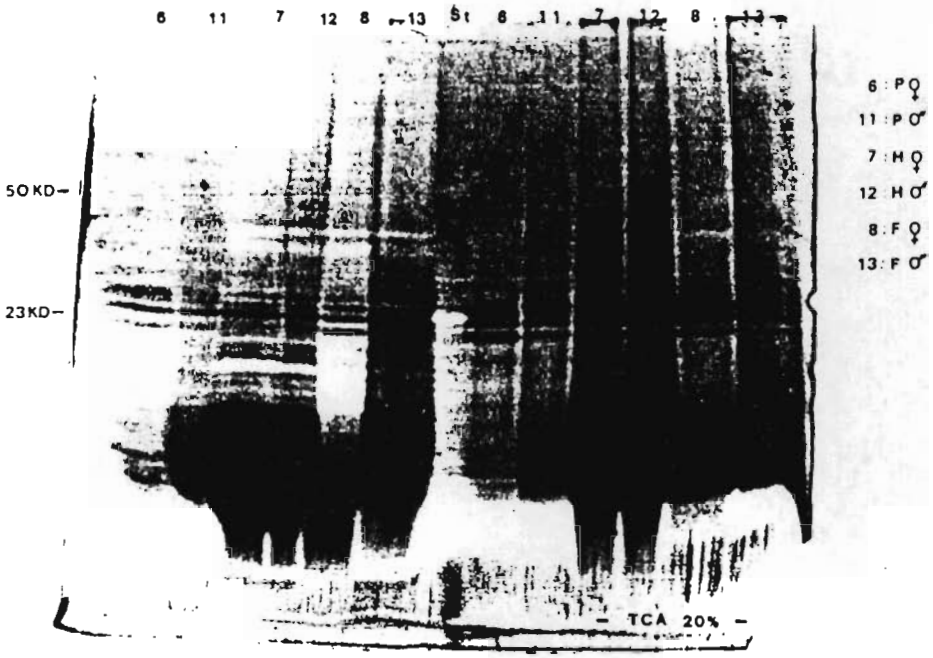
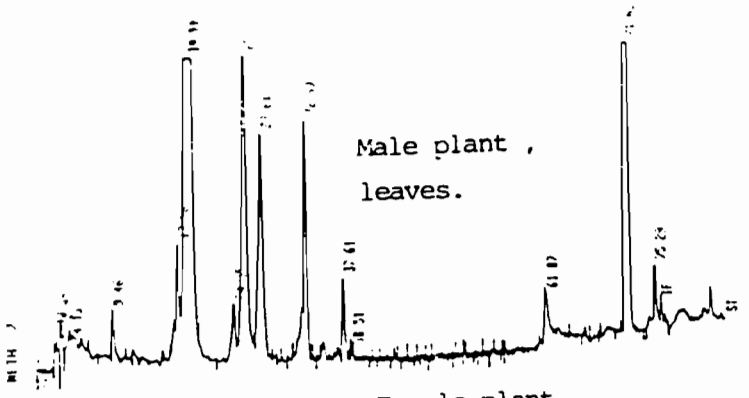
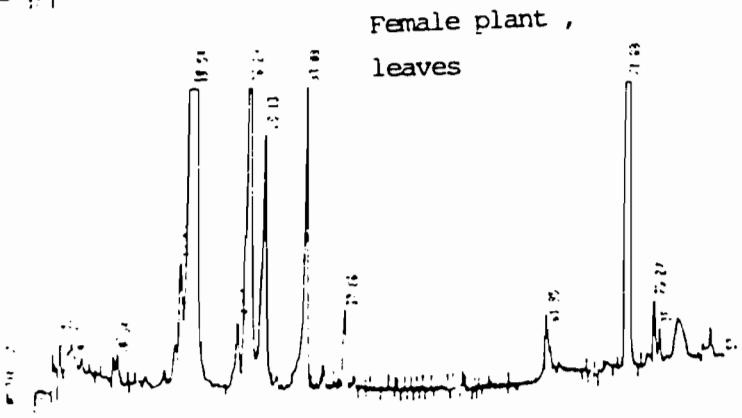
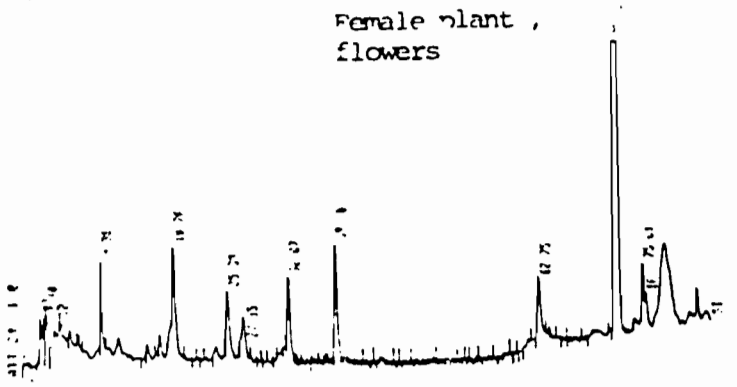
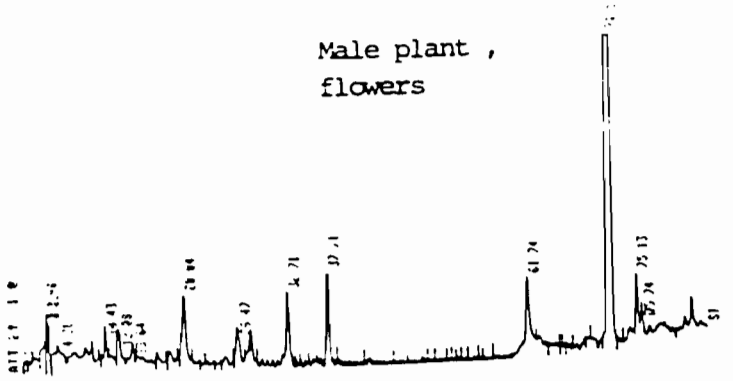


Fig. 7. SDS-polyacrylamide gel electrophoresis of proteins of leaves, petioles, and flowers of female and male plants of *C. candamarcensis*. Only some differences are seen, i.e., a band present in female flowers *8 : F* not seen in male flowers *13 : F*. *F Flowers*; *H* leaves; *P* petioles


Male plant ,
leaves.


Female plant ,
leaves


Female plant ,
flowers


Male plant ,
flowers

3.6 Transfer to Greenhouse or Field

Little work has been done concerning plant performance in the field. Plantlets have been maintained in the greenhouse without problems. Vitrification or abnormal variants have not been detected so far. In colder regions, greenhouses with double polyethylene covers and the use of heating systems permit plantlets to grow (Acevedo, pers. commun.). Further development must be carried out in milder coastal climates.

3.7 Commercial Aspects

Somatic embryogenesis from calli seems to be the quickest, most efficient, and most economical way to propagate *C. pubescens* since the whole callus is embrygenic, and a large number of plants are recovered. Economic estimates have not been worked out in detail, but, considering that large numbers of plants are available through short-term culture, as there is no need to subculture, costs may be low if desired traits can be maintained.

4 Summary and Conclusions

Different micropropagation systems are suitable to regenerate papaya (Jordan et al. 1983; Litz 1984; Jordan 1986, 1989). Proper conditions must be defined for mountain papaya and babaco to reach this result. Information available so far

Table 4. Morphogenic responses found in three species of *Carica*

Species	*C. candamarcensis*	*C. pentagona*	*C. papaya*
Explants			
Hypocotyls	Calli, embryoids, Plantlets		Calli, embryoids
Axillary buds	n.s.	Roots, plantlets	Calli
Pericarp	Calli, embryogenic tissue	No response	n.s.
Leaves	Calli	n.s.	n.s.
Petioles	Roots, calli, embryoids, Plantlets	Calli, embryoids[a]	n.s.
Anthers	Calli (somatic), androgenesis		n.s.
Pith	Calli	Calli	n.s.
Protoplasts	From leaves: fusion products, microcallus, shoot	n.s.	From callus (hypocotyl): Fusion products, microcallus, shoots

n.s.: not studied in our laboratory.
[a] De-differentiated later.

Fig. 8. HPLC phenols (Standard: 6-methoxyflavon). As shown no clear qualitative differences between phenolics of flowers and leaves were found, but quantitative discrepancies: in the leaves of both male and female plants the main peak was RT 19 min, which is 30 times as large as peak RT 37 min and six times as peak RT 33 min; these three compounds show an equal number in flowers. The molecular structure of the phenolic compounds could not be determined. (Jordán and Treutter, unpubl.)

from the experience gained in our laboratory (Table 4) allows breeding work and selection incorporating genetic engineering/protoplast fusion, since protoplasts are available. Chromosome number 2n = 18 in these species may be an advantage to generate rearrangements during recombination experiments with protoplasts.

5 Protocol

1. Babaco regeneration: axillary buds respond to culture in MS 1/2 strength + ascorbic acid, NAA, BA; 0.05 and 0.8 mg/l respectively, then transfer to NN 1/2 strength + addenda, (Cohen and Cooper 1982) and phytohormones.
2. Callus-somatic embryogenesis "mountain papaya": hypocotyl sections on NN medium form large calli (30 days); embryoids (70 days), without subculture. Best medium NN + NAA and BA 1 mg/l each, pH 5.5. Whatman No. 1 paper bridges, 18-h light 57 $\mu E/m^2/s$ (Philips lamps TL 40 W 54), 25 C. Isolated embryoids grow to plantlets/plants on 1/2 strength NN medium 0.5 mg/l IAA + casein hydrolysate 200 mg/l.
3. Cell suspension—somatic embryogenesis "mountain papaya": 30-day calli most suitable. Suspend 60-mg calli pieces in 4 ml NN medium + 1 mg/l NAA, agitate 120 strokes/min, 10 days, light as in (2), 28 C. Check for embryoids, then plate 2 ml in agarose (Sigma A 6013) with NN + BA 1 mg/l only. Keep 4 days in darkness, then at 100 $\mu E/m^2/s$ for microcalli and green embryoids.
4. Protoplast fusion products: *C. pubescens* young green leaves (a) give the highest yield. Cells and protoplast of *C. papaya* cv. Pauna (b) are from hypocotyl calli showing little pigmentation.
   (a) Leaves: disinfection 10 min Captan 0.2%, water rinse, immersed ethanol 60 s; transferred Na hypochlorite 10% 7 min; washed dist. sterile water. Preplasmolysis: Mannitol 13%, 60 min or sucrose 22%, 60 min in CPW salts (Reinert and Yeoman 1982). Digestion, darkness, Cellulase 2%, Macerozyme R-10 0.5%; Mannitol 13 M, MES, 0.45%, $CaCl_2$, mod MS medium, 39 h, 30 C.
   (b) *C. papaya* calli protoplasts: start with preplasmolysis and digestion as in (a). Protoplasts filtered, centrifuged 100 g (three times); passed triple 62 mesh nylon sieve; washed CPW + Mannitol. Plate protoplasts on NN mod medium: 1/1 vol fresh NN/30-day-old callus growth medium + 0.5 agarose (Sigma A-6013), Mannitol 13%, keep dark for 48 h. Add drops osmoticum-free NN to lower Mannitol conc. to 8%. Fusion: mix drops of both suspensions plus microdrops PEG 20000 (Sigma B-2263) on concave slide. Viability check: bromphenol blue 0.1%.

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22 Tropical and Subtropical Small Fruits

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Actinidia spp.

History and importance

Kiwifruit usually refers to *Actinidia deliciosa*; however, the genus is variable and there are a number of distinct taxa. The cultivated species, according to Liang & Ferguson (1984) are *A. chinensis* Planch. var. *chinensis* ($2n = 58$) and *A. deliciosa* (A. Chev.) C.F. Liang and A.R. Ferguson var. *deliciosa* ($2n = 170$ or 174). They belong to the Actinidiaceae (section *Leiocarpae*), and originated in China, where they occur in nature in many areas, especially in the forests of the valley of the Yang Tse River (Rosenberg, 1981; Ferguson *et al.*, 1990).

There has been rapid expansion of kiwifruit production in recent years in several countries (Panorama Económico de la Agricultura, 1985). The largest areas of production are in New Zealand, with approximately 34% of the total, followed by Italy, Chile, France, Japan, and the USA (Morales, 1988; CORFO, 1989).

History of genetics and breeding

The most important varieties originated in New Zealand, i.e. 'Abbott', 'Allison', 'Bruno', 'Hayward' and 'Monty'. However, they did not arise from a breeding programme involving specific crosses. After the species was introduced from China into New Zealand in 1904, selections were made among seedlings; the best selections were spread as horticultural varieties (Zuccherelli & Zuccherelli, 1982).

The major cultivars of New Zealand are derived from a single staminate and two pistillate plants; currently, the most widely grown variety is 'Hayward' (Rosenberg, 1982; Zuccherelli & Zuccherelli, 1982; Kulczewski, 1985; Ferguson, 1990; Ferguson *et al.*, 1990). Nevertheless, Kulczewski (1985) points out that this cultivar is the least precocious and prolific of all existing pistillate selections. There is little information on the horticultural behaviour of other selections (Rosenberg,

1982; Ferguson, 1990; Ferguson *et al.*, 1990).

The supremecy of 'Hayward' effectively means that the entire kiwifruit industry is dependent upon a single genotype, which could render the crop extremely vulnerable to disease and insect pests (Young, 1985; Revilla & Power, 1988; Brook, 1990). This implies that the application of tissue culture and genetic manipulation technologies could broaden the genetic base of the species (Revilla & Power, 1988).

ding mechanisms and limitations

The kiwifruit is dioecious. Pistillate plants produce non-viable pollen, although the flowers appear to be perfect (Ferguson, 1990). Staminate flowers have non-functioning styles. As a result of domestication, Ferguson (1990) has noted that there is an apparent gradual shift toward hermaphroditism. The flowers are probably insect pollinated.

Although the genus contains more than 50 species that have been collected from temperate and subtropical regions, there do not appear to be major barriers that would limit hybridization between species. Suezawa (1989) obtained successful crosses between *A. arguta* ($2n = 4x$), *A. chinensis* ($2n = 2x$) and *A. deliciosa* ($2n = 6x$).

The primary limitation for breeding kiwifruit is the widespread acceptance of 'Hayward' as the epitome of the fruit (Ferguson *et al.*, 1990). Although there is assumed to be great genetic diversity within the genus, this has not been exploited. According to Ferguson (1990), it is possible that production of 'Hayward' can be further optimized by improving cultural conditions. Because of this reliance on a single cultivar, potential pistillate and staminate parents for a long-term breeding programme have not been identified. For example, the contribution of the staminate parent toward fruit quality is not known.

ıtic Cell Genetics/Molecular Approaches

ial

Although many horticultural problems or physiological disorders have not been detected yet, most producers and fruit specialists concur that breeding should address disease and pest problems, plant and fruit traits and postharvest disorders. Many of these will probably be solved using traditional breeding methods. However, biotechnology may offer several advantages, including screening, selection and characterization at the cellular level, embryo rescue of interspecific crosses or for detection and creating genetic variability (Fraser & Harvey, 1986b; Pringle, 1986).

e and pest problems

According to Ferguson (1990), there is little genetic variability among kiwifruit cultivars with respect to disease resistance. The main problems affecting kiwi

plants are *Phytophthora* spp., which cause root rot, bacterial blossom blight (*Pseudomonas viridiflava*), *Sclerotinia* rot of flowers, leaves and fruit, postharvest rots caused by *Botrytis cinerea* and *Botryosphaeria dothidea* and nematodes (Zuccherelli & Zuccherelli, 1982; Sale 1985). Control of *Phytophthora* damage might be possible using cell cultures for studies on pathogen resistance or with phytotoxin. Sacristán (1986) reviewed studies that demonstrated expression at the cell level for resistance of tobacco to *Phytophtora parasitica* var. *nicotianae*. However, with potato, selection for toxin resistance was inconclusive because resistance to the toxin was not well correlated with resistance to the pathogen (Stolle & Schöber, 1984). This has led to the use of other *in vitro* indicators, i.e. phytoalexin accumulation and activity (Sacristán, 1986). There are still many limitations to the use of cell culture systems for the selection of disease-resistant mutants. Alternative approaches such as exploitation of anther culture, somatic hybridization and genetic transformation could be more promising.

The presence of nematodes (*Meloidogyne arenaria*, *M. hapla* and *M. javanica*) on the roots of kiwifruit can severely restrict plant growth, especially under drought stress (Zuccherelli & Zuccherelli, 1982; Sale, 1985). Nematode control in other fruit crops has usually been approached by using appropriate rootstocks. In kiwifruit, however, propagation is by cuttings or by grafting onto seedlings, which are genetically extremely variable. Identification of cultivars or the creation of new genotypes that are resistant to this pest is urgently needed. Insect pests have not become limiting factors in kiwifruit production; however, as plantings continue to expand, pest control becomes more important. Leafroller caterpillars are the most serious pests in New Zealand (Steven, 1990), although scale insects are becoming increasingly important in Australia and Italy (Ferguson, 1990). As the 'Hayward' monoculture expands in time and space, biotechnological strategies for addressing pest problems may be needed.

nt and fruit traits

The root system of kiwifruit is very susceptible to water stress and is dependent on aeration (Sale, 1985). Under waterlogged conditions, roots are more susceptible to infection by *Phytophthora*. Plants with stress-tolerant roots are needed for many potential growing areas. Early fruit maturation is another desirable character which would result in optimum prices in international markets. Most producers agree that single and bigger fruits are a must, and should be considered as a priority in a breeding programme for the species.

Horticulturally important traits such as root architecture, hermaphroditism, yield and maturation time at present cannot be approached through selection in plant cell cultures (Chaleff, 1986). These characteristics should be addressed through traditional breeding methods. However, biotechnological methods are now available which may significantly accelerate the primary search for and selection of valuable genotypes. As an example, high correlation has been found between intensity of fluorescence of chlorophyll 'a' and plant resistance to water stress (Lichtenthaler & Rinderle, 1988). Isozyme and RFLP studies could be useful for identifying desirable genes and to quantify genetic variability in wild populations. With regard to the creation of genetic variants, embryo rescue of inter-

specific crosses, pollen and ovule cultures for haploid production, endosperm culture for triploid plant regeneration and somatic hybridization should all be exploited. Moreover, an improvement programme will greatly benefit from efficient methods of plant regeneration and *in vitro* conservation of selected genotypes, allowing an easy interchange of plant material among breeders.

Postharvest disorders

Kiwifruit is very susceptible to ethylene during storage. A high percentage of the fruit ripens and turns soft during and after cold storage (Sale, 1985). Genetic transformation of kiwi with either the antisense gene for ACC synthase (van der Straeten *et al.*, 1990) or polygalacturonase (Smith *et al.*, 1988) could be a useful strategy for retarding fruit ripening.

Advances

Organogenesis

Several *in vitro* studies have been reported, most of which concern micropropagation from shoot tips or meristems (Standardi, 1981, 1982, 1983; Standardi & Catalano, 1984; Wessels *et al.*, 1984; Andreani, 1986; Monette, 1986a,b, 1987; Piagnani *et al.*, 1986).

Organogenesis from callus has been described with various objectives, e.g. biochemical and morphogenic responses of tissues in culture, the effect of growth regulators on genetic stability of regenerated plants, organogenic potential in long-term cultures and as an alternative for mass propagation (Hirsch & Fortune, 1984, 1986; Barrales *et al.*, 1986; Leva, 1986; Barbieri & Morini, 1987; Monette, 1987; Revilla & Power, 1988; Comparini, 1989; Botti & Comparini, 1990; González *et al.*, 1990).

Organogenesis from callus derived from stem segments and petioles is possible (Harada, 1975; Gui, 1979; Hong, 1981; Barrales *et al.*, 1986; Pais *et al.*, 1987; González *et al.*, 1990), although Pais *et al.* (1987) mention that Augé *et al.* (1982) considered petioles unsuitable. Harada (1975) obtained an organogenic callus from root and stem segments of male and female plants of 'Hayward', using a modified MS medium (Murashige & Skoog, 1962) supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), naphthoxyacetic acid (NOA) and zeatin. Of all growth regulators tested, zeatin (1 $mg \cdot l^{-1}$) was most effective for inducing shoots, which were capable of developing roots and regenerated into vigorous plants. The presence of NAA (0.1–1.0 $mg \cdot l^{-1}$) was most effective for root formation. The organogenic potential of the calli could be maintained for several subcultures, but especially when the media contained cytokinin or NOA. The importance of zeatin has also been emphasized by Brossard-Chriqui & Tripathi (1981) for shoot proliferation. Similar studies have been reported by Barrales *et al.* (1986), Leva (1986), Leva & Magrini (1986) and Barbieri & Morini (1987).

Gui (1979) obtained a higher number of shoots in callus from female plants and demonstrated that var. *hispida* was more prolific than *chinensis*. Other authors

(Brossard-Chriqui & Tripathi, 1984) have compared the morphogenetic ability of fertile stamens (male plants) with sterile stamens (female plants). They concluded that sterile stamens tended to form roots rather than callus and shoots in comparison with fertile ones.

Hong (1981) was able to include callus and plant regeneration from *A. chinensis* leaves, but when using *A. arguta* leaves under the same conditions, only one plant could be regenerated. The effect of genotype on organogenesis has also been reported by Hirsch & Bligny-Fortune (1979). A preliminary study on the organogenic potential of *in vitro* grown kiwi leaves was reported by Abud (1987), who found that shoot formation was optimum when the cytokinin:auxin ratio was 5:1 (0.5 mg zeatin$\cdot l^{-1}$ and 0.1 mg NAA$\cdot l^{-1}$). A recent study by Botti & Comparini (1990) reports that leaves of *in vitro* grown plants subjected for 32 days to modified MS medium complemented with 1 and 2 mg 6-benzyladenine (BA$\cdot l^{-1}$ plus 0.1 mg NAA$\cdot l^{-1}$) induced the greatest amount of shoots. Zeatin (1 and 3 mg$\cdot l^{-1}$) with NAA (0.1 mg$\cdot l^{-1}$) were less effective with the highest shoot number being obtained 53 days after explanting on medium with 3 mg$\cdot l^{-1}$.

Somatic embryogenesis

There have been a few reports of somatic embryogenesis in kiwifruit, using explants such as leaf, stem and root segments (Harada, 1975; Bini, 1979; Abud 1987; Quintino 1989). All of these studies reported differentiation of embryogenic cells or early stages of somatic embryos, but no plant regeneration. Harada (1975) and Bini (1979), using stem and root segments from male and female kiwi plants and the auxins 2,4-D or NAA (0.1–1.0 mg$\cdot l^{-1}$), were able to induce differentiation from callus of globular somatic embryos but failed to regenerate plants. Abud (1987) and Quintino (1989) obtained similar results when leaf segments were used as explants.

Endosperm culture

The culture of immature endosperm from kiwifruit seeds has proven to be the only effective explant source to date for embryoid induction and subsequent plantlet formation. Gui *et al.* (1982) reported that immature endosperm cultured on MS medium, supplemented with zeatin (3 mg$\cdot l^{-1}$), formed embryos which then developed into plants. Likewise, Huang *et al.* (1983) reported the regeneration of triploid plantlets from endosperm of *A. chinensis*. Kin *et al.* (1990) described the regeneration of plants by organogenesis and somatic embryogenesis from *Actinidia* interspecific hybrids.

Promising results in organogenesis were also reported by Gui *et al.* (1982) when culturing immature endosperm on MS medium supplemented with 3 mg zeatin$\cdot l^{-1}$, 0.1 mg NAA$\cdot l^{-1}$ and 400 mg casein hydrolysate$\cdot l^{-1}$. Numerous adventitious buds were induced after 18 days in culture, which later developed into normal shoots.

Haploids

Anther cultures have been tried in kiwifruit with the aim of producing pollen-derived haploids as a tool for breeding programmes (Brossard-Chriqui & Tripathi, 1984; Fraser & Harvey, 1986a; Quintino, 1989). Embryogenesis and organogenesis were achieved from callus tissue of anthers of *Actinidia chinensis* Planch. var. *chinensis* and *Actinidia deliciosa* (A. Chev.) Liang and Ferguson var. *deliciosa* by Brossard-Chriqui & Tripathi (1984) and Fraser & Harvey (1986a), while only organogenesis was obtained by Quintino (1989). Only Fraser & Harvey (1986a) were able to regenerate whole plants from anther-derived embryoids, reporting a genotype effect on the embryogenic potential as male source plants yielded the largest numbers of embryoids.

Protoplasts

Petiole segments were used as primary explants for isolating viable protoplasts by Pais *et al.* (1987) with very high yields (90% survival). Plant regeneration from protoplasts has been reported by Mii & Ohashi (1988) and Tsai (1988) using immature internode segments of actively growing shoots and leaves as primary explants, respectively. Both authors concur that efficient plantlet regeneration only occurred when calli were transferred onto regeneration medium after two sub-cultures on preconditioning medium (Mii & Ohashi, 1988) or using the 'step induction method' (Tsai, 1988). Plant regeneration from protoplasts of long-term callus cultures has also been reported (Oliveira & Pais, 1991).

Molecular genetic marker/transformation studies

There have been several reports concerning the study of isozymes in the genus *Actinidia* (Hirsch & Bligny-Fortune, 1979; Hirsch & Fortune, 1984, 1986). Isozymes were used in these studies principally as biochemical markers for organogenesis from callus (Hirsch & Bligny-Fortune, 1979; Hirsch & Fortune, 1986), to dinstinguish staminate from pistillate plants (Hirsch & Fortune, 1986) and to distinguish between different cultivars (Daoyu & Lawes, 1990). Crowhurst *et al.* (1990) used RFLP polymorphisms in a phylogenetic study of kiwifruit, *A. latifoli*, *A. eriantha* and *A. chinensis*. Based on RFLP analysis of nuclear and chloroplast DNAs, they concluded that the hexaploid *A. deliciosa* is most probably derived from the diploid *A. chinensis*; however, they could not determine whether kiwifruit is an allopolyploid or an autopolyploid.

Genetically transformed kiwifruit plants were obtained following co-cultivation of hypocotyl and stem segments with *Agrobacterium tumefaciens* strain EHA101 harbouring a binary vector which contained the neomycin phosphotransferase and the β-glucuronidase genes (Uematsu *et al.*, 1991). An increased rooting ability was demonstrated by regenerants derived from explants of transgenic plants containing the *rol* genes from *A. rhizogenes* (Rugini *et al.*, 1991).

nona spp.

tory and importance

The Annonaceae is an archaic family of flowering plants of probable New World origin. Among its many species, several are commercially important (George, 1984): *Annona cherimola* Mill. (cherimoya); *A. diversifolia* Saff. (ilama); *A. glabra* (pond apple); *A. montana* Macfad. (mountain soursop); *A. muricata* L. (soursop); *A. purpurea* Moc. et Sessé; *A. reticulata* L. (custard apple); *A. scleroderma* Saff. and *A. squamosa* L. (sugar apple). There is one species from Africa, *A. senegalensis* Pers. (wild custard apple). A hybrid of *A. squamosa* × *A. cherimola* (atemoya) originated in Florida. There are two species of the *Rollinia* genus, *R. deliciosa* Saff. and *R. mucosa* Baill., which are restricted to hot, humid tropical lowlands (Martin *et al.*, 1987). The pawpaw (*Asimina triloba* Dunal.) is a native of North America.

The fruit of these species is composed of many fused carpels with a fleshy receptacle, forming a syncarpium (Schroeder, 1970). Fruit weight can reach up to 4000 g in *A. muricata* and between 200 and 2000 g for *A. cherimola* and most other *Annona* species.

The most important species of the genus include *A. cherimola*, *A. muricata*, *A. squamosa* and the interspecific hybrid atemoya (Ochse *et al.*, 1961; Purseglove, 1968). *Annona cherimola* is grown widely in subtropical regions and in areas with a Mediterranean climate. Spain and Portugal have the largest production of cherimola. Both *A. muricata* and *A. squamosa* thrive in tropical lowlands, and cannot tolerate frost. The atemoya can be grown in the subtropics and in tropical lowlands.

Several biomedical applications of *Annona* alkaloids have been discussed by Kowalska & Puett (1990). Jaramillo (1952), Mendieta & Del Amo (1981) and Simeon *et al.* (1990) have described the antimicrobial activity of stem bark alkaloids, the effectiveness of leaves in the treatment of burns and as an anti-inflammatory agent in the control of several diseases.

ɔry of genetics and breeding

tic diversity

There is great genetic diversity within the genus *Annona*, which reflects the different centres of origin of many of the species. Most of the species, including *A. muricata* and *A. squamosa*, are believed to have originated in the hot humid lowlands of northern South America and Central America, whereas *A. cherimola* originated in the tropical Andean highlands (Popenoe, 1970). The chromosome number for most *Annona* species is $2n = 2x = 14$ or 16 (Bowden, 1948; Thakur & Singh, 1969), with the exception of *A. glabra*, which is tetraploid. Many of the species are apparently sexually compatible, e.g. the atemoya is a natural hybrid between *A. cherimola* and *A. squamosa*. According to Ochse *et al.* (1961), *A. squamosa* can also be hybridized with *A. glabra* and *A. reticulata*. Clift (1977) has also reported the existence of hybrids between *Asimina triloba* and *Annona*

longifolia and *A. obovata*. Thus, there appears to be great potential for genetic manipulation.

Breeding mechanisms

Most species in the Annonaceae are hermaphroditic; however, the flowers are dichogamous and protogynous, thereby preventing self-pollination (Gardiazábal *et al.*, 1985). Fruit set is adversely affected by a high degree of pollen sterility (George, 1984), the negative influence of environmental conditions on flower development and by the absence of effective pollinators (Nagel *et al.*, 1989). Fruit set is usually assured by hand pollination.

Limitations of conventional breeding

Juvenile period

Among the *Annona* species, the length of the juvenile period varies, but is usually 5 years. Full production only begins after 8 years. The juvenile period in scions is greatly influenced by seedling rootstocks, and can be highly variable.

Heterogeneity

All of the commercial varieties of *Annona* are derived from openly pollinated seeds. Because of the nature of the breeding system, seedling populations are extremely heterogeneous. Although this is advantageous for certain broad breeding objectives, it is difficult to 'fix' specific characters in a reasonably short period of time. Because disease and insect pest problems develop rapidly in monocultures in the tropics and subtropics, conventional breeding procedures are consequently ineffective.

Somatic Cell Genetics/Molecular Approaches

Potential

Postharvest problems

Annona spp. fruit are highly climacteric, and ripen very quickly after harvesting. Inability to control ripening and extend the shelf life of these fruit is the most serious horticultural problem, and severely limits the exploitation of potential markets in temperate countries. Controlling the ripening process might be possible by genetically transforming *Annona* spp. for the polygalacturonase gene in the antisense configuration. This has been shown to delay ripening in tomato (Smith *et al.*, 1988; Grierson *et al.*, 1989). In addition, ethylene synthesis could be blocked in plants that have been transformed for the antisense ACC synthase gene (van der Straeten *et al.*, 1990). However, the appropriate regulators for controlling gene expression during fruit ripening should be identified.

econdary product metabolism

Several biologically active metabolites have been isolated from crude seed oils and other plant extracts from the *Annona* species (Da Ponte *et al.*, 1977; Del Fresno *et al.*, 1983; Mariappen & Saxena, 1983). These metabolites have included alkaloids, anthraquinones, phenanthrenes and triterpenoid derivatives. Leboeuf *et al.* (1982a,b) isolated β-carboline alkaloids from *A. montana* which have been shown to be potent antibiotics (McKenna & Towers, 1981; Wu *et a.*, 1987). Insecticidal activity has been found in isolates of *A. squamosa* (Cavin & Rodriguez, 1988; Mariappen & Saxena, 1983, 1984; Naidu *et al.*, 1953; Saxena *et al.*, 1984). Studies to develop efficient recovery of these valuable metabolites from cell suspensions may be worth while for pharmaceutical and insecticidal applications.

old tolerance

One of the major breeding objectives for all of the *Annona* species would be greater cold tolerance. Many tropical species do not thrive in the subtropics, and *A. cherimola* is adversely affected by prolonged cool temperatures, when it is grown in semi-tropical or Mediterranean-type climates. None of the important species can tolerate temperatures below 0°C, although there is otherwise considerable genetic variability within the genus. Transformation of *Annona* spp. for the putative cold tolerance gene (Garcia *et al.*, 1990) could be a useful strategy for addressing this problem.

oidy manipulation

Many of the *Annona* spp., but particularly *A. muricata*, have numerous large and stone-like seeds that have discouraged their consumption as fresh fruit. Seedlessness through manipulation of ploidy levels might be a successful strategy for overcoming this problem. Nair *et al.* (1986) have described the recovery of triploid plants from *A. squamosa* endosperm callus. The field performance of these plants has not been described; however, this approach should be attempted with other *Annona* species.

Other potentially useful approaches for ploidy manipulation could involve the use of haploids for the production of diploids. Nair *et al.* (1983) regenerated haploid ($n = 7$) plantlets from cultured anthers of *A. squamosa*; however, this has not been demonstrated with other species.

Ivances

generation from somatic tissue

Direct shoot organogenesis has been reported from seedling leaf explants of *A. squamosa* (Nair *et al.*, 1984) and from hypocotyl and seedling petioles of *A. cherimola* (Jordán, 1988). The optimum media for stimulating morphogenesis consisted of MS basal formulation with 0.5 $mg \cdot l^{-1}$ each of kinetin and BA for *A. squamosa* (Nair *et al.*, 1984) and 2.0 mg $BA \cdot l^{-1}$ for *A. cherimola* (Jordán, 1988).

Rooting has been sporadic. Regeneration from explants of clonal origin has not been reported.

Haploids

Nair *et al.* (1983) reported that haploid *A. squamosa* could be regenerated from anther callus on Nitsch & Nitsch (1969) medium containing 2.0 mg BA·l^{-1} and 1.0 mg NAA·l^{-1}. Regenerated plants had the haploid ($n = 7$) chromosome number, and survived transplantation.

Triploids

Callus has been initiated from endosperm removed from mature *A. squamosa* seeds on White's medium (White, 1963) supplemented with 0.1 mg kinetin·l^{-1}, 0.2 mg BA·l^{-1}, 1.0 mg NAA·l^{-1} and 1.0 mg gibberellic acid (GA_3)·l^{-1} by Nair *et al.* (1986). After subculturing the callus onto Nitsch medium with 0.5 mg NAA·l^{-1} and 2.0 mg BA·l^{-1}, shoot organogenesis was observed. The regenerants had a triploid chromosome number ($3n = 21$); rooted plantlets were not recovered.

Biochemical and molecular markers

Many of the existing *Annona* cultivars have been derived from seedling trees that did not originate from breeding programmes. They have been vegetatively propagated and distributed to a rapidly developing industry. Inevitably, there is much confusion about these cultivars, and many have been inaccurately named. Ellstrand & Lee (1987) demonstrated that isozymes could be used to identify 15 *A. cherimola* cultivars and to determine the probable hybrid origin of an atemoya cultivar 'African Pride'.

Solanum spp.

History and importance

Solanum muricatum Aiton. (melon shrub, pepino dulce, kachano) and *S. quitoense* Lam. (naranjilla, lulo, morelle de Quito) originated in the Andean highlands (Bukasov, 1982). *Solanum muricatum* has been placed in the section *Basarthrum* of the Solanaceae, which is considered to be the group most closely related to the tuberous species (section *Petota*) (Correll, 1962). Cultivation of the pepino along coastal Peru is thought to date from prehistoric times (Anderson, 1979). It is no longer found in the wild. Heiser (1969) and Correll (1962) have proposed that *S. muricatum* arose from *S. caripense* and *S. tabanoense*, respectively. Anderson (1979) suggested that *S. basendopogon* might be the ancestral species of *S. muricatum*. In contrast, *S. quitoense* (section *Lasiocarpa*) is thought to have been cultivated only in modern times, perhaps as late as as the seventeenth or eighteenth century (Patiño, (1963). It has never been collected in the wild (Heiser, 1971).

Both species are grown widely in the highlands of the tropical Americas, and increasingly in semi-tropical California (USA), Chile and New Zealand. Pollen abortion under long-day conditions has restricted distribution of *S. quitoense* (National Research Council, 1989).

Both *S. muricatum* and *S. quitoense* are considered to be underexploited fruit crops (National Research Council, 1989); however, the economic potential of the former is now being developed rapidly. *Solanum quitoense* has been available as fresh and preserved fruit in Andean South America and in Central America, although its exploitation has been hindered by serious disease and pest problems (National Research Council, 1989).

History of genetics and breeding

Breeding mechanisms

Solanum muricatum plants are either self-incompatible or self-compatible (Anderson, 1979). The chromosome number of *S. muricatum* is $2n = 2x = 24$, although Standring *et al.* (1990) have reported that 'El Camino' is a tetraploid ($2n = 4x = 118$). *Solanum quitoense*, like all other species in the section *Lasiocarpa*, is self-compatible (Whalen *et al.*, 1981). The chromosome number of *S. quitoense* is $2n = 2x = 24$ (Heiser, 1971). Flowering begins 3–4 months after germination with both species.

Limitations of conventional breeding

Both *S. muricatum* and *S. quitoense* appear to be relatively highly inbred species that show little genetic variability (Anderson, 1979; Whalen *et al.*, 1981). When accessions of *S. quitoense* from 13 widely scattered locations in Central and South America were analysed for isozyme polymorphisms, no difference among the accessions was observed (Whalen & Caruso, 1983). Whalen *et al.* (1981) also observed little morphological variation in *S. quitoense*. Although there is great genetic variability for disease resistance and environmental stress tolerance within other species of the sections *Basarthrum* and *Lasiocarpa*, most of these species are sexually incompatible with *S. muricatum* and *S. quitoense*, respectively (Anderson, 1979; Whalen *et al.*, 1981). Low pollen viability, abnormal chromosome behaviour after fertilization and a high frequency of parthenocarpy are often the result of crosses between different cultivars of *S. muricatum* (Grigg *et al.*, 1988).

Somatic Cell Genetics/Molecular Approaches

Potential

Solanum muricatum and *S. quitoense* are both related fairly closely to species that have been used as model systems for the study of morphogenesis *in vitro* and for the development of *in vitro* protocols that are now being used for crop improvement, i.e. *Datura* spp., *Lycopersicon* spp., *Nicotiana* spp., *Petunia* spp., and other

Solanum spp. Both *S. muricatum* and *S. quitoense* are relatively homogeneous, and are fairly isolated, genetically, from other *Solanum* species. Although *in vitro* studies with *S. muricatum* and *S. quitoense* are in their infancy, there exist wonderful opportunities to adapt technologies that have been developed for those model species.

Diseases

Solanum quitoense is susceptible to infection by a number of viruses, including tobacco mosaic virus (TMV) and tobacco necrosis virus (Jimenez, 1982); however, there have been no studies to demonstrate that other viruses which commonly affect solanaceous species might also be implicated. *Solanum muricatum* is reportedly resistant to most virus diseases that affect the family (Anderson, 1979). The root-knot nematode (*Meloidogyne* spp.) is probably the most serious threat to the production of either species. According to Guise (1982), *S. quitoense* production must be shifted after continuous cultivation in a field for 3–4 years, due to the build-up of nematode populations. *Solanum muricatum* is similarly affected.

Because of the lack of genetic variability in the two species, the induction or uncovering of somaclonal variants with enhanced disease and nematode resistance should be pursued. A similar strategy has been demonstrated to be effective for other *Solanum* species, e.g. *S. tuberosum* (Shepard *et al.*, 1980) and *Lycopersicon esculentum* (Evans & Sharp, 1983). Transformation of *S. quitoense* with the coat protein gene for TMV (Nelson *et al.*, 1988) could provide important protection against this disease in *S. quitoense*.

The recovery of somaclonal variants with enhanced resistance to the root-knot nematode could resolve the major production problem of both species. Burke *et al.* (1979) found that regenerants from dihaploid tobacco that was heterozygous for root-knot nematode resistance did not segregate in the expected 1:1 ratio, and indeed segregated in favour of susceptibility. On the other hand, Uhrig & Wenzel (1987) demonstrated that equivalent or better levels of resistance to the white potato cyst nematode could be obtained in haploid regenerants from potato anther cultures. Screening for resistant somaclonal variants might be greatly facilitated if it could be demonstrated that resistance to the root-knot nematode could be correlated with a variant allele of acid phosphatase as was shown by Rick & Forbes (1974) with tomato.

The isolation and culture of protoplasts of *S. muricatum* and *S. quitoense* should be relatively straightforward, particularly as so many related species have been regenerated from leaf protoplasts, including *S. dulcamara* (Chand *et al.*, 1990), *S. tuberosum* (Shepard & Totten, 1977), etc. Recovery of haploid plants from cultured anthers should also be feasible, as this has been described for several *Solanum* species, including *S. melongena* (Raina & Iyer, 1973), *S. tuberosum* (Dunwell & Sunderland, 1973), etc. Overcoming the interspecific and intergeneric sexual incompatibilities that isolate *S. muricatum* and *S. quitoense* from other *Solanum* species could be overcome by somatic hybridization involving diploid or haploid protoplasts. This could be particularly useful for extending the geographical range of cultivation into more tropical and temperate regions.

Advances

Regeneration

Morphogenesis *in vitro* has been studied extensively in species within the Solanaceae, although there have been only preliminary studies involving *S. muricatum* and *S. quitoense*. Regeneration of *S. quitoense* via organogenesis from leaves of *in vitro* grown plants derived from mature selections has been described (Hendrix *et al.*, 1987). Jordán *et al.* (1990a,b) have investigated the regeneration potential of many types of explanted tissues of *S. muricatum*, and have indicated that organogenesis can be induced without difficulty. Recently, Atkinson & Gardner (1991) obtained transgenic *S. muricatum* plants that expressed GUS, NPT II and chlorosulfuron genes.

Conclusion

The fruit crop species that have been the subject of this chapter were little known outside their centres of origin only a few years ago. If the rapid exploitation of kiwifruit is a persuasive example, there is a substantial niche for new and exotic subtropical fruits in temperate countries. Production of kiwifruit has been dependent upon a single cultivar 'Hayward'. The limited genetic base of cultivated *S. muricatum* and *S. quitoense* has also been noted. Biotechnology can increase the genetic variability of these species through the agency of embryo rescue of wide interspecific crosses (particularly involving *Actinidia* spp. and *Solanum* spp.), somatic hybridization, *in vitro* selection and transformation. Because of the high frequency of pests and diseases in tropical and subtropical agroecosystems, aggressive cultivar improvement programmes are essential. Unfortunately, fruit breeding generally has a low priority in underdeveloped countries. The *Solanum* fruit species could well be models for the adaptation of many of the new technologies for plant improvement that have been developed with other solanaceous species.

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Micropropagation note

Regeneration of *Pouteria lucuma* (Sapotaceae) plants *in vitro*

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Abbreviations: AC – activated charcoal, BA – benzyladenine, GA_3 – gibberellic acid, NAA – naphthaleneacetic acid, PVP – polyvinylpyrrolidone, PVPP – polyvinylpolypyrrolidone, TDZ – thidiazuron

The lucuma (*Pouteria lucuma* [R. et Pav.] O.Kze) (Muñoz 1987), a perennial fruit species originating from Peru, is cultivated in Peruvian lowlands, at sea level in Chile and in the south of Ecuador. Currently, its cropping potential is being evaluated in Hawaii, California and New Zealand (National Research Council 1989). Commercial exploitation is limited by frost conditions (Lavin 1989), although it might be grown in Andean highlands between 1500 to 3000 m above sea level.

Lucuma is a vigorous, evergreen tree about 6–12 m tall; the fruit is fleshy, green, approx. 4–10 cm in diameter, with bright yellow pulp and a distinctive flavor. It is used fresh or as flour in drinks, pastry and ice-cream, resembling the flavour of maple syrup (National Research Council 1989).

Due to its long juvenile period (15 years), propagation by grafting is used to bring plants into production within 4–5 years (Reyes 1989). Normally seedling radicles are used for grafting, followed by 18 to 20 months growth in a nursery prior to marketing (Gardiazábal & Valenzuela 1983). To develop large-scale production of uniform rootstocks for grafting, propagation *in vitro* of *P. lucuma* was attempted.

A range of lucuma explants were excised and cultured, viz. shoot tips (6 mm), meristems (0.5 mm), leaf sections (1 cm^2) and roots from 10 cm high plantlets; shoot tips and leaves from 3-year-old 'Montero' plants; pericarp (1 cm^2) from 'Rosalía' fruits; embryos from overripe fruits. Additionally, epicotyl explants were excised from embryos cultured *in vitro* (Pliego-Alfaro & Murashige 1987), cutting transverse sections 0.5 to 0.7 mm long.

Explants were incubated for 5 h in a solution containing antibiotics (Young et al. 1984), fungicides (mancozeb 80% WP 2.4 $g\,l^{-1}$, benomyl 50% WP 0.17 $g\,l^{-1}$, captan 80% WP 1.5 $g\,l^{-1}$), and ascorbic acid (500 $mg\,l^{-1}$). Explants were then rinsed four times in sterile distilled water, and left in ascorbic acid 500 $mg\,l^{-1}$ until implanted in media.

Explants were grown in a range of media: Nitsch & Nitsch (1969) (N); Murashige & Skoog (1962) (MS); Anderson (1980) (A); Lloyd & McCown (1981) (WPM) and White (1963) (W), containing a range of growth regulators (zeatin, BA, kinetin, NAA, GA_3, and TDZ). Gelrite 0.3% (Kelco) was the gelling agent. AC 100–2000 $mg\,l^{-1}$, PVP (PVP-360, Sigma) 1–3 $g\,l^{-1}$, PVPP (P-6755, Sigma) 1–3 $g\,l^{-1}$ and/or ascorbic acid 28–100 $mg\,l^{-1}$, were used to control enzymatic browning. Additionally casein hydrolysate (500 $mg\,l^{-1}$) with glutamine (200 $mg\,l^{-1}$) for leaf and pericarp cultures, and adenine sulfate (40–80 $mg\,l^{-1}$) for leaf and epicotyl cultures were incorporated.

Twenty replicates per treatment were made. Pyrex tubes (125 × 25 mm) with 13 ml of auto-

claved nutrient media, each containing one explant and sealed with 25 cm^2 aluminium foil were used.

Medium pH was adjusted at 5.7 before autoclaving at 121°C for 15 min. Cultures were incubated at 27 + / − 3°C in darkness for 7 days, then in a 16-h photoperiod (light source 57 μmol m^{-2} s^{-1} from cool-white fluorescent tubes).

Histological preparations were made from some cultures after fixing in FAA, embedding in paraffin, sectioning and staining with safranin and fast-green.

Regeneration responses from the different explants used are shown in Table 1.

Direct root formation was obtained from shoot tips of seedlings grown in WPM with 1.61 μM NAA + 0.44 μM BA + 0.01% AC + 28 mg l^{-1} ascorbic acid. Roots grew from the base of the explants after 45 days in culture. The shoots elongated and new leaves developed, thus forming new plantlets.

Meristems of 3-year-old and seedling plants of 'Montero' started callus formation after 30 days culture on MS medium supplemented with NAA (0.54 to 5.37 μM) and zeatin (0.46–22.81 μM). After 45 days culture, calluses of seedling explants cultured in zeatin (4.56 μM) and NAA (0.54 μM) formed roots and 2 weeks later developed shoots: no organogenesis was observed from calluses derived from 3-year-old plant meristems. After 60 days culture, plantlets were transferred to sterile peat moss, but did not survive.

After 45 days culture, leaf sections with attached petioles from seedlings formed abundant calluses in MS medium supplemented with TDZ (4.54 μM) and NAA (5.37 μM). Shoots and roots grew from this callus on the petiole base (Fig. 1) after 30 days in subculture on MS containing half-concentration macroelements, TDZ (0.09 μM), zeatin (0.046 μM), and GA_3 (0.29 μM). In similar treatments with lamina explants, only callus grew. Direct shoot formation was observed from epicotyl transverse sections, after 45 days in A medium with adenine sulfate 40 mg l^{-1}.

Complete plantlets regenerated from embryos cultured on MS medium + AC (100 mg l^{-1}) with-

Table 1. Morphogenic responses induced in different *P. lucuma* explants cultured *in vitro*[a].

Explant	Response	Plant regeneration potential	Culture conditions
Shoot-tips	Elongation[c,d]	++	*MS* + GA_3 (2.89)
	Rooting, plantlets[c]	+++	*WPM* + NAA (1.61) + BA (0.44) + AC (100)
Meristems	Organogenic calluses[c,d], shoots and roots[c]	+++	*MS* + zeatin (4.54) + NAA (0.54) + PVP (3000)
Leaf with petiole	Shoot and root formation[c]	Establishment: Subculture: +++	*MS* + TDZ (4.54) + NAA (5.37) + PVPP (2000) ½ *MS* + TDZ (0.09) + zeatin (0.046) + GA_3 (0.29) + PVPP (3000)
Embryos	Adventitious shoots	+++	*MS* + AC (100) + PVP (1000)
Epicotyls	Direct de novo shoot-formation (no roots)[c]	++	*A* + adenine sulfate (40) + PVPP (3000)
Leaf	Callus formation, without organogenesis[d]	+	*N* + kinetin (23.20) or BA (22.20) + NAA (0.054) + GA_3 (2.89) + PVPP (1000)
Pericarp	Callus formation (later necrosis)	+	*MS* + TDZ (4.54 − 0.45) + NAA (0.54) + PVPP (2000)
Root	Callus formation, without organogenesis[c]	+	W + zeatin (4.56) + NAA (0.54) + TDZ (0.09)

[a] Twenty replicates per treatment. Numbers in parentheses show concentration (μM) for growth regulators and (mg l^{-1}) for AC, PVP, PVPP and adenine sulfate.
[b] Symbols +++: direct or indirect regeneration. ++: scarce morphogenic potential. +: callus only.
[c] Juvenile explant.
[d] 3-year-old plant.

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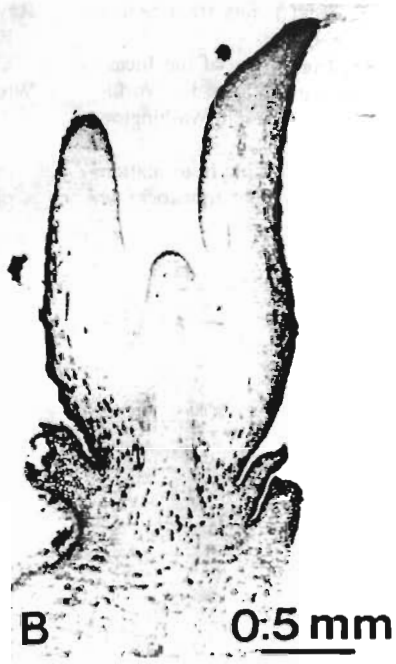

Fig. 1. Close-up and longitudinal section of callus derived from a petiole explant forming shoot and root, established in MS, with TDZ 4.54 μM and NAA 5.37 μM, after subculture in half-strength MS, with TDZ 0.09 μM, zeatin 0.046 μM and GA_3 0.29 μM. Section shows main shoot and axillary bud, both formed *de novo*.

out growth regulators. The formation of multiple adventitious shoots derived from the embryo axis was observed after 90 days in culture.

Plantlets derived from culture *in vitro* were potted in a mixture of peat moss and vermiculite 1:1, and grown in the greenhouse for one year.

Explant browning was the most common culturing problem, varying with explant type, antioxidants used, and the season in which culture was started. Soluble PVP was most effective for small explants (less than 5 mm) and PVPP was more effective for larger explants. Browning in pericarp explants could not be controlled, and the medium gel liquefied.

Preliminary results shown here indicate that shoot tips from juvenile plants are the most efficient material for propagation *in vitro*; no regeneration resulted from adult plant explants. Techniques to optimize transfer to greenhouse conditions must be developed, because a high mortality rate was evident.

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Promotion of Annona cherimola in vitro Shoot Morphogenesis as Influenced by Antioxidants

Förderung der in vitro-Sproßentwicklung bei Annona cherimola durch Antioxidantien

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Introduction

Annona cherimola (Annonaceae) is a fruit species of Andean origin, which is now becoming economically important in several subtropical regions (NATIONAL RESEARCH COUNCIL 1989). There are several cultivars and a series of ecotypes not well classified yet. Selection, improvement, and massive multiplication of elite plants are needed to increase plantation areas under different microclimatic conditions (REGINATO and LIZANA 1980, BERGER and PERALTA 1985).

Up to now, propagation of cherimoya has been successful by means of grafting or budding material derived from seeds, but seedlings are not recommended due to strong variability (GEORGE and NISSEN 1987). Although viability is enhanced by hand pollination in commercial orchards, seed germination is still low – however, it can be slightly increased by the use of phytohormones (SAINTE-MARIE 1987, VARGAS DE LA FUENTE 1986). As in other woody species, presence of phenolics and high polyphenoloxidase activity has been reported in cherimoya fruit (MARTINEZ-CAYUELA et al. 1988), which is described as highly climateric, browning very quickly after picking due to enzyme activity. Browning also affects vegetative propagation very strongly, limiting rooting of cuttings and morphogenic responses in tissue culture. The regeneration potential, as shoot formation and rhizogenesis in hypocotyl and petiole explants of this species, was reported in previous work (JORDAN 1987, 1988).

Here morphogenic responses are described as found in different explants of 3 year old trees, including nodal sections, internodes, embryos, and calli. The effects of several antioxidants, compounds benefiting explant growth, as well as phenolic levels in tissues and media during in vitro culture are also examined.

Materials and Methods

Shoots of the last growth season, from 3 year old plants, cv. Concha Lisa, grown in the greenhouse were kept in the dark for 15 days prior to culture. Partially etiolated twigs down to 10 cm below the apex of each shoot were excised. Nodal explants with a single bud, as well as internodes, were cut from these sections.

Explants were disinfected in Captan plus Benlate, both 0,2 %, stirring gently during 15 min to avoid browning, cut under a sterile antioxidant solution (ascorbic acid 400 mg/l), placed for 5 min in 3 % sodium hypochlorite, and immersed in the same antioxidant solution while awaiting culture.

All treatments for nodes and internodes were carried out using Nitsch basal medium with 3 % sucrose (except the treatment with sorbitol) and supplemented with NAA 0.5 mg/l and BA 2.0 mg/l).

For embryo culture, seeds were submerged for 20 min in Captan 0.2 %. Embryos with cotyledons, about 3 to 6 mm long were removed under the binocular, disinfected in sodium hypochlorite for 4 sec and rinsed in distilled water. They were cultured in combinations of naphtalene-acetic acid (NAA) or 2,4-dichlorphenoxyacetic acid (2,4-D) and benzyladenine (BA) or zeatin (Z).

Culture was done in tubes (13 cm x 2.5 cm) containing 13 ml autoclaved nutrient solution according to NITSCH and NITSCH (1969), including 3 % sucrose or sorbitol, several hormone and antioxidant combinations: amino oxyacetic acid; glutathione (reduced); casein acid hydrolysate; polyvinyl-pyrrolidone (PVP-360 and PVP-6755); all Sigma products, and DIECA (Merck-6689). Explants were placed either on Whatman No. 1 paper bridges, agarose 0.5 % or Gelrite 0.3 %. Cultures remained in darkness during three to seven days and were then transferred to an 18 hours dim light regime of 57 µE m seg. at 25 °C ± 1 °C. Twenty explants were used in each treatment.

Phenol content was determined according to SWAIN and HILLIS (1959), using the Folin-Ciocalteau reagent instead of the Folin-Denis reagent. Total phenolics in explants are expressed as percentage/fresh weight (absorption measured at 730 nm), estimated after standardisation with catechin (Roth Nr. 8822) since thin layer chromatography revealed presence of catechin, epicatechin, biflavanes, besides other flavonoids in tissue. For measurement, 25 mg of fresh or cultured internode tissue, and 1 ml of used nutrient solution were employed. Two absorbance determinations were made for each probe.

Table 1. Effect of substrates and phytohormones on morphogenic responses and browning of Cherimoya nodal sections after 30 days in culture

Einfluß von Nährmedien und Phytohormonen auf Morphogenese und Verbräunung von Nodiensegmenten von Cherimoya nach 30 Kulturtagen

Phytohormones (mg/l)					% Explants			
Substrate	IBA	NAA	BA	GA3	With Sprouts	With Shoots	With Callus	Brown
Agarose	0.0	0.1	1.0	0.0	50	40	40	40
Agarose	0.0	5.0	0.1	0.0	50	20	50	50
Agarose	0.0	0.3	0.1	0.01	50	0	40	30
Gelrite + CH + PVP**	5.0	0.1	0.0	0.0	100	0*	63	8

* Adventitious shoots appeared after 60 days in culture.
** Casein hydrolysate 200 mg/l, PVP (P-6755) 1000 mg/l.

Initiation of meristematic centers and new buds was investigated by means of histology, sectioning explants included in Paraplast to a thickness of 12 µm and staining with safranin/fast green.

Results and Discussion

Nodal explants

In the experiments with single or multiple nodes two kinds of responses were observed. First, the seasonal buds, which are hidden in cherimoya, always resumed growth sprouting and forming leaflets in presence of NAA and BA (Table 1). Later, de novo shoots were formed and appreared at the basal end of each explant. They were easily identified by their whitish colour and hairy aspect due to the presence of septate trichomes. Shoots emerged simultaneously all over the surface of the nodal segment. Media including GA3 inhibited de novo shoot initiation, so this phytohormone was subsequently omitted. In several phytohormone levels tested, nodal sections always showed a tendency to form new shoot or favour sprouting of the preexisting node bud. Cherimoya nodes assayed on high NAA or IBA levels did not form roots but did give rise to new shoots, as rosettes appearing at the base of the explants, while leaflets from the upper axillary buds fell.

New shoots and calli were still formed after 30 days in conditions normally leading to rhizogenesis. In treatments with Gelrite including PVP and casein hydrolysate

Table 2. Morphogenic responses and phenol content of internode explants, after 40 days in culture on media supplemented with different addenda and antioxidant compounds*

Morphogenese und Phenolgehalt von Internodienexplantaten auf unterschiedlichen Nährmedien nach 40 Kulturtagen

Antioxidants/addenda (mg/l)		Phenol		% Shoots Treatment	Average shoots/ explant	% Calli + outgrowth	% Green tissue
		Expl. % F. W.	Med. %				
ucrose	3000 Ctrl.	0.17	0.75	0	0.0	–	–
orbitol	3000	0.20	0.02	29	4.2	–	–
CH.	200	0.28	0.90	18	0.2	–	–
CH.	500	0.24	1.10	14	1.4	–	–
CH 200 + PVP*	100	0.25	0.50	28	19.2	–	–
CH 200 + PVP*	1000	0.17	1.85	67	5.8	–	–
CH 200 + DIECA	100	0.25	0.50	14	1.7	–	–
CH 500 + DIECA	100	0.18	1.30	6	1.0	–	–
DIECA	10	0.11	0.75	0	0.0	–	–
	50	0.14	0.90	0	0.0	–	–
	100	0.16	0.75	14	0.7	–	–
VP**	500	0.08	1.65	33	9.0	–	–
	1000	0.08	n.m	67	1.5	–	–
VP*	500	0.12	n.m	43	2.8	–	–
	1000	0.12	3.25	50	1.3	–	–
itric acid	10	0.21	–	0	–	50	21
	50	0.23	–	0	–	29	21
	100	0.24	–	0	–	0	0
scorb. acid	10	0.11	–	0	–	33	33
	50	0.12	–	41	–	46	76
	100	0.07	–	2	–	70	15
minooxy. ac.	10	0.24	–	14	–	45	4
	50	0.19	–	10	–	57	0
	100	0.23	–	0	–	50	4
lutathione	100	0.27	–	39	–	56	22
	200	0.24	–	30	–	80	45
	300	0.25	–	33	–	67	27
ysteine	100	0.25	–	0	–	80	20
	500	0.24	–	0	–	81	0

PVP-360, ** PVP-6755

browning of nodal explants was strongly reduced, and formation of calli at the distal end was observed after 30 days. Responses after 60 days showed shoots which initiated again from calli.

Internode sections

When exploring organogenesis in 3 year-old tree internodes, a series of hormonal combinations were successful in promoting shoot formation. However, the best hormone levels (NAA 0.5, BA 2.0 mg/l, JORDAN 1988) promoting shoot formation in hypocotyls were not effective for internodes; only calli were formed in those conditions. On the other hand, when antioxidants were added, regeneration of new shoots followed within 40 days of culture. As in nodal explants shoots were easily identified by their characteristic aspect. Meristematic centers were easily detected at an earlier stage showing dense zones surrounded by stach-rich parenchymatic cells. The effect of the incorporation of different antioxidants in the media on browning and levels of phenolics in tissue and nutrient media vs. growth responses is shown in Table 2.

Results indicate that despite the addition of NAA 0.5, BA 2.0 mg/l, no shoots were formed when antioxidants were omitted in controls with sucrose, although replacing sucrose by sorbitol stimulated shoot formation in internodes to 30% approx. giving an average of 4.2 shoots per explant. A greater stimulating effect was performed by casein hydrolysate and both types of PVP, a strong organogenic response was observed as shoot percentage and/or average number of shoots/explant in all cases. Finally, the best combinations were CH plus PVP, giving the highest percentage of shoots per treatment, or average shoots per explant.

Other antioxidants which favoured shoot organogenesis were ascorbic acid (50 mg/l), glutathione (all concentrations) and aminooxyacetic acid. Some of these compounds helped to keep tissues green during the first 40 days in culture.

The amount of total phenols found in fresh explants from the greenhouse (0.28%, not shown) was always higher than that measured in vitro. As expected, PVP and other antioxidants lowered the phenol content in explants. Glutathione (100–300 mg/l) promoted shoot formation although phenolic levels in tissue were kept high, comparable to the in vivo content. Although phenols can be highly concentrated in nutrient media leaching from explants, shoot morphogenesis is not affected in presence of PVP alone or in combination with casein hydrolysate. On the other hand, all concentrations of citric acid, cysteine and DIECA were inhibitory.

Zygotic embryos

Multiple adventitious shoots formed directly on whole embryos excised from seeds, and were distributed regularly along the main axis down to the base, without calli, within 3–4 weeks. This effect was obtained growing embryos in combinations of NAA or 2,4-D together with BA or zeatin, as shown in Table 3.

The best treatment for direct shoot induction in zygotic embryos is 2,4-D as auxin and BA and zeatin as cytokinins. Although shoots were induced with both treatments, those initiated with zeatin were better developed and greener than those obtained with BA, deficient in pigmentation. Basal calli formation was also observed. Growth on Whatman paper-bridges or Agarose was similar.

Table 3. Effect of different phytohormone concentrations and culture substrates on shoot formation of zygotic embryos after 40 days in culture

Wirkungen von Phytohormonen und Nährmedien auf die Sproßbildung an zygotischen Embryonen nach 40 Kulturtagen

	Phytohormones (mg/l)				% Explants		
Substrate	2,4 D	NAA	BA	Z	With Shoots	With Callus	Browning
Whatman	0.0	0.0	0.0	0.0	0.0	7.7	30.3
	0.0	1.0	1.0	0.0	0.0	100.0	0.0
	0.0	1.0	0.0	1.0	0.0	50.0	0.0
	0.1	0.0	1.0	0.0	30.8	46.1	30.8
	0.1	0.0	0.0	1.0	20.0	20.0	0.0
Agarose	0.0	0.02	1.0	0.0	0.0	0.0	0.0
	0.0	0.02	0.0	1.0	12.5	25.0	12.5
	0.0	0.02	1.3	0.0	0.0	0.0	0.0
	0.0	0.02	0.0	1.3	0.0	100.0	0.0

Culturing detached embryos in 2,4-D 0,1 mg/l with BA 1 mg/l or zeatin1 mg/l promoted callus formation; multiple green shoots arose later when these calli were subcultured in IAA 0,05 mg/l with casein hydrolysate 200 mg/l, and omitting 2,4-D. Calli from embryos grown on NAA combined with zeatin or BA were not morphogenic.

Despite *Annona cherimola* browning metabolism, different explants, including nodes, internodes, and embryos form organogenic calli and direct de novo shoots within appropiate phytohormone levels. This morphogenic potential appears to be similar to that informed for *Annona squamosa* (NAIR et al. 1983, 1986), although it seems that in this species the browning problem found in cherimoya explants is not a limitation. In *A. cherimola* the addition of antioxidants appears necessary to trigger direct shoot initiation in 3-year old tree explants. Shoots arise from the nodal and internodal sections and, as in the case of hypocotyls (JORDAN 1988), they form simultaneously showing a tendency to polar gradients, being more abundant and/or appearing earlier at the distal ends of explants.

In woody nodal or internodal explants, browning is the major problem affecting morphogenesis. Although shoots do form in browning tissue, appearing during the first days in culture, the presence of several antioxidants enhanced morphogenesis while tissue remained green. A similar effect is achieved by nurse co-culture of cherimoya with non-browning explants of other species (JORDAN et al. 1990).

The examination of several antioxidants including DIECA and PVP combined with CH in culture, showed a promoting or synergic effect, avoiding browning and simultaneously favouring shoot formation. The addition of CH and PVP in presence of phytohormones was the best combination to maximize the number of shoots per internode explant. Alternatively, sorbitol replacing sucrose, allowed shoot initiation, while Gelrite in combination with PVP and casein hyrolysate also proved to be an adequate substrate reducing browning in this species. The use of higher amounts of IBA or NAA turned tissue brown, de novo shoots did not form and the growth of pre-existing axillary buds in nodes was inhibited.

Assuming that shoots can be formed easily in all kinds of explants and that roots are quite difficult to regenerate as reported earlier for hypocotyl sections (JORDAN 1988), a strategy for the multiplication of selected cherimoya clones [easily recognized by isozymes (ELLSTRAND and LEE 1987)] by micrografting de novo buds formed in vitro on rootstocks derived from seedlings should be assessed. This could prove useful, since conventional budding and grafting techniques seem to be successful in cherimoya (GEORGE and NISSEN 1987).

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Summary

Nodal sections, internodes, and zygotic embryos of *Annona cherimola* 'Concha Lisa' were cultivated in vitro in several phytohormone combinations to trigger morphogenesis. De novo multiple shoot formation was the most common response. Various antioxidant compounds, especially PVP, ascorbic acid, and glutathione enhanced this effect and reduced browning, while DIECA and aminooxyacetic acid were less effective in the concentrations tested. Citric acid and cysteine were not beneficial. Combinations of PVP with casein hydrolysate in presence of NAA 0.5 mg/l plus BA 2.0 mg/l were the best blends to promote shoot formation and yielded the highest number of shoots per explant.

Zusammenfassung

Nodalabschnitte, Internodien und zygotische Embryonen von *Annona cherimola* 'Choncha Lisa' wurden in vitro mit verschiedenen Phytohormonkonzentrationen zur Induktion von Morphogenese kultiviert. Die Sproßbildung wurde durch PVP, Ascorbinsäure und Glutathion gefördert, Zitronensäure und Cystein wirkten nicht optimierend. Die Kombination PVP und Caseinhydrosylat ergab zusammen mit NES (0,5 mg/l) und BA (2,0 mg/l) die höchste Sproßausbeute je Explantat.

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INDUCCIÓN DE RESPUESTAS MORFOGÉNICAS *IN VITRO* MEDIANTE CO-CULTIVO DE DIFERENTES ESPECIES FRUTALES.

MORPHOGENIC RESPONSES AFFECTED BY INTERSPECIFIC *IN VITRO* CO-CULTURES OF FRUIT SPECIES.

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Summary

Morphogenic responses were evaluated in several herbaceous and woody species of economic importance, showing high and low regeneration potential, by means of **in vitro** *co-culture of explants.*

Some pairings showed interesting morphogenic responses: shoots, multiple shoots and roots (babaco-pear apple; lúcumo-pear apple) and inhibition of oxydation processes (kiwi-cherimola).

Responses show that the **in-vitro** *pairing of the culture of one species with tissues of another could favor morphogenic responses which would not be due to the presence of hormones only, this even in species of low* **in vitro** *regeneration potential (babaco).*

INTRODUCCIÓN

Diversas especies frutales requieren ser multiplicadas en forma masiva para cubrir la actual demanda del sector productivo. Sistemas de propagación vegetativa pueden satisfacer esta necesidad, siendo la micropropagación *in vitro* una herramienta de gran utilidad. Actualmente, se han obtenido mediante esta metodología 20.000 plántulas de arándano al cabo de 9 meses de cultivo (Arce, resultados no publicados).

Sistemas convencionales de inducción de respuestas morfogénicas pueden ser mejoradas manipulando *in vitro* niveles y concentraciones de fitohormonas. Sin embargo, en muchas especies leñosas, problemas de estacionalidad, presencia de compuestos fenólicos y, mayormente, la expresión de un metabolismo oxidativo *in vivo* e *in vitro* limitan dicho potencial regenerativo. No obstante dichas limitaciones, hemos observado en nuestro laboratorio que mediante el cultivo simultáneo de explantes de dos especies distintas (co-cultivo), uno con alto potencial regenerativo y otro más recalcitrante, es posible condicionar en el segundo respuestas morfogénicas conducentes a una regeneración (Jordán *et al.*, 1989, enviado).

En este trabajo se presentan resultados preliminares de respuestas morfogénicas de explantes de diferentes órganos de algunas especies herbáceas y leñosas sometidos a co-cultivos interespecíficos bajo condiciones *in vitro*.

MATERIALES Y MÉTODOS

Se utilizaron ocho especies de importancia económica para Chile, cuatro de alto potencial regenerativo: *Solanum tuberosum* (papa); *Solanum muricatum* (pepino de fruta); *Actinidia deliciosa* (kiwi); *Nicotiana tabacum* (tabaco) y cuatro de difícil propagación: *Carica pentago-*

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na (babaco); *Pouteria obovata* (lúcumo); *Carica pubescens* (papayo) y *Annona cherimola* (chiromoyo). Los explantes fueron mantenidos en condiciones de crecimiento en co-cultivo en medio Murashige & Skoog (MS) 1962 con la adición de sacarosa 3% y Gelrite (Kelco) 0,17% y las fitohormonas ácido naftalenacético (ANA); benciladenina (BA); isopentiladenina (IPA); ácido giberélico (GA_3) en distintas combinaciones, ajustando el pH del medio a 5,7. Los medios fueron esterilizados en autoclave a 121°C por 15 minutos. Protocolos de cultivo y regeneración para Caricáceas están descritos en Jordán, 1989.

Se utilizaron como explantes: ápices, entrenudos, yemas axilares y secciones de lámina foliar, los que fueron superficialmente esterilizados con hipoclorito de sodio comercial en un rango de 3% para papa, pepino y tabaco y 6% para babaco, kiwi, lúcumo, papayo y chirimoyo, lavándose repetidas veces con agua destilada estéril. Los explantes fueron cultivados en tubos de vidrio de 12 × 1,8 cm bajo condiciones asépticas, manteniéndose en cámaras de crecimiento a 25°C con un fotoperíodo de 12 hr a una intensidad lumínica de aproximadamente 57 $\mu E\ m^2 s^{-1}$. Las respuestas fueron evaluadas al cabo de 40 días de cultivo. Se realizaron determinaciones de fenoles totales contenidos en los explantes mediante el método descrito

CUADRO I
INTERACCIONES DE CRECIMIENTO Y DESARROLLO EN CO-CULTIVOS DE DIFERENTES ESPECIES *IN VITRO*
Growth responses by means of in vitro co-culture of explants

Especies *Species*	Explante cultivado *Explant*	ANA BA GA_3 *Hormones* (mg/l)	Resultados *Results*	Observaciones *Remarks*
Pepino Babaco	Ápice Ápice	0,1 0,1	DB IR — IR	En babaco formación de callo con raíz
Pepino Babaco	Ápice Ápice	0,2 0,2	DB IR — —	Pepino se forma callo en la base. Babaco se torna café; sin respuesta
Pepino Babaco	Ápice Ápice	1,0 1,0	DB IR — —	Babaco formación de callo
Pepino Lúcumo	Ápice Yema axilar	0,0 0,5	DB IR DB —	Lúcumo desarrolla brotes múltiples
Kiwi Chirimoyo	Entrenudo Entrenudo	0,1 0,1 0,01	DB — — —	En chirimoyo formación de callo sin oxidación
Pepino Chirimoyo	Ápice Ápice	0,1 0,1	DB IR DB —	Chirimoyo se observa oxidación
Kiwi Babaco	Ápice Entrenudo	1,0 1,0	— — — —	Formación de callo en ambas especies
Kiwi Tabaco	Entrenudo Yema axilar	0,1 0,1	DR — — —	Tabaco formación de callo
Pepino Kiwi	Ápice Entrenudo	0,1 0,1	DB IR — —	En kiwi abundante callo en la base
Pepino Tabaco	Ápice Entrenudo	0,1 0,1	DB IR DB —	No hay crecimiento
Tabaco Papa	Entrenudo Entrenudo	0,3 0,1 0,01 Subcultivo s/hormona	I I	Se inhiben mutuamente en el crecimiento y elongación de brotes

DB: Desarrollo de brote; *shoot development*
IR: Inducción de raíces; *root induction*
I : Inhibición; *inhibition*

por Swain & Hills (1959) con el reactivo de Folin-Ciocalteus, midiendo absorbancia a 730 nm.

RESULTADOS Y DISCUSIÓN

Los resultados de diversas combinaciones de cultivos interespecíficos se muestran en el Cuadro 1. En éste se observan respuestas de inducción de raíces y desarrollo de ápices, brotes en yema y hoja; condicionamiento antioxidativo y respuestas morfogénicas diferenciales de los explantes en diferentes niveles hormonales.

En la combinación de babaco con pepino se indujo en el primero la formación de raíces con dosis de 0,1 mg/l de ANA y BA, respectivamente. Esta rizogénesis no se observó con las mismas dosis hormonales cuando el explante se cultivó aisladamente como control o cuando, en condiciones de co-cultivo, fueron modificadas las concentraciones hormonales.

Bajo co-cultivo de pepino y lúcumo en dosis hormonales de 0,5 mg/l de BA (Figura 1c) se indujo la formación de brotes y raíces en

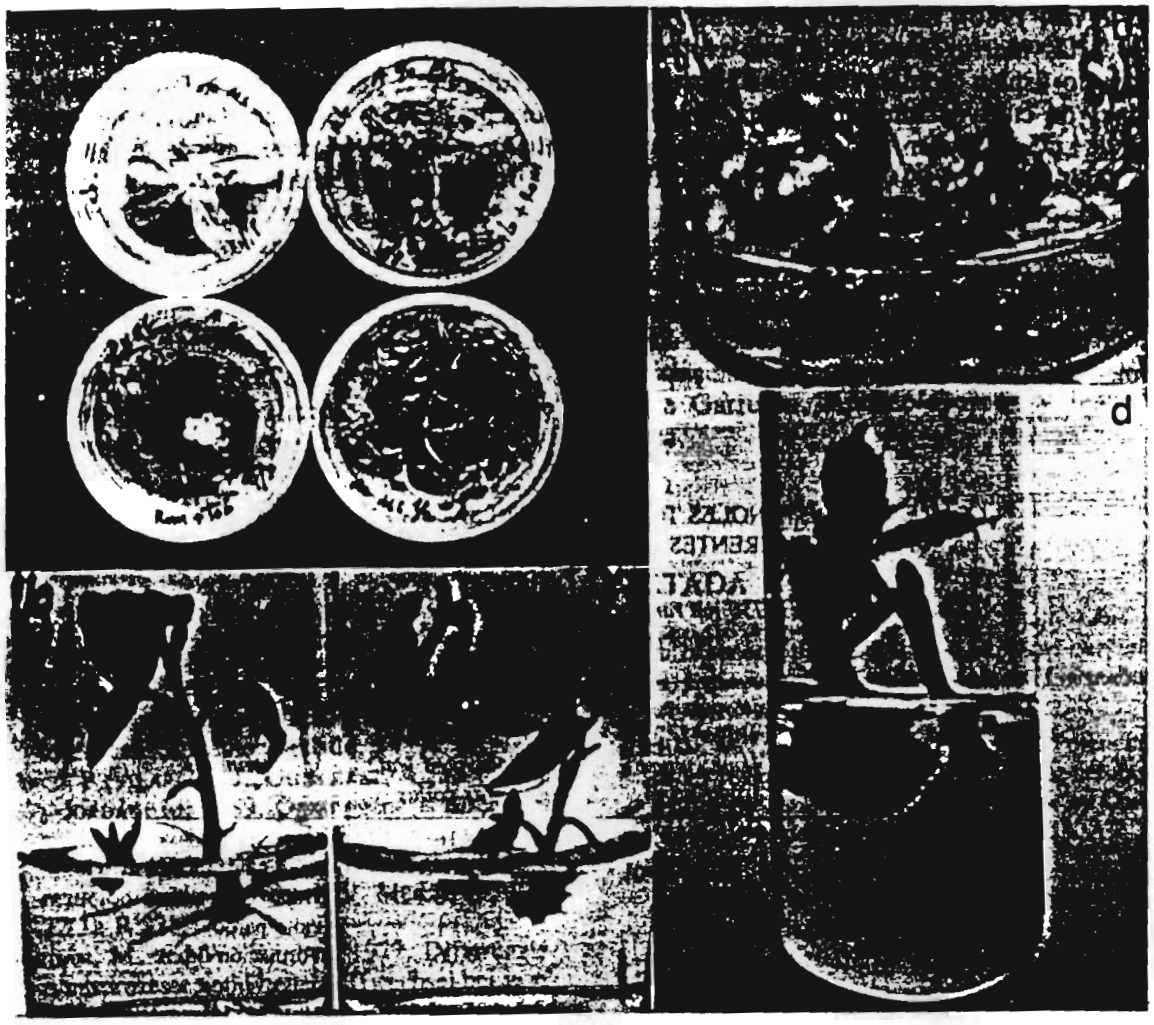

FIGURA 1
CULTIVOS DUALES DE PAPA/TABACO (a), LÚCUMO/PEPINO (c), KIWI/CHIRIMOYO (d) Y ORGANOGÉNESIS DIRECTA DE BROTES A PARTIR DE SECCIONES DE LÁMINA FOLIAR EN BABACO (b). EXPLICACIONES EN EL TEXTO.

Dual cultures of Potato/Tobacco (a); Lúcumo/Pear apple (c), Kiwi fruit/Cherimola (d) and direct shoot-organogenesis from leaf sections in Babaco (b). Explanation in text.

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pepino y se manifestó la inducción de brotes múltiples en lúcumo. Cultivos individuales de yemas de lúcumo en las mismas condiciones condujeron sólo a la elongación de la yema, sin formación de brotes múltiples. En pepino, en cambio, se evidenció una mayor proliferación de brotes y raíces comparado con el co-cultivo con lúcumo. Trabajos preliminares en explantes de pepino habían mostrado respuestas semejantes (Jordán *et al.*, 1989, enviado).

Otra serie de co-cultivos permitieron reducir el efecto oxidativo de algunas especies leñosas bajo condiciones *in vitro*. Así, mientras kiwi muestra un alto potencial regenerativo a corto plazo y sin oxidación, cuyas respuestas morfogénicas habían sido informadas antes (Jordán *et al.*, 1986); en chirimoyo, se observan respuestas morfogénicas con gran empardecimiento tisular (Jordán, 1988). Bajo condiciones de cultivos interespecíficos, entrenudos de kiwi en presencia de dosis hormonales 0,1 mg/l, 0,1 mg/l y 0,01 mg/l de ANA; BA; GA_3, respectivamente, inhibe la oxidación anteriormente manifestada en explantes de chirimoyo (Figura 1d). En kiwi se desarrollaron brotes y callo verde; los explantes de chirimoyo también se mantuvieron verdes. Paralelamente, combinaciones de ápices de pepino, de alta capacidad regenerativa, con ápices de chirimoyo, no mostraron respuestas positivas; el tejido de chirimoyo altamente oxidativo se empardecía al cabo de dos días, en cambio, el crecimiento de pepino continuó sin ser afectado.

Combinaciones papa-tabaco (Figura 1a), pepino-tabaco; pepino-kiwi; kiwi-tabaco y kiwi-babaco no demostraron interacción positiva; sin embargo, en esta última especie es posible inducir brotes múltiples *de novo* a partir de lámina foliar en forma directa con un nivel de citocinina de 5 mg/l de IPA (Figura 1b), que también había sido descrita como promotora para inducir brotes directos en hojas de arándano (Arce *et al.*, 1989), con posible obtención de variantes somaclonales de células foliares (Arce y Jordán, 1989 enviado).

Aunque no se ha estudiado el movimiento de sustancias de un explante *in vitro* a otro, nuestros resultados parecen indicar que algunos compuestos podrían difundir desde un explante hacia el otro y tal vez condicionar respuestas mutuamente. Este fue el caso de la interacción kiwi-chirimoyo, en que se observó inhibición de oxidación en chirimoyo, mientras que al cultivarse aisladamente, éste se empardece rápidamente. También, en el caso particular de

CUADRO 2

CONTENIDO DE FENOLES TOTALES (EXPRESADOS EN ABS. A 730 nm) EN HOJAS DE DIFERENTES ESPECIES SIN CULTIVAR Y CULTIVADAS *IN VITRO**

*Total phenols (Abs. 730 nm range) in leaves of different species prior culture and co-cultured in vitro**

Especie *Species*	Hojas previo cultivo** *Leaf prior culture*	Hoja y callo cultivado** *Cultivated leaf plus callus*	Observaciones *Remarks*
Lúcumo	2.00	0.166	Hoja verde, callo claro
Pepino	0.21	0.38	Hojas verdes, callo pardo
Kiwi	1.00	0.06	Formación de callo verde
Chirimoyo	0.1	0.93	Hoja oxidada, sin callo
Papayo	0.18	0.02	Hoja y callo amarillento

*$\bar{X}$ de dos mediciones.
**40 días de cultivo.

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papa-tabaco, ambos explantes compiten, inhibiendo el desarrollo mutuamente (Figura 1a); sólo explantes individuales y no en combinación realizan morfogénesis de brotes.

Los análisis de contenidos de compuestos fenólicos que fueron realizados en explantes antes y después de haber estado en condiciones de co-cultivo, mostraron cambios importantes en su contenido total presente en hojas (Cuadro 2). En general, aquellas especies de mejor capacidad morfogénica presentan bajos niveles de fenoles y una estabilidad *in vitro*, lo cual no es el caso de explantes de carácter oxidativo que denotan gran variabilidad en su contenido. Los tejidos con tendencia a la oxidación o empardecidos, como es el caso del chirimoyo, muestran una síntesis de compuestos fenólicos muy alta. En hojas de otras especies que permanecen verdes o denotan sólo amarillamiento, con formación de callo o brotes, el contenido de fenoles se mantiene estable o se reduce durante el cultivo. Variaciones semejantes se han descrito vinculadas a la diferente capacidad androgénica de anteras de 2 especies del género *Prunus: P. avium* y *P.* "Pandora" (Jordán y Feucht, 1977), asumiéndose una posible participación de dichos compuestos en el gatillamiento o inhibición de respuestas morfogénicas *in vitro*, especialmente en aquellas de carácter leñoso.

RESUMEN

Se evaluaron respuestas morfogénicas de diferentes especies de importancia económica de tipo herbáceo y arbóreo de alto y bajo potencial regenerativo, a través de co-cultivo de explantes vegetativos *in vitro*.

Algunas combinaciones de cultivos duales mostraron respuestas de interés en la inducción de morfogénesis: brotes, brotes múltiples y raíces (lúcumo-pepino; babaco-pepino) e inhibición de procesos oxidativos (kiwi-chirimoyo).

Las respuestas indican que aun en especies de bajo potencial regenerativo *in vitro* (babaco), el co-cultivo con tejidos (especies) condicionadores podría favorecer respuestas morfogénicas no determinadas exclusivamente por la presencia de hormonas.

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Perspectivas del Cultivo in Vitro de Células y Tejidos Vegetales [1]

Miguel Jordan Z. y
Andrée Goreux B.[2]

I. Introducción

"Cultivos de plantas o tejidos in vitro" corresponde a una denominación general para definir una biotecnología de propagación vegetativa consistente en mantener, diferenciar y regenerar plantas de trozos de tejidos u órganos diversos, o específicamente células o protoplastos vegetales en tubos de ensayo, en condiciones de asepsia, empleando medios nutritivos artificiales que simulan las condiciones existentes en el medio ambiente celular de la planta madre.

En especies que responden rápidamente bajo estas condiciones, es posible obtener millones de copias de una misma planta (clones) en el curso de un par de años bajo condiciones de laboratorio.

No sólo trozos de plantas pueden ser empleados para ello, sino también porciones celulares muy pequeñas y aún células individuales. Así la transferencia de material nuclear y la ingeniería genética, con incorporación de fragmentos de ácidos nucleicos foráneos (a veces no de origen vegetal) mediante vectores, también es posible. Toda la potencialidad que ofrecen las células o tejidos vegetales in vitro permite la selección de plantas con caracteres nuevos deseables, la conservación de germoplasma, la transformación del genoma para la obtención de material transgénico y la obtención de híbridos interespecíficos o nuevos clones con genes más productivos los cuales son heredables (transmisibles y permanentes).

La opción celular o tisular como material inicial de ensayo dependerá de los objetivos y potencialidades de cada sistema bajo condiciones in vitro. El empleo de una u otra en programas de multiplicación y/o mejoramiento vegetal se visualiza a continuación.

II. Material vegetal y condiciones de desarrollo

Según los propósitos el material vegetal elegido puede ser muy variable. Dada la plasticidad que manifiestan las plantas puede usarse prácticamente cualquier célula o tejido de ella.

a) Trozos de tallo que incluyan un nudo (segmento nodal);
b) Meristemas o ápices caulinares que representan el punto de crecimiento del tallo principal, yemas laterales y ápices radicales (punto de crecimiento de la raíz);
c) Embriones viables procedentes de semillas de germinación muy lenta o embriones no viables, resultantes de cruzamientos híbridos;
d) Células somáticas y polen inmaduro;
e) Células desprovistas de pared celular denominadas "protoplastos" derivados de una digestión enzimática de la pared celular.

Para expresar dichos eventos morfogénicos, células o tejidos deben crecer asépticamente en soluciones nutritivas artificiales, formuladas hace ya casi medio siglo. Estas son modificables en concentraciones y contenido, adicionando/eliminando compuestos químicos orgánicos e inorgánicos, según las necesidades de la especie y explante en particular.

La diferencia bajo condiciones in vitro es que en forma aislada, diferentes explantes, como los mencionados más arriba, pueden expresar la totipotencia celular, manifestar diferenciación y conformar órganos, principalmente raíces, y cuando no existen, brotes, para originar una planta de novo, fenómeno restringido al ser constituyentes en la planta.

El factor más importante de dichas soluciones son los componentes denominados fitohormonas, las cuales gatillan diferencialmente respuestas morfogénicas en plantas correspondiendo a compuestos naturales o sintéticos, de acción morfogénica diferencial.

Su acción se visualiza por Ej. si se escoge un trozo de médula de tabaco, un tallo de papa, de kiwi o un hipocotilo de chirimoya; los niveles altos de auxina versus bajos de citocinina originan raíces; lo opuesto, concentraciones altas de citocinina y bajas de auxinas determina la formación de nuevos brotes.

Sin embargo, en muchas plantas leñosas dichas respuestas se ven inhibidas por diferentes compuestos metabólicos, entre los cuales se citan productos fenólicos. Para minimizar este efecto, recientemente se emplean tejidos de otras especies, que provocan efecto antioxidativo cuando se

1 El presente artículo se basa en los siguientes proyectos: FONDECYT 0669-89; UNIDO Project N° DP/RLA/83/003 y AID Grant N° 513-5542-G-SS-9067-00. Se agradece la colaboración técnica de señores C. Roverano, L. Iturriaga y J. Soria.

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santes o mutágenos, inducción de variación somaclonal, fusión de protoplastos de distintas especies, recuperación de embriones híbridos, cultivo de células gametofíticas (polen) y transferencia de material génico diverso a través de vectores (Agrobacterium).

- Un embroide (futura plántula haploide) de tabaco derivado de polen dentro de una antera al cabo de 5 semanas de cultivo.

1) Cultivo de anteras, plantas originadas de polen

La condición diploide (2n) es una norma general aunque no excluyente en el reino vegetal; las plantas pueden variar en su genoma y tener mayor o menor dotación cromosómica. En el primer caso se encuentran poliploides, triploides (3n) o tetraploides (4n) y en el segundo haploide (n).

Una de las biotecnologías promisorias de los últimos años en el campo del mejoramiento genético es la capacidad de reprogramar in vitro el desarrollo de un grano de polen, no hacia la fecundación como ocurre naturalmente in vivo sino hacia la diferenciación de un individuo nuevo de carácter haploide. Las así llamadas "plantas de polen" son derivadas directas de una morfogénesis de los gametos y por lo tanto haploides. Cada planta expresa la genética de un grano de polen y en una antera, por recombinación génica se producen plantas diferentes los que manifiestan un conjunto de posibilidades distintas. Es posible posteriormente duplicar fácilmente su dotación cromosómica y obtener en una sola generación plantas homocigotos diploides. Con ello se puede lograr resistencia a enfermedades, dada por dos alelos. Así los heterocigotos son totalmente aprovechables en programas de cruzamiento, pues todos ellos presentan al menos un gen de resistencia.

Se han logrado resultados espectaculares en trigo, arroz, tabaco, petunia, papa y especialmente en la familia solanácea. En este caso, la técnica es muy simple y no requiere de un equipo sofisticado.

Aunque en algunos casos la frecuencia morfogénica del polen puede ser baja, cada antera cultivada puede contener 50.000 granos de polen, potencialmente viables. En cada cápsula es posible sembrar 30 o más anteras, de tal modo que las respuestas deberían ser prometedoras. En la Fig. 1 se observa una plántula de tabaco haploide derivada de polen, emergiendo de una antera después de 28 días de cultivo.

En Chile se están efectuando estudios en muchas especies, en particular arroz, trigo, cebada y frutales. En China existen variedades comerciales de arroz derivadas del cultivo de anteras, que se han visualizado como superiores en términos de productividad.

2) Cultivo de gametofitos

Existe una segunda fórmula para obtener material haploide y luego diploides homocigotos, aplicable en coníferas, la que en la actualidad ha cobrado gran importancia para las plantas de las distintas especies de carácter plus o elite y de aquellas introducidas comercialmente al país. La fuente de origen haploide corresponde en este caso al denominado gametofito, tejido componente de la semilla donde se localiza el embrión. Se han iniciado intentos de regeneración en Araucaria araucana, induciendo organogénesis en este tejido.

3) Suspensiones celulares

Es posible aislar y cultivar células somáticas. Combinando esta biotecnología con la selección mediante condiciones stressantes (salinidad, compuestos tóxicos, toxinas de patógenos) se logran plantas resistentes a diversas condiciones del medio. Además, se han empleado distintos agentes selectivos, metionina, valina, clorato, para obtener en un caso individuos con mayor producción y contenido de aminoácidos y en el segundo identificar mutantes fisiológicos. A partir de ello, se visualiza otra aplicación, que es la producción de semillas artificiales, en que se recubre cada embrión derivado de suspensiones celulares con una "testa" sintética (alginato u otros compuestos biogradables) pudiendo así sembrar plantas idénticas.

4) Hibridización somática por fusión de protoplastos

Esta biotecnología permite fusionar diversas especies, de distintos géneros e incluso distintas familias, produciendo híbridos imposibles de lograr in vivo. Consiste en eliminar la pared de las células (compuesta de pectina/celulosa) empleando enzimas que la digieren, liberando así protoplastos. El protoplasto mantiene normalmente su metabolismo, aun en este ambiente artificial; y aquí, mediante una serie de manipulaciones, se

s co-cultiva; la multiplicación a través de cultivos nterespecíficos, duales, o co-cultivos. En relación los costos para iniciar un programa de multiplicación, el material vegetal representa, comparatiamente, una inversión mínima. Sin embargo el em fitohormonas (especialmente algunas de ran eficiencia) junto a los reactivos y la energía equerida para esterilizar soluciones representa ya n insumo mayor en el proceso. Adicionalmente adquisición de equipos (un autoclave corriente, ampana de flujo laminar que proveen la asepsia, strumental óptimo y cámaras climatizadas con ientes lumínicas) incide fuertemente en la inverón, tal como se observa en la tabla de costos inçativos.

I. Potencialidades y proyecciones de cultivo in vitro de vegetales

Multiplicación masiva

Diferentes caracteres cualitativamente favobles en cuanto a productividad han sido fijados el pasado mediante selecciones históricas, por ncuentro al azar, o mejoramiento genético, consuyendo plantas elite, las que posteriormente dearon a clones y actualmente son cultivares o vaedades. Así ha ocurrido en el caso de patrones anos y variedades en manzano y muchos otros utales.

Tales plantas elite pueden ser multiplicadas asivamente, obteniendo así innumerables copias plantas que cuenten con características deseles, como algún tipo o color de frutos, mayor o enor contenido de azúcar y fenoles en los frutos, ayor tamaño o vigor de la planta y tolerancia a ndiciones adversas (salinidad, sequía) entre ros factores. Esto es aplicable a todo tipo de culos y permite el plantío inmediato, con lo cual se sibilita la producción rápida, factor importante el mercado. Son ejemplos la intensa plantación kiwi hace algunos años y actualmente el aranno, los que, establecidos en corto plazo permin conquistar un lugar importante en el mercado, n compitiendo con exportadores internacionalente establecidos.

Aun cuando en estas especies se utilizaron cnologías de macro y micropropagación in tro, teóricamente el nivel celular es el sistema e podría, dado un conocimiento previo, maxizar la multiplicación de estas plantas, mediante sistema de suspensiones celulares, embriogéne somática, y "semillas" artificiales. A la fecha, chas potencialidades morfogénicas y de regenección masiva a través de células ocurre sólo en as pocas especies, debiendo preferirse todavía ntros de crecimiento preformados denominados eristemas, secciones nodales o promover la inicción de brotes nuevos mediante organogéne.

Conocidos los requerimientos in vitro, el sistema celular aparece como muy ventajoso económicamente, puesto que millones de plantas pueden ser producidas en un solo matraz.

b) Selección

La multiplicación masiva facilita y acelera la selección de cultivares aptos para distintas condiciones ambientales, al producir copias idénticas, obviando el lento camino de cruce, retrocruce, ensayo en terreno, selección de ejemplares y obtención de nuevas generaciones que deben a su vez ser ensayadas. En el caso de plantas de germinación lenta como las palmas, el proceso de selección de una nueva variedad puede ser acelerado significativamente. Esta rápida selección es aplicable a los productos de otras biotecnologías descritas a continuación.

c) Producción de plantas libres de patógenos

La reproducción vegetativa por generaciones (estacas, mugrones, tubérculos, bulbos) tiende a transmitir y acumular progresivamente diferentes patógenos, en especial virus, bacterias, micoplasmas. Este hecho es muy importante en el caso de Chile, porque un gran porcentaje de las especies de valor económico están afectadas por distintas patologías, lo que reduce la producción y la torna inapta para exportación, además de acortar la vida útil de la planta.

La estrategia del sistema de eliminación de patologías consiste en cultivar zonas de crecimiento en muy activa división celular, las que se encuentran en los ápices de las plantas; el "meristema apical", de 0,1 a 1 mm de diámetro. Este se encuentra libre de patógenos aunque sí están presentes en el resto de la planta madre. In vitro este cúmulo de células puede seguir creciendo en forma ordenada, elongando un brote y desarrollando nuevas raíces, sin modificar sus características genéticas.

Se ha visto que las plantas sanas regeneradas por meristemas junto con aumentar su productividad (un 30% en frutilla) presentan además otro hábito; la verdad es que estamos acostumbrados a ver las especies en su condición de enfermas y desconocemos su potencial productivo cuando se hallan sanas. En la actualidad también se combinan técnicas de termo y quimioterapia con cultivo in vitro para eliminar patógenos y luego obtener plantas sanas.

d) Producción de plantas de características superiores

Las técnicas utilizadas para obtener nuevas variedades y/o alguna característica particular, involucran empleo de mutágenos, regeneración de embriones somáticos tratados con factores stres-

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ɔgra su fusión con otro. Los productos recombi-
antes que contienen material genético de ambos
rogenitores, regeneran la pared vegetal, y siendo
ɔmpatibles dan lugar a un embrión y finalmente a
na plántula, que crece en invernadero y poste-
ɔrmente en terreno. Estos híbridos interespecífi-
ɔs se evalúan, y de ser interesantes desde un
ınto de vista científico o económico, son mul-
ɔlicados in vitro, al presentar un grado de esterili-
ıd variable. Resultado pionero con este sistema
ɔrresponde al denominado "pomato" (patata y
mate), que es más bien una curiosidad; ni las
ıpas ni los tomates que produce son de buena
lidad (y ni se pensó que así fuera, ya que morfo-
gicamente las papas corresponden a un tubércu-
y los tomates a un fruto, correspondiendo a un
deal extremo"). En otros cultivos se han obteni-
ı híbridos interespecíficos interesantes y se tra-
ıja intensamente para mejorar diversos frutales
ediante esta técnica.

Recuperación de embriones híbridos.

Tal como en la hibridización somática por fu-
ɔn de protoplastos, es posible que ocurran fu-
ɔnes o fecundaciones sexuales interespecíficas
e no permiten el desarrollo completo del
ıbrión en el ovario. La temprana extracción del
ıbrión y su cultivo en condiciones in vitro permi-
ı llevar embriones y plántulas a término, pudien-
obtenerse una recombinación génica entre
ıntas normalmente incompatibles. Aunque tales
ıntas ofrecen dificultades como para iniciar una
ıntación comercial, el fruto es tan apreciado que
tifica la inversión. En la práctica, la biotecnolo-
de micropropagación asexual in vitro ha re-
ɛlto el problema.

En la naturaleza, sin embargo, ocurren casos
que, siendo incompatibles al provenir de dos
ɔecies distintas, la fecundación llega a buen éxi-
El Babaco o **Carica pentagona** es un tipo de
ɔaya derivado de este cruzamiento, el cual aun-
ɛ viable produce progenie estéril, por lo que su
ıltiplicación masiva por distintos explantes se
ɛ imprescindible si se quiere instalar una plan-
ión comercial. En Italia existen laboratorios que
dedican a generar este tipo de material, de
ıerdo a la demanda, y han programado multipli-
ɛiones de al menos 1.000.000 de unidades
ıalmente.

Variación somacional

Corresponde a una fuente de variación extra
gran actualidad y muy efectiva, que no es pro-
ɛto de manipulación alguna. Ella consiste en
ɛ algunas células de un conjunto celular (tejido
ıanizado, por ejemplo una hoja) evidencian res-
ɛstas diferentes ante un estímulo **in vitro**.

En la práctica, algunas células gatillan respues-
regenerativas, dando origen a embriones o
brotes diferentes. El análisis bioquímico en primer lugar y las observaciones fenológicas o morfológicas posteriormente muestran que desde un mismo explante se han originado individuos con características distintas. En Brasil se logró, mediante este sistema, obtener selecciones con individuos de caña con mayor contenido de azúcar.

En papas es posible lograr resistencias a virus y patógenos, cambios en vigor, cambios fenológicos y otros factores que deberán ser evaluados por los genetistas.

7) Ingeniería genética

La ingeniería genética ofrece posibilidades en muchos aspectos: identificar y aislar genes útiles para el mejoramiento de cultivos en plantas resistentes a virus, insectos, herbicidas, e introducirlos en otras especies (plantas transgénicas). Básicamente, se utiliza el sistema de plásmido T de **Agrobacterium tumefaciens**. Este microorganismo tiene la capacidad natural de insertar (infectar) parte de su ADN, conocido como T-ADN en células vegetales. Este T-ADN contiene distintos genes: entre otros, los que codifican fitohormonas que inducen la formación de tumores típicos de la agalla del cuello, y los de opina, que provocan la síntesis de compuestos específicos, nopalina u octopina, utilizados por la bacteria para multiplicarse dentro de la planta.

Para trabajar en ingeniería genética con esta bacteria, es necesario eliminar total o parcialmente los genes de fitohormonas, para no generar tumores, y recuperar plantas normales. Los genes del tumor pueden ser reemplazados por los genes que se desee incorporar, junto a genes sintéticos marcadores, para identificar la planta transformada. En principio, si las secuencias de acoplamiento son funcionales, cualquier fragmento de ADN de cualesquier origen, animal o vegetal, puede ser inserto y codificado; primero en la bacteria y luego a nivel de las células de la planta transformada.

A la par con los sistemas de transformación, se ha trabajado mucho en los últimos años para identificar y aislar genes que podrían ser útiles para mejoramiento de cultivos. La alternativa más impactante es la posibilidad de insertar genes provenientes de gran diversidad de seres vivos, genes que se expresan en las plantas transformadas.

Quedan ahora dos problemas por resolver, primero, tan sólo dicotiledóneas y no monocotiledóneas son posibles de modificar por **Agrobacterium**; segundo, las transformaciones logradas en explantes in vitro deben expresarse y heredarse en terreno a través de generaciones para ser provechosas.

IV. Costos aproximados de una unidad de cultivo in vitro.

La implementación de un laboratorio de culti-

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vo in vitro dedicado a la producción masiva de plantas requiere personal especializado, instalaciones, equipos e insumos. En el cuadro N° 2 se entrega una estimación del costo aproximado de estos ítems, para un laboratorio que produzca aproximadamente 1.000.000 de unidades al año.

CUADRO N° 1: RESUMEN DE LAS PERSPECTIVAS DEL CULTIVO IN VITRO.

Biotecnología	Grado de Dificultad	Costo de Implementación	Resultados (%Exito)
Microestacas (1)	sencillo	bajo	90-95%
Microinjerto (2)	sencillo	bajo	90%
Cultivo segmentos/ ápices/yemas	mediano	bajo	90%
Cultivo meristemas	mediano	bajo	80%
Callo/células	mediano	bajo	80%
Anteras	complejo	mediano	30%
Microesporas	complejo	mediano	30%
Protoplastos (3)	complejo	mediano	10%
Fusión de protoplastos	muy complejo	mediano	1%
Microinyección	muy complejo	mediano	
ADN recombinante/ transformación	muy complejo	mediano	

(1) Corresponden a segmentos nodales de tamaño pequeño.
(2) Se refiere al injerto de una porción de ápice caulinar de una especie sobre otra plántula en condiciones postgerminativas, "in vitro".
(3) No interesa el porcentaje de fusión, sino que exista viabilidad o compatibilidad de algunos combinantes para conformar plantas nuevas.

En la actualidad en Chile se han tomado iniciativas de construcción y/o habilitación de laboratorios de investigación y desarrollo para distintas especies frutales, hortícolas y forestales, lo cual indica un gran interés en nuestro país por aplicar tecnologías de avanzada.

CUADRO N° 2: COSTOS APROXIMADOS DE UNA UNIDAD DE CULTIVO IN VITRO (US$).

Costos de Inversión:	
(1) Construcciones	
-Dos cámaras de cultivo, de 50 m2 c/u y anexos	100.000
-Habilitación cámaras, mesones, racks, aislación	15.000
-Invernadero	20.000
(2) Equipo laboratorio	
-Equipo mayor: campanas de flujo laminar, autoclaves, estufas, microscopio, agitadores, congelador	30.000
-Equipo menor: balanzas, horno microondas	3.000
(3) Equipo cámaras de cultivo	
-Unidades refrigerantes/calefactores, control fotoperiodo, refrigerador	10.000
TOTAL INVERSION MINIMA	US$ 178.000
Costos de Operación:	
-Fungibles (material de vidrio, reactivos)	15.000/año
-Gastos operación y mantención equipo	5.000/año
-Gastos operación cámaras	6.000/año
-Personal (Biotecnológo, técnico de laboratorio, de invernadero y vivero)	25.000/año
-Gastos generales	2.500/año
TOTAL COSTO OPERACION	US$ 53.500/año

NOTA: Se deben agregar además costos tales como impuestos, leyes sociales y otros.

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MICROPROPAGACIÓN *IN VITRO* DE ALGUNAS ESPECIES FRUTÍCOLAS DE CHILE

IN VITRO PROPAGATION OF SOME FRUIT SPECIES GROWN IN CHILE

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Summary

Five fruit species of economic importance in Chile: ***Annona cherimola*** *(chirimoya),* ***Actinidia deliciosa*** *(kiwi),* ***Carica pubescens*** *(mountain papaya)* ***Carica pentagona*** *(babaco) and* ***Solanum muricatum*** *(pear apple) were studied as regards their regeneration potential under in vitro conditions. Best explants, culture conditions and hormone levels required to induce morphogenic responses and plant regeneration were determined. It was possible to induce somatic embryos in papaya, adventitious shoots in kiwi starting from calli, direct shoot and root formation in chirimoya hypocotyls, somatic embryoids from microcalli originating from cell suspensions and root formation in nodes or axillary buds in pear apple, kiwi and babaco. All morphogeneses conductive to plantlet regeneration.*

INTRODUCCIÓN

En una serie de especies frutales, la expansiva demanda de cvs. y clones ha requerido contar con métodos de regeneración asexual rápidos y masivos, sin embargo, la respuesta en muchas especies se ve afectada por variaciones estacionales, cambios fisiológicos, de nutrientes, niveles endógenos hormonales vs. inhibidores en tejido y la presencia de patógenos, los cuales afectan fuertemente la propagación clonal. Técnicas de micropropagación *in vitro*, paralelamente, han mostrado proveer de sistemas de multiplicación asexual de alta eficiencia, favoreciendo la expresión de potenciales morfogénicos en diversos tipos de explantes, incluyendo células y tejidos no organizados.

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La capacidad de inducción de respuestas regenerativas *in vitro* en diferentes tejidos de algunas especies frutales de gran potencialidad económica actual, es el objetivo principal de este trabajo.

Las metodologías descritas más abajo abren nuevas perspectivas para la propagación de selecciones o cvs. de chirimoya (*Annona cherimola*), la obtención de regenerantes o formas sexuales más productivas o con mayor contenido de enzimas proteolíticas en papaya (*Carica pubescens*), la multiplicación masiva del babaco (*Carica pentagona*), un híbrido interespecífico estéril de reciente introducción en el país, la reproducción asexual de clones polinizadores y patrones de kiwi (*Actinidia deliciosa*) y el saneamiento de virosis en pepino de fruta (*Solanum muricatum*).

MATERIALES Y MÉTODOS

Se usaron diferentes tipos de explantes para su evaluación *in vitro*. Entre ellos se escogió:

secciones de hipocotilos, secciones internodales, yemas axilares, secciones de pecíolo, hojas, pericarpio y médula (Cuadro 1).

Los medios nutritivos ensayados fueron Nitsch & Nitsch (1969) [N]; Murashige & Skoog (1962) [MS]; Anderson (1978 [A] y Jordán *et al.* (1978) [J], los cuales se utilizaron en combinaciones con diferentes niveles de las fitohormonas ácido naftalenacético (ANA), benciladenina (BA) y ácido giberélico (GA_3). Los medios fueron esterilizados en autoclave a 121°C durante 20 minutos. Los explantes se desinfectaron dos veces con hipoclorito comercial al 5%, lavándolos posteriormente con agua de la llave estéril, y se cultivaron en los medios indicados colocándolos sobre puentes de papel de cromatografía Whatman Nº 1. Se evaluaron respuestas regenerativas cada 30 días antes de iniciar un subcultivo en caso necesario. En algunos ensayos se procedió a realizar cortes histológicos usando "Paraplast" y tiñiendo con Safranina/Fast Green. Los cortes se hicieron a 10 µm.

Con el objeto de promover mayores respuestas morfogénicas en algunas especies frutales, se realizaron algunos ensayos en forma de cocultivos empleando explantes (brotes y callo, respectivamente) de tres especies; entre ellas, *Salonum muricatum* (Sm), *Actinidia chinensis* (Ach) y *Solanum tuberosum* (St), en forma de condicionamiento metabólico, a fin de superar respuestas regenerativas determinadas sólo por hormonas.

CUADRO 1

RESPUESTAS REGENERATIVAS *IN VITRO* DE CINCO ESPECIES FRUTALES

Regeneration responses of five fruit species grown in vitro

Especie	Sistema de regeneración	Explantes	Frecuencia Respuesta regenerativa	Condiciones de cultivo
Annona cherimola	Brotes adventicios múltiples (organogénesis directa).	Hipocotilos	+ + +	*MS*, ANA 0,5 BA 2,0. *N*, ANA 1,0 BA 0,1-1,0.
Actinidia deliciosa	Callo, raíces, brotes adventicios múltiples. Plantas (organogénesis indirecta).	Pecíolos	+	*J*, ANA 1 BA 1, sin subcultivo al cabo de 30 días.
	Callo, raíces y brotes adventicios, plantas (organogénesis indirecta).	Entrenudos	+ + +	*MS* o *A*, ANA 1 BA 1; subcultivo *MS*, ANA 0,0 BA 1,0.
	Desarrollo a plantas, rizogénesis	Yemas	+ + +	*MS* o *A*, ANA, 0,3; BA 1,0 y GA 0,01, sin subcultivo al cabo de 30 días.
Carica pubescens	Callo, embriones somáticos, plantas.	Hipocotilos	+ + +	*N*, *MS J*, ANA 1,0 BA 1,0.
	Callo, raíces, embriones somáticos, plántulas.	Pecíolos	+	*N*, ANA 1,0-5,0 BA 1,0.
	Callo, tejido embriogénico.	Pericarpio	–	
	Callo.	Hojas	–	
	Callo.	Antera	–	
	Callo.	Médula	–	
	Microcallo embriogénico.	Protoplastos y suspensiones	+	*N*, sin hormonas o ANA 1,0; luego BA 1,0 en subcultivo.
Carica pentagona	Desarrollo de yemas, raíces, plántulas.	Yemas axilares Pericarpio	+	*N*/2, ANA 0,05 BA 0,8 (suppl. con carbón activado, hidrolizado de caseína 500 mg/l y sulf. adenina 40 mg/l.
	Embrioides somáticos.	Pecíolo	+	*N*, ANA 1,0 BA 1,0.
	Callo.	Médula	–	
Solanum muricatum	Rizogénesis directa (crecimiento de brotes o yemas simultáneo).	Nudos	+ + +	*J*, ANA 0,3 IBA GA_3 0,01.
	Callo en la base de nudos.	Yemas axilares	+ + +	*J*, ANA 0,3 BA 0,1 GA_3 0,01.

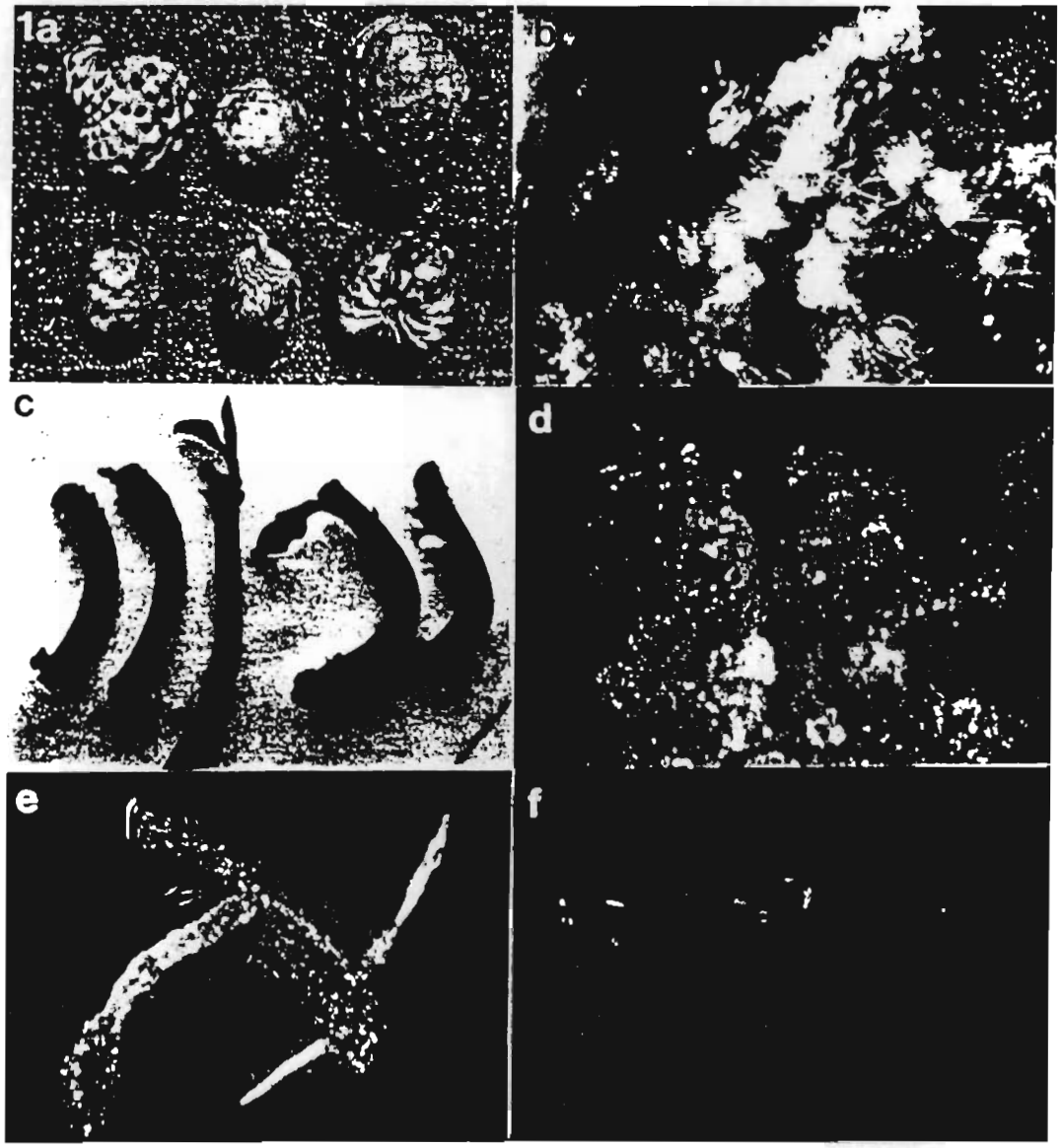

FIGURA 1

1a: DIFERENTES CVS. DE CHIRIMOYA SUSCEPTIBLES DE MULTIPLICAR Y SELECCIONAR *IN VITRO*; ENTRE ELLAS (IZQUIERDA A DERECHA): "BRONCEADA", "CONCHA LISA", "TUMBA SANDIA"; (ABAJO) "JULIANA" Y FINALMENTE "CANARIO"
b. SECCIONES DE HIPOCOTILOS DE CHIRIMOYA CV. "CONCHA LISA" CON FORMACION MULTIPLE DE BROTES DE *NOVO*, SIN MEDIAR CALLO. c: SECCIONES DE HIPOCOTILOS DE CHIRIMOYA CV. "CONCHA LISA" PARCIALMENTE OXIDADOS, PERO CON FORMACION DE BROTES Y RAICES (FLECHA) EN LAS REGIONES PROXIMALES Y DISTALES, RESPECTIVAMENTE.
d: FORMACION DE BROTES MULTIPLES SOBRE CALLO ORIGINADO DE PECIOLO EN KIWI CV. "HAYWARD" A PARTIR DE 30 DIAS DE CULTIVO.
e: SECCIÓN NODAL DE PEPINO DE FRUTA CON FORMACION DE 2 RAICES EN SU PORCIÓN DISTAL.
f: CO-CULTIVO DE SEGMENTOS FOLIARES DE PEPINO DE FRUTA EN DIFERENTES NIVELES DE HORMONAS. CONCENTRACION DE ANA 1 mg/l Y BA 0.01 mg/l DETERMINAN LA FORMACIÓN MÚLTIPLE DE RAICES A PARTIR DE LOS BORDES DE LA LÁMINA FOLIAR.

1a: Several Cherimola cultivars to be multiplied and selected: "Bronceada", "Concha Lisa", "Tumba Sandia", "Juliana", "Canario". b: Hypocotyl section of Cherimola cv "Concha Lisa" showing simultaneous formation. Shoots form directly without calli. c: Hypocotyl sections of Cherimola with shoots and roots at proximal and distal ends. d: Multiple shoot formation in calli grown from petiole in kiwi cv. "Hayward" after 30 days in culture. e: Nodal section of **Solanum muricatum** *showing root formation on its distal portion. f: Dual-cultures of* **S. muricatum** *leaf explants. ANA and BA levesl (10 mg/l and 0.01 mg l, respectively) trigger multiple root formation at leaf edges.*

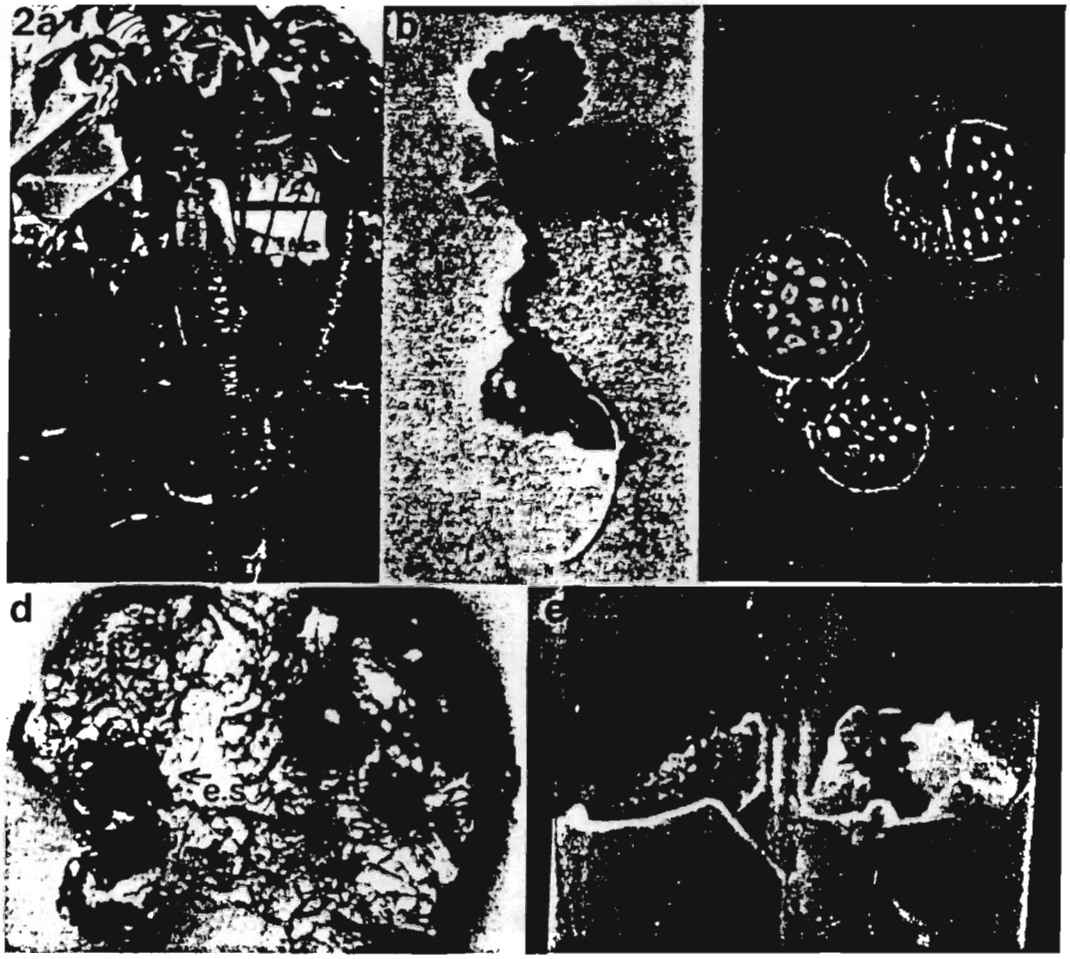

FIGURA 2

2a: PLANTAS DE BABACO b. PLÁNTULA DE BABACO ARRAIGADA *IN VITRO* INICIADA DE UNA YEMA AXILAR c. FUSION DE PROTOPLASTOS DE *CARICA PAPAYA* (PAPAYO TROPICAL) Y *C. PUBESCENS* (PAPAYO MONTAÑES). LAS CELULAS DE *C. PAPAYA* PROVIENEN DE CALLO QUE SOLO PRESENTA PROPLASTIDIOS. LAS DE *C. PUBESCENS* SE OBTUVO DE HOJA DE PLANTA ADULTA, CUYO MESOFILO MUESTRA CLOROPLASTIDIOS NORMALES d. MICROCALLO PLURIESTRATIFICADO ORIGINADO DE SUSPENSIONES CELULARES DE *C. PUBESCENS* MOSTRANDO LOS PRIMEROS ESTADIOS DE FORMACION DE UN EMBRION SOMATICO (FLECHA), QUE APARECE DE COLOR VERDE BAJO LUPA BINOCULAR e. EMBRIONES SOMATICOS MULTIPLES APARECIENDO DE UN CALLO DE *C. PUBESCENS*

2a: Babaco plants. b: Rooted babaco plantlet grown from an axillary bud. c: Protoplast fusion of **C. papaya** *(tropical papaya) and* **C. pubescens** *(mountain papaya). Protoplast cells of* **C. papaya** *were isolated from calli and show only proplastids; those of* **C. pubescens** *derived from mesophyll (adult leaf) have normal chloroplasts. d: Microcalli from cell suspension of* **C. pubescens** *showing first stages of a somatic embryo, appearing green under binocular. e: Multiple somatic embryos arising from a calli of* **C. pubescens**.

RESULTADOS

Todas las especies cultivadas bajo las condiciones específicas indicadas en el Cuadro 1 pudieron ser regeneradas a plántulas sin dificultad. Sólo explantes de babaco mostraron respuestas recalcitrantes.

–*Annona cherimola*: Las mejores respuestas morfogénicas se observaron en secciones de hipocotilos usando el cv. "Concha Lisa", con niveles relativamente altos de citocinina vs. auxina. En estas condiciones los explantes forman brotes múltiples *de novo* en las regiones más proximales en forma directa, sin pasar por callo (Figura 1a,b). Dicha respuesta disminuye hacia la región distal/basal donde las porciones de hipocotilos tienden más a la formación de callo y/o raíz (Figura 1c). Para la iniciación de raíces y producción de plántulas, la mejor respuesta se logró subcultivando las secciones de hipocotilos de 2-3 cm de largo, previa formación de brotes, en presencia de 10 mg/l de ANA (Figura 1c). Secciones de menor tamaño (5 mm) o brotes individuales decaen rápidamente en idénticas condiciones de subcultivo junto con un empardecimiento tisular debido a oxidación fenólica, efecto igualmente descrito en hojas y otros explantes de esta especie (Jordán, 1990). Sin embargo, las secciones de hipocotilos que se manifiestan morfogénicas, muestran síntesis de antocianinas sin empardecer, la cual aumenta con el tiempo de cultivo. De acuerdo a Moran (com. pers.), chirimoya tiene *in vitro* tendencia a formar preferentemente brotes, lo que también es característico en *A. squamosa* (Nair, 1984).

–*Actinidia deliciosa*: Los resultados indicaron una rápida respuesta de crecimiento *in vitro* y alta potencialidad regenerativa a partir de yemas, entrenudos y pecíolos. Al crecimiento de formación de callo puede continuar directamente la inducción de brotes adventicios que se generan en este tejido basal no organizado, no requiriéndose de subcultivo adicional.

Los brotes, de carácter múltiple, se distinguen por una intensa pigmentación verde mientras emergen del callo. Un trozo de pecíolo puede sucesivamente, sin subcultivo, iniciar callo, formar brotes y raíces al cabo de 30 días en el medio J, ANA/BA/GA_3, 0,3/0, 1/0,1 (Figura 1d), semejante a lo observado en papa (Jordán, 1978). La alta capacidad morfogénica concuerda con los resultados de Standardi (1982) y Monette (1986) hallada en ápices y brotes de estas especies.

—*Solanum muricatum*: El pepino de fruta responde análogamente a la condición de cultivo dada por kiwi. Usando regiones nodales o yemas axilares realiza crecimiento y formación de raíces al cabo de 30 días, alcanzando a formar plantas de 10 cm sin requerir de subcultivo (Figura 1e).

En experimentos de cultivos duales monoespecíficos e interespecíficos (Cuadro 2), se desprende que puede ser promovida la organogénesis en callos y el desarrollo de brotes entre *Solanum tuberosum* y *Solanum muricatum* cuando ambos explantes se co-cultivan (tratamientos 2 y 5) en ausencia total de hormona. Igualmente, se observa que al cultivar callo de las tres especies en forma independiente sin hormonas (tratamiento 13, 14 y 15), no se obtiene respuesta, aunque en co-cultivo el callo de una u otra es capaz de desarrollar la neoformación de brotes (tratamiento 5 y 6). Este efecto puede ser adscrito a que las Solanáceas *S. tuberosum*, y aquí especialmente *S. muricatum*, responden morfogénicamente a niveles de auxinas/citocininas endógenas gatillándose la iniciación de brotes o raíces sin demandas de fitohormonas adicionales. En cultivos que incluyen fitohormonas, bajos niveles de citocininas respecto de auxinas, determinan la formación de raíces (Figura 1f). La potencialidad de los co-cultivos tisulares interespecíficos en combinación con fitohormonas para promover morfogénesis en especies de difícil regeneración deberá ser fruto de mayores investigaciones.

—*Carica pubescens*: El papayo puede iniciar crecimiento de tejido no organizado a partir de diferentes explantes y niveles hormonales (Jordán *et al.*, 1983). La posterior regeneración a plántulas puede lograrse a partir de hipocotilos, los cuales inician, desde el callo previamente formado, embriones somáticos al finalizar los 2 meses de cultivo (Figura 2e). Dicho callo también se forma en trozos de pecíolos, conduciendo igualmente a respuestas regenerativas, aunque en menor escala. Explantes de pericarpio pro-

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veniente de fruto inmaduro, aún con clorofila, sólo muestran centros embrionarios, pero no diferenciación a plántulas (Jordán, en prensa). Los hipocotilos fueron los más adecuados para proveer de callo para iniciar suspensiones celulares y experimentos de fusión de protoplastos. La regeneración de embriones somáticos a partir de microcallo originado desde suspensiones celulares se logró suspendiendo células en un medio Nitsch sin hormonas o con sólo 1 mg/l de ANA, subcultivándolas luego en presencia de 1 mg/l de BA en el mismo medio nutritivo (Figura 2d). Finalmente, en las mismas condiciones se obtuvieron productos de fusión de protoplastos de *C. papaya* × *C. pubescens*, aunque a la fecha no se cuenta aún con regenerantes viables entre ambas especies de Caricaceas (Jordán *et al.*, 1986) (Figura 2c).

— *C. pentagona*: Este híbrido inicia a partir de pecíolos de hoja la formación de callo semejante al de *C. pubescens*, es decir, sin pigmentación y con escaso crecimiento. En presencia de ANA/BA 1/1 mg/l el callo inicia formación de embriones somáticos, pero éstos posteriormente se desdiferencian. Sólo a partir de yemas axilares en medio suplementado con hidrolizado de caseína 500 mg/l, ANA 0,05 mg/l y BA 0,8 mg/l, se produce crecimiento del brote con formación de raíz y plántulas, aunque en baja frecuencia (Figura 2a,b).

CONCLUSIONES

Todas las especies examinadas y cultivadas *in vitro* bajo condiciones de nutrientes y niveles hormonales determinados más arriba, son capaces a través de la potencialidad morfogenética existente en sus órganos y/o tejidos particulares de regenerar plantas vía micropropagación asexual. La frecuencia de regeneración está determinada por la velocidad del crecimiento tisular, la inducción de respuestas morfogénicas y problemas vinculados con el metabolismo oxidativo de compuestos fenólicos, especialmente en chirimoya y otras spp. leñosas (Jordán, 1975, 1977). Especies que regeneran raíces a partir de explantes meristemáticos como el pepino de fruto y kiwi, responden con mayor facilidad; estos explantes no empardecen bajo condiciones *in vitro*. Plantas que desarrollan embriones somáticos y/o brotes adventicios de preferencia, ya sea directamente a

CUADRO 2

RESPUESTAS REGENERATIVAS DE CO-CULTIVO DE BROTES (br) CON CALLO (c) EN DOS ESPECIES DE MEDIO MS SIN HORMONA DE CRECIMIENTO.

In vitro *regeneration responses of shoot (br) and callus (c) dual cultures grown in MS-medium without phytohormones.*

Tratamiento	Co-cultivo	Respuesta
1	br St + c St	St, desarrollo brote/St, callo sin respuestas
2	br St + c Sm	St, mucho desarrollo de brote/*Sm, callo forma brote*
3	br St + c Ach	St, desarrollo brote/Ach, sin respuesta
4	br St	St, desarrollo brote (menor que tratamiento 2)
5	br Sm + c St	Sm, desarrollo brote/*St, callo forma brote*
6	br Sm + c Sm	Sm, desarrollo brote/*Sm, callo forma brote*
7	br Sm + c Ach	Sm, desarrollo brote/Sm, callo sin respuesta
8	br Sm	Sm, desarrollo brote (menor que tratamiento 5)
9	br Ach + c St	Ach, desarrollo brote/St, callo sin respuesta
10	br Ach + c Sm	Ach, gran desarrollo brote/Sm, callo sin respuesta
11	br Ach + c Ach	Ach, desarrollo brote/Ach, callo sin respuesta
12	br Ach	Ach, desarrollo brote
13	c St	St, callo sin respuesta
14	c Sm	Sm, callo sin respuesta
15	c Ach	Ach, callo sin respuesta

St: *Solanum tuberosum*; Sm: *Solanum muricatum*; Ach: *Actinidia chinensis*.

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través de un callo, si bien pueden tener una alta tasa de regeneración, deben ser sometidas a mayor tiempo en subcultivo para iniciar, por ej., raíces, lo cual demanda niveles hormonales exógenos y endógenos opuestos a los requeridos para la iniciación de brotes, como es el caso de *A. cherimola*. Esta especie tiende a iniciar brotes, raramente raíces; lo mismo reportan Nair *et al.* (1984), en explantes de hoja de *A. squamosa*. En estacas de *A. cherimola*, la capacidad rizogénica está vinculada al grado de lignificación (Gardiazábal y Karelovic, 1985), lo cual denota también relación con niveles de compuestos fenólicos en los tejidos. Finalmente, *Carica pentagona* regenera con mayor dificultad en relación a lo que describen Cohen y Cooper (1982), siendo necesario más estudios, a fin de contar con sistemas de reproducción masiva para esta especie. El uso de cultivos duales, además, ofrece nuevas potencialidades para promover regeneración, ya que la presencia de algunos explantes en cultivo condiciona la organogénesis de otros, en especial entre las especies de Solanáceas ensayadas.

RESUMEN

Cinco especies frutales de importancia económica en Chile: *Annona cherimola* (chirimoya), *Actinidia deliciosa* (kiwi), *Carica pubescens* (papayo montañés), *Carica pentagona* (babaco) y *Solanum muricatum* (pepino de fruta) fueron estudiados en cuanto a su potencial regenerativo bajo condiciones *in vitro*. Para las cinco especies indicadas se determinaron los mejores explantes, condiciones de cultivo y niveles hormonales conducentes a la inducción de respuestas morfogénicas y de regeneración a plantas. Se logró inducir brotes adventicios en kiwi a partir de callo; formación directa de brotes y de raíces en hipocotilos de chirimoya, embrioides somáticos de microcallo originado de suspensiones celulares e hipocotilos, formación de raíces en nudos y yemas axilares en pepino, kiwi y babaco; todas morfogénesis conducentes a plántulas.

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Regeneración *in vitro* y aplicaciones a través del cultivo de células y tejidos vegetales

In vitro regeneration and applications of cell and tissue culture

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Plant cells by means of their totipotency and aided by *in vitro* culture techniques can be induced to perform morphogenesis leading to somatic embryoids and massive clonal multiplication; microspores or pollen can be triggered to recover haploid plants, then characters expressed via haploidy can be selected and fixed. Protoplasts from different species can lead to recombinations. We report here work done on *Carica pubescens,* where somatic embryoids were obtained from cells; in *Prunus avium* androgenesis leading to pollen calli was triggered, while plants were recovered from *Nicotiana tabacum* anthers. Fusion products were obtained using *C. pubescens* and *C. papaya protoplasts,* leading up to calli and shoots.

Bajo condiciones de cultivo *in vitro,* células vegetales meristemáticas o diferenciadas pueden ser inducidas a modificar sus programas ontogénicos preestablecidos, iniciando procesos de reembrionalización y de regeneración de plantas en forma clonal, masiva y rápida. La totipotencialidad inducible en células y tejidos vegetales hace factible aplicar diferentes biotecnologías permitiendo, entre otras, la disponibilidad de nuevo germoplasma mediante la regeneración de plantas directamente a partir de gametos (microsporas y/o megasporas); la hibridización somática, mediante la fusión de células (intra o interespecies, frecuentemente incompatibles); la introducción de ADN-foráneo y la selección temprana de material con características deseables. Mientras la regeneración *in vitro* de plantas a través de polen (androgénesis) permite la expresión de cambios o recombinaciones producidas durante el *crossing-over* con la generación inmediata de material haploide y la obtención de sus respectivos diploides homozigotos en una sola generación, la fusión somática de protoplastos asume una recombinación génica nuclear y/o citoplasmática, permitiendo la obtención de nuevos genotipos no producibles en la naturaleza por cruzamientos espontáneos o inducidos. En la práctica, ambas biotecnologías entregan un gran potencial de regeneración de nuevas plantas de selección y de amplificación de genes vinculados con resistencias, vigor y muchos otros caracteres deseables, especialmente en cultivos (Narayanaswamy and George, 1982; George and Sherrington, 1984; Gleba and Sytnik, 1984). En este trabajo se muestran potencialidades regenerativas *in vitro* existentes en polen, suspensiones celulares y protoplastos en especies del género *Prunus, Carica* y *Nicotiana.*

MATERIALES Y MÉTODOS

Los estudios de desarrollo de microsporas se condujeron cultivando anteras de *Prunus avium, Nicotiana tabacum* y *Carica pubescens.* Se usó preferencialmente el medio de Nitsch y Nitsch (1969) incluyendo las fitohormonas ac. naftalenacético (ANA) en concentración de 0,0-10,0 mg/l y de bencilaminopurina (BA) en rangos de 0,0-1,0 mg/l. Las anteras se sembraron en tubos colocándolas previa esterilización en hipoclorito comercial al 2% sobre un puente de papel cromatográfico Whatman N° 1 en contacto con el medio nutritivo líquido igualmente esterilizado en autoclave a 121°C por 20 minutos. Previo al cultivo se detectó para cada especie el estadio de desarrollo del polen, mediante un macerado con orceína acética o cortes histológicos, eligiendo la condición de microspora uninucleada por ser ésta la que presenta un mayor potencial morfogénico *in vitro* en la mayoría de las especies estudiadas.

Para el cultivo de suspensiones celulares y de protoplastos de las especies del género *Carica* se empleó el mismo medio indicado anteriormente, solidificando con agarosa al 0,4% usando combinaciones de las hormonas ANA, BA, 2,4-D (2,4-diclorofenoxiacético), Z (zeatina) e IPA (isopentiladenina), todos de la marca Sigma. Las células

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y/o protoplastos fusionados se sembraron luego en placas para lograr una densidad de 10^4-10^5 células/ml aprox. En algunos tratamientos los cultivos celulares se cultivaron paralelamente con un explante de callo de aproximadamente 0,5 cm^3, colocando en el centro de la placa (tejido nodriza) con el objeto de condicionar o gatillar respuestas morfogénicas de las células en suspensión. Las células de *C. pubescens* se obtuvieron por suspensión (agitador recíproco Eberbach, 40 golpes/min) de un callo derivado de hipocotilo de 30 días de formación (no embriogénico), mientras que los protoplastos se derivaron de hojas de plantas juveniles o adultas digeridas en presencia de celulasa 2% y Macerozyme 0,5% en presencia de manitol o sacarosa. En *C. papaya* también se obtuvieron protoplastos a partir de callo (sin cloroplastos desarrollados) bajo las condiciones de enzimas y osmóticos indicados antes. Alícuotas de suspensiones de protoplastos de ambas especies se mezclaron usando polietilenglicol y se sembraron en presencia de diferentes fitohormonas (Jordán, 1986; Jordán *et al.*, 1986).

RESULTADOS Y DISCUSIÓN

Cultivo de microsporas/anteras

Las distintas etapas de desarrollo de las microsporas y su respuesta ante niveles de fitohormonas para *N. tabacum, P. avium* y *C. pubescens* se ilustran en la Tabla I, Fig. 1. Mientras plántulas de tabaco se originaron directamente en varios tratamientos hormonales (Fig. 1f), respuestas de formación de callo en *P. avium* y *C. pubescens* se lograron sólo en concentraciones de ANA y BA 1 mg/l, respectivamente. Cortes histológicos mostraron formación de polen multicelular en polen de *P. avium* con una exina claramente visible (Fig. 1b), posteriormente desarrollo de agregados celulares los cuales crecen desde el interior de las tecas (Fig. 1d) y emergen por la pared de la antera para conformar un callo. En algunos casos proliferación de células de gran tamaño con núcleos prominentes (Fig. 1c) sugiriendo diferentes grados de ploidía fueron frecuentes; en otras anteras, cuatro conglomerados o callos independientes suponen el origen de cuatro microsporas en forma paralela (Fig. 1e). Un desarrollo posterior no se observó por empardecimiento tisular, debido al metabolismo fenólico oxidativo típico de esta especie leñosa y de otras especies arbóreas en general (Phan and Letouze, 1983; Gaspar and Coumans, 1987). En el caso de *C. pubescens,* bajo idénticas condiciones de cultivo, se inició una abundante proliferación de callo desde el interior y exterior de las anteras a partir de los 15 días. A los 30 días, sin mediar subcultivo, se observó la formación en embriones somáticos múltiples en este callo, distinguibles por su color verde. El origen de este callo y la ploidía de los embriones aún debe ser confirmada.

Cultivo de células y protoplastos

Suspensiones celulares de *C. pubescens,* derivadas de un callo crecido por 30 días en un medio Nitsch y Nitsch con 1 mg/l de ANA y BA, respectivamente, mostraron alta capacidad organogénica sembradas en diferentes tratamientos con fitohormonas (Tabla II).

TABLA I

Respuestas morfogénicas de microsporas de *Nicotiana tabacum, Prunus avium* y *Carica pubescens* a partir de cinco semanas de cultivo en medio según Nitsch y Nitsch (1969)

Fitohormonas (mg/l) ANA	BA	Especies	Anteras sembradas	Anteras microsc. examinadas	Respuestas morfogénicas
0,0	0,0	*P.a.*	870	45	Sin crecimiento.
0,1	0,1	*P.a.*	500	60	Sin crecimiento.
0,1	0,1	*N.t.*	460	–	14 plántulas.
1,0	0,0	*P.a.*	270	31	Sin crecimiento.
1,0	0,1	*P.a.*	550	60	Sin crecimiento.
1,0	1,0	*P.a.*	1.280	145	21 anteras con callo de polen.
1,0	1,0	*N.t.*	120	–	4 plántulas.
1,0	1,0	*C.p.*	110	–	2 con callo, realizando embriogénesis múltiple.

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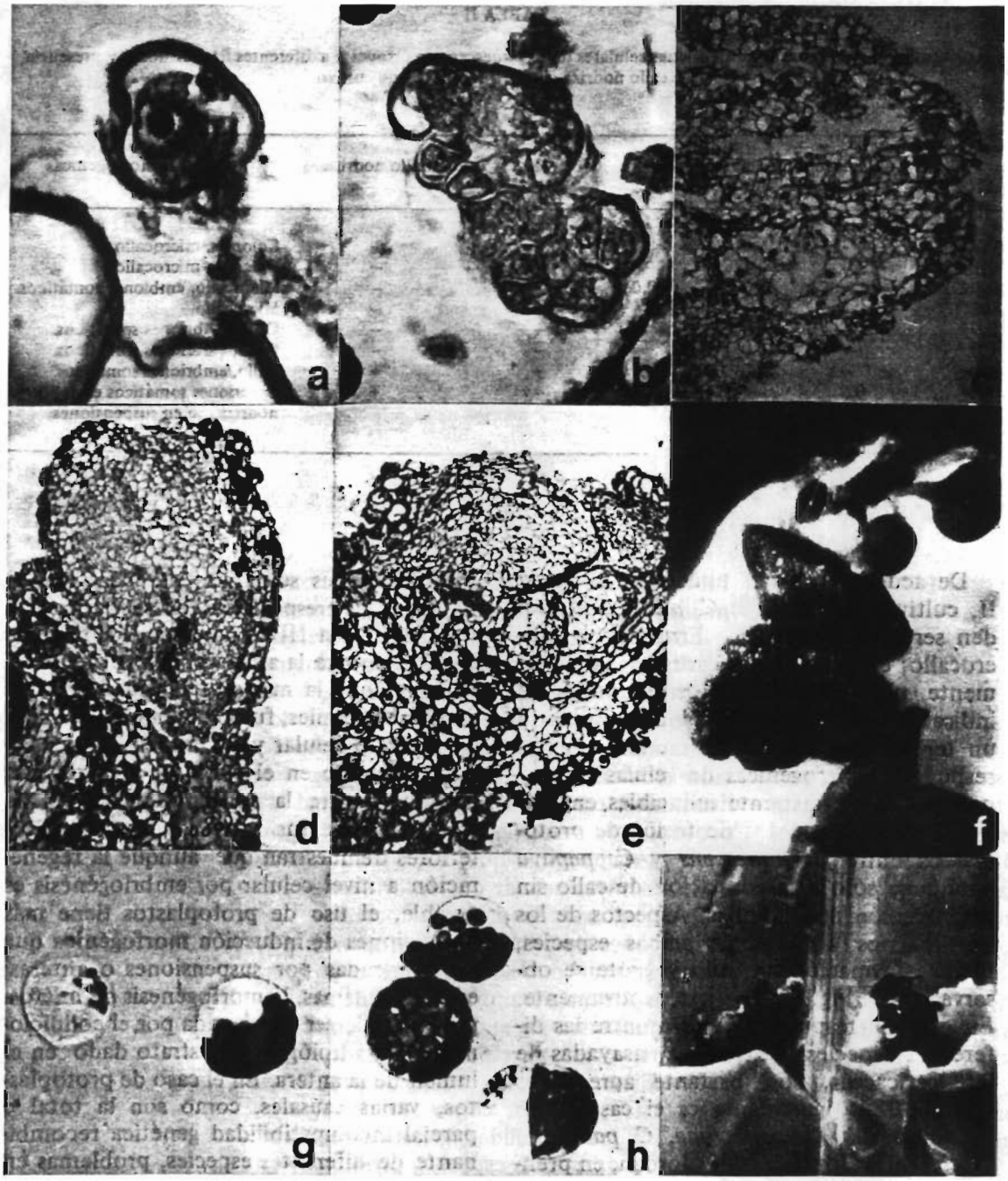

Fig. 1a: Grano de polen binucleado con célula vegetativa (centro) y célula generativa más pequeña (borde).
Fig. 1b: Grano de polen multicelular de *P. avium* en el interior de una antera mostrando desprendimiento de la exina a las 4 semanas de cultivo.
Fig. 1c: Formación de callo de microspora dentro de las tecas con células y núcleos prominentes, después de 7 semanas.
Fig. 1d: Callo emergiendo desde las anteras (región central) y granos de polen no androgénicos (base) en el interior de una teca, después de 8 semanas de cultivo.
Fig. 1e: Cuatro callos en el interior de una teca cada uno originado de una microspora; alrededor polen no androgenico, 8-9 semanas de cultivo.
Fig. 1 f: Plántula de tabaco originada directamente de una microspora sin mediar callo al cabo de 35 días de cultivo.
Fig. 1g: Protoplastos aislados de *C. papaya* con proplastidios (derivada de un callo, izquierda) y de *C. pubescens*, con cloroplastidios, derivados del mesófilo de hojas juveniles (derecha).
Fig. 1h: Fusión de protoplastos entre *C. papaya* (porción izquierda) y *C. pubescens* (porción derecha).
Fig. 1i: Formación de brotes en callo probablemente derivado de un producto de fusión protoplasmática interespecies.

TABLA II

Respuestas morfogénicas de suspensiones celulares de *C. pubescens* en respuesta a diferentes fitohormonas y presencia de callo nodriza después de 80 días de cultivo

Fitohormonas (mg/l)					Callo nodriza	Respuestas morfogénicas
ANA	2,4-D	BA	Z	IPA		
0,1	–	5,0	–	–	–	Colonias, microcallo.
0,1	–	0,1	–	–	–	Colonias, microcallo.
0,02	–	–	2,0	–	–	Microcallo, embiones somáticos.
0,5	–	–	–	0,5	–	Microcallo.
0,0	–	1,0	–	–	–	Callo, embriones somáticos.
1,0	–	1,0	–	–	–	Callo, embriones somáticos.
1,0	–	1,0	–	–	+	Callo, embriones somáticos.
1,0	0,2	–	–	–	+	Embriones somáticos en tejido nodriza, no en suspensiones.

De acuerdo a los resultados de la Tabla II, cultivos celulares en *C. pubescens* pueden ser conducidos a la formación de microcallo, embriones somáticos y, posteriormente, plántulas a través de organogénesis indirecta en cultivos individuales o usando un tejido dual como nodriza. Mientras las respuestas morfogénicas de células en suspensión son fácilmente inducibles en esta especie, los productos de fusión de protoplastos entre *C. pubescens* y *C. papaya* conducen sólo a la formación de callo sin regeneración de plántulas. Aspectos de los protoplastos aislados de ambas especies, fusión, formación de callo y brote se observa en la Fig. 1g, h, i; respectivamente. En general, respuestas *in vitro* entre las diferentes especies y explantes ensayadas de las Caricaceas, son bastante apreciables (Litz, 1984) igualmente es el caso entre *C. pubescens, C. pentagona, C. papaya* y *C. chilensis* (Jordán, 1989; Jordán, en prensa). A pesar de ello, los eventos de aislación de protoplastos, fusión interespecífica y los primeros estadios de desarrollo de un microcallo pueden ser promovidos mediante el uso de material juvenil, medios de digestión y/o de cultivos adecuados y la presencia de antibióticos. Eventualmente, con el cocultivo de tejidos nodriza (callo altamente embriogénico de *C. pubescens*), se podrían gatillar respuestas morfogénicas en productos de fusión de protoplastos en hibridizaciones somáticas de otras combinaciones interespecíficas entre Caricaceas.

En la Tabla III se muestran los mejores resultados para la aislación de protoplastos. Mientras que la más alta densidad se logra en hojas juveniles, fueron la heterogeneidad del tamaño celular y el grado de diferenciación existente en el callo los factores más limitantes para la concentración final de protoplastos a fusionar. Los resultados anteriores demuestran que aunque la regeneración a nivel celular por embriogénesis es posible, el uso de protoplastos tiene más limitaciones de inducción morfogénica que las mostradas por suspensiones o anteras; en estas últimas, la morfogénesis de microsporas puede ser promovida por el condicionamiento fisiológico o sustrato dado en el lumen de la antera. En el caso de protoplastos, varias causales, como son la total o parcial incompatibilidad genética recombinante de diferentes especies, problemas en los procedimientos de aislación y/o requerimientos nutritivos precursores de pared más específicos a los demandados por suspensiones celulares son limitantes para promover fusiones interespecíficas y son variables que deberán ser establecidas específicamente.

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TABLA III

Respuesta al aislamiento de protoplastos de hojas de material adulto y juvenil de *C. pubescens*

Material vegetal	Esterilización	Solución de digestión (Incluye enzimas)	Condición durante digestión	Densidad (Hemacitómetro)	Estado y apariencia
Hoja juvenil	Etanol 70% 1' NaCl 10% 5'	Medio MS (Modif.) + 1,5 ml Gentalyn	Oscuridad T° 30°C, 25 hrs. Sacarosa 20% 500 rpm.	± 27,72 44,3 x 10^4	Protoplastos esféricos, perfecto estado, abundantes.
Hoja adulta	Etanol 70% 1' NaCl 10% 5'	Medio MS (Modif.) + 1,5 ml Gentalyn	Oscuridad T° 30°, 25 hrs. Sacarosa 20% 500 rpm.	2,0 x 10^4	Baja densidad, muchos destruidos.
Callo de hipocotilo*	No se requiere	Medio MS (Modif.) + 1,5 ml Gentalyn	Oscuridad T° 30°C Sacarosa 20% 100-300 rpm, 10'.	3,5 x 10^4	Protoplastos esféricos, perfecto estado, baja densidad.
Callo de pecíolo	No se requiere	Medio MS (Modif.) + 1,5 ml Gentalyn	Oscuridad T° 30°C Sacarosa 20% 100-300 rpm, 10'.	4,0 x 10^4	Protoplastos esféricos, perfecto estado, baja densidad.

* Los callos de hipocotilo y de pecíolo de *C. pubescens* o *C. papaya* crecieron en el medio de Nitsch que contenía 1 mg/l de ANA y BA, respectivamente.

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XXV JORNADAS DE INVESTIGACION DEL CIUNC

IX JORNADAS REGIONALES DE INVESTIGACION

I JORNADAS TRASANDINAS DE INVESTIGACION

10 al 13 de mayo de 199[illegible]

RESUMENES

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plántulas de papa cv. Bintje.- El regulador provocó fuerte inhibición del ulterior crecimiento de las plántulas pero no afectó la tuberización, que se manifestó luego que los testigos comenzaran a hacerlo . - Se deduce que la acción del ácido jasmónico estimulando tuberización es indirecta, a través de la inhibición del sistema radical, generador de giberelinas endógenas que normalmente retardan aquella manifestación. - (Tizio y Goleniowski 1985; Abdala et al, 1992)

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ALTERNATIVAS DE MICROPROPAGACION Y TRANSFORMACION EN DOS ESPECIES DE SOLANACEAS ANDINAS. Jordán, M.; Goreux, A.; Velozo, J.; Obando, M. - Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile

Solanum muricatum (pepino) y Cyphomandra betacea (tamarillo) son dos especies frutales de gran potencial económico debido a la calidad de sus frutos. Sistemas de multiplicación clonal de material seleccionado son necesarios para establecer futuras plantaciones de alta productividad. Diferentes respuestas regenerativas alcanzadas en estas especies se detallan en este trabajo. Secciones nodales, yemas axilares y explantes de hoja de S. muricatum son altamente organogénicos in vitro; varios suplementos hormonales inducen brotes, raíces y plántulas, sin requerimiento de subcultivo. La mejor combinación de fitohormonas para la organogénesis caulinar en hojas y obtención de plántulas fue 0.1 mg/l TDZ y 0.5 mg/l IAA. Trabajos preliminares de transformación se iniciaron también en ápices de embriones en germinación mediante el sistema de bombardeo de partículas, detectándose reacción de B-glucuronidasa (GUS).
Regeneración de plantas de C.betacea se obtiene en un 90% de secciones nodales mediante inducción de raíces y desarrollo de la yema axilar preexistente en presencia de ANA, BA y GA3, todas en concentración de 0.2 mg/l. La formación de múltiples brotes de novo se logró también en secciones de hojas empleando TDZ (2.5 - 10 mg/l) reduciéndose el pardeamiento oxidativo bajo condiciones in vitro.
Se agradece el apoyo de la Dra. M.Sticklen, Michigan State University.

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ORGANOGENESIS Y REGENERACION DE ALGUNAS CARICACEAS IN VITRO. Jordán, M.; Velozo, J.; Goreux, A.; Mujica, A.M. - Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile.

Procesos de regeneración in vitro permiten masificar la propagación de clones y amplificar germoplasma elite. Respuestas morfogénicas inducidas en células y tejidos de tres especies frutales de Caricaceas se informan en este

trabajo. En C. pubescens (sin. C. candamarcensis), embriogénesis somática directa (ESD) y formación de embriones somáticos adventicios son obtenidos a partir de suspensiones celulares derivadas de callo de origen meristemático en medio Nitsch (1969). Las suspensiones se realizaron en medio WPM (1981) incluyendo manitol 3%, 2,4-D 1.1 mg/l y Z 0.4 mg/l por 72 horas en agitador a 100 rpm., posterior subcultivo sin hormonas a 50 rpm. durante 5 meses. Se evidenció también ES indirecta de callo originado en medio Nitsch adicionando las fitohormonas ANA 0.1 mg/l y BA 1 mg/l transcurridas 9 semanas. Resultados semejantes se obtuvieron usando inicialmente hipocotilos. En C. pentagona (babaco) la neoformación de brotes adventicios y estados proembrionales se observó en lámina foliar promóvidos específicamente por TDZ en WPM. Estas respuestas morfogénicas anteriores apoyan trabajos de fusión de protoplastos iniciados en C.papaya.
Se agradece el financiamiento otorgado por el Grant AID N° 513-5542-G-SS-9067-00

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BULBIFICACION (FORMACION DE CABEZAS) "IN VITRO" DE AJO TIPO COLORADO Allium sativum L.. Deromedis, R.; Vega, C.; Verdes, P. - Laboratorio de Genética y Biotecnología Vegetal. F.I.C.E.S - U.N. San Luis.

Allium sativum L. es una especie de amplia difusión, que presenta reproducción asexual.
La variabilidad genética se puede obtener por medio del cultivo "in vitro" de meristemas, con el agregado de 2,4-D, induciéndose variación somaclonal.
La formación de bulbos (cabezas), puede obtenerse en condiciones "in vitro" en medio líquido de Murashige y Skoog, con el agregado de 50 g/l de glucosa, previa vernalización de los microbulbitos durante 15 días a 4° C.
A los seis meses de la brotación comienza a formarse la cabeza, previo un período de 30 días a baja temperatura (7 a 8° C.) e iluminación reducida (8 hs. a 1000 Lux). Al cabo de 10 a 11 meses de la brotación de los microbulbitos se obtuvieron pequeñas cabezas, compuestas por 3 a 5 dientes, constituyendo cada cabeza un clon.
Con este trabajo se trata de completar las etapas de obtención de microbulbitos a cabezas "in vitro" con variación somaclonal, para asegurar posteriores trabajos de mejoramiento genético.

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EVALUACION IN VITRO DE LA RESISTENCIA DE DIFERENTES PATRONES DE CEREZO (Prunus avium) Y DE VID (Vitis vinifera) AL NEMATODE Meloidogyne incognita. Gil de Rubio, F.; Del Toro, M.S.; Castellanos, S. - Cát. de Fruticultura y Laboratorio de Nematología, F.C.A., U.N.C.

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PPLICATIONS OF BIOTECHNOLOGY IN PALMS

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The recalcitrance of most palms to simple horticultural methods of propagation mbined with their economic importance have provided a strong impetus for palm tissue lture studies. Most studies have aimed at clonal propagation and after nearly two decades of search, the large-scale clonal propagation of oil palm and date-palm seem to have almost ıched a stage of practical feasibility. Other palms that appear amenable to clonal propagation rough tissue culture include the sago palm and several rattan species. The large-scale opagation of coconut remains elusive, despite sustained intensive effort by several research oups. Abnormalities often appear several years after field planting and it is necessary erefore to develop early markers for these variants. This is best illustrated in oil palm clones ıere feminization in floral organs has been observed in almost all clones produced by tissue lture. The scope for utilization of ancillary methods such as embryo culture and ropreservation in palm improvement is real, particularly for oil palm, coconut and date-palm, ıere protocols for these methods are available. Regeneration from protoplasts remains ficult in oil palm and has not been explored in other palms seriously. Relatively unexplored enues of palm improvement include haplogenesis and the use of somaclonal variants. e applications of the tools of molecular biology are already evident in RFLP studies in ation to somaclonal variation in oil palm. The scope for genetically engineered palms is well cognized and programs aimed at altering oil composition have been initiated in oil palm.

ICROPROPAGATION OF SOME SUB-TROPICAL SPECIES.

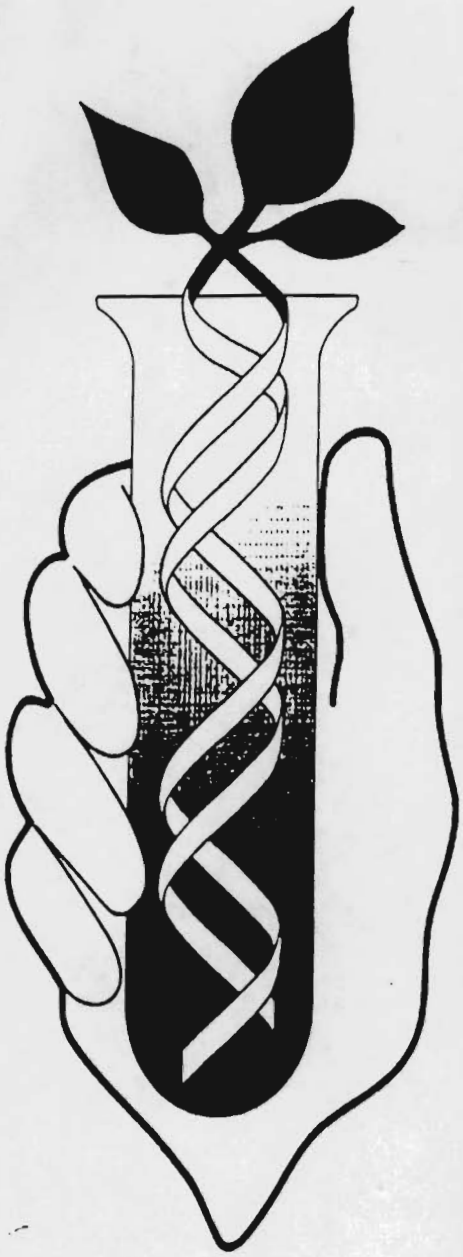
iguel Jordan, Facultad de Ciencias Biologicas, Pontificia Universidad Catolica Chile, Casilla 114-D, Santiago, Chile, FAX 56-2-2225515

Little known species growing in sub-tropical areas may be important to open new rkets; to this end, best quality cultivars are required. Well-known species and their germplasm also susceptible to improvement. Advances concerning the regeneration potential of several cies are examined.

Among tree species, *Annona cherimola* (cherimoya) forms multiple *de novo* adventitious ots in several types of explants tested; best results are found in nodal sections, internodes, bryos and hypocotyls, opening the possibility for massive multiplication. Tissular browning ıld be controlled using soluble and insoluble polyvinylpyrrolidone (PVP), and other ioxidant compounds. In *Pouteria lucuma*, (lucumo) several explants show morphogeni ential; multicellular pollen and supposedly androgenic embryos appear after low temperature uction treatments; a few adventitious shoots originate from leaf sections.

Among herbaceous species, in *Carica pubescens* (a highland papaya) somatic embryos e preferently from hypocotyls; zygotic embryos give calli which originate embryoids in sub-ture. In papaya, anther culture generated androgenic embryos and somatic calli. Cell pensions and interspecific protoplast fusion with Carica papaya have been tried. Also, in *rica pubescens* (Babaco) adventitious shoots are easily regenerated on leaf explants using high ıcentrations of cytokinins; cuttings can also be rooted. In another herbaceous fruit species, *anum muricatum* (pepino, sweet cucumber), it was possible to insert the GUS gene by means he biolistic particle delivery system in developing shoot tissues from germinating seeds. ernatively, work is proceeding to transform plantlets using *Agrobacterium tumefaciens* since have seen regeneration from leaf explants in pepino. Several vegetative regeneration strategies also possible for *Solanum tuberosum* (potato}; as plants can be obtained from leaf sections, have started transformation work on Chilean clones with *Agrobacterium tumefaciens* carrying plasmid pGV 2260 coding for resistance to kanamycin and GUS. Virus-free selections are) very important for this species. The vine *Actinidia deliciosa* (kiwi} is easy to regenerate *in o*; calli form on petioles and internodes, and differentiate into shoots, without sub-culture. o, nodal sections or axillary buds show 100% rooting after 30 days.

knowledgements: This research was supported by PSTC Grant No. 513-5542-G-55-9067-00 from U.S. AID Program of the State Department's Office of the Science Advisor, the Chilean National ence Commission/Fondecyt Grant Nr. 0669/89, and PNUD (Project No.: DP/RLA/ 83/003}.



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ORGANOGENESIS AND REGENERATION OF SOME ANDEAN FRUIT SPECIES.

M. Jordan*, M. Obando, L. Iturriaga, A. Goreux and J. Velozo.
Department of Ecology, Pontificia Universidad Catolica de Chile, Casilla 114-D, Santiago, Chile

Lesser-known Andean species are of potential economic importance, due to their fruit quality, flavor and vitamin content. The regeneration of elite clones is required to establish standard and consistently productive plantations. In vitro experiments on different cultivars and ecotypes of three promising species were conducted in our laboratory. Results have shown, for Annona cherimola (cherimoya), primarily multiple shoots were formed directly from hypocotyls, which showed strong polarity. Callus formation also occurred in different explants, in spite of the high oxidation rate shown by this species. Browning has been strongly reduced by antioxidant supplements. Solanum muricatum (pepino) shows great organogenic potential; leaves cultured with different hormone supplements regenerate roots and shoots, leading to plantlets in 33% of explants. No subculturing is required. Preliminary results show that Cypomandra betacea (tamarillo) axillary bud explants also show high regeneration potential, forming shoots and roots. Recovery of plantlets of these species under in vitro conditions is continuing in our laboratory. Transformation research has also been started.

Acknowledgements. Partial support for this presentation was provided by AID Grant 513-5541-G-SS-9067-00.

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EFECTO DE DAÑO MECANICO Y DE CALCIO EN LA ACTIVACION DE LA VIA FENILPROPANOIDE EN PLANTULAS DE LIMONERO. (Effect of mechanical damage and of calcium in the activation of the phenyl propanoid pathway in lemon seedlings). Castañeda, P. Dep. de Bioq. y Biol.Mol., Fac. Cs. Quím. y Farm., U. de Chile y Dep. Biol., Fac. Cs. Bás., UMCE. (Patrocinio: L. M. Pérez)

Una de las vías metabólicas que se activa en tejidos vegetales frente a diferentes estímulos, es la vía fenilpropanoide. La primera reacción de ésta, es la desaminación de la L-Fenilalanina a ácido trans-cinámico, catalizada por la fenilalanina amonio liasa (PAL). Se han postulado varios posibles segundos mensajeros para la activación de esta vía, entre ellos el calcio.

Seestudió el efecto que tiene el daño mecánico y la influencia que tiene sobre esta respuesta la adición de Ca^{2+} en plántulas de limonero. Para ello se cuantificó la actividad de PAL a diversos tiempos después del daño.

La respuesta de las plántulas de limonero frente a daño mecánico fue bifásica, con máximos de actividad de PAL a las 4 y 8 horas. Cuando se adicionó Ca^{2+} 10 μM, la respuesta sigue la misma tendencia bifásica, pero los máximos se desplazaron a las 3 y 7 horas.

Estos resultadosnos permitensugerir la participación de Ca^{2+} en la activación de la vía fenilpropanoide, posiblemente actuando en la transducción de la señal primaria.

Dirigido por L. M. Pérez. Financiado por proyecto FONDECYT 91/0886.

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REGENERACION IN VITRO DE ALGUNAS ESPECIES FRUTALES ANDINAS. (In vitro regeneration of some Andean fruit species) Jordan, M., Obando, M., Goreux, A. y Veloso, J. Departamento de Ecología, Facultad de Ciencias Biológicas, P. Universidad Católica.

Algunas especies andinas conocidas en mercados nacionales pero sin proyección internacional, son actualmente estimadas de importancia económica dada la calidad, sabor y contenido en vitaminas de sus frutos. La regeneración masiva de material seleccionado correspondiente a variedades y ecotipos es necesaria para iniciar plantaciones en gran escala. En algunas especies, que muestran incompatibilidad sexual, la propagación vegetativa es la única forma de regeneración. Se informa acerca de los resultados de micropropagación en tres especies estudiadas en nuestro laboratorio: Babaco (Carica pentagona), puede ser regenerado a partir de iniciación de brotes en secciones de lámina foliar, y mediante rizogénesis de yemas axilares y de estacas. En pepino (Solanum muricatum), la regeneración de plantas se logra rápidamente a partir de secciones nodales como también por organogénesis de brotes en porciones de lámina foliar hasta un 33% de los explantes. Los resultados de tamarillo (Cyphomandra betacea), demuestran que yemas axilares al igual que hojas pueden regenerar nuevos brotes adventicios (en un 90% de los explantes) e iniciar rizogénesis conducente a plantas. Dado el alto potencial morfogénico existente en estas especies, han sido iniciados trabajos de transformación.

Se agradece el financiamiento otorgado por AID (Proyecto 8007, PSTC Grant No. 513-5542-G-SS-9067-00), y el apoyo de la Dra. M. Sticklen y su grupo, Michigan State University para efectuar ensayos de transformación en pepino.

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PLASTIDOS ESTAN INVOLUCRADOS EN EL MECANISMO DE INDUCCION DE LA FENILALANINA AMONIO LIASA EN *Citrus limon*. (Plastids are involved in the mechanism of phenylalanine ammonia-lyase induction in *Citrus limon*). Chiong, M., Prieto, H. y Pérez, L.M. Depto. Bioquímica y Biol. Molecular. Fac. Cs. Químicas y Farmacéuticas. U. de Chile.

La fenilalanina amonio liasa (PAL) es inducida por numerosos estímulos, que incluyen luz blanca, luz ultravioleta, daño mecánico, infecciones por patógenos, entre otros. Aunque se han clonado numerosos genes de PAL y analizado sus promotores, se desconocen aún los eventos intracelulares involucrados en su activación.

Rifampicina y cloramfenicol, que inhiben transcripción y traducción en sistemas procarióticos y organelares, así como alfa-amanitina y cicloheximida son capaces de inhibir la inducción de PAL en plántulas de limonero (*Citrus limon*) inoculados *in vitro* con *Alternaria alternata*. Se obtuvieron plántulas de limonero completamente etioladas, germinando semillas de limón en presencia del herbicida Norfluorazon o cloramfenicol y cultivando las plántulas bajo luz blanca continua (2000 lux). Cuando estas plántulas son inoculadas con *A. alternata*, ellas son incapaces de inducir PAL.

Callos obtenidos de hipocotilos de plántulas de limonero, inducen la PAL frente a daño mecánico en una cinética de 2 máximos. El primer máximo, 10 horas post-daño, se inhibió con cloramfenicol, rifampicina y cicloheximida. En cambio, el segundo máximo, 20 horas post-daño, se inhibió con cloramfenicol y rifampicina, pero no con alfa-amanitina ni con cicloheximida.

Estos experimentos sugieren la participación de cloroplastos en los eventos intracelulares de inducción de la PAL en *Citrus limon*.

Financiado por FONDECYT 90-0066 y Departamento de Post-grado y Postítulo, U. de Chile PG 009-92 (M. Chiong) y FONDECYT 91-0886 (L.M. Pérez).

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PRODUCCION DE PLANTAS TRANSGENICAS DE PAPA (SOLANUM TUBEROSUM) Y ESPARRAGO (ASPARAGUS OFFICINALIS) VIA AGROBACTERIUM TUMEFACIENS. (Transgenic production of potato (Solanum tuberosum) and asparagus (Asparagus officinalis) plants, via Agrobacterium tumefaciens). Oliger, P., Gutiérrez, A. (Patrocinio: Jordana, X.) Programa Micropropagación Vegetal, Facultad de Agronomía y Ciencias Biológicas, Pontificia Universidad Católica de Chile.

El fitomejoramiento tradicional para la obtención de variedades mejoradas de plantas se ha venido practicando desde que comenzó la agricultura. Sin embargo, ésta ha sido limitada por las barreras filogenéticas que están implícitas en esta metodología. A través de la Ingeniería Genética y utilizando el potencial que ofrece el cultivo in vitro podemos lograr con mayor rapidez introducir características de importancia agronómica en los cultivos.

El objetivo de este trabajo fue el obtener transformación estable de plantas de papa (Solanum tuberosum) y esparrágo (Asparagus officinalis) vía Agrobacterium tumefaciens.

Para esto se infectaron explantes provenientes de cultivo in vitro de estas plantas con una cepa de A. tumefaciens portadora del plasmido pBI 121. Este plasmido contiene los genes de la neomicina fosfotransferasa que confiere resistencia a Kanamicina y de la B-glucuronidasa (GUS).

Las plantas transformadas se seleccionaron en medio de regeneración que contiene Kanamicina y se detectó actividad Gus por ensayo histoquímico.

Actualmente se intenta introducir resistencia a bacterias en estas especies utilizando esta metodología.

(FINANCIAMIENTO PROYECTO DIUC Nº 9107 E)

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Wednesday-April 1 - morning

09:00-12:00 Grantee review sessions

9:00	10.139	Oduro/Furtek
9:30	9.396	Tabora/Balick
10:00	12.303	Janick/Figueira/Enrique
10:30	7.340	Vega de Rojas/Kitto
11:00	9.174	Suarez/Purdy
11:30	9.395	Molina/Strobel

Wednesday-April 1 - afternoon

Travel to Escuela de Agricultura de la Region Tropical Humeda

Wednesday-April 1 - evening

18:00 Dinner

20:00 Reception

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The Board on Science and Technology for International Development (BOSTID) conducts programs with and for developing countries on behalf of the National Research Council through its Office of International Affairs which serves the international interests of the National Academy of Sciences, the National Academy of Engineering and the Institute of Medicine

IN VITRO PROPAGATION AND IMPROVEMENT OF FRUIT SPECIES WITH ECONOMIC POTENTIAL FOR SEMI-ARID ZONES.

AID Grant Nº 513-5541-G-SS-9067-00
Coordinator: Dr. Miguel Jordan, Chile

Within the framework of this project, different micropropagation strategies are researched to be applied to several semi-arid fruit species of economic importance; Cherimoya (Annona cherimola), Lucuma (Pouteria lucuma) and highland Papayas (Carica pubescens) as well as (C. pentagona) Babaco. To this end, several protocols have been developed and tested and most morphogeneses lead to plantlets. Results were checked via histological observation, in different types of explants of the species under study. Among - the best responses, it was possible to regenerate plantlets from meristems, shoot-tips, petioles and embryos in lucuma; to induce adventitious shoots directly from cherimoya and papaya hypocotyls as well as from papaya and babaco leaves. Studies concerning browning metabolism are also carried out by means of addition of antioxidants in vitro.

Nodal sections, internodes and zygotic embryos of Annona cherimola were cultivated in vitro in several phytohormone combinations to trigger morphogenesis. De novo multiple shoot formation was the most common response. Various antioxidant compounds, specially PVP, ascorbic acid and glutathione enhanced this effect and reduced browning, while DIECA, cysteine and citric acid were not effective in the concentrations tested. Combinations of PVP with casein hydrolysate in presence of NAA 0.5 mg/l plus BA 2.0 mg/l were the best blends to promote shoot formation and yielded the highest number of shoots per explant. The use of Gelrite, sorbitol and PVP was beneficial, preventing browning and promoting growth and/or morphogenesis.

In Pouteria lucuma, plantlets were more frequently recovered from meristems, shoot tips, petioles and embryos; these explants showed high morphogenetic ability. Best responses were achieved and direct root formation was obtained from shoot tips of seedlings grown in WPM with NAA + BA + activated charcoal. Axillary buds developed on nodal sections. Also, only leaf explants attached to petioles (basal end) of juvenile material derived from seedlings formed shoots and roots from calli originating at the petiole, following subculture in presence of TDZ, NAA and organic addenda. Soluble PVP and PVPP proved to be the best to reduce browning. Androgenic embryoids were also obtained from anthers.

In Carica pubescens and Carica pentagona, rooting of shoot-tips or cuttings was induced by inmersing in a BA solution during 24 hours. Then, several shoots were observed sprouting from each cutting, especially in the upper part; these shoots were subcultured in vitro for rooting, as regular shoot tip culture. In this way, 10% rooted plantlets were obtained using WPM-medium

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in combination with NAA, BA, and GA3, or without giberellin, but root formation was scarce.

In babaco, 14% rooting was obtained, as well as development of axillary buds, when 10 cm long cuttings, with basal leaves removed, were soaked in an IBA solution and then mantained in tap water for one month in greenhouse conditions. Axillary buds also developed on nodal sections.

Multifactorial experiments to induce morphogenesis from leaves for C. pubescens showed scarce results when using TDZ and NAA, however better responses were found in leaves of babaco which were able to regenerate shoots. Although the whole leaf loses chlorophyll, new shoots were entirely green. Multicellular pollen and androgenic embryoids were also detected after anther culture in papaya.

The following table summarizes the morphogenic events obtained in Lucuma, Cherimoya and Papaya explants.

Explant	Lucuma	Cherimoya	Papaya
Anthers (microspores)	Androgenic embryos, calli	Somatic cells proliferation	Androgenic embryos
Leaves	Shoots, calli*	Calli	Calli, shoots in Babaco & Papaya*
Petiole	Shoots and Roots	Multiple shoots.	Calli
Nodal sections	Rhizogenesis	Multiple shoots	Bud sprouting Calli
Internodes	Shoots	Multiple shoots and calli	Calli and Somatic embryos
Pericarp	Callus	not tested	Calli and embryos.
Zygotic embryos	Adventitious embryos	Shoots, Calli	Calli and somatic embryos
Hipocotyls/ Epicotyls	Multiple shoots	Multiple shoots	Calli and somatic embryos
Cuttings	No response	No response	Roots Both Caricas

*single events

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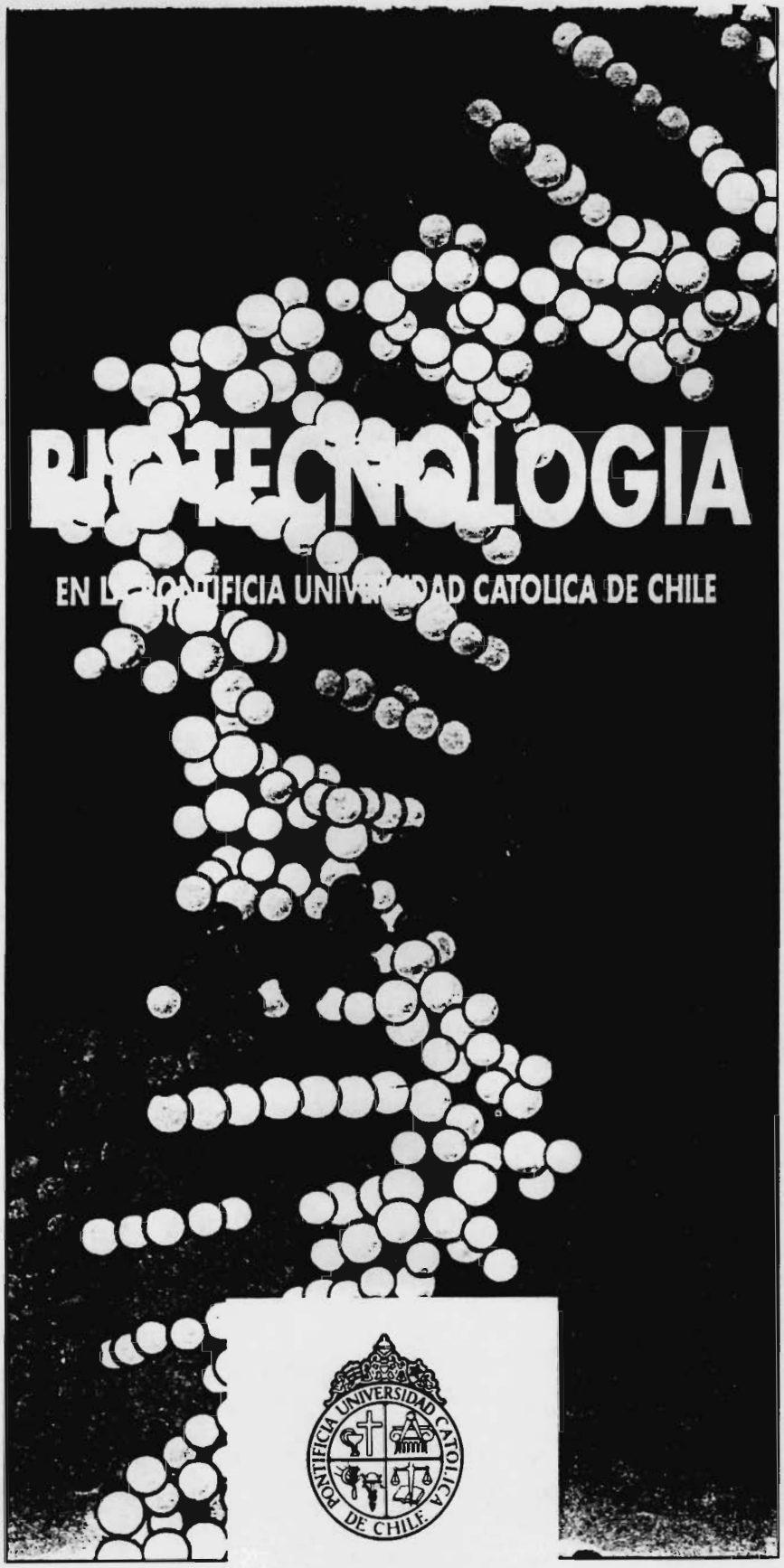

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la flora melífera que las abejas pueden utilizar como fuente de polen y néctar. Esta dependiencia es limitada y temporal si la flora está representada por cultivos, surgiendo como alternativa de gran proyección las comunidades de flora nativa. Dado que las abejas son selectivas, la factibilidad de emprender una producción sostenida depende del conocimiento de los periódos de floración de la flora melífera del lugar. El objetivo del proyecto es detectar las especies vegetales nativas utilizadas por las abejas melíferas como fuente de alimento, a través del reconocimiento específico de los granos de polen que recolectan y de aquellos presentes en el néctar acumulado en las celdillas. La especifidad del polen permite este diagnóstico utilizando la tecnica de Microscopía Electrónica de Barrido. Construyendo los calendarios polínicos y nectaríferos de distintos puntos geográficos se podrá implementar una red fenológica nacional de especies melíferas.

La existencia en Chile de comunidades vegetales de distinta diversidad específica y actividad fenológica a lo largo de gradientes latitudinales y altitudinal determina la posibilidad de utilizar las especies melíferas en periódos distintos del año (transhumancia con panales no sólo a cultivos sino que a zonas de vegetación nativa) lo que lleva a maximizar la utilización de los recursos naturales y en consecuencia aumentar la producción de la actividad apícola.

Proyecto Fondecyt 199/88

MULTIPLICACION EN VITRO DE ESPECIES FRUTALES DE IMPORTANCIA ECONOMICA PARA ZONAS SEMI-ARIDAS

M. Jordán, M.L. González, A. Goreux, L. Iturriaga, G. Montenegro, A.M. Mujica, C. Roveraro, B. Tesser, M.P. Valenzuela, J. Velozo, Departamento de Ecología, Facultad de Ciencias Biológicas P. Universidad Católica de Chile.

En este proyecto se estudian las potencialidades de micropropagación de tres frutales de zonas semiáridas: chirimoya (*Annona cherimola*), lúcuma (*Pouteria lucuma*) y papaya (*Carica pubescens*), de gran importancia económica para Chile.

Estas tres especies frutales, de un alto valor económico y potencial de exportación, corresponden a cultivos muy productivos y rentables en diferentes localidades a lo largo del país, que cuentan con climas carentes de temperaturas extremas. En Chile existen para cada una de estas especies diversos ecotipos, selecciones, clones o variedades locales cuyas características pueden ser de interés para futuros programas de mejoramiento, selección y producción expansiva. La posibilidad de lograr una multiplicación de estos individuos élite, fijando las mejores características genéticas; conducentes a la obtención de material más productivo, resistente a enfermedades y adaptado a condiciones de bajas temperaturas, requieren contar con sistemas de regeneración asexual, rápidos y masivos. Estas tres especies que presentan diferentes respuestas morfogénicas, han podido ser inducidas bajo condiciones *in vitro*, destacándose: la inducción de embriones somáticos múltiples y plántulas derivadas de tejido no organizados totipotentes, a partir de explantes de hipocotilo, embriones y pericarpio, la inducción *de novo* de brotes en diferentes órganos, la generación de embrioides a partir de polen mediante cultivo de anteras, la regeneración a partir de hojas, el establecimiento de suspensiones celulares y la obtención de algunos estadios derivados de la fusión de protoplastos interespecie, aún de capacidad morfogénica limitada.

Financiamiento: AID Nº 8007, PSTC-Grant Nº 513-5542-G-SS-9067, Fondecyt Nº 0669/89

FLAVONOIDES COMO ESTABILIZANTES DE ACEITE DE PESCADO. UNA ALTERNATIVA AL USO DE ANTIOXIDANTES SINTETICOS

F. Leighton, L.A. Loyola *, G. Morales*, J. Sanhueza**, A. Garrido**, C. Skorin, F. Solis de Ovando, C. Necochea, A. Valenzuela**
P. Universidad Católica de Chile * Universidad de Antofagasta ** INTA, Universidad de Chile

Los Flavonoides constituyen una gran familia de compuestos naturales. Entre sus propiedades destacan: una alta afinidad en la unión a polímeros biológicos e iones de metales pesados; el catalizar

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34a. REUNION ANUAL

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CULTIVO " IN VITRO" DE Euphorbia lactiflua Phil. EUPHORBIACEAE.

(In vitro culture of Euphorbia lactiflua Phil. EUPHORBIACEAE).

Mancinelli P.*, Gnecco, S., Pooley, A., Caamaño V. & Beratto, V.

Departamento de Botánica y Departamento de Química. Universidad de Concepción.

E. lactiflua,"Lechero" produce látex que contiene terpenos muy interesantes; actualmente bajo investigación. Como la planta crece en la III Región, parece adecuado utilizar el cultivo de tejidos, para multiplicarla y estudiar su composición.

Explantes foliares de E. lactiflua, recolectados cerca de Caldera, III Región, formaron callos al cultivarlos en Medio Murashige/Skoog con bencilamino purina, ácido naftalenacético y Giberalina (0.5; 1.5 y 1.0 mg l^{-1}). Los callos diferenciaron yemas de los mismos reguladores pero a distinta concentración (1.5; 0.5 y 1.0 mg l^{-1}).

Plántulas derivadas de las yemas formaron raíces al incubarlas con ácido indolbutírico, carbón activado y sacarosa (2mg l^{-1}, 10 g l^{-1} y 15 g l^{-1}), respectivamente.

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ESTUDIO PARA EVALUAR EL USO BIOTECNOLOGICO DE DOS ESTIRPES DE CIANOBACTERIAS NATIVAS DEL NORTE DE CHILE.(Study to assess the biotechnological use of two native strains of cyanobacteria from northern Chile).

Olivares.H., Gomez-Silva.B., Neira.I., Sagua H. y González.J.Instituto de Desierto y Facultad Cs. de la Salud, U. de Antofagasta.

Algunos cuerpos de agua continentales del Desierto de Atacama en Chile son habitats colonizados por microorganismos fotosintéticos adaptados a crecer en condiciones ambientales extremas representando así recursos naturales necesarios de ser estudiados.Este trabajo informa sobre dos cepas de cianobacterias de la región norte con interesantes proyecciones biotecnológicas : *Nostoc sp.* (Llaita) como fuente nutritiva y *Microcystis sp.*(Chiuchiu) como productor de compuestos con actividad anti -*T. cruzi*.
Aislamiento y purificación de las dos microalgas en este estudio se realizaron de acuerdo a Ripka (Methods in Enzymol. Vol.167, 1988). Obtención de extractos con diferentes solventes y su evaluación en actividad anti-*T.cruzi* se llevó a cabo como lo describen González y col.(Phyt. Res. 4: 1-4. 1990). El producto seco que se expende para consumo humano en Arica fué reactivado en el laboratorio y está constituido esencialmente por una estirpe de la cianobacteria *Nostoc sp* filamentosa con abundantes heterocistos. Extractos obtenido con acetato de etilo, éter y metanol a partir de *Microcystis sp.* (aislado de la laguna Chiuchiu,II Región) muestran actividad anti-*T. cruzi* de 90%, 68%, y 56%, respectivamente. Composición molecular y condiciones de cultivo se discuten.

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RESPUESTAS ANDROGENICAS EN ANTERAS DE LUCUMA (Pouteria lucuma), PAPAYA (Carica pubescens) Y CHIRIMOYA (Annona cherimola). (Androgenic potential of lucuma (Pouteria lucuma), papaya (Carica pubescens) and cherimola (Annona cherimola) anthers). Jordán, M., Valenzuela, M.P. y Oyanedel, P. Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. Avenida Libertador B. O'Higgins 340, Santiago, Chile.

En una serie de especies herbáceas y leñosas, mediante un proceso de inducción in vitro, ha sido posible modificar la ontogenia normal de la microesporogénesis conducente a la formación de polen en anteras y reprogramando alternativamente la morfogénesis directa de embriones. Por su naturaleza androgénica estos embriones originados de microsporas son de carácter haploide lo que permite la diploidización posterior de este material y la obtención de plantas homocigotas diploides. Asumiendo que cada planta regenerada, hija de polen, ha sufrido las recombinaciones del crossing-over durante la microesporogénesis la variabilidad de plantas que es posible obtener por esta vía regenerativa permite la producción y selección rápida de material para estudios genéticos y de cruzamientos.

Se presentan los primeros resultados de respuestas androgénicas en estas especies frutales de importancia económica que posibilitan conducir estudios futuros hacia la selección y caracterización de plantas más tolerantes a bajas temperaturas, la caracterización de formas sexuales y la identificación de germoplasma, ecotipos o variedades.

Se agradece el financiamiento otorgado por AID a través del Proyecto 8007, PSTC Grant N° 513-5542-G-SS-9067-00 a M. Jordán.

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REGENERACION IN VITRO DE NOTHOFAGUS ALPINA. (In vitro regeneration of Nothofagus alpina) Velozo, J., Sánchez, P. Depto. Ecología, Facultad de Ciencias Biológicas, P.. Universidad Católica de Chile.

El raulí, Nothofagus alpina, es una especie endémica que se encuentra entre los 100 a 800 m.s.n.m. en la Cordillera de los Andes y la Costa, desde la VII hasta la X Región. Dadas sus características estructurales se presenta como una de las especies nativas más valiosas para la industria maderera. Encontrándose sus poblaciones naturales muy degradadas, su recuperación es un imperativo ecológico; programas de reforestación con material genéticamente mejorado ofrecen perspectivas promisorias para una explotación racional futura. En esta perspectiva, fueron realizados ensayos de micropropagación in vitro, usando explantes de material juvenil de 2 años. Se evaluaron condiciones y medios de cultivo como diversos tipos y niveles de fitohormonas. A partir de secciones de lámina foliar, fue posible la formación directa de brotes transcurridos 60 días de cultivo, previa incubación en oscuridad. El medio WPM fue el más adecuado usando citocininas solas o en combinaciones con auxinas.

En secciones nodales se logró inducir organogénesis indirecta de brotes a partir de callo y paralelamente, crecimiento de yemas axilares y brotes múltiples.

Mediante subcultivo fue posible inducir rizogénesis en los brotes usando medios de cultivo similares con niveles crecientes de auxinas. Formación de raíces se logró también microestacas a los 35 días de incubación en condiciones semejantes. Sobre la base del potencial morfogénico de esta especie, es factible la aplicación de esta tecnología para la producción de plantas en programas de mejoramiento.

Convenio PUC/Bioforest Ltda. (Patrocinio Dr. M. Jordán).

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JORDAN

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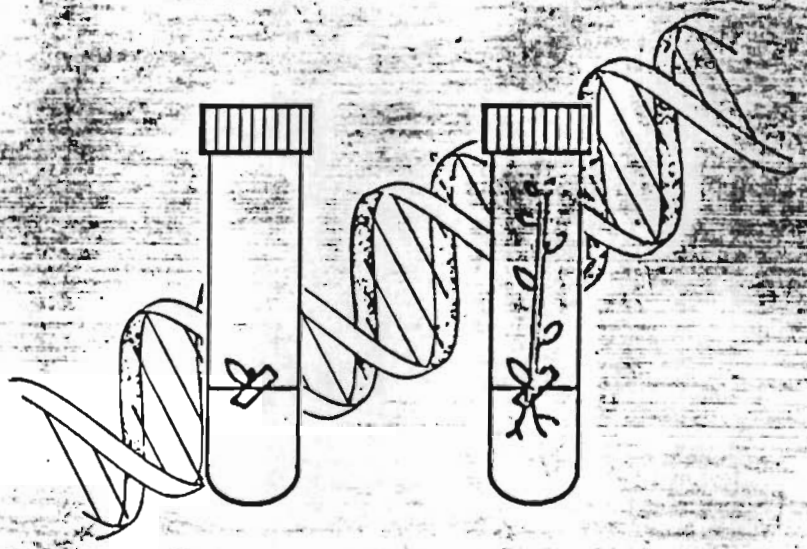
FACULTAD DE CIENCIAS AGROPECUARIAS

Simposio Argentino de Biotecnología Vegetal



LIBRO DE RESUMENES

VAQUERIAS (CORDOBA), 11 y 12 DE JUNIO DE 1991

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POTENCIALIDADES MORFOGENICAS Y MULTIPLICACION IN VITRO DE TRES ESPECIES FRUTALES DE IMPORTANCIA ECONOMICA PARA ZONAS SEMI-ARIDAS EN CHILE.

Jordán, M., Valenzuela, M.P.; Velozo, J., Oyanedel, E., Sánchez, P. y Montenegro, G. Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda Bernardo O'Higgins 340, Santiago, Chile.

Tres especies frutales poco conocidas en mercados internacionales y de gran importancia económica en zonas semi-áridas: Chirimoya (Annona cherimola), Lúcuma (Pouteria lucuma) y Papayo Montañes (Carica pubescens), fueron estudiadas para evaluar el potencial regenerativo de diversos explantes cultivados bajo condiciones in vitro. Diferentes respuestas morfogénicas son inducibles; de entre ellas las más relevantes son, la neoformación de embriones somáticos múltiples con generación de plántulas a partir de tejido no organizado totipotente (callo), hipocotilo, embriones y pericarpio; la inducción directa de brotes a partir de lámina foliar, la diferenciación de embrioides a partir de polen (androgénesis), la formación de embriones somáticos en suspensiones celulares y la inducción de algunos estadios de fusión interespecífica de protoplastos, entre otras morfogénesis expresadas in vitro. Dado que en el país existen para cada especie diversos ecotipos, selecciones locales, clones o variedades cuyas características pueden ser de interés en futuros programas de mejoramiento, la aplicación de sistemas de regeneración asexual o gametoclonal rápidos y masivos permitirían fijar y amplificar las mejores características genéticas de individuos elite.

Se agradece el financiamiento otorgado por AID al Proyecto N° 8007, PSTC-Grant N° 513-5542-G-SS-9067-00 y FONDECYT Proyecto 0669/89 a Miguel Jordán

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EXTRACCION DE N, P Y K POR ALGUNAS MALEZAS COMUNES EN HUERTOS FRUTALES.

Verónica Díaz M., Bruno Razeto M. y Silvia Rojas[1].
(1) Universidad de Chile, Facultad de Cs. Agrarias y Forestales. Casilla 1004-Santiago.

Las malezas compiten con el crecimiento de los árboles frutales por luz, agua y elementos minerales. La cuantificación del grado de competencia es difícil, ya que depende de las especies de malezas su densidad y la fertilidad del suelo, entre otros. Con la finalidad de cuantificar las cantidades de macroelementos que consumen algunas malezas comunes en los huertos de nuestro país, se diseñó un proyecto de investigación, con financiamiento FONDECYT. En una primera etapa se pudo establecer que el consumo de Nitrógeno, Fósforo y Potasio por parte de Sorghum halepense, Cynodon dactylon, Bidens aurea y Echinocloa crusgalli, considerando una infestación inicial de 4 pl/m2, la extracción fluctuaba según la maleza y fertilidad del suelo entre 30-250 u de N,4-36 u de P y 28 a 466 u de K.

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CASTAÑOS : ALTERNATIVA FRUTAL PARA LA ZONA CENTRO SUR DE CHILE

Angélica Salvatierra G.[1] y Jorge Valenzuela B.[2]
(1) Estación Experimental Quilamapu (INIA), Casilla 426, Chillán; (2) Estación Experimental La Platina (INIA), Santiago.

Durante la temporada 1989 se inició una prospección de las áreas productoras de castañas en la VIII Región, principalmente en las zonas de precordillera. Las comunas son Pinto, El Carmen, Quilleco y Antuco.

El objetivo es la búsqueda de individuos que presenten buenas características. Las principales características consideradas fueron tamaño y tabicación del fruto y productividad.

Las plantaciones generalmente son de plantas originadas de semillas. Los huertos existentes en la región son antiguos, generalmente no están sometidos a normas técnicas de manejo y se observa una gran variabilidad en cuanto a precocidad, forma del árbol y calidad de frutos.

Luego de dos temporadas de prospección y evaluación de frutos se han seleccionado algunos individuos que presentan características promisorias.

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INFLUENCIA DE ANTIOXIDANTES EN LA PROMOCION DE BROTES DE ANNONA CHERIMOLA CULTIVADOS IN VITRO.

Liliana Iturriaga[1], Andree Gorroux[1] y Miguel Jordán[1].
(1) Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago, Casilla 114-D.

Annona cherimola (Annonaceae) es una especie de origen andino, existiendo diferentes cultivares y ecotipos. Se requiere mejorar y multiplicar plantas elite para establecer plantaciones en distintos microclimas. Esta especie presenta grandes problemas en el metabolismo oxidativo de los compuestos fenólicos, por lo que el control de este fenómeno es una prioridad para su cultivo. Fueron cultivados in vitro secciones nodales, internudos y embriones cigóticos de chirimoya cv. "Concha Lisa" junto con distintas combinaciones de fitohormonas. La formación de brotes de novo fue la respuesta más usual. Varios compuestos antioxidantes, entre ellos PVP, ácido ascórbico y glutatione maximizaron este efecto y redujeron la oxidación, mientras que DIECA y el ácido aminooxyacético fueron menos efectivos en las concentraciones ensayadas. El ácido cítrico y la cisteínas tuvieron poco efecto. Combinaciones de PVP con hidrolizado de caseína, en presencia de NAA y BA fueron las mejores en cuanto a la formación de brotes por ex plante.

Se agradece el financiamiento PSTC Grant N° 513-5542-G-SS-9067-00 U.S. AID y FONDECYT Grant N° 0669/89 de M. Jordán.

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EL PISTACHO: NUEVA ALTERNATIVA FRUTAL PARA CHILE

Jorge Valenzuela B[1]., Gamalier Lemus S. y Carlos Muñoz Sch.
(1) Estación Experimental La Platina (INIA), Casilla 439-3 Santiago.

En 1981, se germinó semillas de Pistacia atlantica y P. terebinthus en la Subest. Exp.

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SEGUNDO CONGRESO NACIONAL DE BIOTECNOLOGIA

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RESPUESTAS ANDROGENICAS EN ANTERAS DE LUCUMA (Pouteria lucuma) y papaya (Carica pubescens). (Androgenic potential of lucuma (Pouteria lucuma) and papaya (Carica pubescens) anthers). Jordán, M., Valenzuela, M.P., Velozo, J., Oyanedel, P., González, M.L. y Sánchez, P. Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avda. Libertador B.O'Higgins 340, Santiago, Chile.

En una serie de especies herbáceas y leñosas, mediante un proceso de inducción in vitro, ha sido posible modificar la ontogenia normal de la microesporogénesis conducentes a la formación de polen en anteras y reprogramando alternativamente la morfogénesis directa de embriones. Por su naturaleza androgénica estos embriones originados de microsporas son de carácter haploide lo que permite la diploidización posterior de este material y la obtención de plantas homocigotas diploides, Asumiendo que cada planta regenerada, hija de polen, ha sufrido las recombinaciones del crossing-over durante la microesporogénesis la variabilidad de plantas que es posible obtener por esta vía regenerativa permite la producción y selección rápida de material para estudios genéticos y de cruzamientos.

Se presentan los primeros resultados de respuestas androgénicas en estas dos especies frutales de importancia económica que posibilitan conducir estudios futuros de importancia económica que posibilitan conducir estudios futuros hacia la selección y caracterización de plantas más tolerantes a bajas temperaturas, la caracterización de formas sexuales y la identificación de germoplasma, ecotipos o variedades.

Se agradece el financiamiento otorgado por AID a través del Proyecto 8007, PSTC Grant N° 513-5542-G-SS-9067-00 a M. Jordán.

OBTENCION DE PIGMENTOS CAROTENOIDES EN CULTIVO DE TEJIDOS DE ALFALFA (*Medicago sativa*) (*In vitro* obtentibn of carotenoids in *M. sativa*). Figueroa, C., F. Massardo, F. y Zúñiga, G.E. Laboratorio de Fisiología Vegetal, Departamento de Química, Facultad de Ciencia, Universidad de Santiago de Chile. Casilla 5659-2.

El optimización de metodologías que permitan la producción competitiva de pigmentos de uso industrial constituye un área importante de desarrollar. Los pigmentos carotenoides, particularmente el β-caroteno, son ampliamente utilizados en la industria de alimentos como precursores de la vitamina A. En este trabajo se evaluó la producción de pigmentos carotenoides en alfalfa cv. California utilizando la técnica de cultivo *in vitro*.

Para la inducción de callos de utilizaron los medios MS y NN suplementados con NAA/K y 2,4-D en combinación con diferentes explantes. El porcentaje de inducción fue superior en el medio MS en ambas dosis hormonales empleando como explante la plántula completa 1 día después de la germinación. La producción de pigmentos fue dependiente de la dosis de hormona siendo superior con la combinación NAA/K.

El cultivo de tejidos de alfalfa se presenta como una alternativa viable para la producción de pigmentos carotenoides.

Financiado por DICYT, Universidad de Santiago de Chile (Proyecto 8832-ZN).

SIMPOSIO INTERNACIONAL

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Santiago - Viña del Mar 16, 17 y 18 de Abril de 1991

PROMESAS BIOTECNOLOGICAS EN ARBOLES FORESTALES

MIGUEL JORDAN
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Casilla 114-D Santiago, Fax 2225515

Más y mejores especies forestales son consideradas hoy en día posibles de ser plantadas y explotadas comercialmente. Se requieren millones de plantas a corto plazo que deben contar con características genéticas superiores derivadas de material elite. Biotecnologías modernas usadas en estrategias de mejoramiento de diferentes especies agrícolas pueden igualmente significar un avance en especies forestales. Diferentes problemas que afectan la productividad pueden ser potencialmente resueltos a través de un enfoque biotecnológico; entre ellas, una multiplicación clonal in vitro de individuos superiores derivados de programas de selección o de cruzamiento, la producción de árboles de más rápido crecimiento que permitan amortizar más tempranamente la inversión, la selección de clones capaces de formar xilema más tempranamente con mejor calidad de fibra, de fijar su propio nitrógeno o afines a la participación de micorrizas para extraer nutrientes minerales con mayor eficiencia.

Programas genéticos de cruzamiento y selección pueden ser apoyados con la producción de haploides/diploides homozigotos a través de la generación de plantas derivadas de polen, denominada androgénesis, como se está desarrollando en especies frutales. Idealmente se puede visualizar hoy en día la posibilidad de contar con plantas transformadas genéticamente para obtener resistencia a diferentes clases de insectos mediante la inserción de los genes responsables de las toxinas de Bacillus thuringiensis usando como vehículo a Agrobacterium tumefaciens. La inserción directa de genes actualmente también es factible usando un cañón génico, dispositivo que permite disparar microproyectiles de ADN a tejidos diversos con alta eficiencia.

La producción masiva de plantas a partir de individuos mejores derivados de selección, cruzamientos dirigidos o material transformado, depende finalmente del control in vitro del caracter embriogénico en sistemas celulares/bioreactores y, de la organogénesis a nivel tisular. El proceso culmina con la encapsulación automatizada en geles (incluyendo pesticidas, nutrientes y hormonas) de semillas somáticas histológicas idénticas, derivadas de suspensiones celulares para su siembra directa en terreno.

Aunque la ingeniería genética y la biología molecular cuentan hoy en día con estas metodologías para insertar, transferir y detectar genes foráneos logrando plantas transformadas, la gran limitación de respuestas morfogénicas y de regeneración (salvo excepciones) en muchas especies leñosas forestales es casi una constante para la utilización de esta biotecnología en gran escala. Aún se requiere de mayor información básica sobre mecanismos fisiológicos, incluyendo: aspectos de regulación, juvenilidad, floración, reproducción vegetativa y criopreservación tisular como también respecto al metabolismo de terpenos, fenoles y otros ciclos que deberán ser establecidos particularmente.

Se agradece el financiamiento de BIOFOREST Ltda. y Proyecto AID 8007 PSTC Grant Nº 513-5542-G-SS-9067-00.

Biotechnology for Tropical Crop Improvement in Latin America

Proceedings of the
Fourth Conference
of the International
Plant Biotechnology
Network (IPBNet)
San Jose, Costa Rica
January 14-18, 1991

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Development

Colorado State

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objectives are: the formulation of national policies, the promotion of the exchange of information and biological materials, the support and/or co-sponsorship of regional training activities in advanced biotechnology, the development of cooperative research projects among laboratories within the region and from developed countries, and the promotion of the preparation of a "code of conduct" in plant biotechnology. The network coordinating bodies are the regional coordinating committee composed of the subregional coordinators (4), the FAO Technical Secretariat, the Consulting Technical Council and the National Network Coordinators. Priority activities of the network were established for 1991-1992 involving molecular virology, use of RFLP's in plant breeding, regulations and biosafety, a network circular newsletter and coordinating meetings.

IN VITRO PROPAGATION AND IMPROVEMENT OF FRUIT SPECIES WITH ECONOMIC POTENTIAL FOR SEMI-ARID ZONES

Miguel Jordan*, Gloria Montenegro, Maria L. Gonzalez, Andree Goreux, Liliana Iturriaga and Maria P. Valenzuela. Ecology Department, Catholic University of Chile, Santiago, Chile.

Little known species growing in developing countries may be important to open new markets; for this, cultivars of standard quality must be obtained. *In vitro* culture techniques suited to each species/variety must be developed to select and propagate elite material. Advances concerning three species: *Annona cherimola* (cherimoya), *Pouteria lucuma* (lucuma) and *Carica pubescens* (highland papaya) are examined. Explants of the three species were evaluated to establish *in vitro* morphogenic/androgenic potential and capacity to regenerate specific clones.

A. cherimola forms multiple de novo adventitious shoots in all types of explants assayed, best results in nodal sections, internodes, embryos and hypocotyls. Tissular browning could be controlled using soluble and insoluble PVP. In *P. lucuma*, best morphogenesis occurs as multicellular pollen, and supposedly androgenic embryos; calli and/or shoots arise from leaf sections. In *C. pubescens*, somatic embryos arise from immature fruit pericarp and hypocotyls; zygotic embryos give calli which originate embryoids in sub-culture, in papaya, anther culture generated somatic calli only; microspores do not show internal divisions leading to androgenic embryoids.

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EMBRYOGENESIS AND PLANT REGENERATION IN CALLUS CULTURE OF *LINUM AUSTRIACUM* L.

K. K. Joshi* and M. S. Chennaveeraiah[1]. Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu. [1]Department of Botany, Karnatak University, India.

Embryogenesis occurred in the segments of stem, leaf and endosperm calli of *Linum austriacum* L. when cultured on nutrient agar media supplemented with different growth regulators. Callusing in these explants initiated on modified White's medium supplemented with either

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33a. REUNION ANUAL

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RESUMENES DE
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21 - 23 de noviembre de 1990
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MULTIPLICACION IN VITRO DE ESPECIES FRUTALES DE IMPORTANCIA ECONOMICA PARA ZONAS SEMI-ARIDAS. (In vitro regeneration of fruit species with economic importance for semi-arid zones). González, M.L., Iturriaga, L., Mujica, A.M., Oyanedel, E., Valenzuela, M.P., Velozo, J. Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile.

Explantes de tres especies frutales de importancia económica Annona cherimola, (chirimoya), Pouteria lucuma (lúcuma) y Carica pubescens, (papayo montañés) fueron evaluados bajo condiciones in vitro para determinar su potencial morfogénico/androgénico y su capacidad regenerativa tendiente a la multiplicación de clones o selecciones de interés.

A. cherimola forma brotes adventicios múltiples de novo en todos los explantes estudiados, especialmente en secciones nodales, internudos, embriones e hipocotilos. El empardecimiento tisular se pudo controlar empleando el antioxidante PVP en sus formas soluble e insoluble. En P. lucuma las morfogénesis más destacadas corresponden a la formación de polen multicelular y embriones de carácter supuestamente androgénicos cultivando anteras. Secciones de hoja igualmente forman tallo y/o brotes directamente. En C. pubescens se logran embriones somáticos en pericarpio de frutas inmaduras e hipocotilos, mientras que a partir de embriones cigóticos, se obtienen callos, que subcultivados generan embrioides. En papaya el cultivo de anteras solo generó callo de origen somático; las microesporas no evidencian divisiones internas que generen embrioides androgénicos.

Proyecto AID 8007, PSTC Grant N° 513-5542-G-SS-9067-00* y FONDECYT 0669/89

* Coordinador: Dr. M. Jordán; Co-Investigador; G. Montenegro.

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VARIACION SOMACLONAL Y TRANSFORMACION EN PLANTAS DE PAPA. (Somaclonal variation and transformation in potato). Jordán, M., Tesser, B., Stipo, A., Roveraro, C. Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avenida Libertador B. O'Higgins 340, Santiago, Chile.

El plasmido pGV 2260 (pEsc4) que codifica para resistencia a rifampicina y kanamicina fue introducido in vitro en brotes axénicos de papa cv. "Desiree" y otros clones mediante el vector Agrobacterium tumefaciens. Las plantas regeneradas a partir de dichos brotes, mantuvieron su crecimiento y vigor respecto de los controles, a pesar de ser tratadas con kanamicina 50ul/ml durante tres semanas de cultivo. La pigmentación verde se asemejó a la de plántulas no infectadas ni tratadas con antibióticos in vitro. Los controles no co-cultivados con A. tumefaciens, o infectados sin transformación, mostraron clorosis y muerte después de agregar el antibiótico.

En nuestro laboratorio, ha sido posible inducir regeneración de novo, empleando el cv. "Desiree", a partir de discos de lámina foliar sin elementos meristemáticos preformados, logrando individuos que muestran variación somaclonal. La infección directa en hojas permitiría obtener un mayor número de variantes transformados, o la transformación misma sería más eficiente, en especial en clones chilotes que se están seleccionando in vitro. Otra fuente de variación en estudio es el uso de radiación U.V. y agentes mutágenos químicos. Se presentan resultados preliminares de transformación, selección y regeneración en material de papa.

Se agradece el financiamiento de PNUD (Proyecto N° DP/RLA/83/003), Proyecto FONDECYT N° 0669/89 y al CINVESTAV, Irapuato, México.

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"ESTUDIO DE PROCEDIMIENTOS PARA LA OBTENCION DE PLANTAS TRANSGENICAS DE PAPA (SOLANUM TUBEROSUM) (Methods to obtain transgenic potato (Solanum tuberosum) plants). Ulmer P., Arce Pº., Quilódrán A., Arce B.O., y Iriarte F.
Programa Micropropagación Vegetal, Facultad de Agronomía y Ciencias Biológicas, Pontificia Universidad Católica de Chile.

La papa es una especie de consumo masivo en países en vías de desarrollo, cuya importancia radica en el gran aporte en calorías por unidad de superficie cultivada comparada con cualquier otro cultivo. En Chile se pierde actualmente un alto porcentaje de su rendimiento como resultado del daño causado por patógenos hecho que se agrava por las técnicas de propagación vegetativa con que tradicionalmente se multiplica esta especie.

A través de técnicas de ingeniería genética se ha logrado introducir genes de interes que se expresan en células vegetales lo cual complementado con técnicas de cultivo de tejidos permite obtener plantas transgénicas. Actualmente se ha obtenido plantas resistentes a virus, hongos, bacterias, insectos, tolerantes a herbicidas, y se están logrando exitosos resultados en la introducción de genes que aumentan el valor proteico en papa.

El objetivo de esta investigación es estandarizar las condiciones para obtener plantas transgénicas de papa vía Agrobacterium tumefaciens, con el fin de producir a futuro plantas con características agronómicas deseables. Para ellos se utilizaron plasmidos derivados de A. tumefaciens que codifican para resistencia al antibiótico kanamicina en plantas lo que permite seleccionar con relativa facilidad las plantas transformadas. En este trabajo han sido evaluado diferentes medios de regeneración de plantas a partir de lámina foliar (CB3I, M5 y modificaciones) obteniéndose mayor numero de brotes en un tiempo inferior en M5 modificado en comparacion con CB3I.

Se hicieron cocultivos de láminas foliar con tres cepas de A. tumefaciens [LBA 4404: pAG5 140, PJJ 2658, y LBA 4404 desarmado utilizada como control]. Se esta evaluando la transformación a través del crecimiento de los brotes regenerados en medios de selección (kanamicina 50 mg/lt) e inducción de callos, y se investigará la integracion del gen al genoma de la planta y su expresion a través de las tecnicas de Northern, Southern y Western.

Financiamiento: DIUC N° 89029E y PNUD Chi 88 006. Becario RLB.

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CARACTERIZACION Y SECUENCIACION DE GENES MITOCONDRIALES DE *Solanum tuberosum*. (Characterization and sequencing of mitochondrial genes of *Solanum tuberosum*). Zanlungo, S., Dell'Orto, P., Moenne, A. y Jordana, X. Laboratorio de Bioquímica, Fac. Cs. Biológicas, P. Universidad Católica de Chile.

El genoma mitocondrial (mt DNA) codifica para un número reducido de proteinas mitocondriales, estando el resto codificado en el núcleo. En la biogénesis mitocondrial deben estar por lo tanto coordinadas las expresiones génicas nuclear y mitocondrial. En vegetales el mt DNA es de mayor tamaño y complejidad que el de hongos y mamíferos, presentando además una alta frecuencia de eventos de recombinación. Se ha descrito en algunos vegetales el fenómeno de "editing", es decir la modificación postranscriptional del RNA mensajero que hace que la secuencia aminoacídica de la proteina no corresponda a la deducida a partir de la secuencia nucleotídica del gen. *Solanum tuberosum* (papa) es el cuarto cultivo mundial y existe muy poca información sobre la estructura y expresión de su genoma mitocondrial. Hemos iniciado el estudio de la expresión génica mitocondrial en *S.tuberosum* escogiendo como modelos los genes mitocondriales que codifican para el apocitocromo b (cob) y para la subunidad 9 de la ATP sintetasa (atp9), ambos componentes de complejos que tienen subunidades codificadas en el núcleo y en la mitocondria.

Se aislaron por hibridación molecular con sondas apropiadas los genes que codifican para estas proteinas (cob y atp9). Se establecieron mapas de restricción de los genes aislados que fueron luego secuenciados utilizando el método de los didesoxinucleotidos (método de Sanger), subclonando previamente fragmentos pequeños generados por cortes con enzimas de restricción en derivados del fago M13. Se obtuvo la secuencia nucleotídica completa de ambos genes y fue comparada con la de otros vegetales con los cuales se ha observado el fenómeno de "editing". De esta comparación se deduce que probablemente existe "editing" en mitocondrias de *S.tuberosum*. Por último se ha iniciado el estudio de la transcripción de estos genes, analizando por el método de Northern los transcritos.

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TISSUE CULTURE NETWORKING/WORKSHOP MEETING
Davis, California
July 16-20, 1990

Monday, July 16

Chairman: Paiboolya Gavinlertvatana
Discussant: Gustaaf Wattimena

8:25AM Opening Remarks
8:30 Richard Griesbach: Tissue Culture in the Nineties.
9:10 Rebeca Vega de Rojas: Somaclonal Babaco Establishment on Field Conditions.
9:30 Y.M.H.B. Yapabandara and M.D. Dassanayake: *In Vitro* Propagation of Nutmeg and Clove.
9:50 Miguel Jordan: *In vitro* Propagation and Improvement of Fruit species with Economic Potential for semi-Arid Zones.

10:10 Recess

10:40 Pranom Prutpongse and Paiboolya Gavinlertvatana: *In Vitro* Germplasm Conservation and Exchange of Bamboo.
11:00 Pornchai Chuthamas, Pranom Prutpongse, Isara Vongkalung, and Sureeya Tantiwiwat: *In Vitro* Conservation and Propagation of Three Economic Species of Rattans.
11:20 Marc Berthouly and Dennis J. Gray: Somatic Embryos for Germplasm Storage and Clonal Propagation.

11.40 Poster Session

12:30PM Lunch

Chairman: Miguel Jordan
Discussant: Jesse Jaynes

2:00 Antonio Figueira, Anna Whipkey and Jules Janick: Micropropagation of *Theobroma Cacao*, a Recalcitrant Species, with High CO_2 and High Light.
2:20 Oded Reuveni and Ludwig Muller: *In Vitro* Germ Plasm Preservation of Musa Spp.
2:40 J.S. Tahardi and Jacintha Nathan: Commercialization of Clonal Micropropagation of Superior Tea Genotypes Using Tissue Culture Technology.

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IN VITRO PROPAGATION AND IMPROVEMENT OF FRUIT SPECIES WITH ECONOMIC POTENTIAL FOR SEMI-ARID ZONES.

AID Grant Nº 513-5542-G-SS-9067-00
Coordinator: Dr. Miguel Jordan, Chile.

Micropropagation aspects of three semi-arid region fruit species of economic importance; Cherimoya (Annona cherimola), Lucuma (Pouteria lucuma) and a highland Papaya (Carica pubescens, or C. candamarcensis) are being researched within the framework of this project. Other studies, concerning browning metabolism are also carried out. Several protocols, designed for this subject, have been developed so far; some have already been tested. Results, after six months work, show morphogenic responses (which are being checked via histological observation) in several explants of the three species under study. The work was carried out with selected clones obtained at experimental stations and nurseries close to Santiago.

Cherimoya shows mostly formation of adventitious shoots in a series of explants tested, including nodal sections, internodes, embryos and hypocotyls. These shoots appear along the whole explant in culture, depending on the hormonal level, mainly in presence of NAA 0.5 mg/l and BA, 2.0 mg/l. Several shoots form simultaneously in the same explant. In the case of nodes, calli form both at the distal end and along the explant, when the auxin level is raised; the pre-existing axillary bud also develops under these conditions. A series of anti-oxidant compounds were tested, either prior to culture or supplemented in the medium. The inclusion of ascorbic acid and/or PVP, markedly avoids browning and fosters shoot formation. The best anti-oxidant used was PVP 360 in a concentration of 1 g/l, followed by ascorbic acid, also 50 mg/l. Casein hydrolysate enhances shoot formation. Anther culture is difficult because they are very small in this species, and easily damaged by handling. Anti-oxidants did not prevent browning, up to now, but it was possible to obtain some calli growth, protruding from the anther wall.

Lucuma. The most relevant results in this species were the formation of multicellular pollen and androgenic embryoids by means of anther culture. Calli formation also takes place in these explants and others tested, and their origin, androgenic or somatic is being tested. Androgenesis was triggered by the use of NAA and BA, 1 mg/l each, and cold treatment conditions (4-8-11 days at 4º or 8º C.). The exact nature of the androgenic origin of the embryoid is being examined by means of histology. Preliminary results sustain this condition, since growth of other somatic tissue or organs (filaments) does not occur in developing explants, at the hormonal level tested. Besides the appearance of embryoids, calli formation, originating from the

inside of the anthers also occurs; the morphogenic potential of this material is also being checked. Other explants, such as leaf sections, also form calli in presence of NAA 0.02 mg/l and Zeatin 2.0 mg/l. Experiments are underway to induce embryoid formation from calli or directly from leaves. Preliminary results showed that it is possible to obtain roots and shoots directly from leaf explants, without calli mediation, protocols to refine this line of work are underway. For this species, ascorbic acid used in concentrations of 250 mg/l for nodal explants avoid browning and allow callus formation in about 60% of explants tested.

Papaya. In this species, several kinds of explants can easily lead to calli. Zygotic embryos were cultivated in NAA and Z 1.0 mg/l each, or 2,4D 0.1 mg/l plus BA 1.0 mg/l, leading to calli proliferation. These calli were subcultured in Nitsch media, including IAA 0.05 casein hydrolysate, 200 mg/l and Z 1.0 mg/l, and formed somatic embryoids only when Z was included. Anther culture results also showed profuse calli formation in treatments based on Nitsch medium and including NAA and BA 1 mg/l. Anthers form calli, but these issue from connective or other somatic tissue. Browning is less evident in tissue culture in this species than in others. At present, microspores do not show evidences of internal division leading to embryoids. Leaves in culture stay green when using cytokinins; zeatin, followed by kinetin, proved to be better than BA and IPA, so far; and combinations of auxins to trigger morphogenesis is under study.

Browning problems. In this respect, preliminary analyses of leaves, prior and after 40 days culture, showed a reduction of phenolics in papaya and lucuma, but an increase (10 times Abs. 730 nm, Folin-Ciocalteu determination) in cherimoya, causing browing and inhibiting calli formation. (Jordan et al, in press). Results of these analyses suggested the use of antioxidants, as mentioned in the text, and which worked out successfully.

PRESENT WORKPLAN (1990)

1. Systems to induce growth and morphogenic responses from juvenile and adult explants, using tissue and organs.

   - potential morphogenic responses in vitro of leaves, nodal sections, internodes, shoot-tips/meristems, floral sections and embryos of the three species studied.
   - somatic embriogenesis and adventitious shoot formation on zygotic embryos, hypocotyl and nodal sections.
   - micrografting of clones on seedlings (cherimoya, lucuma).
   - rooting of microcuttings (cherimoya).
   - rooting of axillary sprouts from main stem (papaya).

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2. Systems to induce androgenesis.

. anther culture.

3. Systems to induce regeneration from unorganized tissue

. callus culture.
. dual cultures (nurse cultures).

4. Systems to maintain cell suspension cultures, and induce regeneration.

. somatic embryogenesis.
. testing explants responses, in the three species, to obtain cell suspensions and differentiate organs or embryoids.

5. Systems to overcome browning processes in organs and calli of cherimoya and lucuma explants.

. using several anti-oxydants such as citric acid, cysteine, ascorbic acid, amino-oxy-acetic acid, PVP, DIECA, and others in culture, or when slicing explants.
. assaying phenolic compounds in culture (Folin-Ciocalteu, total phenols, thin layer chromatography).

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