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Regeneration of babaco *C. pentagona* Heilborn, Badillo via Tissue Culture

Principal Investigator: Rebeca Vega de Rojas
Grantee Institution: AMDE Corporation
Box 632
Ambato, Ecuador

Collaborator: Sherry Kitto
Institution: University of Delaware
Dept. Plant & Soil Sciences
Newark, Del. 19717

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EXECUTIVE SUMMARY

Babaco, Carica pentagona, is sterile and, therefore, cannot be improved using conventional breeding techniques. One objective of this project was to regenerate babaco plants in vitro and evaluate the regenerated plants for somaclonal variation. Babaco has been regenerated from ovular callus, leaf tissue and peduncle sections. Two embryogenic babaco lines were regenerated from ovular calli. Regenerated plants have been grown outside up to six months under regular enviromental conditions and have demonstrated different characteristics than the babaco stock plant. One somaclonal-plant line appears to be more susceptible compared to a second somaclonal-plant line that appears to have some resistance (based on the ability to produce healthy roots) to root-knot nematode Meloydogine incognita when compared to the parent. The final evaluation of nematode resistance will compare two other Carica spp. micropropagated by tissue culture. The fruiting habit of the regenerants is still unknown. One publication is "in press" with the Journal of American Society for Horticultural Science. The second object of this project involved the introduction and dissemination of tissue culture information throughout Ecuador. One advantage is that AMDE Corp. is located not only roughly in the center of Ecuador but also in a center of biological diversity. Ecuadorian students have had the opportunity to be trained in tissue culture and a number of theses related to babaco or micropropagation have been initiated and/or completed as a result of this AID project. During the course of the project many scientific activities have been held including congresses, workshops, seminars, and training courses, many in coordination with other institutions like the University of Ambato (UTA), FUNDAGRO, PROEXANT, IDEA, PL-480, and international organizations like FAO, and Corporaciòn Andina de Fomento (CAF). As a result, other tissue culture facilities are under development in Ecuador. In the international context, AMDE Corp. laboratories have been recognized for their work with native species and now is the Ecuadorian Contact Point for FAO-RED-BIO (Latin American Biotechnology Network).

RESEARCH OBJECTIVES

One objective was to regenerate babaco using tissue culture techniques in order to upgrade its characteristics. Once regenerated, the objective was then to mass propagate the regenerants and test them for resistance to pests and evaluate them for improved characteristics.

A principal objective also was to establish a tissue culture facility in Ecuador that could serve the international scientific community, as well as Ecuadorian scientists and students.

METHODS AND RESULTS

INTRODUCTION

Babaco originated in the Ecuadorian Andean Highlands. The edible fruits are parthenocarpic and contain flavonoids and digestive enzymes similar to those in papaya. Babaco is dioecious; there are only pistillate plants that are cross-incompatible with related species (Horovitz and Jimenez, 1967). Thus, improvement via conventional breeding is impossible. An alternative method to breeding for improved fruit crops involves the regeneration of somaclonal variants via somatic embryogenesis or organogenesis. Tissue culture regeneration may allow for the selection of new babaco types having pest resistance, cold hardiness, or enhanced fruit characteristics.

Papaya, a more commonly known Carica crop, has been successfully regenerated from callus derived from seedling stem (DeBruijne et al., 1974; Yie and Liaw, 1977), anthers (Litz and Conover, 1978; Tsay and Su, 1985), embryos (Phadnis et al., 1970), and ovules (Litz and Conover, 1982). Other Carica species that have been regenerated by somatic embryogenesis include C. stipulata (Litz and Conover, 1980), and C. candamarcencis (Jordan et al., 1982/83).

Ovules, leaves and peduncles were chosen as explants based on availability and reports of successful regeneration in other crops. Crops that have been regenerated from callus cultures originating from unfertilized ovules include Citrus spp. (Kochba and Button, 1974; Mitra and Chaturvedi, 1972) and Cabernet-Sauvignon grape (Mullins and Srinivasan, 1976). Sugar cane plants regenerated from leaf tissue have contained high variability (Larkin and Scowcroft, 1981) while somatic embryogenesis has been achieved from peduncular cell suspensions in Carica stipulata (Litz and Conover, 1981).

Babaco has been micropropagated in vitro from shoot tips (Cohen and Cooper, 1982) and axillary buds (Cossio, 1988); however, there have been no reports of regeneration. Thus, the objective of this study was to determine the regenerative potential of babaco ovules, leaves and peduncles.

METHODS

Plant material. Babaco plants were grown in the University of Delaware greenhouse. A two-year-old plant was the stock plant during the first year, and was supplemented with new material provided by Dr. R. Litz from the Tropical Research and Education Center of the University of Florida for the second year. Plants were vegetatively propagated from lateral shoots. Flowers tagged at anthesis were labeled as day one for fruit age. Fruit were selected at different stages of development ranging from 23 to 140 days after anthesis (DAA) and were 3 to 20 cm in length.

The three most apical leaves collected from greenhouse grown plants were cut into sections that contained either main-vein or petiole tissue. Leaves of uniform size with petioles intact also

were collected from proliferating shoot cultures maintained under sterile conditions on semi-solid proliferation medium (Vega, 1989). Leaf tissue was cultured abaxial side in contact with the medium. Peduncles were classified as young (< 2 months old) or old (> 2 months old) and ranged from 3 to 15 cm in length.

Surface disinfection, chilling and culture. Fruit were harvested from the greenhouse in the morning except for pretreated fruit that were collected the previous afternoon and chilled at 4C for 1, 3, 5 or 15 days. Fruit were dipped in 95% ethanol and then placed in a solution of 20% household bleach (1.0% sodium hypochlorite) with 0.1% Tween 20 for 20 to 30 min with continuous agitation. Leaf pieces and peduncles (sectioned into 1 cm pieces) were disinfected with 1.0% sodium hypochlorite (household bleach at 20%) plus 0.1% Tween 20 (v/v). After disinfection all explants were washed with three rinses of distilled, sterile water prior to culture. Surface-disinfected fruit were dissected longitudinally on the fruit angles. Only uninjured ovules were cultured longitudinally on the medium surface for measurement and data collection. Calli were subcultured every 5 to 6 weeks and were maintained for more than a year.

Media

Callus. Media used included the basal salts of Murashige and Skoog (1962) (half- or full-strength major salts) or White (1943) (modified to include MS chelated iron) and, unless stated otherwise, the following addenda in mg·liter^{-1}: pyridoxine·HCl, 0.5; thiamine·HCl, 0.4; *myo*-inositol, 1; nicotinic acid, 0.5; sucrose, 30,000 and washed Difco Bacto agar (Difco Laboratories, Detroit), 8,000. Organic nitrogen sources included glutamine (GLU), 400; proline (PRO), 400; adenine sulfate (AdS), 40; or casein hydrolysate (CH), 1,000. Growth regulators used were BA, NAA, IAA, and Dicamba, at different combinations and concentrations ranging from 0.25 to 2 mg·liter^{-1}. Media pH were adjusted to 5.7 to 5.8 prior to being autoclaved at 121C and 124 kPa for 15 minutes. Media were dispensed into 100 x 15 mm (20ml) or 60 x 15 mm (10 ml) petri dishes.

Somatic embryo treatments. Immature somatic embryos were cultured for 1 to 2 days on semi-solid medium containing TIBA at 1 to 4 mg·liter^{-1} or in liquid medium containing half-strength MS salts to eliminate recallusing.

Embryo conversion. Embryo conversion medium consisted of a modified babaco shoot-growth medium (Cossio, 1988) and contained full-strength MS salts and vitamins, a second complement of MS microelements, and the following addenda in mg·liter^{-1}; AdS, 80; CH, 500 ; sucrose, 30,000; BA, 0.1 and agar, 7,000. The pH was adjusted to 5.4 to 5.6 before autoclaving. Medium (25 ml) was dispensed into 55 x 70 mm jars. Converted embryos 3 to 4 cm in length were transplanted into a proprietary peat-lite mix (Redi-Earth, W. R. Grace, Fogelsville, Penn.) and placed in the greenhouse under intermittent mist (6 seconds every 6 minutes).

Leaf regeneration. Leaf regeneration medium consisted of MS salts (half-strength) and vitamins, myo-inositol, 1; glutamine, 400; and sucrose, 30,000. Growth regulators used were benzyladenine (BA) (0, 0.5, 1.0, 1.5), naphthaleneacetic acid (NAA) (0, 0.5, 1.0), or indoleacetic acid (IAA) (0, 1.0, 2.0, 3.0) under different combinations. Media were gelled with 8 g washed Difco Bacto agar per liter. Medium pH was adjusted to 5.6 to 5.7 prior to being autoclaved at 121 C, 124 kPa for 15 min.

Peduncle regeneration. Media recommended for C. stipulata (Litz and Conover, 1980) and C. papaya (Litz and Conover, 1981) were used for peduncle tissue. Media were either gelled or liquid. Growth regulators included BA (0, 0.5, 1.0, 1.5 mg l^{-1}), NAA (0, 1.0, 2.0 mg l^{-1}), or IAA (0, 1.0, 1.5 mg l^{-1}) alone or in combinations. Media were gelled with 7 g washed Difco Bacto agar $liter^{-1}$, pH = 5.6-5.7.

Environmental conditions. Cultures were placed either in darkness or under a 16- or 18-hr photoperiod using cool white fluorescent lamps (50 $\mu mol \cdot s^{-1} m^{-2}$). Light conditions for somatic embryo induction and development consisted of a 16- or 18-hr photoperiod with light intensities of 12, 16 or 24 $\mu mol \cdot s^{-1} m^{-2}$. The temperature was 23±2C and the humidity was ambient.

Histological procedures. Tissues were fixed in FAA (formalin: acetic acid (30%): ethanol, 1:1:10) for 48 hr, dehydrated through an ethanol series and embedded in paraffin. Blocks were cut with a rotary microtome set at 12 to 15 μm, stained with safranin and fast green and mounted with Permount (Berlyn and Miksche, 1976).

Pest identification. Fungus isolation: Diseased babaco tissue was disinfested with 70% alcohol or 5.25% sodium hypochlorite for 1 to 5 min and rinsed with sterile distilled water 2 to 3 times. Small fragments from the advancing edge of young lesions was aseptically taken out with scalpel and transferred onto petri dishes (4 isolation points/dish, 4 replicates, 3 dishes) containing 15 ml of 1.5% potato-dextrose-agar (PDA), pH 7.0 or other suitable agar-gelled medium. If bacterial contamination occurred, acidified PDA (25lactic acid drops $liter^{-1}$) was used. Petri dishes were incubated in a growth chamber at 22-24C.

Pure isolates were made by making single spore isolations from 2% water-agar containing fungus streaks. If required, purification was done by passing hyphae tips from the original isolate to PDA plates or slants and incubating at 22-24C. Sporulated pure cultures were kept at 4C under sterile mineral oil. Identification of the isolated fungus was done using conventional plant pathology literature and keys.

Pathogenicity test protocols. Healthy babaco leaves disinfested as above were placed in plastic containers and inoculated with a) 5 mm-diam. disc of medium colonized by the fungus, with or without wounding, or b) 10^2 - 10^5 conidia/ml of sterile distilled water plus 0.1% Tween 80 (v/v) if necessary. One drop was placed on each inoculation point.

Screening for resistance. Detached babaco leaves placed onto a benzimidazole 68 ppm-agar medium were inoculated with pure foliar pathogen isolates and incubated at laboratory temperature. Records were taken measuring leasion diameter (mm) and estimating the percentage of the foliar area affected (Velasteguì and Ball, 1986).

Babaco plants growing in 10 cm dia. pots containing Meloidogyne-contaminated soil (1500 J2/pot) were used to screen for resistance to the nematode. Conventional root-knot index scale (0 - 5) was applied to determine incidence of infection (Taylor and Sasser, 1980).

Statistical evaluation. Six to twenty ovules were cultured per petri dish depending on the size of ovules and container. Observations were made on the first day after culture and weekly thereafter. Data collected included percent and area of ovules initiating callus and callus growth (area). Callus growth was calculated by multiplying two measurements: D_1 = diameter and D_2 = diameter perpendicular to D_1 (King and Morehart, 1988). There were two or three replications per treatment depending upon the availability of material. All samples were selected for as much uniformity as possible. Experiments were set up in factorial combinations in a randomized design. Data were evaluated using an unbalanced analysis of variance. General Linear Models and Least Squares Means were calculated and step-wise comparisons made to determine significance when applicable.

RESULTS

Regeneration of Babaco [Carica pentagona (Heilborn) Badillo] from Ovular Callus

Explant condition. Babaco flowering occurred in February and continued until November. Fruit of comparable ages were similar in length irrespective of season. The number of ovules per fruit depended on fruit age with 195 to 225 present during the first two months. Ovule length and overall size depended on the age of the fruit and also on location within the fruit. Larger ovules were located at the stylar end. Ovules in fruit over 90 days old were commonly shrunken; however, some resembled mature Carica seeds. Many fruit that originated in the summer had abnormalities such as rounded shape, six sides, and green structures instead of ovules.

Normal fruit were pentagonal, with five locules and the ovules were located in two opposing rows per locule (Fig. 1A&B). Fruit 7 DAA contained young, normal ovules composed of a vascularized funiculus and an outer and inner integument surrounding the nucellus, except at the micropyle, with the nucellus enclosing a megaspore mother cell (Fig. 1B).

Callus origin. Callus appeared to have three origins from ovules: the inner and outer integuments (Fig. 2A) and an inner origin that may be nucellar (Fig. 2B) containing small and

heavily stained cells.

Callus initiation and growth

Ovule size and age. Callus produced per ovule (60 to 90 DAA) after 6 weeks in culture appeared to be directly related to ovule length (Fig. 3) and age (data not shown). Longer and older ovules produced more callus.

Salt formulation. Callus produced per ovule after 6 weeks was significantly greater on White's than on full-strength MS medium (Table 1). In a subsequent experiment, ovules cultured on half-strength MS for 6 weeks had a larger mean callus area than either of the former treatments.

Light compared to dark. Two experiments were conducted to compare the effects of light and dark on callus initiation. Ovules from fruit of four ages chilled (45 and 51 DAA) or non-chilled (66 and 70 DAA); or from chilled fruit of three ages (110, 115, and 130 DAA) were cultured on MS medium supplemented with GLU. In experiment 1, regardless of age or chilling, ovules produced a greater amount of callus in the dark than in the light after 28 days (Fig. 4). In experiment 2, ovules produced similar percentages and areas of callus after 15 days of culture either in the light or dark, but after 45 days of culture, ovules maintained in the light had a significantly greater percentage and area of callus (Table 2). However, callus produced in the light was watery compared to callus produced in the dark which was compact and had a more uniform surface. Subsequent experiments included dark as a condition for callus initiation.

Chilled compared to fresh. Chilling the fruit before culture had a variable effect on ovular callus initiation. In an initial 6-week-long experiment, significantly more callus was initiated from chilled (1 day) ovules greater than 60 days old compared to fresh ovules less than 60 days old (Table 3). A second experiment was set up using ovules from 130 DAA fruit chilled for 0, 3 and 5 days. Although callus initiation was greatest (93%) for the 3-day-chilling period, 86% of fresh ovules produced callus. In a third experiment, 79-88% of the ovules from 130 DAA fruit chilled for 15 days produced callus. Generally, chilled ovules produced callus earlier than non-chilled ovules.

Growth regulators and organic nitrogen

Growth regulators. Ovules from fruit 61 or 87 DAA pretreated by chilling (1 day), and from a 23 DAA fresh fruit were cultured on MS media containing a 2 x 2 x 2 factorial of sucrose (3, 6%), BA (0.5, 1.0 $mg \cdot liter^{-1}$) and NAA (0.25, 0.5 $mg \cdot liter^{-1}$). Sucrose concentration did not affect callus initiation. Callus initiation for all treatments was at least 39% (Fig. 5). The most consistent callus initiation regardless of ovule age or chilling occurred on medium containing 0.25 $mg \cdot liter^{-1}$ NAA and 1.0 $mg \cdot liter^{-1}$ BA.

Organic nitrogen. Ovules from three fruit (110, 115, 130 DAA) were cultured on media containing half- or full-strength MS major salts supplemented with 0.5 $mg \cdot liter^{-1}$ NAA, 1.0 $mg \cdot liter^{-1}$ BA,

and the organic nitrogen sources in mg·liter^{-1}: GLU, 400; CH, 1000; AdS, 40; or PRO, 400. The percentage of ovules that produced callus was at least 67% with no significant difference among treatments (data not shown).

Growth regulators and organic nitrogen. Ovules from 4 fruit (73, 85, 118, 130 DAA) were cultured on half-strength MS media containing in mg·liter^{-1}: Dicamba, 2.0 and BA, 1.0; in combination with GLU, 400; CH 1000; or AdS, 40. The control contained in mg·liter^{-1}; BA, 1.0; NAA, 0.5; and GLU, 400. Callus growth was evaluated after 45 days. Ovules 130 DAA produced the greatest amount of callus when cultured on medium with Dicamba, BA and CH (Fig. 6). However, the greatest production of callus for ovules 73, 85 and 118 DAA was obtained with the control treatment (Fig. 6).

Additional studies with NAA and BA in combination with GLU, CH, AdS, or PRO indicated that callus initiation and growth occurred equally well on all 4 nitrogen sources (data not shown). However, PRO and GLU were preferred due to earlier (PRO) and more consistent (GLU) callus initiation.

Somatic embryogenesis

Embryogenic callus initiation. Regenerative calli originated from 73 to 90 DAA (summer and fall fruit) ovules that had been treated by chilling and cultured on semi-solid medium containing White's or half-strength MS salts, supplemented with MS vitamins, GLU or PRO (400 mg·liter^{-1}), NAA (0.25 - 0.5 mg·liter^{-1}), and BA (1 mg·liter^{-1}) under light (50 μmol s^{-1} m^{-2} with a 16-hr photoperiod) or dark conditions.

Embryogenic callus maintenance. Callus was maintained in the light or dark and subcultured every 5 to 6 weeks. Callus maintenance medium contained 0.5 mg·liter^{-1} NAA and 1.0 mg liter^{-1} BA. During subsequent subcultures the growth regulators were removed from the medium as follows: subculture 1: 0 NAA, 1.0 mg·liter^{-1} BA; subculture 2: 0 NAA, 0.5 mg·liter^{-1} BA; subculture 3: 0 NAA, 0 BA; subsequent 3 subcultures: no growth regulators. Half of the light-grown cultures became green when maintained without transferring for 3 months. At this time, all light grown and some dark grown cultures were placed under one of three light intensities (12, 16,and 24 μmol · s^{-1} m^{-2}) and one of two photoperiods (16- or 18- hr). Callus placed under 12 or 16 μmol · s^{-1} m^{-2} grew and became green compared to callus under 24 μmol · s^{-1} m^{-2} which did not grow. Photoperiod had no effect on callus growth or greening. Green calli were either friable or compact.

Nodular callus and somatic embryo initiation. Compact green callus was cultured on medium containing half-strength MS salts; GLU or PRO at 400 mg·liter^{-1}; sucrose at 30,000, 60,000 or 90,000 mg·liter^{-1}; and BA at 0, 1, 2, or 3 mg·liter^{-1}. Callus initially maintained on medium containing 3% sucrose was subcultured onto media containing 3 or 6% sucrose and subsequently onto media containing 3, 6 or 9% sucrose. After four months, the compact

green calli became nodular (Fig. 7A). Anatomically, these nodules contained small densely staining cells that were actively dividing (Fig. 7B). Proembryonal structures developed when nodular green callus from media containing 3 or 6% sucrose was subcultured onto media containing BA (1 to 3 mg·liter^{-1}) and then onto medium containing 3% sucrose and no growth regulators. Nodular green callus cultures were grown under 16- or 18-hr photoperiods; only 18-hr cultures regenerated.

Somatic embryo development. Globular proembryonal structures turned green and developed leaf primordia when subcultured twice onto medium without growth regulators. Red pigments were always associated with the leaf primordia. The globular proembryonal structures failed to develop further and recallused.

To eliminate recallusing, globular proembryonal structures were cultured either onto semi-solid basal media with TIBA or in liquid half-strength MS medium. TIBA cultures produced a few embryos and many roots. Globular proembryonal structures cultured in liquid half-strength MS medium for a week developed into somatic embryos when subcultured onto semi-solid medium containing in mg·liter^{-1}: either sucrose, 60,000; GA_3, 0.1 and activated charcoal, 2000; or sucrose, 30,000; IAA, 0.5 and CH, 200. Although a greater number of somatic embryos and roots regenerated when cultured on medium with GA and charcoal (Table 4), more normal somatic embryos were produced on medium containing IAA + CH. Anatomically normal somatic embryos were bipolar, with shoot and root initials, and developed from globular- to heart-shaped somatic embryos (Fig. 8A).

Somatic embryo conversion and micropropagation. Somatic embryos with well developed cotyledons and roots (Fig. 8B) were transferred to 55 x 70 mm jars containing somatic embryo conversion medium. Six weeks after initiation somatic embryos reached the 3 leaf stage and had lanceolate leaves. Two 3-cm plantlets were potted in Redi-Earth and placed in the greenhouse under mist. One plant rooted and grew under Delaware conditions. At this point, the project was moved to AMDE Corp. in Ambato, Ecuador.

Regeneration of Babaco from Leaves and Peduncles

Leaf regeneration. In vitro leaf sections were cultured for 6 wk on media containing a 3 x 3 factorial combination of BA (0.5, 1.0, 1.5 mg liter^{-1}) and IAA (1.0, 2.0, 3.0 mg liter^{-1}) plus a control without growth regulators (Table 6). One shoot regenerated in each of 3 media which contained in mg l^{-1}; 1.0 IAA + 1.5 BA, 3.0 IAA + 1.0 BA, or 3.0 IAA + 1.5 BA. Shoots developed from 5% of the leaves and appeared to initiate from the joint between leaf and petiole. Half of the leaves cultured produced callus in the presence of growth regulators.

Selected treatments were repeated as shown in Table 7, with no improvment in regeneration. When the early stages of regeneration were examined histologically vascular connections

were evident.

Peduncles. Preliminary experiments examined White's (Litz and Conover, 1981) versus MS (Litz and Conover, 1980) media and peduncle age. Of 50 peduncle sections cultured for 8 wk, 27 or 54% initiated callus and 1 (2%) young peduncle regenerated a root (data not shown).

Peduncle sections were cultured with combinations of NAA, BA, and IAA. One shoot was regenerated on medium containing 0.5 mg each of BA and IAA $liter^{-1}$.

Peduncle sections were cultured under one of three light intensities; 12, 16, or 24 $\mu mol\ s^{-1}m^{-2}$ with a 16 or 18 hr photoperiod. The greatest amount of shoots (9/16 or 56%) developed from sections cultured under 12 $\mu mol\ s^{-1}m^{-2}$ (Table 8), none of the peduncle sections cultured under 24 $\mu mol\ s^{1}m^{-2}$ developed shoots as most sections yellowed and turned brown. There appears to be no difference in shoot production between peduncles cultured for 16 or 18 hr under 16 $\mu mol\ s^{-1}m^{-2}$ (Table 8).

Peduncle sections were cultured on medium containing BA (0, 0.5, 1.0 mg $liter^{-1}$) and IAA (0, 0.5, 1.0 mg $liter^{-1}$). IAA appears to be required for shoot regeneration (Table 9). The results suggest that increasing BA concentratins reduce shoot regeneration (0 BA = 21%, 0.5 BA = 17%, 1.0 BA = 11%).

There was some callus initiated from the subepidermal layers of the peduncle and rhizogenesis occurred on older peduncle sections. Histological sections of peduncular shoots demonstrated that the bud development was from the vascular bundles.

Establishment of Somaclonal Babaco under Field Conditions

Regenerants recovered from two lines of ovular babaco calli, 6-70 and 8-B1, and massed up by micropropagation demonstrated the ability to root directly in peat-moss. However, because this material is not available in Ecuador, other soil mixes were examined: (1) Brunizem soil (Tierra milagrosa) and sand (50/50); (2) sand and shreaded moss (50/50); (3) "pomina"(volcanic lava, 100); (4) "worm soil" (100); and (5) "worm soil" and sand (50/50). Only Worm soil and worm soil + sand were suitable for root development with 17 to 20% of the 6-70 line plants rooting. Rooted plantlets were transplanted to the same soil and maintained under controlled conditions for two months after which they were moved to the greenhouse. These plants defoliated and died within a month at two greenhouse locations.

As a result of this failure and the inability of line 8-B1 to root directly in greenhouse rooting medium, microcuttings were cultured in vitro in rooting medium for at least two weeks. When root primordia were initiated plants were transplanted to Jiffy pellets. The highest percentage (75 to 80%) of plants survived when they were transplanted into Jiffy pellets, placed in a mini-greenhouse and maintained for a month under the same light and temperature conditions as in vitro cultures. When roots had grown out of the Jiffy pellets, plants were transplanted to pots

and taken to the greenhouse and placed under natural conditions. These plants have survived up to 6 months without problems and have been repotted into bigger containers. Three months after transplanting to field conditions (roots still in containers) the plants have produced axillary shoots, developed thorns in the pedicel bases and produced leaves that are thicker and have an altered shape.

The variables measured to help quantify somaclonal plant survivability were number of branches, shoot length (cm) and diameter (mm), number of leaves, size of the first and second largest leaves in order to obtain foliar area. The length of the third and fourth internodes was measured to evaluate growth rate. The number of plants evaluated were 46, three batches of 12 to 16 plants.

The means of shoot length, number of leaves, shoot diameters and foliar area of the whole population are in Fig. 9. There were little differences between the first and third replications, while the second replication had shorter shoot lengths, fewer leaves and less foliar area (Fig. 10). Internode size for the third and fourth internodes was correlated with the initial shoot length (Fig. 11). There appeared to a lag phase when plants are initially transplanted with plant growth, shoot diameter and shoot length increasing after being transplanted one month (Fig. 12).

Babaco Pests

Diseases: The aim of this research was to identify the main babaco diseases through lab analysis and pathogenicity tests. Plant samples were collected from two babaco growing areas, Agoyàn (Baños) and Huachi Chico both located in Tungurahua province. Samples were collected and preserved until analysis. Preliminary analysis in the laboratory on fresh samples collected from the field have identified 4 fungal pathogens infecting leaves (Oidium sp., Asperosporium sp., Alternaria sp., and Colletotrichum sp.) and 9 unidentified bacterial pathogens infecting both leaves and roots. Identification procedures are still in progress.

Nematodes: One of the most damaging pests of babaco is root-knot nematode (Meloidogyne sp.) which usually causes big crop losses and reduces the productive life of plantations. The aim of this research was to (1) identify the Meloidogyne spp. attacking babaco, chamburo and jigacho, (2) determine the life cycle of Meloidogyne on babaco, chamburo and jigacho, and (3) establish levels of susceptibility/resistance of the three species to Meloidogyne. Meloidogyne incognita was the common species found to be infecting babaco, chamburo and jigacho in the areas sampled. Experiments designed to answer objectives 2 & 3 are underway.

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Table 1. Effect of medium formulation on babaco callus area after 6 weeks in culture.

Medium[z] composition	n[y]	Callus area (mm^2)[x]
Experiment 1[w]		
White's	20	11.4 ± 1.32*[v]
Full-MS	22	8.3 ± 1.22
Experiment 2[u]		
Half-MS	48	14.0 ± 2.17

[z] Murashige and Skoog, 1962, at full-strength and at half-strength major salts, and White's, 1963.
[y] Number of Petri dishes (10-12 ovules/dish).
[x] Mean ± standard error.
[w] Ovules from fruit 87-130 DAA.
[v] * = significant at 5% level by Least Square Means.
[u] Ovules from fruit 90-140 DAA.

Table 2. Light and dark influence on babaco callus initiation and growth.

Environment condition	n[z]	Callus (%) 15 days	Callus (%) 45 days	Callus area (mm^2) 15 days	Callus area (mm^2) 45 days
Light[y]	24	54	93*[x]	7.95	19.85*
Dark	24	40	67	7.73	8.21

[z] Number of Petri dishes (10-12 ovules/dish).
[y] 50 $\mu mol \cdot s^{-1} m^{-2}$.
[x] * = significant at 5% by General Linear Model.

Table 3. Chilling effect on babaco callus initiation in culture.

Treatment	Fruit age (days)	n[z]	Callus initiation[y] (%)
Fresh	>60	13	71 ± 0.55
Fresh	<60	10	43 ± 0.07
Chilled[x]	>60	11	85 ± 0.05*[w]
Chilled	<60	11	52 ± 0.09

[z] Number of Petri dishes (10 to 13 ovules/dish).
[y] Mean ± standard error of the mean.
[x] 4C, 24 hours.
[w] * = significant from fresh <60-day ovules at 5% level by Least Square Means.

Table 4. Babaco somatic embryogenesis from nodular callus.

Callus line	n[z]	Treatment	Regeneration: Proembryos	Embryos	Roots
8-B1	28	GA_3 + charcoal	63	19	3
	26	IAA + CH	111	7	0
6-70	14	GA_3 + charcoal	34	8	1
	12	IAA + CH	30	0	0

[z]Number of petri dishes.
[y]0.1 mg·liter^{-1} gibberellic acid (GA_3) + 1 g $^{-1}$ activated charcoal or 0.5 mg·liter^{-1} IAA + 200 mg·liter^{-1} casein hydrolysate (CH).

Table 5. Regeneration sequence of babaco from ovules.

Regeneration sequence	Special addenda[z] (mg·liter^{-1})		Subculture
Callus initiation	NAA	0.5	1
	BA	1.0	
	GLU or PRO	400	
Callus habituation	NAA	0	2
	BA	1.0	
	GLU or PRO	400	
	NAA	0	3
	BA	0.5	
	GLU or PRO	400	
	NAA	0	4
	BA	0	
	GLU or PRO	400	
Green callus initiation	no subculture 3 months (under light)		
Nodular callus initiation	BA	1, 2, 3	5
	Sucrose	30,000 or 60,000	
	GLU or PRO	400	
Somatic embryo initiation	BA	0	6
	Sucrose	30,000	
	GLU or PRO	400	
Somatic embryo development	GA^3	0.1	7
	Charcoal	2,000	
	or		
	IAA	0.5	
	CH	200	
Embryo conversion	BA	0.5	8
	CH	500	
	AdS	80	

[z] For further media details see Materials and Methods.

Table 6. Shoot regeneration from babaco leaf cultures.

Treatment IAA (mg liter^{-1})	BA	nz	Leaf response Green	Yellow	Callus	Shoot
0.0	0.0	2	4	2	0	0
1.0	0.5	2	2	1	3	0
1.0	1.0	2	2	1	3	0
1.0	1.5	2	1	0	4	1
2.0	0.5	2	2	1	3	0
2.0	1.0	2	4	0	2	0
2.0	1.5	2	4	0	2	0
3.0	0.5	2	2	1	3	0
3.0	1.0	2	0	0	5	1
3.0	1.5	2	0	0	5	1

z Number of jars; 3 leaves/jar.

Table 7. Shoot regeneration from babaco leaves.

Treatment IAA (mg liter^{-1})	BA	nz	Leaf response Green	Yellow	Callus	Shoot
1.0	1.5	8	8	6	7	1
3.0	1.0	8	8	2	11	1
3.0	1.5	8	6	12	6	1

zNumber of jars; 2-3 leaves/jar.

Table 8. Influence of light intensity and photoperiod on peduncle shoot regeneration.

Light intensity (μmol s^{-1}·m^{-1})	Photoperiod (hours)	nz	Shoot number	
12	16	16	9	(56%)y
16	16	24	4	(17%)
16	18	24	3	(13%)
24	16	32	0	(0%)

zNumber of peduncle sections.
yPercent of sections initiating shoots.

Table 9. Regeneration of babaco shoots from peduncles cultured with BA and IAA.

Treatment BA (mg liter^{-1})	IAA	nz	Peduncle response Green	Brown	Shoots
0.0	0.5	4	1	7	4
0.0	1.0	4	2	8	1
0.5	0.0	4	1	11	0
0.5	0.5	4	2	8	2
0.5	1.0	4	4	3	4
1.0	0.0	4	2	10	0
1.0	0.5	4	2	6	1
1.0	1.0	4	3	2	3

zNumber of petri dishes (3 peduncle sections/dish).

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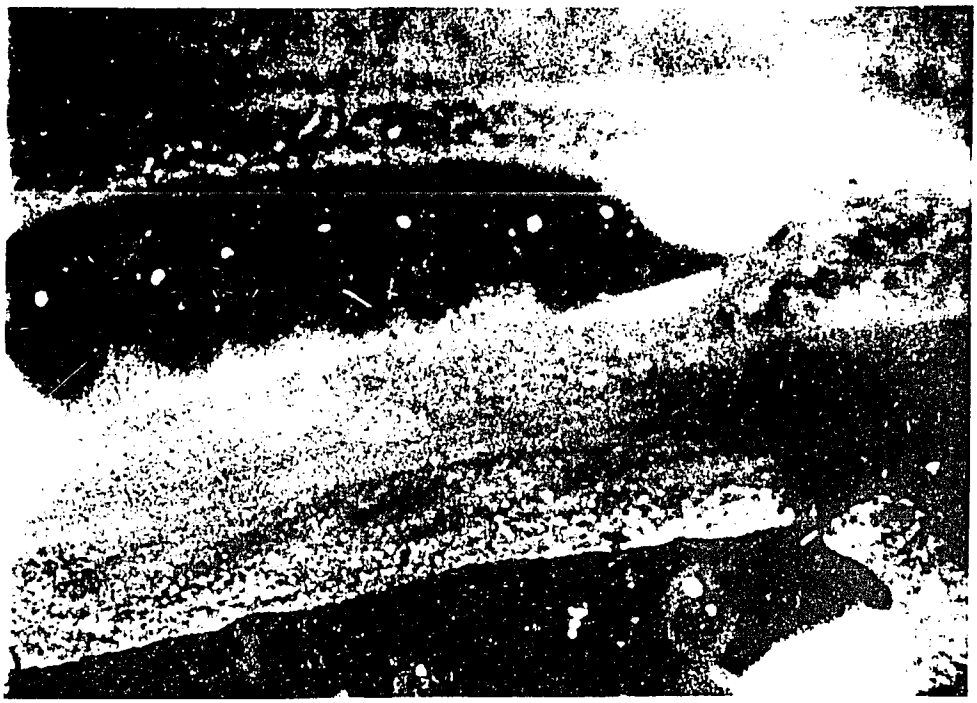
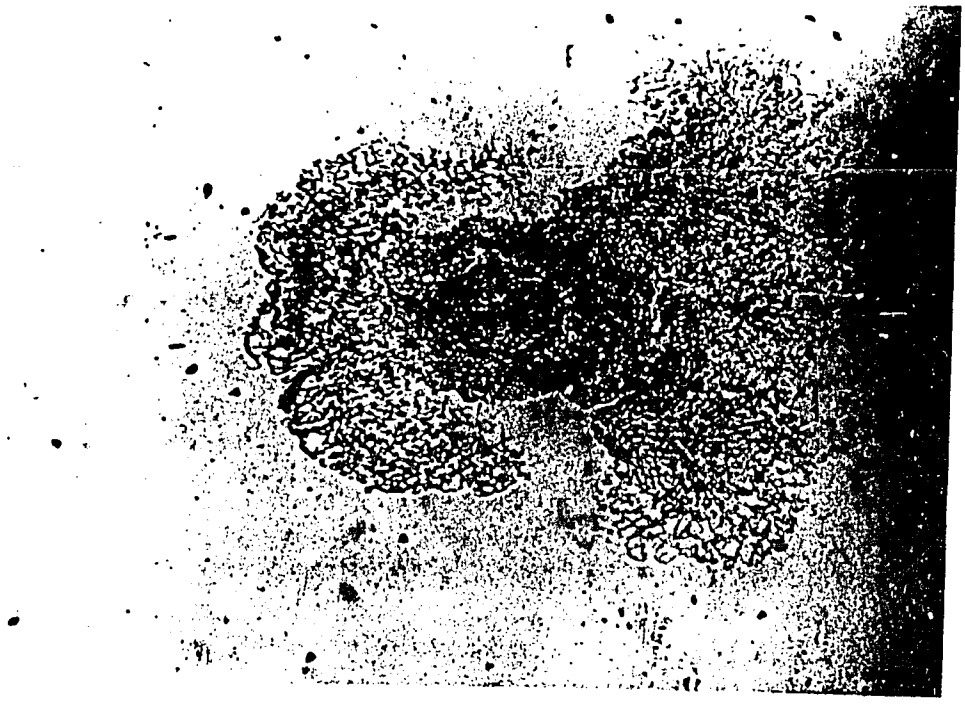


Fig. 1a

Fig. 1b



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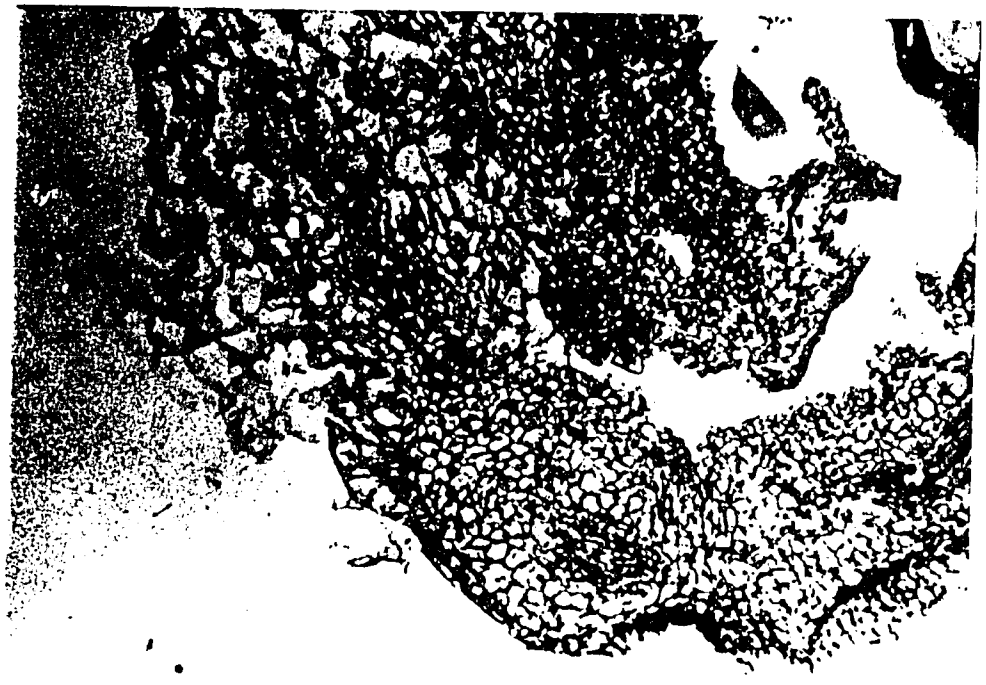

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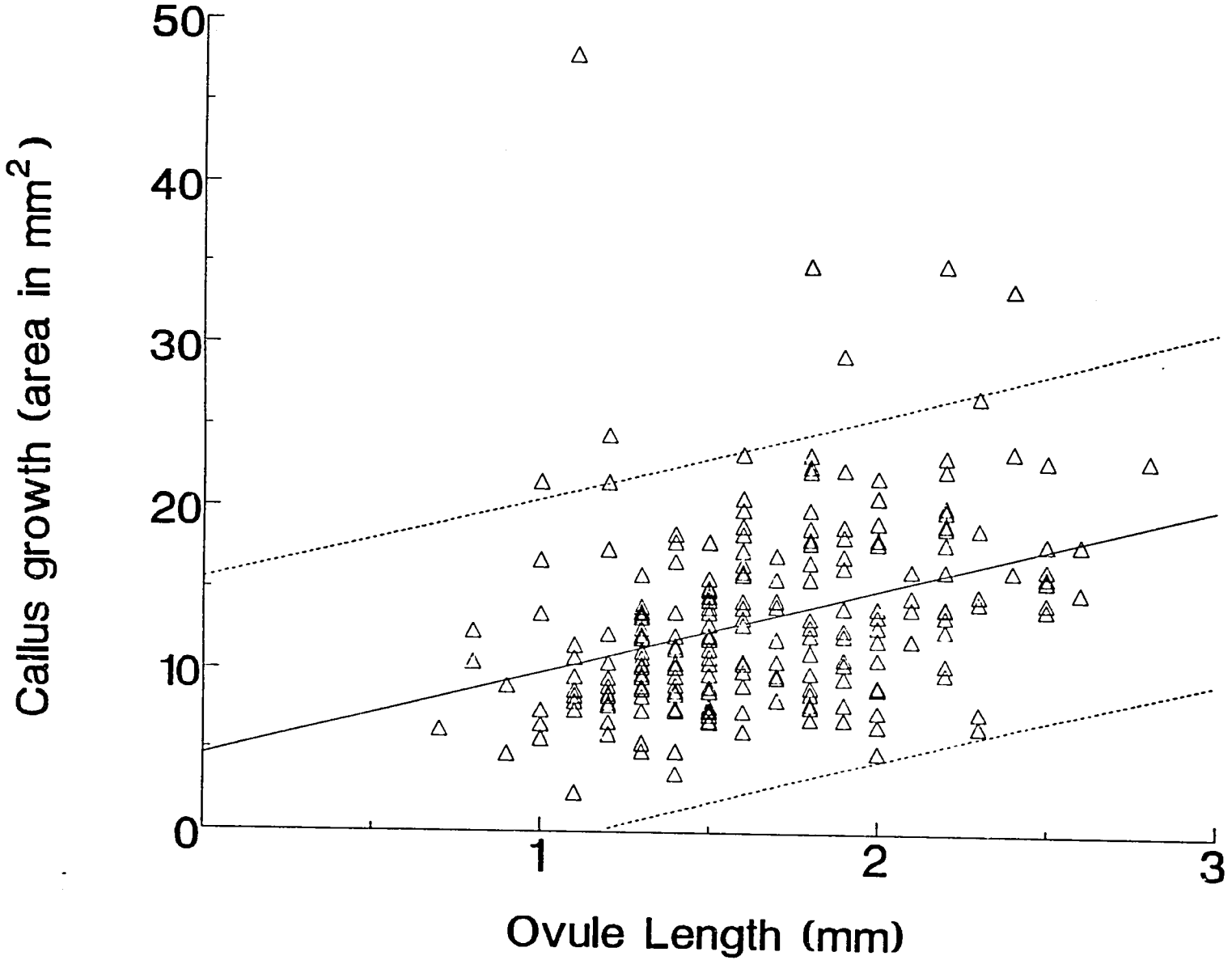

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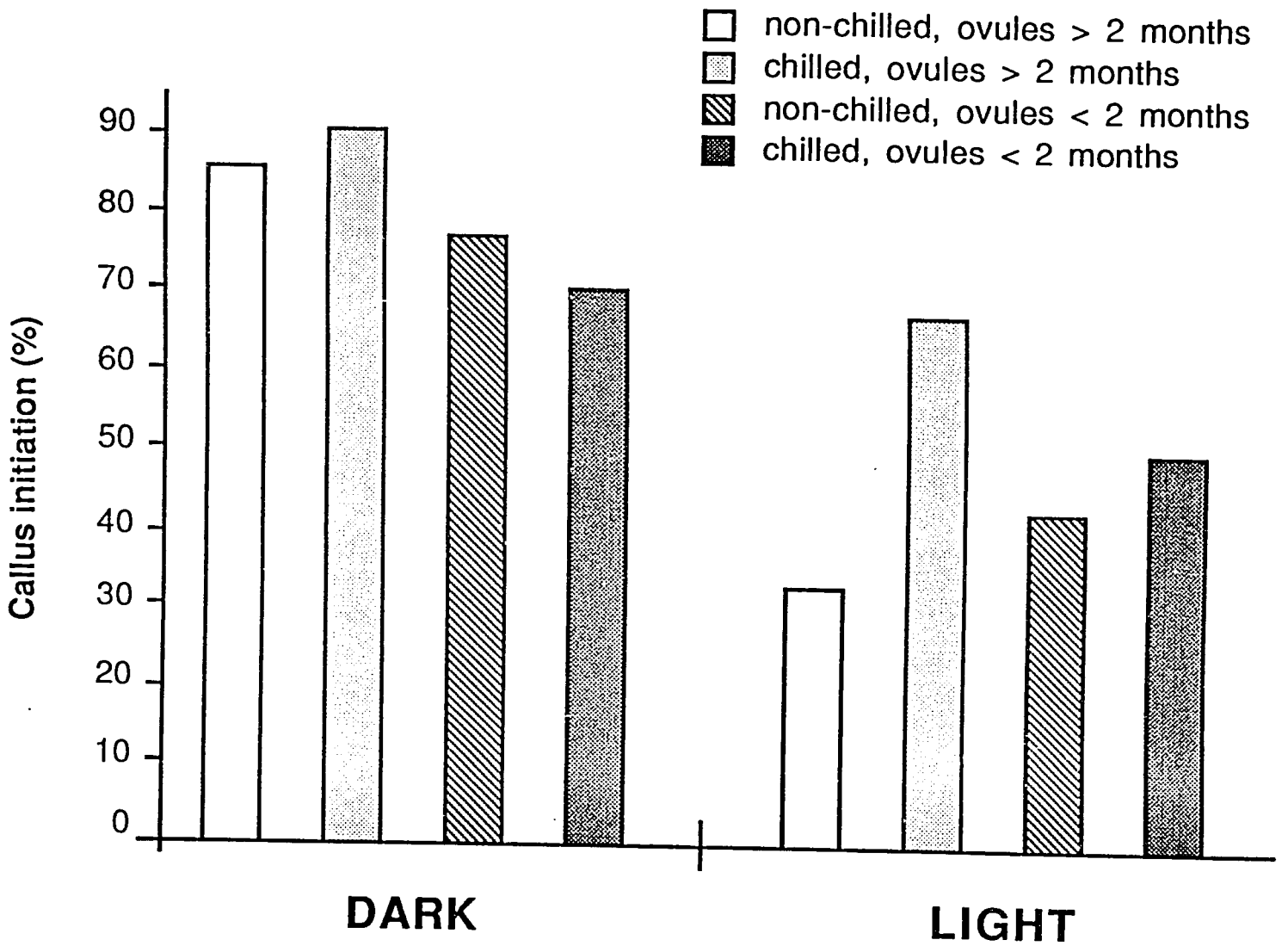

non-chilled, ovules > 2 months
chilled, ovules > 2 months
non-chilled, ovules < 2 months
chilled, ovules < 2 months
Callus initiation (%)
90
80
70
60
50
40
30
20
10
0
DARK
LIGHT

Fig. 4

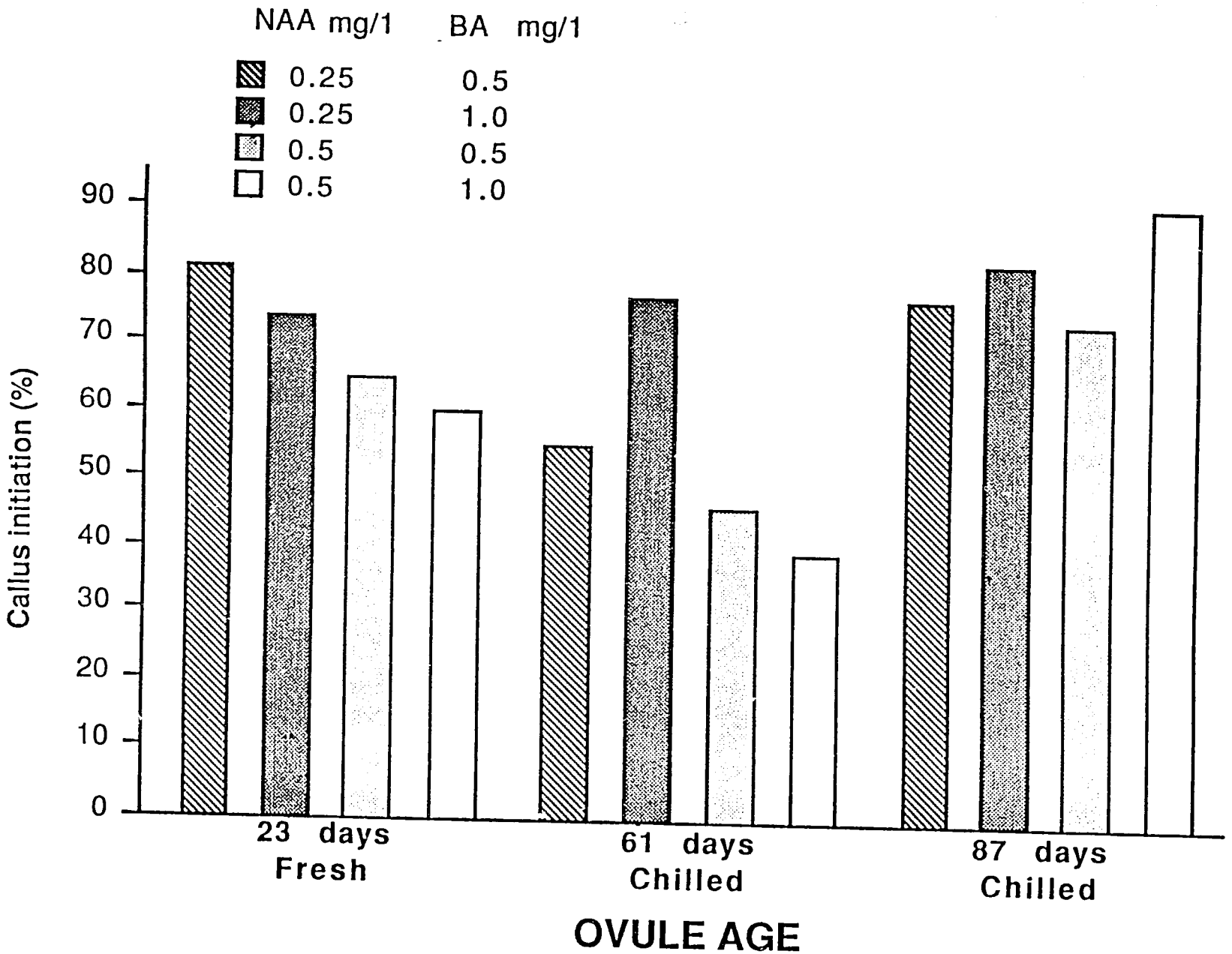

NAA mg/1
BA mg/1
0.25
0.5
0.25
1.0
0.5
0.5
0.5
1.0
Callus initiation (%)
90
80
70
60
50
40
30
20
10
0
23 days
Fresh
61 days
Chilled
87 days
Chilled
OVULE AGE

Fig. 5

Fig. 6

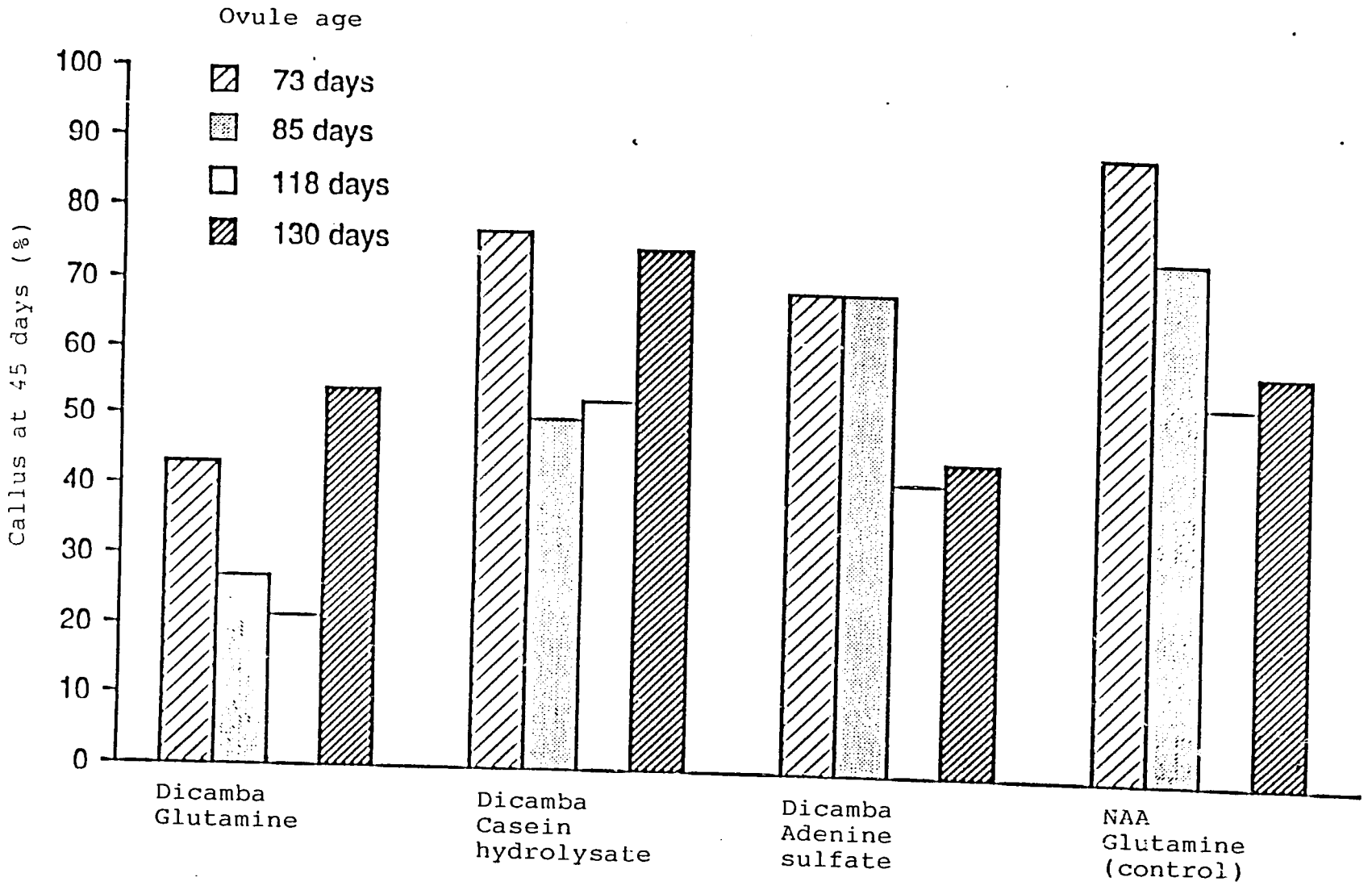

Ovule age
73 days
85 days
118 days
130 days
Callus at 45 days (%)
100
90
80
70
60
50
40
30
20
10
0
Dicamba Glutamine
Dicamba Casein hydrolysate
Dicamba Adenine sulfate
NAA Glutamine (control)

Fig. 7a

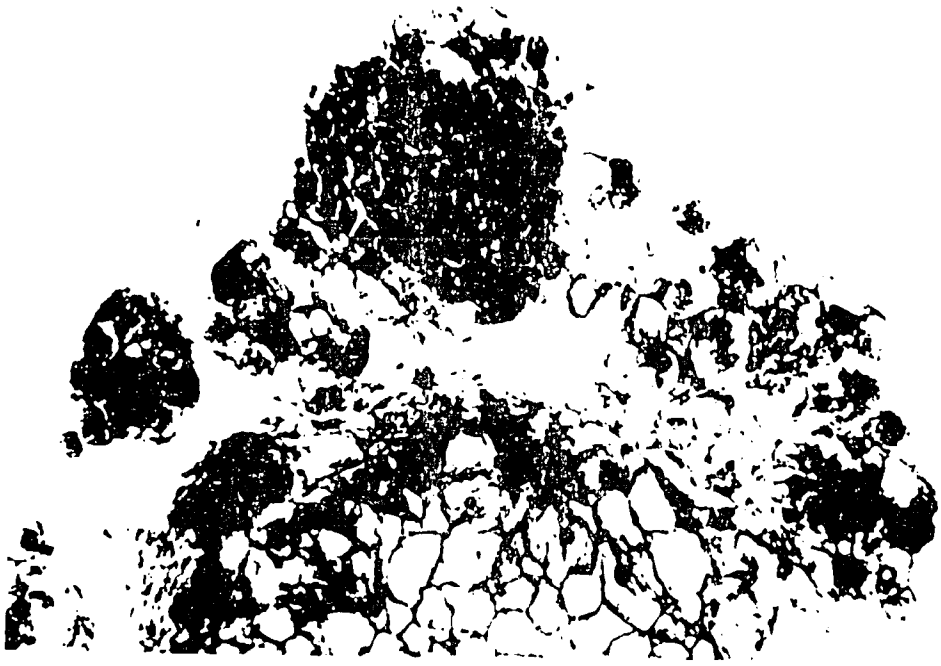


Fig. 7b

Fig. 8a

Fig. 8b

Fig. 9

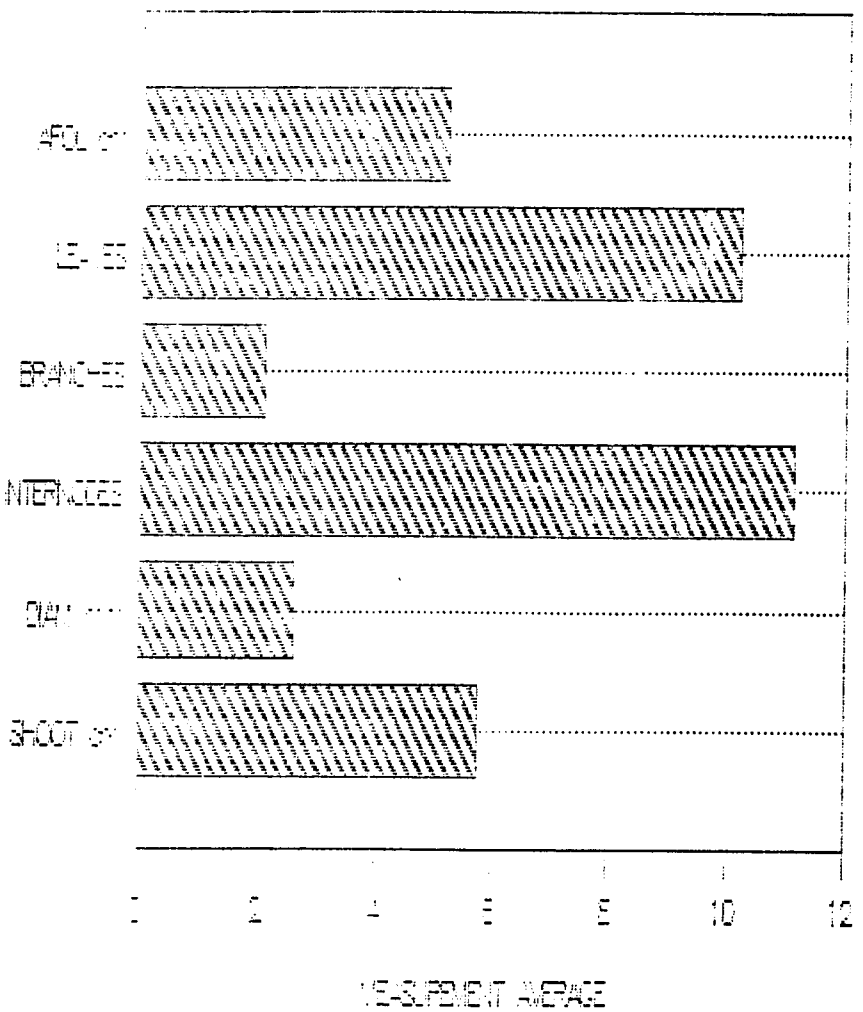


Fig. 10

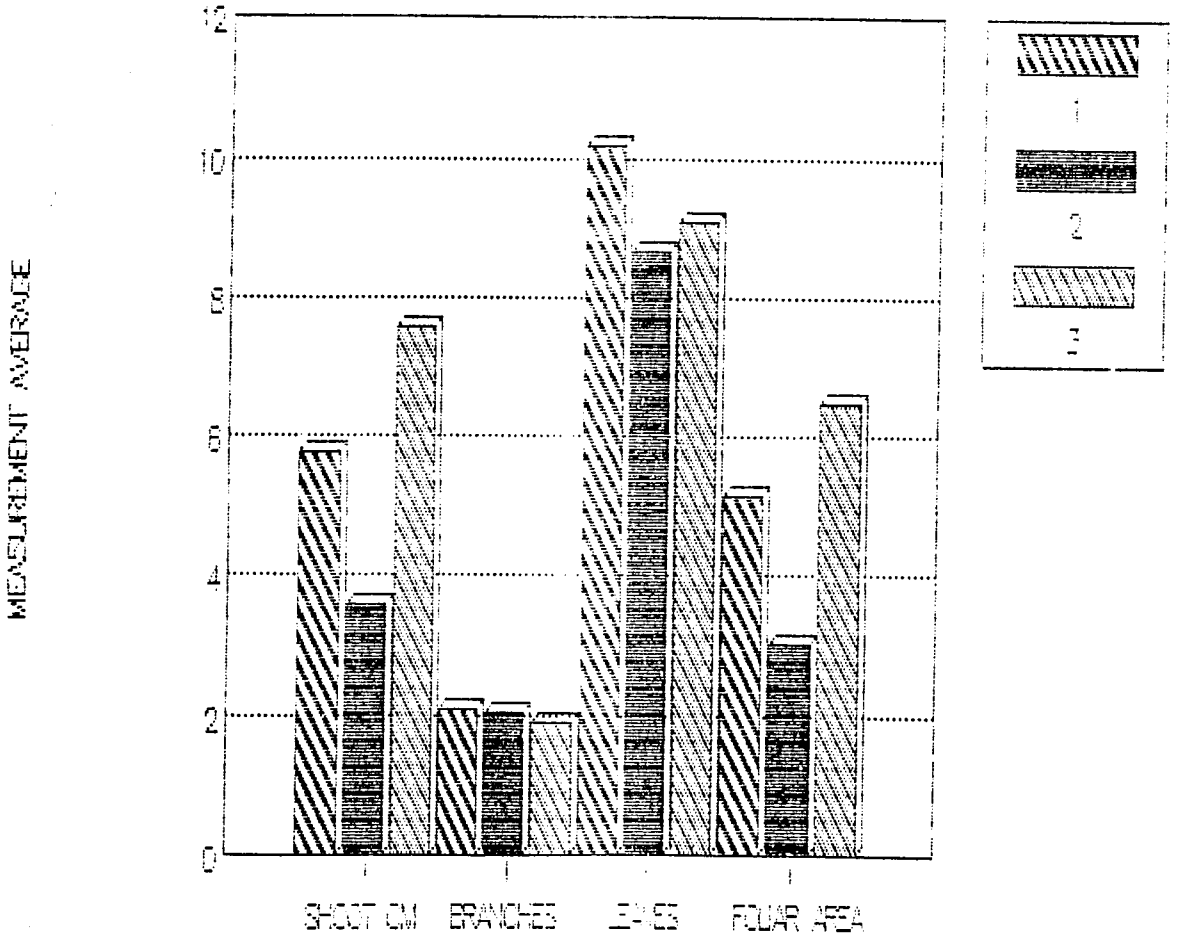

MEASUREMENT AVERAGE
0
2
4
6
8
10
12
SHOOT CM
BRANCHES
LEAVES
FOLIAR AREA
1
2
3

Fig. 11

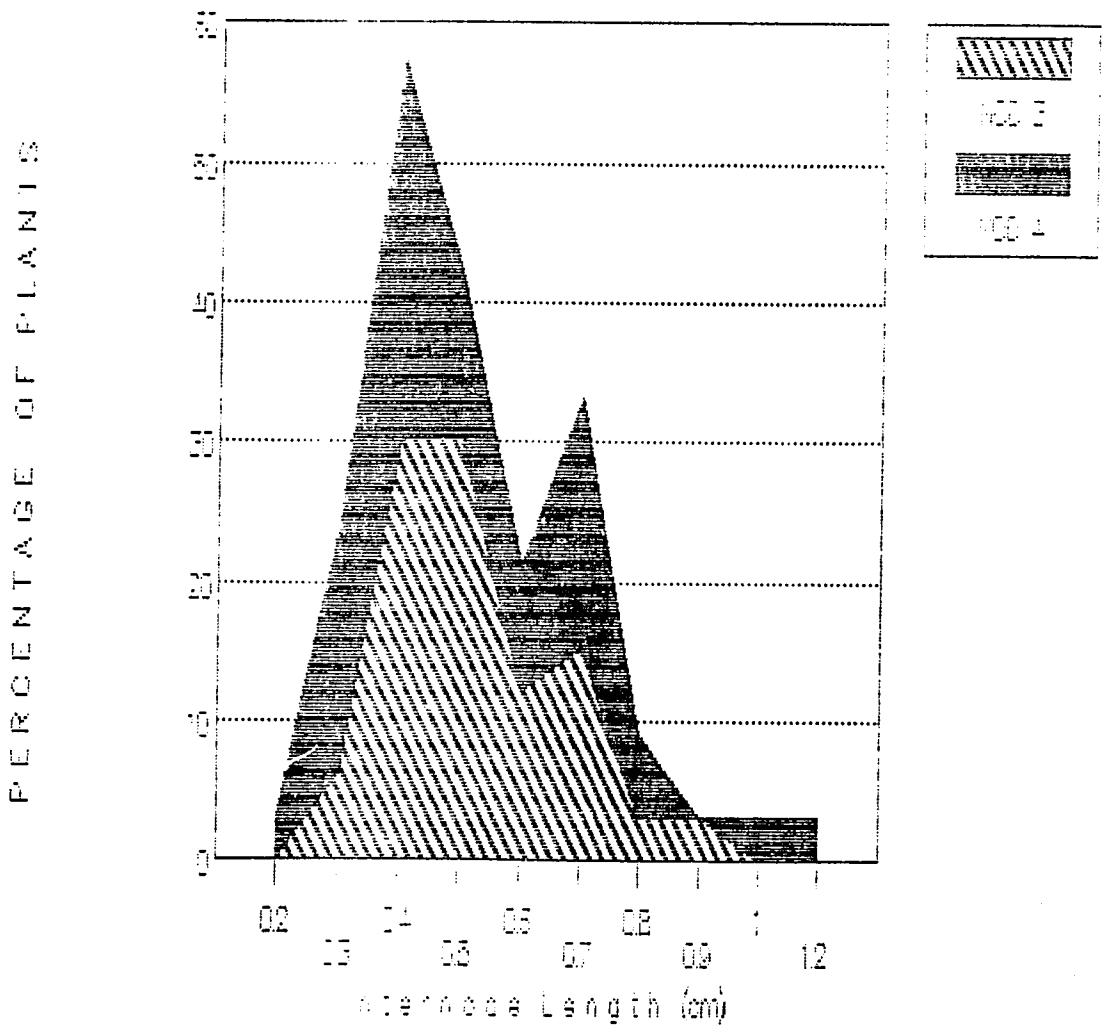
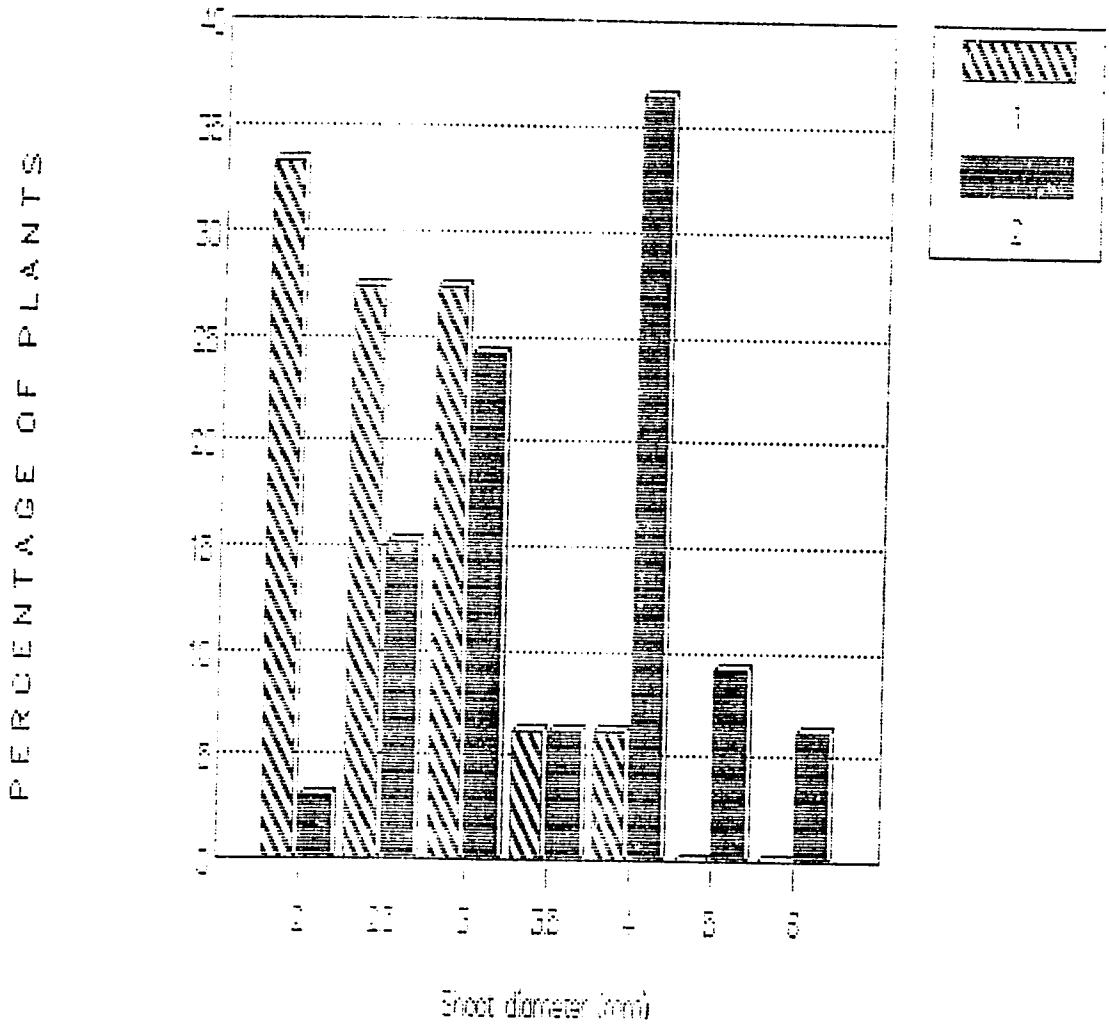

PERCENTAGE OF PLANTS
0
10
20
30
40
50
60
70
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.2
Internode Length (cm)

Fig. 12



IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

During the time span of the project several scientific activities had been held at AMDE:

- 4 Training courses for technical high school teachers,
- 3 Workshops for students, lab technicians, Ag. Ministery extensionists, and growers,
- The First Tissue Culture Congress for 300 participants and international scientists,
- 5 Ecuadoriam laboratories (Maoscap, Cuenca; Univ. of Cuenca, Inexa, Quito; Laboplant, Quito; ESPOCH, Riobamba) received free consulting services and chemicals for their research activities,
- 20 Students from the Univ. of Ambato (UTA) have received training in tissue culture techniques on a regular basis during their senior year at the agriculture university.
- 6 Students from UTA have conducted their research theses for graduation as agriculture engineers,
- 1 student from ESPOCH has conducted her research internship and presented her report for graduation as a chemical technician, and
- AMDE Corp. scientists have presented talks at many events in Ecuador, as well as, internationally.
- Participation at international meetings has helped establish new contacts with other institutions: University of Kentucky; Purdue University, Indiana; Canadian Community Colleges, Ontario; BOSTID, Washington; FAO, Santiago de Chile; CIP, Lima; CIAT, Colombia; CATIE, Costa Rica; IEI, New York; and ACEVIV, Bogotà.
- AMDE has established the Ecuadorean Association of Tissue Culture, Asociaciòn de Cultivadores de Tejidos In Vitro de Ecuador (ACTIVE), and is the represent for RED-BIO (FAO Biotechnology Network).
- AMDE now has two operating tissue culture laboratories and greenhouses, a computer center, and a fax service all of which are available to Ecuadorian and internatiomal scientists.
- The trained personnel have and will continue to provide(d) training services for in country needs.
- 2 AMDE Corp. trained personnel through the project are now cooperating with the Univ. of Ambato and Estate Univ. of Bolìvar teaching and directing joint projects.

PROJECT ACTIVITIES/OUTPUTS

CONGRESSES AND COURSES

<u>Organized, developed and Hosted:</u>
-First Tissue Culture seminar, July 22 1989, with the participation of Dr. S. L. Kitto, international consultant University of Delaware and more than 60 scientists representing different institutions and universities in Ecuador.
- Training Course for university teachers and personnel from private nurseries and public sector with cooperation of Partners of Americas and the University of Kentucky, Dr. Leonard Stoltz.

"Latest Advances in Micropropagation", March 14 -17,1990.
- First Tissue Culture Congress, September 19-21, 1990 with the participation of Dr. S. L. Kitto, international consultant and Dr. T. Evans specialist in plant virology of the University of Delaware; Dr. Jules Janick, Purdue University; & Dr. Margarita Perea, Universidad Nacional de Colombia.
- Tissue culture laboratory practices conducted at AMDE Corp. for senior students of the University of Ambato.
- National training courses for Agricultural Technical High School Teachers:

Sep 25 -26 1989	Micropropagation of fruit trees lab demonstration	Fruit Crops Teachers PROMEET - UTA - AMDE
Nov. 9 -15 1989	Functions of growth regulators in plants	Hort Crops Teachers PROMEET - UTA - AMDE
Feb. 7/90	Storage of germplasm through in vitro systems	Ecology Teachers PROMEET - UTA - AMDE
March 14/90	Biotechnology as a system for resistant ssp.	Pesticides Teachers PROMEET - UTA - AMDE

- National Workshop on "Identification and elination of plant viruses" held in cooperation with the University of Delaware, AGDIA, PROEXANT, Dr. Tomas Evans instructor.
- Training Workshop for Teachers on "Advances in Plant Tissue Culture" held in cooperation with the Universidad Estatal de Bolivar (UEB), University of Delaware, PROEXANT, Dr. Sherry Kitto, Rebeca Vega, Jorge Ibarra, Jorge Fabara and Jaime Rojas, Instructors. June 2-3, 1991, at the UEB, Guaranda, Ecuador.

Attended:

- Tissue culture training for Ing. C. Barberan and Ing. R. Vega de Rojas from August 1988 to March 1989 at the University of Delaware Tissue Culture Lab, directed by Dr. S.L. Kitto in conjunction with Vega de Rojas' babaco research.
- Presentation at the PSTC conference on Biotechnology for Health and Agriculture, Washington, D.C., June, 1988. NEED TITLE
- Poster presentation "Regeneration of Babaco (C. pentagona) through somatic embryogenesis" at the First New Crops conference in Indianopolis, Ind., November, 1988.
- Presentation at the Northeast Regional American Soc. for Hort. Sci. Meeting. Maine, Jan. 1989. "Tissue culture of babaco", R. Vega de Rojas won best graduate paper award.
- Attended AAAS meeting in Boston, Mass., Feb. 1989, International Student R. Vega de Rojas received a "Student travel Award" fron the Institute for International Estudies.
- Presentation at PSTC conference, Davis, Calif., July 23, 1990. "Somaclonal babaco establishment under field conditions".
- R. Vega de Rojas elected Ecuadorean representative to FAO at the Latin American Biotechnology Network conference in Santiago, Chile, Nov. 1990.

- Participation in national conferences and seminars in different locations in Ecuador, as listed:

Oct. 89	Developments in fruticulture Presentation of babaco research	INIAP - COTESU Quito
Nov. 89	Biotechnology Seminar AMDE Representative	CONACYT Quito
Dec. 89	Conference on micropropagation for high school students	C. Benjamin Araujo Patate
May 90	Biotechnology aplications in fruit crops, babaco as a model	I Nac.Con.Fruit Crops SEFRU - UTA -ANDE
Sep. 90	Establishment of tissue culture plants in the field	I Nac. T. C. Congress ACTIVE, ANDE, AMDE, CAF, FUNDAGRO, CONACYT
May 91	Micropropagation of native <u>Caricas</u>	T.C. Seminar ESPOCH, PROEXANT

LAB TOURS

- Many national institutions (DINACE, Pl-480, MAG, ANDE, CFN, FUNDAGRO), high school students from Puyo, Sto. Domingo, two courses from the Agricultural Faculty of Central University, Quito, banana growers from guayaquil have visited AMDE facilities. Visitors received a slide presentation and practical demonstrations.

VISITING SCIENTISTS

- Several workshops were developed with international visitors for evaluacion of the project and for data collection on biothechnology in Latin America, on the following dates:

Oct. 2/89	Dr. Christopher Potter Visit for project evaluation	NAS-AAAS Fellow (USA) Ecology Specialist
Dec.19/89	Dr. Rodolfo Estrada Biotechnology data collection	CIP - Specialist (PERU) NAS- Project Dir.
Feb.9/90	Dr. Donn R. Ward Richard E. Bir James A. Daughtry Visit instalation and facilities	North Carolina (USA) Agr. Extension Service
Mar.19/90	Dr. Leonard Stolz Use facilities for shared tissue culture course	U. of Kentucky (USA) Partners of Américas
July 90	Dr. J. Villalobos	CATIE, Costa Rica
Sep.18/90	Dr. Jules Janick Dr. Margarita Perea Dr. Thomas Evans Dr. Sherry Kitto Conference on T.C.	Purdue University Univ. of Colombia Univ. of Delaware Univ. of Delaware FUNDAGRO
Nov. 90	Dr. Nancy Santana Visiting and given TC seminars for University	ISCAH, Cuba CONFCA, CONUEP

Mar. 91	Dr. L. M. Pierik Visit for future work	Wageningen University
Apr. 91	Dr. Thomas Evans Virus indexing workshop	Univ. of Delaware PROEXANT

THESES

Masters:
- Vega de Rojas, R. "Establishment of techniques and protocols for production of somatic embryos of babaco", University of Delaware, Dec. 1989.

Undergraduate (Ing.):

- Mina, Becquer. "Physical and chemical analysis of babaco cuttings with 3 times of aging, to select the best aging time for rooting", Univ. of Ambato, Jun. 1991.
- Sànchez, Jose. "Babaco grafting systems evaluation upon Chamburo rootstock", Univ. of Ambato, Jun. 1991.
- Cortez, Vìctor. "Initiation of Meloydogine studies on tomato Lycopersicon esculentun var. Jefferson in order to develop protocols for inoculation and evaluation of somoclonal babaco plants", Univ. of Ambato, Nov. 1991 (projected finish date).
- Bermudez, Elsa. "Evaluation of different systems for micropropagation of Tropaelum tuberosum, Univ. of Ambato, May 1991.
- Soria, Tito and Soria, John. "Evaluation of different treatment for rooting babaco cuttings". Univ. of Ambato. May 1991.
- Rojas, Patricia, "Stocks solutions preparation and storagea evaluation" 6 month intership to graduate as a Chemical Technician, Escuala Politècnica dael Chimborazo, Feb. 1991.

FACULTY INVOLVED IN THE BABACO PROJECT
- Dr. Sherry Kitto. Plant Science U. of Delaware
- Dr. Thomas Evans. Plant Virology U. of Delaware
- Dr. Ramiro Velasteguí. Plant Phatology. Specialist UTA.
- Ing. Jorge Fabara. MSc. Fuit Crops Specialist.
  Dean of Ag. Sc. Fac. UTA.
- Ing. Eduardo Fiallos. Andean Crops. Specialist
  Sub-Dean Ag. Sc. Fac. UTA.
- Ing. Jaime Rojas MSc. Project Director UTA.

GERMPLASM COLLECTION
- Establishment of babaco germplasm collection at the Univ. of Ambato, La Viñita and Querochaca consisting on a number of accessions from different areas in collaboration with Ing. Jorge Fabara and students J. Sánchez and B. Mina.
- Selected plant material have been provided to a commercial nursery in California, National Repository Hilo Hawai; and public exhibits as Longwood Gardens, Kenett Square PA; National Aquarium, Baltimore, MA; private gardens in Delaware and Pensylvania, and IRMA tropical research station in Dominica.

CONSULTANT BENEFITS

- Dr. Sherry Kitto, International consultant advised in tissue culture to representants of University of Cuenca, University of Machala, INEXA, MAOSCAP, INIAP, FUNDAGRO, PROEXANT.
- Dr. Kitto also participated actively in the germplasm collection from Loja, in order to complete the accesions of C. stipulata from that parte of the country.

PUBLICATIONS

Refereed

Vega de Rojas, R. and S.L. Kitto. 1991. Regeneration of babaco (Carica pentagona) from ovular callus. J. Amer. Soc. Hort. Sci. In Press.

Vega de Rojas, R. and S.L. Kitto. 1991. Regeneration of babaco from leaf and peduncle tissue. In prep.

Non-refereed

- Rojas, Jaime and Rebeca, Vega de Rojas, UD 1985. Spanish-English publication "Babaco an Exotic Fruit" was published with the cooperation of the Technical University of Ambato. This document was distributed to participants in the tissue culture seminar.
- Rojas, Jaime (ed.). 1991. Memorias del I Congreso Nacional de Cultivo de Tejidos del Ecuador. AMDE Corp., Ambato, Ecuador.
- Vega de Rojas, R. y S.L. Kitto. 1991. La Odisea del Babaco. En preparación.

Abstracts

- Vega de Rojas, R. and S. L. Kitto. 1988. Tissue culture of babaco. Northeast Regional Annual meeting, ASHS. Orno, Maine.
- Vega de Rojas, R. and S. L. Kitto. 1988. Tissue culture of babaco ovules. PSTC Conference on Biotechnology for Health and Agriculture. Washington, D.C.
- Vega de Rojas, R. and S. L. Kitto. 1988. Regeneration of Carica pentagona. New Crops Symposium. Indianapolis, Ind.
- Vega de Rojas, R. 1989. Micropropagaciòn de babaco mediante embriogènesis somàtica. INIAP COTESU I Seminario-Taller sobre investigaciòn, extensiòn y desarrollo de frutales, Quito, Ecuador.
- Vega de Rojas, R. 1990. Somaclonal babaco establishment under field conditions. BOSTID Tissue Culture Networking/Workshop meeting. Davis, Calif.
- Vega de Rojas, R. 1990. Adaptaciòn de plantas micropropagadas-Fase Invernadero. AMDE, CAF, CONACYT. Ambato, Ecuador.

PROJECT PRODUCTIVITY

The project has accomplished all of the goals except for evaluating the fruit quality of the regenerated plants. However, there are enough facilities, trained personnel, and regenerating and proliferating babaco cultures to continue the project. Also, other goals that were not specifically listed in the proposal have been reached through BOSTID contacts like widening our scientific experiences and our contacts with other PSTC projects.

FUTURE WORK

AMDE has initiated a mass propagation system for blackberry (Cherokee, Comanche, Cheyenne, Shawnee, Titan, and thornless Hull) and raspberry (Ruby, Chester, Redwing, Heritage) cultivars new to Ecuador. To date 3,000 micropropaged plants have been distributed to growers and institutions for adaptation testing at different locations to define the growing areas for each cultivar and to provide certified plants for commercial plantings.

Seven in vitro stock-lines of virus-free strawberries have recently been obtained from Ontario, Canada to test under Ecuadorian field conditions.

Seeds from several species of native orchids have been cultured in order to rescue endangered species.

Vitamin and growth regulator stocks will be offerred nationally at reasonable prices so that educational laboratories with limited budgets can initiate tissue culture projects.

AMDE Corp. will continue to conduct training courses according to the needs of growers, institutions and universities.

A number of proposals on micropropagation of banana, oil palm, ornamentals, and berries, as well as one on eliminating viruses from andean tubers have been sent to a number of institutions and exporting companies.

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