Micropropagation and Determination of the *in vitro* Stability of *Annona cherimola* Mill. and *Annona muricata* L.

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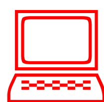

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Botanical and Cultural Aspects

1 Introduction

Annona cherimola and *Annona muricata* belong to the family *Annonaceae*. These subtropical and tropical trees are part of the natural flora in Central- and South-America. The medicinal and nutritional uses have been exploited by the Indians before the discovery of this continent. In America these fruit species have been preserved by tradition, but are still unknown as crop plants.

The commercial acceptance of *A. cherimola* and *A. muricata* as exotic fruits is widespread worldwide, therefore there is a rising interest to expand *A. cherimola* and *A. muricata* plantations in countries where these plants have been introduced such as Australia, California, Chile, Israel and Spain. These countries promote technical knowledge in terms of plantations, marketing and industrialization which is deficient in the countries where these plants are part of the native flora such as Colombia, Peru, Ecuador and the Caribbean Islands.

The yield potential of *A. cherimola* and *A. muricata* in Colombia, Venezuela, Brazil and Peru is promising. In 1992 Colombia reported 1,134 ha planted of *A. muricata* and predicts 16,499.420 kg of fresh produced pulp for 1999, furthermore as far as it is known there are no established technical plantations with *A. cherimola* in Colombia. Chile, the leader in South America of *A. cherimola,* expects a rising promedium of 7,500 tons per year. *A. cherimola* is growing on 2,500 ha in the south of Spain with promising perspectives in the fruit market of the European Community.

The current chapter presents a review of the basic botanical and culture information regarding *A. cherimola* and *A. muricata* as a product of the compilation of isolated world publications.

1.1 *Annona* spp.

The family ***Annonaceae*** Juss., covers more than 130 genera with more than 2000 species, grouped taxonomically, widespread in the subtropics and tropics (Watson and Dallwitz, 1992). Of them 17 genera are distributed in tropical areas. Only four genera, *Annona*, *Rollinia, Uvaria* and *Ansimina* produce edible fruits (Samson, 1986).

The genus ***Annona*** L. Syst. ed. I. (1735) comprises around 120 species, as well as a number of hybrids commonly referred to as „Atemoyas“. The species of this genus are one of the most delicately flavoured fruits when properly ripened. The flesh has a pleasant blend of sweetness and mild acidity with a consistency of baked custard. This latter characteristic is the reason why *Annonas* fruits are called indistinctly ***custard apple*** or ***sugar apple*** and confused with each other frequently (Table 1) (Lizana and Reginato, 1990). The term *Annona* etymologically derives from the Latin *Annona* (yearly produced) (Bourke, 1976) .

Table 1. List of the most well-known species of the Annona genus and their popular assigned names in several languages

Species	Known
Annona cherimola Mill.	Annona blanca, Annone, Anone, Cachiman la Chine, Cherimolia, Cherimolier, Cherimoya, Cherimoyer Frucht, Chirimoya, Chirimoyabaum, Chirimoriñon, Custardapple, Graviola, Jamaikapfel, Pac, Pap, Peruanischer Flaschenbaum, Poox, Poshte, Pox, Rahmapfel, Tsummy, Tukib, Tzumus, Zuckerannone
Annona diversifolia Stafford	Annona blanca, Chirimoya de las tierras bajas, Ilama, Ilama-zapote, Papauce
Annona reticulata L.	Annona colorada, Bullock's heart, Cachiman, Corazón de buey, Custard apple, Mamon, Manzana de ilán, Netzannone, Ochsenherz, Rahmapfel, Ramphala, Reticulata
Annona squamosa L	Anón, Annona blanca, Annone écailleuse, Aritium, Ata, Attier, Cinnamon apple, Corossolier écailleux, Custard apple, Fruta de conde, Noi-na, Pinha, Pomme canelle, Rahmapfel, Riñón, Saramuyo, Schuppenannone, Sitaphal, Sugar apple, Sweetsop, Zimtapfel
Atemoya *	Atemoya, Chirimorriñón, Custard apple
Annona purpurea L.	Cabeza de Negro, Catigüire, Manirote, Soncoya
Annona glabra L	Anona lisa, Chirimoyo de los pantanos, Pondapple
Annona montana L.	Cimarrón, Guanábana de las montañas, Mountain soursop,
Annona muricata L.	Anona de broquel, Anona de puntitas, Araticu-ponhé, Cachiman épineux, Catucho, Coraçao de Rainha, Corosol, Corosol épineux, Corosselier, Durian benggala, Durian blanda, Durian maki, Graviola, Guanábana, Guayabano, Huanábana, Jaca do Pará, Nanqka, Nangka blanda, Nangka longa, Prickly-custard apple, Sauerapfel, Saure sobbe, Seki-kaya-belanda, Sinini, Sorsaka, Soursop, Stachelannone, Sauersack, Sour apple, Toge-Banreisi, Thu-rian-khack, Zapote agrio, Zapote de viejas, Zuurzah

⇒ *The underlined words emphasize the most popular assigned names of the particular species*

⇒ *Atemoya, is a hybrid between (Annona cherimola x Annona squamosa)*

⇒ *Source : Gandhi and Gopalkrishna (1957), Bünemann (1973), Popenoe (1975), Bourke (1976), George (1984), Sanewski (1988), Gardiazabal and Rosenberg (1988), Lizana and Reginato (1990), Leal (1990)* and *de Feo (1992).*

The most important species of the genus are ***Annona cherimola*** Mill., ***Annona muricata*** L., ***Annona squamosa*** L., ***Annona reticulata*** L., and the interspecific hybrid ***Atemoya*** (*A. cherimola* x *A. squamosa*) (Sanewski, 1988). ***Annona diversifolia*** Saff and ***Annona montana*** Macfad. are also important in some countries, but their production chiefly takes places in private orchards. All of them have one thing in common, they are composite fruits, made up of scaly sections that grow together in fir-cone fashion (Samson, 1986).

1.2 *Annona cherimola Mill.*, Gard. Dict. ed. 8 No. 5 (1768)

Synonyms *Annona pubescens* Salisb
Annona tripetala Aiton.

The *A. cherimola,* native to the Ecuadorian Andes, is the most appreciated subtropical fruit tree in the *Annona* genus. (Schroeder, 1956; Farre and Hermoso, 1987; National Research Council, 1989; Correa and Bernal, 1989; Calzada, 1993). The cherimola has long played an important role in the life of the Incas. Terracotta vases modelled after cherimoya fruits have been unearthed from prehistoric graves in Peru.

The *A. cherimola* around the world has acquired only few colonial names, which are only merely variations of the original Quechua[1] word Chirimoya. The Chilean B. Vicuña Mackenna (1875) interpreted from Quechua language the word ”chirimoya”, where ”chiri” means cold and ”moya” means seed, sinus or rounded. He wrote in Spanish the translation ”seno frio de mujer” or ”cold breast of woman” in English (Gardiazabal and Rosenberg, 1988)

Origin and Distribution

The *A. cherimola* is native of the Ecuadorian Andes, South America, (Farre and Hermoso, 1987). It is an important backyard crop throughout Colombia, Ecuador and Peru (Figure1). It has been grown since prehistoric times by Indians from Mexico to Chile. Chileans consider the cherimoya to be their „national fruit” and produced it (notably in the Aconcagua Basin) on a considerable commercial scale (Corfo, 1984).

In other cooler regions of South America such as Bolivia in the department of Cochabamba and surrounding areas the crop flourishes well. In Argentina, the cherimoya, is mostly grown in the province of Tucuman. In Brazil, it is naturalized in the highlands. In other countries of Central America like Guatemala, El Salvador, Costa Rica, Nicaragua, Honduras and Mexico the plant has been naturalized in temperate zones (National Research Council, 1989).

In 1790, the cherimoya was introduced in Hawaii and naturalized on dry upland forests. In 1785, it reached Jamaica and Haiti, where it grows at an altitude ranging from 1,066 m to 1,524 m SL. In the United States, seedlings from different regions were planted in small sections of Southern California and Florida with some difficulties, because cherimoya is not frost tolerant (Morton, 1987).

At the Mediterranean it was introduced by the Spanish conquerors and employed as dooryard. In the province of Granada it gained importance in the 1940*s* when replacement of the old orange trees in the Sierra Nevada mountains started. Actually, Spain is one of the biggest producers of cherimoya fruits. There are also widespread small cherimoya plantations in Italy -province of Reggio Calabria-, Portugal, Madeira (Constantino, 1963; Behr, 1992), Canary Islands, Algeria, Egypt, Israel, Libya, Eritrea and Somalia. In Thailand, Indonesia, Java and the Philippines scattering Cherimoya

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[1] Quechua is the language of Amerindian people of central Peru, constituting the dominant element of the Inca Empire. Today the tongue is widely spoken for approximately 4 millions of people in Peru, Bolivia, Ecuador, Colombia and Argentina.

trees can be found where it is well adapted to lower altitudes (750 m). It was introduced into India and Ceylon in 1880 and there is a small-scale culture in both countries between 457 m and 2,134 m SL., (George, 1984; Morton, 1987; National Research Council, 1989; Behr, 1992).

Figure 1. World distribution of Annona cherimola

Description

A. cherimola is a subtropical, erect and low branched tree (Figure 2), somewhat shrubby or spreading (5-9 m). It has greyish pubescent young branchlets. The leaves are briefly semideciduous (just before spring flowering), alternate, 2-ranked, with minutely hairy petioles (6-12 mm), its form is ovate to elliptic or ovate lanceolate, short blunt-pointed at the apex, slightly hairy on the upper surface, velvety on the underside, and measures between 7-15 x 3-9 cm (Figure 2).

The fragrant flowers are dichogamous, protogynous, often opposite to the leaves. They occur in most cases on one-year branches, on new shoots and in older wood. The number of flowers is variable, up to three flowers can be produced on each bud site. The flowers appear solitary or in clusters (2-3), nodding on short peduncles. They are long and narrow, about 2.5 cm long, with brown or yellowish tomentose outside. The outer petals (6) are three-parted and narrow, the inner ones small and scale-like.

Figure 2. Aspect of Annona cherimola tree, flower, fruit and plantation

The calyx is tubular. The stamens are numerous with fleshy filaments bearing long, adnate, spiral anthers. The pistils are great in nummer, ovuled, crowded on an elongated receptacle (Figure 2).

The cherimoya fruit is composite, formed by fusion of many carpels, conical or somewhat round, apple- or heart-shaped, 10 – 20 cm long and up to 10 cm in width. Its weight ranges between 200 - 600 g. The greenish yellow purple skin is thin or thick, may be smooth with bumps or oblong scales, which overlap each other like roof tiles or fingerprint depressions with a form a complete outer case. It is easily broken or cut open. Pulp is creamy white, juicy flesh and has a pleasant aroma and tasting, sour-sweet flavour, contains numerous hard, brown or black, bean-like, glossy seeds (1-2 cm), which are inedible. The fruit is ripe when the skin begins to blacken (Figure 2) (Bourke, 1976; Morton, 1987)

Bloom, Harvest and Storage

The *A. cherimola* has a dichogamous protogynous flowering behaviour (Figure 3). Flowering is strongly associated with vegetative flushing, with most flowers being produced on the basal nodes of newly emerging vegetative laterals (George and Nissen, 1987a). When the tree is in the flowering stage, all carpels must be pollinated to produce full shaped fruits (Lizana and Reginato, 1990). Fruit set and yield of *A. cherimola* is affected by ineffective pollination (Venkataratnam, 1959; Blumenfeld, 1975).

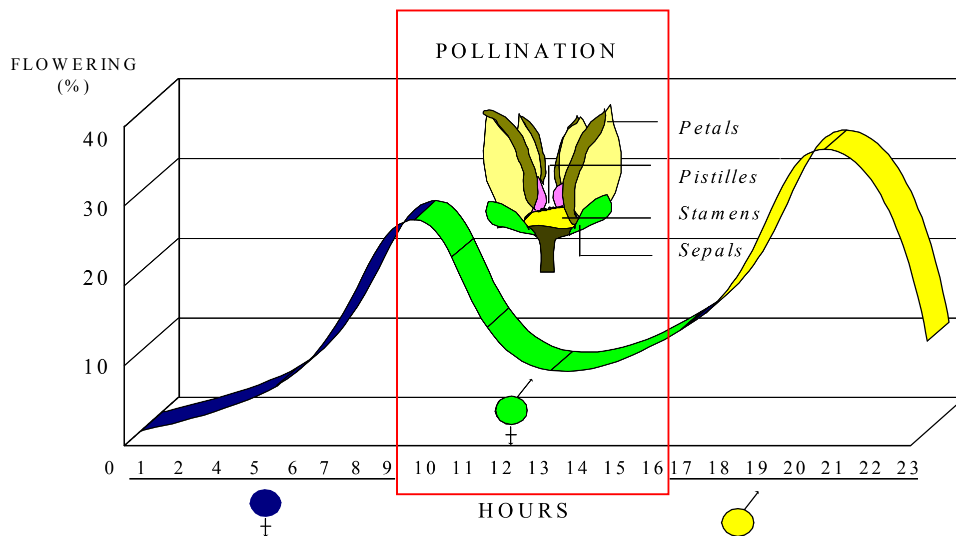


Figure 3. Dichogamous protogynous flowering behaviour on A. cherimola Mill.

Incomplete pollination bears also the risk of shapeless fruit and yield decrease (Thomson, 1970). Flowers are unable to self pollinate (McGregor, 1976), because the male and female organs do not mature simultaneously (Schwarzenberg, 1946). Furthermore flowers are not attractive to insects. However, Thakur and Singh (1965) and Gazit and Eisenstein (1985) reported the presence of *Nitidulis* spp., fruit beetles, which are promoting in natural conditions cross pollination. The commercial production

of cherimoyas actually depends upon fruit management in relation to artificial pollination (Sainte Marie, 1987). Campbell (1979) and Saavedra (1979) tested the use of gibberellic acid (GA_3) and indole-3-acetic acid (IAA) on several *Annona* species. Of these species the *A. cherimola* did not show a positive response however, in the hybrid Atemoya (*A. squamosa* x *A. cherimola*) increased the production of seedless fruits.

The first fruits appears about 100 days after blooming, fruit growth follows the typical sigmoidal pattern, it takes about 9 months in Chile to obtain a ripe fruit (Saavedra, 1979). The *A. cherimola* fruit is best harvested, while it is began to turn in color and is still firm. If it is picked too green, the fruit will soften properly, but its quality is reduced. Sometimes, near to the stem, fruits are waxed to hasten ripening and to improve the custard-like flavour (Thomson, 1970).

This fruit must be eaten within a few days after harvesting (Teubner *et al.* 1990). Maturity is reached when the fruit has grown to its maximum size, changed in colour of middle green to lighter green or yellowish-green (Reginato and Lizana,1980).The cherimoya is a typical climateric fruit during ripening (Biale *et al*, 1954; Kosiyachinda and Young, 1975; Delcura *et al.* 1996). It ripens quickly and may be deteriorated after eight days (Reginato and Lizana, 1980).

The skin browning increases after harvesting, but this is not related to chlorophyll decrease in concentration (Martinez *et al*, 1993). The variation of the skin colour depends on the concentration of phenolic compounds and the activity of the polyphenoloxidase, which catalyzes the oxidation of these phenols to *o*-quinones whose are promoting spontaneously a dark-colored melanine (Coseteng and Lee, 1987; Martinez-Cayuela, 1988).

Cherimoya can be transported short distances at room temperature 18 ± 2 °C and should be protected from rub. When the skin becomes bronze yellow and the peel is susceptible to fall off, handling becomes almost impossible (Lizana and Reginato, 1980). The shelf-life of the fruit is short at 20°C it is less than one week. This characteristic limits the excellent potential marketing of cherimoya far away from the plantation points (Brown *et al*, 1988).

Low temperatures during transport cause chilling and several injures. The cherimoya is not cold resistant and does not tolerate frozt. However, the cold susceptibility differs between cultivars. Low temperatures on refrigerated storages cause chilling injuries on *A. cherimola* fresh fruit (Fuster and Prestamo, 1980). Symptoms of chilling injury include, skin darkening, failure to ripen properly, pulp discoloration and the appearance of pale pink vesicles around the seeds (Gutierrez *et al*, 1992). Changes in total soluble solids and tissue pH in the mesocarp may constitute a biochemical way to assess the occurrence of chilling injury in stored cherimoya fruits (Gutierrez *et al*, 1994). Refrigerated storage proves not effective in prolonging the postharvest life of cherimoya fruits since they do not withstand temperatures below 10-13 °C without developing chilling injury symptoms (Gutierrez *et al*, 1992; Fuster and Prestamo, 1980).

Temperatures in a range of 10 ± 2 °C prolong the postharvest life of the cherimoya fruits. The *A. cherimola* cv. Concha Lisa can be maintained at 10 °C with 5 % O_2 for 43 days and still ripen normally. It remains acceptable after 4 days at room temperature (Palma *et al*, 1992; Alique, 1995). Commercialization of cherimoyas will be possible if ships are equiped with controlled or modified atmosphere chambers rather than fresh air, but little is known about the response of cherimoyas to controlled atmosphere (Kahn, 1983; Palma and Stanley, 1993). The response of *A. cherimola* to cold storage

and waxing have been reported by Lizana and Ararrázabal (1984). Wax allows fruit quality preservation, decreases water loss and stimulates flavor during strorage.

Cultivars

There is a great diversity of *Annona cherimola* due to two factors: Its nature as hermaphrodite, dichogamous-protogynous species and natural regeneration through seeds (George, 1984). Thus numerous selections have been made from *A. cherimola* seedling populations. Conventional plant breeding methods have been applied to improve the cherimoya fruit quality. Selected cherimoya plants are maintained in germplasm orchards by grafting. California, Chile and Australia. Each selection or cultivar is described according to phenotypic characteristics, tree growth and development, type of fruit, weight, number of seeds, quality of pulp and production (for details *cf.* Cordoba, 1967; Thomsom, 1970; Ibar, 1979; Tijero, 1992; Sanewski, 1988; Gardiazabal and Rosenberg, 1988)

The external appearance, surface and colour of the fruit is also used to distinguish some selections in Spain and Colombia (Figure 4; Table 2) (Bourke, 1976; Tijero, 1992). New Zealand reports 223 cultivars of *A. cherimola*, of them only 16 are the promising (Sanewski, 1988). In 1988, Chile introduced 18 cherimoya cultivars (Gadiazabal and Rosenberg, 1988) (Table 3).

Impressa

Smooth

Tuberculata

Mammilata

Figure 4. Classification of some Cherimoya fruits by the surface appearance (Tijero,1992)

Table 2. A. cherimola cultivar characterization by fruit appearance

Cultivar	Surface Appearance	Characteristics
Smooth – Loevis - Lisa	Free from obstacles and hairs	One of the finest of all, sweet, juicy and relatively free of seeds. Ideal to transport
Fingerprint - Impressa	Lines on the tip of a finger or thumb or heart. Smooth surface,	________
Umbonata	Sharp Bulging, swelling, several carpels. The skin is thick	Pulp acid and numerous seeds. Is ideal to prepare cooling drinks and sherbets
Mammillata - Tetillada	Defined fleshly hard nipple-like protrusions, during ripe. The distal area is smooth and the basal area is nipple-like	Common in India and Madeira island. One of the best forms
Tuberculata	Conical protrusions and wart-like tips during ripening	Common in Peruvian markets. Preserved in prehistoric potteries from the graves of the Incas

⇒ *Source : Thomson, 1970; Ibar, 1979* and *Tijero, 1992*

Table 3. The most promising cultivars of A. cherimoya around the world in 1999

Country	Cultivars
Australia	Andrews*, Kempsey*, Mossman*
Bolivia	Bronceada*, Concha Lisa
California	Arthur, Bays*, Bonita, Booth, Carter, Chaffey, Deliciosa, Bumpo, Golden Russet, Haluza Nº. 2, Horton, Libby, Lisa, Loma, McPherson, Mira Vista, Oakwood, Ott, Peru 7752, Peru seed Nº. 24, Pierce, Ryerson, Sabor, Sallmon, Spain,Thomson, Whaley, White*
Chile	Bronceada*, Canaria „canaria clasica“, Capucha*, Concha Picuda, Concha Pesada, Concha Corriente*, Concha Lisa*, Cuero Dechando, Dedo de Dama, Impressa, Juliana, Juniana, Margarita, Piña*, Plomisa, Popocay, Serenense Lisa, Serenense Larga, Terciopelo or Felpa*, Tumba „sandia“*
Colombia	Amarilla, Blanca, Lisa, Negra, Rio Negro, Rugosa, Tocarema
Spain	Basta, Negrito, Pinchua
New Zeland	Bays Mt, Bronceada Mt, Burtons Mt*, Burtons, Chaffey Mt*, Deliciosa Mt, Favourite*, Favourite Mt, Jete, Kent (PK)*,PK 31*, P43 Mt, P52 Mt, Piña ,Mt, PK2 Mt, Q Mt, Reretai Mt*, Smoothey, Spain, White
Peru	Asca, Chavez, Chiuna 1, Chiuna 2, Chiuna 3, Conde Concha, Cumbre, Guayacuyán, Lope Concha, Ñamas, San Miguel, Sander

⇒ ** cultivars with great potential in this country*

⇒ *Source : Richardson and Anderson (1990), Cordoba (1967), Daou and Daou (1991), Dawes et al. (1990), Gadiazabal and Rosenberg (1988), Sanewski (1988), Tijero (1992)* and *Thompson (1970).*

Cultural Aspects

The open-globe pruning is the traditional system for *training* custard apple trees, owing is it elliptical canopy growth (Sawneski, 1988). This practice is common for many semideciduous fruit trees (Westwood, 1993). The basic framework of the tree is developed over the first four years. From then on, pruning is mainly used to maintain tree shape, restrict size, regulate cropping and rejuvenation of trees. Authors like Thomson (1970), Egelhart (1974), Gardiazabal and Rosenberg (1988) and Sawneski (1988) report field work results on pruning.

A. cherimola is a *semideciduous* tree, and different growth cycles are recognized, which are influenced independently by the environmental conditions. The semideciduous cycle starts at the onset of low temperatures. The degree of leaf drop depends on the vigour of the tree and the environmental conditions. For example, the relatively dry and cool conditions in Nambour (Australia) increase leaf drop. Leaves that are not shed in the dormant period will usually be dropped by the emerging shoot in spring (Sawneski, 1988).

Since there has been little research on the *nutrition* of *A. cherimola*, authors like Sanewski (1988) in Australia, Tijero (1992) in Peru and Gardiazabal and Rosenberg (1988) in Chile based their recommendations on field studies.

The *A. cherimola* crop seems to depend on mycorrhizae (arbuscular) for optimal growth, in combination with *Glomus deserticola*. The mycotrophic character of *A. cherimola* has been described for the first time by Azcon-Aguilar *et al.* (1994).

The adjustment of *fertilizer* rates, in particular nitrogen and potassium, is necessary in order to obtain a moderate level of tree vigour. Applications of nitrogen just prior to and after the beginning of flowering appear to enhance fruit set (George, 1988).

The optimum leaf nutrient levels have not been precisely determined for *A. cherimola* and other custard apples. Sanewski (1988) presented suitable approximations that should be used as a guideline only (Table 4).

For determination of the fertilizer needs for established trees, a leaf analysis is more useful than a soil analysis. Nutritional deficiencies in cherimoya yield crops in Chile have been described by Navia and Valenzuela (1978).

Environment

The environmental variables that limit productivity of custard apples are temperature and humidity (George and Nissen, 1992). Soil and location also have a strong impact on the vegetative development of A. cherimola. Hence, the tree must be protected against frost and strong winds. It benefits from summer rainfall and moderate deep, well drained soils (Table 5).

Under natural conditions the cherimoya appears in the subtropical or mild-temperature highlands of the Andes (1,200 - 2,000 m SL). It does not do well in the lowland tropics due to the subtropical climatic conditions.

Table 4. Acceptable leaf mineral concentration in Custard apple

Ion	Concentration
Nitrogen	2.5 - 3.0 %
Phosphorus	0.16 - 0.2 %
Potassium	1.0 - 1.5 %
Calcium	0.6 - 1.0 %
Magnesium	0.25 - 0.5 %
Iron	40.0 - 70.0 ppm*
Manganese	30.0 - 90.0 ppm
Zinc	15.0 - 30.0 ppm
Copper	10.0 - 20.0 ppm
Sodium	< 0.02 %
Chloride	< 0.3 %
Boron	15.0 - 40.0 ppm

⇒ *ppm : parts per million (mg/l)*
⇒ *Source: Sawneski (1988)*

Table 5. Ecophysiological optimal requirements of A. cherimola

Climatic Factors	Range
Elevation	1,200 - 2,500 m SL
Climate	Tropical mild
Temperature	18 - 22 °C
Wind	Moderate
Humidity	60 - 80 %
Soil	Middle-loam
Precipitation	800 - 1,000 mm
PH	6.5 - 7.6
Limitation	Not frost tolerant

The cherimoya tree needs 12-14 hours *light period* and *protection from strong winds and frost*. which interfere with pollination and fruit set, especially when carrying a full yield (Sawneski, 1988). In the flowering period hot dry winds cause pollen and stigma desiccation resulting in reduction of fruit set (Duarte, 1974). The fruit skin is also easily damaged by rubbing and long time exposure to dry winds (George and Nissen,1988; Lizana and Reginato, 1990).

The ambient *mild-temperature* should be 18 ± 5°C during the vegetative cycle and 10 - 18°C in the semi-dormancy after the harvest. Air temperatures influence leaf and vegetative shoot growth. Leaf size increases dramatically with middle warm temperature (Sawneski, 1988).

In areas where low temperatures are expected trees should be protected because even light frost affect fruit quality (Liebster, 1983; National Research Council, 1989; Behr, 1992). The frost sensitivity is related with the biological cycle which is regulated by several exogenous and endogenous factors (Gardiazabal and Rosenberg, 1988). The tree prefers a rather dry environment and does not grow well in extremely wet conditions. neither where rainfall is spread throughout the year (Sawneski, 1988).

Rainfall between 800 - 1.000 mm^3 is desirable. *Irrigation* is essential in most areas, particularly during the flowering, fruit set and early fruit maturation periods. Water stress during any of these periods can result in flower or fruit drop (George, 1984; George and Nissen, 1992). The cherimoya requires for both flowering and fruit development 60 - 80% humidity (Sanewski, 1988).

Relative humidity below 60% limits fruit set and increases flower shedding (Schroeder, 1943). Humitity superior to 60% influences pollination. Saavedra (1977) and Ahmed (1936) demonstrated that the insertion of a drop of water inside the flower at the time of pollination markedly increased fruit set. However during fruit set high environmental humidity benefits the production of sugars and acids but simultaneously enhance diseases, causing problems during its production (Boshell, 1982. Sanewski, 1988).

The cherimoya tree performs well on a wide range of soils in natural conditions (Farre and Hermoso, 1987). The tree grows not successfully on rocky, highly calcareous soils. Loose and sandy loam and well structured clay loam is suitable (George and Nissen, 1992). Higher yield appears to be obtained from trees grown on sand or sandy loam soil types (George, 1987). At any case the cherimoya needs a soil with good aeration and drainage and does not tolerate water logging (Gardiazabal and Rosenberg, 1988).

A neutral or moderately alkaline soil with pH 6.5 to 7.6 appears to be most suitable (Thomson, 1970). The maximum suitable slope is about 15% to operate efficiently on steeper land (Sanewski, 1988). *Soil temperature* has a strong effect on the root development and subsequently on productivity. Soil temperatures between 17 and 22°C promote vegetative growth and development and the cherimoya tree increases its production (George and Nissen, 1987; Gardiazabal and Rosenberg, 1988).

The cherimoya tree can be employed in reforestation and soil recuperation programs. Its rustic nature condition and shallow roots are desirable in non-deep soils. This plant is sensible to lack of calcium but not in nutritional demands (Bridg, 1993a).

Pests and Diseases

There are only a few reports on pests and diseases affecting cherimoya trees. Nevertheless, the publications by Chardon and Toro (1934), Gallego (1950), Rocha (1965), Cuculiza and Torres (1975), Raski (1976), Dominguez (1980), Muñoz (1981), Pinochet (1987), Gardiazabal and Rosenberg (1988), Avilan *et al.* (1989), Martinez and Godoy (1989) Ochoa and Salas (1989) and Tijero (1992), allow to recognize the

following group of cherimoya pathogens:

– Fungi

Ascochyta cherimolaer, *Botryodiplodia theobremae*, *Cercospora annonaceae*, *Cladosporium carpophilum*, *Colletotrichium* spp., *Colletotrichium annonicola*, *Colletotrichium gloeosporioides*, *Corticium salmonicolor*, *Fumagina* spp., *Fusarium solani*, *Gloeosporium*, *Glomerella cingulata*, *Isariopsis anonarum*, *Koleroga noxis*, *Monilia*, *Nectria episphaeria*, *Oidium, Phakopsora cherimolae*, *Phomopsis* spp., *Phomopsis annonacearum, Phyllosticta*, *Phythium* spp., *Phytophtora palmivora*, *Phytophtora parasitica*, *Rhizopus nigricans*, *Rhizopus stolonifer*, *Rhizoctonia* spp., *Rhizoctonia solani*, *Salssetia oleare*, *Sclerotium rolfsii*, *Uredo cherimola and Zignoella annonicola.*

– Insects

Ammiscus polygrophoides, *Anastrepha atrox*, *Anastrepha barandianae*, *Anastrepha bistrigata*, *Anastrepha chiclayae*, *Anastrepha disticta*, *Anastrepha extensa*, *Anastrepha fraterculus*, *Anastrepha oblicua*, *Anastrepha serpentina*, *Anastrepha striata*, *Anastrepha suspensa*, *Apate monachus*, *Bactrocera* spp., *Bephrata maculicollis*, *Brevipalpus* spp., *Ceratitis capitata*, *Cerconota anonella*, *Coccoidea* spp., *Emanadia flavipennis*, *Gelwchiidae* spp., *Heliothrips haemorphoidalis*, *Leosynodes elegantales*, *Lyonetia* spp., *Oiketicus kirby*, *Orthezia olivicola*, *Phyllocnistis* spp., *Pinnaspis aspidistrae*, *Pseudococcus citri*, *Saissetia nigra, Talponia* spp., *Tenuipalpidae*, *Tetranynchus* spp., and *Thrips*

– Nematodes

Cephalobidae spp., *Dorylaimidae* spp., *Gracilacus* spp., *Helicotylenchus* spp., *Hemicycliophora* spp., *Hoplolaimidae* spp., *Meloidogyne incognita* spp., *Pratylenchus* spp., *Paratylenchus micoletzky*. *Rhabditis* spp., *Tylenchorhynchus* spp., and *Xiphinema americanum*.

– Algae

Cephaleuros virescens, Cephalosporium spp., *Paecilomyces* spp.

The CCA „California Cherimoya Association" has been studying the interaction of some cherimoya pests-diseases and the „benefit control pathogens" such as: *Chrysoperla* spp., *Cryptolaemus montrouzieri*. *Cryptolaemus* spp., *Delphastus* spp., *Encarsia formosa*, *Eretmocerous* spp., *Hyppodamia convergens*, *Muscidifurax zaraptor*, *Novius cardinalis*, *Scolorthips sexmaculatus*, *Spalangia endius* and *Trichogramma* sp. (Blehm, 1995).

Propagation

The cherimoya can be reproduced gametically by seeds or multiplicated agamically by cuttings, grafting, marcottage. Micropropagation by tissue culture has been introduced in order to reduce time of propagation, but a micropropagation protocol has not as far is known reported (Sawneski, 1988; Jordan and Botti, 1992; Bridg *et al*, 1994).

The cherimoya *seed propagation* can be managed successfully. The germination of seeds is satisfactory and the germination conditions have been discussed by Duarte *et*

al. (1974), Toll *et al.* (1975); Bridg, (1993) and Bourke, (1976). If seeds are kept dry, they will remain viable for several years (Sanewski, 1988). Germination is expected after the third or fifth week. The plants are transplanted when they are 7 to 10 cm high.

Cherimoya seedling plants are heterozygous and a great segregation is observed in plantations of seedling plants (Bridg, 1993a). The tendency of seedlings to produce inferior fruits has given attention to improve vegetative propagation methods (Morton, 1987). Now seed propagation is made principally to produce cherimoya rootstocks. Seedling trees are optimal, if selected germplasm programs are applied to select elite plants (Tijero, 1992).

If propagation of *A. cherimola* germplasm is made with homozygous plants in which the autofecundation predominates, it is possible to obtain cherimoya clonal lines. Nevertheless, cross pollination does not permit to maintain this process and *vegetative propagation* must be applied (Thomsom, 1970; Sawneski, 1988).

Cherimoya selections and the other custard apples are propagated generally by grafting, whereby the selected scion cultivar is being attached onto seedling rootstock (Table 6). Thus the scion wood and seeds for rootstock should only be taken from selected trees of known performance (Thomson, 1970).

Seedling plants from cherimoya are budded or grafted 100 to 150 mm above ground level when 12 or 24 months old and dormant, using either a whip, whip-tongue, modified side and cleft graft and then allowed to grow to 1- 1.5 m height before being set out on the field (Morton, 1987).

Table 6. Graft compatibilities between the Annona species

	Roostock						
Scion	Atemoya	*A.cherimola*	*A. squamosa*	*A. reticulata*	*A. muricata*	*A. glabra*	*A.montana*
Atemoya	+	+	+	-	-	-	-
A. cherimola	+	+	+	+	?	?	
A. squamosa	+	+	+	+	-	?	?
A. reticulata	?	+	?	+		+	?
A. glabra	?	?	+	+	?	+	?
A. diversifolia	?	?	?	+	+	+	?

⇒ *Symbols : compatible (+), not compatible (-), unknown (?)*
⇒ *Source: Sanewski, 1988*

A grafted plant can be produced in about 18 months if a covered propagation shed is used. When plants are grown in the open the process can take two years or more, Grafted trees begin to bear the second or third year after planting (Thomson, 1970; Sanewski, 1988).

Because rootstocks are produced form seeds, there are large differences in performance among individual trees. The rootstock influences the tree vigour but its tolerance is related with the adverse soil and climatic factors. Yield can vary as much as

100%. Thus it is possible to increase yield significantly by use of superior rootstock clones (Sanewski, 1988).

Cherimoya is the most suitable rootstock, mainly because of its tolerance to bacterial wilt. Preliminary test showed that cultivars „White", „Deliciosa" and „Kempsey" are moderately tolerant, „White" is the most cold-tolerant cultivar and so it is considered the best rootstock, but it has the disadvantage of being very vigorous (Sanewski, 1988). In Colombia grafting is not a widespread practice (Bridg, 1993) but the seedlings from *A. reticulata* and *A. montana* are used as rootstocks, especially when tolerance against drought and moisture soils to be enhanced (Cordoba, 1967).

In Philippines the species *A. reticulata, A. glabra* and *A. squamosa* are reported to be compatible with *A. cherimola*. In moderate climate areas in Madras (India), *A. reticulata* is used as vigorous rootstock with 90% success (Bourke, 1976), *A. glabra* is recommended in Florida (USA) as rootstock in areas with moist soils and it grows better than the graft (Morton, 1987). Grafting between *A. cherimola* und *A. cherimola* could be made without compatibility problems, in spite of the slow growth during the first four years, after which the normal tree size is gained (Tijero, 1982).

A. cherimola grafted on *A. muricata* induces dwarfism, precocity and the fruits are more aromatic. This procedure appears to be very promising, when establishing of new plantations is the objective (Saavedra, 1984). The *A. muricata* roostocks show a good development in litosolic soils (Tijero, 1992). The grafting of *A. cherimola* on *A. squamosa* promotes dwarf trees (Bourke, 1976) and those in North Queensland (Australia) are used as rootstock, because they are more precocious than the cherimoya rootstocks (Sanewski, 1988).

A new propagation technique called „green grafting" has been developed by CSIRO[2]. It involves grafting of young rootstocks with soft, green shoot tips. This grafting can take place within three to six months after sowing the seed. In Australia it appears to be a promising method because it reduces the nursery time (Sanewski, 1988).

Propagation of cherimoya by cuttings is not a widespread practice, nevertheless the advantages of cuttings include high vigour and higher yields in the first few years of production, more uniform orchards and lower cost of trees. Propagation by cuttings does not produce tap-root plants, making the tree susceptible to blow over by strong winds (Bourke, 1976). The "African Pride"cuttings are not as resistant to bacterial wilt as certain cultivars of cherimoya (Sanewski, 1988).

Cherimoya *cuttings* (13-15 cm long and 1- 1.25 cm wide) selected from woody branches in the dormancy stage, are recommended to be planted with 4/5 of its length in the ground and 1/5 exposed (Bourke, 1976). Nevertheless, rooting and disease problems limit this practice considerably. Temperature variations combined with hormonal treatments, IBA, IAA and combination of IBA and Rutina have been applied (Duarte *et al.* 1974; Gardiazabal and Karelovic, 1985; Tazzari *et al*, 1990).

The hormone indolebutyric acid (IBA) is used as solution or powder to enhance rooting. The combination of 50:50 sand and peat or sand and vermiculite is used for the cuttings bed, which is maintained at 25-28 °C. Then over the 4-6 week the cuttings should start to form a callus and root organogenesis and development will be defined after 8-12 weeks. Cuttings are usually ready for potting after 10-16 weeks. Care should be taken not to damage the roots when cuttings are being removed from the bed, Usually

[2] CSIRO : Commonwealth Scientific and Industrial Research Organization, Australia

it takes another 6 months before the plants are ready to be field planted (Sanewski, 1988).

George and Nissen (1986) reported notorious increments on the production of Atemoya (*A. cherimola* x *A. squamosa*) *cv*. „African Pride" (18.7 t/ha) and (9.2 t/ha) of *A. cherimola*, when propagated by cuttings. Flowering, fruit quality and a harvest cycle „two times per year" were superior compared with the production and development of seedling plants. The benefits of cutting propagation are still under examination. It should only be tried on deep, well-drained soilsand protected areas. A success of 60-80% is common reported with „African Pride", others cuttings e.g. „Pink's Mammoth" and cherimoya have proved difficult to root, averaging less than 20% (Sanewski, 1988).

Marcotting (air layering) can also be used with propagated cherimoya cultivars that have been grown from cuttings. Nevertheless cherimoya cannot be propagated easily by this vegetative method. The Queensland Department of Primary Industries, Australia, modified a layering method for propagate cherimoya rootstocks. The roots should develop in four or five months, afterwards the stem can be trimmed and cut from the tree (Sanewski, 1988).

Properties

The cherimoya fruit is used as food product because it can be eaten as a fresh fruit. When fully riped, it is soft to the touch and the stem and attached core can be easily pulled out. The ripe fruit has a fresh pulp, smooth solidity, the taste is sour-sweet and very aromatic, its colour is white (Figures 2; 4).

The fruit may be simply cut in half and the flesh eaten from the "shell", e.g., with a spoon. In some countries the pulp is pressed and used in combination with other fruits to prepare fresh fruit salad or the flesh is blended with milk or water and little sugar to make juice, desserts, ice cream and tropical fruit cocktails (Liebster, 1983; Benk, 1985 Morton, 1987; Bridg,1993).

Usually the cherimoya pulp is very difficult to process due to it is strong phenolization or browning tendency and can not be used for cooking. However, the industrial products derived from pulp extracts encompass great varieties, ranging from chocolate tablets to cosmetics (Figure 5)

Nutritional Value

The cherimoya fruit contains remarkable amounts of calcium, phosphorus, carbohydrates, thiamine, riboflavin, fructose, glucose, sucrose, cellulose, hemi-cellulose, lignin and peptic substances (Table 7).

The cherimoya is very digestive and nutritive with a particular taste as result of the harmonic combination of acids and sugars. The sugars are the product of the starch reduction. The main sugars are glucose (11.75%) and sucrose (9.4%) (Kawamata, 1977).

Figure 5. Some commercial products made with A. cherimola

The principal organic acids are citric and malic acid (Bueso, 1980). For processing it is necessary to select cherimoya fruits with a high content of soluble solids and ascorbic acid (Kawamata, 1977; Vidal-Valverde *et al*. 1982). For immediate consumption, juicy and big sized fruits are preferred.

The aroma is one of the most distinguished characteristics of the cherimoya and related to the ripening development. The pleasant smell is the product of aromatic compounds metabolism by leucine pathway (Schreier *et al*, 1985 ; Lizana y Reginato,1990).

Secondary Produced Metabolites

The *A. cherimola* is also known as medicinal plant. Tea made from leaves and bark is relaxing. The fruit is moderately laxative and benefits the digestion (Garcia-Barriga, 1974).

The cherimoya fruit produces more or less 208 volatile compounds, 23 hydrocarbons, 58 esters, 47 carbonils, terpenoids (mono and sesquiterpens), kar-3-ene, 54 miscellaneous structures of alcohol as *1*-butanol, *3*-metyl-butanol, 1-hexanol, linalol and *3*-metyl butyl ester (Idstein *et al*, 1984; Ekundayo and Oguntimein, 1986; Chen *et al.* 1998a, 1998b, 1998c; 1999).

Since 1970, the cherimoya was used by the natural products industry due to the high presence of secondary metabolites that show antimicrobial activity. The cherimoya leaves are rich in dimeric proantocianidines (Weinges *et al*, 1969). The stems are rich in alkaloids such as liriodenine, anonaine, michelalbine and (+)-reticuline (Urzua and Cassels, 1977; Chen, 1997). Simeon *et al.* (1989) reported 18 alcaloides (9 aporfines, 3 oxoaporfines, 1 proaporfine, 4 tetra-hydroprotoberberines and 1 bencil-tetrahydro isoquinole) from extracts of bark and stems.

Table 7. Food components in 100g of A. cherimola edible portion

Component	Concentration Promedium	
Calories	62.0	kcal
Energy	265.0	kj
Water	74.1	g
Carbohydrates	13.4 - 18.2	g
Ash	0.6 - 1	g
Fat	0.1 - 0.3	g
Crude Fiber	1.5 - 2.0	g
Protein	1.5 - 1.9	g
Calcium	22.0 - 32.0	mg
Minerals	0.8	g
Iron	0.4 - 0.8	Mg
Phosphorus	30.2 - 47.0	Mg
Vitamin A	0.00 - 0.01	UI
Thiamine (B_1)	0.06 - 0.11	Mg
Riboflavin (B_2)	0.11 - 0.14	Mg
Niacin	0.90 - 0.15	mg
Ascorbic Acid	4.3 - 5.0	mg

⇒ *Notes: The edible portion. without skin and seeds is about 60% (Stahl, 1935)*

⇒ *Source: Cordoba, 1967; Morton, 1987; Teubner et al, 1990;*

The seeds of cherimoya are rich in glycoside esteroide (ß-sitosterol-3-glucoside), isoquinoline, azantraquinone and acetogenines all of them grouped in the category of bis-tetrahydrofuran-γ-lactona. The azantraquinone isolate was reported as cleistofoline and the new acetogenine as laherradurineero. There are also cherimolina, dihydrocherimolina and asimicina (Rios *et al.*1989; Villar *et al*, 1982).

Natural Products Industry

The seeds and the leaves of cherimoya are very rich in essential oils and reported as insecticide. The seed juice in ethanol solution kills lice (Jaramillo, 1952). The extract of stems and bark is used as poison to kill fishes and parasites. In tropical areas the extract of seeds, stems and leaves is used as repellent against insects.

The Indians say that the cherimoya leaves could inhibit the development of tumours. Two spoons of pulverised fruit is said to be an antidote against poisoning caused by food and beverages (Garcia-Barriga, 1974).

Extracts of bark and stems from cherimoya are rich in alkaloids and showed antimicrobial activity against gram positive and negative bacteria (Nickell, 1959). The barks contain Annonaine (3µg/ml), which is active against *Klebsiella pneumoniae*, Norushinsunia (100 µ/ml) is active against *Pseudomonas aaeruginosa* and anolobina controls well the yeast *Candida albicans*.

Other alkaloids that are present in high concentration as isoboldine, corypalmine, discretamine and stepholidine were inactive against the micro-organisms tested, the experimental results showed a great effect against bacteria gram positives as *Bacillus subtilis*, *Staphylococcus aureus* and *Mycobacterium phlei* (Simeon *et al*, 1990).

1.3 *Annona muricata* L. Sp. Pl. 536, 1753

A. muricata is the most tropical semideciduous tree with the largest fruits of the *Annona* genus, widespread at the tropic areas of Asia, Central and South America including the Amazon basin. It is known as guanabana (Spanish), graviola (Portuguese), naqka (Austronesian), Stachelannone (German) and soursop (English) (*cf.* Table 1). The Spanish name „Guanábana" is a variation of the Arawak word „Guanahaní", who remember the first Caribean island in the Antilles, landed by Christopher Columbus in 1942 and renamed today San Salvador.

The *A. muricata* is regarded as great delicacy in areas where *A. cherimola* can not grow. In contrast with the other *Annona* spp., the *A. muricata* flowers and fruits more or less continuously during the whole year. The fruit pulp is exceptionally well suited for juice production and the vegetative parts have a medicinal application. The market of soursop is potential at the tropics and undetermined in the word season areas. The demand of soursop in 1999 does not exceed the supply in the productive countries.

Origin and Distribution

A. muricata is native to the Antilles. It grows well below 1,150 m SL in the most tropical micro-ecosystems of Central and South America. In Colombia there are reported more than 1,134 ha of soursop with 15 tons/ha yield

There are established *A. muricata* plantations in Argentina, Australia, Bahamas, Brazil, Bermudas, British Guiana, Colombia, Cuba, Curaçao, Dominican Republic, Florida (USA), French Guiana, Haiti, Hawaii, India, Jamaica, Malaysia, Mexico, Panama, Pacific Islands, Peru, San Salvador, Santo Domingo, South East China, Surinam, Philippines, Trinidad and Tobago, Venezuela and Vietnam (Figure 6) (Morton, 1987).

Figure 6. World distribution of Annona muricata

Description

A. muricata is a tropical evergreen semideciduous fruit tree (4-15 m) (Figure 7), branched near the base, with all parts evil-smelling when bruised. The branchlets are terete, finely wrinkled, scabrous, reddish brown and glabrous, with many round lenticels. The *leaves* are entires with an acute or cuneate base, biserate, short petioled, dark green and shiny, the upper surface is lustrous and leathery or coriaceous with a obovate, or elliptic-oblong form, shortly acuminate apex and narrow transparent margin.

The *flowers* are cauliflowers, regular and pedicled, strong-smelling and borne on the short, axillary, one or two flower branchlets. The pedicel is densely clothed with short hairs, the bract is small. The three *sepals* are almost free, dark green, ovate triangular, coriaceous, densely clothed with small hairs (Figure 7).

The six *petals* are placed in two rows, the three outer ones are the largest, thickly coriaceous, covered with a short tomentum, they are first green and later on pale yellow, their size is 3-5 cm long and 2-4 cm wide. The three inner petals are smaller, alternate with the outer ones, thinner, short clawed towards the base, yellowish 2-4 cm long and 1.5 - 3.5 cm wide (Figure 7).

The numerous *stamens* are borne in many rows on a raised torus and crowned in whorls around the ovaries. The filaments are short, thick and densely pubescent, the connective is thickened and produced beyond the linear anthers. The *ovaries* are numerous densely pubescents, afterwards confluent into a collective berry which bears the styles in the shape of soft reflexed prickles, while the torus develops within the fruit into a robust-uvulate carpohore.

The *fruit* is the largest of the *Annonas* spp., weighing up to 7 kg. It is a syncarpous ovoid or ellipsoid, usually irregular, oblique or curved, heart shaped. It measures between 15-35 cm length and 10-15 cm width. The *skin* is dark green on the immature fruit, becoming slightly yellowish green, glabrous, but bears numerous and fleshy spine-like prickles. The *pulp* is creamy white, fleshy, juicy and subacid with soft, cottony stands that contain many seeds. The seeds are numerous (100 approx.), glabrous with a horny testa and strongly ruminate albumen 2 cm long, obovoid, compressed, shiny with dark brown colour (Figure 6) (Morton, 1987).

Bloom, Harvest and Storage

Like *A. cherimola* the *A. muricata* has a dichogamous protogynous flower behaviour (*cf.* Figure 3). There are several floral stages of soursop during the whole year. The production of the flowers is not uniform but constantly (Moraes, 1979; Escobar and Sanchez, 1993).

The *A. muricata* fruit grows with a typical sigmoid pattern and matures in 100-150 days. The increment of size and weight is proportional to the number of fertilized pistils. The size of the resulting fruits ranges from 4,000- 22,000 cm^3 and weight 0.8-7 kg (Bridg *et al*. 1994). The soursop blooming is influenced directly by the environmental conditions, self-pollination levels as low as 2% have been reported, furthermore the natural pollination by insects is occasional (Gardiazabal and Rosenberg, 1988).

Figure 7. Aspect of Annona muricata tree, flower and fruit and plantation

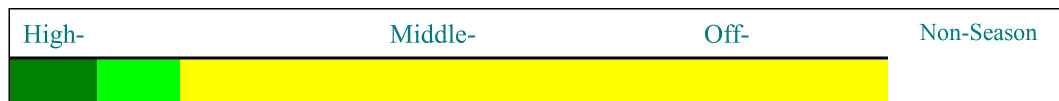
Escobar and Sanchez (1993) reported for first time the presence of *Tapinoma* spp., (*Hymenoptera-Formicidae*) ants and *Ciclocephala signata* (*Coleoptera- Scarabacidae*), *Diabrotica* spp., (*Coleoptera-Chrysomelidae*), *Carpohilus* spp., (*Coleoptera-Nitidulidae*) and *Coleoptera-Staphylinidae,* which are benefiting the natural pollination of soursop in Colombia. However there are no reports on the efficiency of this entomophilous pollination. Thus, artificial pollination is a widely recommended practice in productive commercial *A. muricata* plantations.

The *A. muricata* fruit is best harvested while it is still light green and has grown to its maximum size. The fruits should not ripen entirely on the tree, because they are too heavy and fall down. On the other hand unripe fruits are bitter. The soursop gains maximum yield after the sixth or seventh year of production, because the cauliflower formation is superior to the branched blooming.

The seasonal production of *A. muricata* in the countries where it is commercially cultivated is reported (Table 8). Yields of 10 tons/ha/year are reported in Puerto Rico, 20 tons/ha/year in Hawaii and up to 15 tons/ha/year in Colombia (Escobar and Sanchez, 1993). Furthermore, the existing actual market for *A. muricata* frozen pulp is open and suggests, that the potentialities of this species in the tropical fruit world trade for the next century are great.

Table 8. Seasonal production of A. muricata in several countries

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Colombia												
Florida												
Hawaii												
India												
Mexico												
Puerto Rico												
Indonesia												



Cultivars

A. muricata has been propagated by seeds and as the other *Annona* spp., its pollination problems are affecting fruit quality. In natural conditions the wide range of fruits with different shape, flavour and flesh consistency is interesting in terms of plant selection.

In 1999 there are no selected *A. muricata* cultivars reported, however some general classifications have been tried for the first time in Puerto Rico, considering factors such as fruit form, flavour and consistency (Table 9).

Table 9. Classification of soursop cultivars in Central America

Classification by	Description
Form appearance	Round
	Heart-shaped
	Oblong or Angular
Flavour pulp	Sweet*
	Sweet-sour
	Sour
Flesh consistency	Soft
	Juicy
	Dry

⇒ *The term "sweet" is used in a relative sense to indicate low acidity*

⇒ *Source: (Morton, 1987)*

In other countries like El Salvador a medium-sized, yellow-green, fiberless, soursop is reported and called „*guanábana sin fibra*". It has been vegetatively propagated by the Agricultural Experimental Station of Santiago de las Vegas in Cuba. This selection is one of the most productive in Central America (Morton, 1987).

In Colombia there are no still established selections of *A. muricata* cultivars, there the most relevant considered characteristics on the *A. muricata* tree are bearing growth pattern, brix grade and natural tolerance to some limiting quality diseases like *Colletotrichium* spp., (Escobar and Sanchez, 1993).

Cultural Aspects

The natural growth pattern of *A. muricata* is a canopy. Thus, open-globe pruning system is a desirable training method (Escobar and Sanchez, 1993). In commercial orchards training should be made and the tree size must be reduced to 2.5-3 m.

The basic framework of the tree is developed over the first three years. From then on-pruning is mainly used to maintain tree shape, restrict size, regulate cropping and rejuvenation.

In terms of productivity, the soursop countries are still developing training methods. The unique clear training definition is to open the tree and improve ventilation to reduce the attack of fungi diseases.

Environment

The main climatic factors that are affecting fruit production are: temperature, humidity, rainfall and wind (George, 1984). *A. muricata* is tropical. The tree grows well between 500 and 1,250 m SL. in areas with plenty of sun and protection against strong winds.

Temperature and humidity are the principal factors that are influencing growth and development. The *A. muricata* such as the other *Annona* spp. is not cold tolerant. Temperatures between 25-28 °C and 60-80% humidity are desirable. Trees exposed to temperatures below 12 °C drop their leaves and flowering and pollination are physiologically affected.

The fungi attack increases proportionally with the humidity and temperature, i.e., in Valle del Cauca, Colombia leaf development and fruit production are limited by the fungi Colletotrichium spp., (antracnosis) when 85% or more of ambient humidity is reported at 28-30 °C temperature (Escobar and Sanchez, 1988).

Because of its semideciduous habit the *A. muricata* requires active growth and vegetative development requires 800-1,000 mm^3/year, in the form of well distributed rainfall .

The *A. muricata* grows well in acid, sandy soils, porous, oolitic and preferentially limestone soils. Desirable growth is achieved in semi-dry deep soils (1.20 m), rich in minerals and well drained. Good aeration and drainage are required in any case. The soursop does tolerate lack of water. Soils with pH from 5.5-6.5 appear to be most suitable (Table 10) (Morton, 1987).

Table 10. Ecophysiological requirements of A. muricata

Climatic Factors	Range
Elevation	500 - 1,000 m SL
Climate	tropical
Temperature	25 - 30 °C
Wind	Moderate
Humidity	60 - 70 %
Soil	Limestone
Precipitation	800 - 1,000 mm
PH	5.5 - 6.5
Limitation	Fungi susceptibility

Pest and Diseases

In Queensland (Australia), the principal *A. muricata* pest is the mealybug (*Pseudococcidae* spp.). The soursop is an ideal host for fruit flies like *Anasptrepha suspensa*, *Anastrepha striata* and *Ceratitis capitata*, Red spiders are also problem in dry climates (Morton, 1987).

In Colombia leaves and fruits are attacked by insects *Homoptera* spp., *Acarina* spp., *Lepidoptera* spp., and *Coleoptera* spp., all of them present during the entire biological cycle of *A. muricata* and only the *Lepidoptera* spp., are present in the most juvenil stages. The species *Bephratelloides maculicollis* and *Cerconota annonella* drill throughout the fruit causing several yield problems (Table11) (Escobar and Sanchez, 1993; Donascimiento *et al*. 1998). The principal disease of *A. muricata* is caused by the fungus *Colletotrichium gloesporoides* (antracnosis). The attack of this fungi is general and affects the leaves as well as fruits and flowers. Important beneficial prey aphid agents for *A. muricata* are *Aphidius testataceipes*, *Chrysopa* spp., and *Curinus* spp.

Table 11. Reported A. muricata pathogens in Colombia plantations

Organ	Pathogen
Leaves-Branches	*Aconophora concolor, Aleurodicus giganteus, Aphis gossypii, Aphis spiraecola, Colapsis* spp., *Colletotrichium gloesporioides, Corticum salmolicolor, Corythucha ossypii, Diabrotica* spp., *Empoasca* spp., *Empocasca* spp., *Eriophyes Annonae, Hylesia* spp., *Leucoptera* spp., *Phylephedra* spp., *Sabulodes* spp., *Saissetia coffeae, Salenaspidus articulatos, Scolecotrichum spp., Tetranychus mexicanus, Toxoptora aurantii*
Trunk-Stem	*Atta* spp., *Colletotrichium gloesporioides, Cratosomus bombina, Nasotiternes corniger, Trachyderes interruptus.*
Flower	*Cercospora Annonae, Ciclocephala signata, Colletotrichium gloesporioides, Tecla ortignus, Toxoptora aurantii.*
Fruit	*Antiteuchus hediondus, Antiteuchus tripterus, Bephratelloides maculicollis, Cratosomus inaequalis, Cerconota annonella, Colletotrichium gloesporioides, Rhizopus stolonifer, Toxoptora aurantii.*
Root	*Armillaria mellea, Fomes lamaoensis, Phytophtora spp., Rhizocthonia spp.*

⇒ *Source: Morton (1987); Escobar and Sanchez (1993)*

Propagation

In Colombia as well as in other tropical countries the native orchards of *A. muricata* are comming from old seedling plantations. The soursop is propagated by *seeds*, but the variation among individual trees is often great. Germination of seeds causes no major problems and takes from 15-30 days if humidity is low (approx. 60%) and temperatures above 12 °C are maintained. Pre-treatment of seeds with fungicides are required and 90% of viability is expected after 5 months (Escobar and Sanchez, 1993).

In productive orchards the selected *A. muricata* plants are being propagated by *grafting*. This propagation is a recommended practice in order to maintain clonal selections. The soursop may be either budded, shield-budded, or grafted on rootstocks of the same species. Buds should be taken from mature non-petioled wood of the previous

year shoot growth. Seedlings or budded plants grow rapidly and come into bearing by the third year (Morton, 1987).

Grafting soursop (*A. muricata*) on custard apple (*A. reticulata*) or mountain soursop (*A. montana*) has a moderate dwarfing effect, whereas grafting on pond apple (*A. glabra*) rootstock has a strong dwarfing effect. If grafted on sugar apple (*A. squamosa*) or cherimoya (*A. cherimola*), vitality is limited, while reduction processes at the top of the tree are reported. In Ceylon and India grafting on sugar apple (*A. squamosa)* is preferred (Morton, 1987).

In Colombia there are no published reports on grafting, however the graft compatibility of soursop on soursop has been studied (Escobar and Sanchez, 1993). Cutting propagation of selected types has been implemented in Colombia, but no results are reported because the *A. muricata* has difficulties to induce roots.

Properties

The wood of *A. muricata* tree is pale, aromatic, soft, light in weight and not durable. It has been used for ox yokes because it does not cause hair loss on the neck. In Colombia it is deemed to be suitable for pipestems and barrelstaves. The wood of soursop has a high potential to produce paper pulp. Investigations in Brazil showed a cellulose content of 65 to 76% (Morton, 1987).

A. muricata fruits may be eaten fresh, when the fruit colour has turned to green-yellow. The flesh can be eaten with a spoon and the seeds plucked. Its pulp is cotton white, very succulent and sub-acid, the flavour is acid tang ”crisper”. The seeded pulp may be torn or cut into bits and added to fruit cups or salads, chilled and served as dessert with sugar and milk or cream, to prepare ice cream, nectar and juice. Those are the forms which are most preferred by the consumers (Figure 8). The unripe fruit can be eaten cooked, broiled or fried (Morton, 1987; Ijjász, 1999)

Unlike the other species *A. muricata* is a model fruit for processing. Its pulp does not oxidize, it maintains its pleasing aroma and flavour after frost manipulation to produce soursop pulp. In Colombia the pulp is seeded manually and some difficulties are reported (Escobar and Sanchez, 1993).

Nutritional Value

A. muricata fruit contain 85.5% pulp, 3.3% seeds, 8.9% skin and 2.9% of a flesh receptacle (Paull *et al.* 1982). The soursop taste is the result of a pleasant combination of sugars and organic acids (0.65 - 0.85%), of them, the most outstanding are malic- and citric acid in a ratio of 2:1. The concentration of soluble solids varies between 0.65-0.85 % (Table 12) (Morton, 1987; Escobar and Sanchez, 1993). The *A. muricata* fruit is climacteric, it ripens quickly and consequently the production of ethylene rises from 0.2-0.9 µl/kg/hour to 80-720 µl/kg/hour maximum until the sixth day. During ripening the starch hydrolyses rapidly to sucrose (16.5%), glucose (21.5%) and fructose (17%) (Paull *et al.* 1982; Bruinsma and Paull, 1984).

Figure 8. Some commercial products made with A. muricata

Table 12. Food components in 100g of edible portion of A. muricata

Component	Concentration	
Calories	53.1 - 61.3	kcal
Moisture	82.8	g
Carbohydrates	14.63 - 15.63	g
Ash	0.63 - 60.0	g
Fat	0.31 - 0.97	g
Crude Fiber	0.79 - 1.63	g
Protein	1.0 - 1.22	g
Calcium	10.3 - 22.0	mg
Potassium	45.8	mg
Magnesium	23.9	mg
Sodium	23.0	mg
Iron	0.47 - 0.64	mg
Phosphorus	26.0 - 27.0	mg
Starch	1.62	
Vitamin A	0.0 - 20.0	UI
Thiamine (B1)	0.06 - 0.11	mg
Riboflavin (B2)	0.05 - 0.07	mg
Niacin	1.28 - 22.00	mg
Ascorbic Acid	29.6	mg
Amino Acids		
Tryptophan	11.0	mg
Methionine	7.0	mg
Lysine	60.0	mg

⇒ *Source: Paull (1982), Morton (1987),*

Secondary Metabolites

The soursop or ”guanabana” has been used by the natives of Central and South America as medicine. All parts of the *A. muricata* tree are used in natural medicine in the tropics (Asprey and Thornton, 1955).

The Indians have recorded the use of ”guanabana or graviola” and diverse properties and uses are attributed to each part of the tree: bark, leaves, roots, fruit and seeds. In popular medicine in South America, herbal preparations derived from dried leaves and roots of *A. muricata* are used to control diabetes, as a sedative and are used as antispasmodic. The leaves and green fruits have been identified to combat diarrhoea and used as astringents.

In the Peruvian Andes, prepared tea from *leaves* is used against catarrh and the crushed seeds are used to kill parasites (de Feo, 1992). In the Brazilian Amazon basin, a leaf tea is used for liver problems (Branch and da Silva, 1983). The oil of the leaves and the unripe fruit is mixed with olive oil and used externally for neuralgia, rheumatism and arthritis pain (de Almeida, 1993). In Ecuador the leaf tea is taken as an analgesic and antispasmodic.

In the Peruvian Amazon the *bark roots* and *leaves* are used for diabetes and as a sedative and antispasmodic (Vasques, 1990). Indigenous tribes in Guyana use a leaf and/or bark tea of soursop as a sedative and heart tonic (Grenand *et al,* 1987). Feng *et al,* (1962) reported that bark and leaves extracts of soursop are muscle relaxant and cardio-depressant. Activity as hypotensive, antispasmodic and vasodilator has been assessed. *Roots* of the tree are employed as a vermifuge and the root bark as an antidote for poisoning.

In Jamaica, Haiti and on the West Indies, the *fruit* and/or fruit juice is said to be diuretic and serves as remedy for haematuria and urethritis, it is used against fever, against parasites, as a lactagogue, and against diarrhoea, and the bark or leaves are used as an antispasmodic, sedative, and neuroendocrine for heart conditions, coughs, grippe, difficult childbirth, asthma, asthenia, hypertension and parasites. Pulverised immature fruits, which are very astringent are indicated as a dysentery remedy (Asprey and Thornton, 1955).

Most of the active biocompounds of *A. muricata* are described as monotetrahydrofurans annonaceous acetogenins (Wu *et al,* 1995a; Gleye, 1997a,b; 1998; Yu *et al.* 1998) as: amyl-caproate, amyloid, annomuricins - A, -B, -C, annomutacin, annonacin-10-one, annonacin-10-one, annonacins, annonain, anomurine, anonol, atherosperminine, β-sitosterol, campesterol, cellobioside, citric acid, citrulline, coclaurine, coreximine, dextrose, ethanol, folacin, fructose, gaba, galactomannan, geranyl-caproate glucose, gigantetrocin A-gigantetronenina, goniothalamicin, hydrocianic acid, 2,4-*cis*-R-annonacin-A-one, 2,4-*cis*-*iso*-annonacin, 2,4-*trans*-10-R-annonacin-A-one, 2,4-*trans*-isoannonacin, isocitric acid, lignoceric acid, malic acid, manganese, mericyl alcohol, methanol, methyl-hexano,2-enoate, methyl-hexanoate, muricapentocin, muricatetrocins -A, -B, muricatetrocins A and B, muricatocins -A, -B, -C, muricine, muricinine, muricoreacin, myristic acid, N-*p*-coumaroyl tyramine, Monotetrahydrofuran acetogenin, ρ-coumaric acid, paraffin, potasssium chloride, procyanidin, reticuline, scyllitol, stearic acid, stepharine, stigmasterol, sucrose, tannin, xylosyl-cellulose

Toxicity and Antimicrobial Activity

Several studies have demonstrated that leaf, bark, root, stem and seed extracts of soursop are antibacterial *in vitro* against numerous pathogens (Branch and da Silva, 1983; de Feo, 1992; Bories *et al.* 1991) and the bark has fungicidal properties (Lopez-Abraham, 1979).

The leaf extract showed to be active against malaria. Isoquinoline derivatives found in soursop fruit were showing anti-depressive effects in animals (Hasrat *et al*, 1997).

Methanolic acetogenins extracts of *A. muricata* and *A. cherimola* seeds were isolated and tested against the parasitic activity of the larvae *Molinema desetae* and activity inhibition has been reported (Bories *et al.* 1991).

The cytostatic activity of aqueous, alcoholic and ketonic extracts of *A. muricata* were tested on the fungi (*Ascomiceto*) *Neurospora crassa*, inhibition of growth percentages was reported (Lopez *et al*, 1979).

The bark of the trunk and the seeds of soursop contain certain alkaloids like ”anonina”, ”muricina” and ”muricinina”, which can be used to produce bio-insecticides. It produces hydrocyanic acid. The bark has been used in tanning. Leaves and bark have been used as fish poison (Aguilar *et al.* 1947). The seeds contain 45% of a yellow non drying oil, which is an irritant poison, while pulverised seeds are effective pesticides against lice and aphids (Morton, 1987).

The bio-activity of *A. muricata* leaf compounds is provided by the effect of muricatocins -A and -B, 2,4-trans-10-R-annonacin-A-one, 2,4-cis-R-annonacin-A-one and N-p-coumaroyl tyramine, which significantly enhanced cytotoxicity against the A-549 human lung tumour cell line and the MCF-7 human breast solid tumour cell lines (Wu *et al*, 1995b, 1995c, 1995 d).

The cancer researches are also concentrating on soursop. Several scientific studies have demonstrated the anti-cancerous properties of some phytochemical derivatives from leaves, seeds and stem, which are cytotoxic against cancer cells (Zeng *et al*.1996). A new mono-tetrahydrofuran ring acetogenins from *A. muricata* is cytotoxic against Colon Adenocarcinoma cells, in which it, reached 10.000 times the potency of Adriamycin (Doxorubicin). Chemotherapy drug for some types of cancer, manufactured by Pharmacia & Upjohn (Rieser *et al*, 1996).

2. Aims

Among of the worldwide aims in *A. cherimola* and *A. muricata* are: *Development of technical plantations*, *control of* the typical *diseases* and *promotion* of healthy *high quality trees*.

The exploration, collection, conservation, and evaluation of *A. cherimola* and *A. muricata* germplasm, not only in natural conditions, but also in repository gardens are in 1999 a register priority as result of the neglected knowledge of these species in those countries where they are part of the native flora. Furthermore the application of plant research and breeding programs on *A. cherimola* and *A. muricata* is still in development principally in those countries where they have been introduced.

The *characterization* of *A. cherimola* and *A. muricata* natural genotypes, *selection* and *multiplication* of the elite plants by any propagation system that ensure the preservation of interesting genotypes, are calling the attention of *Annona* cultivators.

The *A. cherimola* plant and fruit are not cold tolerant, thus natural selection, multiplication and *promotion of cold resistant genotypes* from the tropical highlands mountains in South America are a priority not only to transport this fruit long distances in containers with controlled atmosphere, but also to promote its technical development on the native countries.

The *A. muricata* is a fungi disease sensitive plant, thus selection of trees carrying natural resistance against *Colletotrichum* spp. is desirable in all the geographic regions where it is planted. Otherwise, fruits of *A. muricata* seedless and tasting pulp are one of the Colombian farmers additional expectations.

If selected genotypes of *A. cherimola* or *A. muricata* are propagated traditionally by seeds a high genotype variation is expected. Otherwise if conventional vegetative propagation methods are applied, the dichogamous protogyneous flower behaviour in *A. cherimola* and *A. muricata* is promoting a intervarietal crossing. This situation is not only well known in *Annona* spp., as well as in another fruit species with this type of flowering. The natural conservation of a selected genotype by conventional propagation methods has been until now impossible therefore, micropropagation of *A. cherimola* and *A. muricata* by tissue culture is actually the alternative of clonal multiplication.

The *in vitro* multiplication of *A. cherimola* and *A. muricata* selected genotype is hypothetically profitable because the heterozygosity degree is not expected in a clonal multiplication system, the *in vitro* regenerants could be included in further plant breeding programs. This type of vegetative clonal multiplication might be not only safe but also economic in terms of time. The storing and monitoring of selected fruit tree clonal material in smaller units is possible.

The *in vitro* multiplication of *A. cherimola* and *A. muricata* selected germplasm could rapidly increase the existing ancient genotypes, in danger of extinction, at the tropical high- and low-lands in America. Those trees are possibly carrying the competitive commercial characteristic, desirable today on the world trade market of exotic commercial plants.

2.1 Needs for in vitro Culture in *Annona* spp.

Genetic Diversity

It is obvious that a great inherited variation of *Annona cherimola* and *Annona muricata* exists not only in the centres of origin, South- and Central America; but also in the tropics and subtropics areas where they have been successfully adapted and cultured (Jordan and Botti, 1992).

Selections of *A. cherimola* and *A. muricata* differ considerable in phenotype pattern, when they grow at different locations in the same area *e.g.,* the fruit characteristics, plant pattern growth and cropping capacity are modified by the micro-ecosystem conditions (George, 1984; Gardiazabal and Rosenberg, 1988; Sanewski, 1988; Tijero, 1992).

The characterization and use of these non exploited natural variations of *A. cherimola* and *A. muricata* will lead the development of improved and fashion-tailored trees (Reginato and Lizana, 1980).

There are in South America native wild ecotypes of *A. cherimola* and *A. muricata*, well adapted to the natural extreme conditions. Of them, some should be cold resistant and naturally protected against fungi diseases. The identification and massive multiplication of those ecotypes should be the first priority in terms of plant germplasm conservation (Bridg, 1993a).

Procurement clones, showing greater productivity, vigour, cold tolerance and other physiological traits is a great advantage, which is provided by the selection of elite *Annona* spp., cultivars (Jordan, 1988).

The application of Plant Tissue Culture methods in *A. cherimola* and *A. muricata*, will allow the mean development of economically and rapidly producing, large numbers of clonal plants. The micropropagation procedures have also proven to be useful for the physiological and genetic uniform plant maintenance, the research purposes find on it additional benefits (Davis and Keathley, 1992).

Increment of Natural Types

Selection and propagation of productive, healthy *A. cherimola* and *A. muricata* trees from ancient germplasm and establishment of orchards to promote the natural conservation and observation of these species is important in order to impulse the knowledge of them. Otherwise, the development of a successful micropropagation method should be enable to improve the clonal propagation of superior *A. cherimola* and *A. muricata* earliest natural selections in danger of extintion (Cordoba, 1969).

Nevertheless characterization of selected types of *A. cherimola* and *A. muricata* in order to identify either genetic variation or define genetic identity between selections, applying conventional biological methods, with a high probability is a time consuming

process with a high probability of fail information (Anderson and Richardson, 1990; Castro and Maia, 1984; George *et al.* 1986). The application of the new developments in molecular biology based on the DNA technique would identify selections of *A. cherimola* and *A. muricata* in a short period of time with a high probability of success (Ronning and Schnell, 1995).

Long Juvenile Periods

The *Annonas* are slow growing semi-deciduous trees (Samson, 1986) and with exception *A. muricata*, drop their leaves during the cool season and remain bare and dormant for several months. The length of the juvenile period varies between selections, however the first important fruit production begins after eight years. The juvenile period is extremely variable and greatly influenced by seedling rootstock and type of scion (Jordan and Botti, 1992).

Heterogeneity

The out-breeding of the *Annona* spp., which turn in heterozygosis, is a consequence of the dichogamous protogyneous flower behaviour (Sainte-Marie, 1987). The manual pollination is a common practice in productive orchards of *A. cherimola* and *A. muricata* and the human manipulation can also promote plant variation promoting genetic combinations uncommon in the nature (Richardson and Anderson, 1996).

In general the commercial varieties of this genus are coming from openly pollinated seeds (Clark, 1992; Farre *et al*. 1977). Although this is advantageous for certain broad breeding objectives, it is difficult to „fix“ specific characters in a reasonably short period of time (Vargas de la Fuente, 1986).

The *Annona cherimola* and *Annona muricata* are traditionally propagated by seeds, because this is the cheapest and most reliable method for establishing commercial plantations. However, commercial orchards derived from seedling populations of selected material are unsatisfactory because of the high degree of heterozygosity promoted (George and Nissen, 1987). The variations in fruit form, color, cold hardiness, pulp and timber quality are wide-spread by seeds. Consequently clonal propagation of both scions and rootstocks from superior selections is highly desirable (George, 1984).

Propagation of *Annona* by grafting has been made, but with considerable limitations in costs and time. Graft incompatibilities do exist and grafted plants are influenced by the variability imparted by the rootstock. The *A. cherimola* genotypes selected for invasive root systems, for example, cannot be propagated by grafting. Similarly if *A. cherimola* cold-resistant scions are grafted on untried seedling material, they survive at low temperatures but eventually die later on, due cold injury (George, 1984).

Tree Improvement

The *Annona* spp., as well as other woody tree species are problematic, when vegetative propagation of mature trees is desired, once a tree is old enough to determine its merits,

it becomes very difficult or even impossible to root cuttings on a large scale (Gupta *et al.,* 1981).

Proportional with the aging of the orchards the *A. cherimola* and *A. muricata* looses of rooting ability, difficulties with hibernation of rooted cuttings are increasing and changes from plagiotropic to orthotropic growth are accelerating in contrast, the *in vitro* propagation of mature trees by cuttings is suitable (Gupta *et al.,* 1981). Beyond this, the *in vitro* culture itself can exhibit a rejuvenation effect (Mullins *et al.,* 1979).

The main application of the plant tissue culture techniques in *A. cherimola* and *A. muricata* would be the further propagation of a large number of selected superior mature trees. The massive promotion of genotypes not only with invasive root systems, that are difficult to root by conventional methods, but also those which are superior and cold resistance furthermore, the flowering genotypes with desirable fruit in size and pulp qualities, are the primary goals for improvement.

The micropropagation systems allow to test the *Annona* mature genotypes, which can be used to promote a high-performance multi-clonal varieties. The genetic variation typical in *Annona* seedling progenies could be avoided. The micropropagation procedures have also proven to be useful for maintain physiological and genetical uniformity of the plant material (Barlass, 1990).

2.2 Biotechnology Applications on *Annona* spp.

Micropropagation

In vitro propagation from *Annona* spp., has been described by some authors with limited success. The most common limiting problems have been explant selection, avoid or control of phenolics, difficulties to eliminate exogenous and endogenous contaminants without induce tissue damages, low rate of shoot multiplication, sporadic rhizogenesis and hardening (Table 13).

Haploid plants have been reported from anther culture of *A. squamosa* (Nair *et al.* 1983). Triploids have been obtained from endosperm culture of mature *A. squamosa* seeds, shoot organogenesis was induced from callus, rhizogenesis was not successful.

The cytological analysis of roots and leaf tips of regenerated plants has been reported ($3n$ =21) (Nair *et al.,* 1986). Direct shoot organogenesis of *A. squamosa* has been reported from seedling leaf explants, root organogenesis was not reported (Nair *et al.,* 1984).

Micropropagation from nodal explants with axillary buds from *Annona* hybrid (*A. squamosa* x *A. cherimola*) have been reported, rooted plants and field transfer have been promoted (Nair *et al.* 1984). From hypocotyl and seedling petioles of *A. cherimola* shoot organogenesis have been reported (Jordan, 1988) and rooting has been sporadically promoted.

Direct shoot and root formation in *A. cherimola* hypocotyls were studied and the outcomes were reported with regard to the regeneration potential under *in vitro* conditions. Explants, culture conditions and hormone levels required to induce morphogenic responses for examination of plant regeneration have been reported

(Jordan *et al.,* 1990). Adventitious shoot buds developed directly from hypocotyl explants and the rhizogenesis potential of *A. muricata* shoot have been studied (Bejoy and Hariharan, 1992).

The high activity of the polyphenoloxidase on *A. cherimola* (Martinez-Cayuela *et al.,* 1988a, b) and the presence of phenolic compounds on the *in vitro* culture medium coming from the explant in general are limiting the *in vitro* propagation of *A. cherimola* (Bridg, 1993b). The morphogenic responses of *A. cherimola* is affected by phenols (Jordan *et al.,* 1991). Fruits from *A. cherimola* and *A. muricata* species after picking are affected by phenols, the tissues take a brown colour and the fruit is quality reduce (Vargas de la Fuente, 1986).

Table 13. In vitro approaches on Annona spp.

Species	Explant	Regenerated Organs	Author	Country
Annona spp.	mesocarp	callus	Bapat and Narayanaswamy, 1977	India
	endosperm	shoots, roots*		
Atemoya	leaf	shoots	Nair *et al.* 1984a	India
	hypocotyls	rooted shoots	Rasai *et al.* 1994, 1995	
A. cherimola	petioles	shoots, roots*	Jordan, 1988	Chile
	hypocotyls	shoots, roots*	Jordan *et al.* 1991	
	zygotic embryos	shoots	Jordan *et al.* 1992	
	bud	shoots	Tazzari *et al.* 1990	Italy
	bud	shoots	Bridg, 1993b	Colombia
A. muricata	bud	shoots, roots *	Encina *et al.* 1994	Spain
	hypocotyl	rooted shoots*	Bejoy *et al.* 1992	India
	hypocotyl	adventitious bud, shoots, roots *	Lemos and Blake, 1998	Brazil & U.K.
A. squamosa	internodes	shoots, roots *	Lemos and Blake, 1996	
	anthers	haploids	Nair *et al.* 1983	India
	leaf	shoots	Nair *et al.* 1984	
	endosperm	shoot, roots *	Sreelata *et al.* 1986	
	endosperm	shoots	Nair *et al.* 1986	

⇒ * *root formation as regeneration potential under in vitro conditions*

The regeneration of *Annona* spp., from explants of clonal origin has not been reported (Jordan and Botti, 1992). On *A. squamosa* root and organogenesis has been induced form anther, endosperm and leaf explants, as well as in *A. cherimola* hypocotyl, petiole, internode and nodal cuttings. Hypocotyl and nodal cuttings of *Atemoya* (Rasai *et al.* 1994) and *A. muricata* have proved to be suitable for *in vitro* culture. Shoots from hypocotyl and nodal segments of some *Annona* spp., have been successfully rooted (Rasai *et al.* 1995). The effect of arbuscular mycorrhizal fomation have been investigated on *A. cherimola* rooted *in vitro* plantlets (Azcoaguilar *et al.* 1994; 1996).

From hypocotyls of *A. muricata* seedlings adventitious bud and shoot proliferation were achieved, rooting and acclimatization have been studied (Lemos and Blake, 1996).Adventitious shoot regeneration from internodal explants of mature plants of *A. muricata* from meristem culture have been reported, rhizogenesis was not achieved (Lemos and Baker, 1998).

Genetic Variation

Genetic variation among cherimoya cultivars has been studied using isozyme markers, and DNA analysis by Polymerase Chain Reaction and Restriction Fragment Length Polymorphic (PCR-RFLP), Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphic (AFLP).

Mendelian inheritance was demonstrated on five clonally propagated varieties of *A. cherimola* by the linkage of isozymes (Lee and Ellstrand, 1987). Cultivar identification by isozyme variation patterns in 15 varieties of *A. cherimola* and 1 *Atemoya* (*A. cherimola* x *A. squamosa)* has been reported (Ellstrand and Lee, 1987).

The allelic segregation of 13 isozyme loci in hand fertilized heterozygous *A. cherimola* trees was demostrated (Pascual *et al*., 1993). Gametic selection appears to be the main contributer, although zygotic selection seems also to play a part (Perfectti and Pascual, 1996).

Isozymes have been used as genetic markers to characterize more than characterized 200 *A. cherimola* and *Atemoya* (*A. cherimola x A. squamosa)* plants, however the isozyme analysis is limited by the relatively small number of loci (Perfectti and Pascual, 1998 a,b).

The phylogenetics of *A. reticulata*, *A. glabra*, *A. muricata*, *A. montana* and *Atemoya* (*A. cherimola* x *A. squamosa*) were studied by PCR-RFLP. A basic information in the phylogenetic studies of *Annona* spp. could be made using a large number of experimental units (Rahman *et al.* 1997).

The RAPD have been applied to identify the species *A. reticulata*, *A.muricata*, *A. cherimola*, *A. squamosa* and some interspecific hybrids of *Atemoya*, this molecular marker technology showed to be fingerprinting efficient method to identify genotypes within between *Annona* species with an expected Mendelian fashion.

The genetic diversity of 19 *A. cherimola* cultivars classified into five types based on their skin texture was taxonomically identified and differentiated by AFLP, this molecular marker technologie provides a better resolution, AFLP enables to analyse the phylogentic relationships between *A. cherimola* cultivars (Rahman *et al*. 1998).

RAPD markers offer the potential of generating large number of markers representing a random sample of the genome, thereby presents an advantage over isozyme markers (Ronning *et al*. 1995).

Chromosome Number

In *Annona* spp., cytological and karyological studies have been limited due the tendency to form chromosome clumps. Hutchinson (1923) described for *Annona* karyotypes and

outstanding its fixed predominance for taxonomic and phylogenetic relations. Bowden (1948) figured the chromosome complements of nine species of *Annonaceae,* some of the species showed one pair of large chromosomes and one small pair, e.g., *Asimina triloba* and *Annona muricata.* In *Annona cherimola* the two small chromosomes are also reported, one large pair of chromosomes and other pair almost as large can be recognized. The two small chromosomes frequently stuck to or were hidden by larger chromosomes (Table 14).

Bowden (1948) reported the chromosome number (x = 9) and (x = 8) for most of the species of *Annona* genus except *A. glabra* (x = 7) because it may be tetraploid (4n = 28) (Table 14). Sreelata *et al.* (1986) analyzed the roots and young leaf tips of *A. squamosa* cytologically and reported a triploid (3n=21) chromosome number. The *Annona* spp. Have a ruminate type of endosperm which is characteristic of the *Annonaceae* family (Bhojwani and Bhatnagar, 1978).

The *Annonaceae* species shows distinctive cytological characteristics such as chromosome number, range of chromosome size and fixation image (Bowden, 1948) but discrepancies in counting *A. cherimola* and *A. muricata* chromosomes may have been caused by the failure to observe the two small chromosomes which are often obscured by the larger chromosomes. Nair *et al.* (1983) reported that haploid *A. squamosa* could be regenerated from anther callus. Regenerated plants had the haploid (n = 7) chromosome number and survived transplantations

Table 14. Reported chromosome number in A. cherimola and A. muricata

Species	Number	Origin	Established	Author
A. cherimola Mill. (cherimoya)	*2n = 14*			Kumar and Ranadive, 1941
	2n = 16			Bowden, 1948
	2n = 16			Darlington and Wylie, 1956
Annona muricata L. (soursop)	*2n = 16*	Colombian seed plants	Berlin Botanical Garden, Germany	Bowden, 1948
	2n = 16	Colombian seed plants	Walter R. Lindsay, Canal Zone Experiments Gardens, Panama	Bowden, 1948
	2n = 16	Colombian seed plants	F.G. Walsingham, Atkins, Institution of the Arboretum, Soledad, Cienfuegos, Cuba	Bowden, 1948
	n = 7			Kumar and Ranadive, 1941
	2n = 14			Darlington and Wylie, 1956 Simmonds, 1954

3 Micropropagation

The *in vitro* plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organs under sterile conditions and controlled environmental conditions (Murashige and Skoog, 1974).

The *in vitro* culture is often used as *model system* in the study of various physiological, biochemical, genetic and structural problems related to plants. There are several ways to promote the *in vitro* regeneration of a selected plant material relying on the initial material (Torres, 1989):

– Plant culture	⇒	From seedlings or larger plants
– Embryo culture	⇒	From isolated mature or immature embryos
– Organ culture	⇒	From isolated plant organs
– Tissue culture or Callus culture	⇒	From tissues arising from explants of plant organs
– Suspension culture or Cell culture	⇒	From culture of isolated cells or very small aggregates remaining dispersed in liquid medium
– Protoplast culture	⇒	Culture from protoplats, *i.e.*, cells devoid of their retaining walls
– Anther culture or Haploid culture	⇒	Micropropagation of anthers and/or immature polen grain in an effort to obtain a haploid cell or callus line

According to the objectives to search for: mineral salt combinations, plant growth regulators and environmental factors are variables which can be manipulated to promote organogenesis, differentiation and dedifferentiation of the selected initial cultured explant (Torres, 1989 ; de Fossard, 1977). Plant tissue culture techniques have a great potential as a means of vegetatively propagating economically important crops and crops of future potential on a commercial basis (Roca and Mrogiski, 1991).

Micropropagation is a vegetative multiplication system based on the promotion of growth of microcuttings from axillary buds of apically dominant plants, growth of shoots from nodal sections, dissection of axillary shoots on rooting medium and production of small rooted shoots (Drew, 1997).

Micropropagation has been defined as *in vitro* regeneration of plants from organs, tissues, cells or protoplast (Beversdorf, 1990) and the *true-to-type* propagation of a selected genotype using *in vitro* culture techniques (Deberg and Read, 1991).

As shown in the Table 15 the promotion of *in vitro* plants is divided in stages (Murashige, 1974), which describe not only the micropropagation steps but also identify the morphogenic events of the selected *explant* or initial culture plant material in aseptic conditions.

Table 15. Micropropagation steps

Stage	Description	Objective	Activity
0	Stock Plant Selection and Adaptation	Reduction of endogenous tissue contaminants	Definition of the stock or mother plant and source preparation of explants
I	Establishment	Promotion of in vitro aseptic culture	Explant selection, elimination of exogenous contaminants and new in vitro adaptation
II	Multiplication	Somatic cell embryogenesis Enhanced axillary development Adventitious shoot development	Promotion of In vitro cell or tissue organogenesis and rapid differentiation-multiplication of new shoots
III	Rooting	Promotion of in vitro plant regenerants	The rhizogenesis of the new in vitro derived shoots is stimulated under ex vitro or in vitro conditions
IV	Acclimatization	Stimulation of autotrophic metabolism	Hardening of vegetative structures
V	Field	Improvement of the normal development of an in vitro regenerant	Open growth of regenerants

Application

Micropropagation has also been useful for the rapid initial release of new varieties prior to multiplication by conventional methods, e.g. pineapple (Drew, 1980) and strawberry (Smith and Drew, 1990).

Micropropagation is also used to promote germplasm storage for maintenance of disease-free stock in controlled environmental conditions (Withers, 1988) and in long term via cryopreservation (Kartha, 1985).

This type of *in vitro* vegetative propagation has important benefits to produce stable lines in plants that have no named varieties *e.g., Annona* spp. (Bridg, 1993b), *Australian dioecious* papaw genotypes (Drew, 1988; Drew, 1992) where traditional plant breeding has failed. It has also a great potential for the propagation of important crops like: *Cassava* spp., *Oryza* spp., *Phaseoulus* spp., *Solanum* spp., principally (Roca and Mroginski, 1991). The micropropagation of elite or selected plants showed good results which benefit the agriculture, horticulture and forestry (Conger, 1981; Drew, 1997).

Worldwide there is much interest to promote the development of an *in vitro* technology that permits the propagation and breeding of commercial valuable woody, semiwoody, ornamental, basic food, industrial and medicinal plants. These species are in danger of extiction and should receive a priority in terms of gemplasm conservation (Conger, 1981; Deberg and Zimmerman, 1990; Drew, 1997).

Limitation

In Plant Tissue Culture the genetic stability of the *in vitro* produced plants is cause of preocupation when clonal micropropagation is applied in terms of conservation and incrementation of germplasm lines. The *in vitro* promotion of clonal *true-to-type* plants is a priority, as result of the report of *true-off-type* plants comming from *in vitro* culture (Skirvin, 1978).

When an *in vitro* clone multiplication system introduces a high risk of genetic variation, it is not desirable. The culture-derived plants have to represent genetically the source of material (Withers, 1989) and failures to understand this principle have resulted in disastrous consequences for some species (Smith and Drew, 1990b; Beversdorf, 1990).

There are numerous reviews that describe the variability observed on *in vitro* plant regenerants (Reisch, 1983; Evans, 1984; Evans *et al.* 1984). This changes are *in vitro* regenerants with a new genome modifications as the result of culture media traces that are interferring the plant genome e.g., *chimeras* or *genetic off-types* (Krikorian *et al.* 1983; Tilney-Bassett, 1986; Torres, 1989).

The ability to propagate *in vitro free-off* plants or *genetic off-types* is dependent on the technique used during the micropropagation (Pierik, 1987).The tissue culture variability could be a directly generated effect of several and interacting factors on the multiplied cells (Karp and Bright, 1985; Vuylsteke *et al.* 1988; Meier *et al*. 1988).

Murashige (1974) has described three types of *in vitro* culture differentiation, which have been defined earlier for cultures of higher plants and are dependent of the cell-tissue organization of the initial explant:

I. Organized tissue

- The cultures of almost whole plants, embryos, seeds, pre-formed buds and organ cultures are termed as organized cultures
- The organizational characteristic structure of individual cell, organ and tissue of a plant is being maintained
- It closely resembles *in vivo* vegetative propagation by cuttings, division, runners and axillary buds or shoots
- If the organizational structure is not broken identical progeny arises to the original plant material

II. Non Organized tissue

- If cells and/or tissues are isolated from an organized part of a plant and de-differentiation divisions are induced and non-organized growth in the form of a callus tissue is promoted, the original tissue culture material in this case is a non-organized tissue structure.
- If the callus disperses clumps of cells aggregates and/or single cells results also referred to suspension culture a non organized tissue structure is promoted.
- Non-organized growth is mainly induced by the use of *very* high concentrations of auxin and/or cytokinin in the nutrient medium.
- The genetic stability of non-organized cultures is often low.

III. Combination of non-oganized and oganized tissue

- This type of culture is intermediate between types I and II. *Cells* in an isolated organ or tissue are first de-differentiate and then from tissues or a layer of callus tissue, by division from which organs e.g., roots and/or shoots, or even whole individuals, pro-embryos or embryos, often rapidly develops.
- It must be taken into account that organized structures can develop from non-organized cultures either through special techniques or spontaneously.
- In all cases the progeny is often not completely identical with the original plant material.

Protocols for *in vitro* plantlet regeneration via organogenesis could be the basis of the development of a transformation system applied on *Annona* spp. Molecular markers would be useful both for documenting genetic diversity and elucidating the confusion on the name and origin of many commercial cultivars (Jordan and Botti, 1992; Drew, 1997).

3.2 Somaclonal Variation

The term somaclonal variation has been used by Larkin and Scowcroft (1981) and refers to the phenotypic and genotypic variation observed in regenerated plants from any form of cell culture.

When an explant, any plant segment, is subjected to a tissue culture cycle it might be possible to obtain a somaclonal variant (D'Amato, 1978, Rani *et al.* 1995, Hashmi *et al.* 1997). This cycle includes establishment of a dedifferentiated cell or tissue under

defined conditions and subsequent organogenesis and regeneration of plants (Hammerschlag, 1992). The genetic variation has been shown to originate either from the original explant or from exposure to a tissue culture cycle (Skirvin and Janik, 1976) and is often heritable (Larkin *et al.* 1984; Breiman *et al.* 1987).

Factors

The source of somaclonal variation has not been completely explained and the degree and frequency of somatic genetic changes *in vitro* is uncertain (Phillip *et al.* 1990). Variation, as has been shown, is conditioned by source of material, plant genotype, kind of explant, cell segregation of primary explant, development, organogenesis (D' Amato, 1975; Drew, 1997) and number of subcultures (Skirvin and Janik, 1976). All of these factors are correlated with time of culture and culture media (D'Amato, 1964; Evans and Gamborg, 1982; Dolezel and Novak, 1984; Scowcroft *et al.* 1987; Schilde-Rentschler and Roca, 1987).

The importance of culture medium and particularly the exogenous application of plant growth regulators (PGR*s*) has been discussed (Terzi and LoSchiavo, 1990). The embryogenic potential of explants is closely associated with the content of PGR*s*, the balance and concentration among naturally produced and exogenous applied PGR*s*.

Due to the complexity and sophistication of the techniques required for PGR*s* analysis full recognition of these techniques is still in process. Only a few research teams have found links between the embryogenic capacity of plant tissues and a specific endogenous hormonal content (Rajaserakan *et al.* 1987a, b; Wenck *et al.* 1988; Ivanova *et al.* 1994).

Cytokinins required for shoot induction and multiplication may cause abnormalities such as decreased rooting, stunted or compact plants, increased branching or slender stems and leaves (Karp and Bright, 1985). Albinism is an abnormality that often occurs during *in vitro* propagation. The albino plants might be due to the dis-organization of the ribosomal RNA*s* and plastids *in vitro* and (Torres, 1989).

Abnormalities in regenerated plants have been less frequently attributed to auxins (Smith, 1979) as abnormal fertility of the flowers, abnormal petal shapes, decreased vigor, malformed leaves, and increased lateral shoot formation (Bilkey and Cocking, 1981). Other factors that may result in the production of abnormal plants include Gibberellic acid (GA_3), temperature, osmotic potential of the medium and agar or gelrite concentration (Torres, 1989).

Phenotypic changes

Phenotypic variation is expressed principally by changes in morphological characteristics, which can be affected for instance by growth habit, plant height, shoot length, number of primary branches, flower quality, fruit colour, production, uniformity, disease resistance and tolerance to environmental conditions (Skirvin, 1978; Reisch and Bingham, 1981; Evans *et al.* 1984; Hammerschlag, 1992). This variation in plant regenerates has been reviewed in most of the cases for some herbaceous plants, but not for woody species (Economou *et al.* 1981).

Some phenotypic changes observed in *in vitro* derived plants include the presence or absence of pubescence, changes in leaf morphology, dwarfs, loss of pigmentation, and alteration in flower morphology. Reduction or enhancement in plant vigour may also occur in plants propagated through tissue culture. Many of these abnormalities appear to have an epigenetic or physiological basis and are therefore reversible. Variegation in leaves may appear in certain species and disappear in others when plants are propagated *in vitro* (Torres, 1989).

Genetic changes

Somaclonal variation due to karyotypic changes is a widespread phenomenon in plant cell culture, and it might affect plant breeding (Larking and Scowcroft, 1981; Wenzel, 1985). Somaclonal variants in some cases seem to be similar in magnitude to regenerates from cells exposed to mutagenic agents.

Although a number of different types of genetic mutations has been described as the basis for somaclonal variation (Larkin and Scowcroft, 1981), the recognition of genetic and epigenetic changes that occurs during *in vitro* culture and on plants derived from these cultures actually remains to be discussed (Reish, 1983; Krikorian, 1983; Roca and Mroginski, 1991).

New genome arrangements, aneuploids not separable in culture, mitotic break off that conduces to polyploid lines, genome reorganisation by transposoms, amplification or reduction of genes, effects of inversion, trans-location, changes in chromosome number, chromosomal rearrangements, karyotype variation, prevalence of specific plant karyotype in polysomatic plants or tissues, alterations of the interaction nucleus-cytoplasm have been demonstrated as basis of somaclonal variation (Larkin and Scowcroft, 1981; Karp *et al.* 1982; McCoy *et al.* 1982; Orton, 1983).

Screening

The genetic stability or fidelity adviser of *in vitro* produced crop plants are gaining importance (Larkin and Scrowfort, 1981; Goto *et al.* 1998) with special emphasis on chromosome arrangements (D'Amato, 1978; Whiters, 1989).

The characterization of clone regenerants comming from *in vitro* culture by traditional methods such as morphological descriptions, physiological supervisions and cytological studies have been made. These methods are time-consuming processes based on characters which can be affected by the *in vitro* manipulation and it is no easy to differentiate clonal selections or to predict genetic identity with a high probability (Patel and Berlyn, 1982, Meier, 1982, Mo *et al.* 1989).

Moreover, biochemical molecular analysis by isoenzymes and electrophoresis techniques to separate molecules have been developed in order to detect genome modifications (Renfroe and Berlyn, 1984; Mo *et al.* 1989; Shenoy and Vasil, 1992). However, these analytical approaches have serious limitations because are describing only partially the genetic changes and there are difficulties in assess those changes if any, at the DNA sequence level. In addition the evaluation and interpretation of the results cover many years of research and probes are time dependent. (Rani *et al.* 1995; Rani and Raina, 1998).

The DNA molecule maintains its characteristics permanently because it is not conditionated by the time, irrespectively of its source. The molecular DNA technology by the genetic markers is comparatively less complex, quick to perform and requires only a small amount of probe material. Furthermore DNA extraction protocols are simple, suitable and cover a wide spectrum (Rafalski *et al.* 1993a)

Recently several DNA molecular techniques such as Restriction Fragment Length Polymorphic DNA (RFLP) (Shirzadegan *et al.* 1991), Randomly Amplified Polymorphic DNA (RAPD) (Rani *et al.* 1995), DNA Amplification Fingerprinting (DAF) (Vos, 1995) and Arbitrarily Primed PCR (AP) (Vos *et al.* 1993) have been introduced and offer several advantages compared with the cytological, biochemical and physiological methods, where the fundamental and practical questions of plant tissue culture remain unsolved (Williams *et al.* 1990).

The application of DNA molecular markers in genetic mapping, genetic diagnostics, molecular taxonomy and evolutionary studies has been established since 15 years ago, nevertheless DNA polymorphisms origins and causes are still in study (Vos *et al.* 1995).

3.3 Random Amplified Polymorphic DNA

Detection of DNA markers can be based either on Southern hybridisation or Polymerase Chain Reaction (PCR) amplification techniques. In Southern hybridisation the detection of a fragment is based on sequence homology with the DNA probe. Therefore, this method enables the detection of homologous DNA sequences among distantly related species using the same DNA probe. This has applications in research on genome evolution. Otherwise the DNA fragments detection by Southern hybridization is laborious and incompatible with applications which requires a high resolution.

The most commonly used DNA markers are RFLP (Restriction Fragment Length Polymorphisms) because of its reliability and the possibility of detecting multiple alleles of the same locus. The utility of DNA markers for genetic mapping has enabled the location of several genes of agronomic interest within the context of an existing RFLP Polymorphic map (Rafelsky *et al.* 1993).

The PCR recognition of specific sequences by the PCR-primers allows for very little sequence redundancy, therefore, PCR-based markers are generally highly species-specific (Vos *et al.* 1995) Among PCR-based marker technologies two main types of techniques may be distinguished, targeted and random amplification techniques.

The random amplification techniques rely on PCR techniques using arbitrary primers at low annealing temperatures. The amplification products are the primer binding sites. Fragment patterns cannot be predicted, but are primer specific. All these random DNA polymorphisms are generally detected as presence/absence of amplified fragments. These marker techniques are known as mono-allelic DNA markers (Vos *et al.* 1995).

Genetic tests based on PCR (Polymerase Chain Reaction) are simple to perform, but target DNA sequence information is required to design specific primers. The Random Amplified Polymorphic DNA markers (RAPD), have recently been applied in woody species (Goto *et al.* 1998) to asses the reproduction of some segments of the genome, as rapid appraisal of tissue-culture-derived plants (Rani and Raina, 1998). They

have been shown to enhance breeding efforts in annual and perennial crops (Rafelski and Tingey, 1993). They are also effective for cultivar identification (Ronning *et al.* 1995b).

The DNA amplification with RAPD not requires previous knowledge of natural target DNA sequence. The amplification of random DNA segments is made with single primers (usually 10-mers) of arbitrary nucleotide sequence (Williams *et al*. 1990). The assay is not radioactive, requires only nanogram quantities of DNA and is applicable to a broad range of species.

To perform a RAPD assay a single oligonucleotide of an arbitrary DNA sequence is mixed with genomic DNA in presence of a thermostable DNA polymerase mixed with a suitable buffer and then is subjected to temperature cycling conditions "polymerase chain reaction". The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction conditions.

At an appropriate annealing temperature during the thermal cyle the simple primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other and a discrete DNA segment is produced. Allelic variation among individuals is detected as the presence or absence of the multiplication product visualized as a band after PCR and electrophoresis (Rafalski *et al.* 1993b).

The DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide-binding sites on the genomic DNA. Polymorphic bands are noted, and the polymorphisms can be mapped in a segregating population. Often a single primer can be used to identify several polymorphisms, each of which maps to a different locus.

Analysis of variations at the nuclear genome level using RAPD has advantages over RFLP as a single primer produces several loci, covering a larger portion of the genome.

According to Jayasankar *et al.* (1998) RAPD have been used to determine:

- Genetic relationships in *Annona* spp. (Ronning *et al.* 1995), *Theobroma cacao* (Ronning *et al.* 1995) and *Carica papaya* (Drew, 1988).
- To determine phylogenetic relationships in *Mangifera indica* (Schnell and Knight, 1993)
- Cultivar identification (Ronning *et al*. 1995)
- Confirming somatic hybrids following protoplast fusion between *Citrus* spp. (Deng *et al*. 1995)
- Somaclonal variation in regenerants from protoplast, and from embryogenic cultures derived from zygotic embryos in *Vitis* spp. (Schneider *et al.* 1996)
- To identify resistant gene in near-isogenic tomato lines linked to a bacterial resistant (Martin *et al*. 1991)
- To identify selected resistant lines from embryogenic *Mangifera indica* cultures for resistance to *Colletotichum gloesporoioides* (Jayasankar *et al.* 1998)
- For index Avocado viroid species (Ronning *et al.* 1997).

4 Objectives

The purpose of this work is to develop an *in vitro* vegetative multiplication system assuring the clonal propagation or *true-to-type* regenerants for *Annona cherimola* Mill. and *Annona muricata* L., subtropical and tropical semideciduous woody fruit species from selected growing plants in order to improve a new an alternative propagation method that assures the rescue of natural selections.

The micropropagation of *A. cherimola* and *A. muricata* is studied in regard to the physical and chemical factors, related to the cellular dedifferentiation and differentiation processes of the selected plant explant under aseptic conditions until the promotion of *in vitro* plant clone regenerants.

The adaptation and endogenous *preconditioning of the mother plants,* source of explants in the greenhouse, to promote the *in vitro* establishment, is reported.

To advance in the *aseptic in vitro establishment* of *A. cherimola* and *A. muricata,* the effect of some disinfectant solutions in combination with several tissue explants is studied and their effect evaluated on vegetative, semi-woody and woody branches.

To proceed with the micropropagation of *A. cherimola* and *A. muricata,* different nutrient media formulations supplemented with plant growth regulators are evaluated to define the *initial culture conditions* of a selected explant.

A. cherimola and *A. muricata* are phenol reactive plants, the effect of explant size, branch lignification grade, photo-reaction stress in combination with antiphenol solutions is evaluated in terms of explant quality during all the micropropagation steps.

To forward the *A. cherimola* and *A. muricata* newly formed *in vitro* shoots, different salt media formulations in combination with several plant growth regulators are tested and evaluated in terms of shoot *organogenesis* and shoot *differentiation*.

To proceed with *A. cherimola* and *A. muricata in vitro* propagation, the previously formed shoots are stimulated to increase, not only in number and size, but also in height thus, some plant growth regulators in combination with nutrient media are evaluated on the quality of the new *in vitro* formed shoots. The limiting problems such as shoot chlorosis or shoot hyperhydration are prevented in advance.

A. cherimola and *A. muricata* are not root prolific plants, thus nutritional, hormonal and physical factors in the micropropagated shoots are evaluated under *in vitro* and *ex vitro* conditions. To stimulate rhizogenesis and root differentiation auxines, light, mineral salt composition and substrate the principal factors considered.

Shoot hardening of *A. cherimola* and *A. muricata* regenerants and their gradual adaptation to the greenhouse conditions is described in terms of optimal conditions to improve the survival rate of regenerants under *in vitro* conditions.

In order to support the *in vitro* vegetative propagation of *A. cherimola* and *A. muricata*, the quality of the *in vitro* regenerants was screened by the molecular DNA technique of *Random Amplified Polymorphic* DNA (RAPD).

The DNA pattern band between *A. cherimola* and *A. muricata ex vitro* source of material and *A. cherimola* and *A. muricata in vitro* regenerants was compared. The reported results are discussed in terms of genetic stability and somaclonal variation.

Furthermore karyotype chromosome number is being complementarily reported from the plants obtained by means of *in vitro* culture.

To my knowledge this research presents for the first time a clonal micropropagation protocol for Annona cherimola and Annona muricata tested by RAPD. In addition rhizogenesis and genetic stability of the in vitro regenerants is discussed.

5 Micropropagation

Plant Material

Tissue culture experiments were carried out with two kinds of mother *A. cherimola* and *A. muricata* plant material. The initial material or stock mother plants of these subtropical and tropical semideciduous fruit trees were commercial plants in Colombia and Chile (Table 16).

Table 16. Source of A. cherimola and A.muricata experimental material

Species and Selections or Cultivars		Procedence Country (Region)	Type of Material	Material Code	Selection Code
A. cherimola	cv. Felpa	Chile	seeds	T_0	Ch-X
	cv. Bronceada	Chile	seeds	T_0	Ch-Y
	cv. ?	Colombia (Cundinamarca)	seedling*	T_1	Ch-Z
A. muricata	cv. ?	Colombia (Tolima)	seedling*	T_1	Mr-2
	cv. ?	Colombia (Cundinamarca)	seedling*	T_1	Mr-4
	cv. ?	Colombia (Valle del Cauca)	seedling*	T_1	Mr-5

⇒ * one year old plants from seeds of selected native trees

Healthy, one year old, 1 m high and vigorous native Colombian *A. cherimola* and *A. muricata* seedling trees (T_1), growing in the field, were selected based on previous information from the farmers in terms of plant quality, because in Colombia there are no clonal plantations established with *Annona* spp.

These plants were transported from three different regions of Colombia to Berlin without expanded leaves. The trunk, cuttings and roots were covered and protected with moistened paper and kept at a room temperature of 18 ± 3°C for four days and coded or tissue culture work purposes (Table 16).

Selected seeds of *A. cherimola* var. Felpa and Bronceada (T_0) from *Chile* were also used as experimental material (Table 16). Germination treatments were applied in order to break the seed dormancy of these semideciduous species. Seeds with and without coat were immersed in water for 2 days at 20 ± 2°C, not only to improve endosperm hydration and promote embryo emergency but also to select the viable seeds, those seeds which floated were eliminated.

To prevent fungal attack the hydrated seeds were treated with Benomyl[3] 0.03% for 1 hour and then washed with distilled water for 20 minutes.

Temperature and gibberellic acid GA_3 were the experimental variables for *A. cherimola* var. Felpa and var. Bronceda germination seeds, 20 units were used per immersion treatment:

- distilled water and stored in darkness 24h / 4°C
- 250 ppm GA_3 solution and stored in darkness 24h / 4°C
- 500 ppm GA_3 solution and stored in darkness 24h / 4°C
- distilled water and stored in darkness 20 ± 2°C
- 250 ppm GA_3 solution and stored in darkness 20 ± 2°C
- 500 ppm GA_3 solution and stored in darkness 20 ± 2°C

Afterwards all of them were sown in pots filled with quartz sand and maintained at seedling germination nursery conditions, 23 ± 2°C, 95% humidity and 16 h photoperiod. After germination some seedlings started to grow and develop new expanded leaves. These were transplanted into pots and maintained under hydroponic conditions with a commercial solution of Wuxal[4] 1 ml/l during the duration of this study.

The *A. cherimola* (T_0) and (T_1) plants were planted in subtropical greenhouse conditions,18 ± 5° C and 75 % humidity and the *A. muricata* (T_1) plants in tropical greenhouse conditions , 25 ± 5°C and 70 % humidity, at the Fruit Science Department of the Humboldt University, Berlin (Dahlem).

The *A. cherimola* and *A. muricata* plants were pruned three times per year and treated permanently with Benomyl 0.03% to reduce the endogenous contaminants of the tissues such as fungi. After eight months of greenhouse adaptation these plants were transplanted into pots with quartz-sand to prevent wilting.

Explant Selection and Isolation

To induce the establishment of *A. cherimola* and *A. muricata*, different tissues from the T_0 and T_1 selections were evaluated. Factors such as source, age and size were evaluated.

To reduce *in vitro* contamination, all the selected branches were trimmed from the plant and the big open-wide leaves were removed with a scapel in the greenhouse. The branches were segmented into approximately 15 cm long stems and cleaned with running tap water prior to any surface disinfection.

[3] Benomyl $_{(DuPont)}$: Fungicide 0.03 %
[4] Wuxal Top N $_{(Hoechst\ Schering\ AgrEvo)}$: N (140-), P_2O_5 (45-), K_2O (70-). B (0,11-), Cu (0,08-), Fe (0,18-), Mn (0,15-), Mo (0,011-), Zn (0,055-), - g/l.

For *A. cherimola* and *A. muricata* the first year growth- (vegetative), second year growth- (semi-woody) and third year growth- (woody) were used as the source of explants:

- First year branch, soft woody material: meristems (0.1 - 0.5 cm), buds (0.5 - 1 cm), shoot tips (0.5 - 1.5 cm), leaf pieces (0.5 - 1 cm), micro shoots with 1 bud (1 - 3 cm) and macro shoots with 3 buds (5 - 9 cm) were isolated as the primary source of explants
- From the second year old branch, semi-woody material: buds (0.5 - 1 cm), stems with one pre-formed bud (1 - 3 cm) and stems with two or three pre-formed buds (5 - 9 cm) were evaluated
- Third year old branch, woody material: buds (0.5 - 1 cm), stems with one pre-formed bud (1 - 3 cm) and stems with two or three pre-formed buds (5 - 9 cm) were evaluated

The viability of these explants to promote the micropropagation of *A. cherimola* and *A. muricata* was analysed in terms of shoot quality and the potential of *in vitro* regeneration. The explant responses were observed and noted during the manipulation time and after some days of culture:

- Latency: Description of any *in vitro response* of the explant to the aseptic conditions. The explant did not have the potential to promote new cell divisions and subsequently differentiation. The explant did not react to the *in vitro* conditions and reduced its quality during the culture time
- Died: The explant did not survive after wounding. Manipulation problems, excessive concentration of phenolics and also the presence of contaminants were observed.
- Hypersensitive reaction: Production of phenolics or tannins by the explant and toxic accumulation in the culture media, the explant tissues were limited by cell blackening or total necrosis
- Green: The explant survived to the first *in vitro* manipulation, it remained fresh and green. It was not affected by wounding and its growth in the *in vitro* culture media was promoted. No blackening of the *in vitro* culture media was observed. These explants had the potential to induce new *in vitro* organogenesis
- Contamination: The explant survived to wounding, had the intrinsic potential to induce new organogenesis, but was affected by endogenous or exogenous contaminants like bacteria and fungi and the aseptic *in vitro* establishment could not be promoted

Phenolics

As most woody species, the *A. cherimola* and *A. muricata* are sensitive to wounding. The phenol metabolism is stimulated immediately after the cells of the isolated explant are separated from the mother plant.

The hypersensitive reaction is a tissue-protected reaction originating in the oxidation of polyphenol-like compounds and tannins by the polyphenoloxidase activity to *o*-quinones, antimicrobial growth (Martinez-Cayuela, 1986; Martinez-Cayuela *et al*. 1988; Debergh and Read, 1990).

The product of this reaction is a brown/black exudate coming from the explant. The first reactive areas are the tips of the explant, upon wounding. The phenolic compounds diffuse quickly in the tissue culture medium (Bridg, 1993b) and the concentrations are toxic for the explant (Debergh and Read, 1991).

Prevention

Some *A. cherimola* and *A. muricata* plants were maintained in darkness in the greenhouse for two weeks to promote the reduction of the photosynthetic metabolism and subsequently the rapid stimulation of phenolics on the first *in vitro* culture days. Besides, shoot sprouting is expected to be stimulated under *in vitro* conditions.

Use of antioxidants to improve the in vitro culture: The combinations of L-ascorbic acid (AA) (50-150 mg/l), citric acid (CA) (100-200 mg/l) and polyvinylpyrrolidone (PVP) $_{10,000 \text{ Mol. Wt}}$ (2, 4, 8 -ppm) were evaluated. Several concentrations *vs.* exposition time (1, 5, 10 -minutes) were combined with two management methods as follows:

- Immersion of the *A. cherimola* and *A. muricata* branch „source of explants“ in the antioxidant solutions mentioned before, *during the all manipulation time* needed for the explant separation in the laminar air-flow cabinet (1 hour approx.)
- Media supplement to control the hypersensitive reaction when the tissue explant contacted with the establishment medium

Disinfection

Different disinfectant solutions *vs.* exposition time were tested on separated segments coming from the 15 cm long selected branches of *A. cherimola* and *A. muricata* to improve surface disinfection:

- Ethanol 70% (1, 2, 3 min)
- Ethanol 70 % (1 min) + sodium hypochlorite 0.5 % (1, 5, 10 min)
- Sodium hypochlorite 2% and 3% (5, 10, 15, 20, 30 min)

- 2 drops Tween 20 + sodium hypochlorite 0.5% , 2% , 3% for (5, 10, 15, 20 minutes)
- Benomyl 3% (15 min) + sodium hypochlorite 3% (5, 15, 20, 30 min)

Due to the fact that meristem explants are sensitive, the concentration of sodium hypochlorite was reduced to 0.5-1 % since chlorine solution and vapour can, more easily, penetrate the meristem of actively growing shoots which are less tightly bound by leaflets.

Following the surface disinfection with antiseptic solutions, the *A. cherimola* and *A. muricata* selected cuttings source of explants were rinsed in sterile deionized, double distilled autoclaved water five times 5 minutes each, to remove any remaining traces of the disinfectant. The branches were laid on Erlenmeyer flasks (500 ml) with the antioxidant solutions during the explant selection and manipulation in the laminar air flow-cabinet.

The tips of the *A. cherimola* and *A. muricata* became white after the disinfection treatment and they had to be removed with a scapel before any *in vitro* inoculation these white ends or tissue translucent tips were the first negative explant response to the disinfection applied treatment. The respective explant was separated from the branch in sterile conditions in the laminar air-flow cabinet, previously sterilized with UV-light lamps, switched on the night before. All the inoculations were made with sterile instruments. The meristems were separated under a binocular microscope. The sterilized explants were inoculated according to the selected media taking care of its natural polarity and subsequent orientation in the culture media.

Effect of Antibiotics

The *A. cherimola and A. muricata* endogenous contaminants are difficult to eliminate. Some previous *A. cherimola* cultures showed the presence of bacteria during the multiplication step. Then a review of the establishment disinfection conditions was made and previous results showed that the establishment media must be supplemented with antibiotics.

The Nystatin is a well known antifungal agent coming from *Streptomices* spp., and it is recommended in tissue culture because of its fungicidal activity in aqueous solutions. Cefotaxime is one of the most effective antibiotics against *Gram* (-) and *Gram* (+) bacteria species and Rifampicin, characterized by its wide spectrum against *Gram* bacteria and *myco*-bacteria, is the most recommended antibiotic for tissue culture purposes.

These antibiotics were selected because of their differences in spectrum action, 40 µg/ml Nystatin $_{(\text{Sigma N-9767})}$, 95 µg/ml Cefotaxime $_{(\text{Sigma C-7039})}$ and 20 µg/ml Rifampicin $_{(\text{sigma R-7382})}$ were evaluated during the establishment of *A. cherimola* and *A. muricata* selected explants. These were manipulated at room temperature conditions with 0.22 µm sterile millipore cellulose acetate membrane $_{(\text{Sigma F'9643})}$ filters:

- On direct contact with the explant (some drops or ml were added, depending on explant size)
- As a supplement in the mineral basal medium

– By previous soaking (the explant was in contact 5-20 minutes with the antibiotic solution before its inoculation)

Mineral Basal Composition

The Nitsch and Nitsch (1969) (NN-69) and Murashige and Skoog (1962) (MS) were tested to promote induction of new cell divisions in *A. cherimola* and *A. muricata* greenhouse explants during the micropropagation *in vitro* stages (Table 17).

Table 17. Mineral media basal formulation

Composition	Concentration in mg/l	
	MS	NN-69
KNO_3	1,900	
NH_4NO_3	1,650	720
MgSO4 * $7H_2O$	370	185
$CaCl_2$ * $2H_2O$	440	219.9
KH_2PO_4	170	68
H_3BO_3	6.2	10
$MnSO_4$ * $4H_2O$	15.6	18.94
$ZnSO_4$ * 7 H_2O	8.6	10
Na_2MoO_4 * $2H_2O$	0.25	0.25
$CuSO_4$ * $5H_2O$	0.025	0.025
$CoCl_2$ * 6 H_2O	0.025	-
KI	0.83	-
$FeSO_4$ * $7H_3O$	27.8	27.8
Na_2 * EDTA	37.3	37.3
Biotin	-	0.05
Folic acid	-	0.5
Glycine	-	2.0
myo-inositol	100	100
Nicotinic acid	0.5	5.0
Pyridoxine hydrochloride	0.5	0.5
Thiamine hydrochloride	0.5	0.5

According to the micropropagation objectives during the establishment-, multiplication- or rooting- steps, the selected basal media formulations were supplemented with factorial combinations of some growth regulators such as:

- Auxin

4 [3-Indolyl]butyric acid (Sigma I 5386)	IBA	0.49 - 2.46 μM
α-Naphtaleneacetic acid (Sigma N 0640)	NAA	2.69 - 26.85 μM
Indole-3-Acetic acid (Sigma I 2886)	IAA	0.57 - 6.89 μM

- Gibberellin

Giberellic acid (Sigma G 7645)	GA_3	0.29 - 6.06 μM

- Cytokinin

6-Benzylaminopurine (Sigma B 3408)	BAP	2.20 - 8.87 μM
1-Phenyl-3-(1,2,3-thidiazol-5-yl)urea (Sigma P6186)	TDZ	4.44 - 9.08 μM
6-Furfuryl-amino-purine (Sigma K O753)	K	0.46 - 23.23 μM
6-[4-Hydroxy-3-methylbut-2-enylaminopurine] (Sigma Z 0164)	Z	0.46 - 4.56 μM

To compare *in vitro* explant responses, double distilled autoclaved water supplemented with sucrose was used during the establishment. For the multiplication and rooting stage the selected mineral salts and organic nutrients with sucrose and support agent and non growth regulators were used as controls.

The effects of casein hydrolysate (Sigma C 7290) (CH) (100-200 mg l^{-1}) on shoot quality growth and development were evaluated during the multiplication step to avoid *in vitro* chlorosis.

Other supplements such as Sucrose (Sigma 5391) (S) 1% - 3%, Agar (AG) 6 mg l^{-1}, Gelrite (GR) (sigma P 8169) 3 g l^{-1}or Whatman No.1 paper bridges for the liquid medium were evaluated not only on establishment but also on rooting.

The antioxidant substances *L*-ascorbic acid (Sigma A 2174) (AA) (50 -150 mg l^{-1}l) + citric acid (Sigma C 4540) (CA) (100- 200 mg l^{-1}) and Polyvinylpyrrolidone (Sigma P 2307) (PVP) 10,000 Mol. Wt. (1-10 ppm) were added to avoid necrosis of the selected explant on the pre-defined medium during establishment.

The pH of all mediums was adjusted at 5.7 ± 0.1 with an electronic pH meter after adding agar or gelrite into the solution and prior to autoclaving at 120°C and at a pressure of 1.2 kg/cm^2 for 15-20 minutes.

The media were distributed into different glasses: Test-tubes (25 x 150 mm) with 15 to 20 ml medium for the biggest explants and closed with magenta sigma caps. Single or multiple shoots were grown in wide-necked baby-food jars (55 x 95 mm) containing 30 ml medium with magenta sigma B-caps. Disposable plastic petri dishes (90 x 15 mm) with 1.5 ml media were used to establish meristems and leaf young segments as well as small shoot tips.

The effect of parafilm on the closures was evaluated in terms of shoot development, some media were completely closed and then compared with others which were only closed with magenta caps.

Rooting

The well developed shoots of *A. cherimola* and *A. muricata* (3-4 cm long) were excised to improve rhizogenesis under *ex vitro* and *in vitro* conditions.

To promote *in vitro* rhizogenesis 1/4 -, 1/2-, and full strength, Nitsch and Nitsch (1969) medium supplemented with different concentrations and combinations of sucrose (0, 1, 2, 3 %) and plant growth regulators (IBA, NAA and IAA) previous concentration were evaluated.

The *ex vitro* rhizogenesis was stimulated with a commercial growth regulator powder, Rhizopon[5] in two presentations (AA) and (B). The base of the micropropagated shoots were treated with:

- Rhizopon (AA-powder), 0.5 mm for 2 seconds and the excess was shaken off
- Rhizopon (B-tablet), the shoots were immersed in a water solution, 0.5 mm for 24 hours

In both cases the shoots with roots were planted in sterile autoclave quartz sand. Rooting development was improved in a water foggy chamber with 90% humidity and 25± 3°C temperature.

Hardening

The well developed regenerants of *A. cherimola* and *A. muricata* with *in vitro*-formed roots were removed from the culture media and transplanted into *ex vitro* nursery conditions in pots containing quartz-sand and misted every five minutes. They were covered with a funnel to permit transpiration and hardening, 90% humidity reduced slowly to 70% in 30 days, 25 ± 3 °C and 16-h photoperiod remained constant.

The *A. cherimola* and *A. muricata in vitro* shoots were rooted under *ex vitro* foggy conditions in the greenhouse with Rhizopon (-AA) or (-B) and were transferred after 1 ½ months hardening to subtropical greenhouse conditions and maintained there under hydroponic conditions.

Physical Factors

According to the objectives of the experiment, the cultures were maintained at 50-60% relative humidity, 25 ± 3°C temperature, 16-h photoperiod promoted by cool light white lamps (45 µE m^{-2} s^{-1}) intensity or total darkness.

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[5] Rhizopon $_{(Hazerswoude-Holland)}$ AA: Indol-butiric-acid 1%
Rhizopon $_{(Hazerswoude-Holland)}$ B : Naphtalene-acetic-acid 10%

Statistical Analysis

Statistical analysis was performed on the results of each experiment and the data was analysed through the Statgraphics Programme for Windows (ANOVA). Analysis of variance was calculated (Steel and Torrie, 1985). In total 36 or 20 experimental explants were used depending on the objectives of the experiment which were repeated three times

5.1 Random Amplified Polymorphic DNA (RAPD)

Plant Material

Two kinds of material were used as a source of genomic DNA to compare the RAPD on *A. cherimola* and *A. muricata* (T_0) and (T_1) plants (Table 15):

- DNA from mother plants established in the greenhouse and,
- DNA from *in vitro* regenerant clones of the established plants in the greenhouse

DNA Extraction

Genomic DNA was isolated from young leaves of *A. cherimola* and *A. muricata* modifing Rogers and Bendich (1985) method**.** In this procedure, the precipitation of soluble nucleic acids complex was induced in combination with a high concentration of natriumchloride NaCl 0.7 M + CTAB. The reduction of NaCl to 0.4 M permitted the precipitation of CTAB[6]/nucleic acid complex while the solution of polysaccharides remained.

Two grams of fresh weight (FW) border young leaves were ground to a fine powder in liquid nitrogen (-70°C) to construct the nuclei acid complex. Grinding was continued with the addition of pre-warmed buffer 5 ml of CTAB 2% [*2% (W/V) CTAB; 1,4 M NaCl; 100 mM Tris/Base (pH 5.8); 20 mM Ethylenediaminetetraacetic acid (EDTA)*].

The homogenate was incubated at 65°C for 15 minutes, with occasional swirling in polyallomertubes and extracted with the same volume amount of chloroform : isoamyl alcohol (24:1). Samples were centrifuged at 6000 rpm in JA 13.1 for 10 minutes at room temperature to extract the denatured proteins which can be collected in the interphase.

The supernatant was transferred to a fresh tube with 1 ml vol. CTAB 5% [*5% (W/V) CTAB; 350 mM NaCl*] buffer, pre-warmed. The mix was incubated at 65°C for 10

[6] CTAB : hexadecyltrimethylammonium bromide

minutes. Chloroform-isoamyl alcohol was added in an equal volume and swirled. The mix was centrifuged for 10 minutes, 6000 rpm JA 13.1 at room temperature.

The genomic DNA was recovered carefully in corex tubes and mixed with 1 vol. precipitation buffer [*1% (W/V) CTAB; 50 mM Tris/Base (pH 8), 10 mM EDTA*], extraction, closed with parafilm and swirled. After some hours the precipitation of CTAB/nucleic acids complex were visible.

The nucleic acids complex re-suspended in the solution was recovered by centrifugation at 6000 rpm (JA 13.1) for 10 minutes, at room temperature. The precipitate was washed with 2 ml HS-TE Buffer [*1M NaCl; 1 mM Na_2EDTA; 10 mM Tris/HCl (pH 8)*]and warmed for10 minutes at 65°C to be dissolved and separated with 2 Vol. absolute Ethanol (30 minutes in -70°C), centrifuged 10 minutes, 6000 rpm (JA 13.1), 4°C. The supernatant was removed with ethanol and the dried pellet was dissolved in autoclave bi-distilled water.

For the DNA-RNA-CTAB complex, 0.3 ml 5 M ammoniumacetate pH 7,5 (f*inal concentration 2,5M*) was added and incubated for 20 minutes at 0°C. Precipitation of RNA was induced by centrifugation for 10 minutes,13000 rpm, 4°C, supernatants were taken and distributed in new Eppendorf tubes and complete with 2 Vol. *abs.* ethanol. DNA concentration was calculated fluorometrically.

DNA Amplification

The DNA amplification of *A. cherimola* and *A. muricata* was supported by the Polymerase Chain Reaction (Table 18) and synthetic oligonucleotide 10-base primers with 40-60% of Guanosine and Cytidine (GC) distributed uniformily. These are available commercially from the following companies:

- CARL-ROTH, Karlsruhe, Baden-Württemberg. Germany

  B-11, C-04, C-05, C-07, C-11, C-19, G-19, J-19, L-04, P-02, P-05, P-10, P-13, P-20, Q-04, Q-05, Q-06, Q-07, Q-08, Q-09, Q-10, Q-11, Q-12, Q-13, Q-14,Q-17

- Operon Technologies, Alameda, CA. U.S.A.

  OPA-16, OPA-17, OPA-18.

In a DNA-Thermal Cycler (Perkin-Elmer, serial P19516) programmed to 45 cycles, the PCR reactions (25 µl) were improved (Table 19).

DNA Separation

Amplification products were separated by electrophoresis in ethidium bromide stained gels: 1.5 % (w/v) agarose gel soluble in ”TBE” [Tris-borate (TBE) 5X: 54g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0) 1x (0.5 x: 0.045 M Tris borate + 0.001 M EDTA] to provide adequate buffering power.

Table 18. Polymerase chain reaction mix

Reagent	25 µl reaction mixture	Final concentration 25 µl reaction mixture
Annona spp., DNA template	2.5 µl	25 ng
PCR-Buffer [1]	2.5 µl	1x (incl. 1.5 mM $MgCl_2$)
DNTP-Mix [2]	2.0 µl	each 5 µl, 100 µM
50 mM $Mg\text{-}Ac_2$	1.5 µl	3 mM
Taq DNA polymerase [3]	0.2 µl	1 u/µl [4]
Primer[e]- [5'→ 3'] 10 nucleotides	1.5 µl	
Sterile deionized water	14.8 µl	-
Final volume	25.0 µl	-
Mineral parafilm oil	50.0 µl	by PCR tube

⇒ [1] *PCR-Buffer: 20mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol*

⇒ [2] *dNTPs: Mix solution:in mM of 2'-deoxyadenosine 5'-triphosphate sodium salt (dATP), 2'-deoxycytidine 5'-triphosphate sodium salt (dCTP), 2'-deoxyguanosine 5'-triphosphate sodium salt (dGTP), 2'-deoxythymidine 5'-triphosphate sodium salt dTTP*

⇒ [3] *Thermus aquaticus DNA Polymerase (recombinant): Purified from an E.coli train carrying a Thermus aquaticus DNA polymerase overproducing plasmid. Catalyses 5'→ 3' synthesis of DNA and possesses low 5'→ 3' exonuclease activity. Reported to have reverse transcriptase activity*

⇒ [4] *Unit Definition: 1 unit of the enzyme Taq DNA polymerase catalyses the incorporation of 10 nmoles of deoxyrribonucleotides into an acid insoluble form in 30 min at 70°C*

Table 19. DNA amplification steps applied on Annona spp.

Step		Time	Temperature
Initial denaturation		4 min	94°C
Denaturation		20 sec	93°C
Primer annealing		1 min	36°C
Extending		20 sec	72°C
Number of cycles	45		
Extending		6 min	72°C
Cooling			10°C

DNA Separation

Amplification products were separated by electrophoresis in ethidium bromide stained gels: 1.5 % (w/v) agarose gel soluble in "TBE" [Tris-borate (TBE) 5X: 54g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0) 1x (0.5 x: 0.045 M Tris borate + 0.001 M EDTA] to provide adequate buffering power.

RAPD Analysis

The amplification products were visualized and photographed under standard conditions used for ethidium bromide-stained gels on an UV-transiluminator, and identified with reference to the Lambda DNA/ EcoRI+HindIII marker (MBI Fermentas, Germany)

The polymorphic reproducible bands in the agarose-gels were scored as (1) present or (0) absent. PCR reactions were performed five times to establish reproductibility of results.

5.2 Karyotype Observations on *Annona* spp.

For the analysis of mitotic chromosomes of *A. cherimola* and *A. muricata* mother plants and *in vitro* regenerants, the youngest and whitest translucent light root-tips were selected. Each sample consisted of 10 root-tips, 0.5-1.0 cm.

Several treatments were tried for arresting the *A. cherimola* and *A. muricata* chromosomes at metaphase because chromosomes can be visualised in their most condensed form in mother plants and *in vitro* regenerants : *Ice-cold water 4°C/ 24 h and alcohol- acetic acid 3:1 48 h; Bromonapthatlene (6ml/l) 4 h then glacial acetic acid 30 min and 1 N HCl 60°C 10 min; Hydroxyquinoline 0.003 M 4 h then alcohol-acetic acid 3:1 48 h stain aceto-carmine 0.5%+ ferric-III-chloride 10% 1:100 48 h*

The root-tips were taken with forceps and immediately subjected to a suitable treatment. They were incubated for the appropriate time and temperature as described above. Five root-tips per plant and at least 10 cells per root-tip were analysed.

The root tips were stained with Orcein-HCL filtered and with Aceto-carmine. Also feulgen 45% acetic acid was used. This colours the cytoplasm and enables the determination of whether the cell is intact. This background colours are also helpful to determine the chromosomes in *Annona* spp. cells.

A fluorescence method was also applied to see the chromosomes of *A. cherimola* and *A. muricata*. The probes were stained with 2µg ml^{-1} diamino-2-phenylindole (DAPI, Sigma) in PBS (pH 7.4) for 10 min. and analyzed under a fluorescense microscope (Orthoplan, Leitz, Germany) using the epifluorescence optics of filter A (excitation filter, 340 to 380 nm; dichroic mirror, 400 nm; and barrier filter, 430 nm).

6 Micropropagation

6.1 Plant Material

The *seed germination* of *A. cherimola* (T_0) cv. Felpa (Ch-X) and cv. Broceada (Ch-Y) was observed on the 35th day after sowing, in all the tested combination treatments. However the germination percent varied (Table 20).

Table 20. Effect of gibberelic acid and cold temperature shock on seed germination of Annona cherimola cv. *Felpa* and cv. *Bronceada*

Germination Treatments	Previous Temperature shock for 24 h	Percent of Germination			
		A. cherimola cv. Felpa		A. cherimola cv. Bronceada	
		coated-seeds	coatless-seeds	coated-seeds	coatless-seeds
water solution	4°C	78	34	81	43
250 ppm GA_3	4°C	45	35	73	34
500 ppm GA_3	4°C	37	17	54	18
water solution	20 ± 2°C	41	30	67	36
250 ppm GA_3	20 ± 2°C	33	12	56	28
500 ppm GA_3	20 ± 2°C	28	7	45	23

The coated seeds showed a higher germination percent without any pre-treatment of gibberellic acid. The highest percent of seed germination of *A. cherimola* cv. Bronceada and cv. Felpa was obtained with the control treatments or without gibberellic acid.

The *A. cherimola* cv. Bronceada germinated in 78% with a previous cold precondition and without the presence of GA_3. The same situation occurred for *A. cherimola* cv. Felpa in 81%. It is important to note that these seeds were coated.

The coatless seeds of *A. cherimola* and *A. muricata* showed the lowest percent of germination in all the experimental cases. However those which were pretreated 24h/4°C and maintained in a water solution were more viable 34% cv. Felpa and 43% cv. Bronceada when these were immersed in a gibberellic acid solution. Coatless seeds lost quality potential, changed in colour; texture and hardening were also observed in the endosperm. These seeds were also more sensitive to fungi attack during germination.The giberellic acid GA_3-250 ppm broke the seed dormancy more efficiently than GA_3-500 ppm which stimulated the lowest rate of seed germination in both varieties.

The previous low temperature shock 4°C in 24 hours in darkness promoted seed germination if results are compared with those treatments where the seeds were not submitted to cold temperature shock. The germination of *A. cherimola* cv. Felpa and cv. Bronceada was promoted more by pre-shock with low temperatures than by the effect of the gibberellic acid as a plant promotor.

Otherwise the seedling plant adaptation was successfully achieved, 70% of *A. cherimola* (T_1) (Ch-Z) and *A. muricata* (T_1) (Mr-2; Mr-4; Mr-5) Colombian plants survived the transport conditions. They were adapted to the subtropical and tropical greenhouse conditions.

After one month under greenhouse conditions both the *A. cherimola* and *A. muricata* (T_1) plants started to break the dormancy of the latent buds and began the formation of new leaves and branches, plants grew well during the course of this study(Figure 9).

Figure 9. A. cherimola and A. muricata Colombian plants adapted to greenhouse conditions in Berlin

Endogenous Precondition of Material

Previous results showed that the micropropagation of *A. cherimola* and *A. muricata in vitro* are strongly limited by two factors: first the production of phenolics and secondly the presence of endogenous contaminants such as fungi and bacteria.

The presence of endogenous contaminants not only limits the aseptic establishment but also promotes the production of phenolics in the explant. Therefore *in vitro* establishment under these conditions is difficult to achieve.

To avoid the infection of fungi and bacteria during the micropropagation, the *A. cherimola* and *A. muricata* (T_0) and (T_1) plants were treated in the greenhouse with Benomyl (0.03%) systemic fungicide, two times per month.

Applications of Benomyl reduced the percent of fungi expression of *A. cherimola* and *A. muricata* (T_0) and (T_1) plants during the *in vitro* culture (Table 21). The expression of *in vitro* contamination by fungi was less serious if the mother plants were exposed for a long time prior to endogenous systemic fungicides. Four weeks before taking the explants, the greenhouse plants should be irrigated with Benomyl.

The initial *in vitro* contamination of fungi of 100% reduced after the first month of treatment on woody explants to 91% and after two years to 58%. Benomyl systemic treatments only preconditioned the explants to be *in vitro* manipulated. Other surface contaminants from the explant should be eliminated with disinfectant solutions and/or with antibiotics, if some bacteria remain after the precondition and disinfection. The treatments should be applied independently to promote the *in vitro* establishment the *A. cherimola* and *A. muricata* plants should be treated with Benomyl at least four weeks before the first inoculation.

Table 21. Effect of Benomyl 0.03% on A. cherimola and A. muricata in the greenhouse to control in vitro fungi contamination

Species	Explant "branch" cutting with 1 bud	% of in vitro fungi contamination expressed with the Benomyl applications [1] after	
		1st month	1st year
A. cherimola	Vegetative	76	44
	Semiwoody	88	55
	Woody	100	58
A. muricata	Vegetative	80	45
	Semiwoody	91	47
	Woody	100	77

⇒ [1] *Contaminants from the woody explant*
⇒ *In vitro establishment media formulation : NN-69 + S 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*
⇒ *A. cherimola and A. muricata selected explant, woody branches 1-3 cm*
⇒ *The values represent the mean of three independent observations, n=36*

6.2 Initiation of Culture

Explant

The aseptic establishment of *A. cherimola* and *A. muricata* (T_0) and (T_1) plants is not only affected by the presence of contaminants in the *in vitro* culture media. Also the nature of the plant material, the intrinsic potential of the organ source of explants to improve new cell regeneration, size and age are the principal factors which condition the *in vitro* responses.

The seedlings of *A. cherimola* (T_0) and ne year old sedling trees of *A. cherimola* (T_1) and also *A. muricata* (T_1) influenced the establishment of *in vitro* morphogenic responses of the selected explants and subsequently *in vitro* formation of new shoots.

The cuttings either from vegetative, semiwoody or woody branches are the most potential explants, they promote the shoot sprouting of pre-formed buds, the contamination and blackening problems should be treated independently. In addition the aseptic establishment of cuttings was limited by endogenous contamination. Greenhouse treatments with systemic fungicides such as Benomyl only preconditioned the reduction of some *in vitro* contaminants, therefore an aseptic culture from cuttings, such as the initial culture material should be improved with the application of exogenous tissue disinfectant solutions.

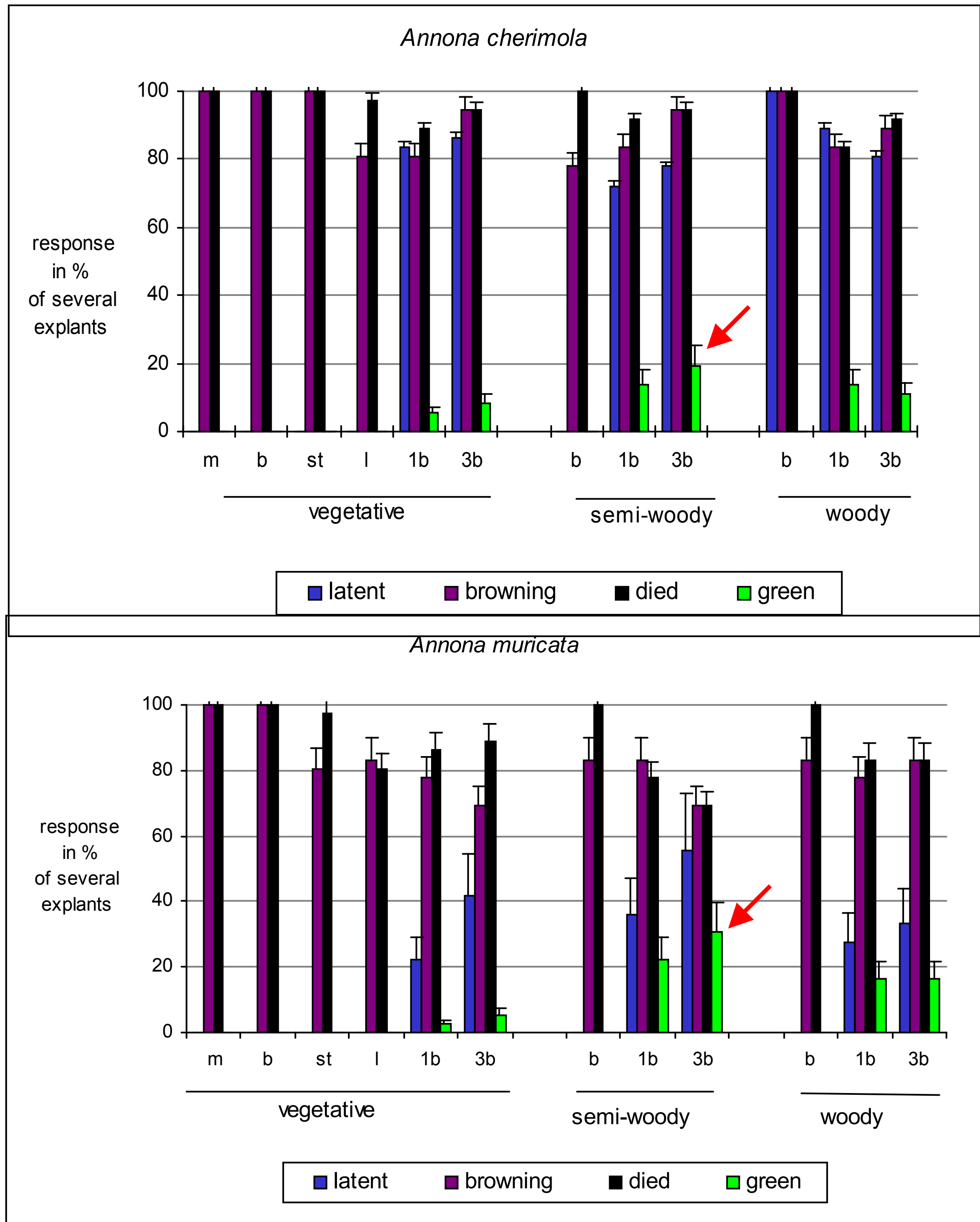
The size of the cuttings showed some differences. Cuttings (1-3 cm) with one bud were *in vitro* less shoot prolific if they are compared with cuttings (5-9 cm) with three buds. Because if *A. cherimola* and *A. muricata* cuttings carry more than one bud, they have more possibilities to promote new *in vitro* shoots.

Semiwoody branches with two or three buds (5-9 cm) from the second year growth with a semiwoody lignification grade were the best source of explants. These semiwoody cuttings promoted the highest percent of new formed shoots. The base of them was affected by blackening and some contaminants however, the buds reserve organs had the potential to promote new shoots (Figure 10).

Vegetative branches of *A. cherimola* and *A. muricata* were used as a source of small explants such as *meristems* (0.1 - 0.5 cm), *buds* (1-3 cm) or *shoot tips* (0.5 -1.5 cm). However these explants were not able to be established under *in vitro* conditions, blackening was the most generalized response of these explants which died within the first week after *in vitro* inoculation (Figure 10).

Meristems of *A. cherimola* and *A. muricata* were very sensitive, wounding induced severe injury, and the physical answer was the promotion of phenolics. These explants started to turn from green to black on the scalpel tip. Thus the *in vitro* establishment from these explants is not reported in this study (Figure 10).

Young *leaf segments* (0.5-1 cm) could be potentially explants to promote new *in vitro* callus formations on *A. cherimola* and *A. muricata*, however the border cells are phenol reactive and a dark brown was observed. Leaves remained green during some weeks but the quality of these explants diminished with the time of culture.



⇒ *m : meristems (0.1-0.5 cm), b : bud (0.5 - 1 cm), st : shoot tips (0.5 - 1.5 cm), l : leaves (0.5 - 1 cm), 1b cutting with 1 bud (1-3 cm), 3b : cutting with 3 buds (5-9 cm), green : possible new shoot formation*

⇒ *Establishment basic media formulation : NN-69 + S 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*

⇒ *The values represent the mean (± SE) of three independent experiments, n = 36*

Figure 10. First in vitro responses of various explants of A. cherimola and A. muricata -after one week of culture-

Other experiments (data not shown) in media supplemented with antioxidant substances and auxines to promote callus formation from leaf segments did not give any *in vitro* response. This might be due to the presence of phenolics in the leaf border. No regeneration from leaf segments of *A. cherimola* and *A. muricata* (T_1) and (T_0) occurred (Figure 11).

The reaction of the phenolics and remanent contamination of the small explants from *A. cherimola* and *A. muricata* could not be established. In tissue culture it is very well known that it is more difficult to induce growth in very small structures such as cells, clumps of cells and meristems, than in larger structures such as leaf or stems of *woody species* compared with herbaceous plants (Pierik, 1986).

Cutting explants from semiwoody branches of *A. cherimola* and *A. muricata* established plants are an optimal initial culture material. They can promote direct bud sprouting. Four new shoots they could be inducted from one bud. If the cutting is large the establishment rate improves if contamination and phenolization are controlled.

Disinfection

The *in vitro* culture of any cell organ and tissue should be made in aseptic conditions (Murashige, 1974) because it is well known that the plant surfaces are habitats for microorganisms (Campbell, 1985). The precondition in the greenhouse with systemic chemicals helped the plants to reduce the percent of endogenous microorganisms but not completely. Thus they should be also disinfected externally.

The optimal disinfection of a selected explant depends on the penetration degree of the disinfectant agent into all contaminated parts (Pierik, 1989). The selected disinfectant solution is effective only when the concentration is toxic for the microorganisms present on the surface but not toxic for the cells of the tissue.

Disinfectant Solutions

Previous experiments showed that *A. cherimola* and *A. muricata* explants are extremely sensitive to calcium hypochlorite and mercury chloride. These solutions in different concentrations limited the *in vitro* establishment of *A. cherimola* and *A. muricata* because the surface cells of these plants are hypersensitive and phenolic oxidation arises in high concentrations (Bridg, 1993).

The sodium hypochlorite was evaluated *concentration vs. time* and in combination with other agents such as: Benomyl 0.03% and Tween 20. The results showed that sodium hypochlorite reduced, in all the cases, the presence of contaminants in the *in vitro* culture media (Figure 11).

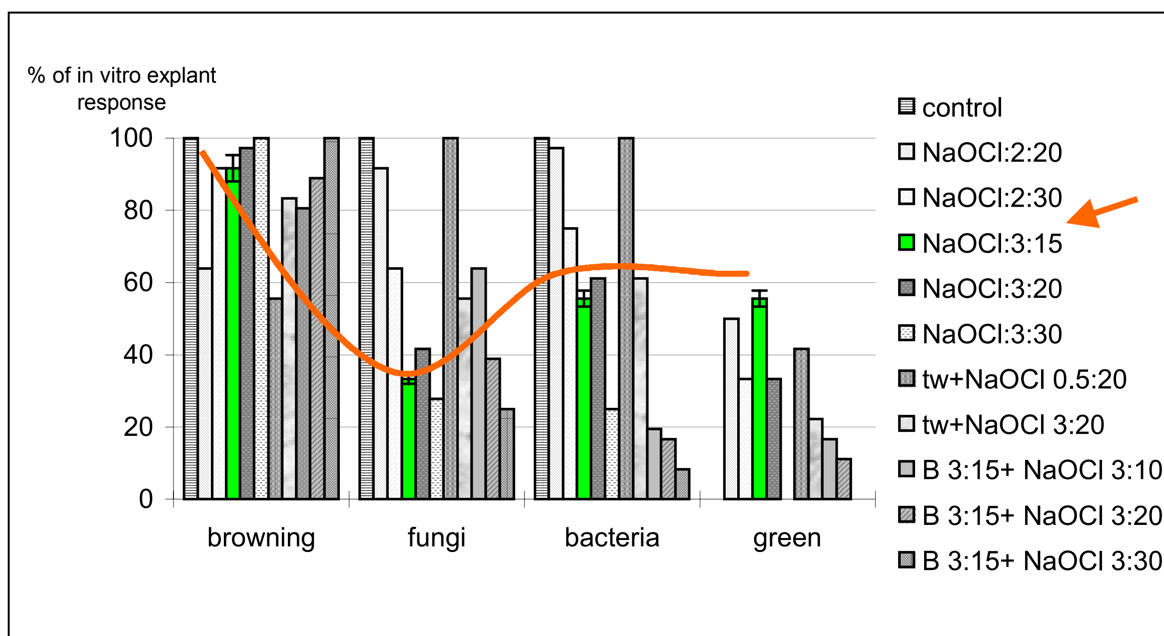
Sodium hypochlorite 2% did not eliminate all the surface contaminant microorganisms, some bacteria and fungi were observed in the culture media, neither the exposition for 20 minutes nor for 30 minutes were effective.

The effect of sodium hypochlorite 3% during 15, 20 and 30 minutes was evaluated, the effect of this disinfectant varies with the exposition time. In all the cases the reduction of surface contaminants were effective**.** The exposition for 30 minutes

injured the explant, the bud sprouting reduced significantly and a blackening in the culture media was observed.

The concentration of *sodium hypochlorite 3% for 15 minutes* helped to reduce the presence of exogenous *in vitro* contaminants. 45% of cultures could be established aseptically, but they were not all aseptic. The endogenous contamination of these tissues became evident after a few subcultures during the multiplication stage. However this treatment was selected to improve the exogenous disinfection of semiwoody branches of *A. cherimola* and *A.muricata* plants, nevertheless this should be complemented.

The combination of *sodium hypochlorite* with *Tween 20* as a wetting agent was made to reduce the surface tension of the cutting explant of *A. cherimola* and *A. muricata* and to improve the surface contact of this bleach solution. The presence of Tween 20 in the disinfection treatments in combination with sodium hypochlorite 0.5%/20 was not aggressive for the explant, however brown exudates coming from the explant were gentle on the tissue culture media (Figure 11). The microorganisms present on the surface of the semiwoody cutting explant of the *A. cherimola* and *A. muricata* explants seems are harbour and low concentrations of sodium hypochlorite had no effect on the explant surface.



⇒ *NaOCl (sodium hypochlorite): concentration "%" : time "minutes", tw: Tween 20 "2 drops",*
⇒ *B: Benomyl 0.03%*
⇒ *Establishment basic media formulation : NN-69 + S 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*
⇒ *The values represent the mean (± SE) of three independent experiments, n = 36*

Figure 11. In vitro response of A. cherimola and A. muricata semiwoody shoots (5-9 cm) to different disinfectant solutions
- after two weeks of culture-

The combination of *Tween 20* and *sodium hypochlorite* 3% by 20 min., registered no differences in terms of reduction of the microorganism contamination in *in vitro* culture. The exposition time of this disinfectant and maybe the presence of Tween 20 made this treatment aggressive and not advantageous in terms of *in vitro* establishment. The bud sprouting also reduced from 45% in sodium hypochlorite 3% / 15 minutes to 25% in this treatment.

Prewashes with ethanol 70% for some minutes to condition the surface of the cutting explant of *A. cherimola* and *A. muricata* to remove the surface air bubbles and improve the action of sodium hypochlorite were ineffective because the *A. cherimola* and *A. muricata* cuttings have a middle lignification grade which makes these explants sensitive to ethanol. The ethanol helps to eradicate some microorganisms on the explant surface, but also the alcohol dissolves the epicuticular layer of the cutting explant (Pierik, 1989) and a high concentration of phenolics was observed. In addition the ethanol promotes some dehydration of the explant surface.

The combination treatments of *sodium hypochlorite* and *Benomyl* 0.03% were effective in terms of aseptic *in vitro* establishment. However this external disinfection combined treatment reduced the percent of bud sprouting. In most of the cases the cutting explant stayed latent for some weeks, while the phenolics substances accumulated in the culture media slowly.

The pre-treatment in the greenhouse of the stock plants with systemic fungicides was not enough to avoid all the endogenous contaminants. The disinfection treatments with sodium hypochlorite helped to disinfect the external surface of the explant but the endogenous contamination should be treated independently.

Semiwoody cuttings of *A. cherimola* and *A.muricata* with three pre-formed buds were the most viable explants to be established under *in vitro* conditions. They could survive the sodium hypochlorite treatment (NaOCl 3%/15 m). Their size (5-9 cm) protects the pre-formed buds from the phenolic products which increase in concentration in the establishment medium. The brown exudates on the base of the explant prevent the directly new bud sprouting (Figure 11).

Antibiotics

Contamination in tissue culture can originate from microorganisms present in the intercellular tissues of the explant. They are able to grow in the plant tissue culture medium (Campbell, 1990). For *A. cherimola* and *A. muricata* the endophytic presence of *Pseudomonas* spp., and *Pseudomonas saccharophila* were corroborated by the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) in the multiplication aseptic media. These endophytic microorganisms came from the internal tissues of the semiwoody cutting explant.

The *in vitro* contamination is one of the most important obstacles to promote the aseptic micropropagation of *A. cherimola* and *A. muricata*. The contaminants of these plants could not be easily eliminated with the sodium hypochlorite disinfectant solution. Therefore nystatin, rifampicin and cefotaxime were evaluated.

The effect of these *antibiotics* supplied *to the explant* showed effective results in terms of bacteria control expression during the establishment. Nevertheless the new

"aseptic" shoots coming from this essay were subcultured in a new medium without any antibiotics and three weeks after *in vitro* conditions a residual bacterial contamination was observed.

If the antibiotics were added in a *liquid double phase* into the culture media, they were effective in terms of the elimination of contaminants. The liquid presentation of the antibiotic allowed it to diffuse into the internal tissues of *A. cherimola* and *A. muricata* cuttings. The shoots coming from this treatment retained their aseptic condition during the shoot multiplication stage and aseptic culture could be improved.

If antibiotics were added into the culture medium as a *media supplement*, the antibiotics diffused from the media into the internal explant tissues of the *A. cherimola* and *A. muricata* semiwoody cutting explant. This action had a bacteriostatic effect, the endogenous contamination could not be seen in the establishment media with antibiotics but after the subculture to antibiotic free media some shoot cultures showed endogenous contamination again.

Rifampicin was most effective against residual endogenous contamination supplemented in a double phase, followed by Cefotaxime. Nystatin is not recommended because of its low effectiveness on *A. cherimola* and *A. muricata* shoot culture initiation (Table 22).

The residual endogenous contamination of the semiwoody cuttings of *A. cherimola* and *A. muricata* which could not be eliminated by the sodium hypochlorite exogenous disinfection were controlled with the supplementation of Rifampicin into the establishment media which improved the establishment of aseptic cultures during the micropropagation.

Table 22. Effect of some antibiotics on the bacterial contamination of *A. cherimola* and *A. muricata*

Species	Branch Type	Number of buds	Aplication-Method								
			Over			Media supplement			Soaking		
			Nys	Cef	Rif	Nys	Cef	Rif	Nys	Cef	Rif
A. cherimola	semi-woody	1b	·	▪	■	·	▪	■	-	-	■
		3b	·	▪	■	·	▪	■	-	-	■
	woody	1b	·	▪	■	·	▪	■	-	-	■
		3b	·	▪	■	·	▪	■	-	-	■
A. muricata	semi-woody	1b	·	▪	■	·	▪	■	-	-	■
		3b	·	▪	■	·	▪	■	-	-	■
	woody	1b	·	▪	■	·	▪	■	-	-	■
		3b	·	▪	■	·	▪	■	-	-	■

⇒ *Establishment basic media: NN-69 + sucrose 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*
⇒ *Nys: Nystatin (40 µg/ml), Cef: Cefotaxime (95 µg/ml), Rif: Rifampicin (20 µg/ml)*
⇒ *1b : shoot 1 bud (1-3 cm), 3b: shoot 3 buds (5-9 cm)*
⇒ *(■) low effect (25%), (■) medium effect (50%), (■) Effective (100%), (-) no effect (0%)*
⇒ *The values are the mean of the three independent observations, n=36*

Hypersensitivity Reactions

When plant tissues are exposed to stress situations such as mechanical injury the metabolism of phenolic compounds is stimulated. Wounding leads to hypersensitive reactions in the neighboring cells but without showing symptoms of injury themselves, and/or to the premature death of specific cells in the environment of the wound or the place of infection (Deberg and Read, 1990). The *A. cherimola* and *A. muricata* were preconditioned in the greenhouse and also the explants were treated with antioxidant substances to prevent phenolics.

Darkness Precondition in the Greenhouse

The *A. cherimola* and *A. muricata* mother plants were kept in darkness for two weeks, in order to improve the initiation of shoot cultures and to reduce the plant metabolism with the purpose of retarding the hypersensitivity of the branch reaction caused by mechanical injury.

The main response of *A. cherimola* and *A. muricata* mother plants in the greenhouse after two weeks of darkness was defoliation of the expanded leaves and partially etiolated, chlorotic cuttings. The culture explants coming from these etiolated cuttings did not promote new *in vitro* shoot cultures because the lack of vigour was the main limitation.

The selected *A. cherimola* and *A. muricata* explants were not strong enough to promote the *de novo* shoot formation and most of them died by necrosis or vegetative vigour decayment after the inoculation. The presence of phenolics in the culture media was not different in comparison with the explant from the plants of *A. cherimola* and *A. muricata* plants growing in normal light conditions (Table 23).

Table 23. Effect of darkness precondition on A. cherimola and A. muricata plants in the greenhouse

Species	Explant[1] (shoot 5-9 cm)	Photoperiod					
		in vitro growth expression		% latent response		% hypersensitivity reaction	
		12/12 [2]	24/24[3]	12/12	24/24	12/12	24/24
A. cherimola	Soft	8.3	0	100	100	97	88.8
	Semiwoody	25	0	100	100	100	85
	Woody	20	0	100	100	100	100
A. muricata	Soft	5.5	0	100	100	100	90
	Semiwoody	0	0	100	100	100	75
	Woody	5.5	0	100	100	100	100

⇒ *Establishment media: NN-69 + sucrose 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*
⇒ *12/12 : 12 hours light + 12 hours darkness during two weeks*
⇒ *24 : 24 hours darkness during two weeks*
⇒ *The values are the mean of the three independent observations, n=36*

Antioxidant Substances

The phenols are phytotoxic products that limit the *in vitro* culture of *A. cherimola* and *A. muricata*. At the place were wounding was made a constant production of phenols

was observed and a blackening ring around the explant. This black ring comes from the oxidation products promoted by the phenols, which limit the *in vitro* morphogenic responses of the selected *A. cherimola* and *A. muricata* explant.

The effect of some antioxidant substances such as polyvinylpyrrolidone (PVP) and citric acid (CA) in combination with ascorbic acid (AA) were evaluated during the *A. cherimola* and *A. muricata* establishment. The manipulation of the antioxidant substances modified its effect on the tissue explant.

The combination of *citric acid* and *ascorbic acid* prevented the phenolics compounds more effectively during the immersion time after the disinfection treatment than polyvinylpyrrolidone solutions. However they had a short period of action and the polyvinylpyrrolidone results were more active in terms of the control of phenols over a period of time. It means that PVP could be supplemented in the culture media to improve the establishment and might be the subsequent step of the micropropagation where the *A. cherimola* and *A. muricata* show shoot blackening problems (Table 24).

The cutting explants from the vegetative branch have the tendency to produce phenols: meristems, buds, shoot-tips and border of leaves of *A. cherimola* and *A. muricata* are sensitive and react to phenols, PVP, CA and AA, in any of the tested cases could stop this reaction (Table 24).

The woody cutting explants of *A. cherimola* and *A. muricata* showed, in the culture medium, a remarkable tendency to produce phenol compounds if they are compared to the response of the vegetative cuttings. The broken cells on the base of the woody cuttings promote the metabolisms of phenols and the oxidation of pre-formed phenolic components such as lignine could then not be stopped in culture media without antioxidate substances.

The Polyvinylpyrrolidone supplemented in the culture media partially reduced the production of phenolics on the woody explants of *A. cherimola* and *A. muricata* however, these explants could not be established because the phenolics were produced in high concentrations (Table 24).

The semiwoody cuttings are more able to be established under *in vitro* conditions, these explants, in combination with the antioxidant substances, promote the direct *de novo* shoot organogenesis from the pre-formed buds. The production of phenols could be controlled because this explant is not at all lignificated and still retains the potential vegetative condition to improve new *in vitro* shoots if carrying pre-formed buds.

The effect of the antioxidant substances varies with its chemical stability, time of action and way that they are supplied. The polyvinylpyrrolidone PVP seems to be more effective if it is supplemented into the tissue culture media. The tested *A. cherimola* and *A. muricata* explants did not show any blackening in the culture media. It is an indicator that PVP in the culture media is a stable component for a long period of time and the cutting explant found it to be to its own benefit (Table 24).

The semiwoody cuttings are more able to be established under *in vitro* conditions, these explants, in combination with the antioxidant substances, promote the direct *de novo* shoot organogenesis from the pre-formed buds. The production of phenols could be controlled because this explant is not at all lignificated and still retains the potential vegetative condition to improve new *in vitro* shoots if carrying pre-formed buds.

Table 24. Effect of some antioxidants on A. muricata explants
-after two weeks of culture-

Branch	Explant[2]	Immediate response: immersion of the explants during time of culturing -air-flow cabinet-					After two weeks of culture: supplemented into the establishment basic media [1]				
		PVP (ppm)			Cit.ac / Asc.ac (mg/l)		PVP (ppm)			Citr.ac / Asc.ac (mg/l)	
		2	4	8	50/100	150/200	2	4	8	50/100	150/200
Soft Woody	m	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	b	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	st	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
	l	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	1b	++++	+++	++	++++	++++	+++	+++	+++	+++	+++
	3b	++++	++++	+++	++++	+++	+++	+++	+++	+++	+++
Semi-Woody	b	+++	+++	+++	+++	++	++	++	++	++++	++++
	1b	++	+	+	+	-	+	-	-	+	+
	3b	++	+	+	-	-	+	-	-	+	+
Woody	b	+++	+++	+++	++	++	++	+	+	++	++
	1b	++	++	++	+	-	++	+	+	++	++
	3b	++	++	++	+	-	++	+	+	++	++

⇒ [1] *Establishment basic media: NN-69 + sucrose 30 g/l + GR 3 g/l : pH 5.7 ± 0.1*
⇒ [2] *Explants: ½ : semi, m : meristems (0.1 - 0.5 cm), b : buds (0.5 - 1 cm), st : shoot tips (0.5 - 2 cm),*
l : leaves (0-5 - 1 cm), 1b : shoot 1 bud, 3b : shoot 3 buds (5-9 cm).
⇒ *Response: (-) not blackening, (+) low intensity, (++) medium blackening, (+++) blackening,*
(++++) high blackening
⇒ *The values represent the mean of two independent observations, n=36*
⇒ *(*) The response of A. cherimola explants were not significantly different, data are also representative*

The effect of the antioxidant substances varies with its chemical stability, time of action and way that they are supplied. The polyvinylpyrrolidone PVP seems to be more effective if it is supplemented into the tissue culture media. The tested *A. cherimola* and *A. muricata* explants did not show any blackening in the culture media. It is an indicator that PVP in the culture media is a stable component for a long period of time and the cutting explant found it to be to its own benefit (Table 24).

Ascorbic acid in combination with citric acid was not effective, in comparison with PVP, against blackening for a long period of time. This antioxidant mix was highly effective over a short period of time.

The immersion woody and semiwoody cutting explants of *A. cherimola* and *A. muricata* did not produce blackening exudates during two hours of immersion. However if citric acid in combination with ascorbic acid was supplemented into the tissue culture media the percent of blackening exudates in the tissue culture media increased in direct proportion to the period of time for establishment. The phenolic production in *A. cherimola* and *A. muricata* semiwoody cutting should be prevented during the *ex vitro* manipulation of the explant and in the *in vitro* culture medium with polyvinylpyrrolidone.

Culture Media

The concentration of two mineral salts: Murashige and Skoog (1962) (MS-62) and Nitsch and Nitsch (1969) (NN-69) were evaluated on *A. cherimola* and *A. muricata* semiwoody macrocuttings (5-9 cm) explants with two or three buds. The establishment media composition was supplemented with sucrose and the effect of the support agent was evaluated. Some semisolid, solid and liquid mediums were tested. The effect of Agar and Gelrite were compared (Table 25).

Due to the fact that the *in vitro* establishment of the *A. cherimola* and *A. muricata* was limited by oxidation and contamination, the combined effect of Polyvinylpyrrolidone and Rifampicin were evaluated as media supplements to promote healthy and aseptic cultures (Table 25). The semiwoody cuttings of *A. cherimola* and *A. muricata* were *in vitro* reactive to the variation of several factors such as:

Liquid media: 44.4 % of *A. cherimola* and 36.1% of *A. muricata* semiwoody cuttings sprouted in liquid Nitsch and Nitsch (1969) basal medium without hormones, an outstanding percent. However in water media without any salt composition the sprouting of the cutting was also high 44.4% for *A. cherimola* and 38.8 % for *A. muricata*. There were no significant differences between these treatments. The sprouting in Murashige and Skoog (1962) liquid media yielded the lowest percent of sprouting 25.0 % for *A. cherimola* and 22.2 % for *A. muricata*, this media has a significantly high mineral salt concentration. The semiwoody macrocuttings of *A. cherimola* and *A. muricata* did not need high concentrations of mineral salts to promote the sprouting of the pre-formed buds.

Carbon source: The presence of sucrose increased discretly the percent of bud sprouting of *A. cherimola* and *A.muricata* in liquid NN-69 and MS-62 media (Table 25). The cutting explants in water plus sucrose yielded the highest percent of bud sprouting 52.7 % *A. cherimola* and 38.8 % *A. muricata,* as well as the quality of the new *in vitro* shoots which did not show the tendency to be hyperhydrated, in contrast to the shoots coming from mediums not supplemented with sucrose.

Solid media with Agar and Gelrite: Both *A. cherimola* and *A. muricata* are sensitive to Gelrite as a media support agent which, looking at them respectively, showed 30.5 % and 22.2 % of bud sprouting in NN-69, in addition cuttings in mediums supplemented with gelrite proved to be less oxidized on the base than those shoots in mediums supplemented with agar.

Semisolid media with Agar and Gelrite: The explant of *A. cherimola* in semisolid NN-69 media supplemented with Gelrite and sucrose sprouted 41.6% and the *A. muricata* 33.3%. The semisolid media influenced the quality of the newly formed shoots, because these shoots showed the tendency to degenerate into a hyperhydrated shoot in the multiplication stage.

Polyvinylpyrrolidone: The concentrations 2, 4 and 10 ppm were evaluated: the *A. cherimola* showed the minimum control of oxidation in NN-69 media supplemented with 2ppm-PVP. 86.1 % of the explants were affected by the blackening exudates. The 10 ppm- PVP showed the highest percent of the cultures without blackening 47.2% and 4 ppm-PVP 41.6%, there were no significant differences between treatments. *A. muricata* showed similar results, the explant base oxidation reduced from 77.7% in 2ppm-PVP to 47.2% in 10ppm-PVP and 4ppm-PVP. Both species reduced the percent

of bud sprouting when in the culture media not only phenol compounds but also microorganisms were present (Table 25).

Table 25. Effect of the culture medium and supplements on A. cherimola and A. muricata semiwoody cuttings -response after 4 weeks of culture-

Salts and Supplements	A. cherimola			A. muricata		
	sprout	fungi or bacteria	phenols	sprout	fungi or bacteria	phenols
mineral salts						
NN-69	44.4	-	-	38.8	-	-
MS-69	25.0	-	-	22.2	-	-
Water	44.4	-	-	36.1	-	-
mineral salts and sucrose						
NN-69 + S 30 g l^{-1}	47.2	-	-	41.6	-	-
MS-62 + S 30 g l^{-1}	30.5	-	-	25.0	-	-
Water + S 30 g l^{-1}	52.7	-	-	38.8	-	-
mineral salts and gel-agents						
NN-69 + Ag 6 g l^{-1}	27.7	-	-	19.4	-	-
NN-69 + GR 3 g l^{-1}	30.5	-	-	22.2	-	-
MS-62 + Ag 6 g l^{-1}	22.2	-	-	22.2	-	-
MS-62 + GR 6 g l^{-1}	25.0	-	-	25.0	-	-
Nitsch and Nitsch (1969) sucrose and two gel agents						
NN-69 + S 30 g l^{-1} + Ag 6 g l^{-1}	33.3	100	100	33.3	100	100
NN-69 + S 30 g l^{-1} + Gr 3 g l^{-1}	47.2	100	88.8	38.8	100	91.6
Nitsch and Nitsch + sucrose + gelrite+ antibiotic						
NN-69 + S 30 g l^{-1} + GR 3 g l^{-1} + PVP 2 mg l^{-1}	38.8	86.1	86.1	30.5	80.5	77.7
NN-69 + S 30 g l^{-1} + GR 3 g l^{-1} + PVP 4 mg l^{-1}	58.3	83.3	41.6	38.8	75.0	47.2
NN-69 + S 30 g l^{-1} + GR 3 g l^{-1} + PVP 10 mg l^{-1}	52.7	80.5	47.2	30.5	72.2	47.2
Nitsch and Nitsch + sucrose + gelrite+ antioxidant + antibiotic						
NN-69+ S 30 g l^{-1} +GR 3 g l^{-1}+PVP 4 mg l^{-1}+Rf 20 µg ml^{-1}	72.2	11.1	19.4	63.8	5.55	27.7

- ⇒ *Number of independent experiments 3, ** Average of in vitro response per explant, n = 36*
- ⇒ *NN-69 : Nitsch and Nitsch mineral basal media composition (1969)*
- ⇒ *MS-62 : Murashige and Skoog (1962)*
- ⇒ *S : Sucrose (α-D-Glucopyranosyl-β-D-fructo-furanoside; saccharose) (Sigma, S-5390)*
- ⇒ *Gr : GelriteTM, Phytagel (Sigma, P-8169)*
- ⇒ *Ag : Agar (Sigma, A-1296)*
- ⇒ *PVP : Polyvinylpyrrolidone (Av. Mol. Wt. 10,000) (Sigma. P-2307)*
- ⇒ *Rf : Rifampicin (Sigma, R-7382)*
- ⇒ *W : Double distilled-de-ionized-autoclaved water*
- ⇒ *- : (data no shown)*

Rifampicin: The addition of Rifampicin to the establishment culture media reduced the percent of contaminated cultures to 11.1 % in *A.cherimola* and 55.5% in *A. muricata*. Aseptic cultures without Rifampicin in a double phase were difficult to achieve. Additionally the highest proliferation rate was found 72.2% for *A. cherimola* and 63.8% for *A. muricata*.

The percent of bud sprouting increased in a number of cultures where PVP was supplemented. The contamination rate was reduced by an outstanding percent when Rifampicin was supplemented into the culture media. These should be selected to improve the aseptic establishment of *A. cherimola* and *A. muricata*.

Plant Growth Regulators

The relation auxine:cytokine is profitable for species difficult to propagate (Torres, 1989). The effect of *6-Benzylaminopurine* (BA) and *Indole-butiric acid* (IBA) on *A. cherimola* and *A. muricata* bud sprouting was evaluated (Table 26).

The mediums substituted with benzylaminopurine were less prolific in this study. The combination of 8.87 : 2.46µM l^{-1} benzylamino purine and indole butiric acid promoted 86 % of the bud sprouting of semiwoody cuttings of *A. cherimola* and *A. muricata.*

Table 26. Effect of BAP and IBA on shoot bud organogenesis of A. cherimola and A. muricata semiwoody cuttings -response after four weeks of culture-

Species	Growth Regulator (µM l^{-1})	% of cultures with induced shoots *	Number of new shoots per bud *
A. cherimola	BA 2.20 : IBA 0.49	75.0 ± 3.2	1.5 ± 0.6
	4.44 : 1.48	80.3 ± 3.5	1.7 ± 0.7
	8.87 : 2.46	86.0 ± 2.1	3.0 ± 0.9
	BA 2.20 -	10.2 ± 2.3	1.0 ± 0.2
	4.44 -	15.3 ± 4.6	1.0 ± 0.7
	8.87 -	17.7 ± 5.9	1.5 ± 0.8
	- : -	72.2 ± 2.1	2.0 ± 0.2
A. muricata	BA 2.20 : IBA 0.49	80.5 ± 1.1	1.7 ± 0.6
	4.44 : 1.48	83.3 ± 2.8	2.0 ± 0.4
	8.87 : 2.46	86.1 ± 1.5	3.0 ± 1.0
	BA 2.20 -	13.8 ± 1.5	1.5 ± 0.2
	4.44 -	13.8 ± 2.0	1.4 ± 0.8
	8.87 -	19.4 ± 1.4	1.4 ± 0.3
	- : -	63.8 ± 2.3	2.3 ± 0.6

⇒ *The values represent the mean (± SE) of three independent experiments. n = 36*
⇒ *BA (6-Benzylaminopurine): IBA (4,-3-Indolyl]butyric acid)*
⇒ Culture media : Nitsch and Nitsch (1969), Sucrose 30 g/l, Gelrite 3g/l, pH 5.7 ± 0.1

Shoot Induction

The semiwoody macro cuttings of *A. cherimola* and *A. muricata* with two or three pre-formed buds were induced to promote direct bud sprouting of shoots in Nitsch and Nitsch (1969) basal media formulation, supplemented with BA and IBA, Rifampicin, PVP and gelrite.

The combined effect of antibiotics, fungicides, antioxidants, support agent and plant growth regulators was evaluated to prove that to overcome the contamination and oxidation of the *A. cherimola* and *A. muricata* explants the establishment of these woody fruit species in *in vitro* conditions could be easily achieved. The reported results (Table 27) obtained with *A. muricata* (Figure 11) were not significantly different for *A. cherimola* (Figure 12).

The selected chemical composition media of the establishment is able to be used for both species. The addition of Benomyl 0.03 mg/l as a media supplement to control contamination was very effective. The media that were supplemented with Benomyl did not stimulate the new formation of shoots from the pre-formed bud of the macro-cutting. The presence of Benomyl is effective against contamination but is also a shoot culture inhibitor.The other supplements such as antibiotic and antiphenol substances saturated the establishment media that seems to be toxic for the selected explant. No contamination, no phenolization and also no shoot proliferation was reported in this essay.

The high number of shoot per node in *A. cherimola* and *A. muricata* was obtained in the control treatment with water, without hormones, and supplemented with antibiotics and polyvinylpyrrolidone (Table 27).

The *A. cherimola* and *A. muricata* gelrite (GR) supplemented media were more shoot proliferant (27.3 explants producing shoots / 36 cultured explants) than those media supplied with agar-agar (Ag) (21.8 explants producing shoots / 36 cultured explants).

There were no significant differences between the explants proliferating in gelrite and the explants proliferating in the liquid medium supplemented with sucrose (26.8 explants producing shoots / 36 cultured explants) (Table 27).

The surface of the stem, the cambium was also stimulated and clusters with a whitish color were observed but no regeneration responses were obtained with this new source of multiplication material. Until four new shoots emerged simultaneously all over the pre-formed bud. This result confirmed the tetrapotential dormant meristem of this semideciduous subtropical and tropical trees (Figures 12; 13).

Effect of Closures

Closures prevent drying out and infection, while on the other hand a change of gases with the "outside air" must be possible to avoid a shortage of O_2 and to prevent an accumulation of gases produced, such as CO_2 and ethylene. The accumulation of ethylene affected the development of *A. cherimola* and *A. muricata* new shoots.

The *in vitro* shoots also developed new leaves, which were induced to fall down during the establishment 45.9 % for *A. cherimola* and 38.9 % for *A. muricata* when the magenta sigma cups of the test-tubes were totally closed with parafilm (Figure 14).

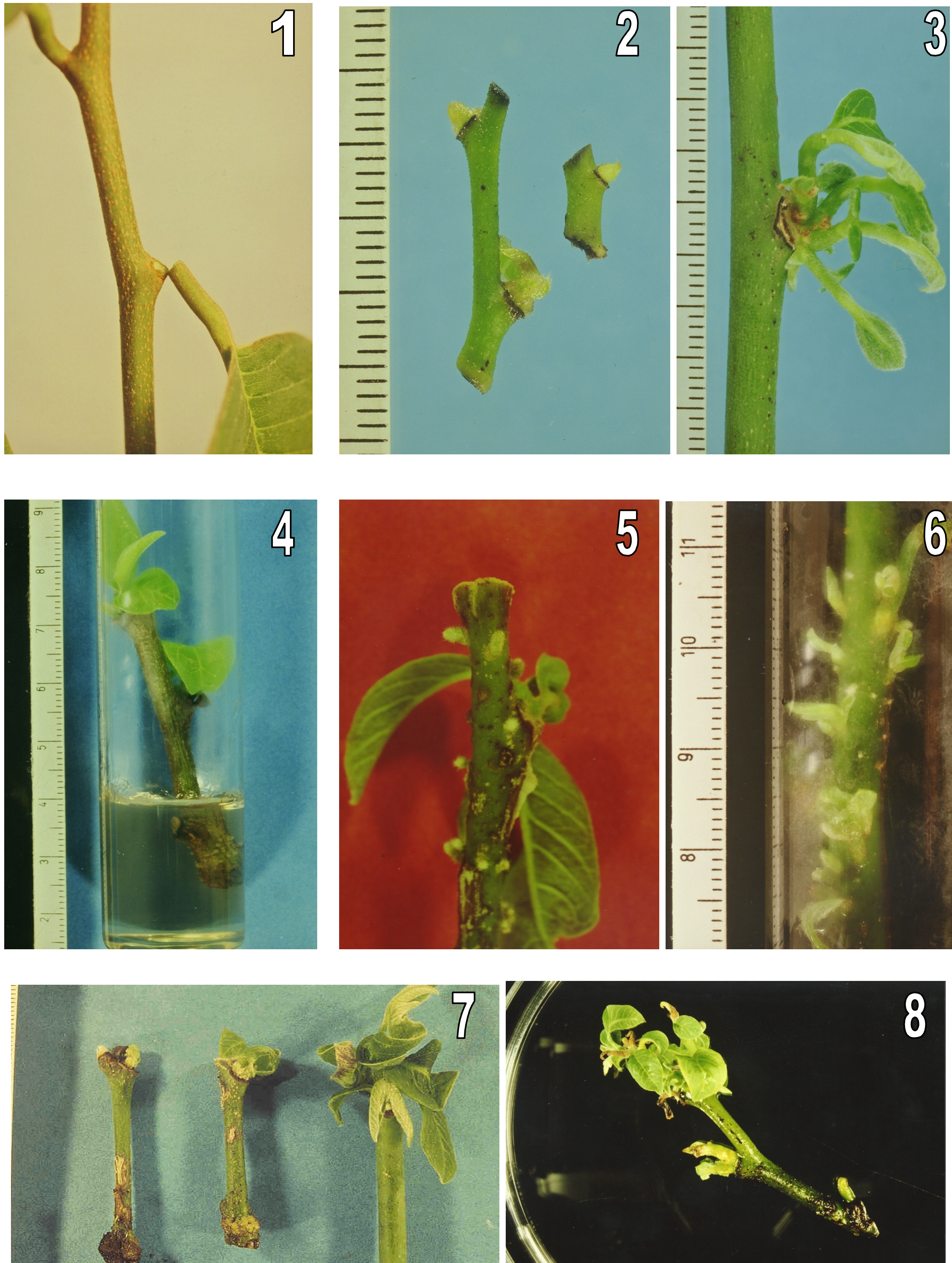
The polypropylene magenta sigma caps performed as diffusible membranes for gases (with the exception of water vapour). If they were closed completely with parafilm during the establishment and multiplication of the *A. cherimola* and *A. muricata* the humidity increased in the test-tube and some new *in vitro* shoots were hyperhydrated, 23.3 % for *A. cherimola* and 18.3 % for *A. muricata* (Figures 14).

The tendency of the *A. cherimola* and *A. muricata* shoots to induce hyperhydrated shoots during the establishment of the test-tubes completely closed with parafilm, was observed also during the multiplication stage.

Table 27. Effect of some supplements on the phenolization and contamination of *Annona muricata* (T$_1$) *semiwoody cuttings (5-9cm) with pre-formed buds* to promote the *in vitro* establishment - *after four weeks of culture-*

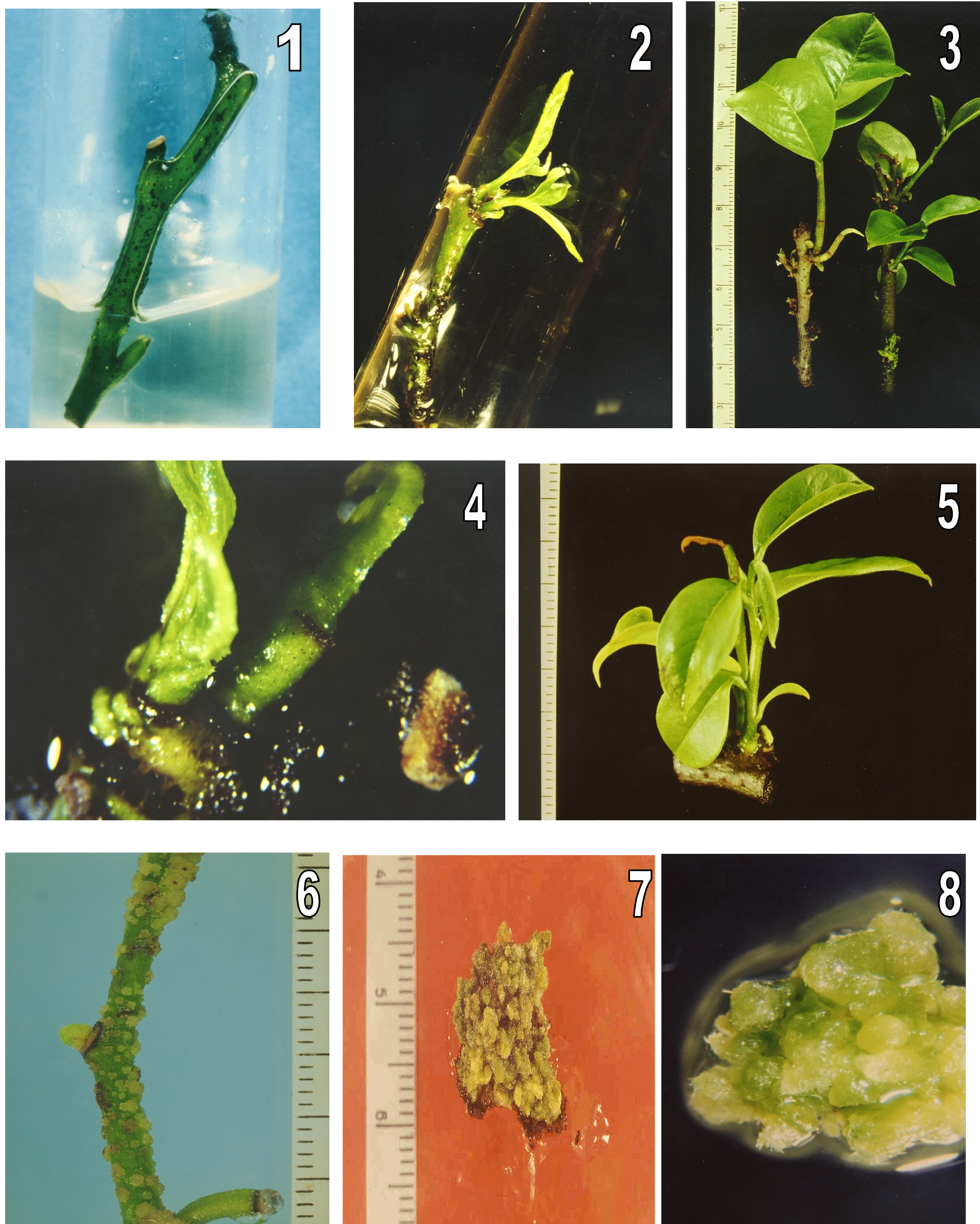
SALTS	BA :IBA	S	G	Ag	Rf	PVP	Cit.ac / Asc.ac	Bmyl	Nr. explants producing shoots	Mean** shoot number per bud	Nr. non aseptic explants	Nr. phenolized explants
NN-69	-	-	-	-	-	-	-	-	22	1.6 ± 0.4	36	36
	8.87:2.46	-	-	-	-	-	-	-	25	2.0 ± 0.5	33	36
	8.87:2.46	30	-	-	-	-	-	-	25	1.6 ± 0.3	30	36
	8.87:2.46	30	3	-	-	-	-	-	26	2.3 ± 0.2	32	36
	8.87:2.46	30	3	-	20	-	-	-	28	2.6 ± 1.0	9	33
	8.87:2.46	30	3	-	-	4	-	-	28	2.6 ± 0.8	35	23
	8.87:2.46	30	3	-	-	-	150:200	-	25	1.3 ± 0.5	36	28
	8.87:2.46	30	3	-	20	4	-	-	33	3.0 ± 0.9	6	8
	8.87:2.46	30	3	-	20	-	150:200	-	30	1.3 ± 1.2	5	23
NN-69	8.87:2.46	30	-	6	-	-	-	-	18	0.6 ± 0.1	32	36
	8.87:2.46	30	-	6	20	-	-	-	20	0.6 ± 0.5	4	36
	8.87:2.46	30	-	6	-	4	-	-	23	1.0 ± 0.4	33	23
	8.87:2.46	30	-	6	-	-	150:200	-	22	1.0 ± 1.1	29	26
	8.87:2.46	30	-	6	20	4	-	-	25	1.8 ± 0.8	15	14
	8.87:2.46	30	-	6	20	-	150:200	-	23	1.0 ± 0.2	10	22
	8.87:2.46	30	-	6	-	-	-	0.3	5	1.0 ± 0.2	0	12
	8.87:2.46	30	-	6	20	-	-	0.3	8	0.6 ± 0.2	0	8
	8.87:2.46	30	-	6	-	4	-	0.3	4	0.6 ± 0.0	0	0
	8.87:2.46	30	-	6	-	-	150:200	0.3	3	0.6 ± 0.5	0	0
	8.87:2.46	30	-	6	20	4	-	0.3	10	0.6 ± 0.2	0	0
	8.87:2.46	30	-	6	20	-	150:200	0.3	2	0.6 ± 0.1	0	0
W	-	-	-	-	-	-	-	-	30	2.3 ± 1.1	36	36
	-	30	-	-	-	-	-	-	36	3.0 ± 0.4	36	36
	-	30	-	-	20	-	-	-	27	3.0 ± 0.1	12	36
	-	30	-	-	-	4	-	-	30	3.0 ± 0.5	36	15
	-	30	-	-	-	-	150:200	-	20	1.3 ± 1.2	36	23
	-	30	-	-	20	4	-	-	20	2.3 ± 0.5	10	10
	-	30	-	-	20	-	150:200	-	25	1.6 ± 1.3	5	22
	8.87:2.46	-	-	-	-	-	-	-	29	2.3 ± 0.8	36	28
	8.87:2.46	-	-	-	20	-	-	-	28	1.6 ± 0.6	5	35
	8.87:2.46	-	-	-	-	4	-	-	30	1.6 ± 0.8	36	10
	8.87:2.46	-	-	-	-	-	150:200	-	22	0.6 ± 0.3	36	22
	8.87:2.46	-	-	-	20	4	-	-	25	1.6 ± 1.0	30	9
	8.87:2.46	-	-	-	20	-	150:200	-	19	1.3 ± 0.5	4	17

⇒ *Number of independent experiments 3. ** Average number of new formed shoots per bud (± SD)*
⇒ *NN : Nitsch and Nitsch mineral basal media composition (1969)*
⇒ *S : 30 g/l Sucrose (α-D-Glucopyranosyl-β-D-fructo-furanoside; saccharose) (Sigma, S-5390)*
⇒ *G : 3 g/l GelriteTM, Phytagel (Sigma, P-8169)*
⇒ *Ag : 6 g/l Agar (Sigma, A-1296)*
⇒ *BA : 8.87 µM (6-Benzylaminopurine) (Sigma, B-9395)*
⇒ *IBA : 2.46 µM (4,-3-Indolyl]butyric acid) (Sigma, I 5386)*
⇒ *PVP : 4 mg/l Polyvinylpyrrolidone (Av. Mol. Wt. 10,000) (Sigma, P-2307)*
⇒ *Asc. ac : 150 mg/l L-Ascorbic acid (Sigma, A-2174)*
⇒ *Cit.ac : 200 mg/l Citric acid (Sigma, C-4540)*
⇒ *Bmyl : Benomyl 0.03 mg/l*
⇒ *W : Double distilled- de-ionized-autoclaved-water*
⇒ *Rf : 20 µg/ml Rifampicin*
⇒ *- : non supplemented*



1. A. cherimola shoot with a non manipulated node showing the location of a pre-formed bud complex with four meristems
2. Bud sprouting of semiwoody cuttings after the control of phenols and contaminants under in vitro culture
3. Direct sprouting of four new shoots per bud on semiwoody cuttings of A. cherimola after four weeks of culture
4. Semiwoody branch of A. cherimola in medium supplemented with Polyvinylpyrrolidone and Rifampicin
5. Adventitious bud formation in Nitsch and Nitsch (1969) medium with BA and IBA
6. Organogenic potential of A. cherimola in optimal establishment conditions
7. Sequence of the formation of multiple shoots without callus formation
8. Multiple shoots in optimal development and size to be subcultured in multiplication medium

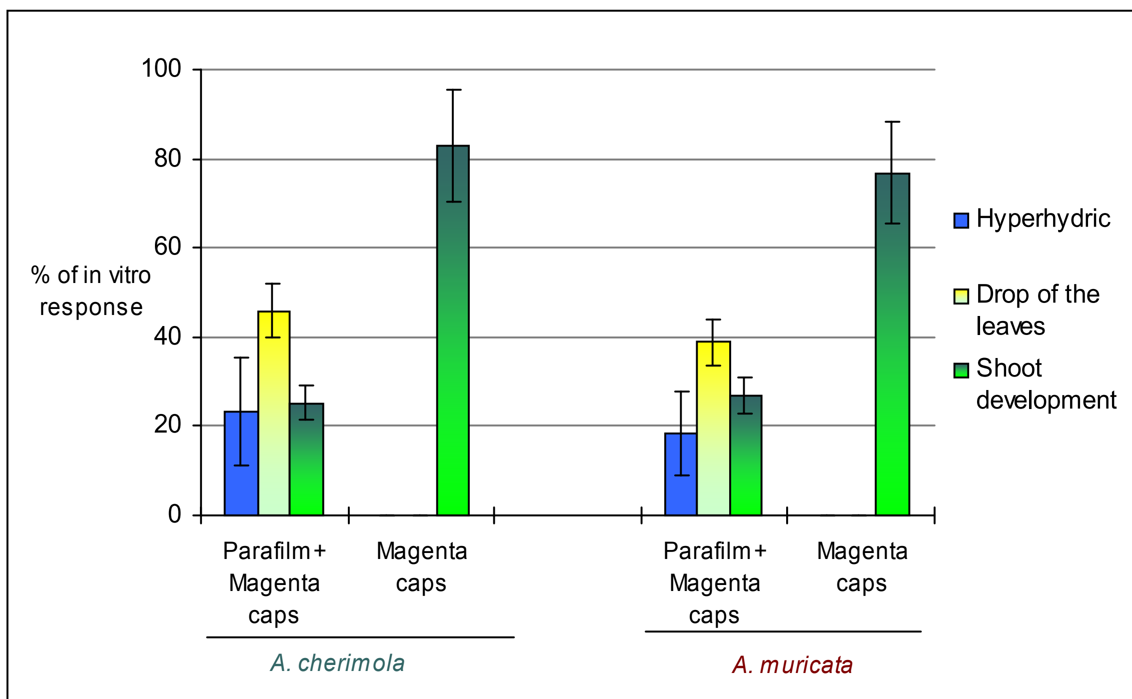
Figure 12. Various stages during the in vitro establishment of A. cherimola



1. A. muricata shoot with a non manipulated node with four meristems
2. Bud sprouting of semiwoody cuttings after the control of phenols and contaminants under in vitro culture
3. Direct sprouting of four new shoots per bud on semiwoody cuttings of A. muricata after four weeks of culture
4. Detail of the multiple shoot formation
5. Organogenic potential of A. muricata microcutting
6. Adventitious callus formation from the meristematic cambium in A. muricata
7. Formation of callus in A. muricata subcultured on NN-69 + 2,4-D (4,53 µM)
8. Formation of multiple buds on A. muricata callus (no regeneration is reported)

Figure 13. Various stages during the in vitro establishment of A. muricata

A. cherimola and *A. muricata* during *in vitro* conditions needed to diffuse some gases produced during organogenesis. It is important to note that the recipients, where the plants were micropropagated, were greatly aromatic. During the *in vitro* root organogenesis a total closed recipient is not recommended, because the purpose is to induce in the explant a gradual adaptation for an improved successful adaptation to the *ex vitro* conditions



⇒ *Establishment media: NN-69+ S 3 % + GR 0. 3 % + B 8.87µM + IBA 2.46 µM + Rf 20 µg/ml*
⇒ *Number of independent experiments 3, Average of number of new formed shoots per bud (± SD)*
⇒ *Total cultured explants: n=36*

Figure 14. Effect of magenta caps and parafilm during the establishment of A. cherimola and A. muricata in test-tubes

It is convenient to initiate growth in medium prepared in test-tubes 25 x 150 mm being not totally closed. Only the magenta Sigma caps were useful. The test-tubes make it easy to monitor the new shoot formation and allow individual treatments of explants.

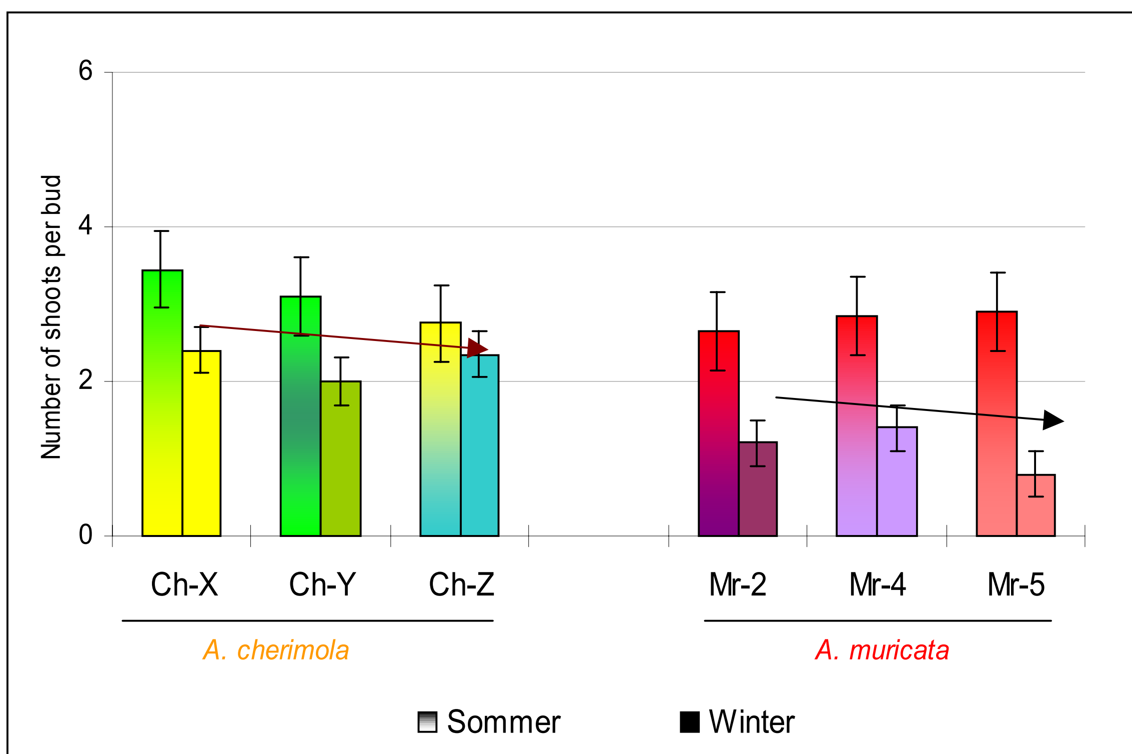
The incubation conditions for *A. cherimola* and *A. muricata* cuttings were 16 h light and 8 h darkness and 26 ± 2 °C as temperature standard. Incrementes of temperature to 30°C or higher can result in condensation on the inner surface of culture vessels and the cultures. This can lead to hyperhydration, a condition where internal leaf air spaces become water-filled and leaf and culture morphology become abnormal. The *A. cherimola* and *A. muricata* the semiwoody cuttings taken in summer were more *in vitro* reactive than cuttings from winter.

Effect of Season

Previous experiments inducing the establishment of *A. cherimola* and *A. muricata* showed that the season had an influence on bud sprouting in *in vitro* conditions. The *A. cherimola* and *A. muricata* selections showed different responses in different seasons. In winter the number of shoots per bud reduced significantly in both species. Thus semiwoody shoots with 3 buds taken from *A. cherimola* and *A. muricata* semideciduous selections were evaluated in summer and winter (Figure 15).

The bud sprouting and subsequently the number of shoots per bud were more effective in summer. From *A. cherimola* the cv. Felpa (Ch-X) 3.45 ± 0.46 was more prolific than the cv. Bronceada (Ch-Y) 3.1 ± 0.42 and the Colombian selection (Ch-Z) 2.75 ± 0.50 showed no great difference in the number of shoots per bud in summer 2.75 ± 0.47 than in winter 2.35 ± 0.33 (Figure 14).

The semiwoody cuttings of *A. muricata* selections (2; 4; 5) were severly affected in terms of bud sprouting in winter. A very low rate of the number of shoots per proliferating bud was registered (Figure 14). The *A. muricata* selections were more proliferating in summer, this species is also the most tropical of the *Annona* spp.



⇒ *Establishment media: NN-69+ S 3% + GR 0.3% + B 8.87µM + IBA 2.46 µM + Rf 20 µg/ml*
⇒ *Number of independent experiments 2, Average of number of new formed shoots per bud (± SD) n=20*

Figure 15. Season effect on bud sprouting of A. cherimola and A. muricata selections

6.3 Multiplication

The new *in vitro* sprouting shoots of *A. cherimola* and *A. muricata* from the pre-formed established buds were transplanted after five weeks of *in vitro* establishment in the multiplication media.

The mineral basal formulation reported by Nitsch and Nitsch (1969) used during the establishment was the basic salt combination during multiplication experiments. Additionally the Woody Plant Medium and Murashige and Skoog (1962) salt formulations were evaluated.

The young shoots with a nodal segment coming from the semi-woody *A. cherimola* and *A. muricata* cuttings were not phenol reactive in the tissue multiplication media therefore antioxidants were excluded.

Effect of Cytokinins

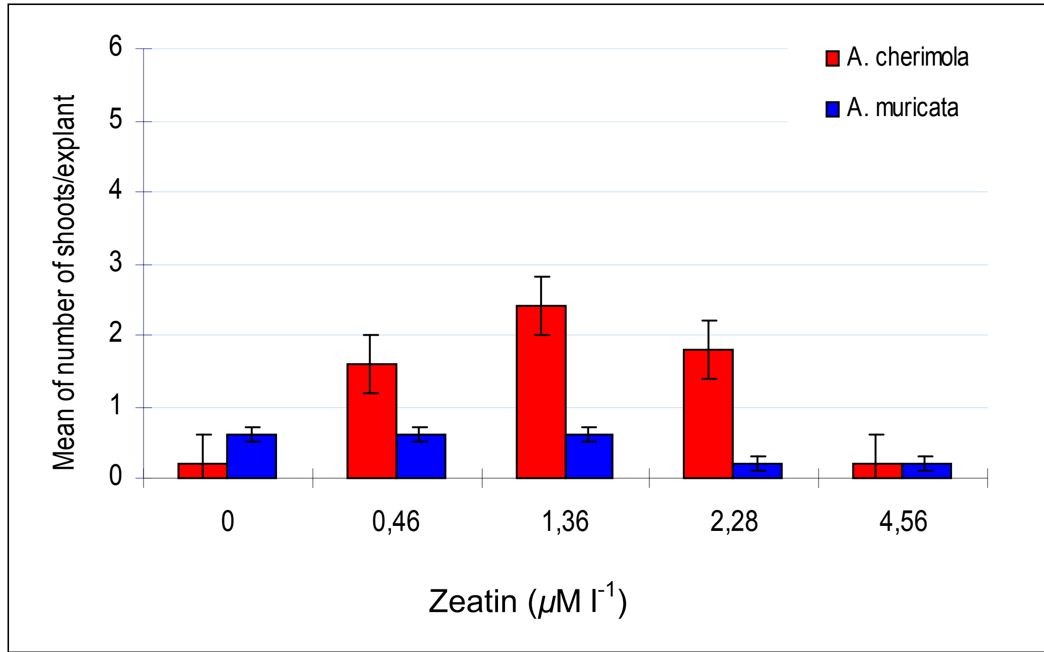
To promote the new shoot multiplication in *A. cherimola* and *A. muricata* different combinations of cytokinins were evaluated in Nitsch and Nitsch (1969) mineral and vitamin combinations in addition to sucrose 30 g l^{-1}, gelrite 3 g l^{-1} the media pH 5.7 ± 0.1. Temperature and photoperiod factors have been previously described (*cf.* Materials and Methods). Finally the cultures were not closed with parafilm.

Zeatin

The *A. cherimola* reported a special sensitivity to different zeatin concentrations. The best results were obtained at 1.36 µM l where 75 % (15/20) of established shoots induced a proliferation rate of 2,4 ± 0,7 new shoots per explant without callus (Figure 16). *A. muricata, on the* contrary, did not proliferate in Nitsch and Nitsch (1969) media supplemented with zeatin. Only a few small new shoot formations were observed in the best cases. The highest mean proliferation rate of *A. muricata* was obtained in the treatment which excluded the zeatin. 1.38 ± 0.3 shoots were induced, in addition the shoot mean proliferation rate reduced proportionally when zeatin concentration increased.

Kinetin

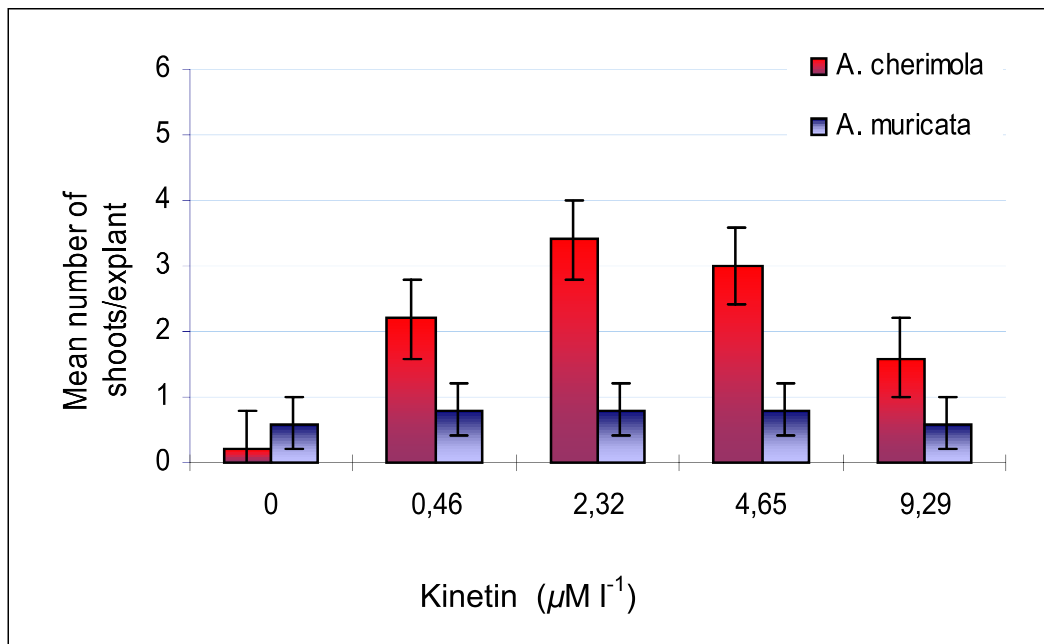
The *A. cherimola* shoots also proliferated in media supplemented with kinetin, the concentration of 2.32 µM is the most proliferating, 60% (12/20) of established shoots induced a proliferation rate with 3.5 ± 0.5 new shoots per explant without callus (Figure 17). *A. muricata* was not shoot proliferant in kinetin supplemented medium, big-green and more expanded leaves were observed.



⇒ *Mean of 3 replicates per treatment - Number of shoots per treatment = 20*

⇒ *Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

Figure 16. Zeatin effect on A. cherimola and A.muricata shoot formation



⇒ ** Mean of 3 replicates per treatment - Number of shoots per treatment = 20*

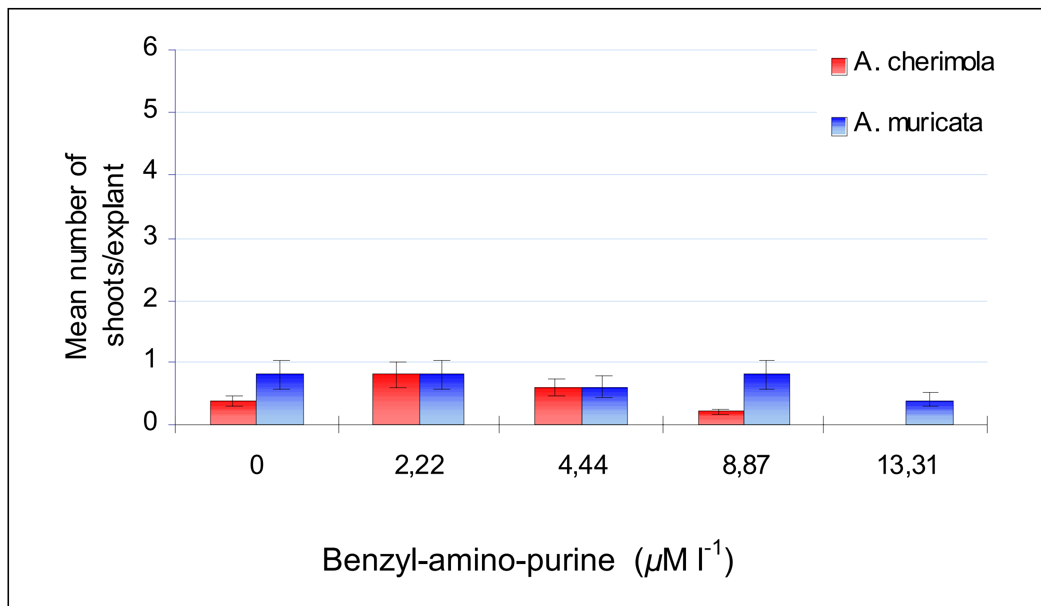
⇒ ** Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

Figure 17. Kinetin effect on A. cherimola and A.muricata shoot formation

Benzyl amino-purine

The cytokinin benzylaminopurine which had a positive effect on shoot induction for *A. cherimola* and *A. muricata* semi-woody cuttings in combination with Indole butyric acid were tested singly to induce shoot multiplication.

As a result, neither *A. cherimola* nor *A. muricata* showed shoot proliferation in a media supplemented with several concentrations of BA (Figure 18) The explants remained green but no new *in vitro* response was observed, less than 5% (1/20) of the shoots induced 0.8 ± 0.2 new shoots per explant were obtained.



⇒ ** Mean of 3 replicates per treatment - Number of shoots per treatment = 20*
⇒ ** Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

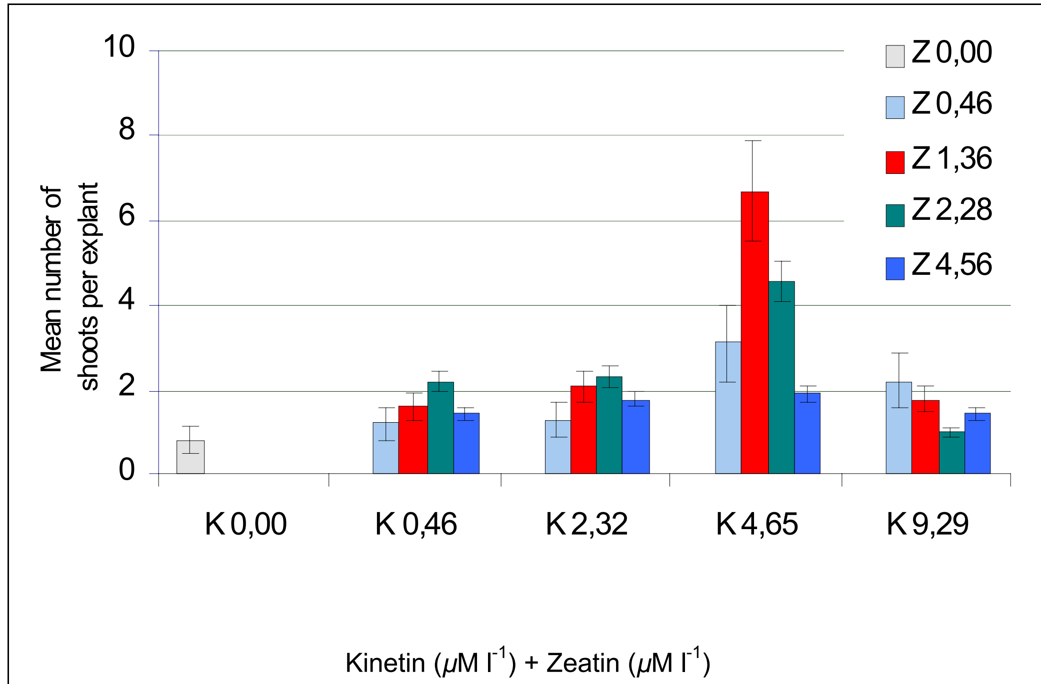
Figure 18. Benzyl-amino-purine effect on A. cherimola and A.muricata shoot formation

Kinetin and Zeatin

In order to increase the mean proliferation rate in *A. cherimola* and *A. muricata*, the combination kinetin and zeatin were evaluated (Figure 19).

A. cherimola reported, in a concentration of 4,65 µM kinetin + 1,36 µM zeatin, 100% shoot proliferation (20/20) with 6,7 ± 1,3 new shoots as a mean proliferation rate (Figure 19). The individual shoots coming from this experiment were also shoot proliferant in the subsequent multiplication subcultures.

The *A. muricata* established shoots did not induce new proliferation shoots. The control medium without citokinin showed the better quality shoots, increasing in size.



⇒ *Mean of 3 replicates per treatment - Number of shoots per treatment = 20*

⇒ *Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

Figure 19. Effect of Kinetin combined with Zeatin on A.cherimola shoot proliferation

Thidiazuron

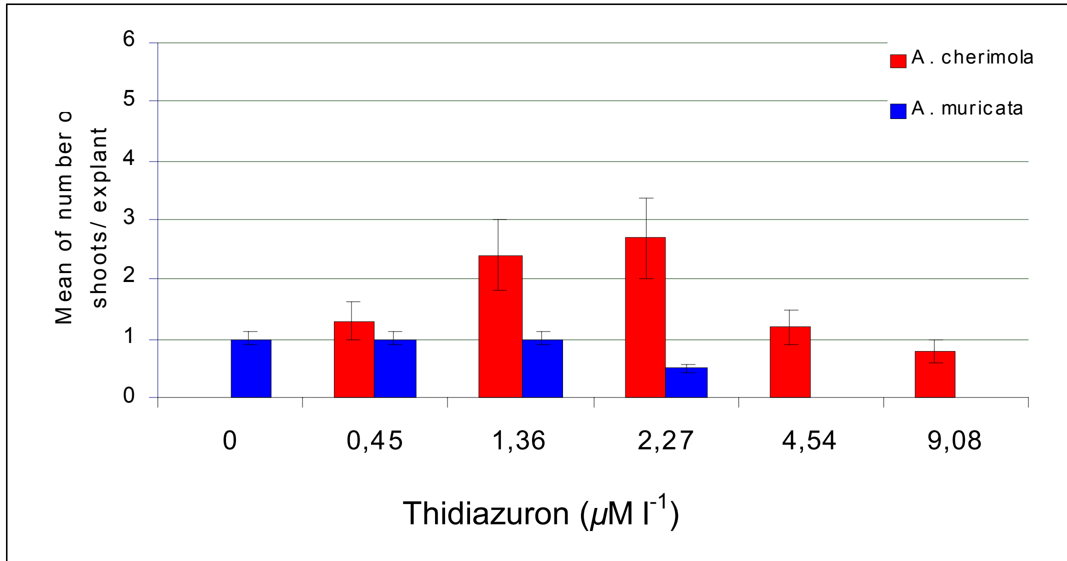
Thidiazuron is a synthetic cytokinin with a benzyl-amino-purine activity but 10,000 times more powerful. Several concentrations were tried to promote the proliferation of *A. cherimola* and *A. muricata.* As a result, at the base of shoots some roseta formations were observed (Figure 22) with organogenesis of multiple leaves which did not induce any regeneration response.

Gibberellic acid

Due to the fact that the *A. muricata* were not shoot proliferating in Nitsch and Nitsch (1969) supplemented with cytokinins, shoot elongation was stimulated in order to induce proliferation through *in vitro* cuttings in this media formulation supplemented with Gibberellic Acid (GA_3) (Figure 21).

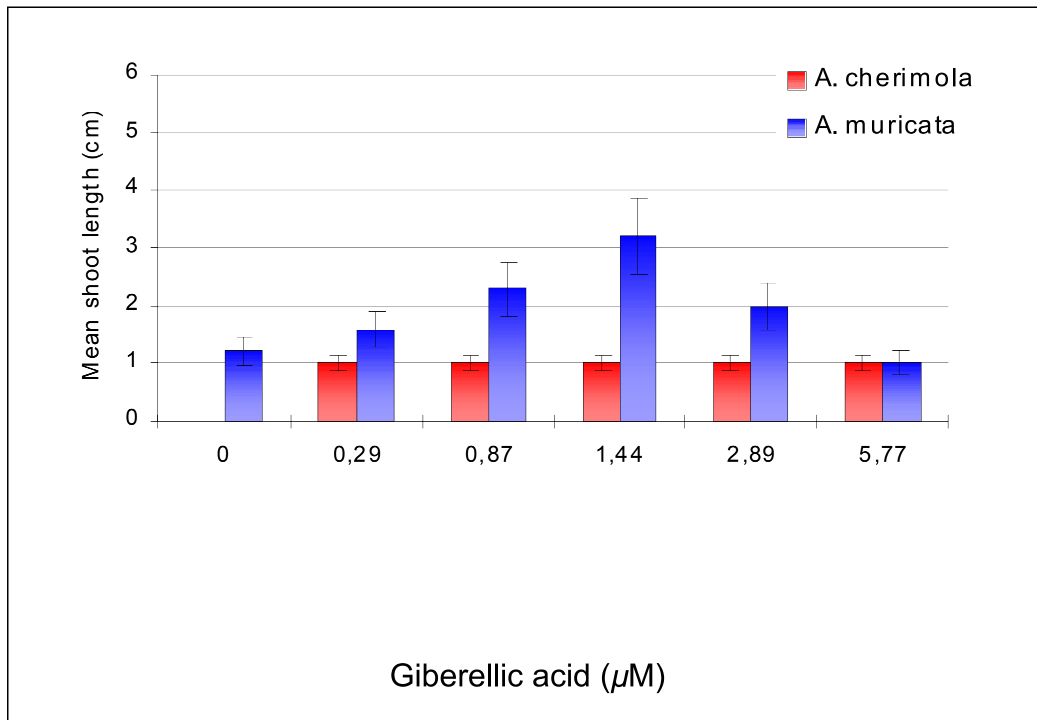
Elongation was performed with 1 cm in length *A. muricata* and *A. cherimola* shoots. The *A. cherimola* did not increase in length and some shoots lost their quality when they remained in this medium supplemented with GA_3

A. muricata increased up to 3.5 cm in four weeks of culture with a mean promedium of 5 ± 3 leaves per shoot and 3 ± 1 internode. The quality of the shoot was optimal and no bud sprouting or adventitious formations were observed.



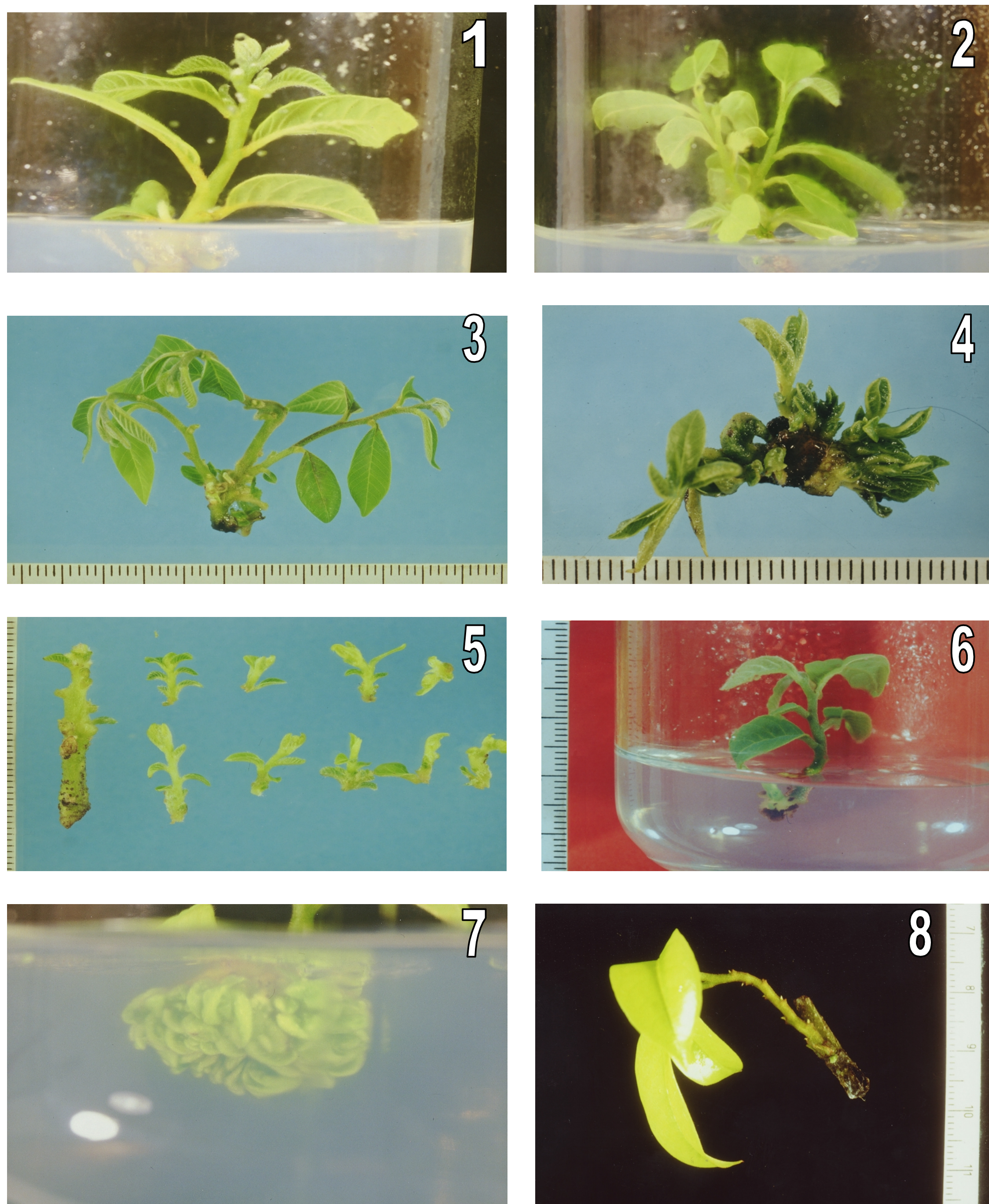
⇒ ** Mean of 3 replicates per treatment - Number of shoots per treatment = 20*
⇒ ** Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

Figure 20. Effect of thidiazuron on A. cherimola and A.muricata shoot formation



⇒ ** Mean of 3 replicates per treatment - Number of shoots per treatment = 20*
⇒ ** Medium: NN-69 + S 3% + Gr 0.3% pH 5-7 ± 0.1*

Figure 21. Effect of gibberellic acid on A. cherimola and A. muricata shoot elongation



1. 2. 3. 4. A. cherimola shoot proliferation on Nitsch and Nitsch (1969) and 2.32 µM K and 1.6 µM Z

5. A. cherimola showing proliferation rate, the same situation is reported for A. muricata

6. 7. A. muricata shoot elongation

9. Effect of thidiazuron 2.27 µM on A. muricata base of the shoot, as well as in A. muricata, no adventitious shoot formation is reported from this type of organogenesis

Figure 22. Several in vitro responses of A. cherimola and A. muricata during the multiplication stage - after four weeks of culture-

Shoot Quality

During the shoot multiplication in a Nitsch and Nitsch (1969) medium, supplemented with zeatine and kinetine, necrotic spots on the leaves and poor growth were observed in all the *A. cherimola* selections after three months of culture before they started to loss the green colour and showed *chlorosis*. The shoots lost their quality and it was not possible to rescue them. A subculture in a fresh medium was tried as a first strategy for not loosing the micropropagated material, but without success.

On the base of many chlorotic explants a white exudate was observed. The presence of some residual bacterial contamination *Pseudomonas saccharophila*[7] was found. The explants with residual contamination during the multiplication were eliminated and a new establishment with reported strategies (*cf. 5.1.2 Endogenous disinfection*) was reviewed, healthy explants were obtained after these experiments.

Chlorosis

Healthy explants of *A. cherimola,* after the third subculture in a defined Nitsch and Nitsch (1969) basic salt medium formulation supplemented with zeatine 1,36μM + kinetin 4,65 μM, started to be chlorotic.

Preliminary experiments, varying the concentration of macro and micro elements as reported by Nitsch and Nitsch (1969), showed that the chlorosis in *A. cherimola* shoots was related to the concentration of nitrogen ions (data not shown).

The effect of ammonium nitrate, potassium nitrate and ammonium carbonate ions supplied in the Nitsch and Nitsch (1969) salt formulation medium were evaluated. In this series of experiments the other media components such as: macroelements, iron, microelements, vitamins, sucrose and gelrite were maintained standard as well as the kinetin and zeatin concentrations (c*f.* Materials and Methods).

The total concentration of (NH_4^- *m*M) and (NO_3^- *m*M) reported in the media of: Lloyd and McClown (1980), De Gref and Jacobs (1979), Murashige and Skoog (1962) and Nitsch and Nitsch (1969) were compared.

The effect of casein hydrolysate, natural complex of nitrogen (Sigma C 7290) in concentrations of 100 - 200 mg l^{-1} was evaluated and compared with the different nitrogen medium formulations described before (Table 28).

The results showed chlorosis and leaf defomation with some necrotic spots in all the tested media where the ions NH_4^- and NO_3^- were not included (Table 28). All this material was eliminated because the quality was poor.

The casein hydrolysate, as an external nitrogen source, promoted growth and the number of shoots without chlorosis, suggesting that the observed chlorosis was due to a deficiency of nitrogen in the mediums (Table 28)

The substitution of the concentration reported by Nitsch and Nitsch(1969), NH_4^- 9.0 mM and NO_3^- 18.4 mM by the concentration reported by Murashige and Skoog (1962) NH_4^+ 20.6 mM and NO_3^- 39.4 mM was enough to maintain the *A. cherimola* cultures green and proliferating (Table 28) (Figure 23).

[7] Analysis made by the „Biologische Bundesanstalt für Land- und Forstwirtschaft . BBA“

With the optimal (NH_4^+ / NO_3^-) balance the number of new shoots preserved their quality. Thus ammonium nitrate reported by Nitsch and Nitsch (1969) was substituted by the ion concentration reported by Murashige and Skoog (1962).

Table 28. Effect of different nitrogen sources during the shoot multiplication of A. cherimola on Nitsch and Nitsch (1969) basic medium *

CH[a] (mg l^{-1})	Supplemented on NN-69		Total Ion * concentration reported by	% cultures without chlorosis **
	NH_4^+mM	NO_3^- MM		
0	0	0	-	4.0 ± 2.6
	5.0	9.8	WPM [b]	41.3 ± 3.3
	6.0	19.8	Dg-J [c]	22.4 ± 2.5
	9.0	18.4	NN-69 [d]	8.3 ± 1.3
	20.6	39.4	MS [e]	100.0 ± 0.0
100	0	0	-	20.0 ± 3.1
	5.0	9.8	WPM	55.4 ± 5.2
	6.0	19.8	Dg-J	25.2 ± 1.8
	9.0	18.4	NN-69	33.1 ± 3.4
	20.6	39.4	MS	100.0 ± 0.0
200	0	0	-	41.6 ± 4.2
	5.0	9.8	WPM	60.2 ± 3.3
	6.0	19.8	Dg-J	32.4 ± 2.5
	9.0	18.4	NN-69	36.3 ± 1.3
	20.6	39.4	MS	100.0 ± 0.0

⇒ *Only the $NH_4^+$and NO_3^- concentration in "mM" vary on the original mineral salts reported formulation " Nitsch and Nitsch, 1969".*
⇒ *The mediums were supplemented with sucrose 3%, Gelrite 3 g l^{-1} and Zeatine + Kinetine as growth regulators*
⇒ *The values represent the mean (± SE) of three independent experiments, twenty explants were used for each experiment*
⇒ *[a] CH : casein-enzymatic hydrolysate (N-Z-Amine A) total nitrogen 13.1% and amino nitrogen 6.5% approx.*
⇒ *[b] Lloyd and McClown (woody plant medium) (1980), [c] De Gref and Jacobs (1979), [d] Nitsch and Nitsch (1969), [e]Murashige and Skoog (1962)*

Subcultures

The continued production of multiple shoots in *A. cherimola* and *A. muricata* was evaluated during the subcultures in the same multiplication medium. The percentage of abnormal plantlets or plantlets not growing well was very low and not significant. Hyperhydration and chlorosis were totally avoided. Callus formations were not observed in any case. There were no significant differences between selections during the multiplication rate (Figure 24).

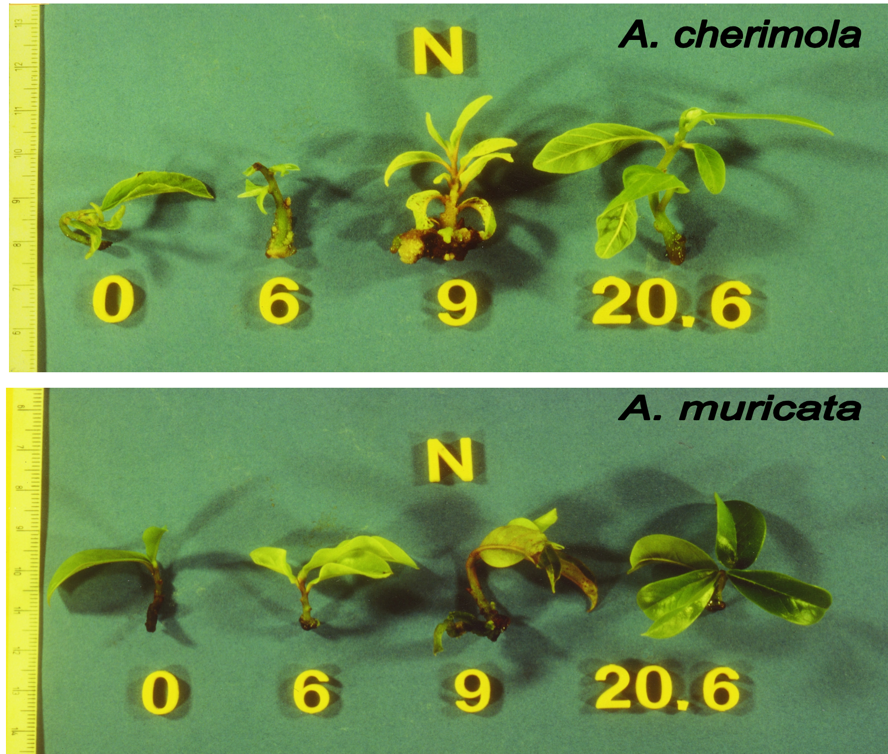


Figure 23. Effect of nitrogen concentration in [µM] on in vitro chlorosis of A. cherimola and A. muricata

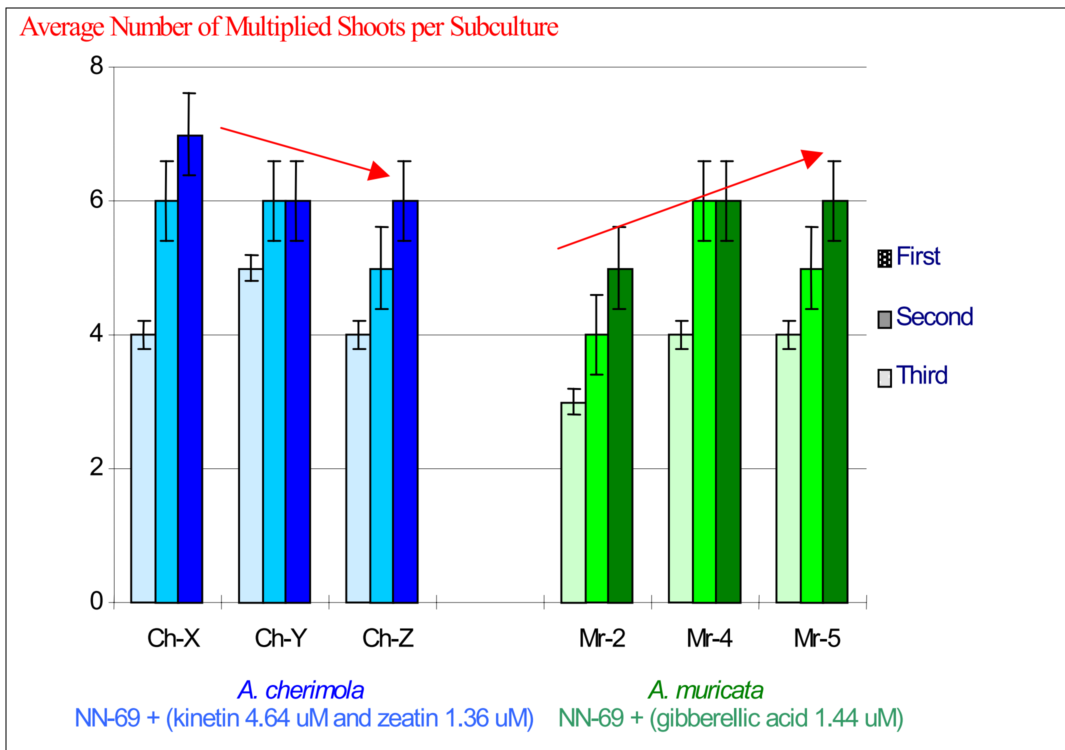


Figure 24. Effect of subculture number on the proliferation of A. cherimola and A. muricata selections - after three four weeks of subculture

6.4 Rooting

To promote rhizogenesis in *A. cherimola* and *A. muricata* previous negative results have suggested that the *in vitro* shoots multiplied *in vitro* should be preconditioned before the application of any root organogenesis treatment.

The number of subcultures and the cytokinin synergistic combination in *A. cherimola* seemed to inhibit the potential of the shoots to promote new roots *in vitro*. Also *A. muricata* showed the same limitation, because the gibberellic acid is known as a root inhibitor in tissue culture.

In vitro

The root organogenesis of *A. cherimola* and *A. muricata* multiplied shoots (3 -5 cm long) was very difficult. The *A. cherimola was* rooted by 43.2% with IBA 4.90 µM and *A. muricata* 36.4% respectively. The *in vitro* medium was ¼ macro salt NN-69 concentration and 1% sucrose.

The concentration of macro-elements reported by Nitsch and Nitsch (1969) was reduced to ¼ of its normal concentration and supplemented with a low concentration of sucrose 1%. For both species the first roots began to emerge after 40 days without callus formation (Table 29).

Table 29. Evaluation of some auxins to improve the rhizogenesis on A. cherimola and A. muricata

s	Growth Regulator µM l^{-1}		Promedium in % of rooted shoots ± SD After 40 days of in vitro culture	
			A. cherimola	A. muricata
1/4	-	-	0	0
	IBA	2.46	0	0
		4.90	43.2 ± 3.6	36.4 ± 5.3
		9.80	0	0
		24.60	0	0
1/4	NAA	2.69	0	0
		5.37	0	0
		10.74	0	0
		26.85	0	0
1/4	IAA	2.85	0	0
		5.71	0	0
		11.42	0	0
		28.54	0	0

⇒ * *The values represent the mean (± SE) of five independent experiments.*
⇒ *Twenty explants were used for each experiment*

The root induction of *A. cherimola* micropropagated explants were tested with the total macro-elements salt concentration (1X) and (1/2) with IBA, NAA and IAA combinations. In all the experiments rhizogenesis was not reported (data not shown).

The *A. cherimola* and *A. muricata* are not root prolific under *in vitro* conditions. The base of the multiplied explant did not improve any callus or rosette formation when they were treated with NAA and IAA or IBA in a high concentration. The shoot explants showed the tendency to improve the production of phenolics which were not present during multiplication.

The concentration of IBA 4.90 µM promoted the rhizogenesis of *A. cherimola* explants only if the mineral basal composition was reduced to ¼ concentration. The *A. cherimola* and *A. muricata* rhizogenesis were evaluated also in the clonal selections (Table 30).

Table 30. Evaluation of in vitro rhizogenesis of A. cherimola and A. muricata selections - shoots conditioned one month in a hormone free media-

Species	Cultivar or selection	¼ NN-69* + 1% S* + 3% GR* IBA 4.90 µM l^{-1}
A. cherimola	Ch-X	41.8 ± 1.5
	Ch-Y	39.2 ± 3.1
	Ch-Z	36.4 ± 5.2
A. muricata	Mr-2	32.4 ± 5.3
	Mr-4	29.8 ± 2.6
	Mr-5	31.6 ± 8.2

⇒ * *The values represent the mean (± SE) of three independent experiments.*
⇒ *Twenty explants were used for each experiment*
⇒ *¼ macro elements reduction, S -sucrose, GR -gelrite*

Ex vitro

Rooting micro-cuttings under semi-sterile conditions can be an easy way to obtain *A. cherimola* and *A. muricata* plantlets. With a pre-dip in 4.90 µM IBA. Rooting percentages of 51.3 % for semiwoody coming shoots were obtained for *A. cherimola* and 58.5% for *A. muricata* .

The *ex vitro* root organogenesis requires careful attention to the physiological condition of the microcuttings and to the rooting humidity atmospheric condition. The first roots appeared after 40 days and then intensive growth and development of the plants proceeded. The highest number of rooted plants in all the selections was obtained when they were treated with Rhizopon (Table 31).

Table 31. Effect of Rhizopon on ex vitro root formation of A. cherimola and A. muricata selections

Species	Selection (code)	Rhizopon **		control *
		NAA 10% (solution) *	IBA 1% (pulver *)	
A. cherimola	Ch-X	0	51.3 ± 2.7	0
	Ch-Y	0	46.7 ± 3.3	0
	Ch-Z	0	41.6 ± 3.8	0
A. muricata	Mr-2	0	56.7 ± 5.1	0
	Mr-4	0	52.1 ± 7.4	0
	Mr-5	0	40.8 ± 1.2	0

⇒ * *The values represent the mean (± SE) of three independent experiments.*
⇒ *Twenty explants were used for each experiment*
⇒ ** *Rhizopon ® (Hazerswoude-Holland)*

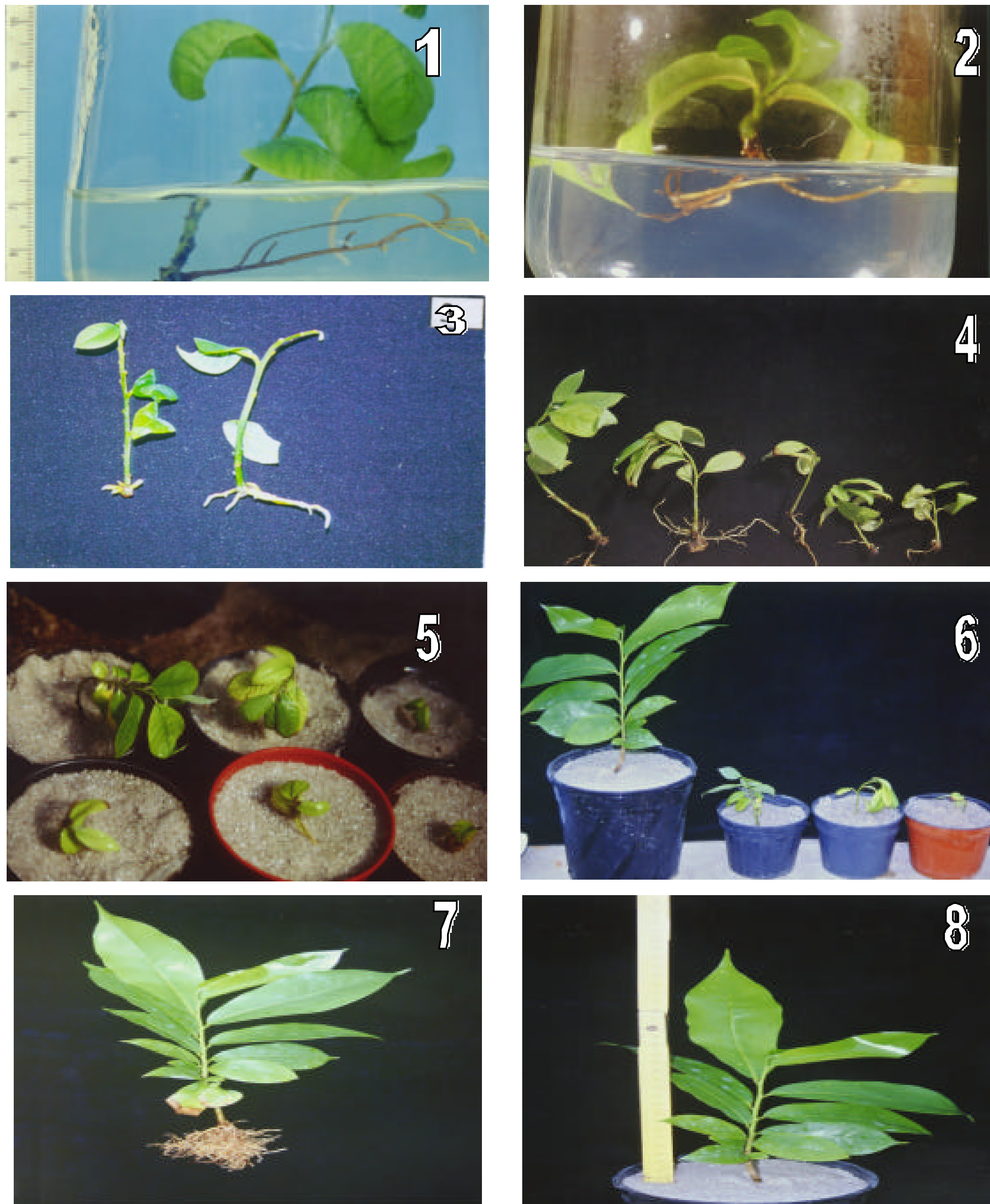
To compare the efficiency of the *in vitro* and *ex vitro* rhizogenesis in *A. cherimola* and *A. muricata* the number of roots and root growth were compared (Table 32).

Table 32. Comparison of the ex vitro and in vitro root organogenesis Of A. cherimola and A. muricata

Species	IBA 4.90 µM	Root emergence in days	Number of roots per shoot	(mm/week) Root growth rate	(%)Rooting efficiency	Callus formation
A. cherimola	*in vitro*	40	3	2.4	21.2	-
	ex vitro	40	5	4.0	45.6	-
A. muricata	*in vitro*	40	3	2.0	25.0	-
	ex vitro	40	7	4.3	55.5	-

6.5 Hardening

The *A. cherimola* and *A. muricata* micropropagation success required that the new regenerant shoots, which are heterotrophic under *in vitro* conditions with a very high humidity (90-100%), began to be autotrophic under *ex vitro* conditions.



1. *In Vitro rooting of A. cherimola after 70 days on ¼ NN-69 salts supplemented with 10% surcrose*
2. *In Vitro rooting of A. muricata after 70 days on ¼ NN-69 salts supplemented with 10% surcrose*
3. *Ex vitro root formation in A. cherimola induced by Rhizopon IBA 1% after 50 days*
4. *Ex vitro root formation in A. muricata induced by Rhizopon IBA 1% after 50 days*
5. *Ex vitro adaptation of A. cherimola*
6. *Sequence of ex vitro growth in quartz-sand pots in A. muricata in vitro regenerant*
7. *A. muricata ex vitro root development*
8. *A. muricata regenerant after four months (autotrophic plant)*

Figure 25. Morphogenic events related with the the root organogenesis of A cherimola and A. muricata under in vitro and ex vitro conditions

After two months in the rooting micropropagation stage, the regenerants were transferred to sterile sand-quartz pots. The acclimatization was promoted in a moderate low humidity (60-70%) in nursery conditions with 23 ± 5 °C.

To increase the acclimatization rate, the pots were covered with an inverted funnel in order to facilitate the O_2 and CO_2 mobilization. About 40% of the *A. cherimola* and *A. muricata* regenerants survived in the *ex vitro* conditions.

Once the *A. cherimola* and *A. muricata in vitro* plants were acclimatized, they did not promote a new shoot growth formation. They seemed to be dormant, the growth in terms of elongation and new leave formation was observed slightly after two months of adapting to the greenhouse conditions.

At the time of acclimatization the shoots elongated, and the leaves expanded and turned deep green. The leaves coming from *in vitro* culture were weak and showed a slight green colour possibly due to the low photosynthetic activity and had a tendency to loose water with extreme facility.

The new *ex vitro* formed leaves were stronger and after two months they were very green showing the new successful photosyntesis activity. It is important to notice that the acclimatization process is dependent on the apical meristem, which stimulates the new leaf formation. If the apical meristem died, because of dehydration or browning, there was no chance to induce an *ex vitro* new growth development. The regenerant could not be adapted and died.

After its perfect adaptation to *ex vitro* conditions the *A. cherimola* and *A. muricata* grew vigorously and after six months they were pruned for the first time. The well adapted plants are still growing with full vigour and uniformity in the greenhouse (Figure 25).

7. Random Amplified DNA Polymorphic (RAPD)

From the isolated *A. cherimola* and *A. muricata* DNA plant cell extract (Figure 26), the total concentration of DNA was determined fluorometrically. To test the quality of DNA extracted, an ethidium bromide agarose (1.5%) gel was run with 0.5 x TBE, 45 volts for 18 hours.

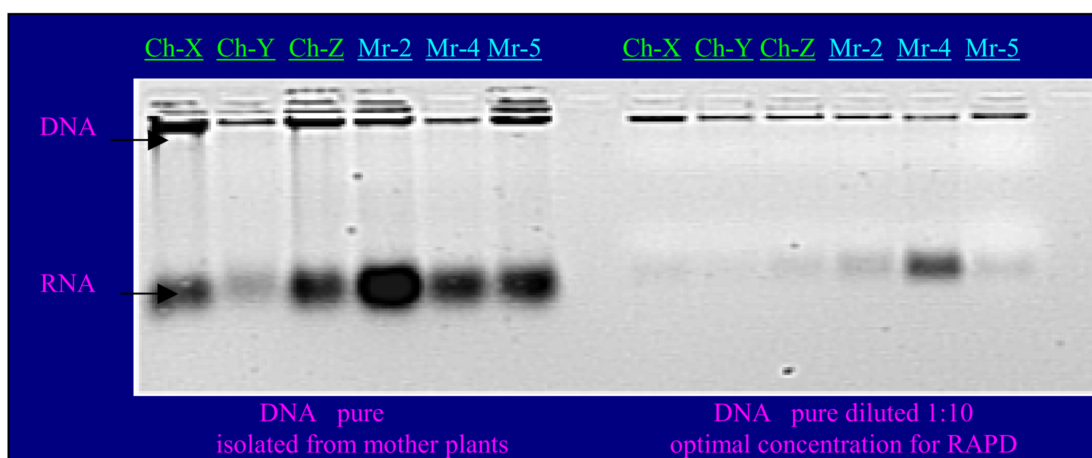


Figure 26.DNA extracted from A. cherimola and A.muricata

DNA Amplification

Genomic DNA of *A. cherimola* and *A. muricata* **mother selections** was amplified with 29 primers (Table 33).

174 scoreable bands yielded from the tested primers in *A. cherimola* and *A. muricata* mother plants established in the greenhouse (Table 33).

The number of RAPD bands scored for *A. cherimola* species was 45 compared with 42 bands for *A. muricata* species.

From the 29 primers only 6 produced clear amplification profiles, scorable and available for the DNA pattern band comparison in all the tested samples with *A. cherimola* selection Ch-A, Ch-Y, Ch-Z and *A. muricata* Mr-2, Mr-4, Mr-5. These primers were the C-5, C-11, Opa-16- Opa-18- Q-4 and Q-12 respectively.

Table 33. Arbitrary Primers used to screen A. cherimola and A. muricata by RAPD
(0) : no pattern, no repetition (1) : pattern and successful repetition

	Primer	Sequence			*A.cherimola*			*A. muricata*		
					Ch-X	Ch-Y	Ch-Z	Mr-2	Mr-4	Mr- 5
1	B-11	5'>	gTA gAC CCg T	<3'	0	0	0	0	0	0
2	C-04	5'>	CCg CAT CTA C	<3'	1	1	0	1	1	1
3	**C-05**	**5'>**	**gAT gAC CgC C**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
4	C-07	5'>	gTC CCg ACg A	<3'	0	0	1	1	1	1
5	**C-11**	**5'>**	**AAA gCT gCg g**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
6	C-19	5'>	gTT gCC AgC C	<3'	0	1	0	1	0	0
7	G-19	5'>	gTC Agg gCA A	<3'	1	0	0	0	1	1
8	J-19	5'>	ggA CAC CAC T	<3'	1	1	0	0	0	0
9	L-04	5'>	gAC TgC ACA C	<3'	1	1	1	0	0	0
10	**OPA-16**	**5'>**	**AgC cAg CgA A**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
11	OPA-17	5'>	gAC CgC TTg T	<3'	0	0	1	0	1	1
12	**OPA-18**	**5'>**	**Agg TgA Ccg T**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
13	P-02	5'>	TCg gCA CgC A	<3'	0	1	1	0	0	0
14	P-05	5'>	CCC Cgg TAA C	<3'	1	1	1	1	0	0
15	P-10	5'>	TCC CgC CTA C	<3'	0	0	0	1	1	1
16	P-13	5'>	ggA gTg CCT C	<3'	0	0	0	0	0	0
17	P-20	5'>	gAC CCT AgT C	<3'	0	0	0	0	0	0
18	**Q-04**	**5'>**	**AgT gCg CTg A**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
19	Q-05	5'>	CCg CgT CTT g	<3'	1	1	0	0	0	0
20	Q-06	5'>	gAg CgC CTT g	<3'	0	0	0	0	0	0
21	Q-07	5'>	CCC CgA Tgg T	<3'	0	1	0	1	0	0
22	Q-08	5'>	CTC CAg Cgg A	<3'	0	0	0	0	0	0
23	Q-09	5'>	ggC TAA CCg A	<3'	0	0	0	0	0	0
24	Q-10	5'>	TgT gCC CgA A	<3'	0	0	0	0	0	0
25	Q-11	5'>	TCT CCg CAA C	<3'	0	1	1	1	0	0
26	**Q-12**	**5'>**	**AgT Agg gCA C**	**<3'**	**1**	**1**	**1**	**1**	**1**	**1**
27	Q-13	5'>	ggA gTg gAC A	<3'	0	0	1	1	0	0
28	Q-14	5'>	GgA CgC TTC A	<3'	1	1	1	1	1	1
29	Q-17	5'>	gAA gCC CTT g	<3'	0	1	1	1	1	1
	Total scoreable (1) bands per mother selection = 87				13	17	15	16	13	13
	Total scoreable (1) bands= 174 with the 29 primers									

Pattern Band Comparison

Five sets of PCRs were carried out with the 6 selected primers to improve the RAPD fingerprinting of each DNA sample from *A. cherimola* and *A. muricata in vitro* regenerants as well as from the mother source of explants or established Colombian and Chilean plants in the greenhouse (Table 34), (Figures 27,28,29,30).

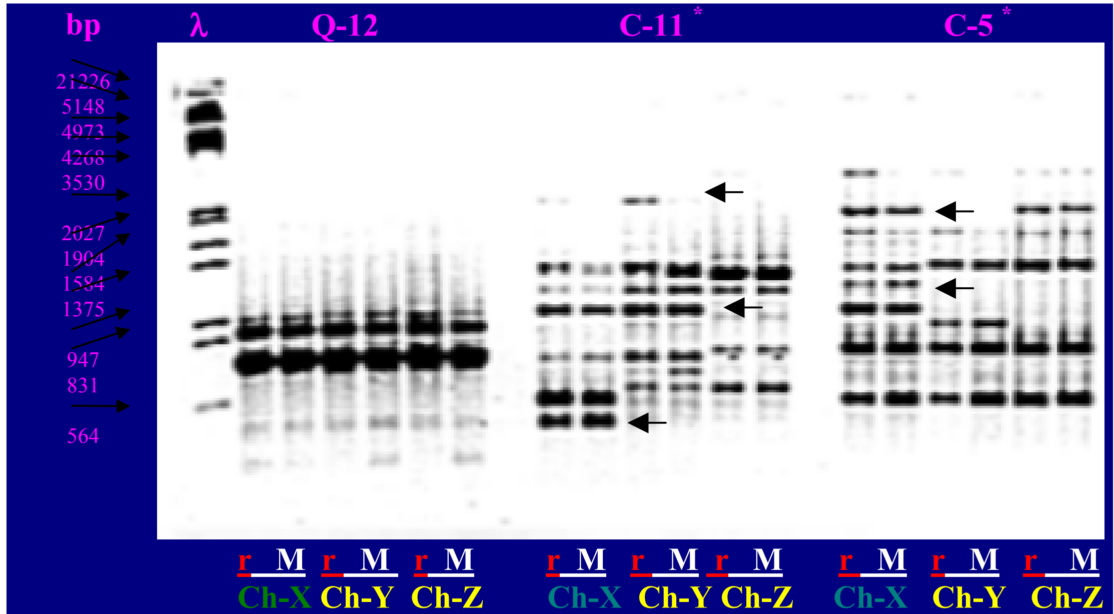
The six tested markers Q-12, C-11, C-5, Q-4, OPA-18, OPA-16 showed **monomorphic bands** in *A. cherimola* (Figure 27; Figure 29) and *A. muricata* selections (Figure 28; Figure 30) when only the intensive bands were compared.

Table 34. Primers used in RAPD analysis and number of scoreable bands in in vitro clonal regenerants and mother plant of A. cherimola and A. muricata

	A. cherimola				*A. muricata*			
Primer	Code	r	M	P	Code	r	M	P
Q-12	*Ch-X*	3	3	**0**	*Mr-2*	7	7	**0**
	Ch-Y	3	3	**0**	*Mr-4*	7	7	**0**
	Ch-Z	3	3	**0**	*Mr-5*	7	7	**0**
C-11 *	*Ch-X*	6	6	**0**	*Mr-2*	2	2	**0**
	Ch-Y	7	7	**0**	*Mr-4*	2	2	**0**
	Ch-Z	5	5	**0**	*Mr-5*	2	2	**0**
C-5 *	*Ch-X*	7	7	**0**	*Mr-2*	5	5	**0**
	Ch-Y	4	4	**0**	*Mr-4*	5	5	**0**
	Ch-Z	5	5	**0**	*Mr-5*	5	5	**0**
Q-4	*Ch-X*	4	4	**0**	*Mr-2*	2	2	**0**
	Ch-Y	4	4	**0**	*Mr-4*	2	2	**0**
	Ch-Z	4	4	**0**	*Mr-5*	2	2	**0**
OPA-18 *	*Ch-X*	4	4	**0**	*Mr-2*	7	7	**0**
	Ch-Y	3	3	**0**	*Mr-4*	9	9	**0**
	Ch-Z	4	4	**0**	*Mr-5*	7	7	**0**
OPA-16 *	*Ch-X*	2	2	**0**	*Mr-2*	5	5	**0**
	Ch-Y	2	2	**0**	*Mr-4*	4	4	**0**
	Ch-Z	2	2	**0**	*Mr-5*	4	4	**0**
Total bands scored *in vitro* and *ex vitro* plants		**72**	**72**	**0**		**84**	**84**	0
Polymorphic scored bands = 0					***Polymorphic scored bands = 0***			

⇒ r : *in vitro regenerant*
⇒ M : *mother plant established in the greenhouse and source of explants*
⇒ P : *Polymorphic bands observed during the DNA amplification*
⇒ Underlined primers * : *Represents Polymorphic bands found by random between A. cherimola and A. muricata selections*

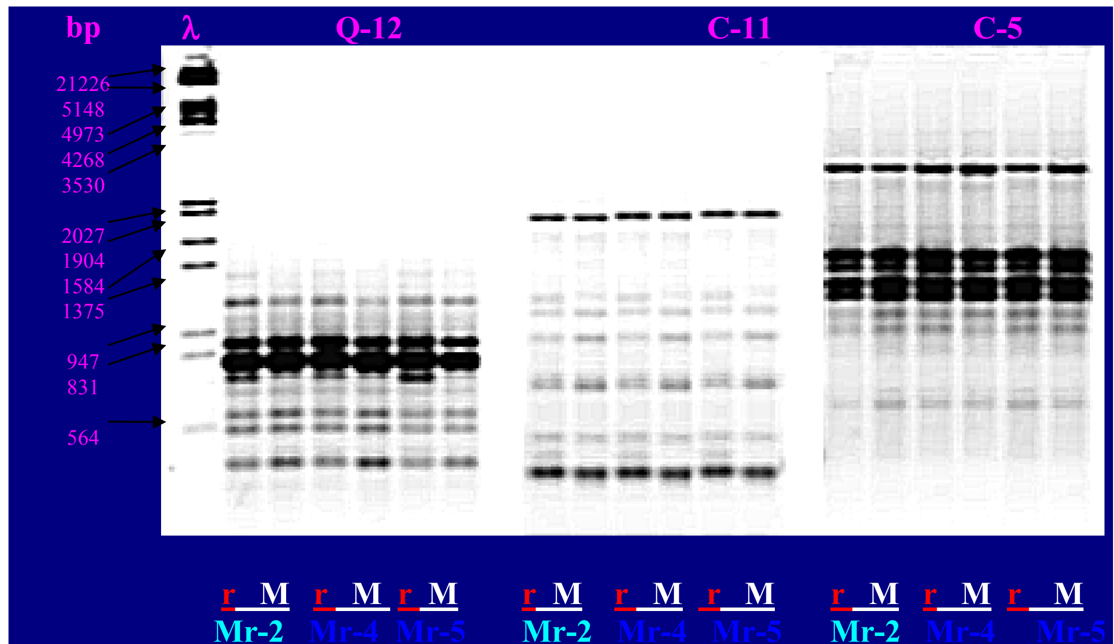
The primers C-11, C-5 and Opa-18 in *A. cherimola* scored polymorphic bands on the var. Felpa, var. Bronceada and the Colombian selection. The primers Opa-18 and Opa-16 in *A. muricata* scored polymorphic bands between the Colombian selections (Table 16; Table 35). The mentioned primers register a variation between bands in terms of intensity, suggesting that they could be used to identify varieties or clone plants by RAPD.



⇒ *bp = base pairs Lambda DNA/ EcoRI+HindIII marker*

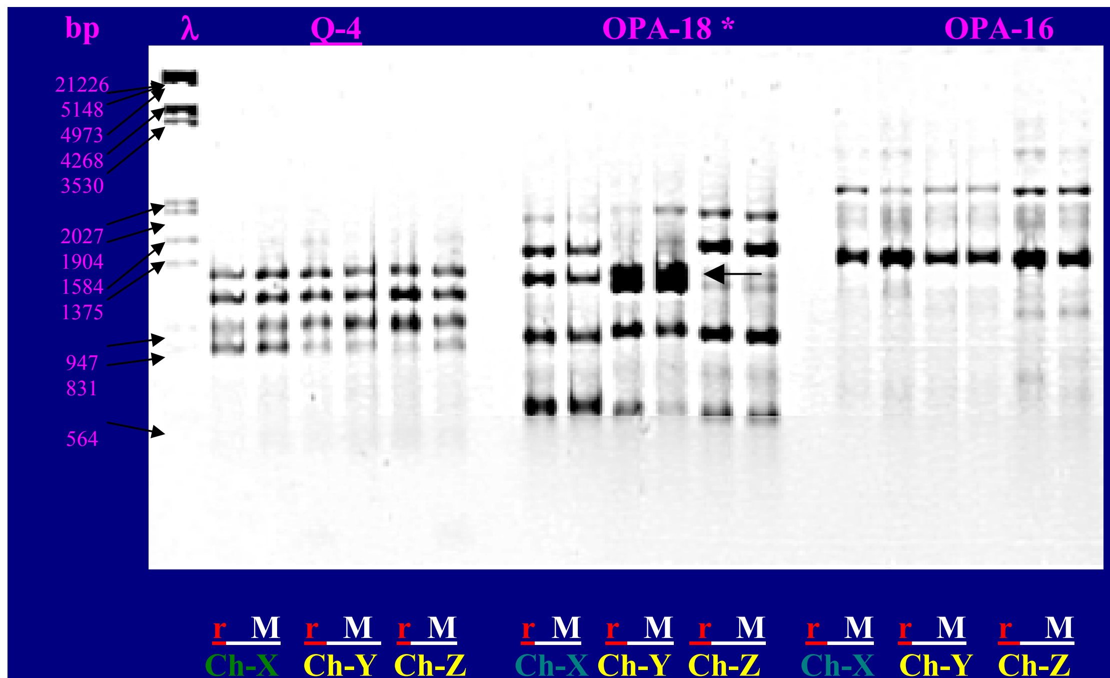
⇒ *: muster band distinction*

Figure 27. Amplification of genomic DNA primers Q-12, C-11, C-5 primers from regenerants and mother plants of A. cherimola



⇒ bp = base pairs Lambda DNA/ EcoRI+HindIII marker

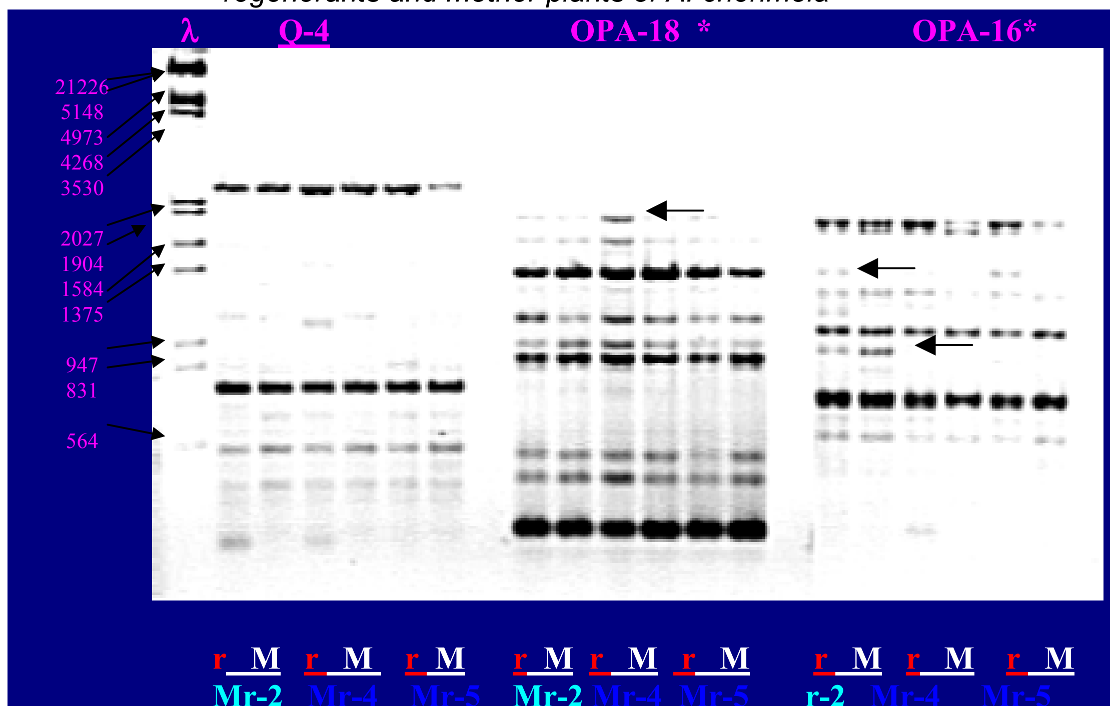
Figure 28. Amplification of genomic DNA primers Q-12, C-11, C-5 primers from regenerants and mother plants of A. muricata



⇒ *bp = base pairs Lambda DNA/ EcoRI+HindIII marker*

⇒ ⟶ *: muster band distinction*

Figure 29. Amplification of genomic DNA primers Q-4, Opa-18, Opa-16 primers from regenerants and mother plants of A. cherimola



⇒ *bp = base pairs Lambda DNA/ EcoRI+HindIII marker*

⇒ ⟶ *: muster band distinction*

Figure 30. Amplification of genomic DNA primers Q-4, Opa-18, Opa-16 primers from regenerants and mother plants of A. muricata

Karyotype observations

The observation of mitotic chromosomes under the light microscope is a rapid and informative method of studying the genomes as a whole. Although the technique, involving the collection, fixation, staining and preparation of chromosome squashes, is easy, the resolution of the *A. cherimola* and *A. muricata* chromosomes were not clear and many observations were required.

The tested methods to visualize the chromosomes of *A. cherimola* and *A. muricata* were ineffective. Only the DAPI-fluorescence method allowed the visualization of the chromosomes but with some difficulties (Figure 29).

The *A. cherimola* and *A. muricata* showed the tendency to clump or stick to one another. A few chromosones were oblique or vertical**,** this limitation was also reported for *Annona* spp. by Bowden (1948).

The number of chromosomes counted were diploid, the *A. cherimola* reported 2n=16; 14 and for *A. muricata* 2n=14; 18 (Figure 30).

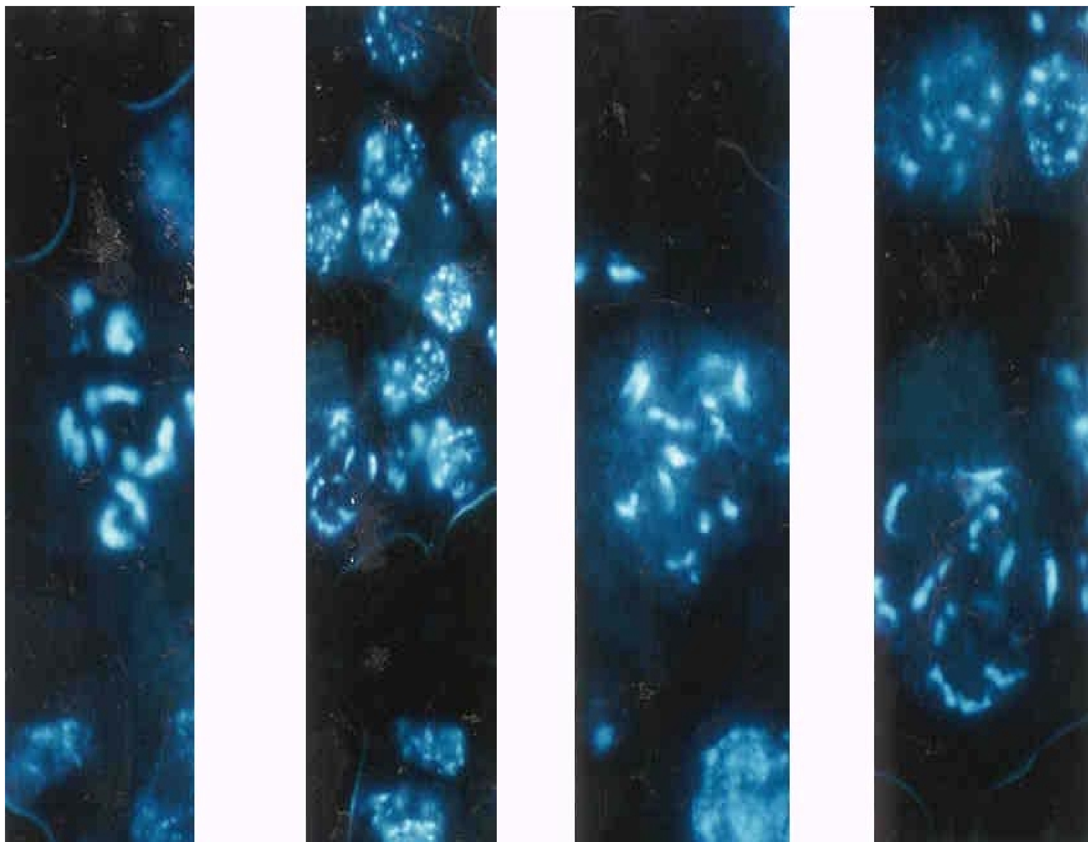


Figure 31. Chromosomes of A. cherimola and *A. muricata in vitro regenerants*

Discussion

Micropropagation

According to Murashige and Skoog (1974) any *in vitro* culture manipulation should start with an elite or known plant material however, there are in nature, dichogamous protogyneous flowering plants such as *A. cherimola* and *A. muricata* which promote intervarietal crossing, thereby making the preservation of elite or selected material impossible.

Cordoba (1969), Gardiazabal and Rosenberg (1988), Sawneski (1988), Gazit and Eisenstein (1985), Sanchez and Torres (1992) and Bridg (1993) did not report the expanded existence of *A. cherimola* and *A. muricata* elite material either in the countries where they are planted for commercial purposes. Most of the present plant material of *A. cherimola* and *A. muricata* comes from seeds and it is well known that seedling populations promote hetereogenity between species (Hartman and Kester, 1894).

There are no examples in woody species, but in the herbaceous ones where an elite material is either a selected phenotype or a specific plant selected for its specific qualities. In forestry and/or fruit trees the elite material would be the bearer of an elite seed, which would be used to initiate a more or less extensive micropropagation program (Deberg and Read, 1991).

To develop the present *in vitro* propagation protocol for *A. cherimola* and *A. muricata* **selected plants were used as mother plants**. The selection was made in connection with the advice of the farmers from three Colombian regions where these plants are believed to be part of the native flora. The farmers have a large knowledge of the phenotypic characteristics of the trees, the quality of the fruit produced and their origin.

One year old seedling trees (T_1) of *A. cherimola* and *A. muricata* were planted in Colombia under open field conditions, their semideciduous natural condition promoted the bud sprouting in the new environmental conditions in the greenhouse in Dahlem-Berlin.

Dehydration of *A. cherimola* and *A. muricata* (T_1) roots was prevented with the defoliation of the leaves, transport in darkness and by protection with moisture paper. The sprouting of semideciduous and deciduous fruit trees after the dormant or stress season is related not only to the endogenous concentration of plant growth regulators, starch and other metabolic products but also to the water content on the internal tissues (Hartman and Kester, 1984).

A good root system assures the sprouting of a deciduous or semideciduous bud once the optimal environmental conditions give the signal in which water transport normalizes the transport of metabolic substances and the latent bud promotes the new vegetative growth of the plant (Westwood, 1978).

Temperature is one of the factors which influence *A. cherimola* and *A. muricata* behaviour, because they are not cold tolerant plants. Ambient temperature variations have been demonstrated to be detrimental to the plant and fruit (Boshell, 1982; Lizana

and Irarrázabal, 1984, Gutierrez *et al*. 1992). The Colombian plants were transported at room temperature to protect cold injury of the vegetative structures .

Chilean seeds of *A. cherimola* cv. Felpa and Bronceada **(T_0)** were also used as a material source (Table 16). Cordoba (1969), Bourke (1976), and Sawneski (1988) reported seed germination problems in *A. cherimola*.

The seeds of tropical tree species from open field conditions are more susceptible to the attack of insects and micro-organisms such as fungi (Hammond *et al*., 1999). This study confirms the beneficial effect of **Benomyl** 0.03% as a **systemic fungicide** on the germination of *A. cherimola* cv. Felpa and Bronceada seeds. No losses by fungi and other seed pathogens were noted during germination. Wunkhaus and Aquinas (1990) evaluated the potential effect of Mancozeb and Dichloran as systemic fungicides combined with shock temperature treatments on *A. cherimola* seed germination.

The low percentage of seed germination in several woody species is related to the seed dormancy characteristic of semideciduous and deciduous fruit trees (Hartman and Kester, 1984). The manipulation of some physical factors to break dormancy have been discussed (Westwood, 1978).

The present study shows the beneficial effect of **pre-treatment of seeds with cold temperature** on seed germination of *A. cherimola* cv. Felpa 78% and cv. Bronceada 81% when coated seeds were stored at 4°C for 24 hours during the imbibition in water. The group of seeds of *A. cherimola* cv. Felpa and cv. Bronceada which were not part of this treatment reduced the rate of germination by 41% and 67% respectively at room temperature conditions. The effect of warm water treatment to improve germination did not increase the rate of germination on *A. cherimola* (Duarte *et al.* 1974).

The results suggest that warm temperatures used as a pre-treatment to break the dormancy of *A. cherimola* seeds did not increase the rate of germination. However a pre-cold temperature shock treatment improved the germination rate (Table 20).

Plant growth regulators such as Gibberellic acid (GA_3) and benzyl-aminopurine (BA) have, in combination with seed stratification, been tested to break dormancy of *A. cherimola* seeds (Vargas, 1986). From the results it can be seen that no high concentrations of plant growth regulators are required to break the seed dormancy of this semideciduous subtropical species.

Toll *et al*. (1975) reported no germination of *A. cherimola* seeds on GA_3 1,000 ppm., treatment. The best germination results were reported on GA_3 500 ppm. Duarte *et al*. (1974) reported the effectiveness of GA_3 on seedling growth but not in seed germination. The concentration of 1,000 ppm GA_3 was shown to be more effective than 10,000 ppm in terms of the promotion of vegetative growth of *A. cherimola* plantlets.

Some seed manipulations during sowing have been suggested to improve germination on semi-deciduous and deciduous woody tree species (Hartman and Kester, 1984). Vincent (1974) and Toll *et al*. (1975) found that scarification, position and direction of the seed polarities are factors which have no appreciable effect on *A. cherimola* seed germination.

In this study, it was found that the coat of *A. cherimola* cv. Felpa and cv. Bronceada seeds have an effect on germination. Those seeds which were coatless showed a low germination rate in all experimental cases (Table 20). This was not only because of the presence of phenolics on the endosperm but also because of the

susceptibility of the seeds to the attack of some fungi species. Otherwise the coat of *A. cherimola* seeds has been reported to be rich in oils (Jaramillo, 1952) and oils are reserve energy to promote the embryo growth and development during germination.

The ecophysiological relation between seed germination preconditions and natural growth condition of the *A. cherimola* native plant in the highland mountains in the South American Andes. It grows in a subtropical mild temperature ecosystem which fluctuates during the year, early morning low temperatures have been registered 4 ± 3 °C for 2 hours and during the day the 25 ± 5°C remains constant (Lauer, 1986). The seeds of *A. cherimola* are exposed not only to high humidity conditions but also to low soil temperatures during the early morning as a consequence of the "helada" (Bridg, 1993a).

The present study suggests that seeds of *A. cherimola* cv. Felpa and Bronceada could break their dormancy if they are stored in dry conditions free from fungi attack, pre-washed with 0.03% Benomyl for 1 hour to avoid endogenous pathogens which limit the germination when plants are grown in open field conditions. Pre-cold temperature shock 4°C/ 24 hours in water promoted imbibition and improved the seed germination rate.

It is well accepted that the *in vitro* propagation of a species is influenced by several factors, the foremost are genotype, age and source of the initial tissues, which, in turn are related to their *endogenous hormonal* status (Bajaj and Pierik, 1984; Meyer, 1983). The day length and temperature had a definitive effect on the formation of new sprouts in T_o and T_1 *A. cherimola* and *A. muricata* plants established in the greenhouse. In order to evaluate the effect of the **photoperiod** on the *in vitro* establishment (Table 23) some plants were maintained in total darkness for two weeks.

This study confirmed the effect of seasons and the light duration of the day, as factors which have a strong influence on the vegetative growth and development of *A. cherimola* and *A. muricata* plants established in the greenhouse, as well as during the *in vitro* culture. These species are sensitive to the photoperiod. Short days, common in winter, influence the leaf angle of the plants and wilting of the branches, vegetative growth is reduced and the principal branch develops an apical dominance, larger leaves were also observed.

The *A. muricata* explants from winter are not shoot prolific if compared with those taken in summer or spring. The *A. cherimola* explants showed the same behaviour but appeared to be a little more resistant to the atmospheric variations in the greenhouse in terms of bud sprouting. The *A. cherimola* is a subtropical species and *A. muricata* grows only in the most tropical areas where short days and low temperatures are never reported.

Plant Tissue Culture is defined as *in vitro* culture of any plant cell, tissue or organ in aseptic conditions (Murashige, 1974). However one of the main difficulties in tissue culture of woody tree species is the **disinfection of the selected explant**, especially when plant material is collected outdoors, as usually plant tissues and organs from these conditions carry endophytic floras, consisting of inter- and intracellular micro-organisms including viruses, viroids, prokaryotes and fungi species (Cassells, 1990).

The experimental *A. cherimola* and *A. muricata* Colombian plants from open field conditions carry micro-organisms which limit the aseptic *in vitro* micropropagation. Several fungi and nematodes species have been reported to live on *Annona* plants growing in the field (Raski, 1976; Muñoz, 1981; Ochoa, 1989).

Figure 9 shows losses from *A. cherimola* and *A. muricata* during the initial establishment of several explants under *in vitro* conditions. Hadelman *et al*. (1987) reported the particular difficulties found when a woody plant from the field is to be used to promote cultures free from contaminants. *In vitro* contamination has two sources of origin, external when it is present on the surface of the explants or endogenous or intracellular when it is capable to grow in tissue culture medium (Lawson, 1986)

The use of systemic fungicides for preconditioning the field plants is a recommended practice in tissue culture of woody species (Conger, 1986). The fungi contaminants on *A. cherimola* and *A. muricata* during the *in vitro* establishment was reduced if constant **applications of Benomyl** 0.03% were made (Table 21). The present study shows on *A. cherimola* and *A. muricata* that irrigating with Benomyl in the greenhouse is not enough to promote the aseptic *in vitro* culture of *A. cherimola* or *A. muricata* explants, however the reduction of fungi contaminants was reported (Figure 9).

The fungi on *in vitro* culture are not always related to the external disinfection of preconditioning treatments. Some fungi reproduce themselves by spores and mycelium which could survive the external disinfected material. The contaminants could be the expression of a latency stage or be natural forms which develop new races in a short period of time in a culture media (Cassells, 1990; Montiel, 1991).

The *in vitro* establishment of *A. cherimola* and *A. muricata* is conditioned, not only by the nature of the selected material and environmental growth conditions, but also by the presence of contaminant micro-organisms such as fungi which have been associated with *A. cherimola* in open field growth (Benitez and del Pilar, 1990). Preconditioning of the plants with Benomyl should be a permanent practice during the time where initial explants are required. The reduction of *in vitro* fungi expression were related to the frequency of Benomyl irrigations.

In principle each plant segment or explant has the potential to induce new growth and development on aseptic conditions and regenerate a complete plant (Pierik, 1989). The *in vitro* culture differentiation is dependent on the cell or tissue conditions of the initial explant (De Fossard, 1977).

Meristems of *A. cherimola* and *A. muricata* vegetative branches were selected as initial culture explants because many *in vitro* cultures were obtained from these explants due its rapid capacity to promote cell divisions.

Meristems are used also to assure the establishment of aseptic cultures (Conger, 1986). Micropropagation of *A. cherimola* and *A. muricata* through meristems in the present study could not be reported because of the high production of phenolic substances, meristems did not survive to the first *in vitro* manipulation. It is well known that the *Annona* spp., are phenol reactive plants after wounding or injury (Martinez-Cayuela *et al*. 1998).

The micropropagation of many fruit tree species have been promoted from young tissues like hypocotyls from seedling plants, shoot tips from vegetative branches and meristems because they are less prone to blackening than older tissues (Conger, 1986; Torres, 1989). However in woody trees the survival rate of the small explants to disinfection treatments is minimal (Miller and Murashige, 1976)

Neither *A. cherimola* nor *A. muricata* could be propagated by bud culture from vegetative branches due to the hypersensitive reaction of this soft tissue explant. Other woody fruit species such as *Feijoa sellowiana* have shown the same limitation during establishment, producing a high concentration of phenolics (Bhojwani *et al.* 1987).

For most woody species a widely applied technique to promote micropropagation is the enhancing of the axillary bud (Conger, 1977), the induction of adventitious buds and callus culture from the tissue of the original explant (Huhtinen, 1976) and the culture of shoots derived from shoot tips and nodal segments as well as the promotion of terminal and axillary buds (Pirre, 1986).

Micro-cuttings (1-3 cm) and **macro-cuttings** (5-9 cm) were found to be an optimal source of explants from vegetative-, semiwoody- and woody -branches of *A. cherimola* and *A. muricata* (Figure 9). Some differences in bud sprouting and production of phenolics and endogenous disinfection from these explants were noted in the present study. The sprouting of new shoots from pre-formed buds and the green and aseptic establishment of *A. cherimola* and *A. muricata* explant were related to the plant position and degree of lignification of the mother branch.

Aghion-Prat (1965) and Jaiswal (1986) noted different regeneration gradients in explants isolated from different plant positions. It is also known that explants from the same plant organ show *in vitro* response variations because there are different ages on the tissues of the same organ (Torres, 1989). This was confirmed also in *Pseudotsuga menziesii* where shoot initials isolated from positions low down on the tree showed better development *in vitro* than the terminal buds and grew faster than axillary buds (Evers, 1984).

The **semiwoody cuttings** from the second year growth were the most potential explants to induce the establishment of *A. cherimola* and *A. muricata*. These explants were phenol reactive on the base but size and plant position benefited their *in vitro* culture and bud sprouting. This response has been described by Stonier (1971) which indicates that the production of phenolic substances decreases right up the base of the explant.

The potential of rejuvenation of one species can be achieved not only by the propagation of vegetative explants, but also by the adventitious shoot formation or new shoot formation from adult shoot tips (Torres, 1989). Woody species have the inclination to inhibit the bud sprouting and produce high concentrations of phenolic substances in woody branches (Chalupa, 1987).

The promotion of new *in vitro* shoots of *A. cherimola* and *A. muricata* from semiwoody cuttings (5-9 cm) with 3 pre-formed buds is reported. This explant is larger, vigorous and carrying pre-formed semideciduous buds with their own supply of energy and growth regulators. The induction of new shoots from these pre-formed buds in the case of *A. cherimola* and *A. muricata* is an alternative to improve the rejuvenation of adult trees.

The micropropagation of tropical and subtropical fruits by stimulation of axillary bud proliferation from cuttings has been reported for several species (Litz and Jaiswal, 1990). The influence of the explant length on the regenerative capacity of apolarly placed bulb scale explants of *Hyacinth* spp., has been discussed (Pierik and Ruibing, 1973).

Cultures from pre-formed buds without callus induces *true-to-type* micro-shoots which through repeated subcultures promotes rejuvenation (Pierik, 1989). *A. cherimola* and *A. muricata* selections could find on this type of initial explants an alternative to improve vegetative multiplication of its selections with a high success of rooting because of rejuvenation. This process results from the increments of cell division and regeneration rate as shown on *Pinus pinaster, Sequoia sempervirens, Vitis vinifera* and *Malus sylvestris* (Hackett, 1985).

The absence of tissue juvenility on the third year old branch of *A. cherimola* and *A. muricata* could be an explanation of why woody- macrocuttings and microcuttings were more difficult to improve in *in vitro* culture, not only because of the hypersensitivity of the explant, high contamination rate and low percentage of shoot regeneration but also because there was no juvenility. The adventitious shoot formation is far more difficult to induce in woody shoots than from semiwoody or juvenile shoots (Zimmerman, 1988 ; Hackett, 1985).

The potentialities of cell division and regeneration decreases from the apical to the basal region according to the lignification grade and age of the branch (Bajaj and Pierik, 1984; Meyer, 1983). The micropropagation of *A. cherimola* and *A. muricata* from semiwoody cuttings has the advantage of promoting new growth of selected trees under *in vitro* conditions and to improve their rejuvenation. These explants are physiologically relatively juvenile and for tissue culture purposes more able to be propagated because they could be manipulated under sterile conditions. The bud sprouting from semiwoody cuttings can be built up to form new shoot structures which leads to organ regeneration (Evans *et al.* 1984).

The micropropagation and the disinfection of one species is strongly dependent on the explant size, source, disinfectant concentration and time of sterilization (Pierik, 1989). The disinfection of *A. cherimola* and *A. muricata* was tested with several concentrations of sodium hypochlorite in combination with Benomyl to avoid all the external *in vitro* contaminants. These treatments were complemented with the effect of Rifampicin as a selected antibiotic to control the bacteria contamination characteristic in plants of tropical and subtropical origin (Young, 1984).

Sodium hypochlorite (NaClO) is one of the common disinfectant solutions that is used in tissue culture to promote surface sterilization and improve the establishment of aseptic cultures (Pierik, 1989). NaClO 3%/15 minutes was the most viable combination in time and concentration to avoid some external contaminants on *A. cherimola* and *A. muricata* semiwoody cuttings.

The *A. cherimola* and *A. muricata* semi-woody cuttings were irreversibly damaged or even killed by too high concentrations or a long exposure time in sodium hypochlorite (Figure 10). In general, woody tree species are sensitive to high concentrations of disinfectant agents (Deberg, 1986; Gordon and Brown, 1988).

In tissue culture it is difficult to interpret results with respect to **endogenous contamination**. When a woody species is tissue culture propagated, it is very difficult to distinguish between endogenous and exogenous bacterial contamination on the culture media (Cassells, 1991). In *A. cherimola* and *A. muricata* the endogenous bacterial contamination observed during the multiplication stage which revealed from the shoot explant disinfection, that the applied methods to improve the disinfection of the semiwoody branch was only effective on the surface of the explant.

In plant tissue culture there are several strategies to avoid internal infections when they can not be eliminated by a simple external disinfection. Since most of the micro-organisms are not present in the meristem, the meristem culture is one of the most common techniques to improve the establishment of aseptic cultures when the principal aim is to produce virus free plants such as in *Passiflora edulis* cv. Flavicarpa (Roca and Mroginski, 1992; Bridg, 1990).

In *A. cherimola* and *A. muricata* the *in vitro* culture through meristems is not possible because of the hypersensitive reaction of this tissue explant and necrosis. The establishment was promoted with semiwoody cuttings (Figure 11; Figure 12) and these large explants have the potential to carry endogenous micro-organisms. Large explants are more difficult to culture because they are more difficult to disinfect (Roca and Mrogisnski, 1989).

The pre-formed shoots from semiwoody branches of *A. cherimola* and *A. muricata* showed an endogenous contamination during the multiplication stage which was very difficult to eliminate. Explants with a thick lignin mantle were difficult to disinfect because not all the surface parts could be penetrated by the sodium hypochlorite solution. Otherwise apparently sterile cultures can arise in a multiplication media if microorganism mutations take place during the establishment (Pierik, 1989).

The endophytic micro-organisms are located in the internal tissues of the plants. In some cases their colonization is permanent and in others it depends on the tissue anatomy where the colonization could be systemic or nearly so. There are the cases which the pathogenic micro-organisms have a mechanism to gain entry into the plant. They may follow a pathogenesis or latency of common plant associated bacteria, e.g., *Agrobacterium* spp., *Bacillus* spp., *Corynebacterium* spp., and *Pseudomonas* spp., which as intercellular endophytic microorganisms escape the effect of surface sterilants (Madoff, 1981; Campbell, 1990).

Young *et al*. (1984) found that no single antibiotics were effective against bacteria endogenous contaminants in shoot cultures of woody plants. The combination of different antibiotics have been more effective than any single chemical, nevertheless the risk of promoting somaclonal variations increases with the use of antibiotics (Scowcroft *et al.* 1987).

Rifampicin, Cefotaxime and Nystatin in the culture media as supplements for a long period of time were evaluated and reduction in the percent of bud sprouting and growth and development in *A. cherimola* and *A. muricata* semi-woody explants was observed (Table 22). Ammirato *et al.* (1990) have questioned the use of antibiotics and did not recommend them for routine use in plant tissue culture (Dodds and Roberts,1982). However antibiotics have been proposed as an alternative method to promote the aseptic establishment of several woody species (Young *et al.* 1984).

Rifampicin in *A. cherimola and A. muricata* semi-woody explants inhibited more effectively the contamination caused by *Pseudomonas* spp. in all the evaluated cases. The concentration of Rifampicin 20 μg/ml was not toxic for the explant. The bud sprouting induced green shoots without callus formation. They were excellent in quality due to the absence of contaminants. The effect of Rifampicin has been also reported by Staritsky *et al.,* (1983) who compared the effects of different antibiotics in the culture media of *Cryptocoryne* spp., and *Cinchona* spp. and Rfampicin was the most effective antibiotic which supported the *in vitro* establishment of these species.

The 20 µg/ml concentration is recommended by Sigma because this concentration is toxic enough for the micro-organisms. Concentrations as high as 25 µg/ml were noted to be toxic for the plant tissue, effective in terms of elimination of bacterial contamination (Tanaka *et al*. 1983; Mathias *et al.* 1987). Rifampicin is a commonly used antibiotic in Plant Tissue Culture because of its wide spectrum (Young, 1984).

The establishment media of Nitsch and Nitsch (1969) supplemented with Rifampicin, controlled the expression of endogenous *Pseudomonas* spp. Gram (-) bacteria, non pathogenic micro-organisms found in *A. cherimola* and *A. muricata* in the micropropagation. The bud sprouting increased in medium supplemented with Rifampicin (Table 22).

Phillip *et al.* (1981) found that Rifampicin was highly effective against bacterial contamination and did not affect rates of cell division and tracheary element differentiation or DNA synthesis in tuber cell cultures of *Helianthus tuberousus.* Pollock *et al.* (1983) found as Rifampicin one of the least toxic antibiotics and Goldberf and Friedman (1981) reported that Rifampicin inhibits prokaryotic RNA synthesis, while DNA is unaffected. The RNA polymerase of nuclear origin in eucaryotic cells is resistant to high concentrations of Rifampicin (Mathias *et al.* 1987).

The use of antibiotics for *in vitro* culture of higher plants is not encouraging (Pierik, 1989), because they are harmful for particular species (Torres, 1989) and they can lead to the selection of resistant micro-organisms (George and Sherrington, 1984).

In the present study, the *in vitro* culture establishment of *A. cherimola* and *A. muricata* without Rifampicin as a media supplement could not be achieved. The RAPD analysis on regenerants found no polymorphism on the DNA isolated from *in vitro* regenerants. The *true-to-type* propagation from pre-formed semiwoody buds was promoted although the establishment medium should be supplemented with Rifampicin.

The substitution of Benomyl in the culture media to control the presence of endogenous contaminants only and in combination with Rifampicin (Table 27) were effective and 100% of aseptic cultures could be register during the establishment of *A. cherimola* and *A. muricata.* Benomyl after the autoclave is degraded to 2-benzimidazole carbamic acid methyl ester which has been determined to be the active component of this fungicide (Maxweell and Brody, 1971).

0.4% of Benomyl was used as a media supplement by Haldelman *et al* (1987) to eliminate persistent contamination and obtain aseptic shoot tip material from *Camellia* spp. This fungicide was combined as well with Rifampicin to improve the traditional disinfection methods. Consequently a reduction of bud sprouting was observed.

However antibiotics and fungicides have not been recommended for routine proceedings in plant tissue culture (Dodds and Roberts, 1982) and a long period or exposure time of Benomyl is not desirable in a micropropagation protocol because it might induce phytotoxicity and *off-type* plants (Thurston *et al*., 1979).

The establishment of *A. cherimola* and *A. muricata* found in this Benomyl and Rifampicin combination is an alternative to improve the promotion of aseptic cultures. However, the bud sprouting was low 0.6 ± 0.5 in a medium supplemented also with antioxidants. Due to the low percentage of new aseptic shoots, Benomyl was excluded from the tissue culture establishment medium and only media supplemented with

Rifampicin were recommended to produce aseptic shoots in a 3.0 ± 0.9 sprouting rate for *A. cherimola* and *A. muricata.*

Most of the hardwood species produce **phenolic compounds** after wounding (George and Sherrington, 1984). The *Annona* spp., as well as other fruit tree species are limited by the excessive **hypersensitivity** of the cells to wounding or mechanical injury (Nair *et al.* 1983, 1986). During fruit conservation and postharvest, high losses were reported due to phenolic brown exudates (Martinez-Cayuela, 1986). The phenolic substances promote tissue blackening which have been reported as an inhibitor of new *in vitro* morphogenic responses in *Annona* spp., (Jordan *et al.* 1990; Bridg, 1993).

A. cherimola is a species rich in tannins, hydroxyphenolics, cathechin, epicatechin and biflavanes (Martinez-Cayuela, 1987). To control phenolics on *A. cherimola,* Jordan *et al*. (1991) compared the effect of some **antioxidant substances** such as citric acid, ascorbic acid, amino-oxiacetic acid, glutathione and cysteine and reported the effect of Polyvinylpirrolydone on internode explants of *A. cherimola.*

Young soft tissues of *A. cherimola and A. muricata* are hypersensitivite, the meristems, shoot tips, segments of leaf, buds and small shoots with one bud, produced after the isolation of a necrotic exudates in *in vitro* culture. The *in vitro* establishment of these small and vegetative explants is limited by the phenolic substances in the culture medium coming from the explant, they are not protected by lignin or its cuticular mantle is thinner. Furthermore the surface of these explants is covered by some microscopic trichomes which are a physical barrier and could prevent the action of NaClO. The presence of contaminants in the *in vitro* culture medium also promoted necrotic exudates on the base of the tissue explant of *A. cherimola* and *A. muricata*.

The *A. cherimola and A. muricata* plant species were characterized by the production of blackening where wounding took place, which released the phenolic content of broken cells. This oxidation at the beginning affected the neighboring cells which were not affected by wounding and did not show symptoms of injury themselves. The phenolic promotes the premature death of specific cells in its own environment (Debergh and Read, 1991).

The hypersensitivite reaction of semi-woody and woody cuttings with pre-formed buds of *A. cherimola* and *A. muricata* could be totally controlled. The use of antioxidant substances such **polyvinylpyrrolidone** (PVP) 4 ppm were effective if it was supplemented in the culture media (Table 23). The PVP in the selected establishment media adsorbs the active phenol-like substances and seems to be more effective than charcoal (Johansson, 1983). Jordan *et al*. (1991) reported also the reduction of the total phenolic concentration of the *A. cherimola* internode explants after 40 days in culture on media supplemented with PVP only and in combination with casein hydrolysate.

Annona explants, after wounding, produced phenols which could not be stopped by **ascorbic acid** and **citric acid** over a long period of time in *in vitro* culture media. They are the most common antioxidant compounds, however they are very labile and are also easily to be oxidized themselves and it is well known that oxidation products are phytotoxic (Martinez-Cayuela, 1982; Debergh and Read, 1991).

The production of phenolic substances is promoted when the oxidation of pre-formed phenolic components of the explant such as phytoalexin or lignin (quinones) or by the synthesis of monomeric or polymeric phenolic derivates begin on the cultured explant (Rhodes and Wooltorton, 1978).

The present study shows that phenolization of semiwoody cuttings of *A. cherimola* and *A. muricata* explants could be controlled well if they are immersed previously in a combined solution of citric and ascorbic acid during the manipulation time to prevent oxidation on the surface of the explant during the inoculation time (Table 24).

The **semiwoody cutting** explants of *A. cherimola* and *A. muricata* have the potential to induce new *in vitro* shoots because they are carrying buds. The mechanical injury caused by the scapel, promotes phenolization on the base and tip of the cutting where the cut was made. The leaf scales of the pre-formed buds protect the meristems of the phytotoxic oxidation products.

The lignification grade in semiwoody cuttings is less intensive than woody cuttings. During the establishment the semiwoody cuttings should be protected from the oxidation reaction with a long-phase antioxidant such as Polyvinylpyrrolydone on establishment, the absence of phenols promotes quality shoots under *in vitro* conditions.

If the meristems of the pre-formed bud from the axillary branching are stimulated**,** the formation of four new shoots per bud could be induced by direct shoot organogenesis which promotes the genetic stability of the selections during the *in vitro* establishment of *A. cherimola* and *A. muricata.* The results suggest that the micropropagation of this species begins with a genetically stable shoot material.

A. cherimola and *A. muricata* are subtropical and tropical plants which grow well on the tropical high-lands with rich mineral soils of volcanic origin (Bridg, 1993) but not on soils with high salt concentrations (Morton, 1987).

Because most plants react positively to the Murashige and Skoog (1962) mineral salt and vitamin combination during *in vitro* culture (Conger, 1986), it was tested during the establishment of *A. cherimola* and *A. muricata*. The mineral basal composition of **Nitsch and Nitsch** (1969) has been reported by Jordan (1998) on *A. cherimola* during the induction and promotion of *in vitro* morphogenic responses.

The *A. cherimola* and *A. muricata* responded better during establishment to the mineral salt formulation of Nitsch and Nitsch (1969) than to the Murashige and Skoog (1962) salt combination which is a middle salt rich medium in comparison with the Nitsch and Nitsch (1969) formulation.

This study shows that the *A. cherimola* as well as the *A. muricata* did not respond well when cultured in Murashige and Skoog (1962) medium. In accordance with Witjaksono *et al.* (1999) most woody species did not respond well to this salt high media formulation.

A. cherimola as well as *A. muricata* did not require a high content of mineral salts to promote the *in vitro* sprouting of the pre-formed buds (Table 25). Other species like those of the genus *Sorbus* that are also deciduous trees and shrubs require high concentrations of salts, not only during the establishment, but also during the multiplication (Chalupa, 1987). The sensitivity of *A. cherimola* and *A. muricata* to salt high conditions has been reported by Ebert (1998).

The shoot cuttings of *A. cherimola* and *A. muricata* used in this study to promote the direct bud sprouting of these species under *in vitro* culture seems to have all the energy and nutritional requirements to induce new growth. Then the culture media

should only provide a low mineral balance that permits the shoot cutting to be in an osmotically balanced environmental condition.

Furthermore 3.0 ± 0.1 was the mean shoot number per bud in *A. cherimola* and *A. muricata* cutting in media supplemented with sucrose 30 g/l. Establishment culture media without sucrose reported a low percent of bud sprouting 1.6 ± 0.6 mean of shoot number per bud. The sucrose in all the tested media in this study is shown to improve the quality of the new *in vitro* sprouted shoot. The shoots from media supplemented with sucrose in any case showed a hyperhydratation or anomalies during their development.

The osmotic relation between the tissue cells of the semiwoody cuttings of either *A. cherimola* or *A. muricata* was promoted by the presence of sucrose which is a very important component in any nutrient medium and its addition is essential for *in vitro* growth and development. Sucrose is a disaccharide used in *in vitro* culture because this sugar is also synthetized and transported naturally by the plant. The sucrose present in the culture medium is rapidly broken down to fructose and glucose by hydrolysis because of extracellular enzymes.

A. cherimola and *A. muricata* shoot cuttings sprouted well in medium without plant growth regulators, because the shoot explants with two or three pre-formed buds themselves have a endogenous plant growth regulator concentration to promote the new shoots.

6-benzyl amino purine 8.87 µM and indol-butiric acid 2.46 µM are required for the semiwoody macro-cutting of *A. cherimola* and *A. muricata* to stimulate bud sprouting and shoot growth and cell elongation. The relation of cytokinin : auxin is very significant for *A. cherimola* and *A. muricata* in terms of shoot quality.

Liquid media and s**emisolid media** of NN-69 for *A. cherimola* and *A. muricata* establishment promote bud sprouting, however these new shoots showed the tendency to be hyperhydrated during multiplication. The hyperhydrated shoots of *A. cherimola* and *A. muricata* showed a poor epidermal development and glassy appearance which restricts its multiplication because leads to metabolic and morphological derangements in the *in vitro* explant.

Species such as *Malus* spp., *Picea sitchensis, Gladioulus grandiflorus* and *Prunus avium* which have been been cultured in a liquid medium, have reported vitreous shoots, aerenchyma disorders, unorganized cortex, thin cell walls, abnormal stomata and low epicuticular wax deposits (John, 1986).

The *A. cherimola* and *A. muricata* hyperhydrated shoots in the multiplication stage produced some phenols in the culture media and a light green color was observed suggesting that chlorophyll content was reduced. Liquid media could promote extra-protoplastic water on the tissues as an effect of high enviromental humidity.

The incubation temperature used during the micropropagation of *A. cherimola* and *A. muricata* cuttings was 25 ± 3 °C and the total closure of the test tube with parafilm (Figure 13) was not favorable for the explant. The gas circulation was stopped, the water vapor increased and the relative humidity in the *in vitro* test tube around the explant promoted an abnormal epicuticular development. Furthermore *A. cherimola* as well as *A. muricata* in natural conditions do not grow well in high humidity conditions (George and Nissen, 1992).

In accordance to Pàques and Boxus (1987) vitrification and hyperhydratation limit in most of the cases the industrialization of micropropagation protocols thus in the present study the *A. cherimola* and *A. muricata* hyperhydrated shoots were eliminated

Solid media of Nitsch and Nitsch (1969) either with agar or gelrite did not promote vitrification or hyperhydratation of shoots on *A. cherimola* and *A. muricata*. The presence of a support agent contributes to the regulation of the humidity which affects the availability of the water status (Deberg, 1983), because the water in a solid medium is bounded.

In this study the *A. cherimola* and *A. muricata* in all the micropropagation did not respond well to a high concentration of **Agar** which not only limited the bud sprouting, but also the new shoot formation and rooting.

The *A. cherimola* and *A. muricata* in this study responded well to a culture medium supplemented with **Gelrite** which was added in a half concentration of agar and showed a clear medium which permitted the evaluation of contaminants more easily.

The *A. cherimola* and *A. muricata* reduced the percent of phenolics on a Gelrite supplemented medium on establishment. Pierik (1989) showed that gelrite gels are more or less free from contaminating materials which could promote phenolic compounds. In comparison with Agar which is seaweed derived, gelrite is a natural anionic heteropolysacharide that forms rigid, brittle agar-like gels in the presence of soluble salts. Gelrite is a polysaccharide comprised of glucuronic acid rhamnose, glucose and O-acetil moieties (Pierik, 1989).

The cuttings with pre-formed buds of *A. cherimola* and *A. muricata* semi-woody tissue, showed regeneration differences on NN-69 medium combined with **Benzyl amino purine (BA)** and **Indole-butiric acetic acid (IBA)** in terms of shoot sprouting.

The *A. cherimola* and *A. muricata* shoots that developed in NN-69, not supplemented with plant growth regulators, showed a high percent of bud sprouting (Table 27) because larger explants such as macro-cuttings used to promote new shoots from pre-formed buds, have a starch reserve which promotes the meristem activity if in the culture condition sugars, minerals and plant growth regulators are added.

A. cherimola and *A. muricata* in NN-69 supplemented with BA and IBA promoted the formation of quality shoots, the balance cytokinin : auxin during the establishment benefit the promotion of healthy shoots, probably due by the regulation of endogenous plant hormones on the bud.

86 ± 2.1 % of *A. cherimola* and 86.1 ± 1.5 % *A. muricata* buds were sprouted successfully in NN-69 media supplemented with BA 8.87 mM and IBA 2.46 mM. This study showed four healthy new shoots formed from pre-formed buds on semiwoody cuttings of *A. cherimola* and *A. muricata* in the presence of plant growth regulators.

The bud sprouting and *in vitro* plantlet differentiation of *A. muricata* from hypocotyl segments have been reported using BA (Bejoy and Hariharan, 1992). However they failed to elongate. To promote shoot elongation the BA (8,9 mM) was combined with NAA (0,54 mM) and further growth into elongated shoots was observed. Even then, all shoots buds did not develop into elongated shoots.

The endogenous growth regulators, age and plant location condition the *in vitro* response of the cutting explant in *A. cherimola* and *A. muricata* as well as in other woody species (Torres, 1989).

The new shoots of *A. cherimola* and *A. muricata* promoted in the highest frequency in a high cytokinin and low auxin concentration (Table 26). In tissue culture there are plants which in principle need neither cytokinins nor auxines for the formation of adventitious shoots (Pierik, 1989).

Most of the plants require cytokinin for shoot formation and benzyl-aminopurine is the cytokinin which shows the most efficient application in the promotion of adventitious shoots. The citokinin:auxin ratio is very important for the shoot formation in many different plant species (Pierik, 1989). Jordan *et al*. (1991) reported from single nodes of seasonal buds the sprouting and forming of leaflets in the presence of NAA 0.5 mg/l and BA 2.0 mg/l with the presence of some calli.

A. cherimola and *A. muricata* promoted not only direct bud sprouting form pre-formed buds of semiwoody cuttings but also clumps of white cells on the surface of the explant. The **cambium** tissue of cuttings was stimulated (Figue 11; Figure 12) in medium NN-69 supplemented with BA and IBA reported concentrations (Table 25). Callus induction and shoot regeneration were tried on *A. cherimola* and *A. muricata* from this meristematic cambium with high levels of auxines such as 2,4-D and NAA , this study shows no regeneration from meristematic cambium callus on *A. cherimola* and *A. muricata*.

In accordance to Pierik (1989), Torres (1989) and Murashige (1976) the year`s season has an effect on the *in vitro* manipulation. *A. cherimola* and *A. muricata* explants coming from actively growing plants in summer are more able to induce morphogenic responses than those coming from winter.

The *A. cherimola* and *A. muricata,* as other semideciduous woody species, show different rates of growth during the year (Sawneski, 1988; George, 1984; Torres and Sanchez, 1992) and under *in vitro* conditions they were affected also by the **year`s season** during establishment and multiplication. The semiwoody explants reduced the sprout percentage in winter and in summer they were more shoot proliferant (Figure 14). In open field conditions the *A. cherimola* have the tendency to burst in spring and loose the leaves in winter (Gardiazabal and Rosenberg, 1988).

The deciduous trees as well as the deciduous trees such as *Malus* spp., *Pyrus* spp. and *Prunus* spp., have an intrinsic biological cycle which under the most optimal environmental conditions promote leaf fall. The *A. cherimola* and *A. muricata* during *in vitro* culture in terms of bud sprouting were affected by the year`s season. The percentage of shoot sprouting from pre-formed buds reduced in winter more significantly for *A. muricata* than for *A. cherimola* (Figure 14).

The *A. muricata* is the most tropical of the *Annonas* and it is extremely affected by the reduction of the photoperiod. The light intensity has a strong influence on the metabolism of the plants and active growing. The endogenous plant growth regulators level in semideciduous and deciduous trees have a different balance during short days and low temperatures (Hartman and Kester, 1984).

During the tissue culture establishment *A. muricata* needs more time to promote bud sprouting than *A. cherimola* which is the most subtropical of the *Annona* species

and adapted to some temperature variations on the highland mountains in natural conditions.

The **proliferation of new shoots** includes the processes of first dedifferentiation and second differentiation which possibly lead to the re-determination and rejuvenation of the new formed cells (Roca and Mroginski, 1991; Conger, 1977).

The organogenic potential of *A. cherimola* and *A. muricata* are different, although these species showed the same shoot sprouting behaviour during establishment in the multiplication stage the *A. cherimola was* shown to be more proliferant than the *A. muricata* which was not proliferant in *in vitro* conditions but elongated well in a Benzyl-aminopurine supplemented media.

The establishment Nitsch and Nitsch media supplemented with 8.87 µM Benzylamino-purine (BA) and 2.46 µM Indole-butiric acid (IBA) showed the highest sensitivity of *A. muricata* to BA levels (Table 26).

Lemos and Blake (1996) reported the effect of BA 9.5 mg/l in combination with NAA concentrations on *A. muricata* where the increments of BA concentrations inhibit the formation of buds per explant. This result is also confirmed in this study as well as the fact that the presence of a cytokinin on the multiplication culture media had no significant effect in terms of number of shoots per explant neither in *A. cherimola* nor in *A. muricata.*

The *A. muricata in vitro* shoot explants have the capability to induce bud elongation and become useful shoots in media supplemented with BA 2.22 µM. In contrast with Lemos and Blake (1988) the increments of BA concentration did not increase the number of shoots per explant in *A. muricata*.

The elongation of *A. muricata* shoot explants in media supplemented with BA was compared to **Gibberellic acid (GA_3)** supplemented media. *A. muricata* shoots in media with GA_3 were intermediate in length and the multiplication rate per shoot reduced from 1 explant : 4 new cuttings with bud in BA medium to 1 explant : 2 new cuttings in BA medium.

The GA_3 as media supplemented has been reported by Jordan *et al* (1991) as an inhibitor of the *de novo* bud promotion in *A. cherimola* and in *A. muricata*. It has been suggested as an inhibitor of bud development (Lemos and Blake, 1996). The use of gibberellins in *in vitro* culture has been reported as not essential for most species or higher plants. Gibberellins promotes elongation of internodes as plant growth regulators but inhibits adventitious shoot formation and adventitious root formation in most of the cases (Boggetti *et al.* 1999)

The comparative effect of **Thidiazuron** (**TDZ)** and **Benzylamino purine (BA)** on shoot proliferation of *A. cherimola* and *A. muricata* was evaluated, although TDZ has recently been used in tissue culture on recalcitrant tissues because of its cytokinin-like activity (Huetteman and Preece, 1993).

TDZ stimulated shoot organogenesis in *A. cherimola* and *A. muricata* (Figure 18) but on the base of the explant multiple adventitious leaves were observed in 0.45 µM, 1.36 µM and 2.27 µM (Figure 11). The reason why TDZ as a pyridil-urea compound has a high activity in low concentrations in woody plants to promote adventitious shoots is until now not clear (Huetteman and Preece, 1993).

TDZ has been reported as a persistent growth regulator in tissue culture and since most tissues are transferred to fresh medium without a regulator the cytokinin activity of TDZ continues, because little is known about the cytokinin plant receptors (Huetteman and Preece, 1993).

The *A. cherimola* and *A. muricata* in this study were shown to be very sensitive to TDZ supplemented medium but the new shoot organogenesis was interfered by a non normal leaf regeneration on the base of the shoot explant. The TDZ supplemented medium was avoided because the principal aim was to produce *in vitro true-to-type* plants.

Although TDZ has been used to promote adventitious shoot formation in many species, this study did not report positive effects on the shoot quality regeneration in *A. cherimola* and *A. muricata*. There is no information (as far as it is known) about the endogenous cytokinin shoot concentration of woody species when they are exposed to synthetic cytokinins in culture media.

The TDZ was developed by Schering AG (Germany) for utilization as a defoliant for *Gossypium hirsuum* L. TDZ is up to 10,000 times a cytokinin activity and in several woody species caused a dramatic increase in the number of shoots and proliferation rate. In concentrations less than 0.1 µM it is capable of inducing adventitious shoots in *Poplar spp*. calluses but also axillary shoot formation is severely inhibited in *Poplar spp*, when thidiazuron is present in all the treatments then it is necessary to transfer shoot-regeneration cultures to TDZ free media for shoot development (Pierik, 1989).

The *A. cherimola* and *A. muricata* new shoots proliferated also in Zeatin (Z) which is reported to be 10 times more powerful than Kinetin (K) and the synthetic cytokinin Benzyl-amino purine (BA). *A. cherimola* is bud proliferating in media supplemented with Zeatin (1.36 µM), whereas *A. muricata* is not bud proliferating in Zeatin supplemented media. These species are not shoot proliferating in media supplemented only with Zeatine.

Encina *et al.* (1994) reported the effect of 1.36 µM of **zeatin** on *A. cherimola* shoot formation, at 0.66 µM wide and longer leaves were reported and in high zeatin concentrations aberrant shoots were observed. This study showed a reduction in the proliferation rate in a zeatin high concentration medium. High quality shoots were observed in a 1.36 µM concentration with a means of shoot number of 2.5 $\pm$ 3 per explant. Also *A. cherimola* was shoot proliferating in a kinetin supplemented media which at 2.32 µM shows 3.5 $\pm$ 5 shoots per explant.

The synergistic effect of **zeatin and kinetin** were tested on *A. cherimola* shoot explants which were high shoot proliferants in Z 1.36µM + K 4.65 µM., 6.8 $\pm$ 0.7 new shoots were induced in this concentration but shoots should only be exposed to this concentration for a short period of time because if several subcultures are promoted in a Z+K supplemented medium no root formation could be induced in the next micropropagation. The *A. cherimola* shoots elongated well in a BA supplemented medium which is a less powerful cytokinin (Krikorian, 1989b).

A. cherimola and *A. muricata* during the multiplication stage showed chlorotic shoots in cytokinin supplemented media, these shoots lost their vigour and reduced the multiplication rate. The formulation of Nitsch and Nitsch (1969) (NN-69) is not a high

salt concentration formulation in comparison with that reported by Murashige and Skoog (1962) (MS-62) and Woody Plant Medium (WPM).

The MS-62 showed the highest concentration in **ammonium and nitrate** (20.6 and 39.4) respectively. In contrast WPM showed the lowest concentration of these ions in its formulation (5 mM and 9.8 mM), the NN-69 has a concentration of these ions (9 mM and 18.4 mM). Therefore these ions were substituted in the formulation of NN-69 to increase the ammonium and nitrate concentration and support the *A. cherimola* and *A. muricata* during their multiplication with a media rich in nitrogen. The other elements such as potassium, magnesium, calcium, sodium, phosphate, sulphate and chloride showed in MS-62, WPM and NN-69 concentrations that did not vary at all.

The catabolic metabolism of ammonium during the photorespiration and the anabolic inorganic nitrogen assimilation are related to the electron transport systems in chloroplast and mitochondria organelles. If there is a non-balance of these ions in the cell it promotes the production of free radicals and ultimately the death of the plant (Miflin and Lea, 1977; Skotut et al. 1978: Krogmann et al. 1959; Puritch and Baker, 1967).

In the present study the *A. cherimola* and *A. muricata* shoot multiplication was stimulated with high levels of ammonium and nitrogen ions (Table 28) in order to preserve the quality of the new *in vitro* produced plant material. The variation of the amount in Nitsch and Nitsch (1969) media formulation from 9 mM to 20.6 mM NH_4^- and 18.4 mM to 39.4 mM NO_3^- promoted shoots without chlorosis on a high cytokinin supplementation.

The casein enzymatic hydrolisate is a nitrogen complex which is used in plant tissue culture to promote growth and as a nitrogen source because it is rich, not only in aminoacids, but also in reduced nitrogen. In this study the supplementation of casein in the culture media increased the number of shoots without chlorosis in the culture media, but not all.

Rooting has been occasionally reported in *Annona* spp., but regeneration from explants of clonal origin has not been reported (Jordan and Botti, 1992). A low rate of rhizogenesis was reported by Tazzari *et al*. (1990) on *A. cherimola* cuttings micropropagated from adult cuttings.

In this study *A. cherimola* and *A. muricata* micropropagated shoots in preliminary rhizogenesis experiments (rooting culture media supplemented with cytokinins and dark stimulation for five days) were tested and did not show any *in vitro* root organogenesis, also no callus formations were observed on the base of the explant.

The *in vitro* media during the micropropagation have been supplemented with sucrose which is a carbon source, essential for *in vitro* growth and development, because phosynthesis is insufficient, due to growth taking place in conditions unsuitable for photsynthesis or without photosynthesis (in darkness). Green tissues are not sufficiently autrophic *in vitro* (Pierik, 1989).

The organogenic potential of plant explants under *in vitro* conditions is closely associated with the content of natural and exogenous applied plant growth regulators (PGRs) (Terzi and LoSchiavo, 1990; Wenck *et al.* 1990). Unfortunately, little is known about this topic due to the complexity and sophistication of the techniques required for PGR analysis. Only a few research teams have found links between the embryogenic

capacity of plant tissues and a specific endogenous hormonal content (Rajasserakan *et al.* 1987; Ivanova *et al.* 1994; Wenck *et al.* 1988), but all these results were obtained in herbaceous plants and not in woody species (Centeno *et al.* 1997).

A. cherimola and *A. muricata* shoots were obtained from the multiplication stage with three subcultures in a rich cytokinin medium during six months after its inoculation. All the media were supplemented with sucrose 30 g/l to improve the photosynthesis of the new vegetative plant organs. The endogenous synthesis of PGRs is related to the photosynthetic activity of the vegetative explant.

Endogenous indole-3-acetic acid (IAA), abscisic acid (ABA) and cytokinins [zeatin (Z) zeatin ribosied, dihidrozeation, dihydrozeatin riboside, N^6-isopentenyl adenine (iP) and N^6- isopentenyladenine riboside] have been evaluated in *Coryllus avellana,* results suggest that the endogenous hormonal balance is a very important factor defining the *in vitro* potential of tissue explants in plant culture (Centeno *et al.* 1997).

A. cherimola shoots were multiplicated in a zeatin:kinetin combination medium and *A. muricata* in a benzyl-amino-purine supplemented medium, the selected shoots to improve rhizogenesis were cultured in a root precondition medium with 20 g/l sucrose and a PGR supplementation because the shoot explants were cultured in a high cytokinin combination medium.

Previous results indicated that rhizogenesis could be stimulated and root formation promoted after this precondition, then mineral salt composition, carbon source concentration and the effect of some auxines were evaluated to promote either *in vitro* or *ex vitro* roots.

The effect of indole-acetic-acid (IAA), naphtalene-acetic-acid (NAA) and IBA (indole-butiric-acid) were evaluated. No roots were observed except with 1% IBA from Rhizopon ® applied on the base of the explant in *ex vitro* conditions. The effect of 0.3% IBA in talcum powder has been reported also on *Betula* spp (Meier-Dinkel, 1992). *A. cherimola* and *A. muricata* had rooted shoots in *ex vitro* conditions in 90% high humidity.

To promote *in vitro* rooting, shoots were treated with IBA 1% (4. 90 μM l^{-1}) with the reduction of macroelements to ¼ and ½ of its concentration. The media used the mineral basal composition of Nitsch and Nitsch (1969), reduction of sucrose concentration was observed also as one of the factors which are related to rhizogenesis in *A. cherimola* and *A. muricata* selections.

The precondition of the rooting media in darkness for five days to promote rhizogenesis by the intensive activity of the IBA in darkness conditions to prevent photo-degradation of this PGR, reported no significant differences among treatments, suggesting that this species needs hormone free media precondition but not darkness when the media are supplemented with IBA.

Bejoy and Hariharan (1992) reported 1-2 roots induced on 80% of the *A. muricata* explants a root organogenesis when the shoots were treated with 14,8 mM IBA. Similarly, on 4.9 mM IBA 85% shoots rooted but with only 1-3 roots. Root organogenesis on *A. squamosa* has been reported when the explants were tried with 98 mM IBA (Nair *et al.* 1984).

In this study 4.90 µM IBA promoted in *A. cherimola* and *A. muricata* roots after 40 days either *in vitro* and *ex vitro.* 3.6 roots per shoot were the registered promedium after the root emergence after 60 days. Encina *et al.* (1994) reported rooting in *A. cherimola* at 500 µM IBA. In this study this concentration did not promote rhizogenesis. The other auxins such as IAA and NAA had no effect on *A. cherimola* and *A. muricata* root organogenesis.

The sucrose and concentration of macroelements salts of Nitsch and Nitsch (1969) in the culture medium should be reduced. Carbohydrates are responsible for inducing changes from the pentose phosphate to the glycolitic pathway in plant tissues and this could explain its effect on rooting (Haissing, 1982).

In accordance to Encina *et al.* (1994) *A. cherimola* reduced the percent of rooting progressively with the increments of sugar in the culture media. *A. cherimola* and *A. muricata* are sensitive to the accumulation of sugar in the culture media. High carbohydrates in the culture media have been reported as root inhibitors in *A. cherimola* (Encina *et al.* 1994), however low concentrations are essential to promote roots in *A. muricata* (Lemos and Blake, 1996).

There are differences between the *in vitro* and *ex vitro* percentage of rooted shoots in *A. cherimola* and *A. muricata*. The number of roots per shoot under *in vitro* conditions of both *A. cherimola* and *A. muricata* was reduced in comparison with the *ex vitro* rooting. These differences may be caused by their different levels of action of metabolic regulators such as ethylene. The *in vitro* shoots of *A. cherimola* and *A. muricata* probably accumulated more ethylene in the baby food jar than the *ex vitro* shoots. Ethylene inhibits root formation of apple shoots under *in vitro* conditions (Mo *et al*, 1989).

For the **Acclimatization** of regenerated plantlets with well established root systems plants were washed carefully to remove the gelrite and transferred to pots (9x9 cm) containing quartz sand. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 25 ± 2°C under 16 h photoperiod. After 4 weeks, acclimatized plants were transferred to the greenhouse.

The pre-acclimatization of the *A. cherimola* and *A. muricata* explants was made in a sterile medium and then they were transplanted successfully into a non sterile quartz sand. Nair *et al.* (1984) reported for *A. squamosa* a pre-transplantation made before acclimatization in the greenhouse on aseptic conditions. Bejoy and Hariharan (1992) transferred the *A. muricata* micro plants directly into non sterile soil.

Hence the present procedure is simple and the autotrophic development of *A. muricata* plantlets was satisfactory. Regenerated plantlets were successfully established in the greenhouse after acclimatization. The plants, thus established, did not exhibit altered phenotypes, there were no morphological visible differences between regenerated plants of *A. cherimola* and *A. muricata* and mother source explants.

Random Amplified Polymorphic DNA (RAPD)

The genetic molecular variability of the DNA sequences in plants could be monitored by RAPD markers because of the wide spectrum of application (Williams *et al.* 1990).

RAPD have been found to be efficient in the detection of genetic variability at the nuclear genome level (Tulseiram *et al.* 1992; Brown *et al*., 1993; Munthali *et al*., 1996).

DNA characterization through RAPD permits us to characterize the relationships and determine likely parentage between species, selections or varieties because they are dominantly inherited markers (Villand *et al.* 1998). The expected segregation in F_1 can be inferred, in some cases, from parental phenotypes as Ronning and Schnell, (1995) reported for *Annona* spp.

The RAPD analysis have been compared with other molecular techniques such as Restriction Fragment Length Polymorphism (RFLP) and isozymes and no differences between results have been found (Munthali *et al*. 1996; Sabir *et al*., 1992).

Genetic variation between *A. cherimola* cultivars has been studied using isozyme markers (Ellstrand and Lee, 1987; Lee and Ellstrand, 1987; Pascual *et al.* 1983; Perfeccti and Pascual, 1996). However isozyme analysis is limited by the relatively small number of loci. RAPD offer the potential of generating large numbers of markers representing a random sample of the genome, thereby presenting an advantage over isozyme markers (Ronning and Schnell, 1995).

29 primers were tested by RAPD to analyse the DNA amplification of *A. cherimola* and *A. muricata in vitro* regenerants comparing results with the DNA amplification of *ex vitro* mother plants. The Q-12, C-11, C-5, Q-4, OPA-18 and OPA-16 showed repeatable and scoreable bands for both species. Because the number of tested plants was reduced the amplification might be the result of the duplication of some parts of the genome.

The DNA amplification of the *A. cherimola* and *A. muricata* experimental plants shows monomorphic bands in all the six tested primers(Figures-36,-37, -38 and -39). Although these results are limited by the number of plants tested, the developed and scorable bands assure the absence of DNA polymorphism in *A. cherimola* and *A. muricata* regenerants, coming from the developed micropropagation protocol presented in the present study (Table 36).

Using the RAPD technique, various investigators have reported somaclonal variation in *Pinus thunbergii* (Goto *et al.* 1998), *Picea glauca* (De Verno, 1999), *Allium sativum* (Al-Zahim, 1999), *Acacia nilotica* (Garg *et al.* 1996) and *Phalaenopsis* spp. (Chen *et al.* 1998) *in vitro* cultured plants from somatic embryogenesis, endosperm culture.

The somaclonal variation promoted by the *in vitro* culture of a selected plant material is related to several factors (Larkin and Scowcroft, 1981). One of them is the natural condition of the initial material „mother plants", because there are, in nature, plants that shows a tendency to induce bud mutations and diversifications than others (Krikorian, 1991) and there are no reasons to suppose other behaviour when they are manipulated *in vitro* , (Conger, 1987; Zimmerman, 1981, Krikorian *et al*., 1983). The causes of the natural mutations on open grown field plants is not clearly explained (Krikorian, 1991).

The cell organization of the selected explant is conditioning from the beginning the *true-to-type* and *true-off-type in vitro* multiplication (De Fossard, 1977). The most stable explants described are: meristems, lateral buds and nodes which could be propagated directly, succeeding somatic embryos and advent buds, while the most

unstable explants are cell callus and protoplasts (Roca *et al*., 1981), because they are not organized structured explants (Scowcroft *et al*., 1987).

The RAPD results suggest that *A. cherimola* and *A. muricata* plants exhibit relative stability during *in vitro* propagation through the direct bud sprouting induced under *in vitro* conditions. Genetic stability has been reported as well as in *Eucalyptus tereticornis* micropropagated by enhanced-axillary-branching-derived plants and tested by RAPD (Rani and Raina, 1998).

The discrete polymorphism revealed by RAPD and between *A. cherimola* and *A. muricata* selections is of future interest not only for phylogenetical information of this species, but also because there are several diverse and discrete fragment lengths, which can serve as characteristic markers for quality control in plant production systems, and for marker-assisted breeding programs

The use of different primer combinations enables the analysis of the presence or absence of polymorphisms. This is enough to distinguish not only cultivars, but different clones belonging to the same cultivar. Moreover, the results allowed the identification of high certainty cultivars, especially in fruit species, that belong to the same variety within the collection, which will save time and reduce the cost of their conservation.

The nuclear genome analysis has played an important role in early genetics. The basic cytological techniques enable an accurate determination of chromosome number and structure either in meiosis or mitosis cell division phases (Karp, 1991).

The observation of mitotic chromosomes under the light microscope is a rapid and informative method of studying the genomes as a whole. The technique involves the collection, fixation, staining and preparation of chromosome squashes. Karp (1991) commented that although the cytology technique is easy to perform, there are some problems determined by species and its particular genome. The *A. cherimola* and *A. muricata* present some difficulties also to visualize the chromosomes by any traditional cytology method.

In a wide range of plant species, aneuploidy and/or changes in the ploidy level of the chromosomes have been considered to play a major role in describing somaclonal variation (Bayliss, 1980; D'Amato, 1985). RAPD and nuclear genome size estimation singly have been effectively used in many studies to detect somaclonal variation on *in vitro* produced plants (Brown, *et al*., 1993; Cecchini *et al*., 1993; Rani, 1995).

In vitro plantlets regenerated from callus and/or meristem cultures exhibited startling differences in the chromosome number in *Populus* species (Rani *et al*., 1995; Sommer and Wetzstein, 1984). Chromosome doubling is a common type of somaclonal variation (Novak, 1980) and has not been observed in *Annona* regenerants in the reported results.

The efficiency of RAPD markers to differentiate the *Annona* spp is in agreement with the conclusions reached by Ronning and Schnell (1995). Further, this study has demonstrated for the first time that RAPD markers applied to *A. cherimola* and *A.muricata in vitro* regenerants screen the quality of the produced plantlets.

This work contributes to the knowledge of the A. cherimola and A. muricata potential fruit species and reports a clonal regeneration protocol with a successful rate of rhizogenesis and without much risk of genetic instability according to the RAPD analysis.

Summary

A. cherimola and *A. muricata* are semideciduous native trees from the tropical highlands of South America and tropical areas of the Caribbean Islands. Both have developed a commercial promise in the fruit trade market, because of their edible fruits and phytochemical products.

The knowledge of these fruit trees has been scattered thus, exploration, collection, conservation and evaluation of *A. cherimola* and *A. muricata* natural genotypes is a priority in Colombia, Peru, Ecuador, Venezuela and El Salvador, countries where they are believed to be part of the native Flora. Furthermore, the promotion of technical plantations with healthy and high quality trees are the principal worldwide aims for these species.

If a selected genotype of *A. cherimola* and *A. muricata* is propagated by seeds a high genotype variation is expected, when conventional vegetative propagation methods are applied, the dichogamous protogyneous flower behaviour promotes an intervarietal crossing. Therefore the conservation of selected ecotypes by the application of conventional propagation methods has been until now impossible, in those regions where they have been introduced because of their qualities such as Spain, Australia, Asia, California and Chile.

The aim of the present study was to review the botanical and cultural aspects of *A. cherimola* and *A. muricata* and develop a reproducible micropropagation protocol preserving the genetic stability of the selections or promote *true-to-types* genotypes in order to open an alternative for this plant species.

Preformed axillary bud sprouting excised from the side branches of four year old plants have been developed with a yield of 4 new shoots per bud in 20 days. The position of the buds on the branches had an effect on the bud break and establishment of cultures under *in vitro* conditions, therefore semiwoody cuttings with three buds were the most suitable explants for multiple shoot proliferation when cultured on a Nitsch and Nitsch (1969) medium containing 8.87 µM of benzylaminopurine and 2.46 µM of indole-butiric acid.

The effect of Benomyl, Rifampicin and some antioxidants like Polyvinylpirrolydone, ascorbic acid and citric acid are discussed. There were no significant differences during the establishment of *A. cherimola* and *A. muricata* in terms of *in vitro* requirements, time and yield of bud new shoots formation.

To improve shoot proliferation and multiplication the effect of benzylaminopurine, kinetin, zeatin and thidiazuron were compared. The NN-69 media supplemented with 2.32 µM kinetin and 1.36 µM zeatin was the proliferant for *A. cherimola*. *A. muricata,* it showed no shoot proliferantion but elongated well in 1.44 µM Gibberellic acid. Both species improved the formation of eight new shoots in 60 days of culture with one subculture after 30 days.

A. cherimola and *A. muricata* multiplied shoots lost their quality and started to be chlorotic in relation to the number of subcultures on the same multiplication media. The variation of ammonium nitrate, potassium nitrate and ammonium carbonate

supplied as salts on the Nitsch and Nitsch (1969) media were evaluated as well as the supplementation of casein hydrolisate and coconut water. The supplementation of 20.6 mM NH_4^- and 39.4 mM NO_3^- improved the promotion of quality and green shoots available for rooting.

The indole-3-butiric acid 4.90 µM promotes rhizogenesis under *in vitro* and *ex vitro* conditions in both cases a previous precondition of the shoots was required. To improve *in vitro* rooting the concentration of Nitsch and Nitsch (1969) macro-salts should be reduced to ¼ with a supplementation of 1 % of sucrose and 3% gelrite. The *ex vitro* rooting is succesfully in quartz-sand substract without plant growth regulators and 90% of relative humidity.

Pattern band comparison by Random Amplified Polymorphic DNA markers was applied to determine the genetic stability of micropropagated shoots of *A. cherimola* and *A. muricata* 4 Colombian and 2 Chile selections. 29 primers were screened, of them 5 primes gave clear reproducible bands. No variation in RAPD banding patterns among the tested shoots. These results verify the genetic stability of the *in vitro* regenerants and tested the *true-to-type* propagation.

Zusammenfassung

A. cherimola und *A. muricata* sind als Halblaubbäume in den tropischen Hochländern Südamerikas und besonders auf den Karibischen Inseln endemisch. Beide besitzen ein großes Potential als Handelsfrüchte aufgrund ihrer wohlschmeckenden Früchte und ihrer Eigenschaften als Heilpflanzen.

Die Forschung über diese Obstarten wurde in Kolumbien, Peru, Ekuador, Venezuela und in der Dominikanischen Republik vernachlässigt. Deshalb sollte die wissenschaftliche Bearbeitung, das Sammeln, die Konservierung und eine neue Bewertung der natürlichen Genotypen von *A. cherimola* und *A. muricata* in jedem Land eine Vorrangstellung erhalten. Die Etablierung von Plantagen mit selektierten Genotypen und die Erzeugung hoher Qualität ist eines der fundamentalen weltweiten Ziele in der Züchtung dieser Arten.

Wenn ein selektierter Genotyp von *A. cherimola* und *A. muricata* über Samen vermehrt wird, entsteht eine hohe Variationsbreite von Pflanzen. Bei konventioneller Stecklingsvermehrung dagegen ist der gleiche Genotyp zu erwarten, aber die dichogame protogyne Blüte bringt eine Kreuzung hervor. Deshalb war es bisher unmöglich, einen selektierten Genotyp in den Regionen Spaniens, Australien, Asiens, Kaliforniens und Chiles, in denen er eingeführt wurde, durch die Anwendung konventioneller Vermehrungsmethoden auf natürliche Weise zu erhalten.

Die gegenwärtige Studie zielt darauf, die botanischen und Pflanzenkulturaspekte der *A. cherimola* und *A. muricata* zu untersuchen und ein *in vitro* Vermehrungsprotokoll zu entwickeln, um die Produktion klonierter Genotypen zu sichern.

Selektierte Bäume aus Kolumbien und Chile wurden *in vitro* durch direkte Sproßknospenentwicklung einer vier Jahre alten Pflanze vermehrt. Die chemischen, hormonellen und physikalischen Faktoren der Wachstumsvermehrung vor und während der *in vitro* Phasen wurden untersucht. Da *A. cherimola* und *A. muricata* in ihrem natürlichen Lebensraum mit Mikroorganismen kontaminiert sind, mußten Antibiotika und Fungizide zugesetzt werden. Um die Bildung von Phenolen zu verhindern, wurde mit mit Zusätzen von Zitronensäure, Ascorbinsäure und Polyvinypirrolydon im Medium gearbeitet. Mit dieser Behandlung entstanden in einer Nitsch und Nitsch-Kultur, die 8,87 µM Benzylaminopurin und 2,46 µM Indol-3-Butiricsäure enthielt, aus einem halbverholzten Explantat mit zwei Knospen innerhalb von zwanzig Tagen vier neue Triebe pro Knospe.

Die Auswirkungen der Hormone Benzylaminopurin, Kinetin, Zeatin und Thidiazuron auf die Vermehrung der Triebe wurden miteinander verglichen. Bei *A. cherimola* wurden durch eine Anreicherung der Nitsch und Nitsch-Kultur mit Zeatin und Kinetin die besten Ergebnisse erzielt. Das Explantat der *A. muricata* zeigte keine Triebe, verlängerte aber im durch Benzylaminopurin angereicherten Medium ihren Sproß.

Während der *in vitro* Vermehrung verloren beide Arten ihre Vitalität und zeigten Chlorose-Erscheinungen. Daraufhin wurden die Ionenkonzentrationen von Bor, Kalzium und Stickstoff in der Nitsch und Nitsch-Lösung untersucht, und es wurde festgestellt, daß nicht Bor und Kalzium, sondern der fehlende Stickstoff für die Chlorose verantwortlich war. Die Anreicherung der Lösung mit 20,6 mM NH_4 und 39,4 mM NO_3 behob dieses Problem und verbesserte die Qualität der Sprosse.

Indol-3-Butiricsäure bewirkte ein Wurzelwachstums unter *in vitro*- und *ex vitro*-Bedingungen. In beiden Fällen mußten die Sprosse vorher in ein Kulturmedium mit 20% Saccharose ohne Hormone gebracht werden. Die Wurzelausbildung unter i*n vitro* Bedingungen gelang am besten in einer ¼ Nitsch und Nitsch-Lösung, wenn sie mit nur 1% Saccharose angereichert wurde. Unter *ex vitro* Bedingungen war das Wurzelwachstum in einem Quarzsandsubstrat bei 90% Feuchtigkeit optimal.

Durch Vergleich des DNS-Bandenmusters bei Verwendung von RAPD-Marker wurde die genetische Stabilität der *in vitro* vermehrten Triebe von *A. cherimola* und *A. muricata* von vier kolumbianischen und zwei chilenischen Klonen analysiert. 29 Primer wurden überprüft. Fünf von ihnen ergaben ein reproduzierbares linienspezifisches Bandenmuster. Für die *A. cherimola* wurden drei Primer identifiziert, die bei verschiedenen Klonen unterschiedliche Muster ergaben, während sich beim Vergleich von Mutterpflanze und Regenerat in allen Tests monomorphe Muster zeigten.

Diese Arbeit stellt zum erstenmal eine *in vitro* Vermehrung von Klonen von *A. cherimola* und *A. muricata* vor, die durch RAPD getestet wurde. Die genetische Stabilität *in vitro* vermehrter Arten wurde gezeigt, so daß die Ergebnisse dieser Arbeit die Züchtung dieser Pflanzen unter Erhaltung der natürlichen genetischen Charakteristik ermöglichen.

Resúmen

A. cherimola “chirimoya ó cherimoya” y *A. muricata* “guanábana, graviola ó soursop” son árboles frutales, semicaducifolios, nativos de las regiones cálidas de los Andes y de algunas áreas del caribe trópical de acuerdo a su origen. Estas especies son potencialmente promisorias a nivel mundial, no sólo en el mercado de las frutas tropicales, sino también en la industria de alimentos procesados. Los compuestos fitoquímicos de sus hojas, tallos y flores tienen una destacada aplicación en la fitomedicina y en la industria de productos cosméticos

Ambas especies son localmente conocidas en los países de América Latina de donde son originarias, y los desarrollos científicos aplicados son de orígen reciente. La exploración, colección, conservación, evaluación y divulgación de genotipos naturales de *A. cherimola* y *A. muricata* en los países donde son especies reconocidas en la Flora Nativa, tales como Colombia, Perú, Ecuador, Venezuela y El Salvador, es una prioridad en términos de redescubrimiento y conservación de germoplasma nativo.

La propagación por semilla de *A. cherimola* y *A. muricata* induce un alto grado de variación genética. Igualmente si un método convencional de propagación vegetativa es aplicado, las características hermafroditas de la flor y su comportamiento dicógamo-protogíneo, promueven el cruce entre individuos ó selecciones. Por lo tanto, la conservación de numerosos genotipos “élite” a través de los metodos tradicionales de propagación es una utopía, no sólo en los países centros de orígen, sino en aquellos donde han sido introducidas y adaptadas a microclimas específicos como es el caso de España, Australia, California, Chile y algunas regíones en Asia.

El presente estudio presenta una revisión y compilación de la información relevante en áspectos botánicos y culturales, que han sido publicados, de manera aislada, sobre la *A. cherimola* y la *A. muricata* a nivel mundial.

Debido a los problemas para propagar y conservar material élite de *A. cherimola* y *A. muricata*, el primer objetivo de éste estudio, aplicando la técnica del cultivo de tejidos vegetales, ha sido desarrollar protocolo que permita la indución y propagación *in vitro* de estas especies, asegurando la preservación de los genotypos naturales o iniciales, es decir, no modificando la estabilidad genética del material micropropagado „*true-to-type*“.

A. cherimola y *A. muricata* son especies que han sido clasificadas como recalcitrantes o difíciles de ser propagadas *in vitro* debido a los problemas de remanentes de contaminación y oxidación. La clonación *in vitro* se inició con la identificación del explante inicial en edad, tamaño y ubicación en la planta que permite, no sólo en un 95%, controlar la típica contaminación y fenolización inicial que limitan las subsecuentes etapas del cultivo *in vitro.* Estacas de árboles de 4 años de edad, con yemas preformadas fueron estimuladas a inducir en condiciones *in vitro* la proliferación de cuatro nuevos brotes por yema en un tiempo de cinco semanas.

El effecto de Rifampicina, antibiótico de amplio espectro, en la contaminación endógena del explante, y el efecto de compuestos anti-oxidantes para facilitar el establecimiento aséptico y supervivencia del explante son discutidos. Este estudio no reporta diferencias metódicas en el establecimiento de *A. cherimola* y *A. muricata* en condiciones *in vitro*.

En la etapa de proliferación de brotes *in vitro* la *A. muricata* es menos proliferante que la *A. cherimola* a nivel de desarrollo e inducción de nuevos brotes laterales. Concentraciones de ácido giberélico permiten la elongación de entrenudos en *A. muricata* con una tasa de proliferación de 1:6 explantes por subcultivo. Los brotes de *A. cherimola* proliferan bien, si el medio de cultivo es suplementado con zeatina y kinetina de 1:7 explantes por subcultivo en termino de cuatro semanas.

Durante la micropropagación tanto *A. cherimola* como *A. muricata* presentaron una clorosis *in vitro* y decaimiento total del explante. De acuerdo con la sintomatología, la formulación del medio de cultivo de Nitsch-Nitsch (1969) fue revisado, y los resultados confirmaron una deficiencia de nitrógeno, el cual fue incrementado de 20.6 μM a 39.4 μM en ambos tipos de suplementación química, amonio y nitrato. Este estudio reporta que tanto *A. cherimola* como *A. muricata* requieren en la etapa de proliferación una mayor suplementación de nitrógeno. La concentración reportada por Murashige-Skoog (1962) fue la más adecuada para proliferar explantes de alta calidad

El enraizamiento clonal de *A. cherimola* y *A. muricata* ha sido reportado esporádicamente tanto en condiciones *in vitro* como *ex vitro.* Este estudio revela que tanto la inducción y desarrollo de raíces esta relacionado con los contenidos endógenos de reguladores de crecimiento. Estas especies están en la etapa de proliferación *in vitro*, están somentidas a niveles altos de concentración de citoquininas exógenas, los contenidos altos de nitrógeno que se requieren en la etapa de multiplicación indican una alta activida fotosintética y metabólica endógena cuyos niveles pueden estar inhibiendo la formación de raíces.

Explantes de *A. cherimola* y *A. muricata* fueron precondicionados para inducir raíces en medio de cultivo con reducción de sales y azúcar por un tiempo mínimo de seis semanas sin ninguna suplementación hormonal. Pasado este tiempo los explantes reaccionan a la concentración de ácido-indol-butirico 1%, tanto en presentación comercial como análitica con una eficiencia de 55.5% y 45.6% de plantúlas enraizadas respectivamente. El enraizamiento *ex vitro* presenta el mayor porcentaje de brotes con raiz inducida con mayores probabilidades de endurecimiento y adaptación a las condiciones *ex vitro* ó de invernadero.

Haciendo uso de la técnica “Random Amplified Polymorphic DNA (RAPD)” se evacuo la estabilidad genética de las selecciones regenerantes *in vitro* de *A. cherimola* y *A. muricat.* 29 primers fueron evaluados, de ellos 6 fueron seleccionados por su patrón de repetición en ambas especies y seleciones. Los resultados confirmaron la estabilidad genética de las plantas micropropagadas de acuerdo a los primers seleccionados.

Esta investigación presenta por primera vez, hasta donde es conocido un protocolo que permite la propagación clonal *in vitro* de *A. cherimola* y *A. muricata*, el cual es confirmado por la técnica molecular RAPD. Con éste protocolo se pueden regenerar y propagar árboles selectos de *A. cherimola* y *A. muricata* y conservar la estabilidad genética de los mismos, resultado de gran importancia en términos de conservación de la diversidad natural.

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