BIOTECHNOLOGY APPLIED TO *ANNONA* SPECIES: A REVIEW

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ABSTRACT -Annonaceae is an ancient family of plants including approximately 50 genera growing worldwide in a quite restricted area with specific agroclimatic requirements. Only few species of this family has been cultivated and exploited commercially and most of them belonging to the genus *Annona* such as *A. muricata, A. squamosa*, the hybrid *A. cherimola* x *A. squamosa* and specially *Annona cherimola*: the cherimoya, commercially cultivated in Spain, Chile, California, Florida, México, Australia, Ecuador, Peru, Brazil, New Zealand and several countries in South and Central America. The cherimoya shows a high degree of heterozygosis, and to obtain homogeneous and productive orchards it is necessary to avoid the propagation by seeds of this species. Additionally, the traditional methods of vegetative propagation were inefficient and inadequate, due to the low morphogenetic potential of this species, and the low rooting rate. The in vitro tissue culture methods of micropropagation can be applied successfully to cherimoya and other Annona sp to overcome these problems. Most of the protocols of micropropagation and regeneration were developed using the cultivar Fino de Jete, which is the major cultivar in Spain. First it is developed the method to micropropagate the juvenile material of cherimoya (ENCINA et al., 1994), and later it was optimized a protocol to micropropagate adult cherimoya genotypes selected by outstanding agronomical traits (PADILLA and ENCINA, 2004) and further it was improved the process through micrografting (PADILLA and ENCINA, 2011).At the present time we are involved in inducing and obtaining new elite genotypes, as part of a breeding program for the cherimoya and other Annonas, using and optimizing different methodologies *in vitro*: a) Adventitious organogenesis and regeneration from cellular cultures (ENCINA, 2004), b) Ploidy manipulation of the cherimoya, to obtain haploid, tetraploid and triploid plants (seedless), c) Genetic transformation, for the genes introduction to control the postharvest processes and the genes introduction to provide resistance to pathogen and insects and d) Micropropagation and regeneration of other wild Annona or related Annonaceae species such as: *Annona senegalensis*, *A. scleroderma*, *A. montana*, *A. reticulata*, *A. glabra*, *A. diversifolia* and *Rollinia* sp.
Index terms: *Annona*, *in vitro*, micropropagation, protoplasts, organogenesis, polyploidy.

BIOTECNOLOGIA APLICADA A *Annona* sp.: UMA REVISÃO

RESUMO - Annonaceae é uma antiga família de plantas, incluindo cerca de 50 gêneros com crescimento em todo o mundo em uma área bastante restrita, com requisitos agroclimáticos específicos. Poucas espécies desta família tem sido cultivadas e exploradas comercialmente e a maioria delas pertencem ao gênero *Annona*, tais como *A. muricata*, *A. squamosa*, o híbrido *A. cherimola* x *A. squamosa* e especialmente a *A. cherimola*: a cherimóia, cultivada comercialmente na Espanha, Chile, Califórnia, Flórida, México, Austrália, Equador, Peru, Brasil, Nova Zelândia e vários países da América do Sul e Central.O cherimoia mostra um elevado grau de heterozigoze e a obtenção de pomares produtivos e homogêneos é necessário para evitar a propagação de sementes desta espécie. Além disso, os métodos tradicionais de propagação vegetativa foram ineficientes e inadequados, devido ao baixo potencial morfogenético e à sua baixa taxa de enraizamento. Os métodos de cultura de tecidos *in vitro* (micropropagação) podem ser aplicados com êxito para cherimoia o outras *Annona* sp. a fim de superar estes problemas. A maioria dos protocolos de micropropagação e regeneração foram desenvolvidos utilizando o cultivar 'Fino de Jete', que é o principal cultivar na Espanha.Primeiramente nós desenvolvemos o método de micropropagação do material juvenil de graviola (ENCINA et al., 1994), e mais tarde otimizamos este protocolo para micropropagação de genótipos de cherimoia em fase adulta, selecionando-os por características agronômicas (PADILLA; ENCINA, 2004) e ainda melhorou-se o processo através da microenxertia (PADILLA; ENCINA, 2011).Atualmente, estamos envolvidos na indução e obtenção de novos genótipos de elevada qualidade, como parte de um programa de melhoramento genético para a cherimóia e outras anonáceas, utilizando e otimizando diferentes metodologias *in vitro*: a) organogênese adventícia e regeneração de culturas celulares (ENCINA, 2004), b) Manipulação 'ploidia' da cherimoia, para obter plantas haplóides, tetraplóides, triplóides (sem sementes), c) transformação genética com introdução de genes objetivando controlar os processos de pós-colheita e para fornecer resistência à patógenos e insetos, d) micropropagação e regeneração de outras espécies selvagens do gênero *Annona* ou da família Annonaceae, tais como: *A. senegalensis*, *A. esclerodermia*, *A. montana*, *A. reticulata*, *A. glabra*, *A. diversifolia* e *Rollinia* sp..
Termos de indexação: *Annona*, *in vitro*, micropropagação, protoplasto, organogênese, poliploidia.

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REVIEW

The Annonaceae is an ancient family of plants that seems to have its origin in Andean American area apparently appears to be natives to the lands of Ecuador and Peru (POPENOE, 1921). This family comprises a good number of tropical and subtropical habitats worldwide, but its further territorial expansion is limited, due to the environmental requirements of the Annonaceae, to geographical areas with very specific characteristics of altitude, temperature, relative humidity and soil.

The most important species at commercial level (nourishment, pharmacy industry) belong to the genera *Annona*, being the most interesting and used species *A. squamosa*, *A. muricata*, *A. cherimola* and the hybrid *A. squamosa x A. cherimola* (GEORGE and NISSEN, 1993). The vegetative propagation of these species present problems of different degree, being its sexual propagation of limited agronomic value due to the high degree of heterozygosis of these species and preventing their propagation by seed.

Among these Annonaceae, in cherimoya (*Annona cherimola*), the most exploited Annona, the morphogenetic capacity is extremely low, showing minimal level of rooting making almost impossible the use of classic vegetative propagation methods, because the induction rate of adventitious roots from scions obtained from adult specimens with agronomic interest is null. This low morphogenetic capacity is the general trend for most of other Annona species even if it happens in different degrees depending on the species.

In order to fix the Annonaceae's vegetative propagation problems, *in vitro* methods have been applied. But unfortunately the limited funds and human resources devoted to this subject never reach the necessary volume to obtain quick and substantial advances.

Micropropagation

As a first step a micropropagation method using juvenile material (ENCINA, 1992; ENCINA *et al*., 1994) was developed, and then using these results and methods as a reference a successful micropropagation system for adult material of cherimoya selected by their elite agronomic traits, was achieved (PADILLA, 1997).

This micropropagation method shows a 50% of rooting and an excellent rate of acclimatization (PADILLA and ENCINA, 2004). Other attempts to propagate in vitro the cherimoya failed due to permanent systemic contamination of the cultures (TAZZARI et al*.,* 1990). After the first success with the micropropagation of cherimoya and after the studies of BEJOY and HARIHARAN (1992), LEMOS and BLAKE (1996a, 1996b) recorded similar results micropropagating another two important Annonaceae species: *Annona muricata* and *Annona squamosa*. As far as it is known, only these three species of genus Annona were successfully micropropagated before 1996 (RASAI et al., 1995) together with the hybrid atemoya or custard apple *A. cherimola* x *A. squamosa* (NAIR et al., 1984a)

Furthermore, in order to improve the plant recovery rate, in the final stage of the micropropagation process some complementary techniques, such as the mycorrhizal inoculation with *Glomus deserticola* were applied (AZCÓN-AGUILAR et al., 1994, 1996), the use of VA fungi improved the success of the acclimatization phase, these treatments resulting in an better growth and development of the micropropagated plants. At present, this knowledge are being applied to other related Annona species such as *Annona senegalensis*, *A. scleroderma*, *A. montana*, *A. reticulata*, *A. glabra*, *A. diversifolia* and *Rollinia* sp. trying to develop methods of *in vitro* propagation.

After achieving efficient micropropagation methods, we are involved in an ambitious program to develop the *in vitro* tissue culture of cherimoya in order to attempt the plant breeding of this species. Our research program includes studies in: Adventitious organogenesis and regeneration of cellular cultures, already finish, and ploidy manipulation and generating of new cultivars (recovery of triploid and tetraploid cherimoya and custard apple plants), genetic transformation and micropropagation of wild Annona species or related Annonaceae species such as Rollinia sp., all of these works still in progress at this date.

Adventitious organogenesis, cellular cultures and regeneration

Some authors such as GEORGE and SHERRINGTON (1984), TAZZARI et al. (1990) studying juvenile *Annona cherimola* and (JORDAN, (1988), JORDAN et al. (1991), JORDAN and BOTTI (1992) studying the cultivar *Concha Lisa* reported very good response in the induction and development of adventitious shoots from hypocotyls, unfortunately with a low rate of rooting success. Working with the cultivar Fino de Jete it was obtained similar results enhancing the rates of adventitious shoot formation and achieving a high rooting percentage (near the 100%) (ENCINA et al*.,* unpublished data). NAIR et al. (1984b) also succeed in promoting organogenesis from callus leaves in *A. squamosa*.

It was also attempted to control the adventitious morphogenesis at other different levels, and was recorded a very good response using juvenile shoots. This material produced a high number of buds (clusters) followed by the growth and development of buds into normal shoots, which can be rooted easily, when were separate from the primary explant after reaching 2 cm long. On the other hand the induction of adventitious shoots from adult explants also showed budding occurrence, but it was very difficult to induce the development of the shoots from the buds. Finally,full regeneration and plantlet recovery was succeeded (ENCINA and PADILLA, in press).

Callus proliferation was successfully induced from young tissues (leaf, hypocotyl), these explants show a good callus formation and the growth rate seems to be influenced by the type of explant and by the growth regulators used. One of the differences in growth and regeneration is a delay of the morphogenetic response (PADILLA and ENCINA, in press). Best results in callus induction and shoot regeneration were provided by the tissues obtained from hypocotyl, achieving the development of the rooted adventitious microshoots regenerated from callus.

Other morphogenetic callus proliferating from seed tissues showed a tendency to regenerate following the embryogenetic way, producing the differentiation of somatic embryos, unfortunately these somatic embryos failed to develop become necrotic and died (ENCINA et al., unpublished).

Protoplast isolation and culture

Protoplasts were successfully isolated from two different explant types: hyperhydric leaves and etiolated hypocotyl tissue, both obtained from *in vitro* germinated seedlings of *A. cherimola* 'Fino de Jete'. Microcallus developed after six weeks. Regeneration from protoplasts derived from leaf callus failed; however, a low rate of regeneration, approximately 0.5%, was obtained from protoplasts derived from hypocotyls, and 50% of regenerated shoots were rooted. Efficiency of this protocol must be increased for it to be useful. This first success on protoplast isolation and culture of an *Annona* sp. was obtained in 2002 (ENCINA, 2004).

Ploidy manipulation: Development of triploid, tetraploid and haploid plants

Methods focused to the manipulation of the ploidy levels in cherimoya and custard apple (atemoya) has been developed with the goal to eliminate the multiple stone seeds present in the fruit, this character of the Annonas fruit is a problem that could discourage the consumption of Annona fresh fruit in some markets. It seems to be possible that a strategy based in the recovery of the modified ploidy cherimoyas (triploid plants) could have success in the production of seedless fruits of the cherimoya and custard apple, as occurs in other species such as *A. squamosa* (NAIR et al., 1986).

Development of cherimoya/atemoya triploid genotypes

Following the results obtained for NAIR et al. (1986) studying *Annona squamosa* we tried to induce callus from endosperm tissue of cherimoya, a tissue characterized to be triploid (3n) and later to induce regeneration from proliferate triploid callus. The results indicate that the regeneration way for the endosperm callus was the embryogenetic way, our callus cultures showing globular somatic embryos. Until now we never reach to overcome the problems of browning, slow growth and gradual degeneration and necrosis of this callus which make impossible following this experimental research line the regeneration of shoots or full triploid cherimoya plants. (ENCINA et al., unpublished).

After the failure of this experimental approach, antimitotics were used to induce failures in the cellular division process in order to obtain triploid regenerates at low rate. We recover a few triploid genotypes following this procedure and at the present time we are progressing in the micropropagation and rooting these triploid genotypes, after verify several times through flow cytometry the stability of their modified ploidy.

Development of cherimoya/atemoya tetraploid genotypes

Our cherimoya breeding program has already developed some tetraploid genotypes from cherimoya and atemoya (cv. Fino de Jete and cv. I-464), using colchicine treatments to duplicate the chromosome number of the diploid cherimoya. It has already recovered triploid and tetraploid plant of cherimoya in some tetraploid lines from atemoya (custard apple) after evaluating by flow cytometry the ploidy level of the resultant shoots issues after antimitotic shocks and treatments. At the present time, more experiments in this way are in progress.

Development of cherimoya haploid genotypes

Some years ago and following the studies of NAIR et al. (1983) with *Annona squamosa*, we worked with limited success in the development and the regeneration of haploid plants from cherimoya

from microspores of cv. Madeira for plant breeding purposes. In our experiments we try to elucidate and select the optimum growth and maturity state of the anther used as explants, correlating it with the level of developing (size) of the cherimoya flower buds. Our data showed that flower buds of 1.5 cm have the best response in callus induction. Further regeneration of the callus was never detected (ENCINA et al., unpublished data).

Genetic transformation

The preliminary steps in the cherimoya genetic transformation via *Agrobacterium* have been carried out. Inoculation techniques have still been developing: the co-culture conditions, the antibiotic application, etc. In this moment we are adapting this knowledge to the regeneration method for cellular cultures previously developed. Our final objective is the control of the physiological processes (ripening, flowering) that will allow a better knowledge of these species of genus Annona and the improvement of specific characteristics of these plants, such as changing the flowering patterns, the tolerance to pathogen and insects, the postharvest characteristics, the quality of the fruits and the capability cross-hybridization with the goal of obtaining new and improved elite varieties. (ENCINA et al., unpublished).

CONCLUSIONS

The future guidelines of the tissue culture applied to the Annona species (cherimoya, atemoya, etc) are focused to the mass micropropagation of selected specimens, and through the development of the regeneration methodologies (organogenesis and embryogenesis) to the agronomic improvement of these species through plant breeding using ploidy manipulation and/or genetic transformation.

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