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Chapter 8

Tissue Culture Techniques for Native Amazonian Fruit Trees

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Additional information is available at the end of the chapter

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1. Introduction

The fruits of the Amazon have attracted great interest in recent years, both nationally and internationally, according to its exotic flavors and pleasant and varied ways to use its pulp by agribusiness [1], pharmaceutical industry [2], high vitamin and antioxidant content [3].

In recent decades, the production of native fruits of the Amazon showed significant growth, mainly due to expansion of area for fruit production. It is noteworthy that this activity has had little impact on native vegetation, since most of the orchards were planted in areas previously occupied by other crops for market problems or environmental issues and pressure for sustainable agriculture, ceased to be interesting for farmers [4].

The Amazon forest has large number of non-domesticated fruit species and a minority being exploited through crop in place of natural occurrence [5]. According to the Brazilian Yearbook of Fruit [6], explored the country are 500 varieties of edible fruit-producing species native and exotic, and of these, 220 are still as untamed. The high rate of destruction of biomes, together with the predatory extraction, result in loss of genetic material of desirable characteristics, [7], with potential for use in food, as an ornamental or in pharmaceutical production can never be known. It is therefore essential to know these species and their growing needs for exploitation on a commercial scale, a rational and sustainable.

However, little efficient production technology and knowledge of native Amazonian fruit tree species exist. Low orchard productivity indicates that Amazonian fruit is underused; underuse, in turn, has hindered its cultivation. In [8] notes that many of the current fruit production systems were developed empirically, which required technology that ensured greater productivity, sustainability, and profitability than the older production systems.

Such technology involves crop management, production of reproducible seedlings, and distribution of the seedlings to farmers. Therefore, the first step in domesticating native fruit species and in introducing them to commercial cultivation is developing seedling propagation techniques.

The sexual propagation of native plants for use in horticulture, is not advantageous because it results in populations with wide variation in the period of maturation. In addition, some species have seeds with dormancy, which compromises the germination and seedling production on a commercial scale, or the recalcitrance of the seeds, preventing their storage for extended periods.

The method of propagation of fruit species most commonly used grafting. This technique, like other methods of vegetative propagation allow the cloning of selected plants directly from nature or from artificial hybridizations, maintaining their desirable traits. Vegetative propagation results in high quality seedlings in orchards and more uniform and earlier, with higher productivity and better quality of fruits [9]. Besides these advantages, the graft is also advantageous because it allows greater control of plant height facilitating the management and harvesting, and the formation of plants with resistance to pathogens in soils and drought tolerant.

2. Tissue culture techniques for native Amazonian fruit trees

For decades numerous studies have been developed with the objective of establishing protocols for micropropagation of fruit species for clonal multiplication of superior individuals, in view of the numerous advantages it offers in vitro propagation.

Besides the application of tissue culture techniques for plant propagation of high agronomic value carriers or rare genes and those at risk of extinction, the technique is also used for cleaning clonal plants through tissue culture. In plant breeding programs, the tissue culture can be used to reduce the time for development of new cultivars and expansion of genetic variability. Among the tissue culture techniques most widely used in plant breeding, there may be mentioned: in vitro selection of resistant /tolerant to various stress factors of the genetic variability from pre-existing or induced somaclonal variation or by use of mutagenic, haploidization, somatic hybridization, and rescue of zygotic embryos obtained from crosses between different species or genera of plants [10]. Tissue culture also stands as an adjuvant in introgression of genes of agronomic interest through genetic engineering.

An application of tissue culture which is very important, especially for native plants is the maintenance and storage of germplasm. However, in many other species, tissue culture techniques have been also widely used for studying the metabolism, physiology, development and reproduction of plants with desirable commercial property of interest [11], like the nutraceutical and pharmaceutical products.

Studies on the application of tissue culture techniques in fruit tree species native to the Amazon, are still incipient. Except for a few species in which native tissue culture studies

are in an advanced stage, such as *Theobroma cacao* and *T. grandiflorum*, for most species have been developed with the aim of improving methods for initial establishment of cultures in vitro (Table 1).

Species	Purpose of the study	Author
Açai (*Euterpe oleracea*.)	Culture of embryos	[12]
Araça (*Psidium spp.*)	Establishment of culture	[13]
Bacuri (*Platonia insignis*)	Establishment of culture	[14], [15]
Cacau (*Theobroma cacao*)	somatic embryogenesis	[16], [17]
	Multiplication of shoots	[18]
	In vitro establishment	[19], [14]
Caja (*Spondias mombin*)	Culture of embryos and callus	[20]
Camu-camu (*Myrciaria dubia*)	Effect of culture media on morphogenetic responses	[21], [22], [23]
Cupuaçu (*Theobroma grandiflorum*)	In vitro establishment	[24], [25]
	Callus	[26], [27]
	somatic embryogenesis	[28], [29]
Inga (*Inga vera*)	Establishment of culture	[30]
Murici (*Byrsonima basiloba*)	direct organogenesis	[7]
Murmuru	In vitro propagation and somatic embryogenesis	[9]
Pupunha (*Bactris gasipaes*)	Cultivation of embryos	[31]

Table 1.

3. *In vitro* establishment and micropropagation

Micropropagation is a high-impact plant tissue culture technique that is more consistent than other tissue culture methods. Micropropagation involves a high reproduction rate over a short time period, which produces plants with excellent phytosanitary quality. The technique involves various steps, from aseptic *in vitro* culture establishment to rooting, culminating in seedling acclimatization [32].

Most studies on the tissue culture of native Amazonian plant species have sought to improve methodologies for initial culture establishment. Disinfection plays a critical role in culture establishment because explant contamination during tissue culturing is

extremely problematic. Explant contamination is most severe in woody species, which include all Amazonian fruit species. The potential for contamination is greatest when the explants are taken directly from the field. Although plants maintained under greenhouse conditions are easily controlled, explants derived from them also have a high potential for contamination. However, even plants maintained in nurseries or greenhouses and subjected to rigorous phytosanitary control can harbor microorganisms, which may limit *in vitro* culture procedures [20]. In [10] cautions that, even before harvesting material from the field, it is essential to take some measures for each step in the culture process, from the laboratory to acclimatization and plant development in greenhouses. The phytosanitary conditions of the mother plant help determine the ease of explant sterilization during isolation.

Several substances have been tested to minimize contamination and facilitate *in vitro* establishment, and chlorine and ethanol-based compounds are now standard disinfection tools (Silva et al., 2005). In some cases, antibiotics are added to the culture medium [29]. Research has demonstrated the success of these active ingredients in disinfecting explants of native fruit tree species when establishing plant tissues *in vitro* for use in studies on reproduction. The active ingredients in disinfectant solutions and their exposure durations vary greatly.

In studying decontamination of floral explants from cupuaçu, [29] recommended immersing the explants in a 0.25% sodium hypochlorite solution for 20 minutes. The authors also observed that adding cefatoxime antibiotic to the culture medium was vital in controlling contamination in these explants.

In studying decontamination of explants from bacuri trees, [29] and his team found that pre-treating the explants in an antifungal solution of carboxin (0.067% p/v), thiram (0.067% w/v), carbendazim (0.17% w/v), chlorothalonil (0.17% w/v), and thiophanate-methyl (0.067% w/v) [15], and immersing them in a 1.75% sodium hypochlorite solution for 30 minutes produced the best results.

In [31] found that the most efficient method for disinfecting the apices of peach palms was to immerse the peach palm explants in a 1.25% sodium hypochlorite solution for 20 minutes. The authors reported that 90% of these explants avoided contamination during *in vitro* culture. In [33] obtained the most promising results for the disinfection of explants from peach palm shoots; he first immersed the shoots in a 50% ethanol solution for 1 minute, and then in a 0.5% sodium hypochlorite solution for 5 minutes.

In Figure 1 you can see the success obtained in vitro establishment of camu-camu (*Myrciaria dubious*) conducted by staff of the Tissue Culture and Fruits Embrapa Roraima and UFRR.

In [34] and [35] note that explant oxidation during the *in vitro* establishment of native plants is common; in camu-camu, explant oxidation is a major obstacle to *in vitro* establishment. In [23] evaluated the efficiency of different concentrations of sodium hypochlorite solutions at different immersion durations in the disinfection of nodal segments of camu-camu. The

authors observed that the lowest rate of contamination occurred when the explants were immersed in a 0.5% sodium hypochlorite solution for 10 minutes, but the rate of oxidation in the tissues was high for all treatments. Similarly, [21] found that immersing the explants in a 2% sodium hypochlorite solution for 10 to 20 minutes provided the best rate of disinfection in camu-camu seeds. However, these treatments killed the seeds.

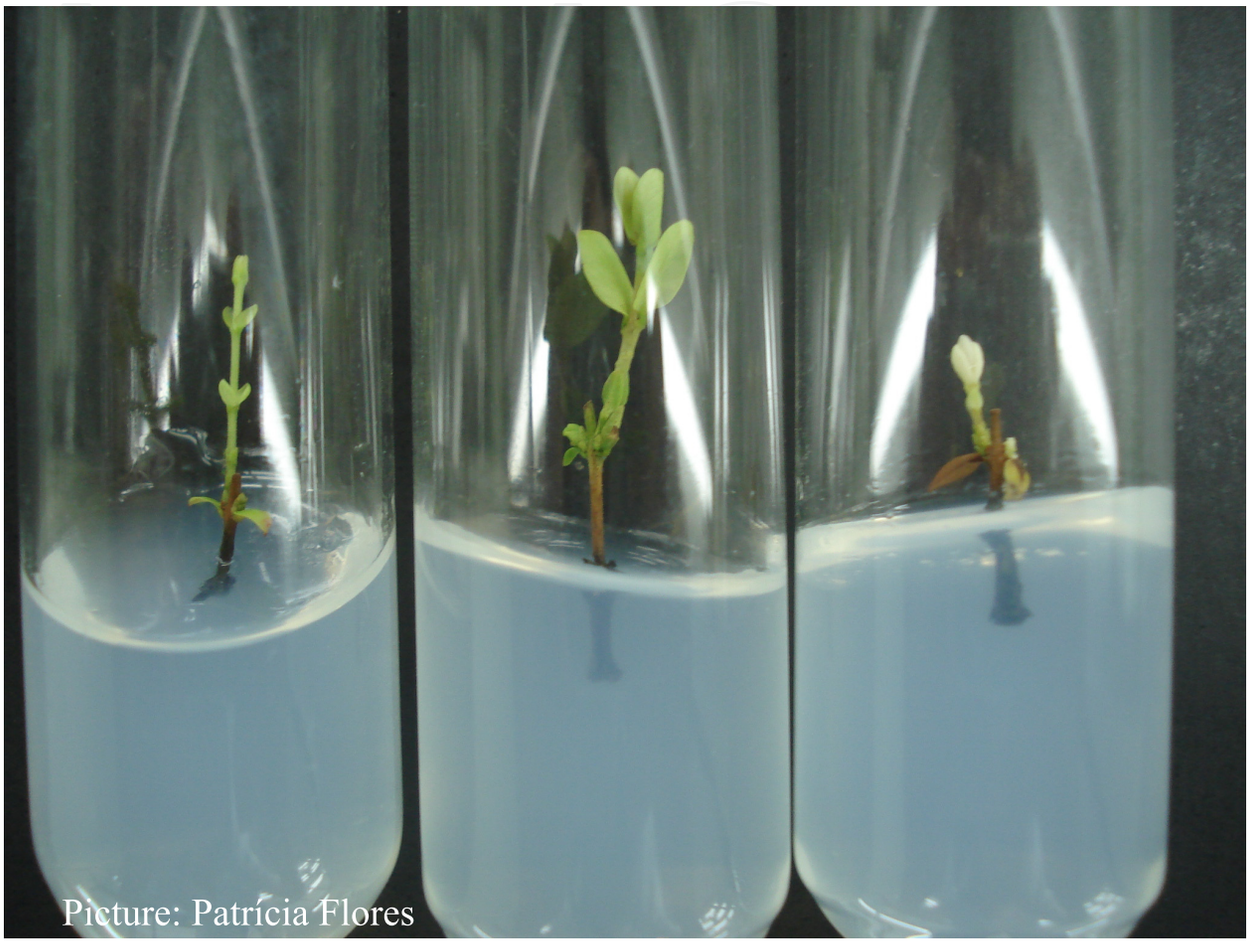

Figure 1. *In vitro* establishment of camu-camu held in Biofactory (UFRR) by the team of Fruits and Tissue Culture of Embrapa Roraima and UFRR. Boa Vista, RR, 2010.

In evaluating the effect of a light regime on the mother plant and the branch type used for the *in vitro* establishment of araça (*Psidium* spp.), [13] observed lower rates of contamination when using nodal segment explants of herbaceous branches, and obtained the highest survival rates when the mother plants were kept in darkness. These results are likely due to the reduced exposure of younger branches to contamination in the environment from which the explants were harvested.

Another way to control explant oxidation during *in vitro* establishment is to reduce the concentrations of some components of the culture medium. In [33] found that reducing the concentrations of NH_4NO_3 and KNO_3 macronutrients, sucrose, and agar by 50% in MS culture medium [36] controlled phenolic oxidation and the proliferation of peach palm explant shoots.

During the reproductive stage of shoots *in vitro*, the culture medium's composition varies with the species and the type of tissue or organ used as an explant. MS is most commonly used in the micropropagation of plant species. However, the Woody Plant Medium (WPM) [37] has been used successfully in woody species, including native Amazonian fruit tree species.

When using growth regulators to induce new shoot growth, native plants generally respond better to a culture medium supplemented with low doses of auxins and/or cytokinins. In [38] obtained excellent results in nance trees using different benzyladenine (BAP) doses for micropropagation. However, high BAP concentrations (above 4.0 mg L^{-1}) were inefficient in inducing the growth of axillary shoots in nodal segments. In [30] found that BAP reduced the number of shoots and leaves in Inga tree explants.

4. Somatic embryogenesis

In somatic embryogenesis, somatic cells or tissues to develop the formation of a complete plant, through a series of stages similar to zygotic embryogenesis.

Most systems via somatic embryogenesis is indirect, in which somatic embryogenesis is induced and maintained through the multiplication. The advantage of this method is that large quantities of somatic embryos can be formed with minimal manipulation and laboratory space.

This process of plant regeneration was successfully achieved in cocoa [16, 17], cupuaçu [26,27] and murici [30].

In indirect somatic embryogenesis, the formation of pro-embryogenic callus is the first step in obtaining somatic embryos and is usually obtained with the culture of embryonic or juvenile explants in medium supplemented with auxin, especially 2,4-D and TDZ [39].

In [31] can be observed callus induction in shoot apices of peach palm. The highest percentage of induction was 60%, obtained by combining 10.0 mg L^{-1} 2,4-D and 3.0 mg L^{-1} BAP.

Some studies have shown the ability of different cupuaçu explants to form callus. In [27] studied the induction of somatic embryogenesis in explants of cupuaçu, concluded that the hypocotyl region proved to be the most responsive of the embryonic axis, forming callus-looking white and bright. The MS medium supplemented with 2,4-D promoted the formation of large callus. Similar results were found by [24], studying the effect of auxin concentration and liquid medium on the development of calluses cupuaçu. The authors observed that the combination of NAA and 2,4-D induced callus formation and root formation in hypocotyl segments, and coconut water in medium without growth regulators favored the rooting and callus formation.

In [40] also studied the induction of somatic embryogenesis in explants cupuaçu and observed that the callus appeared as a process influenced by genotype. The staminode is

shown as a great source of explants for obtaining callus, and the PVP the best alternative to control the oxidation of explants cupuaçu. On the other hand, [41] did not succeed in callus formation using nodal segments cupuaçu in medium supplemented with different concentrations of 2,4 D. Similar results were obtained by [28]. The authors assessed the responses of different morphogenetic cupuaçu explants subjected to various culture conditions in vitro and argue that the absence of induction of somatic embryogenesis observed in culture may be related to several factors such as type and stage of development of the explants, using culture type and concentration of plant growth regulators.

In [25] undertook a study of the induction of callus formation of the hybrid *Theobroma grandiflorum* x *T.obovatum*, which has resistance to disease witches' broom (*Crinipellis perniciosa*), a disease that greatly affects native Amazonian species of the genus Theobroma. Among the explants, the cotyledons were produced more callus tissues in culture medium.

For açaizeiro, [12] reported the conversion can be in vitro isolated zygotic embryos from mature seeds and seedlings complete normal requiring the presence of NAA and BA in the culture medium. The concentration of 2.68 mM NAA combined with 1.11, 1.55 or 2.22 mM BAP promoted the best growth of the seedling shoot. The authors also found that required the presence of NAA and BA in the culture medium for the conversion of zygotic embryos and the early growth of seedlings grown in vitro. The same authors in studies on direct somatic embryogenesis using zygotic embryos of açai, satisfactory results, with a halving of the amount of nutrients in MS medium in the absence of fitoregulators.

5. *In vitro* zygotic embryo culture

In vitro plants require a source of exogenous energy for carrying out the photosynthesis. Sucrose has been used more carbon source being present in fruit culture media at concentrations ranging native 20-40 g L^{-1} [26]. In [9] found that immature embryos murmuru showed higher germination in medium supplemented with 30 g L^{-1} sucrose. For embryos obtained from ripe fruits, 15 g L^{-1} sucrose in the medium was sufficient for it to reach the best germination rates. These results confirm the hypothesis that depending on the species and stage of development of embryos, the presence of carbohydrate in the medium may be in minute concentrations, or even not necessary because of embryos of many species use the energy required for germination in vitro from their own reserves of the embryo [42].

Embryos younger typically require higher concentrations of carbohydrates in the culture medium to sustain germination [43, 44]. In [9] have obtained best results using 3% sucrose in the development of embryonic axes cupuaçu, in accordance with [16, 18, 19 and 45] in studies performed with *Theobroma cacao*.

6. *In vitro* germination of pollen grains

One of the techniques used to obtain new varieties is controlled hybridization in the field and subsequent evaluation of the progenies. Thus, to obtain success in breeding is important

to catch up before going to the field the viability of pollen grains [46]. Thus, for the application of artificial pollination techniques, knowledge of the time of pollen viability and stigma receptivity is crucial for a successful fertilization of the flower [47]. However, the germination of the pollen grain depends on several factors, such as osmotic pressure, concentration and type of sugar, consistency, temperature, humidity, presence of enzymes and phytohormones in the middle [48, 46]. In native fruits, especially those species whose domestication process is more advanced, there are few studies in this line of research.

In [49] Oliveira et al. (2001), studying the viability of pollen *in vivo* and *in vitro* staining method in genotypes of açai, the success observed in *in vitro* pollen germination of different cultivars. The authors found that pollen grains in vivo assai, drawn from floral buds and newly opened, exhibit high viability, being higher in the second stage of evaluation. With respect to time of storage at -100ºC, in vitro pollen showed a reduction in viability with increasing storage time. We also found that the viability of pollen grains varied with genotype and stage evaluated. With this study, the authors still recommend that the pollen of genotypes can be used for controlled pollination without fertilization damages, including, may remain stored for up to one year of storage under the conditions tested in that work.

In [50] tested four concentrations of galactose, glucose, lactose and sucrose, with or without boric acid, for the germination of pollen from cubiuzeiro (*Solanum topiro* Humb. & Bonpl.) and cupuaçu (*Theobroma grandiflorum* Willd. Ex. Spreng. Schummann). The authors found that higher germination rates occurred in 10, 15 and 20% sucrose after 25 hours, no effect of boric acid for the two species.

In [48] in their studies to obtain high levels of pollen from *Theobroma grandiflorum* found that pollen from one flower is not uniform. Therefore, it is recommended to use all anthers of a flower to prepare a sample. It is not necessary to have more than 300 pollen grains per sample. The stage at which the button is to be collected for the purpose of artificial pollination or germination and when all are detached sepals, petals with the extended length, style, stigma and exposed about two hours after the onset of anthesis (Step E) The pollen collected should be used, preferably up to two hours after it is collected, where there was more viable. The author also recommends the optimal medium for pollen germination cupuaçu should consist of 5% lactose, 0.01 H_3BO_3 and 1% agar, pH 6.1. The pollen collected cupuaçuazeiro button at the stadium and can remain viable up to 72 hours after the plant collected and stored at ambient conditions on the button, but at the end of this period, viability is very low, about 5%.

7. Possible tissue culture techniques for native Amazonian fruit trees

There is a myriad of tissue culture techniques for native Amazonian fruit trees. However, this important technology has been seldom used.

Tissue culture techniques can be used for *in vitro* conservation, reproduction, exchange, and genetic resource conservation. These techniques are particularly useful in species that have

recalcitrant seeds, low germination potential, and exclusively vegetative propagation, as well as in endangered species [51, 52].

Ovary cultures are important in selective plant breeding. Obtaining plants from haploid tissues and subsequently doubling the chromosome number through colchicine treatment produces homozygous strains quickly, eliminating the multiple generations of selfing required in conventional selective breeding.

Producing synthetic seeds from somatic embryos offers various advantages for propagating native fruit tree species, including year-round production, while avoiding the risk of losses from adverse weather, biomass degradation, pests, disease, and low production years. Moreover, synthetic seed technology enables more secure maintenance of the clonal identity of the material under laboratory conditions. Synthetic seeds can be sown directly into the field, thus eliminating the need for acclimatization structures, sowing, and nurseries.

Protoplast fusion in somatic plant cells has great potential for combining genomes from sexually incompatible species. Somatic hybrids (artificial polyploids) of different species or genera are obtained through protoplast fusion and the subsequent regeneration of plants. These polyploids can be used as rootstocks in fruit production.

8. Final thoughts

Although there are eminent importance of the native fruits of the Amazon, it appears that only a few studies with applications of tissue culture techniques in the domestication and breeding of these species. The works are practically concentrated in developing protocols for the establishment of *in vitro* plants, plus a few studies with somatic embryogenesis, immature embryo rescue protocols and studies of the germination of pollen grains to support breeding programs.

Despite the reality and effectiveness of the techniques of tissue culture, it is important that some basic difficulties must be overcome to implement satisfactory in woody fruit trees, as is the case of native Amazon. The phenolic oxidation disinfestation and represent the most serious problems during the establishment of in vitro culture of explants woody species.

Suitable types of explants removed at appropriate times can help by having a lower content of endogenous phenols in tissues and, thus, less oxidation. Likewise, minor physical and chemical damage at the time of excision and pest may help reduce the problem of oxidation. Furthermore, the addition of antioxidant compounds as cysteine, ascorbic acid and adsorbents such as activated carbon and PVP can be critical in preventing oxidation, which is more pronounced in the initial stages of cultivation [53]. Additonally, with cultivation at low light intensities, and frequent replacement of the culture medium, the chances of success in the establishment and cultivation of explants of woody species are quite high.

Another important aspect to be highlighted concerns the small number of fruit species native to the Amazon that has been the subject of study and application of techniques of tissue culture. These species can be summed up pretty much to those whose breeding programs are in advanced stage. Thus, it appears that there is still vast field of study to be explored and a significant deficiency of techniques and information that will enable progress in the domestication and breeding of these species.

9. Conclusion

Although native Amazonian fruit tree species are important, few studies on the use of tissue culture techniques for domesticating and improving these species exist. Research has instead focused on developing protocols for the *in vitro* establishment of plants, and some studies have examined somatic embryogenesis, immature embryo rescue, and germination protocols for pollen grains as a basis for selective breeding programs.

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