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Review article

Propagation methods in Babaco plants (*Vasconcella x helbornii*)

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Abstract: Babaco, (*Vasconcellea x helbornii* syn *Carica pentagona),* is an endemic fruit to South of Ecuador and North of Peru, which is becoming very popular on the continuously expanding subtropical fruit market for a few years now. From the nutritional point of view, this fruit is very rich in fiber, it also has a good amount of vitamins, minerals and papain, an enzyme with great digestive and anti-inflammatory properties. However, due to the diseases that attack babaco plants and the sterile character of their fruits, exports have been significantly reduced being considered as an unprofitable agricultural production. New techniques in plant biotechnology as *in vitro* culture could make possible a massive production of free-pathogens babaco plants. In particular, somatic embryogenesis is a rapid way of obtaining genetically identical individuals which is also used in *in vitro* conservation of germplasm and plant genetic improvement. The present document aims to summarize the techniques of obtaining new plants of babaco and evidences the achievements obtained in somatic embryogenesis in the *Caricaceae* family up today.

Keywords: Babaco - *Vasconcellea x heilbornii* - *Cárica pentágona* - *Caricaceae* - *in vitro* culture - Somatic embryogenesis.

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INTRODUCTION

Babaco, [*Vasconcellea x helbornii* (Badillo) Badillo syn. *Carica pentagona*] (Badillo 1993, 2001), is an endemic plant to southern Ecuador and northern Peru, which belongs to the *Caricaceae* family, that is formed by six genera and 35 species (Carvalho & Renner 2012). Probably under human influence, *V. stipulata* (Badillo) was hybridized with *V. cundinamarcensis* (Badillo) resulting in *V. x heilbornii* hybrids including babaco and jigacho (Van Droogenbroeck *et al.* 2004, Coppens d'Eeckenbrugge *et al.* 2014). Babaco is among the so-called mountain papayas, which develop at elevation of 2000 m asl, (Scheldeman *et al.* 2003). It is a shrub plant with semi-perennial culture and a parthenocarpic fruit (it does not have seeds); an elongated and pentagonal berry type, whose size varies from 30 cm long to 15 cm in diameter (Fabara *et al.* 1985).

In recent years, the export of fresh fruits, such as babaco, has been strengthened, so product quality becomes increasingly demanding in terms of organoleptic and phytosanitary characteristics. However, due to the high sensitivity of this crop to temperature and humidity (Janick & Paull 2008), along the diseases produced by the various pathogens that tend to affect the plant and the sterile nature of its fruit, the best yields are obtained under greenhouse conditions since open sky culture result in a not profitable production (Robles-Carrión *et al.* 2016). Although, the production of babaco fruits in greenhouses is a challenge as well. One of the pathogens that causes the greater economic losses in babaco plants is the *Fusarium oxysporum* Schltdl., which produce 'babaco vascular wilt' in fields and greenhouses which can achieve losses of 100% of production (Robles-Carrión *et al.* 2014). To all these reasons, many scientists have attempted to overcome all the limitations regarding babaco plants propagation by the use of alternative techniques as biotechnological techniques with successful results. So far, biotechnological methods have lead to the production of pathogen-free plants, massive propagation (Vasil 1994, Kitto 1997), genetic improvement through mutation induction, *in vitro* selection (Predieri 2001), and genetic engineering (Herrera-Estrella *et al.* 1983), in several plant species including babaco. In the present review, we aim to summarize the state-of-the-art of babaco propagation methods by studying the most relevant works of this plant and its *Caricaceae* relatives.

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Propagation by Stakes

Since, babaco plants have a parthenocarpic fruit (seedless) is limited to asexual reproduction by cuttings, buds, grafts or stakes, which leads to the attainment of a new mature plant at approximately two years (AAIC 2003). For this reason, the spread of diseases increases. Among the most important diseases are the massive bacterial infections of the genus *Erwinia caratovora* (Jones) Waldee and *Agrobacterium* sp. and the fungus *Fusarium oxysporum*. *Vasconcellea* that produces 'babaco vascular wilt' and generates large losses (Scheldeman *et al.* 2003). As a preventive measure, farmers use a large amount of agrochemicals that affect the quality of the fruit, pollute the environment and harm human health (AAIC 2003). Nowadays, this is one of the major problems around babaco production being pointed out by several researches (Scheldeman *et al.* 2003, AAIC 2003, Falconí *et al.* 2006, Chávez 2006, Robles-Carrión *et al.* 2014, 2016). Some efforts have been done on a genetical improvement of babaco by grafting on three varieties of papaya patterns; Hawaiian, Criolla and Maradol papaya, tending to the production of disease-free plants tolerant to *Fusarium oxysporum*. According to Chávez (2006), the best yields, with 85% of successful plant grafted, were obtained by the combination of Hawaiian papaya and babaco plants, followed by papaya "criolla" with 73% of successfully grafted plants, while Maradol papaya and babaco grafts presented the lowest values. Successfully grafted plants showed increased resistance to babble diseases such as 'babaco vascular wilt', contributing to increasing its production.

According to Falconí *et al.* (2006), the length of the babaco stakes to propagate should be between 25 to 30 cm and have a diameter of about 4 to 6 cm. They must have a superior cut in bevel to avoid the accumulation of rainwater and a transverse basal cut to have a greater surface of rooting by applying growth regulators. By means of this method and after several forms of disinfection, a new plant can be obtained at an overall of 10 or 12 months. Soft buds are a widely used form of propagation, which are extracting from growing plants. Shoots can be also produced for this purpose being of a length of 10 cm and a diameter of about 1.5 to 2.5 cm. Then, the upper part of the shoot is cut out to stimulate sprouting to finally induce rooting (AAIC 2003). Although, this technique for obtaining new plants of babaco takes too long time and does not ensure the acquisition of a new plant without infections. Currently, new techniques such as *in vitro* culture are intended to improve these times for the production of the species and to obtain disease-free and genetically improved plants (Soria & Viteri 1999).

Somatic embryogenesis propagation

i. Somatic embryos development: Somatic embryogenesis is a process by which somatic cells are transformed into somatic embryos, which resemble zygotic embryos morphologically, since are carriers of typical embryogenic organs; however, they develop in a different way (Adobkar *et al.* 2012). The greater interest in somatic embryos focuses on their practical applications for large-scale vegetative propagation, particularly due to the possibility of expanding propagation through the use of bioreactors (De Feria 2000). Most probably, somatic embryogenesis can be achieved for all plant species in which an explant, a culture media and the appropriate environmental conditions are used. Some examples are found in forest species, (Celestino *et al.* 2005), fruit trees, (Jordán 2011, Bao-Fundora *et al.* 2013) and ornamental plants (Ribero-Bautista *et al.* 2008). In addition, in the majority of cases, somatic embryos or embryogenic cultures may be cryopreserved, which makes it possible to establish gene banks, constituting a pathway for cryopreservation and regeneration (Engelmann *et al.* 1997). Moreover, embryogenic cultures are used as targets suitable for genetic transformation (Peña & Séguin 2001) and functional genomics, allowing the evaluation of the function of specific genes (Campbell *et al.* 2003).

Somatic embryos can be obtained from different parts of the plant, like as young leaves, hypocotyls, stem, immature seeds, and suspension cell cultures. The cells of this tissue being organized cells are inducted to the disorganization and again to the reorganization of them (Freire 2003). *Caricaceae* somatic embryos have been obtained from different sources of explants, as for example in *C. papaya* have been obtained from leaves, immature seed integuments, mature and immature zygotic embryos and many others (Fitch & Manshardt 1990, Cabrera-Ponce *et al.* 1995, Monmarson *et al.* 1995, Posada-Perez & Koski 2007, Malabadi *et al.* 2011). Somatic embryos of *C. stipulata* were also obtained from peduncle calli (Litz & Conover 1980) and *C. pubescent* from germinated hypochromic callus *in vitro* (Jordán 1986). Although, several studies have been carried out in other *Vasconcellea,* such as Jordán (1986), who worked to achieve the regeneration of plants via somatic embryogenesis of *V. cundinamarcensis* without satisfactory results. Results of Jordán *et al.* (2011), achieved only the formation of multiple buds from nodal sections of *V. chilensis* adult plants. However, babaco has only been *in vitro* propagated from nodal sections (Cohen & Cooper 1982), buds, apical and lateral buds (Cossio 1985). Some attempts have led to the formation of calli from ovules and

leaves (Vega de Rojas & Kitto 1991, Vega de Rojas *et al*. 1993, Quillay 2011), the formation of buds via organogenesis (Jordán & Piwanski 1997), and somatic embryos from cell suspensions obtained from calli (Jordán & Velozo 1996). In addition, an effective protocol for the isolation and fusion of babaco and jigacho protoplasts from callus and leaf respectively (Rivera & Jadán 2010) has been standardized.

One of the challenges, when intend to propagate babaco plants by tissue culture techniques, is the plant material used, which come from plants growing in field conditions, without any sanitary control and exposed to the environment, rending this technique unsuccessful. Newly findings of Jadán *et al.* (2016a), showed a stable protocol to create a bank of donor plants of the babaco hybrid from cuttings, under phytosanitary semicontrolled conditions by the use of Carbendazim fungicide and biostimulant (GERMO-TB01) application to increase the number of axillary shoots. The results of this work allowed to achieve 100% sprouting and root formation in the different stem sections, which could be used in *in vitro* propagation protocols ensuring a high genetic and phytosanitary quality. Furthermore, aiming to solve the problem of microbial contamination of bacteria, which also impede the proliferation of *in vitro* tissues, Jadán *et at.* (2016b), reported the establishment of 68.5% of bacteria-free explants from babaco buds using 1.5% Sodium Hypochlorite for 10 minutes and immersion in a solution of Gentamicin 50 mg l^{-1} and Streptomycin 25 mg l^{-1} for 3 hour.

In the process of induction of somatic embryogenesis, not only the type of explants and genotype must be taken into account, but the effect of the composition of the medium at each stage of the tissue culture is equally very important, since optimal media conditions vary depending on the type of explant and genotype. Although the, (NN) medium (Nitsch & Nitsch 1969) is widely used, but, the most commonly used culture medium for the development of somatic embryogenesis is (MS) Murashige & Skoog (1962) with some modifications. Most researchers working on the development of somatic embryogenesis in species of the *Caricacea* family use half the concentration of the MS salts and 2,4-diclorofenoxiacético, (2,4-D), as a growth regulator, in concentrations from 1–10 mg l^{-1} (Zhu *et al.* 2004). However, other authors have used growth regulators 1-Naphthaleneacetic acid, (NAA), indolacetic acid, (IAA), 6-Benzylaminopurine, (6-BAP; cytokinin), Kinetin and Zeatin (Jordán & Velozo 1996, Cabrera-Ponce *et al.* 1996). In addition, Woody Plant Medium, (WPM), culture medium (Lloyd & McCown 1981) has been used in *in vitro* culture of babaco as well (Jordán & Piwanski 1997).

Recently, Cornejo-Rodriguez *et al.* (2009), evaluated four auxins (NAA, Picloram, 2,4-D and 2,4,5-Trichlorophenoxyacetic acid, (2,4,5-T)) in combination with different concentrations of 6BAP for the induction of papaya calli and different concentrations of 2,4,5-T and Thidiazuron, (TDZ; cytokinin), for the induction of papaya somatic embryos. Concentrations between 1 and 5 mg l^{-1} of auxins such as NAA and 2,4,5-T combined with 1 and 2 mg l^{-1} of 6BAP were found to be the most suitable for callus induction. Cultures pretreated with Picloram during the callus induction phase show greater efficiency in the induction of embryos at concentrations of 1 mg l^{-1} of 2,4,5-T. Further, the use of 1 mg l^{-1} of 2,4,5-T in combination with 2 mg l^{-1} of TDZ, resulted in 81% of embryo induction.

ii. Maduration of somatic embyos: The maturation phase of somatic embryo development is essential to promote germination. In this phase, cell expansion, the accumulation of reserve substances, and tolerance to desiccation are achieved (Parrott 1993). The correct accumulation of reserves leads to an increase in the dry mass of the somatic embryos, which indicates a high quality in the vigor and positively influences its later germination (Fuji *et al.* 1990).

Although, it has been reported embryo maturation is deficient in the absence of ABA (abcisic acid), leading to abnormal morphology and early germination (Fernando & Gamage 2000), Vega de Rojas & Kitto (1991), obtained mature somatic embryonic structures from nodule structures of babaco ovules by transferring them to media with GA3 (0.1 mg l^{-1}) in addition to activated carbon (2.0 g l^{-1}) or casein hydrolysate (200 mg l^{-1}) plus IAA (0.5 mg l^{-1}). Nevertheless, according to Malabadi *et al.* (2011), some varieties of papaya have greater effectiveness in maturation starting from partially dried tissue combined with the addition of ABA.

Somatic embryo germination

Germination of somatic embryos is defined as the radicle and bud developmental process. In many plant species, somatic embryo germination and in consequence the subsequent plant recovery is relatively poor and difficult. Some of the handicaps, which influence low embryo germination, are poor somatic embryo quality, lack of proper maturation desiccation tolerance and dormancy or inhibitors(s) within the embryo (Rtienne *et al.* 1993, Mujib *et al.* 1998). In recent years several innovated methods, such as an addition of osmotic agents,

carbohydrates, sugar alcohols etc., have been used to improve embryo quality before germination (Xing *et al.* 1999, Lipavská & Konrádová 2004, Robichaud *et al.* 2004). Although the production and quality of somatic embryos have been improved in several crop species by using the temporary immersion systems known under the trade name RITA, no many researchers have used the temporary immersion system for the propagation of *Caricaceae* family so far. Some successful results have been observed by Vegas-García *et al.* (2015), which standardized the conditions of initiation, multiplication, rooting and acclimatization of hermaphrodite papaya plants from axillary shoots produced in RITA® temporary immersion vessels. Stem cuttings were obtained by direct treatment of immersion with the subsequent maintenance of cuttings in water with aminoisobutyric acid, (AIB), and 50 mg l^{-1} for the formation of roots (Jordán 2009).

Germination phase has been also improved by the trial out of many protocols by using several combinations of growth regulators and growth media, thus currently; there are many studies which show promising results in *Caricacea* family. Some of them are listed below:

For example, Yie & Liaw (1997), used two methods of *in vitro* cultivation to regenerate papaya plants: from callus and individual apical buds. Callus was induced from stem sections of papaya seedlings in a medium consisting of 1 mg per 1 NAA and 0.1 mg 1^{-1} kinetin. The authors observed that calli regenerated shoots/embryos when transferred to medium with lower concentrations of auxin, 0 to 0.05 mg 1^{-1} IAA, and higher cytokinin concentration, from 1 to 2 mg 1^{-1} kinetin. They also observed the production of multiple outbreaks from decapitated apical buds grown in an average medium supplemented with 0.05 mg 1^{-1} IAA and/or 5 mg 1^{-1} kinetin or 0.5 to 1.0 mg 1^{-1} benzyladenine. The root formation of shoots and embryos derived from callus was produced in a medium with 5 mg 1^{-1} IAA and at a light intensity of 3,000 to 4,000 lux. Plants with roots obtained were established successfully in the soil and in standard conditions of greenhouse effect after passing a phase of acclimatization in which their initial development was induced on wet vermiculite in covered polyethylene pots.

It has been reported, the formation of buds in axillary buds and the formation of calli in leaf sections derived from seedlings through the use of NN medium supplemented with NAA and BAP as well as the increase of the germination time and the percentage germination up to 53% in the presence of hydrogen peroxide in *in vitro* conditions in *V. stipulata* (Vélez-Mora *et al.* 2015).

For rhizogenesis of shoots, a subculture AIB 2.0 mg l^{-1} (Jordán & Piwanski 1997), has been used, although roots have also been induced in the presence of NAA 0.01 mg l^{-1} and BAP 0.1 mg $^{-1}$ and NN nutrients from axillary buds (Jordán *et al.* 1999). In addition, the inclusion of activated charcoal 0.6 g l^{-1} and casein and cysteine hydrolysate helps avoid browning in WPM and MS media, besides improving root proliferation in babaco axillary bud cultures. According to Vaca-Suquillo (2008), buds was obtained by the use of 0.167 ppm IAA and a 50% rooting percentage was obtained by using 0.5 ppm AIB and 1ppm NAA in babaco explants.

Studies of Jordán (2011), showed a high number of outbreak formation from *in vitro* induction in the presence of relatively high levels of TDZ, IAA and organic additions in WPM media, including casein hydrolysate, adenine sulphate and cysteine, in *V. chilensis ex C. chilensis* nodal sections. Somatic shoots and embryos have been obtained by using high concentrations of TDZ, in addition to IAA and gibberellic acid (AG3), from foliar leaf sections in babaco. Likewise, leaf section shoots have been induced in MS medium with TDZ neat or in combination, including casein hydrolysate and adenine sulfate. It has been pointed out that the application of GA3 promotes the germination of seeds in *V. stipulata* (Vélez-Mora *et al*. 2015), *V. cundinamarcensis*, *V. x heilbornii,* by accelerating the transport of nutrients via the endosperm (Scheldeman *et al*. 2003). 0.1 mg l^{-1} BAP, 0.1 mg l^{-1} and GA3 126 mg l^{-1} floroglucinol have been used for the multiplication of axillary shoots in *Carica papaya* L. (De Winnaar 1988) and in *Carica pubescens* (Jordán 1992), as well as combinations of IBA and floroglucinol for the induction of rooting in apical buds in babaco (Jordán & Piwanski 1997), although this combination was not successful as a promoter of *Vasconcellea Chilensis ex Carica chilensis* root (Jordán 2011). Several *in vitro* studies in which floroglucinol has been used have shown that this compound is much more potent than other growth regulators in inducing and enhancing different events of plant development, especially in plants where, not yet, an effective *in vitro* propagation protocol has been standardized (Teixeira da Silva *et al.* 2013). The use of other bioregulatory growth compound, such as Pectimorf®, used as auxins and cytokinins substitute or complement, have been successfully applied in the germination of *Nicotiana tabacum* (Acosta *et al.* 2004), as well as to increase the production of citrus fruits, such as *Citrus macrophyla Western* by the use of somatic embryogenesis techniques (Bao-Fundora *et al.* 2013). Lately, Posada-Pérez *et al.* (2016), had a positive effect on rooting and *ex vitro* acclimatization of papaya shoot (*Carica Papaya*) by the use of Pectimorf®.

Since somatic embryo plants are smaller and weaker than those of zygotic or seed embryos, the conversion rates are much lower in somatic embryos compared to natural seed (Lai *et al.* 1995). One way to optimize maturation and conversion in somatic embryos plants is to try to simulate the natural processes of plant development. In order to obtain an efficient result in the production of plants, traits that take into account the time and type of application of growth regulators, low oxygen concentration and desiccation of mature embryos can be used (Carman 1989). In some *Vasconcellea* species, the use of MS medium salts and/or media with low nitrate levels as NN and woody plant medium allowed survival and good quality of the plants (Jordán 1986, Jordán & Piwanski 1997). Furthermore, the use of treatments to improve the post-germination, such as the application of low temperatures to activate cell development; (Das *et al.* 2002), are highly recommended, since depending on the species, direct induction of somatic embryos germination results in a low percentage of positive results (Parrott *et al.* 1988, Senaratna *et al.* 1990).

Ex vitro acclimatization

A large number of plants produced *in vitro* do not survive the transfer from *in vitro* conditions to an *ex vitro* environment under greenhouse or field conditions (Hazarika 2003, Hazarika & Bora 2010). Anomalies in the morphology, anatomy and physiology of seedlings cultured *in vitro* can be repaired after transfer to *ex vitro* conditions (Maene & Debergh 1987). Hyperhydricity can be controlled in a number of ways including better aeration of the vessel used *in vitro* (Rossetto *et al.* 1992), by reducing cytokinin levels (Williams & Taji 1991), increasing agar concentration (Brand 1993), and by changing the concentration of the components of the medium (Ziv *et al.* 1989). Since the photosynthetic activity is scarce in the *in vitro* cultures, it is necessary to adapt the foliar system to become an active photosynthesis system. Several strategies have been proposed for this adaptation, such as the elimination of carbon sources, the mechanical defoliation of seedlings, the induction of storage organs and/or the use of growth retardants to inhibit leaf growth (Ziv & Lilien-Kipnis 1990). Many efforts have been done on amending this scenario. For example, studies focused on obtaining elite plants of *Vaconcellea Stipulata* resulted in the significant reduction (50%) of hyperhydricity under *in vitro* conditions when gibberellic acid was added at low concentrations in the NN media, allowing to recover up to approximately 80% of viable seedlings (Vélez-Mora *et al.* 2015). Cruz *et al.* (2008) obtained the *in vitro* rooting of transgenic papaya plants by using 2 $mg.l^{-1}$ of indole-3-butyric acid in the culture medium, as well as *ex vitro* rooting with high percentages of survival. The synergistic action of AIB with 9.0 mg l^{-1} Pectimorf® allowed to obtain *in vitro* plants with greater leaf area, fresh weight, number of roots, photosynthetic rate and stomatal conductance, which together with a high percentage of rooting and less percentage of open stomata allowed to reach a 76.2% survival *ex vitro* conditions in *Carica Papaya* seedlings (Posada-Pérez *et al.* 2016).

Genomic aspects of somatic embryogenesis propagation

It is important to take into account plants been regenerated by tissue culture are prone to somaclonal variations with genetic alterations events due to the stress induced by the *in vitro* culture conditions and the mode of regeneration. Genetic stability is an essential requirement for its use (Bhowmik *et al.* 2016). To this to solve, ISSR markers, which have only been used to characterize genetic diversity and gene flow between populations in babaco (Kyndt *et al.* 2005), it has been used to determine the genetic stability of babaco plants propagated *in vitro*, resulted in no differences at the molecular level between the in *vitro* plants and the mother plants (Jadán *et al.* 2017). Although, somaclonal variations events could be an alternative to improve the quality of fruits, either in size (smaller units for export), compact fruits or resistant to pests and/or other diseases (Vega de Rojas & Kitto 1991).

Nowadays, *Vasconcellea* genomic research is very poor, just counting with the development of some BAC libraries of few species. Although, genome sequencing and genetic mapping technology will make possible the identification of genes of interest in *Vasconcellea*, which will open a door in the expansion of new species of this genus (Scheldeman *et al.* 2011). Recently, the first papaya callus transcriptiome profile analysis revealed the presence of highly expressed somatic embryogenesis genes, such as SERK and LEA, hormone-related genes, stress-related genes, and genes involved in secondary metabolite biosynthesis pathways, as well as, transcription factors families as, NAC, WRKY, MYB, WUSCHEL, Agamous-like MADS-box protein and bHLH, which are known to play a important role in somatic embryo development in other species (Jamaluddin *et al.* 2017). In addition, recent studies by Moura-Vale *et al.* (2014), in which they analyzed the differential expression of 6% Polietilenglycol induced treatment of three proteins (enolase, esterase and ADH3), concluded that these three proteins could play an important role in the maturation of the somatic embryos, being able to be used as candidate biomarkers of somatic embryogenesis in papaya. The discovery of genes and proteins expressed in

papaya somatic embryogenesis development provides important information to improve our understanding on genes and proteins associated with somatic embryogenesis in other relative plant species as babaco, which could lead to the optimization of protocols allowing the use of biotechnology for babaco propagation.

CONCLUSION

Somatic embryogenesis is making its way as the most suitable via of plant regeneration to complement classical improvement techniques in various agricultural species such as forestry (Celestino *et al.* 2005), ornamentals (Ribero-Bautista *et al.* 2008), fruit trees (Jordán 2011, Bao-Fundora *et al.* 2013), among others. Many efforts are being devoted to the application of this technique to obtain effective results based on the clonal evaluation and cryopreservation of embryogenic lines to be used in each place and suitable purpose (Engelmann *et al.* 1997). In addition, the latest advances in genomics are being applied to elucidate the metabolic pathways of regeneration processes (Campbell *et al.* 2003, Scheldeman *et al.* 2011, Jamaluddin *et al.* 2017). In light of the low regeneration of babaco plants by conventional methods as stakes, some efforts need to be done in the production of babaco by *in vitro* techniques. To date, the studies carried out in *Caricaceae* family and specifically in babaco plants are scarce, but the emerging possibility of cloning by somatic embryogenesis this species is an idea started by many researchers who have as a purpose the production of high quality and pathogen-free plants. The use of biotechnology as a tool in the propagation of elite plants of this crop can be an alternative via for rapid multiplication, as well as the introduction on a commercial scale of new varieties or hybrids.

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