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OPTIMIZATION OF SOMATIC EMBRYOGENESIS IN *COFFEA CANEPHORA*

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ABSTRACT

Plant tissue culture allows the clonal propagation of *Coffea canephora* on a large scale through somatic embryogenesis. The aim of this study was to optimize the conditions for the induction of somatic embryogenesisin leaves of the Robusta variety. The physiological stage of the leaves – drain or source; the use of the surrounding area ofprimary or secondary veins; and the position of the explants – abaxial or adaxial surface in contact with the culture medium were evaluated. The explants were inoculated in a modified MS culture medium (1/2 macro, 1/4 micronutrients), supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar, 1.0 mg L^{-1} 2iP and 5.0 mg L^{-1} IBA. At 45 days of culture, the occurrence of callus induction and the dry weight of the explants were evaluated and, at 120 days, the average number of cotyledonary embryos per explant. The leaf fragments most responsive to the induction of somatic embryogenesis were taken from drain leaves, with explants containing secondary veins, placed with the adaxial face in contact with the culture medium.

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INTRODUCTION

Somatic embryogenesis is a technique of plant tissue culture from which it is possible to clone plants*in vitro*through vegetative propagation, on a large scale and in a short time, from small non-reproductive plant fragments. Leaf fragments are widely used, due to the fact that the leaves are abundant organs, which can be removed without great damage to the plants, and due to the peculiarity that the leaves, to a large extent, can be obtained throughout the year (Canhoto, 2010). The species *Coffeacanephora* is an allogamous, cross-fertilized plant – what generate great genetic variability (Andrade, 2014), and therefore can benefit from *in vitro* vegetative propagation. Somatic embryogenesis in coffee occurs in two ways: direct somatic embryogenesis, in which embryos originate directly from the plant tissues, without the formation of intermediate stages of callus; and indirect, where somatic embryos originate from calluses, a mass of cells with disorganized growth (Amaral-Silva et al., 2020). The first studies of somatic embryogenesis in the genus *Coffea* were carried out in 1970 by Starisky, who obtained rapid proliferation of calluses in the species *C. arabica*and embryoids and seedlings in explants of *C. canephora* (Pereira et al., 2007). Since the 1990s, somatic embryogenesis has enabled the propagation of selected varieties, Arabica F1 hybrid and Robusta clones, originating from the two cultivated coffee species, *Coffeaarabica* and *Coffeacanephora*, respectively (Etienne et al., 2018). The objective of this study was to optimize the conditions for the induction of somatic embryos from leaves of the Robusta variety of*C. canephora*. The physiological stage of the leaves – drain or source; the use of primary or secondary veins; and the position of the explants – abaxial or adaxial surface in contact with the medium were evaluated.

MATERIAL AND METHODS

The leaves were collected from orthotropic branches of adult Robusta plants in the experimental field of Embrapa Rondônia, in Porto Velho, Brazil, and taken to the Laboratory of Plant Tissue Culture. In the collection, were selected leaves with intense green color, bright and thick cuticle layer, and complete expansion of the leaf blade. They were placed in plastic bags in order to minimize dehydration during locomotion from the collection site to the laboratory. The leaves were observed using a stereomicroscope to remove dust and small particles with the aid of a soft bristle. Under aseptic conditions, the leaves were immersed in a solution of water with a detergent agent for one minute, in sodium hypochlorite (2.4% active chlorine) under agitation for five minutes and then rinsed three times in sterile water.

Table 1. Explant responses (visual aspects and dry weight) to physiological state of the leaves, leaf area used as explant and surface in contact with the medium at 45 days of cultivation

Physiological state of the leaves	Leaf area used as explant	Surface in contact with the medium	Visual aspect of the explants	Dry weight of the explants (mg)
Source	Primaryvein	Abaxial	No induction, darkedges.	108.0 c
		Adaxial	No induction, darkedges.	98.2 c
	Secondaryvein	Abaxial	No induction, darkedges.	80.3 d
		Adaxial	No induction, darkedges.	79.0 d
Drain	Primaryvein	Abaxial	No induction, intactexplants.	100.9 c
		Adaxial	Calluses on all explants, cell proliferation at the ends of the veins.	180.7 b
	Secondary vein	Abaxial	No induction, intactexplants.	80.2 d
		Adaxial	Calluses on all explants, abundant cell proliferation at the edges of the explants.	240.2 a

*Averages followed by the same letter do not differ by Tukey's test (0.05).

Explants of 1.0 cm² were sectioned from the leaf blades and inoculated in a modified MS culture medium (1/2 macro, 1/4 micronutrients), supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar,1.0 mg L^{-1} 2iP and 5.0 mg L^{-1} IBA. In order to evaluate the influence of the physiological stage of the leaves donor of explants in the induction of somatic embryogenesis, they were collected from the second or fourth pair of leaves – drain or source, respectively. The explants were taken from the leaf blade using the surrounding area of primary or secondary veins, and inoculated with the abaxial or the adaxial surfaces in contact with the culture medium. The cultures were kept in a grow room at 25±1°C and 16 hours photoperiod. At 45 days of culture, the percentage of callus induction was evaluated and, at 120 days, the average number of cotyledonary embryos per explant.

RESULTS AND DISCUSSION

The induction of callus at 45 days after the inoculation was intense on the explants taken from drain leaves, placed with the adaxial face in contact with the culture medium,containing primary or secondary veins (Table 1). The other treatments did not result in callus induction. Consequently, at 120 days, cotyledonary embryos were observed only in explants in which callus formation occurred, that is, taken from drain leaves and placed with the adaxial face in contact with the medium. The explants taken from the leaf areas containing primary veins resulted in an average number of 5.4 embryos per explant, while those from leaf areas with secondary veins produced an average of 8.9 embryos per explant. All explants from source-type leaves showed oxidation and necrosis at their edges, which prevented callogenesis in the explants and, consequently, somatic embryogenesis.Despite the fact that theoretically any tissue can present totipotency (ability to generate new individuals), in practice explants that contain a greater proportion of meristematic tissue and are therefore younger are used (Grattapaglia and Machado, 1998). In coffee, the light green leaves of the second pair of leaves can be considered "drain", as they are under intense growth and still have little chlorophyll in relation to the older, dark green leaves of the fourth pair onwards, which are considered "source". This is in agreement with the description that Teixeira (2017) makes of the ideal leaves of *C. arabica* for callogenesis and, consequently, somatic embryogenesis: fully developed, newly expanded leaves, with intense green color and bright adaxial surface. According to this author, the use of leaves from the third pair onwards results in a higher rate of contamination (generally by fungi), a higher rate of phenolic oxidation, a lower rate of primary callus formation and, consequently, less or no embryogenic callus formation. The absence of callogenesis in older leaves can be explained on the basis that highly specialized plant cells hardly differentiate – these can be considered recalcitrant, as they have lost their ability to regenerate new plants. In contrast, cells that still have their ability to differentiate and regenerate are considered competent. According to this concept, competence would be characteristic of younger parts of the plant, while recalcitrance would be gradually more frequent in older tissues (George et al., 2008).

It was observed that no callus induction occurred in the leaf explants that were placed with the abaxial face in contact with the culture medium, thus preventing the subsequent formation of embryos in these explants. On the other hand, calluses were observed in explants whose adaxial face was in contact with the medium. The abaxial surface of *C. canephora* leaves has about six times more stomata than the adaxial surface (Deuner et al., 2011). This fact may be responsible for the success in obtaining callus in the explants whose abaxial face was exposed, allowing gas exchange with the air inside the tubes – as mentioned by George et al. (2008), although photosynthetic and respiratory rates in *in vitro* cultures are generally low, gas exchange is still important and plays a fundamental role in morphogenesis. Pacheco et al. (2012) observed that leaf explants with the abaxial surface in contact with the medium were not able to regenerate plants in sweet passion fruit (*Passifloraalata*), while explants with the adaxial surface in contact with the medium were morphogenic and regenerative. Ma et al. (2009) observed that lychee (*Litchi chinensis*) leaf explants with the adaxial surface in contact with the medium resulted in much more abundant callogenesis than those with the abaxial surface in contact with the medium. Regarding leaf area used as explant, callogenesis was more abundant in explants that contained secondary veins, and consequently more embryos were produced in these explants. The use of leaf explants containing veins is based on the fact that the veins have meristematic regions, such as the procambium, which makes these explants susceptible to differentiation (Santos et al., 2019). The greater success in callogenesis in explants containing secondary veins compared to those containing primary veins is probably due to the fact that younger and less lignified tissues are more prone to callus induction and morphogenesis in general (DornelasJúnior et al., 2018; Verstraeten et al., 2013).

CONCLUSION

The leaf fragments of the Robusta cultivar of *C. canephora*most responsive to the induction of somatic embryogenesis were taken from drain leaves, with explants containing secondary veins, placed with the adaxial face in contact with the culture medium.

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