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INDUCTION OF SOMATIC EMBRYOS IN ROBUSTA AND CONILON VARIETIES OF COFFEA CANEPHORA

[1]Camila Karen Candeira da Silva and *[2]Maurício Reginaldo Alves dos Santos

[1]Universidade Federal de Rondônia, 76801-974, Porto Velho, RO, Brazil
[2]Embrapa Rondônia, 76815-800, Porto Velho, RO, Brazil

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Corresponding author:
Mauricio Reginaldo Alves dos Santos

ABSTRACT

Clonal propagation of *Coffea canephora* varieties is feasible through somatic embryogenesis. The aim of this study was to evaluate the induction of somatic embryos from leaves of *C. canephora*, Robusta and Conilon varieties, in relation to different concentrations of three growth regulators. The explants were inoculated in a MS culture medium with 2iP (isopentanyladenine), IBA (indolebutyric acid) and BA (6-benzylaminopurine), in factorial combinations, at concentrations of 1.0; 2.0; 3.0; 4.0 and 5.0 mg L^{-1}. At 45 days of culture, the percentage of callus induction was evaluated and, at 120 days, the average number of cotyledonary embryos per explant. The most efficient combinations of growth regulators to induce callogenesis in the explants were: 5.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 3.0 mg L^{-1} of IBA + 3. 0 mg L^{-1} of BA – for the Robusta variety; and 5.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 5.0 mg L^{-1} of IBA + 5.0 mg L^{-1} of BA – for the Conilon variety. As for the average number of embryos per explant, the most efficient combinations of growth regulators were: 5.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 2.0 mg L^{-1} of IBA + 3 .0 mg L^{-1} of BA – for the Robusta cultivar; and 4.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 5.0 mg L^{-1} of IBA + 5.0 mg L^{-1} of BA – for the Conilon variety.

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INTRODUCTION

Coffea canephora Pierre ex A. Froehner has two distinct botanical varieties that are commercially cultivated – Robusta and Conilon. Robusta is characterized by erect growth, larger leaves, late maturation, lower drought tolerance and high resistance to pests and diseases. Conilon has shrub growth, early maturation, greater drought tolerance and high susceptibility to pests and diseases (Spinelli *et al*., 2018). From the crossing between these varieties, the genetic variability increases, which makes it possible to obtain hybrids that combine desirable characteristics of both and that present high agronomic performance due to the occurrence of heterosis (Oliveira *et al*., 2018). Plants of the species *C. canephora* have been propagated vegetatively*in vitro* through somatic embryogenesis (Table 1), a technique that allows rapid and large-scale multiplication (Landey *et al*., 2013; Hervé *et al*., 2016). From fully differentiated plant cells can be easily cultured *in vitro* to generate undifferentiated embryogenic cells, which can regenerate whole plants. However, the use of this technique depends on determining the most appropriate concentrations of growth regulators for each genetic material (Santos *et al*., 2013). The objective of this study was to promote somatic embryogenesis from leaf explants of the Robusta and Conilon varieties of *C. canephora* in relation to different concentrations and combinations of an auxin and two cytokinins.

MATERIAL AND METHODS

The leaves of the second pair were collected from orthotropic branches of adult Robusta and Conilonplants 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. Explants of 1.0 cm^2 containing secondary veins were sectioned from the leaf blades and inoculated with the adaxial surface in contact with a modified MS culture medium (1/2 macro, 1/4 micronutrients), supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar and IBA, in factorial combinations with 2iP or BA, all regulators at concentrations of 1.0; 2.0; 3.0; 4.0 and 5.0 mg L^{-1}. 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 highest percentages of callus induction in Robusta variety in the combinations of growth regulators (Tables 2 and 3) were 35.0%, with 5.0 mg L^{-1} IBA + 1.0 mg L^{-1}2iP and 25.0%, with 3.0 mg L^{-1} IBA + 3.0 mg L^{-1} BA. In the combination of IBA and 2iP, the media lacking 2iP did not result in callus induction; and in the combination of IBA and BA, there was no callogenesis were only one of the growth regulators was used. Regarding the Conilon variety, 100% callus induction was reached with 5.0 mg L^{-1} IBA + 1.0 mg L^{-1} 2iP and 5.0 mg L^{-1} IBA + 5.0 mg L^{-1} BA (Tables 4 and 5). It was also observed that at least one of the cytokinins was needed to induce callogenesis. In relation to the occurrence of cotyledonary embryos per explant, the highest values observed in Robusta were 9.36, with 5.0 mg L^{-1} IBA + 1.0 mg L^{-1} 2iP and 6.96 with 2.0 mg L^{-1} IBA + 3.0 mg L^{-1} BA (Tables 6 and 7).In the Conilon variety, 22.99 and 13.89 embryos per explant were obtained with 4.0 mg L^{-1} IBA + 1.0 mg L^{-1} 2iP and 5.0 mg L^{-1} IBA + 5.0 mg L^{-1} BA (Tables 8 and 9). As it was observed for callus induction, in absence of cytokinins there was no callus induction.

Callus induction can be achieved with a hormonal balance guaranteed by combinations of exogenous growth regulators – auxins, cytokinins and eventually gibberellins (Santos *et al*., 2015). In general, cytokinins and auxins, or just one of these classes of growth regulators, may be sufficient to promote induction (Santos *et al*., 2014a). Auxins are capable of initiating cell division and controlling cell growth and elongation processes (Nogueira*et al*., 2008). Often, slightly similar concentrations of auxins and cytokinins in the culture medium promote callus induction, but responses to the interactions of these classes of growth regulators may vary according to the regulator, explant and genotype peculiarities (Cordeiro *et al*., 2007).). They can act together in synergistic interaction or not, leading to dedifferentiation. These interactions have been used and tested in different ways to establish and refine the exact concentrations that should be supplemented in each situation (Santos *et al*., 2014b). The results of this work demonstrate that the presence of cytokinin in the culture medium is necessary for callus induction in leaf explants, which does not occur only in the presence of auxin. Cytokinin is fully involved and related in cell division, its use alone has little or no effect, but the presence of auxin and cytokinins generally results in

Table 1. Somatic embryogenesis in *Coffea canephora*

Explant	Growth regulators	Effect	Reference
Internodalsegments	?	Callusinduction and embryogenesis	Staritsky, 1970
Leaves (disks)	2iP (1.0 mg L^{-1}) + IBA (5.0 mg L^{-1})	Embryos in sixweeks	Piersonet al., 1982
Leaves (disks)	2iP (1.0 mg L^{-1}) or BA (1.1 mg L^{-1}) or KIN (1.1 mg L^{-1})	Embryoids in fiveweeks	Hatanaka et al., 1991
Leaves (1 cm^2)	2,4-D (0.5 mg L^{-1}) + IBA (0.5 mg L^{-1}) + 2iP (2.0 mg L^{-1})	Callogenesis in 14 weeks	Berthouly & Michaux-Ferrieri, 1996
Leaves (50 mm^2)	2iP (1.0 mg L^{-1})	Embryogenesis in four weeks (cell proliferation in one week)	Fuentes et al., 2000
Leaves (1 cm^2)	2iP (1.0 MG L^{-1}) + IBA (5.0 MG L^{-1})	Embryogenesis in 56 days, cotyledonary embryos in 82 days	Santos and Silva, 2020

Table 2. Percentages of callus induction in leaf explantes of Robusta variety, at 45 days after inoculation, in relation to combinations of IBA and 2iP

2iP (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	5.0 Ea	12.5 Da	10.0 Da	17.5 Ca	27.5 Ba	35.0 Aa
2.0	2.5 Ba	5.0 Bb	12.5 Aa	12.5 Ab	15.0 Ab	15.0 Ab
3.0	0.0 Db	2.5 CDb	5.0 BCb	7.5 ABc	10.0 Ac	10.0 Ac
4.0	-	-	-	-	-	-
5.0	-	-	-	-	-	-

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 3. Percentages of callus induction in leaf explantes of Robusta variety, at 45 days after inoculation, in relation to combinations of IBA and BA

BA (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	-	5.0 Bc	5.0 Bc	10.0 Ad	12.5 Ac	12.5 Acd
2.0	-	7.5 Bbc	5.0 Bc	12.5 Acd	15.0 Abc	15.0 Abc
3.0	-	12.5 Ca	17.5 Ba	25.0 Aa	20.0 Ba	20.0 Ba
4.0	-	10.0 Cab	10.0 Cb	15.0ABbc	17.5 Aab	12.5BCcd
5.0	-	-	5.0 Bc	10.0 Ad	12.5 Ac	10.0 Ad

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 4. Percentages of callus induction in leaf explantes of Conilon variety, at 45 days after inoculation, in relation to combinations of IBA and 2iP

2iP (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	30.0 Ea	52.5 Da	72.5 Ca	90.0 Ba	87.5 Ba	100.0 Aa
2.0	17.5 Db	47.5 Cab	72.5 Aa	67.5 Ab	70.0 Ab	57.5 Bb
3.0	12.5 Cbc	55.0 Aa	52.5 Ab	60.0 Ab	27.5 Bc	22.5 Bc
4.0	5.0 Dc	32.5 Bc	50.0 Ab	37.5 Bc	15.0 Cd	12.5 CDd
5.0	5.0 Ec	45.0 Ab	37.5 Bc	37.5 Bc	25.0 Cc	12.5 Dd

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 5. Percentages of callus induction in leaf explantes of Conilon variety, at 45 days after inoculation, in relation to combinations of IBA and BA

BA (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	32.5 Bc	32.5 Bc	32.5 Bc	37.5 Bd	47.5 Ab	55.0 Ad
2.0	40.0 Cc	35.0 Cc	40.0 Cc	62.5 Ac	50.0 Bb	52.5 Bd
3.0	37.5 Cc	62.5 Bb	57.5 Bb	60.0 Bc	77.5 Aa	72.5 Ac
4.0	50.0 Cb	70.0 Ba	57.5 Cb	72.5 Bb	80.0 Aa	85.0 Ab
5.0	70.0 Ea	75.0 CDa	72.5 DEa	82.5 BCa	85.0 Ba	100.0 Aa

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 6. Average numbers of embryos per explant in leaf explantes of Robusta variety, at 120 days after inoculation, in relation to combinations of IBA and 2iP

2iP (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	-	1.17 Da	4.01 Ca	8.10 Ba	7.53 Ba	9.36 Aa
2.0	-	0.75 Eab	2.59 Db	4.99 Cb	6.28 Bb	7.13 Ab
3.0	-	0.36 Dbc	1.11 Cc	3.09 Bc	5.22Ac	3.62 Bc
4.0	-	-	-	-	-	-
5.0	-	-	-	-	-	-

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 7. Average numbers of embryos per explant in leaf explantes of Robusta variety, at 120 days after inoculation, in relation to combinations of IBA and BA

BA (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	-	1.39 Dc	1.68 CDd	2.01 Cd	2.77 Bb	3.51 Aa
2.0	-	2.22 Cb	4.12 Ab	4.15 Ab	2.32 Cc	3.27 Ba
3.0	-	3.57 Ca	6.96 Aa	6.50 Aa	4.39 Ba	2.99 Db
4.0	-	2.20 Db	3.58 Ac	3.11 ABc	3.02 BCb	2.72 Cb
5.0	-	-	1.12 Cd	0.97 Ce	1.79 Bd	2.93 Ab

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 8. Average numbers of embryos per explant in leaf explantes of Conilon variety, at 120 days after inoculation, in relation to combinations of IBA and 2iP

2iP (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	7.13 Ea	8.90 Dc	15.72 Ca	20.66 Ba	22.99 Aa	22.97 Aa
2.0	5.31 Db	12.62 Ca	14.88 Bab	16.99 Ab	17.51 Ab	17.53 Ac
3.0	2.14 Ec	11.13 Dab	13.25 Cbc	13.67 Cc	16.02 Bb	20.32 Ab
4.0	0.97 Dcd	9.92 Cbc	11.65ABc	12.79 Ac	13.00 Ac	10.93BCd
5.0	1.26 Ecd	10.39 Abc	9.13 Ad	5.45 Bd	4.44 BCd	3.25 De

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

Table 9. Average numbers of embryos per explant in leaf explantes of Conilon variety, at 120 days after inoculation, in relation to combinations of IBA and BA

BA (mg L^{-1})	IBA (mg L^{-1})					
	0.0	1.0	2.0	3.0	4.0	5.0
0.0	-	-	-	-	-	-
1.0	4.48 Cd	4.83 Cc	5.19 Cd	5.01 Cd	6.54 Bd	7.32 Ac
2.0	4.92 Cd	5.82 BCc	6.72 Bc	8.68 Ac	8.79 Ac	9.01 Ab
3.0	7.88 Cc	8.13 Cb	9.52 Bab	10.74 Ab	10.84 Ab	10.90 Aa
4.0	8.54 Cb	8.93 b	8.81 Cb	10.55 Bb	11.13 Bb	13.02 Aa
5.0	9.82 Da	10.05 Da	11.29 Ca	12.59 Ba	12.99ABa	13.89 Aa

*Averages followed by the same uppercase letter in rows or lowercase in columns do not differ significantly at 5% probability by Tukey's test.

rapid cell division (Taizand Zeiger, 2013; Kerbauy, 2004). This work also demonstrate the high potential of somatic embryogenesis in cloning selected genetic material. Considering that it is possible to remove 20 leaf explants from a single coffee leaf, the production of 22.99 cotyledonary embryos per explant indicates a large production capacity of 460 new plants cloned from a single coffee leaf.Santos and Silva (2020) observed the formation of 46 cotyledonary embryos per leaf explant in hybrids of *C. canephora* with the supplementation of 2iP and IBA together, and the combination of these growth regulators showed superior results than those obtained with one of them alone.

CONCLUSION

The auxin-cytokinin combination is necessary to the induction of calluses and somatic embryos in the varieties Robusta and Conilon of

C. canephora. Maximum average number of embryos per explant were achieved with 5.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 2.0 mg L^{-1} of IBA + 3 .0 mg L^{-1} of BA – for the Robusta cultivar; and 4.0 mg L^{-1} of IBA + 1.0 mg L^{-1} of 2iP and 5.0 mg L^{-1} of IBA + 5.0 mg L^{-1} of BA – for the Conilon variety.

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