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Callus induction and embryo regeneration in *Coffea arabica* L. anthers by silver nitrate and ethylene¹

Indução de calos e regeneração de embriões em anteras de *Coffea arabica* L. por nitrato de prata e etileno

Adelaide Siqueira Silva^{2*}, José Magno Queiroz Luz², Tatiana Michlovská Rodrigues³, Cecília Alves Bittar⁴ e Leandro de Oliveira Lino⁵

Abstract - The genetic breeding of coffee by conventional methods to obtain new cultivars is time-consuming. Anther culture can yield homozygous lines quickly from bi-haploids. This study used anther cultures of *Coffea arabica* L. cv. Catuai Vermelho 99, and tested the effect of silver nitrate (AgNO₃) and ethylene in callus-formation induction and bi-haploid seedling regeneration. The anthers were inoculated in MS to which 2 mg L⁻¹ 2,4-D had been added with AgNO₃ (5 mg L⁻¹) together with ethylene being introduced or not, for different periods (the control, 2, 4, 6, and 8 days). At the end of 12 days, the anthers were transferred to the regeneration medium, to which 0.108 mg L⁻¹ kinetin had been added. Greater oxidation and intumescence were observed in the first days after exposure to ethylene; the cultivar Catuai Vermelho 99 responded to callus formation and also direct embryogenesis in the presence of ethylene.

Key words - Coffee. Embryogenesis. Anthers.

Resumo - O melhoramento genético do cafeeiro por métodos convencionais é um processo demorado para aquisição de uma nova cultivar. Através da cultura de anteras podem-se obter linhagens homocigóticas rapidamente, oriundas de dihaplóides. O objetivo foi utilizar na cultura de anteras na cv. Catuai Vermelho 99 de *Coffea arabica* L., nitrato de prata (AgNO₃) e etileno para induzir a formação de calos e regenerar plântulas dihaplóides. As anteras foram inoculadas em meio MS acrescido de 2 mg L⁻¹ de 2,4-D com e sem AgNO₃ (5 mg L⁻¹), juntamente com etileno por diferentes dias: (testemunha; 2; 4; 6, e 8). Ao final de 12 dias, estas foram transferidas para o meio de regeneração, acrescido de 0,108 mg L⁻¹ de cinetina. Foi observada uma oxidação e intumescimento maior nos primeiros dias de inoculação em exposição ao etileno, e a cv. Catuai Vermelho 99 respondeu a formação de calos e também a embriogênese direta na presença do etileno.

Palavras-chave - Café. Embriogênese. Anteras.

* Autor para correspondência

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²Departamento de Fitotecnia/POSAGRO/ICIAG-UFU, Avenida Amazonas s/n, Bloco 2E, Sala 01, Caixa Postal 593, Bairro Umuarama, Uberlândia-MG, Brasil, 38.400-902, adelaidelibio@yahoo.com.br, jmagno@umuarama.ufu.br

³Universidade Federal Rural de Pernambuco/Unidade Acadêmica de Garanhuns, Av. Bom Pastor, s/n, Boa Vista, Garanhuns-PE, Brasil, 55.292-270, tatiana_mrodrigues@yahoo.com.br

⁴Programa de Pós-Graduação em Agronomia, Universidade Federal de Uberlândia, Uberlândia-MG, Brasil, ceciliabial@yahoo.com.br

⁵Bolsista do programa de Pós-Graduação em Genética e Melhoramento de Plantas, Universidade Federal de Lavras, Lavras-MG, Brasil, leagrolino@yahoo.com.br

Introduction

Coffee is the second most important commodity in the international market, coming second only to petroleum. Its industry generates more than 100 million jobs around the world. Around three-fourths of all coffee grown enters the international market, and 70% of this coffee is produced by small properties, each of less than 10ha, in more than 60 mostly developing countries (CENTRO DE INFORMAÇÕES..., 2010). World coffee production in 2010 should be around 124 million 60 kg sacs, and according to estimates from Companhia Nacional de Abastecimento (CONAB), Brazil will be responsible for 47 million (38%). Within these 47 million sacs of home production are included 35.3 million sacs of Arabica coffee and 11.7 million sacs of Robusta coffee; the Arabica type therefore, representing about 75% of total Brazilian production (EPTV, 2010).

There are about 100 described species in the genus *Coffea*, but only two, *C. arabica* L. and *C. canephora*, yield fruits with economic importance in the international market; those of *C. arabica* yield a better quality beverage and represent around 70% of the world's production (MINEIRO, 2006).

Biotechnology offers alternative strategies for generating new and improved coffee varieties, including those resistant to environmental extremes, pests and diseases, and with low levels of caffeine, and of uniform fruit maturation. Large improvements in somatic embryogenesis, the development of haploids, and scaled-up micro-propagation have been achieved in the last 5 years (BUZZY et al., 2007). The use of genetically improved cultivars could be in part a solution for these problems, since breeding programs have presented promising results for coffee culture, including yield increases and new cultivars. Conventional breeding techniques have been widely used for this purpose.

A number of attempts have been made to reduce the time needed for embryogenesis in coffee. Triacantanol, silver nitrate (AgNO_3), salicylic acid, thidiazuron and 2IP are the growth regulators most used in coffee embryogenesis for this purpose. According to Giridhar, Indu and Vinod (2004), direct somatic embryogenesis was obtained from hypocotyl explants of *in vitro* regenerated plantlets of *C. arabica* and *C. canephora* in a modified MS medium containing AgNO_3 supplemented with N6 benzyladenine and indole-3-acetic acid.

Contrasting responses to ethylene have been observed during the somatic embryogenic process. Ethylene may stimulate the embryogenic response in some systems, while inhibiting it in others. Both these effects of ethylene have been observed in the coffee embryogenic

response, which is in agreement with the responses generally observed for growth regulators: a stimulant effect at optimum concentration and inhibition at supra-optimum levels. (HATANAKA et al., 1995).

Silver ion is considered one of the most effective inhibitors of this ethylene action. Although its effect is not well understood, it is supposed that the silver ion binds a possible ethylene receptor at the plasma membrane, thus inhibiting the binding of ethylene to this receptor and consequently triggering the specific action of the hormone (TEIXEIRA et al., 2002).

This study evaluated the effect of AgNO_3 and ethylene on anther cultures of *Coffea arabica* L. cv. Catuaí Vermelho 99 in inducing the formation of calli and regenerating bi-haploid seedlings.

Material and methods

The experiment was done in the Biotechnology laboratory of the Instituto de Ciências Agrárias at the Federal University of Uberlândia (UFU), Uberlândia MG, from August 2004 to December 2006. The plant material was *Coffea arabica* L. cultivar Catuaí Vermelho LCH-2077-2-5-99, obtained from the experimental area of Campus Umuarama at the University.

The flower buds were collected between 8 and 9 am, stored in Petri dishes containing tissue paper moistened with distilled water, placed in a Styrofoam box to prevent drying, and taken immediately to the laboratory. Flower-bud size was measured with calipers; 4.5 to 6.0 mm long buds being selected.

The flower buds were wrapped in sterilized cheesecloth and disinfested in 70% alcohol for a few seconds followed by a 1% sodium hypochloride solution for fifteen minutes, with constant stirring. Subsequently, the buds were rinsed three times with sterile distilled water in a flow-hood. The anthers were removed from the flower buds through a side cut with the aid of a stereo microscope, tweezers and scalpel, these being flame-sterilized for each bud. The anthers were dipped in 600 mg L^{-1} ascorbic acid, 0.2% sodium hypochloride and sterile distilled water for a few seconds before seeding in the medium. The culture medium had its pH adjusted to 5.9 and was sterilized in an autoclave at 121 °C at 1 atm. for twenty minutes.

In the first part of the experiment, flower buds of the coffee cultivar Catuaí Vermelho 99 were collected and disinfested. The anthers were removed with care to avoid damage, and were seeded in sterilized Petri dishes containing the medium MS (MURASHIGE; SKOOG, 1962), to which 2 mg L^{-1} 2,4-D had been added. The

experimental design was completely randomized (CRD), with 10 treatments combining the exposure-time to ethylene (2; 4; 6; 8 days and the control) both with and without 5 mg L⁻¹ AgNO₃ in four repetitions, where each experimental unit consisted of one Petri dish containing 25 anthers. Damaged anthers were discarded. A drop of ETHREL[®] was placed in the center of each dish after seeding, with the aid of a sterilized disposable syringe; subsequently the plates were wrapped and placed in the dark for different periods (the control, 2; 4; 6 or 8 days). After the first incubation period the plates were opened in a flow-hood enabling the ETHREL[®] to evaporate completely from the dishes which were then rewrapped and maintained in the dark for up to another eight days before being transferred to the growth room and maintained at 26 °C with 16 hours of light at a photon flux of approximately 170 μmol m⁻² s⁻¹ where they were kept until the twelfth day after inoculation.

In the second part of the experiment at the end of the twelve days, the anthers were transferred to a regeneration medium, called medium R by the authors (Sibi; Dumas de Vaulx; Chambonnet, 1979), supplemented with 0.108 mg L⁻¹ kinetin, where they were kept for 30 days.

The experimental design was completely randomized (CRD), with 10 treatments combining the exposure-time to ethylene (2; 4; 6; 8 days and the control) both with and without 5 mg L⁻¹ AgNO₃ in four replications, where each experimental unit consisted of one Petri dish containing 25 anthers.

Twelve days after inoculation, in the first part of the experiment, the percentages of anthers that were either oxidized, contaminated or contained intumescences and calli was determined, this procedure being repeated thirty days after seeding in the R medium (the second part of the experiment), using the same variables and additionally the pro-embryoid count. The data were submitted for

statistical analysis using the program PROPHET. When a lack of normal distribution of the residues and or the absence of homogeneity of variances were observed, the data were transformed by square root of $\sqrt{x + 1/2}$ and run through the program SISVAR (FERREIRA, 2003). The F test was performed at 5% significance, the averages compared using the Tukey test, and polynomial regression was used for the study of the effect of ethylene.

Results and discussion

In the first part of the experiment, based on the analysis of variance, significant differences were found for the variables oxidation, intumescence and contamination in the presence of ethylene. Only the variable contamination was significantly affected by Silver nitrate (TAB. 1).

As ethylene exposure-time increased, the number of oxidized anthers also increased, becoming stable (4.0605) after approximately two days (FIG. 1), suggesting that ethylene is capable of oxidizing the anthers in a very short time after inoculation; incubation in darkness could also have contributed to this result.

The results obtained in this study are similar to those of Marques (2006). Although some oxidation was observed, anther development was not jeopardized, subsequently presenting calli and thus far, possible direct embryogenesis.

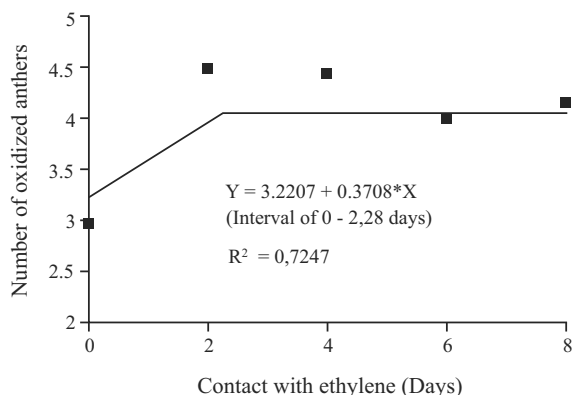
Figueira (2005), studying the same cultivar Catuai Vermelho 99, confirms the hypothesis mentioned above in relation to seeding time and oxidation, observing oxidation of all anthers, even in those maintained in darkness for up to 60 days. Fialho et al. (2005), working with anther culture of *Anacardium occidentale* L. observed better anther development when these were initially kept in the dark. Maintaining the anthers in the dark, although

Table 1 - Analysis of the variance of the average number of anthers that were oxidized (OXID.), those with intumescence (INTUM.) and those contaminated (CONT.), for the cultivar Catuai Vermelho 99, seeded in "MS" culture medium both with and without silver nitrate (AgNO₃) in contact with ethylene (ETHREL[®]) for different periods. UFU/Uberlândia-MG, 2007

Source of Variation	Degrees of Freedom	Mean Squares		
		OXID.	INTUM.	CONT
Ethylene	4	5.7613*	3.5953*	1.4288*
Nitrate	1	0.0695 ^{ns}	0.0088 ^{ns}	1.1810*
Ethylene*Nitrate	4	0.1246 ^{ns}	0.2941 ^{ns}	0.2858 ^{ns}
Error	30	0.3929	0.4040	0.1499
CV (%)		17.55	19.27	38.04

*; ns = Significant and non-significant at 5% significance by the F test, respectively

favorable to their development, was not effective in the present study, since even under that condition they were found to have oxidized in the final evaluation; this result is different from that of Silva (2003), who states that oxidation tends to decrease when *Coffea* anthers are incubated in the dark.



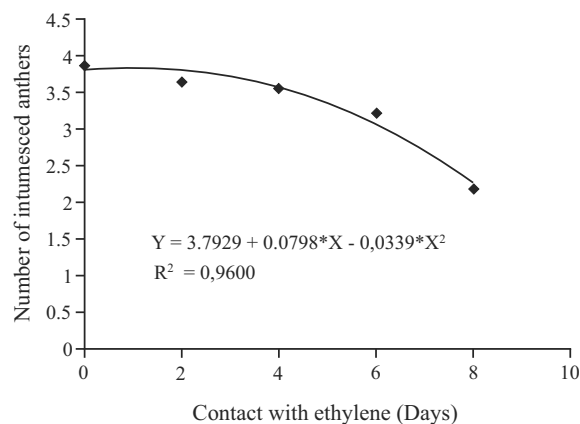
* Parameters of the equation shown for the model $p < 0.05$

Figure 1 - Average percentage of oxidized anthers in contact with ethylene for different periods after seeding. UFU/Uberlândia-MG, 2007

Another factor that may explain explant oxidation is the fact that coffee is a woody species and liberates phenols. For this reason, antioxidant substances such as activated charcoal, polyvinylpyrrolidone (PVP), ascorbic acid or citric acid, should be added to the medium (ANDRADE, 1998).

Development of intumescences was observed while the explants remained in contact with ethylene for 8 days, reaching the smallest average for this variable (2.17) corresponding to 13.19%. The explant had better averages of intumescence in the first periods of contact with ethylene, including the control, the highest average being observed in the control (3.87) (23.25%) at 2 days of contact (3.65) (22.12%), although there were no significant differences between 4 and 6 days in the presence of ethylene (FIG. 2).

When we consider the two-day period, the ethylene-stimulated intumescence of the Cultivar Catuaí Vermelho 99 has an average of (3.87). It is assumed that ethylene induces anther intumescence on first contact; however, as time passes it has the opposite effect, reducing the number of intumescences and leading to explant stagnation. This demonstrates that the studied cultivar is responsive to

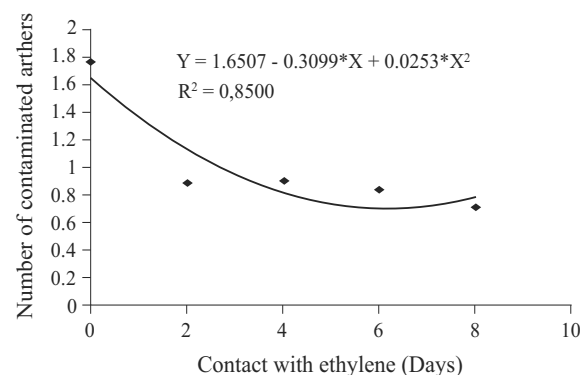


* Parameters of the equation shown for the model $p < 0.05$

Figure 2 - Percentage of intumescenced anthers exposed to ethylene for different contact periods. UFU/Uberlândia-MG, 2007

intumescence and future callus development. The same fact was observed by Figueira (2005) who obtained high indices of intumescence with Catuaí Vermelho 99 anthers soon after seeding.

Contamination was high in the first days after seeding, when the anthers were not exposed to ethylene, reaching 34.61%, which is considered relatively high for plant-tissue culture. No significant differences were found for the other periods of exposure to ethylene (FIG. 3). According to the analysis of variance, the presence or absence of silver nitrate was significant in the contamination. Contamination was lowest in the presence of nitrate, in contrast to the material which had been inoculated in its absence (data not shown).



* Parameters of the equation show for the model $p < 0.05$

Figure 3 - Percentage of contamination after exposure to ethylene for different contact periods. UFU/Uberlândia-MG, 2007

One of the hypotheses for reduced contamination by fungi and bacteria, is that silver nitrate acts as a chemical control agent. Another hypothesis is that it can inhibit anther intumescence, since the explant seeded in its presence showed low development or did not intumescence, as observed during evaluation (data not shown). The contamination level could also be related to the plant genotype, since the anthers were collected from field plants.

In the second part of the experiment, the analysis of variance shows significant values only for the variables of intumescence and callus formation (callosity) (TAB. 2). Due to contamination, treatment T10 (8 days with ethylene/no nitrate) was lost; therefore analysis was carried out only for the remaining treatments.

Anther intumescence was effective in treatments T1, T2, T8 and T9 with averages of 2.71 (17.12%), 3.02 (19.09%), 2.20 (13.89%) and 2.49 (15.78%) respectively; whereas in the other treatments no significant differences were seen (TAB. 3).

Based on these results, it can be stated that the treatments which originated from the control batch in the first part of the experiment and those from the six and eight day contact periods with ethylene, were those that performed best in medium R (TAB. 3). It can therefore be inferred that on first contact with ethylene, followed by a change in growth medium, the explant intumescence, favoring callus formation. This is highlighted in the same table, where the best results for callus formation were also the treatments T1(0/0), T2 (0/1) and T8 (6/1), although they did not differ from treatments T4 (2/1) and T6 (4/1); with callus percentages of 19.85%, 18.73% and 10.04%, respectively; in the last two treatments most of the calli came from those dishes with 5 mg L⁻¹ nitrate. Treatments T3 (2/0), T5 (4/0), T7 (6/0) and T9 (8/0) were the least responsive; coming from media without nitrate (TAB. 3).

Silver nitrate affected intumescence and callosity. The treatment with silver nitrate on the sixth day, was the one which gave the best responses for both variables. According to Kumar, Parvatam and Ravishankar (2009),

Table 2 - Summary of the analyses of variance of the average number of anthers oxidized (OXID.), intumescenced (INTUM.) contaminated (CONT.) and with callosity (CALLI.), of the Catuaí Vermelho 99 cultivar, seeded in culture medium "R" and kinetin (0.108 mg L⁻¹), obtained in the second part of the experiment. UFU/Uberlândia-MG, 2007

Source of Variation	Degrees of Freedom	Mean Squares			
		OXID.	INTUM.	CONT.	CALLI
Treatment	8	1.5480 ^{ns}	2.2413*	0.2855 ^{ns}	0.5150*
Error	20	1.0671	0.7524	0.3686	0.1214
CV (%)		26.50	50.22	0.800	36.15

*; ns = Significant and non-significant at 5% significance by the F test, respectively

Table 3 - Number of intumescenced anthers and those with callosity of the cultivar Catuaí Vermelho 99 as a function of the treatments, in the second part of the experiment, using ethylene (ETHREL®) and silver nitrate (AGNO₃). UFU/Uberlândia-MG, 2007

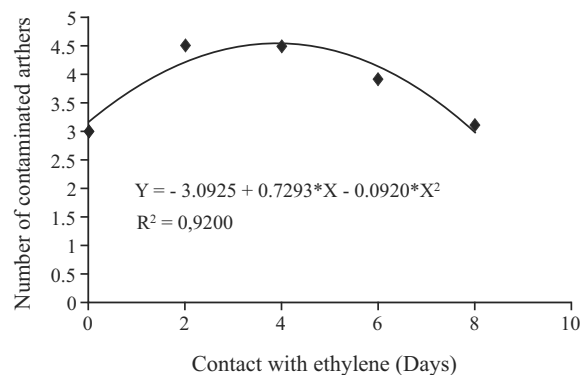
Treatments (Ethylene/AgNO ₃)	Intumescence	Callosity
T1(0/0)	2.7063 a	1.7390 a
T2 (0/1)	3.0179 a	1.6410 a
T3 (2/0)	0.7071 b	0.7071 b
T4 (2/1)	1.1166 b	0.8365 a
T5 (4/0)	1.3212 b	0.7071 b
T6 (4/1)	0.8365 b	0.8365 a
T7 (6/0)	1.4142 b	0.7071 b
T8 (6/1)	2.1959 a	0.8796 a
T9 (8/0)	2.4943 a	0.7071 b
General Average	1.7566	0.9734

Averages followed by the same letters in the column are not significantly different by the Scott-Knott test, at 5% significance

silver nitrate is known to promote multiple shoot formation in different plants. *In vitro* shoot formation was improved by incorporating silver nitrate into the culture medium.

Giridhar, Indu and Ravishankar (2004) stated that AgNO_3 , acetylsalicylic acid (ASA), triacontanol, TDZ and 2iP are commonly used as growth regulators in coffee embryogenesis studies. Giridhar, Indu and Vinod (2004) reported that direct somatic embryogenesis was found in hypocotyl explants of *C. arabica* and *C. canephora in vitro* in MS medium modified with 10-70 mM AgNO_3 , 1.1 mM N- benziladenine and 2.85 mM AIA. Kumar, Ramakrishna and Ravishankar (2007), evaluating the influence of different ethylene inhibitors on somatic embryogenesis, observed that 35-48% of explants responded for embryogenesis under the silver nitrate treatment, 38 ± 7 and 153 ± 27 embryos being produced from each callus mass respectively, whereas only 5% of the control explants responded in a medium devoid of silver nitrate, cobalt chloride and salicylic acid.

Ethylene was significant in anther oxidation, as shown by analysis of the variance (TAB. 4), and presented a quadratic effect. According to Figure 4, as the anther remain in contact with ethylene, oxidation increases, reaching maximum level around four days after seeding, it then decreases, reaching its lowest level on the eighth day. It can therefore be inferred that in the absence of ethylene, or with few days exposure, anther oxidation is observed, decreasing within four to six days; this possibly occurring due to the stabilization of the physiologic process in the conditions present in the medium. According to Pasqual et al. (2002) cited by Figueira et al. (2008), ethylene is a growth-regulator gas produced by every plant cell, and which leads to important physiologic effects; however, with *in vitro* cultivation, the production and action of this gas in growth containers directly affect explant response, which can be either negative or positive.



* Parameters of the equation shown for the model $p < 0.05$

Figure 4 - Percentage of oxidized anthers in contact with ethylene for different periods after seeding. UFU/Uberlândia-MG, 2007

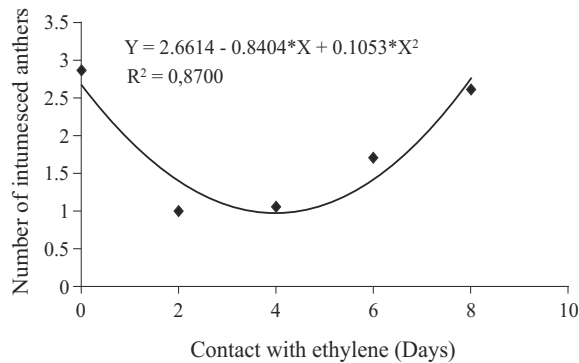
A quadratic effect was also observed for intumescence, similar to that found for oxidation, since the effect of the ethylene was significant (TAB. 4). The effect of the ethylene on the percentage of intumesced anthers after the first days of cultivation is shown in Figure 5, with a relatively large number of intumesced anthers (approximately 3.0) in the absence of ethylene; however, after about four days in contact with ethylene, this average decreases, rising again on the eighth day after seeding. Therefore it would appear that anthers can intumesce in the absence of ethylene or within a few days of contact, and moreover longer exposures to ethylene demonstrated positive effects on anther intumescence.

Ethylene was significant in inducing callosity, with the data being better adjusted by a quadratic regression even though the linear and cubic effects were also significant

Table 4 - Summary of the analyses of variance of the average percentage of anthers oxidized (OXID.), intumesced (INTUM.), contaminated (CONT.) and with callosity (CALOS.), of the cultivar Catuai Vermelho 99, seeded in culture medium R and kinetin (0.108 mg L^{-1}) obtained in the second part of the experiment. UFU/Uberlândia-MG, 2007

Source of Variation	Degrees of Freedom	Mean Squares			
		OXID.	INTUM.	CONT.	CALLI
Ethylene	4	2.80*	4.02*	0.460 ^{ns}	1.00*
1st Degree	1	0.01 ^{ns}	0.00 ^{ns}	0.086 ^{ns}	2.05*
2nd Degree	1	9.95*	13.03*	0.800 ^{ns}	1.03*
3rd Degree	1	0.73 ^{ns}	1.91 ^{ns}	0.030 ^{ns}	0.48*
Error	24	0.94	0.70	0.041	0.11
CV (%)	4	24.85	48.54	0.460	33.79

*, ns = Significant and non-significant, at 5% significance by the F test, respectively

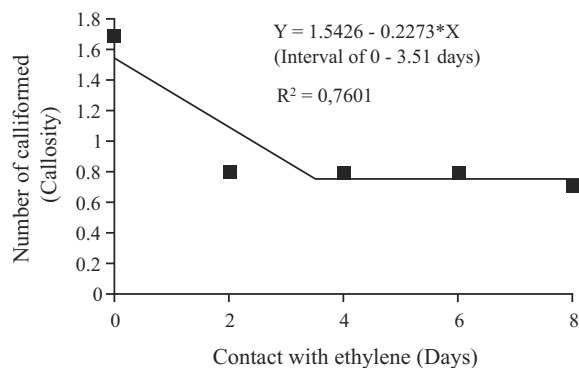


* Parameters of the equation shown for the model $p < 0.05$

Figure 5 - Number of intumesced anthers exposed to ethylene for different contact periods. UFU/Uberlândia-MG, 2007

(TAB. 4). It was possible to observe that callosity stabilized at about 0.7441 after 3.51 days in contact with ethylene. This demonstrates that ethylene has a positive effect only in the first few days of contact, its absence also favoring callus formation (FIG. 6). According to the intumescence observed, the lack of ethylene or few days of contact, as well as the increase in exposure time, was favorable to callosity, demonstrating that ethylene affects both intumescence as well as callus formation.

According to Kumar, Naidu and Ravishankar (2006), the known effects of silver nitrate on somatic embryogenesis support the hypothesis that this product promotes direct somatic embryogenesis and callus formation in indirect somatic embryogenesis, which can be attributed to ethylene regulation during the specific embryogenesis stages of *Coffea*. According to Feria, Jimenez and Barbon (2003),



* Parameters of the equation shown for the model $p < 0.05$

Figure 6 - Number of formed calli after ethylene exposure for different contact periods. UFU/Uberlândia-MG, 2007

exogenous ethylene concentrations and dissolved O_2 concentrations perform an important role in coffee somatic embryogenesis. Jha, Dahleen and Suttle (2007), studying plant regeneration in barley (*Hordeum vulgare* L.), observed that blocking the action of ethylene with silver nitrate during weeks 5-10, almost doubled regeneration in Morex, while Golden Promise increased regeneration 1.5-fold. Luz et al. (1999) obtained direct regeneration of haploid seedlings from pepper anthers in a medium to which 2,4-D, kinetin and $AgNO_3$ had been added, and when exposed to ethylene. In *C. arabica*, embryogenic callus was induced after prolonged culture with cytokinin, with subsequent somatic embryo formation on the embryogenic callus (BRIONES; SOTOMAYOR, 2006).

Nitrate is highly effective in inducing responses for primary and secondary embryogenesis in comparison to other ethylene inhibitors (KUMAR; NAIDU; RAVISHANKAR, 2006). Due to its negative action, there are several studies on ethylene inhibitors, such as silver nitrate ($AgNO_3$) and acetylsalicylic acid (ASA). Silver nitrate, which is a potent inhibitor of ethylene, promoted regeneration in *Triticum aestivum*, *Nicotiana plumbaginifolia*, *Zea mays*, in some *Brassica* genotypes and in *Daucus carota* (HATANAKA et al., 1995). Pereira et al. (2007) studied the direct somatic embryogenesis in *Coffea arabica* L. and found that the combination of kinetin and GA_3 promoted embryo induction. the use of kinetin 8 mg L^{-1} and GA_3 17 mg L^{-1} not associated in medium, with 8.0 mg L^{-1} of ANA promoted higher rates in vitro.

After 30 days of cultivation in medium R to which 0.108 mg L^{-1} 2,4-D had been added, possible direct anther embryogenesis was observed in the treatments T1r2 (MS + 2.0 mg L^{-1} 2,4-D + lacking $AgNO_3$ + the control) and T2r4 (MS + 2.0 mg L^{-1} 2,4-D + 5.0 mg L^{-1} $AgNO_3$ + the control) (FIG. 7; FIG. 8), which were not in contact with ethylene (data not shown).

According to the results, $AgNO_3$ probably together with 2,4-D, caused this direct embryogenesis, in contrast to the other results, showing that ethylene is a good embryogenesis- inducer agent, and that silver nitrate would be beneficial in some cases whilst negative in others. Studies, such as those of Figueira (2005) and Gurel, Gurel and Kaya (2000) report this disagreement over the action of silver nitrate on pro-embryoid induction. Greater somatic induction occurs in media containing silver nitrate, possibly because nitrate blocks the inhibitory endogenous ethylene action in the embryos (BIDDINGTON; ROBINSON, 1991).

Marques (2005) found that the longer the explant remains in the culture medium, the greater is the response in the formation of pro-embryoids, highlighting the



Figure 7 - Formation of direct embryogenesis in anther in treatment T1 (lack of ethylene / lack of AgNO_3) in *Coffea arabica* for the cultivar Catuai Vermelho 99 after 30 days of incubation

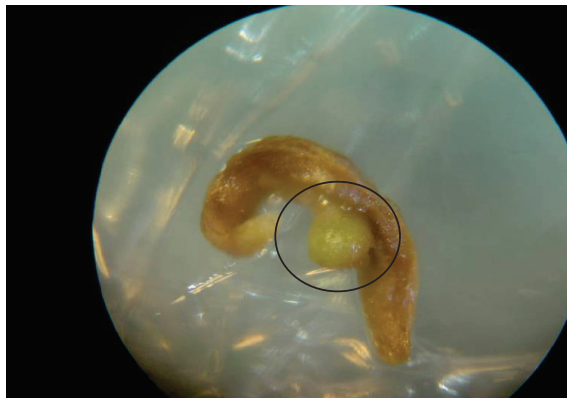


Figure 8 - Formation of direct embryogenesis in anther in treatment T2 (lack of ethylene / presence of AgNO_3) in the *Coffea arabica* cultivar Catuai Vermelho 99 after 30 days of incubation

fact that long seeding times lead to the oxidation of the material. That author obtained 84.5% pro-embryoids from calli in *Coffea arabica*; in contrast, Figueira (2005), working with the concentration of 2.0 mg L^{-1} 2,4-D both with and without silver nitrate, found that 19.8% of the anthers with calli had pro-embryoids, concluding also that silver nitrate was more effective for this induction.

Conclusions

1. Ethylene induced an increase in oxidation and intumescence in the first days after inoculation, with a subsequent stabilization of its effects;

2. The cultivar Catuai Vermelho 99 responded to callus formation and also to direct embryogenesis in the presence of ethylene.

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