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Calli induction in leaf explants of coffee elite genotypes

Indução de calos em explantes foliares de clones elite de café

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ABSTRACT

Three experiments were carried out with the objective of achieving high effectiveness in calli induction from high heterozygosis leaf explants of ***Coffea arabica*** *through indirect somatic embryogenesis. A randomized-block design in a 2x5 factorial arrangement made up of two media [BOXTEL & BERTHOULY (1996) and TEIXEIRA et al. (2004)] and five* ***C. arabica*** *genotypes were used in the first experiment. In the second experiment the embryogenic calli production potential was evaluated in ten genotypes. Each of them was considered as a treatment. In the third experiment the variations in both 2.4-D (2.5 e 20µM) and 2-iP (2.5 e 20µM) concentrations in TEIXEIRA et al. (2004) medium and secondary media were evaluated. Crops were kept in a growth room under darkness, at 25±2°C. The medium described by TEIXEIRA et al (2004) was found to be superior when compared to that described by BOXTEL & BERTHOULY (1996) in the 2.2 and 7.2 genotypes. An opposite behavior was noticed in 4.2 genotype, that is, BOXTEL & BERTHOULY (1996) had medium superiority. Both 3.0 and 5.0 genotypes had the same behavior in both media studied, which shows that the somatic embryo production depends on the genotype. Calli induction depends on the 2-iP and 2.4 D ratio. The 20.0µM of 2.4-D and 20.0µM of 2-iP combination caused the highest embryogenic calli induction rate.*

Key words: ***Coffea arabica****, culture medium, growth regulators.*

RESUMO

Visando a alcançar alta eficiência na indução de calos a partir de explantes foliares de plantas matrizes de ***C. arabica*** *com alta heterozigose, por meio da embriogênese somática indireta, foram instalados três experimentos. O primeiro experimento foi conduzido em esquema fatorial 2x5, constituído de dois meios de cultura (BOXTEL & BERTHOULY, 1996 e TEIXEIRA et al., 2004) e cinco genótipos de* ***C. arabica****. No segundo experimento, foi avaliado o potencial de produção de calos embriogênicos em 10 genótipos, sendo cada genótipo considerado como um tratamento e, no terceiro experimento, foram avaliadas as variações nas concentrações de 2.4-D (2,5 e 20µM) e 2-iP (2,5 e 20µM) nos meios primário e secundário de TEIXEIRA et al. (2004). As culturas foram mantidas a 25°C, sob obscuridade. Para os genótipos 2.2 e 7.2, verificou-se a superioridade do meio de cultura Teixeira et al. (2004) em relação ao meio BOXTEL & BERTHOULY (1996). No genótipo 4.2, observou-se o comportamento inverso, ou seja, a superioridade do meio BOXTEL & BERTHOULY (1996). Os genótipos 3.0 e 5.0 apresentaram o mesmo comportamento em ambos os meios de cultura estudados, evidenciando que a produção de embriões somáticos é fortemente dependente do genótipo. A indução de calos depende da relação de 2-iP e 2.4-D. A combinação de 20.0µM of 2.4-D e 20.0µM of 2-iP promoveu a maior porcentagem de indução de calos embriogênicos.*

Palavras-chave: ***Coffea arabica****, meios de cultura, reguladores de crescimento.*

INTRODUCTION

The introduction of biotechnological methods as a tool for helping genetic breeding programs has been quite useful, mainly in perennial cultures, as

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the coffee tree. An important method of *in vitro* propagation of ***C. arabica*** plants is the somatic embryogenesis, which consists of embryos development from either haploid or diploid somatic cells, without gamete fusion, which enables the accelerated vegetative propagation and the superior clones genetic uniformity showing a great potential to be explored.

Breeding programs have produced hybrid plants witch superior agronomical features. The Siriema genotype, which was developed by the breeding program carried out by Procafé Foundation, is the result of the cross between ***C. arabica*** and ***C. racemosa***. Such genotype it is not only productive but also resistant to both rust and leaf-miner. Another important population has been development by the Epamig/ UFLA/UFV breeding program. It is the result of cross between 'Icatu' and 'Catuai' cvs. It is resistant to either horizontal or vertical rust, has high plant vigor and productivity.

The advances witch have taken place in the last decades in cloning process by using somatic embryogenesis encourages plans for future exploration of coffee heterozygosis through the development of hybrid varieties. This research was carried out with the objective of seeking high effectiveness for calli induction from leaf explants of elite genotypes developed by the genetic breeding programs in Minas Gerais state.

MATERIAL AND METHODS

Well grown leaves belonging to the third pair were collected from the plagiotropic branches of the medium third of adult mother plants and then taken to the tissue culture laboratories where the experiments were conducted. An application of Triazol+Estrobilurina fungicides, trade name Sphere® were applied in the mother plants 24 hours before the explants were collected.

The leaves were immersed in a 70% alcohol solution for one minute and then disinfested by using sodium hypochlorite, at the concentration of 2.4% (commercial concentration), for 15 minutes. Soon afterwards, the leaves were washed three times with autoclaved distilled water.

After the mediums were prepared, their pH was adjusted to 5.6±0.1 by using NaOH 0,1N or HCl 0,1N, before being autoclaved at 121°C and 1atm, for 20 minutes. With the aid of a scalpel the leaves were cut into approximately 1cm^2 squares and their explants inoculated with the adaxial face of the leaf in contact with the culture medium (TEIXEIRA at al., 2004).

According to the protocol described by TEIXEIRA et al. (2004), the leaf explants were initially inoculated in the primary medium (PM). After 30 days in a growth room, in continuous darkness at 25±2°C, they were transferred to the secondary medium (SM), where they remained under the same conditions. A randomized block design was used. Each plot had a Petri dish containing nine explants.

Experiment 1 - Culture mediums and genotypes influence

Greenhouse grown ***C. arabica*** genotypes leaves in F_1 generation produced by the Coffee plant Genetic Improvement Program conducted by UFLA/ Epamig/UFV and resulting from the cross between Icatu x Catuai cvs were used as explants.

The leaf explants were inoculated in the primary medium (PM) (TEIXEIRA at al., 2004) or C medium (BOXTEL & BERTHOULY, 1996). After 30 days in growth room under darkness and temperature of 25±2°C, they were transferred for the secondary medium, SM (TEIXEIRA at al., 2004) or E medium (BOXTEL & BERTHOULY, 1996), respectively, in which they stayed for 180 days, under the same previous conditions.

Test tubes measuring 16x160mm and containing about 15mL of medium were used. It were used six replications (six explants taken as a plot) in a 2x5 factorial arrangement made up of two media (BOXTEL & BERTHOULY, 1996 and TEIXEIRA et al., 2004) and five ***Coffea arabica*** genotypes was used. Embryogenic calli formation was evaluated after 180 days.

Experiment 2 - Calli formation potential

Ten genotypes originating from generations F_3 progenies resulting from the cross between ***C.arabica*** x ***C.racemosa*** were studied. They are described as follows: 4-20, 20-5, 10-1, 5-14, 6-38, 14-8, 14-3, 8-10, 02 and 10-8. Each genotype was considered as a treatment. Six Petri dishes were used. Each one corresponded to a replication. Six months after installation the experiment was evaluate through percentage of explants with embryogenic calli.

Experiment 3 - Influence of 2-iP and 2.4-D levels

The treatments consisted of variations in the 2.4-D and 2-iP concentrations in the PM and SM mediums of TEIXEIRA et al. (2004), described as follows: 2.5μM of 2.4-D and 2.5μM of 2-iP (treatment 1), 2.5μM of 2.4-D and 20.0μM of 2-iP (treatment 2), 20.0μM of 2.4-D and 2.5μM of 2-iP (treatment 3), 20.0μM of 2.4-D and 20.0μM of 2-iP (treatment 4), 20.0μM of 2.4-D and

9.84µM of 2-iP in PM medium and 10.0µM of 2.4-D and 9.84µM of 2-iP in SM medium (treatment 5, TEIXEIRA et al. medium (2004), used as control).

It was used 15 replications. Each one corresponded to a replication. Genotype of 03 leaves was used as explants. Five months after, the experiment installation t was evaluated by means of explants rate containing embryogenic calli.

Statistical analyses

In the first experiment, the adjustments were tested for the four models: the first with no factor (null); the second considering the principal culture medium effect; the third incorporating the principal genotype effect and the last, the interaction. In the second experiment, the adjustments were tested for two models: the first with no factor (null) and the second considering the main effect of the genotype, and in the third one, the adjustments were tested for two models: the first with no factor (null) and the second, considering the effect of the five studied treatments.

Deviance Analysis was used in order to select the most appropriate model. Residue analyses noticed that the best adjustments were obtained with the quasibinomial family and the probit connection function. The variance homogeneity was verified and considered satisfactory. The Scott & Knott averages comparison test was used. Because the percentage data not follow a normal distribution the GLM (*Generalized Linear Model,* Computational program R®, 2008) routine was used to perform the analyses.

RESULTS

Experiment 1- Culture mediums influence and genotypes

Different behavior was noticed only for genotypes inoculated in BOXTEL & BERTHOULY (1996) culture medium. This was showed by the significant effect of the qui-square test at 5% significance. On the other hand, genotypes inoculated in TEIXEIRA et al. (2004) medium did not show any significance, demonstrating that this culture medium does not influence their behavior.

When inoculated in BOXTEL & BERTHOULY (1996) medium, the 4.2 genotype formed 19.44% of embryogenic calli, which is statistically superior to the others treatments (Table 1). In this same culture medium, no embryogenic calli formation was found in 2.2, 5.0 and 7.2 hybrids. The hybrid 5.0 was the only one which did not show any calli formation in TEIXEIRA et al. culture medium (2004).

For 2.2 and 7.2 genotypes TEIXEIRA et al. (2004) culture medium was considered superior when compared to BOXTEL & BERTHOULY (1996) medium. However 4.2 genotype showed an opposite behavior, which means the superiority of BOXTEL & BERTHOULY culture medium (1996). The 3.0 and 5.0 showed the same behavior in both culture media studied.

Those two culture media differ especially in relation to 2.4-D, 2-iP and IBA concentrations. In BOXTEL & BERTHOULY culture medium (1996), 2.4-D the concentration of it doubled from C medium (2.26µM) to E medium (4.52µM). Contrarily, in TEIXEIRA's et al. medium (2004), the 2.4-D concentration reduced by half, from medium PM (20.0µM) to medium SM (10.0µM). In this same protocol, the 2-iP and of IBA concentrations used in PM medium are kept in medium SM. However, those regulators are not used in BOXTEL & BERTHOULY (1996) secondary medium. Those auxin/cytokinin different ratios used, might have influenced on the embryogenic calli formation of the genotypes studied.

Experiment 2 – Calli formation potencial

The qui-square test showed that the genotype influenced the embryogenic calli formation. Significant difference was found among the hybrids studied, with average percentage ranging from 0 (Genotypes 14-8; 14-3 and 8-10) to 53.3% (genotype 5-14). There was high variability for the embryogenic

Table 1 - Percentage of embryogenic calli formation in leaf explants of genotypes 2.2; 3.0; 4.2; 5.0 and 7.2, developed according to the studied culture mediums.

Culture mediums	Embryogenic calli (%)[1]				
	G 2.2	G 3.0	G 4.2	G 5.0	G 7.2
BOXTEL & BERTHOULY (1996)	0.0 b B	6.11 b A	19.44 a A	0.0 b A	0.0 b B
TEIXEIRA et al. (2004)	2.94a A	6.11 a A	5.50 a B	0.0 a A	13.89a A

[1]Averages followed by the same lower case letter in the horizontal and upper case in the vertical do not differ among themselves, by the Scott & Knott test, to 5% of significance.

calli induction (Figure 1). Genotypes 20-5, 10-1, 5-14 and 6-38 showed more embryogenic calli formation than others. These results corroborate with those obtained in Experiment 1 of this research. Yet they reinforce the hypothesis that the production of somatic embryos is strongly dependent on genotypic factors.

According to the protocol, TEIXEIRA et al. (2004) genotypes which presented an average percentage of embryogenic calli induction above 25%, can be used for the production of clonal seedlings on a commercial scale. On the other hand, there is a need to improve the protocol for calli induction of genotypes 14-8; 14-3 and 8-10, which did not show any embryogenic calli formation, in order to reach the clonal multiplication process on a commercial scale.

Experiment 3 - 2-iP and 2.4-D levels influence

The qui-square test at 5% of significance showed significant difference among the studied treatments, by, which showed that the combinations of the studied regulators influenced the embryogenic calli formation. There was high variability for the induction of embryogenic calli in the different 2.4-D and 2-iP concentrations, with percentage average ranging from 2.08 to 38.46% (Figure 2).

It can be noticed that Treatment 4 (20.0µM of 2.4-D and 20.0µM of 2-iP) showed a higher percentage of embryogenic calli, and this reason was statistically superior to Treatment 1 (2.5µM of 2.4-D and 2.5µM of 2-iP), Treatment 2 (2.5µM of 2.4-D and 20.0µM of 2-iP), Treatment 3 (20.0µM of 2.4-D and 2.5µM of 2-iP) and to Treatment 5 (TEIXEIRA et al., 2004 protocol). In general a good response was obtained in embryogenic calli formation. For instance, in Treatment 4 the calli formation rate was 38.46%.

DISCUSSION

The variability in the embryogenic calli formation (from 0 to 19.44%, depending on the genotype) corroborates the result found by SANTANA et al. (2004), who claims that one of the main factors related to somatic embryogenesis in coffee concerns to the genotype influence. The somatic embryogenic potential of leaf explants from greenhouse-grown plants of eight ***C. arabica*** genotypes was investigated by BYESSE et al. (1993) who also found high variability of response for production of somatic embryos among one commercial variety and seven genotypes of ***C. arabica*** Ethiopian wild type. Leaf explants from 15 trees (genotypes) belonging to the F_3, F_4 and F_5 generations of the cross between the ***C. arabica*** cv. 'Caturra' and the 'Timor Hybrid' were used by MOLINA et al. (2002). Large variations in embryogenic capacity among genotypes were detected with rates ranging from 4.8 to 72.7%.

In their first research, about somatic embryogenesis of ***C. arabica***, SÖNDAHL & SHARP (1977) obtained relatively high rates, reaching 60% of embryogenic calli. BOXTEL & BERTHOULY (1996) found that the somatic embryogenesis induction in four genotypes of this species varied from 0% to 10%.

According to BERTHOULY & ETIENNE (1999), many Coffea species still have difficulty in regenerating in tissue culture, in spite of the great progress accomplished in the embryogenic cell induction protocols. The accumulation of phenolic compounds such a melanin, suberin, lignin and cutin around the wood plants excised surface changes the cultivation medium, which makes the metabolite intake

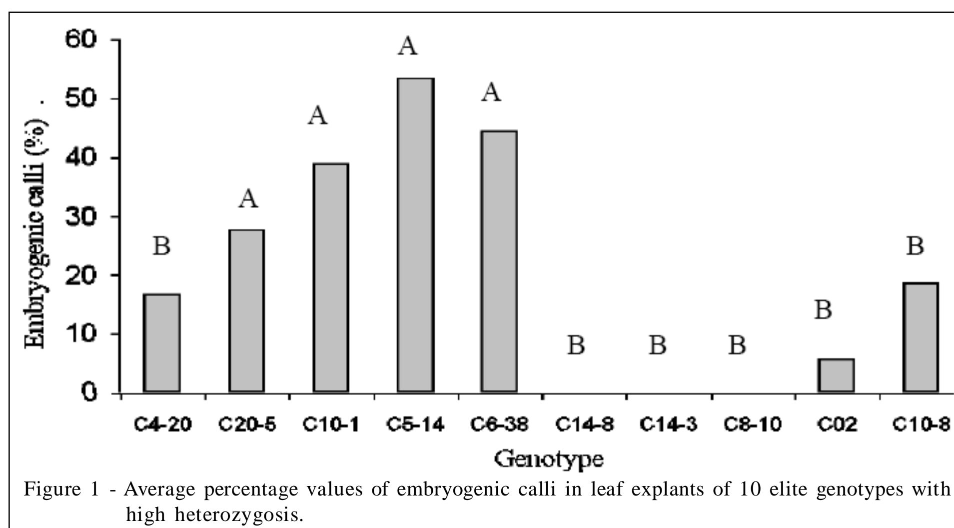

Figure 1 - Average percentage values of embryogenic calli in leaf explants of 10 elite genotypes with high heterozygosis.

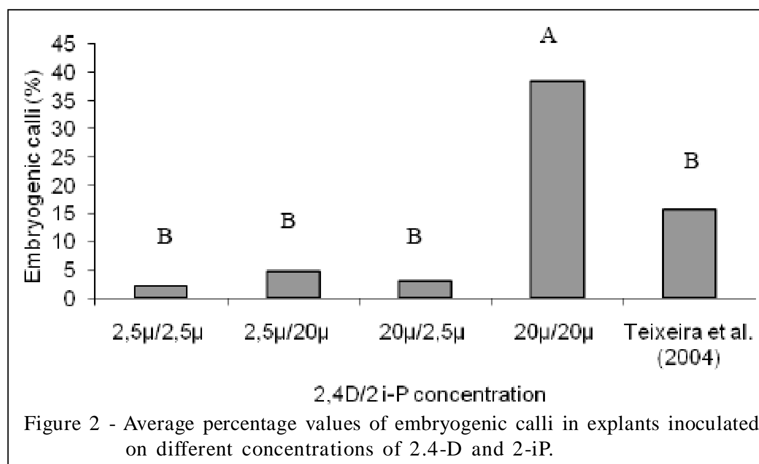

Figure 2 - Average percentage values of embryogenic calli in explants inoculated on different concentrations of 2.4-D and 2-iP.

(COSTA et al., 2007). This influence is clearly seen by the variability observed in the embryogenic calli induction frequency of the genotypes studied in the present research.

Although they generally present a better response to the embryogenic calli induction yet differences among ***C. canephora*** genotypes are frequently noticed (GATICA et al., 2007). According to these authors, the embryogenic calli formation in that species may vary from 0% to 100%, depending on the genotype, the leaf physiological condition, the collection month and the explants age.

The genotypic potential of embryogenic response is also moderated in other woody plants, such as eucalyptus (BRAVO et al., 2008) and also in herbaceous plants, such as soybean (DROSTE et al., 2010) and maize (FERNANDES et al., 2008).

The significant increase in the embryogenic calli formation was only noticed at the highest concentrations of both growth regulators (20.0µM), showing a synergistic effect when the higher 2-iP and 2.4-D doses were used. In a similar way, MACIEL et al. (2003) and PEREIRA et al. (2007) found that combined action between kinetin and auxin stimulates the somatic embryos induction. On the other hand when HATANAKA et al. (1991) studied the effect of plant growth regulators on somatic embryogenesis in leaf cultures of ***C. canephora***, all of the auxins tested (NAA, IBA, IAA and 2.4-D) inhibited the formation of embryos. Yet the maximum number of somatic embryos was obtained on media that contained only cytokinin as a plant growth regulator. 2-iP in a 5µM concentration was found to be more effective.

Abnormal somatic embryos formation, low regeneration frequency, lack of synchronization in growth, no change of these embryos into plants and no repetition of results were found to limit the development of ***C. arabica*** effective to somatic embryos protocol. Researches have been development to overcome such constraints. Therefore, with the optimization of the protocols, somatic embryogenesis has a great potential to develop hybrid varieties which generally show higher productivity than the stable cvs. and carry genes which complement then resistance to diseases and insects.

CONCLUSION

The production of somatic embryos is strongly dependent on the genotype.

The ratio of 2-iP and 2.4-D in both primary and secondary mediums influences calli induction. The 20.0µM of 2.4-D and 20.0µM of 2-iP combination caused the highest embryogenic calli induction rate.

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