Agrociencia

FIELD PHYTOPROTECTION OF *Coffea arabica* MOTHER PLANTS, DISINFECTION AND CALLOGENESIS INDUCTION

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Citation: Joya-Dávila JG, Ruíz-Sesma B, Lecona-Guzmán CA, Ruiz-Lau N, Ruíz-Valdiviezo VM, Rojas-Martínez RI, Gutiérrez Miceli FA. 2023. Field phytoprotection of *Coffea arabica* mother plants, disinfection and callogenesis induction. **Agrociencia** https://doi.org/ 10.47163/agrociencia.v57i2.2707

Editor in Chief:
Dr. Fernando C. Gómez Merino

Received: March 03, 2022.
Approved: January 08, 2023.
Published in Agrociencia: March 15, 2023.

ABSTRACT

In Chiapas, Mexico, there are high-yielding coffee plants adapted to the local climatic conditions. The presence of microorganisms in the explants makes developing protocols for cloning these genotypes *in vitro* difficult. The objective of this work was to evaluate how field and *in vitro* microorganism management affected asepsis and the induction of callogenesis in leaf explants of agronomically important coffee plants. Drought-tolerant genotypes were obtained from aseptic explants and cell cultures using a participatory study, agronomic, and *in vitro* techniques. Five accessions (ITTGj 1-5) were selected from a 21-year-old Borbon cultivar. The present study was conducted over two flowering cycles, evaluating the effect of field management on explant asepsis on the first and the callogenic response caused by the culture medium, the transport solution, and the genotype, on the second. Sixty days before explant collection, biweekly applications of calcium polysulfide (10 % v v^{-1}), copper oxychloride (2 g L^{-1}), and *Zingiber officinale* extract (20 g L^{-1}) reduced fungal contamination by 100 % and bacterial contamination by 90 %. Field explant transportation in 200 mg L^{-1} citric acid and ascorbic acid solution reduced oxidation in explants and *in vitro* cultures. Proembryogenic friable corns were formed with the combination of 4.4 µM BAP (6-benzylaminopurine) and 7.25 µM 2,4-D (2,4-dichlorophenoxyacetic acid) at 45 days of culture, with genotype four showing the best response. Field management was effective for the aseptic establishment of *Coffea arabica* cultures *in vitro*, and the combination of BAP and 2,4-D used was an appropriate growth regulator for the induction of callogenesis.

Keywords: local genotypes, *in vitro* culture, phenology.

INTRODUCTION

Coffee is one of the agricultural export goods that generates significant foreign exchange to producing countries in Africa, Latin America, and Asia, supporting the livelihoods of approximately 125 million farmers (de Sousa *et al*., 2021). In Chiapas,

high production coffee trees adapted to local climatic conditions have been observed, with the potential to increase coffee production per unit area. *In vitro* tissue culture is a biotechnological tool that allows for the mass propagation of local coffee plants while preserving their genetic characteristics (Viltres-Barbán *et al*., 2021).

For the establishment of *in vitro* cultures of *Coffea arabica*, it is necessary to select the genotype to be cloned (based on agronomic attributes), establish phytoprotection protocols for the mother plants prior to explant collection, and *in vitro* disinfection to avoid explant contamination. Because a differential presence and abundance of phyllospheric communities in leaves at different growth stages has been reported, the phenological stage of the plant must be considered (Li *et al*., 2021). In this regard, Lu *et al*. (2022) reported 648 fungi species in coffee plants, 46 % of which are pathogens and 22 % are endophytes. In *Coffea* species, *Trichoderma*, *Mycosphaerella*, *Cladosporium*, and *Xylaria* are mainly reported as endophyte genera; and *Fusarium*, *Alternaria*, and *Phoma* as pathogens (de Sousa *et al*., 2021). In *in vitro* culture, these microorganisms cause contamination.

On the other hand, determining the appropriate concentrations of growth regulators (RGC) and the optimal culture medium for callogenesis induction (López-Gómez *et al*., 2016) is another factor to consider. There are protocols for the propagation of coffee plants through somatic embryogenesis using young leaves collected from plants established in greenhouses; however, reports of agronomic management in the field prior to *in vitro* cultivation are scarce. Arias-Pérez *et al*. (2021) describe the application of chemical fungicides three days before the collection of explants in various coffee plantations, with contamination levels ranging from 40 to 100 %. This justifies the need to evaluate multiple disinfection protocols based on the species, phenological stage, and geographical location of mother plant (Silva *et al.,* 2015). Callogenesis has been induced in coffee plants by combining different types of auxins and cytokinins based on genotype, which required the establishment of the appropriate concentrations and combinations of growth regulators (Sanglard *et al*., 2019). The objective of this work was to evaluate the effect of field and *in vitro* management of microorganisms on explant asepsis and induction of callogenesis in *C. arabica*.

MATERIALS AND METHODS

Participatory selection of plant material

Plant material was collected in two annual flowering cycles beginning in 2019 in coffee plantations in the municipality of Angel Albino Corzo, Chiapas, Mexico. Information on local climate, phytopathogen prevalence, and desired characteristics was gathered and used to choose the criteria for the selection of mother plants (Joya-Dávila and Joya-Dávila, 2020). Subsequently, during the dry season, plantations were visited with a group of producers. Coffee trees showing vigor and tolerance to water deficit were marked. For the development of this work, five eight-year-old accessions were selected from a genotype of the Borbon variety with 21 years of cultivation, located

in the same coffee plantation. Accessions ITTGj-1, 2, and 3 were used to develop a protocol for managing phytopathogens in the field and for *in vitro* disinfection. For cell culture induction, accessions ITTGj-4 and 5 were used.

The coffee plantation is located in a mountainous area at an altitude of 980 m, with a slope of 47.8 %; it has permanent shade, consisting primarly of trees of the genus *Inga* (*I. spuria*, *I. vera*, and *I. laurina*). It is managed organically under rainfed conditions.

Aseptic establishment of *in vitro* explants

Agronomic management of phytopathogens in the field

The percentage of contaminated explants was evaluated in response to three field treatments. For this study, the methodology described by Arias-Pérez *et al*. (2021) and Ortiz-Gómez *et al*. (2017) was adapted; each treatment corresponded to one accession. Spraying was established biweekly 60 days prior to collection, according to the BBCH scale stages for *C. arabica* as reported by Arcila-Pulgarín *et al*. (2002). The first spray (day 0) comprised the FL-57 stage and the fourth (day 45) the FR-70 stage (Table 1). All spraying was carried out within a 10 m radius, including surrounding coffee trees.

Table 1. Fungicide sprays prior to leaf explant collection.

	Treatments†	Spray (Day)						
		0	15	30	45	57	58	59
1	No management	-	-	-	-	-	-	-
2	Precollection	-	-	-	-	PC	OC	PC
3	Biweekly + precollection	PC	PC	OC	EJ	PC	OC	PC

†PC: 10 % calcium polysulfide; OC: copper oxychloride 2 g L^{-1}; EJ: *Zingiber officinale* extract 20 g L^{-1}.

Collection, transportation, and disinfection of foliar explants

Thirty days after flowering (phenological stage FR-71), leaves were collected from the second plagiotropic apical growth (20 per accession) and washed with 1 % Axión® commercial detergent. They were then immersed in fungal antioxidant solution (200 mg L^{-1} citric acid and ascorbic acid + 1 mL L^{-1} Previcur® (Bayer, 530 g L^{-1} propamocarb and 310 g L^{-1} fosetyl)). Using a potentiometer (Thermo Scientific® model Orion 3 Star, CA, USA), the pH of the solution was adjusted to 5.7 with NaOH or 1N HCl. The solutions were autoclaved at a pressure of 103.42 kPa for 15 min. All samples were transported to the plant tissue culture laboratory of the Instituto Tecnológico de Tuxtla Gutiérrez (ITTG).

In a flow hood, the leaves were disinfected with commercial chlorine of the brand Cloralex® (sodium hypochlorite 4–6 %) at 30 % v v^{-1}, followed by alcohol (70 % v v^{-1}) for 10 and 5 min, respectively, under continuous agitation. At the end of each disinfection

step, the leaves were rinsed three times with sterile distilled water. For each treatment, the leaves were sectioned into 1 cm^2 explants without veins or domatia. Four explants were seeded in glass vials of 110 mL capacity with 20 mL of sterile 50 % MS medium (Murashige and Skoog, 1962), its pH was adjusted to 5.7, and it was solidified using 2.5 g L^{-1} of phytagel (Sigma-Aldrich, St. Louis, MO, USA). They were incubated in a bioclimatic chamber at 25±1 °C in complete darkness. After 15 days of culture, the percentage of contaminated explants was evaluated.

Induction of *in vitro* cultures

In a new flowering cycle, the field management, collection, and *in vitro* disinfection protocols were replicated in ITTGj 4 and 5 accessions with two modifications: first, an antibacterial agent was added to the transport solution, and a second solution containing antioxidants was evaluated (Table 2); it was sterilized under the same conditions previously described. The antifungal and antibacterial agents were added in a laminar flow hood using a Whatman® syringe filter. Second, the exposure time to sodium hypochlorite was increased by 5 min.

Table 2. Composition of foliar explant transport solutions.

Solution	Antioxidant		Antibacterial	Antifungal
Gj-1	AC	AA	AG	PV
Gj-2	AC	AA	-	-

AC: citric acid 200 mg L^{-1}; AA: ascorbic acid 200 mg L^{-1}; AG: Agrimycin® 1 g L^{-1}; PV: Previcur®.

Callogenesis induction

Aseptic explants were seeded in 50 % MS culture medium with sucrose (30 g L^{-1}), citric acid, and ascorbic acid (200 mg L^{-1}), supplemented with different growth regulators, and a control without regulator. Growth regulators and culture conditions were established according to the methodology proposed by Sanglard *et al*. (2019). The treatments were: 50 % MS + 4.91 μM naphthaleneacetic acid (ANA); 50 % MS + 7.25 μM 2,4-dichlorophenoxyacetic acid (2,4-D); 4.4 μM 6-benzylaminopurine (BAP) was added to both treatments. The pH of the medium was adjusted to 5.7 and it was solidified with 2.5 g L^{-1} of phytagel (Sigma-Aldrich, St. Louis, MO, USA). The explants were incubated in bioclimatic chamber at 25±1 °C in darkness. After 45 days of culture, the percentage of contaminated explants, oxidized explants, proembryogenic callus induction, and friability were evaluated by the interaction of three factors: A) two genotypes, B) two transport solutions, and C) three culture media, for a total of 12 treatments.

In vitro culture propagation conditions

The biomass multiplication capacity of media one (2,4-D) and two (ANA) was evaluated. 200 mg of callus were cultured on the described media and kept in total darkness at 25±1 °C for six weeks. To test the capacity for embryogenic callus formation, 200 mg of callus were transferred to semi-solid BAP medium (MS + 17.6 μM BAP + 1.8 μM 2,4-D + 30 μM $AgNO_3$). At eight weeks, embryogenic callus induction was assessed.

In the case of liquid medium, the cultures were kept in agitation at 100 rpm in the dark. In the end, cell viability was determined by incubating the cell sample mixed with trypan blue (0.4 %) 1:1 v v^{-1} for one minute; unstained cells were considered viable.

Experimental design and statistical analysis

The experiments were established in a completely randomized design with 20 replicates per treatment in agronomic management. In the case of the *in vitro* culture induction trial, a 3x2x2 factorial arrangement with twelve replicates was used. Percentage data were transformed using sine arc square root. For each variable, analysis of variance and Tukey's mean comparison test ($p \leq 0.05$) were performed using the SAS® OnDemand statistical package.

RESULTS AND DISCUSSION

Participatory selection of plant material and collection season

The institutional linkage with Chiapas coffee growers made access to the local geography and the selection of ITTGj 4 and 5 genotypes as mother plants easier. This is because they are more widely accepted by growers, have higher leaflet production, and show no visible signs of deficit water stress according to the IPGRI (1996) scale in drought (February 2020). Participatory selection uses local germplasm and provides an alternative to the use of introduced coffee plants, which present adaptation challenges and can be a source of pests and diseases entering the country (Joya-Dávila and Joya-Dávila, 2020).

Flowering began in April (second week) in accordance with the onset of the first rains. A single phenological cycle was observed without overlapping, making agronomic task management and programming easier. In the first 45 days after flowering, there were no symptoms of disease in the growing leaflets, which is why explants were collected on day 30. During floral opening, the production and maturation of leaflets useful in *in vitro* tissue culture increased, coinciding with the findings of Marín *et al*. (2018), who reported that FL-65 presented the highest leaf area values.

In a coffee plantation adjacent to the study area, Arias-Pérez *et al*. (2021) reported high rates of fungal contamination (60 %) during the fruiting season when field explants were collected. In this event, the prevalence of pests and phytopathogens increased under the growing conditions in Chiapas and the heavy rainfall which caused landslides and an increase in riverbeds, making access to the coffee plantations difficult; therefore, the recommendation is not to collect at this time of year.

Aseptic establishment of *in vitro* explants

The effect of pre-harvest management in *C. arabica* (first flowering cycle) on the percentage of *in vitro* contamination was recorded (Table 3). Treatment three was the most effective at controlling fungal (100 %) and bacterial (90 %) contamination. In this same species, Ortiz-Gómez *et al*. (2017) reported 100 % asepsis due to the effect of 24 weekly sprays of chemical fungicides (unspecified) applied over a six-month period between the vegetative and flowering stage.

Table 3. *In vitro* contamination by microorganisms in field-collected explants of *Coffea arabica* cv. Borbon.

	Treatments Fungicide spraying (Days)							Contamination (%)	
	0	15	30	45	57	58	59	Fungi	Bacteria
1	-	-	-	-	-	-	-	85A*	70A
2	-	-	-	-	PC	OC	PC	40B	45AB
3	PC	OC	PC	EJ	PC	OC	PC	0C	10B
p =								0.0001	0.0035

*Different letter in the same column indicates a significant statistical difference (Tukey, $p \leq 0.05$). PC: 10 % calcium polysulfide; OC: copper oxychloride 2 g L^{-1}; EJ: ginger extract 20 g L^{-1}.

The management carried out in this work reduces the application of fungicides and provides an alternative for the organic management of mother plants under cultivation conditions; its effectiveness is related to the protective coverage of each application, in accordance with the development and phenological growth of the coffee plant. In this regard, Martins *et al*. (2014) reported that copper protectant fungicides should be reapplied as plants grow to maintain coverage and prevent diseases. On the other hand, Jiménez-Terry *et al*. (2007) demonstrated that field management improves the vigor and survival of explant donor plants of local agronomic importance. It is important to note that the fungicides used are authorized by the new organic products law DOF 07-02-2006 (SENASICA, 2020); therefore, they did not interfere with the traditional management carried out by the producer.

Treatment two, which involved the intercalated spraying of PC and OC three days prior to collection, reduced *in vitro* contamination, 45 % fungal and 25 % bacterial, when compared to the unmanaged treatment. Arias-Pérez *et al*. (2021) reported that a three-day pre-harvest management with Captan® and Agrimycin® on field plants does not fully control the presence of fungi *in vitro* (40 % effectiveness). Similarly, López-Gómez *et al*. (2011) reported that spraying 1 g L^{-1} of Amistar® (Syngenta, Azoxystrobin 50 %) for three days on *Coffea* sp. genotypes resulted in 50 to 100 % aseptic explants, with differential response among genotypes and the highest contamination observed in *C. arabica* genotypes. Cupric and sulfur fungicides acted preventively, forming a protective barrier on the leaf surface against phytopathogens. Copper oxychloride is

used to reduce the incidence of *Hemileia vastatrix* by up to 75 % (Zambolin, 2016); when this compound comes into contact with water, it slowly and continuously releases Cu^{2+} ions that, when absorbed by the spores, inhibit their germination. The ions bind to various chemical groups (hydroxyl, sulfhydryl, and phosphate) found in many enzymes and proteins of phytopathogens, ultimately generating cell damage and membrane loss (Husak, 2015).

Calcium polysulfide has an invigorating effect on *C. arabica*. When applied to leaves, an increased production of cuticular waxes (protective barrier to stress) is observed; Fedyaev *et al*. (2018) reported increased tissue formation in *Triticum aestivum*, possibly due to a decrease in oxidative stress by stimulating catalase and peroxidase enzymatic activity. On the other hand, it inhibits fungal growth under natural conditions and is a precursor of phenol production in bell pepper, which helps to reduce the incidence of *Botrytis cinerea* (Erdem and Karaca, 2020). To avoid possible resistance of phytopathogens to the application of mineral fungicides, the application of ginger extract was included due to its inhibitory effect on the germination of filamentous fungal spores (Joya-Dávila *et al*., 2015).

The unmanaged control had the highest contamination rates in this study. The Previcur® fungicide present in the transport solution, together with the laboratory disinfection protocol alone did not reduce the in vitro presence of microorganisms in the explant; Sanchez *et al*. (2019) found that 100 % *in vitro* asepsis was obtained in six-month-old coffee plants grown in greenhouses without pre-harvest management, indicating that these plants have a lower microbial load than organically managed crops. When compared to monocultures and nursery plants, coffee plantations with shade have more diverse plant-microorganism interactions. In these management systems, the presence of arbuscular fungi and endophytic bacteria has been estimated, which can be problematic when establishing *in vitro* cultures, even though they are beneficial to the plant in *ex vitro* culture (Munroe *et al*., 2015).

In the unmanaged treatment, mycelium and bacteria were observed seven days after the *in vitro* establishment (Figure 1) under the light microscope, exhibiting the same characteristics in all explants (Figure 1A-D), according to the morphotype reported by Rentería-Martínez *et al*. (2018). Based on mycelial color, growth type, and presence of conidiophores and macroconidia, it was identified as *Fusarium* sp.

After disinfection, mycelial growth on explants originates from vascular ducts (Figure 1A) and domatias (Figure 1 E–F). Microorganisms are present in these parts of the leaf, which is why, under *in vitro* conditions, the explant is usually the main source of contamination. To reduce the presence of microorganisms, it is advisable to select young, non-coriaceous leaves and cuticles with a low proportion of waxes, which can be removed with detergent solutions; additionally, remove the nervures and domatias. In this regard, Tafolla-Arellano *et al*. (2013) demonstrated that the composition of the cuticle and its hydrophobic nature determine the type of particles that can lodge on the leaf surface (dust, spores), which increases with maturation, so mature leaves are not recommended.

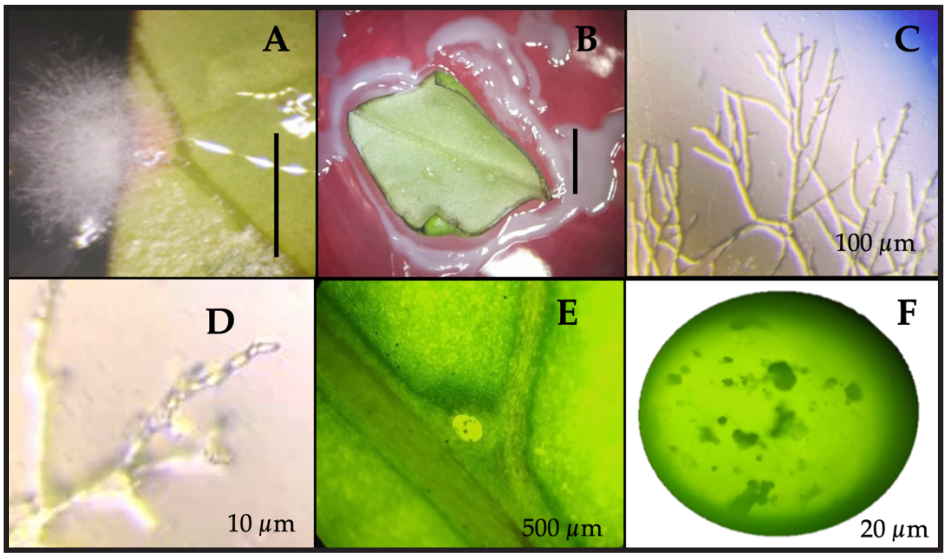

Figure 1. Mycelial and bacterial growth in explants of *Coffea arabica*. A: mycelium in vascular bundles; B: bacteria around the explant; C: filamentous mycelium; D: conidiophores and macroconidia of *Fusarium* sp.; E: domatia, crypt-like exterior view; F: interior of the domatia, presence of particles and spores. Bar corresponds to 0.5 cm.

Induction of *in vitro* cultures

Obtaining aseptic explants

Field handling and *in vitro* disinfection protocols performed in the second flowering cycle resulted in 100 % aseptic explants (Figure 2A).

The vigor expressed in the explants allowed them to withstand the stress of disinfection protocols, a response that may be reflected in their nutritional content. Marín *et al*. (2018) reported that leaves in flowering coffee trees contain higher concentrations of nutrients and chlorogenic acid than leaves in other stages (3-CQA). Luján-Hidalgo *et al*. (2020) reported that coffee plants increase the content of 3-CQA in the leaves in response to the fungus *H. vastatrix*, conferring them resistance, which means they would be using chlorogenic acids in response to phytopathogen attack.

The transport solution had no effect on the disinfection protocol, therefore, under this management, the application of the antibacterial and antifungal agents can be suppressed, leaving only the antioxidants to prevent oxidation of the leaves during transport. On the other hand, in comparison to the first flowering cycle, increasing the immersion time in sodium hypochlorite reduced bacterial contamination to zero. In this regard, López-Gómez *et al*. (2011) found that sodium hypochlorite is effective in controlling *in vitro* contamination. Depending on the tolerance of the coffee plant to the disinfectant and the time of collection, it is necessary to adjust the dose and exposure time according to the genotype evaluated and its phenology. Finally, it was

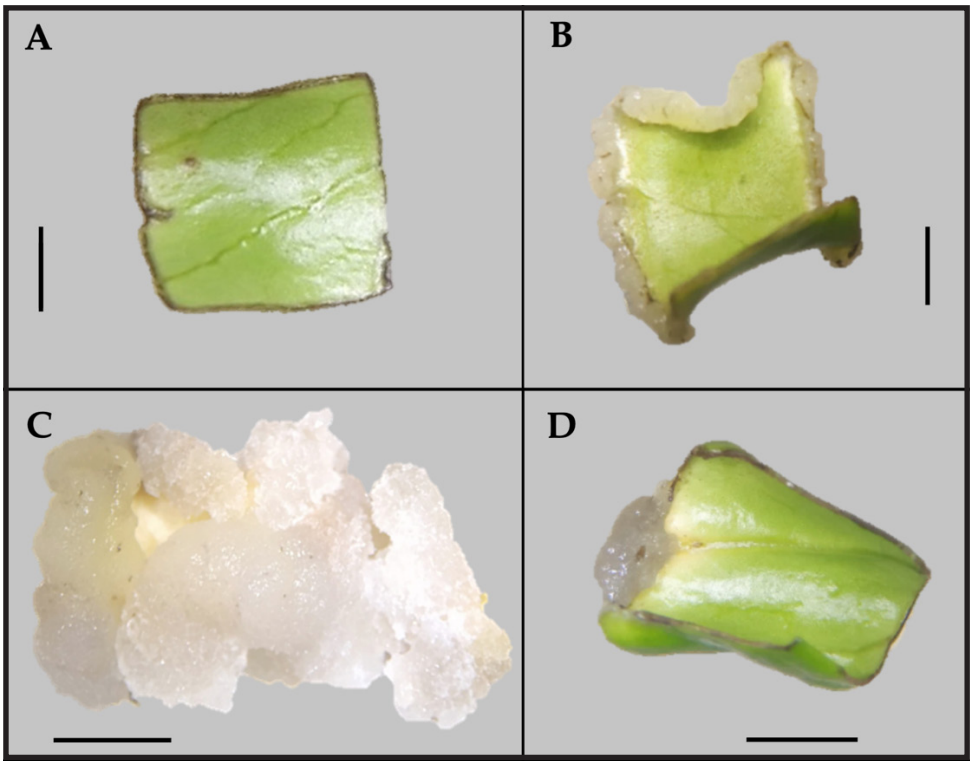

Figure 2. Induction of pro-embryogenic callus in explants of *Coffea arabica* cv. Borbon. A: aseptic leaf explant (day 0); B: callogenesis in leaf explant (day 15); C: embryogenic callus with 2,4-D (day 45); D: aqueous callus with ANA (day 45). Bar equals 0.5 cm.

observed that the genotype does not influence the disinfection process, indicating that this methodology can be used effectively on *C. arabica* cultivars.

Callogenesis induction

The use of 4.4 µM BAP in combination with the auxins evaluated resulted in callogenesis (Table 4); with 2,4-D, induction was observed in all explants, whereas with ANA, induction was genotype dependent, with greater response in ITTGj-4. From the control without regulators, callus formation in response to the cut wound was observed in some explants, but did not proliferate in subsequent days of culture. In contrast to the 2,4-D treatment, where callus formation and cream-colored cell cluster formation were observed in all four explant sections (Figure 2B), slowly growing aqueous callus formed with ANA in one or two explant sections (Figure 2D).

López-Gómez *et al*. (2016), in robusta coffee trees, observed differential response to the use of auxins and cytokinins, including the formation of proembryogenic friable callus and direct embryogenesis. When no RGCs are used in the culture medium, explants do not generate callus. Callus induction is a physiological process that demands growth

Table 4. Percentage of *C. arabica* explants with callogenic response by effect of genotype, growth regulator and transport solution.

Treatments			Callogenesis		Friability	CPE	Oxidation
A	B	C	Induction	Growth			
No regulators	Gj-1	ITTGj-4	25C[†]	6D	0D	0B	0D
2,4-D[¶] + BAP	"	"	100A	100A	100A	100A	40C
ANA[§] + BAP	"	"	92A	39BC	92AB	0B	69ABC
No regulators	Gj-3	"	0C	0D	0D	0B	0D
2,4-D[¶] + BAP	"	"	100A	100A	100A	100A	58BC
ANA[§] + BAP	"	"	92A	43B	92AB	0B	35CD
No regulators	Gj-1	ITTGj-5	17C	4D	0D	0B	0D
2,4-D[¶] + BAP	"	"	100A	100A	100A	100A	100A
ANA[§] + BAP	"	"	58B	25C	67C	0B	94AB
No regulators	Gj-3	"	0C	0D	0C	0B	0D
2,4-D[¶] + BAP	"	"	100A	100A	100A	100A	81AB
ANA[§] + BAP	"	"	75AB	39BC	75BC	0B	69ABC
R^2			0.950	0.913	0.9822	1	0.925
P=	Treatment		0.0001	0.0001	0.0001	0.0001	0.0001
	Growth regulator (A)		0.0001	0.0001	0.0001	0.0001	0.0001
	Transport solution (B)		0.268	0.524	0.5691	-	0.0314
	Genotype (C)		0.014	0.112	0.0081	-	0.0002
	Interaction A*B		0.010	0.023	0.7198	-	0.0131
	Interaction A*C		0.231	0.159	0.0018	-	0.0017
	Interaction B*C		0.268	0.340	0.5691	-	0.2643
	Interaction A*B*C		0.656	0.587	0.7198	-	0.0843

[†]Different letters in the same column indicate significant statistical difference (Tukey, $p \leq 0.05$). [¶]7.25 µM 2,4-D + 4.4 µM BAP; [§]4.91 µM ANA + 4.4 µM BAP; Gj-1: citric acid 200 mg L^{-1} (AC) + ascorbic acid 200 mg L^{-1} (AA) + Agrimycin® 1 g L^{-1} + Previcur®; Gj-3: AC + AA.

regulators for cell division and differentiation, auxins are effective in stimulating cell elongation and vascular differentiation, while cytokinins are critical in stimulating cell division (Kumar *et al*., 2016).

In *C. arabica*, the differential response to RGC could be related to the endogenous level of auxins in coffee plants; exogenous auxins can cancel polarity by diffusing into embryogenic masses, causing the inhibition of the process initiated by endogenous auxins. Therefore, future research would focus on determining the type and appropriate dose of these auxins by evaluating the kinetics of endogenous RGC production in *in vitro Coffea* cultures.

No oxidation occurred in explants grown on MS medium without RGC. Treatments with 2,4-D and ANA resulted in statistically similar partial oxidation, with a higher number of oxidized explants in ITTGj-5; in this genotype, when explants were transported in Gj-3 solution, oxidation decreased. This result is similar to that reported by López-Gómez *et al*. (2011), where they decreased the oxidation of *C. arabica* explants using ascorbic acid and citric acid, prior to *in vitro* disinfection.

The transport solution had no effect on callogenesis, contrary to the genotype and RGC factor. The explants grown in 50 % MS medium + 2,4-D induced friable and proembryogenic callus in both genotypes; the use of ANA induced friable callus, but of watery consistency, with a greater presence in the ITTGj-4 genotype. After six weeks of culture on ANA medium, the callus did not survive; 2,4-D induced callus were friable cream-colored with proembryogenic characteristics (Figure 3A), and when transferred to BAP medium after eight weeks of culture, embryogenic callus induction was observed (Figure 3B).Viable, constantly dividing cells in suspension were obtained (Figure 3C), with a defined nucleus and a negative reaction to the trypan blue test.

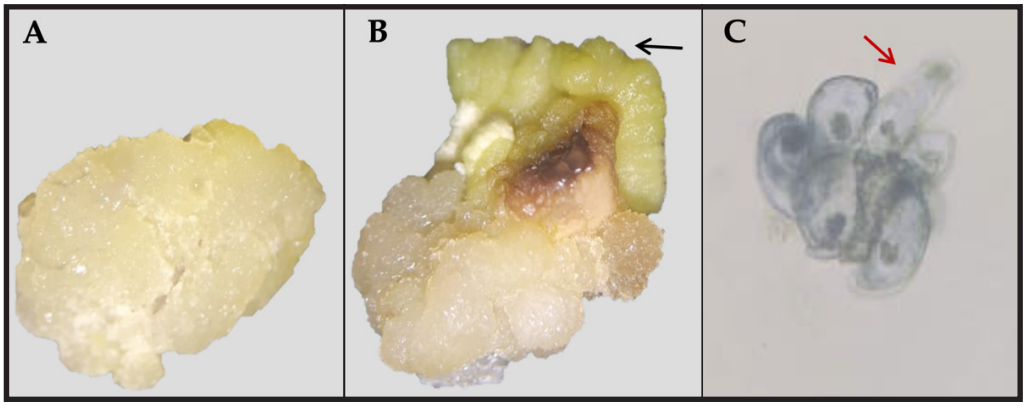

Figure 3. Callus induction of *Coffea arabica*. A: proembryogenic callus in 2,4-D and BAP; B: embryogenic callus in MS medium + 17.6 µM BAP + 1.8 µM 2,4-D + 30 µM $AgNO_3$; C: viable cells in medium with 2,4-D and liquid BAP. Black arrow indicates embryogenic callus; red arrow indicates dividing cells.

Similar results were obtained to those reported by Sanglard *et al*. (2019), who used 2,4-D (9.06 µM) and BAP (4.44 µM) to induce proembryogenic callus. After two months of cultivation, when suppressing the use of 2,4-D, embryogenic callus was induced, indicating that the combination of auxins and cytokinins affect the induction of embryogenic callus in *C. arabica*, a response observed in our experiment. Similarly, the addition of $AgNO_3$ to the MS medium and the growth regulators BAP and 2,4-D stimulated embryogenic callus growth.

CONCLUSIONS

The interaction with coffee growers facilitated a faster selection of genotypes adapted to the climatic conditions of the study area. It was determined that entering the coffee plantation 30 days after the main flowering *C. arabica* is the best time to collect leaf explants. Field management based on phenological stages of the coffee plant, transportation of leaf explants in antioxidant solutions, and *in vitro* disinfection allowed

for aseptic establishment. Finally, the combination and ratio of 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid showed the highest callogenic response and cell culture formation.

ACKNOWLEDGEMENTS

Our thanks to the Consejo Nacional de Ciencia y Tecnología (CONACyT-Mexico) for the support granted through the scholarship for JDJG (CVU. 789022) and the Tecnológico Nacional de México (TecNM) for its financial support.

REFERENCES

Arcila-Pulgarín J, Burh L, Bleiholder H, Hack H, Meier U, Wicke H. 2002. Application of the extended BBCH scale for the description of the growth stages of coffee (*Coffea* spp.). Annals of Applied Biology 141 (1): 19–27. https://doi.org/10.1111/j.1744-7348.2002.tb00191.x

Arias-Pérez E, Lecona-Guzmán C, Gutiérrez-Miceli F, Montes-Molina J, Ruiz-Lau. 2021. Encapsulation of immature somatic embryos of *Coffea arabica* L. for *in vitro* preservation. Phyton 90 (6): 1741–1748. https://doi.org/10.32604/phyton.2021.016004

de Sousa LP, Guerreiro Filho O, Costa Mondego JM. 2021. Differences between the leaf mycobiome of *Coffea arabica* and wild coffee species and their modulation by caffeine/chlorogenic acid content. Microorganisms 9 (11): 2296. https://doi.org/10.3390/microorganisms9112296

Erdem A, Karaca G. 2020. Alternative treatments against gray mold on pepper. Ziraat Fakültesi Dergisi 15 (2): 129–137.

Fedyaev VV, Farkhutdinov RG, Massalimov IA, Tsvetkov VO. 2018. Effect of calcium polysulfide on morphometric, physiological and biochemical processes of wheat plants. Proceedings of Universities Applied Chemistry and Biotechnology 8 (2): 55–62. https://doi.org/10.21285/2227-2925-2018-8-2-55-62

Husak V. 2015. Copper and copper-containing pesticides: metabolism, toxicity and oxidative stress. Journal of Vasyl Stefanyk Precarpathian National University 2 (1): 38–50. https://doi.org/10.15330/jpnu.2.1.38-50

IPGRI (International Plant Genetic Resources Institute). 1996. Descriptors for coffee (*Coffea* spp. and *Psilanthus* spp.). Roma, Italia. 36 p. https://www.bioversityinternational.org/fileadmin/user_upload/online_library/publications/pdfs/365.pdf (Recuperado: enero 2022).

Jiménez-Terry F, Barbón R, La O M, Pérez M, Collado R, Acosta-Suárez M, Alvarado-Capó Y, Agramonte D. 2007. Efecto de la revigorización en el establecimiento *in vitro* de ápices y segmentos nodales de *Cedrela odorata* L. Biotecnología Vegetal 7 (1): 45–51.

Joya-Dávila JG, Joya-Dávila RH. 2020. Mejoramiento genético de cultivares: un enfoque participativo ligado a la soberanía alimentaria. *In* Cigarroa-Vázquez F, Esponda-Hernández W, Mendoza-Nazar P. (eds.), Experiencias para lograr la soberanía alimentaria y sustentabilidad. Universidad Autónoma de Chiapas: Tuxtla Gutiérrez, México, pp: 97–114. https://www.cicav.unach.mx/images/CICAV_2020.pdf (Recuperado: enero 2022).

Joya-Dávila JG, Ramírez-González S, López-Báez O, Alvarado-Gaona A. 2015. Efecto antifúngico de hidrodestilados de *Zingiber officinale* Roscoe sobre *Moniliophthora roreri* (Cif & Par). Ciencia y Agricultura 12 (2): 21–29. http://dx.doi.org/10.19053/01228420.4350

Kumar S, Singh R, Kalia S, Sharma SK, Kalia RK. 2016. Recent advances in understanding the role of growth regulators in plant growth and development *in vitro*-I, conventional growth regulators. Indian Forester 142 (5): 459–470.

Li Y, Zhang Z, Liu W, Ke M, Qu Q, Zhou Z, Qian H. 2021. Phyllosphere bacterial assemblage is affected by plant genotypes and growth stages. Microbiological Research 248: 126743. https://doi.org/10.1016/j.micres.2021.126743

López-Gómez P, Iracheta-Donjuan L, Ojeda-Zacarías Ma. del C, Ducos JP. 2016. Medio de cultivo e inhibidores de etileno en la embriogénesis somática del café. Revista Mexicana de Ciencias Agrícolas 7 (7): 1749–1757.

López-Gómez P, Iracheta-Donjuan L, Castellanos-Juárez M, Méndez-López I, Aguirre-Medina J, Gutiérrez-Díez A, Ojeda-Zacarías Ma. del C, Pérez-Pérez BR. 2011. Variación en la tolerancia a desinfectantes de genotipos élite de *Coffea* spp. cultivado *in vitro*. Revista Mexicana de Ciencias Agrícolas 2 (5): 645–657.

Lu L, Tibpromma S, Karunarathna SC, Jayawardena RS, Lumyong S, Xu J, Hyde KD. 2022. Comprehensive Review of Fungi on Coffee. Pathogens 11 (4): 411. https://doi.org/10.3390/pathogens11040411

Luján-Hidalgo MC, Jiménez-Aguilar L, Ruiz-Lau N, Reyes-Zambrano SJ, Gutiérrez-Miceli FA. 2020. Cambios bioquímicos en respuesta al ataque de roya en plantaciones de café. Polibotánica 49: 149–160. https://doi.org/10.18387/polibotanica.49.10

Marín-Garza T, Gómez-Merino FC, Aguilar-Rivera N, Murguía-González J, Trejo-Téllez LI, Pastelín-Solano MC, Castañeda-Castro O. 2018. Composición bioactiva de hojas de café durante un ciclo anual. Revista Fitotecnia Mexicana 41 (4): 365–372. https://doi.org/10.35196/rfm.2018.4.365-372

Martins V, Teixeira A, Bassil E, Blumwald E, Geros H. 2014. Metabolic changes of *Vitis vinifera* berries and leaves exposed to Bordeaux mixture. Plant Physiology and Biochemistry 82: 270–278. https://doi.org/10.1016/j.plaphy.2014.06.016

Munroe JW, Soto G, Virginio Filho E. de M, Fulthorpe R, Isaac ME. 2015. Soil microbial and nutrient properties in the rhizosphere of coffee under agroforestry management. Applied Soil Ecology 93: 40–46. https://doi.org/10.1016/j.apsoil.2015.04.003

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiologia Plantarum 15: 473–493. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ortiz-Gómez N, Turiño-Peña M, Jiménez-Ferrer L. 2017. Efecto de diferentes variantes para la desinfección en hojas de *Coffea arabica* L. en el establecimiento *in vitro*. Revista Café Cacao 16 (1): 9–14.

Rentería-Martínez ME, Guerra-Camacho MÁ, Ochoa-Meza A, Moreno-Salazar SF, Meza-Moller ADC, Guzmán-Ortiz JM. 2018. Description and comparison among morphotypes of *Fusarium brachygibbosum*, *F. falciforme* and *F. oxysporum* pathogenic to watermelon in Sonora, México. Revista Mexicana de Fitopatología 37 (1): 16–34. https://doi.org/10.18781/r.mex.fit.1808-1

Sánchez K, Cabrera-Pintado R, Jiménez-Dávalos J. 2019. Induction of somatic embryogenesis from foliar explants in three varieties of coffee. Scientia Agropecuaria 10 (2): 259–264. https://doi.org/10.17268/sci.agropecu.2019.02.11

Sanglard NA, Amaral-Silva PM, Sattler MC, de Oliveira SC, Cesário LM, Ferreira A, Carvalho CR, Clarindo WR. 2019. Indirect somatic embryogenesis in *Coffea* with different ploidy levels: a revisiting and updating study. Plant Cell, Tissue and Organ Culture 136: 255–267. https://doi.org/10.1007/s11240-018-1511-9

SENASICA (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria). 2020. Organic Products Law. Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria. Ciudad de México, México. https://www.gob.mx/senasica/documentos/ley-de-productos-organicos (Recuperado: enero 2022)

Silva AT, Barduche D, do Livramento KG, Paiva LV. 2015. A putative BABY BOOM-like gene (CaBBM) is expressed in embryogenic calli and embryogenic cell suspension culture of *Coffea arabica* L. *In Vitro* Cellular and Developmental Biology - Plant 51 (1): 93–101. https://doi.org/10.1007/s11627-014-9643-z

Tafolla-Arellano JC, González-León A, Tiznado-Hernández ME, Zacarías-García L, Báez-Sañudo R. 2013. Composition, physiology, and biosynthesis of plant cuticle. Revista Fitotecnia Mexicana 36 (1): 3–12.

Viltres-Barbán D, Pérez-Pérez JL, Robles-Pastrana J. 2021. Efecto de reguladores de crecimiento en la embriogénesis somática del híbrido de café Velazco-5. Hombre, Ciencia y Tecnología 25 (1): 84–90.

Zambolin L. 2016. Current status and management of coffee leaf rust in Brazil. Tropical Plant Pathology 41 (1): 1–8. https://doi.org/10.1007/s40858-016-0065-9