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EVALUATION OF IN VITRO CULTURE MEDIA FOR THE MULTIPLICATION OF AVOCADO (PERSEA AMERICANA) ROOTSTOCKS CV. HASS, FREE OF PHYTOPHTHORA CINNAMOMI, IN THE DEPARTMENT OF TOLIMA

AVALIAÇÃO DE MEIOS DE CULTIVO IN VITRO PARA A MULTIPLICAÇÃO DE PORTA-ENXERTOS DE ABACATEIRO (PERSEA AMERICANA) CV. HASS, LIVRES DE PHYTOPHTHORA CINNAMOMI, NO DEPARTAMENTO DE TOLIMA

EVALUACIÓN DE MEDIOS DE CULTIVO IN VITRO, PARA LA MULTIPLICACIÓN DE PORTAINJERTOS DE AGUACATE (PERSEA AMERICANA) CV. HASS, LIBRES DE PHYTOPHTHORA CINNAMOMI EN EL DEPARTAMENTO DEL TOLIMA

https://doi.org/10.56238/sevened2025.036-159

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ABSTRACT

Avocado (Persea americana Mill) has become a crop of vital economic importance for Colombia; however, its productivity faces serious limitations due to the incidence of Phytophthora cinnamomi and the genetic variability resulting from seed propagation. The present study aimed to evaluate protocols for the in vitro establishment and multiplication of avocado rootstocks of the Criollo variety, ensuring pathogen-free plant material for the Department of Tolima. The methodology used nodal segments from mother plants previously analyzed by ELISA testing. Two disinfection protocols (P1 and P2) and three culture media (Murashige and Skoog – 50% MS, WPM, and Yasuda) were compared, evaluating contamination, oxidation, and viability variables. The results showed that Protocol 1 presented a significantly lower contamination rate (57.8%) compared with Protocol 2 (79.5%). Although oxidation was successfully controlled (7.31% overall) through the use of antioxidants and activated charcoal, microbial contamination remained the main challenge. It is concluded that the 50% MS medium combined with Protocol 1 provides the highest viability rate for the establishment stage, laying the groundwork for future phases of large-scale clonal multiplication.

Keywords: Plant Micropropagation. Persea Americana. *Phytophthora cinnamomi*. In Vitro Culture. Rootstocks. Explant Disinfection.

RESUMO

O abacate (Persea americana Mill) tem se consolidado como uma cultura de grande importância econômica para a Colômbia; entretanto, sua produtividade enfrenta sérias limitações devido à incidência de Phytophthora cinnamomi e à variabilidade genética decorrente da propagação por sementes. A presente pesquisa teve como objetivo avaliar protocolos para o estabelecimento e a multiplicação in vitro de porta-enxertos de abacate da variedade Crioula, garantindo material livre de patógenos para o Departamento de Tolima. A

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metodologia empregou segmentos nodais de plantas-matrizes previamente analisadas por teste ELISA. Foram comparados dois protocolos de desinfecção (P1 e P2) e três meios de cultivo (Murashige e Skoog – MS a 50%, WPM e Yasuda), avaliando-se as variáveis contaminação, oxidação e viabilidade. Os resultados demonstraram que o Protocolo 1 apresentou percentual de contaminação significativamente menor (57,8%) em comparação ao Protocolo 2 (79,5%). Embora a oxidação tenha sido controlada com sucesso (7,31% no geral) por meio do uso de antioxidantes e carvão ativado, a contaminação microbiana permaneceu como o principal desafio. Conclui-se que o meio MS a 50% combinado com o Protocolo 1 oferece a maior taxa de viabilidade para a fase de estabelecimento, estabelecendo as bases para futuras etapas de multiplicação clonal em larga escala.

Palavras-chave: Micropropagação Vegetal. Persea Americana. *Phytophthora cinnamomi*. Cultivo in Vitro. Porta-enxertos. Desinfecção de Explantes.

RESUMEN

El aguacate (Persea americana Mill) se ha posicionado como un cultivo de vital importancia económica para Colombia; sin embargo, su productividad enfrenta serias limitaciones debido a la incidencia de Phytophthora cinnamomi y la variabilidad genética derivada de la propagación por semilla. La presente investigación tuvo como objetivo evaluar protocolos para el establecimiento y multiplicación in vitro de portainjertos de aguacate variedad Criollo, garantizando material libre de patógenos para el departamento del Tolima. La metodología empleó segmentos nodales de plantas madre previamente analizadas mediante test de ELISA. Se compararon dos protocolos de desinfección (P1 y P2) y tres medios de cultivo (Murashige y Skoog - MS al 50%, WPM y Yasuda), evaluando variables de contaminación, oxidación y viabilidad. Los resultados mostraron que el Protocolo 1 presentó un porcentaje de contaminación significativamente menor (57,8%) en comparación con el Protocolo 2 (79,5%). Aunque la oxidación fue controlada exitosamente (7,31% general) mediante el uso de antioxidantes y carbón activado, la contaminación microbiana persistió como el principal desafío. Se concluye que el medio MS al 50% combinado con el Protocolo 1 ofrece la mayor tasa de viabilidad para la etapa de establecimiento, sentando las bases para futuras fases de multiplicación clonal masiva.

Palabras clave: Micropropagación Vegetal. Persea Americana. *Phytophthora cinnamomi*. Cultivo in Vitro. Portainjertos. Desinfección de Explantes.

1 INTRODUCTION

The avocado (Persea americana Mill) belongs to the Lauraceae family, which comprises about 2200 species, within this family are cinnamon (Cinnamomum verum), laurel (Laurus nobilis) and sassafras (Sassafras albidum). It is a species native to Mexico and Central America and is currently one of the most economically important fruit crops in the tropics/subtropics worldwide. (Bost et al, 2013; Pérez et al, 2015).

In avocado cultivation, the most common practice is to graft the avocado with shoots from a mature tree. The beneficial characters of shoots and roots are integrated by the selection of a rootstock and cuttings with optimal productivity. In the case of the selection of the crop from which the sprout is to be obtained, the vigor of the plant, productivity and quality of the fruit must be taken into account. Rootstocks are selected for their size, salinity tolerance, adaptation to alkaline soils, and resistance to pests and diseases (Hiti-Bandaralage et al, 2017).

Colombia is currently ranked as the third avocado producing country; however, the crop presents productivity and competitiveness problems due to low technological development, as well as deficiencies in marketing channels, lack of plant health guarantees, genetic variability of propagation material, and field losses associated with plant material from poor quality seeds (Restrepo et al, 2018).

2 JUSTIFICATION

The avocado fruit has a high nutrient content, high levels of monounsaturated lipids, proteins, vitamins and beneficial phytochemicals, which make the avocado an important part in the fight against malnutrition and hunger problems in developing countries. In addition to this, due to its oil production, it also plays an important role in the cosmetic and pharmaceutical industry (Chanderbali, 2013).

Colombia has planted 73,986 hectares of avocado with a production of 403,184 tons, ranking as the third producing country and the second in terms of harvested area. The areas of the country where it is most cultivated are Tolima, Antioquia, Caldas, Santander, Bolívar, Cesar, Valle del Cauca, Risaralda, and Quindío, which represent 86% of the country's total planted area (González, 2018). Of the avocado varieties, currently the one with the greatest economic importance in the country is the Hass avocado, as it has increased its exports by more than 413% in less than three years. The second national producer is the Department of Tolima with more than 11,000 tons harvested and has 148 hectares of avocado farms

registered for export (Suarez, 2019). However, one of the problems presented by producers is the incidence of the disease caused by Phytophthora cinnamomi, which has caused losses that accumulate millions of dollars and severely affect the growth, production and international trade of avocados (Litz et al, 2007).

There are two types of rootstock or rootstock in avocado cultivation: rootstock from seed and clonal rootstock. Due to the high level of heterozygosity which creates inconsistency in genetic stability, the use of rootstock from seed is less preferable than clonal rootstock. On the other hand, clonal propagation of avocado rootstocks has been it has become the bottleneck of production, as the method currently used for clonal propagation of rootstocks is costly and time-consuming (Ibarra et al, 2016; Hiti-Bandaralage et al, 2017).

Plant tissue culture has the potential to be an effective and efficient alternative for the clonal production of avocado rootstocks, since it allows the clonal propagation under controlled conditions, of a large number of plants in a short period of time and with little labor, and also guarantees genetic health and stability (Kumar and Loh, 2012). The micropropagation process in avocados has focused mainly on the massive propagation of rootstocks with tolerance to soil-borne diseases and salinity conditions; as well as to revitalize adult material (Ibarra et al, 2016).

Woody plants are generally recalcitrant to tissue culture conditions (Benmahioul et al, 2012) and avocado is no exception to this trend. Several authors have carried out tissue culture techniques for avocado micropropagation; however, factors such as: the explant, conditions of the mother plant, time of collection of the plant material, the cultivar, the composition of the culture medium, concentration of growth regulators, affect the success of micropropagation (Zirari and Lionakis, 1994; Wessels, 1996; Barringer et al, 1996; Dalsaso and Guevara, 1998; Rodríguez et al, 1999; Premkumar et al, 2002; Barceló-Muñoz & Pliego-Alfaro, 2003; Nhut et al, 2008; Zulfiqar et al, 2009; Hiti-Bandaralage et al, 2015; Ibarra et al 2016; Restrepo et al, 2018).

Taking into account the above, the objective of this research is the evaluation of different culture media and growth regulators for the establishment and induction of Sprouts on Avocado Rootstocks Var. Criollo free of Phytophthora cinnamomi, so that Hass avocado producers in the Department can use it as a pattern.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

To evaluate different culture media for the in vitro multiplication of rootstocks of Avocado (Persea Americana) cv. Hass, free of Phytophthora cinnamomi in the Department of Tolima.

3.2 SPECIFIC OBJECTIVES

- Evaluate disinfection protocols for aseptic establishment in avocado cultivar explants.
- Define the culture medium that allows the adequate in vitro multiplication of avocado explants.
- To identify native rootstocks potentially resistant to Phythopthora cinnamomi, in the Department of Tolima.

4 MATERIALS AND METHODS

The research was carried out in the plant biotechnology laboratory of the Agricultural Center "La Granja", National Learning Service in Espinal, Tolima. For the evaluation, nodal segments of native avocado rootstocks obtained from the nursery were used. Prior to obtaining the explants, Elisa tests were carried out for Phytophthora sp. in the mother plants, in order to guarantee plant health for this pathogen.

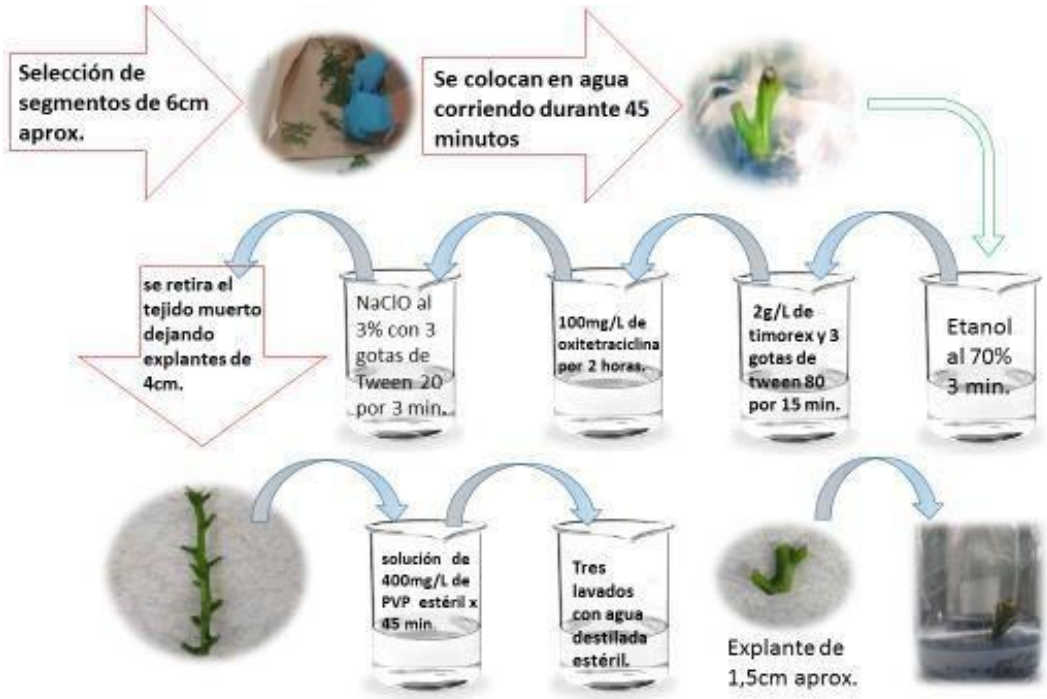
4.1 DISINFECTION PROTOCOLS

Two protocols were carried out to disinfect the explants, adapted from Hiti-Bandaralage et al. (2015), Ibarra et al. (2016), Restrepo et al. (2018). In the first protocol (P1) the segments of approximately 6cm are placed in running water for 45 minutes. Then, in a laminar flow chamber, they are immersed in 70% ethanol for three minutes and rinsed three times with sterile distilled water. The fragments are placed in a solution with 2 g/L of timorex and 3 drops of tween 80 in stirring for 15 minutes, then immersed in 100 mg/l of oxytetracycline for 2 hours. Rinse again three times with sterile distilled water for 5 minutes each and place in magnetic agitation for 3 minutes in 3% sodium hypochlorite solution with 3 drops of Tween 20, then five washes are made with sterile distilled water, the dead tissue is removed leaving explants of 4cm. These are placed in agitation for 45 minutes in a sterile solution of 400mg/l of sterile PVP (polyvinylpyrrolidone). Finally, they are rinsed three times in sterile distilled water and dissected of plant material, removing the oxidized portions at both

ends of the microcutting, leaving approximately 1.5cm of the explant for planting in the culture medium (Figure 1).

Figure 1

Protocol 1 for the disinfection of the explants. The red lines correspond to passages with contaminants, green for transitions and blue for passages and clean areas carried out in the laminar flow chamber



Source: Authors.

In the second disinfection protocol (P2), the nodal segments of approx. 6cm are washed in soapy solution for three periods of 10 minutes each and 3 rinses are performed with sterile water. In the laminar flow chamber they are kept in a solution of 2ml/l of timorex for two hours, followed by a solution of 50mg/l of vancomycin and 100mg/l of cefotaxime for 3 hours, washed with sterile water, then immersed in 96% ethanol for one minute and 1% sodium hypochlorite for 10 minutes. rinses with sterile water. Subsequently, the dead tissue is removed, leaving explants of 4cm in length. The material is left in a solution of 100mg/l of ascorbic acid and 100mg/l of citric acid for 30 minutes, three washes are carried out with sterile distilled water. Finally, fragments of 1.5cm are obtained for sowing in a culture medium (Figure 2).

Figure 2

Protocol 2 for the disinfection of the explants. The red lines correspond to passages with contaminants, green for transitions and blue for passages and clean areas carried out in the laminar flow chamber

Source: Authors.

4.2 IN VITRO ESTABLISHMENT

For in vitro establishment, the culture media MS (Murashige and Skoog, 1962) with 50% macroelements, WPM (Lloyd & McCown, 1980) and Yasuda (Yasuda et al., 1985), supplemented with vitamins and 30 g.L-1 sucrose, were used. Additionally, the implementation of activated charcoal and permanence in darkness for 15 days for the three media was evaluated, as shown in Table 1. The pH was adjusted to 5.75 in all media, they are then sterilized at 1.2 kg cm-2 pressure at 121 °C for 15 min. Once the

Experimental units are incubated at a temperature of 26 ± 2 °C under a photoperiod of 16 light hours depending on the treatment, for two weeks. The variables that were evaluated 15 days after planting were bacterial contamination, fungal contamination, oxidation and viability of the explants. Table 1 shows the treatments used to control the oxidation of plant tissue.

Table 1

Treatments used to prevent oxidation of explants

Treatment	Protocol di sinfection	Photoperiod	Coal d (g/l)	Activate
S0	P1	Photoperiod 12 hours of light	0	
S1	P1	Photoperiod 12 hours of light	2	
S2	P1	Darkness 15 days	0	
S3	P1	Darkness 15 days	2	
S4	Q2	Photoperiod 12 hours of light	0	
S5	Q2	Photoperiod 12 hours of light	2	
S6	Q2	Darkness 15 days	0	
S7	Q2	Darkness 15 days	2	

4.3 OUTBREAK INDUCTION

After fourteen days of in vitro establishment, viable segments were seeded in 50% MyS media, Yasuda and WPM with growth regulators at different concentrations: IBA (indolebutyric acid), BAP (6-Benzylaminopurine) and GA3 (gibberellic acid) for budbreak induction (Table). Added with 30 g/l of sucrose, the pH was adjusted to 5.7 in all media, then sterilized at 1.2 kg cm-2 pressure at 121 °C for 15 min. Once seeding is completed, the experimental units are incubated at a temperature of 26 ± 2 °C under a photoperiod of 16 light hours. The number of shoots per explant, shoot length and number of leaves at 30, 60 and 90 days after subculture were evaluated. To maintain adequate nutrient supplementation and growth regulators, the plants wereTransferred to fresh medium every four weeks, a fine cut was made in the base in each subculture to ensure the continuous absorption of nutrients.

Table 2

Growth hormone treatments for the in vitro multiplication stage

TREATMENT	BAP (mg. L-1)	GA3 (mg. L-1)	IBA (mg. L-1)
S0	0	0	0
S1	0.1	0,1	0
S2	0,5	0,1	0
S3	1	0,5	0
S4	0,5	0	0,1
S5	1,5	0,2	0
S6	2	0	0,5
S7	0,5	0	0,5

S8	0,2	0,2	0
S9	0,5	0,5	0

4.4 STATISTICAL ANALYSIS

For the establishment stage, the qualitative variables evaluated are: contamination, oxidation and viability of the explants at 15 days after planting in the three culture media of this stage and two disinfection protocols, with fifty replications, which were established under a completely randomized design under homogeneous conditions. The variables were analyzed using contingency tables and correspondence analysis for qualitative data. In the shoot induction stage, the following variables will be evaluated: number of shoots, shoot length, and number of leaves at two and three months after the establishment of the explants. An analysis of variance will be performed with the previously transformed data and the differences between the means of treatments are compared using the Tukey test at a 5% probability. Data from both stages will be analyzed using the InfoStat 2008 statistical package (DiRienzo et al, 2008).

4.5 ROOTSTOCK IDENTIFICATION

Creole rootstocks for the Hass avocado were identified in some of the avocado-producing municipalities in the Department of Tolima, which have possible resistance to Phytophthora cinnamomi. This identification was made by Elisa test.

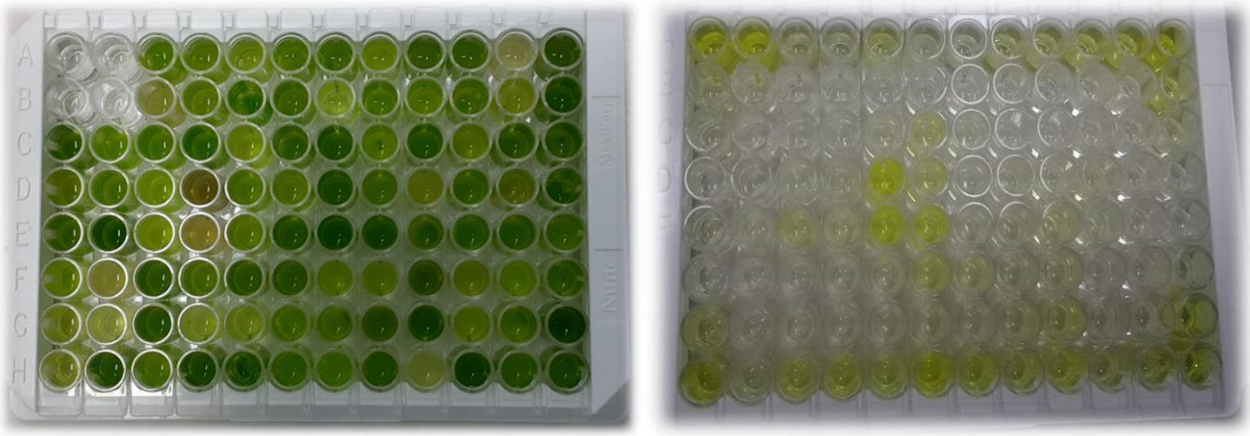
5 RESULTS

5.1 IDENTIFICATION OF FREE ROOTSTOCKS OF PHYTOPHTHORA SP.

Var avocado mother plants were chosen. Criollo that serve as rootstocks, to which an Elisa test was performed with the Phytophthora Pathoscreen kit for the identification of Phytophthora and in this way multiply the plants free of it.

Figure 3

ELISA test on avocado mother plants for the identification of Phytophthora spp



5.2 IN VITRO ESTABLISHMENT

5.2.1 Pollution

The establishment of explants in tissue culture is associated with problems of fungal and bacterial contamination. Measures to reduce contamination include the selection of healthy and stress-free mother plants, as well as the application of fungicides and bactericides to the explants (Barceló and Muñoz, 2003; Hiti-bandaralage, 2017).

At 15 days after planting, the percentage of contamination was 68.74, with bacterial contamination being higher than 40.34% (Table 2), similar results were found by Cooper (1987), with 70% contamination in the Duke variety 7, Dalssaso and Guevara (1988) also obtained a high percentage of bacterial and fungal contamination in the Fuerte variety and Zulfiqar et al. (2009) observed 72% contamination in the Fuerte variety. Of the protocols evaluated, the one with the lowest percentage of contamination was protocol 1 with 57.8% ($p<0.01$), in turn the culture medium with the highest contamination was Yasuda in both disinfection protocols and the medium with the lowest percentage of contamination was MyS using protocol 1 with a significance of <0.05 (Table 2) (Figure 3). which differs from what was found by Ibarra et al. (2016) where Yasuda was the medium with the lowest percentage of contamination (Figure 4).

In woody plants, higher percentages of contamination can occur because they grow in the soil for several years under environmental conditions, so they can be infected by microorganisms both exogenously and endogenously, which often makes in vitro control difficult. This requires establishing one or more sterilization agents that can remove bacteria and fungi from the explant (Ahmad et al, 2003). The disinfection protocols implemented in

this study were adapted from Hiti-Bandaralage et al. (2015), Ibarra et al. (2016), Restrepo et al. (2018), who obtained low percentages of contamination; however, the results obtained in the current research can be due to factors such as the plant health of the mother plant from which the explants were obtained (endogenous contamination), as well as by the manipulation at the time of planting.

Figure 3 shows that protocol 2 is associated with bacterial and fungal contamination; in turn, the MyS culture medium is associated with less bacterial contamination. Correspondence analysis explains 52% of the variables.

Table 2

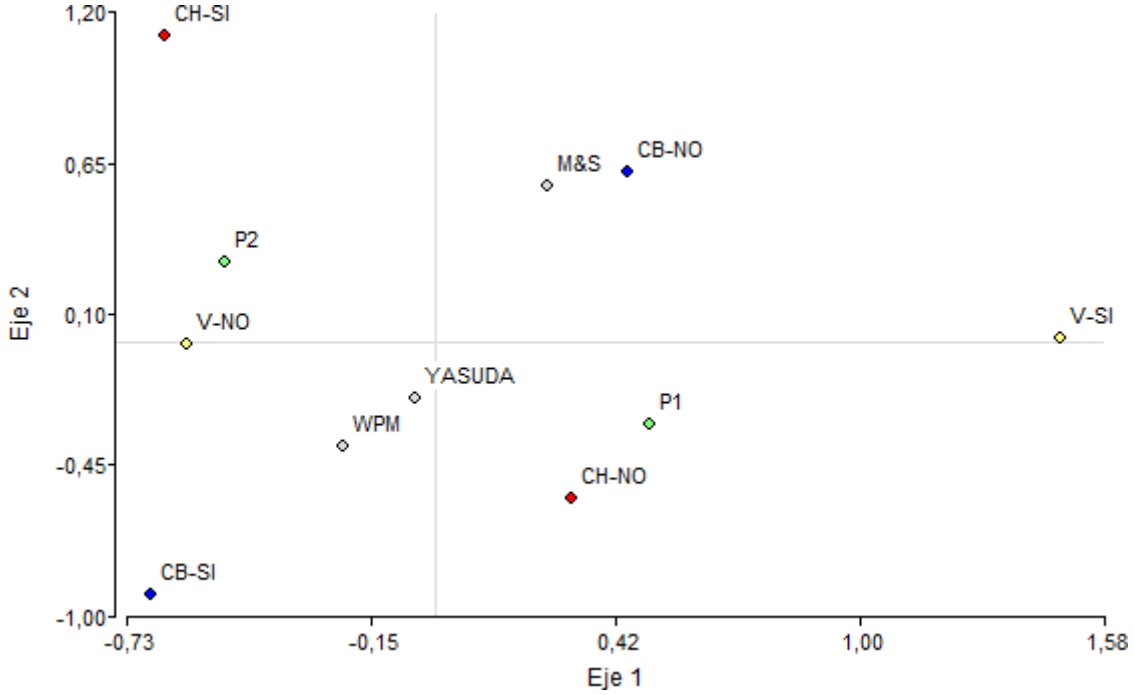
Percentages of contamination, oxidation and viability with the two disinfection protocols and three culture media two weeks after planting

Protocol/medium	Pollution	C. Fungi	C. Bacterial	Oxidation	Feasibility
P1					
50%MyS	39*	35**	20**	2**	47,5
Yasuda	87*	20**	41,58*	12,63**	40**
WPM	61*	15**	54	10**	30**
Total P1	57,80**	23,39**	38,47**	8,14**	39,15**
Q2					
50%MyS	73**	43,5	38,5**	6,50**	19,5**
Yasuda	74**	42*	47	8**	17,5**
WPM	67**	45	41*	5**	17**
Total P2	79,50**	43,50**	42,17**	6,50**	18**

*(p<0.05) **(p<0.01) obtained in the contingency tables. Source: Authors.

Figure 3

Correspondence analysis for the association of the variables: disinfection protocol 1 (P1), disinfection protocol 2 (P2), bacterial contamination (CB), fungal contamination (CH), viability (V), WPM culture medium, Yasuda and MyS. (NO) negative, (YES) positive



Source: Retrieved from the Infostat 2008 statistical program.

5.2.2 Oxidation

In the process of conditioning the explant for in vitro establishment, plant tissues cut primarily from woody plants excrete phenolic compounds that can be oxidized by polyphenoloxidases, peroxidases, or by air. Oxidized phenolic compounds (quinines) have a browning around the cutting surface of the tissue, these quinones have an inhibitory effect on the enzymatic activity of the plant, which ultimately results in tissue death (Bath and Chandel, 1991). Taking into account the above, there are reports in the literature of various pretreatments of the explant to prevent this oxidation (Hiti-bandaralage et al, 2017).

In general, the percentage of oxidation with the different treatments used was low 7.31% (Table 3), being the lowest with treatment 5, in which ascorbic acid, citric acid and activated carbon were used, as reported by Cortes-Rodríguez et al. (2011). However, the other treatments showed significant results to prevent tissue browning, with the use of polyvinylpyrrolidone as reported by Ibarra et al. (2016) and keeping the tissue in darkness in the growth chamber for 15 days reported by Restrepo et al. (2018).

Figure 4 shows that all variables are associated with zero oxidation, with the use of photoperiod and protocol 2 being more associated with citric acid and ascorbic acid as antioxidant agents.

Table 3

Percentage of oxidation obtained with the different treatments with antioxidant agents

Treatment	Oxidation percentage
S0	2,67**
S1	17,36**
S2	2**
S3	10,96**
S4	4**
S5	1,33**
S6	15,33**
S 7	5,33**

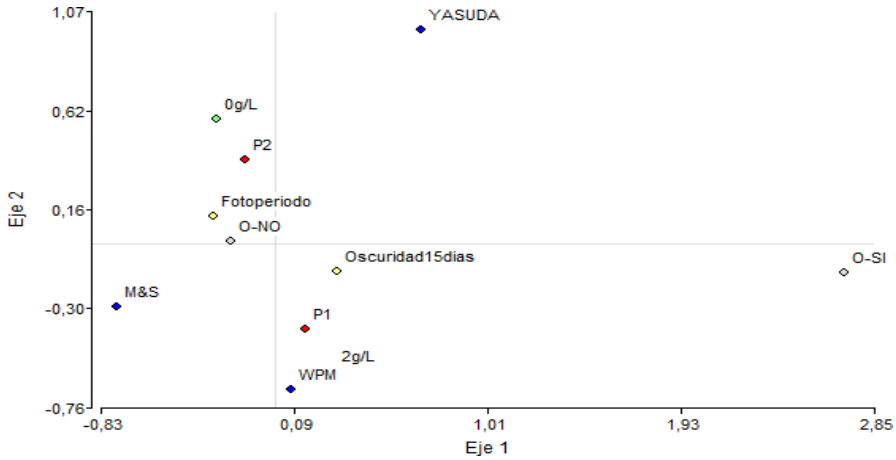
*(p<0.05) **(p<0.01) obtained in the contingency tables. Source: Authors.

5.2.3 Feasibility

The viability of the explants was 28.49%, being higher for protocol 1 and for the 50% MyS culture medium, and lower for the WPM culture medium. This viability is affected by oxidation and contamination processes, as they cause tissue death (Table 2) (Figure 3).

Figure 4

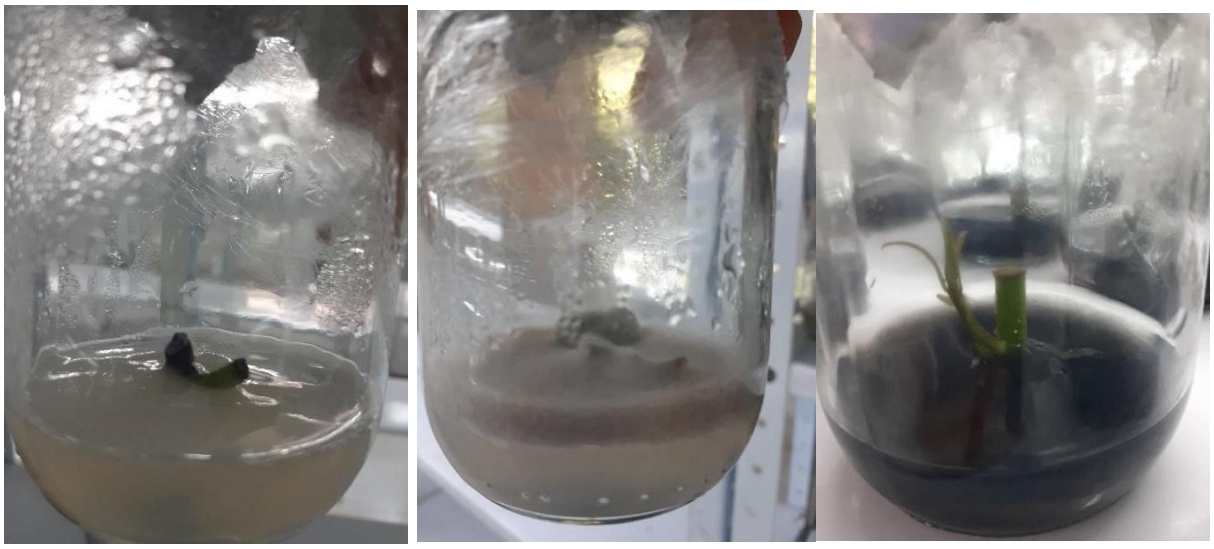
Correspondence analysis for the association of the variables: disinfection protocol 1 (P1), disinfection protocol 2 (P2), Oxidation (O), WPM culture medium, Yasuda and MyS. Activated charcoal 2g/l and activated charcoal 0g/l, Photoperiod in quarter growth and darkness in quarter growth for 15 days, (NO) negative, (IF) positive



Source: Retrieved from the Infostat 2008 statistical program.

Figure 5

a) bacterial contamination, b) fungal contamination, c) viable explant in culture medium



Source: Authors.

6 CONCLUSIONS

Disinfection protocol 1 was the one with the highest percentage of viability. The three culture media in the establishment stage allowed the development of the explants; however, the 50% MyS culture medium had the highest viability. The percentage of contamination was high while oxidation was very low, this indicates that the different treatments to prevent oxidation were efficient, presenting a slight improvement with the treatment in which ascorbic acid and citric acid were used. On the other hand, the high incidence of contamination may be due to various factors such as the plant health of the mother plants, handling at the time of sowing and the disinfection protocols used. Because it is an ongoing project, it is expected to obtain results from the use of different growth hormones in the multiplication stage.

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