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IN VITRO induction of callus from foliar explants in rocoto (*CAPSICUM PUBESCENS RUIZ & PAV.*)

Inducción *IN VITRO* de callos a partir de explantes foliares en rocoto (*CAPSICUM PUBESCENS RUIZ & PAV.*)

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Abstract

Rocoto (*Capsicum pubescens* Ruiz & Pav.) is a native plant of Peru, used in the national gastronomy with great nutritional value; it has pharmaceutical and medicinal properties. The crop is susceptible to diseases caused by phytopathogens, which are spread by seeds of infected cultivars or seedlings. Through the use of biotechnological tools such as *in vitro* plant tissue culture, it is possible to obtain plants free of pathogens, of good quality and great agronomic potential. Therefore, the present work aimed to develop a methodology for the *in vitro* induction of callus from hot pepper leaves. *In vitro* germinated rocoto seedlings were used, from which the first true leaves were selected, which were sectioned into 1 cm explants and placed in different MS culture media added with 2,4-dichlorophenoxyacetic acid (2,4-D). The induction response to calllogenesis was evaluated in five treatments with different concentrations of 2,4-D (0, 0.25, 0.5, 0.75 and 1 mg l^{-1}), under conditions of 25 °C in the dark for 35 days. The culture media added with 0.75 and 1 mg l^{-1} of 2.4-D allowed to obtain 100% induction of calluses in the hot pepper leaves with 81% and 86%, respectively of grade 3 callus formation. This study, pioneer for the species, is good for the potential use of breeding programs.

Keywords: Biotechnology, plant tissue, callogenesis, leaves, germination.

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Resumen

El rocoto (*Capsicum pubescens* Ruiz & Pav.) es una planta oriunda de Perú, empleada en la gastronomía nacional, tiene un gran valor nutricional, y presenta propiedades farmacéuticas y medicinales. El cultivo es susceptible a enfermedades causada por fitopatógenos, los cuales son diseminados por semillas de cultivares o almácigos infectados. Mediante el uso de herramientas biotecnológicas como el cultivo de tejidos vegetales *in vitro* se puede obtener plantas libres de patógenos, de buena calidad y de gran potencial agronómico. Por lo tanto, el presente trabajo se planteó como objetivo desarrollar una metodología para la inducción *in vitro* de callos a partir de hojas en rocoto. Se emplearon plántulas de rocoto germinadas *in vitro*, de las cuales se seleccionaron las primeras hojas verdaderas que fueron seccionadas en explantes de 1 cm y colocadas en diferentes medios de cultivo MS adicionadas con ácido 2,4-diclorofenoxiacético (2,4-D). Se evaluó la respuesta de inducción a callogénesis en cinco tratamientos con diferentes concentraciones de 2,4-D (0; 0,25; 0,5; 0,75 y 1 mg l^{-1}), en condiciones de 25 °C en oscuridad durante 35 días. Los medios de cultivo adicionados con 0,75 y 1 mg l^{-1} de 2,4-D permitieron obtener 100 % de inducción de callos en las hojas de rocoto con 81 % y 86 % respectivamente de formación de callos de grado 3. Este estudio, pionero para la especie, abre expectativas en programas de mejoramiento para su potencial uso.

Palabras clave: Biotecnología, tejido vegetal, callogénesis, hojas, germinación.

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1 Introduction

Rocoto pepper (*Capsicum pubescens* Ruiz & Pav.) is a herbaceous plant with a perennial life cycle belonging to the *Solanaceae* family. It is originary from the Andean region of Peru, but it is also found in its wild form in the mountains and high jungle. This species differs from the other species belonging to the *Capsicum* genus by presenting purple flowers and by its black seeds. The fruit can vary in shape, size and color, but it is characterized by being spicy and for this reason it is very used in the Peruvian gastronomy. In addition, rocoto pepper presents pharmaceutical and medicinal properties related to capsaicin (Caballero et al., 2017).

Two varieties of rocoto have been defined in Peru, forest rocoto (*rocoto de monte*) and orchard rocoto (*rocoto de huerta or serrano*). It is known as forest rocoto because it is cropped in the central forest; it has a larger fruit size, so there is a greater demand of this variety for the preparation of the filled rocoto. The other variety is orchard rocoto and it is cropped in all the areas of the country, mainly in the Andean valleys. The fruit of this variety is smaller and it is very spicy, reason for which it is used for sauces (Valdez, 2017).

The national production of rocoto has increased at an annual rate of 5% mainly because of the great gastronomic demand developed in the country; for this reason it is currently considered a product of national importance (Sardón, 2015). However, this crop is sensitive to wilt, root decay and other diseases caused by *Fusarium oxysporum*, *Phytoptora capsici*, *Risotonia solanacearum* among other phytopathogens. It is also sensitive to damage caused by viruses that can provoke yellowing in the nerves, deformations in leaves and fruits, enanism, lack of vigor and fall of the leaves. These diseases are normally spread through seeds from infected cultivars or seedbeds, because seeds from previous crops are normally used in new crops; and some viruses are spread with the contact among plants (Lucana, 2012; Hernández et al., 2019b).

It is important to strengthen the value chain of rocoto, and the idea is to obtain qualified and certified seeds or pathogen-free seedlings in order to maintain more uniform crops with higher quality and production. Biotechnology tools are considered as alternatives for the production of aseptic plant material with *in vitro* plant tissue culture techniques (Robledo and Carrillo, 2004; Sanatombi and Sharma, 2007; Orlinska and Nowaczyk, 2015). These techniques allow the proliferation of cells from an explant (plant fragment that can be meristems, axillary buds, leaves, roots, anthers and even microspores) in a culture medium equipped with nutrients, vitamins, and in some cases with hormones (Levitus et al., 2010; Vélez et al., 2010; Venkataiah et al., 2016). Under appropriate conditions, these explants will induce the formation of calluses, which are amorphous or disorganized masses of undifferentiated cells. The importance of the callus lies in its irregular growth functionality with the potential to form organs or embryos under appropriate conditions (Alayón et al., 2006; Pérez et al., 2009; Terra et al., 2009; Smith, 2012; Rashmi and Trivedi, 2014).

The potential of calluses as a pathway for organogenesis and indirect embryogenesis is an alternative for breeding programs in rocoto, because these methods in some species or genotypes when introduced *in vitro* culture media with different concentrations of phyto-hormones or combinations may induce somaclonal variation, allowing new characteristics to be obtained or the elimination of some unwanted trait (Sala and Labra, 2003; Rodríguez et al., 2014). But without these somaclonal variations, promising genotypes of good performance and good quality can be multiplied, as is being developed in the *in vitro* cultivation of plant tissues in different species of the genus *Capsicum* (Marín, 2012; Gómez, 2016; Gutierrez-Rosati and Vega, 2017; Izquierdo et al., 2017).

Therefore, the aim of this research is to develop a methodology for inducing calluses *in vitro* from rocoto leaf segments.

2 Materials and methods

This research was carried out at the facilities of the Plant Biotechnology Laboratory of the Professional School of Biology with the specialization on Biotechnology, located at the National University José Faustino Sánchez Carrión, Huacho, Lima, Peru.

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2.1 Disinfection of the vegetal material

Rocoto seeds obtained from mature fruits present in the laboratory were used. The seeds were washed with water and commercial detergent for 5 minutes, and then the disinfection process was conducted in laminar flow chamber using the protocol established by Hernández et al. (2019a). The seeds were first immersed in 70% of ethanol for 1 minute, then were immersed in a 2% sodium hypochlorite solution for 15 minutes in constant agitation. After that time, three rinses were made with sterile distiled water, then seeds were placed on filter paper and three seeds were placed per test tube using MS culture medium (Murashige and Skoog, 1962).

All treatments were kept in the growth chamber (Plant Growth Chamber, LGC-5201 G, LabTech) in total darkness conditions at 25 °C, with relative humidity of 75 ± 2%. When germination began, they were switched to a photoperiod of 16 hours of light and 8 hours of darkness.

MS culture medium consisted of salts described by Murashige and Skoog (1962), with myoinositol (100 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine HCL (0.5 mg l^{-1}), thiamine HCL (0.1 mg l^{-1}), glycine (2 mg l^{-1}) and sucrose (30 g l^{-1}). Before adding agar agar (7 g l^{-1}) the pH was adjusted to 5.7 ± 0.1 using the Lab 850 potentiometer (SI Analytics). Subsequently, autoclave (BKM-Z18N, Biobase) was sterilized at 1.2 Bar of pressure and 121°C for 20 minutes.

2.2 Induction of calluses

The first two green leaves were selected from the seedlings germinated *in vitro*; these were segmented into 1-cm explants and five segments were introduced by MS culture with myoinositol (100 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine HCL (0.5 mg l^{-1}), thiamine HCL (0.1 mg l^{-1}), glycine (2 mg l^{-1}), sucrose (30 g l^{-1}) and auxin 2.4-D at different concentrations (Table 1). Then pH was adjusted to 5.7 ± 0.1 and agar (7 g l^{-1}) was added. It was then autoclaved at 1.2 Bar pressure and 121°C for 20 minutes.

All treatments were kept in the growth chamber (Plant Growth Chamber, LGC-5201 G, LabTech) in total darkness conditions at 25°C and with relative humidity of 75 ± 2% for 35 days, with observations every seven days to differentiate the induction progress.

Table 1. Treatments for inducing calluses in rocoto leaves.

Treatment	2.4-D (mg l^{-1})
T1	0
T2	0.25
T3	0.5
T4	0.75
T5	1

2.4-D = Dichlorophenoxyacetic acid 2.4

2.3 Experimental design and statistical analysis

It was a completely randomized design (CRD) with five treatments, using 15 replications per treatment, and the experimental unit was made up of each segment of rocoto leaf. The percentage of callus induction in rocoto leaves and the callus grade on the Santana scale Santana (1982) were evaluated (Table 2). The data obtained were submitted to Analysis of Variance (ANOVA) and mean comparison was made with Tukey test ($p \leq 0.05$), using the statistical package of the R program (version 4.0.3 for Windows).

3 Results

The methodology used for the disinfection of rocoto seeds allowed to obtain 100% of contamination-free seeds in the culture media, permitting the germination by bacteria or fungi to compete with the growing seedlings.

The induction of calluses from foliar explants in rocoto was observed from the seventh day in the treatments with 0.75 and 1 mg l^{-1} of 2.4-D (T4 and T5, respectively) forming grade 1 calluses. The treatments with 0.25 and 0.5 mg l^{-1} of 2.4-D showed the formation of calluses in rocoto leaves during the second week in the induction medium. After 35 days, MS culture medium with 0.75 and 1 mg l^{-1} of 2.4-D presented the highest percentages of callus formation with 100% callus induction in rocoto leaves (Figure 1), showing significant differences with the other treatments (Figure 2).

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Table 2. Description of the scale used by Santana (1982)

Grade	Callus induction	Observation
0	None formation of callus	-
1	Slight formation of callus	Slight enhancement in the edge of the explant
2	Formation of callus	Proliferation of cells in the edge of the explant, without creating a mass
3	Significant formation of callus	Formation of a callus mass

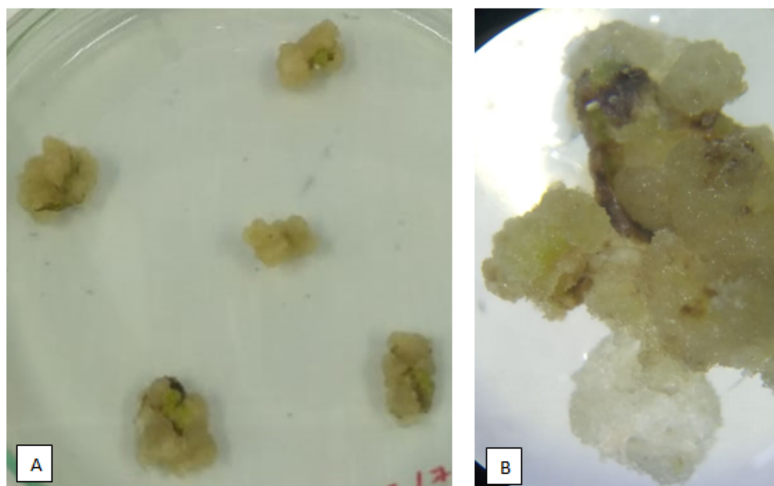

Figure 1. A. Calluses at 35 days formed from rocoto leaves in basal medium MS with 0.75 mg l^{-1} of 2.4-D. B. Callus induced at 35 days with treatment 1 mg l^{-1} of 2.4-D with yellowish coloring, translucid zones and friable consistency.

The treatments with higher percentages of grade 3 calluses were 0.75 and 1 mg l^{-1} culture medium of 2.4-D with 81% and 86%, respectively; however, there were no significant differences between the two treatments (Table 3). Also treatment 0.5 mg l^{-1} of 2.4-D with 57%, while treatment 0.25 mg l^{-1} of 2.4-D showed a higher percentage of grade 2 calluses.

The morphological characteristics of calluses were: light yellow coloring and bleaching or translucid zones in some explants, and friable consistency of the callus. Calluses began their formation at the cutting sites (edges) of the explants until completely covering the surface of the explants in the T3 and T4 treatments. Hence, it can be indicated that rocoto has good response to callus induction, rapid formation and growth (Figure 3).

Table 3. Percentage of the formation degree of calluses in rocoto leaves.

Treatment	Callus formation by scale degree (%)			
	0	1	2	3
T1	100 a	0 c	0 c	0 d
T2	0 b	18 a	58 a	24 c
T3	0 b	7 b	46 a	57 b
T4	0 b	0 c	19 b	81 a
T5	0 b	0 c	14 b	86 a

Means with different letters differ significantly according to Tukey´s test p<0,05

4 Discussion

In vitro callus formation was induced in rocoto foliar explants with MS culture medium with the presen-

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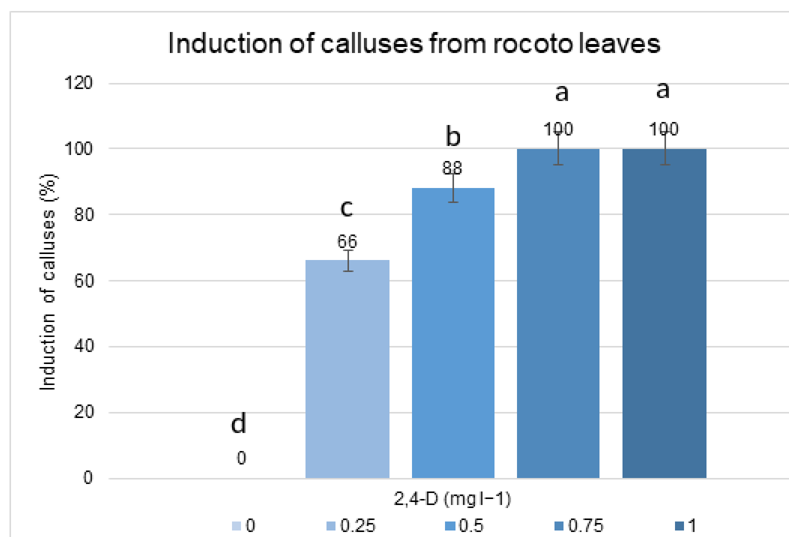

Figure 2. Percentage of *in vitro* callus induction from rocoto leaves. Means with different letters differ significantly according to Tukey's test for p<0.05.

ce of 2.4-D; the appearance of calluses was translucent and compact. The induction of calluses in *Capsicum* leaves is very favorable compared with other types of explants (Solís-Ramos et al., 2010; Alva-Guzmán et al., 2014; Argüelles et al., 2020). As mentioned by Rodríguez et al. (2014) and Espinosa et al. (2012) the induction of callus in leaves mostly depends on the type of explant and hormone used, unlike other explants. An evident increase of callus is seen when the concentration of 2.4-D is increased, in addition, this type of hormone influences the appearance of calluses.

The addition of auxins and their concentration in the culture medium may generate a higher percentage of callus induction depending on the species or genotype used, but the action of auxins is related to the presence of compounds present in the culture medium that have a greater influence on cell development and differentiation (Feeney et al., 2007; Meiners et al., 2007). This is because these endogenous compounds in *in vitro* culture have drastic changes in the cellular environment, which generate stress effect that can cause cell reorganization and form a mass of undifferentiated cells (Feher et al., 2003; Shriram et al., 2008).

Calluses that generated in the first weeks started at the edges of the explants, and gradually progressed in the explant over the course of the days. On day 35 the explants were covered and there was an increase in the size of the callus. Smith (2012), states that calluses are generated from the cutting zone of explants, an area that is in direct contact with the growth regulators that influence an accumulation of auxins, stimulating a continuous mitotic division that generates the gradual formation of small tissue until covering the explant largely or completely.

The effect of auxin 2.4-D on rocoto foliar explants resulted in callus induction, which could be related to physiological actions that are activated in the explant by exogenous auxin in the culture medium. Taiz and Zeiger (1998) determined that induction is expressed by genes encoding protein factors by binding auxins to external and internal receptors. Azcón-Bieto (2008), discovered that protein factors allow increasing plasticity and softening the cell wall, resulting in dilatation of the cell by turgor pressure, increasing in size until the cell wall opposes resistance. Additionally, the induction of calluses is influenced by enzymes that activate or repress gene transcription by the physiological actions of the cytokines; these enzymes are the histidine phosphotransferases, which are activated by the binding of the cytokines to histidine kinase receptors (Müller and Sheen, 2007).

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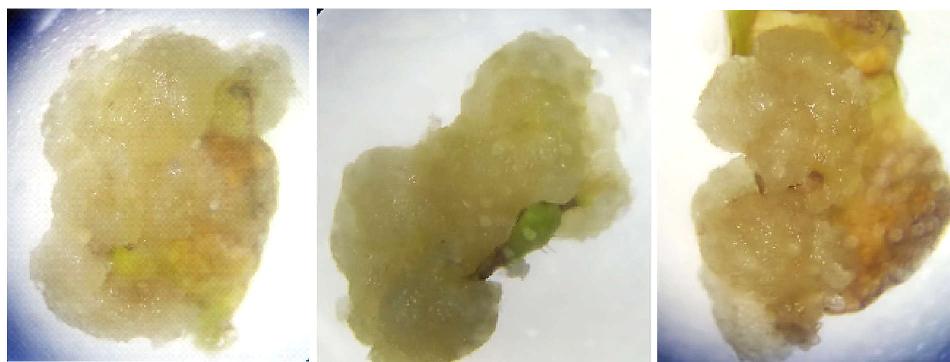

Figure 3. Calluses obtained in foliar rocoto explants, induced in MS culture medium with 2.4-D (0,75 mg l^{-1}).

The formation of rocoto callus in the first week can be an important finding because it provides information of having a sort of rapid induction effect. who generated calluses in Alva-Guzmán et al. (2014), quienes generaron callos en *Capsicum chinensis* from the tenth day in the induction medium, using 0.5 and 1 mg l^{-1} of 2,4-D.

The importance of auxin 2.4-D, being the main phytohormone used in *in vitro* conditions for the induction of calluses, is highlighted with a large number of reports of its addition in the culture medium for species of the same genus as rocoto, as well as for other genera (Larson et al., 2006; Terra et al., 2009; Hernández and Díaz, 2019; Hernández et al., 2020).

The age of the explants used should also be taken into account, since the first true leaves of rocoto were the ones influencing the study with a higher induction potential as stated by Alleweldt and Radler (1962), indicating that the physiological age of explants is inversely proportional to their organogenic potential.

These results represent an important advance in the application of biotechnological techniques in genetic improvement programs of rocoto, providing information for the potential use of calluses of this species in cell cultures to obtain secondary metabolites of pharmacological interest. It is a basis for future research in the induction of indirect somatic embryogenesis, indirect organogenesis, rhizogenesis, isolation of protoplasts, among others.

5 Conclusions

A methodology was developed for the *in vitro* induction of calluses from rocoto leaves, obtaining the highest induction percentage and formation of calluses in grade 3, using MS culture medium with 0.75 and 1 mg l^{-1} of 2,4-D.

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