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ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

EFFECT OF 2,4-DICHLOROPHENOXYACETIC CONCENTRATION ON *IN VITRO* CALLUS INDUCTION USING COTYLEDONS OF ROCOTO (*CAPSICUM PUBESCENS* RUIZ & PAV.)

EFECTO DE LA CONCENTRACIÓN DE 2,4-DICLOROFENOXIACÉTICO EN LA INDUCCIÓN DE CALLOS *IN VITRO* UTILIZANDO COTILEDONES DE ROCOTO (*CAPSICUM PUBESCENS* RUIZ & PAV.)

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ABSTRACT

The rocoto (*Capsicum pubescens* Ruiz & Pav.) is a herbaceous or shrubby plant, it differs from other species of the genus Capsicum by its purple corolla and black seeds. Peru is considered as its center of origin, where it is widely used in medicine, pharmaceutical industry, and especially in gastronomy. In the present work a methodology was developed to determine the effect of 2,4-dichlorophenoxyacetic (2,4-D) concentration on *in vitro* induction callus from cotyledons of rocoto cv. Serrano. Rocoto seeds were germinated *in vitro*, of which the cotyledons were cleaved in the first week and placed in the MS culture media added with 2,4-D. Five treatments with different concentrations of 2,4-D were evaluated. The best results were obtained with the treatments that 0.75 and 1 $mg \cdot L^{-1}$ were added for callus induction, achieving 72% callus proliferation at 21 days. This study aims to be the basis for future studies related to the culture of tissue in rocoto, allowing to indicate the potential use of callus of rocoto in the induction of somatic embryos and isolation of protoplasts.

Keywords: 2,4-D – cotyledons – *Capsicum pubescens* – callus – *in vitro*

RESUMEN

El rocoto (*Capsicum pubescens* Ruiz & Pav.) es una planta herbácea o arbustico, se diferencia de otras especies del género *Capsicum* por su corola de color púrpura y semillas negras. Se considera al Perú como su centro de origen, donde es muy empleado en la medicina, industria farmacéutica, y sobre todo en la gastronomía. En el presente trabajo se desarrolló una metodología para determinar el efecto de la concentración de 2,4-diclorofenoxiacético (2,4-D) en la inducción de callos *in vitro* a partir de cotiledones de rocoto cv. Serrano. Se germinaron in vitro semillas de rocoto, de las cuales a la primera semana se escindieron los cotiledones y fueron colocados en los medios de cultivo MS adicionado con 2,4-D. Se evaluó cinco tratamientos con diferentes concentraciones de 2,4-D. Los mejores resultados fueron obtenidos con los tratamientos que se adicionaron 0.75 y 1 $mg \cdot L^{-1}$ para la inducción de callos, logrando a los 21 días 72% de proliferación de callo. Este estudio pretende ser base para futuros estudios relacionados al cultivo de tejido en rocoto, permitiendo indicar potencial uso de callos de rocotos en la inducción de embriones somáticos y aislamiento de protoplastos.

Palabras clave: 2,4-D – cotiledones – *Capsicum pubescens* – callos – *in vitro*

INTRODUCTION

The rocoto (*Capsicum pubescens* Ruiz & Pav.) is described as a herbaceous or shrub, with woody trunk and dichotomous branching, has alternating, rough and pubescent leaves. It is characterized more by its purple or purple corolla, smooth and persistent fruit, and especially by black seeds. It is found in the wild in the Andes of Peru, based on proof of being its center of origin; in Peru there are also two well-defined cultivars of rocoto: serrano and monte. It has 24 chromosomes, of which one pair is acrocentric, it also has a high percentage of self-incompatibility (Guevara *et al*., 2000; Valdez, 2017).

The applications that are given to rocoto are oriented to gastronomy as fresh food, sauce or pasta, also to pharmaceutical and medicinal products with the extraction of the enzyme capsaicin for the preparation of drugs that are intended to relieve gastric pain. Therefore, the production of rocoto in Peru has been increasing year after year, being the main destination of the fruits for Peruvian gastronomy due to the gastronomic boom of recent years, which is why the rocoto has increasingly positioned itself as a flagship product in Peru (Sánchez, 2015; Sardón, 2015; Caballero *et al*., 2017).

It should be taken into account that this crop has susceptibility to diseases caused by *Phytophthora capsici*, *Fusarium oxysporum*, *Risotonia solanacearum*, anthracnose, wilting and root rot, which generate damage to different plant structures, such as lack of vigor, yellowing of the ribs, deformations and leaf falls, abortion in the fruits or their fall, or death of the plant (Lucana, 2012; Hernández *et al*., 2019b). In addition, some of the symptoms may be related to diseases caused by viruses, like the tomato spotted wilt virus (TSWV), alfalfa mosaic virus (AMV), pepper mild mottled virus (PMMoV), Peru tomato virus (MTV) and Tomato mosaic virus (ToMV), the latter by dissemination on contact between plants, also by seeds from infected cultivars or seedlings (Valdez, 2017; Vallejo-Gutiérrez *et al*., 2019).

Being important to strengthen the rocoto value chain is to obtain seeds or seedlings free of pathogens, and therefore have great production. Within the alternatives of aseptic plant material, biotechnological tools are present, with tissue culture techniques, being the technique of callogenesis or embryogenesis often inducing somaclonal variation (Orlinska & Nowaczyk, 2015).

In this way, genotypes of good quality can be multiplied, as has been developed in the tissue culture and regeneration of *in vitro* seedlings of different species of the genus *Capsicum* (Sanatombi & Sharma, 2007), but no tissue culture

work has been reported. in *C. pubescens*.
The present investigation was carried out with the objective of developing a methodology to determine the effect of 2,4-dichlorophenoxyacetic concentration on *in vitro* callus induction from cotyledons of rocoto cv. Serrano.

MATERIALS AND METHODS

This research was carried out in the facilities of the plant biotechnology laboratory of the professional school of Biology with a mention in Biotechnology, located at the José Faustino Sánchez Carrión National University, Huacho, Lima, Peru.

Disinfection of plant material
Rocoto seeds were used from the fruits present in the greenhouse of the Plant Biotechnology laboratory of the School of Biology. The fruits were opened and the seeds were extracted, to which a first wash with more detergent water was carried out than to remove which waste still present from the fruit, for which a brush was used to remove any possible contaminant, the rinsing was continued of the seeds for detergent removal. Then the seed disinfection process began, using the protocol established by Argüelles *et al*. (2019), transferring all the material to the laminar flow cabinet and continuing to immerse the seeds in 70% ethanol for 60 s, then transfer the seeds to a 2% sodium hypochlorite solution for 15 min with constant agitation. Subsequently, they were transferred to sterile distilled water to remove traces of sodium hypochlorite, so the rinsing was done three times, and planting was continued in the test tubes containing the MS culture medium, placing 3 seeds per test tube. They were kept in photoperiod of 16 hours of light and 8 hours dark, with a temperature of 25 ± 1° C and relative humidity of 75 ± 1%.

The MS culture medium consisted of salts and vitamins described by Murashige & Skoog (1962) (Table 1), but was used at half of its concentration; 7 $g \cdot L^{-1}$ agarose, 30 $g \cdot L^{-1}$ of sucrose were added and the pH was adjusted to 5.7. Subsequently, it was sterilized in an autoclave at 1.2 Bar pressure and a temperature of 121 ° C for 20 min.

Table 1. Composition of the Murashige and Skoog medium (MS).

Components	Concentration ($mg \cdot L^{-1}$)
KNO_3	1900
NH_4NO_3	1650
$CaCl_2$	332.2
$MgSO_4$	180.7
$KH_2P\ O_4$	170
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	37.26
$FeSO_4.7H_2O$	27.8
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
Glycine	2
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1

Callus Induction

After seven days of sprouting, the seedlings of rocoto split the cotyledons, placing five segments of the cotyledons per plate with MS culture medium (Table 1), which was used at half of its concentration and It was supplemented with five 2,4-D treatments (Table 2), in addition all treatments consisted of 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ agarose. All plates were incubated at 25 ° C, in total darkness and with relative humidity of 75 ± 2% for 21 days.

Table 2. Treatments for the callus induction on rocoto leaves.

Treatment	2,4-D ($mg \cdot L^{-1}$)
T_1	0
T_2	0.25
T_3	0.5
T_4	0.75
T_5	1

2,4-D = 2,4-Dichlorophenoxyacetic acid.

Experimental design and data análisis

The data analyses were conducted using statistical package agricolae of the free software R (version 3.6.1 for Windows). Each treatment consisted of 10 plates with five explants each, using each explant as an experimental unit. To observe the significant differences in the induction percentages, an analysis of variance (ANVA) was used and the comparison between the means was made according to the Tukey test ($p \leq 0.05$).

RESULTS

With respect to the disinfection of rocoto seeds, the methodology used allowed to obtain 100% free of contamination.

In callus induction from cotyledons of rocoto, callus formation was observed from day 11 on the induction medium T_4 and T_5 (Fig. 1), From day 15 on induction media, it was observed that the first 4 treatments had callus formation, but treatments T_4 and T_5 had a percentage of callus induction greater than 50%, while treatment T_1 did not present callus induction. At day 21, the treatment with greater callus induction was obtained and in less time the treatments with 0.75 and 1 $mg \cdot L^{-1}$ of 2,4-D with 72% (Fig. 2) with a translucent and compact appearance (Fig. 3).

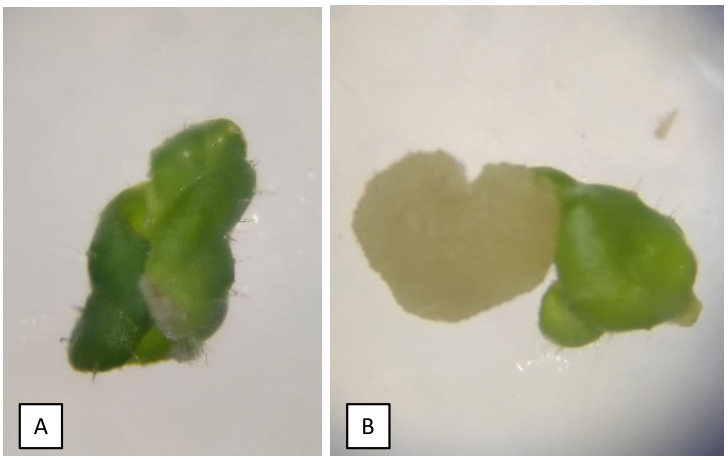

Figure 1. A. Rocoto leaf explants on day 11 in the callus induction medium. B. The callus increases in size for the second week in the induction medium.

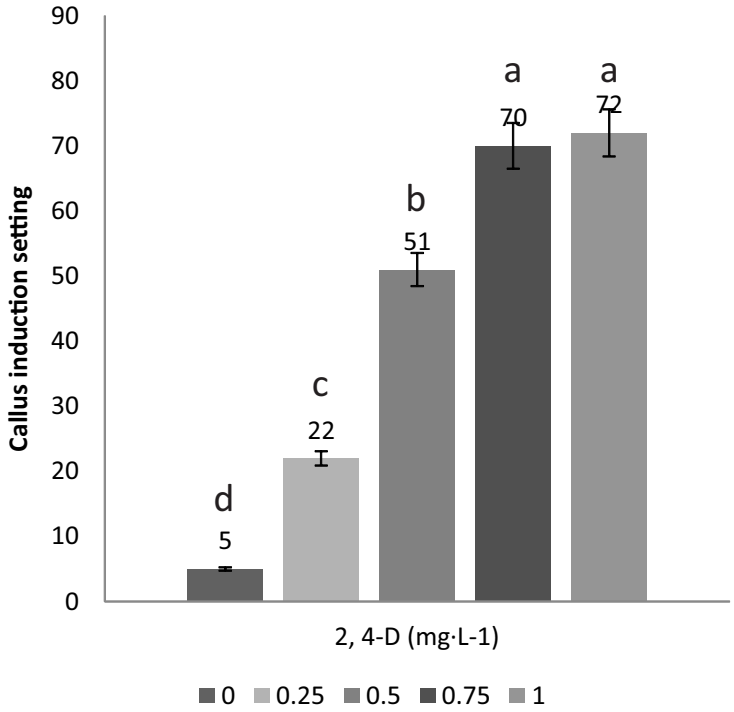

Figure 2. Percentage of callus induction *in vitro* from rocoto leaves at 21 days.

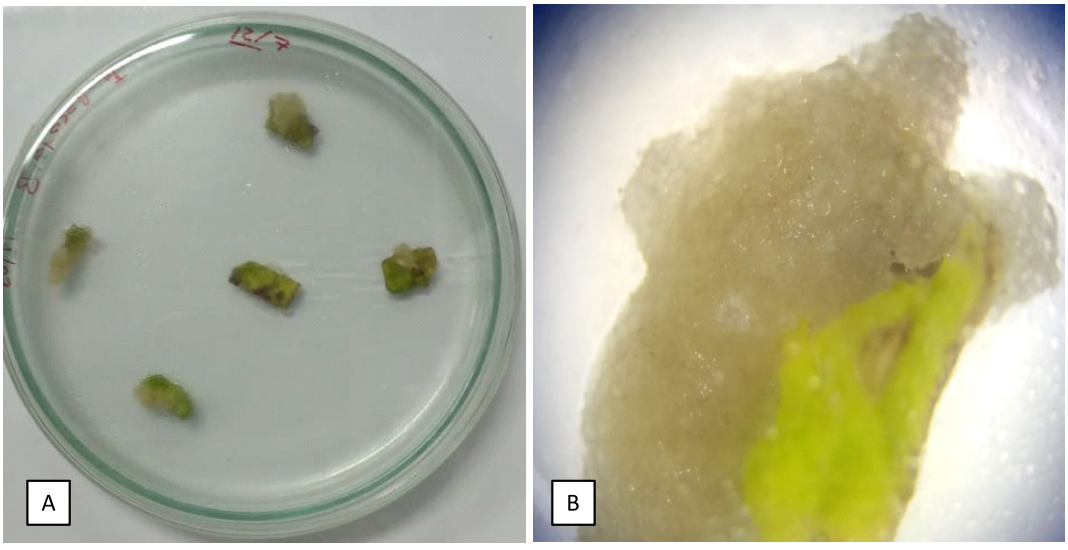

Figure 3. A. Callus formed from rocoto leaves in basal MS medium with 0.75 mg·L^{-1} of 2,4-D. B. Callus induced at 21 days with the 1 mg·L^{-1}2,4-D treatment with a translucent and compact appearance.

DISCUSSION

The method of disinfection of rocoto seeds was effective when immersed in 70% ethanol and then with sodium hypochlorite, which is a substance commonly used in the superficial disinfection of plant material in the process of introduction in the *in vitro* culture for presenting A positive effect in the elimination of resistant microorganisms, due to the effects of chlorination (Ramirez *et al*., 2002), is effective in most pathogenic bacteria, but with an unpredictable action against fungi and viruses. The death of microorganisms is due to the direct combination of chlorine with the proteins of cell membranes and enzymes, this because chlorine destroys organisms by being inactivated by oxidation of cellular material. Likewise, in the presence of water gives off nascent oxygen (O_2) that oxidizes organic matter (Folgueras *et al*., 2001). In addition, stirring the explants together with the disinfectant helps to reduce the surface tension of the water, thus allowing greater contact of the explant with the disinfectant solution (Abdelnour & Escalante, 1994; Hernández *et al*., 2019a).

On the other hand, ethanol acts by denaturing proteins, dissolving lipid layers and as a dehydrating agent, it is lethal to bacteria, but irregular to fungi and viruses, it does not act on spores; when combined with antiseptics of another group has a greater germicidal action (Folgueras *et al*., 2001).

It was possible to induce callus from cotyledons of rocoto by starting auxin 2,4-D in different concentrations in the MS culture medium, with which calluses with translucent and compact appearance were observed. As a result of this induction, we have that callus formation is highly dependent on the type of explants, and on the type and concentration of phytohormone, in the case of calluses from leaf segments unlike other explants, a higher percentage of callus formation by increasing the concentration of 2,4-D also having it have an effect on the appearance of the calluses, also presenting a linear effect (Rodríguez *et al*., 2014).

The culture medium supplemented with different concentrations of growth regulators generates different responses in the explants, when using auxins, it will have participation in cell development and differentiation, affecting *in vitro* culture considerably since these endogenous compounds modify the cellular environment, thus generating a stress and cellular reorganization that leads to the formation of a mass of undifferentiated cells (Fehér *et al*., 2003).

The greatest number of calluses formed were obtained from the treatment added with 0.75 and 1 $mg{\cdot}L^{-1}$ of 2,4-D, in comparison to other authors that recommended the addition of a high combination of cytokinins and auxins, or only the addition of auxins can favor the callus induction, in addition to the appearance of callus is related to the type of hormone used during induction. In the case of the growth regulator that has more reports of callus induction is auxin 2,4-D (Larson *et al*., 2006; Feeney *et al*., 2007; Meiners *et al*., 2007; Shiram *et al*., 2008; Hernández & Díaz, 2019).

These results represent an important advance in the use of biotechnological techniques for the genetic improvement of rocoto, which opens expectations for the potential use of callus of this species for the induction of somatic embryos and isolation of protoplasts.

A methodology was developed to determine the effect of 2,4-dichlorophenoxyacetic concentration on *in vitro* callus induction from cotyledons of rocoto cv, Serrano, in which using the MS culture medium added with 0.75 and 1 $mg{\cdot}L^{-1}$ of 2,4-D achieved the highest callus induction with more than 70% at 21 days.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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