

***In vitro* anther culture of rocoto (*Capsicum pubescens* Ruiz & Pav.)**

Cultivo in vitro de anteras de rocoto (Capsicum pubescens Ruiz & Pav.)

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ABSTRACT

The rocoto (*Capsicum pubescens* Ruiz & Pav.) is a crop of great importance in Peru because it is in great demand within the national gastronomy. This crop has no defined varieties or cultivars, but it is represented by two significant genotypes: Serrano and Monte. It is necessary to obtain homozygous lines to define a cultivar; for this reason, the alternative of using biotechnological tools through another culture under *in vitro* conditions is presented to obtain homozygous individuals. Nevertheless, the response of another culture depends on each genotype under study, and it is necessary to establish a basis for the establishment and generation of somatic embryos from rocoto anthers. For the reasons described above, the present research aimed to induce somatic embryos from *in vitro* culture of rocoto anthers. Flower buds of the Serrano genotype were used to determine the flower bud stage with the highest percentage of uninucleated microspores and determine the treatment with the highest percentage of disinfection in rocoto flower buds, and determine the treatment that allows the induction of somatic embryos from rocoto anthers. The IR4 group of flower buds presented the highest percentage of microspores in the uninucleate stage with 81.3%. Treatment D4 achieved the highest disinfection of flower buds with 89.01%. A total of 18.27% of anthers with somatic embryo formation were obtained using treatment T2.

Keywords: Ecotypes, microspores, uninucleate, somatic embryos, flower bud.

RESUMEN

El rocoto (Capsicum pubescens Ruiz and Pav.) es un cultivo de gran importancia en el Perú, con gran demanda dentro de la gastronomía nacional. Este cultivo no presenta variedades o cultivares definidos, pero está representado por dos genotipos principales: Serrano y Monte. Para definir un cultivar es necesario obtener líneas homocigóticas, por lo que se presenta la alternativa de utilizar herramientas biotecnológicas a través del cultivo de anteras en condiciones in vitro, para obtener individuos homocigóticos. Pero la respuesta del cultivo de anteras depende de cada genotipo en estudio, y es necesario establecer una base para el establecimiento y generación de embriones somáticos a partir de anteras de rocoto. Por las razones descritas anteriormente, la presente investigación tuvo como objetivo inducir embriones somáticos a partir del cultivo in vitro de anteras de rocoto. Se utilizaron botones florales del genotipo Serrano para determinar el estado del botón floral con mayor porcentaje de microsporas en estado uninucleadas, para determinar el tratamiento con mayor porcentaje de desinfección en los botones florales de rocoto y para determinar el tratamiento que permite la inducción de embriones somáticos a partir de anteras de rocoto. El grupo IR4 de botones florales presentó el mayor porcentaje de microsporas en estado uninucleado con un 81,3%. El tratamiento D4 logró la mayor desinfección de botones florales con un 89,01%. Con el tratamiento T2 se obtuvo un 18,27% de anteras con formación de embriones somáticos.

Palabras clave: Ecotipos, microsporas, uninucleados, embriones somáticos, botón floral.

Introduction

Peru has a great variety of *Capsicum* species that are being cultivated nationally, of which four are the most commercialized: paprika (*Capsicum annuum* L.), yellow chili (*C. baccatum*), rocoto (*C. pubescens*), and panca chili (*C. chinense*). Due

to the wide variety and productivity of chili peppers and peppers, Peru has managed to rank among the top ten countries in the production and export of *Capsicum* (Rojas *et al.*, 2020).

The rocoto is a crop of national gastronomic importance, presenting two major genotypes (Serrano and Monte), which differ in size and spiciness.

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However, both are in great demand in the markets for sauces and typical national dishes, such as *rocoto relleno arequipeño* (Meckelmann *et al.*, 2013). Within the pharmaceutical industry, it is also relevant for presenting capsaicinoid alkaloids and carotenoids (Vera-Guzmán *et al.*, 2011).

The rocoto crop presents problems of biotic stresses such as diseases and pests, which affect the production and economy of farmers dedicated to this crop. Infection and damage to plants will depend on each genotype of rocoto; some may have a certain degree of resistance, and in susceptible genotypes the plants can collapse. In order to deal with these problems, it is necessary to identify genotypes that are resistant to these types of stresses to form part of rocoto breeding programs (Pineda *et al.*, 2021).

Despite the great national importance of rocoto cultivation, no cultivars or varieties defined by genetic improvement programs have yet been obtained with resistance and tolerance to abiotic and biotic factors; farmers use seeds from previous harvests (Guevara *et al.*, 2000). The reason that makes it challenging to obtain homozygous lines is that the type of pollination that presents the rocoto presents cross-pollination and be self-incompatible; due to this, there have been no defined cultivars of rocoto or certified seeds, using only the seeds from a previous production (Saborío and Da Costa, 1992; Zewdie *et al.*, 2001).

The alternative to finding efficiency in conventional breeding methods whose main disadvantage is the large number of self-pollination cycles or crosses until homozygosity is achieved (Miyajima *et al.*, 2021). Biotechnological tools within their applications constitute an alternative to overcome these types of disadvantages, such as the *in vitro* anther culture technique for obtaining haploid or double haploid plants, which allows obtaining pure or well-defined lines from a single generation, so it could be successful in obtaining homozygous genotypes of rocoto in a short time (Seguí-Simarro and Nuez, 2008). This will make it possible to obtain high-yielding progenitors with lower susceptibility or resistance to phytopathogens to produce new varieties.

Therefore, the present research is focused on laying the foundations on the subject of tissue culture in rocoto from another culture since there are no works focused on the genetic improvement of this species using biotechnological techniques. Thus, the present research aims to induce somatic

embryos from *in vitro* culture of rocoto (*Capsicum pubescens* Ruiz & Pav.) anthers. The research will provide knowledge from protocols that allow the formation of rocoto embryos from anthers under *in vitro* conditions to regenerate plants with great agronomic potential in the near future and to initiate breeding programs in rocoto from pure lines to obtain cultivars with characteristics depending on the demand of both farmers and consumers.

Materials and methods

Plant material

The Serrano or Huerto genotype of rocoto (*Capsicum pubescens* Ruiz & Pav.) was used in the experiment. The rocoto plants were grown under greenhouse conditions, and the mother plants were selected 4 weeks after the appearance of the first flower buds.

Correlation between flower bud size and microspore development stage

Flower buds of different sizes were randomly selected and visually divided into five classes according to flower bud to determine the correlation between flower bud size and microspore developmental stage, petal, and sepal size. Flower buds were taken to the laboratory, and measurements (mm) of flower buds, petals, sepals, sepal/petal ratio index, and petal/flower bud were taken.

The stage of microspore development was determined by staining with 0.5% acetic-orcein. A drop of acetic-orcein was placed on the isolated anthers; then, a glass rod was used to apply pressure to the anthers to burst it and release the microspores. After a few minutes, the slide was placed under the microscope, and the stage of microspore development was observed. The number of microspores/anthers was obtained by the ratio between the total number of cells found in each stage of development (counted in five fields).

Disinfection of plant material

The selected rocoto flower buds were collected and transferred to the laboratory to perform the disinfection process in different treatments. The flower buds were first immersed in 70% alcohol for one minute, then transferred to disinfectant solutions

of different treatments (Table 1) for 10 minutes under constant agitation. After the established time, three rinses were performed with sterile distilled water for five minutes each rinse, and then the anthers were extracted to be placed on plates with MS (Murashige and Skoog, 1962) culture medium at half concentration, added with sucrose (60 g/L) and agar (5 g/L) with 5.7 pH.

All explants were placed in a growth chamber at 25 °C, with a photoperiod of twelve hours of light, for 14 days, during which evaluations were made. The percentage of disinfection of rocoto anthers and the percentage of partially or entirely necrotic anthers were evaluated.

Induction of somatic embryos via anther culture

The previously disinfected rocoto anthers were introduced into different induction culture media (Table 2) to determine the culture medium with the highest induction percentage from the rocoto anthers. The culture media consisted of macronutrients and micronutrients described by Murashige and Skoog (1962) at half concentration, added with myoinositol (100 mg/L), glycine (2 mg/L), nicotinic acid (0.5 mg/L), pyridoxine HCL (0.5 mg/L), thiamine HCL (0.1 mg/L) and sucrose (60 g/L); before adding the agar (5 g/L),

Table 1. Treatments with disinfectant solutions of rocoto flower buds.

Treatment	Disinfectant solution
D1	NaClO 1%
D2	NaClO 2%
D3	NaClO 3%
D4	NaClO 4%

Table 2. Treatments for somatic embryo induction in rocoto anthers.

Treatment	2,4-D (mg/L)	KIN (mg/L)
T1	0	0
T2	0.1	0.1
T3	0.1	1
T4	1	0.1
T5	1	1

2,4-D = 2,4-dichlorophenoxyacetic acid; KIN = Kinetin.

the pH was adjusted to 5.7. The media were then transferred to the autoclave to be sterilized at 1.2 Bar pressure and 121°C for 20 minutes.

All culture media were placed in a growth chamber in total darkness at 35 °C for the first four days (Dumas de Vaulx *et al.*, 1981; Parra-Vega *et al.*, 2012). Subsequently, the growth chamber conditions were modified with 12 hours of light and 12 hours of darkness, and 25 °C for eight weeks, the evaluation of somatic embryo induction was carried out after eight weeks, and the culture media were renewed every two weeks.

Statistical analysis

The design used was a completely randomized design (CRD) in all treatments, with 10 replicates per treatment and the experimental unit consisted of a Petri dish with 30 rocoto anthers. The data were processed using the free software R (version 4.0.3), using Analysis of Variance (ANOVA), and for the results with significance, a comparison between means was performed with the Tukey test ($p \leq 0.05$).

Results and discussion

Correlation between flower bud size and microspore development stage

The rocoto flower buds were grouped according to the morphological relationship between petals and sepals, forming five groups (Table 3). This point is crucial because the maturation of microspores presents changes parallel to the growth of flower buds. The correlation of size change and maturation allows the classification of flower buds that can indicate a higher percentage of microspores at the desired stage of development without invasively damaging the anthers.

The grouping of flower buds of rocoto genotype Serrano concerning their morphology between petals and sepals allowed the selection of the group indicated for presenting a higher percentage of microspores in a uninucleate state of great importance for the research. This morphological relationship allows working with an established group of flower buds without the need to damage each anther before introduction. The grouping of flower buds is considered a good indicator of the stage of microspore development due to the current relationship between the morphology of flower buds

Table 3. Classification of rocoto flower buds by morphological relationship.

Ratio index	Flower bud (mm)	Sepal (mm)	Petal (mm)	Sepal/Petal	Petal/Flower bud
RI1	2.35 c	2.12 c	0.23 e	9.22 a	0.09 c
RI2	2.82 c	2.14 c	0.68 d	3.15 b	0.24 b
RI3	4.14 b	2.52 b	1.62 c	1.56 c	0.37 b
RI4	4.89 b	2.56 b	2.34 b	1.09 d	0.48 a
RI5	6.65 a	3.04 a	3.61 a	0.84 d	0.54 a

Means with different letters differ significantly according to Tukey test for $p < 0.05$.

with the maturation of anthers and consequently the stage of microspore development in rocoto (Hegde *et al.*, 2017).

The percentage of microspores in the uninucleate stage was evaluated from these flower bud groups, determining which group based on their ratio index (RI) had the highest percentage of microspores in the uninucleate stage. Group RI4 presented the highest percentage of microspores in the uninucleate stage with 81.3% (Figure 1), followed by RI3 with 59.6%, while RI1, RI2, and RI5 were the groups with the lowest percentage of microspores in the uninucleate stage with 5.7%, 14.9%, and 11.8% respectively, among the last two groups there was no significant difference. This result allows us to determine that the RI4 group of flower buds are optimal for the induction of somatic embryos because they present a higher percentage of microspores in the uninucleate state.

Group RI4 was the group selected for the research because it presented the highest percentage of microspores in the uninucleate stage (81.3%); these results are similar to those shown by Hegde *et al.* (2017), grouped into three different groups

anthers of *Capsicum annuum* L., determining that group 2 was formed by flower buds of the same length of sepals and petals, this group presented 80% of microspores in the uninucleate stage.

Alves *et al.* (2015) grouped *Capsicum* spp. anthers in three groups, in which group 2 was characterized by presenting similar lengths between sepals and petals with 1.50 mm and 1.45 mm respectively, representing a sepal/petal and petal/floral bud ratio of 1.03 and 0.76 respectively, in this group presented microspores in the uninucleate stage higher than 95%. This ratio agrees with the result obtained by Dumas de Vaulx *et al.* (1981), who used anthers in *Capsicum annuum* L., where microspores were in the late uninucleate stage equivalent to a size with sepals and petals of equal length.

Disinfection of plant material

In the evaluation corresponding to 14 days of in vitro establishment, the results analyzed by analysis of variance allowed determining that at least one treatment differed from the others in the percentage of disinfection, while in the percentage of necrosis, there was no significance between treatments. The best results were obtained with treatments D3 and D4 regarding the percentage of disinfection, with 83.68% and 89.01%, respectively (Table 4).

Treatment D4, which consisted of 4% sodium hypochlorite solution, allowed obtaining the highest percentage of disinfection of flower buds without damaging the anther tissue. This result is superior to that used by Comlekcioglu (2021), who obtained positive disinfection results and no damage in anthers of *Capsicum annuum* L. using calcium hypochlorite solution (15%) for 15 minutes. Ozsan and Naci (2017) performed disinfection of flower buds of *Capsicum annuum* L. with an alcohol solution (70%) for sixty seconds, followed by 15 minutes in sodium hypochlorite solution (10%),

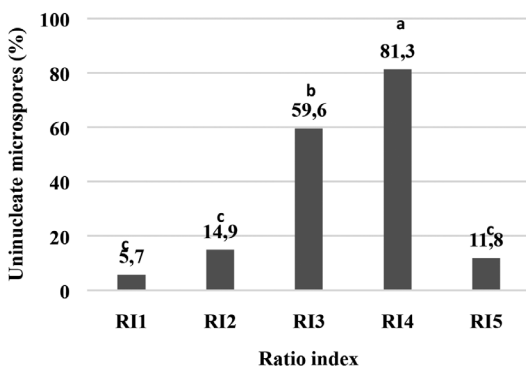


Figure 1. Percentage of microspores in uninucleate state by ratio index groups in rocoto flower buds. Means with different letters differ significantly according to Tukey test for $p < 0.05$.

Table 4. Comparison of disinfection and necrosis of rocoto flower buds 14 days after in vitro establishment.

Treatment	Disinfectant solution	Disinfection (%)	Necrosis (%)
D1	NaClO 1%	27.34 c	0 a
D2	NaClO 2%	52.67 b	0 a
D3	NaClO 3%	83.68 a	0 a
D4	NaClO 4%	89.01 a	0 a

Means with different letters differ significantly according to Tukey test for $p < 0.05$.

obtaining results similar to those obtained in the present research.

Induction of somatic embryos via anther culture

Allus and somatic embryo formation were induced in rocoto anthers by adding the growth regulators 2,4-D and KIN to the culture media; the treatment without the addition of growth regulators showed no response in rocoto anthers. The presence of friable regions in the callus was observed in rocoto anthers, and these regions were generally white and translucent, which characterized them as an embryogenic callus.

There was a significant difference between treatments, with higher percentages of callus induction in treatments T4 and T5. In rocoto anthers, up to 52.65% of the anthers induced callus in the T4 medium. This medium contained the highest concentration of auxin (2,4-D 1 mg/L) and the lowest concentration of cytokinin (Kinetin 0.1 mg/L) among the four media used (Table 5).

The formation of callus in the anthers of rocoto was related to the response to the addition of 2,4-D and Kinetin in the culture media, especially due to the characteristic of the auxin 2,4-D, which when found exogenously in the culture medium

induces cell proliferation in the explants, as well as cell disorganization, which is characteristically observed as callus concerning the concentration of the growth regulator and the genotype of the explant under study (Argüelles *et al.*, 2020; Hernández-Amasifuen *et al.*, 2021).

The results obtained in callus formation in rocoto anthers present similarity with the results demonstrated by Alves *et al.* (2015), who induced *Capsicum* spp. callus formation, presenting the best results of 36% and 24% of callus formed using culture media added with 4.65 mg/L KIN plus 4.52 mg/L 2,4-D and 23.25 mg/L KIN plus 22.6 mg/L 2,4-D, respectively. Hegde *et al.* (2017) obtained 53.33% callus formation in anthers of *Capsicum annuum* L. variety Indra and 51.11% in cultivar Bharat, adding 7.5 mg/L KIN and 0.2 mg/L NAA. Luitel and Kang (2013) induced callus in anthers of mini paprika (*Capsicum annuum* L.) using MS culture medium added with 2,4-D (0.1 mg/L) and KIN (0.1 mg/L), obtaining up to 53.2% in the cultivar Vine Sweet - Red, but in the same cultivar, they managed to induce up to 78.5% of callus by changing the culture medium. Ozsan and Naci (2017), using MS culture medium added with NAA (5 mg/L) and BAP (0.5 mg/L), induced 12.05% callus formation in anthers of *Capsicum annuum* L. cultivar Belissa, while with the same culture medium in cultivars

Table 5. Somatic embryo induction response in rocoto anthers.

Treatment	2,4-D (mg/L)	KIN (mg/L)	Callus induction (%)	Somatic embryo induction (%)
T1	0	0	0 c	0 d
T2	0.1	0.1	11.06 b	18.27 a
T3	0.1	1	8.76 b	5.28 c
T4	1	0.1	52.65 a	3.39 c
T5	1	1	45.75 a	11.19 b

Means with different letters differ significantly according to Tukey test for $p < 0.05$.

Benino and Kanyon they obtained 0% and 2.29% callus formation respectively.

The culture of anthers of rocoto anthers was carried out in MS medium with the addition of growth regulators. The effect of each treatment in inducing the formation of somatic embryos in rocoto anthers was determined firstly, the need to add phytohormones that regulate growth or influence the culture media, and secondly, that there was a significant response in the induction of somatic embryos in the treatments with a balance between 2,4-D and KIN. Table 5 shows T2 as the treatment with the highest somatic embryo formation with 18.27%, representing five to six somatic embryos per plate. The second-best treatment was T5, which was presented in the culture medium with a hormonal balance of 2,4-D (1 mg/L) and KIN (1 mg/L), obtaining 11.19% of anthers with somatic embryo formation, which corresponds to three to four anthers with somatic embryos per plate. In the T1 treatment, which was a culture medium without added growth regulators, there was no induction of somatic embryos in any rocoto anther.

The formation of somatic embryos in the anthers of rocoto was observed from the sixth week in treatment T2, while the other treatments that presented somatic embryo formation were observed from the seventh week after establishment in the culture media. The somatic embryos were identified as having a whitish color when they emerged from the anthers of rocoto, and as the days went by, growth was observed in the emerged embryos, forming the characteristic of cotyledonary, globular, and torpedo embryos.

The induction of somatic embryos in rocoto anthers was achieved with the addition of 2,4-D and KIN, auxin, and cytokinin (growth regulators), respectively, presenting the T2 treatment (0.1 mg/L 2,4-D and 0.1 mg/L KIN) the superior results with 18.27% of anthers in the formation of somatic embryos. The results are close to those obtained by Comlekcioglu (2021), with 18% of somatic embryos in anthers of *Capsicum annuum* L. cultivar Diyar, using MS culture media added with NAA (4 mg/L) and BAP (0.5 mg/L).

Grozeva and Nankar (202) obtained results superior to the present research with the formation

of 54 somatic embryos (corresponding to 27% of somatic embryogenesis) in anthers of *Capsicum annuum* L. cultivar Stryama using MS culture media added with 2,4-D (0.1 mg/L) and Kinetin (0.1 mg/L); in the same research using the same culture media but they did not manage to form somatic embryos from anthers of *Capsicum annuum* L. cultivar Zlaten Medal 7 (0% of somatic embryos per anther). Cultivar Zlaten Medal 7 (0% somatic embryos per anther), showing that the induction of somatic embryos is genotype-dependent, that is to say, that each genotype needs the previous standardization of the culture medium with the nutrients and growth regulators necessary for the generation of organogenesis or induction of somatic embryogenesis.

Alves *et al.* (2015) obtained inferior results using 4.65 mg/L KIN plus 4.52 mg/L 2,4-D and 23.25 mg/L KIN plus 22.6 mg/L 2,4-D with 12% and 2% of somatic embryo formation respectively in anthers of *Capsicum annuum* L. Parra-Vega *et al.* (2012), using culture media similar to the present investigation, obtained superior results in the cultivar Herminio of *Capsicum annuum* L. with 22% cultivars, but inferior results in the cultivars Coyote, Quito, and Vélez, with 10%, 4% and 4% of somatic embryos, respectively.

Vélez *et al.* (2010) obtained lower results than the present research with 2.23% of somatic embryos in anthers of *Capsicum annuum* L. cultivar Miahuateco, using MS culture medium added with BAP (0.1 mg/L) and 2,4-D (1 mg/L). Koleva-Gudeva *et al.* (2007) obtained superior results with 33% of somatic embryos in anthers of bell pepper (*Capsicum annuum* L.).

Conclusions

It was determined that the T2 treatment (0.1 mg/L 2,4-D and 0.1 mg/L KIN) is the optimal treatment to induce somatic embryos from *in vitro* culture of rocoto anthers with 18.27% of anthers with somatic embryo formation. Furthermore, the T4 treatment (1 mg/L 2,4-D and 0.1 mg/L KIN) allows callus formation in rocoto anthers with 52.65% anthers with the presence of callus.

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