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PLANT TISSUE CULTURE

Micropropagation of *Physalis angulata* L. and *P. chenopodifolia* Lam. (Solanaceae) *via* indirect organogenesis

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Abstract

The husk tomato (*Physalis* spp.) is an exceptional commercial crop for its nutritional and medicinal properties where the whole plant is used. This has led to the search for new micropropagation methods to accelerate plant production in the field. *P. angulata* and *P. chenopodifolia* were micropropagated *via* shoot proliferation of axillary buds and indirect organogenesis. Shoot multiplication was performed on Murashige and Skoog (MS) basal medium supplemented with 2.22, 4.43, or 6.65 µM 6-Benzylaminopurine (BAP) combined with 2.32, 4.64, or 6.96 µM Kinetin (Kin). For *P. chenopodifolia*, the largest number of new shoots was obtained by adding 4.64 µM Kin (10.47 ± 2.25 shoots per explant); for *P. angulata*, the best treatment was obtained with a combination of 4.43 µM BAP and 2.32 µM Kin (8.47 ± 2.91 shoots per explant). Indirect organogenesis was performed by placing leaf sections of both *Physalis* species on MS medium supplemented with 2.22, 4.43, 6.65, or 8.87 BAP combined with 1.13, 2.26, or 3.39 µM 2,4-dichlorophenoxyacetic acid (2,4-D). *P. chenopodifolia* showed the highest number of new indirect shoots (37.14 ± 3.54) with the addition of 1.13 µM 2,4-D and 6.65 µM BAP; *P. angulata* had the highest result (22.71 ± 2.5 shoots per explant) with 1.13 µM 2,4-D and 4.43 µM BAP. Stimulation of root induction was obtained in different mediums with auxins 1.07, 2.68, 5.37, or 8.05 µM 1-Naphthaleneacetic acid (NAA) and 1.41, 2.85, 5.70, or 8.55 µM Indoleacetic acid (IAA). The regenerated plantlets resulting from the rooting process were acclimatized and transferred to the greenhouse. The average of shoots per explant in the indirect organogenesis method was higher than the axillary bud culture method. These results could provide an efficient alternative for the micropropagation and conservation of these species with high commercial potential.

Keywords Regeneration · Shoot multiplication · Rooting · Husk tomato berries · Conservation

Introduction

Physalis spp. (Solanaceae) are distinguished by an additive and inflated calyx, which expands completely around the fruit (Soares *et al*. 2009). The genus includes more than 90 species distributed throughout the American continent (Stehmann *et al*. 2015); approximately 70 of these species grow in Mexico where this country is the place of origin, diversity, and distribution of the taxon (D'Arcy 1991; Martinez 1998; Vargas-Ponce *et al*. 2011). Recently, *P. angulata* has been widely cultivated in an artisanal manner in central Mexico due to its nutritional and medicinal properties (Valdivia-Mares *et al*. 2016, Vargas-Ponce *et al*. 2016). Conversely, *P. chenopodifolia* has several uses, taking advantage of the whole plant; roots, stems, leaves, and fruit calyces are used in traditional medicine, and their sweet-bitter fruits are highly consumed (Valdivia-Mares *et al*. 2016).

Germplasm conservation through *in vitro* techniques is commonly used for species with high pharmaceutical and commercial potential, such as *P. angulata* and *P. chenopodifolia* (Romo-Paz *et al*. 2021). This technique allows germplasm to multiply, conserves genetic variability, and allows the study of the species' properties under controlled conditions without affecting wild populations (Bertoni *et al*. 2010).

Recent studies in different plant species where micropropagation techniques have been used have shown that this technique can be a solution for commercial

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exploitation, propagation, and conservation of selected species, improved genotypes, or elite specimens (Afroz *et al*. 2010; Delgado-Aceves *et al*. 2021). The biotechnological interest in the *Physalis* genus has increased in recent years due to it being rich in nutrients and compounds of pharmacobiological interest, such as phytosterols, withanolides, carotenoids, phenolic compounds, and physalins (Puente *et al*. 2011; Salcedo-Pérez *et al*. 2015; Lima *et al*. 2020). It also shows important antibiotic, antioxidant, anticancer, and anti-inflammatory properties (Reyes-Reyes *et al*. 2012; da Silva *et al*. 2015). However, there are few studies on the management, conservation, micropropagation, and plant regeneration of selected species of the *Physalis* genus with high commercial potential. Escobar-Guzmán *et al*. (2009) described the capacity of *P. ixocarpa* Brot. to regenerate embryos and plants from anther cultures treated with different levels of 2,4-D and vitamin B12 was tested. In the case of *P. minima* L., Mungole *et al*. (2011) reported the induction of callus *in vitro* and the regeneration of shoots from leaves with different concentrations of 2,4-D, Kinetin, and BAP, either alone or in combination. In *P. peruviana* L., rhizogenesis and organogenesis were induced from cultured leaves and nodes with treatments containing BAP and Kin (Otroshy *et al*. 2013). For *P. angulata* L, various methods have been used to generate propagation of clones *in vitro* including the use of phytohormones, such as BAP, Zeatin, Gibberellic acid (GA_3), IAA, Kin (Kumar *et al*. 2015; 2016), or different concentrations of sucrose and light spectra (Santos *et al*. 2020). On the contrary, for *P. chenopodifolia* Lam., there is no published information on the *in vitro* management of the species to the knowledge of the present authors.

Despite the great potential for use in the pharmaceutical industry, the commercial extraction of these chemical compounds is still difficult mainly because of two factors: the high genetic variation caused by the cross-pollination that predominates within *Physalis* genus and the low production of secondary metabolites in wild plants (Trillos *et al*. 2008). Thus, *in vitro* propagation techniques are promising tools for large-scale multiplication of selected varieties with a higher content of bioactive compounds and for *in vitro* production of high added-value compounds (de Oliveira *et al*. 2013).

Organogenesis is an important *in vitro* tissue culture technique that induces genetic changes and increases the plant multiplication rate for conservation (Su and Zhang 2014). Organogenesis is the ability to regenerate entire organs or plants from cells that rearrange to develop a meristem upon stimulation (Hnatuszko-Konka *et al*. 2021). It includes the formation of buds, shoots, or radical meristems directly from explants (direct organogenesis) or after cell proliferation and the generation of a callus (indirect organogenesis) (Lardon and Geelen 2020; Shin *et al*. 2020). In morphogenetic competition, explants develop the ability to respond to organ-inducing hormonal signals, a process known as dedifferentiation (Hnatuszko-Konka *et al*. 2021). Auxins and cytokinins are the main phytohormones involved in this process. These plant growth regulators (PGRs) need to be applied simultaneously or sequentially because they coordinately guide the formation of organ-generating structures through complex intracellular crosstalk (Duclercq *et al*. 2011; García-Pérez *et al*. 2020). This process also depends on the capability of the cells and tissues to respond to these changes (Sugiyama 2014). This study aimed to determine an effective procedure for mass multiplication of *P. angulata* and *P. chenopodifolia* through whole-plant regeneration from callus induction of leaf segment explants.

Materials and methods

Plant material and culture conditions The biological material was established *in vitro* from dried seeds of *Physalis angulata* L. (Voucher OVP 544) and *P. chenopodifolia* Lam. (Voucher OVP 539). Seeds are deposited at the University of Guadalajara, at the National Laboratory for Plant Identification and Characterization (LaniVeg).

The plant material (50 seeds per species) was disinfected with 70% ethanol for 1 min, and then commercial sodium hypochlorite (Cloralex®, Monterrey, Mexico) (25%, *v/v*) was applied (1.5% active chlorine), plus two drops of liquid detergent for 15 min in constant agitation, followed by washing three times with sterile distilled water. Plant material was established on basal Murashige and Skoog (MS) medium (25%) (Sigma-Aldrich, St. Louis, MO) (Murashige and Skoog 1962) supplemented with L2 vitamins (Sigma-Aldrich) (Phillips and Collins 1979), 3% (w/v) sucrose (Golden Bell®, Zapopan, Mexico), and 0.8% (*w/v*) agar (Sigma-Aldrich); pH was adjusted to 5.8 ± 0.02 (Mascarenhas *et al*. 2019). The medium was sterilized by autoclaving at 121 °C for 15 min. The seeds were kept in dark conditions for 1 d, then they were maintained under diffuse light conditions (27 µmol m^{-2} s^{-1}) with a 16-h photoperiod, at 27 ± 2 °C (Silva *et al*. 2016).

Micropropagation Aseptic explants of *P. angulata* and *P. chenopodifolia* (axillary buds) were established for shoot proliferation in basal MS medium, supplemented with L2 vitamins (Phillips and Collins 1979), and complemented with different combinations of PGRs, 0.0, 2.22, 4.43, and 6.65 µM BAP (Sigma-Aldrich), combined with 0.0, 2.32, 4.64, and 6.96 µM Kin (Sigma-Aldrich). Three stem segments (1 to 2 cm) with one axillary meristem each were established per container, seven replications per treatment for a total of 16 treatments. The culture media were enriched with 3.0% (*w/v*) sucrose (Golden Bell®) and 0.8% (*w/v*)

agar (Sigma-Aldrich); pH was adjusted to 5.8 ± 0.02. Shoot proliferation of axillary buds' data were documented and analyzed 4 wk after culture initiation. These *in vitro* propagation experiments were replicated in three cycles. Data from all three cycles were pooled, and average proliferation rates over time were determined. For indirect organogenesis, explants (leaf sections) of approximately 0.5 cm in length were used; these explants were obtained from the 4-wk-old plants propagated *in vitro*. Explants were individualized and established in sterile plastic Petri dishes, containing 20 mL of basal MS medium supplemented with L2 vitamins (Phillips and Collins 1979) and several combinations of PGRs (cytokinins and auxins): 0.0, 2.22, 4.43, 6.65, and 8.86 µM BAP (Sigma-Aldrich) combined with 0.0, 1.13, 2.26, and 3.39 µM 2,4-D (Sigma-Aldrich). Three leaf segments were established per container, and seven replicates per treatment were inoculated for a total of 20 treatments. Sucrose (Golden Bell; 3.0% *w/v*) and agar (Sigma-Aldrich) (0.8% *w/v*) were added to all culture media. The pH was adjusted to 5.8 ± 0.02. The medium was sterilized by autoclaving at 121 °C for 15 min. The explants were maintained under diffuse light conditions (27 µmol m^{-2} s^{-1}) with a 16-h photoperiod at 27 ± 2 °C. Percentage of explants with callus, percentage of explants forming shoots, and number of shoots per explant were recorded and analyzed 4 wk after culture initiation.

Histological analysis Fresh organogenic calluses samples were dissected; to fix the samples, the plant material was treated with 70% *v/v* ethanol. Subsequently, the tissue was placed in a solution of polyethylene glycol (BioReagent®, Toluca, Mexico) (PEG, 1450 molecular weight) and deionized water in a 1:4 proportion, as described by Delgado-Aceves *et al*. (2021). Subsequently, the plant tissue was embedded in the PEG solution, and cuts were made with a rotary microtome (YIDI® Jinhua, China) to obtain 15 µm sections of the samples; then, in order to observe structures, the tissues were double stained, as described by Gupta and Durzan (1987), applying 0.5% (1:1 *w/v*) acetocarmine and 0.5% (1:1 *w/v*) Evan's blue. The samples were then analyzed under an optical microscope (Carl Zeiss® White Plains, N Y).

Rooting and acclimatization Micropropagated accessions of *P. angulata* and *P. chenopodifolia* were established in a rooting experiment, in basal MS medium supplemented with L2 vitamins and auxins in the following concentrations: 0.0, 1.07, 2.68, and 5.37 µM NAA (BioReagent®, Toluca, Mexico) or 0.0, 1.41, 2.85, and 7.70 µM IAA (Sigma-Aldrich). Three healthy shoots from the indirect shoot multiplication (2 cm long) were established per container; seven replications by treatment were placed in the culture medium for a total of nine treatments. Sucrose (Golden Bell; 3.0% *w/v*) and 0.8% *w/v* agar (Sigma-Aldrich) were added to all culture media. The pH was adjusted to 5.8 ± 0.02. The medium was sterilized by autoclaving at 121 °C for 15 min. The explants were maintained under diffuse light conditions (27 µmol m^{-2} s^{-1}) with a 16-h photoperiod at 27 ± 2 °C. Number of roots present per explant and their corresponding length were recorded 4 wk after treatment. Healthy plantlets with well-developed shoots and roots were transferred to plastic pots containing sterile perlite and peat moss (1:1). Potted plantlets were covered with transparent domes to ensure high humidity and were maintained under diffuse light conditions (27 µmol m^{-2} s^{-1}) with a 16-h photoperiod at 27 ± 2 °C. The plantlets were watered every 3 d with 1/8-strength MS basal salt solution supplemented with L2 vitamins without sucrose. The plastic dome was removed after 2 wk to acclimatize plants to greenhouse conditions. After 4 wk, plantlets were maintained in the greenhouse under full sunlight (Swartwood and Van-Eck 2019).

Statistical analysis A completely randomized design was used to carry out all experiments. The significance of differences among factors in micropropagation (levels BAP-Kin), indirect organogenesis (levels BAP-2,4-D), and rooting (levels NAA-IAA) experiments was determined by two-way analysis of variance (ANOVA). Fisher's LSD test was performed when significance was detected to differentiate among means ($P < 0.05$). Statistical analyses were carried out using the Statgraphics Centurion XVI® statistical software 16.2.4.

Results and discussion

Plant material and culture conditions The seeds of *P. angulata* exhibited 92% germination and those of *P. chenopodifolia* 96% germination after 4 d of inoculation on the medium supplemented with 25% MS basal salts supplemented with L2 vitamins. These results demonstrated the high capacity of *Physalis* spp. to develop under reduced *in vitro* conditions, as reported by Rodrígues *et al*. (2013) and da Silva *et al*. (2021) who performed *in vitro* culture of *P. peruviana*, *P. minima*, and *P. ixocarpa* explants with 25, 50, and 75% MS basal salts.

Micropropagation Micropropagation of *P. angulata* and *P. chenopodifolia via* multiplication of axillary buds on MS basal medium supplemented with L2 vitamins and different combinations and concentrations of PGRs was tested. In all treatments, both species showed shoot proliferation (Fig. 1).

The ANOVA showed a significant difference ($P \leq 0.05$) among factors (BAP and Kin) and a positive interaction between PGRs ($P \leq 0.05$) in *P. angulata* culture. The highest average number of new shoots in this species was found in

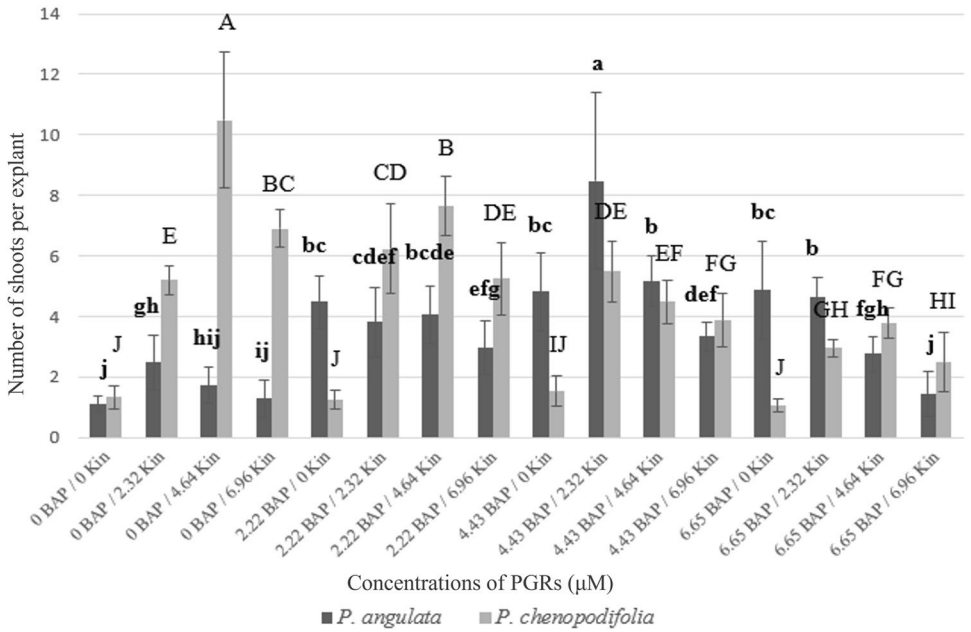

Figure 1. Effects of 6-benzylaminopurine (BAP) and Kinetin (Kin) on micropropagation of *Physalis angulata* L. and *P. chenopodifolia* Lam. *via* multiplication of axillary buds (data recorded after 4 wk). The means followed by the same *letter(s)* are not significantly different according to the Fisher's LSD test (*P. angulata*, *lowercase letter*; *P. chenopodifolia*, *capital letter*). Data represent means ± standard deviation (SD).

the treatment with 4.43 µM BAP and 2.32 µM Kin in which 8.47 ± 2.91 shoots per explant were counted. The interaction between BAP and Kin has been favorable for bud multiplication of various plant species (Mungole *et al*. 2011; Handique and Bhattacharjee 2000). Conversely, *P. chenopodifolia* showed the best result with the addition of Kin alone; 10.47 ± 2.25 shoots per explant were obtained in basal medium supplemented with 4.64 µM Kin only ($P < 0.05$).

Cytokinins are known to induce axillary shoot formation (Jafari *et al*. 2011; de Oliveira *et al*. 2019). Indeed, PGRs, such as BAP or Kin, induce multiplication in solanaceous plants like *Solanum lycopersicum* (= *Lycopersicon esculentum*), *Capsicum annum*, and *S. tuberosum* (Harish *et al*. 2010; Kumar *et al*. 2011; Kazemiani *et al*. 2018). The results in the present study were similar to those of previous reports (Romo-Paz *et al*. 2021), demonstrating the multiplication capacity of the *Physalis* after several micropropagation cycles.

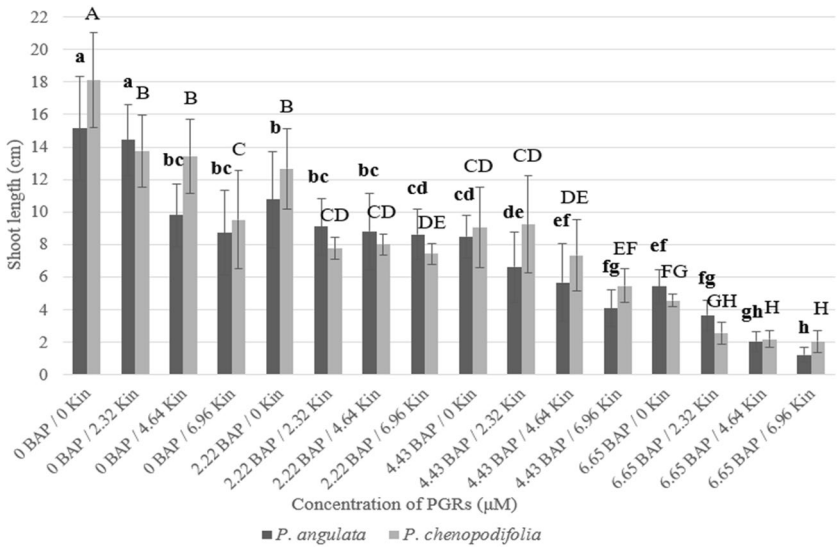

Figure 2. Effects of 6-benzylaminopurine (BAP) and Kinetin (Kin) on the stimulation of the length of the micropropagated shoots of *Physalis angulata* L. and *P. chenopodifolia* Lam. (data recorded after 4 wk). The means followed by the same *letter(s)* are not significantly different according to the Fisher's LSD test (*P. angulata*, *lowercase letter*; *P. chenopodifolia*, *capital letter*). Data represent means ± standard deviation (SD).

In contrast, the highest shoot length was obtained without PGRs in both species ($P < 0.05$). These results confirmed the findings of Huetteman and Preece (1993) and Brassard *et al*. (1996), stating that cytokinins commonly inhibit shoot elongation. *P. angulata* and *P. chenopodifolia* had the maximum lengths of 15.14 ± 1.2 and 18.09 ± 1.1 cm, respectively, on media without cytokinins; furthermore, the length of the shoots decreased as the concentration of the PGRs increased (Fig. 2).

Additionally, the effect of 2,4-D combined with BAP in the micropropagation of *P. angulata* and *P. chenopodifolia* *via* indirect organogenesis was investigated. Leaf explants cultured on MS basal medium formed a yellow-to-green friable callus (Fig. 3), followed by numerous shoot buds in most treatments with both PGRs (Table 1). This result was similar to those reported for other species, such as *P. minima* (Sandhya and Rao 2016) and *P. peruviana* (Hernández-Villalobos and Chico-Ruíz 2020). The highest number of new indirect shoots was found in *P. chenopodifolia*; the treatment with 1.13 µM 2,4-D and 6.65 µM BAP generated 37.14 ± 3.54 shoots per explant ($P < 0.05$). For *P. angulata*, the best result was observed with 1.13 µM 2,4-D and 4.43 µM BAP in the basal medium; this treatment generated 22.71 ± 2.5 shoots per explant ($P < 0.05$). In both species, adding both BAP and 2,4-D to the medium stimulated the induction of shoots, and the interaction between these PGRs was significant ($P < 0.05$). These results contrast with those of Gupta and Chandra (1987), who found that the formation of shoot buds in *P. ixocarpa* leaf explants was induced with BAP (0.5 to 5.0 mg L^{-1}) addition alone.

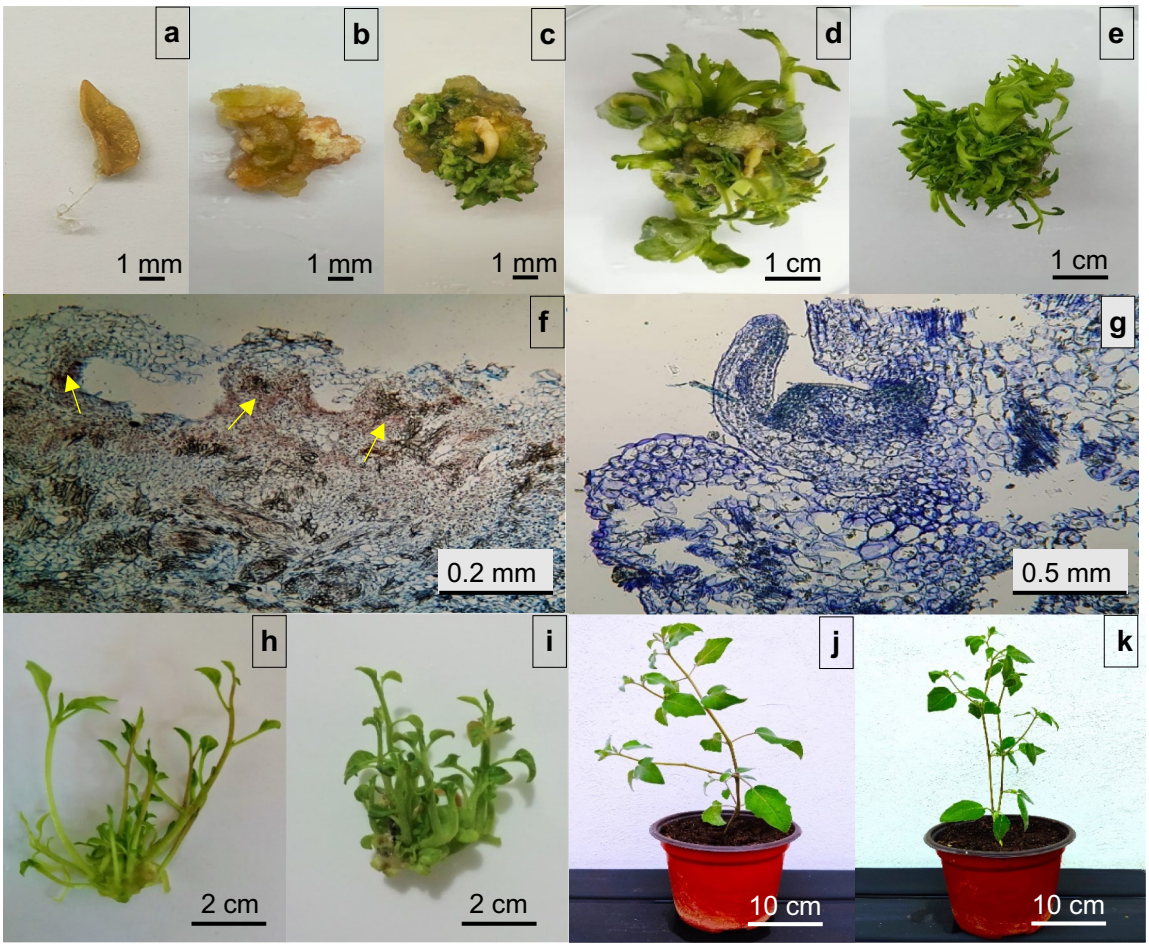

Figure 3. Anatomical characterization and histological analysis of organogenic callus of *Physalis angulata* L. and *P. chenopodifolia* Lam.). (*a*) Leaf explant (1 wk). (*b*) Callus formation with friable characteristics (2 wk). (*c*) Development of meristematic structures from organogenic callus (3 wk). (*d*) Shoots of *P. angulata* from leaves treated with 1.13 µM 2,4-D and 4.43 µM BAP (4 wk). (*e*) Shoots of *P. chenopodifolia* from leaves treated with 1.13 µM 2,4-D and 6.65 µM BAP (4 wk). (*f*) Histological section of a *P. angulata* callus with signs of cell development and proliferation prior to the development of shoots (*yellow arrows*). (*g*) *De novo* meristem from organogenic callus of *P. chenopodifolia*. (*h*) Section of callus with shoots of *P. angulata* before obtaining rooting material. (*i*) Section of callus with shoots of *P. chenopodifolia* before rooting experiments. (*j*) Rooted and acclimatized *P. angulata* plantlets (6 wk after acclimation). (*k*) Rooted and acclimatized *P. chenopodifolia* plantlets (6 wk after acclimation).

Table 1. Effects of Murashige and Skoog medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) on indirect organogenesis — stimulation from leaf explants of *Physalis angulata* L. and *P. chenopodifolia* Lam. (data analyzed after 4 wk)

PGRs		*P. angulata*				*P. chenopodifolia*			
2,4-D (µM)	BAP (µM)	Explants with calluses (%)	Calluses with shoots (%)	Number of shoots per explants ± SE*		Explants with calluses (%)	Calluses with shoots (%)	Number of shoots per explants ± SE*	
0.00	0.00	0	0	0	**f**	0	0	0	**f**
0.00	2.22	14.28	0	0	**f**	0	0	0	**f**
0.00	4.43	14.28	0	0	**f**	14.28	0	0	**f**
0.00	6.65	28.57	0	0	**f**	28.57	0	0	**f**
0.00	8.86	57.14	0	0	**f**	57.14	0	0	**f**
1.13	0.00	100	0	0	**f**	100	0	0	**f**
1.13	2.22	100	85.71	3.57 ± 0.89	**de**	100	85.71	1.42 ± 0.36	**ef**
1.13	4.43	100	100	22.71 ± 2.50	**a**	100	100	18 ± 2.05	**c**
1.13	6.65	100	100	16.4 ± 3.08	**b**	100	100	37.1 ± 3.54	**a**
1.13	8.86	100	71.42	5.57 ± 1.60	**cd**	100	100	29.8 ± 2.96	**b**
2.26	0.00	100	14.28	0.42 ± 0.42	**ef**	100	0	0	**f**
2.26	2.22	100	57.14	0.85 ± 0.34	**ef**	100	0	0	**f**
2.26	4.43	100	85.71	6.42 ± 1.70	**cd**	100	100	18.7 ± 2.61	**c**
2.26	6.65	100	100	8.57 ± 1.65	**c**	100	85.71	9.14 ± 1.80	**d**
2.26	8.86	100	0	0	**f**	100	57.14	4 ± 1.57	**e**
3.39	0.00	100	0	0	**f**	100	0	0	**f**
3.39	2.22	85.71	42.85	0.85 ± 0.40	**ef**	57.14	0	0	**f**
3.39	4.43	100	28.57	1.14 ± 0.70	**ef**	28.57	0	0	**f**
3.39	6.65	57.14	0	0	**f**	71.42	42.85	0.57 ± 0.29	**ef**
3.39	8.86	85.71	0	0	**f**	42.85	0	0	**f**

*Data represent means ± standard error (SE). Means followed by the same *letter(s)* within a *column* are not significantly different at $P < 0.05$ according to Fisher's LSD test

Table 2. Effect of NAA (1-naphthaleneacetic acid) and IAA (Indoleacetic acid) on root induction of micropropagated plantlets of *Physalis angulata* L. and *P. chenopodifolia* Lam. (data analyzed after 4 wk)

PGRs		*P. angulata*				*P. chenopodifolia*			
NAA (µM)	IAA (µM)	Number of roots per explants ± SE*		Root length ± SE (cm)*		Number of roots per explants ± SE*		Root length ± SE (cm)*	
0.00	0.00	7.47 ± 0.52	**e**	13.60 ± 1.05	**a**	8.42 ± 0.61	**d**	17.85 ± 1.73	**a**
1.07	0.00	27.47 ± 2.01	**a**	8.80 ± 0.69	**b**	21.60 ± 1.44	**bc**	9.76 ± 0.62	**bc**
2.68	0.00	23.90 ± 2.17	**ab**	4.28 ± 0.49	**d**	24.57 ± 0.89	**b**	9.52 ± 1.14	**bc**
5.37	0.00	19.10 ± 1.31	**bc**	3.04 ± 0.20	**de**	32.19 ± 2.50	**a**	4.47 ± 0.49	**d**
8.05	0.00	18.90 ± 2.36	**c**	1.42 ± 0.21	**e**	25.14 ± 1.70	**b**	4.59 ± 0.73	**d**
0.00	1.41	15.19 ± 1.19	**cd**	9.30 ± 0.75	**b**	23.57 ± 1.38	**b**	11.52 ± 1.06	**b**
0.00	2.85	19.57 ± 1.89	**bc**	7.42 ± 0.90	**bc**	25.47 ± 0.90	**b**	8.90 ± 0.84	**bc**
0.00	5.70	15.90 ± 1.47	**cd**	6.60 ± 0.62	**c**	26.14 ± 3.80	**b**	8.00 ± 1.91	**c**
0.00	8.55	12.38 ± 1.46	**d**	2.95 ± 0.44	**de**	16.57 ± 1.18	**c**	2.64 ± 0.42	**d**

*Data represent means ± standard error (SE). Means followed by the same *letter(s)* within a *column* are not significantly different at $P < 0.05$ according to Fisher's LSD test

Leaf explants of *P. angulata* and *P. chenopodifolia* barely exhibited sprout regeneration when the auxins or cytokinin were added alone in MS basal medium, supplemented with L2 vitamins. Callus initiation was not obtained in all treatments. According to Phillips and Garda (2019), the production of a pluripotent callus is closely linked to the addition of 2,4-D to the culture medium. Moreover, a high cytokinin concentration promotes shoot regeneration from callus cells because cytokinins mediate early loss of root identity, root primordia disorganization, and initiation of shoot development (Shin *et al*. 2020). Thus, hormone equilibrium and crosstalk between auxins and cytokinins are crucial for determining cell fate and patterning (Cheng *et al*. 2013).

Established explants on media supplemented with low concentrations of 2,4-D and different BAP concentrations showed the highest frequency of shoot formation. These findings agreed with those of Ramar *et al*. (2014) and Rezanejad and Hosseini (2019), who observed that, due to cellular pluripotency, the regenerated explant of other solanaceous species (*S. americanum* and *P. alkekengi*) dedifferentiated the tissues, returning them to their proliferative condition (Greb and Lohmann 2016).

Indirect shoots were induced along the entire surface of the explants (Fig. 3, *d* and *e*). Shoots induced by these PGR interactions at different concentrations did not show morphological alterations. The shoot buds obtained on BAP and 2,4-D medium were excised and grown to complete plants on 100% MS medium supplemented with L2 vitamins.

Histological analysis Histological analysis of the organogenic callus originating from disorganized leaf explants allowed the present authors to characterize the tissues anatomically and structurally. The shoots obtained were presented asynchronously and abundantly, which allowed the present authors to observe differentiation zones and meristem formation of both species (*P. angulata* and *P. chenopodifolia*) (Fig. 3, *f* and *g*).

In the early stages of the culture, the organization of the callus with large asymmetric vacuolated cells was observed. The cells that comprise it show continuous proliferation and high mitotic activity where many differentiation zones were identified on the periphery of the callus (Fig. 3, *f*). Subsequently, in advanced stages of the crop, it was identified that the formation of apical meristems begins from these differentiation zones, presenting a central zone, procambium, protoderm, and defined leaf primordia (Fig. 3, *g*).

Rooting and acclimatization Root formation of *P. angulata and P. chenopodifolia* was observed in all the treatments with NAA or IAA (Table 2). The results showed a significant difference ($P \leq 0.05$) between the two factors. These auxins had already been used for rooting in *Physalis* plants (Ramírez-Malagón and Ochoa-Alejo 1991). The first roots emerged from the basal segment of the shoot 5 to 6 d after culture with no callus formation. The highest number of roots was observed in regenerated *Physalis* shoots when cultured on MS medium (Murashige and Skoog 1962) supplemented with L2 vitamins (Phillips and Collins 1979) and with NAA. The highest number of roots was registered on *P. chenopodifolia* 32.19 ± 2.5 ($P \leq 0.05$) with a media containing 5.38 µM NAA; *P. angulata* showed a maximum average of 27.47 ± 2.01 ($P \leq 0.05$) roots per shoot in the medium with 1.07 µM NAA. Conversely, IAA treatments yielded a lower number of roots; *P. angulata* generated 19.57 ± 1.85 roots per explant in the treatment with 2.85 µM IAA, and *P. chenopodifolia* generated 31.3 ± 2.93 roots in the treatment with 5.70 µM IAA.

Most studies using NAA and IAA for root induction have achieved a high rooting frequency (De Klerk *et al*. 1997; Ozel *et al*. 2006; Afroz *et al*. 2010). These auxins stimulate cell division; however, although they are essential for the

initial induction of roots, their presence at certain concentrations can inhibit root development (Mills *et al*. 1997; Singh *et al*. 2008), as observed for the highest concentrations of NAA and IAA (Table 2). Thus, the balance between the substances added to the medium should be standardized to optimize the rooting of the explant (Borchetia *et al*. 2009), and the association between the rooting percentage and the endogenous-exogenous auxin levels in the plantlets must also be considered (Blakesley *et al*. 1991).

The highest root length was obtained without PGRs in *P. chenopodifolia* (17.85 ± 1.73 cm) while *P. angulata* recorded a maximum average of 13.6 ± 1.05 cm. The roots observed in the treatments with the highest concentration of NAA (8.05 µM) and IAA (8.55 µM) were small, thick, yellow–brown, and devoid of root hairs. Rooted shoots were transferred to plastic pots and successfully established and grown in greenhouse conditions (Fig. 3, *j* and *k*) with a survival rate of 90% for *P. angulata* and 94% for *P. chenopodifolia*.

Conclusion

The interaction between BAP and Kin was favorable for the development of *P. angulata*, stimulating the proliferation of buds and inhibiting their elongation. For *P. chenopodifolia*, the best result was observed with the addition of Kin alone. Moreover, leaf explants were shown to be effective for shoot regeneration *via* indirect organogenesis. The histological analysis demonstrated this process and allowed the multiple-cellular origin of buds to be defined. The successful production of multiple indirect shoots and the formation of roots *in vitro* depended on the effect of different PGRs (2,4-D and BAP and NAA or IAA, respectively) and growing conditions. The standardized method for mass multiplication of these two *Physalis* species through callus induction and plant regeneration could be used to produce and isolate medicinally important secondary metabolites, such as physalins and withanolides. To the best of the present authors' knowledge, this is the first report regarding *in vitro* whole-plant regeneration by indirect organogenesis of *P. angulata* and *P. chenopodifolia* from leaf segment explants.

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Author contribution FJ-RP wrote the manuscript. FJ-RP, JD-OF, and L-DA carried out *in vitro* propagation experiments. FJ-RP and JD-OF designed and performed organogenesis experiments. FJ-RP and L-DA took and processed the photos. O-VP: seed collection and manuscript reviewing. LP, JF-ZN, E-SP, and L-DA conceived the review. All authors reviewed and approved the final manuscript.

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