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Full Length Research Paper

Isolation and regeneration of protoplasts from leaf explants of *Rhyncholaelia digbyana*

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A protocol for the isolation and regeneration of protoplasts from leaf explant of *Rhyncholaelia digbyana* is presented. The protoplasts were isolated using hemicellulase enzymes at 1.5, 2.25, and 3% (w/v), pectinase at 0.5 and 0.75% (w/v) and cellulase at 1 and 2% (w/v). Protoplast counting was carried out with a Neubauer camera and an optical microscope at 40X, and viability was determined with Evans blue dye at 0.025% (w/v). The protoplasts were cultivated following the standard plate method, using the K&M media with 0.06 M of saccharose, 2.3% of Gelrite and plant growth regulators. It produced a yield of 386250±1875 protoplasts/g of tissue using an enzymatic combination of 1.5% (w/v) of hemicellulase, 0.5% (w/v) of pectinase and 1% (w/v) of cellulase with an incubation time of 4 h. The colonies were observed after two months of culture and the highest number of colonies (1.66±0.50) was obtained when the protoplasts were cultured in Kao medium with 4.53 µM of 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.912 µM of Zeatin and 3.10 µM of 6-Benzylaminopurine (BA). The nuclear DNA content estimated by flow cytometry for *R. digbyana* was 27.39±3.8 pg of DNA equivalent to 26.79×10^9 pb and the number of mitotic chromosomes counted was 2n=40.

Key words: Isolation, *in vitro* culture, plant regeneration, flow cytometry.

INTRODUCTION

Orchids belong to the family Orchidaceae and their diversity in the world is comprised 28,484 species, classified in more than 1,000 plant genera (Dodson, 2016; WCSP, 2017). These plants are cosmopolitan and present a diversity of forms, sizes, colors, and aromas. However, their main attribute is their floral structure (Tamay et al., 2016). The ornamental characteristics and long life span of the flower place them among the families with greater commercial importance. In 2015, the Department of Agriculture of the United States reported that the sales of orchids occupied 1st place in commercialized ornamental plants, dominating this category with 288 million dollars, a 5% increase in comparison with 2014; with *Cymbidium, Cattleya, Dendrobium,* and *Phalaenopsis* representing the most cultivated genera (Suárez and Téllez, 2015; Murguía et al., 2016).

Rhyncholaelia digbyana is a species that belongs to the

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subfamily Epidendroideae, tribe Epidendreae and subtribe Laeliinae, it has a flower measuring 20 cm in diameter with white to greenish coloring and a citric fragrance, whose genetic potential has not yet been explored and which could be used to improve or produce new varieties of orchids with better ornamental characteristics which can compete in the national and international markets. One way to accomplish genetic improvement in plants is through direct transfer of DNA (Vallejo and Estrada, 2002), such as the technique of protoplasts which has permitted the production of plants with enhanced ornamental characteristics (Hossain et al., 2013).

In the family Orchidaceae, most studies on protoplasts have focused on the genera *Dendrobium* and *Phalaenopsis.* In 1990, the first isolation of protoplasts in *Dendrobium hawaiian*, using leaf tissue, was reported (Kuehnle and Nan, 1990), also involving callus and leaf tissue for *Phalaenopsis* species (Sajise and Sagawa, 1991; Kobayashi et al., 1993). Cells in suspension of *Phalaenopsis* have also been used for the isolation of protoplasts (Shrestha et al., 2007). In *Dendrobium crumenatun* and *Dendrobium* var. *'*Queen Pink*',* it was reported that better results are obtained with the use of leaf tissue (Lee et al., 2010; Aqeel et al., 2016).

The size of the *R. digbyana* genome has been shallow studied. Furthermore, there are no existing reports of the isolation of protoplasts and *in vitro* regeneration. According to Leitch et al. (2009), the techniques available in orchids for the analysis and evaluation of the genetic diversity, with the aim of contributing to their conservation, are limited. Therefore, given the ornamental importance of these plants and the need to expand our knowledge of their genome, in this study the nuclear DNA content was estimated by flow cytometry and a chromosome count was carried out. This work also represents the 1st report describing the establishment of conditions for the isolation and regeneration from protoplasts, in order to establish the basis for future genetic improvement of the species.

MATERIALS AND METHODS

Plant materials

The number of chromosomes was determined from young roots with meristems and the nuclei for the estimation of DNA content and the protoplasts were isolated from the leaf tissue of *in vitro R. digbyana* plantlets provided by CIATEJ, Sede Sureste. The *R. digbyana* plantlets were maintained in Phytamax^{TM} (P-0931-L SIGMA) culture media under photoperiod conditions of 16/8 h with a light intensity of 60 $\mu mol^{-2}s^{1}$ and a temperature of 23±2°C.

Weighing and cutting of plant tissue prior to the enzymatic treatment

One gram of *R. digbyana* leaves was weighed for each enzymatic treatment; these were placed in a Petri dish and a scalpel was used to cut thin transversal strips of approximately 3 mm, which were then placed in an Erlenmeyer flask.

Protoplast isolation

The process of protoplast isolation was carried out under aseptic conditions in an ESCO laminar flow cabinet. The solution for the isolation of protoplasts consists of different concentrations of cell wall degrading enzymes (Table 1), such as hemicellulase, pectinase, cellulase and an osmotic solution consisting of 0.2 mM of KH_2PO_4, 0.1 mM of KNO_3, 10 mM of $CaCl_2{\cdot}2H_2O$, 0.1 mM of $MgSO_4{\cdot}7H_2O$, 0.1 µM of KI, 0.1 µM of $CuSO_4{\cdot}5H_2O$ and 7 mM of Mannitol. The pH of the solutions was adjusted to 5.8. A total of 24 treatments were performed and for one of them the enzymatic mixture and the osmotic solution were added by filtration, procuring a 1:1 proportion, after which each treatment was incubated under conditions of darkness at 25±2°C and agitation at 50 rpm for 4 and 6 h.

Purification of protoplasts

After cell walls digestion, the cells and the enzymatic mixture were filtered through a 60 µm nylon sieve and the filtrate was centrifuged at 750 rpm for 5 min. The supernatant was eliminated using a micropipette. The protoplast sediment was re-suspended in 6 ml of the osmotic cleaning solution with mannitol at 7 mM and 4 ml of osmotic cleaning solution with saccharose at 6 mM, after which it was centrifuged at 300 rpm for 4 min. The fraction of viable protoplasts was formed in the interphase of the osmotic solution with mannitol and the osmotic solution with saccharose. These protoplasts were recovered using a micropipette and re-suspended in 5 ml of osmotic solution with saccharose, after which a final centrifugation was applied at 500 rpm for 4 min. Finally, the purified protoplasts were prepared to determine their yield and viability, and to carry out their culture and regeneration.

Determination of protoplast yield

The number of protoplasts obtained was determined with the use of a Neubauer camera (Osorio et al*.,* 2016), under a compound microscope. The cells without cell walls were observed at 40× and the number of protoplasts observed was registered. The total yield of protoplasts was calculated using the following equation of Aqeel et al. (2016).

$$\text{Protoplast yield} = \frac{\text{Total number of cell} \times \text{Total volume of the cells in suspension}}{4 \times \text{Weight of leaf tissue (g)}}$$

Protoplast viability

In order to evaluate the viability of the protoplasts the cells were stained with Evans blue dye at 0.025% (w/v) (Botero et al., 2011). A sample of 50 µl of protoplasts was taken in solution and mixed with 25 µl of Evans blue at 0.025% (w/v). The viable cells were observed under an optical microscope at 40×.

Culture and regeneration of protoplasts

Protoplast culture was carried out at a density of 1.0×10^4 cells/ml, with the technique known as standard plate method (Shrestha et al., 2007) which involved suspending the purified protoplasts in 2 ml of K&M culture media (Kao and Michayluk, 1975), supplemented

Table 1. Average protoplasts isolate of *R. digbyana* in each enzymatic combination and incubation time.

Enzymatic combination			Average number of protoplasts	
			Incubation	
%p/v Hemicellulase	%p/v Pectinase	%p/v Cellulase	4 h	6 h
3.00	0.75	2.00	173750±2864.10^{i}	44375±2864.10^{b}
1.50	0.75	2.00	220000±2165.00^{m}	51250±3903.10^{c}
2.25	0.50	2.00	206250±1875.00^{k}	61875±1875.00^{e}
3.00	0.50	2.00	206875±2165.00^{k}	56875±1082.50^{d}
1.50	0.75	1.00	272500±2864.10^{ñ}	73125±1875.00^{f}
2.25	0.75	1.00	255625±2165.00^{n}	71250±1875.00^{f}
3.00	0.50	1.00	310000±1082.50^{o}	78750±1875.00^{g}
1.50	0.50	1.00	386250±1875.00^{q}	88125±1875.00^{h}
2.25	0.75	2.00	188125±2165.00^{j}	37500±1875.00^{a}
3.00	0.75	1.00	217500±1875.00^{lm}	63750±1875.00^{e}
1.50	0.50	2.00	215000±1082.50^{l}	63750±1875.00^{e}
2.25	0.50	1.00	323125±2165.00^{p}	80625±1875.00^{g}

The values represent the average ± standard deviation of protoplasts isolated from 3 repetitions per treatment in 3 repeated experiments; averages followed by the same letters are not significantly different (P <0.05).

with 0.06 M of saccharose, 2.3% of Gelrite and 3 different concentrations of plant growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D), zeatin and 6-benzylaminopurine (BA) (Table 3). The culture media with the protoplasts was placed in a Petri dish, forming a disk, and left to solidify; in this way, the protoplasts became incrusted. Subsequently, the protoplasts were sub-cultivated, dividing the disc into 6 equal parts. Each part was transferred to a flask with culture media of the same composition and concentration. Finally, the cultures were left to sit in darkness at a temperature of 25 ± 2°C. After the 1st week of culture, the K&M media were replaced with fresh media. The formation of colonies or division of protoplasts was evaluated by direct observation using a compound microscope and the evaluation commenced from the 1st week of culture.

Estimation of the nuclear DNA content by flow cytometry

For the isolation of nuclei, 100 mg of *R. digbyana* leaf and 100 mg of *Glycine max* (L.) Merr. 'Polanka' leaf were weighed (reference pattern with 2C = 2.50) (Doležel et al., 1994); they were then cut with a razor in a Petri dish with 1000 µL of a solution of WPB (0.2 M Tris HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1% PVP-10, 1% (w/v) Triton X-100, pH 7.5) (Loureiro et al., 2007). The nuclear sample was then filtered with a 40 µM nylon mesh and 50 µl of propidium iodide were added (1 mg/1 ml) to stain the nuclear DNA. After an incubation period of 15 min, the sample was analyzed in a BD Accuri C6® flow cytometer equipped with a blue and red laser, 2 light scattering detectors and 4 fluorescence detectors with optimized optical filters. The data were obtained using the BD Accuri™ C6 Plus software in the format of FCS 3.1; 3 samples were analyzed in triplicate per day over a period of 3 days; each sample was analyzed with its respective standard of reference.

Once average, 2C fluorescence peaks of *R digbyana* and *G. max* (L.) Merr 'Polanka' were obtained and the nuclear DNA content was estimated as follows:

$$\text{Estimation of nuclear DNA (pg)} = \text{Reference DNA content (pg)}\left(\frac{\text{Mean position of G1 sample peak}}{\text{Mean position of G1 reference peak}}\right)$$

The estimated value of the nuclear DNA was converted to base pairs, taking into consideration that 1 pg of DNA corresponds to 0.978×10^9 pb (Doležel et al., 2003).

Cytology

Pretreatment and fixing

In order to carry out the cytological studies, young roots with meristems were collected from *in vitro R. digbyana* plantlets at 8 h; they were rinsed in water and placed in vials containing 1 mL of 8-hydroxyquinoline. The vials were then left to sit in darkness for 5 h at room temperature, after which the 8-hydroxyquinoline was removed and the roots were rinsed 3 times with distilled water prior to fixing in a mixture of ethanol: chloroform: cold acetic acid (6:3:1). The roots were stored in refrigeration for 48 h, after which the fixing agent was discarded, and the roots were rinsed twice in ethanol at 70% (w/v).

Hydrolysis

The roots treated with fixative and then rinsed with 70% ethanol (v/v) were subsequently washed 3 times with a mixture of 75 mM KCl and 7.5 mM of EDTA (pH 4). The apices of the roots were extracted, eliminating the differentiated tissue with the aid of a scalpel and a NIKON stereoscopic microscope (zoom of 4 and observation of 640 LP/mm). The apices were placed in 200 µL of 1 N HCl and incubated for 5 min at 50°C, after which they were centrifuged at 3000 rpm for 1 min and the supernatant was discarded. The obtained pellet was incubated in 100 µL of Vizcozyme® and 100 µL of citrate buffer 1X (0.1 M of $Na_3C_6H_5O_7$ and 0.1 M of $C_6H_8O_7$) for 30 min at 37°C, and was then centrifuged at 3000 rpm for 1 min and the supernatant was discarded. The pellet was then re-suspended in 400 µL of 75 mM of KCl and 7.5 mM of EDTA (pH4) at room temperature for 15 min and centrifuged at 3000 rpm for 1 min after which the supernatant was discarded. Finally, the pellet was re-suspended in cold ethanol at 70% (w/v) and stored at 4°C.

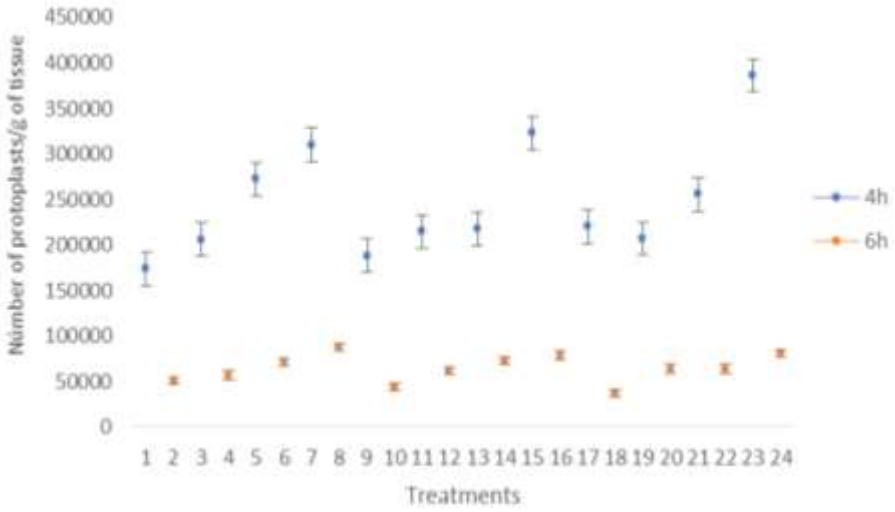

Figure 1. Average number of protoplasts of *R. digbyana* obtained at 4 and 6 h of incubation in different enzymatic mixtures.

Staining and observation of chromosomes

Seven microliters of protoplast suspension, obtained from the hydrolysis described previously, was placed on a slide and left to dry at room temperature, after which 7 µL of acetic acid at 45% (w/v) was also left to dry at room temperature. Finally, slides were rinsed with 96% (w/v) absolute ethanol and dried at room temperature. Seven microliters of carbol fuchsin was used to stain the sample, a cover glass was put in position and the slide was gently warmed over a Bunsen flame.

Image analysis

The images of the protoplasts and chromosomes were captured with the INFINITY ANALYZE® software; however, the images of the chromosomes were edited with the free software ImageJ (https://imagej.nih.gov/ij/index.html) (ImageJ, 2018).

Statistical analysis

All of the experiments were repeated 3 times. In order to evaluate the effect of the treatments on the production of the protoplasts, a 3×2×2×2 design was used (3 concentrations of hemicellulase, 2 concentrations of pectinases, 2 concentrations of cellulose, and 2 exposure times). The regeneration treatments from the protoplasts were evaluated using a 3^3 design (3 factors: 2,4-D, Zeatin and BA, evaluated at 3 concentrations). The data were evaluated using analysis of variance (ANOVA) and the averages were compared using the Tukey test with an interval of 95% ($p<0.05$). All the statistical analyses were performed using STATGRAPHICS Centurion XVI software.

RESULTS AND DISCUSSION

Effect of incubation time on the number and viability of the protoplasts

The types of enzymes and their concentrations had a considerable influence on the quantity of the obtained protoplasts in *R. digbyana.* The highest number of protoplasts was obtained at 4 h of incubation (Figure 1) using the combination of 1.5% (w/v) of hemicellulase, 0.5% (w/v) of pectinase and 1% (w/v) of cellulase, with a yield of 386250±1875 protoplasts/g of tissue (Table 1). At 6 h of incubation, the highest yield was 88125±1875 protoplasts/g of tissue. The lower number of obtained protoplasts at 6 h of incubation may have been since there was more time for digestion of the cell wall and possible damage to the protoplast tissue. The protoplasts obtained at 4 h of incubation presented an oval shape and the organelles had been observed grouped in a point within the membrane (Figure 2a). When the incubation time was increased to 6 h, the yield and viability of the protoplasts was reduced, and it was possible to observe circular protoplasts with organelles dispersed inside the membrane (Figure 2b). These results concur with the earlier reports of Khentry et al. (2006), Lee et al. (2010) and Aqeel et al. (2016), who indicated that the optimal time for the isolation of the protoplasts in *Dendrobium* was 4 h. After 6 h of incubation, there was a reduction in the yield and viability of the protoplasts due to the prolonged period of incubation, which causes the protoplasts to rupture.

The results of this work are in contrast with those obtained by Kanchanapoom et al. (2001), who reported that 3 h is an adequate time to obtain the highest yield of protoplasts in species such as *Dendrobium*. According to Prasertsongskun (2004), the protoplasts were isolated efficiently using 0.5% of pectinase. This result concurs with that obtained in *R. digbyana,* but differs in the incubation time, since Prasertsongskun affirms that 10 h is the optimal time to obtain a greater yield of protoplasts in *Vetiveria zizanioides* (Nash). Sherestha et al. (2007)

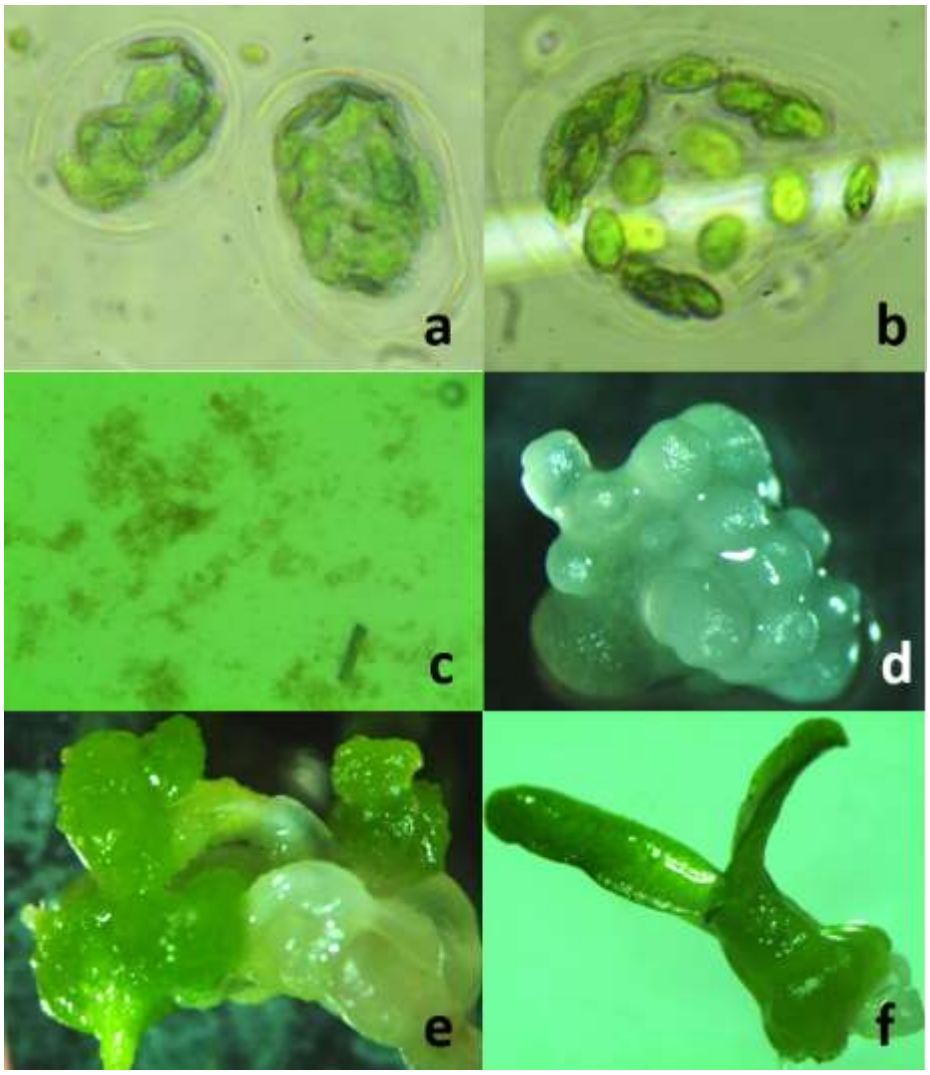

Figure 2. Protoplasts of *R. digbyana*. (a) Isolated at 4 h of incubation. (b) Protoplast isolated at 6 h of incubation. (c) Formation of the micro-colonies of protoplasts after 4 weeks of culture. (d) Callus derived from the protoplasts with somatic embryo type structures after 2 months of culture. (e) Development of the somatic embryo type structures at 3 months of culture. (f). *R. digbyana* shoots regenerated from protoplasts, after 5 months of culture.

reported that after 7 h of incubation, the protoplast yield begins to fall in *Phalaeonopsis.* However, in this study it was found that this occurs after 4 h of incubation, and thus differs entirely from the affirmations of these authors.

Regeneration and formation of colonies from protoplasts

The micro-colonies of protoplasts were clearly observed 4 weeks after the establishment of the culture (Figure 2c). The regeneration was not obtained in all the established treatments (Table 3). According to the Tukey test of mean separation at 95%, it was determined that the 2,4-D did not present significant statistical differences between its 3 levels evaluated (Table 2). However, the addition of the 2, 4-D to the culture media may favor the formation of protoplasts colonies, as was reported by Kobayashi et al. (1993) who affirmed that 2, 4-D in culture media is essential for the regeneration of *Phalaenopsis*. Nonetheless, for both Zeatin and BA, significant statistical differences were observed between their levels, showing means of 0.66 for Zeatin and 0.60 for BA (Table 2). In accordance with these data, it was possible to determine that with the combination of 4.53 µM of 2, 4-D, 0.91 µM of Zeatin and 3.10 µM of BA, a higher number of protoplast colonies was obtained (Table 3).

Therefore, the most important factor for the successful protoplasts culture could be the optimal concentration of the growth regulators and not the source of protoplasts,

Table 2. Averages of the protoplast colonies obtained of *R. digbyana*, according to the concentration of 2, 4-dichlorophenoxyacetic acid, zeatin and 6-benzylaminopurine.

2,4-D (µM)	Colonies of protoplasts	Zeatin (µM)	Colonies of protoplasts	BA (µM)	Colonies of protoplasts
0.45	0.45[a]	0.91	0.66[b]	1.77	0.37[b]
2.26	0.33[a]	2.28	0.49[b]	3.10	0.60[c]
4.53	0.37[a]	3.64	0.00[a]	4.44	0.18[a]

The values represent the average of protoplast colonies from 4 repetitions per treatment in 3 repeated experiments; averages followed by the same letters are not significantly different Tukey (P <0.05). 2, 4-D2; 4-Dichlorophenoxyacetic acid; BA: 6-benzylaminopurine.

Table 3. Average number of protoplast colonies *R. digbyana* obtained according to the combined concentration of plant growth regulators.

Treatment	2,4-D (µM)	Zeatin (µM)	BA (µM)	Colonies of protoplasts
T1	4.53	3.64	4.44	-
T2	0.45	3.64	4.44	-
T3	2.26	3.64	4.44	-
T4	4.53	0.91	4.44	0.66±0.50[ab]
T5	0.45	0.91	4.44	-
T6	2.26	0.91	4.44	-
T7	4.53	2.28	4.44	1.00±0.86[ab]
T8	0.45	2.28	4.44	-
T9	2.26	2.28	4.44	-
T10	4.53	3.64	1.77	-
T11	0.45	3.64	1.77	-
T12	2.26	3.64	1.77	-
T13	4.53	0.91	1.77	-
T14	0.45	0.91	1.77	0.66±1.00[ab]
T15	2.26	0.91	1.77	1.33±0.50[bc]
T16	4.53	2.28	1.77	-
T17	0.45	2.28	1.77	1.33±0.50[bc]
T18	2.26	2.28	1.77	-
T19	4.53	3.64	3.1	-
T20	0.45	3.64	3.1	-
T21	2.26	3.64	3.1	-
T22	4.53	0.91	3.1	1.66±0.50[c]
T23	0.45	0.91	3.1	1.00±0.00[bc]
T24	2.26	0.91	3.1	0.66±0.50[ab]
T25	4.53	2.28	3.1	-
T26	0.45	2.28	3.1	1.11±1.05[bc]
T27	2.26	2.28	3.1	1.00±1.50[b]

The values represent the average ± standard deviation of protoplast colonies from 4 repetitions per treatment in 3 repeated experiments; averages followed by the same letters are not significantly different Tukey (P <0.05). 2, 4-D2; 4-Dichlorophenoxyacetic acid; BA: 6-benzylaminopurine.

as reported by Sajise and Sagawa (1991). Grzebelus et al. (2011) also reported that the combination of 2, 4-D and Zeatin, besides favoring the development of protoplast colonies, also allowed the induction of somatic embryogenesis without needing to transfer the colonies back to a culture media. This result concurs with that obtained in this study, given that after 2 months of culture, it was possible to observe callus derived from the protoplasts with somatic embryo type structures (Figure 2d). After 3 months of culture, callus with structures type of somatic embryos began to develop (Figure 2e). After 5 months of culture, shoots of *R. digbyana* differentiated and with defined aerial structures were observed (Figure 2f). The obtained results differed from those reported by

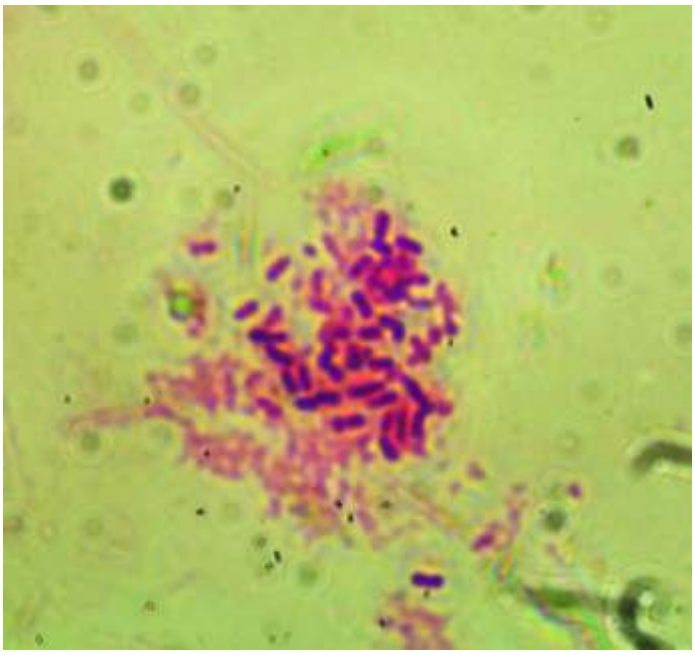

Figure 3. Chromosomes of *R. digbyana* observed through 100X microscope

Shrestha et al. (2007), who affirmed that the addition of plant growth regulators to the culture medium did not produce any beneficial effect on the growth of the colonies.

Estimation of nuclear DNA content by flow cytometry

The peaks of the obtained 2C fluorescence from the histograms were 26936.67±5096.982 for *R. digbyana* and 5391.77±1047.2 for the standard *Glycine max* (L.) Merr. 'Polanka', obtaining an estimated average for the nuclear DNA content for *R. digbyana* of 27.39±3.8 pg of DNA (26.79 ± 10^9 pb). To date, the DNA content for this species has not been estimated. However, Jones et al. (1998) reported the estimate of nuclear DNA content nuclear in some species of the subtribe Laeliinae; they mentioned that the size of the genome can vary widely within this sub-tribe. In another report, Leitch et al. (2009) mentioned that the subfamily Epidendroideae, in which *R. digbyana* is included, obtained the highest size range of the genome.

Cytological studies

Root extraction in the morning was fundamental for the observation of metaphase extended chromosomes (Figure 3). The number of observed chromosomes in mitotic cells was observed to be 2n=40. This result coincides with that reported by Felix and Guerra (2010), who also mentioned that the tribe Epidendreae is subdivided into two main subtribes: Pleurothallidinae Lindl. and Laeliinae Benth. According to Tanaka and Kamemoto (1984) for Pleurothallidinae Lindl., the number of chromosomes is only known in 4 of the 28 genera that it comprised; however, for Laeliinae Benth, the number of chromosomes is only known for 14 of the 45 genera, both subtribes with a predominance of n = 20 in most of the genera.

Conclusion

The isolation conditions influence the efficient number of protoplasts obtained from leaf tissue. Based on the present experimental results, the best treatment to obtain the highest yield was 1.5% of hemicellulase, 0.5% of pectinase and 1% of cellulase at 4 h of incubation with a production of 386250 protoplasts/g of tissue. In this study, the regeneration of *R. digbyana* from protoplasts was achieved after 2 months from the establishment of the culture. Moreover, during this same procedure, somatic embryo type structures were obtained using the standard plate method, K&M culture medium, 2,4-D, Zeatin and BA and 2.3% of Gelrite as gelling agent (Figure 2d). To the best of the authors' knowledge, this is the 1st report of the isolation and regeneration of protoplasts in *R. digbyana.* The estimate of DNA by flow cytometry is also reported for the first time. Thus, this protocol could be used to explore the possibility of creating somatic hybrids of orchids and could also serve as a basis for further research in gene manipulation, particularly in the study of protoplast fusion.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Aqeel R, Zehra M, Kazmi S, Khan S, Kayani H, Mirbahar A (2016). A study on the isolation of protoplasts from mesophyll cells of *Dendrobium* queen pink. Pakistan Journal of Botany 48(2):693-697.

Botero C, Restrepo C, Urrea AI (2011). Response of three tomato genotypes to in vitro culture and protoplast isolation. Actualidades Biológicas 33(94):35-49.

Doležel J, Doleželová M, Novák FJ (1994). Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). Biologia Plantarum 36(3):351.

Doležel J, Bartoš J, Voglmayr H, Greilhuber J (2003). Nuclear DNA content and genome size of trout and human. Cytometry 51:127-128.

Dodson CH (2016). Orchid plants. Encyclopedia Britannica. http://www.britannica.com/plant/orchid.

Felix LP, Guerra M (2010). Variation in chromosome number and the basic number of subfamily Epidendroideae (Orchidaceae). Botanical Journal of the Linnean Society 163(2):234-278.

Grzebelus E, Szklarczyk M, Baranski R (2011). An improved protocol for plant regeneration from leaf-and hypocotyl-derived protoplasts of carrot. Plant Cell, Tissue and Organ Culture 109(1):101-109.

Hossain MM, Kant R, Van PT, Winarto B, Zeng S, Teixeira da Silva JA (2013). The application of biotechnology to orchids. Critical Reviews in Plant Sciences 32(2):69-139.

Image Processing and Analysis in Java (Image J, 2018). Software used on April 24, 2018 in https://imagej.nih.gov/ij/index.html.

Jones WE, Kuehnle AR, Arumuganathan K (1998). Nuclear DNA content of 26 orchid (Orchidaceae) genera with emphasis on *Dendrobium*. Annals of Botany 82(2):189-194.

Kao KN, Michayluk MR (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126(2):105-110.

Kuehnle AR, Nan GL (1990). Factors influencing the isolation and culture of protoplasts from Hawaiian *Dendrobium* cultivars. In Proceedings of the 13th World Orchid Conference, Auckland, New Zealand pp. 259-262.

Kobayashi S, Kameya T, Ichihashi S (1993). Plant regeneration from protoplasts derived from callus of Phalaenopsis. Plant tissue Culture Letters 10(3):267-270.

Kanchanapoom K, Jantaro S, Rakchad D (2001). Isolation and fusion of protoplasts from mesophyll cells of *Dendrobium pompadour*. Science Asia 27(1):29-34.

Khentry Y, Paradornuvat A, Tantiwiwat S, Phansiri S, Thaveechai N (2006). Protoplast isolation and culture of *Dendrobium* Sonia “Bom 17”. Kasetsart Journal - Natural Science 40:361-369.

Loureiro J, Rodríguez E, Doležel J, Santos C. (2007). Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Annals of Botany 100(4):875-888.

Leitch IJ, Kahandawala I, Suda J, Hanson L, Ingrouille MJ, Chase MW, Fay MF (2009). Genome size diversity in orchids: consequences and evolution. Annals of Botany 104(3):469-481.

Lee CS, Kiong AL, Mahmood M (2010). Optimization of protoplast isolation protocols using in vitro leaves of *Dendrobium crumenatum* (pigeon orchid). African Journal of Agricultural Research 5(19):2685-2693.

Murguía J, Leyva OR, Lee HE, Galindo ME, Pardío VT, Llarena RC (2016). Orchids production systems (Orchidaceae) in Veracruz, Mexico. Agro Productividad 9(6):62-66.

Osorio J, Bustamante E, Macareno M, Hernandez E, Beltrán J (2016). Aislamiento enzimático de protoplastos a partir de mesófilo de Dioscorea alata cultivar “Pico de Botella”. Temas Agrarios 15(1):58-70.

Prasertsongskun S (2004). Isolation and culture of suspension protoplasts of vetiver. Journal of Science and Technology 26(3):411-416.

Sajise JU, Sagawa Y (1991). Regeneration of plantlets from callus and protoplasts of Phalaenopsis sp. Malayan Orchid Bull 5:23-28.

Shrestha B, Tokuhara K, Mii M (2007). Plant regeneration from cell suspension-derived protoplasts of *Phalaenopsis*. Plant Cell Reports 26(6):719.

Suárez G, Téllez B (2015). *Dendrobium* 'Tropical Classic'. New hybrid orchid from Cuba. Cultivos Tropicales 36(1):132.

Tanaka R, Kamemoto H (1984). Chromosomes in orchids: counting and numbers. Orchid Biology: Reviews and Perspectives 321-410.

Tamay L, Cruz J, García E (2016). Diversity and use of orchids. Bioagrociencias 9(1):1-6.

Vallejo FA, Estrada EI (2002). Mejoramiento genético de plantas (No. Doc. 21583) CO-BAC, Bogotá.

World Checklist of Selected Plant Families (WCSP) (2017). Date of consultation: June 26, 2018 in http://wcsp.science.kew.org/incfamilies.do.