Journal of the Professional Association for Cactus Development

Characterization of the morphometry, germination process, phytochemicals, and antioxidant capacity of seeds of three species of the genus *Cylindropuntia* (Cactacea) of Chihuahua state

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Citation: Reyes-Corral, V. I., González-Fernández, R., Quiñónez-Martínez, M., Rodrigo-García, J., Garza-Vallejo-, G. I., and Valero-Galván, J. 2022. Characterization of the morphometry, germination process, phytochemicals, and antioxidant capacity of seeds of three species of the genus *Cylindropuntia* (Cactacea) of Chihuahua state. *Journal of the. Professional Association for Cactus Development. 24*: 251-268. https://doi.org/10.56890/jpacd.v24i.506

Associate Editor: Pablo Misael Arce-Amezquita

Technical Editor: Tomás Rivas-García

Received date: 07 May 2022
Accepted date: 08 September 2022
Published date: 20 October 2022

Abstract. *Cylindropuntia* is a genus of cylindrical cacti found in northern Mexico. This study aimed to describe the fruit and seed morphometry, characterize the germination process, as well as determine the seed phytochemicals in three species of the genus *Cylindropuntia* from the state of Chihuahua in México: *C. imbricata*, *C. spinosior*, and *C. leptocaulis*. To measure the morphometric parameters, 20 fruits and 20 seeds from each fruit were used for each specie. For the characterization of the germination process, two scarification treatments were applied, and the germination was allowed for 42 days. Different parameters of the germination process were calculated as the germination percentage, mean germination time, germination speed, and mean germination speed. The concentration of reducing sugars, total phenols, tannins, flavonoids, antioxidant activity (DPPH and FRAP), and protein content were determined from seeds under basal conditions. Results revealed significant differences ($p \leq 0.05$) for morphometric parameters in the fruit and seed of the three species examined, showing *C. imbricata* the highest values in both tissues, followed by those obtained for *C. spinosior* and *C. leptocaulis*, respectively. The germination percentage was higher in *C. leptocaulis* (13.3%), although achieved in a longer mean germination time (27.7 days), facing *C. spinosior* that presented the lowest values for the germination percentage (3.3%), mean germination time (4.6 days), and germination speed index (0.024 days). Seeds from *C. leptocaulis* showed the highest values for reducing sugar content (31.82 mg glucose·g^{-1} of dry weight) and the antioxidant activity determined by DPPH (26.37 mg CE·g^{-1}of dry weight), whereas *C. spinosior* seeds showed the highest ones for the total phenols content (3.08 mg GAE·g^{-1} of dry weight) and the antioxidant activity determined by FRAP (11.7 mmol TE·g^{-1} dry weight). Besides, *C. imbricata* showed the highest value in the protein content (5.6 mg protein·g^{-1}). This study provides the first data on seed phytochemicals and information on the germination process of these three species of *Cylindropuntia*, showing differences among them in the diverse parameters measured.

Keywords: *Cylindropuntia* fruits, *Cylindropuntia* seeds, seed germination index, seed phytochemicals.

Introduction

The Cactus family are native plants of the American continent that adapt to diverse areas like arid or semi-arid places or even jungles (Arias *et al*., 2012) because it has morphological and physiological adaptationsuch as the

crassulacean acid metabolism (CAM). Thus, they commonly inhabit dry environments, whether arid, semi-arid, or deciduous forest. These plants can be treelike or shrubby, with persistent green leaves in some stages of theirdevelopment, and can have glochids, spines, or both. The variation of these characteristics, jointly with those of the tubercles, leaves, ribs, surface, and areoles are influential in their taxonomy classification (Anderson, 2001; Arial *et al.,* 2012). The flowers have undergone specific modifications that differentiate them from the other dicotyledons in their organ number, size, and structure, or even eliminating some. Together with the families of Agavaceae, Crassulaceae, and Euphorbiaceae, the cacti identify as succulent xerophytic plants (Anderson, 2001). Some authors consider that the Cactaceae family may be composed of about 1500 to 1600 species distributed between 110 to 122 genera (Lebgue *et al*., 2011) or even classify them into 124 genera (Pinto and Scio, 2014). In Mexico, about 669 species are identified, corresponding to 63 genera (Lebgue *et al*., 2011), of which the most abundant are *Opuntia*, *Ferocactus, Mammillaria*, *Coryphantha, Echinocereus*, and *Cylindropuntia* (Hunt, 2016).

The *Cylindropuntia* species, better known by their popular word as chollas, are tree-like or shrub-like plants with erect and indefinite growth, some branched, with cylindrical articulated stems, and without longitudinal ribs (Anderson, 2001). The areoles show a variable shape and present glochids and spines with deciduous, papery, epidermal sheaths that fall off entirely (Anderson, 2001). Their reproduction is usually vegetative, occurring through stem segments, which occasionally scatter by zoochory and anemochory (Anderson, 2001; Martínez and Molina, 2013), but even so, these segments can also prostrate and produce roots or develop lateral roots (Anderson, 2001; Martínez and Molina, 2013).

The genus *Cylindropuntia* is native to North America, documenting around 30 species in Mexico (Hunt, 2016). In other regions of the world, *Cylindropuntia* species are part of the introduced vegetation, considering them together with other genera as invasive plants. So, they are designated opportunistic plants that invade regions with fluctuating vegetation, such as areas disturbed by agricultural activities such as pastures dedicated to livestock and agriculture and those dedicated to water extraction (Deltoro-Torró *et al*., 2014). Furthermore, in several ecosystems, *Cylindropuntia* species cause the displacement of native plants. Some examples of these invasive species are *C. fulgida* v. *fulgida*, *C. imbricata*, *C. leptocaulis*, *C. prolifera*, *C. palida*, and *C. rosea* (Deltoro-Torró *et al*., 2014). However, depending on the region where these plants are grown, these species have various uses. For example, plants can function as hedges due to their prickly nature. The dried cladodes take advantage to make handicrafts, as they are woody and have holes that give them an ornamental character, and the glochids and spines are employed ceremonially. Its gum has been utilized as chewing gum, and in the dry season, the fruits are exploited as forage (Anderson, 2001; Bustamante and Búrquez, 2005). As a possible new utility, in a previous study, we evaluated the potential inhibitory effect of the aqueous and ethanolic extracts of shoots from *C. imbricata* and *C. leptocaulis* on mycelium growth from *Fusarium* sp. and *Aspergillus* sp. We found, on the contrary, that these extracts stimulated the development of these two phytopathogenic fungi (Cid-Lucero *et al.*, 2021).

In the Cactaceae family, a high germination percentage is associated with the size of the testa when functioning as a regulator of seed imbibition (Souza and Marcos-Filho, 2001). However, the physical and morphological dormancy of the seed is the most influential factor in seed germination in *Opuntia* and *Cylindropuntia* species (Mandujano *et al.,* 2005; Orozco-Segovia *et al*., 2007). Also, an innate and enforced dormancy regulated by environmental factors such as temperature and light has been indicated for seeds of *Opuntia* spp. (Olvera-Carrillo *et al.*, 2003; Podda *et al*., 2017; Reyes-Agüero *et al.*, 2005). To increase the germination percentage in the *Opuntia* species, in

previous studies, seeds were subjected to pre-treatments such as a low concentration of sulphuric acid and mechanical scarification (Podda *et al*., 2017; Potter *et al*., 1984; Sánchez-Venegas, 1997). Furthermore, knowledge about the seed germination parameters could explain how the species inhabit different environments. Unfortunately, only a few studies have determined the seed germination parameters in the cacti family. To date, no research has deeply addressed the seed germination of the *Cylindropuntia* species with a comparative analysis using both approaches.

C. leptocaulis, *C. spinosior*, and *C. imbricata* (Knuth) are the three major *Cylindropuntia* species growing in the Chihuahuan Desert (Guzmán *et al*., 2003). Though the fruits from these species are collected and consumed by local people of these regions, aspects of ecology such as sexual reproduction, seed phytochemical content, and antioxidant capacity are slightly known. The *Cylindropuntia* species have a reproductive adaptation and prolific fruits. They are characterized by having branched vegetative structures and are less prone to predation, which favors their establishment because this genus has an ovary immersed in a modified stem where there is living meristematic tissue in the areoles (Vázquez-Delfín *et al.*, 2005). An observational study of the sexual and vegetative reproductions in *C. leutocaulis* revealed that sexual reproduction was very restricted, the clonal establishment the most frequently, and this took place under shrub canopy (Flores-Torres and Montaña, 2012). In a previous study, the evaluation of the different gibberellic acid concentrations and the photoblastic behavior of seeds from *C. imbricata* and *C. leptocaulis* collected at Mapimi (Durango, Mexico) showed that the concentration of 1,500 ppm promoted a 30% germination of *C. imbricata* seeds, however, no effect was observed on the *C. leptocaulis* seeds. In addition, the light impact increased germination in both species (Rojas-Aréchiga *et al*., 2011).

This study aims to characterize the fruit and seed morphology, the germination process, and the seed phytochemical content of three *Cylindropuntia* species that growth in the state of Chihuahua, Mexico. This information may provide knowledge for detecting mechanisms that promote germination and have important implications, especially on the propagation of these species for conservation and management strategies.

Materials and Methods

Plant material

Four plants were selected for each *Cylindropuntia* species. The *C. leptocaulis* plants were located at Sierra El Presidio in Samalayuca Medanos Valley, south of Juarez City, Chihuahua, Mexico (31°23'03"N y 106°24'0"O), and those ones for *C. spinosior* and *C. imbricata* were found at the Central Park of Juarez City, Ciudad Juarez, Chihuahua (31°41'13"N 106°25'40"O) (Figure 1). Twenty fruits were randomly collected from each of the four individuals for each species in 2019, between May and November.

Morphometric analysis

The twenty fruits taken from each of the four individuals of each species were washed, photographed over a sheet of millimeter paper, and weighed individually on an analytical balance (Denver Instrument Apx-200). Subsequently, each fruit was made a cross-section to remove the seeds manually, and then they were counted. The pulp was also extracted and weighed. Twenty seeds were taken at random per individual and washed, and then their weight was individually measured. Afterward, they were photographed over a sheet of millimeter paper, separating them by fruit and individual. Images were processed using the ImageJ® software to measure the area, perimeter, width, and length of each fruit and seed. Finally, data were filed in a database (Núñez-Gastélum *et al*., 2018).

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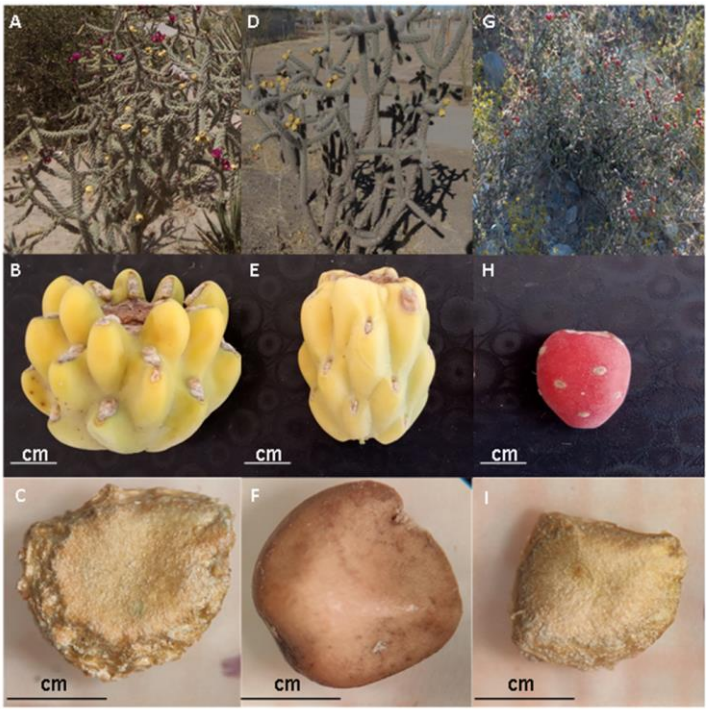

Figure 1. Comparative of the morphology of the three *Cylindropuntia* species. View of the complete plant, fruit, and seed from *C. imbricata* (A, B, and C, respectively), *C. spinosior* (D, E, and F, respectively), and *C. leptocaulis* (G, H, and I, respectively).

Seed germination

Ninety random seeds of each species were taken and divided into three groups of thirty seeds. Each group was subjected to different types of scarification treatments: 1) mechanical: each seed was scarred on at least one side with the help of sandpaper; 2) chemical: the seeds were submerged in H_2SO_4 at 98% (v/v) for 30 min; 3) control: seeds without treatment (Flores-Torres and Montaña, 2012). After scarification treatments, the seeds were disinfected. Briefly, each batch of seeds was submerged in a 70% (v/v) sodium hypochlorite solution for 3 min, then rinsed with abundant distilled water for 1 min. Immediately later, they were immersed in a solution of fungicide (Captan 1 g 20 mL^{-1}) for 2 min to be subsequently sown (Navarro *et al*., 2008). Next, seeds were placed in groups of 10 in sterilized Petri dishes, using 25 g of pre-sterilized sandy-type soil as a substrate. Finally, plates were placed in a bioclimatic chamber at 25 °C, and a photoperiod of 12 h light/darkness was applied. The progress of each seed in each dish was examined every seven days for 42 days, accounting for the number of seeds germinated. Seeds not germinated after this period were considered non-viable or latent, and those with a root protrusion of 1 mm or more were labeled as germinated (Baskin and Baskin, 2014). To find out the differences among the species, the average germination rate, average germination time, germination speed index, and percentage of germination were calculated using the methodology proposed by Souza *et al*. (2016).

Seed protein extraction and quantification

Seed proteins were extracted from 8 g of seeds by applying the TCA/acetone-phenol method (Valero-Galvan *et al*., 2014). The final pellet of protein was solubilized in 50 µL of a solution of 7 M urea (Jalmek Scientific, Nuevo León, México). The insoluble material was removed by centrifugation. Finally, proteins solubilized in the supernatant were quantified according to the Bradford method using BSA as the standard (Merck®, Toluca, Estado de México, México) (Ramagli and Rodriguez, 1985).

Phytochemicals content and antioxidant activity quantification from seeds

For the phytochemicals and antioxidant activity determination, a standard extract was obtained according to the method proposed by Álvarez-Parrilla *et al.* (2011) with minor changes. Briefly, from each sample, 0.25 g of seeds were manually ground in a mortar using a pestle until a fine powder was obtained. Then, 10 mL of 80% (v/v) methanolic solution (JT Baker®, Fisher Scientific, West Palm Beach, FL, USA) was added to the pulverized and blended with the help of the same pestle. Finally, the homogenate was recovered into an assay tube with the help of a micropipette. Later, the sample was stirred and sonicated for 30 min at 4 °C in darkness. Afterward, the extract was centrifuged at 3,500 rpm for 15 min at 4 °C, and the supernatant was collected into a new test tube. This methodology was repeated twice, and the two supernatants were mixed and brought to a final volume of 25 mL. Samples were stored at –20 °C until further analyses.

Reducing sugar content was determined according to the methodology from Ávila-Núñez *et al*. (2012). Briefly, 100 µL of each standard extract was blended individually with 300 µL of the DNS reagent (3,5-dinitrosalicylic acid) in an assay tube. Then, samples were incubated at 80 °C in a dry bath for 10 min, and immediately later, the mixture was cooled in an ice bath for 5 min. Next, 250 µL of each sample was taken and placed in a 96-well microplate, and the absorbance was measured at 540 nm. A calibration curve (y=1.0145x-0.0023, R^2 = 0.9999) was performed using glucose as the standard, and results were expressed as mg glucose per g^{-1} of dry weight (DW) (mg glucose·g^{-1} DW).

The total phenols determination was carried out using a spectrophotometry approach, according to Georgé *et al.* (2005), with minor variations. Briefly, 100 µL of each extract was taken and placed into a microtube of 2 mL, and 500 µL of the Folin–Ciocalteu reagent (10% v/v) was added, and the reaction was allowed to proceed for 2 min at room temperature. Then, 400 µL of Na_2CO_3 was added, and the mixture was incubated at 50 ºC for 15 min. Later, samples were cooled in an ice bath for 2 min, and 250 µL of the mixture was collected and placed in a 96-well microplate. Finally, absorbance was measured at 740 nm. A calibration curve (y = 6.3705x+0.0172, R^2 = 0.9888) was performed using gallic acid as the standard, and results were expressed as mg gallic acid equivalents (GAE) per g^{-1} of DW.

The antioxidant capacity was measured by a spectrophotometric approach, employing two methods: the first based on ferric reducing antioxidant power (FRAP) and the second based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (190 µM in 80% v/v methanol; Merck®, Toluca, Estado de México, México), according to the methodology proposed by Moreno-Escamilla *et al*. (2017). For FRAP assay, 24 µL of the extract was taken and placed in a 96-well microplate. Later, 180 µL of FRAP reagent was added, and mixtures were incubated at 37 ºC for 30 min. The absorbance was measured at 595 nm every minute for 30 min at room temperature. For quantification, a calibration curve (y=0.0028x+0.1523, R^2 = 0.9988) was performed using TROLOX reactive as the standard, and results were expressed as mmol TROLOX equivalents per g^{-1} of dry weight (mmol TE·g^{-1} DW).

For the DPPH assay, 25 µL of each extract was taken and placed in a 96-well microplate. Then, 200 µL of the DPPH solution was added, and the reaction was allowed to proceed for 30 min at room temperature in the dark. The absorbance was measured at 517 nm every minute for one hour at room temperature. For quantification, a calibration curve (y=0.1653x+3.6578, R^2 = 0,9857) was performed using TROLOX reactive as the standard, and results were expressed as mmol TROLOX equivalents (TE) per g^{-1} of DW (mmol TE·g^{-1} DW).

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All absorbances were measured in a BioRad xMark™ Plus Microplate Absorbance Spectrophotometer (Hercules, CA, USA), and data were acquired using the Microplate Manager 6.0 (Tokyo, Japan) computer software. All determinations were carried out in triplicate.

Statistical and clustering analysis

The data matrix obtained from fruits and seed measurements was processed using the IBM SPSS Statistics Base 22.0 software. A one-way ANOVA and Duncan multiple comparison tests were applied with a significance level of 95%. The cluster analysis was determined using the web-based NIA Array Analysis Tool using the methodology determined by Sharov *et al*. (2005).

Results and Discussion

Fruit and seed morphometry

Results from fruit morphological characterization showed significant differences among the three species of *Cylindropuntia* ($p \leq 0.05$) (Table 1). *C. imbricata* showed the highest values for most morphological fruit characteristics, having the largest fruit, while *C. leptocaulis* presented the lowest values, producing the most undersized fruit of the three species (Table 1 and Figure 1).

Table 1. Fruit morphometric analysis from the three *Cylindropuntia* species.

Fruits characteristics	Species			Anova ($p \leq 0.05$)
	C. leptocaulis	*C. imbricata*	*C. spinosior*	
Total weight (g)	0.8±0.1^{c}	13.3±2.5^{a}	8.9±3.4^{b}	0.000
Shell (g)	0.4±0.1^{c}	10.6±2.1^{a}	7.4±3.4^{b}	0.000
Pulp (g)	0.3±0.14^{c}	2.7±1.2^{a}	1.4±0.7^{b}	0.000
Length (mm)	14.5±1.2^{c}	33.6±6.3^{a}	30.5±3.6^{b}	0.000
Width (mm)	11.2±0.7^{c}	25.3±7.5^{b}	29.9±4.1^{a}	0.000
Length/width ratio	1.3±0.13^{a}	1.3±0.2^{a}	1.04±0.2^{b}	0.000
Area (mm^2)	143.4±18.9^{b}	779.2±357.9^{a}	747.3±142.7^{a}	0.000
Perimetry (mm)	43.1±2.7^{c}	156.48±36.0^{a}	99.3±8.8^{b}	0.000

Data are expressed as the mean ± standard deviation (n = 20) and were analyzed using a one-way ANOVA ($p \leq 0.05$). Different letters (a–c) show the result of the Duncan test, indicating a significant difference at $p \leq 0.05$ (displayed in the row of each morphological characteristic evaluated).

The species *C. imbricata* measured on average 3.36 cm long and 2.53 cm wide, similar to those reported in the descriptions made by Deltoro *et al.* (2014) and Pinkava (1999), in which these authors mention that the measurements of the *C. imbricata* fruits ranged from 2.4 cm to 4.5 cm long and 2 cm to 4 cm wide. The *C. spinosior* fruits showed average values of 3 cm long and 2.9 cm wide, similar to those obtained by Deltoro *et al.* (2014) and Pinkava (1999), who described values from 2.5 cm to 5 cm long and 2 cm to 3 cm wide. Last, *C. leptocaulis* fruits presented measures with an average of 1.45 cm long and 1.11 cm wide, similar to those reported by Pinkava (1999), in which the data oscillated from 0.9 cm to 2.7 cm long and 0.6 cm to 1.2 cm wide. In a previous study, fruits of five communities of *O. jaliscana* collected in the Jalisco State (Mexico) showed a length from 3.79 cm to 4.32 cm and a width from 3.02 cm to 3.53 cm (López-Borja *et al*., 2017). Therefore, fruits from *C. spinosior* (3.05 cm long, 2.99 cm wide) and *C. imbricata* (3.36 cm

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long, 2.53 cm wide) were comparable to *O. jaliscana* to those from *C. leptocaulis*, whose measurements were for both parameters.

In the morphological analysis of the seeds, most of them had significant differences ($p \leq 0.05$), except for the seed length, indicating that the three species revealed some similarities (Table 2). *C. leptocaulis* seeds showed the lowest values for most morphological characteristics, *C. imbricata* seeds presented the highest weight, area, and perimeter measures, and those from *C. spinosior* had the highest width values (Table 2 and Figure 1).

Table 2. Seed morphometric analysis from the three *Cylindropuntia* species.

Seeds characteristics	Species			Anova ($p \leq 0.05$)
	C. leptocaulis	***C. imbricata***	***C. spinosior***	
Weight (g)	0.007±0.002^{c}	0.020±0.002^{a}	0.018±0.003^{b}	0.000
Length (mm)	4.10±0.4^{a}	4.10±0.2^{a}	4.20±0.2^{a}	0.189
Width (mm)	3.40±0.3^{b}	3.87±0.2^{a}	3.80±0.2^{a}	0.000
Length/width ratio	1.19±0.13^{a}	1.08±0.07^{b}	1.08±0.07^{b}	0.000
Area (mm^2)	11.1±1.8^{c}	14.1±1.3^{a}	13.4±1.5^{b}	0.000
Perimetry (mm)	12.4±1.0^{c}	13.2±0.6^{a}	12.9±0.7^{b}	0.000

Data are expressed as the mean ± standard deviation (n = 20) and were analyzed using a one-way ANOVA ($p \leq 0.05$). Different letters (a–c) show the result of the Duncan test, indicating a significant difference at $p \leq 0.05$ (displayed in the row of each morphological characteristic evaluated).

Cactaceae family seeds are usually smaller than 1 cm, ranging from 0.2 mm (*Blossfeldia liliputana* specie) to 7.5 mm (*Pereskia bleo*) in length (Rojas-Aréchiga *et al.,* 2013; Leuenberger, 1986), and their weight varies from 0.016 mg (*Mammillaria bocasana*) to 56.04 mg (*Opuntia basilaris*) (Flores *et al*., 2006). In this study, seeds from *Cylindropuntia* species showed similar values to *O. jaliscana* seeds for length (3.7 mm to 4.3 mm). However, the values for width were higher than those of *O. jaliscana* (3.3 mm to 3.5 mm) (López-Borja *et al*., 2017). In a previous study, Núñez-Gastélum *et al*. (2018) determined the length, width, and area of *O. polyacantha, O. engelmannii, O. phaeacantha*, and *O. macrocentra* seeds. Concerning the first parameter, the three *Cylindropuntia* species seeds presented similar values to *O. macrocentra* (4.33 mm), as all the seeds ranged between 4 mm to 4.5 mm. Regarding width, seeds from the three *Cylindropuntia* species exhibited similar measures to *O. phaeacantha* (3.77 mm) and *O. macrocentra* (3.72 mm). Finally, *C. spinosior* seeds showed an average area (13.35 mm^2) like those from *O. macrocentra* (13.15 mm^2), and *C. imbricata* seeds (14.05 mm^2) were closer to those values obtained for *O. phaeacantha* (14.04 mm^2). However, for *C. leptocaulis* seeds, no similarities showed to any of the four species of *Opuntia* or the other two species of *Cylindropuntia* having the smallest size. Despite presenting similar length and width measurements, the most relevant difference found among the seeds of the three *Cylindropuntia* species was in the area and the perimeter parameters, where *C. imbricata* and *C. leptocaulis* showed the highest and lowest values, respectively. An explanation for these findings may be due to the seed morphology found because *C. imbricata* seeds showed a subcircular form while *C. leptocaulis* seeds have a square shape (Pinkava, 1999). Some studies have earlier reported a variation in the seed morphometry in Opuntioideae, which could be related to the seed origin and moist areas (Romo-Campos *et al*., 2010). Furthermore, the seed mass had

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no association with the germination characteristics in cacti; nevertheless, heavier seeds produced more voluminous and cylindrical seedlings (Sosa-Pivatto *et al*., 2014).

Evaluation of the germination process

The most efficient scarification process was the chemical method using 98% (v/v) H_2SO_4, providing a germination percentage of 10% in 14 days. *C. leptocaulis* seeds showed the highest germination percentage (13.3%), followed by those from *C. imbricata* (10.0%) and *C. spinosior* (3.3%) (Table 3). In a previous study, Flores-Torres and Montaña (2012) tested mechanical and chemical scarification pre-treatments jointly with a control group without pre-treatment in *C. leptocaulis*. These authors found that the best-promoted germination was mediated by the chemical scarification method, obtaining the best germination percentage. These results are similar to our research. Nonetheless, our data differ from those obtained for the control group and mechanical scarification pre-treatment. In the present study, the three species increased the germination percentages compared to those presented by Flores-Torres and Montaña (2012). The chemical scarification applied over prolonged periods may be more efficient in interrupting the dormancy or latency of seeds due to the fact that this pre-treatment resembles the process located in the digestive tract of animals, promoting better germination than mechanical scarification methods (Flores and Jurado, 2009). Other treatments for promoting germination were also tested, including different gibberellic acid concentrations and the photoblastic behavior of *C. imbricata* and *C. leptocaulis* seeds from the Mapimí Biosphere Reserve (Durango State, Mexico). Results showed that a solution over 1,500 ppm of gibberellic acid promoted a 30% germination of *C. imbricata* seeds but had no influence on the *C. leptocaulis* seeds. However, the light effect increased the germination percentage in both species (Rojas-Arechiga *et al*., 2011). For evaluating germination data, a multivariate generalized linear model was applied (Tables 3 and 4). Significant statistical differences were observed in all the parameters evaluated at the species level (Table 3).

Table 3. Multivariate generalized linear model effect on germination rate index (GRI), mean germination time (MGT), mean germination rate (MGR), and germination percentage (G) considering the three *Cylindropuntia* species.

Germination index	Species			Anova ($p \leq 0.05$)
	C. leptocaulis	***C. imbricata***	***C. spinosior***	
GRI (days)	0.05±0.004^{b}	0.07±0.005^{a}	0.02±0.004^{c}	0.00
MGT (days)	27.7±1.5^{a}	14.6±1.9^{b}	4.6±1.5^{c}	0.00
MGR (days^{-1})	0.04±0.004^{b}	0.07±0.005^{a}	0.02±0.004^{c}	0.00
G (%)	13.3±1.2^{a}	10.0±1.4^{a}	3.3±1.2^{b}	0.00

Data are expressed as the mean ± standard deviation (n = 20) and were analyzed using a one-way ANOVA ($p \leq 0.05$. Different letters (a–c) show the result of the Duncan test, indicating a significant difference at $p \leq 0.05$ (displayed in the row of each germination indices evaluated).

C. imbricata presented the highest values in the germination rate index (0.05 days), followed by *C. leptocaulis* (0.05 days), and *C. spinosior* showed the lowest values (0.02 days). Furthermore, *C. leptocaulis* presented the highest values of average germination time (27.7 days), followed by *C. imbricata* (14.6 days), and *C. spinosior* presented the lowest ones (4.6 days). These results showed that the seeds of *C. spinosior* tended to germinate faster than seeds from the other two species, while *C. leptocaulis* seeds were the slowest to sprout. Furthermore, *C. leptocaulis* specie presented the highest germination percentage (13.3%), followed by *C. imbricata* (10%), and *C. spinosior* showed a limited germination percentage (G) (3.33%) (Table 3).

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The comparison of the scarification pre-treatments showed a significant statistical difference in the mean germination time (MGT) and mean germination rate (MGR) (Table 4). The highest MGT happened in the control seeds (22.6 days), which was significantly different from values obtained for the chemical (14.4 days) and mechanical (10 days) pre-treatments, between which were no significant differences. In the case of MGR, the chemical pre-treatment showed the lowest values (0.035 day^{-1}), while the highest ones were observed in the control (0.059 day^{-1}) and mechanic (0.047 day^{-1}) pre-treatments, without no significant differences.

Table 4. Multivariate generalized linear model effect on germination rate index (GRI), mean germination time (MGT), average germination rate (MGR), and germination percentage (%G) considering scarification pre-treatments applied to seeds of the three *Cylindropuntia* species.

Germination index	Seed pre-treatments			Anova ($p \leq 0.05$)
	Control	Chemical (H_2SO_4)	Mechanic	
GRI (days)	0.059±0.004[a]	0.051±0.004[a]	0.047±0.004[a]	0.149
MGT (days)	22.6±1.7[a]	14.425±1.7[b]	10±1.7[b]	0.000
MGR ($days^{-1}$)	0.059±0.004[a]	0.035±0.004[b]	0.047±0.004[a]	0.002
G (%)	10±1.3[a]	10±1.3[a]	6.666±1.3[a]	0.151

Data are expressed as the mean ± standard deviation (n = 20) and were analyzed using a one-way ANOVA ($p \leq 0.05$). Different letters (a–c) show the results of the Duncan test, indicating a significant difference at $p \leq 0.05$ (displayed in the row of each germination indices evaluated).

In this study, the variation in the MGT was significant between the three *Cylindropuntia* species and those previously reported for other genera of the cacti family. The species tested in this research showed higher values for seed germination than those determined for *Pereskia aculeata* (5.27 days) and *P. grandifolia* (11.57 days) (Souza *et al*., 2016), and those found for *Astrophytum capricorne* (6 days)*, A. myriostigma* (6 days)*,* and *A. ornatum* (5 days) (Muro-Pérez *et al.*, 2013).

Determination of phytochemical content and antioxidant capacity from seeds

Statistically significant differences ($p \leq 0.05$) were observed in reducing sugar, total phenolics, and protein contents, and in the antioxidant capacity among the seeds of the three species analyzed (Table 5). The *C. leptocaulis* seeds presented the highest values in the reducing sugar content (31.8 mg glucose·g^{-1}), while *C. imbricata* revealed the lowest ones (1.01 mg glucose·g^{-1}) (Table 5). The *C. spinosior* seeds showed the highest values for total phenolics (2.6 mg GAE·g^{-1}), while *C. imbricata* presented the lowest ones (1.01 mg GAE·g^{-1}) (Table 5). Finally, the *C. imbricata* seeds showed the highest values in the protein content (5.6 mg proteins·g^{-1}), while *C. leptocaulis* exhibited the lowest ones (0.25 mg proteins·g^{-1}) (Table 5). Although no information on the contents of reducing sugar, total phenolics, and protein and on the antioxidant capacity in seeds of these three species of *Cylindropuntia* has been reported, variations in these determinations were found in other related species, including the *Opuntia* genus.

For the reducing sugar content, our results showed values higher in seeds of the three *Cylindropuntia* species than those for *O. joconostle* (0.009 mg·g^{-1}), *O. matudae* (0.014 mg·g^{-}1), *O. microdasys* (0.0129 mg·g^{-1}), and *O. macrorhiza* (0.0048 mg·g^{-1}) (Chahdoura *et al*., 2015; Morales *et al*., 2012). These authors also found that fructose was the most abundant soluble sugar in seeds of these species, while glucose and sucrose showed as inferior composites (Chahdoura *et al*., 2015, Morales *et al*., 2012). Sugar content also varied according to the plant tissue. Values from *Opuntia* seeds were higher than those observed in the respective cladodes and flowers of *O. microdasys* and *O. macrorhiza* (Chahdoura *et al*., 2014 a, b; Chahdoura *et al*., 2015). These results suggest that sugars could be mobilized by anabolic pathways to synthesize storage polysaccharides in the *Opuntia* seeds (Chahdoura *et al*., 2015).

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The phenolic content found in plant seeds has emerged as a desirable characteristic for human consumption due to the numerous benefits of these bioactive molecules because these could be used for their anticarcinogenic, antihypertensive, anti-inflammatory, antiallergic, antifungal, and antioxidative activities. In this study, the analysis of phenolic content demonstrated that seeds of *Cylindropuntia* plants of the state of Chihuahua could be a good source of phenolic compounds. In this study, values for total phenolics were higher than those measured in seeds from *O. microdasys* (0.36 mg GAE·g^{-1}), *O. macrorhiza*, and *O. ficus-indica* (0.95 mg GAE·g^{-1}) (Chahdoura *et al*., 2015; Amrane-Abider *et al*., 2018), although were lower than those detected in seeds from *O. polyacantha* (10.78 mg GAE·g^{-1}), *O. engelmannii* (12.55 mg GAE·g^{-1}), *O. phaeacantha* (12.87 mg GAE·g^{-1}), *O. macrocentra* (12.89 mg GAE·g^{-1}), *O. joconostle* (50.43 mg GAE g^{-1}), and *O. matudae* (59.48 mg3.GAE g^{-1}) (Morales *et al*., 2012; Núñez-Gastélum *et al.*, 2018). Few studies have reported the phenolic composition in these plants. In *Opuntia* sp., ferulic and chlorogenic acids were the most abundant compounds found in seeds (Chahdoura et al., 2015; Amrane et al., 2018). Besides, this composition also varied depending on the plant tissue analyzed. In the fruit epicarp of *C. imbricata*, 15 different acidic polyphenols were identified (Coutiño-Laguna *et al.*, 2022).

Seed proteins are essential in the development of germination because a series of proteases are activated, and the storage proteins are hydrolyzed and consumed (Ohanenye *et al*., 2020). Regarding the protein content, results determined in this study were higher than those from seeds of *O. joconostle* (0.0212 mg·g^{-1}) and *O. matudae* (0.0345 mg·g^{-1}), although lower than those detected in seeds of *O. polyacantha* (11.47 %), *O. engelmannii* (14.75 %), *O. phaeacantha* (10.45 %), and *O. macrocentra* (11.45 %) (Núñez-Gastélum *et al.*, 2018).

Some methods have been used to measure the antioxidant capacity of Cactaceae seeds. In this study, the antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. Although these methods are based on the identical mechanism of propensity to donate hydrogens (Katsube *et al.*, 2008), the quantification showed a variation in the averages depending on the specie and the method used to determine these chemical compounds. Phenolic compounds scavenge DPPH by their ability to form o-quinone intermediates upon free radical H-atom abstraction and its subsequent disproportionation. Values calculated by the DPPH assay confirmed that seeds of the three *Cylindropuntia* species presented a quenching capacity and a hydrogen donor capacity, oscillating from 26.37 mmol TE·g^{-1} to 12.02 mmol TE·g^{-1}. *C. leptocaulis* seeds presented the highest antioxidant activity (26.37 mmol TE·g^{-1}), while *C. spinosior* showed the lowest ones (12.02 mmol TE·g^{-1}) (Table 5). These results were higher than those observed in seeds from *O. polyacantha* (2.25 µmol TE·g^{-1}), *O. engelmannii* (3.41 µmol TE·g^{-1}), *O. phaeacantha* (4.30 µmol TE·g^{-1}), and *O. macrocentra* (3.81 µmol TE·g^{-1}) (Núñez-Gastélum *et al.*, 2018).

FRAP assay determines the ability of the sample to reduce Fe^{3+} (ferric ion) to Fe^{2+} (ferrous ion) in the presence of antioxidants. This study also showed slight variation among the three species from 5.6 to 11.7 mmol TE·g^{-1}. The *C. spinosior* seeds presented the highest antioxidant activity (11.7 mmol TE·g^{-1}), while *C. imbricata* showed the lowest ones (5.6 mmol TE·g^{-1}) (Table 5). The higher antioxidant activity observed in the seeds of *C. spinosior* could be related to its equally increased levels of phenolic content (Table 5).

Table 5. Content in reducing sugars, total phenols, and protein, and antioxidant capacity determined by the DPPH and FRAP tests in seeds from the three *Cylindropuntia* species.

Characteristics	Specie			Anova ($p \leq 0.05$)
	C. leptocaulis	*C. imbricata*	*C. spinosior*	
Reducing sugars *	31.8±1.2[a]	8.1±0.8[c]	12.4±2.0[b]	0.000
Total phenolics **	2.6±0.6[a]	1.0±0.1[b]	3.1±0.3[a]	0.003
DPPH ***	26.37±12.9[a]	13.08±7.6[b]	12.02±8.9[b]	0.000
FRAP ****	10.7±0.1[a]	5.6±0.1[b]	11.7±1.8[a]	0.001
Proteins *****	0.25±0.02[b]	5.6±0.40[a]	4.2±0.60[a]	0.008

* mg glucose·g^{-1} of dry weight; ** mg gallic acid equivalents (GAE)·g^{-1}of dry weight; *** mg catechin equivalents (CE)·g^{-1}of dry weight; **** mmol TROLOX equivalents (TE)·g^{-1} of dry weight; ***** mg of proteins·g^{-1} of dry weight. Data are expressed as the mean ± standard deviation (n = 20) and were analyzed using a one-way ANOVA ($p \leq 0.05$). Different letters (a–c) show the results of the Duncan test, indicating a significant difference at $p \leq 0.05$ (displayed in the row of each phytochemical characteristic).

Likewise, the phenolic and protein content and the antioxidant capacity may change under different conditions. The content of total phenolics and proteins increased throughout the seed storage time in *O. ficus-indica* (Tlili *et al.*, 2011). The total phenolics, proteins, and antioxidant capacity were also augmented in cladodes in an *Opuntia ficus-indica* cultivation supplemented with vermicompost and arbuscular mycorrhizal (Lahbouki *et al.*, 2021). Furthermore, they were increased along the germination process in other seed plants such as sorghum, quinoa, and amaranth (Jimenez *et al*. 2019; Singh *et al*., 2019). The phenolics accumulation could be due to the cell wall breakdown during germination leading to the liberation of bound phenolic compounds (Azeez *et al*., 2022; Singh *et al*., 2019). Indeed, the biosynthesis of secondary metabolites, although controlled genetically, specific investigation recommends that the phenols content in *Opuntia* cacti could be influenced by genotype, climatic conditions, post-harvest factors, harvesting time, agronomic practices, and storage conditions (Agostini-Costa, 2022; Mounir *et al*., 2020). On the other hand, the protein increase probably is due to the release of protein in the seed structure during the breakdown of the starch granule by α-amylase during germination (Azeez *et al*., 2022) or perhaps due to protein synthesis during germination (Chinma *et al.*, 2021).

Some studies have shown that the highest antioxidant properties of *Opuntia* seeds agree with their highest flavonoids and phenolics contents (Chahdoura *et al*., 2015; Lahbouki *et al.*, 2021; Núñez-Gastélum *et al.*, 2018; Morales *et al*., 2012; Valero-Galvan *et al.*, 2021). In this study, the data of phytochemicals quantifications correlated among them. The phenolic content and the antioxidant activity determined by the FRAP assay showed a positive correlation (r=0.997, p=0.0001). However, the antioxidant activity determined by the DPPH assay revealed no correlation with the phenolic content (r=0.225, p=0.560) but correlated positively with the content of reducing sugars (r=0.972, p=0.0001).

Hierarchical clustering and principal component analysis

Data generated for the three *Cylindropuntia* species were subjected to cluster analysis to establish groups of specie and distances among them and principal component analysis (PCA), using the NIA array analysis tool (Figure 2). The hierarchical groups were obtained by Ward's clustering method using squared Euclidean distances, which showed the separation of the species in two well-defined clusters (Figure 2A). The first cluster was formed by the *C. spinosior* and *C. imbricata*

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species, resembling fruit and seed morphometry, germination index, and seed phytochemical composition. The second principal group is only involved by *C. leptocaulis*, separating from the other two *Cylindropuntia* species (Figure 2A). The PCA explained 99% of the total variability in the first two components. This analysis also revealed a good separation among the three *Cylindropuntia* species (Figure 2B). Furthermore, the first principal component (PC1) explained 85.4% of all the variation, being negatively correlated with this component the characteristics corresponding to the fruit area (-0.999), the total fruit weight (-0.989), the seed weight (-0.994), and the protein content (-0.995), and positively the antioxidant activity determined by the DPPH test (0.996). The second principal component (PC2) showed 14.5% of the total variation, and the average germination rate correlated positively with this component.

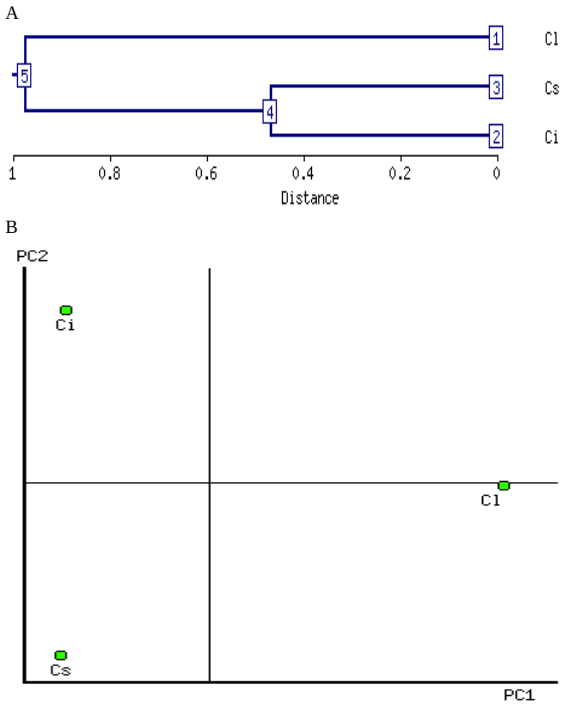

Figure 2. Dendrogram of the hierarchical clustering (**A**) and PCA plot (**B**) were obtained from data analysis from the fruit and seed morphometric parameters, seed phytochemical composition, and seed antioxidant capacity of the three *Cylindropuntia* species. Cl: *C. leptocaulis*, Cs: *C. spinosior* and Ci: *C. imbricata*.

Conclusions

The *C. leptocaulis*, *C. imbricata*, and *C. spinosior* species showed significant differences ($p \leq 0.05$) in the parameters established to characterize the morphology of fruits and seeds as weight, width, length, perimetry, area, and length/width ratio, as well as in protein content, total phenolics, and antioxidant activity in seeds. In general, *C. imbricata* and *C. spinosior* presented the bulkiest fruits and seeds, while *C. leptocaulis* showed the smallest ones. Regarding germination, the chemical method using 98% (v/v) H_2SO_4 promoted a germination percentage of 10% in 14 days. The *C. leptocaulis* seeds showed the highest germination percentage (13.3%), followed by *C. imbricata* (10%), and *C. spinosior* (3.33%). Finally, the *C. leptocaulis* seeds showed the highest value for reducing sugar, the *C. spinosior* seeds exhibited the highest ones for total phenolics, and the *C. imbricata* seeds showed the highest content of proteins.

Acknowledgments

We would like to thank to the Departamento de Ciencias Químico Biológicas of the Universidad Autónoma de Ciudad Juárez for the use of the laboratory and their facilities to perform the characterization of Cylindropuntia species.

Ethics statement

Not applicable

Funding

This work was published with the support of the Institute of Innovation and Competitiveness of the Ministry of Innovation and Economic Development of the State of Chihuahua.

Declaration of competing interest

The authors declare that they have no competing interests.

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