Effect of substrate and gibberellic acid (GA_3) on seed germination in ten cultivars of *Opuntia* sps.

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ABSTRACT

The seeds of *Opuntia* sps show low germination capacity and high latency, so this study compares different inert and synthetic substrates *in vitro* with the addition of gibberellic acid (GA_3) to stimulate seed germination in ten cultivars of *Opuntia* sps. (*O. megacantha, O. amyclaea, O. streptacantha, O. albicarpa* and *O. ficus-indica*). The photoperiod 16/8 h, light/darkness had a positive stimulating effect on the seed germination rate, while an inert substrate (pumice:peat moss:sand, 1:1:1 v/v) showed on average 20% germination. The use of *in vitro* Gamborg medium increased the germination rate to 40%. The addition of 25 mg L^{-1} gibberellic acid (GA_3) to Murashige and Skoog (1962) medium, prepared to ¼ basal salt concentration, increases the germination rate across all cultivars, up to 90% at four weeks. GA_3 was shown to play an important role in breaking the latency of the seed, and increased amylolytic activity during seed germination in *Opuntia* sps.

Keywords: cactus pear fruit, scarification, substrate, *in vitro* germination, α-amylase

INTRODUCTION

Plants of the *Opuntia* genus are native to several ecosystems, from arid zones at sea level to territories of great elevation, such as the Peruvian Andes; from tropical regions in Mexico where temperatures never fall below 5 ºC, to regions in Canada where winter temperatures drop below -40 ºC. This remarkable adaptability can be an interesting genetic resource for very diverse ecological zones (Nobel, 1999). Vegetative reproduction using the cladodes is the most important and used method for both natural and cultivated populations in Mexico (Pimienta, 1990). However, in other arid zones, such as East Texas, natural populations are established by seeds. The latter method has several advantages, among which are: the creation of individuals with a different genetic makeup; it is the only available method if a species is difficult to reproduce by vegetative means, and it lowers the overall operating costs. Its main drawbacks are the difficulty in obtaining viable seeds, and the growth and development of the plants take a longer time (Reyes *et al*., 2005). Despite the importance

and advantages of this method, there are few studies evaluating the germination in *Opuntia*, since as stated before, cultivation is usually done by the propagation by cladodes (Mondragón and Pimienta-Barrios, 1995). However, it is important to consider propagation by seeds as a strategy for the long term conservation of these cacti, as well to keep and increase the genetic diversity.

Seeds of *Opuntia ficus-indica* L. Miller, like many other species of *Opuntia*, have a very low germination rate, mostly due to its lignified pericarp which contains 55% of lignin and cellulose, surrounding the embryo with 90-95% of the seed weight, blocking the radicular protrusion (Werker, 1997; Habibi *et al*., 2008). Scarification methods show mixed results. It is known that exposing the seeds to mineral acids the temperature increases, and this phenomenon acts as a metabolic signal, initiating the first stages of germination, or denaturing germination inhibitors (Potter *et al*., 1984). Avilés (2001) reports that the best scarification method for *Stenocereus gummosus* is the treatment with concentrated sulfuric acid, resulting in germination rate of 63%. Crisóstomo, (2009) achieved almost 100% germination at 20 d in *Astrophytum ornatum* seeds treated with 15% sulfuric acid and placed in 50% MS media. In MS media alone, very few seeds germinated after three months. Gibberellins are phytoregulators synthesized in several plant tissues, but especially so in tissues of active growth, such as embryos or meristems. Another important role is the induction of hydrolytic enzymes such as α-amylase and proteases in seeds, and so increases the mobility of the endosperm. Gibberellic acid (GA_3) induces the synthesis of α-amylase, which is responsible for the processing of the starch reserves present in the seed during germination. Thus, GA_3 is widely used as a promoter or inducer of germination in several plant species (Tigabu and Odén, 2001). In Cactaceae, there are few reports on the use of gibberellins as germination promoters, and the results are quite heterologous. Deno (1994) reports that in *Opuntia* sps, GA_3 is an indispensable promoter. Sánchez-Venegas, (1997) showed similar results in concentration of 40 mgL^{-1} in *O. joconostle*. Olvera *et al*., (2003) reported no significant effect in germination at concentrations up to 1000 mg L^{-1} in *O. tomentosa*. The aim of this research was to determine the optimal germination conditions in ten cultivars of *Opuntia* sps, as well as evaluate the effect of gibberellic acid as promoters of seed germination.

MATERIALS AND METHODS

Opuntia sps samples

Seeds of ten mature fruits of *Opuntia* sps (Table 1, Fig. 1) were collected in the “Las Papas de Arriba” field, located in Ojuelos, Jalisco State, Mexico, between 21°43´30” and 21°43´77” N, 101°39´48” and 101°43´42” W, with an average altitude of 2260 m. The climate in this area is classified as dry template, with an average rain fall of 390-450 mm annual, and a mean temperature of 16-18 ºC; rainfall occurs mainly in summer from May to September (Pimienta, 1990).

Seed Extraction and cleaning

The pulp of the fruit was extracted and washed several times with water at 60 ºC on a 150 µm mesh until only the seeds remain. Afterwards, the seeds were treated with 10% phosphoric acid (H_3PO_4) for 10 min at room temperature, followed by two water rinses to remove the remaining acid. Finally, the seeds were left to dry on absorbent paper, and were used six months after extraction.

Table 1. Characteristics of the *Opuntia* spp. cultivars used in the germination study.

Scientific Name	Common name	Use[a]	Pulp color	Seeds per Fruit[b]	Abortive seeds (%)[b]
O. megacantha Salm-Dyck	"Amarillo Plátano"	F	Orange	290±45	17±5
O. amyclaea Tenore	"Burrona"	F	Light Green	197±30	75±10
O. streptacantha Lemaire	"Cardona"	F-Fo	Red	ND	ND
O. amyclaea Tenore	"Cristalina"	F	Light Green	255±48	50±8
O. megacantha Salm-Dyck	"Huesuda"	F	Orange	192±27	12±5
O. megacantha - Salm-Dyck	"Naranjón Legítimo"	F	Orange	ND	ND
O. megacantha Salm-Dyck	"Pico Chulo"	F	Orange	265±38	20±6
O. albicarpa Sheinvar	"Reyna"	F	Light Green	240±40	55±14
O. albicarpa Sheinvar	"Rojo Ojuelos"	F	Red	344±35	21±4
O. ficus-indica (L.) Miller	"Copena Torreoja"	TP	Red	215±33	28±9

[a] Cultivar use as according to Mondragon-Jacobo and Pimienta-Barrios (1995). F=Fruit; Fo=Feed; TP=Triple purpose.

[b] Mean ± standard deviation based on measurement of 20 fruits from at least ten plants.

ND= not determined.

Inert substrate (IS)

A mixture of pumice (3 mm mean size), peat moss (*Sphagnum* sp) and sand was prepared in a 1:1:1 (v/v/v) relation. The mixture was moistened and sterilized in an autoclave at 121 ºC for 30 min.

Inert substrate plus vermicompost (IS + H)

A mixture of pumice, peat moss (*Sphagnum* sp) and vermicompost (*Eisenia foetida*) was prepared in a 1:1:1 (v/v/v) relation. The mixture was sterilized by placing in a microwave oven for 3 minutes. 20 seeds per cultivar were soaked in distilled water for 24 h. Afterwards, they were placed in expanded polystyrene pots in either substrate (IS or IS+H) and kept in greenhouse conditions.

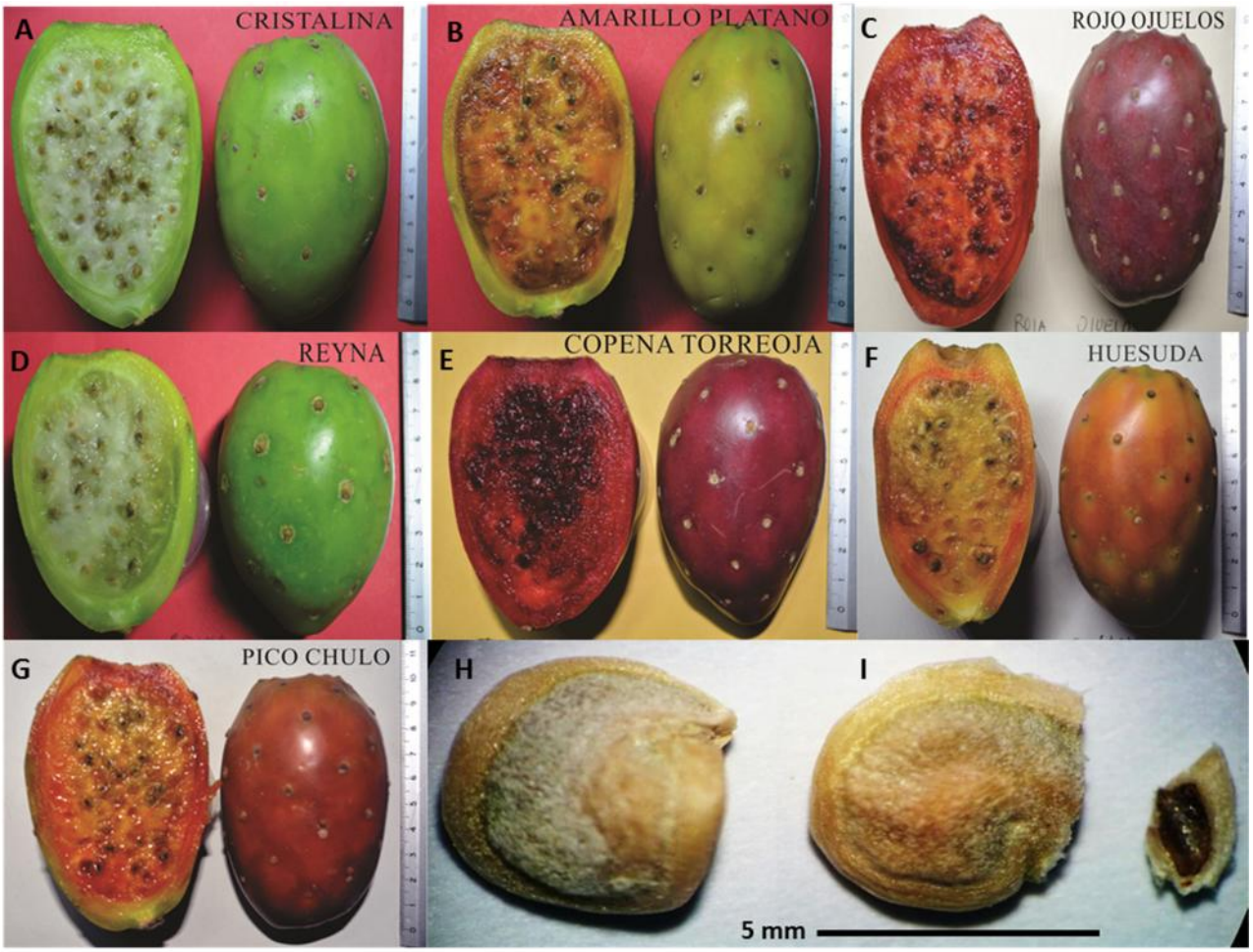

Figure 1. Fruits of different *Opuntia* sps. used in the germination experiments **A-G**. Cutout detail on the *Opuntia* seed coat (**H-I**).

Scarification (S)

Scarification was performed as described by Altere *et al*. (2006). Concentrated sulfuric acid was added to a group of seeds for 5 min. The seeds were rinsed twice with distilled water and were incubated with 6% hydrogen peroxide for 30 min. After this time, the seeds were placed in Petri dishes containing wet cotton in the bottom and filter paper on top as seeding surface. Each dish contained 20 seeds, with three repetitions per cultivar, and were incubated at 26 ºC with 16 h light and 8 h darkness cycles.

***In vitro* medium germination**

Murashige and Skoog medium (MS)

The scarified seeds were disinfected by immersion in 5% sodium hypochlorite (w/v) for 10 minutes, and then rinsed three times with distilled, sterile water. The seed cover was clipped with pliers (Figure 1) in sterile conditions, and the seeds were placed in 100 mL Erlenmeyer flasks with Murashige and Skoog, (1962) media with 7.5 g L^{-1} agar, and adjusted to pH 5.8, and were incubated at 26±1 ºC with 24 h cycle, 16 h of light and 8 h of darkness, with a light intensity of 58 μE.

MS plus gibberellic acid (GA_3)

MS medium was prepared at 25% of total salt concentration with 7.5 g L^{-1} agar, to which 25 mg L^{-1} of gibberellic acid was added. The seed cover was clipped with pliers in sterile conditions, and the flasks were incubated 28 ºC ± 1with 12 h light/8 h dark cycles with a light intensity of 58 μE.

Gamborg liquid medium (B5).

Seeds were disinfected with 5% sodium hypochlorite (w/v) for 24 h. The seed cover was broken with pliers in sterile conditions. Twenty seeds were then placed for four weeks in 30 ml of liquid B5 medium (Gamborg *et al*., 1968) plus 1.95 mg L^{-1} kinetin and 0.099 mg L^{1} 2,4-dichlorophenoxyacetic acid (2,4-D), and placed in an orbital agitator set at 100 rpm. After the four weeks of primary culture elapsed, the germinated embryos subcultured, applying the same base medium without any growth regulators.

Protein extraction and ~-amylase activity determination

The seeds were milled until a fine powder was obtained. This powder was defatted by mixing with an equivalent volume of acetone and vigorous agitation. The resulting mixture was centrifuged at 2150 rcf for 5 min. The supernatant was discarded, and the precipitate left to dry in an aluminum tray at room temperature. Once the acetone evaporated, the resulting powder was placed in an microtube and two volumes of 10 mM, pH 7.2 phosphate buffer were added, keeping it 12 hours at 4 ºC to achieve the extraction of soluble proteins. The samples were then centrifuged at 16800 rcf for 5 min, and the supernatant was recuperated. This solution was used to quantify the protein present in the seed and analyze for α-amylase activity. Protein concentration was determined as described by Bradford (1976) using bovine serum albumin as the standard. In all extracts α-amylase activity was determined as described by Bandini *et al*. (2009); using 1% (w/v) starch as the substrate. One α-amylase activity unit was defined as the amount of enzyme required to produce a microgram of maltose in 30 min at 35 ºC. Maltose was measured by the 3,5-dinitrosalicilic acid (DNS) method Bernfeld (1995). A blank was assayed concurrently with the reaction mixture. All assays were performed in triplicate.

Statistical analysis

For each cultivar three repeats of 20 seeds each were tested. Seeds were monitored daily, and the experiment was followed for 4 weeks. Germination was considered to have occurred once the radicle sprouted. To the germination data (as percentage of germination) an

analysis of variance (ANOVA) and a comparison of means using Tukey test, with $p < 0.05$ by means of the software SPSS V2.0.

RESULTS AND DISCUSSION

Considering that all specimens are domesticated species, the number of seeds in all cultivars (Table 1) was fairly homogenous (averaging 250 per fruit); however, there was a large difference in the number of abortive seeds, from 17 to 75%. Reyes-Agüero *et al*. (2005) reported that the average number of seeds per fruit varies considerably, from 55 in *O. brunneogemmia* (F. Ritter) C. Schlindwein to 208 in *O. rastrera* (Weber). These authors suggest that in fruit cultivars in Mexico, with different degrees of domestication and wild types, fruits contain from 16 to 518 seeds. The number of seeds per fruit is a morphological variable that can be used to differentiate between *Opuntia* species, by discrimination analysis, as well as fruit size and pulp color (Chessa, 2010).

The seed inability to germinate under the correct conditions (temperature, moisture and gases concentration) is considered the latency, or dormancy, of the seed Immersion in 10% phosphoric acid was an effective method to clean the pulp adhered to the seed cover, due to the breakage of the glyosidic links of complex carbohydrates, increasing their solubility and promoting germination. Inert substrate (pumice and sand) and inert plus vermicomposting substrates are commonly used in the nursery due to its characteristic of retaining moisture. The germination rate on these substrates was 33.8% and 16.4% respectively (Figure 2). Therefore, the imbibition water is not sufficient to trigger a uniform germination process.

The hard seed cover in the genus *Opuntia* may act as mechanical protection of the embryo, instead of being an impermeable element (Werker, 1997). Our germination data (Figure 2) shows that the dormancy is not caused by a mechanical barrier blocking the radicular protrusion, as no treatment, neither chemical, with H_2SO_4 or H_2O_2, or mechanical, by performing a cut in the seed cover (Figure 1 H-I), was enough to break the latency, getting only a 4.4% of germination (S).

Sulfuric acid has been used in other *Opuntia* species to increase the germination rate Olvera-Carrillo *et al.* (2009). However, the cover of the embryo does not act only as a mechanical barrier, but may influence the germination indirectly. Altere *et al*. (2006) reported, the use of sulfuric acid influenced germination negatively, as the number of seeds that germinated was lower than the other methods tested.

Another type of dormancy in these species has a physiological origin that has been eliminated in part by two agents: light as a photoperiod treatment and the use of hydrogen peroxide. Forced dormancy is regulated by environmental conditions, such as light, and seeds are ready to germinate immediately after environmental limitations are lifted (Vázquez-Yáñez and Orozco Segovia 1996). Positive photoblastic response is common among *Cacti*, including the *Opuntia* genus (Rojas-Aréchiga and Vázquez-Yáñez, 2000). The hydrogen peroxide solution, acting as an oxidizing agent, regulates the primary, or innate, latency, as

demonstrated by Altere *et al*. (2006). This latency, which prevents the germination of the seed inside the mother plant and after a time period after its dispersion, may be caused by the embryo's asphyxia due to the hardness of the teguments. However, our study did not present this behavior, as it was the treatment that resulted.

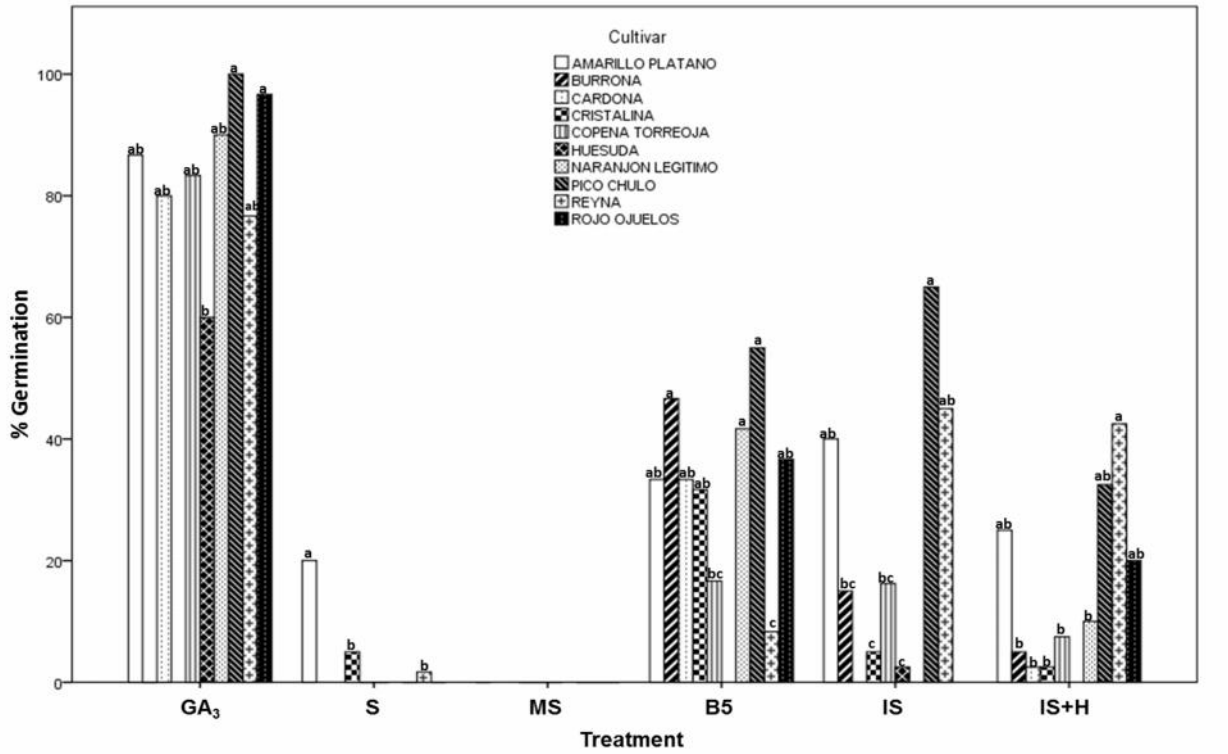

Fig. 2. Germination percentage for seeds of ten cultivars of *Opuntia* spp after 28 days. Treatments: (**GA_3**) MS 25% plus 25 mg L^{-1} of gibberellic acid, (**S**) scarification with H_2SO_4, (**MS**) Murashige and Skoog basal salts, (**B5**) Gamborg basal salts, plus 1.96 mg L^{-1} kinetin and 0.099 mg L^{-1} 2,4-D, (**IS**) pumice: peat moss: sand (**IS+H**) Peat Moss: Pumice: Vermicompost. Different letters between cultivars in treatment are significantly different by Tukey at $p<0.05$.

A photoperiod 16/8 h was required for seeds to germinate in the *in vitro* treatments, and is a shared characteristic with other *Opuntia* species. Monteiro *et al.* (2005) demonstrated the importance of the light treatment in the germination of *O. stricta* and *O. tomentosa* seeds. The best results for *Opuntia* sps germination were obtained in the *in vitro* media GA_3, reaching 85% germination, and B5 with 33.7% (Figure 2). In general, seed covers are impermeable to water, and when placed in *in vitro* media this may not be an issue as they are very high in moisture; however, water presence alone is not sufficient to induce germination, as evidenced by medium MS, where no germination was observed in the evaluated time. It is known that gibberellic acid can participate in the remobilization of nutrients or germination inhibitors George (2008) and Masuoka (2003) report that gibberellic acid induces the mobilization of the seed reserves by inducing hydrolytic enzymes such as α-amylase and

proteases, facilitating the mobilization of the endosperm and the rate of germination. This phenomena was observed in our study, where the cultivars that germinated earliest (2 d), such as Cardona, Rojo Ojuelos, Naranjon Legitimo, Pico Chulo and Copena Torreoja, followed by Burrona, show the highest amylolytic activity (Figure 3), whereas in cultivars such as Amarillo Platano, Reyna, Huesuda y Cristalina the radicle did not appear until the third week, both being higher in the treatment with GA_3 compared to the MS medium alone. Thus the speed of germination is influenced by the specific activity of α-amylase, where the cultivars with the highest germination speed show the highest value of enzymatic activity.

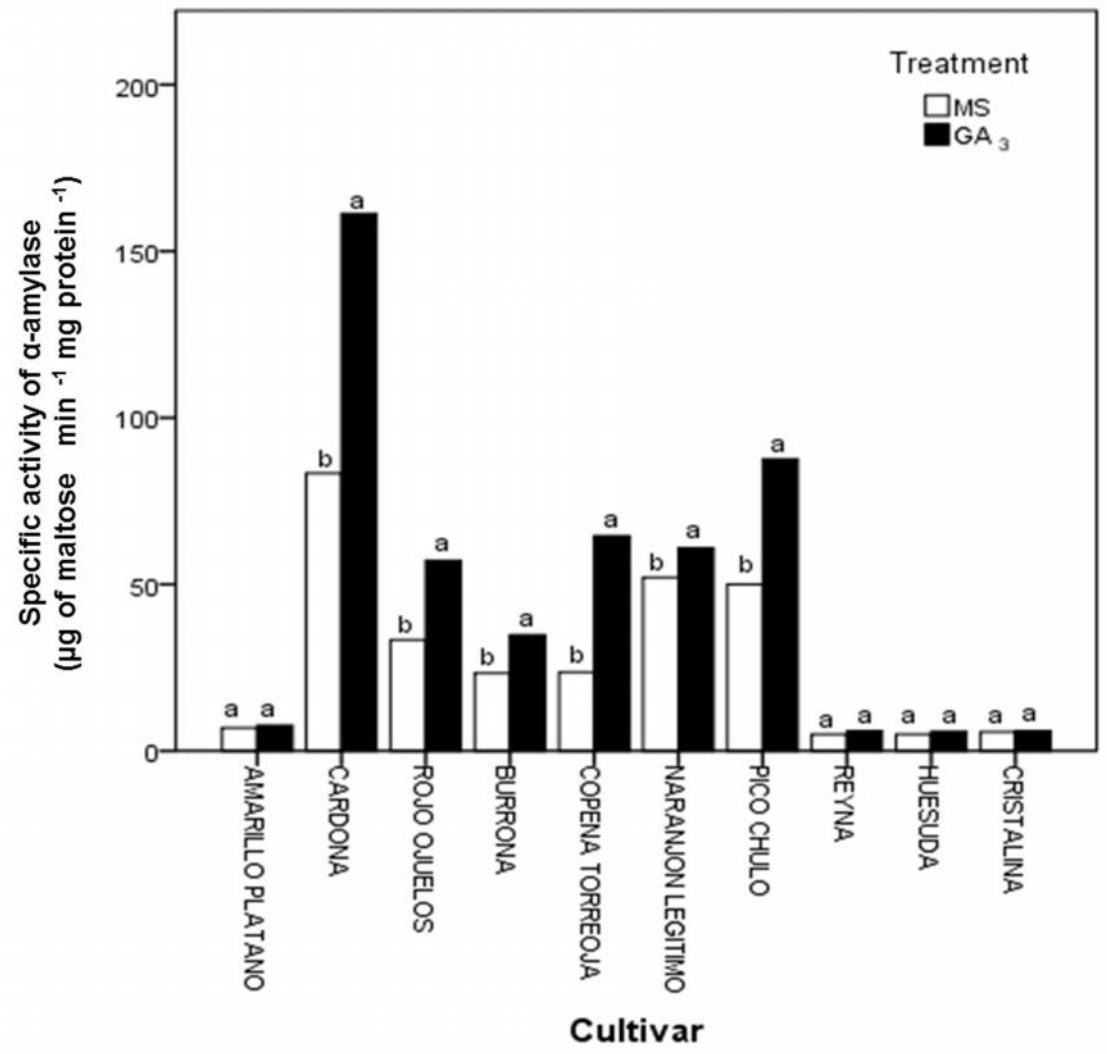

Figure 3. Specific activity of α-amylase in *Opuntia* spp seeds after seven days established on (**MS**) basal salts of Murashige and Skoog (1962) and (**GA_3**) MS at 25% medium plus 25 mg L^{-1} of gibberellic acid. Different letters between treatments are significantly different by Tukey at $p < 0.05$.

Olvera *et al*. (2003) has reported that concentrations of 1000 mg L^{-1} of GA_3 in *O. tomentosa* do not promote seed germination, but in *O. joconostle*, at 40 mg L^{-1} of GA_3, has a promoter effect previous to the seed coat bursting Sánchez-Venegas (1997). In *O. rastrera*, *O. microdasys* and *O. macrocentra*, the addition of 200 mg L^{-1} of GA_3 does not have a significant effect in the germination of the seeds Mandujano *et al*., (2007). In our study, the addition of 25 mg L^{-1} of GA_3 to the medium was enough to stimulate the germination in all the tested *Opuntia* cultivars.

CONCLUSIONS

Both inert substrates showed a similar behavior in the germination of the ten varieties of *Opuntia* tested, averaging 10-55% germination rate. Germination was increased to an average of 40% by mechanical scarification (breaching of the cuticle), while the sulfuric acid treatment had no effect on germination. Under *in vitro* conditions, with a 16/8 h photoperiod, a low percentage of germination in seeds was induced. It was demonstrated that gibberellic acid plays an essential role in the promotion of germination (above 90%) in seeds of *Opuntia* sps., as in the remobilization of the carbohydrate source, by increasing the amylolytic activity in the germinated seeds.

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REFERENCES

Altere, M., Trione, S., Guevara J.C. and Cony, M. 2006. Stimulation and promotion of germination in *Opuntia ficus-indica* seeds. Journal of Professional Association for Cactus Development 8, 91-100.

Avilés, A., Morales, R., Treviño, N., Oranday, C. and Verde, S. 2001. Datos sobre germinación de *Stenocereus gummosus* mediante métodos de escarificación. XV Congreso Mexicano de Botánica.

Bandani, A.R., Kazzazi, M. and Mehrabadi, M. 2009. Purification and characterization of midgut α-amylases of *Eurygaster integriceps*. Entomol. Sci. 12, 25–32.

Bernfeld, P. 1955. Amylase α and β, In Methods in Enzymology. (Colowick SP, Kaplan NO, ed.), Academic Press Inc, New York 1:149-158.

Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Chessa, I. 2010. Discriminant analysis of morphological descriptors to differentiate the *Opuntia* genotypes. VIIth International Congress on Cactus Pear and Cochineal October 17-22, Agadir (Morocco).

Crisóstomo, S. M. 2007. Evaluación de la capacidad morfogenética en explantes longitudinales de *Astrophytum ornatum*. XVIII Congreso Mexicano de Botánica. Zacatecas, Zacatecas. México. Octubre 16-19

Deno, N.C. 1994. The critical role of gibberellins in germination and survival of certain cacti. Cactus and Succulent Journal 66, 28-30.

Flores, J. and Jurado, E. 2003. Are nurse-protégé interactions more common among plants from arid environments? Journal of Vegetation Science 14, 911-916.

Flores, J., Arredondo, A. and Jurado E. 2005. Comparative seed germination in species of *Turbinicarpus*: An endangered cacti genus. Natural Areas Journal 25, 183-187.

Flores, J., Jurado E. and Arredondo A. 2006. Effect of light on germination of seeds of Cactaceae from the Chihuahuan Desert, México. Seed Science Research 16, 149-155.

Flores, J., Jurado, E. and Jiménez-Bremont, J.F. 2008. Breaking seed dormancy in specially protected *Turbinicarpus lophophoroides* and *Turbinicarpus pseudopectinatus* (Cactaceae). Plant Species Biology 23, 43-46.

Gamborg, O. L., Miller R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soyben root cells. Exp. Cell. Res. 50,151-158.

George, E. F. 2008. Plant Propagation by Tissue Culture. 3rd Ed. Springer. 227-248.

Habibi, Y., Heux, L., Mahrouz, M. and Vignon, M. R. 2008. Morphological and structural study of seed pericarp of *Opuntia ficus-indica* prickly pear fruits. Carbohydrate Polymers 72,102–112.

Mandujano, M. C., Golubov, J. and Rojas-Aréchiga, M. 2007. Efecto del ácido giberélico en la germinación de tres especies del género Opuntia (Cactaceae) del Desierto Chihuahuense. Cactáceas y Suculentas Mexicanas 52, 46-52.

Matsuoka, M. 2003. Gibberellins signaling: How do plant cells respond to GA signals? J. Plant Growth Reg. 22, 123-125.

Mondragón-Jacobo, C. and Pimienta-Barrios, E. 1995. Propagation. In Barbera, G., P. Inglese, and E. Pimienta-Barrios (eds.). Agro-ecology, cultivation and uses of cactus pear. FAO International Technical Cooperation Network on Cactus Pear. pp. 64-70.

Monteiro, A., Cheia, V.M., Vasconcelos, T. and Moreira, I. 2005. Management of the invasive species *Opuntia stricta* in a Botanical Reserve in Portugal. Weed Research 45, 193-201.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 437-497.

Nobel, P.S. 1999. Photosynthetic characteristics of CAM succulents with high productivity. In: G. Garab, ed. Photosynthesis: Mechanisms and Effects, Vol. V. Kluver Academic Publishing, Dordrecht. The Netherlands. pp. 3955-3960

Olvera, Y., Márquez-Guzmán, J. Barradas V.L., Sánchez-Coronado, M.E. and Orozco-Segovia, A. 2003. Germination of the hard seed coated *Opuntia tomentosa* SD., a cacti from the Mexico Valley. J. Arid. Environ.55, 29-42.

Olvera-Carrillo, Y., Méndez, I., Sánchez-Coronado, M.E., Márquez-Guzmán, J., Barradas, V.L., Huante, P. and Orozco-Segovia, A. 2009. Effect of environmental heterogeneity on field germination of *Opuntia tomentosa* (Cactaceae, Opuntioideae) seeds. J. Arid. Environ.73, 421–427.

Orozco-Segovia, A., Márquez-Guzmán, J., Sánchez-Coronado, M.E., Gamboa De Buen, A., Baskin, J.M. and Baskin, C.C. 2007. Seed anatomy and water uptake in relation to

seed dormancy in *Opuntia tomentosa* (Cactaceae, Opuntioideae). Ann. Bot. 99, 581–592.

Pimienta, B. E. 1990 El Nopal Tunero. Colección Tiempos de Ciencia. Universidad de Guadalajara. Guadalajara, México. 246 p.

Potter, R.L., Petersen, J.L. and Ueckert, D.N. 1984. Germination responses of *Opuntia* spp. to temperature, scarification and other seed treatments. Weed Science 32, 106–110.

Rojas-Aréchiga, M. and Vázquez-Yañes, C. 2000. Cactus seed germination: a review. Journal of Arid Environments 44, 85-104.

Reyes-Agüero, J.A., Aguirre, J.R. and Valiente-Banuet, A. 2005. Reproductive biology of *Opuntia*: A review. J. Arid. Environ.64, 549-585.

Sánchez-Venegas, G. 1997. Germinación, viabilidad y características distintivas de la semilla de *Opuntia joconostle* Weber, forma cuaresmero. Cactáceas y Suculentas Mexicanas 42,16-21.

Tigabu and Odén 2001. Effect of scarification, gibberellic acid and temperature on seed germination of two multipurpose Albizia species from Ethiopia. Seed Science and Technology 29,11-20.

Vazquez-Yañes, C. and Orozco Segovia A. 1996. Physiological Ecology of Seed. Dormancy and Longevity in the Tropical Rain Forest. pp. 535-554. In: Mulkey, S., R.L. Chazdon, and A.P. Smith (eds). Physiological Ecology of Tropical Forests. Chapman & Hall

Werker, E. 1997. Seed Anatomy. Stuttgart; Gebrüder Borntraeger. 424 pp.