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Viability and germination of *Cucurbita okeechobeensis martinezii* seeds an endangered wild species from México

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ABSTRACT

Cucurbita okeechobeensis martinezii **(L.H. Bailey) is an endangered Mexican wild species. To contribute to its conservation, specimens were prospected in three areas of cloud forest in Veracruz, Mexico. A completely randomized design was established to evaluate the viability of TTZ seeds and the effect of three LED light spectra on the in vitro germination of the collected specimens. Murashige and Skoog's medium supplemented with 2 mgL^{-1} gibberellic acid (GA_3) was used. The results showed differences in seed viability among the specimens evaluated. The highest values of seed viability were observed in the specimens from the San Marcos site. The positive effect of the red LED light spectrum on the germination percentage and growth of germinated seedlings *in vitro* was confirmed. These results may contribute to the conservation and propagation of this valuable genetic resource.**

Keywords: *Cucurbita*, seeds*, in vitr*o, LED lights, conservation, endangered species.

INTRODUCTION

The genus *Cucurbita* has 12–15 species and approximately 20 taxa considered subspecies, most of them distributed and with a center of origin in Mexico (Lira *et al*., 2016). They are monoecious, creeping, climbing, and sub-shrubby plants. Their flowers are pollinated by various insects, which favors gene flow and hybridization between related wild and cultivated species (Montes-Hernández and Eguiarte, 2002).

This genus is noted for its economically important food species. In addition to their food use, pumpkins can be used for industrial, commercial, medicinal, and traditional purposes, as well as containers for handicrafts (Cerón, 2010).

In Mexico, 11 wild species of this genus are present, but there are not enough representative accessions of them. *C. okeechobeensis* subsp. martinezii (L.H. Bailey), T.C. Andres & G.P. Nabhan, ex-T.W. Walters & D.S. Deckham, & G.P. Nabhan, ex-T.W. Walters, 2002, is one of these wild species. At present, two subspecies have been recognized: one of them is endemic to the region around Lake Okeechobee in Florida, and the other to the state of Veracruz in Mexico (Andres *et al*., 1988). In Mexico, this species has been found in humid areas near streams from southern Tamaulipas to northern Oaxaca and Chiapas, as well as in the states of San Luis Potosi, Querétaro, Puebla, and Veracruz (Nee, 1993; Lira, 2001).

Today, this species is included in the Mexican Official Standard NOM-ECOL-059-2010 (Semarnat, 2010) as well as in the list of conservation concerns (G1, critically endangered); this taxon is also included in the United States endangered species list (USFWS, 2019).
In México, this species has been considered a weed in cultivated areas and has been displaced from its natural areas due to land use change. Similarly, being considered a weed, it has been constantly eliminated from cultivation areas, and a decrease in the number of pollinators in the study habitat cannot be ruled out (Lira *et al*., 2009).
Like other wild *Cucurbita* species, *C. okeechobeensis* is resistant to biotic and abiotic factors. This makes it a valuable resource for the introgression of its genes for resistance to diseases or other abiotic factors into other edible pumpkin species (Khoury *et al.,* 2020).
On the other hand, its fruits have been used as a substitute for soap as well as in traditional medicine to cure dermatitis and burns. Infusions of its fruits are used for their properties as laxatives, emetics, antidiabetics, antioxidants, anti-inflammatories, and for the treatment of dysentery, among other conditions (Osuna, 2005). Although these medicinal advantages have not yet been formally corroborated, there is information on the use of extracts from the fruit of this species to inhibit cell proliferation *in vitro* in human cancer cell lines (Morales-Vela *et al.,* 2020).
Based on the records of accessions in the Squash Network of the National Seed Inspection and Certification Service (SNICS), the presence of only three accessions of this species in the germplasm banks of the SNICS in Mexico has been indicated (Rios-Santos *et al*., 2018).
It is therefore of great importance to carry out prospecting work and implement actions for the conservation of these valuable endemic genetic resources in Mexico.
Despite its importance, there is not enough information on this endemic species of Mexico. For this reason, it was proposed to develop this work, for which fruits of this species were collected at three sites in the cloud forest zone, near the municipality of Xalapa, Veracruz.
Seed viability was evaluated by the TTZ method, and the effect of different LED light spectra on *in vitro* seed germination was determined, with the objective of contributing to the conservation and propagation of this valuable wild genetic resource in Mexico.

MATERIALS AND METHODS

In the period between October and January 2021, sampling was carried out in the cloud forest area near the municipality of Xalapa, Veracruz, Mexico. In this area, it has been reported that specimens of this species can be found on shrubs of species such as *Coffea arabica* L., *Platanus mexicana* Moric., *Juglans pyriformis* Liebm., and other species of the Veracruz low deciduous forest (Nee, 1993).
Specimens of this species were located between 1000 and 1500 m.a.s.l. (Conabio, 2016) in the area adjacent to the road "Las Trancas-Coatepec", belonging to the municipality of "Emiliano Zapata" in Xalapa, Veracruz, on the Camino Real to "Puerto Rico", municipality of Coatepec, as well as on the road "San Marcos", Xico, Veracruz (Figure 1).
A total of 17 specimens were collected at the three study sites and transferred to the Institute of Biotechnology and Applied Ecology, of the "Universidad Veracruzana" for study. The viability of the collected seeds per study site was evaluated according to International Seed Evaluation Standards (ISTA, 2014).

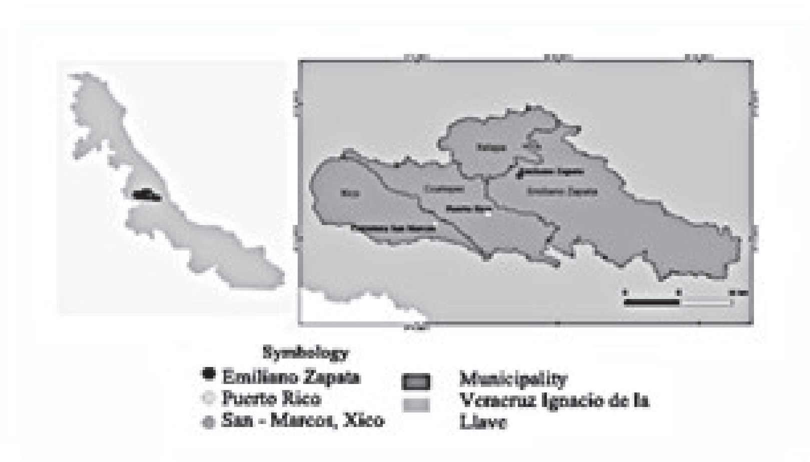

Figure 1. Locations of the sites of *C. okeechobeensis* collected in Xalapa, Veracruz.

The cover of each of the seeds was removed manually, and a 1 mm deep transverse incision was made to allow staining of the embryo. In this way, they were submerged in a freshly prepared 1% solution of 2, 3, 5-triphenyl tetrazolium (TTZ). The submerged seeds were kept for 24 hours in the dark, at room temperature (25 ºC), without shaking. After this time, excess moisture was removed on blotting paper, and the percentage of viability of the seed was evaluated based on the intensity of the staining of the embryo. Seeds showing at least 25% reddish-pink staining were considered viable, and those showing no staining were considered non-viable. Subsequently, the viability percentage of the seeds was determined using the formula proposed by Salazar-Mercado *et al.* (2020).

$$\text{Seed viability percentage} = \frac{Number\ of\ viable\ seeds}{Total\ number\ of\ seeds} \text{ x } 100$$

Effect of LED lights on *in vitro* seed germination

Based on the viability results of the evaluated seeds, viable material was selected to evaluate *in vitro* germination. The seeds selected were kept under constant agitation for 24 hours in a 1 mgL^{-1} gibberellic acid (GA_3) solution. Subsequently, the seed coat was removed before being disinfected for 15 minutes. In a sodium hypochlorite (NaClO) solution at 15%, add 100 µL of Tween-20 (Sigma-Aldrich, St. Louis, Missouri, USA) and 0,5 mL of Microdyne®, per 100 mL of sterile distilled water. The seeds were then rinsed three times with sterile distilled water.
The effects of three different LED light spectra: white (400–750 nm), red (700–800 nm), and blue (400–500 nm), on in vitro seed germination and plantlet morphometric traits were evaluated. For this purpose, 50% Murashige and Skoog (1962), MS medium supplemented with 30 gL^{-1} sucrose, 2 mgL^{-1} GA_3, and 2.5 gL^{-1} Phytagel (Sigma-Aldrich, St. Louis, Missouri, USA) as a gelling agent was used. The pH of the culture medium was adjusted to 5.8 with 0.5 N NaOH and subsequently sterilized at 121 °C for 15 min in an autoclave (FE-84 299 Felisa®, Mexico) at 1.5 $kgcm^{-2}$.
The cultures were incubated at a temperature of 22 ± 2 °C. After 22 days, the germination percentage of each treatment was evaluated with an LED light spectrum. At 3 weeks of culture, morphometric characteristics (length and thickness of stem and roots, number of leaves, number of roots, and length and width of leaves) of germinated seedlings were evaluated for each LED light treatment. The data were analyzed by ANOVA, followed by Tukey's test ($P < 0.05$). The germination percentage for each treatment, the LED light

spectrum, and viability are plotted graphically. In all cases, the STATGRAPHICS program, Centurion XIX, was used.

RESULTS AND DISCUSSIONS

A very limited number of *C. okeechobeensis* specimens (a total of 17) were found in the three sites studied, corresponding to a cloud forest ecosystem near the city of Xalapa, Veracruz. The presence of specimens of this wild species in these sites is consistent with what has been reported, given the actual and potential distribution of *Cucurbita* in Mexico (Rios-Santos et al., 2018). Likewise, it confirms the distribution indicated by several authors (Nee, 1993; Lira et al., 1995; Lira, 2001) that goes from sea level to 1500 meters above sea level, both on the banks of streams and in areas with primary or secondary vegetation (coffee plantations, cane fields, and other crops).
Regarding the viability with the TTZ method, we observed variations in the viability percentages of the seeds, finding values of 48, 53, and 75 % viability in the three study sites (Figure 2). The seeds evaluated in the "San Marcos" site (Xico) presented a viability of 75 percent, which contrasted with the 48 percent viability detected in the "Puerto Rico" site (Coatepec) (Figure 2).

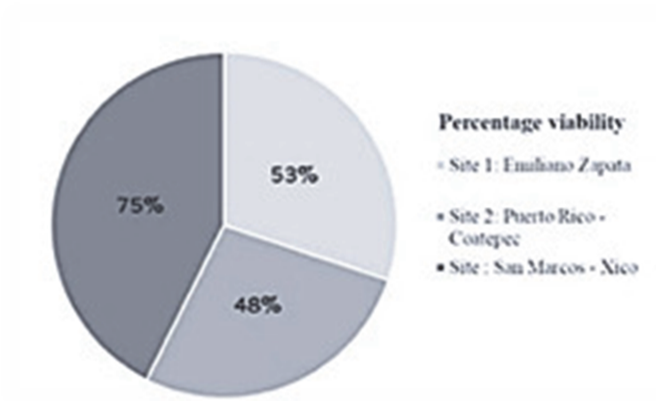

Figure 2. Variation in the TTZ viability of *C. okeechobeensis* seeds from three study sites.

The variation in TTZ staining observed in the seeds of the specimens evaluated, could be due to the presence of increased degradation of cell membranes by lipid peroxidation and non-enzymatic peroxidation, which could have contributed to the degradation of seed viability (Hidayat and Ridhawati, 2020) observed in the seeds of the specimens from the "E. Zapata" and "Coatepec" sites. Specimens from the San Marcos-Xico Road showed the highest percentage of seed viability. Previous studies have indicated that seeds from this population also present larger sizes, so it is possible that the higher viability of seeds from this site is due to this, considering that Steiner *et al.* (2019) indicated that they could contain a higher content of starch and other energy reserves necessary for germination and survival.
The variability of this study agrees with Eguiarte *et al.* (2018) regarding the fact that domesticated *Cucurbita* species found in Mexico present high genetic variation in their populations. Similarly, an effect of the different LED light spectra evaluated on seed germination *in vitro* was observed. The highest germination percentage was obtained under the red-light spectrum (Figure 3). The specimens from the population of San Marcos-Xico Road showed the highest percentage of seed viability. Previous studies have indicated that specimens from this population also have larger seed sizes. It is possible that the higher viability of seeds from this site is due to this characteristic, considering

what Steiner *et al.* (2019) indicated: larger seeds could contain a higher content of starch and other energy reserves necessary for germination and survival. The variability of this study agrees with Eguiarte *et al.* (2018) regarding the fact that domesticated *Cucurbita* species found in Mexico present high genetic variation in their populations. Similarly, an effect of the different LED light spectra evaluated on *in vitro* seed germination was observed. The highest germination percentage was obtained under the red-light LED spectrum (Figure 3).

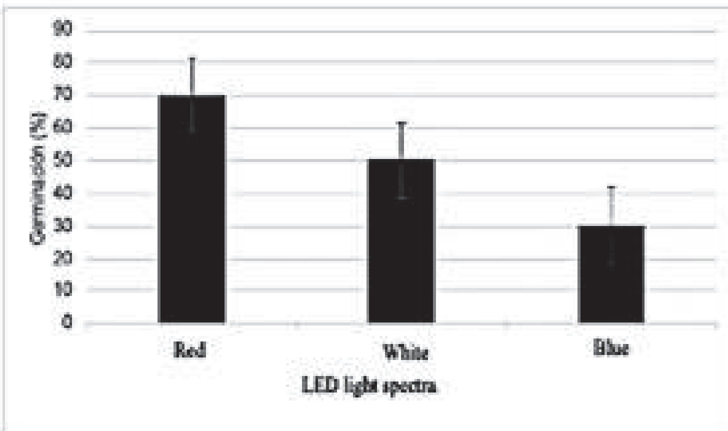

Figure 3. Effect of LED lights (red, white, and blue) on the percentage of *in vitro* germination of *C. okeechobeensis* seeds obtained after 22 days. Error bar: confidence interval

These results confirm the findings of Schmidt *et al*. (2020), who found that when *Cucurbitaceae* seeds were exposed to different LED spectral qualities, they showed differential responses in germination, growth, and development processes. Light induction of germination is known to be mediated by phytochrome B and other phytochromes, that sense the relationship between the red (600–700 nm) and far-red (700–800 nm) light spectra (Stutte, 2009). Seed germination is mainly induced by the action of monochromatic red light (RL) (Bae and Choi, 2008). In this regard, several authors have evidenced the stimulating effect of the red LED light spectrum on seed germination. Wang et al. (2021) reported that red LED light promoted the germination of *Momordica charantia* L. (bitter gourd) seeds, which maintained a high germination potential. This work also confirmed the higher growth shown by seedlings germinating under the red LED light spectrum (Table 2).

Table 2. Effect of LED light spectra on morphological characteristics of *in vitro* germinated *C. okeechobeensis* seedlings.

*LED light	LS (mm)	LH (mm)	ST (mm)	NL	LL (mm)	LW (mm)	NR	LR (mm)
W	20±7b	10±5b	5±1a	2–1b	15±7ab	10±5b	5-0b	40±9b
B	15±8d	6±1d	2±1d	2–1b	14±8b	7±4d	3–1c	20±4c
R	60±12a	50±8ª	5±2a	4–1a	14±7b	10±5b	6-0a	60±7a

** LED light : B: white; A: blue; R: red. LS: seedling length; LH: hypocotyl length; ST: stem thickness; NL: number of leaves; LL: leaf length; LW: leaf width; NR: number of roots; LR: length of the main root. Values represent the mean ± standard error. According to Tukey's test (P< 0.05), different letters show differences.*

The results obtained confirm the findings of Solano *et al.* (2020) on the positive effect of the red LED light spectrum, on plant germination, growth, and development. It is noteworthy that under the blue LED light spectrum, germinated seedlings showed lower growth compared to seedlings from seeds germinated under the red LED light treatment (Figure 4).

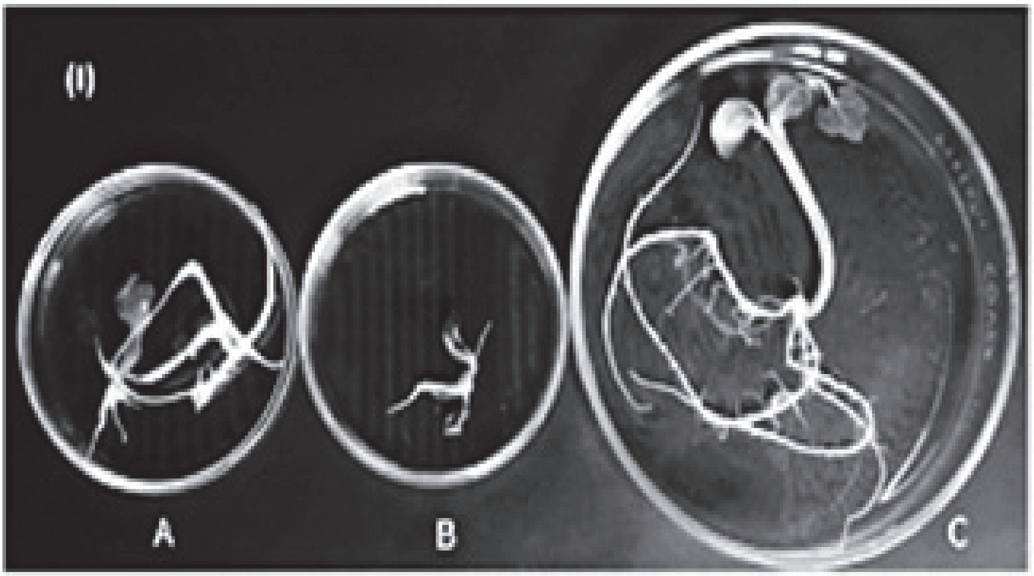

Figure 4. *In vitro* germination of *C. okeechobeensis* seeds at 14 days under different LED light spectra. A) white LED light; B) blue LED light; and C) red LED light

The results of the present work also confirm the usefulness of the application of LED lights, compared to white fluorescent lights, to promote not only germination but also the *in vitro* growth of various plant species (Xu *et al*., 2020; De Araujo *et al.,* 2022). Finally, these results could contribute to the development of future conservation work for this valuable genetic resource.

CONCLUSIONS

The presence of specimens of *C. okeechobeensis* was confirmed at three sites (Emiliano Zapata, Coatepec, and San Marcos-Xico) in the cloud forest near Xalapa, Veracruz, Mexico. Differences in seed viability were observed at the three sites evaluated. The highest seed viability was observed at the site located in San Marcos-Xico, Veracruz. It was found that the red LED light spectrum favorably influenced the germination and growth of germinated seedlings. The results obtained may contribute to increasing the chances of successful conservation of this valuable genetic resource.

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