VARIABILITY IN MORPHOLOGICAL CHARACTERISTICS, VIABILITY AND GERMINATION OF *Cucurbita okeechobeensis* subsp. martinezii.

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Abstract. *Cucurbita okeechobeensis martinezii* (L.H. Bailey) is an endangered wild species from Mexico. To contribute to its conservation, we conducted a search for specimens in three areas of the cloud forest in Veracruz, Mexico. A completely randomized design was established to evaluate the morphometric variation, the viability of the seeds from the different locations and the effects of three spectra of LED light on their germination *in vitro*, using Murashige and Skoog medium with 2 mgL^{-1} of gibberellic acid. (GA_3). Differences were detected in morphological and seed viability between the evaluated sites. The highest values of seed length and viability were observed at the San Marcos site. The positive effect of the red LED light spectrum on the germination percentage and the growth of germinated seedlings *in vitro* was confirmed. These results can contribute to the conservation and propagation of this valuable genetic resource.

Keywords: Conservation, wild species, Cucurbita, seeds, in vitro germination, LED lights.

INTRODUCTION

The genus *Cucurbita* has 12 to 15 species and approximately 20 taxa considered subspecies, most of them distributed 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, but *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. Also known as "calabacilla", "moriche", and "morchete", it is distributed from southern Tamaulipas, northern Oaxaca, and Chiapas, in addition to the states of San Luis Potosi, Querétaro, Puebla, and Veracruz, in humid sites near streams (Nee, 1993; Lira, 2001), and has been considered a weed in cultivated areas and by land use change, so it has been displaced from its natural areas. Today, this species is included in the Mexican Official Standard NOM-ECOL-059-2010 (SEMARNAT, 2010) as well as in the list of conservation concern (G_1, critically endangered); this taxon is also included in the United States endangered species list (USFWS, 2019). Hence the importance of taking actions to contribute to safeguarding the genetic diversity of this important endemic genetic resource of Mexico.

Like other wild cucurbits, it is more resistant to biotic and abiotic factors, which makes it a valuable species for the introgression of disease resistance genes or other abiotic factors into other edible pumpkin species (Khoury *et al.*, 2020). On the other hand, the fruits have been used as a substitute for soap, and in traditional medicine to cure dermatitis and burns, in the same way, infusions of the fruit are used for their properties as laxatives, emetic, antidiabetic, antioxidant, anti-inflammatory, and for the treatment of dysentery, among other conditions (Osuna, 2005). Although these applications have not been formally corroborated; It was recently published that extracts from the fruit of this species inhibit cell proliferation *in vitro* of human cancer cell lines (Morales-Vela *et al*., 2020).

However, despite its relevance, there is not enough information on this endemic species from Mexico. Therefore, it was proposed to carry out a search for this species in three sites in the cloud forest area, near the municipality of Xalapa, Veracruz. To evaluate the variation in the morphometry and viability of its seeds, and the effect of different LED light spectra on seeds germination *in vitro* of this species, which could contribute to safeguarding this valuable wild genetic resource of Mexico.

MATERIALS AND METHODS

During the months of October to January of 2021, we conducted surveys in the cloud forest area near the municipality of Xalapa, Veracruz since, as mentioned in the literature, 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 deciduous forest in Veracruz (Nee, 1993).

Three sites with specimens of this species were located between 1000 and 1500 m.a.s.l. altitude (CONABIO, 2016). The first site was in the area adjacent to the "Las Trancas-Coatepec" road, belonging to the municipality of "Emiliano Zapata" in Xalapa, Veracruz; the second was on the royal road to "Puerto Rico", municipality of Coatepec; and the third site, on the "San Marcos" road, Xico, Veracruz (Fig. 1).

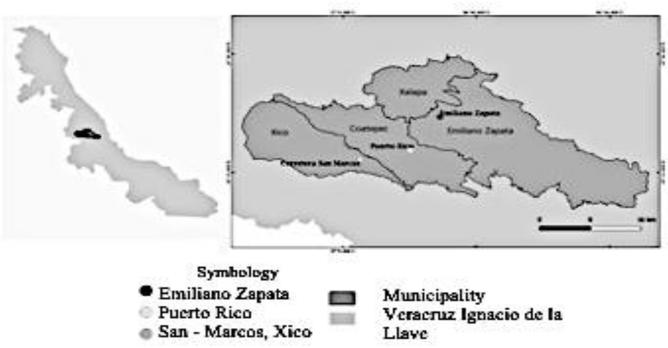

Fig. 1. Location of the sites of *C. okeechobeensis* were collected in Xalapa, Veracruz. Source: Badillo (2021)

A total of 17 fruiting specimens were collected (five at Site I, seven at Site II, and five at Site III), which were taken to the Institute of Biotechnology and Applied Ecology of the University of Veracruz to extract and evaluate their seeds. On this basis, one hundred seeds were randomly selected from the fruits collected for each site to evaluate the morphological characteristics (length, width, and thickness) of each of the seeds; on this basis, their length-to-width ratio was determined. Similarly, the viability of the seeds collected by each study site was evaluated according to the international seed evaluation rules (ISTA, 2014). For this purpose, the seed coat (Testa) was manually removed and 30 seeds from each site were transversely incised to facilitate embryo staining in a 1% solution of 2,3,5-triphenyl tetrazolium (TTZ). After 24 h of the staining process under dark conditions, the percentage of viability of the seeds was evaluated, based on the color intensity of the embryos. Seeds that showed at least 25% pink-red staining were considered viable, and those that did not show staining were considered non-viable. On this basis, the percentage of seed viability was determined for each study site by applying the formula proposed by Salazar-Mercado *et al.* (2020).

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

Effect of LED lights on *in vitro* seed germination Based on the results of seed viability, a total of 150 seeds were selected to evaluate their *in vitro* germination. The seeds were kept under constant agitation for 24 h in a solution containing 1 mgL^{-1} of gibberellic acid (GA_3). Subsequently, the seed coat was removed prior to disinfection for 15 min in a 15% solution of sodium hypochlorite (NaClO), containing two drops of Tween-20 (Sigma-Aldrich, St. Louis, Missouri, USA) and 10 drops of Microdyn, per 100 mL of sterile distilled water. The seeds were then rinsed three times with sterile distilled water.

A completely randomized design was used to evaluate the effects of three different LED light spectra, white (400-750 nm), red (700-800 nm) and blue (400-500 nm). For this purpose, 50% MS culture medium (Murashige and Skoog, 1962) 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 prepared. 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 $kgcm^{-2}$. 20 mL of medium was distributed in each culture flask to then proceed to the sowing of five seeds/flask and five flasks/treatment. The cultures were incubated, at a temperature of 22 ± 2 °C. After 22 days, the germination percentage was evaluated for each treatment of the LED light spectrum.

The morphological data of the variables: length, width, thickness, and width length ratio of seeds from each of the three sites under study, as well as the evaluated data (mm) of morphometric characteristics (length and thickness of stem and root, number of leaves, number of roots, and length and width of leaf) of germinated seedlings, for each LED light treatment, were analyzed by analysis of variance (ANOVA) of simple rank followed by a Tukey's test ($P \leq 0.05$). The germination percentage for each treatment, LED light spectrum, and viability are graphically plotted. The STATGRAPHICS program, Centurion XIX, was used in all cases.

RESULTS AND DISCUSSIONS

The presence of a limited number (17) of specimens of *C. okeechobeensis* was found in the three sites (five in Site I, seven in Site II, and five in Site III) surveyed in the cloud forest in Xalapa, Veracruz. The presence of specimens of this species in these sites is consistent with what was expected given the actual and potential distribution of this wild species of *Cucurbita* in Mexico (Rios-Santos *et al.*, 2018). Similarly, it confirms the indications of several authors (Nee, 1993; Lira *et al.*, 1995; Lira, 2001) that these can be found in Veracruz, distributed from sea level to 1500 m.a.s.l., both on the banks of streams and in areas with primary or secondary vegetation (coffee plantations, sugarcane plantations, and other crops). It is possible that the constant temperature changes experienced in recent years in the habitat of this species, mainly due to different anthropogenic activities (land-use change, greenhouse effect, erosion, and increased soil pollution) (ECLAC, 2020), could have influenced the presence of few fruiting specimens. Although it is not ruled out that this could also be due to problems with pollinators in the study habitat (Lira *et al.*, 2009).

Significant differences were detected for most of the morphological variables of the seeds evaluated in the three study sites (Table 1), as expected, considering that these are wild specimens. These results agree with Lira *et al.* (1995), who observed that the genus *Cucurbita* is characterized by the morphological variability of its seeds. It was interesting to note that site III (San Marcos-Xico) had the highest values for the variables: length and seed length/width ratio. It is possible that this is because, unlike the other two study sites, the specimens from this site developed in a shallow soil type, with a feozem haplandic texture, good drainage, and higher rainfall (between 2000 mm and 2500 mm) (Lira *et al.*, 1995), all of which could have contributed to a better absorption of nutrients and to the morphological differences observed in their seeds.

Table 1.

Variation in morphological characteristics of *C. okeechobeensis* seeds at the three study sites.

Site I: Emiliano Zapata- Xalapa	10.79 ± 0.06^{b}	5.46 ± 0.04^{c}	1.71 ± 0.02^{a}	1.98 ± 0.01^{a}
Site II: Puerto Rico- Coatepec	10.62 ± 0.06^{b}	5.92 ± 0.04^{a}	1.74 ± 0.02^{a}	1.80 ± 0.01^{b}
Site III: San Macos -Xico	11.15 ± 0.06^{a}	5.76 ± 0.04^{b}	1.72 ± 0.02^{a}	1.94 ± 0.01^{a}
Values represent the mean ± standard error. Different letters present statistical differences according to Tukey's test (P ≤ 0.05).				

On the other hand, the existence of variation in seed viability percentages was observed. Values between 48 and 75% viability were found in the three study sites (Fig. 2). The seeds evaluated at the site of "San Marcos" (Xico) showed a 75 percent viability, which contrasted with the 48 percent viability detected at the site of "Puerto Rico" (Coatepec) (Fig. 2).

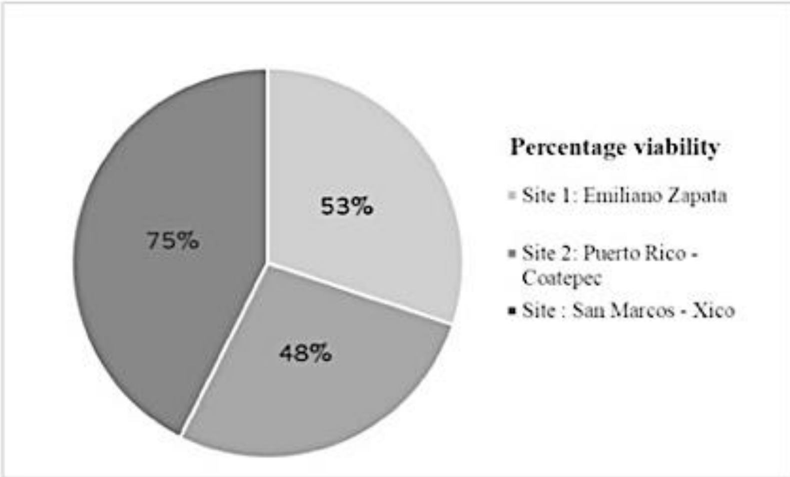

Fig. 2. Variation in the percentage viability of *C. okeechobeensis* seeds from three study sites evaluated by the TTZ method

The variation in staining observed in the seeds originates from the interaction of live cells with the TTZ chloride solution. Thus, the release of hydrogen ions from the dehydrogenase enzymes produces a red, stable, and non-diffusible substance called triphenyl formazan red in the living cells, which is a clear indicator of the respiratory activity of the mitochondria and of cell viability (ISTA, 2014). Thus, it was possible to detect a variation in seed viability across study sites. It is possible that the presence of a higher percentage of non-viable seeds detected in the "E. Zapata" and "Coatepec" sites could be indicative of the degradation of cell membranes by lipid peroxidation and non-enzymatic peroxidation, which are factors that contribute to the degradation of seed viability (Hidayat and Ridhawati, 2020).

The population of the site located on the "San Marcos"-Xico road showed the longest seed length and the highest percentage of viability. In this regard, it has been indicated that seed size is an important physical indicator of seed quality, which can affect germination and plant growth (Xu *et al.*, 2014). It is possible that this is due to a higher content of starch and other energy reserves needed for germination and survival (Steiner *et al.*, 2019). Regarding the variability in morphometric characteristics and viability of seeds, the observed in the evaluated sites agrees with what was expected considering what was indicated by Eguiarte *et al.* (2018), who mention that the domesticated species of *Cucurbita* distributed in Mexico have high genetic variation in their populations, like that of their wild relatives. 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 spectrum (Fig. 3).

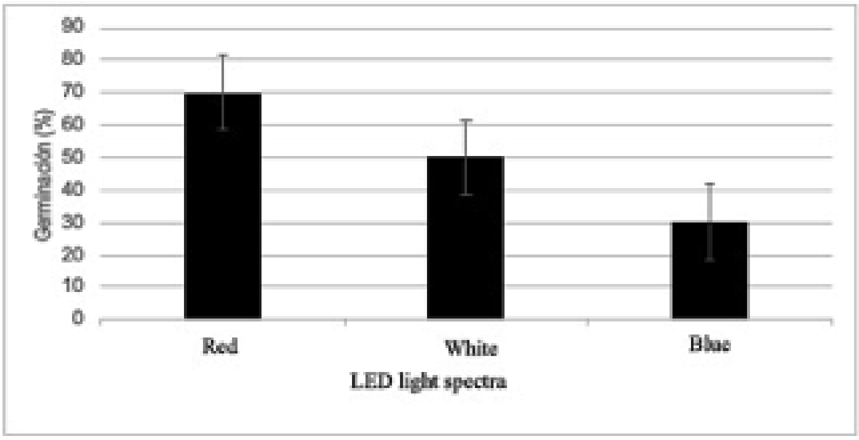

Fig. 3. Effect of LED lights (Red, White, and Blue) on the percentage of *in vitro* germination of *C. okeechobeensis* seeds obtained after 22 days. Error bar: confidence interval

These results corroborate what has been reported by other authors, who have indicated the stimulatory effect of the red LED light spectrum on seed germination. In this regard, Wang *et al*. (2021) reported that red light promoted the germination of *Momordica charantia* (bitter gourd) seeds and maintained a high germination potential. It is known that the phytochrome (photoreceptor) system promotes germination and in turn is sensitive to red light; it has also been reported that, for this type of seed, the low concentration of active phytochrome, due to exposure to periods of darkness or rapid exposure to red radiation, is sufficient to initiate the germination process (Marcos-Filho, 2015).

Similarly, the higher growth of germinated seedlings under the red LED light spectrum was confirmed in this work (Table 2). These results also confirm what was indicated by Lira *et al.* (2020) that the quality of light has a positive influence on the processes of germination, growth, and development of plants.

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

***LED light**							
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 main root. Values represent the mean ± standard error. Different letters present differences according to Tukey's test (P≤ 0.05).**

The results obtained also confirm the indications of Solano *et al*. (2020) on the positive effect of the red LED light spectrum on plant germination, growth, and development. It should be noted that under the blue light spectrum, germinated seedlings showed lower growth in general compared to seedlings from seeds germinated under the red LED light treatment (Fig. 4).

Fig. 4. *In vitro* germination of *C. okeechobeensis* seeds at 14 days under different LED light spectra. A) White LED light, B) Blue LED light, C) Red LED light

Finally, this work confirms the usefulness of the use of light-emitting diodes (LED) and the red-light spectrum in the *in vitro* germination and growth of germinated seedlings, which could be useful for the development of future conservation and sustainable use of this valuable genetic resource.

CONCLUSIONS

The presence of specimens of *C. okeechobeensis* was confirmed in three sites (Emiliano Zapata, Coatepec, and San Marcos-Xico) of the cloud forest near Xalapa, Veracruz, Mexico. Differences were observed in the morphological characteristics and viability of the seeds in the three sites evaluated, as well as a higher viability of the seeds in 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.

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