Colegio de Postgraduados

Effect of *Moringa oleifera* Lam, leaves in the *in vitro* germination of *Guarianthe aurantiaca* (Bateman) Dressler & W.E. Higgins

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ABSTRACT

Objective: To evaluate an alternative culture medium based on moringa (*Moringa oleifera*) leaves and enriched with growth regulators that could improve seed germination efficiency in a controlled environment. This alternative method was compared with conventional methods; in addition, the benefits of using nutrient-dense natural components for the species *G. aurantiaca* were analyzed.

Design/Methodology/Approach: Dehydrated moringa leaves were used to prepare the *in vitro* culture media. Their effect on the germination of *G. aurantiaca* was evaluated using a completely randomized experimental design. Meanwhile, an analysis of variance and Tukey's test were used to compare means. The variables included different moringa leaf (7, 10, and 15 g/L^{-1}) and growth regulator concentrations. The dependent variable was germination percentage.

Study Limitations/Implications: The availability of *G. aurantiaca* seeds was a limiting factor.

Results: After 47 days, the MS medium with GA_3 (0.1 mg/L^{-1}) achieved the highest germination rate (91%). Ninety-seven days after sowing, the moringa leaf-based medium, with a 10 g/L^{-1} concentration and enriched with GA_3 (0.1 mg/L^{-1}), achieved an 89% germination rate.

Findings/Conclusions: The culture medium enriched with moringa leaves is an efficient alternative for the *in vitro* germination of *G. aurantiaca*. These results established for the first time that the moringa-based culture medium is an effective alternative for the germination and conservation of this species.

Keywords: *Guarianthe aurantiaca*, micropropagation, organic extracts.

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INTRODUCTION

Orchids belong to one of the most diverse monocotyledon plant families: Orchidaceae. This family includes approximately 700-800 genera and a total of 35,000 species (Piña, 2020). Orchids are mainly distributed in warm and humid regions, but they cannot be found in deserts and cold areas. Mexico is a megadiverse country that stands out by its high percentage of endemic orchids (López and Rangel, 2018). There are about 1,260 orchid species and they are distributed in 170 genera. Mexico has 444 endemic species, which account for 40% of its orchid diversity (Tejeda Sartorius *et al.*, 2017; López and Rangel, 2018). The Mexican states with the highest orchid diversity are

Oaxaca, Chiapas, Michoacán, Guerrero, Puebla, Morelos, Jalisco, San Luis Potosí, and Veracruz (Morales, 2019).

Guarianthe aurantiaca is an epiphytic orchid that grows in warm weather (20-30 °C) and at 300-1,800 m.a.s.l. (Zaragoza, 2013). Although this species is not included in the NOM-059-SEMARNAT-2010, a decrease in its population has been recorded (Mondragón, 2009). As a result of the small size of its seeds and its dependance on mycorrhiza, it has a low natural germination rate (2-3%); consequently, the *in vitro* cultivation of plant tissue is a key tool for its conservation (Pérez-Martínez, 2016; Valdés-Infante *et al.*, 2012).

In recent years, protocols for the germination and growth of orchid seeds have been developed using organic extracts such as water and coconut milk (Gutiérrez-Zavala *et al.*, 2021), banana homogenate (Utami and Hariyanto, 2019), iron chelate (Bertolini *et al.*, 2014), pineapple juice and purée (Camilo, 2017), potato homogenate or potato dextrose agar (De Stefano *et al.*, 2022), and media with almond milk and tomato (Calevo *et al.*, 2020; Ng and Saleh, 2010). All these organic extracts are growth regulators that favor *in vitro* germination (De Stefano *et al.*, 2022).

Moringa (*Moringa oleifera*) is native to India and it has adapted to tropical climates. It is known for its high content of minerals (calcium, iron, potassium, and zinc) and essential vitamins (beta-carotene, folic acid, A, B12, and C) (Flores, 2021; Glover-Amengor *et al.*, 2016; Ndhlala and Tshabalala, 2023). Moringa has bioactive compounds, such as glycosides, isothiocyanates, glucosinolates, and flavonoids (apigenin) which provide it with antioxidant and anticancer properties (Patil *et al.*, 2022; Perveen *et al.*, 2023; Sreeja *et al.*, 2021). In addition, under controlled conditions, moringa leaf extract improves germination and reduces microbial contamination (Ndhlala and Tshabalala, 2023; Patil *et al.*, 2022). It also has phytohormones (such as auxin and cytokinin) that favor cell and root growth (Perveen *et al.*, 2023; Sreeja *et al.*, 2021). Meanwhile, its antioxidant and antimicrobial properties minimize oxidative stress (Glover-Amengor *et al.*, 2016; Flores, 2021). Therefore, the objective of this study was to use moringa as an *in vitro* culture medium to improve the germination and development of *G. aurantiaca*.

MATERIALS AND METHODS

Experiment location

This study was carried out in the Ciencias Químico-Biológicas lab of the Instituto Tecnológico Superior de Coalcomán (ITSC), located at 18° 47’ 27” N and 103° 10’ 31” W, Ciudad de Coalcomán de Vázquez Pallares, Michoacán, Mexico.

Plant material collection

The orchid seed capsules of *G. aurantiaca* were collected at 1,000 m.a.s.l, in the surroundings of the municipal seat of Coalcomán de Vázquez Pallares (18º 78’ 33” N and 103º 16’ 67” W). The capsules reached maturity at approximately five months. This information was established based on the monitoring of this species and the recording of its flowering and pollination stages, as well as the phenological characteristics of its fruits.

The pruning tool used to collect the leaves was previously disinfected with alcohol (70%). The leaves were washed with distilled water. Afterwards, the leaves were dehydrated at 60 °C for 48 hours. Finally, they were crushed in a mortar with a pestle.

Culture media

The *in vitro* culture media were MS (100%; Murashige and Skoog, 1962) and a moringa-based medium (7 g/L^{-1}, 10 g/L^{-1}, and 15 g/L^{-1}). All the media received 30 g L^{-1} of sucrose, 2 g L^{-1} of activated carbon, and growth regulators such as Bencylaminopurine (BAP; 0, 0.05 and 0.1 mg/L^{-1}) and gibberellic acid (GA_3; 0, 0.1 and 1. 0 mg/L^{-1}). The pH was adjusted to 5.75±1 with a 1N concentration of NaOH and NaCl. Figatel (2.5 g L^{-1}) was used as gelling agent. Subsequently, the media were steam sterilized, at 121 °C for 15 minutes in an autoclave.

Disinfection of orchid seed capsules

The capsules were washed under running water and immersed in a solution of 70% ethanol for 15 minutes. Subsequently, they were placed in a 20% NaOCl solution for 30 minutes. Afterwards, three washes with sterile distilled water were performed to remove the solutions. Finally, the capsules were flamed up. An inoculating needle was used to inoculate the seeds, spreading them in Petri dishes with culture media. The experimental units were kept at 28±2 °C, during a photoperiod of 16 h (light) and 8 h (darkness). Changes in the experimental units were monitored during the incubation period. In addition, the physiological changes of the seeds (germination and initial development of the seedlings) were recorded.

Evaluated variables

The dependent variable was quantitatively determined. The seeds per cm^2 were those in which the embryo emerged from the coat seed, causing the testa rupture, the protocorm formation, and the appearance of the first leaf primordia. The independent variables were 100% MS and the 7, 10, and 15 g/L^{-1} moringa media, enriched with BAP (0, 0.05, and 0.1 mg/L^{-1}) and three GA_3 (0, 0.1, and 1.0 mg/L^{-1}).

Experimental design and statistical analysis

The experimental design consisted of a randomized complete block design. Thirty-six treatments with a 4×3×4 three-way factorial design were conducted, with four repetitions each (36×4). The study factors included three BAP concentrations (0, 0.05, and 0.1 mg/L^{-1}) and three GA_3 concentrations (0, 0.1, and 1.0 mg/L^{-1}), resulting in a total of 9 treatments with the 100% MS and each of the moringa concentrations (7, 10, and 15 g/L^{-1}). The data were set forth as the mean ± the standard error of the mean (SEM). The dependent variable and the germination percentage were subjected to a one-way ANOVA, based on a general linear model (GLM). The Kolmogorov-Smirnov and the Levene tests were priorly used to verify the assumptions of normality and the homogeneity of variance, respectively. Welch's ANOVA was applied in heteroscedasticity cases to correct unequal variances and to guarantee the validity of the analysis. Using the Minitab 2021

and SPSS 2022 statistical software, treatments with significant differences ($P<0.05$) were subjected to a mean comparison test (Tukey's test with 95% reliability), in which $*P<0.05$ and $**P<0.001$ were statistically significant.

RESULTS AND DISCUSSION

In stage 0, *G. aurantiaca* seeds had not germinated yet, because they had been recently placed in the *in vitro* culture medium (Figure 1A). The seeds were still adapting to the new environment, but no germination signs were visible yet. In stage 1 (4 days after sowing), the volume of the seeds started to increase, due to water and nutrient absorption, a process known as imbibition (Figure 1B). In stage 2 (day 18), the embryo had swollen and the color of the seeds had clearly started to turn green —a sign of photosynthetic activity and embryo development (Figure 1C). This change is particularly noticeable in culture media supplemented with a 0.1 mg/L^{-1} concentration of gibberellic acid (GA_3). As the germination process unfolded, most of the seeds in stage 3 had developed an intense green color and the embryo was significantly thicker and longer. In this stage, known as protocorm development, seeds occupy more space and the testa begins to break, a clear sign that germination is underway, leading to the formation of the first leaf primordia (Figure 1D). This process ended approximately 47 days after the sowing and was followed by the development of the second leaf primordia (Figure 1E). Finally, the first seedlings started to develop (Figure 1F).

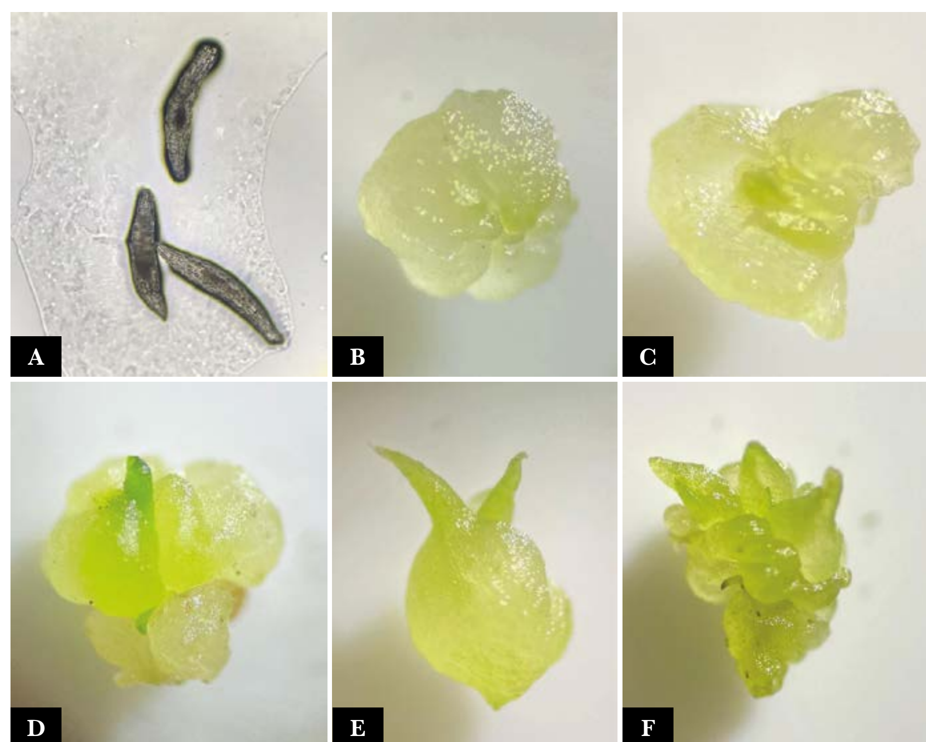

Figure 1. Germination of *G. aurantiaca*. A) Seeds with a viable embryo. B) Imbibition: water and nutrient absorption. C) First signs of green and protocorm development. D) Emergence of the second leaf primordia. F) Germinated seeds. Michoacán, Mexico (2024).

The data analyzed showed the dispersion and core tendency values of the germination variable (%). The ANOVA clearly shows that the germination percentage variable recorded significant differences ($p<0.05$) between treatments, with the MS (100%) treatment at 47 days and the moringa treatment (7, 10, and 15 g/L^{-1}) at 98 days (Table 1).

Tukey's multiple comparison test (95% reliability) confirmed that treatment 4 (0.1 mg/L^{-1} of MS+GA_3) is statistically different from the other treatments: it causes a better germination (91%) than the control (42.67%) in *G. aurantiaca* (Figure 2). A 7 g/L^{-1} concentration of moringa showed significant differences ($p<0.05$) with regard to the control (Table 1). Figure 2 shows significant responses (**) by T2 (0.05 mg/L^{-1} of BAP) and T4 (0.1 mg/L^{-1} of GA_3). T4 recorded an 80% germination, while control only achieved a 43% germination (Figure 2). Meanwhile, T5 (0.05 mg/L^{-1} of BAP) was also highly effective, with a 70% germination (Figure 2). Table 1 shows that similar results were obtained with the 10 mg/L^{-1} moringa concentration, recording highly significant differences (**), with a 95% reliability for the response variable ($p<0.05$) of T4 (0.1 mg/L^{-1} of GA_3). For its part, control recorded a 90% germination (Table 2), indicating a highly effective germination boost (Figure 2). T5 (0.05 mg/L^{-1} of BAP) was also effective, recording a 75% germination.

The interaction between factors resulted in significant statistical differences for the 15 g/L^{-1} moringa-based culture medium (Table 1). These results are significant (**) for the control group, T2 (0.05 BAP), T3 (0.1 BAP), T4 (0.1 GA_3), T5 (0.05 BAP and 0.1 GA_3) and 9 (0.1 BAP and 1.0 GA_3). T4 (0.1 GA_3) obtained an 86% germination, while T5 (0.05 mg/L^{-1} of BAP) recorded a 78% effectivity. Meanwhile, T3 (0.1 mg/L^{-1} of BAP) achieved a 50% germination and the combination of 0.05 mg/L^{-1} of BAP and 0.1 mg/L^{-1} of GA_3 resulted in 45% germination. These results suggest that 0.1 mg/L^{-1} of GA_3 and 0.05 mg/L^{-1} of BAP are the most favorable concentrations for the *in vitro* germination de *G. aurantiaca*, while higher GA_3 concentrations do not significantly improve the process.

All the treatments achieved the *in vitro* germination in a MS medium. Aung *et al.* (2022) reported similar results with the use of MS in orchids, because mature seeds of the *G. aurantiaca* species have reserves of nutrients and endogenous growth regulators that help germination (Santiago-Jerónimo *et al.*, 2024). Meanwhile, De Stefano *et al.* (2022) achieved a 90% germination at 98 days with moringa leaves, confirming their potential substitution with the micro and macroelements of MS salts. Therefore, they corroborated that the use of organic extracts improves the *in vitro* germination process, as a result of its content nutrients which are essential for the development of physiological processes (Ndhlala and Tshabalala, 2023). These elements include calcium (Ca), iron (Fe), potassium (K), and zinc (Zn), as well as vitamins, amino acids, sugars, and phenolic compounds

Table 1. Descriptive statistics results as a response to the germination percentage variable.

Concentration	Time Days	F	Sig.	Dev. Stand.	R^2 square	R^2 (adjusted)	R^2 (pred)
MS al 100%	47	21.743	0.000	31.510	86.56%	82.58%	76.11%
Moringa 7 g/L^{-1}	98	57.839	0.000	19.570	94.49%	92.85%	90.20%
Moringa 10 g/L^{-1}	98	82.183	0.000	16.567	96.06%	94.89%	92.99%
Moringa 15 g/L^{-1}	98	132.354	0.000	13.340	97.51%	96.78%	90.20%

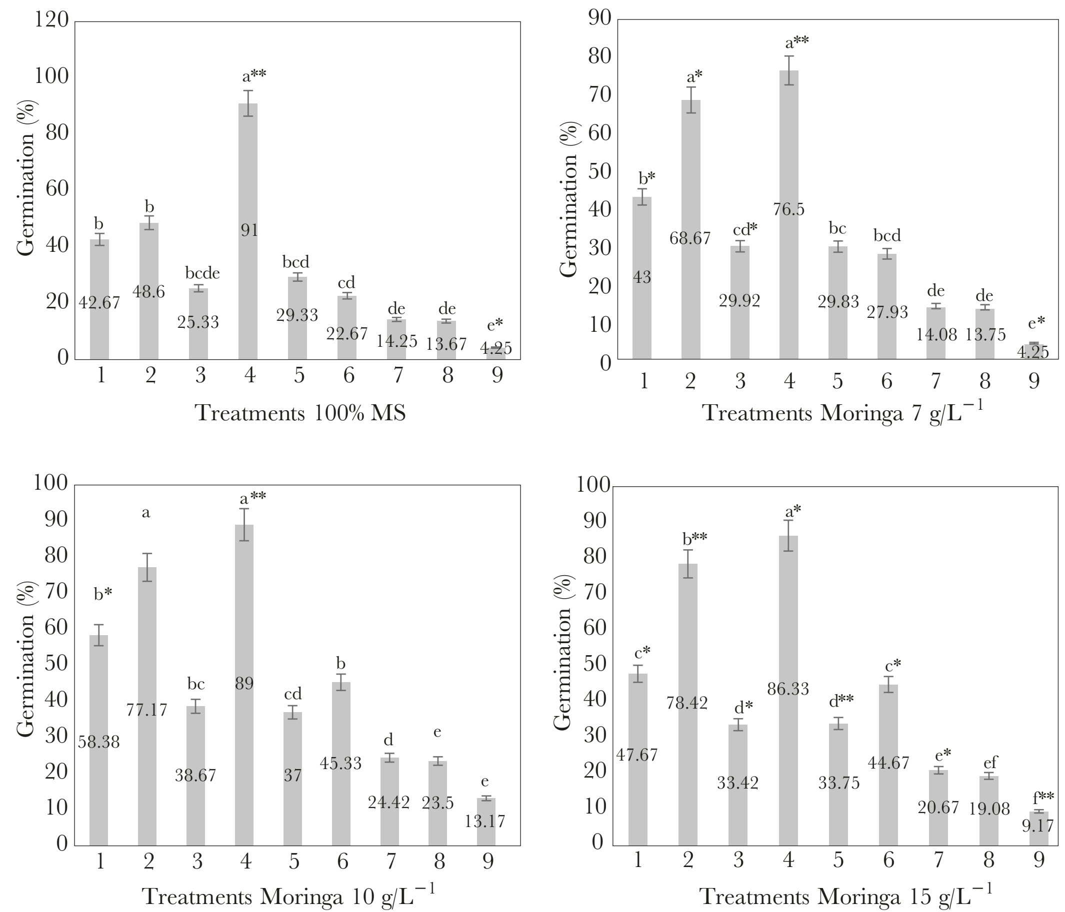

Figure 2. Effect of the MS culture medium and moringa leaves concentration (7, 10, and 14 g/L^{-1}) with various combinations of the BAP (0, 0.05, and 0.1 mg/L^{-1}) and GA_3 (0, 0.1, and 1.0 mg/L^{-1}) growth regulators, as a response to the *in vitro* germination behavior of *G. aurantiaca*. Means that do not share a letter have significant differences ($P<0.05$* and $P<0.001$**). Michoacán, Mexico (2024).

(Nunthanawanich *et al.*, 2016). These results match the findings of Álvarez (2021) who applied banana, pineapple, and coconut milk to *Vanilla planifolia* Jacks for 90 days. Choza *et al.* (2016) reported similar results with the combination of coconut milk, apple purée, and banana purée, which effectively helped the in vitro germination of the *Stanhopea tigrina* Bateman and *Cattleya* sp. orchids. Adding a mixture of tomato and banana pulp to the medium achieves a 70% germination in 30 days for *Catasetum integerrimum* and *Brassabola* sp. (Victoriano *et al.*, 2016). In a similar study, Salazar (2012) germinated *Cattleya mendelii* orchids in a coconut milk-enriched MS culture medium.

GA_3 (1.0 mg/L) recorded the highest percentage with MS (91%) and a 10 g/L^{-1} concentration of moringa leaves (89%). Recent research also supports these findings, concluding that moderate concentrations of GA_3 are particularly effective with several plant species (Salazar-Mercado, 2012).

Meanwhile, the effectiveness of 0.05 mg/L^{-1} of BAP with MS (48%) and moringa (78%) could be attributed to its capacity to stimulate cell proliferation and tissue differentiation in plants. As a cytoquinine, BAP promotes cell division and shoot development, which

are essential for germination and the initial growth of seedlings (Ariza *et al.*, 2018). In this study, these synergic effects explain the high germination percentages recorded by moderate BAP concentrations.

However, treatments that combined BAP and GA_3 particularly with the highest GA_3 concentration (1.0 mg/L^{-1}) recorded lower germination percentages, perhaps as a consequence of antagonistic effects between high concentrations of these growth regulators. Prior studies have proved that excessive regulator concentrations can cause phytotoxicity, inhibiting seed development and resulting in lower germination (Davies, 2010). The presence of phytoxicity in these treatments could have interfered with the normal physiological processes of the seeds, consequently reducing germination.

Likewise, the combination of 0.1 mg/L^{-1} of BAP and 1.0 mg/L^{-1} of GA_3 were less effective and only recorded a 9% germination. These unexpected results could indicate that these specific concentrations created an unfavorable environment for the seeds: perhaps seed metabolism was inhibited or the enzymes required for germination could not be activated.

CONCLUSIONS

A culture enriched with moringa and the BAP and GA_3 growth regulators was an efficient medium for the in vitro germination of *G. aurantiaca*. The application of 0.1 mg/L^{-1} of GA_3 and 0.05 mg/L^{-1} of BAP in both media was the most effective way to boost species germination, achieving 90 and 80% germination percentages, respectively.

These findings contribute to the understanding of the physiological elements involved in the germination of *G. aurantiaca*, proving that the application of specific concentrations of BAP y GA_3 can optimize the *in vitro* germination process.

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