Ornamental Horticulture
ISSN 2447-536X | WWW.ORNAMENTALHORTICULTURE.COM.BR
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

ARTICLE

# Effect of different pollination schemes on the fruit morphometric characteristic of *Vanilla planifolia* Jacks.

Efeito de diferentes esquemas de polinização nas características morfométricas dos frutos de *Vanilla planifolia* Jacks.

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**Abstract**: *Vanilla planifolia* Jacks. is an orchid of great economic interest, classified as endangered. The limited genetic variation of this species has been primarily attributed to its clonal propagation. Genetic improvement through pollination schemes allows for improving the characteristics of fruits, seeds, and seedlings, increasing resistance to biotic and abiotic stress. Therefore, it was proposed to develop the present work to evaluate the effect of three pollination schemes at different times on the morphometric characteristics of the fruit. 65 flowers in the anthesis phase were randomly selected and subjected to the following treatments: 1) natural pollination with emasculated flowers, 2) manual pollination by autogamy, and 3) geitonogamy, in a vanilla plantation located in Mesa de Guadalupe, Veracruz, Mexico. The percentage of set and retained fruits, growth dynamics (length and diameter), weight, and shape index of the fruits were evaluated for each pollination scheme and on different days after pollination (DAP). Fruit formation by natural pollination was not observed, which shows the absence of apomixis and fertilization by any pollinating agent in this vanilla plantation. No differences were observed in the set and retention of fruits obtained by autogamy, and geitonogamy. The growth dynamics show that the fruits reach their maximum length at 40 DAP for both pollination schemes. Greater length, shape index, and weight were observed in the fruits obtained by geitonogamy at 40 DAP. These results show that inbreeding has no apparent effects on capsule production in *V. planifolia* but does influence its morphometric characteristics.
**Keywords**: autogamy, geitonogamy, mating system, Orchidaceae, self-pollination.

**Resumo**: *Vanilla planifolia* Jacks. é uma orquídea de grande interesse econômico, classificada como ameaçada de extinção. A limitada variação genética desta espécie foi atribuída principalmente à sua propagação clonal. O melhoramento genético por meio de esquemas de polinização permite melhorar as características de frutos, sementes e mudas, aumentando a resistência ao estresse biótico e abiótico. Assim, foi proposto que o presente trabalho fosse desenvolvido para avaliar o efeito de três esquemas de polinização diferentes momentos nas características morfométricas do fruto. Foram selecionadas aleatoriamente 65 flores em fase de antese e submetidas aos seguintes tratamentos: 1) polinização natural com flores emasculadas, 2) polinização manual por autogamia, e 3) geitonogamia, em plantação de baunilha localizada em Mesa de Guadalupe, Veracruz, México. A percentagem de frutos fixados e retidos, a dinâmica de crescimento (comprimento e diâmetro), o peso e o índice de forma dos frutos foram avaliados para cada esquema de polinização e em diferentes dias após a polinização (DAP). Não foi observada a formação de frutos por polinização natural, o que mostra a ausência de apomixia e de fertilização por qualquer agente polinizador nesta plantação de baunilha. Não foram observadas diferenças no pegamento e na retenção dos frutos obtidos por autogamia e geitonogamia. A dinâmica de crescimento mostra que os frutos atingem seu comprimento máximo aos 40 DAP para ambos os esquemas de polinização. Maior comprimento, índice de forma e peso foram observados nos frutos obtidos por geitonogamia aos 40 DAP. Estes resultados mostram que a endogamia não tem efeitos aparentes na produção de cápsulas em *V. planifolia*, mas influencia as suas características morfométricas.
**Palavras-chave**: autogamia, autopolinização, geitonogamia, Orchidaceae, sistema de acasalamento.

## Introduction

Vanilla (*Vanilla planifolia* Jacks.), a member of the Orchidaceae family, is cultivated to obtain vanilla extract from its fruits, which is characterized by its flavor and fragrance derived from vanillin (4-hydroxy-3-methoxybenzaldehyde) (Arya et al., 2021). This compound has significant international economic value and is widely used in the food, cosmetic, pharmaceutical, and perfumery industries (Herrera-Cabrera et al., 2022).

One of the main reasons for the high demand for *V. planifolia* is due to its fruits having the most complete aromatic profile compared to other commercially available species (Peña-Barrientos et al., 2023). However, the species faces challenges in its sexual propagation due to low rates of pollination and seed germination (Hernández-Apolinar et al., 2016). As a result, asexual propagation through cuttings has become the preferer method, leading to a decrease in genetic variability and increased susceptibility to biotic and abiotic factors (Bautista-Aguilar et al., 2021). These factors have contributed to the species being classified as endangered (EN) by the International Union for Conservation of Nature (IUCN) (Vega et al., 2017).

*V. planifolia* is a plant with hermaphrodite flowers and an allogamous reproduction system. Studies have been conducted on the pollination ecology of this species, and it has been reported that the bee *Melipona beecheii* Bennet is a pollinator (Dressler, 1981). However, there is currently no documented evidence of efficient pollination by Melipona bees for orchids (Lubinsky et al., 2006). It is worth noting that *V. planifolia* is rarely visited by any insect species, and recent research has shown that only *Eulaema* spp. and *Euglossa* spp. are involved in its pollination (Hernández-Apolinar et al., 2016).

Dressler (1981) has suggested that the excessive use of agrochemicals has led to a loss of interaction between these organisms and vanilla, resulting in a loss of its pollinator. Consequently, vanilla plantations rely on manual self-pollination to produce fruits. However, this method results in low percentages of *in vitro* germination (<10%) (Yeh et al., 2021). Inbreeding depression can lead to reduced seed quality, viability, and germination (Ross and Travers, 2015). The absence of endosperm in the seeds, along with their black, hard, and crusty testa, further inhibits germination (Yeh et al., 2021).

The genotypes of pollen have a significant impact on the development of the embryo and endosperm, as well as the physical characteristics of fruits (Saini et al., 2022). It is well-established that the origin of pollen affects fruit set rates, maturity, size, weight, shape, diameter, and composition (Deng et al., 2022), as well as the number of seeds produced (Hashemabadi et al., 2023).

Geitonogamy refers to the fertilization of ovules with pollen from a flower on the same plant or a genetically identical clone (Fattorini and Glover, 2020; Saini et al., 2022). This type of pollination results in greater genetic diversity compared to asexual reproduction (such as vegetative reproduction and apomixis) due to the potential for allele recombination during meiosis (Fattorini and Glover, 2020; Kundu and Karmakar, 2022).

**Corresponding author:** liglesias@uv.mx | https://doi.org/10.1590/2447-536X.v31.e312822 | Editor: Michele Carla Nadal, Universidad Viña del Mar, Chile | Received: Sep 30, 2024 | Accepted: May 26, 2025 | Available online: June 30, 2025 | 

The effects of geitonogamy can have a significant impact on the size and shape of fruits, seed quality, germination capacity, and the expression of desirable traits (Saini et al., 2022).

Understanding how different pollination types affect fruit development and quality can greatly enhance production efficiency of *V. planifolia*. Likewise, studying reproductive strategies through various pollination schemes is crucial for developing genetic improvement programs. The objective of this study was to evaluate the effects of different pollination schemes (natural pollination, autogamy, and geitonogamy) on the morphometric characteristics of the fruits in *V. planifolia*.

## Materials and methods

### Study site

The study was conducted in a vanilla plantation located in "Mesa de Guadalupe", Veracruz, Mexico. The coordinates of the site are 19.566607 latitude, 96.698592 longitude, and 841 meters above sea level. The plantation covers an area of 242 $m^2$ (11 x 22 m) and consists of 200 *V. planifolia* plants, spaced 1.5 m apart in rows and 0.5 m between plants. The crop was established under a 50% shade mesh, using mulatto sticks (*Bursera simaruba* L.) as stakes.

### Pollination treatments

Three pollination schemes were carried out: 1) natural pollination, where the pollinia were manually removed from the flowers (emasculation); 2) autogamy, where the pollinia were placed on the stigma of the same flower; and 3) geitonogamy, where the pollinia were transferred to the stigma of another flower of a clone plant. For each treatment, 65 flowers in the anthesis phase were randomly selected from 30 plants (Fig. 1A). The pollination process was carried out manually (Fig. 1B), and the flowers in the autogamy and geitonogamy treatments were covered with tulle bags. Pollination took place in March during the early hours of the day (06:00 a.m.).

### Evaluations

After 7 days after pollination (DAP), the percentage of fruits set by each pollination treatment was determined. Subsequently, at 35 DAP, the percentage of retained fruits was determined. The growth dynamics of the fruits in terms of length and diameter were evaluated on 55 fruits at 7, 21, 35, 40, and 45 DAP for each pollination scheme. The shape index (length/diameter) was determined using the data collected at 40 DAP. Additionally, eight fruits were randomly collected at 40 DAP for each pollination scheme and transferred to the laboratory to evaluate their weight.

### Statistical analysis

A Chi-square test was performed to determine the differences between pollination schemes in the percentage of fruit set and retained fruit. Maximum fruit growth, length, and diameter were determined for each pollination scheme with a one-way analysis of variance (ANOVA) followed by a Duncan's test ($p \leq 0.05$). To compare fruit length, diameter, shape index, and weight variables between autogamy and geitonogamy at 40 DAP, Student t-tests were performed ($p \leq 0.05$). These statistical analyses were performed with Statgraphics Centurion (version 19, for Windows), and SigmaPlot (version 12, for Windows) software.

## Results

After 7 DAP, it was observed that the ovaries of the flowers from the natural pollination scheme did not increase in length or diameter (Fig. 1C). Subsequently, the tissue became necrotic, and at 21 DAP, it completely detached from the inflorescence. This indicates that neither an apomixis event nor fertilization by any pollinating agent occurred in this vanilla plantation. No significant differences were observed ($X^2 = 0$; G.L. = 1; $p$ = 1) in the percentage of fruits set (Fig. 1D) by autogamy and geitonogamy. At 35 DAP, premature fruit drop was observed in both autogamy and geitonogamy crosses, but there were no statistically significant differences ($X^2 = 1.878$; G = 1; $p$ = 0.1705) (Table 1).


**Fig. 1.** Development of pollination schemes in *V. planifolia*. A) flowers in anthesis, B) manual pollination, C) unfertilized ovaries, and D) fruit formation at 40 Days After Pollination (DAP).

**Table 1.** Relationship of the percentage of set fruits and retained fruits of *V. planifolia* under different pollination schemes.

| Treatments | Set fruits (%) | Retained fruits (%) |
|---|---|---|
| Natural pollination | 0 | 0 |
| Autogamy | 92.30 a | 98.33 a |
| Geitonogamy | 92.30 a | 93.33 a |

The Chi-square test was performed for all variables. Different letters represent statistically significant differences.

The growth dynamics, specifically the length of the fruits, showed a significant increase for both autogamy and geitonogamy to 40 DAP (Fig. 2), with average lengths of 16.40 and 17.38 cm, respectively (Fig. 3).

Furthermore, it was observed that the fruit diameter increased over time, reaching an average of 1.06 cm and 1.04 cm for autogamy and geitonogamy, respectively, at 40 DAP (Fig. 4).

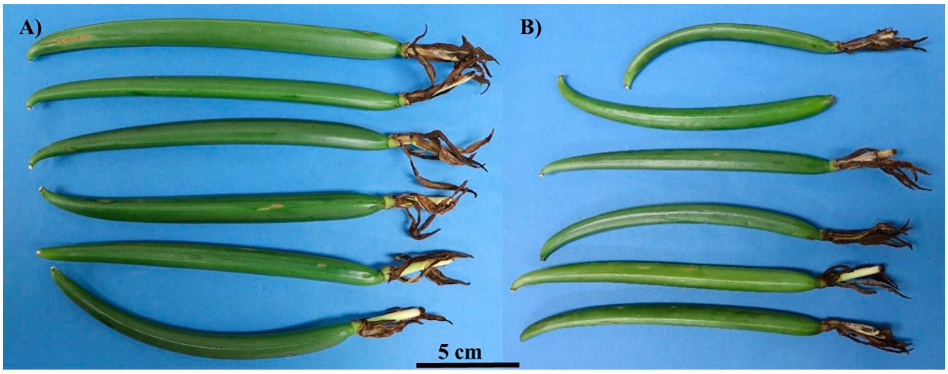


**Fig. 2.** Fruits obtained 40 days after pollination (DAP), A) geitonogamy and B) autogamy.

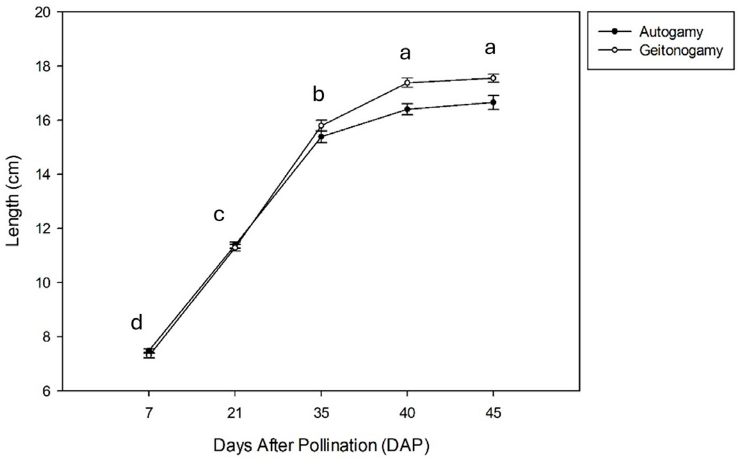


**Fig. 3.** Dynamics of growth in the fruit length of *V. planifolia* under different pollination schemes. Different letters represent statistically significant differences (Duncan, $p \leq 0.05$). The data represents the mean values and the bars the standard error.

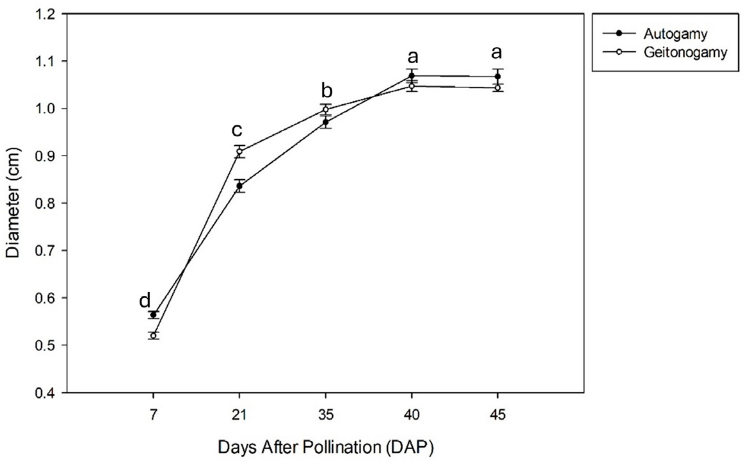


**Fig. 4.** Dynamics of fruit diameter growth in *V. planifolia* under different pollination schemes. Different letters represent statistically significant differences (Duncan, $p \leq 0.05$). The data represents the mean values and the bars the standard error.

When comparing the length, diameter, shape index, and weight of the fruits obtained by autogamy and geitonogamy at 40 DAP using the student's t-test, significant differences were found in the length, shape index, and weight, with higher values obtained by geitonogamy. For diameter, no significant differences were found (Fig. 5).

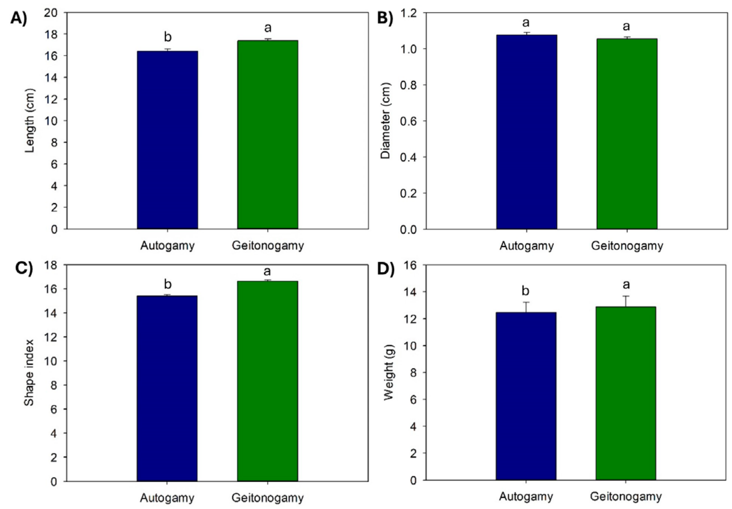


**Fig. 5.** Effects of autogamy and geitonogamy on fruit morphology. A) length, B) diameter, C) shape index (length/diameter), and D) weight of *V. planifolia* fruits collected at 40 Days After Pollination (DAP). A student's t-test was performed, and significant differences ($p > 0.05$) were observed in length, shape index, and weight; no differences were found in the diameter of the fruits.

## Discussion

*V. planifolia,* like many orchids, is a self-compatible species (Andriamihaja et al., 2020). However, pollination without the assistance of pollinators is challenging due to the presence of the rostellum, a floral structure that creates a physical barrier between the pollinia and the stigma, preventing their direct contact. As a result, both self-pollination and natural cross-pollination require a pollinating agent to transfer the pollinia to the stigma (Johnson and Edwards, 2000). In its native habitat, specific pollinators perform this important function. However, in areas where *V. planifolia* is commercially cultivated, far from its natural range, these native pollinators are often absent. This makes natural pollination impossible and necessitates human intervention to ensure the species' reproduction (Pemberton et al., 2023).

In this study, no fruits were recorded as a result of natural pollination, which may indicate a decline in pollinator populations, as previously suggested by Dressler (1981). However, current scientific evidence does not definitively support this hypothesis (Lubinsky et al., 2006). Recent research has aimed to identify potential pollinators, with Pemberton et al. (2023) highlighting *Euglossa dilemma*, *E. tridentata*, and *Apis mellifera* as possible candidates. This finding opens new avenues for exploring the reproductive ecology of *V. planifolia.*

No fruit formation was observed without manual or natural pollination, indicating that reproduction through apomixis did not take place. This finding aligns with reports from other regions where vanilla is produced, where asexual reproduction has similarly not been documented (Pemberton et al., 2023). Apomixis has been proposed as an alternative reproductive strategy for species with high levels of polyploidization that are subjected to elevated stress conditions (Kaushal et al., 2018), such as *V. planifolia* (Borbolla-Pérez et al., 2017). However, the occurrence of this phenomenon is rare, which makes it challenging to evaluate (Reutemann et al., 2022).

In this study, there were no significant differences in the percentage of fruit set between autogamy and geitonogamy. Fruit set is influenced by various pollination factors, including the quantity, quality, and viability of pollen (Abbate et al., 2023), as well as the source of pollen and successful fertilization (Deng et al., 2022). Self-pollination can also result in poor fruit set due to gametophytic incompatibility (Deng et al., 2022); however, the *Vanilla* genus has been observed to have high self-compatibility (Andriamihaja et al., 2020). Generally, cross-pollination is known to increase fruit set (Deng et al., 2022). However, in several orchid species it has been reported that self-pollination by autogamy does not affect fruit set (Capó et al., 2022), results like those obtained in this work.

It has been noted that *V. planifolia* exhibits premature fruit abortion, with losses of up to 40% of the fruits (Borbolla-Pérez et al., 2017). In this study, we found that at 35 DAP, there were low percentages of premature fruit drop in both autogamy (1.67%) and geitonogamy (6.67%) pollination schemes. This phenomenon has been linked to various abiotic, biotic, metabolic, agronomic, and reproductive factors, including changes in temperature and low precipitation during fruit development (Borbolla-Pérez et al., 2017). Previous research has also suggested a correlation between low genetic diversity and premature fruit abortion in xenogamies plants like *V. planifolia*, as self-fertilization can result in the expression of deleterious recessive alleles during early seed development (Faast et al., 2011). However, our results do not support this hypothesis.

Yeh et al. (2021) reports that *V. planifolia* fruits typically reach their maximum length at 35 DAP. Additionally, our observations demonstrated that the fruits at 40 DAP reached their maximum length. However, while the diameter of the fruits can continue to increase up to 49 DAP (Yeh et al., 2021), our study found that the maximum diameter was reached at 40 DAP. Furthermore, significant differences were observed in the length (16.40 and 17.38 cm for autogamy and geitonogamy, respectively), shape index (15.38 and 16.62 for autogamy and geitonogamy, respectively), and weight (12.46 and 12.87 g for autogamy and geitonogamy, respectively) of the fruits obtained at 40 DAP in the different pollination schemes. These findings suggest that the source of pollen has an impact on fruit characteristics. Barreda-Castillo et al. (2023) discovered noteworthy variations in the length, diameter, weight, and shape index of *V. planifolia* fruits resulting from both self-pollination and inter- and intraspecific crosses with *V. pompona*, providing further evidence in support of this theory.

According to Abbate et al. (2023), there is a correlation between the production of smaller capsules and a decrease in the number of fertilized ovules and, therefore, in the number of seeds produced. In orchids, pollen aggregates to form two pollinia, with one pollinia often being enough to fertilize all the ovules in a flower (Johnson and Edwards, 2000). When pollinating vanilla, pollinia are transferred as a single unit, providing a consistent amount of pollen grains (Faast et al., 2011), so similar characteristics would be expected in all fruits. However, self-pollination and mating between closely related species have been found to increase the risk of inbreeding depression, which can lead to a reduction in seed number, viability, and germination capacity (Faast et al., 2011). In contrast, Travers et al. (2018) found that in the orchid *Platanthera praeclara* Sheviak & Bowles, autogamy (self-pollination) did not affect fruit size compared to allogamy (cross-pollination). However, they did observe that only 18% of the seeds obtained through autogamy were viable, compared to 92% viability in seeds obtained through cross-pollination. It is possible that other factors, such as resource availability and habitat environmental characteristics, may also play a role in seed production and viability (Faast et al., 2011). Further research is needed to fully understand this aspect.

On the other hand, mating systems have a significant impact on the levels of diversity and genetic structure of populations, which are important factors to consider in conservation efforts (Faast et al., 2011). Cross-pollination is the primary source of genetic variation in offspring, while self-pollination results in greater variation compared to asexual reproduction (such as vegetative reproduction and apomixis) due to the potential for recombination of alleles during meiosis (Fattorini and Glover, 2020). However, self-pollination still results in less variation than cross-pollination (Kundu and Karmakar, 2022). Negative effects of self-pollination by geitonogamy have been reported, but these effects depend on the directionality of pollination (within inflorescences, between inflorescences, and between clones) (Eckert, 2000) and the degree of clonality (Hu et al., 2015). In contrast, geitonogamy has been shown to increase fruit set and seed germination compared to autogamy and even xenogamy (Johnson et al., 2009).

As is well known, the prevailing opinion is that clonality reduces genetic diversity (Franklin et al., 2021). However, other authors have suggested that clonal populations may have comparable or even greater genetic variation than non-clonal ones (Ellestad et al., 2022). Recent studies have even reported higher levels of heterozygosity in cultivated *V. planifolia* compared to wild specimens (Favre et al., 2022). This increased heterogeneity in *V. planifolia* plantations can be attributed to various factors, such as point mutations (Favre et al., 2022), hybridization, polyploidy (Ellestad et al., 2022), and a high level of epigenetic variation that may have developed in response to adverse environmental conditions (Rajpal et al., 2022).

Both genetic variation and epigenetic regulation can be inherited through meiosis and/or mitosis, although they are not directly related (Rajpal et al., 2022). This indicates that self-pollination via geitonogamy may offer advantages over autogamy by producing fruits and seeds with superior traits due to potential genetic and epigenetic variations present in clonal plants. Furthermore, this type of pollination may enhance fruit set, improve fruit retention, and increase fertilization efficiency. The data obtained in this study shows that *V. planifolia* can produce fruits under different pollination methods, provided that the pollination is done manually. It was observed that fruits reach their maximum length and diameter 40 days after pollination, and the pollination method significantly influences the morphometric characteristics of the fruit, including weight, length, and shape index.

## Conclusions

The results of this study demonstrate that the pollen source has a significant impact on certain morphological characteristics of vanilla fruits, specifically their length, shape index, and weight. Notably, fruits produced through geitonogamy pollination exhibited better morphometric traits compared to those resulting from autogamy. This enhancement indicates improved seed viability and germination capacity, marking a significant advancement in the sexual propagation of this species. These findings present new opportunities for the strategic use of pollination methods in agriculture and for genetic improvement programs focused on *V. planifolia*.

**Acknowledgments**

The authors would like to express their gratitude to the Institute of Biotechnology and Applied Ecology of Veracruz University for their support in making this study possible. We would especially like to thank the Secretariat of Science, Humanities, Technology, and Innovation (SECIHTI, Mexico) for financing the master's program of ZEFV and BGCR.

**Author Contribution**

**ZEFV**: Methodology, Conceptualization, Investigation, Writing - Original Draft. **BGCR**: Methodology, Investigation. **LGIA**: Conceptualization, Supervision, Writing - Review & Editing.

**Conflict of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data Availability Statement**

Data will be made available upon request to the authors.

**Declaration of generative AI and AI-assisted technologies in the writing process**

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

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