

EFFECT OF POLLEN SOURCE ON GERMINATION OF *VANILLA PLANIFOLIA*, *V. POMPONA* (ORCHIDACEAE), AND THEIR INTERSPECIFIC HYBRIDS

¹ JOSÉ MARTÍN BARREDA-CASTILLO¹, ² EMERSON RICARDO PANSARIN², ¹ JUAN L. MONRIBOT-VILLANUEVA¹,
³ MIGUEL ÁNGEL LOZANO-RODRÍGUEZ³, ^{1*} JOSÉ A. GUERRERO-ANALCO^{1*}, AND ⁴ REBECA ALICIA MENCHACA-GARCÍA^{4*}

¹ Red de Estudios Moleculares Avanzados, Instituto de Ecología (INECOL) A.C., Xalapa, Veracruz, Mexico.

² Departamento de Biología, Faculdade de Filosofia, Ciências e Letras, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil.

³ Facultad de Ciencias Agrícolas, Universidad Veracruzana, Xalapa, Veracruz, Mexico.

⁴ Centro de Investigaciones Tropicales (CITRO), Universidad Veracruzana, Xalapa, Veracruz, Mexico.

*Corresponding author: joseantonio.guerrero@inecol.mx; rmenchaca@uv.mx

Abstract

Background: *Vanilla planifolia* and *V. pompona* exhibit low seed germination rates. Although their fruits are obtained through manual self-pollination, the effect of intra- and interspecific cross-pollination on their fruits and seeds remains unknown. Hybrids organisms are considered potential alternatives to traditional cultivation.

Questions: How does cross-pollination affect germination in *V. planifolia* and *V. pompona*? Do hybrids seeds exhibit greater germination than their parental species?

Studied species: *Vanilla planifolia*, *V. pompona*, *V. planifolia* × *V. pompona*, *V. pompona* × *V. planifolia* (Orchidaceae)

Study site and dates: Mesa de Guadalupe, Alto Lucero, and La Concepción, Jilotepec, Veracruz, Mexico, in April 2024.

Methods: Manual pollinations (self-pollination, geitonogamy, and metaxenia/xenia) were performed, and fruits from natural pollination were collected. Hybrids were only self-pollinated. Fruits and seeds were characterized, and seeds were planted *in vitro* under a 16:8 hours light-dark photoperiod, and total darkness.

Results: Higher germination values occurred in natural pollination (67.83 % in *V. planifolia* and 44.66 % in *V. pompona*) and metaxenia/xenia (63.2 and 51.66 %, respectively), along with larger fruits and seeds. Geitonogamy exhibited intermediate results (24.46 and 22.79 %, respectively), while self-pollination resulted in the lowest values (4.82 % in *V. planifolia* and none in *V. pompona*). Hybrids produced larger fruits, but their seeds did not germinate. Light exposure enhanced germination.

Conclusions: Intra- and interspecific cross pollination promotes germination in vanilla. Hybrid fruits could represent a commercial alternative to traditional vanilla crop.

Keywords: Geitonogamy, metaxenia, natural pollination, self-pollination, xenia.

Resumen

Antecedentes: *Vanilla planifolia* y *V. pompona* presentan bajas tasas de germinación. Aunque sus frutos son obtenidos mediante autopolinización manual, el efecto de la polinización cruzada intra- e interespecífica en sus frutos y semillas es desconocido. Organismos híbridos son considerados alternativas potenciales al cultivo tradicional.

Preguntas: ¿Cómo afecta la polinización cruzada la germinación en *V. planifolia* y *V. pompona*? ¿Semillas de híbridos presentan mayor germinación que sus especies parentales?

Especies de estudio: *Vanilla planifolia*, *V. pompona*, *V. planifolia* × *V. pompona*, *V. pompona* × *V. planifolia* (Orchidaceae)

Sitio y años de estudio: Mesa De Guadalupe, Alto Lucero, y La Concepción, Jilotepec, Veracruz, México, en abril de 2024.

Métodos: Se realizaron polinizaciones manuales (autopolinización, geitonogamia, metaxenia/xenia), y se recolectaron frutos de polinización natural. Los híbridos solamente fueron autopolinizados. Se caracterizaron frutos y semillas, estas se sembraron *in vitro* bajo fotoperiodo 16:8 horas luz-oscuridad, y oscuridad total.

Resultados: La germinación más alta ocurrió en polinización natural (67.83 % en *V. planifolia* y 44.66 % en *V. pompona*) y en metaxenia/xenia (63.2 y 51.66 %, respectivamente), asociadas a frutos y semillas mayores. La geitonogamia exhibió resultados intermedios (24.46 y 22.79 %, respectivamente), mientras que la autopolinización resultó en menores valores (4.82 % en *V. planifolia*, nula en *V. pompona*). Los híbridos produjeron frutos más grandes, pero semillas sin germinación. La exposición lumínica favoreció la germinación.

Conclusiones: La polinización cruzada intra- e interespecífica promueve la germinación en vainilla. Los frutos de los híbridos representarían una alternativa comercial al cultivo tradicional.

Palabras clave: Autopolinización, geitonogamia, metaxenia, polinización natural, xenia.

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Vanilla planifolia Andrews (PL) is an orchid of commercial importance worldwide (Ranadive 2018), whose fruit has reached a price of up to 600 dollars per kilogram (Hernández-Hernández 2018). Vanilla fruits are highly valued for their aromatic quality, which is attributed to a combination of phenolic compounds, being vanillin the most important (Pérez-Silva *et al.* 2021). Despite its economic importance, PL is classified as a species under special protection in Mexico and globally protected by the International Union for Conservation of Nature (IUCN) Red List (Armenta-Montero *et al.* 2022). This is attributed to factors such as habitat loss, overexploitation, and genetic erosion, as its cultivation is carried out asexually through stem cuttings (Menchaca-García 2018). On the other hand, *V. pompona* Schiede (PO), a species belonging to the aromatic vanilla clade, produces fruits that also contain vanillin (albeit in smaller amounts than PL), along with other phenolic compounds that give it a unique aromatic profile, granting it significant potential in the food and perfume industries (Maruenda *et al.* 2013, de Oliveira *et al.* 2022, Ravier *et al.* 2024).

The production of vanilla fruits in both PL and PO relies mainly on the manual self-pollination of flowers, due to low rates of natural fruit set, which typically do not exceed 10 % of the flowers produced (Soto-Arenas & Dressler 2009, Pansarin & Pansarin 2014). Flowers of *Vanilla* species show a rostellum that prevent direct contact between the male and female structures, suggesting a possible adaptation to avoid self-pollination (Lubinsky *et al.* 2008, Zhang *et al.* 2021, de Oliveira *et al.* 2022). However, pollination in *Vanilla* commonly involves the manual transfer of the pollen mass to the stigma of the same flower, inducing self-fertilization (Barreda-Castillo *et al.* 2024).

PL and PO have been multiplied through stem cutting propagation (*i.e.*, asexual reproduction), which has caused, in the case of PL, genetic erosion and, consequently, greater susceptibility to biotic and abiotic stress conditions (Watteyn *et al.* 2020, Armenta-Montero *et al.* 2022). Although obtaining individuals from seed ameliorates genetic erosion, seeds obtained through self-pollination in PL have shown *in vitro* germination rates of up to 5 % (Yeh *et al.* 2021), while germination has not been reported in PO (Menchaca-García *et al.* 2011). This could suggest, along with the mechanical barriers (*i.e.*, rostellum), a possible genetic self-incompatibility (Suetsugu 2015, Zhang *et al.* 2021, de Paiva-Neto *et al.* 2022, Ackerman *et al.* 2023).

In contrast, it has been reported that cross-pollination (*i.e.*, allogamy) can promote the production of viable and bigger seeds (São Leão *et al.* 2019). Allogamy can occur in two ways: either through xenogamy (pollination between flowers of different individuals) or through geitonogamy (pollination between flowers of the same individual) (Bateman 2020, de Oliveira *et al.* 2022). To date, it has been considered that xenogamy exhibits the best effects on fruit and seed production (Caballero-Villalobos *et al.* 2017, Emeterio-Lara *et al.* 2018, São Leão *et al.* 2019); nonetheless, pollination by geitonogamy has also resulted in the production of seeds with higher germination rates in some orchid species (Humaña *et al.* 2008, Johnson *et al.* 2009, Buragohain *et al.* 2016).

In addition, xenia and metaxenia have been considered as a possible alternative for the improvement of vanilla cultivation (Barreda-Castillo *et al.* 2024). Xenia and metaxenia are understood as the effect of foreign pollen (different species, but from the same genus) on the formation of seeds and fruits, respectively (Sabir 2015, MacInnis & Forrest 2020). It has been reported in other important crops, such as cucumber and grape, that seeds resulting from xenia are typically larger, along with higher germination rates and seedling development (Olfati *et al.* 2010, Dhakad *et al.* 2024), something needed in *Vanilla*. In turn, metaxenia allows to produce larger fruits or even fruits with biochemical changes such as increased concentrations of sugars or phenolic compounds (Suaib *et al.* 2020, Shahsavari & Shahhosseini 2022). Considering that the flowering of PL and PO coincide phenologically (Barreda-Castillo *et al.* 2023a) and that they are usually cultivated together (de Oliveira *et al.* 2022, Ravier *et al.* 2024), pollination by metaxenia/xenia could be considered as an alternative for this crop.

Related to xenia is the production of hybrid organisms, as it is an interspecific pollination (Kanade *et al.* 2024). In this sense, hybrids *V. planifolia* × *V. pompona* (PL × PO) and *V. pompona* × *V. planifolia* (PO × PL), obtained by Menchaca-García *et al.* (2011), have exhibited resistance to diseases such as root-stem rot caused by *Fusarium oxysporum* f. sp. *vanillae* (Barreda-Castillo *et al.* 2023b), as well as drought tolerance (Barreda-Castillo *et al.* 2023c); so, it has been considered that they might be an alternative to traditional vanilla cultivation. However, the sexual reproduction capacity of these particular organisms is still unknown. It was hypothesized that cross-pollination, par-

ticularly metaxenia/xenia and natural pollination, would result in larger fruits in PL and PO, as well as bigger seeds with higher germination rates, when compared to geitonogamy and self-pollination, with values equal or even higher than those obtained from their interspecific hybrids. Therefore, this study aimed to determine the effect of pollen source on the germination of *Vanilla planifolia*, *V. pompona*, and their interspecific hybrids in field conditions, with the goal of promoting seedling production from seeds.

Materials and methods

Study site. This study was conducted in two sites: Environmental Management Unit “Vainillales Tlali Nantli” (SEMARNAT-UMA-IN-VIV-0281-VER/19), located in Mesa de Guadalupe, municipality of Alto Lucero (-96° 42' W, 19° 34' N) at 851 m asl; and in an experimental vanilla plantation, located in La Concepción, municipality of Jilotepec (-96° 54' W, 19° 36' N) at 1,000 m asl. These sites belong to the Veracruz state in Mexico, and they are 21.28 km apart between them. Natural pollination has been recorded in both sites. Even though there is no evidence of natural pollinator of PL, it has been suggested that Euglossini bees could pollinate it (Soto-Arenas & Dressler 2009, Pansarin & Pansarin 2014). On the other hand, for PO it has been identified three species of Euglossini: *Eulaema cingulata* Fabricius, *E. meriana* Olivier, and *E. bombiformis* Packard (Pansarin 2023). Both *E. cingulata* and *Euglossa* spp. have been recorded in the study area (Lozano-Rodríguez *et al.* 2022). The species are cultivated outdoors, which are established on *Inga vera* Willd. and *Bursera simaruba* (L.) Sarg. trees as living tutors. Additionally, both species and hybrids are cultivated in greenhouse. Both sites have plants that were planted more than 10 years ago. The cultivation is carried out using organic matter as substrate, at 25 (± 5) °C, with weekly irrigation.

Breeding systems. Fruits from PL and PO were obtained through controlled manual pollinations: self-pollination and geitonogamy. Xenogamy was not carried out, as it was not possible to determine genetic differences among the individuals, as vanilla cultivation is usually done through stem cuttings. Additionally, cross-pollinations were performed between the species PL and PO in order to obtain xenia and metaxenia. Also, flowers of both species cultivated outdoors were left to observe the effect of natural pollination. In addition, the hybrid organisms PL × PO and PO × PL were self-pollinated for fruit production. Manual pollinations were carried out during April 2024, and during the same period natural pollination was monitored. Thus, in this study, we analyzed 10 treatments: four in PL (self-pollination, geitonogamy, natural pollination, and metaxenia/xenia), four in PO (self-pollination, geitonogamy, natural pollination, and metaxenia/xenia), along with self-pollination in PL × PO and PO × PL.

Morphological characterization of vanilla fruits. Five fruits from each treatment were harvested (n = 50) 45 days after pollination, the moment when the fruits reach their maximum length (Parra-Quezada 1987, Menchaca-García 2018, Barreda-Castillo *et al.* 2023a), and the highest seed germination percentage has been reported, as the seeds complete their development before the hardening of the seed coat (Menchaca-García 2018, Yeh *et al.* 2021). Total length (cm), diameter (cm) and fresh weight (g) were evaluated. Shape index of the fruits was calculated, defined as the ratio between length and diameter (Barreda-Castillo *et al.* 2023a).

Vanilla seed germination. The vanilla fruits from each treatment were washed with soapy solution and rinsed in tap water twice, carefully removing the floral remains from the ends. Then, they were immersed in a 2 % commercial bleach solution for 15 minutes, followed by immersion in 96 % ethyl alcohol for 10 minutes. The fruits were transferred to aseptic conditions in a laminar flow hood, flamed and later longitudinally dissected with a surgical scalpel. Seeds were extracted from the middle part of the fruits for *in vitro* culture. Murashige & Skoog (1962) culture medium was used in 235 mL jars, with 25 mL of culture medium. Each jar was used as an experimental unit, with 10 replicates per treatment (n = 100). There were ca. 200 seeds per jar. Five jars were exposed to light and five to darkness, the light exposure followed a photoperiod of 16 hours of light and 8 hours of darkness. In both cases, the seeds were incubated at 25 ± 2 °C. The germination of the seeds was evaluated 100 days after *in vitro* planting, when the

highest germination percentage in *Vanilla* has been reported (Menchaca-García 2018, Yeh *et al.* 2021). Both the germination percentage and the germination index were calculated using the formulas reported by Pierik *et al.* (1988):

$$\text{Germination Percentage} = (100) \left(\frac{b + c + d}{a + b + c + d} \right) \quad (1)$$

$$\text{Germination Index} = (10) \left(\frac{b + 2c + 3d}{a + b + c + d} \right) \quad (2)$$

where a, b, c, and d represent the frequency of each growth and development stage of the embryo: a = non-germinating stage; b = the embryo grows or swells without breaking the seed coat; c = the embryo emerges from the seed coat; and d = the embryo fully emerges from the seed coat. When germination does not occur the germination index is zero, while when all seeds germinate, *i.e.* 100 % germination is obtained, and all embryos are fully developed (stage d), the germination index is 30 (Pierik *et al.* 1988). Seed germination was observed through the jar to avoid contamination from handling. A graphical representation of the development stage of germination is shown in [Figure 1](#).

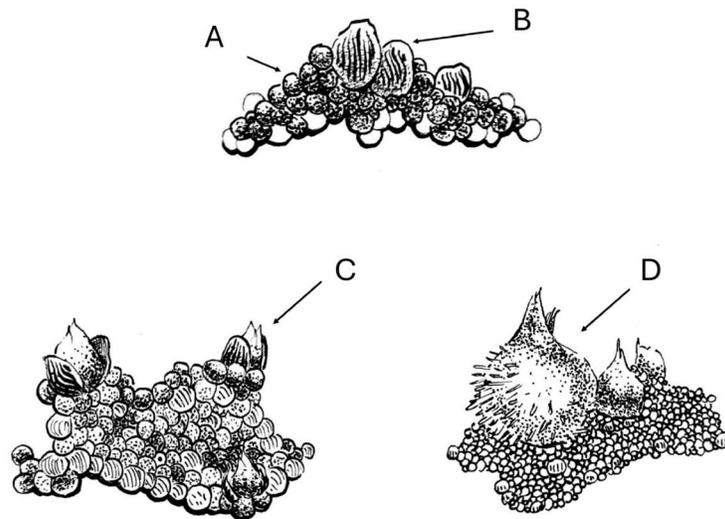


Figure 1. Stages of germination development in *Vanilla*. A: non-germinating stage; B: the embryo grows or swells without breaking the seed coat; C: the embryo emerges from the seed coat; D: the embryo fully emerges from the seed coat. The figure is for illustrative purposes only; the structures are not to scale. Figure constructed from own data and from Pierik *et al.* (1988). Figure made by Erik Polaris Flores Ojeda.

Morphological characterization of vanilla seeds. Thirty remaining seeds from the middle part of the fruits from each treatment were collected ($n = 1,500$). Length (μm) and width (μm) were evaluated, and shape index of seeds was calculated, defined as the ratio between length and width. The shape index, in both fruits and seeds, is a ratio between length and width, being dimensionless and a reflection of the proportions of the fruit/seed. Seed volume (mm^3) was calculated using the formula proposed by Arditti & Ghani (2000):

$$\text{Seed volume} = (4) (3^{-1}) (\pi ab^2) \quad (3)$$

where $a = (\text{length value}) (2^{-1})$, and $b = (\text{width value}) (2^{-1})$. Although the formula for calculating seed volume involves the length and width values, similar to the shape index, this calculation reflects the absolute size of the seed in volume units, not only a ratio. Seed measurements were taken using a Leica DM500 light microscope, with Leica Application Suite EZ software. Both the morphological characterization of fruits and seeds, as well as germination, were carried out at the facilities of the Orchidarium of Universidad Veracruzana (SEMARNAT-UMA-IN-VIV-0129-VER/11).

Statistical analysis. Concerning the morphological characterization of vanilla fruits, a two-factor arrangement was used: pollination type and vanilla genotype. Length, diameter, weight, and shape index were analyzed using two-way analysis of variance (ANOVA), *post hoc* Tukey ($P = 0.05$). Related to the morphological characterization of seeds, the same two-factor arrangement was used, and the variables length, width, shape index, and volume were analyzed using ANOVA, *post hoc* Tukey ($P = 0.05$).

In relation to seed germination, pollination type, vanilla genotype and light exposure were considered as factors. Germination percentage was analyzed using a generalized linear model (GLM) with a binomial distribution and logit link function ($P = 0.05$), while germination index was analyzed using a GLM with a Poisson distribution and log link function ($P = 0.05$). In all cases, the statistical software R v. 4.0.3 was used (R Core Team 2023), with packages *Agricole* (de Mendiburu 2023) for ANOVA, *car* (Fox & Weisberg 2019) and *gmodels* (Warnes *et al.* 2022) for GLM, and *Rmisc* (Hope 2022) and *ggplot2* (Wickham 2016) for graphics.

Results

Morphological characterization of vanilla fruits. The morphological parameters evaluated regarding the formation of vanilla fruits were affected by the pollen source. Contrasts between vanilla fruits are shown in [Figure 2](#). The longest fruits were obtained in natural pollination and metaxenia, followed by geitonogamy and self-pollination in both PL ([Figure 2A-D](#)) and PO ([Figure 2E-I](#)) ($F_{3,46} = 326.86$, $P < 0.01$). PL \times PO fruits showed a length similar to PL obtained by self-pollination, while PO \times PL exhibited higher values than the other hybrid and PL and PO under self-pollination ([Table 1](#)). Related to fruit diameter, metaxenia in PO exhibited the highest value, followed by natural pollination and geitonogamy, while no significant differences were observed in PL among the different pollination types ($F_{3,46} = 218.791$, $P < 0.01$). Fruits from both hybrids exhibited higher values than PL, but lower compared to PO ([Table 1](#)). The weight of vanilla fruits was also influenced by pollen source, with metaxenia fruits from both species being the heaviest, followed by natural pollination and geitonogamy ($F_{3,46} = 7196$, $P < 0.01$). PO \times PL fruits exhibited greater weight than PL \times PO fruits, and both hybrids showed higher values than PL, but lower compared to PO ([Table 1](#)). Fruit shape index exhibited an increase in metaxenia and natural pollination in PL, whereas in PO resulted in a reduction, compared with the other pollen sources ($F_{3,46} = 187.949$, $P < 0.01$). Both hybrids exhibited higher values than PO, but lower than PL ([Table 1](#)).

Morphological characterization of vanilla seeds. Seed length, width, and volume were influenced by the pollen source ([Figure 3](#)). It should be highlighted that developed seeds (up to before seed coat hardening) were observed in all treatments ([Figure 3](#)). Seeds from xenia and natural pollination in PL and PO exhibited the greatest values in length ($F_{3,1496} = 122.678$, $P < 0.01$) and width ($F_{3,1496} = 47.782$, $P < 0.01$), while seeds from self-pollination were the smallest. Hybrids showed no differences in seed length between them, with PO \times PL exhibiting higher width than PL \times PO ([Table 2](#)). Seed volume was affected by pollen source, since xenia and natural pollination produced seeds with the highest volume in both PL and PO ($F_{3,1496} = 220.757$, $P < 0.01$). Seeds from PO \times PL exhibited higher volume compared to PL \times PO seeds ([Table 2](#)). No significant differences in seed shape index were found among groups ($F_{3,1496} = 2.117$, $P = 0.0962$) ([Table 2](#)).

Vanilla seed germination. Germination percentage was influenced by pollen source ($\chi^2_{3,96} = 29.116$, $P < 0.05$) ([Table S1](#)). The highest values were recorded in seeds from natural pollination and xenia in PL and PO. Geitonogamy exhibited intermediate values, and the lowest values were found in self-pollination in PL, whereas no germination was recorded in seeds obtained by self-pollination in PO and neither in the hybrids ([Table 3](#)). Germination index was also affected by the pollen source ($\chi^2_{3,96} = 19.366$, $P < 0.05$) ([Table S2](#)). Again, seeds from natural pollination and xenia exhibited the greatest values and therefore greatest development. ([Table 3](#), [Figure 4](#)).

Light exposure was a significant factor in germination, as seeds exposed to a photoperiod exhibited higher germination percentages ($\chi^2_{1,98} = 9.955$, $P < 0.05$) and index values ($\chi^2_{1,98} = 124.436$, $P < 0.05$) (Supplementary material,

Pollen source effect on *Vanilla* germination

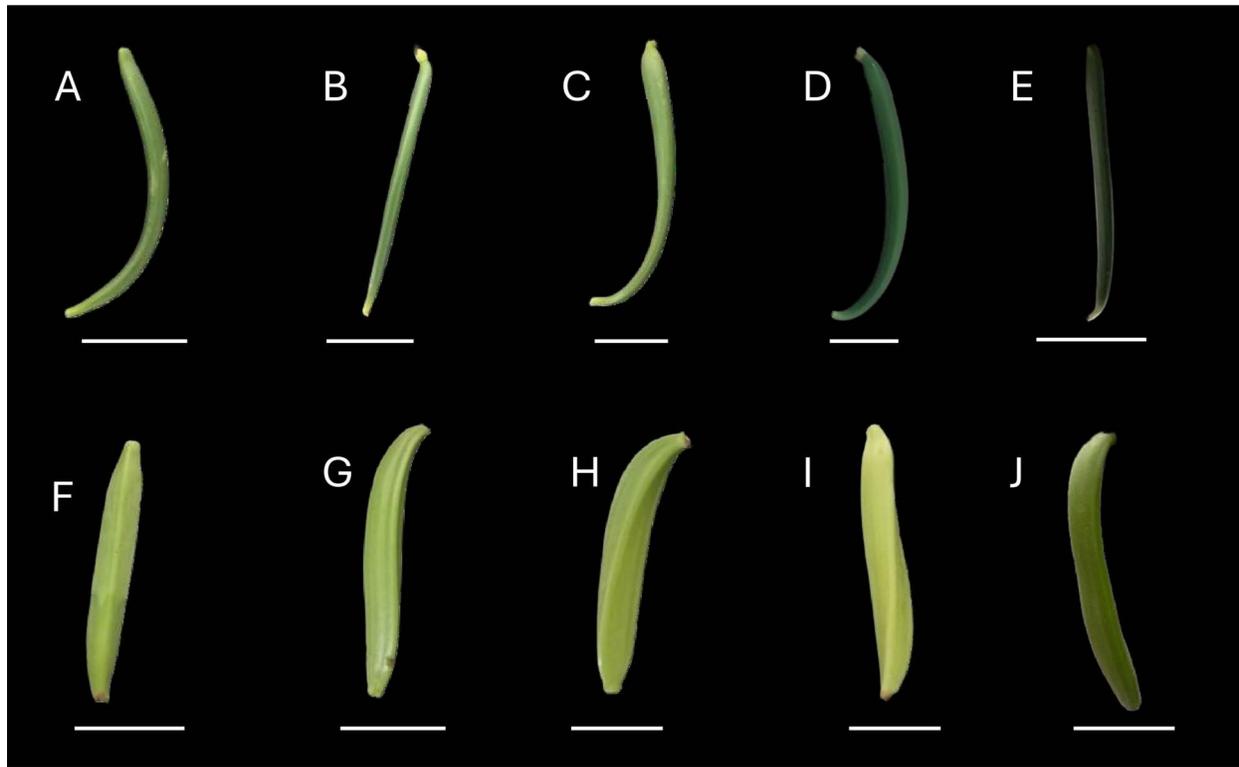


Figure 2. Fruits of *Vanilla planifolia* (A-D), *V. planifolia* × *V. pompona* (E), *V. pompona* (F-I), and *V. pompona* × *V. planifolia* (J), resulting from self-pollination (A, F, E, J), geitonogamy (B, G), natural pollination (C, H), and metaxenia (D, I). Scale bar = 5 cm.

Table 1. Morphological characterization of fruits of the species *Vanilla planifolia* (PL) and *V. pompona* (PO), and the hybrids *V. planifolia* × *V. pompona* (PL × PO) and *V. pompona* × *V. planifolia* (PO × PL), resulting from self-pollination, geitonogamy, natural pollination, and metaxenia. Data are shown as mean ± standard error (n = 50). Different letters indicate significant differences between treatments ($P < 0.05$), according to ANOVA.

Genotype	Pollination	Length (cm)	Diameter (cm)	Weight (g)	Shape index
PL	Self-pollination	12.56 ± 0.17e	1.08 ± 0.07g	5.22 ± 0.09j	11.80 ± 0.63b
	Geitonogamy	14.76 ± 0.10c	1.32 ± 0.08f	7.54 ± 0.16i	11.33 ± 0.62b
	Natural	18.26 ± 0.13b	1.32 ± 0.06f	12.4 ± 0.18e	13.93 ± 0.56a
	Metaxenia	19.46 ± 0.13a	1.46 ± 0.09f	15.72 ± 0.12g	13.53 ± 0.80a
PO	Self-pollination	11.62 ± 0.09f	2.08 ± 0.04d	16.34 ± 0.19d	5.59 ± 0.07e
	Geitonogamy	13.24 ± 0.11d	2.3 ± 0.03c	23 ± 0.07c	5.76 ± 0.08e
	Natural	14.38 ± 0.19c	2.46 ± 0.05b	24.3 ± 0.07b	5.85 ± 0.09e
	Metaxenia	14.46 ± 0.12c	2.8 ± 0.04a	25.58 ± 0.09a	5.17 ± 0.05f
PL × PO	Self-pollination	12.62 ± 0.20e	1.82 ± 0.07e	9.26 ± 0.08h	6.96 ± 0.21d
PO × PL	Self-pollination	13.6 ± 0.19d	1.7 ± 0.05e	13.26 ± 0.14f	8.03 ± 0.23c

Tables S1 and S2). In PL, light exposure increased germination percentage by 1.2 times in self-pollination, 3.2 times in geitonogamy, 2.5 times in natural pollination, and 2.3 times in xenia. Besides, in PO increased 1.8 times in geitonogamy, 2.1 times in natural pollination, and 2.4 times in xenia (Table 3). The germination index also increased with photoperiod, in PL by 1.3 times in self-pollination, 3.3 times in geitonogamy, 3.4 times in natural pollination, and 3 times in xenia. In addition, in PO increased 1.7 times in geitonogamy, 2.3 times in natural pollination, and 3.6 times in xenia (Table 3). Protocorm formation was recorded in both species in seeds from geitonogamy, natural pollination, and xenia, but only when they were exposed to photoperiod (Figure 4).

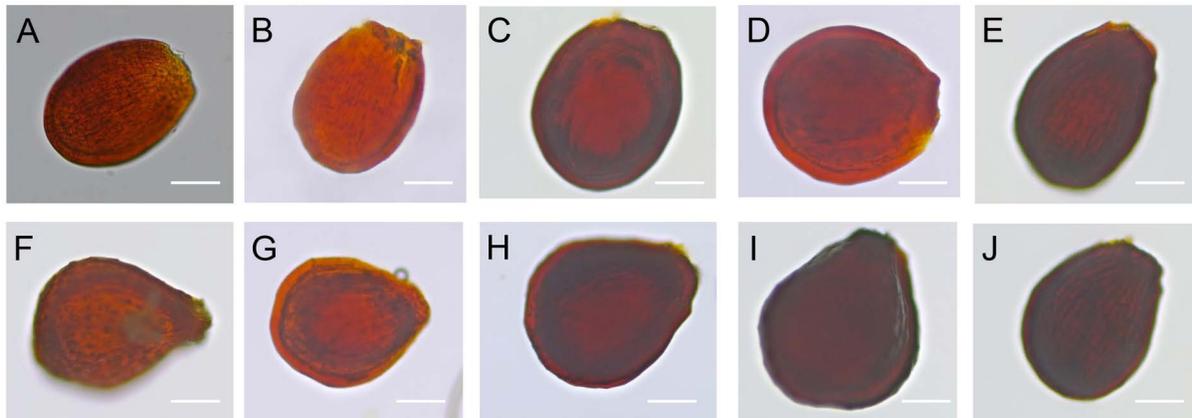


Figure 3. Seeds of *Vanilla planifolia* (A-D), *V. planifolia* × *V. pompona* (E), *V. pompona* (F-I), and *V. pompona* × *V. planifolia* (J), resulting from self-pollination (A, F, E, J), geitonogamy (B, G), natural pollination (C, H), and xenia (D, I). Scale bar = 100 µm.

Discussion

Fruit and seed production in species. To date, only effects of self-pollination on fruit and seed production had been reported for both PL and PO, along with low germination percentages (Menchaca-García 2018). For the first time, it is shown that the pollen source affects fruit and seed production in PL and PO, seed germination is finally achieved in PO, and comparisons with their interspecific hybrids are made. Although fruits were obtained in all treatments (species and hybrids), this does not imply the production of viable seeds, as the mere placement of the pollinium on the stigma induces fruit formation, due to auxins like indole-3-acetic acid, as reported in *Restrepia*, *Dendrobium*, and *Cymbidium* (Arditti & Knauff 1969, Arditti 1992, Promyou *et al.* 2014, Millner *et al.* 2015, Doorn & Ketsa 2021).

Seed formation was recorded until testa development in all treatments, suggesting that seed development was not ceased, whether or not the ovules were fertilized (Brandvain & Haig 2005, Rodrigues & Borba 2023, Wang & Filatov 2023). Testa development is known to be independent of embryo development (Chaudhury *et al.* 2001, Dumas & Rogowsky 2008, Arathi 2011, Rogo *et al.* 2023), which might happen in *Vanilla*, a genus characterized by its hardened testa (Lee & Yeung 2023). Since fruit and seed production were observed in species and hybrids, differences among treatments should then be related to pollen source in the pollination process and therefore linked to issues of genetic material recognition (Brandvain & Haig 2005, Zhang *et al.* 2021, Rodrigues & Borba 2023). Our results suggest that self-incompatibility could be manifested during fertilization, particularly in embryo development.

Effect of self-pollination. Historically, both PL and PO have been manually self-pollinated in order to enhance fruit production, given the low natural fruit set (Soto-Arenas & Dressler 2009). Although self-pollination induced fruit formation in both species, PL produced seeds with low germination percentage, or none at all in PO, suggesting partial self-incompatibility (Chabert *et al.* 2024, Polizelli-Ricci *et al.* 2024). This may also explain the smaller fruits and seeds resulting from self-pollination. Viable seeds induce biochemical changes, particularly auxin production, in

order to promote their self-development (Arditti 1992, Millner *et al.* 2015). This also increases fruit size by modifying the maternal tissues responsible for seed nutrition (Chaudhury & Berger 2001, Chaudhury *et al.* 2001, Arathi 2011). In the absence of viable seeds, fruit growth would be mainly related to the auxins from the pollen mass. The reduced germination in PL and the absence in PO would explain the smaller size of self-pollinated fruits, compared to those from other pollen sources.

Effect of geitonogamous pollination. Geitonogamy showed slightly better results than self-pollination in fruit and seed production, as well as germination. Similar results were found in *Chloraea crispa* Lindl., *Eulophia alta* (L.) Fawc. & Rendle, and *Phaius tankervilleae* (King & Pantl.) Karthik. (Humaña *et al.* 2008, Johnson *et al.* 2009, Buragohain *et al.* 2016). Although geitonogamy is technically cross-pollination, as it involves the exchange of pollen between flowers (Lanzino *et al.* 2023), it resembles autogamy, since the pollen comes from the same organism (Kropf & Renner 2008). However, pollen production involves genetic variation, and within this subtle variability, geitonogamous pollination (by realizing cross-pollination) may result in slightly greater genetic variability compared to self-pollination (Willmer 2011). This subtle genetic difference might be the reason why the germination percentage increased in PL, and germination was finally achieved in PO.

Effect of natural pollination. It is considered superior to manual pollination, since it results in larger fruits and seeds with higher viability in Orchidaceae (Metsare *et al.* 2015, Emeterio-Lara *et al.* 2018, São Leão *et al.* 2019). These benefits are related to pollinators, mainly euglossine bees in Orchidaceae (de Oliveira *et al.* 2022), due to they perform cross-pollination, particularly xenogamy (Scopece *et al.* 2014). Nevertheless, it is not possible to assert that natural pollination in vanilla is synonymous with xenogamy, since the pollen source during natural pollination was not tracked in this study, and most vanilla plantations are usually established through stem cuttings (Hernández-Hernández 2018). Thus, genetic differences among individuals must be verified in order to confirm a xenogamy effect (Tremblay *et al.* 2005, São Leão *et al.* 2019, Wurz *et al.* 2021), in addition to verify pollen load.

Natural pollination produced larger fruits and seeds with higher germination, compared to geitonogamy, possibly related to pollinators depositing more and better-positioned pollen on the stigma, enhancing fertilization (Chautá-

Table 2. Morphological characterization of seeds from the species *Vanilla planifolia* (PL) and *V. pompona* (PO), and the hybrids *V. planifolia* × *V. pompona* (PL × PO) and *V. pompona* × *V. planifolia* (PO × PL), resulting from self-pollination, geitonogamy, natural pollination, and xenia. Data are expressed as mean ± standard error (n = 1,500). Different letters indicate significant differences between treatments ($P < 0.05$), according to ANOVA.

Genotype	Pollination	Length (µm)	Width (µm)	Shape index	Volume (mm ³)
PL	Self-pollination	301 ± 2.38f	227.42 ± 2.27e	1.32 ± 0.009a	0.0082 ± 0.00023g
	Geitonogamy	318.96 ± 2.66e	237.26 ± 2.73d	1.34 ± 0.017a	0.0095 ± 0.00022f
	Natural	335.32 ± 2.18d	257.39 ± 3.08c	1.31 ± 0.032a	0.0118 ± 0.00032e
	Xenia	347.38 ± 2.91c	261.12 ± 2.94c	1.33 ± 0.012a	0.0125 ± 0.00035e
PO	Self-pollination	361.69 ± 5.27b	276.41 ± 6.96b	1.31 ± 0.032a	0.0151 ± 0.00039c
	Geitonogamy	368.11 ± 4.83b	275.82 ± 7.12b	1.34 ± 0.035a	0.0152 ± 0.00040c
	Natural	389.65 ± 5a	298.86 ± 6.94a	1.31 ± 0.028a	0.0189 ± 0.00049b
	Xenia	402.19 ± 5.15a	306.95 ± 7.4a	1.31 ± 0.027a	0.0203 ± 0.00044a
PL × PO	Self-pollination	355.42 ± 4.19bc	245.27 ± 6.76d	1.37 ± 0.048a	0.0117 ± 0.00046e
PO × PL	Self-pollination	365.91 ± 4.57bc	263.75 ± 4.4bc	1.39 ± 0.029a	0.0135 ± 0.00047d

Mellizo *et al.* 2012, Klatt *et al.* 2014, Eraerts *et al.* 2020, Wurz *et al.* 2021). In orchids, this precision could be related to specific structures in floral-pollinator interaction, touching the reproductive structures at the precise angle and direction, unlike manual pollination which may damage the pollen mass (Ackerman 1983, Suetsugu & Fukushima 2014, Baguette *et al.* 2020). Besides, pollen grains desiccation during transport may enhance their activity (Pacini & Hesse 2002, Firon *et al.* 2012). Pollination success in *Vanilla* is more limited by the brief viability of the stigma than by pollen viability (Divakaran *et al.* 2006, 2016). While pollinator behavior was not recorded in this study, these results contribute to promote agroecological practices that favor pollinator abundance (Nicholls & Altieri 2013).

Effect of metaxenia/xenia. Although a preliminary study exists on the metaxenia effect in vanilla fruits (Barreda-Castillo *et al.* 2023a), to best of our knowledge, this is the first formal report on the study of the effect of xenia in vanilla seeds. Metaxenia altered fruit shape index in both species, increasing in PL and decreasing in PO, both compared to self-pollination in their respective species. This uneven effect has been reported previously (Dhakad *et al.* 2024), potentially related to a difference in vigor between the pollen donors (Keulemans *et al.* 1996, Dhakad *et al.* 2024). PO is more vigorous than PL (Soto-Arenas & Dressler 2009, Flores-Jiménez *et al.* 2017), which could explain why shape index increases in PL (when PO is the pollen donor), whereas it decreases in PO (when PL is the pollen donor).

Table 3. Germination percentage and germination index of seeds, exposed to light and darkness, from the species *Vanilla planifolia* (PL) and *V. pompona* (PO), and the hybrids *V. planifolia* × *V. pompona* (PL × PO) and *V. pompona* × *V. planifolia* (PO × PL), resulting from self-pollination, geitonogamy, natural pollination, and xenia. Data are presented as mean ± standard error (n = 100). Different letters indicate significant differences between treatments ($P < 0.05$), according to GLM.

Genotype	Pollination	Condition	Germination percentage	Germination index
PL	Self-pollination	Light	4.82 ± 0.35h	0.5 ± 0.02g
		Darkness	3.89 ± 0.34h	0.39 ± 0.03h
	Geitonogamy	Light	24.46 ± 1.13de	2.49 ± 0.13cd
		Darkness	7.54 ± 0.44g	0.75 ± 0.04f
	Natural	Light	67.83 ± 0.81a	9.29 ± 0.027a
		Darkness	26.63 ± 1.12d	2.74 ± 0.12c
	Xenia	Light	63.20 ± 2.96a	8.88 ± 0.46a
		Darkness	28.10 ± 2.23d	2.91 ± 0.23c
PO	Self-pollination	Light	0 ± 0i	0 ± 0i
		Darkness	0 ± 0i	0 ± 0i
	Geitonogamy	Light	22.79 ± 0.84e	2.46 ± 0.11d
		Darkness	13.01 ± 0.52f	1.43 ± 0.05e
	Natural	Light	44.66 ± 1.29c	5.28 ± 0.17b
		Darkness	21.06 ± 0.78e	2.30 ± 0.09d
	Xenia	Light	51.66 ± 1.83b	8.29 ± 0.46a
		Darkness	21.94 ± 1.35e	2.27 ± 0.14d
PL × PO	Self-pollination	Light	0 ± 0i	0 ± 0i
		Darkness	0 ± 0i	0 ± 0i
PO × PL	Self-pollination	Light	0 ± 0i	0 ± 0i
		Darkness	0 ± 0i	0 ± 0i

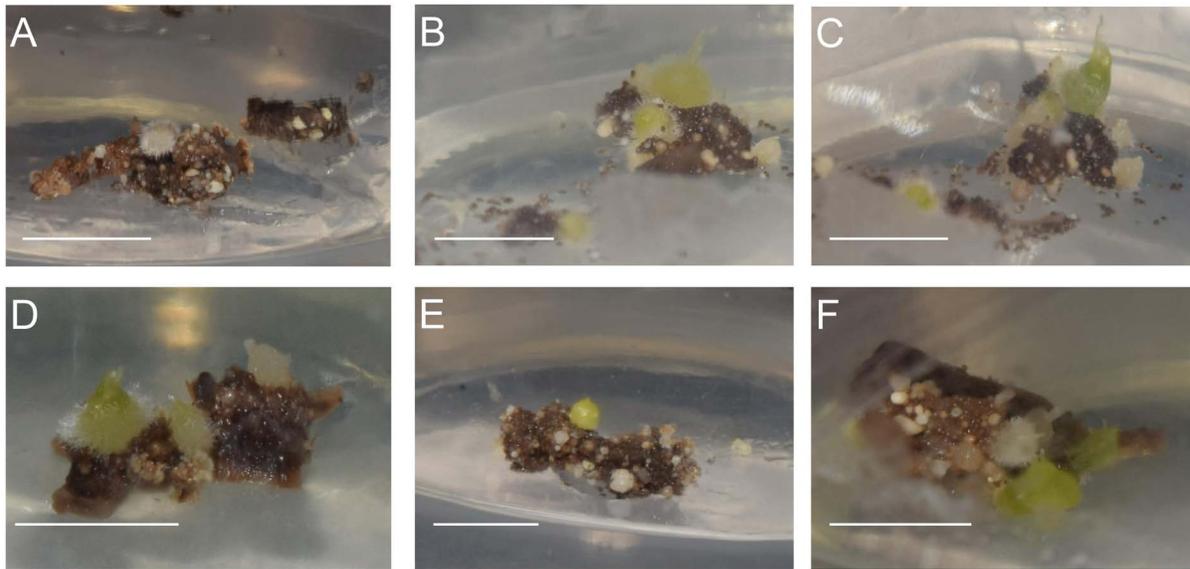


Figure 4. Germination of seeds from the species *Vanilla planifolia* (A-C), and *V. pompona* (D-F), resulting from geitonogamy (A, D), natural pollination (B, E), and xenia (C, F). Scale bar = 1 cm.

Proposed mechanisms for metaxenia/xenia include volatile compounds from pollen from a different species triggering chemical signaling in the ovary (Deng *et al.* 2022). Xenia often results in larger seeds and higher germination percentage (Olfati *et al.* 2010, Sabir 2015, MacInnis & Forrest 2020), possibly related to a greater paternal gene control during seed development, suppressing maternal gene regulations and thereby leading to the production of larger seeds (de Jong & Scott 2007). Xenia seeds tend to produce a greater concentration of auxins, cytokinins, and gibberellins than those seeds obtained by self-pollination (Kanade *et al.* 2024), enhancing metaxenia effects and also self-promoting germination. These effects have been recorded in crops such as apple (Bodor *et al.* 2008, Militaru *et al.* 2015), date (Swingle 1928, Shahsavari & Shahhosseini 2022), strawberry (MacInnis & Forrest 2020), dragon fruit (Mizrahi *et al.* 2004), cucumber (Olfati *et al.* 2010), grape (Sabir 2015, Dhakad *et al.* 2024), pear (Cheng *et al.* 2020), and maize (Suaib *et al.* 2020), among others. However, it remains unknown whether these mechanisms would operate in the same way in orchids. Xenia is directly related to the production of hybrid organisms, as it requires the participation of two species (Preston & Pearman 2015, Goulet *et al.* 2017). This is significant in Orchidaceae, the plant family with the highest hybridization potential (Fiorini *et al.* 2023); yet, the process of xenia remains unknown.

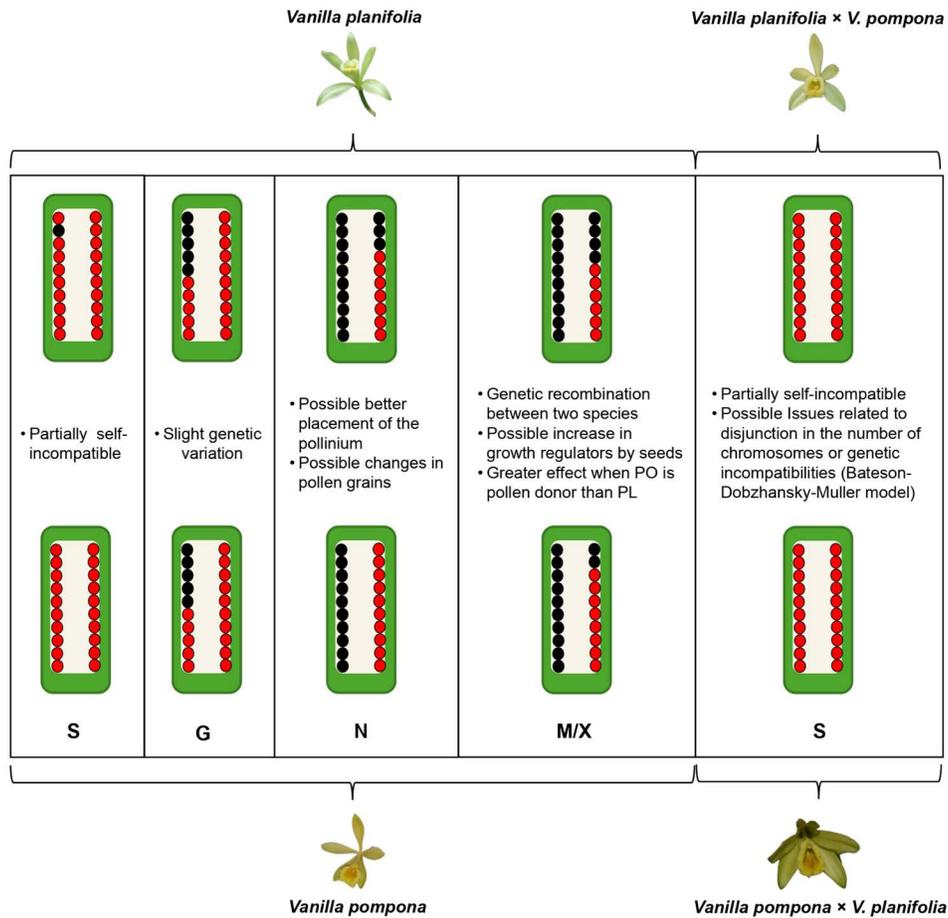
Metaxenia/xenia could be an immediate improvement strategy for vanilla producers, as PL and PO are companion species that flower synchronously (Ravier *et al.* 2024). Since vanilla farmers are already skilled in manual pollination (Cameron 2011, Hernández-Hernández 2018), so, they could cross-pollinate PL and PO. It is also suggested to consider other *Vanilla* species as pollen donors in order to determine if they exhibit similar effects, indirectly obtaining new hybrid organisms with potential new traits, contributing to the development of breeding programs for *Vanilla* species (Watteyn *et al.* 2023).

Fruit and seed production in hybrids. PO × PL produced larger fruits than PL × PO, and fruits from both hybrids were larger than PL fruits obtained from traditional pollination, suggesting that hybrid production could be a strategy for improving PL cultivation, at least in terms of morphological traits and stress resistance (Divakaran *et al.* 2006, 2016, Chambers 2019, Grisoni & Nany 2021). PO traits were more pronounced when it was the maternal species, likely due to a higher gene expression of traits from the ovule donor species (Havkin-Frenkel & Belanger 2018, Barrera-Castillo *et al.* 2024). This phenomenon is possibly related to plastid/mitochondrial genome transfer (Daniell *et al.* 2021, Park *et al.* 2021), or even to epigenetic regulation (Baulcombe & Dean 2014, Kumar & Singh 2016).

Seeds from PL × PO were larger than those from PL produced by self-pollination, while seeds from PO × PL were similar in size to those from PO obtained by self-pollination. However, hybrid seeds exhibited no germination. Although hybrids may eventually become into new species (Li *et al.* 2021, Evans *et al.* 2023), some may face germination difficulties due to issues related to the meiosis process (Dumas & Rogowsky 2008, Pinheiro & Cozzolino 2013, Pinheiro *et al.* 2016, Marques *et al.* 2018, Shang *et al.* 2020, Arida *et al.* 2021), or genetic incompatibilities, according to the Bateson-Dobzhansky-Muller model (Orr & Turelli 2001, Arida *et al.* 2021). Despite the idea that hybrids could potentially replace their parent species (Chung *et al.* 2005), the lack of germination in hybrid seeds might suggest the absence of ecological competition.

Both hybrids have shown resistance to water stress (Barreda-Castillo *et al.* 2023c), and to root-stem rot caused by *F. oxysporum* f. sp. *vanillae* (Barreda-Castillo *et al.* 2023b). In the present study is added new information related to their fruits, specifically about their morphological characterization. Thus, pending to determine the content of aromatic compounds, the use of these vanilla hybrids is proposed as an alternative to traditional vanilla cultivation (Havkin-Frenkel & Belanger 2018, Chambers 2019), since they could produce larger fruits while also being tolerant plants, along with the fact that their seeds fail to germinate and therefore would not pose a risk of ecological competition. A summary about the effect of pollen source in fruit and seed production in PL, PO, and their interspecific hybrids is shown in [Figure 5](#).

Figure 5. Effect of pollen source in *Vanilla planifolia*, *V. pompona*, and their interspecific hybrids. The effect of self-pollination (S), geitonogamy (G), natural pollination (N) and metaxenia/xenia (M/X) is highlighted. Red circles indicate empty seeds inside the fruit, whereas black ones indicate viable seeds. The proportion of empty seeds to viable seeds shown in each treatment was calculated based on the germination percentage observed under a 16:8 light:dark photoperiod in the present study. The figure includes data from the present study and from the literature (Johnson & Edwards 2000, Chaudhury & Berger 2001, Orr & Turelli 2001, Pacini & Hesse 2002, Olfati *et al.* 2010, Arathi 2011, Pinheiro & Cozzolino 2013, Millner *et al.* 2015, Sabir 2015, Emeterio-Lara *et al.* 2018, Zhang *et al.* 2021, Deng *et al.* 2022, Chabert *et al.* 2024, Kanade *et al.* 2024).



Other factors affecting germination in Vanilla. The use of immature seeds (collected 45 days post-pollination) improves germination, since the embryo is fully developed and the testa has not yet hardened (Swamy 1949, Menchaca-García 2018, Yeh *et al.* 2021). Besides, vanilla fruits reach their maximum length at the same time when the fertilization process is completed, and they only increase in weight until harvest (Swamy 1949, Parra-Quezada 1987, Vargas-Hernández *et al.* 2021, Yeh *et al.* 2021). In addition, the germination percentage of self-pollinated seeds in PL aligns with previously reported values in the literature (Menchaca-García 2018, Yeh *et al.* 2021), and higher values were obtained when pollen came from a different flower, supporting the idea that pollen source influences the production of viable seeds.

Light exposure also affects germination and seedling development, with better results obtained under a 16:8 light/dark photoperiod for both PL and PO, indicating a positive photoblastic response (Johnson *et al.* 2011, Nikabadi *et al.* 2014, Baczek-Kwinta 2022). Darkness exposition does not inhibit germination but limits seedling development. This is likely related to phytochrome activation (Chory *et al.* 1996), promotion gibberellin production (García-Martínez & Gil 2001), or a possible degradation of germination-inhibiting metabolites (Zhang *et al.* 2013). However, future studies should focus on analyzing these issues in detail.

This is the first study where the effect of different pollen sources in PL and PO on fruit and seed development is analyzed. It is considered that both PL and PO are partially self-incompatible species, so cross-pollination is recommended to obtain viable seeds and larger fruits. *Metaxenia* produced larger fruits in PL, whilst *xenia* resulted in larger seeds with the highest germination percentage in both species, so cross-pollination between PL and PO is also proposed as an immediate breeding strategy in vanilla. Hybrid organisms exhibited bigger fruits compared to those obtained in PL by traditional pollination, although their seeds did not germinate. Nevertheless, their potential use as an alternative to traditional vanilla cultivation is proposed.

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Supplementary material

Supplemental data for this article can be accessed here: <https://doi.org/10.17129/botsci.3679>

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