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In vivo and *in situ* characterization of the growth of polynic tubes in Japanese plums

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ABSTRACT: The objective of this study was to characterize the reproductive compatibility among cultivars and selections of Japanese plum through controlled pollination, both *in vivo* and *in situ*, to obtain a profile of reproductively compatible cultivars. *In situ* and *in vivo* self-pollinations were carried out in an F1 population of Japanese plum, and *in vivo* pollinations were conducted on the pollen-grain receptor cultivars: Laetitia, Zafira and Fortune, and the pollen-grain donor cultivars: Piamontesa, Selection Plum 86-13, Harry Pickstone, Black Amber, Bruce, SC-17, Laetitia, SC-15, SC 7, Zafira, Amarelinha, América, Reubennel and Fortune, to verify the percentage of germination of pollen grains and growth of pollen tubes in the pistils of the recipient plants. The *in situ* and *in vivo* assays for the F1 population confirm the self-compatibility of genotypes 13 and 35. The Laetitia cultivar is self-incompatible; however, it is compatible with the Selection 'Plum 86-13'. 'Zafira' is self-incompatible. 'Fortune' is compatible with 'Amarelinha', 'SC-7', 'America', 'Harry Pickstone' and 'Reubennel', but it is self-incompatible.

Key words: pollination; *Prunus salicina*; reproductive biology; Rosaceae, self-incompatibility gametophytic

Caracterização *in vivo* e *in situ* do crescimento de tubos polínicos em pistilos de ameixeiras japonesas

RESUMO: O objetivo deste estudo foi caracterizar a compatibilidade reprodutiva entre cultivares e seleções de ameixeira japonesa por meio da polinização controlada, *in vivo* e *in situ* para obter um perfil de cultivares reprodutivamente compatíveis. Executou-se autopolinizações *in situ* e *in vivo* numa população F1 de ameixeira japonesa e polinizações *in vivo* nos cultivares receptores de grãos-de-pólens: Laetitia, Zafira e Fortune, e os cultivares doadores de grãos-de-pólens: Piamontesa, Seleção Ameixa 86-13, Harry Pickstone, Black Amber, Bruce, SC-17, Laetitia, SC-15, SC-7, Zafira, Amarelinha, América, Reubennel e Fortune, para verificar a percentagem de germinação dos grãos-de-pólen e crescimento dos tubos polínicos nos pistilos das plantas receptoras. Os ensaios *in situ* e *in vivo* para a população F1 confirmam a autocompatibilidade dos genótipos 13 e 35. A cultivar Laetitia é autoincompatível, porém é compatível com a Seleção SA 86-13. 'Zafira' é autoincompatível. 'Fortune' é compatível com 'Amarelinha', SC-7, 'América', 'Harry Pickstone' e 'Reubennel', mas é autoincompatível.

Palavras-chave: polinização; *Prunus salicina*; biologia reprodutiva; Rosaceae; autoincompatibilidade gametofítica

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Introduction

The flower, as the reproductive organ, has favored Angiosperms for millions of years by increasing genetic variability through pollination between plants (Sokoloff et al., 2018). The sexual reproduction of plants, associated with reproductive compatibility (Cardoso et al., 2018), biochemical signals (Cui et al., 2022), pollinating agents, and environmental conditions (Shrestha et al., 2018), is essential to maintain plant biological diversity within botanical families and to develop intrinsic characteristics for the survival of the species. However, from an economic point of view, some of these aspects pose obstacles to the production of certain Prunoides due to their own reproductive characteristics, hindering large-scale production.

In the Rosaceae family, many species show gametophytic self-incompatibility (GSI) to prevent self-pollination (Du et al., 2021), and to increase genetic variability in offspring populations. GSI has been identified in species of the genus *Prunus* (Gordillo-Romero et al., 2020) as being governed by multiallelic loci containing the "*S*" alleles. (Sterility loci), which express ribonuclease enzymes (RNases) secreted in pistils (Torres-Rodrígues et al., 2020) and F-Box proteins located in pollen tubes (Fernandez i Marti et al., 2021). After pollination, the pollen tubes grow towards the pistil, but if the genetic identity of the pollen tubes matches that of the pistils, RNases will degrade male gametophyte RNA, interrupting pollen tube growth and egg fertilization (Fernandez i Marti et al., 2021).

The majority of Japanese plum cultivars (*Prunus salicina* Lindl.) exhibit GSI and, being an allogamous species, depend on compatible pollinating plants that not only possess genetic compatibility, but also floral synchrony to achieve a good fruit set rate and good fruiting in the orchard (Milica et al., 2022). Thus, it is necessary to conduct pollination, pollen grain germination, and pollen tube growth tests in Japanese plum cultivars, aiming to identify the best combinations of plants for optimal reproductive compatibility, aiming to obtain the best genotypes for the orchard and guide research on genetic improvement of the species.

Cultivars such as Laetitia, Fortune, Zafira, Amarelinha, Harry Pickstone, Reubennel, are examples of cultivars well adapted to the climatic conditions of Brazil (Castro et al., 2008), with a moderate chilling requirement below 7.2 °C (200 and 1,000 hours) (Anzanello, 2020). These cultivars demonstrate good productivity in orchards, as long as they have at least 10% pollinating plants and exhibit floral synchrony, since Japanese plum plants display gametophytic self-incompatibility (Castro et al., 2008). In the present study, they were studied alongside other plum cultivars and selections to investigate the reproductive compatibility profile.

The present study investigated the reproductive compatibility between cultivars and selections of *Prunus salicina* Lindl. through controlled pollination, both *in vivo* and *in situ*, to obtain a profile of cultivars compatible with each other.

Materials and Methods

The study was carried out at the Estação Experimental de Videira (EEV) - Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI), Videira, SC Brazil, and at the Laboratório de Anatomia Vegetal of Departamento de Botânica of Universidade Federal de Pelotas - RS, Brazil, for two consecutive years, year 1 (2015) and year 2 (2016) on plants aged 12 years, obtained from grafting. In the field trials (*in situ*), the self-pollination of an F1 population resulting from the cross between Japanese plum cv. Rebelatto[1] (male parent) and cv. Fortune (female parent) was conducted. Forty-five seedlings, identified with the numbers 1 to 45 and obtained from grafting, were used. Flowering branches at the balloon stage were bagged with TNT (fabric-non-woven) for a week (during the anthesis period). After 40 days of bagging, fruit set was verified, as proposed by De Conti et al. (2013).

For the laboratory tests (*in vivo*), branches with approximately 50 flowers from the F1 population were collected, and self-pollinations and controlled pollinations were perfomed using various combinations of plum trees: 'Laetitia' (female parent) pollinated with 'Piamontesa', Selection 'Plum 86-13' (SA 86-13), 'Harry Pickstone', 'Black Amber', 'Bruce', and ' Laetitia' (male parents); 'Zafira' (female parent) pollinated with 'Black Amber', Selection 'SC-17', 'Harry Pickstone', Selection 'SC-15', Selection 'SC-7', and 'Zafira' (male parents); Fortune (female parent) pollinated with 'Amarelinha', Selection 'SC-7', 'America', 'Harry Pickstone', 'Reubennel', and 'Fortune' (male parents). After 120 hours of pollination, all pistils were collected into 2 mL tubes and fixed in a solution of formaldehyde, acetic acid and 70% alcohol (FAA-1:1:8). They were stored in a refrigerator at 4 °C, later softened with 8N sodium hydroxide, and colored with 1% resorcinol according to the method of Wilson & Brown (1957).

For the analyses, 15 pistils/combination from pollination in the *in vivo* assays were mounted between a slide and a coverslip and visualized under a light microscope, using a 10X eyepiece and 40X objective. The pollen grains germinated on the stigma surface, pollen tube growth (PTG), and the classification of PTG levels from 1 to 6 were performed, where N1: pollen tube inside the stigma; N2: pollen tube in 1/3 of the style; N3: pollen tube in 2/3 of the style; N4: pollen tube in 3/3 of the style; N5: tube inside the ovary and N6: pollen tube close to the ovule, as proposed by Bandeira et al. (2011).

In the *in vivo* tests, a completely randomized design was adopted, with three replications of five pistils of each combination. The data were submitted to analysis of variance and the means were compared using the Tukey test at 5% of probability level, with the aid of the SISVAR software

[1] Rebelatto is a genotype collected from a farmer, probably the result of crossing 'Amarelinha' with 'Carazinho'. In this case, the cultivar Carazinho presents the characteristic of self-compatibility.

(Ferreira, 2011). The variables pollen grain germination percentage and PTG were transformed by the square root equation √(x + 1), where “x” corresponded to the observed data.

Results and Discussion

Effective fruit set data for the F1 population of Japanese plums varied considerably between the two years of evaluation when the plants were 12 years old. In year 1 (2015), out of the 45 self-fertilized plants, only the seedling n° 11 showed significant fruit set (6.7%), followed by seedlings numbered 2, 9, 21, and 38, each with 2.0%. Seedlings n° 1, 28, 45 presented 1.3% of fruit set, while seedlings n° 25 and 42 had 0.7%. In year 2 (2016), 11 seedlings fructified; however, they were not the same as those registered in 2015, except for the seedling n° 9 with 8% fruit set. In year 2, the seedling n° 26 showed 19.3% fruiting, followed by seedlings n° 15 (14%), 13 (13.3%), 30 (7.3%), 35 (6.7%), 32 (4.7%), 31, 37, and 40, each with 1.3%, respectively (Table 1).

The data obtained from *in situ* pollination showed low fruit set for most plants in year 1 (except seedling n° 11, with 6.7%) and high fruit set in year 2 in seedlings n° 9, 13, 15, 26, 30, and 35, with percentages above 5%. Bandeira et al. (2011) obtained fruiting percentages close to 5% in Japanese plum genotypes, while Jia et al. (2008) obtained between 8 and 14% of fruit set, demonstrating that percentages of effective fruit set above 5% are normally adequate for a genotype to ensure good production of plums.

Years of regular cold, high temperatures in the spring, and reproductive compatibility favor good fruiting of plum trees, as occurred in studies by Jia et al. (2008) when carrying out reproductive tests similar to ours with Black Amber cultivars. They tested the Hongxinli, Miili, and Black Amber cultivars within a period of four days after pollination in the temperate climate conditions of China.

Japanese plum flowering is abundant, and the branches generally have 1-5 flowers per flowering bud. This density of flowers promotes, under appropriate conditions, abundant fruit set when there is floral synchrony and reproductive compatibility between pollen donor and recipient plants (Bandeira et al., 2011; Wangchu et al., 2021). However, the change this for abundant fruit set when there are unfavorable meteorological variations, lack of floral synchrony, and gametophytic self-incompatibility in many Japanese plum cultivars.

The low effective fruit set is reported by several farmers in the southern region of Brazil in recent years for species of the genus *Prunus* (peach, plum and nectarine trees), a fact associated with unfavorable environmental conditions, such as excessive rain and irregular cold in winter, which occurred in both years of evaluation, according to Centro de Informações de Recursos Ambientais e de Hidrometeorologia de Santa Catarina (CIRAM) of EPAGRI records (Figure 1A-Figure 1B; Figure 2A- Figure 2B, respectively).

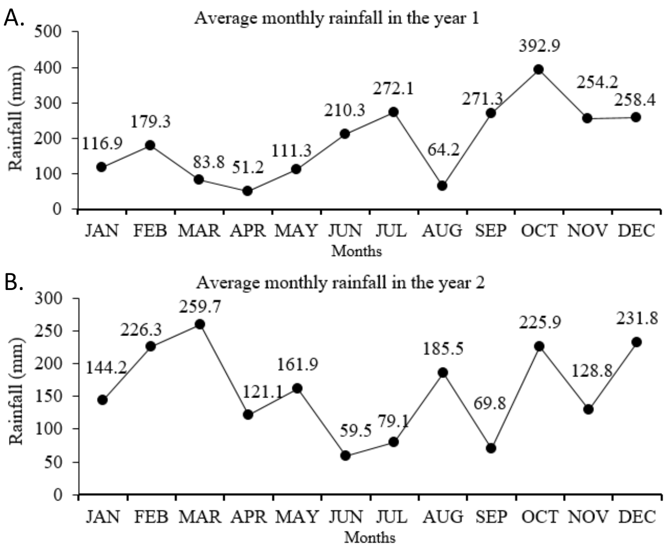
These adverse weather changes for *Prunus* species compromise flowering and stone fruit production (Fadón, et al., 2023), especially in allogamous *Prunus* species. Under climatic conditions that favor excess humidity in the environment, the pollen grains become heavy, do not reach the floral stigma, and consequently, do not bear fruit.

The low winter chill accumulation is also a limiting factor for the production of Prunoids (Fadón et al., 2020). Based on CIRAM-EPAGRI data, there was a low accumulation of cold hours in year 1, in Videira, SC, Brazil (Figure 2A). Such climatic condition possibly influenced the overcoming of dormancy and flowering in most plants of the F1 population in the present study. According to the study by Fadón et al. (2020), the reduced occurrence of temperatures below 7.2 °C, which prevents the accumulation of cold between 430 and 630 hours, can lead to flowering deficit, lack of floral

Table 1. Fruiting percentage of the F1 population of *P. salicina in situ* in EEV - Videira - SC, Brazil.

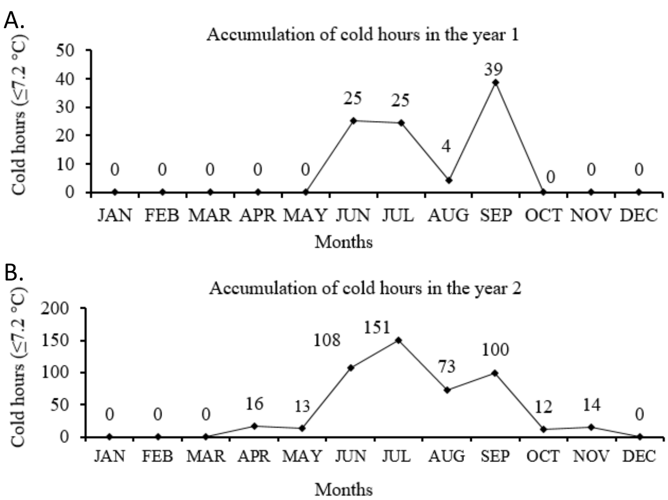
ID[1]	NF[2]		%F[3]		PCL[4]	ID	NF		%F		PCL	ID	NF		%F		PCL
N.	1	2	1	2		N.	1	2	1	2		N.	1	2	1	2	
1	2	0	1.3	0.0	SF	16	0	0	0	0	N	31	0	2	0.0	1.3	SF
2*	3	0	2.0	0.0	SF	17	0	0	0	0	N	32	0	7	0.0	4.7	SF
3	0	0	0.0	0.0	N	18	0	0	0	0	N	33	0	0	0.0	0.0	N
4*	0	0	0.0	0.0	SF?	19	0	0	0	0	N	34	0	0	0.0	0.0	N
5	0	0	0.0	0.0	N	20	0	0	0.0	0	N	35*	0	10	0.0	6.7	SF
6*	0	0	0.0	0.0	N	21*	3	0	2	0	SF?	36	0	0	0.0	0.0	N
7	0	0	0.0	0.0	N	22	0	0	0	0	N	37*	0	2	0.0	1.3	SF
8	0	0	0.0	0.0	SF?	23	0	1	0	0.7	SF?	38*	3	0	2.0	0.0	SF
9	3	12	2.0	8.0	SF	24	0	0	0	0.0	N	39	0	0	0.0	0.0	N
10	0	0	0.0	0.0	N	25*	1	0	0.7	0.0	?	40	0	2	0.0	1.3	SF
11	10	0	6.7	0.0	SF	26*	0	29	0	19.3	SF	41*	0	0	0.0	0.0	N
12	0	0	0.0	0.0	N	27	0	0	0	0.0	N	42	1	0	0.7	0.0	SF?
13*	0	20	0.0	13.3	SF	28	2	0	1.3	0.0	SF	43	0	0	0.0	0.0	N
14	0	0	0.0	0.0	N	29	0	0	0.0	0.0	N	44	0	0	0.0	0.0	N
15*	0	21	0.0	14.0	SF	30*	0	11	0.0	7.3	SF	45*	2	0	1.3	0.0	SF

[1] ID - Identification number of the seedlings in the present study and in the row located in the EEV - Videira - SC, Brazil. [2] NF - Number of fruits produced in the years 1 and 2. [3] %F - Fruiting percentage in the year one (1) and year two (2). [4] PCL - Compatibility level: SF - Self-fertile; N - Non-self-fertile; SF? - Doubtful self-fertility. (*) Total number of individuals self-pollinated *in vivo* at the of EEV – Videira - SC, Brazil, in year 1 and that it was possible to evaluate the PTG in the Laboratório de Anatomia Vegetal of Universidade Federal de Pelotas - RS, Brazil.



Source: CIRAM-EPAGRI - Videira - SC, Brazil.

Figure 1. Average monthly rainfall data for the year 1 and year 2.



Source: CIRAM-EPAGRI - Videira - SC, Brazil.

Figure 2. Data on the accumulation of cold hours with temperature ≤ 7.2 °C of the year 1 and year 2.

synchrony between plants, floral abortion, and absence of fruits, depending on the plum cultivar.

Another important factor associated with plum production is reproductive compatibility, which can significantly contribute to effective fruit set. The individuals of the F1 population evaluated originate from the female parent 'Fortune', a cultivar that has S-alleles linked to gametophytic self-incompatibility (GSI) (Abdallah et al., 2019), a characteristic that may have been inherited in most plants in the population characterizing an impeditive factor for self-pollination, and consequently, for fruiting.

In the *in vivo* tests, the seedlings 2, 4, 6, 13, 15, 21, 25, 26, 30, 35, 37, 38, 41, and 45 were evaluated, and 100% germination of pollen grains on the stigma was obtained, showing the high germination capacity. When evaluating the PTG in the pistils, from the inside of the stigma (N1) and along the style's structure (N2, N3, N4, N5, and N6), it was found that seedlings 4, 6, 21, 30, and 35 showed incidences of PTG by 100% at the N1 level, with higher values compared to plant 25 (91%). In N2, the growth of pollen tubes in genotypes 2, 37, and 38 presented percentages of 3, 2, and 1%, respectively. In N3, male gametophyte growth occurred in plants 2, 4, 15, 25, 26, 37, 41, and 45 with values of 9, 2, 24, 25, 8, 9, 17, and 10%, respectively, with a significant incidence of PTG in plants 15 and 25. In N4, only seedlings 2, 4, 13, 25, and 26 showed 5, 1, 12, 11, and 9%, respectively, and in N6, PTG occurred in plants 13 and 35 with 13 and 6%, respectively, indicating possible self-fertility (Figure 3).

Under controlled laboratory conditions (*in vivo*), a profile of results was obtained from directed pollination with combinations of previously selected cultivars and selections. For 'Laetitia', there was a germination of pollen grains above 99.5%, higher than values observed for 'Piamontesa'. For 'Zafira' and 'Fortune', the percentage of germination was 100% for all combinations (Figure 4A, 4B and 4C, respectively).

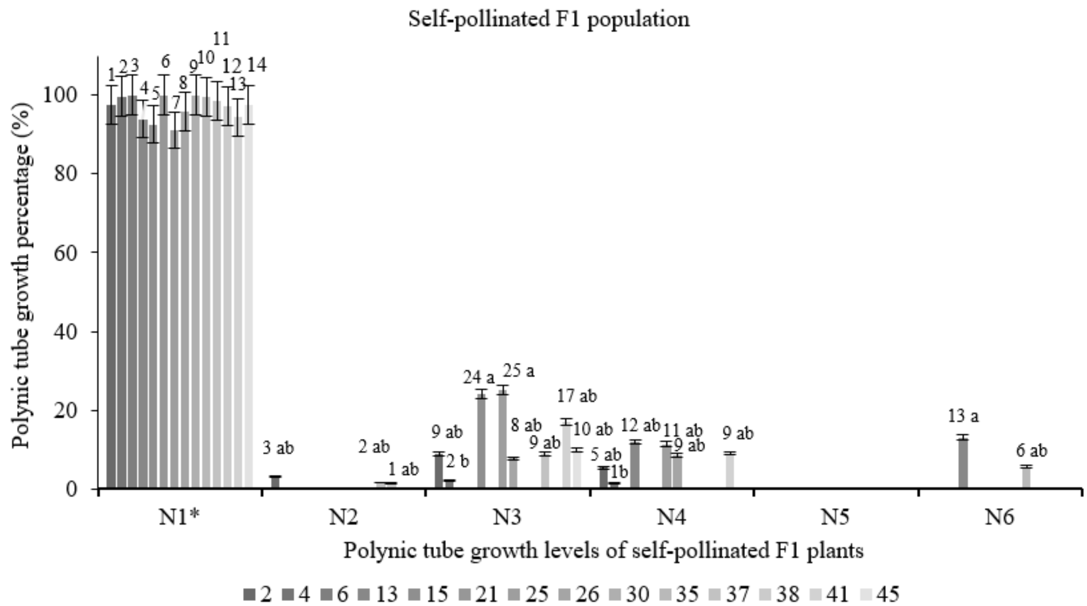
All pollinators used for the 'Laetitia' receptor showed the growth of pollen tubes inside the stigma (N1), with 'Piamontesa' being the pollinator that presented PTG with the highest incidence in this location (Figure 5A). In N2, the mean values of PTG were lower compared to N1, and only 'Piamontesa' showed no tube growth in N2. All pollinators showed PTG in the second and middle third of the style, corresponding to N3 and N4, respectively. For N5 and N6, only the 'SA 86-13' showed growth percentages of pollen tubes, with values of 9 and 13%, respectively, considered the only pollinator compatible for 'Laetitia' in the present study, and which is the main pollinator used by producers in the region of Videira, SC, Brazil.

For the receptor 'Zafira', the growth of pollen tubes of all pollinators tested at level N1 was observed. In N2, only 'Black Amber', 'SC-17', 'SC-15', and 'Zafira' showed PTG, and decreased growth of male gametophytes as the levels along the style were evaluated, as in the N3 levels, N4, and N5, respectively (Figure 5B).

For the 'Fortune' receptor, all pollinators presented PTG in N1. For level N2, there was a decline in growth capacity; however, the pollen tubes continued to lengthen in the style, reaching levels 3, 4, 5, and even level 6, with the exception of cultivars América and Fortune in which it was evidence of PTG near the ovum was not recorded at level 6 (Figure 5C).

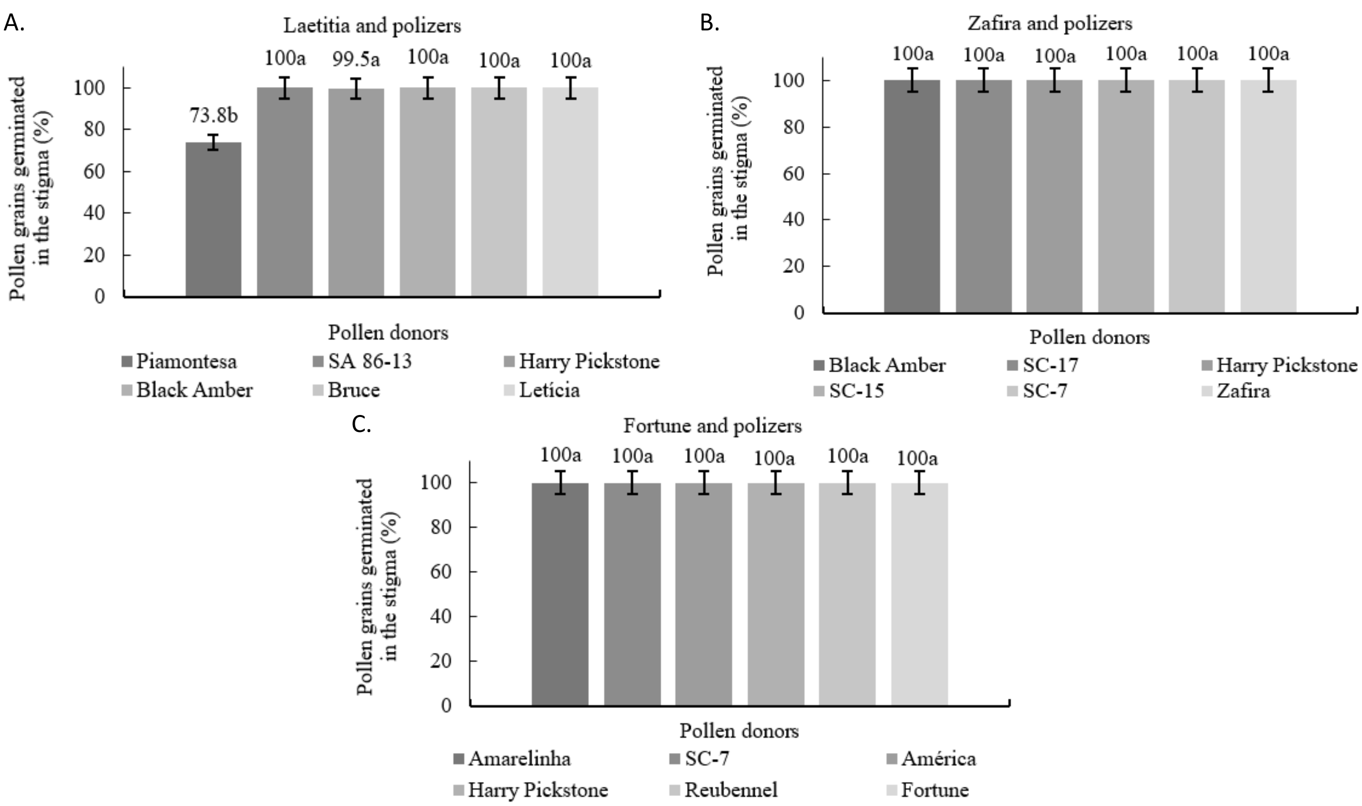
With the percentage of pollen grains germination obtained in the tested cultivars, viability and high germination capacity were verified in the analyzed pistils. Bandeira et al. (2011) obtained about 70% of pollen grain germination, corroborating the data obtained in the present study and ensuring that these percentages are adequate to ensure the continuity of pollen tube growth along the stigma/style in Japanese plum trees.

It is worth mentioning that temperature and humidity influence pollen grain germination. In vitro studies with *P. persica* showed that temperatures around 25 °C are necessary



(a) Means differ statistically. Different letters differ significantly at 5% probability by the same Tukey test. N1*: (1) 2 = 98 ab; (2) 4 = 100 a; (3) 6 = 100 a; (4) 13 = 94 ab; (5) 15 = 93 ab; (6) 21 = 100 a; (7) 25 = 91 b; (8) 26 = 96 ab; (9) 30 = 100 a; (10) 35 = 100 a; (11) 37 = 98 ab; (12) 38 = 97 ab; (13) 41 = 94 ab; (14) 45 = 97 ab. The bars represent the standard error of the mean.

Figure 3. Growth levels of pollen tubes in plant pistils: 2, 4, 6, 13, 21, 25, 26, 30, 35, 37, 38, 41, and 45 of *P. salicina* in the EEV - Videira - SC, Brazil.

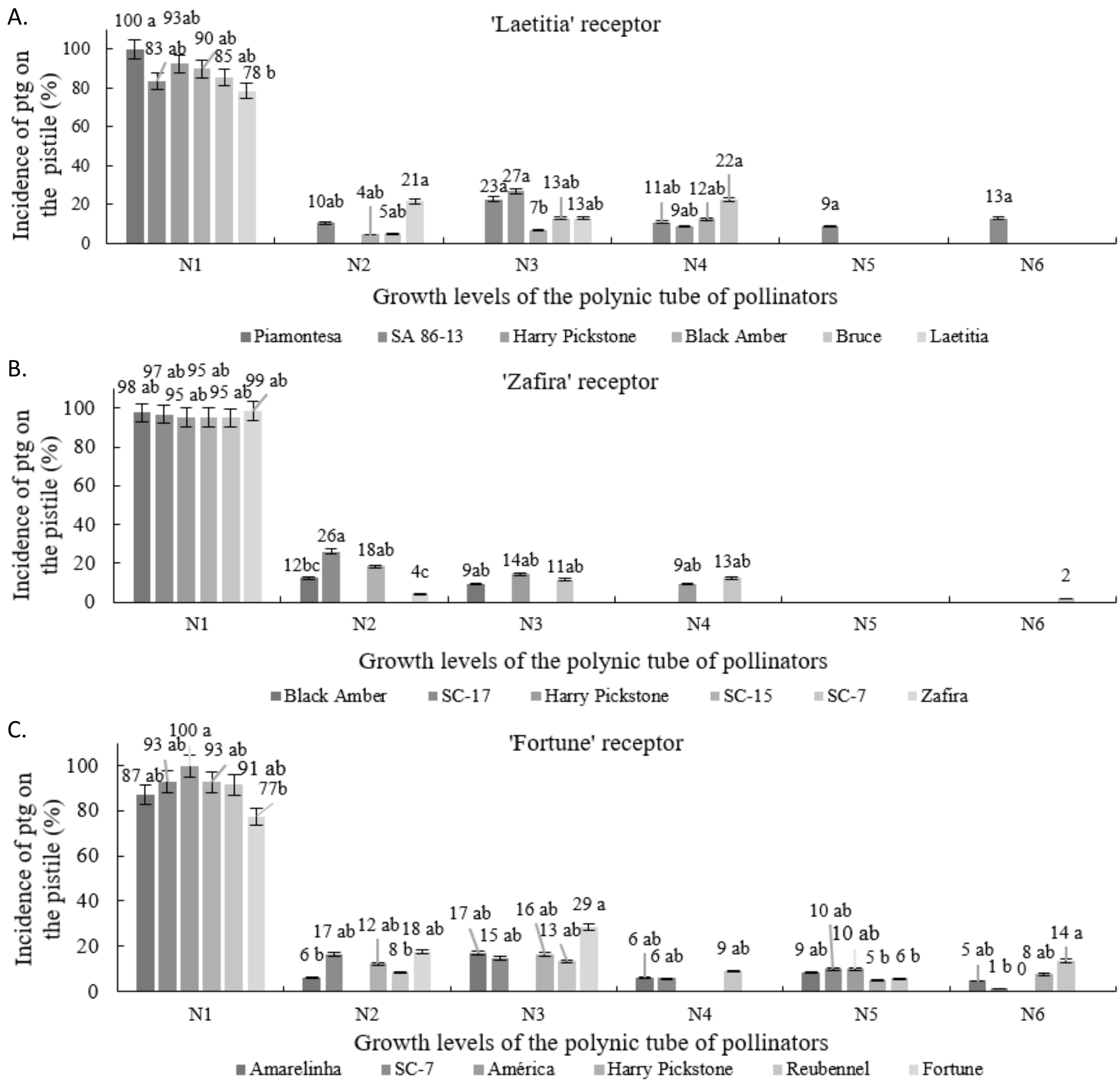


(a) Means with the same letters do not differ significantly. Different letters differ significantly at 5% probability by the same test. The bars represent the standard error of the mean.

Figure 4. Percentage of pollen grain germination in the stigma of flowers of cultivars Laetitia (A), Zafira (B), and Fortune (C) - EEV - Videira - SC, Brazil.

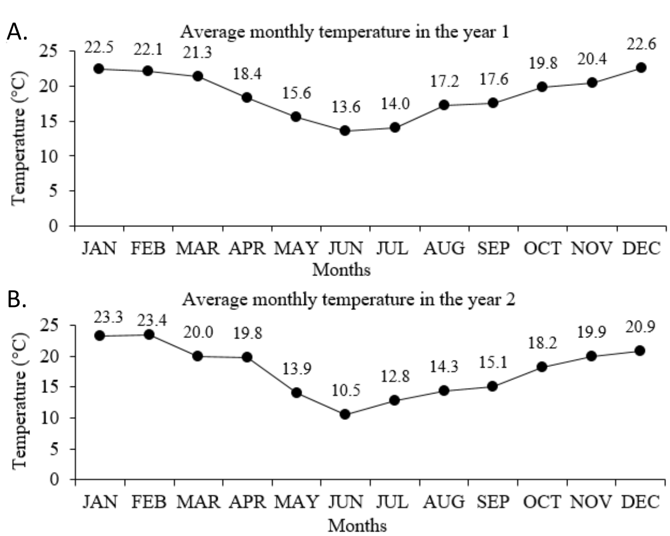
for germination above 60%, with the same values found for *P. avium* (Milatović & Nikolić, 2017). *In situ* and *in vivo* analysis performed in the EEV, the recorded temperatures were around 20 °C, enough to obtain a high percentage of pollen grain germination (Figure 6A and 6B).

The growth of pollen tubes inside the pistil stigma (N1) in the F1 population and in the cultivars and selections showed that the cytotoxic effect of the pistil RNases was not yet efficient to stop the growth of the tubes. CTP is aided by clathrin vesicles found in the tubes themselves,



(a) Means differ statistically. Different letters differ significantly by Tukey test at 5% probability. The bars represent the standard error of the mean.

Figure 5. Growth levels of pollen tubes in *P. salicina* pistils in recipient cultivars Laetitia (A), Zafira (B), and Fortune (C), EEV - Videira - SC, Brazil.



Source: CIRAM-EPAGRI - Videira - SC, Brazil.

Figure 6. Monthly average temperature data for the year 1 and year 2.

which massively absorb RNases, degrading them, thus favoring the initial growth of the male gametophyte (Zhao et al., 2022).

Pollen tubes grow between the tissues of the pistil structure, which under conditions of compatibility mediated by biochemical signaling networks, favors the growth of pollen tubes and the fusion of gametes (Robichaux & Wallace, 2021). However, as pollen tubes grow, RNase concentrations increase along the interior of the stylet to degrade their RNA. This occurs when the genetic identity of the pistil and the pollen tubes are the same, causing an interruption of the growth of the pollen tubes in the pistil, preventing the continuity of the growth of the male gametophyte inside the pistil (Fernandez i Marti et al., 2021).

When the pollinations are compatible, the reticulated and regular deposition of callose occurs in the basal region of the pollen tubes with the function of protecting and allowing the continuity of the growth of the pollen tubes until the egg (De Conti et al., 2013).

Figures 3, 4A, 4B and 4C, show that between N2 and N6, the development of pollen tubes is impaired in the F1 population and in the recipient cultivars Laetitia, Zafira, and Fortune, respectively. The PTG decreases as the inner levels of the pistils are analyzed, and few cultivars are suitable to be used as pollinating plants for the pollen-receiving plants adopted in this study. Therefore, the aptitude of pollinating plants involves the question of pistil acceptability, due to the recognition of reproductive structures, especially RNase enzymes.

RNases are enzymes expressed by S-alleles, a multi-allelic locus, which, according to Gordillo-Romero et al. (2020), may present allelic series "S-alleles" and corroborated by Souza et al. (2019) where the authors identified and sequenced some of these S-alleles, such as "Sh", in 'Laetitia', 'Golden King', and 'SA 86-13', confirming the genetic similarity of the alleles of these cultivars and verifying the semi-incompatibility between these cultivars. Molecular studies and pollination assays are important and integrative to trace a compatibility profile between cultivars, considering the floral synchrony of pollen grain donor and recipient plants for successful Japanese plum fruit production.

Conclusions

The *in situ* and *in vivo* tests for the F1 population confirm the self-compatibility of genotypes 13 and 35, respectively.

The Laetitia cultivar is self-incompatible, but compatible with Selection 'SA 86-13'; 'Zafira' is self-incompatible.

'Fortune' is compatible with 'Amarelinha', 'SC-7', 'America', 'Harry Pickstone' and 'Reubennel', but is self-incompatible.

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Compliance with Ethical Standards

Author contributions: Conceptualization: ROS, VJB; Data curation: ROS, VJB, MAD; Formal analysis: ROS, VJB, MAD, JAF; Investigation: ROS, VJB, MAD; Methodology: ROS, VJB, MAD, JAF; Project administration: VJB, MAD, ROS; Resources: VJB, MAD; Supervision: ROS, VJB, MAD, JAF; Writing - original draft: ROS, VJB; Writing - review & editing: ROS, VJB, MAD, JAF.

Conflict of interest: The authors declare that they have no conflict of interest.

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