Influence of incompatible pollen grains on the reproductive success of Ipomoea asarifolia (Desr.) Roem. & Schult. (Convolvulaceae) in Restinga – RN, Brazil

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Abstract

The deposition of incompatible pollen grains in the stigma can interfere with the performance of compatible pollen grains and compromise the reproductive success of the species in the community. We investigated the influence of incompatible intra- and interspecific pollen grains on the reproductive success of *Ipomoea asarifolia*, based on analyses of natural pollination and controlled experiments. Our hypothesis is that the presence of incompatible pollen grains in the stigma negatively interferes with the formation of fruits and seeds in *I. asarifolia*. Fruit yield was significantly higher in cross-pollination than in natural pollination and mixed pollination experiments of compatible (intraspecific) + incompatible (intra and interspecific) pollens. And seed yield was significantly higher in cross-pollination than between the two mixed pollination experiments. Fruit production was not significant between cross-pollination and natural pollination, nor between natural pollination and mixed pollination experiments. However, fruit and seed production was higher in cross-pollination than in other situations. And experiments with incompatible amounts of pollens do not form fruits. Few fruits and seeds are formed in the presence of incompatible pollens (intra and interspecific), which may be interfering with reproductive success in *I. asarifolia*, especially in the long term. We reinforce the importance of the selection of reproductive displacement in the species to minimize the flow of incompatible pollens and their reproductive interferences.

1 Introduction

Reproductive isolation is common to many species, constituting an important condition for maintaining evolutionary independence (Coyne and Orr 2004; Ortiz-Barrientos et al. 2009). There are several reproductive barriers that can act by disrupting interspecific interbreeding and among them are differences in geographic distribution, phenology, pollinators and floral adaptations and impediments in the germination and fertilization of interspecific pollen grains (Hopkins 2013; Baack et al. 2015). On the other hand, sympatric species with similar floral attributes can invest in flowering synchrony to increase the attractiveness and frequency of pollinators, especially in disturbed environments where there is a shortage of pollinators (Otárola and Rocca 2014). However, this pollination strategy can generate competition for pollinators and result in sharing of floral visitors and the flow of pollen grains between species (see Otárola and Rocca 2014; Runquist and Santon 2013; Randle et al. 2018).

In this case, incompatibility reactions can act by preventing the recognition of interspecific pollen, avoiding hybridization (Hopkins 2013; Baack et al. 2015). However, even if the integrity of the species is maintained, the incompatible pollen flow can have negative consequences for its reproduction (see Morales and Traveset 2008). Interspecific pollen grains can physically block the stigma and adherence of intraspecific pollen (Caruso and Alfaro 2000; Da Silva and Sargen 2011; Dickinson et al. 2012; Runquist and Stanton 2012). In addition, in some species it can compromise stigmatic receptivity (Waser and Fugate 1986) and inhibit germination and development of pollen tubes (Thomson et al. 1981; Galen and Gregory 1989; Murphy and Aarssen 1995; Bronw and Mitchell 2001; Arceo-Gómez and Ashman 2011). Intraspecific pollen commonly excels in the germination and fertilization of ovule over interspecific pollen,

which is another important reproductive barrier (Rieseberg et al. 1995; Fishman and Wyatt 1999; Howard 1999; Campbell et al. 2009). However, when interspecific pollen grains are in high amounts in the stigma, the effectiveness of this reproductive barrier may be compromised (Morales and Traveset 2008; Jakobsson et al. 2008; Briggs et al. 2016). Briggs et al. (2016), in studies with *Delphinium* sp. (Ranunculaceae), found that a large amount of interspecific pollens resulted in greater reproductive damage. In some species even smaller amounts of interspecific pollen can be harmful (e.g. Arceo-Gómez and Ashman 2011; Moreira-Hernández et al. 2019). Self-pollination in self-incompatible species can also cause reproductive interference, resulting in stigma obstruction. This type of pollination is common in species with hermaphrodite flowers (Webb and Lloyd 1986; Barrett and Harder 1996), especially when reproductive structures are close (Miller et al. 2002).

Kill and Ranga (2003), verified that *Ipomoea asarifolia* is self-incompatible and presented high fruit formation under natural conditions in the Caatinga. However, *Ipomoea* species share similar floral attributes (see Marinho et al. 2021) and when they occur in sympathy there is a probability of interspecific pollen flow. In a study in a coastal environment, Santos et al. (in prep.) found that *I. asarifolia* occurs in sympathy with *I. brasiliana*, have small and close populations, with overlapping blooms for a long period and share the same main pollinators, which transfer pollen in the same region of the body. These factors are strong indications that pollen flow may occur between *I. asarifolia* and *I. brasiliana* (see Campbell and Motten 1985; Silvertown et al. 2005; Kudo 2006). Although it has been proven that there is a displacement of reproductive characters that can reduce the interaction between these species in the study area, this constitutes a set of reproductive barriers of partial isolation, which do not totally prevent contact and, consequently, pollen flow between *I. asarifolia* and *I. brasiliana* (Santos et al. in prep.). In addition, the proximity of reproductive structures can result in deposition of autopollen (Kiil and Ranga 2003).

Thus, *Ipomoea asarifolia* is an interesting model to investigate the influence of incompatible inter- and intraspecific pollen grains on the success of compatible pollen (see Morales and Traveset 2008). The present study aimed to analyze the production of fruits and seeds from natural pollination and experiments of manual cross-pollination and pollination with mixture of pollen grains (intraspecific and interspecific) in individuals of *I. asarifolia* located in Restinga. Our hypothesis is that the presence of incompatible pollen grains in the stigma negatively interferes with the formation of fruits and seeds in *I. asarifolia*.

2 Material and Methods

Data collection and field observations were carried out in the Jenipabu Environmental Protection Area (APAJ), located in the state of Rio Grande do Norte, Brazil. The APAJ consists of 1,881.89 hectares which is composed of a Restinga ecosystem (see Correia et al. 2020). The climate is considered tropical rainy, with rainfall in winter and in the dry period of summer. The average temperature is 26.6º C and the average annual rainfall is 1456.6 mm, with an average temperature of 26.6º C and relative humidity of 70% (Nuc-Idema 2009). The management plan of APAJ (2009), divides area into seven geoenvironmental

units: Fixed Dunes, Mobile Dunes, Deflation Plain, River Plain, Flood-Marine Plain, Coastal Deck and Beach Zone (Nuc-Idema 2009). The experiments were carried out in areas of fixed and mobile dunes and coastal board (Marinho et al. 2021), which are areas that suffer great influence from the environment, with strong winds and high temperatures and anthropogenic activities related to tourism and subsistence agriculture (Nuc-Idema 2009). The species *Ipomoea asarifolia*, whose genus corresponds to the most representative of the family Convolvulaceae, was studied (Muñoz-Rodríguez et al. 2019; Wood et al., 2020) and, in the study area, plays an important ecological role in the maintenance and fixation of dunes and other local ecosystems (Nuc-Idema, 2009). *Ipomoea asarifolia* is considered self-incompatible (Kill and Ranga 2003) and has hermaphrodite flowers that have reproductive structures with similar heights (Santos et al. in prep.).

To evaluate the reproductive system and the success in the reproduction of *I. asarifolia* in the study area, the following experiments were performed: I. Apomixis in buds in pre-anthesis that were emasculated and isolated until the end of anthesis (N = 30); II. Natural pollination (control) (N = 49) in freshly opened, marked flowers left free for pollination (Radford et al. 1974); III. Manual self-pollination crosses in previously bagged flowers (N = 32); IV. Manual cross-pollination in flowers previously bagged and pollinated with pollen from other individuals about 100 meters away (N = 55). In all experiments flowers were distributed in three populations, and in approx. 10 to 15 individuals, all about 30 m apart. Subsequently, they were followed for 30 days and the formation of fruits and seeds was recorded (Radford et al. 1974).

To analyze the influence of incompatible pollen on fruit and seed formation, manual crosses were performed in *Ipomoea asarifolia* using mixtures of pollen grains in approximate amounts of PIntraC (compatible intraspecific pollen) x PIntraI (incompatible intraspecific pollen) (N = 31 flowers) or PIntraC x PInterI (incompatible interspecific pollen) (N = 25 flowers). Manual crosses were also performed with mixtures of pollen grains in 2x higher amounts of PIntraI (N = 32 flowers) and PInterI (N = 36 flowers). The compatible intraspecific pollens came from flowers of other populations (distant ca. 100 meters from the receiving population) and the incompatible intraspecific pollens from the crossed flower itself (i.e., autopollen). For the PInterI crosses, pollen grains of *Ipomoea brasiliana*. This species occurs in sympathy with *I. asarifolia*, both have similar floral attributes, have overlapping flowering and share a high frequency of pollinator visits, which are considered main for both species and transfer pollen grains in the same region of the body (Santos et al. in prep.). To determine the appropriate amounts to perform the pollen mixtures, the grains were counted directly under a microscope (N = 10 buds/species, 5–10 individuals). *Ipomoea asarifolia* presents an average of 2739.1 ± 174.0 (average ± d.p.) grains, while in *I. brasiliana* the average is approximately twice as high (4476 ± 429,6).

Thus, the following experiments were carried out with the mixtures of pollens:

I. 2 anthers of PIntraC + 1 anther of PInterI (approximate quantities of PIntraC x PInterI);
II. 1 anther of PIntraC + 1 anther of PIntraI (approximate quantities of PIntraC x PIntraI);
III. 1 anther of PIntraC + 1 anther of PInterI (2x greater quantity of PInterI in relation to PIntraC);

IV. 1 anther of PIntraC + 2 anthers of PIntraI (2x greater quantity of PIntrarI in relation to PIntraC).

The crossings were performed with the aid of eppendorf and brush. The experiments were carried out on flowers of approx. 10 to 15 individuals about 30 m apart and distributed in three populations. The flowers used were previously emasculated and isolated (with “voil” bags) and after crosses were marked and checked for fruit and seed formation after a period of 30 days.

The G2×6 test was used, with Cramer correction, followed by a pair-to-peer post-hoc analysis (adjusted by the Bonferroni method) to verify the association between fruit production and the presence of incompatible pollen grains (general chi-square result), verifying the differences in each level of combination of these two factors (comparison between pairs). Since the seed production was very low from the fruits formed in the experiments of crosses with pollen mixture in approximate quantities (PIntraC x PInterI and PIntraC x PIntraI), the sum of these seeds produced in these two experiments was made to form a single category. As in the experiments 2x PInterI x PIntraC and 2x PIntrarI x PIntraC there was no seed formation (there was no fruit formation), these did not enter into statistical analysis. For the statistical analysis, the results of seeds produced were compared to the number of ovules produced in the species (4.0 ± 0.0; mean ± .sd – information previously collected). Thus, the G 2x2 test was applied, with Cramer correction, followed by a pairwise post-hoc analysis (adjusted by the Bonferroni method) to verify the association between seed production and the presence of incompatible pollen grains, verifying the differences in each level of combination of these two factors (i.e., natural pollination, cross-pollination, PIntraC x PInterI, PIntraC x PIntraI, 2x PInterI x PIntraC, 2x PIntraI x PIntraC). The G test was performed with the DescTools package (Signorell 2023); pairwise post-hoc analysis was performed using the pairwise Nominal Independence function of the r companion package (Mangiafico, 2022).

3 Results

From the crosses of the reproductive system, it was found that *Ipomoea asarifolia* is self-incompatible and does not produce fruits by apomixis (Table 1). An association was observed between fruit production and the presence of incompatible pollen grains in the stigma ($G = 81.4$, $df = 5$, $p < 0.001$). According to the pair-to-peer post-hoc analysis, fruit yield in manual cross-pollination was significantly higher than natural pollination and each of the mixed pollination experiments. Among the other pairs of experiments there was no significant difference.

Table 1
Experiments of manual pollination, control and mixing of incompatible pollen grains (intra and interspecific) in *Ipomoea asarifolia* at APA Jenipabu - RN, Brazil.

Experiments	Flower/Fruit (%)	Seed (%)
Manual self-pollination	32 / 0 (0)	0
Apomixis	30 / 0 (0)	0
Natural pollination	49 / 13 (26,5)	39 (75)
Cross-pollination	55 / 32 (58)	112 (93)
Pollen grain mixture I (approximate quantities PIntraC x PInterI)	32 / 2 (6,2)	4 (50)
Pollen grain mixture II (approximate quantities PIntraC x PIntraI)	25 / 1 (4)	2 (50)
Pollen grain mixture III (2x PInterI x PIntraC)	32 / 0 (0)	0 (0)
Pollen grain mixture IV (2x PIntraI x PIntraC)	36 / 0 (0)	0 ()

For seed production, an association was observed between ovule production and seed production (G = 10.7, df = 2, p = 0.004). Seed production in cross-pollination was significantly higher than between the two mixed pollination experiments (PIntraC x PInterI and PIntraC x PIntraI). Seed production was not significant between cross-pollination and natural pollination, as well as between natural pollination and mixed pollination experiments. In all situations, fruit and seed production was higher in cross-pollination (Table 1). The mixtures of pollen III (2x PInterI x PIntraC) and IV (2x PIntraI x PIntraC) did not form fruits (Table 1).

4 Discussion

The results of this study confirm our hypothesis that the presence of incompatible pollen grains in the stigma of *I. asarifolia* flowers negatively interferes in fruit production, consequently in seed production. The percentage of fruits and seeds produced was low in the experiments of mixing more incompatible pollen (intra and interspecific) in similar amounts and there was no production when this relationship was twice as high with the amount of incompatible pollen deposited in the stigma. In all situations of pollen mixing there was a significant difference in fruit production when compared to manual cross-pollination. The condition of the presence of incompatible grains in the stigma can be aggravated when the species is self-incompatible, as in the species studied. In addition, *Ipomoea asarifolia* has close reproductive structures (Santos et al. in prep.), which contributes to self-pollination in the species (see

Miller et al. 2002). In this case, the proximity between the reproductive structures facilitates the deposition of pollen itself, interfering in the germination of the grains in the stigma and reproduction of the species (Kawagoe and Suzuki 2005; Parra-Tabla and Bullock 2005; Navarro et al. 2012). Parra-Tabla and Bullock (2005), in studies with *I. wolcottiana*, a self-incompatible species, also noted that the proximity between the reproductive structures favored the self-deposition of pollen and proved its reproductive interferences. Deposition of incompatible pollens in the stigma can obstruct and influence stigmatic receptivity (Caruso and Alfaro 2000), inhibit germination and pollen tube formation in compatible pollens (Brown and Mitchell 2001; Parra-Tabla and Bullock 2005), or even, in situations of late-acting incompatibility, can lead to ovules abortion, reducing the number of seeds (e.g. Kawagoe and Suzuki 2005; Da Silva and Sargent 2011; Navarro et al. 2012; Moreira-Hernadez et al. 2019). In the case of the species studied here, even small amounts of interspecific pollens can add to the deposits of pollen itself and compose a large amount of incompatible pollens and interfere with the reproductive success of the species in the study area.

In *I. asarifolia*, interference of incompatible pollen grains, either by self-pollination (due to nearby reproductive structures) or by pollen from other species (pollinator action) has reflected in the natural production of fruits, which was lower when compared to manual cross-pollination. In the study area, *I. asarifolia* occurs in sympathy with *I. brasiliana*, forming close populations and both share the main pollinators, mainly the bee Centris sp. which constitutes the main pollinator in both species (Santos et al. in prep.). This situation favors the interspecific pollen flow between *I. asarifolia* and *I. brasiliana* (Aizen 2006; Cheptou and Avendaño 2006; Otárola and Rocca 2014; Norton et al. 2015; Randle et al. 2018). Still, according to Santos et al. (in prep.), *I. asarifolia* and *I. brasiliana* present displacement of reproductive floral characters, which may have evolved in response to the sharing of pollinators and function as prezygotic reproductive isolation barriers, which can reduce the exchange of pollen between these species. However, the displaced characters between *I. asarifolia* and *I. brasiliana* do not totally isolate them, functioning as partial reproductive isolation barriers (Santos et al. in prep.). Thus, there is still the high possibility of interaction between species and consequently pollen flow between them.

Regarding seed production, there was a significant difference when compared to cross-pollination and pollen mixture pollinations, but there was no difference in seed production between natural pollination and pollen mixing experiments. Although there was no difference, numerically a lower overall seed production (natural pollination + pollen mixtures) is perceived when compared with seed production in cross-pollination (without interference of incompatible pollen). That is, this condition reinforces the interference of incompatible pollen grains in the stigma, a situation that may be occurring in the study area due to the sharing of pollinators. The amount of interspecific pollen deposited in the stigma constitutes an important factor in defining its impacts on reproduction (Morales and Traveset 2008). Considering that the studied species shares pollinators and presents overlapping flowering with *Ipomoea brasiliana*, the reproductive success in *I. asarifolia* depends on strategies that avoid the frequency of arrival of incompatible pollen in the stigma, demonstrating the importance of the evolution of the displacement of reproductive characters between these species so that the reproductive interferences from this interaction are mitigated and the populations of *I. asarifolia*, via sexual reproduction, are kept in

the study area (Morales and Traveset 2008; Pfennig and Pfennig 2010). Even if *I. asarifolia* is able to reproduce asexually (Kill and Ranga 2003), it does not offer genetic variability to individuals and long-term population maintenance.

Our study provides important information about the reproductive success and consequences of the deposition of incompatible intra and interspecific pollens for the reproduction of *I. asarifolia* in a Restinga area, which suffers constant environmental and natural disturbances, and this species is of great ecological importance for the maintenance of dunes in the study area. *Ipomoea asarifolia* showed low fruit formation under natural conditions, suggesting that some factor may be interfering with the reproductive success of this species in the study area. Our results showed that very few or no fruits were formed in the presence of incompatible pollens (intra and interspecific), leading to losses in the reproductive success of both fruit and seeds, especially in the long term. This evidence reinforces the importance of selection of the displacement of reproductive characters in the studied species, as well as can be extrapolated to other species that also share pollinators.

Declarations

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Author contributions AVL, NMA and MTB carried out the research conceptualization and AVL and NMA the conceptualization. BYMS and KMC carried out the methodology and investigation. BYMS did the original draft, and AMMS and AVL did the formal analysis and writing (revision and editing). AVL provided overall supervision of the study.

Data Availability The set of raw data generated in this study can be made available by e-mail request to the author for correspondence.

Conflict of interests We declare that there is no conflict of interest in this study.

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