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REPRODUCTIVE BIOLOGY - ORIGINAL ARTICLE

Reproductive biology and hybridization of *Physalis* L. species

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Abstract

The hybridization of the species belonging to the *Physalis* L. genus has been seldom to date, preventing the combination of distinct characteristics. Thus, assessments concerning basic reproductive biology and inter-specific compatibility of crosses are required for breeding. The present study aimed to evaluate pollen viability, stigmatic receptivity, and the compatibility of self-pollinations and inter-specific crosses in *Physalis* species. *P. angulata* L., *P. ixocarpa* Brot., *P. pruinosa* L., *P. peruviana* L., *P. pubescens* L., *P. minima* L., and *P. daturifolia* Lam, were investigated, each represented by one accession. The viability of pollen grains was determined through in vitro germination. Stigma receptivity was determined using 3% hydrogen peroxide (H_2O_2) with flower buds in pre-anthesis and anthesis, every two hours, from 6:00 am to 6:00 pm, a'. A total of 50 artificial crosses were performed for each hybrid combination and self-pollination. Pollen germination ranged between 43.65 and 75.30% among *Physalis* species. Stigma receptivity was greater during anthesis for all species and the time of day influences receptivity. Self-compatibility for all *Physalis* species was observed, and fruit fixation occurred in most inter-specific crosses, except when *P. daturifolia* was used in hybrid combinations.

Keywords Breeding · Hybridization · Inter-specific crossing · Seed production · Self-compatibility

1 Introduction

Physalis L. is a small exotic fruit belonging to the Solanaceae family (Jagatheeswari 2014). It originates in Andean countries and can be grown in tropical and subtropical regions (Sun et al. 2017). Some *Physalis* species have edible fruits with relevant chemical properties, including a considerable amount of β-carotene content and the high content of soluble solids, flavonoids, carotenoids, alkaloids and terpenes (Feng et al. 2016; Silva et al. 2016a; Saavedra et al. 2019), which make them an important plant genetic resource (Silva et al. 2016b).

The inter-specific variability of *Physalis* allows for plant morphology diversity and variations in fruit size, shape, and chemical characteristics (Aliero and Usman 2016). *Physalis* exhibits some edaphoclimatic peculiarities that do not allow for wide cultivation (Salazar et al. 2008; Fischer et al. 2014). However, sexual propagation allows genotype development improvement through heterosis (Silva et al. 2016b) and may enable the emergence of more productive plants adapted to different climatic conditions, as well as other features of interest.

The most exploited commercial species are *P. peruviana* L. (Fischer et al. 2014) and *P. ixocarpa* Brot., cultivated mainly in Mexico (Zamora-Tavares et al. 2015). Other species, despite having fruits with low commercial potential, can be used to donate genes in the development of more adapted and stable genotypes, with important nutritional components (Lima et al. 2013, 2020).

Exploring interspecific genetic variability may allow the insertion of genes that improve the agronomic productivity of plants. In addition to this gain, the main advantage of

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hybridization and obtaining new genotypes is the aggregation of traits to plants in terms of resistance to diseases and, above all, in increasing the concentration of compounds of nutritional and pharmacological interest in the fruits (Amaro et al., 2017; García-Fortea et al. 2019), improving their quality and adding value to production at the time of marketing. The hybridization of species belonging to the *Physalis* genus has been seldom to date, and basic reproductive biology and intra- and inter-specific crossbreeding studies are required for the establishment of a breeding program.

Knowledge of aspects concerning pollen grain viability and stigma receptivity is essential for performing artificial crosses (Fu et al. 2017; Soares et al. 2018). One of the most practical and viable ways of determining the viability of pollen grains is in vitro germination (Nogueira et al. 2015). By exploring stigma-related aspects, it is possible to determine its most receptive time of day (Crispim et al. 2017). A receptive stigma can receive pollen grains and allow their germination. A quick and inexpensive technique for determining stigma receptivity is through the use of 3% hydrogen peroxide (H_2O_2) (Zambon et al. 2018), as stigmas in contact with this compound emit air bubbles that allow for receptivity estimations based on their activity.

The *Physalis* species are mostly self-compatible (Azeez and Faluyi 2018) and generally autogamous (Djakbé et al. 2017). However, some may display self-incompatibility in reproductive characteristics (Peña-Lomelí et al. 2018). In general, this aspect may vary from one species to another and requires a better understanding. In *Physalis*, pollen can be incompatible when it contains one or more alleles present in the pollen grain and the stigma (Pandey 1957). Concerning inter-specific crosses, F_1 genotypes exhibit high heterosis that promotes genetic gains (Bedinger et al. 2011). However, regarding inter-specific *Physalis* crosses, basic aspects regarding compatibility for fruit formation and seed viability to generate new individuals still require elucidation.

Considering the above-mentioned information, the present study aimed to evaluate pollen grain viability, stigma receptivity, and self-pollination, and inter-specific cross-compatibility in *Physalis* species.

2 Material and methods

2.1 Experimental material

Seven botanically identified *Physalis* L. species were used: *P. angulata* L., *P. ixocarpa* Brot., *P. pruinosa* L., *P. peruviana* L., *P. pubescens* L., *P. minima* L. and *P. daturifolia* Lam. Each species was represented by an accession represented by seeds from different plants, but from the same origin and currently belonging to the didactic collection of the Western Parana State University. The seedlings were obtained through seeds in expanded polystyrene trays comprising 128 cells containing a commercial substrate based on bio-stabilized pine bark and kept in a greenhouse until exhibiting four fully expanded leaves. Thirty seedlings of each species were transplanted to 8 dm^3 pots containing sieved soil and used for the next experimental steps. The soil in the pots was corrected in advance, according to chemical soil analyses, through the application of calcitic limestone to raise the base saturation to 80% and maintain a 4:1 ratio between calcium and magnesium. To perform basic planting fertilization, 15 g of nitrogen, phosphorus, and potassium (NPK) fertilizers were used as a 04–20-20 formula and 7.0 g of simple superphosphate per plant were applied. The plants were maintained in a greenhouse, with the objective of standardizing the cultivation conditions among the species. In addition to favoring the development of plants by restricting the stresses caused by external environmental conditions and facilitating the control of hybridizations through the elimination of the visit of natural pollinators. The plants were conducted with the main stem, tutored employing vertical cuttings. Irrigation was carried out according to plant water requirements through micro-drippers. Phytosanitary control was performed by preventive spraying with thiamethoxam, copper oxychloride + mancozeb, and azoxystrobin + difenoconazole. Leaf sprays were performed weekly with the commercial fertilizer Nutrioxi CAB 105®.

2.2 Pollen grain viability

When the plants reached their reproductive stage, 20 flowers of each species in full anthesis were collected at 9:00 am, placed in Petri dishes, and sent to the Plant Tissue Culture Laboratory. To release the pollen grains from the anthers, the flowers were placed face down. Pollen grains were evaluated in vitro for germinative viability (Silva et al. 2017). According to this methodology, 1 g of culture medium was used to prepare 8 g of agar, 140 g of sucrose, 514.8 g of calcium nitrate, 484.8 mg of boric acid, and distilled water and pH adjusted to 5.4.

The pollen grains of the flowers were distributed on Petri dishes containing 20 ml of the culture medium with the aid of a soft bristle brush and incubated in a BOD chamber, at 25 °C for germination. After 24 h, the pollens were observed for germination under 100-fold magnification using a microscope. The pollen grain was considered to have germinated when the length of the pollen tube was greater than the grain diameter. Eight repetitions were used, each represented by a count of 400 pollen grains. The percentage of pollen germination was obtained based on the number of germinated pollen grains concerning the total number of pollen grains.

2.3 Stigma receptivity

The stems of *Physalis* flower buds were evaluated in two stages, pre-anthesis and anthesis, collected from flowers every two hours, during the day between 6:00 am and 6:00 pm, for three days (Zambon et al. 2018). The stigmas were placed on coverslips, covered by drops of (H_2O_2) at 3%, and observed under an under microscope for the formation of air bubbles in the entire stigma region. The faster the emission of air bubbles, resulting from the breakdown of peroxide by the action of the peroxidase enzyme, the greater the receptivity of the stigma. (Kearns and Inouye 1993). Stigmas were considered very receptive (score 4), when many air bubbles were released at high speed, making it impossible to perform a visual count of the emitted air bubbles, receptive (score 3) when bubbles were released at medium speed, allowing for visual counting of the emitted air bubbles, slightly receptive (score 2), when only a few bubbles were released at low-core speed, and non-receptive (score 1), when no bubbles were formed. All evaluations were carried out in triplicate using five stigmas.

2.4 Inter-specific crossings

Crosses were performed after determining pollen viability and the best stigmatic receptivity time. All crosses and their reciprocals were performed in a diallel crossing scheme, generating 42 hybrid combinations. In addition to the crossings, parental self-pollination was also carried out.

A total of 50 artificial crosses were performed for each hybrid combination and 50 manual self-pollination was performed for each parent from the beginning of flowering, totaling 2450 pollinations. Using tweezers and a hand-held vibrator, the pollen grains of flowers in full anthesis were removed and placed in adequately identified containers. Subsequently, the flower buds were emasculated before the anthesis, using sharp tweezers to facilitate bud opening and removal of the anther cone. Immediately after emasculation, the flowers were manually pollinated, by leaning the stigma on the container containing the collected pollen. The same procedure was carried out for self-fertilization, which in turn used pollen from another flower of the same species. After pollination, the flowers were adequately protected and identified.

Ripe fruit was collected between 30 and 40 days after pollination, depending on the genotype. To evaluate the paired combination of species, three indices were determined at each crossing, namely the fruit fixation index, number of seeds, and the percentage of seed germination. The fruit fixation index of artificial crosses was determined by counting the fruits developed from artificial pollination, expressed as a percentage. In sequence, the number of seeds per fruit was obtained by manual counting. To obtain the germination percentage, four replicates of 25 seeds were used, sown in gearbox boxes containing paper moistened with distilled water at 2.5 times its weight, according to recommendations. Then, placed in a germinator with constant temperature of 25 °C and photoperiod of 8 h. Seeds meeting the botanical criteria of root protrusion were considered germinated.

2.5 Statistical analyses

The stigma receptivity data and the percentage of germinated pollen grains and seeds were transformed into $(x/100)^{1/2}$ arc sine, and subsequently subjected to an analysis of variance by the F test. Using residual variances, data were tested for normality and homogeneity. When the F test was significant and the assumptions of normality and homogeneity were met, the data were subjected were subjected to means comparison tests ($p < 0.05$ level). The Tukey HSD test was used to compare the means of in vitro germination of pollen grains and the evaluation times of stigma receptivity to *Physalis* species. Student's *t* test was used to compare pre-anthesis and anthesis stigmas. The results of the fruit fixation index and number of seeds were not submitted to any statistical analyses. All statistical analyses were performed using the statistical program genes (Cruz 2013).

3 Results

3.1 Pollen grain viability

P. ixocarpa exhibited the highest percentage of germinated pollen grains in vitro (75.34%), followed by *P. pruinosa* (60.10%) and *P. mínima* (59.60%), which were not significantly different ($p < 0.05$). *P. angulata* (57.62%), *P. pubescens* (54.62%) and *P. daturifolia* (51.53%) were not significantly different, although they occupied an intermediate position, resembling *P. angulate*, *P. pubescens* and *P. peruviana*, the latter exhibiting the lowest germination (Fig. 1).

3.2 Stigma receptivity

With flower buds in pre-anthesis, the highest stigma receptivity for *P. angulata* was at 8:00 am, 10:00 am, and 12:00 pm (receptive). For *P. ixocarpa,* this was observed at 10 am (very receptive) and for *P. pruinose*, at 6:00 am, 8:00 am, 10:00 am and 12:00 pm (receptive). For *P. peruviana*, highest stigma receptivity was noted at 10 am and 12 pm (receptive), for *P. pubescens*, at 4 pm (very receptive); for *P. minima*, at 12 pm, 2 pm and 4 pm (very receptive), and for *P. daturifolia*, at 12 pm and 2 pm (very receptive). When in full anthesis, all species had a very receptive stigma in at least one of the assessed schedules. For the full anthesis of the species *P. angulata*, the best receptivity was at 4 pm, for

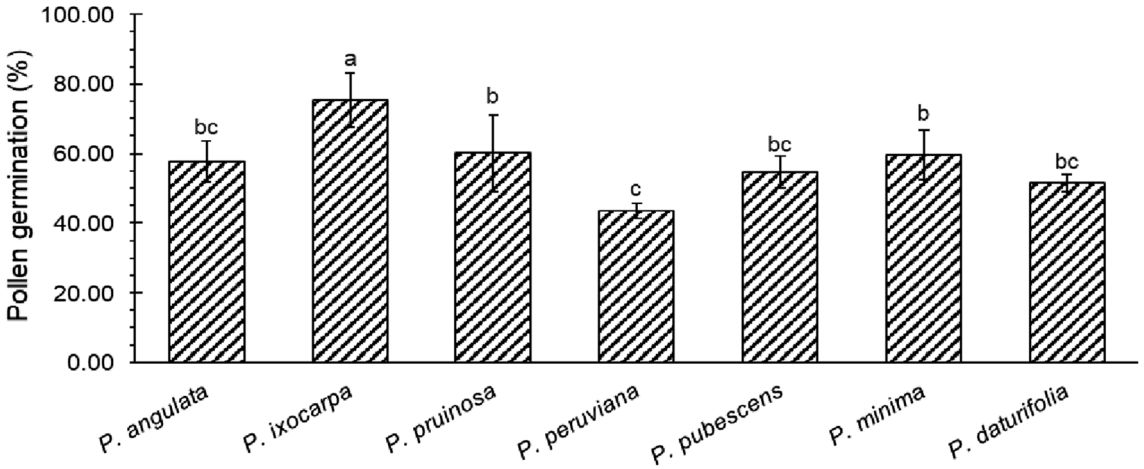

Fig. 1 In vitro germination of pollen grains in *Physalis* spp.. Different letters indicate a significant difference between treatments at $p < 0.05$ level (Tukey HSD test). Bars indicate the standard deviation of the means. Results represent the mean of eight replicates ($n = 8$) and each represented by a count of 400 pollen grains

P. ixocarpa, *P. peruviana,* and *P. daturifolia* from 8 am to 6 pm, for *P. pruinosa* and *P. pubescens* from 8 am to 4 pm, and for *P. minima* from 10 am to 6 pm (Fig. 2). For all species, the best receptivity for stigma occurs when the flowers are in full anthesis (Fig. 3).

3.3 Cross compatibility

Self-compatibility was observed for all investigated *Physalis* species, with *P. peruviana* and *P. pubescens* as the most noteworthy, exhibiting 100 and 94% fruit fixation, respectively. For self-pollinated *P. ixocarpa, P. minima, P. angulata. P. pruinosa* and *P. daturifolia*, the fruit fixation index ranged from 58 and 76%, and seed development was observed for all self-pollinated *Physalis* species (Table 1).

Fruit fixation was observed in most inter-specific hybrid combinations. The most noteworthy fruit fixation rates were noted for *P. peruviana* and *P. pubescens*. As these are female parents, high rates of fruit fixation in combination with most species were observed, except when they received pollen from *P. minima* and *P. daturifolia*.

No fruit fixation was observed when *P. daturifolia* was used as the pollen donor in combination with *P. minima* and *P. pubescens*. Additionally, no fruit fixation was observed when *P. daturifolia* received pollen from *P. pruinose*, and when *P. pruinosa* received pollen from *P. peruviana* (Table 1).

Hybrid seeds did not develop in cases where *P. daturifolia* was used as a pollen donor and fruit fixation occurred. The same was noted when *P. peruviana* was used as a pollen donor for *P. ixocarpa* and *P. minima*, for *P. ixocarpa* pollen in combination with *P. pruinosa* and *P. minima*, and *P. pubescens* pollen in combination with *P. ixocarpa* (Table 1).

Self-pollinations generated an average number of seeds per fruit ranging from 83.00 (*P. ixocarpa*) to 170.00 (*P. peruviana*). Concerning the hybridizations, the highest number of seeds were obtained for the following combination: *P. pubescens* combined with the pollen of *P. angulata, P. pruinosa, P. peruviana,* and *P. ixocarpa*, and *P. peruviana* combined with pollen from *P. pruinosa, P. pubescens, P. ixocarpa,* and *P. angulata*, with some seeds per fruit of ≥ 119.00. The other hybrid combinations generated fruits containing less than 60 seeds (Table 1).

3.4 Seed germination

The percentage of seeds germination obtained from interspecific *Physalis* species crosses and self-fertilization was higher than 84.25%, with no significant difference between crosses ($p < 0.05$). All seeds germinated from the fifth day after sowing.

4 Discussion

Variations between the viability of *Physalis* species pollen grains were observed. However, except for *P. peruviana* (43.60), all the other investigated species exhibited an in vitro pollen grain germination percentage above 50% (Fig. 1). In general, good pollen should exhibit germination rates greater than 50%, with well-developed pollen tubes (Scorza et al., 1995). Besides, all species exhibited high fruit fixation rates when donating pollen to themselves (Table 1), demonstrating that pollen did not limit artificial crossings.

The concentrations used to compose the cultural medium for pollen grain germination testing led to satisfactory results

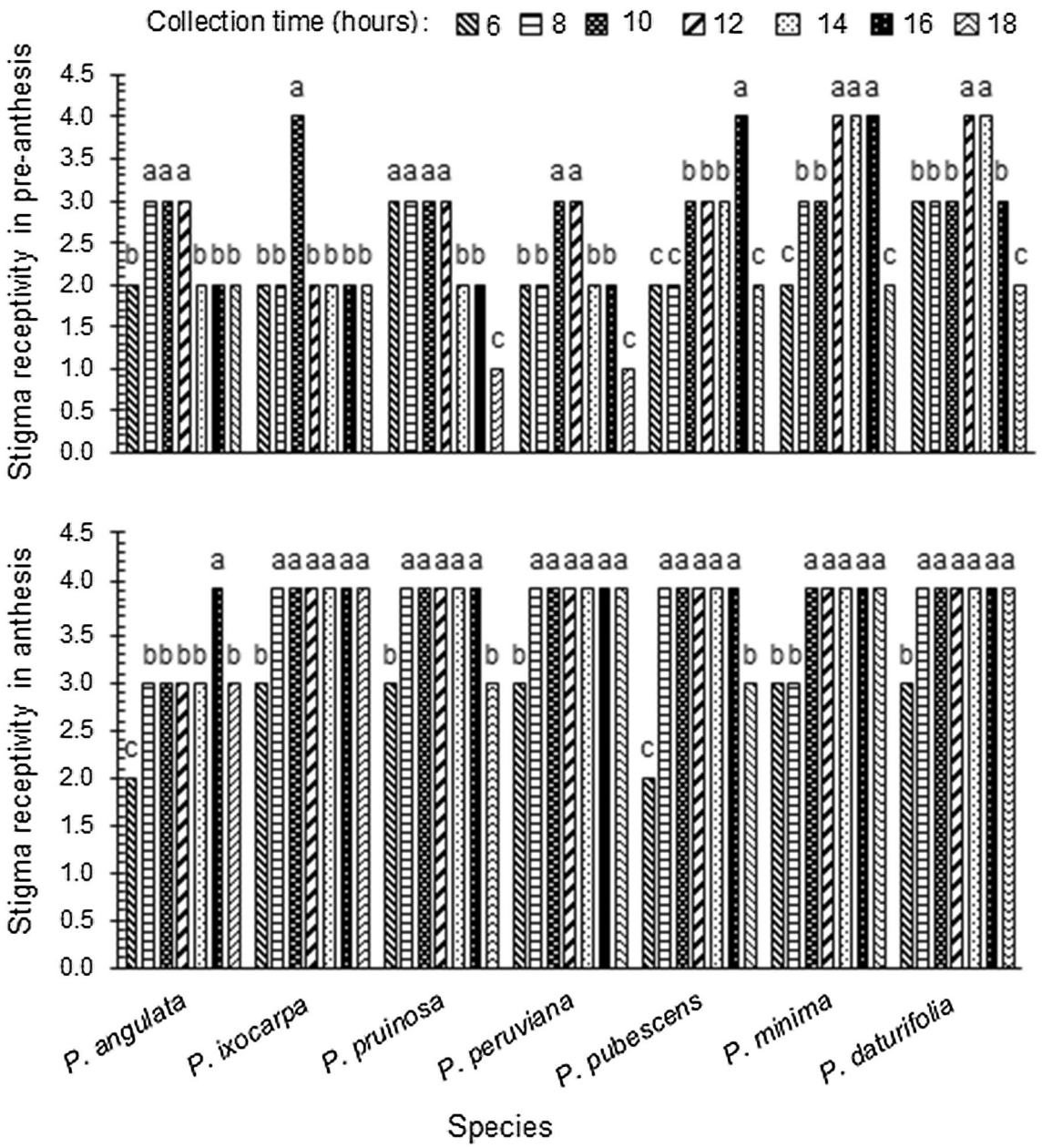

Fig. 2 Stigma receptivity in *Physalis* spp. at pre-anthesis and anthesis at different collection times. Different letters indicate a significant difference between treatments at $p < 0.05$ level (Tukey HSD test). Results represent the mean of five replicates ($n = 5$)

and can be used in new research concerning in vitro germination. The culture medium used to test pollen grain germination was developed exclusively for *Physalis* species (Silva et al. 2017), leading to highly reliable results. In vitro pollen germination studies simulate as much in vivo conditions as possible, exhibiting an excellent ability to induce pollen grain development and generally superior to other methodologies (Sakhanokho and Rajasekaran 2010; Nogueira et al. 2016; Pereira et al. 2018).

In the present study, the use of H_2O_2 identified a positive response for all species, where all stigmas produced air bubbles. When comparing flower phases, anthesis provided greater receptivity to pre-anthesis (Fig. 3), since the stigma tends to be more receptive to receive pollen in flowers in an advanced stage of development (He et al. 2017). However, regarding breeding programs, the pre-anthesis of auto compatible and autogamous species is more relevant to artificial crossbreeding, as it is necessary to avoid undesirable pollen grains. In most autogamous species, pollen grains are viable or can fertilize the plant itself only when the flower is in full anthesis (Zeist and Resende 2019).

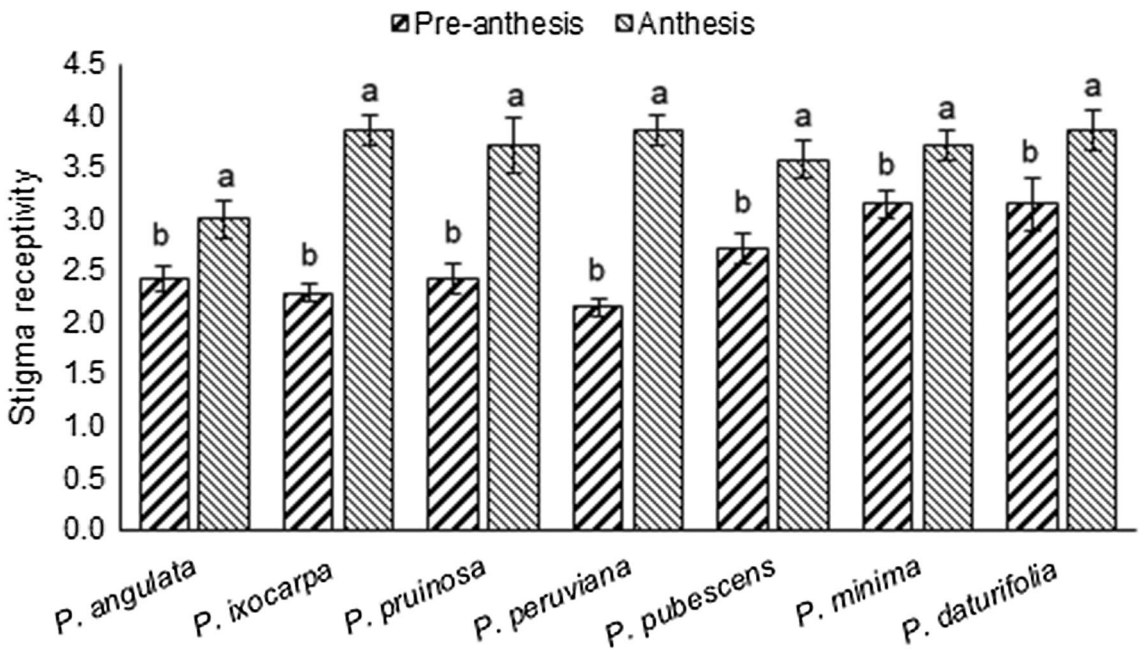

Fig. 3 Stigma receptivity of *Physalis* spp. at pre-anthesis and anthesis. Different letters indicate a significant difference between treatments at $p < 0.05$ level (t student test). Bars indicate standard deviation of the mean. Results represent the mean of five replicates ($n = 5$)

Table 1 Fruit index (FI) and number of seeds per fruit (NS) in self and interspecific artificial of *Physalis* spp. crosses

Male parent	Female parent						
	P. angulata	*P. ixocarpa*	*P. pruinosa*	*P. peruviana*	*P. pubescens*	*P. mínima*	*P. daturifolia*
FI (%)							
P. angulate	**64.00**	40.00	32.00	76.00	84.00	12.00	12.00
P. ixocarpa	28.00	**70.00**	16.00	80.00	100.00	24.00	4.00
P. pruinosa	24.00	4.00	**68.00**	76.00	72.00	4.00	0.00
P. peruviana	72.00	48.00	0.00	**94.00**	64.00	24.00	16.00
P. pubescens	20.00	12.00	10.00	76.00	**100.00**	8.00	8.00
P. mínima	28.00	12.00	8.00	8.00	16.00	**58.00**	8.00
P. daturifolia	8.00	8.00	4.00	4.00	0.00	0.00	**76.00**
NS ($Fruit^{-1}$)							
P. angulate	**127.00**	9.00	18.00	119.20	189.00	24.40	47.00
P. ixocarpa	51.00	**83.00**	0.00	130.00	120.40	0.00	48.00
P. pruinosa	21.00	5.50	**122.00**	155.80	186.00	30.50	–
P. peruviana	40.50	0.00	–	**195.00**	168.80	0.00	31.00
P. pubescens	57.75	0.00	11.00	144.40	**151.20**	8.00	25.00
P. mínima	28.00	16.00	26.00	24.50	21.50	**85.60**	14.00
P. daturifolia	0.00	0.00	0.00	0.00	–	–	**135.40**

Results represent the mean of fifty replicates ($n = 50$)

To obtain greater success in the generation of new hybrids, it is important to establish the times of the day when the stigmas are most receptive, so that the pollen of interest can be deposited (McInnis et al. 2006; Makwana and Akarsh 2017). When the flowers were in pre-anthesis, stigma receptivity was proven to be a more limiting factor, with the best receptivity observed in only some specific moments (Fig. 2). This is the first data on stigma receptivity in *Physalis* species, and likely to be of interest in studies aimed at increasing artificial crossing and controlled self-fertilization efficiency in *Physalis*.

Compatibility between crosses is considered successful when fruit fixation and viable seed formations are observed (Igic and Busch 2013; Muñoz-Sanz et al. 2020). The biggest incompatibility problems between *Physalis* species were associated with *P. daturifolia* as a pollen donor, which led to fruit fixation in only some hybrid combinations and did not allow for seed development. However, when *P. daturifolia* was used as a pollen receptor, fruit fixation and seed formation were observed in most hybrid combinations. *P. daturifolia* exhibits 2n = 20 chromosomes (Madhavadian 1968), while the other species explored in this study are reported to have 2n = 24 (Venkateswarlu and Rao 1979; Zlesak 2009; Wahua and Sam 2013; Azeez et al. 2019), which may have contributed to the low inter-specific compatibility observed for *P. daturifolia*. Additionally, reproductive barriers that result from plant evolution of plants and stimulate the existence of genetic mechanisms to avoid hybridization with related species must also be taken into account (Li et al. 2010). These barriers can be expressed before or after fertilization, characterized as unilateral incompatibility or incongruity between species, and, in some cases, the pollen of one species may be incompatible with the pistils of another related species, while no reproductive barrier occurs in reciprocal crossing (Bedinger et al. 2011).

Some hybrid combinations, despite presenting fruit fixation, did not allow for seed formation. This may be based on the occurrence of post-zygotic obstacles that cause endosperm degeneration and, consequently, the death of the hybrid embryo still inside the fruit (Li et al. 2010; Baek et al. 2015). In general, aspects that control intra- and inter-specific compatibility in Solanaceae go far beyond physiological barriers (Bedinger et al. 2011; Chalivendra et al. 2013), mostly comprising genetic mechanisms that still require further studies.

Studies on *P. ixocarpa* report that this species, despite displaying hermaphroditic flowers, is an obligatory allogamous (Pandey 1957), due to self-incompatibility mechanisms (Peña-Lomelí et al. 2018). However, the present study indicates that *P. ixocarpa* is self-compatible and displayed incompatibility only in some hybrid combinations. The present study corroborated with Azeez and Faluyi (2018), who identified *P. angulata* and *P. pubescens* as self-compatible species with has high compatibility in inter-specific crosses. However, the same authors did not observe fruit fixation for the *P. angulata* × *P. peruviana* combination, which was successful concerning fruit fixation and seed formation in the present study. In general, variations in inter-specific compatibility or even self-pollination within a species due to the explored genotype may occur (Bedinger et al. 2011; Baek et al. 2015).

Despite the variation in fruit fixation and the number of seed results, embryos develop normally in most inter-specific crosses, allowing for the germination of hybrid *Physalis* seeds. This facilitates the development of new *Physalis* genotypes without the need for embryo rescue techniques. The same was noted for self-fertilization, with seed germination similar to those observed in other assessments (Ozaslan et al. 2017; Figueiredo et al. 2020).

Because few commercial genotypes are available, most of which are still based on species and ecotypes, crossbreeding is required to improve commercial and nutritional *Physalis* characteristics. Inter-specific crosses are the fastest way to allow heterosis and the incorporation of useful genes, thus aiding in fruit production and commercialization (Sękara et al. 2007; Mallet 2008). Certainly, the findings reported herein regarding *Physalis* species reproductive biology and conditions that maximize crossbreeding fixation rates of fixation are of interest to genetic improvement programs, contributing to literature information and constituting an important scientific basis for the development of new studies.

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Author contributions ARZ conceived and designed the experiments. DFS contributed with discussions and made the plants available used in the experiments. ADS, DFS, MHSL, GJAO, and BRT performed the experiments. ADS, ARZ, DFS, JNMO, and KY prepared the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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