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Self-, cross- and interspecific pollinations in *Passiflora capsularis* and *P. rubra*[1]

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ABSTRACT – (Self-, cross- and interspecific pollinations in *Passiflora capsularis* and *P. rubra*). This study aimed to characterize the reproductive system of *Passiflora capsularis* L. and *P. rubra* L. *In vivo* controlled pollinations, *in vitro* pollen germination and pollen-ovule (P:O) ratio evaluation were conducted. In self-pollination, intraspecific and interspecific pollination, *P. capsularis* showed means of 62.5, 68.7 and 48.4% of fertilized flowers, while in *P. rubra*, the averages were 67.2, 62.5 and 46.9%, respectively. For *in vitro* germination, 52.2% of *P. capsularis* pollen grains germinated while in *P. rubra*, the percentage was 64.4. The P:O ratio was 22.4 for *P. capsularis*, and 27.4 for *P. rubra*, which included them in the category of obligatory autogamous. *Passiflora capsularis* and *P. rubra* can reproduce both by self-pollination and cross-pollination, and crossings between the two species succeeded though the success rate was lower than 50%. The characteristics of the reproductive system of both species allow the use of greater range of options on breeding methods for production of ornamental *Passiflora* plants.

Key words - *in vivo* pollination, *in vitro* germination, passion flower, pollen-ovule ratio

RESUMO – (Autopolinização, polinização cruzada e polinização interespecífica em *Passiflora capsularis* e *P. rubra*). O presente trabalho objetivou caracterizar o sistema reprodutivo de *Passiflora capsularis* L. e *P. rubra* L. Foram realizadas polinizações controladas *in vivo*, germinação *in vitro* do pólen e razão pólen:óvulo (P:O). Nas autopolinizações, polinizações intra-específicas e interespecíficas, *P. capsularis* apresentou em média 62,5, 68,7 e 48,4% de flores fertilizadas, enquanto em *P. rubra* a média foi de 67,2, 62,5 e 46,9%, respectivamente. Na germinação *in vitro*, 52,2% dos grãos de pólen de *P. capsularis* germinaram, enquanto em *P. rubra* o percentual foi 64,4%. A razão P:O foi 22,4 para *P. capsularis* e 27,4 para *P. rubra*, que as incluiu na categoria de autógamas obrigatórias. *Passiflora capsularis* e *P. rubra* podem se reproduzir por autopolinização e polinização cruzada, e cruzamentos entre as duas espécies foram bem sucedidos mesmo com baixa taxa de sucesso (menos de 50%). As características do sistema reprodutivo de ambas as espécies permitem a utilização de maiores opções de métodos de melhoramento para produção de plantas ornamentais de *Passiflora*.

Palavras-chave - germinação *in vitro*, maracujazeiros, polinização *in vivo*, razão pólen-óvulo

Introduction

The Passifloraceae is composed by 19 genera (Bernacci *et al*. 2003) and about 700 species (Feuillet 2004). The plants are herbaceous or woody, and extrafloral nectaries can be detected (Barroso *et al.* 1978). The most striking morphological characteristic of the family is the presence of corona filaments in the flowers, which supports the group monophyly (Judd *et al*. 1999). *Passiflora* is distributed mainly in tropical and subtropical regions, but they better develop in the climate of the Americas and Africa (Cronquist 1981, Judd *et al*. 1999). According to Cervi (2005), the genus comprises about 520 species, but new species have been discovered (Cervi 2006, Nunes & Queiroz 2007, Viana 2009). Brazil has a privileged status on the genetic resources of *Passiflora*, which presents a wide genetic variability available for use in breeding programs (Meletti *et al*. 2000). Among the Brazilian states, Bahia stands out showing significant occurrence of *Passiflora*, where 45 species were reported by Nunes (2002).

There is a wide diversity of reproductive strategies in angiosperms present in different communities (Richards 1986). Intraspecific and interspecific crossings comprehend important tools in genetic breeding programs. They are fundamental for the development of new varieties, once hybridization increases heterosis by the occurrence of new combinations of genes encoding agronomically important traits (Borém 1999, Paterniani 2001). Self-pollination may lead to loss in genetic variability due to inbreeding depression, which is a phenomenon aggravated in rare species (Reis *et al*. 2005).

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Many *Passiflora* species are usually self-incompatible, requiring cross-pollination (Bruckner *et al*. 1995). *Passiflora* flowers attract a wide range of pollinators, but their pollination is mainly performed by insects, such as carpenters bees of the genus *Xylocopa* Latr., bees of the genus *Ptiloglossa* Smith, and wasps and moths (Koschnitzke & Sazima 1997). A mechanism to increase genetic variability is the sexual reproduction, which allows individuals to better respond to changing environments (Li *et al*. 2002). Plants often show a diversity of mating systems ranging from obligatory autogamy associated with self-compatibility, to obligatory xenogamy associated to self-incompatibility, thus allowing variations in the transition between autogamy and allogamy (Iuchi & Lopes 1997). Decades ago, *Passiflora* species were primarily considered xenogamous, conditioned by self-incompatibility (Semir & Brown 1975).

Nowadays, there are reports of self-compatibility in wild and cultivated species, as *P. edulis* Sims (Menzel & Simpson 1988, Souza *et al*. 2010). Americans and Europeans have cultivated ornamental passion flowers for years, and hybrids that show exotic beauty due to new combinations of colors have been used to decorate glasshouses and gardens (Abreu *et al*. 2009). Hybridization is a very important technique for plant breeding since it enables the recombination of alleles, thus allowing the production of new genetically superior materials (Bruckner 1997). The interspecific hybridization in passion flowers in Brazil has been primarily directed to the transfer of favorable characters of other species to *P. edulis,* especially resistance genes (Viana *et al*. 2003). Few studies have used this technique to develop ornamental hybrids (Abreu *et al*. 2009).

Sexual hybridization in passion flowers can be successfully carried out both among plants of the same species and among related species due to weak incompatibility barriers (Meletti *et al*. 2005). However, some crosses become unviable, a condition that can be solved by using biotechnological techniques, such as protoplast fusion, among others (Bruckner & Otoni 1999, Marcelino *et al*. 2007).

Passiflora hybrids usually show exuberant flowers that are different in colours and size, and favorable for indoor decoration, whilst the genitors are small or do not have colored flowers (Ulmer & MacDougal 2004). *Passiflora capsularis* L. and *P. rubra* L. are wild species found in Brazil, which can be used in breeding programs aiming to produce ornamental hybrids since they have small and abundant flowers, and foliage and fruits in colors and shapes favorable to ornamentation. These two species are closely related, and morphologically similar. The leaves of *P. capsularis* and *P. rubra* are similar so that, in the absence of flowers or fruits, it is difficult to distinguish them. The main differences between the two species remain in the ovary and the fruit. The ovary of *P. rubra* is densely covered with long trichomes that may be white or, more rarely, brown, which usually persist in the fruit. The ovary in *P. capsularis* is merely puberulent and its short hairs often disappear in the ripe fruit. *Passiflora capsularis* fruits are green and always more stretched, while in *P. rubra* fruits are crimson red and show greater variation in length and width, thus being more obovate (Sousa & Meletti 1997).

This study aimed to estimate the outcrossing level in *P. rubra* and *P. capsularis* species, which shows a tight association with the breeding system and may support crossings involving these species in the production of ornamental plants.

Material and methods

Genotypes – Plant material was composed of four accessions of *P. capsularis* and four accessions of *P. rubra*. The accessions of *P. capsularis* were collected in the Atlantic forest of Southern Bahia state, at Morro da Viúva, PRNP Serra Bonita, Camacan, BA, Brazil, 14°47' S, 39°02' W, 789 m asl (accession BGP296), and also seeds from Empresa Brasileira de Pesquisa Agropecuária – Embrapa Cerrados, Planaltina, D.F. (accession BGP295), Universidade Estadual do Norte Fluminense Darcy Ribeiro – UENF, Campos dos Goytacazes, RJ (accession BGP260) and Instituto Agronômico – IAC, Campinas, SP (accession BGP297). The accessions of *P. rubra* were also collected at RPPN Serra Bonita (accession BGP265), at the same locality of *P. capsularis*, and also seed from Universidade Estadual Paulista – UNESP, campus de Jaboticabal, SP (accession BGP292), UENF (accession BGP293) and IAC (accession BGP294).

Cultivation conditions – Seeds were germinated in trays of 128 polystyrene cells. When the first true leaves were developed, the seedlings were transplanted to polyethylene bags with a volume of 1 L, containing clay (argile) soil. After reaching 4.0 cm height, plants were transplanted into black pots of 43 L, containing clay soil and substrate in a 3:1 ratio. Plants were grown in greenhouse at the Active Germplasm Bank of *Passiflora*, located at coordinates 14°39' S, 39°10' W; 78 m asl. Pruning was carried out monthly, and fertilization with NPK (4-14-8) was performed every 60 days. Irrigation was accomplished through an automatic drip system. Pest control was conducted with chemical pesticides Decis® and Vertimec®. Fungi were controlled by spraying products containing copper. Pests and diseases were controlled with

simple preventive measures, not affecting the reproductive cycle of plants.

In vivo pollination – Flowers were tagged and bagged one day before opening. Although the beginning of flowers anthesis on *P. capsularis* was between 5:00 and 6:00 a.m. and in *P. rubra* it occurred between 5:30 and 7:30 a.m., all pollinations were performed at the same period, between 8:00 and 9:00 a.m.: a) Self-pollination – four flowers from each accession (16 flowers) were pollinated using anthers and stigmas of the same flower; b) intraspecific cross-pollination – four flowers from each accession were emasculated in the beginning of anthesis and the stigmas (without pollen grains) were pollinated with pollen from the other accessions; c) interspecific cross-pollination – four flowers of each accession (per species) were emasculated before anthesis; anthers were mixed to form a bulk of the four accessions, which was used to pollinate all the flowers, hence making reciprocal crosses (*P. capsularis* vs. *P. rubra*; *P. rubra* vs. *P. capsularis*). Pollinations were performed with the help of tweezers as to carefully lean the anthers to the stigmas; the flowers were protected with paper bags for 24 hours after pollination. Five days after pollination, the fertilization index was verified, considering the fertilized flower that had initiated fruit development. After that, fruits were covered with a nylon mesh to protect against fall during ripening (Bruckner & Otoni 1999).

In vitro pollen germination – Pollen germination test was performed as described for *P. edulis* Sims (Bruckner *et al*. 2000): 0.10 g L^{-1} boric acid (H_3BO_4), 50 g L^{-1} sucrose, 0.3 g L^{-1} calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$); 0.2 g L^{-1} magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and 0.1 g L^{-1} potassium nitrate (KNO_3). One anther per flower was collected between 8:00 and 9:00 a.m., being the anther slightly hit on the slide to release pollen grains (PG) on a drop of germination medium previously autoclaved at 121 °C for 15 minutes and mounted under a glass cover. The material was incubated in oven at 28 ± 1 °C for 24 hours. We considered a PG germinated if it presented a pollen tube at least two times larger than its diameter.

Pollen-Ovule Ratio (P:O) – The P:O ratio was used as an indicator of the preferred mode of reproduction of the plant (Dafni 1992). The ovules and pollen grains were stained and counted according to Dafni (1992). The estimate of the reproduction mode for each species was carried out according to the classification of Cruden (1977): Cleistogamy (< 5.4); obligatory autogamy (5.5 to 39.0); facultative autogamy (39.1 to 396.9); facultative allogamy (397.0 to 2588.0) and obligatory allogamy (> 2588.0).

Statistical analysis – The experimental design used was totally randomized, consisting of four plants (treatment) maintained in protected cultivation. Replications were four for *in vivo* pollination and five for *in vitro* germination and P:O ratio, represented by each flower, totalizing 16 flowers per type of *in vivo* pollination and species, and 20 flowers per species for *in vitro* germination and P:O ratio. Data were subjected to a descriptive analysis and variance analysis using the software GENES (Cruz 2006).

Results

In vivo pollination – *P. rubra* and *P. capsularis* presented fruit set in over 60% of flowers self- and cross-pollinated and lower average values for interspecific pollination, in which the fruit set was above 46% for both species (table 1). The intra- and interspecific crosses showed no significant difference ($P < 0.05$) between species as for different treatments of pollination.

In vitro pollen germination – The results indicated significant differences ($P < 0.05$) among genotypes for the *P. capsularis* and also between the two species for the PG germination rate (tables 2 and 3).

Pollen-Ovule Ratio (P:O) – According to the mean values observed for the P:O ratio, *P. capsularis* and *P. rubra* were classified as obligatory autogamous. There

Table 1. Mean percentage values of fertilization index for self-pollinations (Self-), intra-specific (Intra) and interspecific (Inter) cross-pollinations in *Passiflora capsularis* and *P. rubra*, considering four replications (plants) (♀ = female genitor; POL = pollination; sd = standard deviation; AC = accession).

Species	POL	% Mean* (± sd) of accessions				
		AC 260	AC 295	AC 296	AC 297	Mean
P. capsularis	Self-	56.2 ± 0,95	68.7 ± 0,95	75.0 ± 0,81	50.0 ± 1,41	62.5
	Intra	68.7 ± 1,50	62.5 ± 1,00	68.7 ± 0,50	75.0 ± 0,81	68.7
	Inter ♀	56.2 ± 0,50	37.5 ± 0,57	50.0 ± 1,15	50.0 ± 0,81	48.4
		AC 265	AC 292	AC 293	AC 294	
P. rubra	Self-	68.7 ± 0,50	56.2 ± 1,25	62.5 ± 1,29	81.2 ± 0,95	67.2
	Intra	81.2 ± 0,50	43.7 ± 0,95	62.5 ± 1,00	62.5 ± 1,29	62.5
	Inter ♀	56.2 ± 0,50	50.0 ± 0,81	43.7 ± 0,50	37.5 ± 1,00	46.9

* Results are means of four repetitions (flowers) per plant.

Table 2. Mean values for number of *in vitro* pollen grain (PG) germination in *Passiflora capsularis* and *P. rubra*, considering four replications (plants) (sd = standard deviation; AC = accession; Germ = germinated; M = mean).

Species	Mean* (± sd) of accessions			
	AC	Nº PG Germ	% PG Germ	PG Total
P. capsularis	260	1939.0 ± 110.3	51.4	3775.7
	295	1929.5 ± 64.8	52.8	3651.7
	296	1997.2 ± 74.4	51.3	3895.0
	297	1762.7 ± 166.9	53.4	3300.0
	M	1907.1	52.2	3655.6
P. rubra	265	1703.5 ± 141.8	69.6	2448.0
	292	1538.5 ± 139.2	66.9	2300.7
	293	1671.5 ± 149.8	63.9	2615.7
	294	1454.5 ± 118.5	57.1	2549.5
	M	1592.0	64.4	2478.5

* Results are means of four repetitions (flowers) per plant.

Table 3. ANOVA summary for *in vitro* germination in *Passiflora capsularis* and *P. rubra*.

Source of variation	DF	Mean squares
P. capsularis	3	40644.41*
Error	12	12444.54
CV (%)		5.84
P. rubra	3	54765.33 ^{NS}
Error	12	18999.33
CV (%)		8.66
Between species	1	796953.12*
Error	30	2118.52
CV (%)		8.50

DF = degrees of freedom; CV = coefficient of variation. * = Significance at the 5% ($P < 0.05$) probability level by F test. ^{NS} = not significant.

was no significant difference ($P < 0.05$) between species for the number of ovules and PG number, while there was a significant difference ($P < 0.05$) between species for PG number. The variation in the number of PG observed among species resulted in a significant difference by F test ($P < 0.05$) for the values of pollen-ovule ratio that was observed within and between species (tables 4 and 5).

Discussion

P. capsularis and *P. rubra* can reproduce both by self-pollination and cross-pollination and that crossing between the two species were well succeeded even

Table 4. Mean values for the number of pollen grains (PG), number of ovules and pollen-ovule ratio (P:O) in *Passiflora capsularis* and *P. rubra*, considering four replications (plants) (sd = standard deviation; PL = plants; M = mean).

Species	AC	Mean* (± sd) of accessions		
		PG	Ovules	P:O
P. capsularis	260	3031.4 ± 698.5	126.2 ± 23.6	24.0
	295	2342.8 ± 235.9	116.4 ± 19.7	20.1
	296	2949.6 ± 590.3	127.2 ± 11.9	23.2
	297	3207.4 ± 272.9	141.0 ± 15.2	22.4
	M	2882.8	127.7	22.4
P. rubra	265	3703.8 ± 428.4	145.8 ± 17.6	25.4
	292	4338.0 ± 504.0	145.6 ± 20.9	29.8
	293	3638.6 ± 317.7	132.8 ± 18.5	27.4
	294	3552.6 ± 685.7	131.2 ± 28.9	27.1
	M	3808.3	138.9	27.4

* Results are means of four repetitions (flowers) per plant.

Table 5. ANOVA summary for the variables number of pollen grains (PG), number of ovules, and pollen-ovule ratio (P:O) in *Passiflora capsularis* and *P. rubra*.

Source of variation	DF	Mean squares		
		PG	Ovules	P:O
P. capsularis	3	7050848.9 ^{NS}	511.8 ^{NS}	5.6180*
Error	16	241659.9	330.5	0.00002
CV (%)		17.05	14.23	2.22
P. rubra	3	642804.8 ^{NS}	314.9 ^{NS}	6.5202*
Error	16	252195.8	482.3	0.00005
CV (%)		13.18	15.81	2.57
Between species	1	8564577.0*	1243.2 ^{NS}	49.6506*
Error	38	314411.9	407.5	3.0413
CV (%)		16.76	15.14	6.99

DF = degree of freedom; CV = coefficient of variation. * = Significance at the 5% ($P < 0.05$) probability level by F test. ^{NS} = not significant.

with a lower fertilization rate. Reproduction in most passion flowers occurs through cross-pollination due to the flower morphology, the anthers being located below the stigma; types of PG that are usually heavy and sticky; and, mainly, due to self-incompatibility in which pollen is unable to fertilize the ovules of the same plant, hence assuming that different plants may or not be compatible with each other (Bruckner *et al*. 2002, Souza *et al*. 2004).

Crossability tests on *P. capsularis* populations from Catas Altas, MG, Brazil, showed this species as self-compatible (Faria & Stehmann 2010). Reproduction in Passifloraceae may involve both self-compatible and self-incompatible systems. Self-incompatibility is understood as a mechanism that provokes allogamy and increases genetic variability (Varassin & Silva 1999). Other species such as *Byrsonima coccolobifolia* Kunth (Malpighiaceae) have mixed reproductive system with high allogamy and autogamy levels. This system combines the advantages of self-pollination and cross-pollination, ensuring a high level of adaptability of population to the present environmental conditions associated with the maintenance of high evolutionary potential through recombination, which enables the species to colonize new and extensive areas (Scariot *et al*. 1991).

Variation in breeding systems is observed among *Passiflora* species and other genus of Passifloraceae: *Mitostemma glaziovii* Mast. can reproduce by xenogamy (95% of the tested flowers) but it may also occur self-pollination and geitonogamy in a lower percentage (Benevides 2006); *Passiflora alata* is a species exclusively xenogamous, thus requiring cross-pollination for fruit set; on the other hand, *P. suberosa* L. is self-compatible with around 50% of fruit set when self-pollinated (Benevides 2006).

Some self-compatible species have a back up in case cross-pollination fails, *i.e.*, if no pollen from a distinct individual reaches the stigma, the flower may be able to self-fertilize, hence ensuring seed production (Hmeljevski *et al*. 2007). The possibility of autogamy occurrence in the species studied would cause a reduction in the frequency of heterozygous forms and consequently increase the proportion of homozygous types. This occurrence should favor the generation of lineages for the production of hybrids (Allard 1999), besides the formation of seeds without the need for pollinators (Cardoso 2007).

In vitro germination of PG in *P. capsularis* and *P. rubra* was below 65%. This is one of the methods for verifying the ability that PG have to emit pollen tubes and for fertilization. However, this method is influenced by different factors. There are species differences regarding the conditions required for pollen germination, mainly involving the constituents of the culture medium, temperature, and incubation time. Moreover, pollen viability is also influenced by development stage of the flower as for the collection of pollen and the storage conditions (Franzon *et al*. 2005).

Very low indexes of *in vitro* germination were obtained in *P. suberosa*, whereas for *P. edulis* f. *flavicarpa*, used as a control, the result was nearly 100% germination (Cruz *et al*. 2008). The average percentage of *P. suberosa* germination was negatively influenced by number of hours after anthesis, with a trend for percentage decrease during the period of flower opening (Cruz *et al.* 2008). Within just two hours after opening, the germination of pollen decreased 64%, with the highest average

germination obtained at 7:00 am., and the lowest at 05:00 p.m.. The same was observed by Souza *et al*. (2002) in *P. edulis* f. *flavicarpa* using chemical tests to assess pollen viability.

In *P. capsularis* and *P. rubra*, the values obtained were close to those observed for the control on *P. edulis* f. *flavicarpa*, which was only 76.9% of *in vitro* germination. This value differs from those found for the cultivated species by Bruckner *et al*. (2000) and Cruz *et al*. (2008). Such fact can be attributed to changes in the conditions of culture medium or procedure of *in vitro* cultivation, or even the manipulation of pollen during the experiment (Silva *et al*. 1999, Buckner *et al*. 2000). Results obtained in *P. capsularis* and *P. rubra* corroborate with the previous studies, since the low *in vitro* germination index obtained in this study may be related to the specific culture medium (Bruckner *et al*. 2000).

No viability or artificial *in vitro* germination test is quite satisfactory, especially after pollen has been stored, for the following reasons: the chemical tests employ dyes that react with chemical constituents or structures whose presence may not reflect the ability of the pollen to germinate; samples of PG that germinate *in vitro* may not produce sufficient elongation of the pollen tube as to affect fertility (Franzon & Raseira 2006). On the other hand, for some species pollen samples that appear to be non-viable when tested *in vitro* can produce large percentage of seeds, and the pollen stored can differently germinate in repeated samples or in different culture media (Miranda & Clement 1990). Maintaining the germination ability of pollen depends not merely on the inherent characteristics of the species, but also on storage conditions (Miranda & Clement 1990, Frazon & Raseira 2006).

In some cases, when spontaneous pollination occurs, autogamy allows the formation of seeds without the need for pollinators. This form of reproduction is used in breeding programs aiming to obtain homozygous lineages to produce hybrids from their crossings (Iuchi 1994). Allogamy may allow maintaining or increasing the hybrid vigor of the species by the occurrence of new combinations of genes that determine the traits of agronomic interest, as noted in *Ocimum canum* Sims (Lamiaceae), once allogamy has allowed greater production of essential oils that are widely used by pharmaceutical companies (Amaral *et al*. 2007).

The pollen-ovule ratio for brevistylus and longistylus flowers of *Psychotria barbiflora* DC (Rubiaceae) corresponded to the type of reproductive system involving the facultative xenogamy (Teixeira & Machado 2004). However, other methods such as the controlled pollinations and the analysis of pollen tube growth used to evaluate the reproductive system in *Psychotria barbiflora* confirmed that the species showed obligatory xenogamy, thus depending on the efficiency of pollinators for their maintenance (Teixeira & Machado 2004). These results were similar to those obtained in our studies for *P. capsularis* and *P. rubra* that were categorized as obligatory, according to values obtained by the P:O ratio (Cruden 1977). But, when comparing these results to those of controlled pollination, it was observed that both species can reproduce either by self-pollination or by intraspecific pollination.

Although the P:O ratio has been widely used to characterize the breeding system of many species, the factors that determinate the P:O ratio are not clear (Wyatt 1984). Vasek & Weng (1988) suggested that the interpretation of P:O ratio is not the only way to characterize the reproductive system of a species and that methods of evaluating the P:O ratio must be standardized at family level. Characterization of the reproductive system in *P. capsularis* and *P. rubra* could be used for establishing genetic breeding strategies aiming to produce inter-specific hybrids.

In conclusion, the use of *in vitro* germination for tests on pollen of *P. capsularis* and *P. rubra* using medium and conditions described in the methodology provides satisfactory results when compared to *in vivo* germination. Analysis on the P:O ratio proved to be inefficient in determining the reproductive system in the two passion flower species studied since they were classified as obligatory autogamous.

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