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Biology-Ecology

Reproductive biology of espinheira-santa (*Monteverdia ilicifolia*, Celastraceae)

Biologia reprodutiva da espinheira-santa (*Monteverdia ilicifolia*, Celastraceae)

Tângela Denise Perleberg[I], Rosa Lia Barbieri[II], Márcio Paim Mariot[I], Tamires Ebeling da Silva[I], Josiane Mendonça Vitória[III], Patrick da Silva Silva[III], Rafaela de Sousa Corrêa de Magalhães[III]

[I] Instituto Federal de Educação, Ciência e Tecnologia Sul-rio-grandense, Pelotas, RS, Brazil
[II] Embrapa Clima Temperado, Pelotas, RS, Brazil
[III] Universidade Federal de Pelotas, Pelotas, RS, Brazil

ABSTRACT

Monteverdia ilicifolia, popularly known as espinheira-santa, is a medicinal plant native of Brazil used in the treatment of gastritis and gastric ulcers. This paper aimed to evaluate floral morphology and biology, to characterize the reproductive system and to describe the reproductive phenology of *M. ilicifolia*. Floral morphology and biology analysis on espinheira-santa plants from an Active Germplasm Bank were performed. The reproductive system was evaluated through self-pollination, apomixis, cross-pollination, natural pollination and geitonogamy tests. Reproductive phenophases were followed for two years and correlated with climatic variables. All evaluated flowers are morphologically hermaphrodites; however, three floral types were found in terms of sexual expression: functionally female flowers, functionally male flowers and hermaphrodite flowers. Functionally male and functionally female flowers occur in different plants, characterizing an evolutionary process for dioecy in this species. The species presented a preferably allogamous reproductive system. The reproductive period occurred from June to February, considering that the reproductive phenophases are correlated with temperature and length of day.

Keywords: Active Germplasm Bank; Floral biology; Reproductive phenology

RESUMO

Monteverdia ilicifolia, conhecida popularmente como espinheira-santa, é uma planta medicinal nativa do Brasil utilizada no tratamento de gastrites e úlceras gástricas. Este trabalho teve como objetivos avaliar a morfologia e biologia floral, caracterizar o sistema reprodutivo e descrever a fenologia reprodutiva de

M. ilicifolia. Foram feitas análises de morfologia e biologia floral em plantas de espinheira-santa de um Banco Ativo de Germoplasma. O sistema reprodutivo foi avaliado por meio de testes de autopolinização, apomixia, polinização cruzada, polinização natural e geitonogamia. As fenofases reprodutivas foram acompanhadas durante dois anos e correlacionadas a variáveis climáticas. Todas as flores avaliadas são morfologicamente hermafroditas, no entanto foram encontrados três tipos florais quanto a expressão sexual: flores funcionalmente femininas, flores funcionalmente masculinas e flores hermafroditas. As flores funcionalmente masculinas e funcionalmente femininas ocorrem em diferentes plantas, caracterizando um processo evolutivo para a dioicia nessa espécie. A espécie apresentou sistema reprodutivo preferencialmente alógamo. O período reprodutivo ocorreu de junho até fevereiro, sendo que as fenofases reprodutivas estão correlacionadas com a temperatura e o comprimento do dia.

Palavras-chave: Banco Ativo de Germoplasma; Biologia floral; Fenologia reprodutiva

1 INTRODUCTION

Monteverdia ilicifolia (Mart. ex Reissek) Biral, popularly known as espinheira-santa, is a sub-shrubby to arboreal plant, native of Brazil, that occurs predominantly in the states of the Southern Region (CARVALHO-OKANO, 2005; GUARINO et al., 2019). The species is widely used in Brazilian popular medicine (DUTRA et al., 2016) presenting proven action in to fight gastritis and gastric ulcers (CARLINI, 1988; JORGE et al., 2004). For this reason, it appears in the National List of Essential Medicines (Rename) of the Ministry of Health (MINISTÉRIO DA SAÚDE, 2020).

However, the strong anthropic action, without adequate management criteria and the lack of information regarding the characterization of germplasm, has led to the loss of this important genetic resource, promoting the reduction in the natural populations of *M. ilicifolia* and the consequent reduction of its genetic variability (STEENBOCK; REIS, 2004; MARIOT, 2005). In order to contribute to the characterization and conservation studies of the espinheira-santa genetic diversity, Embrapa Temperate Agriculture, in partnership with the Sul-Rio-Grandense Federal Institute of Education, Science and Technology keeps an Active Germplasm Bank of Espinheira-Santa that conserves 129 accessions of *M. ilicifolia* from several regions of Rio Grande do Sul (MARIOT, 2005). This Germplasm Bank is an easy access tool to conduct research related to species.

Due to its great medicinal importance, most of the papers on *M. ilicifolia* are restricted to the areas of pharmacology and pharmacochemistry, with little relation to ecological and management aspects (RADOMSKI; BULL, 2010). In that respect, there are still gaps and many questions to be answered about the knowledge of the reproductive biology of this species. Studies based on the reproductive system and the presence of morphologically monoclinous and functionally diclinous flowers have pointed out that *M. ilicifolia* is in the evolutionary process on the way to a probable dioecious (CARVALHO-OKANO, 1992; STEENBOCK, 2003; MAZZA et al., 2011). The identification of functionally female flowers and functionally male flowers within the same population, occurring in separate plants, corroborates this hypothesis (STEENBOCK, 2003; MAZZA et al., 2011). However, monoclinous flowers, with normal stamens and pistils, are also present, indicating an intermediate stage of sexuality of this species (CARVALHO-OKANO, 1992).

The sexual system known as dioecy is characterized by the presence of male and female individuals in a plant population (RENNER; RICKLEFS, 1995; LENZA; OLIVEIRA, 2004). The appearance of dioecious populations may have been promoted by the action of different evolutionary forces on hermaphrodite individuals throughout their evolution. And, the evolution of unisexuality in angiosperms can be considered as an allogamy promoting system whose primary function is to obtain success in reproduction in the most different habitats (BARRETT, 2002). In *M. ilicifolia*, the functional behavior of diclinous flowers is corroborated by the species' high crossbred rates (STEENBOCK, 2003; PERECIN et al., 2004). In this sense, dioecy is a mechanism that increases genetic variability and reduces endogamous depression in the population (CHARLESWORTH; CHARLESWORTH, 1978).

Detailed studies of floral biology and morphology and of the reproductive system, associated with the monitoring of flowering and fruiting phenology are extremely important for understanding the evolutionary process of the sexual system in *M. ilicifolia*. In that note, this paper aimed to evaluate floral morphology and biology, characterize the reproductive system and to characterize the reproductive system and to describe the reproductive phenology of *M. ilicifolia* at the Active Germplasm Bank of Espinheira-Santa of Embrapa Temperate Agriculture/Sul-Rio-Grandense Federal Institute of Education, Science and Technology (IFSul).

2 MATERIAL AND METHODS

2.1 Study area

The work was performed at the Active Germplasm Bank of Espinheira-Santa of Embrapa Temperate Agriculture/Sul-Rio-Grandense Federal Institute of Education, Science and Technology, in Pelotas, Rio Grande do Sul. The Bank is installed in two areas. At the Experimental Cascata Station of Embrapa Temperate Agriculture (31°61’ S and 52°52’ W) where 107 accessions of *M. ilicifolia* are maintained. The place is 25 km from the downtown of Pelotas and it belongs to the physiographic region of Southeast Hillside, with an altitude of 160 m. The other area is at Campus Pelotas - Visconde da Graça of the Sul-Rio-Grandense Federal Institute of Education, Science and Technology (31°42’ S and 52°18’ W) where 22 accessions of *M. ilicifolia* are maintained. This area is 8.5 km from the downtown of Pelotas and it is located on the Coastal Plain, 7 m above sea level. Each accession corresponds to the progeny of a single matrix plant whose seeds were collected from November 2002 to January 2003 in municipalities in Rio Grande do Sul (MARIOT, 2005). The Active Germoplasm Bank conserves a total of 280 plants of espinheira-santa.

The climate in both areas is of the Cfa type, humid subtropical, with hot summers and no dry season, according to Köppen classification (MORENO, 1961). The data from the climatological normals (1971-2000) for the municipality of Pelotas show that there is regularity in precipitation throughout the year, with a monthly average of 113.9 mm. The average annual temperature is 17.8°C, with absolute maximum temperatures above 39ºC, between November and January, and absolute minimum temperatures below 0ºC, between June and August (SCHÖFFEL et al., 2016).

2.2 Floral biology

At first, the sexual characterization of 280 plants of the Active Germoplasm Bank was performed for two consecutive years by observing the flowers in loco using a magnifying glass with five times magnification. Functionally female plants that had, mostly, flowers with evident ovary, short stamens and whitish anthers, and functionally male plants that had, mostly, flowers with developed stamens, dark yellow anthers and non-evident ovary were considered (MAZZA et al., 2011), as well as the presence of pollen in large quantities in the anthers. The production of a large amount of fruit for the characterization of functionally female plants was also considered. The occurrence of hermaphrodite flowers (with evident ovary and developed stamens with dark yellow anthers) was also recorded.

The number of inflorescences was counted in five branches of 10 plants of each sex, totaling 50 branches per sex. The total and daily number of flowers in anthesis by inflorescence was monitored daily in 30 inflorescences of each floral type (considering floral type: functionally female flowers and functionally male flowers) in a total of 10 plants, previously bagged, starting with the count of the number of flower buds and ending when the last flower of each inflorescence opened.

In order to evaluate the morphometry of the functionally male flowers and functionally female flowers, an experiment was performed in a completely randomized design organized in a unifactorial scheme, with four repetitions. The treatment factor tested was the accessions, with functionally female flowers (Ac116F, Ac117F, Ac118F2, Ac118F4, Ac127F1, Ac127F2, Ac129F4, Ac130F, Ac134F1, Ac134F3, Ac136F e Ac137F) and functionally male flowers (Ac116M, Ac117M, Ac123M1, Ac130M, Ac136M e Ac137M). For the morphological characterization of these floral types, the following measurements of floral morphometry were performed using a precision digital caliper (0.01mm): peduncle length (PL), flower length (FL) (from the base of the corolla to the apex of the pistil or stamen), corolla diameter (CD), petals width (PW), petals length (PetL), calyx diameter (CalD), stamens length (SL) (from the base of the corolla to the top of the anthers), anther length (AL), nectar disc diameter (NDD), style length (StyL) (from the base of the corolla to the stigma) and stigma diameter (SD).

The comparison between accessions (with functionally female and male flowers) was performed using multivariate analysis through the principal component method. Principal component analysis (PCA) was extracted from a correlation matrix. This way, the information enclosed in the original variables was projected into a smaller number of underlying variables called Principal Components (PCs). The criterion for reduction of variables (PCs) used was recommended by Jolliffe (2002), this criterion establishes that a few main components that includes at least between 70 and 90% of the total variation must be retained. After selecting the number of PCs, their respective eigenvalues were obtained, with their corresponding eigenvectors. The graphic procedure adopted was the biplot, based on the scores of the selected principal components. The presence of correlations between the study variables was analyzed using Pearson's correlation coefficient (r).

For the characterization of floral types, color of the flowers, presence of odor, floral symmetry, petals and sepals' concrescence, number of petals, sepals and stamens, anther dehiscence, type of ovary, the number of locules and eggs by ovary and presence of nectar disc were recorded. To verify the presence of odor, functionally female and functionally male flowers were kept separated in covered glass containers for 10 minutes (KEARNS; INOUYE, 1993), when 10 people were asked, through their sense of smell, to check for an odor.

Pollen viability was tested by the aceto-carmine method (KEARNS; INOUYE, 1993) on 90 functionally male flowers, collected from 10 functionally male plants, and 90 functionally female flowers, collected from 10 functionally female plants. From each flower, five anthers were used, inasmuch, for functionally male, 100 pollen grains per flower were counted, and, for functionally female, all the pollen grains of each flower were counted. Pollen grains that redden were considered viable.

For each of the following analysis, 30 functionally female flowers and 30 functionally male flowers were used, previously bagged and from 20 plants of the Active Germoplasm Bank. The time of floral anthesis was defined by observing the flowers at two-hour intervals between 7 am and 6 pm and at a single 12-hour interval between 6 pm and 6 am. As the floral anthesis occurred, it was verified if there was production of nectar in the laboratory using a stereomicroscope. For longevity determination, flowers were monitored for 15 days. Stigma receptivity was tested by the hydrogen peroxide method, 3% at the time of anthesis, 24 hours, 48 hours, and so on until 15 days before anthesis (KEARNS; INOUYE, 1993). The moment of anther dehiscence was observed after floral anthesis at two-hour intervals, recording the number of dehiscent anthers in each interval. The period that the anthers keep pollen was followed by floral anthesis until no more pollen was found in the selected flowers.

2.3 Reproductive system

To determine the reproductive system of *M. ilicifolia*, the following treatments were performed: 1) Spontaneous self-pollination: 110 flowers of each floral type were isolated; 2) Manual self-pollination: 110 flowers of each floral type were manually pollinated with their own pollen; 3) Apomixis: 110 flower buds of each floral type, in pre-anthesis, were emasculated; 4) Cross-pollination between different accessions: 110 flowers of each floral type were emasculated and received pollen from functionally male flowers from different accessions; 5) Natural pollination occurred in the Active Germoplasm Bank: 110 buds of the two floral types, in pre-anthesis, were marked and available to floral visitors; so far for each treatment, 11 plants of each sex were used, with 10 flowers per plant being bagged for each treatment; 6) Geitonogamy: 14 flowers of each floral type were emasculated and pollinated with pollen from functionally male flowers from the same plant; 7) Pollination of hermaphrodite flowers: 14 hermaphrodite flowers (total flowers found) were emasculated and received pollen from functionally male flowers from another plant.

For each test, branches with flower buds in pre-anthesis were isolated with fine mesh fabric bags and kept bagged until the flower fell or fruit formation and maturation. The ripe fruits from the treatments were harvested to verify the number of seeds and their viability, as confirmed by the emergency test (MARIOT et al., 2009).

2.4 Reproductive phenology

The monitoring of reproductive phenophases of floral bud, floral anthesis, unripe fruit and ripe fruit was performed monthly between June 2013 and May 2014 (first year) and between June 2014 and May 2015 (second year), in 100 plants of the Experimental Cascata Station of Embrapa Temperate Agriculture and 180 from Campus Pelotas - Visconde da Graça plants. Two parameters were evaluated: the activity index and the Fournier intensity index.

The activity index refers to the percentage of individuals in a certain phenophase, indicating the beginning and end of the phenological event and the synchrony between the plants evaluated for a given phenological event. To estimate this index, data of presence (1) or absence (0) of the phenophase were used in relation to the total number of plants (N) in the sample (BENCKE; MORELLATO, 2002). The Fournier (1974) intensity index refers to the intensity of occurrence of a given phenophase. The intensity of phenological events was estimated individually using a semi-quantitative scale of five categories (from 0 to 4), with a 25% interval between them (FOURNIER, 1974).

To verify the existence of a correlation between the reproductive phenophases (number of individuals exhibiting a certain phenophase) and the climatic variables (average, minimum and maximum temperature, precipitation and length of the day), Spearman's (ZAR, 1999) correlation (rs) was made, using the SAS software.

Exsiccates from the evaluated accesses were deposited at the Herbarium of Embrapa Temperate Agriculture with the voucher numbers ECT0000648 to ECT0000660.

3 RESULTS

3.1 Floral Biology

In the Active Germplasm Bank of Espinheira-Santa, 176 (63%) functionally female plants were identified, inasmuch 20 of these had functionally male flowers in small numbers, and 104 (37%) functionally male plants, with 47 of these plants having a small number of functionally female flowers. A small number (about 20 flowers) of hermaphrodite flowers was recorded in two plants of the Active Germplasm Bank.

In functionally male plants an average of 18.6 inflorescences per branch was observed, with 11.7 flowers per inflorescence. And, in functionally female plants, an average of 15.1 inflorescences per branch and 10 flowers per inflorescence. The monitoring of floral development showed that 89.6% and 87.1% of functionally male and female buds, respectively, originate flowers, with one to two flowers opening per day in each inflorescence. And, regardless of the floral type, the flowering period was 45 days on average for everyone.

Statistical analysis showed that the first two principal components contributed with 80.55% of the variability in the data set (Table 1). The remaining generated PCs (PC3 to PC11) progressively produced smaller eigenvalues and did not significantly explain the variability of the data. Therefore, only the first two PCs were used for extra studies. Analyzing the eigenvectors corresponding to PC1, the variables that showed greater discriminatory power were 0.34 for the petals length, 0.33 for the corolla, calyx and nectar disc diameter. In PC2, the main eigenvectors were 0.55 for stylus length and 0.54 for flower length (Table 1).

Table 1 – Eigenvectors corresponding to the two main components (PC1 and PC2) for dependent variables referring to the tested accessions of *M. ilicifolia*

Dependent variables	**PC1**	**PC2**
Peduncle length (PL)	0,302	0,231
Flower length (FL)	0,214	0,544
Corolla diameter (CD)	0,326	0,122
Petals width (PW)	0,314	0,187
Petals length (PetL)	0,338	0,137
Calyx diameter (CalD)	0,332	0,089
Stamens length (SL)	0,321	- 0,282
Anther length (AL)	0,302	- 0,238
Nectar disc diameter (NDD)	0,329	0,073
Style length (StyL)	- 0,216	0,553
Stigma diameter (SD)	- 0,289	0,355
Eigenvalue	7,04	1,82
Variation (%)	64,01	16,54
Accumulated variation (%)	64,01	80,55

Considering that the first two principal components explained a large proportion of the total variation (80.55%), it was possible the plotting of the scores and loads of the components related to the studied accessions (Figure 1). The formation of four distinct groups was verified, showing the differentiation between the floral types according to the dependent variables evaluated.

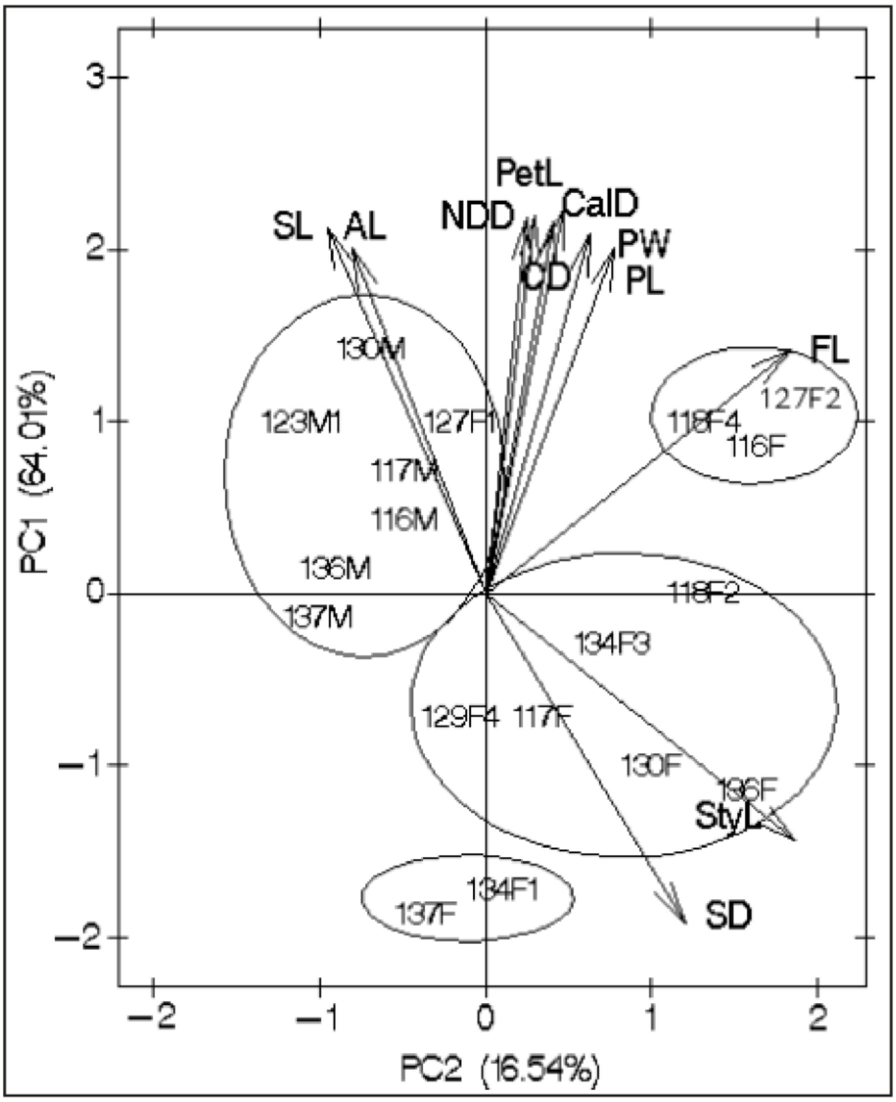
The first group included all accessions with male flowers and one accession with female flowers (Ac127F1). The variables that had the greatest contribution to the grouping of these accessions were the stamens (SL) and anthers (AL) length (Figure 1), whose values found were the highest among the evaluated accessions.

The other groups presented only accessions with female flowers. The variable floral length (FL) was the main responsible for the separation of accessions 118, 127 and 116 in the second group (Figure 1), whose flowers were the largest among the female and greater than the male. The accessions 118F2, 134F3, 129F4, 117F, 130F and 136F formed the third group, in which the stylet length (StyL) and stigma diameter (SD) variables had the greatest influence on the observed discrimination (Figure 1), and they presented average intermediates values for the floral characters evaluated. The last group was composed of two accessions 137F and 134F1, which presented the lowest values for the evaluated characters.

In the accessions with functionally male flowers the average stamens length was 1.9 cm and the average anthers length was 0.9 cm, while in the accessions with functionally female flowers the average stamens length was 1.4 cm and the average anther length was 0,7 cm. Regarding the average values of the stylet length and the stigma diameter, in the accessions with functionally feminine flowers, was 1.2 cm and 0.5 cm, respectively. While, in the accessions with functionally male flowers the values were 0.6 cm and 0.3 cm, respectively.

The correlation analysis reinforces the results obtained in the PCA. A positive correlation was found between the stamens length (SL) and the anthers length (AL) ($r = 0.72$, $p = 0.0007$) and a negative correlation between the stamen length (SL) and the style length (StyL) ($r = -0.81$, $p < 0.0001$) and the stigma diameter (SD) ($r = -0.78$, $p = 0.0001$).

Figure 1 – Plotting scores and loads of the main components (PC1 and PC2) for variables peduncle length (PL), flower length (FL), corolla diameter (CD), petal width (PW), petals length (PetL), calyx diameter (CalD), stamens length (SL), anther length (AL), nectar disc diameter (NDD), stylet length (StyL) and stigma diameter (SD) referring to the tested accessions



According to the statistical analysis performed, to differentiate functionally male and functionally female flowers, the following characteristics can be analyzed: in functionally male flowers, the stamen length is two to five times greater than the style length, whereas in functionally female flowers the difference between the stamens length and the style length is small, never being twice or more. The functionally female flowers have a style longer than 1 cm, while in functionally male flowers this value does not exceed 0.85 cm. Besides these, other easy-to-observe features can be used to differentiate the floral types of *M. ilicifolia*, presented hereafter.

The analysis of floral morphometry allowed the identification of two floral types in terms of sexual expression: functionally male flowers and functionally female flowers. A third type (hermaphrodite flowers) was found, however, with a reduced number of flowers, around 20, and no statistical analysis was performed. The three floral types found (functionally male flowers, functionally female flowers and hermaphrodite flowers) present inconspicuous flowers, on average 5.98 mm in diameter, yellowish-green in color and do not exhale a perceptible odor to human smell. They are actinomorphous, pentamerous, dialipetals and gamosepalous, with a fleshy intrastaminal nectar disc that surrounds the gynoecium and produces nectar. Gynoecium is bi-carpellate and bi-locular. The five stamens are aternipetalous, they are inserted in the nectar disc and the anthers have longitudinal dehiscence.

The functionally male flowers have developed and verticalized stamens and yellow anthers with a large amount of pollen. The ovary is immersed in the nectar disc and it can have two ovules (in 37.5% of the flowers), three ovules (in 25% of the flowers) or four ovules (in 37.5% of the flowers) (Figure 2a).

In functionally female flowers, the stamens are horizontalized or verticalized and, in this case, they acquire a horizontal position immediately after anthesis, becoming adpressed to the petals. The anthers are white or light yellow. The ovary is evident or partially immersed in the nectar disc and it contains four ovules in all the flowers (Figure 2b).

Hermaphrodite flowers were recorded in two plants of the Active Germplasm Bank of Espinheira-Santa, in a reduced number, about 20 flowers per plant. These showed intermediate characteristics to the two floral types described previously regarding the style and stamens. The stamens are verticalized and they are from the same size as the style, the anthers are yellowish and produce pollen, the ovary is protruding or partially immersed in the nectar disc (Figure 2c).

Figure 2 – Floral types of *Monteverdia ilicifolia* (Celastraceae), Pelotas, RS, Brazil - a. functionally male flowers; b. functionally female flowers; c. hermaphrodite flowers. Photos a and b: Paulo Lanzetta. Photo c: Tângela Denise Perleberg

The anthesis of functionally male and functionally female flowers is diurnal. In 80% of the flowers, the anthesis starts at 6 pm on one day and it is completed at

6 am on the other day, when the petals are completely distended and the sexual organs are exposed. In the other flowers (20%), the opening of the petals starts at 6 am and the anthesis is completed throughout the day. Without contact with pollinators, both types of flowers can last up to nine days until senescence.

The stigma of functionally male flowers is receptive from the third day in 20% of the flowers and from the sixth day of floral anthesis in 80% of the flowers. Most functionally female flowers (80%) have receptive stigma already in the anthesis, while in others the stigma is receptive from the second day of anthesis, remaining receptive until floral senescence.

The production of nectar occurs from the first day of anthesis, increasing until the sixth day, when it begins to cease in the functionally female flowers and extending to the senescence of the flower, even when there was no more pollen in the anthers, in the functionally male flowers.

Anther dehiscence, in 80% of functionally male flowers, occurs on the first day of floral anthesis, from 6 am to 2 pm, and, in 20% of flowers, floral anthesis occurs between 2 pm of a day and 10 am of the following day. Pollen is released until the sixth day after anther dehiscence. In functionally female flowers, the anther dehiscence process occurs on the first day of floral anthesis and the release of pollen, when it is present, occurs until the second or third day after anther dehiscence. All the analyzed anthers of the functionally male flowers showed a large amount of pollen, while in 37% of the functionally female flowers no pollen was found in the anthers and in the other flowers, on average, four pollen grains per flower were found. However, pollen analysis, using acetocarmine, showed that 73% of pollen grains of the two floral types are viable.

3.2 Reproductive system

Only functionally female flowers by cross-pollination and geitonogamy and hermaphrodite flowers by cross-pollination formed fruit. The percentage of fruiting from natural pollination in the Active Germoplasm Bank was similar to that of cross-pollination between different accessions (Table 2). The seeds from the fruits formed in the treatments were viable by the emergency test (Table 2).

Table 2 – Tests of the reproductive system of *Monteverdia ilicifolia* (Celastraceae) from Active Germplasm Bank of Espinheira-Santa from Embrapa Temperate Agriculture/Sul-Rio-Grandense Federal Institute of Education, Science and Technology, Pelotas, RS, Brazil

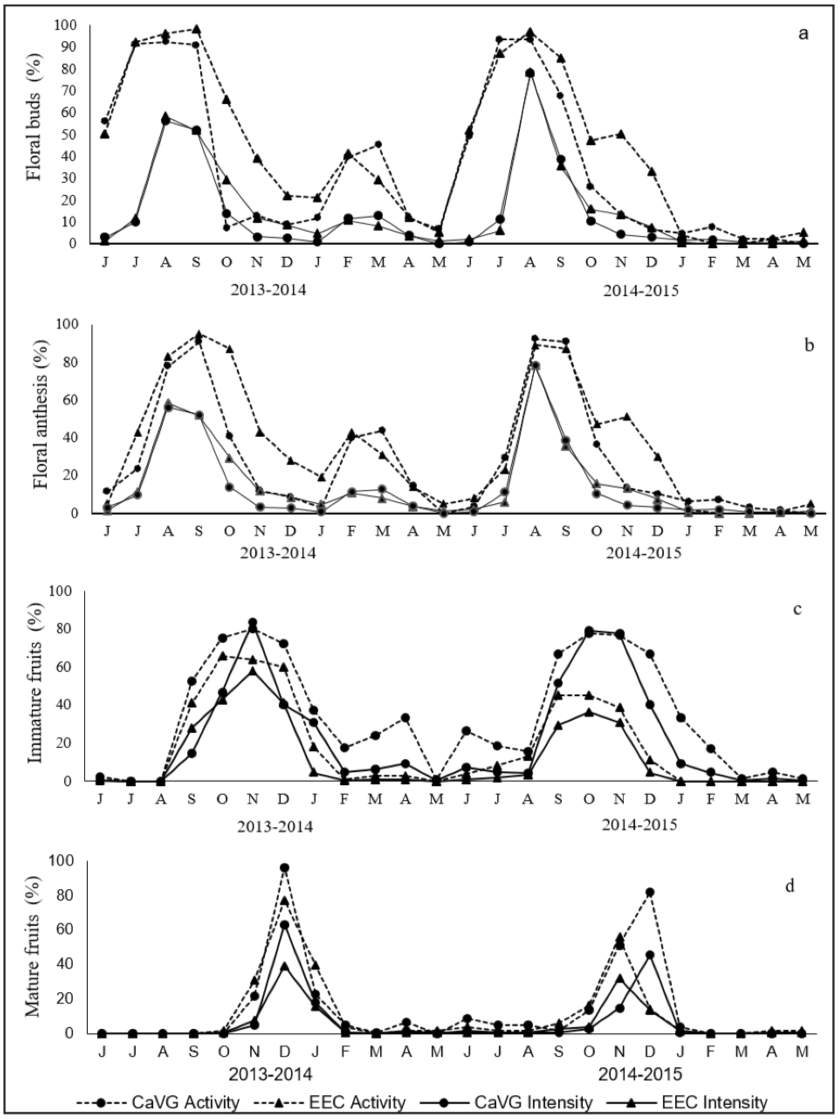
Tests	**Functionally Female Flowers**			**Functionally Male Flowers**	**Hermaphrodite Flowers**	
	Flowers (n) /Fruits (n)	Success (%)	Seeds (n)/ Viability(%)	Flowers (n)/Fruits (n)	Flowers (n)/ Fruits (n)	Success (%)
Spontaneous self-pollination	110/0	0	-	110/0	NP	-
Manual self-pollination	110/0	0	-	110/0	NP	-
Apomixis	110/0	0	-	110/0	NP	-
Geitonogamia with PFMF	14/3	21,4	NP	14/0	NP	-
Cross-pollination between different accessions with PFFF	140/3	2,1	5/80,0	140/0	NP	-
Cross-pollination between different accessions with PFMF	140/60	42,9	109/75,2	140/0	14/8	57,0
Natural pollination occurred in the Active Germoplasm Bank	130/64	49,2	114/89,5	130/0	NP	

PFFF = pollen from functionally female flowers. PFMF = pollen from functionally male flowers. NP = not performed

3.3 Reproductive phenology

The reproductive period of *M. ilicifolia* started in June with the emission of flower buds and it extended until February, with the production of ripe fruits in the two years of evaluation.

Figure 3 – Reproductive phenology of *Monteverdia ilicifolia* (Celastraceae) from Active Germplasm Bank of Espinheira-Santa from Embrapa Temperate Agriculture/Sul-Rio-Grandense Federal Institute of Education, Science and Technology, Pelotas, RS, Brazil, from June 2013 to May 2015 - a. floral button phenophase; b. floral anthesis phenophase; c. immature fruit phenophase; d. mature fruit phenophase



Continuous line = Fournier Intensity Index; dashed line = Activity Index

The highest intensity of flower buds occurred in August, in the two years of evaluation and in both areas. The greatest activity was observed from July to September, for both areas, in the first year and, in the second year, in August at Experimental Cascata Station of Embrapa Temperate Agriculture and in July and August at Campus Pelotas - Visconde da Graça (Figure 3a).

The floral anthesis started in July and the highest intensity occurred in August for both areas and years, decreasing considerably in the following months. Maximum activity was recorded in September in the first year and in August in the second year, for both áreas (Figure 3b). In February and March of the first year of evaluation, there was a second flowering peak, but the intensity of the event was not significant and only at Campus Pelotas - Visconde da Graça it resulted in the formation of some ripe fruits (Figure 3b). Spearman's correlation index showed that there was a correlation between flower bud phenophases and floral anthesis with temperature and length of the day (Table 3).

Table 3 – Spearman correlation (r_s) between reproductive phenophases and climatic variables for *Monteverdia ilicifolia* (Celastraceae) from Active Germplasm Bank of Espinheira-Santa Embrapa Temperate Agriculture/Sul-Rio-Grandense Federal Institute of Education, Science and Technology, Pelotas, RS, Brazil. Values were significant at $p < 0.05$; ns = not significant at the $p < 0.05$ level

Climatic variables	**Reproductive phenophases**			
	Floral button	**Floral anthesis**	**Immature fruit**	**Mature fruit**
Minimum temperature (average)	-0,78	0,76	ns	ns
Average temperature	-0,65	0,76	0,64	0,81
Maximum temperature (average)	ns	0,78	0,73	0,56
Day length (h)	0,79	0,79	-0,72	-0,75
Precipitation	ns	ns	-0,72	ns

Higher peaks of intensity and activity of unripe fruits were observed in October and November (Figure 3c). Fruit maturation occurred from November to January in the first year, and from October to December in the second year (Figure

3d). The maximum activity and the highest intensity of ripe fruits occurred in December in the first year and, in the second year, in November for Experimental Cascata Station of Embrapa Temperate Agriculture and December for Campus Pelotas - Visconde da Graça. There was a correlation between unripe fruits and all the climatic variables analyzed (Table 3). Whereas, for ripe fruits there was a correlation between temperature and length of the day (Table 3).

4 DISCUSSION

4.1 Floral biology

Several results found in this study can be interpreted as evidence that *M. ilicifolia* is on the way to the sexual separation of flowers and individuals. Starting with the occurrence of individuals with functionally female flowers and individuals with functionally male flowers. Previous studies had already mentioned the presence of different floral types in *M. ilicifolia*, suggesting that the flowers would be functionally female or functionally male, revealing a tendency to dioecy (CARVALHO-OKANO, 1992; STEENBOCK, 2003; MAZZA et al., 2011). However, it is important to note that this process is not complete yet, since functionally female flowers (very few) have been registered in functionally male plants and the opposite has also occurred. As well as, hermaphrodite flowers in two individuals from the Active Germoplasm Bank.

The difference in the number of functionally male and functionally female flowers in the inflorescences can be considered a sexual dimorphism that occurs in dioecious plants and that influences male and female reproductive success (BARRETT; HOUGH, 2013; FRANCESCHINELLI et al., 2015). The production of a larger number of functionally male flowers by inflorescence or per individual is a characteristic of dioecious species, which increases the sexual display of male individuals (BAWA, 1980), helping pollinators in the initial task of finding *M. ilicifolia* flowers, since they are inconspicuous, they do not present a noticeable color and odor. However, in a second moment, the similarity between the flowers of the two floral types in shape, size and color, may have the function of facilitating the recognition by pollinating agents, allowing pollen to flow between plants (BAWA, 1980; FREEMAN et al., 1997).

The absence or reduction in the number of pollen grains in the anthers (a maximum of four grains in a flower) and the reduction in the stamens size may indicate the regression of the male sexual organ in functionally female flowers. In the same way that the reduction in the number of ovules from four to two or three and the style size may indicate the regression of the female sexual organ in functionally male flowers. This way, it is likely that a process of evolution is taking place towards the unisexuality of flowers and the establishment of dioecy based on hermaphroditism, as described by Charlesworth (2002).

The process of sexual separation may be earlier in functionally male flowers, since the few pollen grains found in functionally female flowers have the same viability percentage as those found in functionally male flowers. In contrast, the non-formation of fruits from functionally male flowers observed in the Active Germoplasm Bank may indicate that the ovules of these flowers are unviable as suggested by Carvalho-Okano (1992), or that the fertilization process is not completed. Functionally male flowers of two dioecious species, Citharexylum myrianthum Cham. (Verbenaceae) and Cabralea canjerana (Vell.) Mart. (Meliaceae), also do not produce fruit, although they have well-developed ovules (ROCCA; SAZIMA, 2006; FRANCESCHINELLI et al., 2015).

The maintenance of rudimentary organs of the opposite sex in functionally male and female flowers may represent an intermediate stage in the evolution from hermaphroditism to dioecy and that the separation of the sexes is a relatively recent phenomenon (BAWA; OPLER, 1975; ROTTENBERG, 1998). The registration of some hermaphrodite flowers in two plants of the Active Germoplasm Bank reinforces this hypothesis. The occurrence of hermaphrodite flowers, with functional stamens and pistils, which produce fruits, is registered for several dioecious species (BAWA; OPLER, 1975; LENZA; OLIVEIRA, 2005; CESÁRIO; GAGLIONE, 2008; FERNANDES et al., 2012) and for *M. ilicifolia* also (CARVALHO-OKANO, 1992).

The sexual ripening of some functionally male flowers before the functionally female flowers, and vice versa, may be a trace of the dichogamy that the ancestral hermaphrodite flowers of *M. ilicifolia* presented, probably, to prevent or decrease self-fertilization. Since dioecious species, in most cases, evolved from self-compatible species in response to selective pressure to promote crossbreeding (BAWA; OPLER, 1975). However, currently, floral types of this species would tend to synchrony in the maturation of sexual organs, which occurs on the first day of anthesis, promoting the genic flow between floral types.

From these evaluations, it is possible to say that the separation of sex in the flowers and plants of *M. ilicifolia* is incomplete, since some characteristics can be associated with a hermaphrodite ancestry while, other characteristics, tend to dioecy. According to Bawa (1980), this inconsistency in sexual expression in one or both sexes during the establishment of dioecy based on hermaphroditism can be predicted by the theoretical models that trace the evolution of dioecy. Although all *M. ilicifolia* flowers are morphologically hermaphrodites, there are three floral types in terms of sexual expression: functionally male flowers, functionally female flowers and hermaphrodite flowers. The differentiation between floral types can be made by comparing, in the same flower, the stamens and the style length, color and position of the anthers, amount of pollen of the anthers.

4.2 Reproductive system

The results obtained with the tests to evaluate the reproductive system indicate that *M. ilicifolia* presents a preferably allogamous reproductive system, needing a pollinating agent to reproduce. However, with the formation of fruits with viable seeds by geitonogamy, the species is self-compatible.

Although the average viability of *M. ilicifolia* seeds is around 90% (SCALON et al. 2005), the percentage of viability of seeds from functionally female flowers present in functionally male plants, although lower than the average, indicates that these flowers can guarantee the reproduction of the species if there are no functionally female plants in the population.

4.3 Reproductive phenology

The reproductive behavior of *M. ilicifolia* is seasonal, annual and regular, corroborating with Mazza et al. (2011). The flowering pattern observed is classified by Gentry (1974) as cornucopia, where there is a continuous production of flowers over several weeks, thus promoting the pollination efficiency. This cornucopia flowering strategy allows pollination by different groups of organisms with different types of foraging by making flowers available for a long period (OTÁROLA; ROCCA, 2014).

It was found that temperature and length of day are important factors for the beginning and continuity of the reproductive period of *M. ilicifolia*. Studies performed in subtropical regions have shown that there is a greater correlation of phenological events with temperature and length of day than with precipitation, due to the greater annual amplitude of these variables in these locations (MARQUES et al., 2004; MARCHIORETTO et al., 2006).

The greatest production of flower buds occurs at low temperatures. While the maximum activity and intensity of the flower anthesis' phenophase coincides with the period of the year when the average temperatures start to rise. These results are in accordance with the described for *M. ilicifolia* in Paraná, where flowering occurred in the transition between a colder and a warmer period (MAZZA et al., 2011). This increase in temperature allows pollinators to act, promoting the fertilization of flowers and the consequent formation of fruits.

The relation between the reproductive period of *M. ilicifolia* and the length of the day is even more evident in the anticipation of flowering at higher latitudes. In the two evaluated areas (Experimental Cascata Station = 31°61’ S; Campus Pelotas - Visconde da Graça = 31°42’ S), the start of reproductive phenophases was anticipated in one month, when compared to natural populations of *M. ilicifolia* in Mixed Ombrophilous Forest (27º47’ S), in the municipality of Erechim, Rio Grande do Sul (MALYSZ; ZANIN, 2011), and in the Irati National Forest (25º21’ S), in the municipalities of Fernandes Pinheiro and Teixeira Soares, Paraná (MAZZA et al., 2011), where there are smaller latitudes. Mariot (2005) observed that the espinheira-santa presented fruit maturing between November and December in the south of Rio Grande do Sul and in January in the northeast of the same state.

5 CONCLUSIONS

The flowers of *M. ilicifolia* are morphologically hermaphrodites, however they can be classified into three floral types in terms of sexual expression: functionally male, functionally female or hermaphrodite flowers. These last ones are very rare. Differentiation between floral types should be made by comparing the stamens length with the style length, the stamens position (vertical or horizontal), the color of the anthers (white or dark yellow) and the amount of pollen in each flower (up to four grains or countless grains). The sexual separation in the flowers and plants of *M. ilicifolia* is not complete, as floral biology reveals, characteristics related to hermaphroditism and to dioecy. Reproductive phenophases are strongly influenced by temperature and the length of the day. The reproductive system is preferably allogamous. These advances in knowledge about the reproductive biology of *M. ilicifolia* may provide subsidies for evolutionary ecological studies, mainly related to dioecy, involving the reproduction ecology of this species.

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Authorship Contribution

1 – Tângela Denise Perleberg
Doutora em Agronomia
tangelaperleberg@gmail.com – https://orcid.org/0000-0001-7323-7701
Contribution: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing

2 – Rosa Lia Barbieri
Doutora em Genética e Biologia Molecular
lia.barbieri@gmail.com – https://orcid.org/0000-0001-8420-9546
Contribution: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing

3 – Márcio Paim Mariot
Doutor em Agronomia
marciomariot@gmail.com – https://orcid.org/0000-0001-5673-9232
Contribution: Conceptualization, Methodology, Writing – original draft, Writing – review & editing

4 – Tamires Ebeling da Silva
Graduada de Tecnologia em Gestão Ambiental
tamires_ebeling@hotmail.com – https://orcid.org/0000-0001-7829-1248
Contribution: Investigation, Methodology

5 – Josiane Mendonça Vitória
Doutoranda em Sistemas de Produção Agrícola Familiar
josiane_mendonca@hotmail.com – https://orcid.org/0000-0002-1792-482X
Contribution: Investigation, Methodology

6 – Patrick da Silva Silva
Mestrando em Ciência e Tecnologia Agroindustrial
patrick._silva@hotmail.com – https://orcid.org/0000-0002-3242-7864
Contribution: Investigation, Methodology

7 – Rafaela de Sousa Corrêa de Magalhães
Mestre em Sistemas de Produção Agrícola Familiar
magalhaes.rsc@gmail.com – https://orcid.org/0000-0003-2260-4326
Contribution: Investigation, Methodology

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