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Breeding systems and reproductive success on *Salvia smyrnaea*

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Abstract: The reproductive ecology of *Salvia smyrnaea* Boiss. (Lamiaceae) on Mount Nif along with their breeding system and reproductive success were examined. *S. smyrnaea*, distributed over Mount Nif (İzmir Province) at an altitude of 1510 m, is the study material of this research. To detect the stigma receptivity, the Perex test was applied to 25 young and mature flowers. To detect pollen viability, the MTT method was used. To detect the fertilisation type of the taxon *S. smyrnaea*, 5 different fertilisation methods were tested on the flowers of the plant. Breeding success was assessed through nutlet formation. While the enzyme activity was calculated as approximately 14.2% with an average of 71 ppm in 25 young flowers to which Perex tests were applied, it was approximately 60% with an average of 300 ppm in the mature flowers. In the mature flowers, the highest pollen viability was 10.29%, whereas this rate was 70.27% for the young flowers. In our study, the self-incompatibility rate of *S. smyrnaea* was calculated as (ISI) = 22/24 = 0.91 (between 0.2 and 1) and it was detected as partially self-incompatible. This result was in line with the fact that the taxon is protandrous.

Key words: *Salvia smyrnaea*, reproductive success, self incompatibility, pollen viability, Labiatae

Salvia smyrnaea türünün tozlaşma sistemi ve üreme başarısı

Özet: *Salvia smyrnaea* Boiss. (Lamiaceae) türünün Nif Dağı populasyonundaki üreme ekolojisi, özellikle üreme sistemi ve üreme başarısı araştırılmıştır. Çalışma materyalini Nif Dağında (İzmir) 1510 m yükseklikte yayılış gösteren *S. smyrnaea* oluşturmaktadır. Stigma olgunluğunu belirlemek için 25'er adet genç ve olgun çiçekte Perex testleri uygulanılmıştır. Polen canlılığı için MTT yöntemi kullanılmıştır. *S. smyrnaea* taksonuna ait tozlaşma tipini belirleyebilmek için, bitkinin çiçekleri üzerinde 5 farklı tozlaştırma işlemi yapılmıştır. Üreme başarısı, nutlet oluşturulması temelinde incelenmiştir. Perex test uygulanan 25 adet genç çiçekte ortalama 71 ppm ile enzim aktivitesi %14,2 olduğu saptanırken, 25 adet olgun çiçekte ortalama 300 ppm ile enzim aktivitesi % 60 olarak tespit edilmiştir. Olgun çiçeklerde en yüksek polen canlılığı % 10,29 iken, genç çiçeklerde bu değer % 70,27 olarak tespit edilmiştir. Çalışmamızda *S. smyrnaea* türünün, kendine uyumsuzluk değeri (ISI) = 22/24 = 0,91 (0,2 ile 1) olarak hesaplanmış ve kısmen kendine uyumsuz olarak belirlenmiştir. Bu sonuç taksonun proterandrik olması ile uyumlu bulunmuştur.

Anahtar sözcükler: *Salvia smyrnaea*, üreme başarısı, kendine uyumsuzluk, polen canlılığı, Labiatae

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Introduction

Lamiaceae is a family with a cosmopolitan distribution and has 250 genera and approximately 7000 taxa worldwide (Kahraman et al., 2009). *Salvia* L. is the largest member of Lamiaceae with its more than 900 species. *Salvia* is one of the most important and largest genera in Lamiaceae and is referred to as sage. Its medicinal properties have been known about since ancient times and due to these medicinal properties it is thought to derive from the Latin word "Salveo", which means "life saver" or "therapeutic". It has a global distribution and is represented with approximately 500 species in South and Central America, 250 species in Central Asia and Mediterranean, and 90 species in Eastern Asia (Walker et al., 2004). It is reported that *Salvia* species distributed in Asia originated from Anatolia (Hedge, 1982; Davis et al., 1988; Vural & Adıgüzel, 1996). Lamiaceae is represented in Turkey by 45 genera and 574 taxa, 256 (44.5%) of which are endemic (Kahraman et al., 2009). However, in recent revision studies, it is reported that the genus *Salvia* is represented by 97 taxa, 51 (52.5%) of which are endemic. *Salvia smyrnaea* is one of the endemic species belonging to the section Hymenosphace of the genus *Salvia*. Ekim et al. (2000) determined the conservation status of *Salvia smyrnaea* as endangered (EN) according to the threat categories defined in the Red Data Book of Turkish Plants. Celep et al. (2010) also determined the threat category of *Salvia smyrnaea* as endangered [EN B2ab(i,ii,iv): area of occupancy less than 500 km^2, known at no more than 5 locations], but he employed the criteria in the latest version of IUCN Red List Categories Version 3.1 (2001).

The study material includes *Salvia smyrnaea* Boiss. according to the *Flora of Turkey* by Hedge (1982). *S. smyrnaea* is distributed in 2 areas in Turkey, namely, Kemalpaşa District of İzmir Province and Söke District of Aydın.

The morphological characteristics of *S. smyrnaea* are as follows: perennial herb with a woody rootstock; stems ascending erect, unbranched, 9-16(22) cm, densely eglandular-villous and glandular-pilose. Life form is chamephytes. Leaves ovate oblong - oblong, 2-5.5 × 1-2 cm; pedicel 3-5 mm; calyx tubular campanulate and broadens approximately ±15 mm in a slightly purple fruit (16-17 mm), the upper lip tridentate; corolla violet-blue and 30-35 mm, lips almost equal; nutlets pale brown.

It is endemic to the Mediterranean region. It is very similar to *S. cadmica* Boiss. regarding the hair coat and the appearance. *Salvia smyrnaea* differs from *S. cadmica* in the way that it has oblong leaves, its calyx widens very little in fruit and the colour of its corolla is violet-blue.

There have been several studies conducted on the pollination of *Salvia* taxa. Wester and Claßen-Bockhoff (2007) investigated pollen transfer mechanisms and floral diversities in bird-pollinated *Salvia* species. While Miyajima (2001) investigated the pollination ecology of *Salvia splendens* Sellow ex Roem. & Schult., Sanchez et al. (2002) studied the pollination ecology and seed production of *Salvia splendens*. The pollination ecology of *Salvia nipponica* Miq. was investigated by Miyake and Sakai (2005), of *Salvia brandegeei* Munz by Barrett et al. (2000), of *Salvia haenkei* Benth. by Wester and Claßen-Bockhoff (2006), and of *Salvia verbenaca* L. by Navvarro (1997). On the other hand, in Turkey, mostly morphological, anatomical, karyological characteristics (Kandemir, 2003; Baran et al., 2008; Kahraman et al., 2009; Eşiz-Dereboylu et al., 2010) chemical contents, and microbial effects (Başer, 2002; Nakiboğlu, 2002; Gören et al., 2006) of *Salvia* taxa have been investigated.

Reproduction ecology is of fundamental importance in establishing conservation strategies of taxa, particularly with small populations and in determining the obstacles in the production of their reproductive structures (Gross et al., 2003; Tandon et al., 2003). To this end, in our study, reproductive ecology, reproductive system, and reproductive success of *S. smyrnaea* population on Mount Nif were investigated.

Materials and methods

Study area

The study area is Mount Nif (1510 m), located in the west of İzmir Province between 38°22′ and 38°23′N longitudes, and between 27°21′ and 27°22′E latitudes. *S. smyrnaea* population on Mount Nif is distributed between the altitudes of 1036 m and 1510

m. Its flowering takes place in May and it is distributed in open rocky and gravelly areas. A voucher specimen was deposited at the Ege University, Sciences Faculty, Biology Department Herbarium (EGE, herb. no: 40791).

Stigma receptivity and pollen viability

Each collected flower was separately placed in an Eppendorf tube, stored in a cool box, and transported to the laboratory. In the laboratory, each flower was dissected and the anthers and stigmas were removed so that they could be tested.

In order to determine stigma receptivity, Perex tests (Merck chemical 16206) were used in 25 young flowers and 25 mature flowers. In this solution the colour of the tissues turned into dark orange from light yellow in 1-4 min in the presence of hydrogen peroxide. The kit has a colour chart indicating the concentration range of 10-500 ppm peroxide. As a measure of stigma receptivity, average enzyme activity was estimated based on a colour chart prepared especially for this test. One drop of solution was dropped over stigma samples collected from flowers and buds, and then in 1-4 min, stigma receptivities were determined in accordance with the colour scale showing hydrogen peroxide (H_2O_2) concentration (10-500 ppm) (Dafni & Maues, 1998). In this test, the highest hydrogen peroxide (H_2O_2) concentration (500 ppm) was considered as 100% enzyme activity.

For the pollen viability, the MTT (diphenyl tetrazolium bromide) method was used. In this test, the colour of pollen grains turned dark purple or black in the presence of dehydrogenase enzyme. Those pollen grains that were stained were considered as viable pollen, while those that were not stained were accepted as unviable. In order to be used in the test, fresh pollen was collected from plants. One percent MTT solution was first mixed with 5% sucrose and then used for pollen staining. Pollen preparations were incubated at 30 °C after they were stained. The preparations were then examined under light microscopes at 160-400× magnification (Rodriguez-Riano & Dafni, 2000). The percentage viability of the pollens collected from the anthers of young and mature flowers was calculated in 5 repeats. Five hundred pollen grains were used.

Breeding system

Pollination treatments, designed to investigate the breeding system of *S. smyrnaea* based on Dafni (1992), were performed from 16 May to 21 June 2009. Flowers were bagged with fine nylon mesh during the budding stage to exclude pollinators. For hand pollination, bags were opened and the following treatments were administered:

1- For autogamy, flowers were bagged without emasculation.

2- In order to determine whether there was apomixis, flowers were bagged with fine nylon mesh after they were emasculated.

3- For xenogamy, pollen collected from flowers in the male phase was manually pollinated with cross-pollen (we transferred pollinia with a toothpick from a flower to the stigma of another flower), emasculated and re-bagged.

4- For geitonogamy, pollen collected from the flowers of the same plant in the male phase was manually pollinated with cross-pollen (we transferred pollinia with a toothpick from a flower to the stigma of another flower), emasculated and re-bagged.

5- To estimate fruit production under natural conditions, 180 flowers underwent no procedure and were exposed to pollinator visits (control).

Except for control observations, 10 flowers were used for each treatment and fruit development was checked until the nutlets matured. In June, before nutlet dispersal, marked flowers that underwent pollination were separately bagged and brought to the laboratory where the number and the rates of the nutlets formed in each calyx were calculated.

Then, in order to determine the degree of self-incompatibility, the number of the fruits set through geitonogamy was divided by the number of fruits set through xenogamy and the index of self-incompatibility (ISI) was established (Ruiz-Zapata & Arroyo, 1978). In the index, ISI value >1 represents self-incompatible, between 0.2 and 1 partially self-incompatible, <0.2 mostly self-incompatible, and 0 completely self-incompatible.

Reproductive success

Investigation of reproductive success was based on nutlet formation. Thirty different individuals were marked while the plant was flowering, and the number of racemes per plant, the number of verticillasters per raceme, and the number of flowers per verticillaster were calculated and recorded. In the individuals we enumerated, nutlets per flower in 180 flowers were counted and recorded before seed dispersal. Actual seed count (ASC) was calculated by multiplying the average number of flowers (NF) per plant by the average number of nutlets (NN) per flower produced in natural conditions [(ASC) = NF × NN]. Members of the family Lamiaceae can produce a maximum of 4 nutlets per flower (Heywood, 1978). Therefore, the potential number of the seeds (PNS) was estimated by multiplying the average number of flowers per plant by 4 [(PNS) = NF × 4]. Here, the average number of flowers per plant was calculated by multiplying the number of racemes (NR) per plant by the average number of verticillasters per raceme. The percentage of reproductive success of the taxon was calculated with the equation of ASC × 100/PNS.

Statistical analysis

For reproductive success and morphological data, standard descriptive statistics were performed for each quantitative parameter. Chi-square tests were performed in order to compare the frequencies of developed and aborted fruits between breeding system treatments. SPSS (version 10, SPSS Inc., Chicago, IL, USA) was used.

Results and discussion

The results of Perex tests used to assess the enzymatic activity in the stigmas of young and mature flowers were expressed as percentages ranging from light yellow colour formation (10 ppm), in which enzymatic activity is the lowest, to dark orange colour formation (500 ppm), in which enzymatic activity is the highest. At the end, the test was performed on 25 young and 25 mature flowers, and the percentages of hydrogen peroxide concentrations (ppm) in flowers based on the colour scale were assessed as follows: 10, 50, and 100 ppm in 12%, 36%, and 52% of the stigmas of young flowers, respectively, and 100, 200, 300, and 500 ppm in 8%, 24%, 48%, and 20% of the stigmas of mature flowers, respectively. When the Perex test was administered, the mean enzyme activity was determined as 14.2% (71 ppm) in young flowers, whereas it was determined as 60% (300 ppm) in mature flowers (Figure).

Pollen viability test performed with the pollens collected from the anthers of both young and mature flowers revealed that, of the 500 pollens counted in young flowers, 230 were stained, while, of the 500 pollens counted in mature flowers, only 41 were stained. After the pollen viability tests were performed 5 times on 500 pollen grains of young flowers and 500 pollen grains of mature flowers, the viability percentage was determined as 57.42 ± 10.00 in young flowers and 7.58 ± 2.53 in mature flowers.

If the androecium matures before the gynoecium and produces pollens in a hermaphrodite flower, this is called protandry. If the gynoecium matures before the androecium and produces pollens in a hermaphrodite flower, this is called protogyny (Percival, 1965). Protandry is common in the family Lamiaceae (Owens & Ubere-Jimenez, 1992), which hinders self-pollination. It is reported that, in self-compatible protandric species, pollen viability somehow decreases in time, whereas stigma compatibility increases (Rodriguez-Riano & Dafni, 2007). In pollen viability and stigma receptivity tests simultaneously conducted on the stigmas and anthers of young and mature *S. smyrnaea* flowers, it was determined that enzyme activity in the stigmas

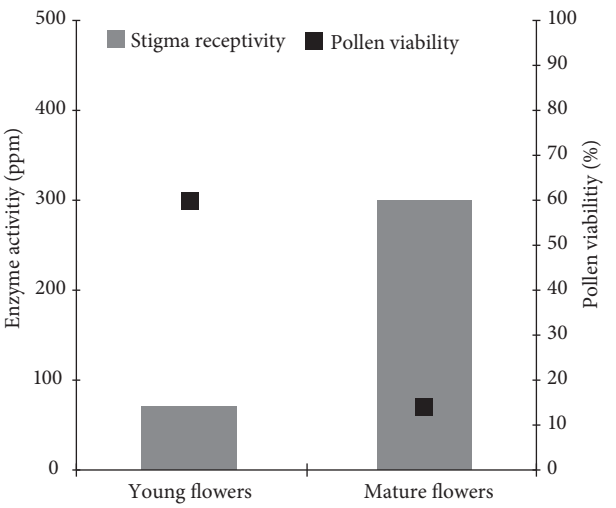

Figure. Pollen viability (%) and stigma receptivity (as measured by enzyme activity, ppm) in *S. smyrnaea* flowers.

of young flowers (stigma lobes closed) was 14.2% whereas the viability of the pollens collected from the anthers of the same flowers was 57.42 ± 10.0%. However, in the mature flowers (stigma bifurcate), enzyme activity was determined to be 60%, whereas pollen viability was 7.58 ± 2.53%. The results of pollen and stigma viability tests we conducted revealed that flowers belonging to *S. smyrnaea* were protandric-hermaphrodite. In a study conducted on *Asperula daphneola*, another taxon endemic to the same mountain, it was reported that stigma enzyme activity was 400 ppm 1 day before the flowers opened and 320 ppm when the flowers were in full bloom. The study also reported that pollen viability was 89% 1 day before the flowers opened and 72% when the flowers were in full bloom. In Perex tests performed on the flowers of *Minuartia nifensis* McNeill, an endemic species distributed on Mount Nif, it was reported that enzyme activity in hermaphrodite flowers was 27 ppm 2 and 3 days before the flowers opened, 55 ppm 1 day before they opened, and 70 ppm when the flowers were in full bloom. In pollen viability tests carried out at the same time, it was found that pollen viability was 74% 3 days before the flowers opened, 92% 2 days before they opened, 88% 1 day before they opened, and 85% when the flowers were in full bloom (Gücel & Seçmen, 2008, 2009).

After various pollination treatments, the lowest fruit set was observed in control flowers exposed to pollination (50.8%). Seed set in self-pollinated (geitonogamy) and cross-pollinated (xenogamy) flowers was 50% and 60%, respectively. However, no fruit set was observed in the flowers in autogomy and apomixis experiments (Table 1). There were significant differences between the treatments, with (Pearson $\chi^2 = 23.053$, df = 6, P = 0.001) or without the geitonogamy treatment (Pearson $\chi^2 = 11.043$ df = 3, P = 0.011). Less fruit set in control treatments than in geitonogamy was considered significant (Pearson $\chi^2 = 13.299$, df = 3, P = 0.004).

At the end of our pollination experiments, the index of self-incompatibility (ISI) was calculated as (ISI) = 22/24 = 0.91 (between 0.2 and 1) and determined as partially self-incompatible. This result was consistent with the fact that the taxon was protandric. In several pollination studies conducted on the members of the family Lamiaceae, it was shown that the plants were self-compatible (Owens & Ubere-Jimenez, 1992). In a pollination study conducted on *Salvia verbenaca,* it was reported that the average weight of the seeds produced through self-pollination (geitonogamy) was 20% more than that of the seeds produced through cross-pollination (xenogamy) and that this genus was facultative xenogam (Navarro, 1997).

At the end of the reproductive success studies, the mean number of racemes per plant in *S. smyrnaea* (NR) was 13.56 ± 9.35, the mean number of verticillasters (NV) per raceme was 4.83 ± 1.20, the mean number of flowers (NF) per verticillaster was 3.80 ± 1.57, and the mean number of nutlets (NN) per flower was 2.03 ± 1.34 (Table 2). Using these data, it was determined that the actual seed count (ASC) and the potential number of the seeds (PNS) were 505.22 and 995.52, respectively. In many studies, it was reported that as the number of open flowers increased

Table 1. Fruit and seed set in treated flowers.

Treatments	Number of the flowers used	Number of the nutlets formed (%)	Number of the nutlets per flower (Mean ± SD)
Geitonogamy	10	22 (55.0%)	2.20 ± 1.13
Xenogamy	10	24 (60.0%)	2.40 ± 1.17
Apomixis	10	0	0
Autogamy	10	0	0
Control	180	366 (50.8%)	2.03 ± 1.34

Table 2. Number of racemes, verticillasters, flowers, and nutlets in *S. smyrnaea*.

	n	Mean ± SD	Min.- Max.
Number of racemes/plant (NR)	30	13.56 ± 9.35	1-40
Number of verticillasters/raceme (NV)	30	4.83 ± 1.20	4-8
Number of flowers/verticillaster (NF)	50	3.80 ± 1.57	1-6
Number of nutlets/flower (NN)	180	2.03 ± 1.34	0-4

Standard deviations: SD, Minimum: Min., Maximum: Max.

so did the number of pollinators' visits and, therefore, with the high seed/ovule ratio, genotypic diversity would increase in future generations of that genus (Harder & Barrett, 1995; Robertson & Macnair, 1995; Ishii & Sakai, 2001). On the other hand, it was also reported that, due to self-pollination (geitonogamy) occurring in many open flowers of racemes, external fertilisation might decrease (Harder & Barrett, 1995; Harder et al., 2000).

During these experiments, it was observed that flowers were visited by one species of the genus Anthophora, a member of the Apidae (Apinae, Anthophorini) family. Anthophora is found in all the temperate and tropical regions in the world, except Madagascar and Indonesia (Michener, 2000). The genus *Anthophora* is one of the largest in Apidae, with over 450 species and 14 different subgenera.

As a result of the data we obtained, the reproductive success rate of the plant was calculated as 50.74% with the ASC × 100/PNS equation. The highest fruit set during pollination was determined at xenogamy, geitonogamy, and control experiments, in that order. This suggests that the 55% fruit set at geitonogamy should not be ignored and that geitonogamy, acquired adaptation in the survival of *S. smyrnaea,* would be considerably effective.

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