REPRODUCTIVE SUCCESS IN *DAPHNE GNIDIUM* (THYMELAEACEAE)

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ABSTRACT

Daphne gnidium L. is a circum-Mediterranean evergreen shrub. The production of flowers and fruits of single individuals within a population were recorded. Despite very high efforts along the flowering season, all plants seemed to show a different allocation of resources during the reproductive process. All plants bore flowers with a complete male function while the female often failed to produce well formed fruits. According to anatomical analyses of reproductive material fallen down to the ground, pre- and a post-zygotic degenerations were observed. These events had no significant correlation with environmental parameters and plant dimension. The complex of data suggests an evolutionary pathway of *D. gnidium* toward dioecy. Multiple patterns of gender variation are common to other members of the Thymelaeaceae (i.e. *Thymelaea hirsuta*, *Daphne sericea* and *D. laureola*), and always caused very low fruit production like the studied species.

KEY WORDS

Reproductive success, *Daphne gnidium* L., fruit/flower production, Thymelaeaceae, sexual expression.

INTRODUCTION

The adaptive phenological, morphological, anatomical and physiological features of shrubs from Mediterranean ecosystems exert a critical influence on the survival of these plants (Aronne & De Micco, 2001; Hanley & Lamont, 2002). Among emerging patterns in the reproductive biology of these species, it has been found that fleshy fruits and low seed production are significantly associated with dioecy (Aronne & Wilcock, 1994). An example of this trend is offered by Thymelaceae, particularly *Thymelaea* and *Daphne* from the Mediterranean Basin and southern Europe (Cornara et al., 2005; Caporali et al., 2006; Roccotiello et al., 2009). This family shows high variation in mating systems and it has been studied for a long time as an example of multiple pathways to dioecy. The *Thymelaea* genus shows variable sexual expressions at the flower/plant level together with sexual segregation within populations, which are interpreted as evolutionary trends to a secondary dioecism within the family (Tan, 1980; Dommée et al., 1995; Minuto et al., 2005). The *Daphne* genus shows a particular breeding system resulting in low fruit productions (*D. sericea* – Barbi, 2008) and variable sexual expressions (*D. laureola* – Alonso, 2004; Alonso, 2005; Medrano et al., 2005).

D. gnidium is a sclerophyllous, evergreen, hermaphroditic sub-shrub, with pollen grains transported by flying insects and seeds dispersed by birds (Herrera, 1987b; Herrera et al., 1998). This species shows an unusual low fruit/flower production in many sites of its distribution range, as reported by ecological (Herrera, 1981; Jordano, 1982; Herrera, 1984, 1985, 1986, 1987b; Jordano, 1987; Herrera, 1988; Guitian & Guitian, 1990) and reproductive biology studies (Roccotiello et al., 2009). The individuals of *D. gnidium* bear hermaphroditic flowers that mature red fleshy fruit (24% of the flowers produced) bearing one seed per fruit with only 48% of them with regular embryo (Roccotiello et al., 2009); the species has been interpreted as monoecious. However, despite evidence of a low reproductive activity recorded in many places, the breeding system of the species had never been investigated in depth before. The aim of this study was to disclose the factors responsible for the low production of fruits in some individuals, and to compare the breeding system and reproductive success of this species with those of other Thymelaeaceae.

MATERIALS AND METHODS

Study sites

A natural population from the North Tyrrhenian area (NW Italy) was studied from June 2005 to October 2007 (three flowering seasons). The study site 'Piani di S. Giacomo - Savona' (44°22'49"N 8°37'14"E; 50 m a.s.l.), harbouring about 250 plants, was characterised by maquis vegetation. The mean annual precipitation was 1062.1 mm, and the mean annual temperature was 15.6°C (Vagge, 1999).

For the studied period mean daily precipitation and mean daily temperature (Table 1) were also obtained from the meteorological station of Lerca (44°24'00"N 8°39'00"E; 98 m a.s.l.), 3.5 km far from the study area (ARPAL, 2008).

Tab. 1. Meteorological data along 2005-2007 in the study area: monthly rainfall (mm) and monthly mean Temperature (°C). Data from Agenzia Regionale per la Protezione Ambientale (ARPAL, 2008).

Year	Month	Rainfall (mm)	T (°C)
2005	Jun	53.6	21.7
	Jul	27.0	22.8
	Aug	228.6	22.0
	Sep	180.4	19.8
	Oct	54.8	15.8
2006	Jun	5.2	20.2
	Jul	56.8	25.3
	Aug	209.6	21.5
	Sep	347.2	20.2
	Oct	105.0	17.6
2007	Jun	90.4	20.6
	Jul	0.2	22.4
	Aug	94.0	22.0
	Sep	132.4	19.3
	Oct	32.2	16.2

Fruit/flower production

Within the population, a study plot (180 m^2), randomly selected and containing 38 individuals, was delimited and each plant was permanently labelled with an identification number. The volume of each plant was approximated to a cylinder after measuring height and foliage diameters. Plants were checked twig by twig, twice a month, in order to count the number of flowers and fruits produced by each individual. The productivity of each individual was defined as the total number of flowers and fruits standardised by volume unit (dm^3). In order to quantify and to examine reproductive material (flowers and fruits) fallen to the ground, from June to November 2006 a cloth (4 m^2) was placed under each of 3 individuals, selected as low, mean and high flower and fruit producers according to a previous study (Roccotiello et al., 2009). Correlation analyses on productivity data were performed with Spearman rank correlation by using Statistica 8.0 software (StatSoft, 2007). Individuals' productivity was represented with a 3D scatterplot.

Flower anatomy and functionality

Flowers and fruits were collected twice a month (*N*=400 from plants; *N*=300 from cloths) and a random sample (*N*=100) was analysed with anatomical investigations to verify its functionality. Specimens were fixed in FAE (formalin-acetic acid-ethanol), alcohol dehydrated and embedded in glycol-methacrylate resin (Technovit 7100, Heraeus Kulzer GmbH &Co.). Longitudinal sections were cut at 8 μm intervals, mounted serially, and stained with toluidine blue (Sigma), pH 4.4 (O'Brien & McCully, 1981). Observations were accomplished with an optical microscope Leica DM2000.

RESULTS

Flower and fruit production

A total of 31 individuals, adjacent to the 38 plants of the analysed population, bloomed during the study period. The total productions of flowers (F), ripening fruits (rFr) and mature fruits (mFr) during the three years are reported in Table 2.

Tab. 2. Production of flowers and fruits in the population during the three studied seasons. The total number of flowers (F), ripening fruits (rFr) and mature fruits (mFr) is reported for each year. The percent ratios between ripening fruits and flowers (rFr/F), mature fruits and flowers (mFr/F), and mature fruits and ripening fruits (mFr/rFr) are also shown.

	F	**rFr**	**mFr**	**rFr/F** %	**mFr/F** %	**mFr/rFr** %
2005	17865	5693	3262	31.87	18.26	57.30
2006	17138	7667	1218	44.74	7.11	15.89
2007	8577	4197	1090	48.93	12.71	25.97
TOT	43580	17557	5570	40.29	12.78	31.73

The production rate (Fr/F) was variable from 7.11 to 18.26%. In 2005 and 2006 the flowering maximum occurred during the second half of August or at the beginning of September, more or less coincident with precipitation maxima for the studied period. In the same years, fruit ripening occurred in the same month of flowering maxima, or one month later (Fig. 1). In 2007, there was a higher precipitation level in June respect to the previous years, and a marked summer drought was recorded (Table 1). In the same year, lower productions of flowers and fruits were observed. A higher mature fruit production was related to a higher rainfall average ($r = 0.545$, $p \leq 0.05$).

The individuals showed a highly variable reproductive activity, as summarised in Table 3. The reproductive success of the population (12.78%), calculated on the global production of mature fruits (5570), was very low compared to the flower production (43580).

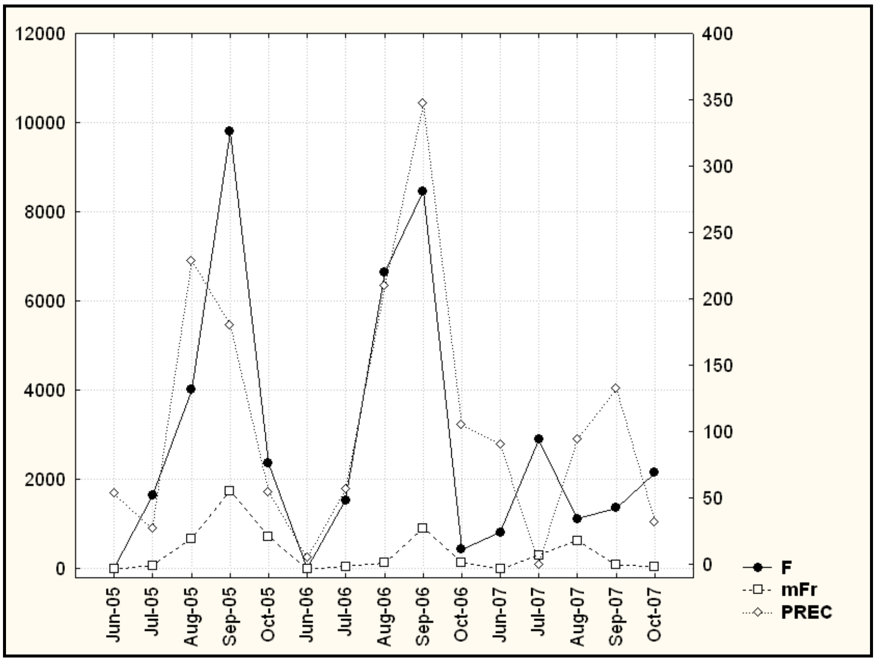

Fig. 1. Comparison between monthly precipitation (dotted line), flower production (continuous line) and fruit production (hatched line) during the three years.

The production tendency was not clearly defined but it showed an anomalous trend over the years. In general, as showed by the 3D scatterplot (Figure 2), most of plants (*N* = 24) produced a rate of mature fruits lower than the mean value (< 12.78%) in comparison with their quite high flower production. Only 7 of the remnant individuals ripened a number of fruits higher than the mean average recorded during the study period. A relationship between plant volume and fruit/flower production was found (r = 0.547 in 2005, r = 0.512 in 2006, r = 0.480 in 2007, r = 0.554 over the whole period; $p \leq 0.05$). However, the productivity for each plant (Table 3) related to the volume unit (dm^3) revealed that 13 plants bore more than 10.28 F/dm^3 (population's mean value) but only 4 of them had a higher value of mFr/dm^3 than the mean

(1.31). The non parametric analysis of data revealed a strong relationship between flower and fruit production (r = 0.842 in 2005, r = 0.672 in 2006, r = 0.656 in 2007, r = 0.832 over the whole period; $p \leq 0.001$) with a higher probability to find mature fruits on plants with a higher flower production.

Tab. 3. Total production of *D. gnidium* plants (*N*= 31) during the three flowering seasons. Plants without flowers (*N*=7) were not reported. Total number of flowers (F), ripening fruits (rFr) and mature fruits (mFr) reported for each year. N° = plant identification number; V = volume of each individual expressed as m^3; % = total fruits/total flowers percent rate per individual; F/dm^3 = number of flowers per volume unit – values higher than mean in light grey; Fr/dm^3 = number of fruits per volume unit – values higher than mean in dark grey. Overall population production, in terms of total volume, and mean flowers and fruits produced for volume unit, is summarised in the last two rows.

N°	V	F	rFr	mFr	%	F/dm^3	Fr/dm^3
9	0.23	5369.00	2192.00	864.00	16.09	23.34	3.76
10	0.12	2534.00	1839.00	586.00	23.13	21.12	4.88
12	0.40	8697.00	3638.00	1850.00	21.27	21.74	4.63
1	0.33	4320.00	1764.00	679.00	15.72	13.09	2.06
31	0.01	39.00	7.00	1.00	2.56	3.90	0.10
38	0.01	100.00	18.00	2.00	2.00	10.00	0.20
16	0.08	1507.00	489.00	42.00	2.79	18.84	0.53
19	0.04	691.00	168.00	8.00	1.16	17.28	0.20
13	0.10	1518.00	430.00	117.00	7.71	15.18	1.17
18	0.20	2964.00	952.00	112.00	3.78	14.82	0.56
15	0.02	343.00	192.00	18.00	5.25	17.15	0.90
37	0.04	514.00	106.00	8.00	1.56	12.85	0.20
32	0.04	464.00	197.00	48.00	10.34	11.60	1.20
24	0.08	848.00	6.00	0.00	0.00	10.60	0.00
6	0.20	1833.00	977.00	33.00	1.80	9.17	0.17
33	0.28	2399.00	1497.00	308.00	12.84	8.57	1.10
23	0.14	1158.00	232.00	80.00	6.91	8.27	0.57
11	0.05	411.00	147.00	23.00	5.60	8.22	0.46

N°	V	F	rFr	mFr	%	F/dm3	Fr/dm3
26	0.25	1728.00	325.00	17.00	0.98	6.91	0.07
21	0.54	3207.00	1407.00	517.00	16.12	5.94	0.96
22	0.09	355.00	201.00	12.00	3.38	3.94	0.13
7	0.43	1482.00	257.00	192.00	12.96	3.45	0.45
30	0.01	30.00	6.00	0.00	0.00	3.00	0.00
17	0.08	215.00	169.00	25.00	11.63	2.69	0.31
20	0.12	305.00	136.00	20.00	6.56	2.54	0.17
36	0.19	430.00	111.00	2.00	0.47	2.26	0.01
28	0.04	66.00	15.00	5.00	7.58	1.65	0.13
35	0.01	12.00	5.00	0.00	0.00	1.20	0.00
27	0.04	16.00	3.00	0.00	0.00	0.40	0.00
29	0.07	18.00	5.00	1.00	5.56	0.26	0.01
34	0.04	7.00	2.00	0.00	0.00	0.18	0.00
Tot	4.24	43580.00	17493.00	5570.00	12.78	9.82	130.14

During the whole period of 2006, the cloths confirmed a great loss of reproductive material by plants (Figure 3). Most of the fallen material was collected on the cloths and only the 7.35% of ripening fruits was globally lost. More than a half of flowers and ripening fruits fell down and the majority of mature fruits were collected on the cloths (Figure 3). Some seeds were also collected and recorded as mature fruits, since they probably derived from developed fruits which had lost the pericarp.

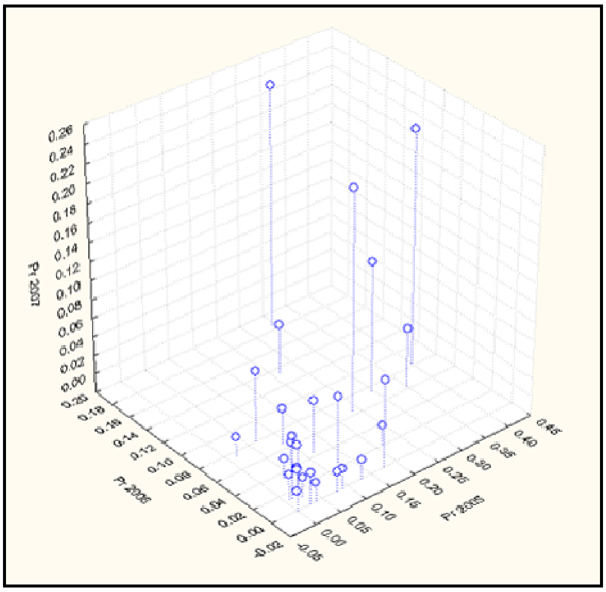

Fig. 2. 3D Scatterplot representing individuals' productivity along the three flowering seasons.

	F		rFr		Fr	
Plant	100		40,68		12,86	
Lost	↓	→ 1,22	↓	→ 2,19	↓	→ 3,94
Cloth	58,1		20,03		14,52	

Fig. 3. Mean results for flowers (F), ripening fruits (rFr) and mature fruits (Fr) collected on the cloths placed under selected plants. Values are expressed as percentage and in the case of ripening fruits still present on plants they are referred to the original total number of flowers (100%) (F) and for mature fruits (mFr) still on the plants, to the total number of ripening fruits (rFR).

Flower anatomy and functionality

The quality of production was verified by anatomical investigations on flowers and fruits collected on plants and cloths. All the collected material always revealed a normal anatomy of anthers at the different developmental stages, with the production of well-formed pollen grains.

90% of flowers fallen down after anthesis showed stigma and style degeneration and, frequently, ovule cells appeared collapsed and stretched at a pre-zygotic phase (Figure 4B, D). In the remnant 10% of the fallen flowers the reproductive organs did not seem to be damaged. The flowers still born by plants (40%) continued the ripening process; however, only a part of them showed a regular embryo development (31.63%) as confirmed by sections of green fruits collected on plants (Figure 4C). Nevertheless, most apparently normal, the 80% of mature fruits showed embryo degeneration with collapsed cotyledons and embryo (Figure 4F). A very low fraction of mature fruits (2.57% of the starting number of flowers) collected both on plants and cloths showed a regular embryo development (Fig. 4E).

DISCUSSION

Flower and fruit production

The reproductive process in *D. gnidium* showed a generally larger investment of resources by functional hermaphrodite flowers to fruit and seed formation as indicated by fruit production within the population: more than half of flowers fell down immediately after anthesis, and a very limited number of fruits reached a complete ripeness (8%). Low fruit production might indicate a low efficiency of pollination syndrome (Whelan & Goldingay, 1989), but it has been previously shown in *D. gnidium* populations from S Spain (Herrera, 1987a) and Italy (Roccotiello et al., 2009) that pollinators do not play a main role in breeding processes.

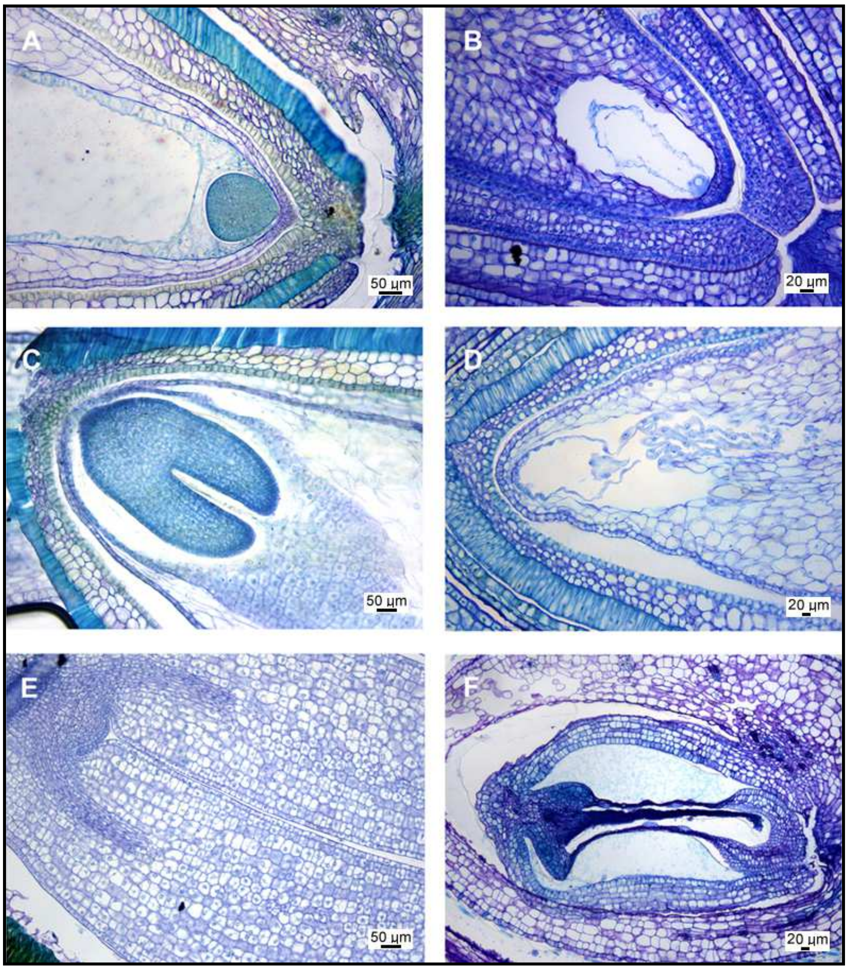

Fig. 4. Gametophyte and embryo development, TBO staining. **A**, Flower with regular developing seed where the epidermal cells are already transformed in a hard integument; **B**, Flowers collected on the cloths showing gametophyte degeneration; **C**, regular embryo development in ripening fruits; **D**, embryo degeneration in ripening fruits collected on plants; **E**, regular embryo development in mature fruits; **F**, embryo degeneration in mature fruits collected on plants.

Our present data on plant productivity reveal a different reproductive effort of this species. The use of cloths placed under selected plants showed a loss of 92.65% of flowers and fruits The small quantity of lost material (7.35%) was probably due to the cloths not wide enough to collect all flowers and fruits in windy days. In addition fruits, because of their weight, could be submitted to a “catapult” effect by twigs. Finally, a predation by birds might be also assumed, as previously reported in literature for *D. gnidium* (Herrera, 1984) and the co-generic *D. sericea* (Aronne & Wilcock, 1994; Aronne et al., 1996), but the phenomenon is probably stochastic according to the low rate of missing fruit on the cloths.

Flower anatomy and functionality

The analysis of flowers and fruits fallen down to the ground showed that the development of the female gametophyte is frequently arrested during anthesis, as confirmed by the 90% of fallen flowers (Roccotiello et al., 2009). In addition, a post-zygotic degeneration was found in ripening fruits sampled on the ground. In the first case a reproductive barrier preventing self-pollination might be suggested (Chaudhury et al., 2001), in the second case an apomictic process might induce the embryo to collapse at different developmental steps (Roccotiello et al., 2009). It seemed to be a difference in pollen allocation between functional hermaphrodite flowers and flowers with male function (giving mainly pollen) as demonstrated by our data on high number of pollinated flowers in *D. gnidium* which did not ripe. A generally larger investment of resources by true hermaphrodite flowers to seed and fruit formation could represent a step toward dioecy, as previously discussed by Aronne & Wilcock (1994).

The low fruit production due to pistil and embryo degeneration has been already recorded elsewhere in the species distribution range (Herrera, 1987a; Guitián & Guitián, 1990; Roccotiello et al., 2009). These data indicated the absence of relationship between climate and reproductive success. However, the present data showed the influence of rainfall on the ripening process.

By considering productivity per dm^3, not all individuals with a flower production higher than the average (*N*= 13) had always a correspondent high fruit production (*N*= 4). This effect might be related to environmental factors inducing post-zygotic degeneration. It has been demonstrated that in the Mediterranean environment shrub species are often affected by resources limitation, therefore lower flower production could end up with a variable percentage of fruits (Aronne & Wilcock, 1994).

Despite vegetative propagation in *D. gnidium* was reported in literature (Herrera, 1987a), it has never been observed in our study, but there is an evident fire resistance resulting in high shoots production from the base with a consequent higher competitiveness in the maquis vegetation.

Comparison with other Thymelaeaceae and Mediterranean shrubs

Ecological conditions are likely to exert a high pressure on the reproductive fitness of *D. gnidium*, with a general low rate of fruit production throughout the distribution range of the species (Herrera, 1987a; Guitián & Guitián, 1990). The ecological features of the species are shared with other species typical of the Mediterranean maquis, like *Fraxinus ornus* and *Phyllirea angustifolia* (Oleaceae). Curiously, these two latter species are interested by different levels of androdioecy (Lepart & Dommée, 1992; Dommée et al., 1999). *Phillyrea angustifolia* (Oleaceae) is a wind-pollinated shrub showing a variable ratio of males to hermaphrodites, according to the geographic area (Lepart & Dommée, 1992; Traveset, 1994). In *Fraxinus ornus* the occurrence of a functional androdioecy with male and hermaphrodite individuals is also reported (Dommée et al., 1999).

Similarly to the these latter species, the Thymelaeaceae family that includes *D. gnidium* might have evolved a high variation in mating systems (Dommée et al., 1995), also involving gender behaviours linked to reduced fruit production (Herrera et al., 1998; Alonso, 2005; De la Bandera & Traveset, 2006). The presence of dioecious, hermaphrodite, monoecious, and gynodioecious species in the family, suggests that this taxon is undergoing an evolutionary change in mating systems throughout its distributional range. According to this view, most of the Mediterranean species belonging to Thymeleaceae show low reproductive rate: *T. hirsuta* ripens a fraction as small as 10% of flowers (Cornara et al., 2005), *D. sericea* 33% (Barbi, 2008), and *D. laureola* from 14% to 33% (Alonso & Herrera, 2001).

D. gnidium, like its congener *D. sericea* (Aronne et al., 1996; Barbi, 2008), might be interpreted as a hermaprodite species with low reproductive success due to female defects in pre- and post-zygotic processes. Also, the different productivity observed among individuals might be viewed as an attempt to develop control mechanisms in the breeding system.

In conclusion, our data on the reproductive success of *D. gnidium* suggest that this shrub species is involved in a process of gender transformation under the pressure of the Mediterranean environment.

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