



TEMPERATURE AND HUMIDITY AFFECT POLLEN VIABILITY AND MAY TRIGGER DISTYLY DISRUPTION IN THREATENED SPECIES

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ABSTRACT - Within the ongoing trend of rapid climate changes there is an urgent need to assess the vulnerability of the endangered species for evaluating their conservation status and planning effective actions. *Primula palinuri* Petagna is a relict chasmophyte classified as Vulnerable due to its decreasing populations. The species evolved a distylous syndrome: a reproductive system that maintains a high genetic variability by enhancing cross-pollination. Using an experimental approach, we compared the effect of three different temperatures and three levels of relative humidity on pollen viability of both short-styled and long styled flowers of *P. palinuri*. Data highlighted that temperature and humidity significantly affect pollen viability. Notably, pollen of short-styled flowers showed a significant higher viability than the long-styled morph in any of the tested treatments. Such results raised the concern for distyly equilibrium disruption in this species, a phenomenon that in isolated populations may cause a sharp raising of the risk of local extinction. Overall, results on this *Primula* species suggest that in a scenario of climate changes the effect of environmental factors on pollen functionality of any endangered distylous species should not be overlooked for their vulnerability assessment and conservation status evaluation.

KEYWORDS: *PRIMULA PALINURI*; CLIFF SPECIES; CLIMATE CHANGES; PLANT REPRODUCTION; PLANT CONSERVATION; IUCN.

INTRODUCTION

It is well known that climate changes can drastically increase the risk of extinction of many species presently surviving in restricted geographical areas (Brook et al., 2008). Such a risk is enhanced when their generation turnover is limited by the occurrence of bottlenecks in the fulfilment of the reproductive cycle and production of new individuals (Aronne, 2017). At species level, reproductive traits as dichogamy, heterostyly and self-incompatibility resulted evolutionary successful to enhance outcrossing and increase genetic diversity of the offspring (Cardoso et al., 2018). However, within the scenario of rapid climate changes such traits can result as a constraint to achieve successful reproduction because of the complex interactions with environmental factors.

Distyly is the most common type of heterostyly and is characterized by individuals presenting two floral morphs, which reciprocally differ in the heights of anthers and stigmas within flowers; consequently, in the same population there are plants with flowers with long styles and short stamens (long-styled or pin morph), and others with short styles and long stamens (short-styled or thrum morph) (Cardoso et al., 2018). Distyly was reported at first by Darwin (1877) in *Primula* species and thereafter reported in 26 families (Naiki, 2012). The hypothesis that heterostyly evolved to promote outcrossing has been widely studied and verified (e.g., Barrett, 1992; Lloyd & Webb, 1992; Stone & Thomson, 1994).

Species of the *Primula* genus represent the classic study model for in-depth genetic studies of heterostyly syndrome (e.g. Mather, 1950; Guggisberg et al., 2006; Naiki, 2012). In Europe, *Primula* species belonging to the sect. *Auricola* diversified under the selective pressures of the climate conditions of the Pleistocene and more than 20 species are endemic to restricted areas of high mountains (Zhang & Kadereit, 2004). Throughout the Pleistocene, cold periods caused the migration of the populations to lower altitudes and latitudes and warm phases caused range contraction, local extinction, and consequent genetic isolation (Zhang & Kadereit, 2004; Crema et al., 2013). At present, all species of the sect. *Auricola* live in mountain habitats (Zhang & Kadereit, 2004) with the exception of *Primula palinuri* Petagna which is the only Mediterranean and maritime species in the whole genus (Richards, 2003). *P. palinuri* is an endemic species and has a small fragmented geographical range confined to a narrow belt of the Tyrrhenian coast of southern Italy (Pizzolongo, 1963; Ricciardi, 1973; Aronne et al., 2014). It is a relict chasmophyte reported in the IUCN (International Union for Conservation of Nature) Red List as Vulnerable (VU) species with decreasing population (Rossi et al., 2013). The main threats to the species are human impact, fire, competition with invasive alien species and landslides (Uzunov et al., 2008). Most of the plants of *P. palinuri* live in soil pockets inside rock fractures, on vertical or sub-vertical north-facing limestone cliffs, most of which are sea plunging (Aronne et al., 2014; Strumia et al., 2020).

Identification of the most critical phases in lifecycle of *P. palinuri* pointed to seed germination and seedling establishment as bottlenecks in the turnover of subsequent generations (Aronne et al., 2010; Aronne, 2017). Moreover, specific studies highlighted that seeds and seedlings of *P. palinuri* are less adapted to salted conditions of the sea cliffs than other cliff species co-occurring in the same habitat (Strumia et al., 2020).

Studies on pre-dispersal biology showed that the reproductive success of *P. palinuri* is high: plants flower every year and produce hundreds of seeds. Data on floral morphology proved the occurrence of the fully distylous syndrome: the reciprocal position of stigma and anthers in the flower as well as the intra-morph incompatibility and the necessity of crosspollination between the two morphs to succeed in seed development (Aronne et al., 2013). Plants have an overall flowering period starting in winter (end of January) and ending in spring (mid of April) and single flowers show an extraordinary long anthesis of over a month (Aronne et al., 2014). The long flowering period and the flower longevity have been interpreted as results of two contrasting selective pressures exerted by progressive climate warming: the first bounds the anthesis to Winter temperatures by pollen functional traits; the second extends the flowering season through Spring by the pollination rate increase (Aronne et al., 2014). Previous studies highlighted that pollen viability

is significantly lowered by increasing temperature and air humidity, envisaging a rising risk of extinction in the ongoing trend of climate changes (Aronne et al., 2014). The distyly syndrome in *P. palinuri* is not limited to the reciprocal position of anthers and stigmas but is complemented by differences in pollen size and exine ornamentation (Aronne et al., 2013). However, data on possible different effects of environmental factors on pollen functionality of the two flower morphs are not available.

Considering that in isolated populations any stochastic event altering the equal proportion of the two morphs in favour of a single genet can cause a sharp raising of the risk of local extinction (Endels et al., 2002; Kéry et al., 2003; Brys et al., 2004), we compared pollen functionality of long-styled and short-styled flowers of *P. palinuri* under different combinations of environmental factors. More specifically, using an experimental approach we assessed the effects of different temperatures and humidity values on pollen viability of the two morphs for a time period comparable to flower anthesis.

MATERIALS AND METHODS

Plants of *Primula palinuri* Petagna mainly occur on ledges and crevices of north-facing vertical cliffs along a narrow coastal area of about 65 km long and 1 km wide between Palinuro (40°02'04"N - 15°17'08"E) and Capo Scalea (39°50'47"N - 15°46'44"E).

In the field, we harvested a total of 30 pendulous flowers whose phenological stage corresponded to beginning of the anthesis. More specifically, for each of the two floral morphs we sampled one flower per plant, from five plants, from three different populations. Flowers were individually labelled and immediately transferred to the laboratory. Here, we collected and carefully mixed the pollen from all the flowers obtaining a bulked pollen sample for each of the two flower morphs. We experimentally tested the effects of temperature, relative humidity (RH) and time on pollen functionality. Pollen functionality was assessed as pollen viability and measured by means of the diaminobenzidine (DAB) reaction (Dafni et al., 2005).

The choice of the environmental parameters followed the criteria reported by Aronne et al. (2014). More specifically, we used three different treatments of RH (0, 48 and 100%) and temperature (6, 18 and 30 °C). Different conditions of RH were obtained in a sealed glass chamber (20×20×20 cm) according to Aronne et al. (2006). In particular, the condition of 100% RH was obtained by lining the chamber with moist filter paper, that of 48% RH with a saturated water solution of NaI and that of 0% RH with silica gel. The experiment was performed according to a factorial design in which humidity

and temperature conditions were cross linked. Overall, we used 2 flower morphs x 3 temperatures x 3 humidity values x 4 time periods. Per each treatment we prepared 3 slides and scored a minimum of 250 grains per slide. Pollen was spread onto distinct microscope slides, placed into the sealed glass chambers, and transferred in three incubators set at different temperatures (VELP®, FOC 200IL). We checked pollen viability at different time intervals that resembled the long anthesis of a single flower of *P. palinuri*. More specifically, after 1, 11, 20 and 32 days, one slide for each of the nine combinations of temperature and humidity was taken out and scored for pollen viability.

At the end of each treatment, to assess pollen viability we added one droplet of DAB solution on each pollen sample, gently warmed the slides with a gas lighter, waited about one minute for staining reaction, and mounted with a cover slip (Dafni, 1992). We scored the samples using an Olympus® BX-60 light microscope and classified as viable only the dark-brown grains.

Statistical analysis

Descriptive statistics were used to calculate central tendency, dispersion and distribution of data. To apply parametric inferential statistic, the symmetry of data was checked; in case of asymmetry, the logarithmic transformation was performed (Sokal & Rohlf, 1995). The effect of each of the independent variables (temperature, RH and time) was tested separately using one-way ANOVA. If a statistical difference between treatments resulted from the one-way ANOVA, the post-hoc multiple comparison analysis (Bonferroni $P < 0.05$) was performed.

RESULTS

Overall data on the effects of temperature and humidity on the pollen of the two flower morphs showed no morphological abnormalities and uniformly reacted to the DAB enzymatic staining. During the scoring, only few doubtful cases were found and excluded from the count. In the whole experiment, we classified as viable or not viable more than 54000 single pollen grains.

Results of the ANOVA showed that viability gradually decreased during the 32 days long period. Differences in pollen viability resulted highly significant (F-ratio = 7.31, $P < 0.001$ for short-styled and F-ratio = 4.64, $P < 0.001$ for long-styled) for pollen of both flower morphs (Figure 1).

More specifically, the post hoc tests showed that viability of pollen from short-styled flowers significantly decreased from 70.1% after one day to 42.2% after one month; that

of pollen from long-styled flowers decreased from 51.3% to 30.7% in the same interval (Figure 1). Focusing on each time interval, data clearly showed that percentage of pollen viability of short-styled flowers was significantly higher than that of long-styled flowers and this difference persisted over time (Figure 1).

Considering the effect of RH, the ANOVA main effect was significant (F-ratio = 383.67, $P < 0.001$ for short-styled and F-ratio = 325.45, $P < 0.001$ for long-styled). However, the post hoc analysis showed that in both morphs, viability did not change when pollen was kept at 0% RH and 48% RH, while it drastically decreased when grains were kept at 100% RH (Figure 2). Moreover, comparing viability in each of the three humidity treatments, viability of pollen of short-styled flowers was always significantly higher than that of pollen of long-styled flowers (Figure 2).

Pollen viability was less affected by temperature (Figure 3): the ANOVA main effect was significant only for pollen from long-styled flowers (F-ratio = 5.56, $p < 0.001$). In such cases viability significantly decreased (down to 31.4%) only when pollen was incubated at 30 °C. In addition, considering each temperature treatment, differences between the morphs were always significant with pollen of short-styled flowers being more viable than that of long-styled flowers (Figure 3).

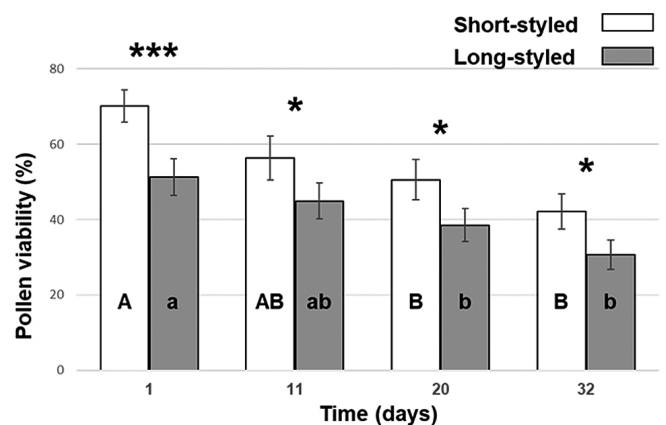


Figure 1. Pollen viability (mean % \pm SE) of short-styled (white bars) and long-styled (grey bars) flowers at different time intervals (1, 11, 20, 32 days). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same time are reported above each couple of bars (* = $P < 0.05$; *** = $P < 0.001$). Letters represent the result of the post-hoc test (Bonferroni, $P < 0.05$) of the one-way ANOVA performed to compare pollen viability at different time within short-styled flowers (capital) and long-styled flowers (lowercase); times with the same letters are not statistically different.

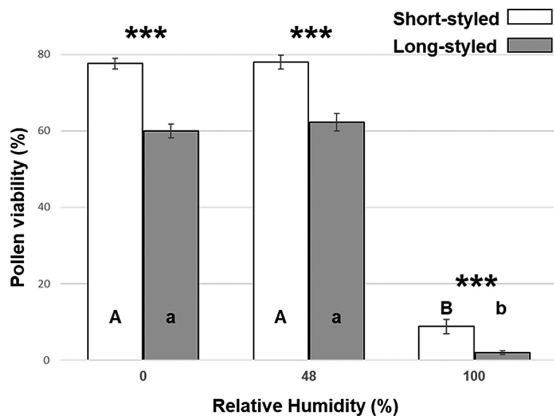


Figure 2. Pollen viability (mean % \pm SE) of short-styled (white bars) and long-styled (grey bars) flowers at different RH (0, 48 and 100%). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same RH are reported above each couple of bars (***) = $P < 0.001$). Letters represent the result of the post-hoc test (Bonferroni, $P < 0.05$) of the one-way ANOVA performed to compare pollen viability at different RH within short-styled flowers (capital) and long-styled flowers (lowercase); RH values with the same letters are not statistically different.

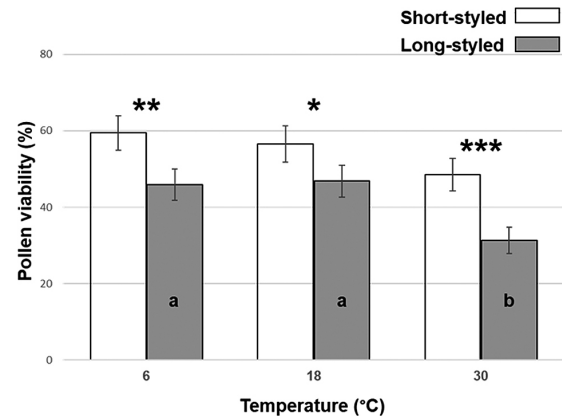


Figure 3. Pollen viability (mean % \pm SE) of short-styled (white bars) and long-styled (grey bars) flowers at different Temperature (6, 18 and 30 °C). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same Temperature are reported above each couple of bars (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$). Letters represent the result of the post-hoc test (Bonferroni, $P < 0.05$) of the one-way ANOVA performed to compare pollen viability at different Temperature within short-styled flowers (capital) and long-styled flowers (lowercase); Temperature values with the same letters are not statistically different.

DISCUSSION

Single pollen grains are autonomous, haploid and genetically different organisms constituted only by two (max three) incorporated cells; they must survive and interact with the environment throughout the period from pollen release to pollen transfer onto the stigma and subsequent pollen tube development. During plant reproduction, pollen is thus exposed to natural selection and can influence the genetic constitution of the resulting diploid sporophytic generation (Mulcahy, 1979).

Our data showed that pollen of *P. palinuri* is affected by both high temperature and humidity confirming results reported in a previous study (Aronne et al., 2013). Similar effects of temperature and humidity on pollen functionality were reported for other Mediterranean coastal species (Aronne, 1999). Our results further highlight that pollen functionality should also be taken into account in the overall evaluation of the future conservation status of this endemic species. This is particularly relevant considering the ongoing climate changes, in which the gradual increase of the temperature is complemented by other environmental changes and by the increase of both intensity and frequency of extreme weather events, including heat waves (Hedhly, 2011).

The most significant contribution of our results refers to the differences in pollen functionality between the two flower morphs. Distyly has polyphyletic origins (Naiki, 2012) and is considered a trait that enhances intermorph cross-pollination

and genetic variability (e.g., Lloyd & Webb 1992; Stone & Thomson, 1994; Barrett, 2002). As for most of the distylous species, also in *P. palinuri*, in addition to the reciprocal position of anthers and stigma, different pollen sizes and exine ornamentations characterize the two morphs (Aronne et al., 2013). Our data highlighted that pollen of the two morphs interacts differently with the environmental factors raising the concern for the risk of a distyly equilibrium disruption. Populations of distylous species at equilibrium have short-styled flowers and long-styled flowers equally represented; however, deviation of morph frequencies from 1:1 ratio (anisoplethy) has been frequently reported in distylous species (Kéry et al., 2003). They are generally associated to: a) demographic stochastic processes (Kéry et al., 2003); b) morph-specific differences in mating patterns and self-compatibility levels (Barrett & Husband, 1990); c) morph-specific selection in favour or against one morph type (Kéry et al., 2003).

Unequal frequencies of morphs were reported for *Primula* species living in isolated populations and fragmented habitats (e.g. Endels et al., 2002; Jacquemyn et al., 2002; Brys et al., 2003). In such contexts, biased morph ratios can disrupt pollen processes contributing to reduced reproductive success (Matsumura & Washitani, 2000; Brys et al., 2004). Morph anisoplethy can exert negative demographic and genetic consequences for the populations and consequently increase the extinction risk of small populations (Washitani, 1996; Jacquemyn et al., 2003; Kéry et al., 2003).

A fully distyly syndrome has been reported for *P. palinuri* (Aronne et al., 2013). However, a slight (although statistically not significant) prevalence of short-styled inflorescences was highlighted in all checked populations (Aronne et al., 2013). Our data on pollen functionality clearly showed that pollen from short-styled flowers performed better than that from long-styled flowers in all treatments of temperature and humidity and throughout the whole anthesis period. If such a prevalence will persist in time, a scenario of significant skewed morph ratios in the few isolated populations can lead to a loss of population genetic diversity by increasing the frequency of the most fertile short-styled morph.

Even if *P. palinuri* is currently classified as Vulnerable (Rossi et al., 2013) the decreasing trend of the population is well known and the risk of extinction of local populations may rise sharply, especially considering the effects of climate change. Skewed ratios in favour of long-styled morph was reported in fragmented populations of *Primula veris*, but no significant relationship was found between genetic patterns and stochastic morph bias (Van Rossum & Triest, 2006); such results raised the hypothesis that individual longevity of the plants in the population and clonality can slow down the expression of the biased morph frequencies. The two morphs are rather balanced also in the populations of *P. palinuri* (Aronne et al., 2010; De Micco & Aronne, 2012; Strumia et al., 2020); however, our results suggest that in this species differences in pollen functionality can favour one of the two morphs in isolated populations. Considering that pollen is highly and finely affected by the interaction with environmental factors, in a scenario of climate changes the role of pollen for long term survival of distylous species should be not overlooked.

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