Article

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Comparing flower biology in five species of *Gagea* (*Liliaceae*) from southern Italy

Abstract

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The reproductive traits in *Gagea* are affected by both abiotic and biotic environmental factors. However, further intrinsic drivers, like the ploidy level, could also have a significant influence on the sexual efficiency of these geophytes. Here, we present a comparative study involving the reproductive biology of five species of *Gagea* occurring in southern Italy, and representing different ploidy levels. The experimental investigations included flower phenology, pollen quality (i.e., morphology, viability, and germination), and stigma receptivity. Such data were then combined with information on seed-set available from previous work. All the studied species showed a gradual pollen presentation, and an early and durable stigma receptivity. Such traits represent effective functional adaptations to prevent pollen limitation under the unpredictable weather dynamics typical of the early blooming season of these plants. The use of a diluted substrate favoured the rate of pollen germination in vitro and, likely, it represents a further adaptation versus adverse environmental conditions (i.e., rain damage on exposed pollen). However, the rates of pollen viability and germination were generally low and appeared related to the ploidy level. Data inherent pollen quality (i.e. morphology, viability and germination) and seed-set indicated the hexaploid *G. lutea* as the most fertile species, while the heptaploid *G. fragifera* had the lowest scores for all the considered traits. Finally, by considering the isolation and reduced size of the studied population, the unexpected low fertility found in the diploid *G. minima* was possibly due to inbreeding depression related to the relict status of this species in southern Italy.

Key words: *Gagea*, Early-flowering plants, Inbreeding depression, Ploidy level, Pollen germination, Pollen presentation, Pollen viability, S Italy, Stigma receptivity.

Introduction

The reproductive success of early-flowering herbs is strongly constrained by the physical conditions characterizing their habitat at blooming time. These plants should cope with two opposite needs. Indeed, they should flower during the first suitable period, in order to limit competition for resource and pollinators but, on the other hand, they are also forced to flower under still unpredictable weather dynamics (Schemske & al. 1978).

The occurrence of unsuitable weather can affect the sexual performance of plant species in different ways, e.g., causing damage to reproductive structures (Percival 1955; Eisikowitch & Woodell 1975; Jones 1976; Eisikowitch 1979; Pacini & Franchi 1984; Corbet & Plumridge 1985; Corbet 1990), or inhibiting pollinator activity and efficiency (Pacini & Franchi 1984; Eisikowitch & al. 1992; Dafni 1996). Furthermore, habitat features may influence the flowering rates within the populations (Eisikowitch 1979; Dafni & al. 1981; Gargano & al. 2007), as well as patterns and effectiveness of plant-pollinator interactions (Gargano & al. 2017). As a consequence, ecologists and evolutionary biologists often considered these constraints as relevant drivers of phenological (Dafni & al. 1981; Harder & Thomson 1989; Thomson & Thomson 1992; Petanidou & al. 1995), morphological (Nishikawa & Kudo 1995; Nishikawa 1998), and physiological (Eisikowitch & Woodell 1975; Corbet 1990; Beardsell & al. 1993; Dafni & Firmage 2000) adaptations in early-flowering, as well as in late-flowering (Dafni 1996), plants. The role played by hybridity, polyploidy, and vegetative propagation in regulating the sexual fertility of flowering plants should be also considered (Wells 1979; Ness & al. 1990; Sato & al. 1993; Burton & Husband 2000; Nepi & Franchi 2000).

Elsewhere, we evaluated the possible relationships between habitat features and reproductive behaviour of some early-flowering species belonging to the genus *Gagea* from Calabria (southern Italy) (Gargano & al. 2007). This fieldwork provided evidences that agreed with the above reported considerations. In fact, most of the evaluated reproductive traits (phenology, amount of clonality, and seed production) affected by abiotic and biotic environmental factors. Furthermore, although a poor efficiency of sexual processes characterized all the studied species, the levels of sexual recruitment were connected to the ploidy level of the species. Artioploids, such as triploid (*G. lojaconoi*, *G. granatellii*) and heptaploid (*G. fragifera*) species, showed pollen malformed in higher rates and lower seed-set than the perissoploids, such as tetraploid (*G. peruzzii*) and hexaploid (*G. lutea*) plants.

In this paper, we present the results of further research carried out on the same target species, with the addition of *G. minima*, a diploid (Peruzzi & Aquaro 2005) and biogeographically interesting species (Peruzzi & Gargano 2005), and the exclusion of *G. granatellii* because of its very low flowering rates. This work is focused on pollen and stigma biology, which often reflect the pressure of genetic, environmental and/or conservation constraints (Thomson & Thomson 1992; Beardsell & al. 1993; Willis 1993; Thomson & al. 1994; Dafni 1996; Carr & Dudash 1997; Dieringer 1999; Dafni & Firmage 2000; Nepi & Franchi 2000). We carried out experimental investigations aiming to compare flower phenology, pollen quality (morphology, viability, and *in vitro*/on stigma germination), and stigma receptivity among the five selected species.

Materials and Methods

Studied species

All the considered species are small bulbous geophytes with yellow, mainly erect, radiate flowers.

Gagea sect. *Didymobulbos* (K. Koch) Boiss.

G. fragifera (Vill.) Ehr. Bayer & G. López – Pedunculate suprabasal axillary bulbils are present only in young (not yet flowering) individuals, while one basal bulblet occurs over its whole life cycle. It is an Eurasiatic species (Peruzzi & al. 2011), growing in mountainous to subalpine meadows and at the margins of beech forests from 1,000 to 1,900 m a.s.l. (Peruzzi & Gargano 2005; Gargano & al. 2007). The studied population (at ca. 1,200 m a.s.l.) is heptaploid, with $2n = 84$ chromosomes (Peruzzi & Aquaro 2005).

G. lojaconoi Peruzzi – There is only one basal bulblet during its whole life cycle. It is a Central Mediterranean species (Tison & al. 2013), growing in garrigues from 100 to 1000 m a.s.l., which was formerly misidentified with *G. chrysantha* Schult. & Schult. f. (Peruzzi & Gargano 2005; Gargano & al. 2007). The studied population (at. ca. 200 m a.s.l.) is triploid, with $2n = 36$ chromosomes (Peruzzi 2003, under the name *G. chrysantha*).

G. peruzzii J.-M. Tison – Sometimes a single axillary suprabasal bulbil is present. On the contrary, there is always a single basal bulblet for each individual. It is a species endemic to the Pollino National Park (Tison & al. 2013; Roma-Marzio & al. 2016), growing in rocky habitats from 500 to 1300 m a.s.l., which was formerly misidentified with *G. bohemica* (Zauschn.) Schulf. & Schult. f. by Peruzzi & Gargano (2005) and by Gargano & al. (2007). The studied population (at ca. 1,000 m a.s.l.) is tetraploid, with $2n = 48$ chromosomes (Peruzzi 2003, under the name *G. bohemica*).

Gagea Salisb. sect. *Gagea*

G. lutea (L.) Ker Gawl. – Many basal bulbils are present only in young (flowering) individuals, while adult flowering plants are unable for vegetative propagation (Schnittler & al. 2009). It is an Eurosiberian species, becoming rare southwards along the Italian peninsula. It grows in meadows at the margins of beech forests, from 900 to 1,500 m a.s.l. (Peruzzi & Gargano 2005; Gargano & al. 2007). The studied population (at ca. 1,200 m a.s.l.) is hexaploid, with $2n = 72$ chromosomes (Peruzzi & Aquaro 2005).

Gagea sect. *Minimae* (Pascher) Davlian.

G. minima (L.) Ker Gawl. – In this species, many basal bulbils occur only in young (not yet flowering) individuals, while one basal bulblet is present over its whole life cycle. It is an Eurosiberian species, showing a scattered distribution in peninsular Italy (Bernardo & al. 2013; Falcinelli & al. 2016) and reaching the southern limit of its range in Calabria, where it is currently known for a couple of populations (Roma-Marzio & al. 2016). It grows in cold places, rich in nitrates, from 950 to 1,900 m a.s.l. (Peruzzi & Gargano 2005). The studied population (at ca. 1,900 m a.s.l.) is diploid, with $2n = 24$ chromosomes (Peruzzi & Aquaro 2005).

Experimental protocols for studying flower biology

The flower phenology was monitored from the flower opening to its wilting, in order to record data related to the timing of pollen exposure and stigma morphology. Preliminary observations allowed us to identify three different flower phases related to anther dehiscence: F1) pollen not yet exposed, F2) pollen exposed on the anthers of the first stamens whorl, and F3) pollen exposed on all the anthers. Such flower phases were used as reference for further experiments on flower biology.

Data about pollen malformation were derived from Gargano & al. (2007), who studied the same populations, for four out the five taxa. This data-set was completed by carrying out qualitative observations under light microscope on about 600 pollen grains of *G. minima*.

Pollen viability was checked for each flower phase by the MTT test (Norton 1966; Khatun & Flowers 1995), which detects the presence of dehydrogenase. For each species, pollen from different anthers was placed on microscope slides, and it was covered by a drop of stain. The stained grains were checked after 50 minutes. For each species, an amount of 100 pollen grains × 5 replicates was collected on different flowers for each flower phase.

According to Brewbaker & Kwack (1963), *in vitro* pollen germination was checked by a solution composed by boric acid (100 mg/l), calcium nitrate (300 mg/l), magnesium sulfate heptahydrate (200 ml/l), and potassium nitrate (100 mg/l). A variable amount of sucrose was added to this base-solution to create a series of six different sucrose concentrations (5, 10, 20, 30, 40, and 50%), in order to investigate pollen germination under different osmotic regimes. The germinated grains were counted after 24 hours of treatment and the experiment was composed by 12 trials for each test. In *G. fragifera*, *G. lutea*, *G. minima*, and *G. peruzzii* the test was carried out on pollen from all the three flower phases, while for *G. lojaconoi* only indiscriminate pollen was used, because of the low number of available flowers.

Following Macior (1983), the presence of pollen grains on the stigma of flowers at different phases was identified by using a mixture of methyl green and phloxine-B (in 50% of alcoholic solutions). Once stained, grains with pollen tube were counted as germinated. For each species, this procedure was repeated for six flowers per flower phase.

Finally, the tests of stigma receptivity were performed directly in the field by means of the Perex test (Dafni & Maues 1998); by evaluating the occurrence and concentration of peroxides on the stigma surface, this test allows to check the receptive area of the stigma and its level of receptivity. For each species, the tests of stigma receptivity were repeated on six flowers per flower phase, with the only exception of *G. peruzzii*, since the flowers of this species wilted very quickly.

Data analysis

Analyses of variance (One-way ANOVA and Two-way ANOVA) were carried out for each variable (pollen viability, pollen germination *in vitro*, stigma receptivity and pollen germination on the stigma) by considering the species, the flower phase, and the sucrose concentration (for *in vitro* pollen germination) as factors. A Pearson correlation with two-tailed test of significance was applied in order to highlight the relationships between pollen/stigma traits and flower phases. All statistical tests were performed by using the package SPSS® 26.0 for Windows.

Results

Three main phases, related to anther dehiscence, were identified in all species (Fig. 1). The first phase (F1) concerned open flowers with pollen not yet exposed (Fig. 1A); an

intermediate phase (F2) was characterized by the dehiscence of the three most external anthers (Fig. 1B); finally, in the third phase (F3), the pollen was exposed in all the six anthers (Fig. 1C). The stigma appeared to be yellowish in all the phases, but during F2 and F3 its surface assumed a papillose and glutinous appearance.

The results of the MTT test are provided in Table 1. The mean amount of viable pollen had the highest score in *G. lutea*, followed by *G. lojaconoi* and *G. peruzzii*, while the lowest viability was recorded in *G. minima* and *G. fragifera*. Significant negative correlations between pollen viability and flower phase resulted in *G. minima* ($r = -0.918$; $P < 0.001$), *G. lojaconoi* ($r = -0.618$; $P = 0.014$), and *G. fragifera* ($r = -0.570$; $P = 0.027$), while no significant relationships was found in *G. peruzzii* ($r = 0.503$; $P = 0.056$) and *G. lutea* ($r = -0.144$; $P = 0.608$). The two-way ANOVA performed for pollen viability (Table 2) revealed that the difference among the species for pollen viability ($F = 18.992$; $P \leq 0.0001$) was only marginally influenced by flower phase ($F = 4.339$; $P = 0.0168$). According to correlation tests, the one-way ANOVA showed that the largest variation of pollen across flower phases occurred in *G. minima* ($F = 32.308$; $P \leq 0.0001$); anyway, significant variations of pollen among flower phases were detected in all the taxa (Table 2).

The results of *in vitro* pollen germination tests are summarized in Table 4. The amount of germinated pollen was lower than the fraction resulted potentially viable by MTT staining. Pollen germination rates significantly varied among species ($F = 6.483$; $P \leq 0.0001$; Table 4). Also in this experiment, *G. lutea* reached the highest values of fertility and *G. fragifera* the lowest (Table 3). Instead, in contrast to the MTT test, *G. peruzzii* showed pollen germination higher than *G. lojaconoi*.

As expected, the sucrose concentration of germination solutions severely affected the rate of pollen germination ($F = 20.406$; $P \leq 0.0001$; Table 4). In fact, 95% of germinations occurred at sucrose concentration between 5 and 30% (Table 3). The two-way ANOVA (Table 4) indicated that the species have different pollen germination patterns in relation to sucrose concentration ($F = 22.672$; $P \leq 0.0001$). Indeed, *G. fragifera* reached the germination peak at 5% of sucrose, *G. lutea* and *G. lojaconoi* at 10%, *G. minima* and *G. peruzzii* at 20% (Table 3). Furthermore, in *G. lutea* the pollen germinated only in a narrow range of sugar concentration, as no germination at all occurred at sucrose concentration > 30% (Table 3).

Also the flower phase strongly influenced pollen germinations *in vitro* ($F = 27.633$; $P \leq 0.0001$; Table 4). Two-way ANOVA highlighted different patterns among species ($F = 29.691$; $P \leq 0.0001$; Table 4). Strong negative correlations between in vitro pollen germination rates and flower phase were found in *G. minima* ($r = -0.395$; $P < 0.001$) and *G. lutea* ($r = -0.352$; $P < 0.001$), while no significant pattern was detected in *G. peruzzii* ($r = -0.059$; $P = 0.392$) and *G. fragifera* ($r = -0.011$; $P = 0.869$).

The stained area and the disposition of the papillae suggested that the receptive stigmatic surface was restricted to the stigma apex. In all the specimens, the stigma was receptive over the whole flowering period (Table 1). However, the two-way ANOVA evidenced significant differences in terms of receptivity level among species ($F = 28.08$; $P \leq 0.0001$) and flower phases ($F = 4.125$; $P = 0.0198$). As showed in Table 1, the lowest stigma receptivity was found in *G. minima*; while in *G. lutea* the receptivity was strongly linked to the flower aging, as it gradually increased from F1 to F3 ($r = 0.682$; $P = 0.002$).

Fig. 1. Flowers of *Gagea lojaconoi* in phase 1 (FP1), with no pollen exposed (A); phase 2 (FP2), with pollen exposed in the three most external anthers only (B); phase 3 (FP3), with pollen exposed in all the six anthers (C). Pictures shot 19 February 2006 by L. Peruzzi.

In the two-way ANOVA, the amount of germinated pollen on the stigma significantly varied among the species ($F = 15.727$; $P \leq 0.0001$), and the highest amount of germinated pollen was found on stigmas of *G. lutea* (Table 1). Instead, no effect was induced by the flower phase ($F = 0.961$; $P = 0.3869$), with the only exception of *G. minima*, in which the fraction of germinated grains were strongly related to the flower phase ($r = -0.652$; $P = 0.003$; Table 1).

Discussion

Flower phenology: ecological and evolutionary implications

The sequential anther dehiscence observed in the studied species could be interpreted as a strategy aiming to extend the temporal pollen availability. Many authors highlighted that early-flowering plants often share various adaptations to face pollen limitation, such as anthesis extension (Nishikawa & Kudo 1995; Nishikawa 1998), long-lived pollen, early and durable stigma receptivity (Beardsell & al. 1993; Dafni & Firmage 2000). Although Harder & Thomson (1989) reported that simultaneous pollen presentation can be expected if pollinator visits are infrequent, it was argued that pollen longevity plays a basic role in the adoption of a gradual or simultaneous pollen presentation (Thomson & al. 1994). Indeed, Thomson & Thomson (1992) highlighted that a gradual pollen presentation can be a successful strategy under low frequency of pollinators, in plants with short pollen longevity. The low scores of pollen viability (Table 1) and pollen germination (Table 3) suggested a rather low pollen fertility in the studied species. In addition, the exposed pollen (collected from flower phases F2-F3) often expressed lower viability and germination than pollen collected just before exposure (flower phase F1) (Tables 1, 2, 3). Furthermore, only a little fraction of germinated pollen was detected on the analysed stigmas (Table 1). All these results suggest that environmental conditions severely affect pollen viability in *Gagea*. Our results also agree with Thomson & al. (1994), which see a gradual pollen presentation as the best adaptation in facing pollen limitation in case of short pollen longevity.

Pollen and stigma biology: ecological and evolutionary implications

As found in other early-flowering geophytes (Peruzzi & al. 2012), the studied species showed highest pollen germination at low sugar concentration, with absence of pollen bursting (Tables 3-4). According to Dafni (1996), such patterns are congruent to an adaptation versus mechanic and osmotic damage caused by the frequent rainfalls typical of the spring Mediterranean season. Indeed, rain is known as a potentially limiting factor for different aspects of plant sexual reproduction, due to its direct (Percival 1955; Eisikowitch & Woodell 1975; Jones 1976; Eisikowitch 1979; Pacini & Franchi 1984; Corbet & Plumridge 1985; Corbet 1990) and indirect (Pacini & Franchi 1984; Eisikowitch & al. 1992; Dafni 1996) effects on reproductive structures.

Our data on stigma receptivity and *in vivo* pollen germination evidenced that the stigma of the investigated species has an early (simultaneous to flower opening) and durable receptive phase (Table 1). This finding has a particular interest if we consider that the genus *Gagea* is proterandrous, given that male gametophytes develop earlier than embryo sacs (Caparelli & al. 2006 and literature cited therein). According to Beardsell & al. (1993) and Dafni & Firmage (2000), these traits of stigma biology could also reflect adaptations to optimize pollination processes in early-flowering plants.

Genetic influence on the observed fertility patterns

A general low pollen viability was evident in all the taxa (Table 1), being the mean viable pollen never higher than 33.8 ± 4.6%. The levels of pollen fertility further decreased in the procedures of *in vitro* pollen germination, where the highest score (reached by *G. minima*) was 21.00 ± 7.7% (Table 3). The discrepancy between pollen viability and germination tests suggest that the MTT can overestimate pollen fertility.

Table 1. Mean scores (%) ± Standard deviation for stigma receptivity (SR), pollen viability (PV) and pollen germination on the stigma (PS) in the studied *Gagea* species. * = incomplete data (see the text).

Species	Flower phase	SR	PV	PS
G. fragifera	1	86.7 ± 20.7	12.0 ± 1.5	0.8 ± 1.2
	2	93.3 ± 16.3	7.6 ± 1.7	1.9 ± 2.8
	3	86.7 ± 20.7	8.9 ± 1.1	0.9 ± 1.6
G. lojaconoi	1	86.7 ± 21.0	33.8 ± 4.6	1.3 ± 1.1
	2	100.00 ± 0.0	21.0 ± 6.5	4.4 ± 2.7
	3	93.3 ± 16.3	21.0 ± 8.3	2.7 ± 1.7
G. peruzzii	1	100.0 ± 0.0*	15.0 ± 5.8	0.00*
	2	100.0 ± 0.0	25.8 ± 5.9	0.00*
	3	100.0 ± 0.0	23.6 ± 5.8	8.7 ± 4.5
G. lutea	1	40.00 ± 12.6	24.2 ± 5.4	11.2 ± 5.6
	2	67.00 ± 30.1	32.0 ± 1.2	19.2 ± 9.8
	3	86.7 ± 20.7	22.2 ± 4.6	9.5 ± 7.4
G. minima	1	43.3 ± 8.2	22.2 ± 3.3	7.8 ± 5.7
	2	36.7 ± 19.6	14.6 ± 3.2	1.8 ± 1.4
	3	50.0 ± 11.0	6.0 ± 3.1	0.7 ± 0.8

Table 2. Variance analyses of pollen viability among the studied *Gagea* species and flower phases. PV = pollen viability (%).

Analysis type	Target	Factor	Dependent variable	F	P
Two-Way ANOVA	all species	species	PV	18.992	≤ 0.0001
		flower phase		4.3394	0.0168
One-Way ANOVA	*G. fragifera*	flower phase	PV	13.057	0.001
	G. lojaconoi			6.211	0.014
	G. peruzzii			4.814	0.029
	G. lutea			7.603	0.007
	G. minima			32.308	≤ 0.0001

Table 3. Mean pollen germination (%) ± standard deviation in relation to the flower phase (FP) and sucrose concentration in the studied *Gagea* species; in *G. lojaconoi* relationships between pollen germination and flower phase are not considered.

Species	FP	[5]	[10]	[20]	[30]	[40]	[50]
G. fragifera	1	1.4 ± 0.9	1.5 ± 1.1	0.3 ± 0.3	0.4 ± 0.4	0.00	0.00
	2	2.4 ± 1.7	1.0 ± 0.8	0.8 ± 0.5	0.3 ± 0.3	0.3 ± 0.3	0.2 ± 0.2
	3	2.3 ± 1.1	0.3 ± 0.5	0.4 ± 0.5	0.1 ± 0.3	0.2 ± 0.4	0.00
G. lojaconoi		0.5 ± 0.8	8.4 ± 2.6	0.4 ± 0.7	0.33 ± 0.4	0.1 ± 0.5	0.2 ± 0.8
G. peruzzii	1	5.7 ± 4.4	3.9 ± 1.7	9.8 ± 5.2	1.5 ± 1.4	0.8 ± 1.3	0.00
	2	8.8 ± 5.0	5.3 ± 2.6	4.0 ± 1.8	2.3 ± 1.6	0.2 ± 0.4	0.1 ± 0.4
	3	8.9 ± 3.8	4.4 ± 2.7	3.1 ± 2.7	0.9 ± 0.9	0.5 ± 1.1	0.4 ± 1.0
G. lutea	1	16.9 ± 2.9	19.4 ± 8.6	7.0 ± 3.8	0.2 ± 0.5	0.00	0.00
	2	7.8 ± 3.8	6.4 ± 3.2	5.3 ± 2.7	0.2 ± 0.5	0.00	0.00
	3	5.2 ± 2.3	3.9 ± 1.2	1.8 ± 2.2	0.1 ± 0.3	0.00	0.00
G. minima	1	10.3 ± 5.8	5.4 ± 4.3	21.0 ± 7.7	1.5 ± 2.3	0.6 ± 1.3	0.3 ± 1.0
	2	1.1 ± 2.5	8.8 ± 5.9	4.4 ± 3.1	0.00	0.00	0.00
	3	0.4 ± 0.9	1.5 ± 2.2	1.9 ± 2.1	0.00	0.00	0.00

Overall, the cytoplasmatic staining techniques allow the evaluation of some structural features of pollen, but not its real physiological adequateness. As a consequence, part of the stained pollen can be unviable (Nepi & Franchi 2000).

However, significant differences occurred among species, in terms of both pollen viability and *in vitro* germination (Table 2 and Table 4). Both pollen traits agreed in indicating highest and lowest pollen fertility respectively in *G. lutea*, on one side, and *G. minima*/*G. fragifera*, on the other side. Instead, for *G. lojaconoi* and *G. peruzzii* the patterns of pollen viability and pollen germination disagreed. This discordance even increased when comparing the outcome of MTT test with those related to pollen germination on the stigma (Table 1), as the two protocols produced congruent patterns only for *G. lutea* and *G. fragifera*. This is not surprising, because each test is related to different aspects of pollen fitness, but none of them can evaluate simultaneously all of its components

(Thomson & al. 1994; Dafni & Firmage 2000). *In vitro* and on stigma pollen germination produced comparable scores in all species, with the exception of *G. minima*.

Our results on pollen viability in *G. lutea* are congruent with those reported by Zhang & al. (1995), and with data from one of the two populations studied by Zarrei & Zarre (2005). The generally scarce pollen fertility observed in the studied species can be also related to their overall low sexual efficiency and frequent vegetative propagation (Gargano & al. 2007). In particular, contrasting patterns of sexual vs. clonal propagation are particularly frequent in the genus *Gagea*, and usually are species-specific (Schnittler & al. 2009; Pfeiffer & al. 2012; Schnittler & al. 2013; Beisenova & al. 2015).

In addition, variations in the ploidy level among the studied species can provide further explanations for the detected fertility patterns. Indeed, data on pollen viability and seed-set indicated the hexaploid *G. lutea* and the heptaploid *G. fragifera* as the highest and lowest fertile species, respectively (Table 5). When comparing *G. peruzzii* (tetraploid) and *G. lojaconoi* (triploid), although the former species showed higher pollen quality, the latter actually produces more seeds (Gargano & al. 2007). Therefore, the sexual fitness in *G. peruzzii* seems to be limited by factors differing from pollen fertility that could involve problems in the development of female gametophytes (Caparelli & al. 2006, under the name *G. bohemica*). A possible explanation for that could rely also on the allopolyploid hybrid origin of this species (Peterson & al. 2009; Tison & al. 2013). Moreover, as suggested by our difficulties in finding pollinated stigmas, pollen limitation may have contributed in limiting the seed-set of *G. peruzzii*. Finally, as previously hypothesized (Gargano & al. 2007), the fruit ripening of *G. peruzzii* could be affected by resource limitation due to its quickly-drying habitat. Such a habitat feature is recognized to promote vegetative propagation (Dafni & al. 1981). Accordingly, vegetative individuals in the *G. peruzzii* population were more numerous than in the population of *G. lojaconoi* (Gargano & al. 2007).

Overall, our data also agree on previous work on the influence of ploidy level on sexual fertility in *Gagea* (Zarrei & Zarre 2005). The observed fertility patterns are also congruent with further relevant biological and evolutionary features of the genus *Gagea*, including the frequency of vegetative propagation and the role played by hybridization events in its evolutionary history (Peruzzi 2008; Peterson & al. 2009, 2011, 2016; Tison & al. 2013). Substantially, our findings evidenced that the studied species undergo common genetic constraints on male fertility, such as hybridization (e.g. Wells 1979; Ness & al. 1990), polyploidy (Burton & Husband 2000; Sato & al. 1993) and high rate of vegetative propagation (Nepi & Franchi 2000).

Conservation implications of the observed fertility patterns

Overall, all the studied species may suffer for environmental variations, because the frequent vegetative propagation can reduce their evolutionary flexibility against habitat changes. However, our data emphasized higher concerns for *G. minima*. In spite it was the only diploid species in our study, it produced a high amount of malformed pollen (Table 5), and pollen viability/germination were strongly influenced by flower phase (Table 2, 5), as its pollen fertility severally decreased after pollen exposure. Because during the phase F1 pollen is not available for pollinators, such data justifies the reduced amount of germi-

Table 4. Analyses of variance for *in vitro* pollen germination among studied *Gagea* species in relation to the % of sucrose and flower phase. VPG = *in vitro* pollen germination (%); * = *G. lojaconoi* is excluded from this analysis.

Analysis type	Target	Factor	Dependent variable	F	P
One-way ANOVA	all species	species	VPG	6.483	≤ 0.0001
		[sucrose]		20.406	≤ 0.0001
	all species*	flower phase		27.633	≤ 0.0001
Two-way ANOVA	all species	species		8.983	≤ 0.0001
		[sucrose]		22.672	≤ 0.0001
	all species*	species		22.382	≤ 0.0001
		flower phase		29.691	≤ 0.0001

Table 5. Ploidy levels, pollen gross morphology and seed-set data for the studied *Gagea* species. *Data from Gargano & al. (2007). GP = good pollen; BP = evidently malformed pollen; S/O = seeds/ovules ratio.

Species	Ploidy level	GP/BP	S/O
G. fragifera	7*x*	1.51*	0.009*
G. lojaconoi	3*x*	2.46*	0.019*
G. peruzzii	4*x*	3.98*	0.003*
G. lutea	6*x*	8.38*	0.028*
G. minima	2*x*	2.59	//

nated pollen detected on the stigmas (Table 1). In addition, *G. minima* had the lowest scores of stigma receptivity, which was also affected by flower aging, as the germinated pollen grains drastically decreased on stigma of F2/F3 flowers. Because the studied *G. minima* population occurs in a high-mountain context, the reduced fertility found in this species could be partially due to severe environment conditions (temperature, water availability), as found in *G. lutea* (Bohdanowicz & al. 2005). On the other hand, the relict status of this species in southern Italy may also play a role. Indeed, the isolation and small size of the studied population may promote the rise inbreeding depression, whose detrimental effects were demonstrated also on pollen fertility (e.g. Willis 1993; Carr & Dudash 1997; Gargano & al. 2011).

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