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Pollen limitation and effects of local patch density on reproductive success in the alpine herb *Inula royleana* (Asteraceae)

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Abstract In alpine species depending on insects for pollination, the decrease of pollinator abundance and activity, due to more hostile weather and climatic conditions, could limit reproductive success because of failures in pollination. We examined the breeding system and pollen limitation (PL) using self- and cross-pollinations and pollen supplementations in *Inula royleana* in alpine Kashmir Himalaya. In addition, we also investigated how natural variation in local patch density and floral neighborhood affect species reproductive success. Supplemental hand-pollinations with outcross pollen resulted in 19 % increase in seed output above natural levels, thus demonstrating PL in this species. The breeding system in *I. royleana* was self-incompatible as caged flower heads, and the selfed?? capitula produced no or very few seeds compared to seed set in open-pollinated capitula. Consistent with the conspecific density in flowering plants, percent seed set differed significantly among patches. Seed set increased with local density of conspecifics, indicating that the reduced plant density in sparse patches may reduce plant reproductive success. Further in patches of low density, number of flowering conspecifics in the immediate vicinity of a focal plant (positive relationship) and distance to the nearest flowering conspecific plant (negative relationship) was found linearly related with greater PL resulting in reduced seed set and fecundity in isolated plants.

Keywords Alpine habitat · Density · Pollen limitation · *I. royleana* · Self-incompatibility

Introduction

The immobility of plants exerts an important influence on mating and reproductive success which is commonly 'context dependent' and relying on pollen vectors, most often insects, to deposit sufficient compatible pollen onto stigmas for ovule fertilization and seed production. Plant reproductive success varies widely within and among natural populations, and studies of factors affecting seed set of flowering plants have examined two proximate limiting factors: pollen limitation (PL) and resource limitation (Kunin 1993; Agren 1996), both of which can be affected by population and/or plant characteristics. PL is a common proximate cause of low seed set (Burd 1994), and it has been estimated that 62–73 % of all biotic pollinated species studied experience PL (Ashman et al. 2004a). Many reviews have examined the frequency, the causes, and the consequences of PL

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(Larson and Barrett 2000; Ashman et al. 2004a; Knight et al. 2005a; Vamosi et al. 2006). PL occurs when seed production is less than what would be achieved if overall quantity or quality of pollen deposited onto stigmas were increased (Ramsey and Vaughton 2000). Pollen quantity may be limiting if pollinators are rare, or if plants compete for the services of pollinators (Johnston 1991; Barrett et al. 1994; Groom 1998). Pollen quality may be limiting, despite adequate pollination, if pollinators deposit self or incompatible pollen on stigmas, which invokes lower ovule fertilization and seed production (Waser and Price 1991; Byers 1995; Totland et al. 1998).

Some species are more predisposed to being pollen limited than others. Considering plants with a self-incompatible (SI) mating system, seed set is crucially affected by pollen supply from other conspecific individuals. Moreover, in small and isolated populations the diversity of pollen genotypes is further reduced and it is more likely that flowers will receive incompatible pollen (Byers and Meagher 1992; Byers 1995). Thus, in a wild population of SI insect-pollinated species, variations in local floral density, due to heterogeneous spatial distribution of individual plants, may affect the seed set of individuals (House 1992). In addition, for alpine species depending on insects for pollination, the decrease of pollinator abundance and activity, due to more hostile weather and climatic conditions (Korner 2003), could further limit seed sets because of failures in pollination. Therefore, alpine plants might experience severe PL and reduction in reproductive success (Bingham and Orthner 1998; Arroyo et al. 2006).

Many plant species do not exist as large continuous populations but rather as isolated patches that are embedded in a landscape matrix and separated by unsuitable types of vegetation (Steffan-Dewenter and Tscharntke 1999). Patchy habitats often are small in size and are spatially isolated, and these aspects are ecologically important as the plants in small patches are often less attractive to pollinators and therefore, receive fewer pollinator visits and a lower proportion of compatible pollen (Kunin 1993; Steven et al. 2003; Waites and Agren 2004; Hegland and Boeke 2006). The size, density and spatial isolation of patches therefore influence pollinator behaviour (Kunin 1993; Fritz and Nilsson 1994; Mustajarvi et al. 2001; Goverde et al. 2002), with consequences for pollen dispersal and plant reproduction. Nearly, all studies of the effects of density on plant reproduction have found that both pollination and reproductive success decrease in sparse populations (Kunin 1997; Roll et al. 1997; Bosch and Waser 2001), and this is likely to have a greater impact for SI species which cannot compensate for lower pollinator abundance by selfing.

Here, we examined the degree of PL and compatibility system for an alpine herbaceous perennial, *Inula royleana*. Since the breeding system of the species was previously unknown, this study was a good opportunity to explore it. Given the abiotic environmental conditions, alpine species are frequently thought to suffer from PL (Totland and Eide 1999; Garcıa-Camacho and Totland 2009) and PL often is related to the activity of pollinators (Totland 1997). A pollination experiment was used to test whether seed set in *I. royleana* is reduced by PL and, if so, whether pollen quantity or quality is responsible. Moreover, given the small, fragmented nature of *I. royleana* populations, we examined natural variation in local patch density of flowering plants and its effects on reproductive success. This species was suitable for this study because the selected population exhibited considerable variation in patch density. We quantified species seed set over 3 years and examined the relationship between local patch density and reproductive success. More specifically, the following questions were investigated: (1) Is *I. royleana* SI and does seed set depend on pollinators? (2) Is seed production in this alpine herb pollen limited? (3) How does patch density affect reproductive success? (4) Does conspecific flowering plant density affect the reproductive success in focal plants?

Materials and methods

Study organism and site description

Inula royleana D.C., (Asteraceae) is a montane perennial herb native to the high alpine ranges of N. W. Himalaya. It mainly grows at altitudes between 3400 and 3500 m a.s.l. and occurs in nearly all alpine community types above the tree line across the Kashmir Himalaya which experiences severely cold climates, fast blowing winds and high humidity all year round. All fieldwork and collection took place on Apharwat and Khilanmarg area, an alpine tundra site with an area of 4–5 km^2 just, 50 km west of the

Srinagar city (34°01′N, 74°32′E) which is dominated by Junipers at an elevation of 3430 m. This site is characterized by a snow-free period of 160–180 days lasting from early-June to mid-November.

The plant begins its life cycle immediately after the snow-melt in the late spring or early summer as a basal rosette of leaves arising from a stout caudex that grows 4–5 cm below the soil surface. Plants grow well under patches of full sunlight conditions among the Junipers and therefore restricted to relatively small and isolated patches within the Junipers. In all reproductive plants, a single bright yellow solitary capitula emerges per growing season. The inflorescence is borne on a stout and erect, 20–40-cm-long hairy peduncle. Mean number of disc florets per capitulum was 489.63 ± 27.41 with each floret having a single basel ovule. The mean date of flowering (first flower) is late July to early August in our study site and seed maturation (small achene) occurs in October.

Controlled pollinations

We performed PL and self-incompatibility experiments on individuals located throughout Apharwat and Khilanmarg area during July 2011. To determine if seed set in *I. royleana* was pollen-limited, we conducted a pollen supplementation experiment. If seed production in pollen-supplemented flowers was increased relative to naturally pollinated flowers, the plant was considered to be pollen limited at that place and time (Zimmerman and Pyke 1988). We randomly selected 40 different plants, each assigned to one of two treatments: open-pollinated with no supplemental pollen added (control), or open-pollinated with supplemental pollen added (pollen supplementation). In the supplementation treatment, the supplemental pollen was a mix of pollen collected from at least five other individuals separated by at least 15 m. To accomplish cross pollen addition, we collected a large quantity of florets with dark orange pollen-laden anthers from the five different plants that were at least 10 m apart and carefully brushed these across the receptive stigmas of florets of capitula on the supplemental pollination plants. Seed heads were harvested 4 weeks after pollination just before the dehiscence of achenes. We counted for each capitulum the numbers of unfertilized ovules, aborted, and viable seeds and differences among different seed classes were tested with ANOVA. Viable seeds were 5–7 cm, reddish brown, expanded and lanceolate in outline. Aborted seeds were distinguished from unfertilized ovules as the latter were narrower, unexpanded, and less than 2 mm long, whereas the former were light brown, lengthened and 3–4 mm long. Seed set (viable seeds only) obtained in control and supplement treatments were used to calculate a PL index expressed as: $PL = 1 - C/PS$, where C and PS represent the mean seed set of the control and supplement treatments, respectively (Larson and Barrett 2000).

To measure self-incompatibility in the *I. royleana*, we conducted a pollinator exclusion experiment using nylon mesh bags to prevent pollinators (mainly bumblebees) from visiting experimental capitula. This method effectively excludes insect visitors but does not alter the microclimate surrounding the developing capitulum (Kearns et al. 1998). Pollinations were conducted over 60 plants and floral heads were randomly assigned to one of the following three treatments: (1) Floral buds were labelled and left untouched (unbagged) under open pollination conditions (control). After the flower heads had withered, they were bagged with fine nylon mesh to prevent dispersal of the seeds. For the other two treatments, flower heads were caged at the start of the experiment with bags made of fine nylon mesh (mesh size ca. 0.8 mm) to prevent insects from visiting. (2) Bagged floral heads were labelled and left untouched to investigate the possibility of autonomous self-pollination (autogamic pollination); (3) Bagged flower heads were hand-pollinated with pollen from the same flower head (selfed). Hand self pollinations were carried out by gently transferring pollen from the anthers to the receptive stigma with small paintbrushes. Experimental capitula were collected after achene maturation and analyzed as described above. ANOVA was used to analyze the effects of pollinator exclusion and pollen supplementation on the mean percentage seed set and Tukey post-hoc tests (differences of least squares means) were used to identify treatments that differed significantly.

Patch density and reproductive success

Further we intend to determine whether natural variation in local patch density of conspecific flowering plants influences reproductive success in *I. royleana*. To this end we chose 32 separate patches of varying conspecific densities showing either a low (sparse plots) or a high (dense plots) density of flowering conspecifics so as to have two contrasted groups of local density. The 32 patches (17 dense and 15 sparse) were selected subjectively, but they always differed at least five to eightfold in density. ''Dense'' patches averaged 7.81 plants/m^2 (range 5.44–11.27, SD = 2.15, $N = 17$ plots) and ''sparse'' patches averaged 1.44 plants/m^2 (range 0.71–3.8, SD = 1.06, $N = 15$). The distance between low and high density plots varied from 5 to 25 m. Composition of the rest of the flora was qualitatively similar in paired dense and sparse areas. Within each plot 1–5 focal plants were selected per plot and individually labelled, for a total of 57 focal plants (32 in dense plots and 25 in sparse plots). Since the size of the plots vary, so we selected the focal plants proportionally as for every five conspecific flowering plants we selected one focal plant to record the seed set. For each selected focal plant, the conspecific density measured as the number of flowering plants of *I. royleana* within a 2-m radius of each focal plant and the distance to the nearest flowering conspecific were noted down. To avoid pseudo-replication, we chose a subset of focal plants such that their nearest neighbors were not shared by other focal plants. About 3 weeks after the labelling of focal plants, the marked capitula were collected at maturity, just before opening and release of the achenes. We counted for each capitulum the numbers of unfertilized ovules, aborted, and viable seeds as described earlier. We repeated the study in 2012 and 2013 and the data were collected for both patch types and individual focal plants in the same way as described above. The entire data set thus consisted of reproductive success and density data from three flowering periods i.e. from 2011 to 2013. Differences among dense and sparse patches in mean proportion seed production were tested with ANOVA. The conspecific density for each focal plant and proportional seed set was also log-transformed, and linear regression was used to determine if floral density around focal plants predicted the seed set.

Results

ANOVA comparing pollen supplemented capitula with capitula on control plants showed a significant effect of the pollen addition treatment on seed set. Supplemental hand-pollination increased the mean seed output of *I. royleana* by 19 % ($\chi^2 = 242.19$, $P < 0.001$; Fig. 1) (supplementation: $\bar{X} = 66.91$ %, SD = 17.47, $N = 20$; control: $\bar{X} = 47.58$ %, SD = 13.64, $N = 20$, $F = 17.29$, $P < 0.05$) indicating strong PL with a PL index of 0.29. In both control and supplemented heads, many of the seeds consisted of the pappus and seed coat, with no endosperm. Supplemented plants produced significantly fewer ($F = 13.05$, $P < 0.001$) empty seeds than did the control plants. Further, the pollen supplementation treatment in *I. royleana* shows little variations in PL among individuals indicating that pollen quality did not affect reproductive success.

The self-incompatibility experiment demonstrated that the *I. royleana* is strongly SI as bagged flower heads, and the selfed capitula produced no or very few achenes compared to seed set in open-pollinated capitula (Fig. 1). Seed set in the autogamic and selfed treatments were statistically indistinguishable, averaging 0.08 and 0.13 %, respectively, whereas seed set in open pollinated capitula (control) averaged 47.58 % (Mean seed set: bagged = 33.24, SD = 4.01, $N = 20$, selfed = 47.02, SD = 3.88, $N = 20$, control = 232.99, SD = 13.64, $N = 20$, $F = 381.6$, $P \leq 0.001$). Also many achenes derived from autogamic and selfed capitula apparently did not contain viable seeds and Tukey post-hoc tests showed that unbagged control flowers produced significantly fewer empty achenes than those produced by the two self-pollination (autogamic and selfed treatments) treatments ($F = 143.61$, $P = 0.009$).

Consistent with the conspecific density in flowering plants, percent seed set differed significantly among patches. In all 3 years, seed set was significantly

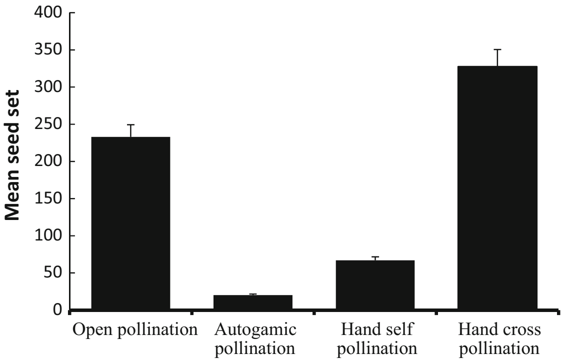

Fig. 1 Mean seed set produced in each pollination treatment tested for *Inula royleana*

greater in high density patches than in low density patches (percentage seed set 55.01 and 40.49 %, respectively, $F = 164.23$, $P \leq 0.003$; Figs. 2, 3). At dense plots, the density ranged from 5.44–11.27 plants/m^2, with 80 % (26 of 32) of the selected focal plants in dense patches having <7 individuals within a 2 m radius. In contrast, the local density at sparse plots ranged from 0.71–3.8 plants/m^2, with only 12 % (2 of 25) of focal plants having <7 individuals within a 2-m radius. We examined the influence of the distance of the nearest neighbor to individual focal plants and the number of flowering individuals with in a 2-m radius on seed set of the focal plants. In low density patches, a strong distance effect appears in the number of achenes per capitula as a function of nearest flowering conspecific neighbor, as the proportion of florets that set seed was linearly related to the nearest neighboring conspecific (Fig. 4b; $r = -0.69$, $\chi^2 = 378.54$, $P = 0.006$). The seed set of each focal plant also increased linearly with the increase in the number of flowering individuals in its immediate neighborhood ($r = 0.79$, $\chi^2 = 287.57$, $P = 0.005$, Fig. 3b). The regression of achene production on distance to the nearest neighbor and number of flowering conspecific neighbors within a 2 m radius was statistically significant ($r^2 = 0.063, N = 25, P < 0.001$) for low density patches. However, both the distance of the nearest neighbor to individual focal plants ($r = 0.39$, $\chi^2 = 422.03$, $P = 0.018$; Fig. 4a) and the number of flowering individuals with in a 2-m radius ($r = 0.46$, $\chi^2 = 197.01$, $P = 0.016$; Fig. 3a) had a marginal significant effect on the seed set of focal plants, in high density patches.

Discussion

Our results of the hand-pollination experiments consistently showed that *I. royleana* requires pollinator visits in order to produce seeds, as 17 of the 20 plants set no seed following pollinator exclusion while three plants setting small amounts of seed. Moreover, flower heads that were hand-pollinated with self-pollen also produced no or very few seeds indicating that *I. royleana* is predominantly SI. The low number of seeds obtained sporadically in some selfed and bagged capitula could be the result of accidental pollen transfer (insect penetration) or may be due to some leakiness in the SI system, as is commonly observed in SI species (Levin 1996; Stephenson et al. 2000) and has been reported in other species of Asteraceae (Ferrer and Good-Avila 2007). These sporadic breakdowns can be attributed to specific characteristics of a sporophytic self-incompatibility system (Lewis 1994; Hiscock 2000). Multiallelic sporophytic self-incompatibility is common in Asteraceae (Luijten et al. 1996; Richards 1997).

Reproduction appears to be pollen limited in *I. royleana.* Supplementary pollination consistently increased seed production in *I. royleana*. PL occurs widely in plant species (Burd 1994; Larson and Barrett 2000; Totland 2001; Ashman et al. 2004b; Knight et al. 2006; Lazaro and Traveset 2006) particularly in SI plants (Larson and Barrett 2000; Ramsey and Vaughton 2000). Furthermore, given the abiotic environmental conditions in alpine habitats, the abundance of insect pollinators is low compared to most lowland habitats (Arroyo et al. 1985; McCall and

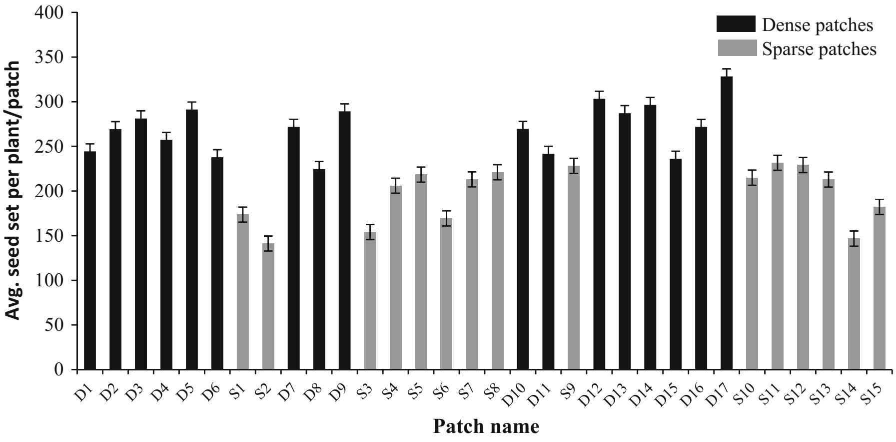

Fig. 2 Relationship between patch density and reproductive success in *Inula royleana. Horizontal axis* corresponds to the patch density and *error bars* are SE

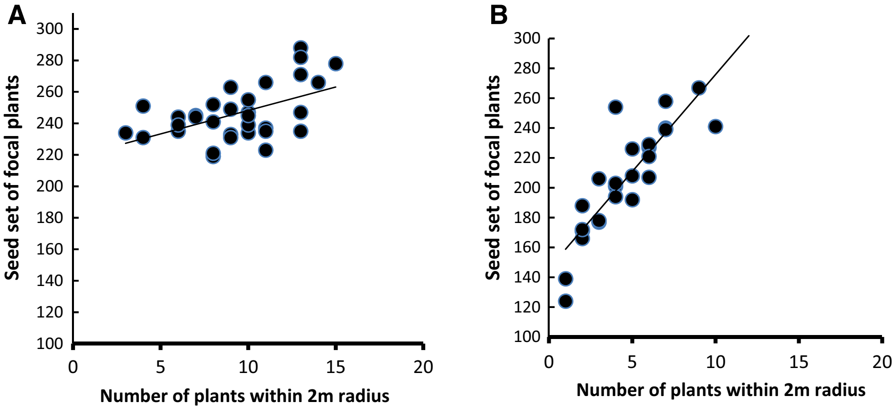

Fig. 3 The relationship between reproductive success and the number of conspecific flowering individuals within 2-m radius from the focal plants in **a** dense and **b** sparse patches of *Inula royleana*

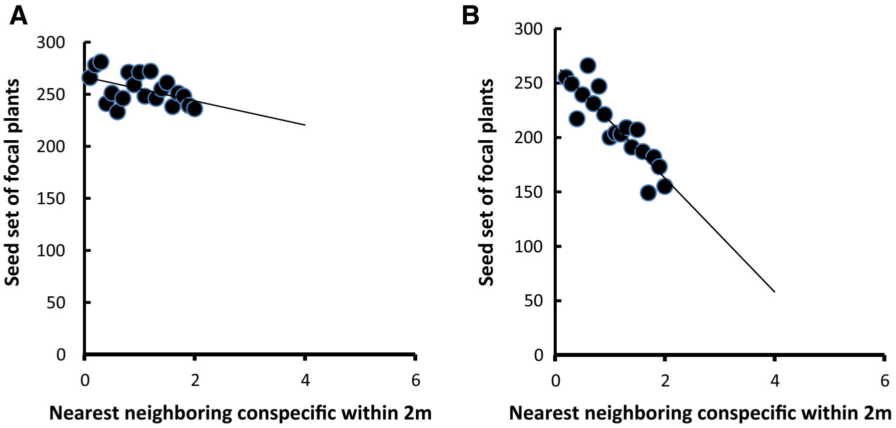

Fig. 4 The relationship between reproductive success and the nearest neighboring conspecific within 2 m radius from the focal plants in **a** dense and **b** sparse patches of *Inula royleana*

Primack 1992; Totland 1993) and considerable reviews also suggest that alpine tundra plants frequently suffer from PL (McCall and Primack 1992; Totland and Eide 1999; Garcia-Camacho and Totland 2009). Moreover, the *I. royleana* population is located in a landscape with heavy encroachment by woody junipers which have effectively divided what once may have been a continuous population into smaller patches. Furthermore, plants produced solitary capitula per plant. Thus, the patchiness of the habitat in combination with solitary capitula produced per plant resulted in a smaller floral display, which may have been less attractive to pollinators.

To determine the effects of patch density on fecundity of plants, we measured reproductive success for a total of 92 plants (49 in dense patches and 43 in sparse patches) that varied dramatically in local conspecific density. Natural seed set varied substantially within and among the patches and was probably related to differences in flowering plant density. Fewer achenes developed per capitula in sparse patches than in high density patches. Many studies of plants that rely on cross-pollination for their reproduction have shown that pollination success and seed set were positively affected by the local density of flowering conspecifics (Feinsinger et al. 1991; Kunin 1993; Van Treuren et al. 1994; Roll et al. 1997; Bosch and Waser 1999; Colling and Matthies 2004). Lower reproductive success in sparse patches as compared to dense ones can either be attributed to a decreased availability of pollinators (pollinator limitation: Wilcock and Neiland 2002; Knight et al. 2005b) or to a decreased quantity and/or quality of the pollen deposited on stigmas (pollen limitation: Kunin 1993, 1997) with decreasing local conspecific density. We found some evidence that the lower seed set in plants of sparse patches may have been caused by pollen limitation. Low pollen ''quality'' can result from a high fraction of heterospecific pollen in the pollen loads brought by insects to stigmas, or from a high fraction of incompatible conspecific pollen (Kunin 1993). Because *I. royleana* is self-incompatible, the increase in seed production with density suggests that the majority of pollen deposited on stigmas was outcrossed pollen deposited by pollinators and was not heterospecific or self-pollen deposition. Sparse patches are likely to be less attractive to pollinators than dense patches and this may lower pollinator visitation, cause pollen to be limiting and reduce seed set (Jennersten 1988). Conspecific individuals at higher densities may therefore serve to alleviate the negative effects of density-dependent seed set. Our results are in agreement with the findings of Wolf and Harrison (2001); Kirchner et al. (2005) and Wagenius (2006) i.e. that seed set of plant species in fragmented populations is related to the area and density of patches of flowers within the populations.

Further in patches of low density, number of flowering conspecifics in the immediate vicinity (within 2 m) of a focal plant (positive relationship) and distance to the nearest conspecific plant (negative relationship) is associated with greater PL in all 3 years of this study, resulting in reduced seed set and fecundity in isolated plants. This implies that the significant variation in reproductive success of focal plants is at least partially attributable to small scale variation in focal plant neighborhood. Thus, the decrease in pollen receipt due to isolation appears to limit seed set in isolated plants in already low-density patches (Gonzalez-Varo et al 2009). The mean intensity of PL in *I. royleana* may therefore depend not only on local density but also on its population spatial structure. However, it is worth stressing that this work needs to be further strengthened with more exhaustive sampling of populations and further work is required to determine whether the observed patterns in reproductive success of *I. royleana* are the result of a behavioural change of pollinators at variable population sizes and plant densities.

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