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Running head: Flower orientation on slopes in angiosperms

Title: Flowers adaptively face down-slope in ten forest-floor herbs

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Summary

1. An animal-pollinated plant living on a slope should orientate its flowers down-slope towards the more open space if by doing so it receives more pollinator visits and thereby achieves increased reproductive success.
2. We measured flower orientation relative to slope direction on individuals of ten species of forest-floor herbs in cool temperate forests in Japan. For one of these species, Erythronium japonicum, we also manipulated flower orientation to test experimentally for effects on both male and female reproductive function.
3. In all ten species, flowers were preferentially orientated down-slope. This pattern was more pronounced in plants growing on steeper slopes.
4. Our field experiment demonstrated that flower orientation relative to slope affected fruit and seed set and pollen dispatch in *Erythronium japonicum*. Specifically, flowers orientated up-slope achieved a lower reproductive performance on steep slopes.
5. We conclude that down-slope orientation of flowers was a general phenomenon among the species that we studied, and that this behaviour may be adaptive in enhancing plant fitness through pollination.

Key words: down-slope flower orientation, *Erythronium japonicum*, forest-floor herb, reproductive success, slope direction and angle, vertical orientation

Introduction

Because plants are sessile, they needs pollen vector for sexual reproduction in most cases. With showy tepals and/or fragrance, animal-pollinated flowers attract their pollinators from a distance. Attraction size, which involves both flower size and number, often affects the frequency of pollinator visitation (de Jong & Klinkhamer 1994, Conner & Rush 1996, Harder & Barrett 1996, Ohashi & Yahara 2001), indicating that pollinators use showy tepals as a kind of flower trademark. To make flowers more attractive for pollinators, how to orient attraction-related tepals to their pollinators would be an important for some species (Patino et al. 2002). In this sense, flower orientation is one aspect of the floral attraction.

Flower orientation is considered to have evolved under pollinator-mediated selection and/or under natural selection due to abiotic environmental factors, such as rainfall and temperature (Hocking & Sharplin 1965; Kevan 1975; Kudo 1995; Neal et al. 1998; Giurfa et al. 1999; Huang et al. 2002; Galen & Stanton 2003; Ushimaru & Hyodo 2005; see also Patino et al. 2002). Flower orientation includes two dimensions: flower angle (Neal et al. 1998; Tadey & Aizen 2001; Huang et al. 2002; Ushimaru & Hyodo 2005) and flower direction (Kudo 1995; Zavala-Hurtado et al. 1998; Kita & Wada 2000; Patino et al. 2002). Flower angle is up-down variation in which en face surface of flowers orients (i.e. upward, vertical, oblique and downward), while flower direction is to which point flowers orient on the compass (east, south, west, north or tracking the path of the sun, i.e., heliotropism). In some species, flower angle and direction are closely connected to each other. For example, angles of heliotropic flowers are usually upward (or inclined) to increase internal flower temperature, which in turn promotes pollinator attraction, pollen tube growth and seed maturation (Hocking & Sharplin 1965; Kevan 1972, 1975; Kudo 1995; Patino et al. 2002; Galen & Stanton 2003).

A plant on a slope experiences a heterogeneous spatial environment (see Ishii and Higashi 1998). When a plant grows up vertically, there spreads larger space in the downslope side than in the upslope side for the individual (Fig. 1). Assuming that pollinators distributed uniformly in the space, we expected the number of pollinator distributing in downslope side to be larger than that in upslope side. This leads to that the probability of pollinators approaching the flowers would be higher from the downslope side than from the upslope side, especially on a steeper slope. If a plant with a vertical flowering stem has flowers that face toward a pollinator-abundant open space to increase pollinator visitations, we can predict the following flower-orientation patterns on a slope: most flowers will face downslope, and this pattern should be most common on steeper slopes; moreover, flowers that face upslope will have lower reproductive success.

These patterns are more likely to be observed in plants with vertical and oblique orientated flowers because these flowers are considered to restrict the approach direction of visiting insects; i.e., pollinators should always approach these flowers from the front (Neal et al. 1998; Ushimaru & Hyodo 2005). Furthermore, plants with flowers near the ground should exhibit these patterns more strongly than flowers on taller plants because flowers growing at greater heights may be less influenced by spatial heterogeneity on a slope.

In this study, we examined the above predictions of the relationship between slope conditions and flower direction in ten herbaceous species with vertical or oblique flowers to test and to generalise our hypothesis. We then examined our prediction on the relationship between flower direction and reproductive success in a natural *Erythronium japonicum* Dence. population by conducting a field experiment in which flower direction was artificially manipulated. Based on our results, we discuss the evolution of flower orientation in angiosperms on slopes.

Materials and methods

Study species

We examined the flower orientation on slopes in the following seven species that have vertical or oblique-oriented flowers: *E. japonicum*, *Hosta sieboldii* (Paxton) J. Ingram (Liliaceae), *Asiasarum sieboldii* (Miq.) F. Maek. var *sieboldii* (Aristlochiaceae), *Viola eizanensis* (Makino) Makino (Violaceae), *Shortia uniflora* (Maxim.) Maxim. var. *kantoensis* Yamazaki (Diapesiaceae), *Corydalis lineariloba* Sieb. et Zucc. var. *lineariloba* (Papaveraceae) and in the Ogawa Forest Reserve (OFR) and *Calanthe reflexa* Maxim. (Orchidaceae) in the Ashu Experimental Forest and *Monotropastrum globosum* H. Andres at Mt. Higashiyama, *M. globosum* H. Andres f. *roseum* Honda at Mt. Kirishima and *Monotropa uniflora* L. at Yada Hills and Iwakura (Table 1; Data of Monotropaceae species are after Imamura and Ushimaru, submitted). Species were deliberately selected to include both monocots and dicots and both actinomorphic and zygomorphic flowers. Individuals of *E. japonicum*, *A. sieboldii* and *S. uniflora* var. *kantoensis* have a single-flowered inflorescence, while those of *V. eizanensis*, *M. globosum* H. Andres, *M. globosum* f. *roseum* and *M. uniflora* often have a few (and sometimes more than five) single-flowered inflorescences. *Calanthe reflexa* and *C. lineariloba* have many flowers (usually more than five) per inflorescence. *Hosta sieboldii* usually has one or two opening flowers per inflorescence.

For *E. japonicum*, we examined in further detail the relationship between flower orientation and slope, and we therefore provide some further details on its floral biology as follows. *E. japonicum* occurs on the forest floor in cool temperate forests of Japan. This perennial herb rarely exhibits clonality and reproduces sexually by ant-dispersed seeds (Ohkawara et al. 1996). In April, a sexual individual of *E. japonicum* produces a single flowering shoot, which has a pinkish, bisexual flower that lasts ca. 2 weeks (Ishii & Sakai 2000). A flower bud, which emerges with two leaves, faces toward just above the scape and then its peduncle bends to orient the flower face obliquely. The species is self-incompatible and requires insect vectors for pollination (Ishii & Sakai 2000). This species is mainly pollinated by relatively large bees, such as *Xylocopa*, *Tetralonia*, *Nomada* and *Bombus* species (Utech & Kawano 1975). However, we have observed *E. japonicum* flowers most frequently visited by small-sized bees in the OFR (Ushimaru et al. 2003). Flowers are adichogamous and pollen receipt is saturated within 3 days after corolla opening in *E. japonicum* while pollen dispatch continues for 10 days (Ishii & Sakai 2000). Fruits of this species mature approximately 1 month after anthesis. In 2001, we conducted an experimental study on an *E. japonicum* population in an old-growth deciduous forest in the OFR. The blooming season began in late March and lasted until late April.

Measurements of slope and flower conditions

Herbaceous plants are likely affected by microscale sloping landforms. We therefore measured slope direction and angle in the microhabitat (20 cm x 20 cm) of each individual and flower orientation for each flower using a clinometer (Showa Sokki Co., Tokyo, Japan; Fig. 1). We then calculated the angular distance between the slope and flower directions (ADSF; $0° \leq \text{ADSF} \leq 180°$) for each flower (Fig. 1). We measured two to five flowers from the bottom of the inflorescence and five flowers from the top of the inflorescence in *C. reflexa* and *C. lineariloba*, respectively (we selected early blooming flowers of each inflorescence). When an individual had more than one flower, we calculated the mean ADSF of flowers as the ADSF for the individual of *V. eizanensis*, *H. sieboldii*, *C. lineariloba*, *C. reflexa* and Monotropaceae species.

First, flowers were categorised into four ASDF classes: ADSF = 45˚ (A), 45˚ < ADSF ≤ 90˚ (B), 90˚ < ADSF ≤ 135˚ (C) and 135˚ < ADSF ≤ 180˚ (D). We counted the number of individual in each class and compared the natural distribution pattern of ADSF to a uniform distribution using a chi-square test for each species. Our hypothesis predicts that the number of individuals is highest in the class A and lowest in the class D. We then examined correlations between slope angle and ADSF to test our hypothesis in ten species.

Relationship between ADSF and female reproductive success in *Erythronium japonicum*

We investigated the relationship between reproductive success and natural variation in ADSF in 146 flowers whose ADSF was measured in the above examination. One or two-days old flowers were tagged on 13 April 2002. We checked fruit set for 146 tagged flowers on 11 May 2002. The relationship between ADSF (0-180 in the field) and fruit set was examined using a logistic regression analysis with fruiting (1) and non-fruiting (0) as a dichotomous response. We also collected fruits of these flowers, and the number of seeds were counted for each fruit in the laboratory. A simple linear regression of the number of seed per fruit on ADSF was performed to test their relationship.

Direction-change experiment in Erythronium japonicum

To examine experimentally the effect of downslope flower orientation on female reproductive success in *E. japonicum*, we conducted a direction-change experiment in the OFR. We arbitrarily chose and tagged 108 flowers whose ADSFs were <20° on five slopes (one north-, two east-, one south- and one west-facing slope). The direction-change to upslope orientation was conducted by fixing the flower stalk with a wire. Flower stalks of controls were also fixed by wires. We changed the ADSFs of 56 flowers to more than 160° (upslope direction) and left the remaining 52 flowers intact as controls on 1-3 April 2002. The slope angle of each flower was also measured. We monitored fruiting and seeding of these 108 flowers to check female success.

The effect of direction change on male reproductive success was also examined. Male reproductive success has often been measured by counting the number of remaining pollen grains on anthers that is assumed to negatively correlate with the siring success (Ishii & Sakai 2001). We collected a single anther in each flower from 29 and 27 control and 30 and 29 direction-changed flowers at 3 and 10 days after anthesis, respectively. Single anthers from 20 newly opened flowers were also sampled. These anthers were stored in 1.0 mL of 70% ethanol. Pollen grains easily detached from anthers in the solution. We estimated the number of pollen grains per anther by counting the pollen number in a 5.0-μL drop under a microscope three times per solution. We multiplied the estimated number by six to calculate the number of remaining pollen grains per flower.

We tested the influence of the treatment and slope angle on fruit set with a generalized linear model (GLM) with binomial errors and a logistic link, in which experimental treatment (control/direction-changed), slope angle, and interaction between them were independent variables. The effect of treatment, slope angle, and their interaction on the number of seed per fruit was examined using GLM with gaussian errors. We also used GLM with a gaussian error structure to test the effect of treatment and days after anthesis (3days/10days) on the remaining number of pollen grains per flower. In this model, we did not use slope angle and interactions between slope angle and other variables as explanatory variables according to the result of preliminary analysis based on model selection with Akaike's Information Criteria (AIC).

Results

Distribution of ADSF and relationship between ADSF and slope angle in herbs

We found a general trend among ten herb species that most flowers faced downslope. In *E. japonicum*, *A. sieboldoii*, *S. uniflora*, *V. eizanensis* and *H. sieboldii*, 50.0–76.7% of flowers faced downslope within a 45° deviation (Fig. 2). These distribution patterns differed significantly from a uniform distribution. Most *C. reflexa*, *C. lineariloba* and Monotropaceae individuals had a <90° ADSF of the inflorescence (Fig. 2), which also differed significantly from the uniform distribution. Thus, species whose individuals always had one or two flowers showed strong downslope orientation while others showed weak trends.

Although the ADSF of single flowers or the ADSF of the individual varied largely for each species, they were negatively correlated with slope angle in all species (Fig. 3). For all species, flowers (plants) with an ASDF >90° were rarely found on steep (>25° angle) slopes (Fig. 3).

Reproductive success in *E. japonicum* on slopes

About 65% of tagged flowers (96 flowers) produced fruits in the field. Logistic regression analysis revealed no significant relationship between the ADSF and fruiting success (d.f. = 1, $\chi 2 = 0.863$, P = 0.35). The average seed set for 146 tagged flowers was 30.2 ± 6.68 (SE). The number of seed per fruit did not have significant relationship with the ADSF (coefficient = -0.014, r = 0.000, P = 0.95).

The percentage of fruiting flowers was 66.7% and 73.0% for direction-changed and control flowers, respectively. The mean number of seeds per fruit was 19.8 ± 18.4 (SD) for direction-changed flowers and 24.1 ± 18.6 for controls. We found experimental treatment had a marginally significant effect on fruit set and the number of seeds per fruit (Table 2). On the other hand, the interaction between treatment and slope angle significantly affected both fruit set and the number of seeds per fruit (Table 2, Fig. 4). This means that fruit and seed set decreased with increasing slope angle in direction-changed flowers but not in control flowers (Fig. 4).

Direction-changed flowers contained more pollen grains than controls both at 3 days and 10 days after anthesis, and the number of remaining pollen grains decreased over time (Fig. 5). Both experimental treatment and days after anthesis had significant effects on the remaining pollen number (Table 2). This indicates that pollen dispatch was lower in direction-changed flowers throughout the anthesis. At 3 days after anthesis, 23.1% of the pollen grains remained on the anthers in direction-changed flowers, while 13.8% remained on control flowers.

Discussion

Effect of downslope orientation on reproductive success in *Erythronium japonicum*

Our field experiment revealed that direction-changed (upslope-oriented) treatment did not strongly reduce female success in *E. japonicum*. However, we found that the significant effect of the interaction between direction-changed treatment and slope angle on female reproductive success, such that upslope flower orientation significantly decreased fruiting and seeding as the slope steepness increased, whereas no such trend was found for downslope-oriented flowers. This means that the disadvantage of upslope orientation increases with slope angle. In E. japonicum, about 11 pollen grains per stigma are enough to produce a fruit (Ishii & Sakai, 2000). It is likely that upslope-oriented flowers rarely received pollen from pollinators on steep slopes.

Pollen dispatch was significantly lower in upslope-oriented flowers than in downslope-oriented flowers. Thus, the direction-change treatment also diminished male reproductive success in Erythronium flowers on the slopes. Small-sized bees tend to land directly on the anthers of Erythronium to collect pollen grains (Ushimaru et al. 2003). Thus, our experimental results are consistent with our prediction that flowers facing upslope should have lower reproductive success. Thus, the downslope flower orientation of E. japonicum may be an adaptation to spatial heterogeneity produced by slope environments.

Trends in flower orientation of herb species on slopes

Flowers of all investigated herb species exhibited a downslope flower orientation independent of slope direction. We rarely observed flowers whose angular distance between the slope and flower directions (ADSF) was more than 135°. This result verifies our predictions that most flowers face downslope and that this trend is common among forest floor herbs, although some deviations (more than 30% of individuals had ADSFs >45°) were found in every species.

These deviations could be explained by slope angle. On gentle slopes, ADSF varied largely, but flowers rarely had ADSFs >90° on steeper slopes (angle ≥25°). We found a significant negative correlation between slope angle and ADSF in seven species. This result is concordant with the prediction that downslope orientation should be most common on steeper slopes. Thus, our field data support the generality of our hypothesis that flower direction of vertical or oblique oriented flowers is influenced by the slope direction and angle. Natural variation of ADSF did not influence female success in 146 intact Erythronium flowers in the field, suggesting that the natural ADSF might more or less adapt to each micro sloping environments.

More than half of the C. reflexa and C. linearioba individuals had ADSFs >45°. These species have many flowers on each inflorescence, so that early-blooming flowers may reduce free space for other flowers of the same inflorescence; i.e., the downslope side is not available for late-blooming flowers. Furthermore, on gentle slopes, the orientation varied substantially among flowers in the same inflorescence. On a flat plain, a multidirectional orientation would be more attractive than a single-directional orientation for species with many flowers because pollinator approaches can be expected equally from every direction on flat land, unlike on slopes. The adaptive significance of multidirectional flower orientation on flat ground may be investigated in the light of the evolution of floral display.

Downslope flower orientation

In this study, we showed that downslope orientation was a general phenomenon among herbs with vertical or oblique flowers and that this trend was more conspicuous on steeper slopes. The field experiment revealed that downslope flower orientation influenced both female and male success in *E. japonicum*. These results together support our postulate that downslope orientation in vertical (or oblique) flowers has evolved to enhance pollen receipt and dispatch mediated by pollinators under heterogeneous spatial conditions on slopes. Downslope orientation in vertical flowers would facilitate pollinator behaviours in several ways. In terms of finding flowers, a downslope orientation may increase the attractiveness of a given flower by facing toward open space, consequently increasing pollinator visitations. In terms of approaching flowers, a vertical flower orientation limits the pollinator approach course (Neal et al. 1998; Ushimaru & Hyodo 2005), which may be secured by downslope orientation. Furthermore, advertisement area recognized by pollinators can be changed by pollinators' approach course (Dafni 1994). An change in flower orientation may affect approach course and consequently reduce attractiveness of a given flower. A downslope flower orientation may also help pollinators assess the flower and facilitate landing. Pollinators usually land on flowers after they assess pollen and/or nectar availability (see Lunau 2000). An upslope orientation makes it more difficult for pollinators to access anthers and other floral organs, so that they cannot determine food availability on flowers. This would lead to a rejection of flowers by pollinators before landing. Furthermore, because landing points are controlled by vertical orientation (Ushimaru & Hyodo 2005), a downslope orientation may make landing points easily accessible by pollinators. These behaviours were not examined in our experiment because of infrequent pollinator visitations to *Erythronium* flowers. Future studies should attempt to examine this issue using different flowers with frequent pollinator visitations. Especially, more attention should be paid for whether attraction size (advertisement area) is affected by flower orientation on the slope or not. Most heliotropic flowers attract pollinators with heat as a floral reward (Hocking & Sharplin 1965; Kudo 1995), and there have been a few evidences showing that flower orientation itself increases pollinator attraction (e.g. Patino et al. 2002).

Another question remains to be answered; i.e., how do flowers face downslope? Kita and Wada (2000) reported that *E. japonicum* tends to orient its flowers toward brighter areas (e.g., toward canopy gaps) in a forest on a flat plain. Artificial shading on the downslope side changes flower direction upslope in some individuals of *M. globosum* (Imamura & Ushimaru, submitted). These results suggest that flower orientation is determined using a light cue. Thus, flowers may orient toward brighter areas, in this case, downslope.

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Figure captions

Fig. 1

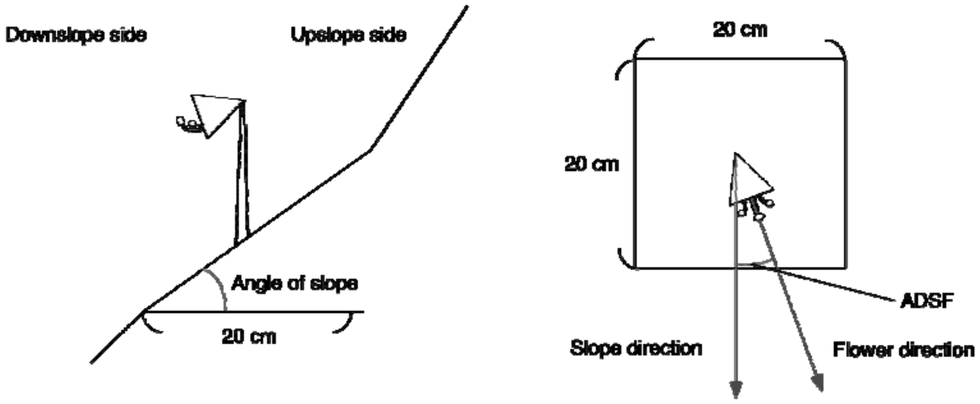

Fig. 1. Measurements of flower direction and slope direction and angle. The angular distance between the slope and flower direction (ADSF) was calculated as in this figure.

Fig. 2

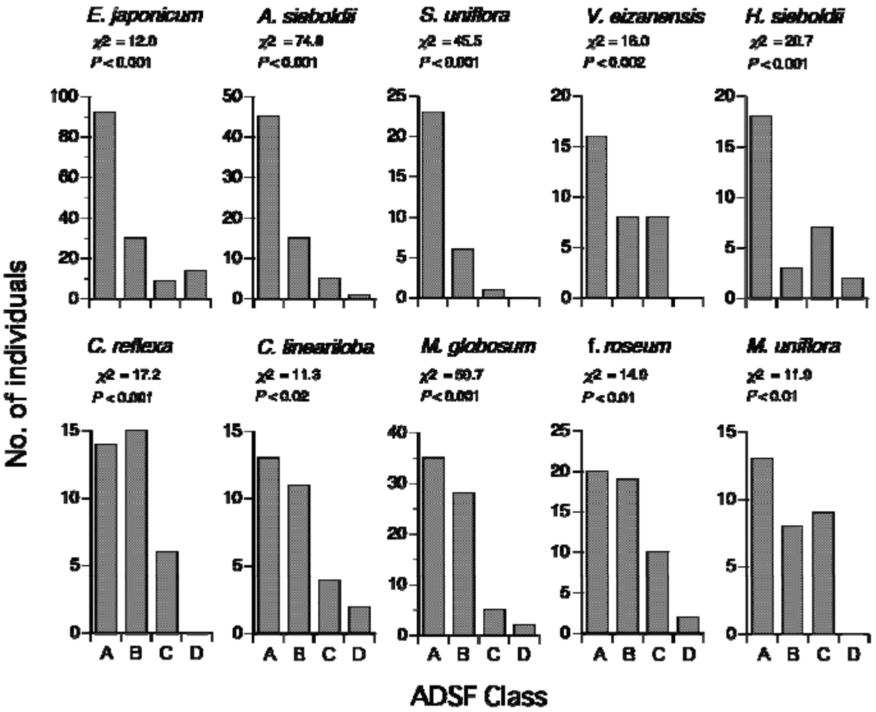

Fig. 2. Frequency distribution of the angular distance between the slope and flower direction (ADSFs) for ten forest herb species. Flowers were categorised into four ASDF classes: ADSF = 45˚ (A), 45˚ < ADSF ≤ 90˚ (B), 90˚ < ADSF ≤ 135˚ (C) and 135˚ < ADSF ≤ 180˚ (D).

Fig.3

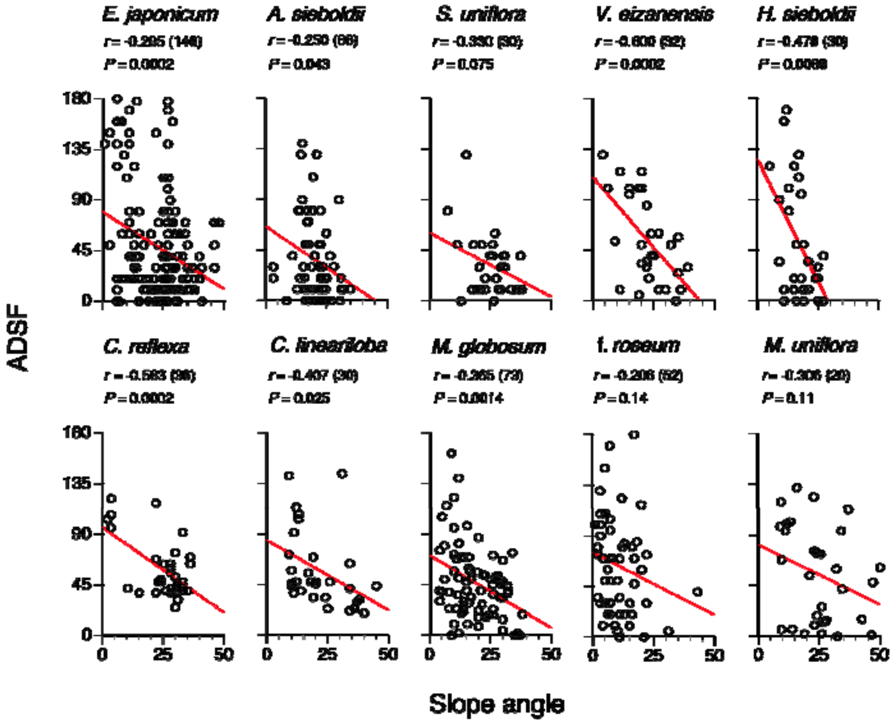

Fig. 3. Relationship between slope angle and the ADSF in ten forest herb species. For multi-flowered individuals, plots indicate the ADSF of an individual. The sampling number is shown in parentheses.

Fig. 4

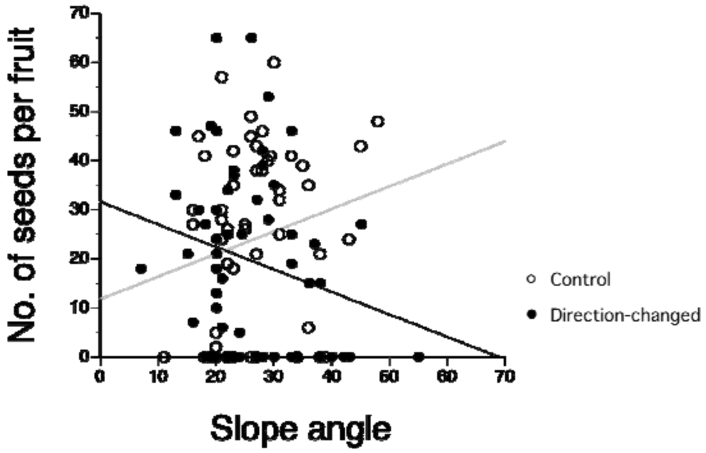

Fig. 4. Relationship between slope angle and the number of sired seeds per fruit in experimental *Erythronium* flowers. Black regression line was for direction-changed flowers (black circles) and gray line for control flowers (open circle). The number of seeds per fruit significantly decreased with increasing slope angle (see text and Table 2).

Fig. 5

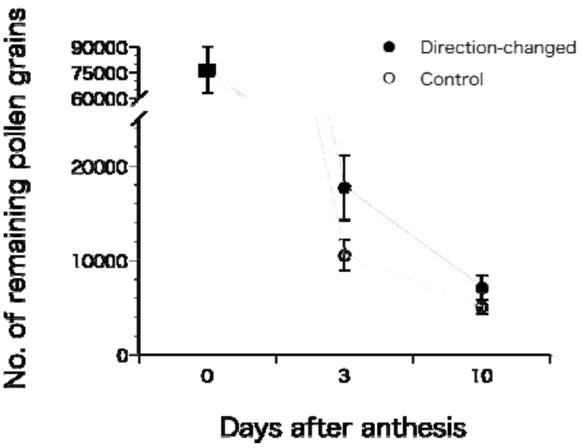

Fig. 5. Effect of direction-change treatment on pollen dispatch in *Erythronium japonicum*. The open circle indicates the mean number of pollen grains on intact flowers. The black circle and square indicate the number of remaining pollen grains on direction-changed (upslope-oriented) flowers and on control (downslope-oriented) flowers, respectively.

Table 1. List of plant species that were investigated.

Family Species	Flower angle	[a]Floral symmetry	Site	Location	No. of slopes	[b]Slope direction	No. of flowers (individuals)	Date
Monocots								
Liliaceae								
Erythronium japonicum	Decline	Ac	Ogawa forest reserve	36°56'N, 140°35'E	13	30-350°	146 (146)	Apr., 2002
Hosta sieboldii	Vertical	Zy	Ogawa forest reserve	36°56'N, 140°35'E	1	80-120°	33 (30)	Aug.,2002
Orchidaceae								
Calanthe reflexa	Vertical	Zy	Ashu experimental forest	35°18'N, 135°43'E	2	20-300°	170 (36)	Aug., 2001
Dicots								
Aristlochiaceae								
Asiasarum sieboldii var *sieboldii*	Incline-decline	Ac	Ogawa forest reserve	36°56'N, 140°35'E	3	0-340°	66 (66)	May, 2002
Papaveraceae								
Corydaris lineariloba var. *lineariloba*	Vertical	Zy	Ogawa forest reserve	36°56'N, 140°35'E	4	50-290°	150 (30)	Apr., 2002
Violaceae								
Viola eizanensis	Vertical	Zy	Ogawa forest reserve	36°56'N, 140°35'E	3	20-280°	50 (32)	Apr., 2002
Diapensiaceae								
Shortia uniflora var. *kantoensis*	Vertical	Ac	Ogawa forest reserve	36°56'N, 140°35'E	1	320-10°	30 (30)	Apr., 2003
Monotropaceae								
Monotropa uniflora	Vertical-decline	Ac	Yada Hills	34°40'N, 135°43'E	1	70-350°	38 (26)	Sep., 2000
			Iwakura-hanazono-cho	35°04'N, 135°48'E	1	30-60°	5 (3)	Sep., 2000
Monotropastrum globosum	Vertical-decline	Ac	Mt. Higashiyama	34°59'N, 135°47'E	3	0-360°	243 (73)	Apr., 2001
M. globosum f. *roseum*	Vertical-decline	Ac	Mt. Kirishima	31°55'N, 130°50'E	1	110-350°	111 (52)	June, 2002

[a] Ac = actinomorphic, Zy = zygomorphic

[b] North = 0° (360°), east = 90°, south = 180°, west = 270°.

Table 2. Effects of experimental treatment, slope angle and their interaction on female reproductive success analyzed and the effects of treatment and days after anthesis on male reproductive success by generalized linear models (GLM).

Response	Factor	Coefficient	Standard error	z value	P
Fruit set					
	Treatment	3.0071	1.5435	1.95	0.051
	Slope angle	0.0450	0.0444	1.01	0.311
	Treatment X slope angle	-0.1230	0.0566	-2.17	0.030
	Intercept	-0.1719	1.1725	-0.15	0.883
No. of seeds per fruit					
	Treatment	19.8062	11.7399	1.69	0.092
	Slope angle	0.4591	0.3275	1.40	0.161
	Treatment X slope angle	-0.9172	0.4249	-2.16	0.031
	Intercept	11.8168	9.0896	1.30	0.194
Remaining pollen number					
	Treatment	-4307.8	1992.2	-2.16	0.031
	Days after anthesis	-863.6	284.6	-3.03	0.002
	Intercept	17209.8	2300.5	7.48	< 0.001