Effects of simulated herbivory on pollen tube growth rate and siring ability in *Nicotiana alata* (Solanaceae)

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The environment in which a pollen donating plant grows is very important as it may influence pollen performance. Here we examine effects of herbivory on pollen performance in two cultivars of *Nicotiana alata* with red and white flowers. Plants used as pollen donors, were subjected to varying degrees of simulated herbivory and were compared to control plants. We grew pollen from all individuals *in vitro* to evaluate inherent male competitive ability. Hand pollinations were made to study the effects of simulated herbivory on *in vivo* siring ability, which in turn is the result of interactions between both the male and the female reproductive functions. Herbivory had an impact on both *in vitro* and *in vivo* pollen performance. In both cultivars, *in vitro* pollen tube growth rate from the control plants was higher than that in pollen from moderately and highly treated plants. The siring ability of the red cultivar decreased with increase in herbivory intensity, while the white cultivar was positively affected. The differences found between the two cultivars suggest that the genotype of the pollen grain influences how pollen from individual plants reacts to different environmental factors.

Key words: gene flow, genetic variation, pollen performance.

INTRODUCTION

When pollen grains are deposited on the stigma they germinate and produce pollen tubes that grow towards the ovules for fertilization (Herrero & Hormaza, 1996). Competition for ovule fertilization takes place when there are more pollen grains germinating on the stigma than there are unfertilized ovules. In many instances pollen loads deposited on the stigma by pollinators are large enough to lead to pollen competition (Snow, 1986; Spira *et al*., 1992; Quesada *et al*., 1996). Pollen competition occurs both between pollen grains from the same pollen donor and among pollen from different donors (Mulcahy, 1979; Willson, 1990; Delph & Havens, 1998). One of the most important factors that influence the competitive ability of pollen grains is the pollen tube growth rate (Snow & Spira, 1991a,b; Pasonen *et al*., 1999; Skogsmyr & Lankinen, 1999). Environmental conditions of the pollen donor plant

during pollen development, such as leaf herbivory and soil fertility may affect speed of pollen germination and pollen tube growth rate (Young & Stanton 1990; Lau & Stephenson 1993, 1994; Quesada *et al*., 1995).

Previous studies on *Cucurbita texana* and *Lobelia siphilitica* have demonstrated that herbivory has a negative effect on *in vivo* pollen tube growth rates and siring ability (Quesada *et al*., 1995; Mutikainen & Delph, 1996). Pollen tube growth, however, is dependent on both inherent traits and the nutrients available in the pistil (Sari-Gorla *et al*., 1995). It is therefore difficult to separate the male and female effects on pollen performance by *in vivo* studies. One way to do this is to grow pollen *in vitro*, which gives information about the inherent (male) effect, as well as *in vivo*, where the male and female effects interact. In the current study, we examined the effects of herbivory on pollen performance both *in vitro* and *in vivo*. We were specifically interested in seeing if there was any variation in the way different cultivars respond to herbivory, since this has implications for the persistence of resistant genes in a population. We used *Nicotiana alata*, an annual plant that has a

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well studied induced defence system, making it a good candidate for herbivory studies.

MATERIALS AND METHODS

Plant material

We used the hermaphroditic, cultivated *Nicotiana alata* (Link and Otto, Solanaceae). It has sweetly scented flowers that release most of their scent in the evening, attracting moths (Carter, 1982). Flower colour varies between white, red, green, pink and yellow. It is easy to grow in greenhouse conditions and produces flowers continuously for a period of almost three months. Seeds for this study were obtained from Svalöf Weibull Trädgård, Sweden. These were inbred lines of two cultivars of *Nicotiana alata* which differ in flower colour, producing either red or white flowers. The lines are homozygous for flower colour and the red gene is dominant over the white.

Experimental design

We planted seeds of the two cultivars of *Nicotiana alata* in small pots for germination. After a month, we transplanted 30 plants of each cultivar to three-litre pots to act as pollen donors and another 30 plants of the white cultivar to serve as recipient plants. The soil used was base fertilized with all the nutrients required for plant growth and there was therefore no need for additional nutrient application. Pots were watered from below using automated emitters. We kept the temperature in the greenhouse constant at 23°C, with a light and dark cycle of 12 h. At the stage of flower bud formation, we assigned ten plants from each cultivar to each of three different treatments: (i) no leaf damage (controls), (ii) moderate leaf damage in which 10 leaves were cut, and (iii) high treatment whereby 20 leaves were cut. We performed the cutting by removing 1/7 of a leaf daily for seven days, in order to mimic natural herbivory by insects.

In vitro pollen tube measurements

After treating the plants, we estimated the gametophytic vigour by pollen germination *in vitro*. We collected one flower from each individual donor plant and smeared its pollen as evenly as possible on slides containing Kwack's basal pollen germination medium (Brewbaker & Kwack, 1963), and store them in a dark chamber for 3 h at 24°C. We applied a few drops of 70% ethanol to terminate pollen tube

growth before measuring them under the microscope. We selected ten pollen tubes at random from each flower and measured them using an ocular micrometer. We estimated pollen tube growth rate of the individual flower by taking the mean of the 10 measurements. The repeatability of this method has been tested in another experiment on the same species and we found it to be good (unpublished data).

Cross pollination

In order to determine the effect of leaf damage on siring ability, we conducted controlled crosses with donors from plants in the different treatments. We tested the effect of herbivory on the white pollen donor with the following crosses, ($White_{control}$ + $Red_{control}$), ($White_{moderate}$ + $Red_{control}$), ($White_{high}$ + $Red_{control}$), and on the red pollen donor with the crosses ($Red_{moderate}$ + $White_{control}$), (Red_{high} + $White_{control}$). The ($White_{control}$ + $Red_{control}$) crosses were used as controls in both sets of experiments. Each of the five crosses was made with all 30 maternal plants of the white cultivar.

One day before pollination we emasculated and bagged the maternal flowers that would open the following day. The next morning we collected an equal amount of pollen from the two donor plants on separate microscope slides, mixed them thoroughly and applied to the stigma of a pistillate flower of the recipient plant. We covered the stigma completely with mixed pollen to ensure competition among pollen tubes for access to the ovules (Quesada *et al.*, 1991; Lau & Stephenson, 1993, 1994). The size of each pollen load was adjusted based on visual estimates. As the grain size of the pollen from both lines was the same, any deviation from 50% pollen load size ought to be normally distributed and would serve to hide effects of the herbivory treatment rather than bias the results.

Paternity analysis

In all we managed to obtain 150 seed capsules from the five crosses. These were harvested and nine offspring per cross were grown until they flowered, after which we noted flower colour.

Statistical analysis

Statistical analysis was done with SPSS for Windows version 10. We used one-way analysis of variance (ANOVA) to determine the effects of herbivory on

pollen tube growth rate between treatments in the two cultivars as well as to check for the effect of herbivory on siring ability. We performed an independent t-test to compare the pollen tube growth rates between the two cultivars. Analysis of covariance (ANCOVA) was run to check if herbivory influences the relationship between pollen tube growth rate and siring ability. To investigate the relationship between ratios of pollen tube growth rates and proportions of offspring sired we used Spearman's Rank Order Correlation. We arcsine-transformed the offspring proportions before the analysis so as to meet the assumptions of normality. Pollen tube ratios were expressed as a function of *in vitro* pollen tube growth of one genotype over the other. For example, the red flowered offspring pollen tube ratio was determined by the red pollen tube growth rate/white pollen tube growth rate, and *vice versa*.
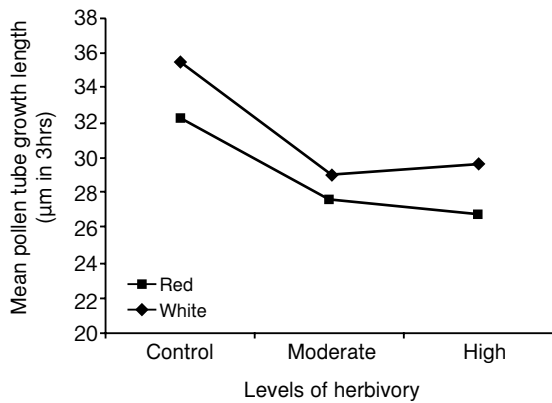
RESULTS

Effects of leaf damage on pollen tube growth rate in vitro

Measurements of pollen tube growth following different kinds of herbivory treatments showed that herbivory had a negative effect on *in vitro* pollen tube growth rate. In both cultivars, pollen from control plants had higher pollen tube growth rates than pollen from the moderately and highly treated plants (Fig. 1, one-way ANOVA, Red: $F_{2,\ 27} = 5.603$, $p = 0.009$ and White: $F_{2,\ 27} = 9.464$, $p = 0.001$). However, white cultivar showed a higher overall pollen tube growth rate than red cultivar (Fig. 1, t-test, df $= 58$, $p = 0.033$, n $= 60$).

Effects on siring ability

Herbivory treatment affected the ability of pollen donors to fertilise ovules (siring ability) using the

FIG. 1. *In vitro* pollen tube growth rate of the pollen donors when subjected to different degrees of herbivory.
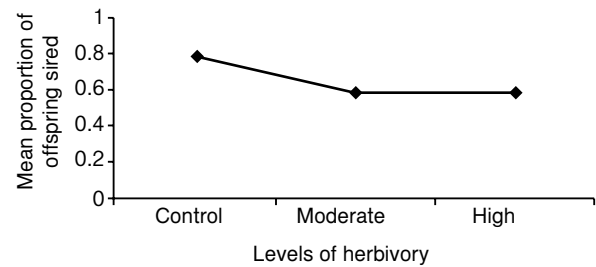

FIG. 2. Siring ability measured as the proportion of offspring sired by the red donor at three different herbivory levels. Note that the proportions sired by the white cultivar are the reverse of those shown here.
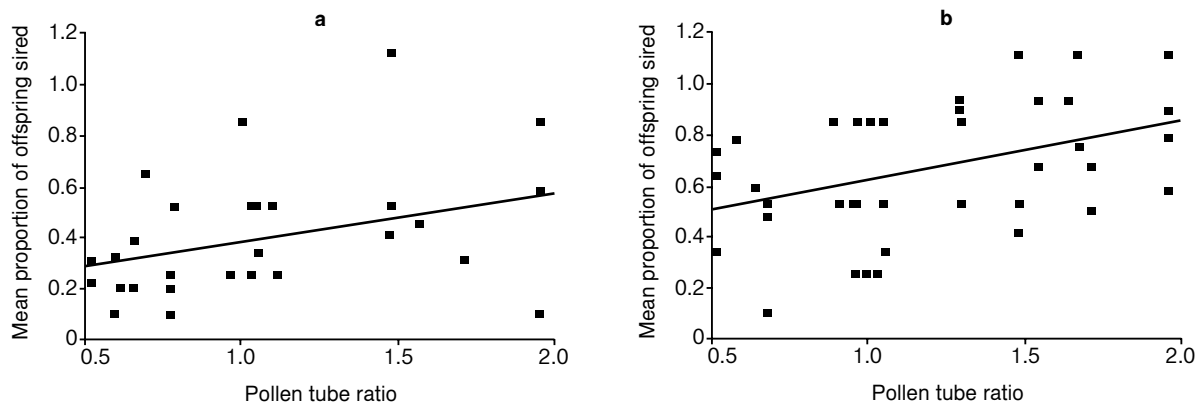

FIG. 3. (a) Positive correlation between pollen tube growth rates and offspring sired in the red cultivar when plants were not treated. Pollen with fast tube growth rates sired more offspring than pollen with slow pollen tube growth rates. (b) Positive correlation between pollen tube growth rates and offspring sired in the white cultivar in the absence of herbivory. Pollen with fast tube growth rates sired more offspring than pollen with slow pollen tube growth rates.
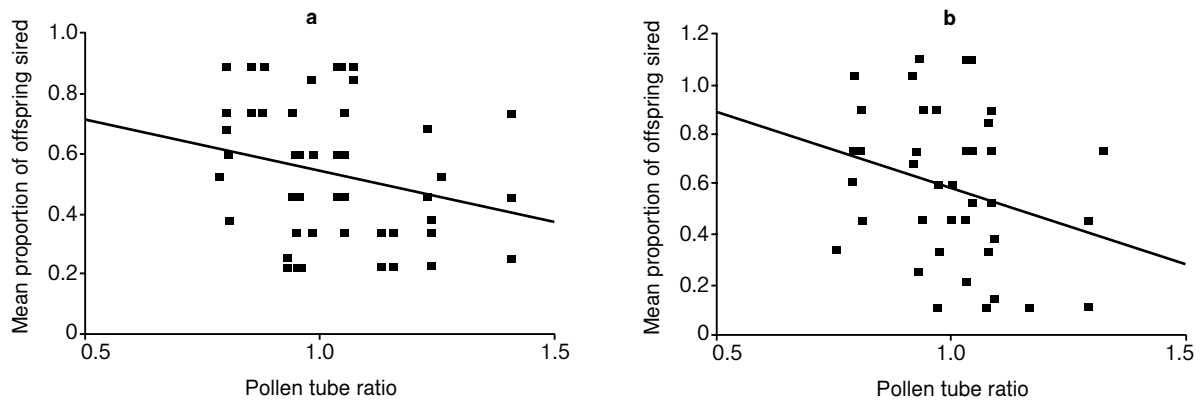
FIG. 4. (a) In the white cultivar, moderate treatment has a negative effect on the relationship between pollen tube ratio and siring ability as individuals with slow pollen tube growth rates sired more offspring. (b) Negative correlation between pollen tube growth rates and siring ability of the white cultivar when plants were highly treated.
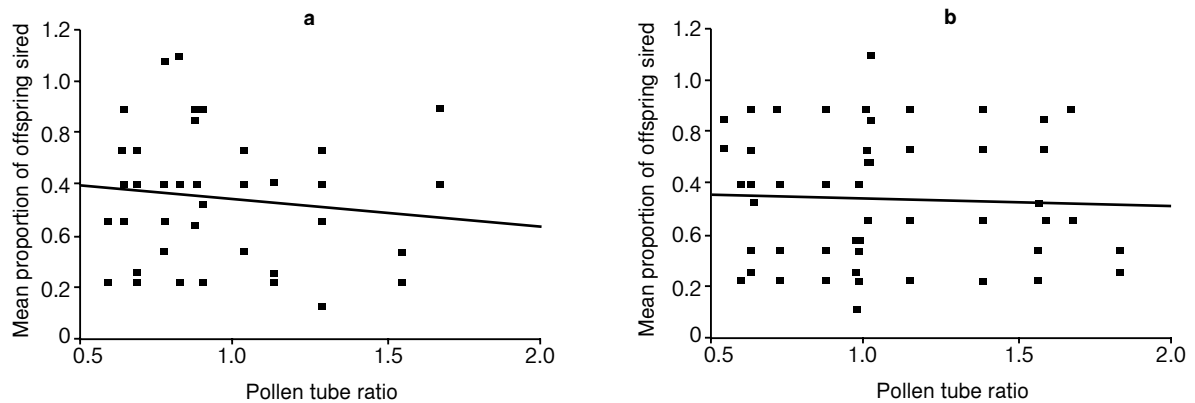

FIG. 5. (a) No correlation between pollen tube growth rates and siring ability in the moderate treatment of the red cultivar. (b) No correlation between pollen tube growth rates and siring ability in the red cultivar when plants were subjected to high treatment.

TABLE 1. ANCOVA test on pollen tube ratio variation in siring ability of the two cultivars as a function of herbivory (n = 300)

Source	df	MS	F	p
		Offspring sired		
Treatment	4	0.292	4.930	0.001
Cultivar	1	0.759	12.808	0.001
Pollen ratio	1	0.237	4.003	0.046
Treatment x Pollen ratio	4	0.304	5.137	0.001
Treatment × cultivar	4	0.197	3.326	0.01
Error	285	0.05926		
Total	300			

proportion sired by the red cultivar (Fig. 2, one-way ANOVA, Red: $F_{2, 87} = 5.633$, $p = 0.005$). The degree of herbivory furthermore affected the relationship between siring ability and pollen tube growth rate, measured *in vitro* (Table 1).

In the control plants, pollen tube growth rates were positively related to siring ability of both cultivars (Red: $r = 0.420$, $n = 30$, $p = 0.010$, White: $r = 0.346$, $n = 30$, $p = 0.03$ (Figs 3a,b).

Conversely, when the white cultivar was subjected to herbivory, a negative correlation was seen between pollen tube growth *in vitro* and siring ability in the pistil in both moderate and high treatments (Moderate: $r = -0.432$, $n = 60$, $p = 0.001$, High: $r = -0.346$, $n = 60$, $p = 0.007$). Thus, when the white cultivar increased its relative siring ability, the individuals with the lowest pollen tube growth rate *in vitro* were most successful (Figs 4a,b).

In the red cultivar, on the other hand, there was no relationship between performance *in vitro* and *in vivo* when the plants were subjected to herbivory (Red: $r = -0.100$, $n = 60$, $p = 0.446$, White: $r = -0.045$, $n = 60$, $p = 0.732$). In this case, when the relative siring ability decreased, the effect of pollen tube growth rate *in vitro* was lost (Figs 5a,b).

DISCUSSION

In this study, we found that simulated herbivory had an effect on both *in vitro* and *in vivo* pollen performance in *Nicotiana alata* plants. In both cultivars, *in vitro* pollen tube growth rate from the control plants was higher than that in pollen from moderately and highly treated plants. The siring ability of the red cultivar decreased when the plants were subjected to herbivory, while the white cultivar was positively affected. There was a positive relationship between pollen tube growth rates and siring ability in the control plants of both cultivars. Moderately and highly treated plants showed a negative relationship between pollen tube growth rates *in vitro* and siring ability in the white cultivar, but we found no relationship in the red cultivar. Overall, we found that the two cultivars reacted differently to herbivory regarding pollen performance.

Pollen performance is known to play an important role in determining male reproductive success in natural populations (Travers, 1999). However, environmental conditions during pollen development and pollen tube growth influence pollen performance (Stephenson *et al*., 1992; Delph *et al*., 1997;

Pasonen *et al*., 2000; Lankinen, 2001). In *Nicotiana alata,* we found a difference in pollen tube growth rates between treatments of the two cultivars studied. Pollen grown *in vitro* from the controls of both cultivars showed higher pollen tube growth rates than pollen from moderately and highly treated plants. Similar results have previously been reported in the gynodioecious *Lobelia siphilitica*, where herbivory seems to have an effect on pollen tube growth rates *in vivo* (Mutikainen & Delph, 1996). Slower pollen tube growth rates could be the result of the reduced ability of damaged plants to provide their pollen with resources needed in germination (Stephenson *et al*., 1992).

The seed siring ability of the two cultivars of *Nicotiana alata* was also affected by herbivory. In the red cultivar, pollen from control individuals sired more offspring than pollen from individuals subjected to herbivory. Related findings have been reported in some studies where partial defoliation led to differences in seed-siring ability between pollen from defoliated and undamaged plants of *Cucurbita texana* (Quesada *et al*., 1995). As with germination ability, leaf damage might also interfere with resource allocation at the fertilization stage, leading to poor siring ability. The white cultivar, on the other hand, sired relatively more offspring with increasing herbivory. Improved *in vivo* pollen performance after leaf damage has also been found in *Cucurita pepo* L. spp. *Texana* (Avila-Sakar *et al*., 2003). It has been suggested that leaf damage in this case leads to a reduction in the number of pollen grains per stamen, which could actually improve the quality and quantity of the resources available as stored reserves in the remaining pollen grains.

We found that pollen tube growth rates *in vitro* were positively related to siring ability when plants were not subjected to herbivory. Previous studies on *Hibiscus moscheutos*, *Cucumis melo* and *Viola tricolor* also reported similar findings (Snow & Spira, 1991a; Susin & Alvarez, 1997; Skogsmyr & Lankinen, 1999). On the other hand, a negative relation was found in the white cultivar when plants were subjected to herbivory, whereas no relationship was found in the red. This suggests that pollen tube growth rate *in vitro* is not always a good indicator of seed-siring ability, especially when plants are stressed. It might be that the environment in which both the pollen-donating and the pollen-receiving plants grow plays a role in siring ability. Even in the absence of herbivory, differences in pollen tube

growth rates among various strains of inbred culti-vated plants have been reported (Pfahler, 1967; Mulcahy, 1971; Sari-Gorla *et al.*, 1975; Ottaviano *et al.*, 1980). This variation is due to the difference in the competence or efficiency of the genes expressed by the vegetative nucleus that are responsible for the metabolism of the stored reserves. These reserves are used for the growth of the pollen tube and many of the necessary pollen-stigma and pollen-style in-teractions (Tanksley *et al.*, 1981; Willing & Mas-carenhas, 1984; Taylor & Hepler, 1997; Graaf *et al.*, 2001). When plants are undamaged, pollen perfor-mance in the pistil resembles that *in vitro* giving rise to the positive relationship between the two mea-surements of pollen performance, i.e. pollen tube growth rate and siring ability. However, individuals with a high rate of metabolism may perform better *in vitro* where nutrients are sufficient than in a pistil. *In vivo*, where nutrient competition is high, individ-uals with fast-growing pollen tubes may sire less seeds than individuals with slow-growing pollen tubes and lower nutrient requirements. This is pos-sible if there is a trade-off between a high growth rate in nutritious circumstances (i.e. in a growth medium) and competitive ability when nutrients are limited (i.e. in the pistil). Similar results have been obtained in *Cucumis sativus* (Haileselassie *et al.*, 2005).

In this study, pollen from the red cultivar was more successful, probably reflecting partial self-in-compatibility. *Nicotiana alata* is known to have a ga-metophytic self-incompatibility system controlled by a single multiallelic S locus (Nettancourt, 1977). In this system, the growth of self-pollen tubes is arrest-ed in the style or occasionally in the ovary (Cornish *et al.*, 1987). As is evident from the fact that the plants are the product of inbred lines and that around 30% of the offspring were sired by the white cultivar, this self-incompatibility does not pose a complete barrier to self- fertilization in this species.

The differences found between the two cultivars in this study suggest that the genotype of the pollen grain influences how pollen from individual plants reacts to different environmental factors.

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