Journal of Pollination Ecology, 8(8), 2012, pp 52-58

IMPACT OF CAPITULUM STRUCTURE ON REPRODUCTIVE SUCCESS IN THE DECLINING SPECIES *CENTAUREA CYANUS* (ASTERACEAE): SMALL TO SELF AND BIG TO FLIRT?

Laurent Penet*, Benoit Marion & Anne Bonis

UMR CNRS 6553, ECOBIO, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes Cedex, France

Abstract—Attracting pollinators and achieving successful reproduction is essential to flowering plant species, which evolved different strategies to cope with unpredictable pollination service. The ability of selfing is most widespread and represents a reproductive insurance under varying conditions. In this study, we investigated reproductive success in *Centaurea cyanus*, a self-incompatible declining Asteraceae species. We measured seed set under outcrossing and autonomous selfing and assessed the impact of capitulum structure (i.e., the number of disc florets) on reproductive success. We report that the incompatibility system is either flexible or evolving a breakdown in this species, since autonomous selfing often resulted in production of few seeds. We also show that capitulum structure has a strong impact on reproduction, with smaller inflorescences presenting a better ability to self than larger ones, while larger inflorescences performed better than smaller ones when cross-pollinated. Variable capitulum structure in this Asteraceae species may therefore represent a reproductive strategy to achieve efficient reproduction under diverse pollination environments. Our results also suggest that this declining species might be disrupting its auto-incompatibility system in response to reduced habitats and declining population sizes.

Keywords: Asteraceae, capitulum structure, Centaurea cyanus, *ray and disc florets, reproductive success, self-incompatibility*

INTRODUCTION

Plant reproduction has fascinated scientists since ages (Darwin 1876). The phenotypic diversity evolved to achieve sexual reproduction in Angiosperms is simply dramatic and resulted, for instance, in extreme specialization of flowers into pollination syndromes (Hermann and Kuhlemeier 2011) and sophisticated mechanisms of selfing (Fenster and Martén-Rodríguez 2007). At the same time, many species evolved various strategies to avoid selfing: self-incompatibility (Levin 1996), dichogamy (Freeman et al. 1997) and separation of sexes on or between individual plants (Narbona et al. 2011), because selfing influences the probability of unmasking mutation loads in progeny and usually reduces individual plant fitness (Goodwillie et al. 2005). Self-pollen deposition may, however, be unavoidable and for self-incompatible species this often results into a decrease in seed production (e.g., Kameyama and Kudo 2009). The effect of self-pollen deposition is two-fold: first it represents a loss of pollen available to outcrossing (i.e., pollen discounting; Busch and Delph 2011), and second it interferes with outcross-pollen on stigmas (Dai and Galloway 2011). These effects may be enhanced in case of pollen limitation and in small populations (Busch and Schoen 2008). Plant mating system is mainly a pollinator driven process, though plant characteristics (e.g., asexual reproduction; Navascués et al. 2010) or other ecological interactions also play a role (e.g., nutriment availability; Helenurm and Schaal 1996), interactions with herbivores (Ivey and Carr 2005) or florivores (Ashman and Penet 2007; Penet et al. 2009). All these interactions are potentially threatened by the effects of climate change (Schweiger et al. 2010), such as loss of diversity (Sander and Wardell-Johnson 2011) and changes in pollinator specialization (Fontaine et al. 2008), and we may face a dramatic change in pollination service in the near future (Potts et al. 2011). It is therefore crucial to increase our knowledge about pollination and reproduction of plant species (Mayer et al. 2011), and particularly for rare or invasive species (Powell et al. 2011) to allow for a sensible management.

In rare or declining plant species, an understanding of mating system and pollination biology in addition to causes of decline is crucial to help in implementing conservation guidelines (Biesmeijer et al. 2011). Indeed, assessing selfing rates and plants tolerance to selfing is a first step toward managing viable reintroductions or reinforcement (Leducq et al. 2010), because it will bring light to density and relatedness effects in small populations (i.e., impacts of inbreeding depression and Allee effects; Ågren 1996; Leducq et al. 2010). In self-incompatible species, these effects are altered, because direct selfing is reduced (Busch et al. 2010), but consanguinity may still impact fitness via bi-parental inbreeding (Uyenoyama 1986; Elam et al. 2007). Many self-incompatible species have actually somehow a plastic expression of incompatibility and incompatibility breakdown has been reported in several families (Igic et al. 2008; Busch and Urban 2011). Ability to self in incompatible species is

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Received 29 November 2011, accepted 21 March 2012

*Corresponding author; email: laurent.penet@gmail.com

indeed associated with repeated extinction and colonization demographic events and it is often greater in small populations (Reinartz and Les 1994). Here we chose to investigate reproductive success in *Centaurea cyanus*, a self-incompatible species experiencing a dramatic decline in its native range since a few decades (e.g., Sutcliffe and Kay 2000; Pausic et al. 2010;Ulber et al. 2010), and considered as an invasive outside its native range (Muth and Pigliucci 2007; Jursik et al. 2009).

Like other species of Asteraceae, *Centaurea cyanus* expresses variation in capitulum size and structure (Jursik et al. 2009). Its capitula are structured with deep blue sterile ray florets advertising for pollinators and less showy fertile disc florets (Boršic et al. 2011). Variation in capitulum structure would reflect alternative investments into pollinator attraction vs. seed production, and we were interested in investigating the effect of reproductive efforts on reproductive success. In this study, we investigated reproductive success under controlled conditions and we asked the following specific questions: 1) how variable is capitulum composition in ray and disc florets, hereafter capitulum structure? 2) Is *C. cyanus* able of autonomous self-pollination and seed production? 3) Does capitulum structure variation impact seed set differently under outcross- and self-pollination? We were particularly interested in determining if variation in capitulum structure may represent a reproductive strategy in this species.

MATERIAL AND METHODS

Plant species

Centaurea cyanus (cornflower) is an annual plant from the Asteraceae family (= Compositae, Angiosperm Phylogeny Group 2003) with a wide distribution. The species originated in Caucasus (Boršic et al. 2011) and dispersed as a weed species associated with cereal crops since prehistorical times (Rösch 1998). Though initially not native to Europe, the species is now well naturalized and part of the flora. It may thus not be considered an alien anymore, given its ancient colonization in Europe.

Centaurea cyanus is generally plastic with regard to many traits (Muth and Pigliucci 2007) and grows either in crop fields (especially associated to wheat or canola) or along field margins, where it is expressing a usually high competitive ability (Wassmuth et al. 2009). Germination occurs in fall (Stilma et al. 2009). Flowering occurs from June to mid-summer and each plant produces lose inflorescences bearing many capitula typical of Asteraceae. Sterile peripheral ray florets are deep blue and surround several whorls of fertile tubular disc florets, each with an ovary of a single ovule. *Centaurea cyanus* is pollinated by diverse species of insects, though mostly bees (Carreck and Williams 2002) and it is reported as self-incompatible as a vast majority of species in Asteraceae (Charlesworth 1985).

Pollination treatments

Seeds from many (30 - 40) individuals of wild *C. cyanus* were sampled from discontinuous patches from three adjacent fields in Macon (Upper Normandy) during summer 2009 and bulked for storage at +4 C. We sowed seeds in autumn at the greenhouse facility of ECOBIO (University of Rennes 1) and transplanted young plantlets into pots. We started the experiment in spring 2010 when experimental individuals were in bloom (n = 17 unrelated plants). We recorded the number of ray and disc florets of each experimental capitulum at opening. Capitula were individually marked and assigned to either manual cross-pollination or autonomous self-pollination. Each experimental plant received both treatments.

For cross-pollination, we collected pollen from several unrelated pollen donors (6 - 10 non-experimental plants). We used a gentle brush to cover receptive stigmas emerging from disc florets with the mix of pollen, thus outnumbering self-pollen grains already present on stigmas due to protandry (L. Penet, personal observation). In the selfing treatment, capitula were left without further manipulation until harvest. We collected achenes before their release from the capitulum. Each achene contains a single seed; we therefore estimated and counted the number of plump fertile seeds. Unfortunately, part of the capitula dried before maturation due to unexpected high temperatures in the greenhouse. For these, we therefore differentiated successfully fertilized ovules based on size and we considered those as seeds in further analyses. This maturation issue is unlikely to have biased our results since a similar number of capitula were damaged in both treatments and no difference in seed production was found between mature and damaged capitula within pollination treatments (data not shown). We collected 5 - 11 capitula per plant resulting in 64 cross- and 68 self-pollinated capitula.

Statistical analyses

We calculated reproductive success as the proportion of seeds per capitulum (i.e., seed set) and tested for a difference between cross- and self-pollination treatments. We conducted a two-way full-factorial analysis of covariance of seed set with pollination treatment as fixed factor and the number of disc florets (i.e., capitulum structure) as covariate. We added individual plant to account for maternal effects; the Ancova assumptions of homoscedasticity and normality of residuals were met. We included an interaction between pollination treatment and capitulum structure because variation in number of disc florets is expected to influence local pollen availability for autonomous selfing and probability of self-pollen transfer. We found that the interaction was significant. To correlate the number of disc florets and seed set while taking into account maternal effects, we conducted separate analyses of variance of seed set for outcross- and self-pollination treatments, with individual plants as sole factor, and extracted the residuals. We then calculated the correlation between the number of disc florets and the residuals for seed set for both pollination treatments. All analyses were conducted with R statistical package (R Development Core Team 2011).

RESULTS

Capitulum structure was highly variable both within and among *Centaurea cyanus* plants. The total number of florets

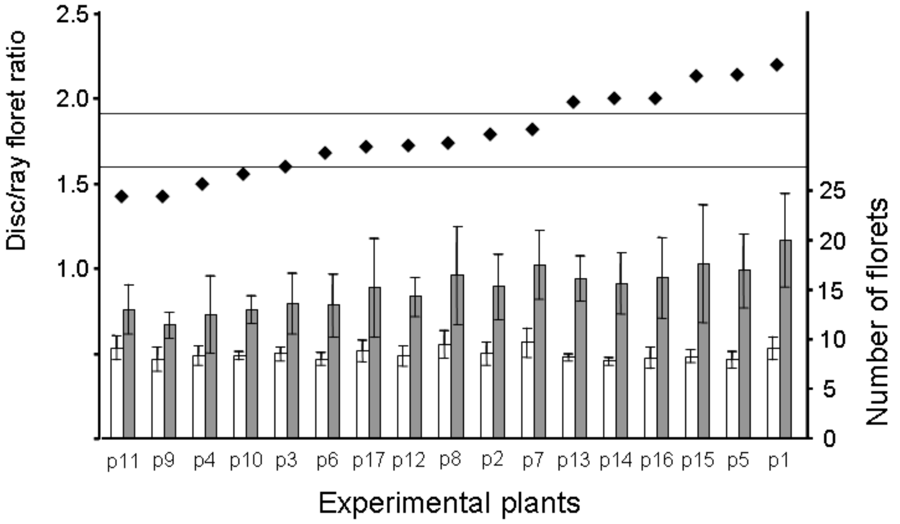

FIGURE 1. Variation in disc to ray florets ratio and number of floret types per inflorescence in the experimental *Centaurea cyanus* plants. Plants are ranked from lowest to largest individual mean disc/ray florets ratio (left y-axis); horizontal lines delineate lower and upper values segregating population in thirds. Mean (± SE) number of ray florets, open bars, and disc florets, filled bars, per plant are presented (right y-axis).

per capitulum varied from 13 to 40 (mean ± se: 23.92 ± 0.42, n = 132) and the average number of florets at the plant level varied from 19 to 29 with a strong maternal effect ($F_{16,115} = 2.72$, $P = 0.001$, n = 132; Fig. 1). Overall, we found that capitula bore a significantly greater number of fertile disc florets than sterile ray florets (grand-means ± se: 15.19 ± 0.54 and 8.49 ± 0.13, respectively, n = 17; paired t -test, $t = 13.11$, $P < 0.0001$) and that variance was also greater for the number of disc florets than for the number ray ones (Levene test, $F_{1,32} = 18.23$, $P = 0.0002$). The ratio of disc to ray florets at the plant level varied continuously (Fig. 1).

The proportion of disc florets that set a seed was significantly affected by pollination treatment (Table 1). Despite a reported self-incompatibility system, most autonomously self-pollinated capitula (93%) set a few seeds though significantly fewer than outcross-pollinated capitula (mean ± se seed set: 0.21 ± 0.02, and 0.66 ± 0.02, respectively). Interestingly, we found that the actual number of disc florets on the capitulum influenced seed set differently between pollination treatments (significant interaction in Table 1). Seed set increased with number of disc florets following cross pollination whereas it decreased with increasing number of disc florets in autonomously self-pollinated capitula (Fig. 2). Both correlations were statistically significant taking into account the maternal plant effect ($r = 0.36$, $P = 0.004$, and $r = -0.30$, $P = 0.013$, for outcrossing and selfing treatments, respectively). We found a significant maternal effect on seed set (Table 1), and high variation in pollination treatment effect among plants (after controlling for variance in number of disc florets, Fig. 3).

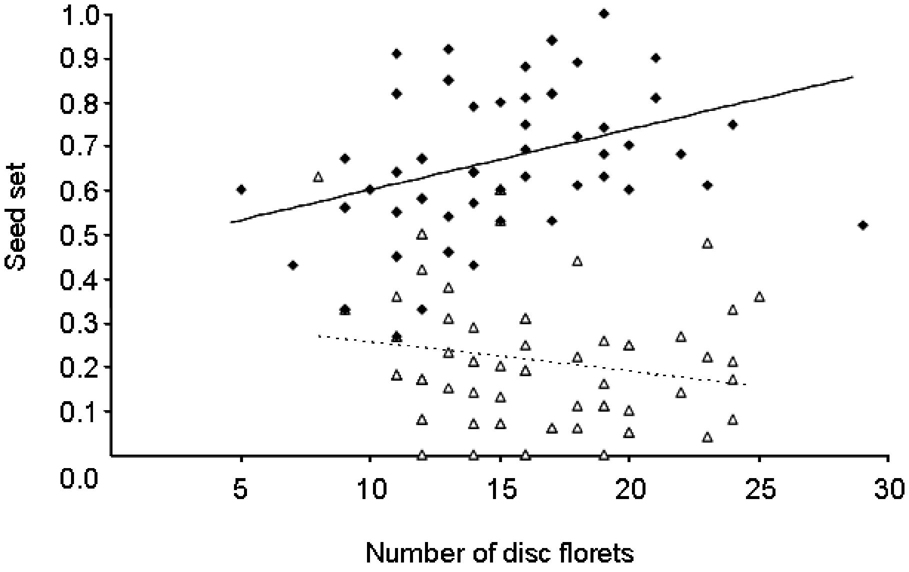

FIGURE 2. Correlation between seed set and number of disc florets per inflorescence following outcrossing and selfing in *Centaurea cyanus*. Pollination treatments: outcrossing (dark diamonds and continuous line), and autonomous selfing (white triangles and dotted line).

Source of variation	df	Mean Square	*F*-value	*P*-value
Pollination treatment	1	6.21	265.8	**< 0.0001**
Number disc florets	1	0.04	1.82	0.180
Treatment * Nb discs	1	0.33	14.22	**0.0002**
Experimental plant	16	0.05	2.01	**0.018**
Residuals	112	0.02		

TABLE 1. Analysis of covariance of seed set per capitulum. Pollination treatments were manual outcrossing and autonomous selfing and the number of disc florets per capitulum was used as covariate.

DISCUSSION

Centaurea cyanus is classified as a self-incompatible species, but we document here that most capitula produced seeds following autonomous selfing under controlled conditions. Seed set was nevertheless low, and significantly smaller than in the outcross pollination treatment with high variation in ability to self among plants (Fig. 3). Most surprisingly, seed set was strongly influenced by capitulum structure though differentially between pollination treatments: small capitula with few disc florets performed better than large ones under selfing, and the reverse was found for large capitula with many disc florets (Fig. 2). This suggests that variation in capitulum structure might reflect a reproductive strategy to ensure reproduction. We discuss these findings in the light of conservation biology for this declining species.

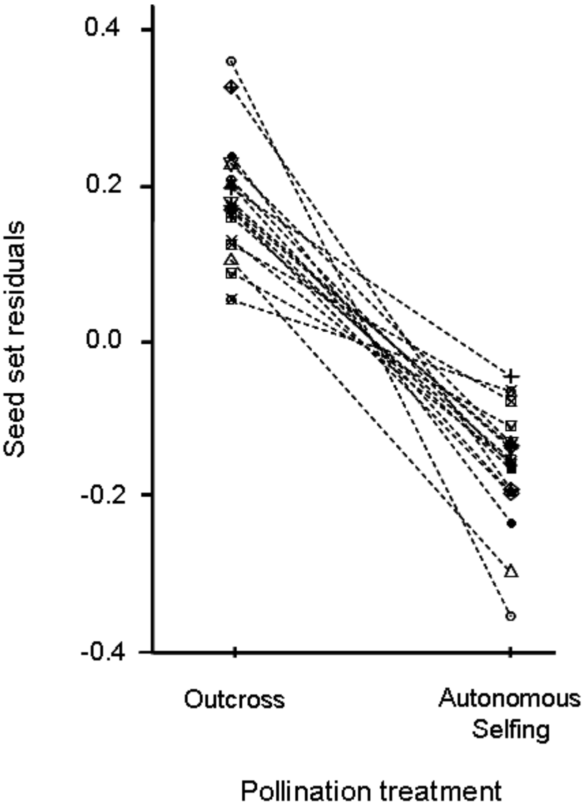

FIGURE 3. Individual plants' response to cross-pollination and autonomous selfing in *Centaurea cyanus*.

Seed set following autonomous selfing was low but non negligible since on average 21% of available ovules were fertilized and up to 50% of seed may be achieved. Thus, even if the plant would receive no visit from pollinators during its reproductive life, it would certainly ensure the production of enough seeds to allow for persistence in the field (at plant level, the average of 21% seed-set under selfing translate into several hundred seeds). Self-incompatible pollination systems are already known to be flexible and to vary depending on environmental factors (Reinartz and Les 1994), and many allegedly self-incompatible species are often reported as able to self (e.g., Mena-Ali and Stephenson 2007). Most interestingly, stigmas bend outward after they emerge from the stamen crown in *C. cyanus*, thus loading themselves with self pollen. This mode of stigma growth might have been selected for because it improves pollen receipt when pollinators visit the capitulum, but it may also be considered a striking pre-adaptation to selfing because it dramatically increases the odds of receiving self-pollen if anthers are still loaded (e.g., Penet et al. 2009). Moreover, a species experiencing population local decline – like cornflower – may be expected to evolve toward increased selfing ability, especially if pollination service becomes unpredictable (Goodwillie et al. 2005). The extent of inbreeding depression (i.e., the lower fitness of selfed progeny compared to outcrossed ones) is an important prospect of study to explore the consequences of evolving self-compatibility in this species.

Capitulum structure had a strong impact on seed set and capitula with fewer disc florets performed better than larger ones under autonomous selfing, while the opposite was found under outcrossing (Fig. 2, larger capitula performed better than smaller ones). In the outcross pollination treatment, we would have expected the reverse, since pollen loads were probably higher in smaller inflorescences, and it is easier to leave a flower un-visited when brushing inflorescences with pollen experimentally in a larger capitulum. This may reflect negative consequences of high competition for fertilization on stigmas if pollen loads were indeed greater in smaller capitula, or that stigmas in smaller capitula were less efficient in pollen receipt. Indeed, the number of disc florets, or inflorescence size, may also reflect individual variation in flower allometry, which is known to translate into reproductive differences between flowers (i.e., herkogamy, differential stigma size; Nishihiro and Washitani 2011), and into decreased pollen receipt and fertilization efficiency. To our knowledge, this is the first time that differences in capitulum structure are directly related to mating efficiency in Asteraceae, usually, an increase in inflorescence or flower size results in increased geitonogamy (i.e., pollinator-mediated selfing; e.g., Klinkhamer et al. 1989;) or an increase in seed predation (Fenner et al. 2002). This variation might thus reflect different reproductive strategies: small capitula, which are also probably less attractive to pollinators (Fenner et al. 2002), would still ensure reproduction via selfing, when pollination service is insufficient or uncertain. On the other hand, the larger and more attractive capitula would result in efficient outcross reproduction under good pollination conditions.

At the individual plant level, capitulum structure as a reproductive strategy appears more strongly than at population level: investment in reproduction (disc florets) compared to advertising (ray florets) showed a continuum in experimental plants (Fig. 1), and was essentially due to variation in disc flower number. Plants with lower values of disc:ray floret ratio (ratio < 1.6) may thus be considered as better "selfers" and those with higher values (ratio > 1.9) as mostly outcrossing. Nevertheless, individual plants showed high inter-capitulum variation in number of disc flowers (Fig. 1), even when they tended to have larger capitula, so that plants do not entirely rely on a single reproductive strategy (selfing or allogamy). When accounting for the influence of capitulum structure, plants showed differences in their response to selfing or outcrossing pollination treatments (Fig. 3), with some individuals being more self-incompatible than others. This gives further evidence that self-incompatibility is probably evolving a disruption and it opens the door to selection on other reproductive modes, like reproductive insurance via autonomous selfing, in this species (Reinartz and Les 1994).

This study documents a link between capitulum structure and reproductive strategies in an Asteraceae species probably experiencing a breakdown in its self-incompatibility system. Stigma bending during female phase is possibly pre-adaptive and facilitates evolution towards increased selfing ability. Autonomous selfing may be selected for in small populations, especially if individuals are related and consanguinity interferes with incompatibility (Busch and Schoen 2008). Since *Centaurea cyanus* is declining throughout its range due to massive use of herbicides, and even considered as locally endangered (Sutcliffe and Kay 2000; Pausic et al. 2010), these results open several pathways for further investigation. It would be of interest to evaluate levels of inbreeding depression experienced by selfed progenies, and whether it varies among lineages with differences in self-compatibility (e.g., Collin et al. 2009). Then, the extent and generality of an incompatibility breakdown within the species' range may drive local persistence of populations, especially if selfing ability is correlated to population size.

Finally, self-incompatibility breakdown and selfing-ability could be influenced by:

(1) Visits of different pollinator species and their relative efficiency to cross-pollinate (e.g., Ostevik et al. 2010); (2) inflorescence size and competition for pollination; (3) how visits translate into seed-set in natural conditions (e.g., as in Nienhuis and Stout 2009) and under which conditions a newly evolving selfing ability would perform better than outcross. Indeed, natural *Centaurea cyanus* seed set may be lower *in natura* than seed sets for our cross pollination treatment. Investigating these interactions between plants and pollinators would inform us as to how the ecology of pollination could drive adaptations to new modes of reproduction.

ACKNOWLEDGEMENTS

The authors thank Carine L. Collin and two anonymous referees for comments on early versions of the manuscript; and Thierry Fontaine and Fouad Nassur for help in the greenhouse facility. This study is dedicated to Jean-Marie Penet (1950-2010), who made early suggestions about investigating mating consequences of variation in capitulum structure.

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