Studies on the pollination biology of the tropical forage legume shrub *Cratylia argentea*

MARIA BYSTRICKY[1], RAINER SCHULTZE-KRAFT[1,2] AND MICHAEL PETERS[2]

[1] *Universität Hohenheim, Stuttgart, Germany*
[2] *Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia*

Abstract

Cratylia argentea is a high-quality forage legume shrub, with particular potential for the subhumid tropics owing to its adaptation to acid and infertile soils and drought tolerance. However, lack of knowledge about the ability of this species to self-pollinate makes purity maintenance of accessions difficult. Studies were conducted to investigate whether the gametes of *C. argentea* flowers are self-compatible, and to verify the dependence of reproduction on insects. Pod set and seed production were assessed in a pollination experiment with 4 treatments (spontaneous self-pollination, artificial self-pollination, insect pollination and artificial cross-pollination). Our results (0–1.2% pod set after selfing, 3.4% after insect pollination and 20.3% after artificial crossing) suggest that *C. argentea* is mostly self-incompatible. Seed:ovule ratios were 0.63 with insect pollination and 0.78 with artificial crossing. We found that pollen grains mature before the stigma becomes receptive, which suggests that the species is cross-pollinated. We also found evidence that *C. argentea* depends on large solitary bees for pollination, based on flower structure and the stigma becoming receptive only after tripping of the flower. *Xylocopa frontalis* and 2 *Centris* species were identified as key pollinators. In view of its simplicity, the research procedure utilised is suggested for other tropical legume species pollinated by large insects.

Correspondence: Maria Bystricky, Technische Universität München, Weihenstephaner Steig 22, 85350 Freising, Germany. E-mail: mbystricky@googlemail.com

Introduction

Cratylia argentea is a tropical forage shrub legume, native to South America, occurring in Brazil, Peru and Bolivia south of the Amazon River in open habitats with a well-pronounced dry season (Queiroz 1991). It possesses useful agronomic and nutritional characteristics, including adaptation to acid soils of low to medium fertility, drought tolerance, good regrowth potential after cutting and high forage nutritional value with low levels of anti-nutritive factors (Argel and Lascano 1998; Peters and Schultze-Kraft 2002). Its major potential lies in the subhumid tropics, where selected genotypes were released as commercial cultivars in Costa Rica (Argel *et al*. 2001) and Colombia (Lascano *et al*. 2005). In those countries, as well as in Venezuela and Nicaragua, where no formal release has taken place, the species is used successfully for grazing or cutting, in freely draining savanna soils.

Information regarding the reproductive biology of *C. argentea,* including its rate of outcrossing, is limited. Self-pollination is common in other members of the Papilionideae in spite of the flowers being adapted to insect pollination (Faegri and van der Pijl 1971; Kalin-Arroyo 1981). Self-pollination as well as cross-pollination can depend on insect visits. An important functional feature in papilionoid flowers is tripping. This term is used to describe the process when the keel petals are pressed down by the force of an alighting insect, releasing the staminal column with the gynoecium in the centre. Tripping can lead to the rupturing of a cuticle on the stigma surface, so that an exudate which is accumulated beneath is released. This exudate stimulates the pollen grains to germinate (Shivanna and Owens 1989) – the stigma is receptive for pollen.

Previous observations (R. Schultze-Kraft, unpublished data) in Colombia with a single, completely isolated *C. argentea* plant, revealed abundant flowering and visits by insects capable

of tripping and transporting pollen, but no pod formation. This first indication of self-incompatibility was supported by the results of another study in Colombia at the Centro Internacional de Agricultura Tropical (CIAT), in which high levels of genetic diversity within cluster groups of *C. argentea* germplasm were found (Andersson *et al.* 2007). Such intra-accession variability is a characteristic of predominantly out-crossing, self-incompatible species (Nybom 2004). An earlier study with *C. argentea* conducted in Brazil, however, indicated that both autogamous and allogamous pollination occurred (*i.e.*, pollination with the plant's own pollen, and pollination with foreign pollen, respectively), leading to the formation of fertile seeds (Queiroz *et al.* 1997).

Knowledge about the reproductive system of a species is crucial for appropriate germplasm management, particularly for the development of propagation methods, for maintaining the purity of accessions, and for breeding programmes. Given the ambiguity of findings from previous observations, the objective of the present study was to determine the level of self-compatibility in *C. argentea*, and the role of insects in pollination.

Materials and methods

Location of the study and plant material

The pollination experiment was conducted from March to July 2006 at CIAT in Colombia. The study site was located at Palmira, near Cali (3°30'N, 76°21'W; elevation 965 m asl) and has an average annual temperature of 24°C and annual rainfall of 1000 mm (distributed bimodally). The soil is a fertile but poorly draining Vertisol with pH 6.7, 2.4% organic matter content and a high content of plant available P (55 mg/kg, Bray II).

For the study, *C. argentea* cv. Veranera, a blend of accessions CIAT 18516 and CIAT 18668, was used. A description of agronomic and genetic characteristics of this variety can be found in Andersson *et al.* (2006 resp. 2007).

Studies

Flower development: stigma and pollen maturity. To allow for conclusions on autogamous or allogamous pollination, the timetable of stigma and pollen maturity was investigated. Anthers opening and releasing viable pollen grains simultaneously with the flower's stigma becoming receptive for pollen can be an indication of autogamous pollination (Dafni 1992; Kull 2000). Thirty-seven closed flower buds, in their development within 48 h of opening, were collected, dissected and examined under a stereoscope to assess the moment of anther dehiscence. The percentage of viable pollen grains in open anthers was also determined in these flowers.

Likewise, open flowers from 10–15 plants were collected at different times of the day over a period of 13 days, totalling 15–20 flowers per hour between 08.00 and 17.00 h, to determine stigma receptivity and pollen viability. Anthesis, *i.e.*, the period during which flowers are open, usually lasts one day in *C. argentea*. To determine stigma receptivity to pollen, the stigmas of the collected flowers were dissected, immersed in a drop of a 3% hydrogen peroxide (H_2O_2) solution and examined under a stereoscope (*cf.*, Hoc *et al.* 1993; Dafni and Maués 1998; Etcheverry 2005). The anthers of the same flower were placed into a drop of acetic carmine (1 g carmine in 100 ml of 45% acetic acid) on a microscope slide. They were then lightly squeezed to expose pollen grains. This procedure resulted in staining of viable pollen grains and the percentage of non-viable pollen grains was determined by counting under a light-optical microscope.

Thirty flowers on 2 plants were protected from insect access to determine whether stigma receptivity depended on insect tripping. The anthers of 10 flowers were clipped off the day before the flowers opened, and stigma receptivity of these was determined after simulating insect tripping (see paragraph below). Twenty flowers from the same plants were used to estimate stigma receptivity without insect tripping.

Pollination: self-compatibility vs *self-incompatibility.* A pollination experiment with 4 treatments was completed to determine the percentage of pod set. For 3 of the 4 treatments, fly screen cages were placed over 3 individual plants to prevent insect access. The treatments were:

- Spontaneous self-pollination (SSP): 200 flowers were tagged on plants in cages. These were left without further treatment to assess whether pods were formed spontaneously by autogamous pollination.

- Artificial self-pollination (ASP): 172 flowers on plants inside the cages were treated using the 'cosquilleo' method (Escobar 1991). This consisted of a manual simulation of insect tripping by pressing down the keel petals with tweezers, thus rupturing the cuticle on the stigma surface and making the stigma receptive for pollen, and covering the stigma with pollen from the same flower's anthers.
- Insect pollination (IP): 178 flowers from 15 plants, which had recently opened and, based on visible traces on the petals, had been visited by pollinators, were tagged (all plants were outside the cages).
- Artificial cross-pollination (ACP): 138 flowers from the aforementioned plants inside the cages were used. Anthers were clipped off in the buds 1 day before anticipated anthesis, and the flowers were pollinated with pollen from other plants when open the next day (Temple and Smithson 1987).

The number of pods obtained from each treatment was recorded and an 'index of self-incompatibility' (ISI) was calculated by dividing the percentage of pod set after self-pollination (mean value of spontaneous and artificial self-pollination) by the percentage of pod set after cross-pollination (Dafni 1992). This index was used to draw conclusions on the level of self-incompatibility of *C. argentea*.

Seed:ovule ratio. After collecting the pods produced by the pollination treatments, the numbers of seeds and non-fecundated ovules within the produced pods were recorded. As low pod numbers from the self-pollination treatments prevented statistical interpretation, the results are based only on insect pollination and artificial cross-pollination. Since the number of pods obtained from insect pollination was equally low, 30 additional pods were collected from other plants in the plot where flowers had been tripped, as indicated by traces on the petals left behind by visiting insects. Thus, random pods from the plot were produced by the same 'pollination treatment' as those from flowers labelled as 'insect pollinated' (see treatment 'Insect pollination' above). The non-fecundated ovules were visible as empty spaces in the row of seeds in the pods. The seed:ovule ratio, *i.e.*, the number of seeds divided by the total number of ovules in a pod, was used as a further indicator of pollination success (Bawa and Buckley 1989).

Pollinating insects. Plants in field plots with open flowers were observed for 2–3 hours on 2 days. All insects seen activating the pollination mechanism were caught and subsequently identified by entomologists of CIAT and the Universidad del Valle in Cali. Subsequently, recording of insect behaviour over 5 days (results not presented) verified that the collected insects comprised all species visiting *C. argentea* flowers.

Statistical analyses

Statistical analyses were performed using the SAS programme version 9.1 (SAS Institute Inc. version 2002–2003). With the results of the pollination experiments, χ^2-test and Fisher's exact test were conducted as tests for independence of nominal data. Each of the data pairs of the pollination treatments (SSP-ASP, SSP-IP, SSP-ACP, ASP-IP, ASP-ACP and IP-ACP, see section above) was tested at a significance level of P=0.05. A t-test was performed for the data set 'seed:ovule ratio' (P=0.05). Log- and logit-transformations of these values were required as the data were not normally distributed.

Results

Flower development: stigma and pollen maturity

Pollen viability. Pollen was found to be viable at the moment of anther dehiscence in the closed bud, remaining so during the day of anthesis. On average, 95.5% of pollen grains were viable during this period. There was no measurable change of the percentage of viable pollen grains during the day of anthesis (data not presented).

Stigma receptivity. There was no set pattern for stigma receptivity (data not presented) during the day of anthesis. Instead, stigma receptivity was stimulated by insect tripping: stigmas of flowers protected from insect access showed no reaction with H_2O_2 when left without further treatment (data not presented). After simulating insect tripping, a reaction with H_2O_2 (bubbles being formed on the stigma surface) was clearly visible through the stereoscope.

Pollination: self-compatibility vs *self-incompatibility*

Pollination success (pod formation) in the self-pollination treatments (spontaneous self-pollination and artificial self-pollination) was very low and was much lower than after artificial cross-pollination (Figure 1). Insect pollination also produced very few pods (significantly different from spontaneous but not from artificial self-pollination, P<0.05).

The 'index of self-incompatibility' (ISI) values of the *C. argentea* flowers was 0.03 (values for single plants lay between 0.0 and 0.04). Dafni (1992) gives the following classification of ISI values: ISI > 1, self-compatible; ISI = 0.2–1, partially self-incompatible; ISI = 0–0.2, mostly self-incompatible; ISI = 0, completely self-incompatible. Based on these standards, the gametes of *C. argentea* flowers can be classified as 'mostly self-incompatible'.

Seed:ovule ratio

Figure 2 shows the results for insect pollination and artificial cross-pollination. On average, the collected pods contained 9.4 ovules, and the number of seeds per ovule ranged from 0.63 to 0.78. Insect pollination resulted in a lower seed:ovule ratio, *i.e.*, a lower number of seeds within pods, than artificial crossing (P<0.05).

Pollinating insects

All insects which tripped flowers, *i.e.*, those which entered the flower from the front and pressed down the keel, belonged to the order of Hymenoptera. *Xylocopa frontalis* (Anthophoridae) and 2 *Centris* species (Anthophoridae) were the most frequent species. Other insects, such as 2 *Megachile* species (Megachilidae; the individual species could not be identified), *Apis mellifera* (Apidae; only seen once), and 1 species from the family Vespidae, occasionally visited the flowers. These species were smaller and may have had difficulties working the pollination mechanism when accessing the nectaries in the interior of flowers.

Discussion

This study has provided valuable information on the floral biology and mating system of *C. argentea*, which will be of great benefit in maintaining the purity of germplasm and in breeding
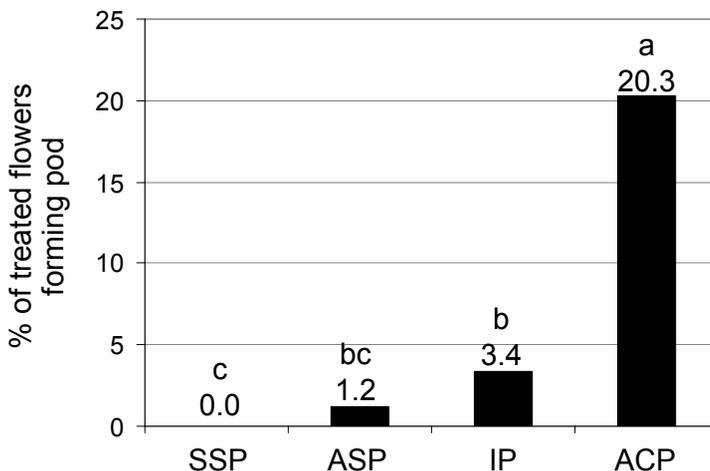

Figure 1. Percentage of flowers of *C. argentea* forming pods after 4 pollination treatments. Bars with a common letter do not differ significantly at P=0.05. SSP: spontaneous self-pollination; ASP: artificial self-pollination; IP: insect pollination; ACP: artificial cross-pollination.

programs. Since this species is largely self-incompatible and relies on insects for cross-pollination, to maintain purity of accessions it is important to realise that the pollinating insect species found for *C. argentea* fly distances in excess of several hundred metres (Gonzalez and Engel 2004; Ricketts 2004). Thus, plot distances currently used for seed increase of collections or in plant nurseries should be re-considered – even if, as in the case of the CIAT Genetic Resources Unit, plot distances between accessions are as large as 300 m. Accessions should be completely isolated for purity maintenance. Pooling of some genetically very similar accessions, as proposed by Andersson *et al*. (2007), seems to be justified.

The finding that pollen grains are viable at the moment of anther dehiscence and remain so during the day of anthesis, while the stigma remains unreceptive until tripping, leads to the conclusion that insect pollination is favoured (Dafni 1992; Kull 2000). As anthers mature before the stigma, self-pollination is prevented and cross-pollination by visiting insects is fostered. From the very beginning, visiting insects encounter mature pollen grains, which they transport to other flowers. The stigma becomes receptive only when it is ensured that foreign pollen has reached it, *i.e.*, when its surface cuticle is ruptured following tripping by a visiting insect. In contrast, studies with other *Cratylia* spp. (*C. hypargyrea* and *C. mollis*) showed that stigmas were receptive from the beginning of anthesis, *i.e.*, without tripping (Queiroz 1991; 1996).

It was surprising that pod set following insect pollination was also relatively unsuccessful in this study, with levels similar to those in self-pollinated treatments. The question arises why plants, which were cross-pollinated artificially, produced so many more pods than those pollinated by insects. Bawa and Buckley (1989) concluded that, with the mixture of a plant's own and foreign pollen, which is carried and deposited by pollinating insects, the success rate is lower than with pure foreign pollen. A common feature of pollination with pollen from other flowers of the same plant in self-incompatible species is that the plant's own pollen grains occupy space on the stigma surface and prevent access from foreign pollen tubes to fertilise ovules (Snow *et al*. 1996). The low success of insect pollination and the low ISI values obtained, in addition to the even lower pod production after self-pollination treatments, are evidence for *C. argentea* flowers being largely self-incompatible.

Interestingly, in a similar study with *C. argentea* conducted at the Planaltina Research Station near Brasília, Brazil, Queiroz *et al*. (1997) obtained a higher level of self-compatibility than we did: 7.2% pod set after artificial self-pollination, while spontaneous self-pollination produced pod set not significantly different from zero. Pod set after artificial cross-pollina-
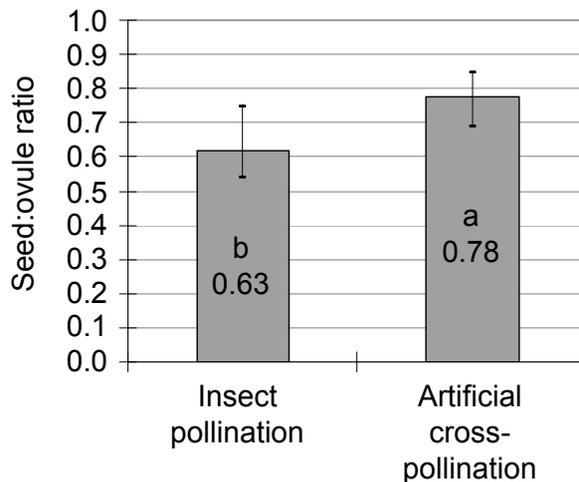

Figure 2. Seed:ovule ratio in pods of *C. argentea* formed after 2 pollination treatments. Bars indicate a 95% confidence interval. Different letters indicate a significant difference at P=0.05.

tion occurred in 14.9% of flowers (compared with 20.3% in the present study). In a treatment similar to our insect-pollination treatment, those authors measured 8.1% pod set compared with 3.4% in the present study. They did not mention, however, whether this figure differed significantly from artificial selfing or crossing. Overall, in their study self-pollination led to higher, and cross-pollination to lower, success than in our work, although cross-pollination was more successful than self-pollination in both studies. Queiroz *et al.* (1997), however, did not state the number of manipulated flowers, nor did they give any information about the genetic material used or site conditions. Thus, we can only speculate why our results and theirs differ. A contributing factor could be different environmental conditions in central Brazil and south-west Colombia. According to Faegri and van der Pijl (1971), self-incompatibility can decline under certain circumstances, such as extreme environmental conditions. Other reasons may be differences between the genetic material or methodology, or different plant ages: according to Frankel and Galun (1977), in some species, self-incompatibility can be significantly reduced at the end of the growing season or in very mature plants.

While pod formation in artificially cross-pollinated and insect-pollinated plants differed greatly (20.3 *vs* 3.4%), the differences in seed:ovule ratios were much smaller (0.78 *vs* 0.63), though also significant ($P<0.05$). According to Bawa and Webb (1984) and Bawa and Buckley (1989), cross-pollinated, self-incompatible Leguminosae often have high flower, pod and seed abortion than self-compatible species. This means that such species have low ratios of fruits:flowers on the whole plant as well as low ratios of seeds:ovules in individual pods. In comparison with results obtained by Bawa and Buckley (1989) and Snow *et al.* (1996) with other species, our seed:ovule ratio for 'natural pollination' (*i.e.*, insect pollination) of *C. argentea* was within the range of other insect- or cross-pollinated species. Similar to pod formation, seed formation within pods seems to be diminished by the mixture of own and foreign pollen carried by insects compared with pod formation with pure foreign pollen.

Conclusions

This study has shown that pollination in *C. argentea* is insect-dependent. This can be concluded from flower development and results of a pollination experiment. We conclude the species is mostly self-incompatible, as without pollen from other plants, successful pollination in *C. argentea* was extremely limited.

The simplicity of the research procedure used in this study makes it suitable for use with other tropical legume species with a largely unknown mating system and in which large insects are involved in pollination.

Acknowledgements

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