Flora 208 (2013) 412–419

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Pollination and self-interference in *Nothofagus*

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ARTICLE INFO

Article history:
Received 16 January 2013
Accepted 1 June 2013
Available online 27 July 2013

Keywords:
Nothofagus
Phenology
Pollen release
Stigma receptivity
Self-interference
Anemophily

ABSTRACT

Interference between male and female functions within a monoecious plant may hinder crossing and decrease seed set. We assessed the probability of self-pollination and the effect of self-pollination on cross-pollination for two self-incompatible species: *Nothofagus obliqua* and *N. nervosa*. The probability of self-pollination was studied by tracking the phenologies of staminate and pistillate flowers, including an analysis of stigmatic receptivity. Pure and mixed pollinations were performed in order to evaluate the effect of self-pollination upon cross-pollination. Phenological observations suggest that self-pollination is highly likely in both species. Compared to pure cross-pollination, the application of self-pollination prior to cross-pollination resulted in lower numbers of germinated pollen grains for both species, and also in a lower production of viable seeds in *N. nervosa*. The low proportion of viable seeds often observed in natural populations of *N. obliqua* and *N. nervosa* may be related to self-pollination.

Introduction

In monoecious plants (with both sexes in each individual), self-pollination within the same individual may interfere with the crossing with conspecific individuals (Barrett, 2002). Since most monoecious species are self-incompatible (Allen and Hiscock, 2008), self-pollination may impose physical or chemical barriers to cross-pollination hindering the production of viable seeds (Barrett, 2002; Cesaro et al., 2004; Dai and Galloway, 2011). It has been argued that the intra-individual interference between male and female functions (self-interference) is an important selective force influencing plant breeding. Both the spatial and temporal intra-individual separations of male and female functions have been interpreted to evolve as mechanisms that tend to reduce self-interference (Barrett, 2002). Accurate information about the phenologies of staminate and pistillate flowers and the mechanism of self-incompatibility are necessary in order to assess the degree of self-interference in each species.

Knowledge about the degree of temporal overlapping between pollen release and pistillate flowers receptivity is essential in the study of self-interference. The ripening of staminate flowers is often evidenced by changes in the morphology and color of anthers, as well as by pollen release. Stigmatic receptivity is more difficult to identify since it is determined by secretions promoting pollen hydration and germination, and pollen-tube growth (Herrero and Hormaza, 1996; Sanzol et al., 2003; Waites and Ågren, 2006). In some species, the period of stigmatic receptivity is evidenced through morphological changes affecting the stigmas (Oddie and McComb, 1998; Tal, 2011; Yi et al., 2006). In many other species this period is not externally apparent, so that indirect techniques based on the detection of enzymatic activity related to receptivity are often applied (Dafni and Maués, 1998; Jones, 2002). A reliable way of evaluating stigmatic receptivity is the application of sequential hand-pollinations followed by the assessment of viable-seed production and/or pollen-grain germination on the stigmas (Oddie and McComb, 1998; Ofosu-Anim et al., 2006; Page et al., 2006; Sanzol et al., 2003). The description of flower phenology and the degree of temporal overlapping between male and female functions for a given species contribute to the reproduction of plants with commercial and/or conservation value (Tooke and Battey, 2010).

Nothofagus species are dominant in temperate forests of the Southern Hemisphere (e.g. Veblen et al., 1996). Their diaspores are one-seeded, indehiscent fruits (achenes). High quantitative and qualitative inter-annual variations in seed production are commonly observed in natural populations of these species (Donoso et al., 2006a,b; Marchelli and Gallo, 1999). The production of empty seeds was found to be high in many populations of *Nothofagus* species (Bahamonde et al., 2011; Marchelli and Gallo, 1999; Martínez-Pastur et al., 1994). Although the amount of empty seeds is often related to damage by insects (Carrillo and Cerda, 1987; Marchelli and Gallo, 1999), non-damaged empty seeds are common, which could be related to pollen limitation,

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http://dx.doi.org/10.1016/j.flora.2013.07.002

self-pollination and/or self-interference. *Nothofagus* species are anemophilous, so the small size of stigmas and the sessile nature of the pistillate flowers may limit pollen capture. Moreover, the development of both flower types on the same shoots (Puntieri et al., 2009; Torres et al., 2012) would promote the highly ineffective self-pollination (Riveros et al., 1995b). However, as the period of stigmatic receptivity has not been investigated, a possible overlapping between the periods of pollen release and stigmatic receptivity within an individual has not been confirmed. Moreover, the effect of self-pollination on viable seed production has not been tested experimentally in *Nothofagus* so far.

In the present work we selected two *Nothofagus* species in order to assess (A) the time of stigmatic receptivity through the application of sequential pollinations and the evaluation of pollen germination, (B) the intra-individual overlapping between the periods of pollen release and stigmatic receptivity, and (C) the degrees of self-incompatibility and self-interference. The selected species, *Nothofagus obliqua* (Mirb.) Oerst. and *Nothofagus nervosa* (Phil.) Krasser (=*N. alpina* (Poepp. et Endl.) Oerst.) are two of the economically most valued *Nothofagus* species due to their fast growth and high wood quality (Donoso et al., 2006a,b; Tuley, 1980). Expanding the current knowledge about the reproductive biology of these species is necessary in order to understand their natural regeneration dynamics and support domestication programs.

Materials and methods

Flowering trees of *Nothofagus obliqua* and *N. nervosa* with accessible flower-bearing branches (at less than 5 m on the trunk) are not easy to find. Moreover, inter-annual variation in flower production within each tree complicates the selection of suitable trees. Due to these difficulties, this study was carried out in three stages on different trees. In such stages we addressed: (A) the period of stigmatic receptivity, (B) flowering phenology of reproductive shoots and (C) interference in cross-pollination and seed production due to self-pollination.

Stigma receptivity

The analysis of stigmatic receptivity was made on hydroponically-maintained flowering branches in order to simplify the periodic observation of flowers and their isolation in prevention of unwanted pollinations. Preliminary tests showed that shoots of *N. obliqua* placed in hydroponic culture present normal bud-break and flowering. In September 2009, in an area of 80 ha close to Lácar Lake (Lanín National Park, Argentina), four *N. obliqua* trees with flowering buds on accessible branches were identified. *Nothofagus nervosa* was not included since trees with high flower production could not be found in that year. Before budbreak, five 17–50 cm long flowering branches of each selected *N. obliqua* tree were cut and placed in water immediately afterwards. An additional *N. obliqua* tree located at San Carlos de Bariloche (brought from another natural population of this species in Argentina) was used as pollen donor. Branches were taken to a polycarbonate greenhouse and with external ventilation. Each branch was placed individually in 500 cm^3 bottles filled with running water. Temperatures in the greenhouse ranged between 8 and 25 °C during the experiment. Every 48 h, bottles were refilled with water and 1 cm of the proximal end of each branch stem was cut while keeping it under water. After bud-break, all shoots of these branches were emasculated. Staminate flowers from the tree used as pollen donor were cut short before pollen release and placed in open petri dishes at room temperature (15–20 °C). Once anthers opened, pollen was placed in small jars at 5 °C with silica-gel in order to maintain a fresh and dry environment, and avoid fungal growth and pollen clumping. Previous studies indicate that pollen grains of *N. obliqua* stored at 5 °C maintain their viability for up to three months (Báez et al., 2002).

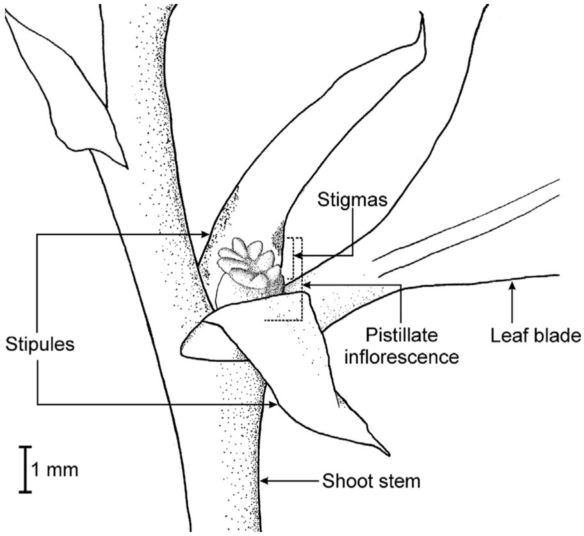

Fig. 1. Semi-schematic representation of a trimerous pistillate inflorescence of *Nothofagus obliqua* at anthesis.

Pollination was applied to the pistillate flowers of one branch per tree at one of the following times: at the time of anthesis (0 DAA), three days after anthesis (3 DAA), six days after anthesis (6 DAA), ten days after anthesis (10 DAA), and 15 days after anthesis (15 DAA). Pistillate flowers of *Nothofagus* species have a rudimentary perigonium, so that we considered that the anthesis of these flowers corresponds with the unfolding of the subtending leaf and their stipules, which makes the stigmas accessible to pollen (Fig. 1). Only the most proximal pistillate inflorescence in each shoot was pollinated since pistillate inflorescences on different nodes of a shoot do not reach anthesis simultaneously. Pollination was applied once for each treatment with a thin painting brush. In all, 278 pistillate flowers from 93 inflorescences were pollinated. Flowers were fixed in FAA 24 h after pollination and stored for 90 days. Fixed flowers were softened in 1.5 M NaOH for 24 h, then gently washed with distilled water, stained for 24 h in a 0.1% (w/v) solution of aniline blue in 0.1 M K_2POH_4, mounted on glycerine and squashed. For each flower, germinated grains were counted with a fluorescence microscope (Olympus BX51) equipped with a HBO 50 W lamp, and a filter for UV excitation (BP 330–385 nm, BA 420 nm; Fig. 2). The three flowers of each inflorescence were considered individually.

Phenology of flowering shoots

In order to describe the phenology of flowering shoots, *N. obliqua* and *N. nervosa* trees were selected at the “Unidad de Mejoramiento Ecológico, Genético y Forestal”, EEA INTA Bariloche, situated about 70 and 100 km south of the nearest natural population of *N. obliqua* and *N. nervosa* respectively. Based on bibliographic information, mean temperature and precipitations at the nursery are similar to those registered in natural populations of *N. nervosa* and *N. obliqua* at Lanín National Park (Conti, 1998). In spring 2011, three *N. obliqua* and two *N. nervosa* trees were chosen based on the presence of flowering buds. Because of the scarcity of trees with flowers in the studied season, three reproductive *N. nervosa* × *N. obliqua* hybrid trees were also included. For each tree, three shoots extended one year before sampling (2010–2011), hereafter referred to as *parent shoots*, were chosen among those with reproductive buds. Phenological observations were made on all annual shoots derived from these parent shoots. Altogether, 98, 64 and 77 annual shoots