Ghent University
Faculty of Sciences
Academic year 2015-2016

Far-reaching effects of root herbivory on pollinator-interactions and plant performance in *Cynoglossum officinale*

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Sciences

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Date of public defense: 25 January 2016

Ghyselen C. 2016. Far-reaching effects of root herbivory on pollinator-interactions and plant performance in *Cynoglossum officinale*. PhD thesis. Ghent University, Ghent (Belgium).

The research reported in this thesis was conducted at the Terrestrial Ecology Unit, Biology Department, Faculty of Sciences, Ghent University, Ghent.
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This research was funded by a grant of the Research Foundation – Flanders (FWO).

Drawings were made by Milena Sonneveld
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Dankwoord

Een wezel die mijn pad kruist, de donker roodpaarse bloemetjes van veldhondstong, hommels die op mijn schouder komen zitten op zoek naar warmte, zicht op zee achter een duinheuvel,... Ik heb veel natuurschoon gezien tijdens mijn doctoraatsonderzoek en ik ben mijn promotoren en de beheerders aan de Belgische kust zeer dankbaar dat ze mij de kans gaven op zo’n mooie plek veldwerk te mogen doen. Een doctoraat is natuurlijk meer dan alleen veldwerk, en ik ben vele mensen dankbaar voor hun hulp en steun hierbij.

Eerst en vooral wil ik mijn promotoren bedanken. Mijn promotor Dries Bonte wil ik bedanken voor de kansen en de vrijheid die hij me heeft gegeven. Om te kunnen onderzoeken wat mij fascineerde en om me mijn weg te helpen vinden in statistiek, het geven van presentaties, het schrijven van artikels...

Mijn co-promotor Rein Brys wil ik danken voor de vele nuttige en interessante besprekingen over de uitwerking van mijn onderzoeksonderwerp, de uitvoering van experimenten, het uitwerken van manuscripten en voor het steeds zorgvuldig nakijken en bijschaven van mijn manuscript-teksten. Zijn grote gedrevenheid en aanstekelijk enthousiasme voor plant-ecologisch onderzoek waren een welgekomen bron van inspiratie en motivatie.

Bea Bossuyt, Maurice Hoffmann en Eduardo de la Peña wekten tijdens de Bachelor- en Masterjaren in de opleiding biologie mijn interesse voor bestuivings- en plantenecologisch onderzoek. Hen wil ik bedanken voor die inspirererende momenten tijdens de lessen, excursies, terrestrische stage en mijn Master-proef.

Renate Wesselingh ontmoette ik in de eerste maanden van mijn doctoraatsonderzoek. Ik wil haar bedanken om haar ervaring en kennis over veldhondstong met mij te delen, me de planten die ik gebruikte voor het experiment in 2011 te schenken. Ook de introductie op het zeer leuke en interessante SCAPE-congres in Zweden in 2010 was aangenaam.

Klaas Vrieling, alsook de andere leden van het leescommité van mijn doctoraatsthesis wil ik danken voor hun vele interessante opmerkingen ter verbetering van mijn doctoraatsproefschrift.

Luc Lens wil ik bedanken voor het leiden van de zeer fijne onderzoeksgroep waarin ik heb mogen werken. De techniekers van de Terec: Hans, Viki, Angelica en Pieter, wil ik bedanken voor hun inzet en steun. In een onderzoeksgroep met een grote turnover zijn jullie de vaste waarden, de ruggengraat van de Terec. Een groot deel van het onderzoek van de Terec bouwt op jullie praktische, inhoudelijke en administratieve werk. Jullie betekenen heel veel voor de onderzoeksgroep en hebben ook veel betekend voor mij, bedankt daarvoor! Ook alle andere collega’s van de voorbije jaren op de Terec en de Limno wil ik bedanken. Het was een genoegen om met zo’n toffe, intelligente en natuurgezinde mensen collega’s te zijn: Martijn, Boris, Debbie, Bram D., Bram V.T., Steven, Annelies, Charlotte D.B., Valerie, Tom, Greet, Carl, Frederik, Liesbeth, Natalie, Lynda, Johan, Katrien, Tanja, Helena, Jeroen, Lies, Jasmijn, Karen, Daan, Bram S., Irene, Dries, Lander, Adriana, Laurence, Alejandro, Catia, Jacob, Dirk, Thijs, Leen, Els, Yoeri, Jorunn,... en iedereen die ik nog vergeten ben!

Een aantal instansties en onderzoeksgroepen wil ik bedanken voor het mogelijk maken van mijn veld-, labo- en statistisch werk: het Agentschap voor Natuur en Bos, het Vlaams Instituut voor de Zee (in het bijzonder Frank Broucke en Michiel Tjampens), het Instituut voor Natuur- en Bosonderzoek (in het bijzonder Sam Provoost en Wouter Van Landuyt), het ForNaLab, het Vanfleterenlab (in het bijzonder Sasha De Henau), de onderzoeksgroep Pteridologie (in het bijzonder Olivier Leroux en Guy Van der Kinderen) en het FIRE-initiatief van de Ugent (in het bijzonder Bart Jacobs).

Mijn veldwerk en praktisch werk werd af en toe een stuk aangenamer gemaakt door de hulp van Leen, Tomas, Lucien, Jelle en Charlotte V.M., bedankt voor jullie aangename gezelschap!

Mama en papa, vele dingen die jullie me mee gaven hebben me enorm geholpen bij het verwezelijken van deze thesis. Bedankt voor alles!

Verder wil ik ook mijn familie, vrienden en kennissen bedanken om de voorbije jaren een bron van steun, plezier en inspiratie te zijn voor mij: mijn oma’s en opa’s, mama en papa, Sophie, Matthias, Bernadette, Guido, Emmy, Michael, Joke, Johan, Katrien, Jacob, mijn nichtjes en neefjes, Isabelle, Laura, Elias, Milena, Emile, Eline, Charlotte, Matthias, Koen, Jens, Janne, Lieven, Sofie, Esther, Gorik, Leen, Tomas, Iris, Noah, Klaas, Karel, Petra, Floriane, Bendix,...

In het bijzonder wil ik Michael, Sophie en Milena bedanken voor hun grote hulp en tips bij de layout van dit boekje en van de uitnodiging voor mijn doctoraatsverdediging.

Dan mijn dochtertje Janne, jou wil ik bedanken om mij in die laatste maanden van thesis schrijven toch af en toe mee naar buiten te nemen en om me steeds instant relativering van mijn ‘kojojaats’-zorgen te bieden!

Tot slot bedank ik graag Willem Laermans. Als mijn steun en toeverlaat heb je dit de voorbije jaren met glans volbracht. Je was en bent mijn rots in de branding. Bedankt voor de enorme steun die ik aan jou heb!

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1. General introduction

Céline Ghyselen

PLANT-INSECT INTERACTIONS

It is widely known that the plant kingdom and the class of insects represent approximately half of all known species of multicellullar organisms. Green plants make up the most voluminous compartment of living matter, whereas insects lead in number of species (Schoonhoven et al., 2005). Insects and plants belong to different trophic levels that are tightly associated and have been evolving together for more than one hundred million years, with varying levels of interaction leading to the selection of characters that are studied today in every branch of biology, from biochemistry and genetics to behavior and ecology (Bernays in Schoonhoven et al., 2005).

Green plants serve as the primary source of energy-rich compounds for heterotrophic organisms, and are therefore indispensable for animal life, including that of insects (Schoonhoven et al., 2005). Insects, on the other hand, may exert one of the major forces on plants and are responsible in shaping the great diversity in the plant world (Ehrlich and Raven, 1964; Marquis, 2004). It is in the interactions between insects and plants that herbivory and pollination reach their highest degree of specialization. As Schoonhoven et al. (2005) put it: "Probably no other interactions between two groups of organisms comparable in type and extent can be found elsewhere in the living world, thus rendering insect-plant interactions a unique and scientifically very fruitful area of biological research."

The antagonism-mutualism spectrum

The interactions between insects and plants vary from antagonism to mutualism. Herbivory, in which insects consume and thereby harm plant tissues without any benefit for the plant, is obviously considered an antagonistic interaction. On the other side of this spectrum is pollination, an interaction in which insects also consume plant products, such as pollen and floral nectar, but in return transfer pollen among flowers. Because pollinators and flowering plants can barely survive without each other, this is an exemplary case of mutualism (Schoonhoven et al., 2005). In between these two extremes of the spectrum, there exists a plethora of intermediate interaction types. Seed predators, for instance, may whilst gathering seeds, also disperse them (Brew et al., 1989). Another example are nectar robbers which sometimes visit flowers via the 'legitimate' entrance but most of the time pierce the floral corolla to 'steal' the floral nectar without touching the flower's reproductive structures (Irwin et al., 2010). Even in interactions between the same two interacting species, the ecological outcome can vary from mutualism to antagonism depending on the community context. For instance, the moth *Greya politella* is a highly host-specific pollinating floral parasite of *Lithophragma parviflorum*. During oviposition in the ovary, female moths also pollinate the flowers and after hatching, the larvae feed on a small subset of the developing seeds. This mutualism can be shifted to commensalism or even antagonism depending on the presence of effective co-pollinator species in the community (Thompson and Fernandez, 2006).

Above- and belowground multitrophic interactions

Recent scientific data suggest that the total biomass of life below-ground is much more extensive than all that is observed above-ground. Plant roots and insects form a substantial element of this unseen world and intimate interactions between insects and plants are likely to mirror the aboveground relationships (Schoonhoven et al., 2005). Some root feeders consume the smaller rootlets and live in the soil, whereas others bore directly into the taproot. Certain cicadas and some aphid species even pierce the roots to imbibe their liquid food (Wolfe, 2001).

Up until now root herbivory has received less attention than aboveground insect feeding, because of difficulties in adequately measuring this. Nonetheless, there is increasing evidence that root herbivores can also strongly impact plant fitness (Blossey and Hunt-Joshi, 2003), since root damage may result in inadequate uptake of water, nutrients and minerals, and thereby reduce the growth of above-ground plant parts (Maron, 2001). As such, root-infesting insects may affect their above-ground counterparts (but also vice versa) via changes in their host's chemistry or physiology (Van der Putten et al., 2001). Consequently, through these systemic changes in plant characteristics, plant-insect interactions both above- and below-ground are cross-linked (Moran and Whitham, 1990; Bezemer et al., 2004). Because induced plant responses can also modify herbivore-predator interactions above- and below-ground, insect-plant interactions may affect multitrophic interactions more comprehensively than is usually considered to be the case (Van der Putten et al., 2001).

Geographical structure in plant-insect interactions

Plant-insect interactions, and by extension also the multitrophic interactions linked to them, are most likely characterized by a geographical structure (Thompson, 1999). This follows from the assumption that the geographical distributions of a focal plant species and its interacting species differ in accordance with the abiotic and/or biotic factors of importance for each species (Parmesan et al., 2005). This geographical variation in species distributions at different spatial scales might imply that the same plant species interacts with certain species at one locality, while the composition of interacting species differs markedly at another locality. Insect pollinator communities, for instance, often vary geographically (Moeller, 2006; Gomez et al., 2009). This geographical structure in plant-insect interactions can have important implications. As already mentioned above, geographical differences in pollinator communities might cause the intimate plant-insect interaction between the moth *Greya politella* and its host plant *Lithophragma parviflorum* to shift from mutualism to antagonism (Thompson and Fernandez, 2006). Another implication is the existence of geographical structuring in the total selection pressure exerted on plant populations, in which each population experiences a specific total selection pressure resulting from the differences in composition, abundance and diversity of the interacting community present in that plant population (Gomez et al., 2009).

IMPACT OF HERBIVORY ON PLANT FITNESS

Because herbivores consume plant tissues, it is evident that they affect plants. At early life stages insect herbivory can result in plant death, but mature plants are seldom killed (Schoonhoven et al., 2005). Nonetheless, insect herbivory can have strong effects on plant characteristics without killing them (Crawley, 1997). Apart from the direct effects resulting from damage or removal of plant tissues like leaves, stems, flowers or fruits (Adler et al., 2001; Cardel and Koptur, 2010), a lot of indirect effects of herbivory have also been documented. Feeding may namely result in changes in shoot growth (Andersen, 1987; Nötzold et al., 1998; Poveda et al., 2003; Hunt-Joshi et al., 2004), flower size (Mothershead and Marquis, 2000; Ivey and Carr, 2005), floral display (Samocha and Sternberg, 2010), flowering phenology (Nötzold et al., 1998; Poveda et al., 2003), production and composition of nectar (Fang et al., 2006; Samocha and Sternberg, 2010), plant volatile emission (Kessler et al., 2011, Lucas-Barbosa et al., 2011), and extrafloral nectar production (Heil, 2004), but also in infection with viruses and other diseases transferred by herbivores (Schoonhoven et al., 2005). Because herbivory can affect all these vegetative and floral plant characters, it can be easily assumed that it may also influence foraging behavior and visitation rates by pollinators. However, apart from the potential pollinator-mediated effects of herbivory on plant reproduction (which will be the subject of a subsequent section in this chapter), herbivory can also have more direct effects on plant reproduction. Typically, the impacts of herbivory on plant reproduction have been assessed by measuring fruit and seed production (Prins et al., 1992; Adler et al., 2001; Hunt-Joshi et al., 2004), but herbivory can also affect male reproductive success, either directly by feeding on pollen or male reproductive organs (Rodriguez-Rodriguez and Valido, 2011), or indirectly by inducing reduced flower or pollen production (Quesada et al., 1995; Samocha and Sternberg, 2010), or lower pollen quality that is, for instance, expressed in reduced pollen tube growth rate (Mutikainen and Delph, 1996). However, only few studies exist on this subject (but see Quesada et al., 1995; Mutikainen and Delph, 1996).

IMPACT OF POLLINATION ON PLANT FITNESS

In most plants, successful pollination is the primary step in sexual seed formation. Most of the angiosperm species rely on insect pollination for pollen dispersal and pollen receipt (Wilcock & Neiland, 2002), and their foraging activity and behavior can have an important role in this process. For instance, as a result of lower pollinator visitation rate the quantity of pollen deposited on the stigma and the diversity of pollen donors may be reduced. This decreases the probability of sampling a genetically compatible sporophytic father (Paschke et al., 2002), and the potential for pollen tube competition in the pistil. Pollen tube competition can promote fertilization by genetically superior fathers, either through direct competition between the growing pollen tubes or through female choice by which growth of pollen tubes is regulated (Skogsmyr and Lankinen, 2002). The intensity of pollen competition is often positively related to seed or fruit maturation (Niesenbaum and Casper, 1994) and to seed quality (Skogsmyr and Lankinen, 2002).

Also the quality of the pollen load can be reduced if pollinators visit several flowers on the same plant, thereby delivering self pollen to the stigmas (=geitonogamous pollination; Ashman et al., 2004). The latter may cause self-incompatibility reactions at the stigma surface, in the style or even in the ovary (Herrero and Hormaza, 1996; Sage et al., 2006), or if fertilization takes place, it may result in seed abortion or seeds of poorer quality when there is inbreeding depression (Owen et al., 2007). When an inadequate amount of pollen or poor quality pollen is deposited on the stigma, seed quantity and quality may thus be reduced and female plant fitness may decrease (Ashman et al., 2004).

For male plant fitness the foraging activity and behavior of pollinators has also important consequences. If a plant receives more pollinator visits, more pollen will be exported and the proportion of seeds sired by this plant will be higher (Stanton et al., 1986). If, however, more flowers are visited in a sequence on the same plant, more pollen will be ‘lost’ before pollinators leave the plants and then pollen export is reduced (de Jong et al., 1993).

IMPACT OF POLLINATION ON OFFSPRING FITNESS

Pollinator foraging behavior can also have an impact on the fitness of the resulting offspring. As a consequence of lower pollinator visitation rate, the diversity of pollen donors and the intensity of pollen tube competition may be reduced as explained above and this may negatively affect offspring quality, because the chance of having a genetically ‘very good’ sporophytic father is then smaller (Skogsmyr and Lankinen, 2002). As a consequence of geitonogamous pollination in self-compatible plants, a certain percentage of the offspring will result from self-fertilization if seed abortion due to early-acting inbreeding depression has not taken place. These selfed offspring may show reduced vitality and a lower capacity for adaptation when there is inbreeding depression (Keller and Waller, 2002).

HERBIVORY EFFECTS ON POLLINATION

Since the publication of Strauss’s paper (1997) highlighting the indirect way in which plant fitness can be affected by herbivory through its effect on pollinators, the number of studies on herbivore-pollinator interactions increased exponentially. Herbivores can cause changes in plant characteristics, as documented above, and these may in turn cause shifts in pollinator foraging behavior. The foraging behavior of pollinators is mostly measured in terms of duration of visits, number of visits and number of flowers visited in a sequence, and herbivory can influence these variables both in a positive (Poveda et al., 2003) or in a negative way (Lehtilä and Strauss, 1997). In these ways, herbivory can ultimately affect pollinator mediated pollen deposition (Mothershead and Marquis, 2000).

Aboveground herbivory

Insects may consume every anatomical part of plants, though most studies that assessed the impact of herbivory on pollinator foraging behavior to date focused on effects of leaf herbivory (e.g. Lehtilä and Strauss, 1997; Strauss, 1997; Suarez et al., 2009; Hladun and Adler, 2009; Kessler et al., 2011). Although it is very likely that impacts of other types of herbivory on pollinator foraging behavior exist, these are a bit neglected in literature. Stem borers, for instance, can reduce stem height, biomass and percentage of flowering stems (Haefliger et al., 2006). Sucking herbivore species, like spittlebugs and mirid bugs, may reduce flower size (Ivey and Carr, 2005), or flower and nectar production (Samocha and Sternberg, 2010). Flower consumers may, on the other hand, directly reduce floral display (Mahoro, 2002) and nectar robbers may directly reduce the nectar content in flowers without pollinating these (Irwin et al., 2010). Some insects feeding on plant sap produce honeydew, which can attract ants that 'patrol' the total plant and also visit its flowers. This has been found to reduce the duration of pollinator visits and disrupt pollination services (LeVan and Holway, 2015).

Belowground herbivory

Belowground herbivory and its resulting impact on aboveground biotic interactions, such as plant-pollinator interactions, are rarely studied (De Deyn and Van der Putten, 2005; Barber and Soper Gorden, 2014). Among the insects, there are, however, many representatives within several orders that rely on belowground plant parts for food: Coleoptera, Diptera, Homoptera, Lepidoptera, Neuroptera, Heteroptera and Orthoptera. In most cases, only immature stages of these insects feed on belowground plant parts. Typically the last instar or adult emerges from the soil and the adult then either does not feed or, if herbivorous, relies on aboveground plant tissues. The root damage inflicted by belowground insect herbivores may cause malfunctioning of some basic root functions such as mineral and water absorption and conduction, synthesis of products necessary for normal growth and development of the shoot, and/or storage and reallocation of metabolites and photosynthates (Andersen, 1987). It can thus be expected that belowground herbivory can indirectly affect the attractiveness of a plant to pollinators. Surprisingly, three out of the four published studies that have measured pollinator behavior in relation to root herbivory to date, documented enhanced pollinator attraction in response to root herbivory. The mechanism for this increased attraction is unknown, as none of these studies found changes in flower number, size or nectar production as a result of root herbivory (Poveda et al., 2003, 2005; Barber et al., 2011). In another study no change in pollinator preference could be observed in response to enhanced root herbivory (Hladun and Adler, 2009).

Artificial versus real herbivory

Studies investigating herbivory effects on pollination can roughly be divided into two classes: the ones that study effects of artificial herbivory, mostly by clipping leaves mimicking leaf herbivory (Suarez et al., 2009; Parra-Tabla and Herrera, 2010; Brody and Irwin, 2012;

Munguia-Rosas, 2015), and the ones that study effects of real herbivores. Artificial herbivory has the benefits of being practically straightforward and well measurable. On the other hand, the amount of damage under such artificial herbivory can easily exceed natural levels of damage (Parra-Tabla and Herrera, 2010). It is also questionable whether the manner of harming plant tissues is comparable to that of real herbivores, the biggest difference being that the saliva of herbivores can activate defence responses and can also induce the plant to attract natural enemies of the herbivores (Paudel and Bede, 2015). Studies that used real herbivores also not always reflect natural situations: sometimes a generalist herbivore is selected that is not specifically related to the focal plant species in natural circumstances (Cozzolino et al., 2015). However, studies investigating herbivore and plant species that do interact with each other in natural situations are also well represented (e.g. Hladun and Adler, 2009; Lay et al., 2011; Russell-Mercier and Sargent 2015).

Geographical structure in herbivore-pollinator interactions

Like plant-insect interactions, herbivore-pollinator interactions can also vary substantially geographically, although the number of papers that focused on this topic is rather small. Gomez et al. (2009), for instance, studied small-scale geographical differences in pollinator communities and in herbivory, and quantified the effects on selection pressure exerted on *Erysimum mediohyspanicum* plant populations. Kalske et al. (2012), on the other hand, investigated local adaptation to generalist pollinators and specialist herbivores in four populations of

Vincetoxicum hirundinaria in a naturally fragmented landscape. However, these two studies do not evaluate the interaction between herbivores and pollinators. I know of only two studies that do investigate this interaction in a geographical context. The first study, of Lay et al. (2011), reported on the study system of *Erysimum capitatum* and the diverse groups of pollinators and herbivores visiting this plant, and documented differences in herbivore-pollinator interactions between populations and over time. The second study, of Parra-Tabla and Herrera (2010), reported spatial variation in the relationships between defoliation, floral traits and male and female pollination success.

Consequences of herbivory effects on pollinator foraging behavior

Herbivory may affect pollinator foraging behavior, and this can have far-reaching consequences on plant fitness. However, this effect on pollinator foraging behavior is not always translated into effects on seed production because this depends on whether seed production is pollen-limited or not. Pollen limitation of seed production is traditionally assessed using supplemental hand pollination experiments (Ashman et al., 2004). This method has been used frequently to assess effects of herbivory on pollination success. In some studies pollen limitation and thus a pollinator-mediated effect of herbivory on female fitness was demonstrated (Mothershead and Marquis, 2000), whereas others did not find any sign of pollen-limited female reproduction (Hladun and Adler, 2009; Barber et al., 2012). Although the link between herbivory effects on pollination and seed or fruit production is already fairly often made, the intermediate steps in the process from pollen deposition to seed maturation are seldom studied in detail. For instance, up until now only few researchers have studied pollen tube growth (Parra-Tabla and Herrera, 2010) or selective fruit abortion (Niesenbaum, 1996) in relation to herbivore-pollinator interactions. Also the consequences of herbivory on pollinator visitation rate or the amount of geitonogamous pollination for male reproductive fitness and/or offspring fitness remain largely unexplored.

STUDY SYSTEM

To address some of the issues raised above, I chose the plant species *Cynoglossum officinale*, its pollinators and its specialist root weevil *Mogulones cruciger* as study system. This study system is well suited to study herbivory effects on pollination for a number of reasons. First, Prins et al. (1992) documented a reduction of 30% in reproductive effort when the roots of these plants are infested by *M. cruciger* larvae. The authors of this study hypothesized that during feeding on the root tissue, larvae consume the resources that otherwise are used for seed production, or damage the transport system within the plant, which may also negatively affect reproductive output. The larval root herbivory occurs mostly just before and during flowering of *C. officinale*, hence it can be expected that this type of herbivory also affects plant and flowering traits and consequently pollinator attraction. Pollinators, on the other hand, are of great importance for *C. officinale* plant fitness, both for pollen export and pollen receipt, because without pollinator visitation only very few seeds develop (Klinkhamer and de Jong 1987; de Jong et al., 1990).

Cynoglossum officinale

Cynoglossum officinale is a herbaceous monocarpic perennial that occurs in sand dunes and in ruderal habitats, such as rangelands (de Jong et al., 1990). It grows on both calcium-rich and weakly acid soil and is a typical drought and nitrogen indicator (Ellenberg, 1974). The species is widely distributed throughout Europe, except the extreme north and south. In North America it is believed to have been introduced by colonists in the 19th century, where it is now an invasive pest species (de Jong et al., 1990; Schwarzlaender, 1997). *C. officinale* grows at a range of densities, from isolated plants up to dense patches of several hundreds of individuals, and seedlings are usually strongly clustered around the parent plant (de Jong et al., 1990). In the first year seedlings develop a taproot and a basal rosette of leaves. First, there is vegetative growth for two or more years during which photosynthates are stored in the taproot. If plants reach a certain threshold size before vernalization in winter, they will flower in the next spring (de Jong et al., 1986). Early in spring, the flowering stalk then starts to elongate from the rosette. On the flowering stalk partial inflorescences (cymes) develop from the axils of alternate stem leaves, each with up to 35 but often no more than 10 flowers. Flowering occurs from mid May to the end of June and starts at the top of the plant. Usually two to three flowers are simultaneously open on each cyme. The corolla has five lobes which are fused at the lower part of the cylindrical tube. Stamens are inserted in the upper part of the tube just beneath the five hairy scales at the throat. A nectar gland is at the base of the ovary (de Jong et al., 1990). Flowers are reported to be protogynous (Kerner, 1895 in Lloyd, 1986), however, de Jong et al. (1990) contest this and pose that the flowers are homogamous. Each flower has four ovules and may therefore give rise to four nutlets, though in the field on average only one nutlet per flower matures (Klinkhamer and de Jong, 1987). *C. officinale* is a self-compatible species with a very limited capacity to reproduce autonomously and it therefore greatly depends on animal pollination for successful seed production (de Jong et al., 1990; Vrieling et al., 1999). In natural populations, pollination does not limit seed set (de Jong and Klinkhamer, 1989). Self pollination produces viable seeds (Vrieling, 1999), however, whether selfing leads to inbreeding depression in offspring vitality is not well studied.

Pollinators

The pollinators of *C. officinale* are bees and bumble bees. Several species have been reported: *Bombus hortorum, B. hypnorum, B. lapidarius, B. pascuorum, B. pratorum, B. ruderatus, B. terrestris* and *Apis mellifera* (Manning, 1956; Klinkhamer et al., 1989). These visitor species gather nectar and/or pollen from *C. officinale* flowers (Koster, 2007). During nectar feeding the flower visitor pushes its tongue through the opening left by the scales of the corolla throat, and at the same time it can deposit pollen on the stigma (de Jong et al., 1990).

Pollinators mostly visit multiple flowers in sequence on this plant (Klinkhamer et al., 1989).

Mogulones cruciger

Mogulones cruciger is an important specialist herbivore of *C. officinale* in Europe and in North America it is used as a biological control agent (Prins et al., 1992; Schwarzlaender, 1997). It can complete full development on species within closely-related genera in the Boraginaceae, but it prefers *C. officinale* as a host (De Clerck-Floate and Schwarzlaender, 2002). From spring on adult weevils emerge from their cocoons in the soil and feed on leaves of *C. officinale*. Three to ten weeks after emergence females start laying eggs in the base of petioles or in the shoot base (Schwarzlaender, 1997). Large plants are preferred for oviposition, probably because the larvae are then assured sufficient food to complete their development (Prins et al., 1992). Most of the ovipositions occur in early spring, but a smaller share occurs in late spring or in early autumn. The resulting larvae from oviposition in autumn are not able to complete their development before winter, but they hibernate in the roots and completed development in early spring when temperatures increased. The seasonal fecundity per female is on average 181 eggs. Under greenhouse conditions, eggs develop over a period of 15 days, after which they hatch. The larvae, which pass through three instar stages, migrate through the root crown into the taproot where they feed on root tissue. The larvae generally feed for 31 days in the root and then they move to the soil for pupation. During the larval stage, especially the second and third instars (from day 9 to 31 of larval development) are most damaging to the taproot of the host plant (Schwarzlaender, 1997).

Fig. 1.1. Illustrations of the study system. *Cynoglossum officinale* (first two rows): individual plant in its natural context, detail of a flower, *Bombus pratorum* and *Osmia bicornis* (respectively) visiting a flower, the fruits with one aborted seed in the lower fruit. *Mogulones cruciger* (third row): adult root weevil, its larva feeding in a taproot, its larva ex situ.

OBJECTIVES AND OUTLINE OF THE THESIS

The general aim of this PhD is to investigate the interaction between root herbivory by a specialist root weevil (*Mogulones cruciger*) and the process of pollination, and ultimately reproductive output in the monocarpic *Cynoglossum officinale*. Based on previous findings in which root herbivory by *M. cruciger* was found to reduce the reproductive effort of *C. officinale* plants (Prins et al., 1992), I wanted to investigate whether root herbivory also affected the pollinator visiting behavior, and reproductive investment and output. To do so, I combined an experimental set-up with observations in a natural field situation to be able to compare and explore what is exactly happening in this herbivore-plant-pollinator interaction system in nature. In the field, I investigated several *C. officinale* populations at the Belgian coast and explored geographical patterns of the interactions with its specialist root weevil and with its pollinators. Herewith I could shed light on an additional research question, namely, whether there is geographical structure in the plant-insect interactions studied. Finally, I also wanted to examine further reaching effects of the impact of herbivory on the process of pollination. Therefore, two other additional research questions handled the effects of herbivory on the intermediate steps between pollen deposition and seed maturation, and possible consequences of this herbivore-pollinator interaction on offspring fitness.

In chapter two I describe geographical variation in the interactions between *C. officinale* and *M. cruciger* on the one hand, and between *C. officinale* and its pollinators on the other, in natural populations at the Belgian coast. I also report the results of an observational study in the field in which I investigated the interaction between root herbivory and pollination and the effect of root herbivory on plant reproduction. I hypothesize that there is potential for geographical structuring in the total selection pressure exerted on plant populations of *C. officinale* at the Belgian coast if 1) the interacting species have specific preferences for certain plant phenotypes and significantly impact plant fitness, and 2) the geographical distributions of these species differ. In that case, the intensity and/or direction of the total selection pressure exerted by the interacting species are expected to vary between *C. officinale* populations.

Chapter three describes the consequences of root herbivory for plant-pollinator interactions and reproductive output under experimental conditions. Several plant traits that are possibly related to pollinator attractiveness were measured. I also surveyed pollinator visitation frequency and the amount of geitonogamous pollination of the experimentally treated plants in the field. To unravel the link between possible herbivory effects on pollination and seed production, I quantified pollen deposition and pollen tube growth, and performed supplemental hand pollinations to assess whether seed set was pollen-limited. With these experiments, I tested the hypothesis that there exists a pollinator-mediated effect of root herbivory on reproductive output in *C. officinale*.

In chapter four I focus on the different steps that occur during the process from pollen deposition to seed maturation. I hypothesized that these different stages may be influenced by herbivory via its effect on geitonogamous pollination and thereby the proportion of self pollen delivered to the stigma, and/or via its effect on the nutritional capacity of the maternal plant. To test this, I subjected plants to experimental root herbivory and performed hand pollinations (outcross, self and open pollination) on separate cymes of each plant. Afterwards, I assessed pollen germination, pollen tube attrition, seed abortion and seed maturation. This study allows us to investigate in more detail what the consequences are of herbivory effects on pollinator foraging behavior.

The fifth chapter also continues on our findings that there is less geitonogamous pollination in plants that are infested by *M. cruciger* root herbivory. Here, I was interested in possible fitness costs of geitonogamous pollination in *C. officinale* on several life stages from maternal seed set to offspring seed set. In offspring derived from self pollination versus outcross pollination I assessed maternal seed set and weight, germination, seedling growth and size, survival, flowering behavior and offspring seed set.

In the general discussion I first give an overview of the main findings of this PhD research. Then I compare the impact of root herbivory on female reproductive fitness in the observational field study versus the experimental study. The effects of root herbivory on both quantitative and qualitative pollen limitation of seed production are also discussed. Further, I deal with some effects of root herbivory on male reproductive fitness which can be deduced from the results. Finally, I discuss the implications of *M. cruciger* root herbivory for evolution of plant traits in *C. officinale* before I suggest some perspectives for future research.

“Als de snuitkever over de tongen gaat, boer pas op je bestuivers”

Willem Laermans

2. Is there potential for geographical structuring in the total selection pressure exerted on plants in the Belgian herbivore-plant-pollinator system of *Cynoglossum officinale* and its specialist root herbivore *Mogulones cruciger*?

Céline Ghyselen, Rein Brys and Dries Bonte

ABSTRACT

Both mutualists and antagonists can exert specific selection pressures on plants. The geographical distribution of a focal plant species and its interacting species may, however, differ and this may result in geographical variability in intensity and direction of the total selection pressure exerted on plants. In this chapter we investigate the potential for this geographical structuring in *Cynoglossum officinale* populations interacting with the root weevil *Mogulones cruciger* and with pollinating bees and bumble bees at the Belgian coast.

We assessed the relative abundance of root herbivory by *M. cruciger* larvae in *C. officinale* plants in five local populations during three consecutive years. We also investigated preference of ovipositing root weevils and the impact of root herbivory on plant fitness to investigate whether *M. cruciger* exerts selection pressure in these populations. Finally, we tested whether the interaction between *C. officinale* and *M. cruciger* influences pollinator visitation rates and/or the impact of pollinators on plant fitness in the field.

The relative abundance of *M. cruciger* differed markedly between populations, but also between years. *Mogulones cruciger* preferred plants with a large root crown diameter and a large flowering stem height for oviposition, and had a negative impact on seed set. Root herbivory negatively influenced pollinator visitation, but seed set was not pollen-limited both in non-infested and in infested plants.

We conclude that the intensity of selection pressure exerted by *M. cruciger* at the Belgian coast most likely varies between *C. officinale* populations and in time. Further monitoring of flowering plant size and relative abundance of *M. cruciger* root herbivory in these populations on the long term is necessary to evaluate the existence of geographical structuring in total selection pressure exerted on plant populations at this scale.

INTRODUCTION

Flowering plants generally experience interactions both with mutualistic organisms, such as mycorrhizal fungi and pollinators (Jacquemyn et al., 2012; Gomez et al., 2009), and with antagonistic organisms, such as pathogens, herbivores, nectar robbers and seed predators (Schoonhoven et al., 2005; Maron, 1998; Irwin et al., 2010; Kolb et al., 2007), which can exert certain selection pressures on plant populations (Cariveau et al., 2004; Gomez et al., 2009). Directional selection pressure exerted by these organisms infers that they have a specific preference for certain plant phenotypes in the population and that the interaction has an impact on plant fitness.

The geographical distribution of a focal plant species and its interacting species may, however, both differ in accordance with the abiotic and/or biotic factors of importance (Parmesan et al., 2005). For instance, insect communities that interact with a certain plant species often strongly vary at broad as well as at local spatial scales (Moeller, 2006; Gomez et al., 2009), because insect species have restricted geographical ranges and because their patch occupancy differs in heterogenic landscapes (Maes and Bonte, 2006). Such spatial variation at different scales might imply that the same plant species interacts with certain species at one locality, while the abundance and occurrence of interacting species can differ markedly at another locality.

Spatial differences in communities that comprise antagonistic and/or mutualistic interactors, may lead to spatial mosaics of total selection pressures exerted on plant populations (Gomez et al., 2009). Selection pressures may strongly vary between populations, if, for instance, in one population plants are exposed to a strong selection pressure exerted by an antagonist, while in another population this antagonist is missing or is much less abundant. If multiple organisms are interacting with one plant species, plants may experience variable selection pressures at the same time. As such, one interacting species can influence the selection pressure exerted by another in several ways, firstly, due to the fact that one interacting species may affect the impact of another interacting species on plant fitness. In the study of Gomez et al. (2009), for instance, this occurred in a plant-pollinator-herbivore system, where the selection exerted by ungulates weakened or in some populations even canceled out the pollinator-mediated selection occurring on plant traits. Second, the species interacting with the plant can also directly or indirectly interact with each other, as is the case, for example, in ants that are attracted to plants by honeydew-producing aphids and that disturb pollinator visitation (LeVan and Holway, 2015). In this way there might exist a mosaic of plant populations, each experiencing a specific total selection pressure resulting from the differences in composition, abundance and diversity of the interacting community in that plant population (Thompson and Fernandez, 2006). Especially in habitats that are fragmented and consist of many small and isolated patches important differences in composition, abundance and diversity between communities are expected (Tscharntke et al., 2012).

In this study we investigated natural populations of the monocarpic *Cynoglossum officinale* at the Belgian coast. This species is exposed to an antagonistic and several mutualistic interactions with insect species that may vary in their distribution and occupancy at different spatial scales. The Belgian dunes consist of heterogeneous landscapes in which patch occupancy was shown to differ greatly among different arthropod species (Maes and Bonte, 2006). These dunes are also highly fragmented because of urbanization (Provoost and Bonte, 2004), resulting in habitat patches that strongly vary in size and isolation. The antagonistic species interacting with *C. officinale* that we studied here is the specialist root weevil *Mogulones cruciger.* . The mutualistic species of *C. officinale* in this study were its pollinators, namely several bumble bee species (*Bombus hortorum, B. hypnorum, B. lapidarius, B. pascuorum, B. pratorum, B. terrestris*) and the bee species *Apis mellifera* and *Osmia bicornis*. These species are generalists and are abundantly present all along the Belgian coast (waarnemingen.be). A pantrap census at four locations (Knokke, Zeebrugge, Bredene and Oostduinkerke) and pollinator observations at a fifth location (De Panne) indeed confirmed that those pollinators are abundantly present in the study populations (Ghyselen et al., unpublished results).

There are multiple indications from studies in other countries that these antagonistic and mutualistic interactors have specific preferences for certain plant phenotypes of *C. officinale*. From the root herbivore *M. cruciger* it is, for instance, known that the adult females prefer large bolting plants for oviposition in populations growing in The Netherlands, Hungary and Germany (Prins et al., 1992; Schwarzlaender, 1997). Root herbivory by *M. cruciger* larvae had a significant impact on plant fitness: it decreased reproductive output by 30% in The Netherlands (Prins et al., 1992). Pollinators, on the other hand, are documented to prefer plants with a large floral display size: both the number of approaches and the number of flowers visited after an approach increased with the number of open flowers in a Dutch study population (Klinkhamer et al., 1989) and in an experimental population with natural pollinators from the surrounding dune area at the Belgian coast (Ghyselen et al., 2016; chapter three). Pollinators affect plant fitness in *C. officinale* in that without any pollinator visitation very few seeds develop (Klinkhamer and de Jong 1987; de Jong et al., 1990), however, seed set was not limited by pollinator visitation in study populations in the Netherlands (de Jong and Klinkhamer, 1989), and in four field populations (Ghyselen et al., unpublished results) and an experimental population in Belgium (Ghyselen et al., 2016; chapter three). Whether the impact of root herbivory by *M. cruciger* influences visitation of pollinators and/or the effect of pollinators on plant fitness under natural circumstances is not studied yet (but see Ghyselen et al., 2016; chapter three, for an experimental study on this).

In this chapter we investigate the study system of *C. officinale* with both antagonistic and mutualistic interactors at the Belgian coast to assess whether there is potential for geographical structuring in total selection pressure exerted on plant populations in this area. A primary factor determining the existence of such geographical structuring is spatial variation in the strength and outcome of the interactions with organisms that act as selective pressures (Gomez et al., 2009). Since hardly anything is known about the specialist root herbivore *M. cruciger* in Belgian *C. officinale* populations, we mainly focus on this antagonistic interaction and also on its effects on plant-pollinator interactions. More specifically, we tested whether: 1) the occurrence and distribution of *M. cruciger* differs among *C. officinale* populations and between years, 2) *M. cruciger* exerts a selection pressure on *C. officinale* populations, and 3) the interaction with *M. cruciger* influences pollinator visitation and/or the impact of pollinators on plant fitness.

MATERIAL AND METHODS

Study system

Cynoglossum officinale is a herbaceous monocarpic perennial that occurs in sand dunes and ruderal habitats such as range lands. The species is widely distributed throughout Europe, except the extreme north and south (de Jong et al., 1990; Schwarzlaender, 1997). In their first year, plants develop a taproot and a basal rosette of leaves. First, there is vegetative growth for two or more years during which photosynthates are stored in the taproot. When plants reach a certain threshold size before vernalization in winter, they will flower in the next spring (de Jong et al., 1986). Early spring, the flowering stalk then starts to elongate from the rosette. On the flowering stalk partial inflorescences (cymes) develop from the axils of alternate stem leaves, each with up to 35 but often no more than 10 flowers. Flowering starts at the top of the plant and usually two to three flowers are simultaneously open on each cyme. Flowering occurs from mid May to the end of June (de Jong et al., 1990). Each flower has four ovules and may therefore give rise to four nutlets, though in the field only one nutlet per flower on average matures (Klinkhamer and de Jong, 1987). Nutlets are densely covered with small hooks which allow animal dispersal, however, half of the nutlets are dispersed within 0.6 m of the parent plant (de Jong et al., 1990; Rademaker and de Jong, 1999).

Pollinators are of great importance for the reproductive success of *C. officinale* (Klinkhamer and de Jong, 1987). Pollinator communities visiting *C. officinale* flowers consist of bees and bumble bees. These visitor species gather nectar and/or pollen to feed their larvae (or those of the colony) and their selves (de Jong et al., 1990, Koster, 2007). In Belgian sand dunes not many other species flower as early as *C. officinale* does, as a consequence, this plant species is an important food source for its visitor species (personal observation).

The root weevil *Mogulones cruciger* is an important specialist herbivore of *C. officinale* in Europe (Prins et al., 1992; Schwarzlaender, 1997). From spring on adult weevils emerge from their cocoons in the soil and feed on the leaves of *C. officinale*. Three to ten weeks after emergence females start laying eggs in the base of petioles or in the shoot base. Most of the ovipositions occur in early spring. *C. officinale* plants that have reached the threshold size to flower, already start bolting at that time and this is probably visible for ovipositing weevils (C. Ghyselen, personal observation). The larvae migrate through the root crown into the root where they feed on the root tissue for about three to four weeks before migrating into the soil to pupate (Schwarzlaender, 1997). Large plants are preferred for oviposition, probably because the larvae are then assured sufficient food to complete their development. During feeding the larvae consume the resources that otherwise can be used for seed production, or damage the transport system within the plant, which may also negatively affect reproductive output (Prins et al., 1992).

Sampled plants and study designs

During three consecutive years from 2010 to 2012, we performed a spatiotemporal survey on *C. officinale* plants of five populations at the Belgian coast: Knokke (N 51.36497, E 3.35032), Zeebrugge (N 51.32669, E 3.16910), Bredene (N 51.25369, E 2.97080), Oostduinkerke (N 51.13851, E 2.69207) and De Panne (N 51.07848, E 2.56575) (in 2010 the population in De Panne was not incorporated in the survey). For each population, an observation window was mapped out using ArcGIS® software (Esri, 2011), at each location more or less in accordance to the shape of a local population (or in

Knokke to the shape of the enclosure around the nature reserve), within which 60 sample plants were randomly selected each year per population. Of each sample plant, we mapped GPS coordinates and collected the roots after seed ripening. In the lab we measured root crown diameter and cut open the roots to assess the presence of root damage caused by *M. cruciger* larvae. Some of the roots were already in such a state of decomposition whereby it was impossible to assess presence of root damage caused by *M. cruciger* larvae, these were then categorized as 'NA' and were further discarded in the analyses.

In 2010 we followed the sample plants from rosette stage in April until seed ripening in August. From these plants we measured initial root crown diameter (measured before flowering stem elongation), flowering stem height, number of cymes, duration of flowering and floral display size (registered every four days). Due to unavoidable natural and human disturbance we lost a number of replicates in this year: 5 in Knokke, 4 in Zeebrugge, 21 in Bredene and 4 in Oostduinkerke.

Spatiotemporal survey of M. cruciger occurrence

To investigate the occurrence and distribution of *M. cruciger* at the Belgian coast the data on presence of root damage from 2010 to 2012 were used. We performed generalized linear model analyses to assess whether the occurrence of root damage caused by *M. cruciger* differed between local populations and/or years. The response variable 'presence of root damage' which was measured in each plant was binomially distributed. Population, year and their interaction were explanatory variables and root crown diameter (measured after seed ripening) was added as a cofactor in the model.

Correlations between plant traits and presence of root damage

To investigate the correlations between plant traits and occurrence of *M. cruciger* root herbivory we performed general/generalized linear mixed models on the data of 2010. First we assessed whether the probability for a plant to be infested by *M. cruciger* increased with root crown diameter. Then, we tested whether flowering stem height, number of cymes, duration of flowering (number of days) and mean floral display size were correlated with *M. cruciger* infestation by including each of these variables as explanatory variable in a model with initial root crown diameter as cofactor. The response variable for each model was presence of root damage and this followed a binary distribution. Location was incorporated as random effect in each model.

Impact of M. cruciger *on plant fitness: reproductive output in relation to root damage*

In the spatiotemporal survey which is explained above, we randomly selected 16 to 23 sample plants per population in the years 2010 (populations Knokke, Zeebrugge, Bredene and Oostduinkerke) and 2012 (populations Knokke, Zeebrugge, Bredene, Oostduinkerke and De Panne) in which we counted the total number of flowers and seeds that were produced. We performed generalized mixed models to test whether the total number of flowers and seeds produced and the proportional seed set differed between infested and non-infested plants. We specified a Poisson distribution for total number of flowers and seeds and a binomial distribution for proportional seed set which was calculated as the total number of seeds divided by the total number of ovules (=total number of flowers multiplied by four). Herbivory was the explanatory variable and root crown diameter (measured after seed ripening) was added as cofactor in the model. Year and location nested within year were specified as random grouping variables.

Pollinator visitation in relation to root damage by M. cruciger

To test whether the interaction between *C. officinale* and *M. cruciger* influences the preference of pollinators, we performed a small-scale field experiment in a *C. officinale* population in De Panne in June 2010. We selected 20 flowering plants based on the occurrence of oviposition marks on the stem and leaves, 13 of them were infested by *M. cruciger* whereas the others (seven) were not infested and function as controls. We measured root crown diameter and counted the number of open flowers (= floral display size) in each study plant. We observed each of these 20 plants four times ten minutes to survey pollinator visitation. The number of pollinators visiting the focal plant per ten minutes and the number of flowers visited per pollinator foraging bout were registered. Pollinator observations were performed during two days in early June by multiple observers at the same time. The ten-minute observation periods were randomly allocated to the study plants and observers during these two observation days. We analyzed the difference between infested and non-infested plants for the number of pollinators visiting the plant and the mean number of flowers visited per pollinator foraging bout per 10 minutes using generalized linear mixed models. For both the response variables we specified a Poisson distribution. Because there were four observation periods per plant, plant ID was specified in the model as random grouping variable. Presence of root herbivory was the explanatory variable and root crown diameter and floral display were added as cofactors in the model.

Impact of pollinators on plant fitness in relation to root damage by M. cruciger

In the spatiotemporal survey which is explained above, we randomly selected 17 sample plants per population in 2010 (populations Knokke, Zeebrugge, Bredene and Oostduinkerke) on which we performed an experimental hand pollination treatment to investigate whether the interaction with *M. cruciger* influences the level of pollen limitation. To quantify the level of pollen limitation, we designated in each plant an open pollinated treatment and a pollen supplemented treatment to two separate cymes to avoid confounding effects of differential resource allocation between flowers of different pollination treatments within the same cyme (Wesselingh, 2007). On each of the two cymes 2 to 14 flowers were labeled and these were all left uncovered available for natural open pollination. The labeled flowers on the pollen supplementation treatment cyme received extra pollen gathered from three randomly chosen other plants from the same population. Before seed ripening we lost 9 sample plants due to unavoidable natural and human disturbance: 1 in Knokke, 2 in Zeebrugge, 5 in Bredene and 1 in Oostduinkerke. After seed ripening we counted the number of fully grown seeds per treated flower.

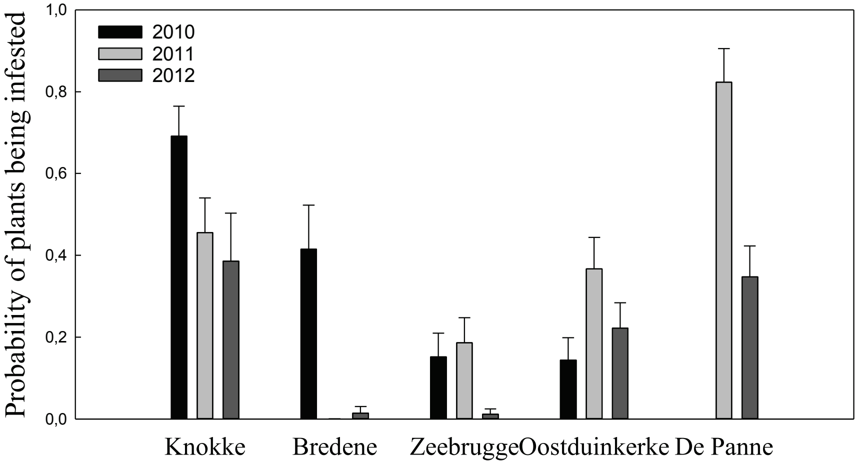
We tested whether the amount of pollen limitation differed between infested and non-infested plants in a generalized linear mixed model. Pollination treatment, presence of root damage and their interaction were the explanatory variables and the proportion of matured seeds per four ovules in a flower was the binomially distributed response variable. Initial root crown diameter and relative flower position on the cyme were added to the model as covariates. We specified location, plant ID and plant ID*pollination treatment as random effects.

RESULTS

Spatiotemporal survey of M. cruciger occurrence

There were significant differences in the occurrence of root damage caused by *M. cruciger* larvae both between populations and years ($F_{4,\,664} = 10.61$, $P < 0.0001$ and $F_{2,\,664} = 9.19$, $P = 0.0001$ respectively). The interaction between population and year was also significant, indicating that the differences between populations were not consistent over the three years and vice versa ($F_{7,\,664} = 3.30$, $P = 0.0018$; Fig. 2.1). The chance to find damage in the roots again increased significantly with root crown diameter ($\beta = 0.14$, $SE = 0.03$, $F_{1,\,664} = 19.36$, $P < 0.0001$). Maps with indications of infection status and root crown diameter per plant of each population in each year can be found in the supplementary information section [Fig. S2.1, Supplementary information].

Fig. 2.1. Estimated chance of plants being infested by *M. cruciger* larvae in five local *C. officinale* populations at the Belgian coast during three years (N = 679). In 2010 no plants were surveyed in the location of De Panne. In 2011 the estimated chance of infestation in the local population of Bredene was only 0.00000006614, therefore this is not visible on the graph.



Correlations between plant traits and presence of root damage

The probability for a plant to be infested by *M. cruciger* increased significantly with initial root crown diameter ($\beta = 0.11$, $SE = 0.043$, $F_{1,\,178} = 6.17$, $P = 0.01$). When initial root crown diameter was controlled for, flowering stem height was still positively correlated with the probability of *M. cruciger* attack ($\beta = 0.04$, $SE = 0.02$, $F_{1,\,164.5} = 4.88$, $P = 0.03$), but number of cymes ($\beta = 0.04$, $SE = 0.02$, $F_{1,\,162.2} = 2.25$, $P = 0.14$), duration of flowering ($\beta = -0.007$, $SE = 0.03$, $F_{1,\,163.9} = 0.07$, $P = 0.79$) and mean floral display size ($\beta = -0.01$, $SE = 0.02$, $F_{1,\,174} = 0.33$, $P = 0.57$) were not related to *M. cruciger* root infestation.

Impact of M. cruciger on plant fitness: reproductive output in relation to root damage

The total number of flowers produced did not differ between infested and non-infested plants ($F_{1,\,130.4} = 0.64$, $P = 0.42$; Fig.2.2a). Root crown diameter was positively correlated to the number of flowers produced per plant ($\beta = 0.09$, $SE = 0.01$, $F_{1,\,129.6} = 84.25$, $P < 0.0001$).

The total number of seeds produced was significantly higher in non-infested plants compared to infested plants ($F_{1,\,122.3} = 5.00$, $P = 0.03$; Fig.2.2b), and significantly increased with root crown diameter ($\beta = 0.11$, $SE = 0.01$, $F_{1,\,130.7} = 83.10$, $P < 0.0001$).

The proportional seed set was significantly higher in non-infested plants compared to infested plants ($F_{1,\,129.2} = 4.56$, $P = 0.03$; Fig.2.2c), and significantly increased with root crown diameter ($\beta = 0.03$, $SE = 0.01$, $F_{1,\,129} = 7.57$, $P = 0.007$).

Fig. 2.2a. Model means and SE's for total number of flowers produced in *C. officinale* plants (N = 134) that were non-infested versus infested by *M. cruciger* larvae. Inset: estimate and SE of the difference in log total number of flowers between non-infested and infested plants.

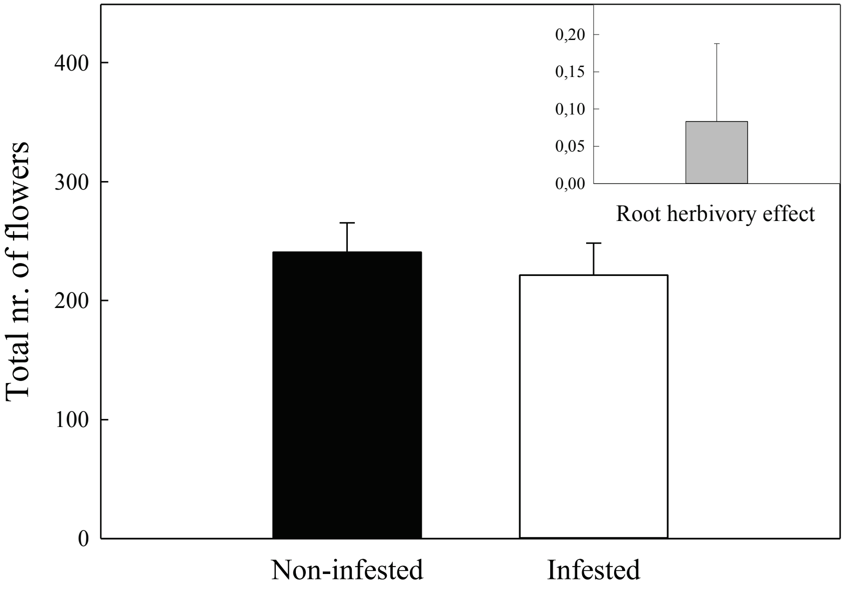


Fig. 2.2b. Model means and SE's for total number of seeds produced in *C. officinale* plants (N = 134) that were non-infested versus infested by *M. cruciger* larvae. Inset: estimate and SE of the difference in log total number of seeds between non-infested and infested plants. *P < 0.05

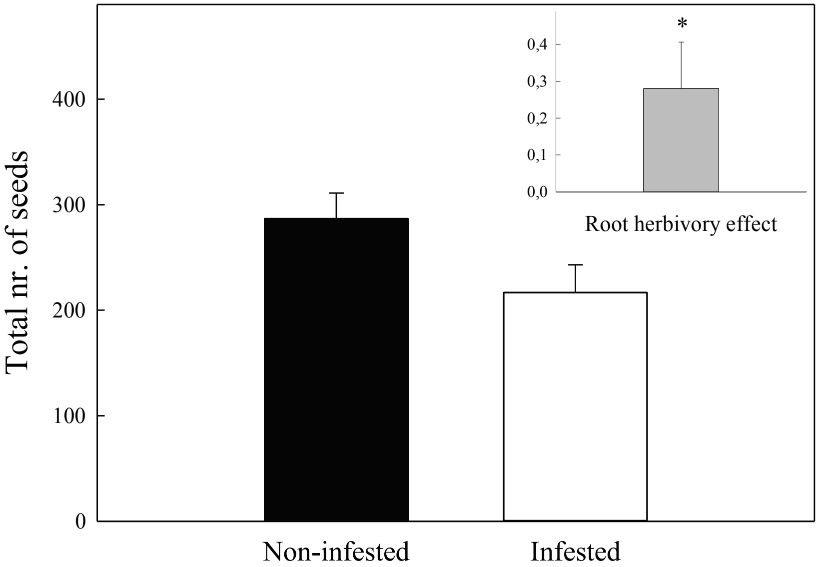
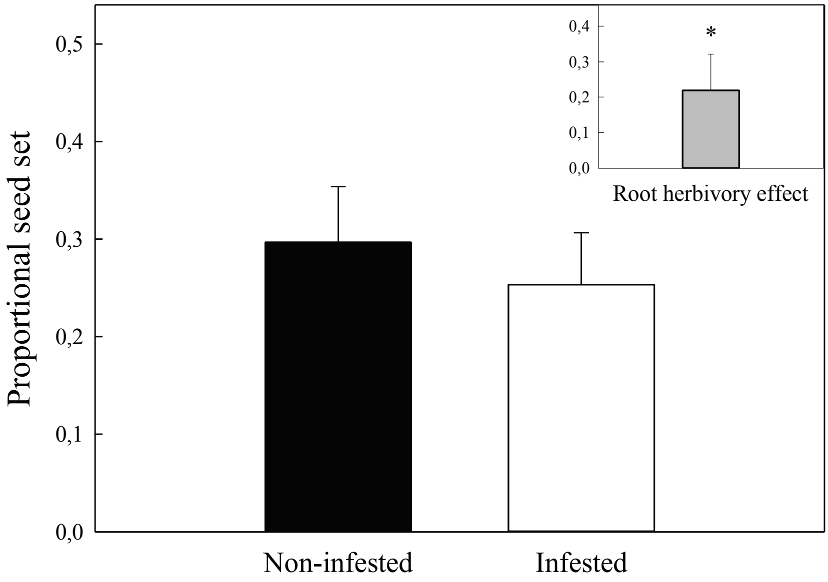


Fig. 2.2c. Model means and SE's for proportional seed set in *C. officinale* plants (N = 134) that were non-infested versus infested by *M. cruciger* larvae. Inset: estimate and SE of the difference in logit proportional seed set between non-infested and infested plants. *P < 0.05



Pollinator visitation in relation to root damage by M. cruciger

The number of pollinators visiting a plant per 10 minutes did not differ significantly between infested and non-infested plants ($F_{1,\,12.1} = 1.76$, $P = 0.21$; Fig. 2.3a). There was also no significant relationship between the number of visiting pollinators and root crown diameter ($\beta = 0.070$, $SE = 0.079$, $F_{1,\,13.36} = 0.78$, $P = 0.39$) or floral display ($\beta = 0.007$, $SE = 0.005$, $F_{1,\,10.62} = 2.11$, $P = 0.17$).

The mean number of flowers visited per pollinator foraging bout tended to be higher in non-infested plants compared to infested plants ($F_{1,\,14.54} = 3.80$, $P = 0.07$; Fig. 2.3b). The number of flowers visited per foraging bout increased significantly with root crown diameter (β = 0.138, $SE = 0.065$, $F_{1,\,17.44} = 4.45$, $P = 0.05$) and non-significantly with floral display ($\beta = 0.007$, $SE = 0.004$, $F_{1,\,13.14} = 3.27$, $P = 0.09$).

Fig. 2.3a. Model means and SE's for number of pollinators visiting *C. officinale* plants that were non-infested (N = 7) versus infested (N = 13) by *M. cruciger* larvae.

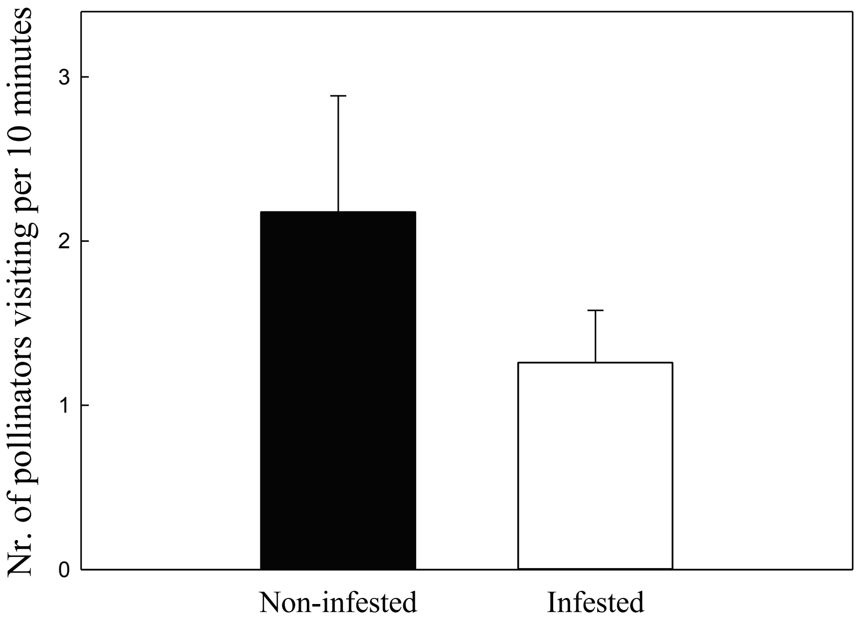
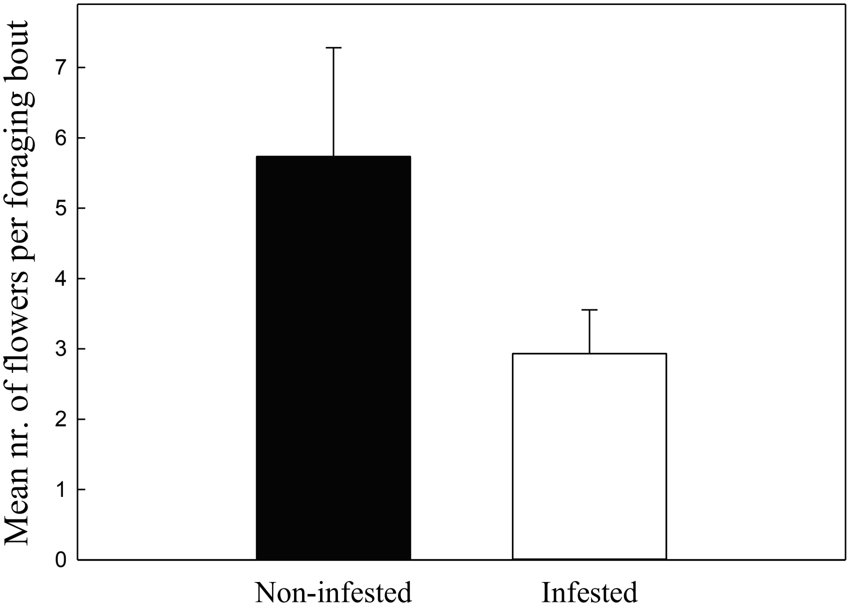


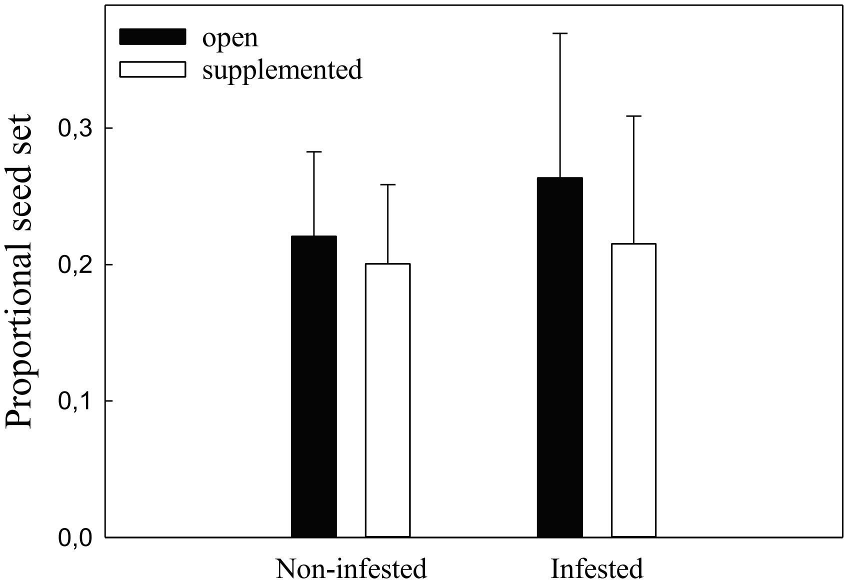
Fig. 2.3b. Model means and SE's for mean number of flowers visited per foraging bout in *C. officinale* plants that were non-infested (N = 7) versus infested (N = 13) by *M. cruciger* larvae.



Impact of pollinators on plant fitness in relation to root damage by M. cruciger

Flowers that were supplemented with pollen did not have higher proportional seed set than flowers that did not receive extra pollen, both in non-infested as in infested plants ($F_{1,\,51.24} = 0.76$, $P = 0.39$), indicating that seed set was not pollen-limited. The interaction between pollination treatment and herbivory was also not significant ($F_{1,\,45.11} = 0.12$, $P = 0.73$), and there was no difference in seed set per treated flower in response to root herbivory ($F_{1,\,37.66} = 0.09$, $P = 0.77$; Fig. 2.4). The proportional seed set was not significantly related to initial root crown diameter ($F_{1,\,39.68} = 1.48$, $P = 0.23$), but it decreased significantly the further away from the main stalk the flower was positioned on the cyme ($\beta = -0.52$, $SE = 0.04$, $F_{1, 470.3} = 136.50$, $P < 0.0001$).

Fig. 2.4. Effect of supplemental outcross pollination versus open pollination on proportional seed set in *C. officinale* plants (N = 54) that were non-infested versus infested by *M. cruciger* larvae.



DISCUSSION

In this study we investigated the potential for geographical structuring in total selection pressure exerted on plants in the Belgian herbivore-plant-pollinator system of *C. officinale*, its specialist antagonist *M. cruciger* and its mutualists, pollinating bees and bumble bees. , its specialist antagonist M. cruciger and its mutualists, pollinating bees and bumble bees. The geographical distribution of the focal plant species and its interacting species differed: in some of the sampled *C.*

officinale populations root herbivory by *M. cruciger* was much more abundant than in others and this also differed between years. The second premise, that the interacting species exerts selection pressure was also satisfied. The specialist antagonist *M. cruciger* had a specific preference for large plants and impacted plant fitness by reducing reproductive output significantly in the studied *C. officinale* populations. Although the interaction with *M. cruciger* influenced the number of flowers visted per pollinator visit, this did not affect seed production since neither the non-infested nor the infested plants were pollen-limited. We conclude that the intensity of the selection pressure exerted by *M. cruciger* is expected to vary between *C. officinale* populations over time.

Occurrence and distribution of M. cruciger at the Belgian coast

There were plants infested by *M. cruciger* larvae in each of the five sampled populations. However, the proportion of plants attacked differed significantly between populations (Fig. 2.1). The proportions of plants attacked in The Netherlands (22% to 61%; Prins et al., 1992) and in Hungary and Germany (56.7% to 95.5%; Schwarzlaender, 1997), were most comparable to those found in our study populations in Knokke and De Panne. There were also differences in incidence of plant attack between the different years (Fig. 2.1) and these might be related to differences in abundance and density of *C. officinale* plants. Schwarzlaender (1997) stated that although the population density of *C. officinale* shows large fluctuations between years (van der Meijden et al., 1992), *M. cruciger* was always found equally abundant at all of their field sites in 1992 and 1994, regardless of actual plant density. If this was also the case in our study area, differences in plant density between years could have caused the differences in proportion of plants attacked because we always sampled about 60 plants independently of the plant density in that local population. Apart from this, the significant interaction between population and year indicates that the relative abundances of *M. cruciger* in the sampled populations fluctuate independently from each other.

Selection pressure exerted by M. cruciger

We similarly observed a positive correlation between root crown diameter and *M. cruciger* root herbivory as already found in *C. officinale* populations in the Netherlands, Hungary and Germany (Prins et al., 1992; Schwarzlaender, 1997). Because *C. officinale* seedlings are often clustered around the parent plant (Rademaker and de Jong, 1999), it can be expected that genetically related plants (half sibs/full sibs) of the same age and thus also of similar size are spatially aggregated. Since the distribution pattern of *M. cruciger* larvae is also aggregated (Schwarzlaender, 1997), the correlation between root crown diameter and larval attack found in the field might originate from such spatial cohort effects. However, point pattern analyses on our data indicate that this is not the case, because the radii at which plants with similar root crown diameters are clustered (2.5 m and 6.5 m) do not concur with the radii at which plants with the same infection status cluster (4 m and 10 m) [Fig. S2.2, Supplementary information]. The postulation that *M. cruciger* selects plants with a large root crown diameter for oviposition therefore holds.

When plants with the same root crown diameter were compared, plants with a larger flowering stem height were more likely to be attacked by *M. cruciger* larvae. Possibly plant height is another cue for ovipositing females to assess a plant's food supply in its root. Other measured plant traits like number of cymes, duration of flowering and mean floral display size appear later in spring at a time when most *M. cruciger* eggs are already laid, probably therefore these traits did not correlate with root infestation.

In our study area root herbivory caused a significant reduction of 32% in the total amount of seeds produced when root crown diameter was held constant. This result is comparable to the reduced number of seeds in response to *M. cruciger* root herbivory (with straw weight held constant) found in the coastal dunes of Meijendel (Prins et al., 1992). In this latter study, seed weight was also measured and the calculated reproductive effort (= seed weight/straw weight) was 30% lower in response to root herbivory. Since ovipositing females had specific preferences for large plants and the larval root herbivory significantly impacted plant fitness in our study area, we can conclude that *M. cruciger* exerts selection pressure on *C. officinale* plants in the Belgian dunes.

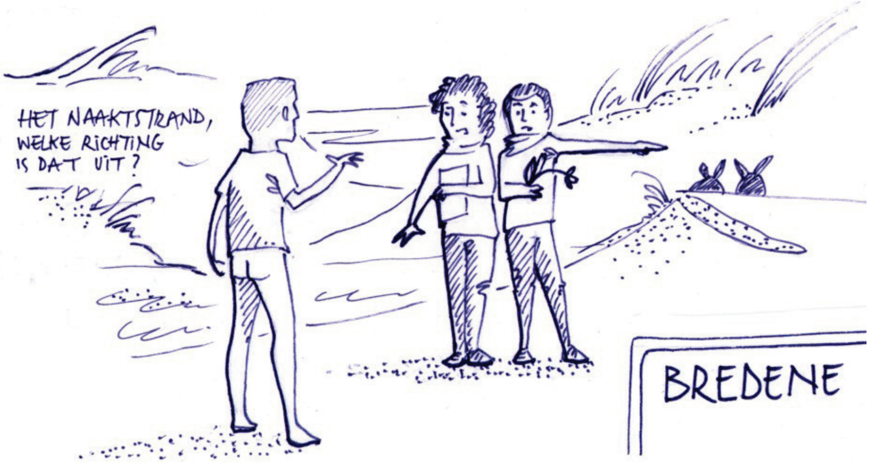
Influence of M. cruciger on pollinator visitation and impact on plant fitness

There were tendencies for root herbivory to affect pollinator visitation behavior. Especially the number of flowers visited per foraging bout showed a strong tendency: this was 51% lower in infested plants compared to non-infested plants with the same root crown diameter and floral display size. Since our sample size was rather small and there is also experimental proof of lower pollinator attraction in response to *M. cruciger* root herbivory (Ghyselen et al., 2016; chapter three), we can assume that *M. cruciger* may have an impact on pollinator preference in the field. As was also concluded in Ghyselen et al. (2016; chapter three), *M. cruciger* root herbivory probably changes the plant's phenotype that is apparent for pollinators, making it less attractive.

Our results indicate that seed set was not pollen-limited both in non-infested as in infested plants. At least through this aspect of plant fitness, pollinators can thus not exert a selection pressure. However, the quality of seeds might still be affected by pollinators, via the quantity and diversity of pollen that they deposit on the stigma (Skogsmyr and Lankinen, 2002). Another aspect of plant fitness is the amount of pollen that is exported to other plants and that can sire seeds. Although not measured in this study, this 'male fitness' can still be affected by the influence of herbivory on pollinator visitation (Strauss et al., 1996). Further research on this subject will enhance the comprehension on the role of this mutualistic interactor in the total selection pressure exerted on *C. officinale* plant populations.

Conclusion

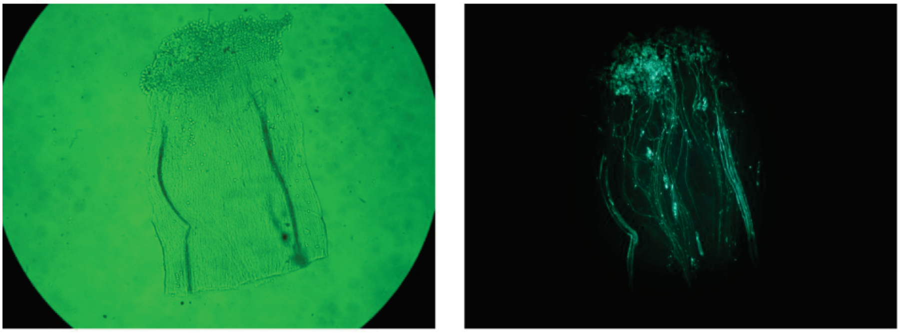
The selection pressure exerted by *M. cruciger* in our Belgian study populations and the observations of differences in relative abundance of *M. cruciger* root herbivory between the sampled populations led us to conclude that there is potential for geographical structuring in the total selection pressures exerted on *C. officinale* plant populations in the Belgian dunes. It would be interesting now to investigate the consequences of these geographical differences in intensity of selection pressure. Because *M. cruciger* prefers large plants, strong selection pressure would result in plants with a lower threshold size to flower. In populations where *M. cruciger* is absent, on the other hand, a higher threshold size would be selected for because large plants have more flowers and produce more seeds (Klinkhamer and de Jong 1987). This process has been studied and was confirmed at a biogeographic scale: Wesselingh et al. (1997) found that *C. officinale* plants in Holkham, England where *M. cruciger* is most probably absent, had a higher threshold size than those in Meijendel, The Netherlands. Given the expected dispersal limitation of herbivore insects in the fragmented coastal dunes (Maes and Bonte, 2006), this process is likely also to be prevalent at much smaller geographical scales. This can, however, only be demonstrated by means of long-term spatial explicit monitoring of both infection rate and threshold size in this study area.



Milena Sonneveld

3. Is there a missing link? Effects of root herbivory on plant-pollinator interactions and reproductive output in a monocarpic species

Céline Ghyselen, Dries Bonte and Rein Brys



Adapted from:

Ghyselen, C., Bonte, D., Brys, R. 2016. Is there a missing link? Effects of root herbivory on plant–pollinator interactions and reproductive output in a monocarpic species. Plant Biology **18**:156–163.

ABSTRACT

Herbivores can have a major influence on plant fitness. The direct impact of herbivory on plant reproductive output has long been studied, and recently also indirect effects of herbivory on plant traits and pollinator attraction received increasing attention. However, the link between these direct and indirect effects has seldom been studied. In this study we investigated effects of root herbivory on plant and floral traits, pollination success and reproductive outcome in the monocarpic perennial *Cynoglossum officinale*.

We exposed 119 *C. officinale* plants to a range of root herbivore damage by its specialist herbivore *Mogulones cruciger*. We assessed the effect of herbivory on several plant traits, pollinator foraging behavior and reproductive output and to unravel the link between these last two we also quantified pollen deposition and pollen tube growth and we applied a pollination experiment to test whether seed set was pollen-limited.

Larval root herbivory induced significant changes in plant traits and had a negative impact on pollinator visitation. Infested plants were reduced in size, had fewer flowers and received fewer pollinator visits at plant and flower level than non-infested plants. Also seed set was negatively affected by root herbivory, but this could not be attributed to pollen limitation since neither stigmatic pollen loads and pollen tube growth nor the results of the hand pollination experiment differed between infested and non-infested plants.

Our observations demonstrate that although herbivory may induce significant changes in flowering behavior and resulting plant-pollinator interactions, it does not necessarily translate into higher rates of pollen limitation. The observed reductions in reproductive output following infection can mainly be attributed to higher resource limitation compared to non-infested plants.

INTRODUCTION

Herbivory is one of the most important environmental variables affecting plant fitness and is known to induce the evolution of multiple mechanisms of plant resistance and tolerance (Marquis, 1992; Strauss and Agrawal, 1999). The impact of herbivory on plant reproduction has long been investigated and numerous studies found positive (Lennartsson et al., 1998; Fang et al., 2006), neutral (Lucas-Barbosa et al., 2013), or negative (Prins et al., 1992; Maron, 1998; Hunt-Joshi et al., 2004) effects on reproductive output.

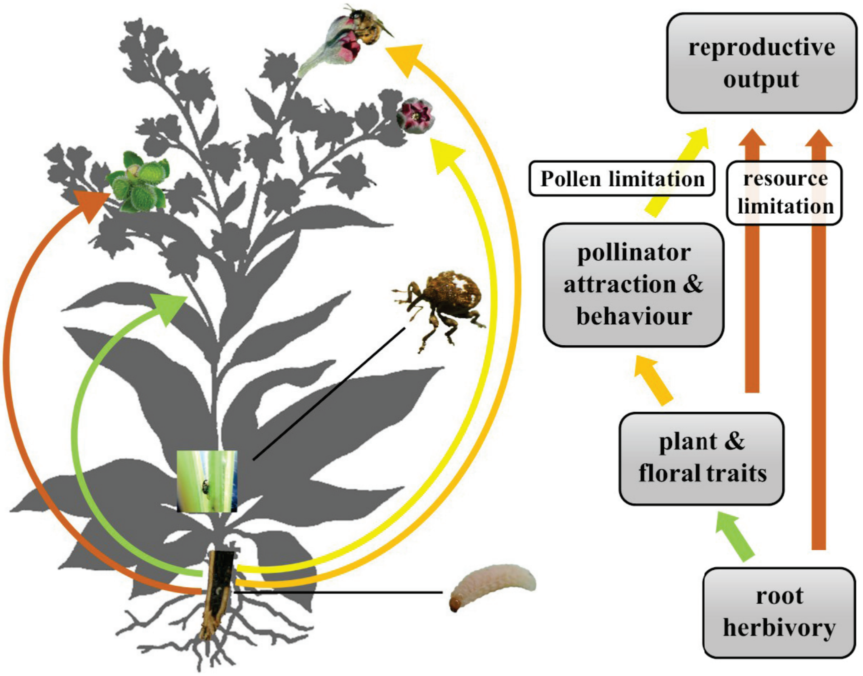
Apart from the direct impact of herbivory on plants, such as the destruction of flowers and/or fruits (Adler et al., 2001; Cardel and Koptur, 2010), it can also have far reaching indirect effects affecting interactions with other organisms and ultimately influencing plant fitness (Strauss, 1997; Ohgushi, 2005; Barber et al. 2012). Plant-pollinator interactions are one of these, and can be affected by herbivore induced changes in, for instance, nectar production (Fang et al., 2006; Samocha and Sternberg, 2010), flower size (Mothershead and Marquis, 2000; Ivey and Carr, 2005), floral display (Samocha and Sternberg, 2010), flowering period (Poveda et al., 2003), plant volatile emission (Kessler et al., 2011; Lucas-Barbosa et al., 2011) or plant architecture (Hunt-Joshi et al., 2004). Herbivore induced changes in the above mentioned floral traits can in turn cause shifts in pollinator foraging behavior and ultimately pollinator mediated pollen deposition. Most studies that assessed the impact of herbivory on pollinator foraging behavior to date focused on effects of aboveground herbivory (Lehtilä and Strauss, 1997; Suarez et al., 2009; Hladun and Adler, 2009; Kessler et al., 2011), whereas belowground herbivory and its resulting impact on aboveground biotic interactions, such as plant-pollinator interactions, is omnipresent but seldom studied (Poveda et al., 2003; Poveda et al., 2005a; De Deyn and Van der Putten, 2005; Barber and Soper Gorden, 2014).

To elucidate whether herbivory may truly affect plant reproductive output via its impact on plant-pollinator interactions, it is necessary to assess pollen limitation (Ashman et al., 2004). The past three decades pollen limitation was traditionally assessed using supplemental hand-pollination experiments, and this method has already been used to assess effects of herbivory on pollination success in some studies (Mothershead and Marquis, 2000; Hladun and Adler, 2009; Barber et al., 2012). However, this approach may under certain conditions suffer from confounding effects of resource limitation and/or reallocation of resources among flowers to developing seeds (Knight et al., 2006; Wesselingh, 2007). In this context, Alonso et al. (2012) recently highlighted that the determination of pollen tube numbers can offer important insights in the actual level of pollen limitation as it is the functional link between pollen arrival and seed production. Although this may provide additional insights on the confounding effects of resource allocation on seed production, up until now only few researchers have performed pollen tube quantification in relation to herbivore-pollinator interactions (but see Parra-Tabla and Herrera, 2010).

In this study we investigate effects of belowground herbivory on plant traits, pollinator behavior, pollen limitation and plant reproductive success in the monocarpic perennial *Cynoglossum officinale* L. (Boraginaceae) (Fig. 3.1). Each flower of *C. officinale* develops four ovules. Flowers produce large amounts of nectar and are mainly visited by bees and bumble bees (De Jong et al., 1990; personal observation). *C. officinale* is a self-compatible species but in the absence of pollinator visitation only very few seeds develop (Klinkhamer and Dejong, 1987; Dejong et al., 1990). An important specialist herbivore of *C. officinale* in Europe is the root weevil *Mogulones cruciger* Herbst (Prins et al., 1992; Schwarzlaender, 1997). Adult females of this weevil lay their eggs mostly in early spring in petioles or in the shoot at the base of preferably large and bolting plants. From there the larvae migrate into the taproot where they feed on the vascular tissue until they mature and leave the root for pupation in the soil in early summer. The average seasonal fecundity per female is 181 ± 18.9 eggs. Under greenhouse conditions, eggs develop over a period of 15.1 ± 0.6 days, after which they hatch. The larvae generally feed on the root for 31 days until they move to the soil for pupation. During the larval stage, especially the second and third instars (from day 9 to 31 of larval development) are most damaging to the taproot of the host plant

(Schwarzlaender, 1997). Root herbivory by this weevil can be easily quantified and the amount of vascular tissue eaten in infested plants ranged from 2% up to 90% in Belgian *C. officinale* populations in 2011 (C Ghyselen, unpubl. results). In an earlier observational study, Prins et al. (1992) already documented a reduction of 30% in reproductive output when *C. officinale* plants were infested by these root weevil larvae. Because *C. officinale* usually flowers from mid-May until the end of June (Dejong et al., 1990), the larval root herbivory occurs mostly just before and during flowering. Hence we can expect that this type of herbivory can have far reaching effects on plant and flowering traits and consequently on pollinator attraction and reproductive output. To test this hypothesis, we experimentally subjected *C. officinale* plants to larval root herbivory by *M. cruciger* and investigated its effects on plant traits, pollinator foraging behavior and plant reproductive success. Additionally, we quantified pollen loads and number of pollen tubes and we assessed pollen limitation through supplemental hand pollinations in order to unravel the link between pollinator attraction, pollen limitation and reproductive output. More specifically we tested whether larval root herbivory by *M. cruciger*: 1) causes effects on plant traits that are potentially important for pollinator attraction, 2) influences pollinator foraging behavior, 3) affects stigmatic pollen loads and numbers of pollen tubes entering the ovary, 4) causes pollen limitation and 5) impairs reproductive output.

Fig. 3.1. Study system with *Cynoglossum officinale*, its specialist herbivore *Mogulones cruciger* and its pollinators. Adult *M. cruciger* weevils oviposit in the leaf and main stems of *C. officinale*. The larvae then migrate into the root where they feed on root tissue. In this study we investigate whether this root herbivory affects plant traits, pollinator foraging behaviour, pollen load and number of pollen tubes, total seed set and pollen limitation of seed set.



MATERIAL AND METHODS

Experimental design

Herbivory treatment—We performed two experiments with potted plants in two consecutive years (2011 and 2012). For the first experiment, 79 plants were grown from seeds originating from eight different populations (two in Alberta, Canada; three in British Columbia, Canada; one in Holkham, England; one in Trieste, Italy; and one in Meijendel, The Netherlands). These plants were reared for one year in separate 2-L containers filled with a 50:50 mixture of dune sand and potting soil in growth chambers and were put outside during winter (2010-2011) for vernalization (prerequisite for flowering in the next growing season: de Jong et al., 1986). In the second week of April 2011, we exposed 47 of these plants to adult root weevils (two to four weevils per plant) by caging them on each of the focal plants for 14 days, while the other 32 plants served as control group growing under the same conditions. During these two weeks the weevils were able to infect plants assigned to the herbivore treatment.

Because in the second year (April 2012), no potted plants grown from seedlings were still available, we dug out 40 plants that had reached the threshold size for flowering (Dejong et al., 1986) from a large *C. officinale* population located at the Belgian coast. Based on visual inspection these plants were not infected yet by *M. cruciger* weevils, which was confirmed when root damage was recorded at the end of the experiment. These plants were transplanted into 10-L pots in early spring and after one week 20 of these plants were exposed to adult root weevils (four per plant during five to six days in the fourth week of April) by caging them on each of the focal plants, while the others ($N = 20$) served as control group growing under the same conditions.

Adult weevils used for the herbivore treatments were collected from a natural population in the natural reserve 'De Westhoek' in Adinkerke a few days before the treatments started. After each of these treatments was applied in the growth chambers, all weevils and cages were removed from the plants and plants were placed outside in a weevil-free experimental garden at the Belgian coast in Wenduine. Aboveground herbivory during weevil exposition was minimal and therefore not taken into account. Secondary infection during the flowering period and seed ripening was very unlikely, since the larval development and pupal stage before adult emergence already take about two to three months (Schwarzlaender, 1997). We omitted eleven plants from the total sample due to lack of flowering (one of the sample exposed to herbivory and one belonging to the control treatment) or early mortality (five belonging to the herbivore treatment and four of the control treatment).

Plant traits—Before the onset of the herbivory experiment, the root crown diameter of every plant was measured with a digital caliper as a proxy of initial plant size.

To assess whether root herbivory causes changes in plant performance and flowering behavior, we measured the following plant traits during the flowering season: total number of flowering days, floral display size (i.e., the number of open flowers counted on a daily basis), total number of flowers per plant developed during the entire flowering season and plant size (i.e., stem height). Due to rabbit herbivory, we were not able to determine the total number of flowers of nine plants.

Pollinator visitation—In order to evaluate the effect of root herbivory on pollinator foraging behavior, we observed insect flower visitation rates to the study plants. During flowering, all study plants (both the herbivory and control plants) were put on a lawn in the above mentioned experimental garden, which is located in the middle of a dune area where natural pollinators of *C. officinale* are common. Plants were randomly assigned to three and two experimental arrays of 19 to 27 individuals in 2011 and 2012 respectively, in which plants were located at a distance of about 0.5 m from each other. Each array was left unchanged during the entire flowering period, and was observed for pollinator visitation during periods of 30 minutes between 1100 h and 1800 h. In total there were 26 observation periods for each of the three experimental arrays in 2011 (from 30 April until 25 May) and 31 observation periods for each of the two experimental arrays in 2012 (from 19 May until 14 June). During each 30-min observation interval, every flower visitor that was noticed foraging on the focal plants was followed until it left the array. When more than one flower visitor was present at the same time, only the first visitor was followed. Besides the fact that simultaneous foraging of pollinators was a rather scarce event, no differences in visitation behavior between the first and following (simultaneous) visitors are expected to occur, hence this protocol provides a good proxy for pollinator visitation within the array. Per pollinator foraging bout, we recorded visitor species, plant IDs of those individuals that were visited and number of flowers visited per plant. Based on these data, we calculated the number of pollinator visits to infested and non-infested plants per 30 min interval (=sum of all the plant visits registered per plant ID), as well as the average rate of geitonogamy per plant (=average number of flowers visited per plant visit) within that array. The visitor species consisted of six bumble bee species and one solitary bee species [Supplementary Information, Table S1] and are all presumed to be effective pollinators of *C. officinale*.

Pollen limitation: Stigmatic pollen loads and pollen tube growth—To quantify stigmatic pollen loads and the number of pollen tubes entering the ovary, we cut off pistils just above the ovary of four to eight withering flowers in each of the non-infested and infested plants (N=39) in the experiment of 2012. Styles were fixed in ethanol-acetic acid (9:1) during one hour at room temperature and afterwards we stored them in 70% ethanol in a refrigerator. Before the styles were softened for about 17 hours in 0.5 mol m^{-3} NaOH, they were rinsed in decreasing concentrations of ethanol (58%, 29% and 14.5%) and finally in distilled water. Hereafter, they were rinsed three times in distilled water again and stained for three to four hours in 0.05% aniline blue solution in phosphate buffer pH 8.5 before squashing them in a 10% glycerine solution under a cover slip. To determine the total number of pollen grains on the stigmatic surface and the total number of pollen tubes at the end of the style, each style was scanned at 200 × with a Leitz Diaplan epifluorescence microscope (the Optical Institute, Wetzlar, Germany) with a UV filter set (420-470 nm excitation, 490-535 nm emission) (protocol adjusted from Brys et al., 2008).

Pollen limitation: Supplemental hand-pollination experiment —Using the supplemental hand-pollination approach we quantified pollen limitation at plant level in each of the non-infested and infested plants (N=108). We supplementally pollinated one to eight flowers (that were not prevented from natural open pollination) of one to two cymes manually with outcross pollen gathered from at least three randomly chosen other study plants. At the same time, but on another one to two cymes located at similar positions on the same plant as the supplementally pollinated flowers, we designated one to eight flowers to a control open pollination treatment. After seed ripening the number of full-grown seeds per treated flower was counted and proportional seed set was calculated by dividing the number of full-grown seeds per flower by the number of ovules (n=4).

Cymes develop from the axils of alternate stem leaves (Dejong et al., 1990) which make the cymes photosynthetically more or less independent. Therefore, we interpreted a cyme as an integrated physiological unit (Watson and Casper, 1984), within which reallocation of resources occurs but exchange of resources between these units is not expected to be substantial (Wesselingh, 2007). Hence, confounding effects of resource reallocation among cymes to developing seeds are expected to be of minor importance using this design.

Plant reproductive output—After seed ripening the total number of full-grown seeds per plant was counted and proportional seed set was calculated by dividing the total number of full-grown seeds by the total number of ovules (=total number of flowers multiplied by four).

Quantification of herbivore damage—After seeds were counted (July-August), all focal plants were excavated and roots were washed and cut open. For each plant, we recorded whether the roots where infested with *M. cruciger* larvae, and when infested, the percentage of root damage was quantified by estimating the amount of vascular tissue in the tap root that was eaten by the larvae. Of all the plants that were exposed to the herbivore treatment, 85.25% showed root damage by the weevil larvae. The percentages of root damage in infested plants ranged between 15-100% (mean = 59%, *SE* = 4.85) in the experiment of 2011 and 5-65% (mean = 31%, *SE* = 4.66) in 2012.

Statistical analyses

The impact of root herbivory on plant traits, pollination success and reproductive output was analyzed following a dual approach by defining root herbivory first as a presence/absence categorical fixed effect and second, by treating root herbivory as a continuous fixed effect in infested plants. Only the plants that showed signs of root herbivory were categorized as infested plants, thus 'root herbivory present', and all other plants were categorized as non-infested. Because the experimental study plants of 2011 originated from different populations, we included population as a random effect in the models.

Also the year of experiment, 2011 or 2012, was incorporated as a random effect in all models, except for the pollen load and tube analyses for which there were only data for 2012. We added the initial root crown diameter as fixed covariate in the full models to correct for this factor that might be correlated to the dependent variables that we want to test. For all analyses mixed effects models and generalized mixed effects models with a backwards selection procedure were used to come to the most parsimonious model. We corrected for over- or underdispersion where necessary. All statistical models were run in SAS (SAS Institute Inc. 2008. SAS/STAT® 9.3 User's Guide. Cary, NC: SAS Institute Inc.) using PROC MIXED and PROC GLIMMIX with estimation of the degrees of freedom by the Kenward Rodgers procedure.

Plant traits—The dependent plant variables were number of flowering days, total number of flowers, stem height and daily floral display, and each of these variables were analyzed in a univariate model. In the daily floral display model, 'plant ID' was included as grouping variable since floral display size was measured every day of the flowering period. We specified a Poisson error distribution and log link for all plant traits, except for stem height for which a normal error distribution and identity link was specified.

Pollinator visitation—For the pollinator visitation data, the unit of replication was the 30-min observation period and 'plant ID' and 'observation date' were included in the random factors to correct for dependent replicates within plant individuals and temporal effects respectively in each model. Also visitor species was included as a random factor to take into account possible differences in foraging behavior between species. For the dependent variables, number of pollinator visits per 30 minutes and mean number of flowers visited per plant visit, a Poisson error distribution with log link was specified. For both these variables models were also run with floral display size added as a fixed covariate to assess the effect of root herbivory on pollinator visitation independent of the number of open flowers on that plant.

Pollen limitation: stigmatic pollen loads and pollen tube growth—Because the samples consisted of styles of several flowers per plant, we included the grouping variable 'plant ID' as random effect in the model to correct for the dependence within plant individuals. We specified a Poisson error distribution with log link for the data on pollen load and pollen tubes. Also here, there were additional models tested with floral display size added as a fixed covariate to assess the effect of root herbivory on pollen loads and tubes independent of the number of open flowers on that plant.

Pollen limitation: supplemental hand-pollination experiment —For the analyses of pollen limitation, the proportional seed set (number of full-grown seeds / number of ovules) of open pollinated versus pollen supplemented flowers was compared in response to root herbivory. Therefore, hand pollination treatment and its interaction with root herbivory were added as fixed covariates. Because the unit of replication here is proportional seed set per treated flower and multiple flowers per plant were treated, we included the grouping variable ‘plant ID’ as random effect to correct for dependence within plant individuals. We specified a Binomial error distribution with logit link for proportional seed set.

Plant reproductive output—For the analyses of plant reproductive output, the proportional seed set of the whole plant (number of full-grown seeds / number of ovules, binomial distribution with logit link) was the dependent variable.

RESULTS

Plant traits

When infested (52 plants in total), plants showed a significantly different vigor and flowering behavior compared to their non-infested congeners (56 plants in total) (Table 3.1). The number of flowering days did not differ significantly between infested and non-infested plants (Fig. 3.2a). The total number of flowers per plant (Fig. 3.2b) and stem height (Fig. 3.2c) were both significantly lower in infested plants compared to non-infested plants. For daily floral display size no differences could be noted (Fig. 3.2d). In the infested plants, plant traits were not significantly related to the percentage of root damage (Table 3.1; Fig 3.3).

Table 3.1. Results for the fixed effects presence of root damage and percentage of root damage of the plant trait models. Model details in Material and methods section.

	Presence of root damage	**Percentage of root damage**
Number of flowering days	$F_{1, 99.35} = 2.75$ $P = 0.1001$	$F_{1, 40.90} = 0.69$ $P = 0.4124$
Total number of flowers	$F_{1, 87.65} = 8.5$ $P = 0.0045$	$F_{1, 36.66} = 1.84$ $P = 0.1836$
Stem height	$F_{1, 99.5} = 4.54$ $P = 0.0356$	$F_{1, 38.4} = 0.54$ $P = 0.4679$
Daily floral display	$F_{1, 91.05} = 1.82$ $P = 0.1806$	$F_{1, 39.74} = 0.09$ $P = 0.7622$

Fig. 3.2. Effect of root herbivory on a) number of flowering days, b) total number of flowers, c) stem height and d) daily floral display in *Cynoglossum officinale*. Means and standard errors calculated on raw data, except for daily floral display: means and standard errors from model. Model results in Table 3.1. * P < 0.05, ** P < 0.01

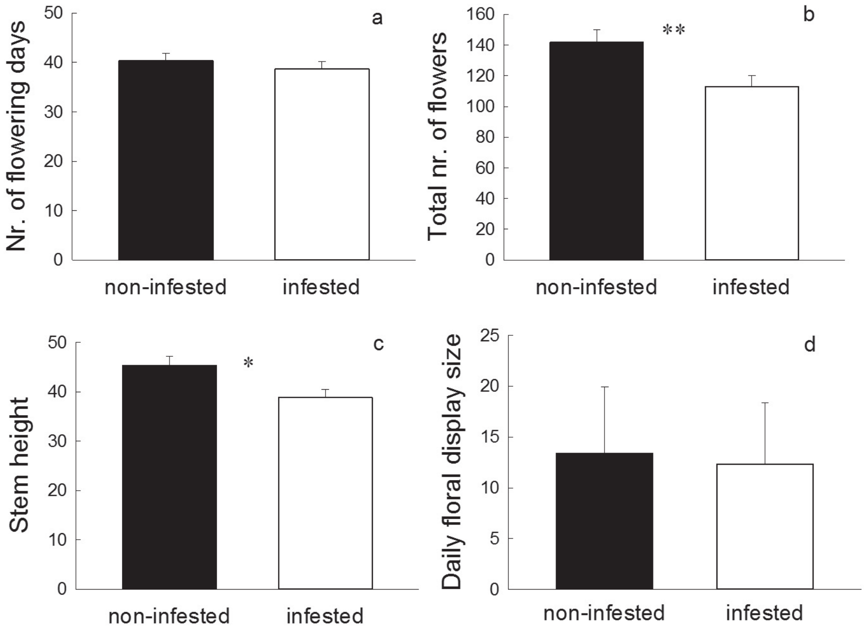
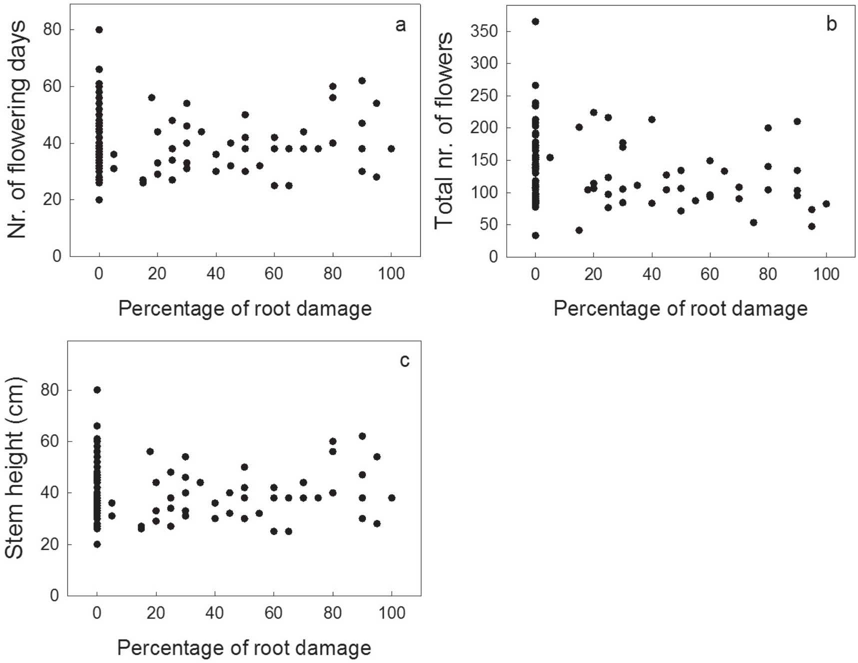


Fig. 3.3. Effect of percentage of root damage on a) number of flowering days, b) total number of flowers, c) stem height in *Cynoglossum officinale*. Model results in Table 3.1.



Pollinator visitation

Infested plants attracted fewer pollinators than non-infested plants ($F_{1, 76}$ = 6.22, P = 0.015) (Fig. 3.4b). In the infested individuals, plants with increasing levels of root damage attracted fewer pollinators (β = -0.005, SE = 0.002, $F_{1, 34.52}$ = 8.38, P = 0.007). Although the number of pollinator visits was strongly and positively related to floral display size (Table 3.2; Fig. 3.4a), the effect of root herbivory on pollinator visitation frequency was still present, though not that strong, when floral display size was added in the model as a covariate (Table 3.2).

The number of flowers visited per foraging bout was lower in infested plants than in non-infested plants ($F_{1,\ 117.7} = 6.47$, $P = 0.012$) (Fig. 3.5b). In the infested plants, the number of visited flowers that an insect visited per foraging bout was not significantly correlated with the percentage of root damage ($\beta = -0.002$, $SE = 0.002$, $F_{1,\ 65.41} = 0.93$, $P = 0.339$). When floral display size was incorporated in the models, the number of flowers visited per foraging bout was also significantly lower in infested plants than in non-infested plants and there was no correlation with percentage of root damage (Table 3.2). Also here, the total number of flowers visited per foraging bout was strongly positively correlated with floral display size (Table 3.2; Fig. 3.5a).

Fig. 3.4. a) Combined effect of root herbivory and floral display in *Cynoglossum officinale* on the number of pollinator visits per 30 minutes (full curve = non-infested plants, dotted curve = infested plants). Model results in Table 3.2. b) inset: estimate and standard error of the difference in the log number of pollinator visits per 30 minutes between non-infested and infested plants. Model results in text. * P < 0.05

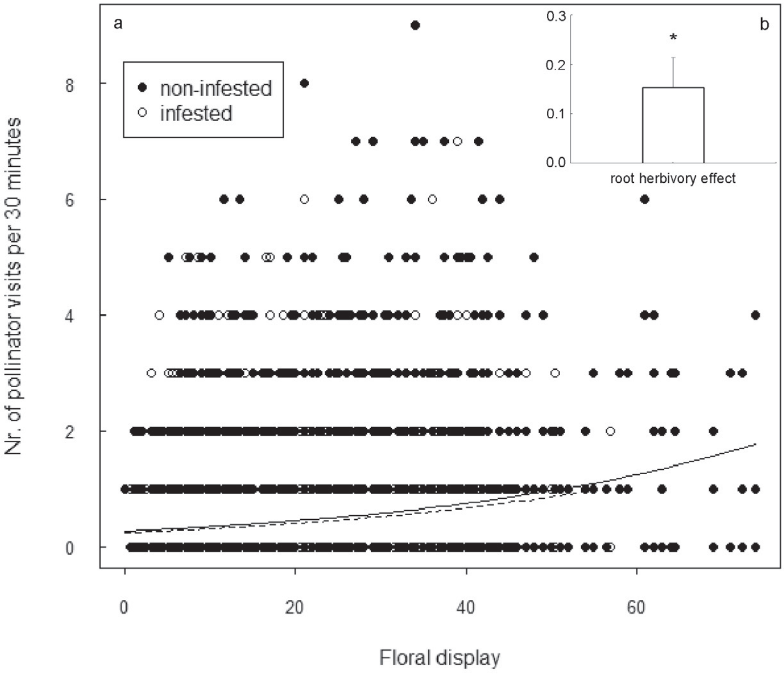
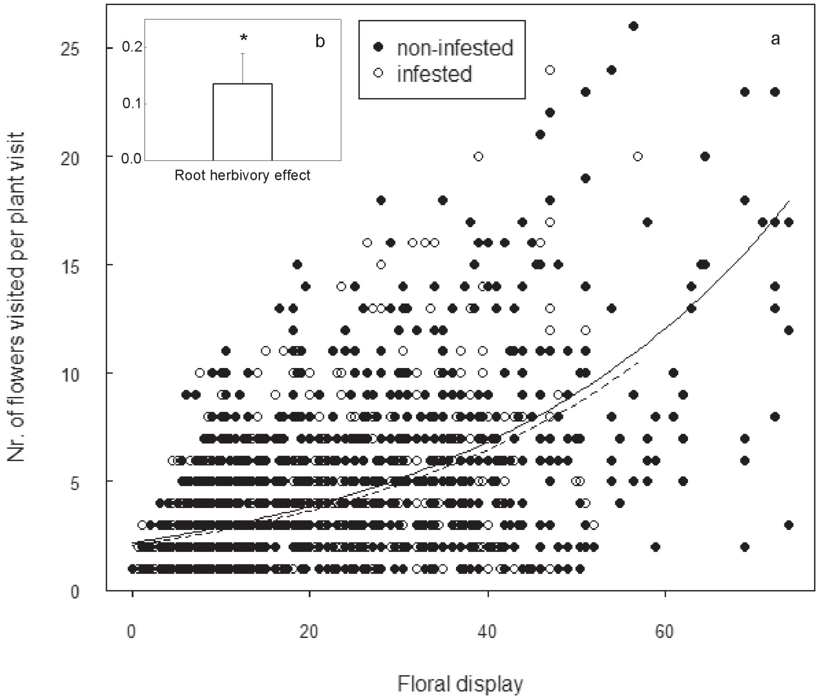


Fig. 3.5. a) Combined effect of root herbivory and floral display in *Cynoglossum officinale* on the mean number of visited flowers per plant visit (full curve = non-infested plants, dotted curve = infested plants). Model results in Table 3.2. b) inset: estimate and standard error of the difference in the log mean number of visited flowers per plant visit between non-infested and infested plants. Model results in text. * P < 0.05



Pollen limitation

Stigmatic pollen loads and pollen tube growth—Overall, the number of deposited pollen grains on the stigma was on average 418.6 ± 15.0 and the average number of pollen tubes reaching the ovary was 31.72 ± 0.93. Stigmatic pollen loads and the number of pollen tubes did not differ between infested and non-infested plants (pollen loads: $F_{1,\ 37.63} = 0.80$, $P = 0.376$; number of pollen tubes: $F_{1,\ 36.17} = 0.63$, $P = 0.434$), nor were they related to the percentage of root damage in infested plants (pollen loads: $\beta = -0.002$, $SE = 0.006$, $F_{1,\ 13.47} = 0.11$, $P = 0.742$; number of pollen tubes: $\beta = 0.00008$, $SE = 0.005$, $F_{1,\ 12.83} = 0.00$, $P = 0.987$). Also when corrected for floral display size there were no differences between infested and non-infested plants in stigmatic pollen loads and the number of pollen tubes reaching the ovary, and there was no relation with percentage of root damage (Table 3.2). Pollen loads and the number of pollen tubes were significantly positively related to floral display size (Table 3.2).

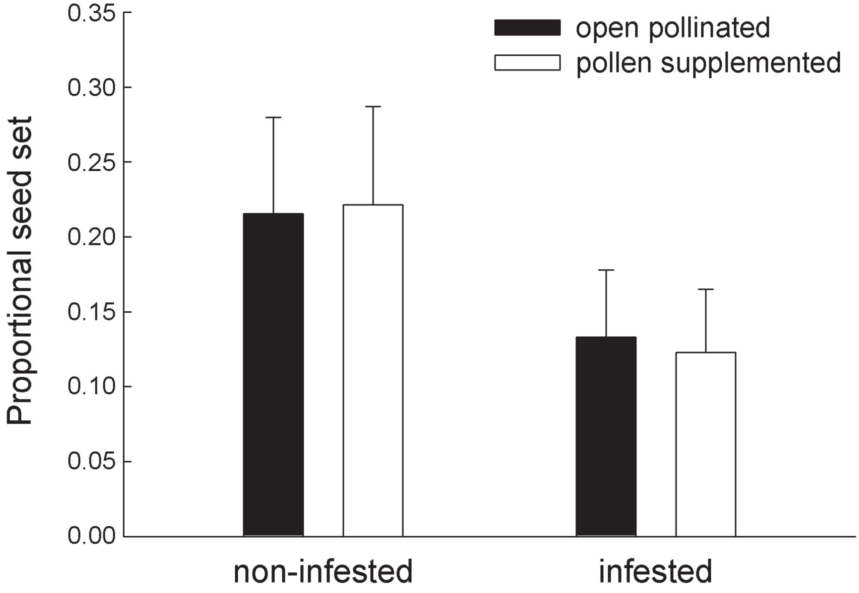
Table 3.2. Results of the generalised mixed models with floral display size as a covariate, for the effects of root herbivory and floral display size in *Cynoglossum officinale* on number of pollinator visits per 30min intervals, mean number of visited flowers per plant in each foraging bout, stigmatic pollen load and number of pollen tubes entering the ovary.

	Presence of root damage	**Percentage of root damage**
Number of pollinator visits		
Root herbivory	$F_{1, 78.72} = 3.70$ $P = 0.058$	$F_{1, 41.4} = 4.76$ $P = 0.035$
Floral display size	$F_{1, 945.5} = 142.99$ $P < 0.001$	$F_{1, 368.4} = 53.01$ $P < 0.001$
Number of visited flowers per plant visit		
Root herbivory	$F_{1, 83.03} = 2.52$ $P = 0.116$	$F_{1, 47.36} = 0.07$ $P = 0.798$
Floral display size	$F_{1, 188.9} = 485.55$ $P < 0.001$	$F_{1, 47.36} = 0.07$ $P = 0.798$
Stigmatic pollen load		
Root herbivory	$F_{1, 38.79} = 0.26$ $P = 0.610$	$F_{1, 12.82} = 0.05$ $P = 0.822$
Floral display size	$F_{1, 177.5} = 5.71$ $P = 0.018$	$F_{1, 86.14} = 3.91$ $P = 0.051$
Number of pollen tubes		
Root herbivory	$F_{1, 35.80} = 0.00$ $P = 0.986$	$F_{1, 12.08} = 0.03$ $P = 0.877$
Floral display size	$F_{1, 116.8} = 24.69$ $P < 0.001$	$F_{1, 71.55} = 4.8$ $P = 0.032$

Supplemental hand-pollination experiment—The seed set of open pollinated flowers did not differ significantly from that of pollen supplemented flowers (categorical root herbivory: $F_{1, 952.9} = 0.02$, $P = 0.9006$; continuous root herbivory: $F_{1, 387.3} = 0.01$, $P = 0.9381$) (Fig.

3.6) and no interaction between hand pollination treatment and root herbivory occurred (categorical root herbivory: $F_{1,\,953.4} = 0.28$, $P = 0.5973$; continuous root herbivory: $F_{1,\,385.8} = 0.09$, $P = 0.7684$) (Fig. 3.6).

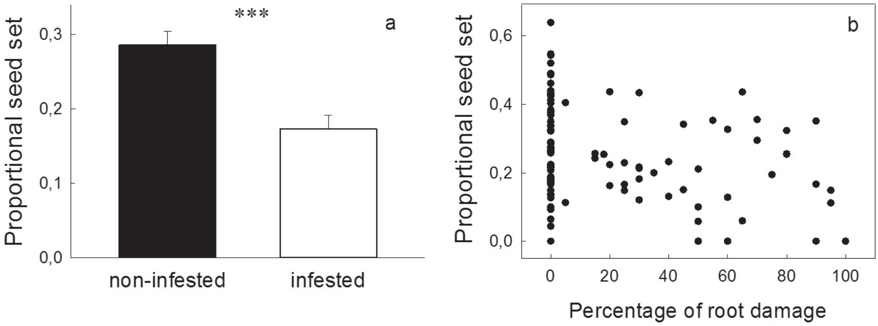
Fig. 3.6. Effect of supplemental outcross pollination vs. open pollination on proportional seed set in non-infested and infested *Cynoglossum officinale* individuals. Model results in text.



Plant reproductive output

Infested plants had a significantly lower seed set than non-infested plants ($F_{1,\,100.2} = 14.02$, $P = 0.0003$) (Fig. 3.7a), and when infested, seed set significantly decreased with percentage of root damage ($\beta = -0.010$, $SE = 0.004$, $F_{1,\,42.99} = 5.25$, $P = 0.0270$) (Fig. 3.7b).

Fig. 3.7. Proportional seed set in relation to a) presence of root damage, and b) percentage of root damage. Means and standard errors calculated on raw data. Model results in text. *** P < 0.005



DISCUSSION

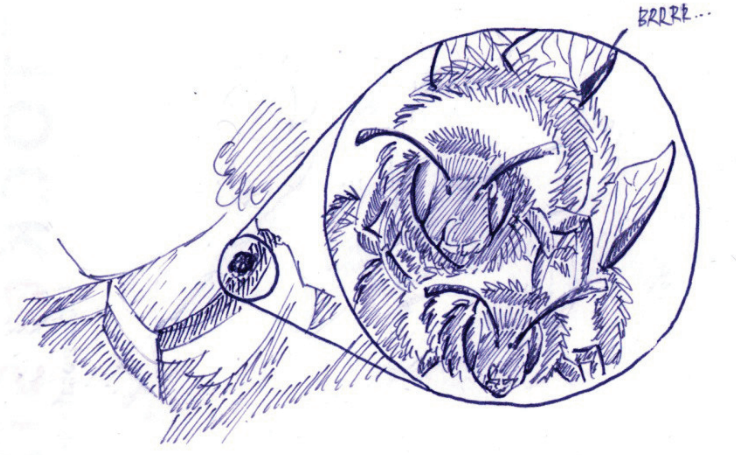
Interactions between herbivores and pollinators can have important consequences for plant fitness. In order to evaluate these effects properly, an integrative approach is necessary in which not only herbivory effects on plant traits and pollinator visitation are investigated, but also the consequences on pollination success and total reproductive output are incorporated (Barber and Soper Gorden, 2014). By using such an integrative approach, we found that root herbivory by *M. cruciger* larvae on the monocarpic *C. officinale* negatively affected several plant traits, pollinator attraction and total reproductive output. However, the negative effect on reproduction could not be attributed to pollen limitation and consequently there is no causative link between reduced pollinator visitation on infested plants and their overall reduction in reproductive output.

Generally, our study revealed that infection by the root herbivore *M. cruciger* significantly affected the flowering behavior and plant size of *C. officinale*. When infested, plants had fewer flowers and were smaller than non-infested plants. Similar indirect effects of aboveground herbivory were also found on number of flowers (Mothershead and Marquis, 2000; Hladun and Adler, 2009; Samocha and Sternberg, 2010) and plant height (Poveda et al., 2003; Hunt-Joshi et al., 2004). Although fewer insights are available on the consequences of belowground herbivory on plant traits, a decrease in shoot growth rate has often been reported in response to root herbivory (Andersen, 1987). In *Lythrum salicaria*, for instance, Nötzold et al. (1998) detected a reduction in flowering period and plant size in response to root herbivory by its specialist weevil, *Hylobius transversovittatus*. In the same plant-root weevil system, an overall reduction in leaf, stem and inflorescence biomass, and in the number of stems was additionally reported by Hunt-Joshi et al. (2004). Barber et al. (2011) found a decreased production of female flowers in *Cucumis sativus* after enhanced root herbivory by the weevil *Acalymma vittatum*. Root damage may cause the malfunctioning of some basic root functions such as mineral and water absorption and conduction, synthesis of products necessary for normal growth and development of the shoot, and/or storage and reallocation of metabolites and photosynthates (Andersen, 1987). Especially the latter function can be expected to have a determining role for aboveground plant vigor and flower production in the monocarp *C. officinale*, since photosynthate production during the first year of vegetative growth is stored in the tap root. Once the threshold size for flowering has been reached, aboveground plant growth and flower production are dependent on the amount of resource reserves stored in the root (Prins et al., 1992; Wesselingh and de Jong, 1995). Surprisingly, we did not find any association between the percentage of root damage and plant traits in infested plants. This might, at least partly, be explained by the fact that 1) a small amount of damage can already suffice to disrupt the basic root functions, and 2) we assessed the percentage of root damage after seed ripening, which is not necessarily related to the amount of root damage at the time of aboveground plant development. Furthermore, when most substantial root damage occurred (about three weeks after the start of the herbivore treatment), most plants had already started flowering (personal observation).

Infested *C. officinale* plants that were exposed to natural pollination received fewer pollinator visits than non-infested plants, a reduction that appeared to be strengthened by the amount of root damage. Also in infested plants, fewer flowers were visited per foraging bout than in non-infested plants. Flowers were visited by bumble bees and a solitary bee [Supplementary information, Table S3.1], which are known to select on plant and floral display size (Manning, 1956; Klinkhamer et al., 1989; Lehtilä and Strauss, 1997; Suarez et al., 2009). Besides the plant traits measured in our experiment, other pollinator attraction traits could also have been influenced by root herbivory and may in turn have affected pollinator behavior too, such as emission of volatile organic compounds (Kessler et al., 2011) and nectar content or composition (Samocha and Sternberg, 2010). Especially the latter deserves further investigation in this study system since root herbivory may induce changes in the pyrrolizidine alkaloid distribution of infested plants (Van Dam and Vrieling, 1994) that could potentially increase the pyrrolizidine alkaloid content in flowers and their nectar. The presence of pyrrolizidine alkaloids in nectar has, for instance, been shown to have a deterrent effect on honey bees (Reinhard et al., 2009). Contrary to our findings, Poveda et al. (2003; 2005a) observed a higher flower visitation of pollinating insects to plants that were exposed to root herbivores than to plants without any signs of herbivory in the annual *Sinapsis arvensis*. In the study of Hladun and Adler (2009), on the other hand, no changes in floral traits nor in pollinator preference could be observed in response to enhanced root herbivory by larvae of the beetle *Acalymma vittatum* in the annual *Cucurbita moschata*. These authors presumed that their study plants must have been able to (over)compensate for the root damage, whereas in our study system this was apparently not the case. Besides the higher number of pollinator visits in non-infested *C. officinale* plants, there were also more flowers visited per foraging bout and this may potentially result in higher rates of self pollen deposition onto the stigmas due to geitonogamy. Although Melser and Klinkhamer (unpublished data in Melser and Klinkhamer, 2001) found indications that there were no differences in number of seeds produced following self versus outcross pollination in *C. officinale*, the resulting offspring did show slight differences in survival (but their reproductive performance was roughly equal). Hence, further research on the selfing percentage in *C. officinale* in response to root herbivory and its consequences for plant fitness of the offspring would be of interest.

The negative impact of root herbivory in *C. officinale* on pollinator attraction and visitation behavior did neither translate into differences in stigmatic pollen deposition nor into differences in the number of pollen tubes that entered the ovary. A possible explanation could be that the stigmas of both the infested and non-infested plants were more or less saturated with pollen grains, so that more pollinator visits in the non-infested plants did not result in larger stigmatic pollen loads. The mean number of pollen grains on the stigma (418.6 ± 15.0) was fairly high and the average number of pollen tubes (31.7 ± 0.9) entering the ovary far exceeded the four ovules available per flower. This together with the results of the supplemental hand pollination treatment led us to conclude that the study plants did not experience pollen limitation. Despite the absence of pollen limitation, we found that reproductive output was clearly reduced in infested compared to non-infested plants. The results of the hand pollination experiment revealed that even pollen supplemented flowers showed lower seed production in infested plants (Fig. 3.6). This indicates that seed production is most likely reduced by the depletion of stored resources in the tap root. The higher the percentage of root damage caused by the larvae, the lower seed set turned out to be, which corroborates the hypothesis that the root weevil larvae deplete the resources intended for seed production. In their study on aboveground herbivory effects in *Cucumis sativus* Barber et al. (2012) also found decreased plant-pollinator interactions and reduced reproduction in damaged plants, but enhanced pollination had no influence on plant reproductive success. Similarly, they concluded that the direct impact of herbivory was more detrimental for plant reproduction than the indirect costs of deterring pollinators from damaged plants.

Although many ecologists have investigated herbivory effects on plant-pollinator interactions in the past two decades (Lehtilä and Strauss, 1997; Mothershead and Marquis, 2000; Poveda et al., 2003; Kessler et al., 2011), only few of them have comprised effects on plant traits, pollinator foraging behavior, pollen limitation and ultimately seed set in one study. In their review paper on how belowground organisms influence plant-pollinator interactions, Barber and Soper Gorden (2014) state that despite the extensive research on the direct effects of belowground organisms on plants, the consequences of these effects for plant fitness through changes in pollination services are almost entirely unknown. Here we addressed this question and found that root herbivory negatively affected plant-pollinator interactions and seed production. However, our experimental plants were not pollen-limited and the reduced seed production was attributed to resource limitation as a direct result of root herbivory. These findings emphasize the need to use an integrative approach when studying herbivory effects on plant-pollinator interactions. Future research on this subject, especially on plant species that are more prone to pollen limitation, may provide further interesting insights on the link between indirect and direct effects of root herbivory on plant fitness.



TIJDENS BESTUIVER-OBSERVATIES OP EEN KOUDE LENTEDAG...

Milena Sonneveld

4. The influence of herbivory on pre- and post-zygotic stages of reproduction following open, self and outcross pollination

Céline Ghyselen, Dries Bonte and Rein Brys

Adapted from:

Ghyselen, C., D. Bonte, and R. Brys. 2015. The influence of herbivory on pre- and postzygotic stages of reproduction following open, self, and outcross pollination. American Journal of Botany **102**:2013-2019.

ABSTRACT

Herbivory affects pollination success and reproductive output in plants. However, the different stages in the process from pollination to seed maturation have hardly been investigated within the context of herbivory. Herbivory might affect these stages via its effect on geitonogamous pollination and thereby the proportion of self pollen delivered to the stigma, and/or via its effect on the nutritional capacity of the maternal plant.

In the monocarpic *Cynoglossum officinale*, we experimentally subjected plants to root herbivory and exposed them to natural open pollination in combination with self and outcross hand-pollination. We quantified pollen germination, pollen tube competition intensity, pollen tube attrition, fruit set and seed initiation, abortion, and maturation.

Although root herbivory did not affect pollen germination or pollen tube attrition, fruit set and seed initiation and maturation were negatively affected by herbivory, but for seed initiation this was only the case in outcross- and open pollinated flowers. The intensity of pollen tube competition positively affected seed initiation, but only in infested plants.

Our study demonstrates that herbivory did not affect the early stages following pollination, but significantly impacted later post-pollination stages like fruit set and seed maturation, and the presence of selection based on pollen tube competition intensity on zygote devel-

opment. Our findings suggest that decreased nutritional capacity of the mother plant in response to root herbivory rather than herbivory effects on pollen quality was responsible for the lower fruit and seed production in infested plants.

INTRODUCTION

Herbivory can cause shifts in pollinator foraging behavior by inducing changes in plant traits that influence pollinator attraction (Lehtilä and Strauss, 1997; Kessler et al., 2011; Barber et al., 2012). In this way herbivory may alter the quantity of pollen deposited on the stigma by influencing for instance pollinator visitation rate (Lehtilä and Strauss, 1997; Ghyselen et al., 2016, chapter three; Russell-Mercier and Sargent, 2015) or duration of flower visits (Barber et al., 2012). Additionally herbivory may also impact the quality of deposited pollen by affecting the number of flowers visited sequentially on a plant (Steets et al., 2006; Ghyselen et al., 2016, chapter three; Russell-Mercier and Sargent, 2015), thereby affecting the proportion of self pollen delivered to the stigma through geitonogamous pollination.

In many plant species, the proportion of self versus outcross pollen deposited on the stigma can have an important impact on pollen tube growth. For instance, in species characterized by a self-incompatible breeding system, the pistil actively constrains pollen germination or pollen tube growth of self pollen (Herrero and Hormaza, 1996). Also in self-compatible plant species, pollen tube growth of self pollen can be hampered, and the presence of self pollen on the stigma may even constrain pollen tube growth of outcross pollen in that same pistil (Cruzan, 1990). During growth, pollen tubes depend on the resources available in the style and as such the pistil can affect pollen tube growth by varying the timing and amount of nutritional support (Herrero and Hormaza, 1996). It can thus also be expected that the nutritional capacity of the recipient plant and factors that influence this capacity, such as herbivory, may affect pollen tube growth.

If the number of pollen grains growing in the style exceeds the number of ovules available in the ovarium, male gametophytes may compete for successful fertilization (Herrera, 2002). The haploid genotypes of pollen are expressed during growth toward the ovule (Becker et al., 2003). Hence, pollen tube competition can promote fertilization by genetically superior or compatible fathers, either through direct competition between the growing pollen tubes or through female choice by which growth of pollen tubes is regulated. The intensity of pollen competition is often connected to offspring quality (Skogsmyr and Lankinen, 2002). Due to this process, even in self-pollinated plants pollen competition may promote offspring of better quality by allowing discrimination against pollen carrying recessive deleterious alleles that are also found in the mother plant (Lankinen and Armbruster, 2007).

Also at the stage where the fittest and/or fastest pollen tubes reach the ovary, selection can act independently of pollen competition before fertilization can take place. This can, for instance, occur via late acting ovarian self-incompatibility, where ovules that would otherwise have participated in fertilization are disabled during growth of self pollen tubes in the style (Sage et al., 2006). After fertilization, seeds develop until they are fully matured, though some developing seeds may be aborted for several reasons. Selfed zygotes can, for instance, be aborted due to early acting inbreeding depression (Owen et al., 2007). But also embryos of lower quality derived from outcross-fertilization can be selectively aborted (Marshall and Ellstrand, 1988). As a consequence, plants can preferentially mature seeds of flowers in which the intensity of pollen competition was highest to enhance fitness of its resulting offspring the most (Niesenbaum and Casper, 1994). When, however, resources become constrained for the maternal plant, seed abortion rates tend to increase (Marshall and Ellstrand, 1988; Niesenbaum, 1996). Nonetheless, the selectivity based on pollen tube numbers does not necessarily increase with resource scarcity because other factors like positional patterns in resource acquisition might also determine seed abortion (Niesenbaum, 1996).

In summary, there are several stages in the process from pollen deposition to seed maturation at which selection on pollen, pollen tube growth or zygote development can occur. It can be expected that these different aspects of pre-dispersal selection are influenced by herbivory via its effect on the proportion of self pollen deposited on the stigma and/or via its effect on the nutritional capacity of the maternal plant. However, these aspects are hardly addressed in a context of herbivory (but see Niesenbaum, 1996). In this study we investigate the impact of root herbivory on the process of fertilization and seed development following variable pollination treatments in the self-compatible monocarpic *Cynoglossum officinale* L. (Boraginaceae). Root herbivory was caused by the weevil *Mogulones cruciger* Herbst (Coleoptera: Curculionidae), which is an important specialist herbivore feeding on the roots of *C. officinale* (Prins et al., 1992; Schwarzlaender, 1997). This study system is perfectly suited to the objectives of our study since it was already shown that this root herbivory significantly affects the number of flowers visited sequentially on *C. officinale* plants (Ghyselen et al., 2016; chapter three). Besides this effect on geitonogamous pollination, root herbivory also induced changes in plant traits and reduced reproductive output in *C. officinale*, suggesting a decrease in nutritional capacity of the plant due to root damage (Prins et al., 1992; Ghyselen et al., 2016, chapter three). To disentangle herbivory effects on fertilization success and seed production due to pollen quality and/or nutritional capacity, we combined open pollinations with self versus outcross hand-pollinations on each of the infested and non-infested plants in an experimental array. We surveyed several stages in the process from pollen deposition to seed maturation to investigate whether root herbivory affects: 1) pollen germination and pollen tube attrition, 2) fruit set and seed initiation, abortion and maturation, and 3) the relationship between pollen tube competition intensity and fruit set, seed initiation and maturation.

MATERIAL AND METHODS

Study system

Cynoglossum officinale is a herbaceous monocarpic perennial that occurs in open, disturbed sites such as sand dunes and range lands. It is self-compatible but it greatly depends on bees and bumblebees to increase its seed production (de Jong et al., 1990; Vrieling et al., 1999). Quantitative pollen limitation is unlikely in this species since supplemental addition of pollen in naturally pollinated flowers did not enhance seed set in natural as well as in experimental populations (de Jong and Klinkhamer, 1989; Ghyselen et al., 2016, chapter three; C. Ghyselen, Ghent University, unpublished data). Furthermore, stigmatic pollen loads often largely exceed the four ovules available per flower in naturally pollinated experimental plants (Ghyselen et al., 2016, chapter three), indicating that pollen competition is a frequently occurring event. However, also seed abortion frequently occurs (Klinkhamer and de Jong, 1987; de Jong and Klinkhamer, 1989) and it can be selective, resulting in offspring of better quality (Melser and Klinkhamer, 2001).

Adult females of the weevil *Mogulones cruciger* lay their eggs in petioles or in the shoot at the base of *C. officinale* plants in spring. Eggs develop over a period of 15 days, after which they hatch. The larvae then migrate into the taproot where they feed until they mature after 31 days and leave the root for pupation in the soil in early summer. During the larval stage, especially the second and third instars from day 9 to 31 of larval development are most damaging to the taproot of the host plant (Schwarzlaender, 1997).

Experimental plants and herbivory treatment

In April 2012 we dug out 40 plants from a large *C. officinale* population located at the Belgian coast (N 51.23926 - 51.29911, E 2.93347 - 3.07463). All plants had reached the threshold size to flower (de Jong et al., 1986) and were not yet infected by *M. cruciger*. Twenty individuals were exposed to four adult root weevils per plant during five to six days by caging them on each of the focal plants, while the others (*N* = 20) served as control group growing under the same conditions. All weevils and cages were removed after the treatments and plants were placed outside in a weevil-free experimental garden at the Belgian coast. The herbivory treatment was conducted three weeks before the onset of flowering so that larval herbivory occurred already from the beginning throughout the course of the flowering season from mid-May to end June (de Jong et al., 1990). After seed ripening in July-August, all plants were excavated and roots were washed and cut open. For each plant, we recorded whether the roots were infested with *M. cruciger* larvae. Only the plants that showed signs of root herbivory were categorized as infested (*N* = 16), all other plants were categorized as non-infested (*N* = 23). In the infested plants, root damage was on average 31% (range 5% - 65%). One plant was omitted from the sample due to early mortality.

Pollination treatments

In each of the infested and non-infested plants we designated 8 flowers per cyme to one of three pollination treatments (24 flowers per plant): 1) bagged and hand pollinated with outcross pollen obtained from three other study plants originating from the same population; 2) bagged and hand pollinated with self pollen, and 3) open pollination by natural pollinators of *C. officinale* from the surrounding dune area. For bagging the flowers, bridal veil with a mesh size of 1 mm was used. Since flowers were only bagged a few days during anthesis, we assume that this procedure does not influence fertilization and/or seed development. Each treatment was conducted on eight flowers of a separate cyme to avoid potential effects of differential resource allocation between flowers of different treatments within the same cyme (Wesselingh, 2007). The position of these three treatment cymes varied between the third and ninth cyme (counting started from the top cyme of the plant), with the open pollinated cyme in each plant at a higher position than the hand-pollinated cymes while outcross- and self-pollinated cymes were alternately higher or lower relative to each other. Due to rabbit herbivory, five experimental plants of which two non-infested and three infested with *M. cruciger* larvae, were damaged and preventively omitted from further analyses.

Pollen germination and pollen tube attrition

To investigate herbivory effects on pollen tube growth, we cut off one pistil per pollination treatment just above the ovary in each study plant. Pistils were fixed in 9:1 ethanol-acetic acid and stored in

70% ethanol. They were prepared for epifluorescence microscopic examination by softening them in 0.5 mol/m3 NaOH and staining them in 0.05% aniline blue solution in phosphate buffer pH 8.5 (Ghyselen et al., 2016; chapter three). For each style, we recorded the number of conspecific pollen grains and the numbers of pollen tubes in the stigma and at the base of the style. We calculated pollen germination as the proportion of conspecific pollen grains from which pollen tubes grew into the stigmatic tissue, and pollen tube attrition in the style as the proportion of germinated pollen that did not reach the base of the style.

Fruit set and seed initiation, abortion and maturation

After seed ripening, we counted the number of ovules, filled seeds and aborted seeds in each of the treated flowers. Filled seeds were large and firm, while aborted seeds were smaller and/or shriveled. We considered a flower that produced at least one filled seed as a flower that had set fruit. Proportional fruit set was then calculated as the number of fruits divided by the number of treated flowers. We calculated seed initiation as the proportion of ovules resulting in filled or aborted seeds, seed abortion as the proportion of initiated seeds that aborted and seed maturation as the proportion of ovules resulting in filled seeds.

Statistical analyses

All analyses were performed in SAS 9.4. (SAS Institute Inc., 2013) using PROC GLIMMIX with estimation of degrees of freedom by the Kenward Rodgers procedure. We corrected for overdispersion where necessary and used a backwards selection procedure for the effects of herbivory, pollination treatment and their interactions with each other and -for the pollen tube competition analyses- with the number of pollen tubes at the stigma. For all analyses root crown diameter measured prior to the herbivory treatments, cyme position and flower position on the cyme were included as covariates to correct for any differences in initial plant size between infested and non-infested plants, and to reduce confounding effects of cyme and flower position.

Pollen germination and pollen tube attrition–To assess whether herbivory affected pollen germination and pollen tube attrition depending on pollination treatment, we used generalized linear mixed models. We first analyzed the number of conspecific pollen grains and because this significantly differed between pollination treatments (Fig. 4.1a), we included this as a covariate in the models for pollen germination and pollen tube attrition. In each model pollination treatment (open, outcross and self pollination), herbivory (infested vs. non-infested) and the interaction between both factors were included, with plant ID additionally entered as a random grouping variable. For number of pollen grains on the stigma a Poisson distribution was specified and for pollen germination and pollen tube attrition a binomial distribution was specified.

Fruit set and seed initiation, abortion and maturation—To check whether herbivory or pollen quality may influence fruit set and zygote development, we compared fruit set and seed initiation, abortion and maturation of all treated flowers in response to herbivory, pollination treatment and their interaction using generalized linear mixed models. To correct for dependent replicates within plant individuals, plant ID, cyme nested within plant ID and plant ID * pollination treatment were added as random effects in these models, and a binomial distribution was specified.

Pollen tube competition—To test whether plants selectively set fruit and initiate and mature more seeds in flowers with higher intensity of pollen tube competition, we compared fruit set, seed initiation and maturation in relation to the number of pollen tubes at the stigma. Besides herbivory, pollination treatment and their interaction, we also incorporated the interaction of the number of pollen tubes with herbivory and with pollination treatment to test whether the selectivity based on pollen tube numbers changes in response to herbivory, and to test for differences in the effect of pollen tube competition intensity between outcross-, open and self-pollinated flowers respectively. We used generalized linear mixed models with plant ID as random grouping variable and a binomial distribution.

RESULTS

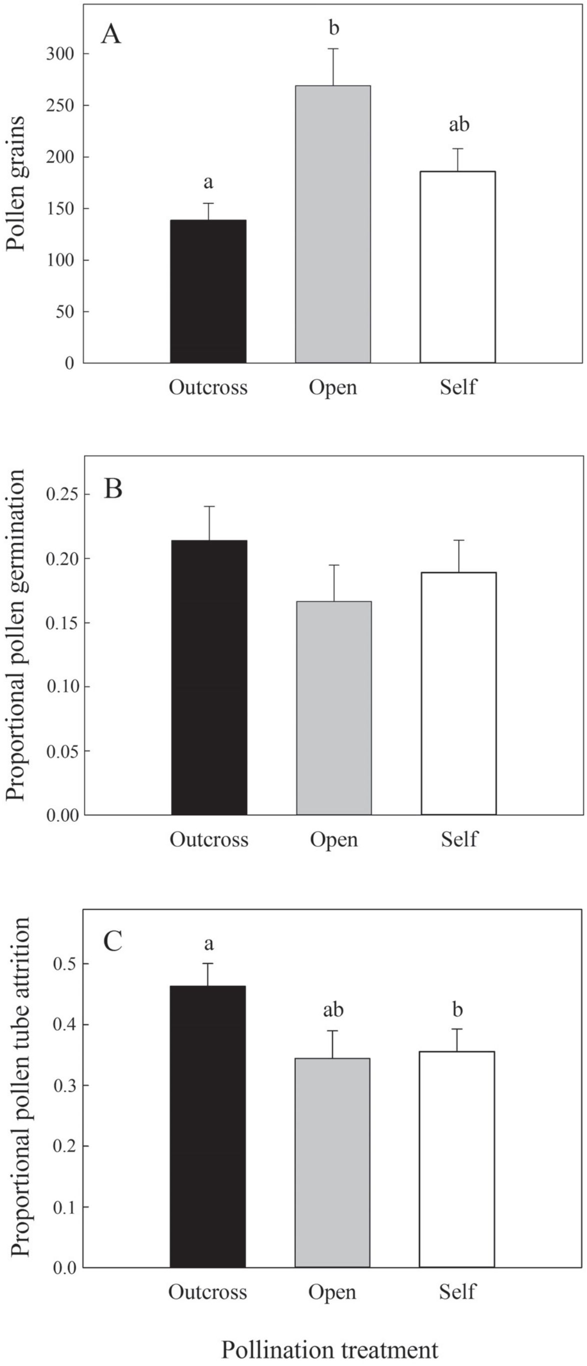
Pollen germination and pollen tube attrition

The number of conspecific pollen grains on the stigma was not affected by herbivory ($F_{1, 25.86} = 1.44$, $P = 0.24$) nor by its interaction with pollination treatment ($F_{2, 54.49} = 0.71$, $P = 0.50$). Pollination treatment, on the other hand, significantly affected the amount of pollen grains deposited on the stigma ($F_{2, 68.97} = 8.31$, $P = 0.0006$), with open pollination resulting in the highest and outcrossing in the lowest stigmatic pollen loads (Fig. 4.1a).

Pollen germination was not affected by herbivory ($F_{1,\,23.67} = 0.01$, $P = 0.91$), nor by pollination treatment ($F_{2,\,71.06} = 0.78$, $P = 0.46$; Fig. 4.1b) or the interaction between both factors ($F_{2,\,55.11} = 1.02$, $P = 0.37$).

Pollination treatment significantly affected pollen tube attrition ($F_{2,\,64.61} = 4.26$, $P = 0.02$), with a higher proportional number of pollen tubes that did not reach the ovary in outcross-pollinated flowers compared to self-pollinated flowers and open-pollinated flowers (Fig. 4.1c). Again pollen tube attrition rates were neither significantly affected by herbivory ($F_{1,\,18.09} = 0.75$, $P = 0.40$) nor by its interaction with pollination treatment ($F_{2,\,47.88} = 0.33$, $P = 0.72$).

Fig. 4.1 (next page). Effect of pollination treatment (outcross, open and self pollination) in *Cynoglossum officinale* plants on (A) average number of conspecific pollen grains on the stigma, (B) average proportion of conspecific pollen grains that germinated and resulted in pollen tube growth into the stigmatic tissue, and (C) average proportion of pollen tubes that failed to reach the base of the style. Error bars represent standard errors. Different letters indicate significant differences between pollination treatments ($P_{Tukey\ adj} < 0.05$).


A
Pollen grains
0
50
100
150
200
250
300
a
b
ab
Outcross
Open
Self
B
Proportional pollen germination
0.00
0.05
0.10
0.15
0.20
0.25
Outcross
Open
Self
C
Proportional pollen tube attrition
0.0
0.1
0.2
0.3
0.4
0.5
a
ab
b
Outcross
Open
Self
Pollination treatment

Fruit set and seed initiation, abortion and maturation

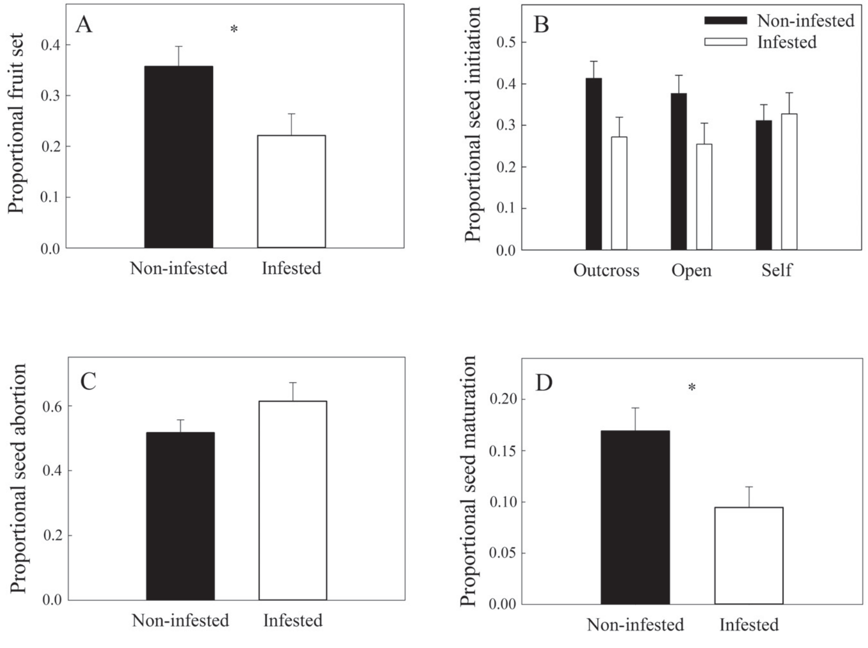
The proportion of treated flowers that set fruit was higher in non-infested plants compared to infested plants ($F_{1, 29.09} = 4.85$, $P = 0.04$; Fig. 4.2a). Fruit set did not differ among pollination treatments ($F_{2, 390} = 1.76$, $P = 0.17$) and there was also no significant interaction between pollination treatment and herbivory ($F_{2, 576.8} = 2.14$, $P = 0.12$).

The proportion of initiated seeds tended to be affected by herbivory ($F_{1, 30.33} = 2.85$, $P = 0.10$), though this effect also depended on pollination treatment as indicated by the significant interaction effect between herbivory and pollination treatment ($F_{2, 510.4} = 3.56$, $P = 0.03$; Fig. 4.2b). Five pairwise comparisons using $\alpha = 0.01$ to correct for multiple comparisons showed that seed initiation in non-infested plants compared to infested plants was higher in outcrossed flowers ($t_{73.16} = 2.20$, $P = 0.03$) and in open pollinated flowers ($t_{46.16} = 2.07$, $P = 0.04$), but did not differ in selfed flowers ($t_{59.76} = -0.27$, $P = 0.79$). Seed initiation in non-infested plants, on the other hand, was significantly higher in outcrossed compared to selfed flowers ($t_{704.5} = 2.64$, $P = 0.009$) while it did not differ between selfed and outcrossed flowers in infested plants ($t_{712.1} = 1.07$, $P = 0.28$). Pollination treatment itself did not influence the proportion of initiated seeds ($F_{2, 362.4} = 0.24$, $P = 0.79$).

The proportion of aborted seeds was neither affected by herbivory ($F_{1, 28.81} = 1.91$, $P = 0.18$; Fig. 4.2c), nor by pollination treatment ($F_{2, 59.38} = 0.24$, $P = 0.79$), and both factors did not interact significantly ($F_{2, 55.04} = 0.08$, $P = 0.92$).

The proportion of matured seeds was higher in non-infested plants compared to infested plants ($F_{1, 29.4} = 5.49$, $P = 0.03$; Fig. 4.2d). Pollination treatment did not significantly affect the proportion of matured seeds ($F_{2, 398.6} = 0.27$, $P = 0.76$), nor did it interact significantly with herbivory ($F_{2, 555.9} = 1.60$, $P = 0.20$).

Fig. 4.2. Impact of root herbivory by *Mogulones cruciger* (non-infested vs. infested) in *Cynoglossum officinale* plants on (A) average proportion of treated flowers that set fruit, (B) average proportion of ovules resulting in initiated seeds. The herbivory effect depended on pollination treatment (outcross vs. open vs. self pollination). (C) Average proportion of initiated seeds that were aborted. (D) Average proportion of ovules resulting in matured seeds. Error bars represent standard errors. Significant differences are indicated: * P < 0.05



Pollen tube competition

The interactions between the intensity of pollen tube competition on the one hand and pollination treatment or herbivory on the other were not of significant importance for fruit set ($F_{2, 76} = 0.40$, $P =$

0.67 and $F_{1,\,78} = 0.68$, $P = 0.41$ respectively). Fruit set was also not influenced by the intensity of pollen tube competition ($F_{1,\,80} = 0.53$, $P = 0.47$).

The proportion of initiated seeds increased significantly with intensity of pollen tube competition ($F_{1,\,72.77} = 4.65$, $P = 0.03$), but this was only the case in infested plants ($\beta = 0.039$, $SE = 0.019$ compared to $\beta = 0.008$, $SE = 0.010$ in non-infested plants; $F_{1,\,68.34} = 2.17$, $P = 0.15$). The relationship between the intensity of pollen tube competition and the proportion of initiated seeds was not affected by pollination treatment ($F_{2,\,65.82} = 0.21$, $P = 0.81$).

The interactions between the intensity of pollen tube competition on the one hand and herbivory or pollination treatment on the other were not of significant importance for seed maturation ($F_{1,\,78} = 0.67$, $P = 0.41$ and $F_{2,\,76} = 0.31$, $P = 0.73$ respectively). Seed maturation was also not influenced by the intensity of pollen tube competition ($F_{1,\,79} = 0.03$, $P = 0.86$).

DISCUSSION

In our study, herbivory did not affect the early stages after pollination, such as pollen germination and pollen tube attrition. However, later stages, like fruit set and seed maturation, were significantly affected by herbivory. Herbivory also influenced the presence of pollen tube competition driven selection on seed initiation.

Pre-zygotic stage of reproduction

Herbivory did not affect pollen germination or pollen tube attrition in the open pollinated flowers and also not in the hand pollinated flowers in which quality of the deposited pollen was controlled for. Although pollen tubes become dependent on the resources available in the style during their growth (Herrero and Hormaza, 1996), our postulation that herbivory might affect pollen tube growth by decreasing the nutritional capacity of the recipient plant did not hold. Possibly at the time of pollen tube growth, infested plants did not experience that much resource limitation because the damage in the roots was not that extensive yet and this did not limit the resources available in the style to support pollen tube growth. In nature, a small proportion of ovipositions occur already in autumn and early spring, but the vast share of ovipositions occurs in April (Schwarzlaender, 1997). Consequently, the amount of root damage at the time of pollen tube growth will in most cases not be larger than in our experiment such that root herbivory by *M. cruciger* may rarely impact early reproductive processes in *C. officinale*.

We did find a significant effect of pollen quality (self vs. outcross) on pollen tube attrition, with outcross-pollinated flowers experiencing 30% more pollen tube attrition than self-pollinated flowers. However, other studies documented contrasting or neutral effects of pollination treatment on pollen tube growth. For instance, in the self-compatible *Erythronium grandiflorum*, a decreased number of pollen tubes was documented to reach the base of the style after self pollination (Cruzan, 1990), whereas in the largely self-sterile *Cyrtanthus breviflorus* no difference in growth between self and outcross pollen tubes was observed (Vaughton et al., 2010). Although it is known that *C. officinale* is able to produce seeds after self pollination (Klinkhamer and de Jong, 1987; Vrieling et al., 1999), until now no insights were available on the effects of pollen quality on pollen germination and pollen tube attrition in this species.

The difference in pollen tube attrition between outcross- and self-pollinated flowers that we observed in *C. officinale* might be explained by the composition of the pollen pool. Cruzan (1990) for instance reported that the attrition of certain pollen tubes may depend upon the source of other pollen tubes growing in the same style in *Erythronium grandiflorum*. Given the observation that in open pollinated flowers of an experimental *C. officinale* population selfing rates were on average 0.32, ranging from 0 to 0.70 (Vrieling et al., 1999), it might be possible that pollen tube competition is more important in outcrossed flowers than in open and self-pollinated flowers. For the latter two, selection of compatible pollen at the stage of pollen germination might be of larger importance (Fig. 4.1b and 4.1c). In the open pollinated flowers and to a lesser extent also in the self-pollinated flowers, the stigmatic pollen load was also higher than in outcross-pollinated flowers (Fig. 4.1a). This could have caused a stigmatic clogging effect, which can impede pollen germination and pollen tube growth of pollen grains (Snow, 1986; Thomson, 1989). Under such conditions, it can be expected that only the fittest pollen grains succeed to germinate. In the outcross-pollinated flowers, on the other hand, selection probably did not yet or to a much lesser extent occur at this stage. Another plausible explanation might be that some of the crosses in the outcrossed flowers may have suffered from outbreeding depression that is reflected in higher pollen tube attrition. The crosses were made with pollen from three other study plants. Because these study plants came from a large population, probably some of the crosses were between plants that would under natural circumstances not cross because the distance between them was too large.

Post-zygotic stage of reproduction

Herbivory provoked reductions in the proportions of fruit set and matured seeds over all pollination treatments. All in all, the treated flowers on non-infested plants set 61% more fruits and produced 79% more matured seeds than those on infested plants (Fig. 4.2a and Fig. 4.2d). We hypothesize that the impact of root herbivory on reproduction in this plant species is due to a decrease in nutritional capacity because the larvae 1) consume the resources that were stored in the tap root during the years of vegetative growth, and/or 2) damage the transport system within the plant, as was also suggested in the studies of Prins et al. (1992) and Ghyselen et al. (2016; chapter three).

The number of initiated seeds tended to be affected by herbivory, though this effect depended on pollination treatment. In outcross- and open pollinated flowers, a lower number of seeds were initiated in infested plants compared to non-infested plants, while selfed flowers did not show such a difference (Fig. 4.2b). Probably herbivory affected the number of initiated seeds by limiting the resources available for seed production. Although we do not know whether the ovules in which no seeds were initiated were actually fertilized, the omnipresence of pollen tubes in the styles of flowers that did not initiate any seeds (C. Ghyselen, Ghent University, personal observation), in combination with earlier indications that there is more abortion than meets the eye in *C. officinale* (de Jong and Klinkhamer, 1989), suggests that these ovules were fertilized and aborted at a very early stage of zygote development.

The lower number of initiated seeds in self-pollinated flowers compared to outcross-pollinated flowers in non-infested plants might point to selective abortion of some of the selfed seeds owing to early-acting inbreeding depression (Husband and Schemske, 1996). In open pollinated flowers of non-infested plants we found an intermediate proportional seed initiation compared to outcross- and self-pollinated flowers of non-infested plants, which probably indicates the presence of a mix of outcross and self pollen in these flowers. In the infested plants we did not find an obvious intermediate proportional seed initiation in open pollinated flowers. Though we expect a lower proportion of self pollen deposition onto those stigmas compared to open pollinated flowers of non-infested plants, our results do not unequivocally support this hypothesis, possibly because the negative effect of herbivory through a decreased nutritional capacity overrides an effect of pollen quality. An important note is that in this study we compared flowers that were hand-pollinated with either only outcross or only self pollen, while open pollinated flowers of *C. officinale* most probably receive a mixture of both (Vrieling et al., 1999). In some other self-compatible species outcross pollen tubes may reach ovules more quickly than self pollen tubes, thereby reducing self-fertilization in flowers with a mixture of both types of pollen (Hessing, 1989; Aizen et al., 1990). In the self-compatible *Erythronium grandiflorum* significantly more seeds were sired by outcross pollen following simultaneous application of equal amounts of self and outcross pollen due to mechanisms operating after ovule fertilization (Rigney et al., 1993). To further investigate a possible effect of herbivory on pollen quality —that is, the proportion of self vs. outcross pollen that arrives on the stigma— and its consequences for seed initiation, the use of genetic markers is needed to determine the parentage in flowers that received a certain proportion of self versus outcross pollen, and in open pollinated flowers in response to herbivory in *C. officinale*.

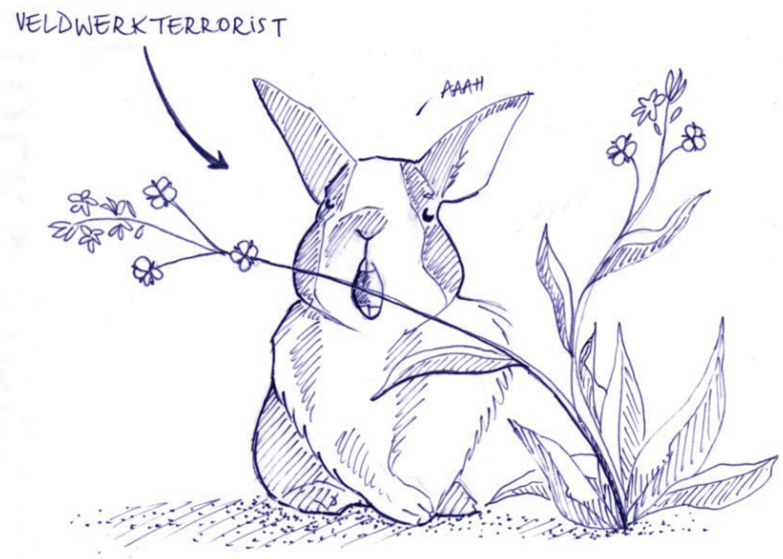
Seed abortion was not affected by herbivory in our experiment, but there was a trend toward more aborted seeds in infested compared to non-infested plants (Fig. 4.2c). Since the quality of deposited pollen did not influence seed abortion, again a decreased nutritional capacity causing higher seed abortion in infested plants is the most plausible explanation for this observation. It has indeed been shown that abortion rates tend to increase when resources become more constrained due to herbivory or other causes (Marshall and Ellstrand, 1988; Niesenbaum, 1996).

Pollen tube competition

In an earlier study on *C. officinale* we found that the mean number of pollen tubes growing in the style was almost eight times higher than the number of ovules in open pollinated flowers (Ghyselen et al., 2016; chapter three), indicating a large potential for pollen tube competition. It was already known that *C. officinale* can selectively abort seeds, resulting in offspring of better quality (Melser and Klinkhamer, 2001) and that intensity of pollen competition is generally positively linked to offspring quality (Skogsmyr and Lankinen, 2002). Here we found a positive relationship between the number of pollen tubes and seed initiation success, indicating that zygotes of lower quality are preferentially aborted, though only in infested plants. Our results thus empirically confirm the hypothesis that the degree of competition driven selection at the level of pollen tube growth, may increase when resources become more limited due to herbivory (Niesenbaum, 1996).

Conclusion

In this study herbivory did not affect early stages in the process from pollination to seed maturation, but it did impact later stages like fruit set, seed initiation and maturation, and the presence of pollen tube competition driven selection on zygote development. Our findings suggest that the impact of herbivory on pollen quality as previously documented (Ghyselen et al., 2016, chapter three), was not responsible for the observed differences in fruit and seed production. Instead, a decreased nutritional capacity of the mother plant due to root herbivory most probably caused higher zygote and fruit abortion, and is presumed to be primarily responsible for the observed reduction in female reproductive success. Our study stresses the importance of assessing the impact of herbivory at the different stages in the process from pollination to seed maturation to better understand the potential cause(s) of herbivory effects on final reproductive output.



Milena Sonneveld

5. Fitness costs following self pollination at different life stages in the monocarpic *Cynoglossum officinale*

Céline Ghyselen, Rein Brys and Dries Bonte

ABSTRACT

In hermaphroditic plant species, self pollination may incur fitness costs when there is inbreeding depression. Many species therefore developed breeding systems or flowering strategies to enhance outcrossing and attract pollinators. As a by-product of increased attraction of pollinators, plants are, however, often inevitably subjected to increased geitonogamous self pollination. In this study we investigate the consequences of self pollination at several life stages in the predominant outcrossing *Cynoglossum officinale*. We assessed maternal seed set and weight, germination success, seedling size, survivorship, flowering behavior and seed set of offspring derived from experimental self pollination versus outcross pollination. Our results indicate relatively small fitness costs of self pollination for certain life stages. For instance, outcrossing resulted in larger seedlings and more successful reproductive output in the offspring compared to selfing. However, since these differences between outcrossed and selfed progeny were only marginally significant and multiple variables were tested separately, these results may have arisen by chance. Consequently, it cannot be concluded that there were clear signs of inbreeding depression in this study. Nevertheless, under real life circumstances sibling competition might enhance differences between outcrossed and selfed progeny in seedling size, mortality and reproductive output. Further research to fitness costs of self pollination at the different life stages in *C. officinale* under natural circumstances is thus needed to

elucidate whether inbreeding depression is of importance for this plant species in nature.

INTRODUCTION

The majority of flowering plants in the plant kingdom are characterized by hermaphroditic flowers (Barrett, 2002). At anthesis, stigmas of this type of flowers can be exposed to several modes of self pollination. Selfing can occur within (autogamy) or among (geitonogamy) flowers on an individual plant, and within-flower selfing can be either vector-mediated (facilitated) or autonomous (Goodwillie et al., 2005). Geitonogamous pollination occurs when pollinators visit multiple flowers and transfer pollen in a sequence on the same plant (Hessing, 1988; De Jong et al., 1993). This kind of flower-visiting behavior occurs widely in different pollinator species (Pyke, 1978, 1981; Waser, 1982). Flowering strategies that plants developed to improve outcrossing success and to attract more pollinators, such as large floral display size (Klinkhamer et al., 1989; Harder and Barrett, 1995; Vrieling et al., 1999), high nectar production (Klinkhamer and de Jong, 1993) etc., however, often increase the amount of geitonogamous pollination (De Jong et al., 1993; Harder and Barrett, 1995). The mechanism of dichogamy, in which pollen and stigmas are presented at a different time within a flower, may diminish geitonogamous pollination depending on the direction of dichogamy (protandry or protogyny), the order of flower development (acropetalous or basipetalous) and the movement pattern between flowers of foraging pollinators.

Geitonogamy is, however, only excluded in the relatively rare case of absolute synchronous dichogamy, in which all open flowers are in the same phase (male or female) (Lloyd and Webb, 1986). As a result, most plant species that have more than one hermaphrodite flower open at the same time, can often not avoid geitonogamous pollination (Snow et al., 1996). On the other hand, other factors can additionally influence the amount of geitonogamous pollination in plants indirectly, via, for instance, variable types of herbivory (Steets et al., 2006). As such, the amount of geitonogamous pollination can vary largely between plant individuals, populations and species.

The deposition of self pollen may lead to self-fertilization, and in self-compatible plants this can impose fitness costs when such species are vulnerable for inbreeding depression (Hessing, 1988; de Jong et al., 1993). Inbreeding depression is the reduction in viability and/or fecundity of selfed offspring relative to outcrossed offspring. Outcrossing plant species often express more inbreeding depression throughout their whole life cycle in comparison to self-fertilizing species (Husband and Schemske, 1996). Inbreeding depression may also vary among different populations according to their genetic structure (Charlesworth and Charlesworth, 1987). To investigate whether self pollination has fitness costs, the offspring from the same maternal plant of self-fertilization should be compared to its counterpart in this context, namely the offspring of outcross-fertilization resulting from crosses between plants from that same population.

Here, we investigate fitness effects of self pollination at several life stages in four coastal populations of *Cynoglossum officinale*. This research question is especially relevant for this species since it is a self-compatible species that, due to a very limited capacity to reproduce autonomously, greatly depends on insect pollination, mainly bees and bumble bees, to realize successful pollination and seed production (de Jong et al., 1990; Vrieling et al., 1999). These pollinators, however, mostly visit multiple flowers in sequence on this plant (Klinkhamer et al., 1989; Ghyselen et al., 2016; chapter three). The actual selfing rate estimated by DNA fingerprinting in an experimental set up in this species ranged between 0 and 0.70 with an average of 0.32 (Vrieling et al. 1999), and this could predominantly be ascribed to self-fertilization through geitonogamous pollination. Previous work on this species also revealed that the amount of geitonogamous pollination can vary largely, depending on floral display size (Klinkhamer et al., 1989; Rademaker and de Jong, 1998) and on the impact of root herbivory presence (Ghyselen et al., 2016; chapter three). However, whether selfing imposes a fitness cost in *C. officinale* is not well studied. Melser and Klinkhamer (2001) mention in their paper unpublished data which indicate that selfing and outcrossing produced on average an equal number of seeds and that the resulting offspring showed slight differences in survival but had a roughly equal reproductive output. Unfortunately, Melser and Klinkhamer have as yet not put forward these results in a publication.

In this study, we investigate the effect of self versus outcross pollination on female reproductive output and on offspring fitness to investigate whether selfing imposes fitness costs in this plant species. We performed experimental pollinations in four coastal populations of *C. officinale* and assessed maternal seed set and weight, and germination success, seedling size, survivorship, flowering behavior and seed set of the offspring.

MATERIAL AND METHODS

Study species

Cynoglossum officinale is a monocarpic perennial in which flowering is strongly size-dependent. This species is characterized by a threshold size below which plants do not flower, and most plants in the field only reach this size after two or more years of growth (de Jong et al., 1986). On the flowering stalk, partial inflorescences (cymes) develop from the axils of alternate stem leaves, each with up to 35 but often no more than 10 flowers. Flowering starts at the top of the plant and usually two to three flowers are simultaneously open on each cyme. The daily floral display size measured in our four study populations ranged from 1 to 314 with an average of 31 (Ghyselen et al., unpublished results). Each flower develops four ovules (de Jong et al., 1990). It was reported that the flowers are protogynous (Kerner, 1895 in Lloyd, 1986), however, de Jong et al. (1990) contest this and pose that the flowers are homogamous. The roots of *C. officinale* plants can be infested by the specialist root weevil *Mogulones cruciger*. This type of root herbivory decreases the number of flowers visited sequentially on *C. officinale* plants (Ghyselen et al., 2016; chapter three).

Study plants and experimental pollinations

In spring 2010, we selected four *C. officinale* populations at the Belgian coast: Knokke (N 51.36497, E 3.35032), Zeebrugge (N 51.32669, E 3.16910), Bredene (N 51.25369, E 2.97080) and Oostduinkerke (N 51.13851, E 2.69207). Per population, we selected 15 study plants on which hand-pollinations were performed during peak flowering. On each study plant, manual outcross and self pollinations were performed. Two different cymes were selected per plant, and in each of these marked cymes, all flowers received the same pollination treatment (selfing or outcrossing) to avoid confounding effects of differential resource allocation between flowers (Wesselingh, 2007). Each of these selected cymes was bagged with bridal veil prior to anthesis of the treated flowers to prevent pollinator visitation and contamination of the pollination treatments. Treated flowers were first emasculated by removal of the corolla onto which the stamens are inserted (de Jong et al. 1990; Quilichini et al., 2001). Then they were hand pollinated by rubbing three anthers from flowers of three other study plants from the same local population onto the stigma in outcross treatment flowers and three anthers from flowers of the study plant itself onto the stigma in self treatment flowers. Each of the populations was visited every four days and additional flowers were pollinated if possible. This resulted in 1 up to 12 treated flowers per cyme (on average 6 flowers).

Fitness measurements and offspring rearing

After seed ripening we counted the number of full-grown nutlets per treated flower and calculated maternal seed set per flower by dividing the number of nutlets by the total number of ovules (=four). Due to unavoidable natural and human disturbance, we lost 13 plants from the experimental setup. Of the remaining 47 plants, only 26 plants produced nutlets for both the outcross and self pollination treatments. Eight plants produced no nutlets for both the outcross and self pollination treatments, six plants produced no nutlets for the outcross treatment and seven plants produced no nutlets for the self treatment. After harvesting, we moisturized the nutlets for one day in cold conditions (5°C) after which the pericarp was removed. Each seed (in total 349 seeds) was then weighted and sown in a petri-dish filled with a moist 50:50 mixture of dune sand and potting soil. All samples were placed in the growth chamber, and germination was assessed nine days after sowing. After this period of time no more additional seeds germinated and all seedlings were transplanted into rectangular trays of 0.10 x 0.40 x 0.45 m filled with the same moist sand/soil mixture. In each of these containers (11 in total), seedlings were arranged in 6 rows of each time 6 seedlings so that the spacing between them and between seedlings and the border of the container was 5.5 to 6.5 cm. Seedlings of the same maternal plant were arranged next to each other, so they went mostly in the same container. The containers were placed in a random design in the growth chambers and were watered twice a week.

One month after transplanting, seedling growth was tracked by measuring the number of leaves, length of the largest leaf and root crown diameter every seven days during 5 consecutive weeks. Four weeks after the last measurement, when seedlings were 3 months old, plants were transplanted to separate cylindrical 2-L containers filled with the moist sand/soil mixture. Because of logistic restrictions, a random selection was made from the remaining offspring to obtain a smaller and more balanced design (i.e., equal number of self and outcross offspring per mother plant). On each of these transplants we then measured the number of leaves, length of the largest leaf and root crown diameter when offspring was 3 (February 2011) and 11 (October 2011) months old, after which plants were put outside to be vernalized during winter time (2011-2012). Because none of the offspring plants started to flower after this vernalization period, most probably because the threshold size to flower was not yet reached (de Jong et al., 1986), we further reared these plants in the growth chamber. In winter 2012-2013 and in summer 2013 a teaspoon of fertilizer pellets (ECOstyle Siertuin-Extra) was added to each plant container.

In November 2013 all surviving offspring plants were put outside in an experimental garden at the Belgian coast in Wenduine to vernalize (since autumn 2011, 21 outcrossed and 19 selfed offspring plants died). In spring 2014 flowering was surveyed in each plant. Flowering plants were left outside in the garden where they were subject to open pollination by natural pollinators of *C. officinale* from the surrounding dune areas. From the plants that flowered, we assessed floral display size and the duration of flowering on a weekly basis and flowering stem height, the number of cymes, the total number of flowers and the total number of full-grown nutlets after seed ripening. Offspring seed set was calculated by dividing the total number of full-grown nutlets by the total number of ovules (= total number of flowers x four). Survival of all offspring plants was measured at the end of the first growth year (before the first winter) and in summer 2014. At several time points during rearing of the offspring, the offspring plants of which all their counterparts (outcross or self) of the same maternal plant had died were discarded since pollination treatment could not be compared anymore within the same maternal plant.

Statistical analyses

All analyses were performed in SAS 9.4. (SAS Institute Inc., 2013) using PROC GLIMMIX or PROC MIXED with estimation of the number of degrees of freedom by the Kenward Rodgers procedure. Where necessary, we corrected for under- or overdispersion. For each variable we ran a model with pollination treatment, population and their interaction as explanatory variables. Insignificant interactions or variables were dismissed following a backward selection procedure. In each model maternal plant and its interaction with pollination treatment were specified as random grouping variables.

Seed traits and germination success—For the response variable proportional maternal seed set per flower a binomial distribution was specified, and in this model we also included flower position on the cyme as cofactor. We specified a normal distribution for seed weight.

For germination we specified a binomial distribution, and we included seed weight as cofactor.

Seedling growth and size—We specified a Poisson distribution for number of leaves and a normal distribution for length of the longest leaf and root crown diameter. We ran a model to analyze the growth of each of these variables from week 5 to week 9, therefore the numbering of the weeks was added as a cofactor in the model and offspring plant identity was specified as random effect to correct for the repeated measures. Seedling sizes at 3 and 11 months were analyzed in separate models. In each of these models we included seed weight and the label of the rectangular container in which seedlings were reared as cofactors whenever these were significant.

Survival and reproduction—Both survival in the first year and lifetime survival (until summer 2014) were binomially distributed. In the models of survival in the first year we included seed weight as cofactor. The response variable 'flowering in 2014' was also binomially distributed. For flowering stem height we specified a normal distribution, and for floral display size, number of flowering days, total number of flowers and number of cymes we specified a Poisson distribution. For floral display size, which was measured every week, we also specified offspring plant identity as random grouping variable in the model to correct for the repeated measures. Offspring seed set was binomially distributed.

Inbreeding depression indices—To quantify the level of inbreeding depression (further denoted by *ID*) for each maternal plant in the study, we calculated an *ID* index that compares the relative performance of selfed and outcrossed progeny: $\delta = (w_o\text{-}w_s) / max\ (w_o,w_s)$, where w_o is the average performance of outcrossed progeny, w_s is the average performance of selfed progeny and $max\ (w_o,w_s)$ is the average performance of the progeny group with the highest average performance (Agren and Schemske, 1993). For each variable we then calculated the overall mean inbreeding depression index and 95% confidence intervals (95% *CI*'s) using the bootstrap procedure (n = 1000 bootstraps) implemented in PopTools (Hood, 2010). For each maternal plant that had both outcrossed and selfed offspring flowering in 2014, we calculated total fitness resulting per pollination treatment as the product of proportional maternal seed set, germination success, lifetime survival, total number of flowers produced and offspring seed set. For this variable of total or cumulative fitness, we also calculated the mean inbreeding depression index and the 95% *CI*.

RESULTS

Seed traits and germination success

Maternal seed set did not differ significantly between self-pollinated and outcross-pollinated flowers and this was the same in all four populations (Table 5.1). Maternal seed set was strongly related to flower position: seed set decreased with distance from the main stalk (β = -0.35, SE = 0.04, $F_{1,\,419.2}$ = 63.63, $P < 0.0001$). Seed weight and germination also did not differ in response to pollination treatment in all four populations (Table 5.1). The probability to germinate significantly increased with seed weight ($\beta = 570$, $SE = 201$, $F_{1,343} = 8.08$, $P = 0.005$).

Seedling growth and size

The number of leaves increased significantly during the five weeks that we measured seedling growth ($\beta = 0.151$, $SE = 0.003$, $F_{1, 885.5} = 45.56$, $P < 0.0001$). There were no significant differences between offspring resulting from outcross pollination and offspring resulting from self pollination in the slope of this increase ($F_{1, 887.2} = 1.10$, $P = 0.30$), nor in the intercept ($F_{1, 11.59} = 0.25$, $P = 0.63$). The interaction between population and pollination treatment was not significant for the number of leaves during these five weeks ($F_{3, 10.3} = 0.43$, $P = 0.74$).

Also the length of the longest leaf increased significantly with time ($\beta = 7.38$, $SE = 0.17$, $F_{1, 887} = 1940.29$, $P < 0.0001$). The slope of this increase did not differ in response to pollination treatment ($F_{1, 887} = 1.37$, $P = 0.24$). Though the length of the longest leaf was in each week higher in offspring resulting from outcross pollination compared to offspring resulting from self pollination, the difference in intercept was not significant ($F_{1, 13.2} = 2.04$, $P = 0.18$). The interaction between population and pollination treatment was not significant ($F_{3, 11.3} = 0.49$, $P = 0.70$).

Root crown diameter also increased significantly with time ($\beta = 0.13$, $SE = 0.005$, $F_{1, 867} = 779.61$, $P < 0.0001$) and neither the slope of this increase ($F_{1, 866} = 0.16$, $P = 0.69$) nor the intercept ($F_{1, 13.7} = 0.20$, $P = 0.66$) differed significantly between offspring resulting from outcross pollination and offspring resulting from self pollination. The interaction between population and pollination treatment was not significant ($F_{3,\ 12.6} = 0.36$, $P = 0.78$).

Selfed and outcrossed seedlings did not differ in number of leaves both at three months and at 11 months in all four populations (Table 5.1). The length of the longest leaf was larger in all four populations in offspring resulting from outcross pollination compared to offspring resulting from self pollination when seedlings were three months old. When seedlings were 11 months old this difference was no longer significant (Table 5.1). Root crown diameter did not differ significantly between outcrossed and selfed seedlings both at three months and at 11 months in all four populations (Table 5.1).

Survival and reproduction

Survival in the first year as well as until summer 2014 did not differ significantly (Table 5.1). Survival in the first year was positively correlated to seed weight ($\beta = 122$, $SE = 49$, $F_{1,\ 123.1} = 6.32$, $P = 0.01$). Pollination treatment did not affect any of the flowering traits we measured in all four populations (Table 5.1). Offspring seed set (resulting from open pollination) tended to differ in response to pollination treatment in all four populations, with higher seed set in offspring resulting from outcross pollination compared to offspring resulting from self pollination (Table 5.1).

Inbreeding depression indices

For offspring seed set and lifetime survival the inbreeding depression indices were the highest (0.24 and 0.19 respectively). However, only the 95% *CI* of offspring seed set was strictly positive (Table 5.1). The 95% *CI*'s for number of leaves at 11 months and for root crown diameter at 11 months did also not include negative values and the 95% *CI* for length of the longest leaf at 3 months contained only a very small percentage of the negative values (-0.005 to 0.17; Table 5.1). Except for the variables flowering in 2014 and total number of flowers mean inbreeding depression indices were always positive, indicating that on average the outcrossed progeny outperformed the selfed progeny (Table 5.1). The mean inbreeding depression index for total fitness was 0.21 and the 95% *CI* for this was -0.10 to 0.50.

Table 5.1 (next page). Results of the statistical analyses for the different variables measured: number of plant individuals (*N*), means ± *SE*'s of outcrossed (outcross) and selfed progeny (self), mean inbreeding depression index (δ), 95% confidence intervals for the mean inbreeding depression index (δ 95% *CI*), model results for the effect of pollination treatment (cross type) and for the interaction between pollination treatment and population (cross type x population).

Response variable	***Outcross***	***Self***	***δ***	***δ 95% CI***	***Cross type***	***Cross type x population***
Maternal seed set (N = 47)	0.148 ± 0.030	0.152 ± 0.031	0.01	-0.20 to 0.23	$F_{1,\,32.09} = 0.03$ $P = 0.86$	$F_{3,\,29.15} = 0.08$ $P = 0.97$
Seed weight (gram) (N = 349)	0.0135 ± 0.0011	0.0125 ± 0.0011	0.03	-0.13 to 0.19	$F_{1,\,42.5} = 0.45$ $P = 0.50$	$F_{3,\,41.3} = 0.67$ $P = 0.58$
Germination (N = 352)	0.96 ± 0.06	0.95 ± 0.07	0.11	-0.09 to 0.30	$F_{1,\,343} = 0.01$ $P = 0.92$	$F_{3,\,340} = 0.03$ $P = 0.99$
Nr. of leaves seedling 3 months (N = 191)	3.66 ± 0.19	3.51 ± 0.19	0.04	-0.09 to 0.15	$F_{1,\,9.673} = 0.61$ $P = 0.45$	$F_{3,\,12.5} = 0.31$ $P = 0.82$
Nr. of leaves seedling 11 months (N = 143)	6.87 ± 0.67	6.81 ± 0.68	0.16	0.00 to 0.32	$F_{1,\,127.7} = 0.02$ $P = 0.89$	$F_{3,\,5.884} = 0.16$ $P = 0.92$
Length longest leaf seedling 3 months (N = 186)	122.52 ± 5.26	109.99 ± 5.51	0.08	-0.01 to 0.17	$F_{1,\,16.2} = 4.98$ $P = 0.04$	$F_{3,\,15.7} = 0.26$ $P = 0.86$
Length longest leaf seedling 11 months (N = 139)	182.45 ± 14.91	169.01 ± 15.35	0.11	-0.03 to 0.27	$F_{1,\,9.23} = 0.87$ $P = 0.38$	$F_{3,\,9.88} = 0.22$ $P = 0.88$
Root crown diameter seedling 3 months (N = 186)	2.43 ± 0.11	2.34 ± 0.11	0.01	-0.07 to 0.09	$F_{1,\,15.6} = 0.66$ $P = 0.43$	$F_{3,\,14.4} = 0.13$ $P = 0.94$
Root crown diameter seedling 11 months (N=143)	8.18 ± 0.43	7.79 ± 0.45	0.10	0.02 to 0.17	$F_{1,\,12.6} = 1.19$ $P = 0.30$	$F_{3,\,12.1} = 0.20$ $P = 0.90$
Flowering in 2014 (N = 79)	0.64 ± 0.12	0.71 ± 0.12	-0.08	-0.38 to 0.23	$F_{1,\,8.799} = 0.30$ $P = 0.59$	$F_{3,\,65.32} = 1.11$ $P = 0.35$
Floral display size (N = 44)	6.00 ± 0.85	6.72 ± 0.97	-	-	$F_{1,\,35.02} = 0.61$ $P = 0.44$	$F_{3,\,31.16} = 1.58$ $P = 0.21$
Flowering stem height (N = 44)	25.22 ± 2.14	25.65 ± 2.19	0.04	-0.10 to 0.21	$F_{1,\,34.4} = 0.06$ $P = 0.81$	$F_{3,\,31.6} = 1.83$ $P = 0.16$
Nr. of flowering days (N = 44)	43.96 ± 7.06	42.28 ± 7.41	0.03	-0.21 to 0.27	$F_{1,\,39.42} = 0.03$ $P = 0.85$	$F_{3,\,32.94} = 0.88$ $P = 0.46$

Response variable	***Outcross***	***Self***	***δ***	***δ 95% CI***	***Cross type***	***Cross type x population***
Total nr. of flowers (N = 44)	62.01 ± 7.58	69.38 ± 8.62	-0.02	-0.18 to 0.18	$F_{1, 36.76}$ = 0.85 P = 0.36	$F_{3, 31.8}$ = 0.72 P = 0.55
Nr. of cymes (N = 44)	7.90 ± 0.70	8.39 ± 0.78	0.04	-0.12 to 0.18	$F_{1, 9.048}$ = 0.27 P = 0.62	$F_{3, 10.68}$ = 1.24 P = 0.34
Offspring seed set (N = 44)	0.21 ± 0.02	0.16 ± 0.02	0.24	0.11 to 0.39	$F_{1, 6.485}$ = 3.19 P = 0.12	$F_{3, 30.15}$ = 1.81 P = 0.17
Survival first year (N = 204)	0.79 ± 0.06	0.71 ± 0.08	0.00	-0.20 to 0.22	$F_{1, 6.005}$ = 1.23 P = 0.31	$F_{3, 8.051}$ = 0.07 P = 0.98
Lifetime survival (N = 204)	0.48 ± 0.05	0.39 ± 0.06	0.19	-0.04 to 0.43	$F_{1, 195.3}$ = 1.66 P = 0.20	$F_{3, 187.5}$ = 0.29 P = 0.83

DISCUSSION

Our results indicate relatively small fitness costs of self pollination for certain life stages. Since these differences between outcrossed and selfed progeny were only marginally significant and multiple variables were tested separately, these results may have arisen by chance. Consequently, it cannot be concluded that there were clear signs of inbreeding depression in this study.

Offspring establishment

Both the *ID* indices and the results of the statistical models indicated differences in seedling size. The statistical model results were sometimes slightly different than the results from the *ID* indices, because of the incorporation of cofactors and random effects in the statistical models. The differences found in seedling size at several developmental stages during establishment can be assumed to have important consequences for total plant fitness, since plant size is often highly correlated with survivorship (e.g. Lovett Doust and Lovett Doust, 1988; Silvertown and Lovett Doust, 1994). Also in *C. officinale* this correlation exists: summer survival, when most seedlings are about three months old, depends significantly on size in this species (Wesselingh et al., 1997). Under natural circumstances, seedling mortality is an important vital rate in the population demography of *Cynoglossum officinale* (de Jong and Klinkhamer, 1988) and is much higher than under the optimal and competition free conditions in our growth chamber setup. Moreover, in our experiments, the mean percentage of mortality was 21 % for outcrossed offspring compared to 29 % for selfed offspring, whereas previous work of de Jong and Klinkhamer (1988) revealed that this could be up to 49.3%, 78.0% and 72.0% in three consecutive years (between 1982 and 1984) under natural conditions in the field. It is generally admitted that studies in the greenhouse or growth chamber underestimate the magnitude of inbreeding depression (Husband and Schemske, 1996; Cheptou et al., 2000). In natural situations, it can thus be expected that the differences in seedling size between selfed and outcrossed offspring induce a higher difference in seedling mortality than found here. Moreover, when there is sibling competition this difference in seedling size may also lead to asymmetric competition in which larger individuals obtain a disproportionate share of the resources (for their relative size) and suppress the growth of smaller individuals (Weiner, 1990). In *C. officinale* half of the seeds are dispersed within 0.6 m of the parent plant (Rademaker and de Jong 1999), so both selfed and outcrossed offspring are likely to grow strongly clustered and at high densities around the parent plant. In *Impatiens capensis* and in *Plantago coronopus*, it is for instance shown that when selfed and outcrossed offspring were grown intermixed, selfed offspring became dominated and suppressed by their outcrossed relatives (Schmitt and Ehrhardt, 1990; Koelewijn, 2004). In *C. officinale* seedling weight and survival only decreased at densities above 10 seedlings per dm^2, which are relatively rare in the field (de Jong and Klinkhamer, 1988; Rademaker and de Jong 1999). Still, it cannot be ruled out that, even at moderate densities that occur in the field, selfed offspring are suppressed by their outcross relatives around the parent plant.

Offspring fertility

Because all offspring plants were subjected to open pollination, the lower seed set in selfed offspring compared to outcrossed offspring might ensue that selfed offspring were less attractive for pollinators and therefore had lower seed set compared to crossed offspring. However, since seed set in *C. officinale* has not been reported to be pollen-limited (de Jong and Klinkhamer, 1989), also not in experimental plants subjected to open pollination in the same experimental garden as was used in this study (Ghyselen et al., 2016; chapter three), this difference in seed set might also result from reduced fertility in selfed plants. In a study on *Echium vulgare* offspring plants derived from selfing also produced fewer seeds than outcrossed offspring plants (Melser et al., 1999). Asymmetric competition might again aggravate this cost of self pollination because this kind of competition might enhance the difference in plant size between selfed and outcrossed offspring (Weiner, 1990). In *C. officinale* it is known that larger plants produce more seeds per flower (Klinkhamer and de Jong, 1987). However, size at flowering is determined by the quality and quantity of the growth period before the winter period in which the plants vernalize if they have reached the threshold size to flower (de Jong et al., 1986). As such, outcrossed offspring may, for instance, already reach the threshold size to flower in their second growing season, resulting in fairly small flowering plants, while selfed offspring might just not reach the threshold size in the second growth season and overshoot this largely by the end of the third growth season, resulting in large flowering plants (Vrieling et al., 1999). Our results indicate no difference in probability of flowering during the experiment and no difference in flowering size between selfed and outcrossed offspring. Consequently, the difference in seed set cannot be attributed to plant size here.

Consequences of fitness costs of self pollination under natural circumstances

Several authors have suggested and/or shown that geitonogamous pollination imposes fitness costs in terms of lower seed set or lower offspring fitness due to inbreeding depression (Hessing, 1988; de Jong et al., 1993). However, others argue that the costs associated with geitonogamy are relatively small because pollinators often visit only a limited number of the flowers that are open on a plant (e.g. Best and Bierzychudek, 1982), and pollen carryover allows outcross pollen to reach most of these stigmas (Robertson, 1992). Here, we performed self hand pollinations by rubbing the anthers of flowers of the same plant onto the stigma, meaning that there was no possibility for outcross pollen carryover. Therefore, our results might overestimate the fitness costs of geitonogamous pollination in *C. officinale* in natural situations. Moreover, differential pollen germination, pollen tube growth or seed abortion can occur when there is a mix of self and outcross pollen deposited on the stigma, possibly resulting in more mature outcrossed seeds than expected from the self vs. outcross pollen proportion on the stigma (Cruzan, 1990 and references therein; Owen et al. 2007). Nevertheless, although it is not known whether these differential post-pollination processes occur in *C. officinale*, the study of Vrieling et al. (1999) showed that the number of selfed seeds per flower increase with increasing floral display and that this relationship is accurately predicted by pollen dynamics and pollinator behavior alone.

As argued in the first paragraph of this discussion, the results of our study do not indicate clear signs of fitness costs for *C. officinale* plants that receive large amounts of geitonogamous pollinator visits. However, under real life circumstances sibling competition might enhance differences between outcrossed and selfed progeny in seedling size and mortality and thereby also in reproductive output. Further research to fitness costs of self pollination at the different life stages in *C. officinale* under natural circumstances can thus elucidate whether inbreeding depression is of importance for this plant species in real life circumstances.

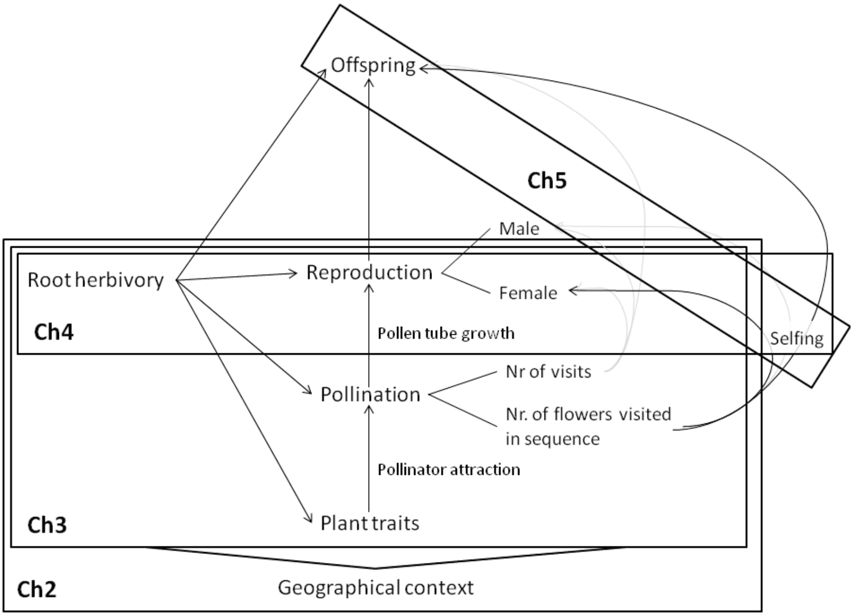
“The bees help the plants to make love”

An Armenian physician about pollination

6. General discussion

Céline Ghyselen

Fig. 6.1. Schematic representation of possible effects of root herbivory on plant performance. The frames indicate the aspects that were studied in each chapter (black arrows: relationships studied in this thesis, gray arrows: relationships that are not studied in this thesis).



OVERVIEW OF THE MAIN FINDINGS

Root herbivory effects on plant traits, pollination and reproduction

The study of Prins et al. (1992) in which a negative effect of *M. cruciger* root herbivory was found on reproductive output in *C. officinale*, provided the inspiration to delve into the subject of herbivory effects on pollination in this PhD research. The main research question, whether root herbivory affected pollination and whether this caused the reduced reproductive output found, was answered both in an observational and an experimental study. The field results showed that root herbivory tended to decrease the number of flowers visited in a sequence on the same plant. Seed production was significantly reduced in response to root herbivory (chapter two), however, this was not pollen-limited in four sampled populations (Ghyselen et al., unpublished results). The results of the experimental study were similar; here we found a significant negative effect of root herbivory both on the frequency of pollinator visitation and on the number of flowers visited in a sequence on the same plant. Seed production was also not pollen-limited, but was still significantly reduced in response to root herbivory. The additional analyses on pollen deposition and pollen tube growth corroborated the finding that the herbivory effect on seed production was not pollinator-mediated (chapter three). Consequently, it can be concluded that there is a negative herbivory effect on pollinator attraction in this study system, but this does not cause the reduced reproductive output.

The correlations between plant traits and root herbivory were different in the observational compared to the experimental study (chapter two and chapter three). This can be explained by the fact that in the observational study root weevils oviposited in plants of their own preference, whereas in the experimental study root weevils were forced to oviposit on plants that were randomly assigned to the herbivory treatment (cfr. Figs. 6.2 and 6.3 which are discussed in a subsequent section).

Geographical structure in herbivore-pollinator interactions

The results in chapter two showed that there is geographical and temporal structure in the relative abundance of *M. cruciger* root herbivory. It would consequently be interesting now to also study geographical differences in the effect of *M. cruciger* on pollinator foraging behavior. Papers in which this subject is addressed in other species are rare, but the studies of Lay et al. (2011, 2013) provide a nice example. Here, the preference of pollinators depends on herbivory in some populations, while in other populations herbivory does not influence pollinator attraction. This study, however, differs from ours in that the diversity of herbivore and pollinator species interacting with the focal plant species is much larger.

Root herbivory effects on the stages between pollen deposition and seed maturation

The results of chapter four demonstrated that root herbivory effects did not affect the early stages after pollination, like pollen germination and pollen tube attrition, but it did affect later stages such as fruit set and seed maturation. Our findings also indicated that the herbivory effect on amount of geitonogamous pollination (chapter two and chapter three) does not affect any of the stages studied. Rather, a decreased nutritional capacity of the mother plant was responsible for the effects of root herbivory on fruit set and seed maturation, as was also hypothesized in Prins et al. (1992).

Consequences for offspring fitness of the herbivory effect on geitonogamous pollination

The results of chapter two and three showed that infested plants receive less geitonogamous pollination visits compared to non-infested plants and this may result in proportionally less selfed seeds per flower (Vrieling et al., 1999). In chapter five I documented relatively small fitness costs of self pollination for certain life stages. Outcrossing resulted in larger seedlings and higher reproductive output in the offspring compared to selfing. But because multiple variables were tested, these results could have arisen by chance and consequently it cannot be concluded that there was inbreeding depression in our study. However, under real life circumstances the differences between outcrossed and selfed progeny may be enhanced due to the effects of sibling competition on seedling size, mortality and reproductive output. Nevertheless, whether the difference in the amount of flowers visited in a sequence between infested and non-infested plants (chapter two and chapter three) would then be large enough to result in a difference in mean offspring fitness between infested and non-infested plants still remains questionable.

CONSEQUENCES OF ROOT HERBIVORY

For female reproductive fitness

Both in the observational study as in the experimental study I explicitly measured female reproductive fitness as the total number of seeds produced (chapter two and chapter three). To illustrate differences between the observational and the experimental study, I plotted the total number of seeds produced against the root crown diameter (Figs. 6.2 and 6.3). The effect of *M. cruciger* preference for plants with large root crown diameters (RCD) is apparent from the ranges in RCD in the observational study: plants with an RCD below ca 7.5 mm are not selected for oviposition. What also can be observed is that the relative difference between infested and non-infested plants is much higher in the experimental study compared to the observational study. At an RCD of 15 mm, for instance, the total number of seeds is ca 410 in non-infested plants and ca 340 in infested plants in the observational study, while it is ca 130 in non-infested plants and ca 65 in infested plants in the experimental study. This means that root herbivory in the observational study caused a decrease of 17% in the total number of seeds produced, whereas in the experimental study a reduction of 50% was observed. Two possible and non-exclusive explanations for this difference between the observational and the experimental study are that 1) the root herbivory to which we subjected the study plants in the experiment was more intense, that is, the percentage of root damaged was higher than the root herbivory in natural field situations, and 2) the experimental plants were generally smaller than the observational plants (see Figs. 6.2 and 6.3) and therefore produced a lower total amount of seeds which caused the relative (though not the absolute) effect of root herbivory on total seed production to be larger. The much higher number of infested plants that produced no seeds (mostly because they died before seed maturation) in the experimental study compared to the observational study corroborates these hypotheses, and is probably also responsible for the difference in slopes between non-infested and infested plants in the experimental study. As a result, I infer that the effects of root herbivory on plant traits, pollinator foraging behavior and seed production found in our experimental study are probably exaggerated when compared to natural field situations in which the root damage inflicted by *M. cruciger* larvae is most likely less intense and in which plants are generally somewhat bigger when flowering. However, variation between populations in intensity of root damage inflicted was clearly observed in the five observational study populations (Ghyselen et al., unpublished results; chapter two), thus at least in some populations the root herbivory effects might be comparable to those found in our experimental study.

Figure 6.2. Correlation between total number of seeds produced and root crown diameter measured after seed maturation in non-infested versus infested plants of the observational study (chapter two).

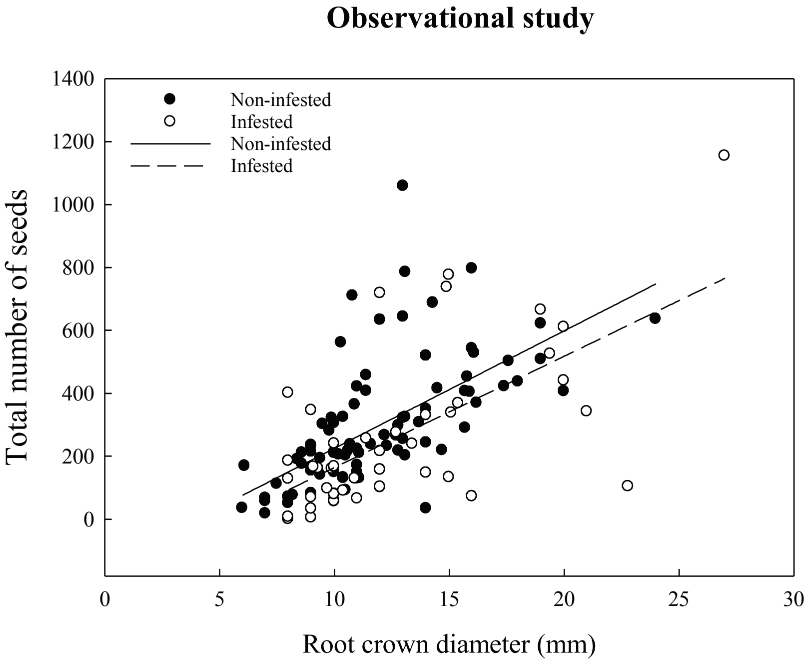
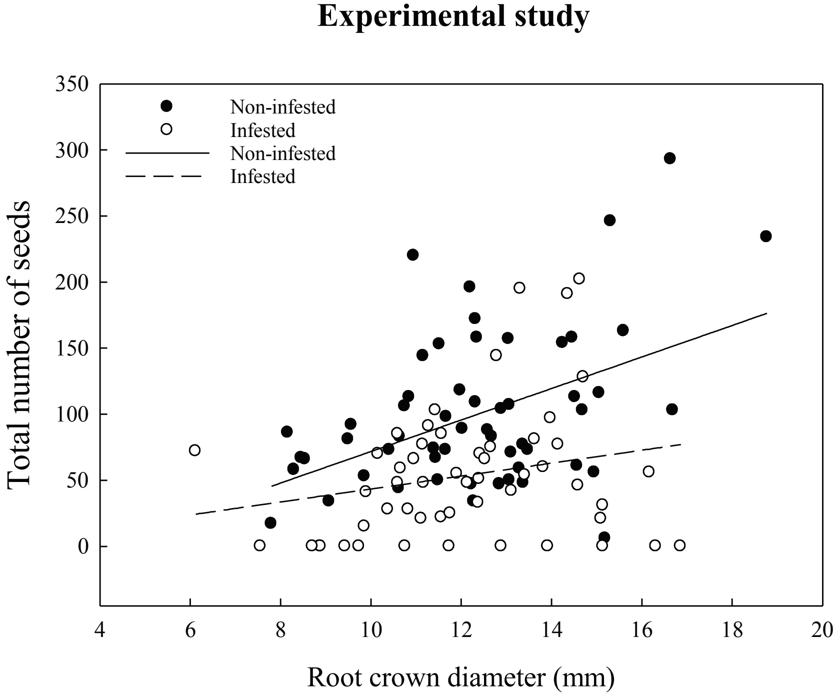


Figure 6.3. Correlation between total number of seeds produced and root crown diameter measured after seed maturation in non-infested versus infested plants of the experimental study (chapter three).



Seed quality is also an important aspect of female reproductive fitness that can be affected by root herbivory. Here, counteracting effects may act. On the one hand, the results of chapter four (Figs. 4.2b and 4.2c) indicated that seed abortion was higher in infested plants compared to non-infested plants. In many plant species, and also in *C. officinale,* seed abortion can be selective, resulting in seeds and offspring of better quality (Melser and Klinkhamer, 2001). On the other hand, infested plants attract less pollinators than non-infested plants (chapter two and three, Figs. 2.3, 3.4 and 3.5), which may result in a lower diversity of pollen deposited on the stigma. This decreases the probability of sampling a genetically compatible sporophytic father (Paschke et al., 2002), and the potential for pollen tube competition in the pistil. The intensity of pollen competition is often positively related to seed or fruit maturation (Niesenbaum and Casper, 1994; Ghyselen et al., 2015, chapter four) and to seed quality (Skogsmyr and Lankinen, 2002). So the latter scenario would lead to seeds of less quality while the first scenario may lead to higher quality seeds in infested plants.

For pollen limitation of female reproductive fitness

Female reproductive fitness can be limited by the quantity as well as by the quality of pollen deposited on the stigma (Ashman et al., 2004). If pollen limitation is assessed using the traditional pollen supplementation method it is hard to distinguish the effects of quantitative and qualitative limitation (Aizen and Harder, 2007). In this thesis I used several methods to be able to assess whether the study plants were quantitatively and/or qualitatively pollen-limited in response to herbivory. In chapter three, I used the traditional pollen supplementation method and I quantified pollen loads and pollen tubes in the style. These results led me to conclude that seed production was not quantitatively limited in the study plants.

In chapter four, we explicitly tested whether pollen quality – that is, self vs. cross pollen- affected seed production (this is called ‘seed maturation’ in chapter four) and, both in the non-infested as well as in the infested plants, this was not the case. In this study I also compared outcross-pollinated flowers with open-pollinated flowers. To assess pollen quality limitation, often seed production resulting from pure outcross pollination is compared with that of natural open pollination (Aizen and Harder, 2007; Vaughton and Ramsey 2010). In our study plants, the seed production of outcross-pollinated flowers did not differ from that of open pollinated flowers, neither in non-infested nor in infested plants (chapter four). It can thus be concluded that root herbivory did not provoke any quantitative or qualitative pollen limitation of seed production in our study plants.

For male reproductive success

Root herbivory affected both the number of flowers and pollinator foraging behavior (chapter two and chapter three) and this can have impacts on male reproductive success. Infested plants produced fewer flowers than non-infested plants, it naturally follows that infested plants then also produced less pollen. A decrease in pollen production in response to herbivory was also found in the study of Quesada et al. (1995), but there, this was the result of reduced production of both staminate flowers and pollen grains per flower. The visitation frequency in infested plants was lower, resulting in less pollen being exported from the flowers. Both of these aspects lead to a lower proportion of seeds sired in infested compared to non-infested paternal plants. This may, however, be counteracted by the effect of root herbivory on the amount of geitonogamous pollination. Fewer flowers in a sequence were visited in infested plants compared to non-infested plants. This means that in these infested plants less pollen will be deposited within the plant, and the fraction of the removed pollen that is actually exported is increased (de Jong, 1993; Klinkhamer and de Jong, 1993).

For evolution of plant traits

The preferences of both the specialist root weevil *M. cruciger* and the pollinators of *C. officinale* for large plants (Prins et al., 1992; Schwarzlaender, 1997; Klinkhamer et al., 1989), and the impact of *M. cruciger* root herbivory on female reproductive fitness (Prins et al., 1992) were documented before, and were also confirmed during this PhD research. Although seed production has always been found to not be pollen-limited in *C. officinale*, pollinators might still impact plant fitness via male reproductive output (Harder and Wilson, 1994). The threshold size to flower in *C. officinale* is a heritable trait (Wesselingh and de Jong, 1995) that shows phenotypic variation within populations (Wesselingh et al., 1993). It is thus likely that natural selection occurs on this trait and that the direction of this selection is in part determined by the presence and abundance of *M. cruciger* and potentially also of the pollinators of *C. officinale.* and of the pollinators of C. officinale. Of course also other factors, such as size-dependent growth and survival are of importance for natural selection on threshold size (Wesselingh et al., 1997).

The results of chapter two indicated that the intensity of selection pressure exerted by *M. cruciger* varies between *C. officinale* populations over time in our study area. As a result, spatial and temporal mosaics of total selection pressures exerted on *C. officinale* populations might exist in which in populations where *M. cruciger* is abundant, there is strong selection pressure for lower threshold size, while in others *M. cruciger* is rare and the selection pressure against large flowering plants is much weaker. Because there are also temporal fluctuations in the spatial differences, these mechanisms stabilize the interaction between *M. cruciger* and *C. officinale* at a regional scale (Wang and Loreau, 2014), and promote genetic variation for threshold size (Thompson, 1999).

PERSPECTIVES FOR FUTURE RESEARCH

The results of this PhD study contribute to the knowledge on the herbivore-plant-pollinator system of *C. officinale*, its specialist root weevil *M. cruciger* and its pollinators. This knowledge is a good foundation to continue further research. Below, I propose some suggestions for further research that appear of particular interest in the light of the results of this PhD research:

1. The results of chapter two provide a take-off to further investigate geographical differences in the intensity of selection pressure exerted by *M. cruciger* in *C. officinale* populations. To investigate if these spatial differences found are relevant for possible evolutionary consequences on threshold size, both the infection rate and the threshold size to flower need to be spatially monitored in the long term. As already mentioned in a previous section of this discussion, it would also be interesting to study geographical differences in the effect of *M. cruciger* on pollinator foraging behavior.

2. In one of the sections above we discuss the possible consequences for male fitness of the root herbivory effects found in our study. However, herbivory in paternal plants might also affect aspects of pollen performance, for example germination ability or pollen tube growth (Quesada et al., 1995; Mutikainen and Delph, 1996). It would be interesting to study these kinds of effects of root herbivory on *C. officinale* in an experimental set-up. Furthermore, it would also be interesting to study the consequences of the root herbivory effect on pollinator foraging behavior for male fitness, for example via experiments with dye powder as an analog for pollen transfer (Adler and Irwin, 2006) or via paternity analyses (Bernasconi, 2003).

3. As mentioned above, I question whether the herbivory effect on the amount of geitonogamous pollination is large enough to result in a difference in mean offspring fitness between infested and non-infested plants. An observational study in which non-infested versus infested maternal plants and their offspring are analyzed genetical-

ly to assess the selfing rate (Vrieling et al., 1999) seems suitable here. Then, ideally, the different life stages of these same offspring could be followed in situ in the field (to also assess effects of sibling competition) and average offspring fitness per maternal plant could be calculated. This average offspring fitness can then be compared to the selfing rates and infection status of the maternal plant.

GENERAL CONCLUSION

The results of this PhD research documented significant interactions between the root weevil *M. cruciger* and the pollinators of *C. officinale*. However, this was not the cause of the reduced seed production in response to root herbivory since seed production was not pollen-limited. Rather a decreased nutritional capacity of the maternal plant due to larval depletion of resources in the root is at the cause of this. There was substantial geographical and temporal variation in the interaction between *M. cruciger* and *C. officinale* in the study populations at the Belgian coast, and this may possibly result in spatial and temporal mosaics of total selection pressures exerted on *C. officinale* populations. Further research in which both the infection rate and the threshold size to flower are spatially monitored in the long term is needed to investigate this hypothesis. Finally, the effects of *M. cruciger* root herbivory on pollinator foraging behavior, namely a decreased pollinator visitation frequency and a decreased amount of geitonogamous pollination visits in infested plants, have further implications for male reproductive fitness and possibly also for offspring fitness.

7. Summary

Herbivory and pollination are probably the two most important biotic interactions that impact plant fitness. The interactions between herbivores and pollinators have only recently received much attention. Regarding this subject, belowground herbivory has been largely neglected and also further reaching effects of the impact of herbivory on the process of pollination have received little attention. In this thesis, I investigated the effects of root herbivory by the specialist root weevil *Mogulones cruciger* on plant performance, pollinator interactions and sexual reproduction in the monocarpic *Cynoglossum officinale*.

In the second chapter I investigated whether there is potential for geographical structuring of the total selection pressure exerted on plants in the Belgian herbivore-plant-pollinator system of *C. officinale* and its specialist root herbivore *M. cruciger* populations at the Belgian coast. Significant spatial and temporal variation in the relative abundance of *M. cruciger* root herbivory was found in the study populations. *M. cruciger* showed clear preference for large plants and also affected pollinator foraging behavior in the field. Although female reproductive output was not pollen-limited, root herbivory significantly decreased seed set. Consequently, it can be expected that the intensity of selection pressure exerted by *M. cruciger* varies spatially between populations and also temporally.

In chapter three the consequences of root herbivory for plant-pollinator interactions and reproductive output are tested under experimental conditions. Here, a negative effect of *M. cruciger* root herbivory on certain plant traits and on pollinator attraction was found.

Non-infested plants received more pollinator visits and also more flowers were visited in a sequence on the same plant (=geitonogamous pollination) compared to infested plants. However, experimental pollen supplementation and quantification of pollen deposition and pollen tube growth showed that seed set was again not pollen-limited. There was a significant reduction in seed set in response to root herbivory, but this could be attributed to higher resource limitation due to larval damage and depletion of the root tissue.

In chapter four I investigated whether root herbivory might affect the different steps that occur during the process from pollen deposition to seed maturation via its effect on pollen quality through geitonogamous pollination, and/or via its effect on the nutritional capacity of the maternal plant. This study demonstrated that herbivory did not affect the early stages following pollination such as pollen germination and pollen tube growth, but significantly impacted later post-pollination stages like fruit set and seed maturation. Our findings suggest that decreased nutritional capacity of the mother plant in response to root herbivory rather than herbivory effects on pollen quality was responsible for the lower fruit and seed production in infested plants.

Chapter five also continues on the findings that there is less geitonogamous pollination in plants that are infested by *M. cruciger* root herbivory and handles possible fitness costs of self pollination in *C. officinale* on several life stages from maternal seed set to offspring seed set. Offspring derived from self pollination were smaller at seedling stages and had lower seed set compared to offspring derived from outcross pollination.

In conclusion, the results of this thesis documented significant interactions between the root weevil *M. cruciger* and the pollinators of *C. officinale*, but this was not the cause of the reduced seed production in response to root herbivory. There was substantial geographical variation in the interaction between *M. cruciger* and *C. officinale* in the study populations at the Belgian coast, and this may result in geographical structuring of the total selection pressure exerted on plants. The interaction between *M. cruciger* and the pollinators of *C. officinale* has further implications for male reproductive fitness and possibly also for offspring fitness.

8. Samenvatting

Herbivorie en bestuiving zijn hoogstwaarschijnlijk de twee meest belangrijke biotische interacties voor de fitness van planten. Sinds enkele jaren is er meer aandacht voor de interacties tussen herbivoren en bestuivers. Hierbij werden ondergrondse herbivorie en ook de verdere effecten van de impact van herbivorie op het bestuivingsproces echter grotendeels verwaarloosd. In dit doctoraatsonderzoek onderzocht ik de effecten van wortelherbivorie door de specialistische snuitkever *Mogulones cruciger* op plantkenmerken, interacties met bestuivers en de seksuele reproductie in de monocarpe plantensoort veldhondstong (*Cynoglossum officinale*).

In het tweede hoofdstuk onderzocht ik het potentieel voor ruimtelijke mozaïeken in de totale selectiedrukken die veldhonstong populaties aan de Belgische kust ondervinden. Er was zowel ruimtelijke als temporele variatie in de relatieve abundantie van *M. cruciger* wortelherbivorie in de studiepopulaties. *M. cruciger* vertoonde een duidelijke voorkeur voor grote planten en had een impact op het foerageer gedrag van bestuivers in het veld. Hoewel de zaad-productie niet pollen gelimiteerd was, verminderde wortelherbivorie de zaadzetting significant. Bijgevolg kan er verwacht worden dat de intensiteit van de selectiedruk uitgeoefend door *M. cruciger* zowel ruimtelijk als temporeel varieert.

In het derde hoofstuk worden de gevolgen van wortelherbivorie voor plant-bestuiver interacties en voor reproductieve output getest onder experimentele condities. Er werd een negatief effect van wortelherbivorie op sommige plantkenmerken en op bestuiveraantrekking gevonden. Planten die niet geïnfecteerd waren door *M. cruciger* larven ontvingen meer bestuiverbezoeken en hier werden ook meer bloemen sequentieel op dezelfde plant bezocht vergeleken met geïnfecteerde planten. De resultaten van experimentele pollensupplementatie en van de kwantificatie van pollendepositie en pollenbuisgroei toonden echter aan dat zaadproductie ook hier niet pollen gelimiteerd was. Er was een significante vermindering van zaadzetting als gevolg van wortelherbivorie. Dit kon toegeschreven worden aan een lagere nutritionele capaciteit door de larvale schade aan het wortelweefsel.

In hoofstuk vier onderzocht ik of wortelherbivorie de verschillende stappen in het proces van pollendepositie tot zaadrijping beïnvloedde. Enerzijds via zijn effect op de pollenkwaliteit ten gevolge van geitonogame bestuiving, en/of via zijn effect op de nutritionele capaciteit van de moederplant. Deze studie toonde aan dat herbivorie geen effect had op de vroege stadia volgend op bestuiving zoals pollenkieming en pollenbuisgroei, maar wel op latere stadia zoals vruchtzetting en zaadrijping. Onze bevindingen geven aan dat de verlaagde nutritionele capaciteit van de moederplant ten gevolge van herbivorie, en niet het effect van herbivorie op pollen kwaliteit, verantwoordelijk was voor de lagere vrucht en zaad productie in geïnfecteerde planten.

Hoofdstuk vijf bouwt ook verder op de bevinding dat er minder geitonogame bestuiving is in planten die aangetast zijn door *M. cruciger* wortelherbivorie. Het behandelt mogelijkse fitnesskosten van zelfbestuiving in veldhondstong voor de verschillende levensstadia van maternale zaadzetting tot zaadzetting van de nakomelingen. Nakomelingen afkomstig van zelfbestuiving waren kleiner als kiemplant en hadden een lagere zaadzetting vergeleken met nakomelingen van kruisbestuiving.

De resultaten van dit doctoraatsonderzoek toonden significante interacties aan tussen de snuitkever *M. cruciger* en de bestuivers van veldhondstong. Dit was echter niet de oorzaak van de verminderde zaadproductie. Er was aanzienlijke geografische variatie in de interactie tussen *M. cruciger* en veldhondstong in de studiepopulaties aan de Belgische kust. Dit kan mogelijks resulteren in ruimtelijke mozaïeken in de totale selectiedrukken die veldhonstong populaties ondervinden. De interactie tussen *M. cruciger* en de bestuivers van veldhondstong heeft verdere implicaties voor mannelijke reproductieve fitness en mogelijks ook voor de fitness van nakomelingen.

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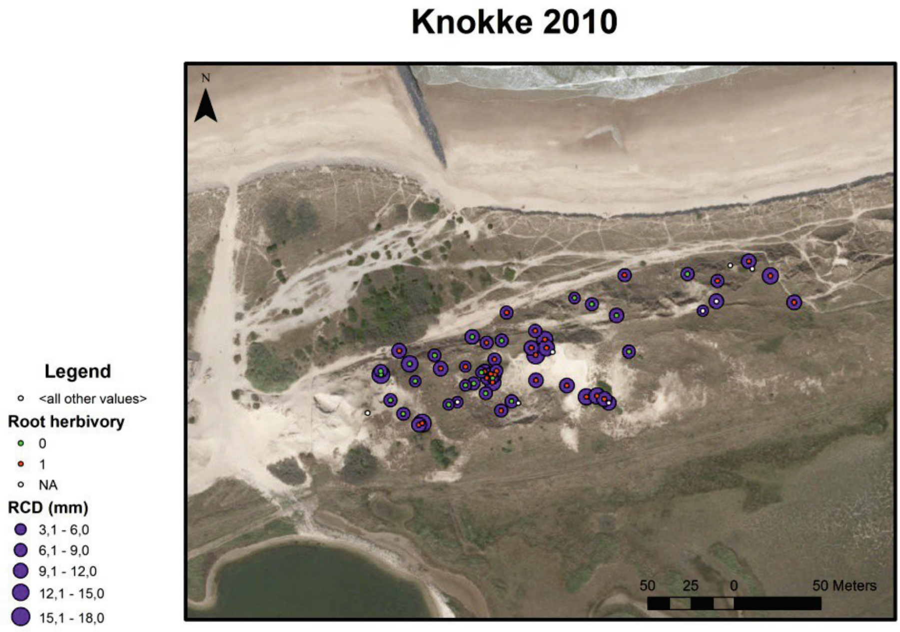
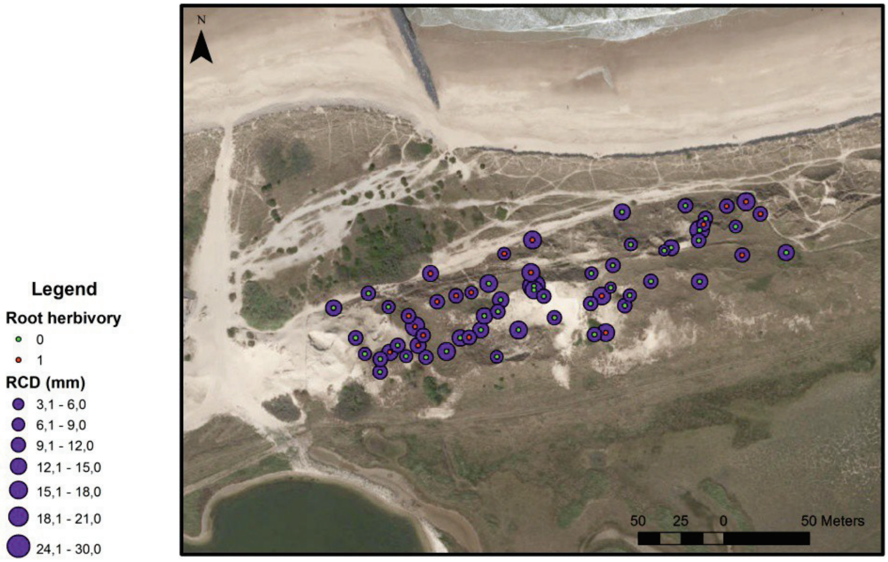
10. Supplementary information

Fig. S2.1. Geographical maps of local study populations with indications of infection status and root crown diameter (in mm) for each sampled plant.





Knokke 2011

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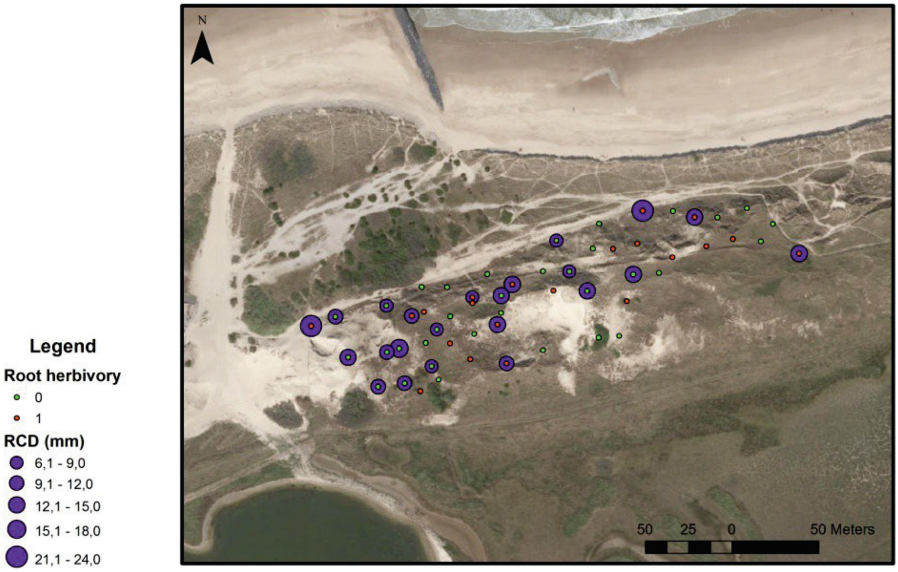
Knokke 2012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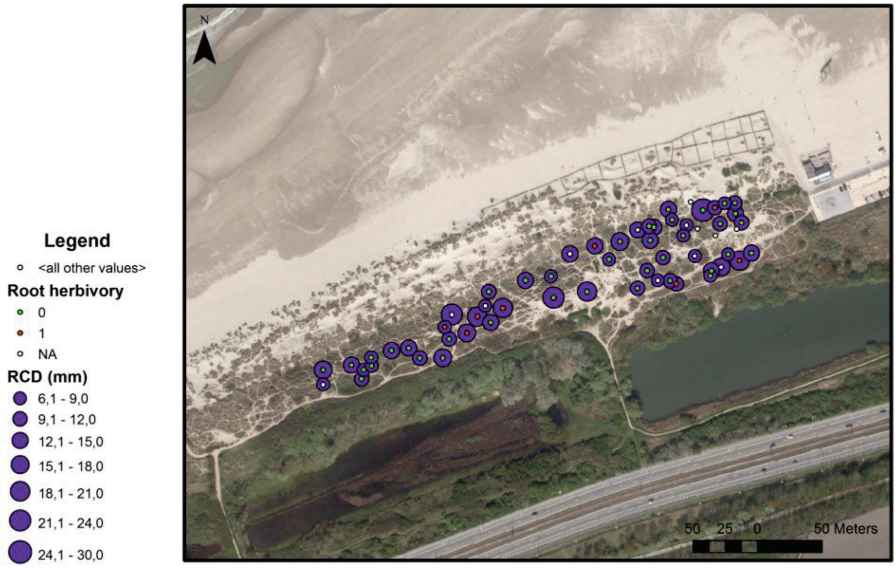
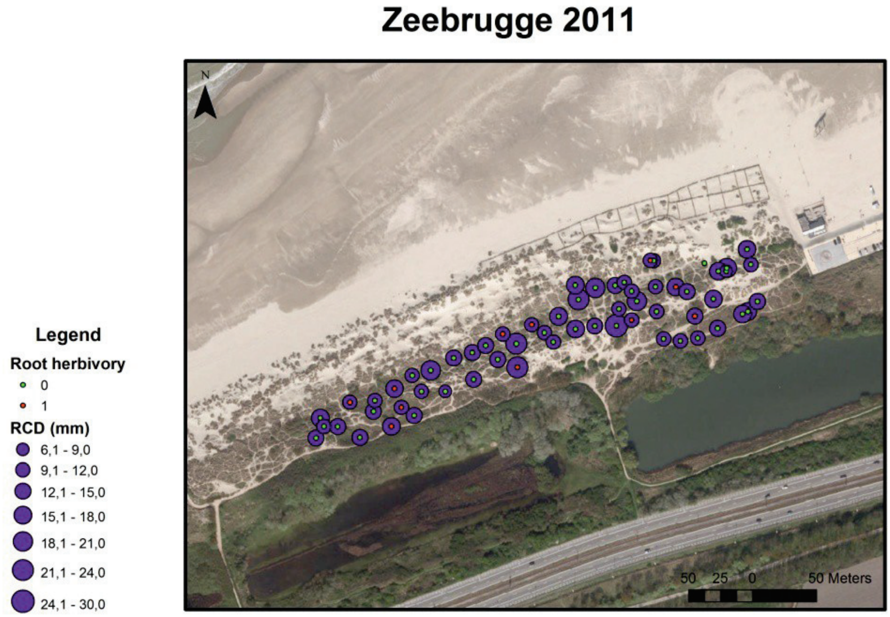
Zeebrugge 2010

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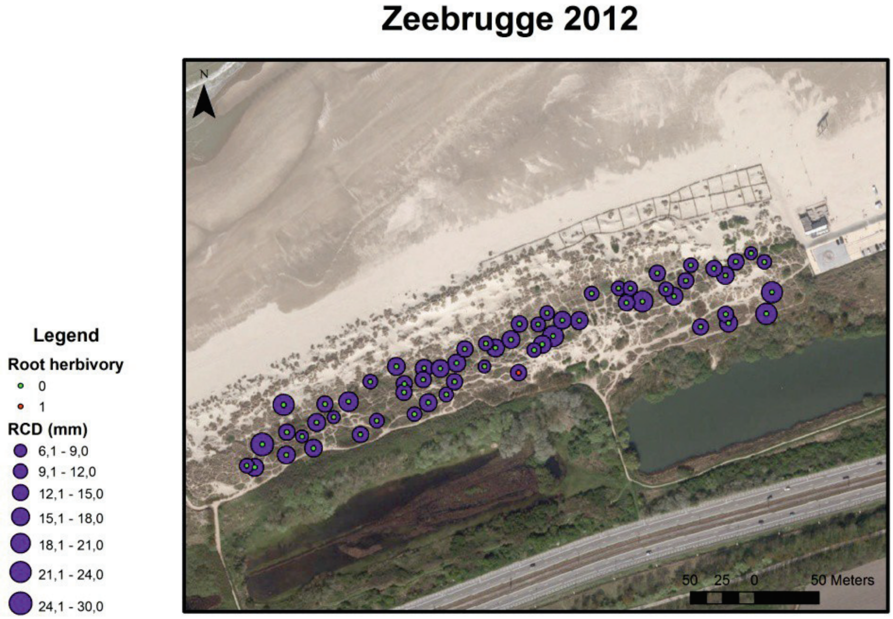
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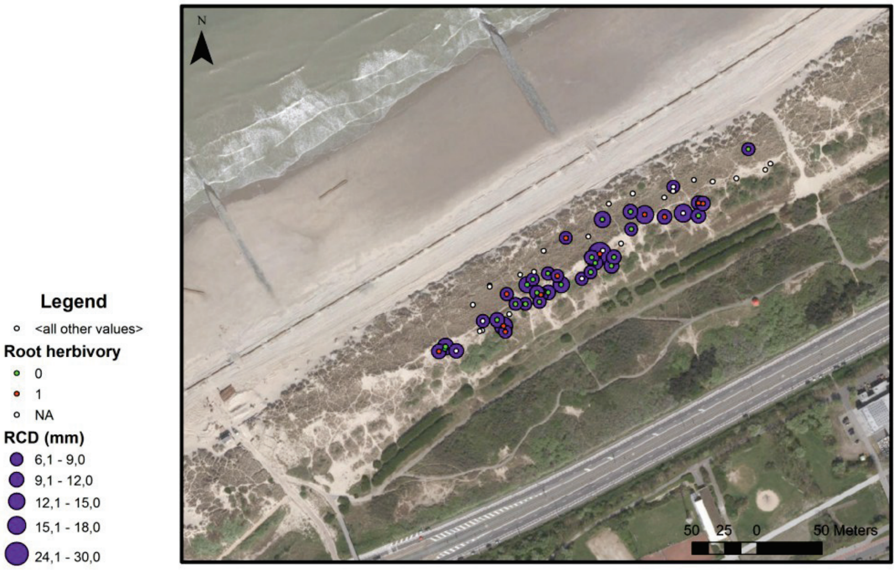

Bredene 2010

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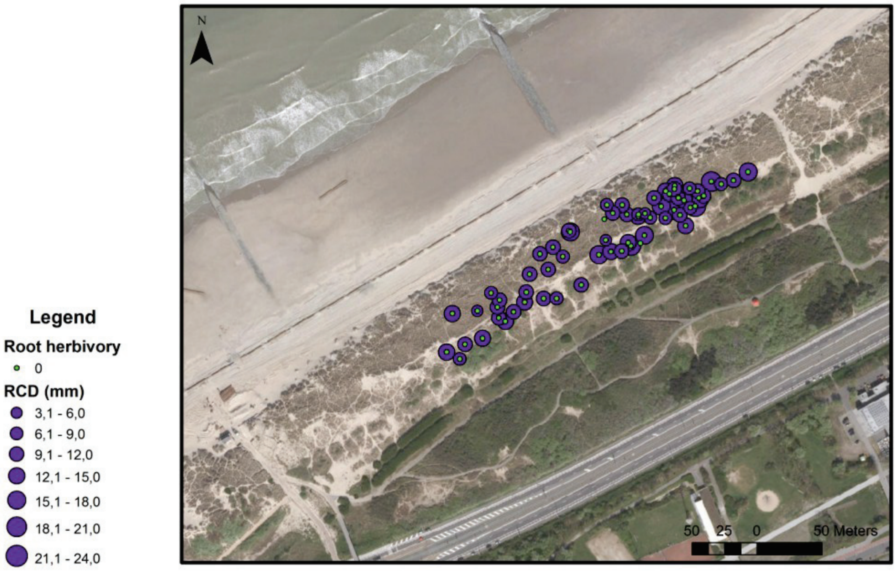

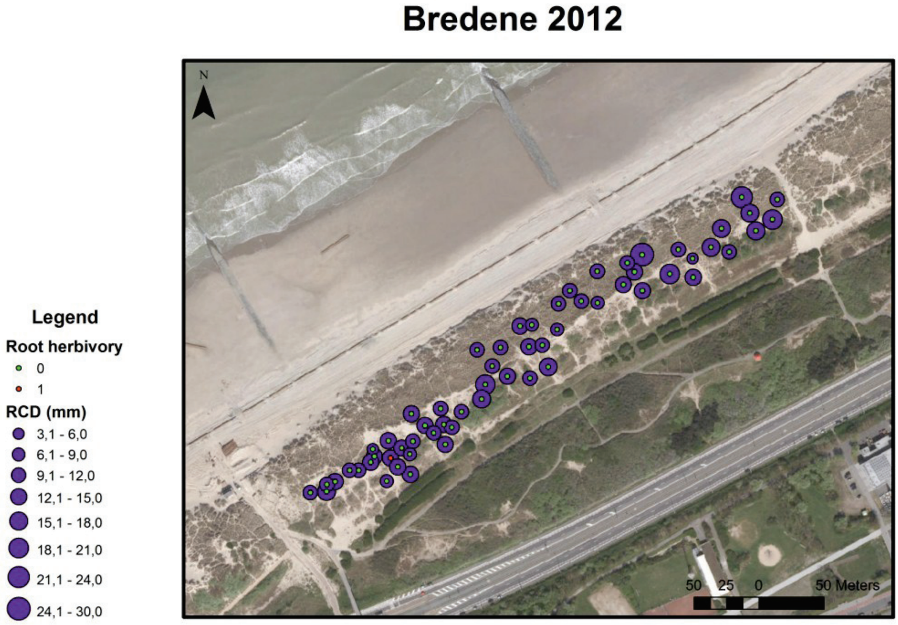
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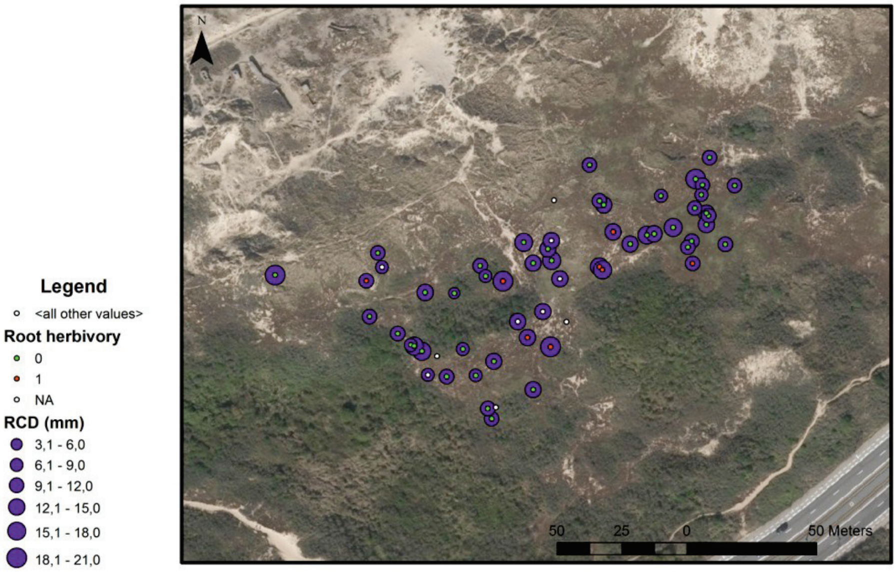
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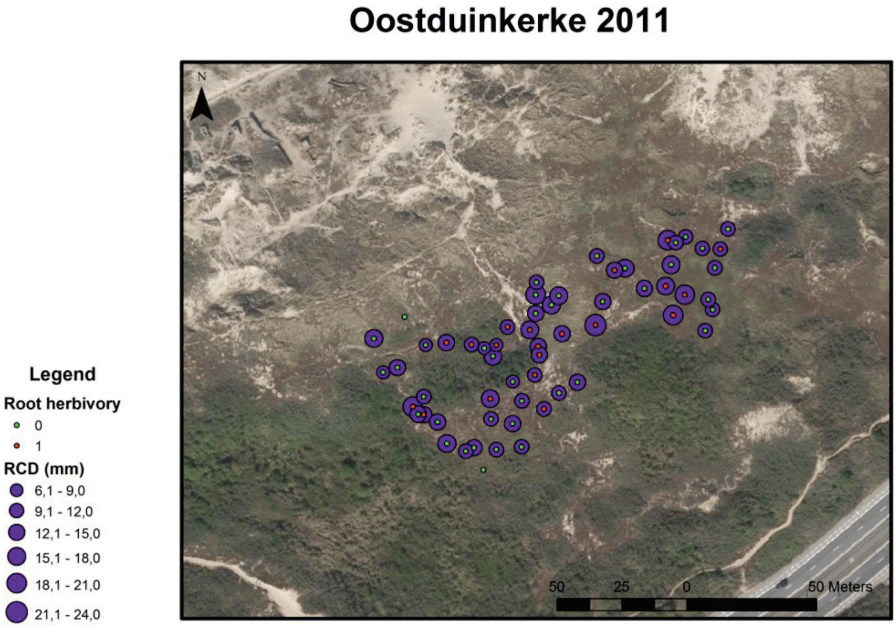
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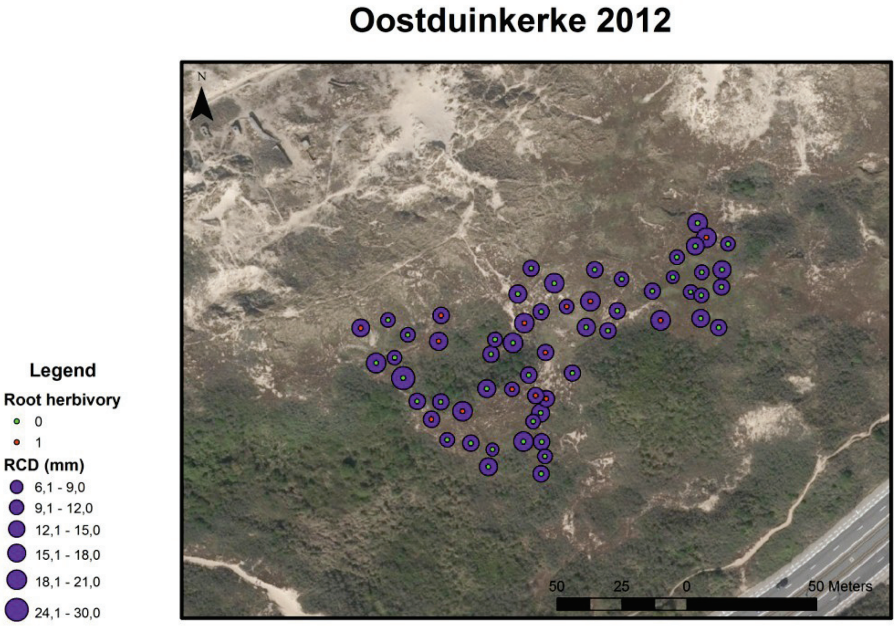
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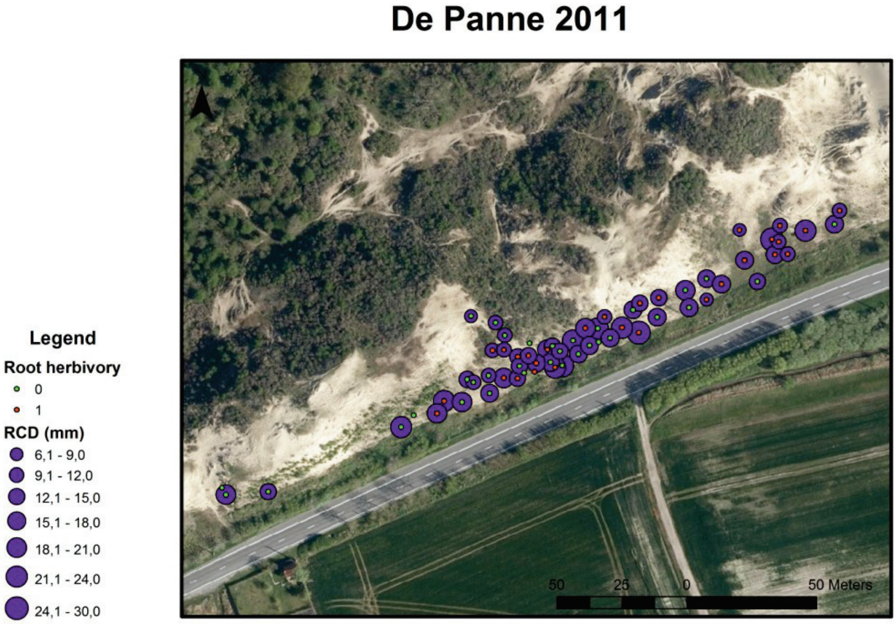

De Panne 2011

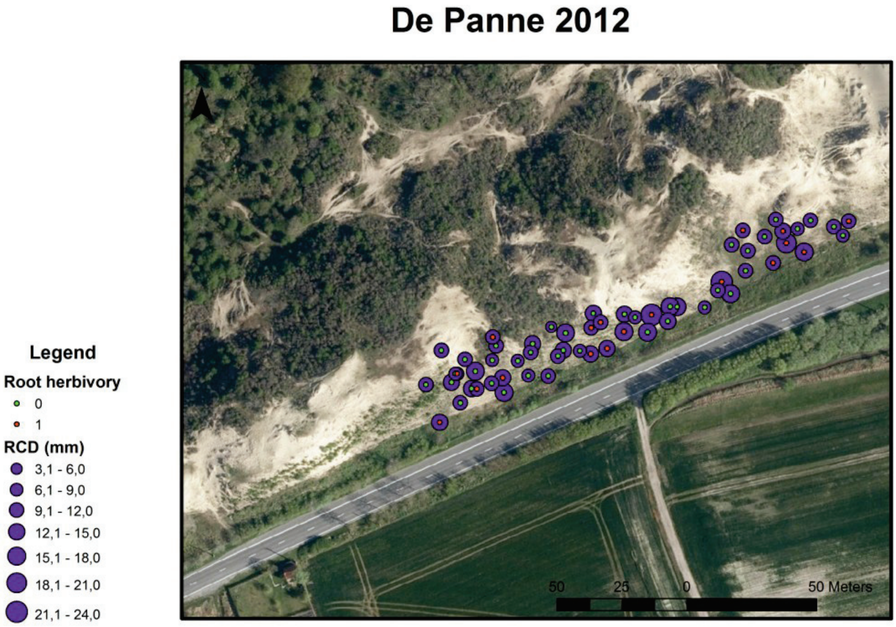
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Fig. S2.2. Graphs showing the spatial correlation for infection status (herb) and root crown diameter (RCD) between plants at a particular radius (r). When the curve is above 1.0 it is more likely that plants have the same infection status or root crown diameter when they are at a distance of that particular radius from each other. When the curve is below 1.0 it is more likely that plants have a different infection status or root crown diameter when they are at a distance of that particular radius from each other.

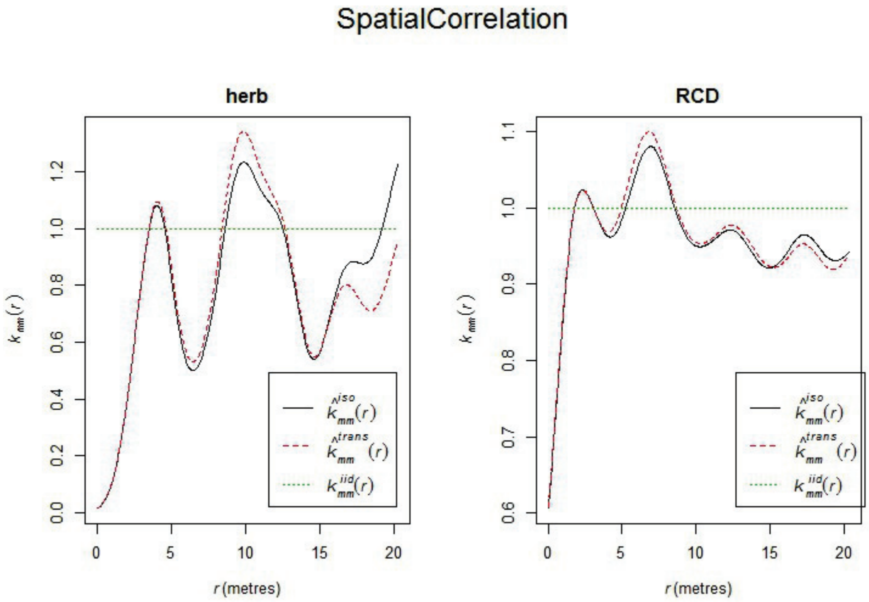

Table S3.1. Numbers and proportions of plant visits made by the different flower visitor species in *Cynoglossum officinale*. We categorized flower visitors that we could not identify under ‘anonymous’.

Flower visitor species	**Number of plant visits**	**Proportion of plant visits (%)**
Bombus hortorum L.	116	3.25
Bombus hypnorum L.	275	7.70
Bombus lapidarius L.	451	12.63
Bombus pascuorum Scopoli	239	6.69
Bombus pratorum L.	1766	49.47
Bombus terrestris L.	122	3.42
Osmia bicornis L.	256	7.17
anonymous	345	9.66