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Ecology of *Arabidopsis thaliana* : local adaptation and interaction with herbivores

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Ecology of *Arabidopsis thaliana*:

Local adaptation and interaction with herbivores

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Ecology of *Arabidopsis thaliana*:

Local adaptation and interaction with herbivores

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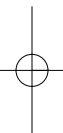
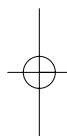
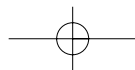
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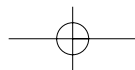
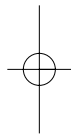
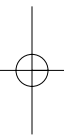
Dr. Ir. N.M. van Dam (NIOO-KNAW)

This thesis is dedicated to
my mother, father, wife and son Mohammed



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General introduction

1

It is unlikely that one single plant species completely escapes herbivory. Especially the forestry literature reports on large-scale total defoliation and the resulting mortality of trees. Many agricultural studies also describe the enormous impact of herbivores on production parameters. But even species that are not selected for productivity and do not grow in monocultures are continuously under attack, and consequently under selective pressure for resistance. The effects of leaf herbivory on plant fitness are not always equivocal. Some leaves, usually older leaves, have already fulfilled their photosynthetic function and losing them will hardly affect a plant. Other leaves, usually the younger ones, still have a high potential contribution to make to plant growth. Loss of these leaves may have a larger impact on plant fitness, although some plant species demonstrate spectacular levels of tolerance. The most direct effect of herbivory is loss of flowers or seeds. Many herbivores feed on seeds (Hulme and Benkman, 2002). Compared to other plant tissues, seeds generally have a higher energy content than roots, stems or leaves. Not surprisingly, seeds are highly sought after and this may explain why granivory (seed predation) is widespread.

Several field studies have identified granivores as having a considerable impact on seed populations (Crawley, 1992). High rates of predation, often greater than 50%, are typical of many plant species in a number of different ecosystems (Hulme and Benkman, 2002). Granivores have been suggested as agents of natural selection that influence seed traits (Benkman, 1999).

The following four questions are the main themes of this thesis, which deals with evolutionary ecology and the population dynamics of the annual plant species *Arabidopsis thaliana* (L.) Heynh:

- What is the impact of herbivory on the population dynamics of *A. thaliana*?
- What is the role of herbivory as an agent of natural selection?
- Does the diversity of glucosinolates affect the acceptability of *A. thaliana* as food for naturally occurring specialist herbivores?
- How do chemical compounds in *A. thaliana* affect leaf herbivory, illustrated by a generalist and a specialist herbivore?

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Why Arabidopsis thaliana?

Arabidopsis thaliana (L.) Heynh., wall cress or mouse-ear cress, is native to Europe and central Asia and is now naturalized at many places all over the world (Al-shehbaz and O’Kane, 2002). Hoffmann (2002) described the biogeography of the species in detail and showed that low spring and autumn temperatures, and high temperatures (average month temperature $>22^{\circ}\text{C}$) with low precipitation in summer, limit its distribution range. However, within these limitations, *A. thaliana* has a wider climatic amplitude than other well-investigated species of the Brassicaceae, and it has an impressive latitudinal range from 68°N (North Scandinavia) to 0° (mountains of Tanzania and Kenya), which makes it a suitable species for analyzing variation in adaptive traits (Koornneef et al., 2004).

Arabidopsis thaliana was originally selected as a model system for research in genetics (Redei, 1992). For geneticists, the main reason to focus on *A. thaliana* was simply its short life cycle and small size. Once enough knowledge of the genetics and physiology of the plant had accumulated, it naturally became the favorite organism for molecular and then for developmental botanists (Dean, 1993; Pyke, 1994; Anderson and Roberts, 1998). This has led to the establishment of a large research community with access to important biological and molecular resources (Meinke et al., 1998).

Ecologists and evolutionary biologists have been slower at capitalizing on the wealth of information available about *A. thaliana* (Pigliucci, 2002). However, the increasing database of information about the physiology, molecular biology, genetics, and developmental biology of *A. thaliana* make it an attractive study subject for evolutionary ecologists as well (Pigliucci, 2002).

Herbivores and population dynamics

Despite the fact that plants can compensate or even overcompensate for sizable amounts of damage, in general, tissue removal by herbivores decreases plant fitness (Bigger and Marvier, 1998). Herbivores remove on average 18% of all terrestrial plant biomass and 51% of aquatic biomass (Cyr and Pace, 1993). Insect herbivores with different feeding modes can differentially affect plant fitness, and it is not always easy to predict which type of herbivore causes most injury (Strauss and Zangerl, 2002). Field studies have shown that seed pred-

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ators have a considerable impact on seedling populations (Crawley, 1992) and therefore can affect dynamics of populations. The role of seed-predators in the dynamics of plant populations has received detailed attention (Andersen, 1989; Crawley, 1992; Hulme, 1998). Seed predation may play a minor role in the demography of plants (Strauss and Zangerl, 2002) if 1) plants regenerate primarily by vegetative means; 2) seed losses due to predators are buffered by the presence of a large persistent seed bank; 3) regeneration is microsite-limited rather than seed-limited and/or 4) high seed mortality is compensated by less intraspecific competition later in the life cycle.

The relation between the population dynamics of *A. thaliana* and its natural herbivores is largely unknown. *Arabidopsis thaliana* offers great potential for improving our understanding of the evolution of plant defences (Mitchell-Olds, 2001; Koornneef et al., 2004). However most studies of the interaction between *A. thaliana* and insects focused on laboratory experiments and on herbivores that have no significant effect on the plant in the field. It would be imperative to integrate such studies with *Arabidopsis*' own natural herbivores and this demands more knowledge about natural populations (Kliebenstein, 2004).

Arabidopsis thaliana and local adaptation

Plants not only stand and wait to be counted (Harper, 1977) but they are also ideal for transplant experiments to study local adaptation experimentally. Transplant experiments as pioneered by Clausen et al. (1940, 1948) provided evidence of a home site advantage for local genotypes against alien genotypes, illustrating the role of adaptation and genetic differentiation (Antonovics, 1971). As selection may vary among life history traits, it is therefore important to study the complete plant life cycle with respect to selection (van Groenendael, 1985). Adaptation has been demonstrated as a results of differences in many environmental characteristics, for example for heavy metals (McNeilly and Antonovics, 1968), soil type (Snaydon, 1970), salinity (Antlfinger, 1981), water availability (van Groenendael, 1985; Farris, 1987). For an extensive review see Linhart and Grant (1996). The role of herbivores in local adaptation, however, is largely unknown (but see Prins, 1989). *Arabidopsis thaliana* has a wide climatic amplitude, which makes it suitable for analyzing variation in adaptive traits (Koornneef et al., 2004). Several studies have demonstrated variation

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in resistance traits (Mauricio, 1998), in flowering time and in important morphological and physiological characters in *A. thaliana* (for a review see Koornneef et al., 2004). However, it is unknown whether these variations are the result of genetic adaptation and lead to a home site advantage for local genotypes. Griffith et al. (2004) tested life-history variation and adaptation in *A. thaliana* plants in North America. They did not find significant differences in performance in a common garden for plants from different populations. However, the common garden and greenhouse experiments do not directly address the potential adaptive nature of genetic variation (Rice and Mack, 1991). Antonovics and Primack (1982) argued that field transplants lead to a more realistic assessment of genetic and environmental effects because they also include biotic factors that might be crucial for the development of local adaptation.

Glucosinolates and interactions between plant and insect herbivores

Glucosinolates are present in sixteen families of dicotyledonous angiosperms including a large number of edible species (Fahey et al., 2001). At least 120 different glucosinolates have been identified in these plants. All the many hundreds of cruciferous species investigated are able to synthesize glucosinolates (Kjær, 1976). Among the Brassicaceae, the genus *Arabidopsis* contains 36 different glucosinolates (Hogge et al., 1988; Brown et al., 2003). These nitrogen- and sulfur-containing secondary metabolites are derived from a variety of protein amino-acids (methionine, tryptophan and phenylalanine) and their chain-elongated analogues (Halkier and Du, 1997). Glucosinolate biosynthetic pathway has been described in detail (Graser et al., 2000).

The principal biological activities of glucosinolates are mediated by hydrolysis of products, formed when tissue disruption brings glucosinolates into contact with myrosinase, a thioglucosidase. After glucosinolate cleavage, the resulting unstable aglycone generates numerous compounds (isothiocyanates, nitrils, epithiocyanates, and thiocyanates) with diverse biological activities (Kliebenstein et al., 2001). Myrosinase hydrolysis products can serve as oviposition and feeding stimulants for insects specialized on glucosinolate-containing plants, but act as toxins or feeding deterrents toward generalist insect herbivores (Giamoustaris and Mithen, 1995). Thus, any given glucosinolate may have positive or negative impacts on plant fitness

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depending upon the insect herbivore present. In the face of such heterogeneous selection pressures, it is not surprising that glucosinolates show extensive genetic variation within and among plant species (Rodman, 1980; Daxenbichler et al., 1991).

Insects as selective forces

Many of the secondary metabolites in plants act as defense against herbivores (Fraenkel, 1959) and it is often postulated that these compounds have evolved under selective pressure of insect herbivory (Ehrlich and Raven, 1964). This assumption has been under dispute because secondary compounds can have multiple functions and defense against insect herbivores may have evolved as a by-product of other ecological functions (Muller, 1969). Furthermore, it has been argued that plant chemistry is not the main factor determining plant-insect interactions (Bernays and Graham, 1988) and that the impact of insects on plant fitness may be too weak to impose selection on plant defense traits (Bernays and Graham, 1988; Jermy, 1993). However, recent experimental field studies have shown that total glucosinolate concentration in *Arabidopsis thaliana* and concentration of two individual alkaloids in *Datura stramonium* are under active selection pressure by insects (Mauricio and Rausher, 1997; Shonle and Bergelson, 2000). These studies provide strong evidence for the potential role of insects in the evolution of plant secondary metabolites. Considering insects as selective forces, there are three possible nonexclusive explanations for the diversity in structurally related compounds within a single plant species: 1) an evolutionary arms race, 2) selection pressure by different herbivores and 3) synergism for related compounds.

An evolutionary arms race: The first hypothesis states that new compounds could have evolved in a continuous evolutionary arms race between plants and herbivores. Plants that produce new compounds are able to escape herbivory and insects, in turn, adapt to these compounds (Berenbaum and Feeny, 1981; Miller and Feeny, 1983).

Selection pressure by different herbivores: The second hypothesis that I tested in this thesis, on the evolution of the diversity of related compounds is based on differences in herbivore susceptibility towards compounds. Relative effects of related compounds may differ between insect species and the diversity may be maintained under selection pressure of different herbivores (Simms, 1990; Mithen et al., 1995; van der Meijden, 1996).

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Synergism for related compounds: The third hypothesis states that the effects of a mixture of related compounds is more effective than would be expected by adding the effects of the individual compounds in the mixture. In other words, related compounds may act synergistically (Adams and Bernays, 1978; Lindroth et al., 1988; Berenbaum et al., 1991).

Alternatively, the diversity in secondary metabolites could also be the results of a selectively neutral process. If new compounds do not increase or decrease the plant's resistance against herbivores and the production of new compounds does not involve extra costs, these compounds could remain within a population.

Outline of the thesis

This thesis will first describe the impact of *Arabidopsis*' own natural herbivores and other environmental factors such as water, disturbance, and nutrient on population dynamics (chapter 2). We will see that *A. thaliana* populations growing in different habitats (dune and inland) are differently affected by herbivory. We studied if this variation is genetically or environmentally based and whether naturally occurring herbivores played an important role in adaptation of *A. thaliana* populations (chapter 3). To test whether differences in herbivory are due to environmental influences or to plant genotype, we set up a reciprocal transplant experiment. To examine the causes of differences in plant defence we compared herbivory on fruits with data on glucosinolate concentration of seeds in the field. For this reason we analyzed plants chemically with Nuclear Magnetic Resonance (NMR) spectrometry and High-Performance Liquid Chromatography (HPLC). These results are discussed in chapter 4. Finally, in chapter 5 we examine how glucosinolates in leaves affect two different herbivores, both a generalist and a specialist.

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Herbivory and abiotic factors affect population dynamics of *Arabidopsis thaliana* in a sand-dune area*



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Population dynamics of the annual plant *Arabidopsis thaliana* (L.) Heynh. was studied in a natural habitat of this species at the coastal dunes of the Netherlands. The main objective was to elucidate factors controlling population dynamics and the relative importance of factors affecting final population density. Permanent plots were established and plants were mapped to obtain data on survival and reproductive performance of each individual, with special attention to herbivore damage. In experimental plots we studied how addition of water, addition of nutrients, artificial disturbance and natural herbivores affected survival and growth.

Mortality was low during autumn and early winter and high at the time of stem elongation, between February and April. A key-factor analysis showed the highest correlation between mortality from February to April and total mortality. The specialist weevils *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) were identified as the major insect herbivores on *A. thaliana*, reducing seed production by more than 40%. These herbivores acted in a plant size-dependent manner, attacking a greater fraction of the fruits on large plants. While mortality rates were not affected by density, fecundity decreased with density, although the effect was small. Adding water reduced mortality in rosette and flowering plant stage. Soil disturbance did not increase seed germination, but did have a significant positive effect on survival of rosette and flowering plants. Seed production of *A. thaliana* populations varied greatly between years, leading to population fluctuations, with a small role for density-dependent fecundity and plant size-dependent herbivory.

A genetic map of all five chromosomes of *Arabidopsis thaliana* is available. The life-cycle of this species is very short and seed production is prolific. These characteristics explain why *A. thaliana* is now so widely used as a model organism in plant biology. Yet we know little about its natural population biology. Population studies on *A. thaliana* offer great potential for improving our understanding of the evolution of plant defences (Mitchell-Olds, 2001; Koornneef et al., 2004). However, the relation between the population dynamics of

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A. thaliana and its natural herbivores is largely unknown. Most laboratory studies focused on the interaction of *A. thaliana* with lepidopteran larvae, which probably do not present a significant herbivory load in the field (Kliebenstein, 2004). It would be imperative to integrate such studies with *Arabidopsis*' own natural herbivores and this demands more knowledge about natural populations.

Herbivores can greatly affect the performance of plants and their population densities (Crawley, 1983). Evidence of herbivory attack on *A. thaliana* in the field comes from the study of Mauricio and Rausher (1997), who quantified damage and found that herbivores exerted a strong detrimental effect on plant fitness. They did not quantify, however, which herbivore caused most damage.

Mortality during different life stages may have a different effect on plant population dynamics. Harper (1977) stressed that, when the density of seeds is such that density-dependent processes will thin the population, seed predation may simply remove seeds that have no future, and were doomed to die. If this is the case, even very high seed mortality may have negligible effects on population density. Recruitment may also be limited by the availability of suitable sites for germination, growth, and reproduction (Harper, 1977; Bergelson, 1990a,b; van der Meijden et al., 1992; Houle, 1996). Small-scale disturbance provides a release from competition with established vegetation and may play an important role in determining the abundance of suitable microsites for germination and establishment. Several authors argued that the number of recruits is a function of both seed production and microsite availability (Klinkhamer and de Jong, 1989; Eriksson and Ehrlén, 1992). Again few data are available for *A. thaliana*.

Myerscough and Marshall (1973) showed in a laboratory study that increased density of *A. thaliana* (strain 'Estland') negatively affected plant growth, mortality and fecundity. It is unknown whether such high densities, in the same life stages, are found in natural populations.

Arabidopsis thaliana is a winter annual and one would expect that, like in other winter annuals (Beatley, 1967), conditions (water and nutrients) during peak growth in early spring are critical for its success.

The habitat of *A. thaliana* in Meijendel, a coastal sand dune area in the Netherlands, exists already for many hundreds of years in a similar way. One can expect that the plant co-evolved with its herbivores in this area. We address the following questions. 1) During which life stage is mortality highest and how does this affect total

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mortality over the whole life span? 2) Is fecundity density dependent? 3) Which factors cause mortality in different life stages? 4) Do specialist herbivores act in a plant size-dependent manner? We studied demography in permanent quadrants and experimentally tested effects of factors like watering, artificial small-scale disturbance, addition of nutrients and natural herbivores on recruitment and growth.

MATERIALS AND METHODS

Habitat description

Arabidopsis thaliana (L.) Heynh. (Cruciferae) is a small annual plant originating from Europe and is now widely distributed in many parts of the northern-temperate zones of the world (Ratcliffe, 1961). One type of habitat of *A. thaliana* in the Netherlands is the coastal sand dune area. Our study site is Meijendel, north of The Hague (latitude 52° 08' N, longitude 4° 22' E). In these dunes *A. thaliana* grows in two different types of sites. It is locally common along roads in the old dune system that was formed between c. 3000-5000 years ago and that is still visible in the landscape as long stretches of sand that run parallel to the coast. It also occurs locally on the more calcareous new dunes that formed in top of the old soil profile c. 800 years ago. At Meijendel these new dunes cover 4 to 5 km from the beach to the inland verge. The observations in this paper only concern the latter habitat, the new dunes.

Descriptive demography

Demographic information was collected in four populations in the new dunes, each covering an area of 4-10 m². Populations number 1, 2 and 3 were near an unpaved road used by hikers (1 m from the road), population number 4 was further away (about 20 m) from the road. All populations were within 20 m of woody vegetation with trees like *Populus nigra*, *P. alba*, *Betula pubescens* and *Crataegus monogyna*. The sand was covered with moss, grasses and small herbs with about 10 percent open soil. Accompanying species included *Erophila verna*, *Cardamine hirsuta*, *Rubus caesius* and *Calamagrostis epigejos*, with small *Hippophae rhamnoides* shrubs nearby.

Randomly chosen (35) permanent plots, each 20 cm by 20 cm, were positioned in populations in March 2002. Every fortnight, from

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October 2002 to June 2003, the fate of each plant of *A. thaliana* was recorded. During these counts attention was given to damage to the plants and to mortality and its causes.

Seed production in each plot was measured indirectly. We counted the number of fruits per plant in each plot. The number of seeds per fruit was estimated in May 2002 by counting the seeds on 146 individual plants, sampled adjacent to the permanent plots. Also seed predation was measured on these latter plants. This yielded an estimate of the average number of seeds per damage fruit (S_d) and seeds per undamaged fruit (S_u). The number of undamaged (u) and damaged (d) fruits together produce $uS_u + dS_d$ seeds so that a fruit has, on average, $S_{tot} = (uS_u + dS_d) / (d + u)$ seeds. Seed production per plot is then $F_{plot} S_{tot}$ in which F_{plot} is the total number of fruits, counted in the plot.

Key factor

To show that mortality occurring during a certain life stage affects total mortality over life, one needs to show that its effects are not offset by other mortality factors acting later in the life of the plant. This problem can be tackled by key-factor analysis (Morris, 1959; Varley and Gradwell, 1960; Silvertown, 1982). We define k_i as the difference between the logarithm of numbers per unit area before and after its action: $k_i = \log(N_i) - \log(N_{i+1})$, in which i is an index of the life stage. For each mortality k_i is correlated with total mortality K during the life cycle of an organism ($K = \sum k_i$). The k -factor with highest correlation with K is called the key factor (Varley and Gradwell, 1960). The method has mainly been applied to long time series of insect populations. Here we use the method as applied by de Jong and Klinkhamer (1988), for a single cohort monitored over one year at a large number of sites. Mortality in different stages of the life cycle can then be correlated with total mortality of the cohort at that site, from seed stage until reproduction. Used in this way, the method still indicates if mortality in a particular life stage is relevant for the number of plants that survives until reproduction at that site or that its effects are offset by other factors. To increase the data set, two control populations from an experimental study (seeds sown but without further experimental treatment) were also included in the analysis.

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Density-dependence

Density-dependence was studied in the 35 permanent plots that were set up for descriptive demography. To determine density-dependent fecundity, a Spearman's rank correlation coefficient was calculated for the relation between the number of flowering plants per plot, estimated without error, and the number of fruits per plant. In the case of density-dependent mortality, a problem with this approach is that the number of seedlings is estimated with some error. This error appears both in the dependent and independent variable, which confounds the regression. It is therefore appropriate to test for proportionality between, for instance, number of seedlings (X) and number of subsequent flowering plants (Y). This test was outlined by Klinkhamer et al. (1990) and involves an F -test for deviations from slope $b = 1$ on a plot of $\log(X)$ versus $\log(Y)$. The same test was used to evaluate whether the number of unattacked fruits was proportional to total fruits, i.e. if attack rate of fruits depends on fruit number, estimated with some error.

Experimental demography

In an experimental field study we tested the effects of watering, artificial small-scale disturbance, addition of nutrients (in liquid form) and natural herbivores on the different life stages of *A. thaliana*, from seed to seed production. On 15 October we sowed 100 seeds on 13 cm $\times$ 13 cm field plots, surrounded by a plastic shield (5 cm high) to prevent seed dispersal to outside the plots by wind or rain. The seeds used in this experiment were 6 months old. By this time seeds lose dormancy and in a lab trial 90% of the seeds immediately germinated under wet conditions. Apart from a control treatment (10 plots with 100 seeds per plot) we used the following treatments:

Small-scale disturbance: disturbance performed by A) scratching the soil with a mini-rake (5 plots) and also by B) compacting the soil with foot force (5 plots).

Water: we added water per plot, except when it was raining or in December, January and February (5 plots). In October, in total 400 ml water was supplied to each plot, in November 200 ml, in March 400 ml and in April 400 ml. Average rainfall in March and April 2002 was 35.1 and 55.7 mm, respectively. Adding 400 ml water per plot in our experimental treatment is comparable to 24 mm rainfall. The

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dune area occasionally and unpredictably suffers from droughts in spring and summer (Noë and Blom, 1982) and has a lower nutrient soil content compared to other habitats of *Arabidopsis thaliana* such as inland sites. Water and nutrients are therefore probably the limiting factors for growth in this area.

Nutrients: we added Hoagland solution instead of water (5 plots). The solution contained 167 mg/l N, 31 mg/l P and 282 mg/l K (Steiner, 1968). The volume given was the same as for the water treatment.

The number of seedlings was recorded and individual seedlings were mapped every day between the emergence of the first seedlings on 21 November and 10 December. Later the fate of the seedlings was recorded every fortnight. At the end of the growing season all experimental plants were harvested and the number of fruits was counted.

To identify which herbivores are responsible for pre-dispersal seed damage, plants were transplanted in April to each of 5 plots (5 plants per plot) covered with a net (mesh width 0.25 cm), 10 plots with a net with a smaller mesh width (0.05 cm) and 10 control plots. The net with 0.05 cm mesh width excludes among others the specialist herbivores *Ceutorhynchus* spp., whereas the net with 0.25 cm mesh width does not. During and after seed set plants were examined for extent and type of herbivory.

RESULTS

Descriptive demography

Census data for four populations are plotted in Fig. 1 as the mean percentage survivorship in each population. Only 1.03% of the seeds produced in the plots was observed to turn into a seedling in autumn. Despite this low germination percentage, we found a positive rank correlation between number of seeds formed in the plots in July 2002 and seedlings recruited in the following autumn (Spearman rank correlation $\tau = 0.397$, $P < 0.05$). The survivorship curves showed low seedling mortality during autumn and early winter and most of the seedling mortality took place between February and April. Table 1 summarizes data on the components of reproduction in the 4 populations. The seedling population on all plots reached a maximum of 302

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individuals on 1 November. The highest number of individuals in any plot was 62, while the lowest number was zero. Before 15 February 30% of the seedlings died. The major cause of seedling mortality at this time appeared to be rain-drag and erosion on bare plots situated on slopes (e.g. populations 2 and 4). Between 15 February and late April 68% of seedlings died. From late April until seed set the mortality was only 1.5%. Approximately 68% of seedlings alive in November died before flowering.

Comparison of fruit production per plant and seeds per fruit showed that the number of fruits was quite variable, averaging 6.85 in 2002 and only 3.35 in 2003, whereas seeds formed per fruit was more constant, averaging 27.02 in 2002 and 25.2 in 2003 (Table 1). Direct observations on four populations showed that the specialist herbivores *Ceutorhynchus atomus* and *C. contractus* (Curculionidae), almost in equal numbers, were the major herbivores on *Arabidopsis* in this area. Table 2 shows seed production and the effect of infestation by these weevils on the number of seeds. The measured seed damage was inflicted by both adults consuming flowers and fruits and by larvae feeding on the seeds within the fruits.

Key factor analysis

Among all life stages, seedling mortality between 15 February and late April had the highest correlation with total *K* (Table 3) and is therefore the key factor.

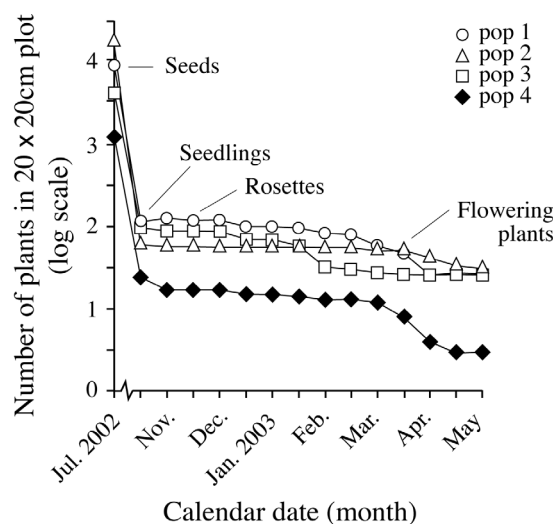


FIGURE 1. Survivorship curves of *Arabidopsis thaliana* in a sand dune area.

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TABLE 1. Life table of *Arabidopsis thaliana* populations. Each plot is 20 cm × 20 cm. (n = replicate plots, m = percentage of mortality until the next stage in the table, a Chi-square analysis was used for testing the differences between populations in mortality).

Life stage	Population 1 $n = 8$ m		Population 2 $n = 8$ m		Population 3 $n = 14$ m		Population 4 $n = 5$ m		Total $n = 35$ m		Sig.
Flowering plants	86	–	78	–	99	–	36	–	299	–	
Total seed production (No. fruits)	12762 (429)	30	10135 (538)	60	28923 (930)	37.5	3580 (153)	66.5	55400 (2050)	42	$P < 0.05$
Seeds after predation	8908	99	4073	98	18103	99.5	1205	98	32289	99	ns
Seedlings in November 2002	121	12	96	39	61	1.5	24	37.5	302	20.5	$P < 0.05$
Seedlings on 15 February 2003	106	73	59	56	60	32	15	73	240	58	$P < 0.05$
Plants in late April	29	3.5	26	0	41	5	4	25	100	4	$P < 0.05$
Flowering plants	28	–	26	–	39	–	3	–	96	–	
Total seed production (No. fruits)	1825 (73)	–	1235 (65)	–	4844 (173)	–	220 (11)	–	8124 (322)	–	

TABLE 2. Effect of infestation by *Ceutorhynchus atomus* and *Ceutorhynchus contractus* (Curculionidae) on the mean ($\pm$ SE) number of seeds per fruit and total seed production of *Arabidopsis thaliana*. SE refers to the standard error of the average of the 35 plots. The value for each trait, followed by a different character is significantly different (ANOVA, Tukey test).

Phenotypic trait	Population 1	Population 2	Population 3	Population 4	Sig.
Fruit production per plant	6.1 $\pm$ 1.1	9.4 $\pm$ 2.4	10.4 $\pm$ 1.0	5.2 $\pm$ 1.4	ns
Fraction damaged fruits	0.4	0.7	0.5	0.8	–
Seeds per undamaged fruit	29.7 $\pm$ 0.4 a	18.8 $\pm$ 1.2 b	31.1 $\pm$ 1.0 a	23.4 $\pm$ 1.7 b	$P < 0.05$
Seeds per damaged fruit	9.1 $\pm$ 0.9 a	3.1 $\pm$ 0.5 b	7.4 $\pm$ 1.0 a	3.5 $\pm$ 0.4 b	$P < 0.05$
Potential seed production per plant	181	177	324	122	–
Seed production per plant after seed predation	127	70	203	42	–

Density dependence

Plants in dense plots produced less fruits compared to plants in low density plots, indicating negatively density-dependent fecundity in these natural populations of *Arabidopsis* (Spearman's rank correlation, $\tau = -0.506$, $P < 0.05$, Fig. 2A). Survival from seedling to flowering plant

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TABLE 3. Key factor analysis for six *Arabidopsis thaliana* populations. r = Pearson correlation of mortality in life stage i , k_i with total mortality K . Significance: * = $P < 0.05$.

Life stage	r
Seed production – Seeds after predation	0.725
Seeds after predation – Seedling in November	0.234
Seedlings in November – Seedlings 15 February	-0.186
Seedling 15 February – Flowering plants in late April	0.845*

seemed to be reduced at densities of 40–60 seedlings per plot (20 cm by 20 cm), but this result was not statistically significant (Spearman's rank correlation, $\tau = 0.024$, $P > 0.05$) and such high densities are not common in our study site (Fig. 2B). The correlation between total fruit per plant versus seeds per damaged fruit (Spearman's rank correlation, $\tau = 0.018$, $P > 0.05$) and/or seeds per intact fruit (Spearman's rank correlation, $\tau = 0.070$, $P > 0.05$) was not significant. Instead, we found that the number of damaged fruit increased more than proportionally to total fruits (slope on log-log plot was 1.489 which is significant greater than one, $F = 16.22$, $P < 0.001$, Klinkhamer et al., 1990). This means that percentage damaged fruits increases with total number of fruits (Fig. 2C). Apparently herbivores were more attracted or fed disproportionately more on fruits on large plants.

Experimental demography

Table 4 shows the effects of addition of nutrients, watering, scratching and compacting the soil on seed germination, survivorship, and

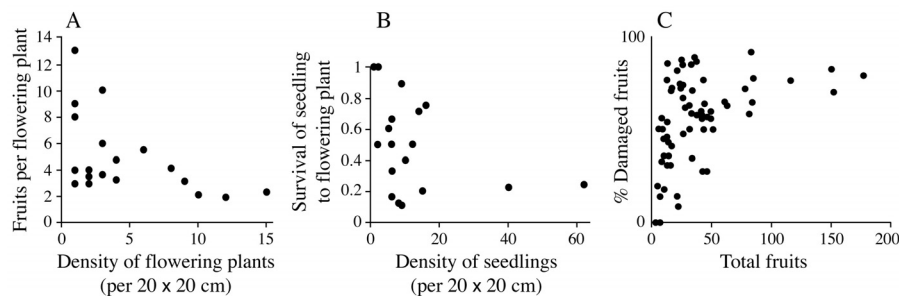


FIGURE 2. The relation between (A) density of flowering plants and fruits per plant, (B) between density of seedlings and survival from seedling to flowering plant and (C) number of fruits per plant and % damaged fruits in *Arabidopsis thaliana*.

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seed production of *A. thaliana*. Applying Steiner solution had a strong significant positive effect on both seed production and survival. Water had a significant effect on seed germination and survival. Disturbance (scratching the soil) only had a significant effect on survival (ANOVA, Tukey test, $P < 0.05$, Table 4, Fig 3).

Ceutorhynchus atomus and *C. contractus* (Curculionidae) were responsible for seed and fruit damage in *A. thaliana*. These weevils

TABLE 4. The effects of artificial disturbance, watering and addition of nutrients on seed germination, survival and seed production per plant for *Arabidopsis thaliana* (mean values per plot $\pm$ SE). The values in each treatment, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$).

	Seed germination (%)	Survival (%)	Seed production
Nutrients and water $n = 5$	10.4 ± 1.6 a	74.2 ± 8.7 a	936.0 ± 318.7 a
Water only $n = 5$	11.0 ± 3.1 a	85.8 ± 6.3 a	151.9 ± 48.1 b
Scratched soil $n = 5$	3.0 ± 0.6 b	72.0 ± 11.6 ab	342.5 ± 234.9 ab
Compacted soil $n = 4$	1.7 ± 0.8 b	12.5 ± 12.5 c	18.0 ± 18.0 b
Control $n = 14$	1.7 ± 0.4 b	26.9 ± 10.1 c	49.1 ± 23.3 b

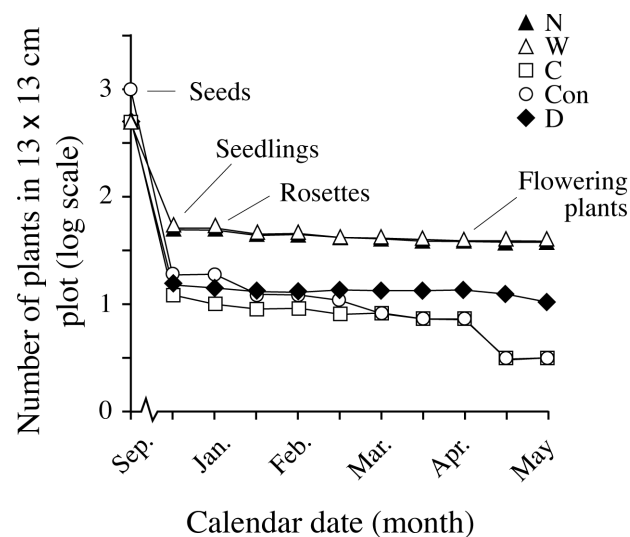


FIGURE 3. Survivorship curves of *Arabidopsis thaliana* in experimental demography (N = nutrients, W = water, C = compact soil, Con = control, D = disturbance).

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had a strong detrimental effect on seed production. Plants protected by a net that excluded the beetles, produced 2417 seeds ($SE = 219$), significantly more than the 1450 seeds ($SE = 131$) produced by plants that experienced natural damage by these weevils (ANOVA; $F = 14.318$, $df = 1$, $P < 0.001$).

DISCUSSION

In which life stage does mortality occur and how does it affect final population density?

Rosette mortality between February and the beginning of bolting in April was most important for the success of the 2002 cohort. Mortality during this stage was the key factor and had a major affect on total population density. Mortality in the seed stage was of less importance for final density. Some mortality during this stage was due to predation before seed dispersal. However, more work needs to be done to explore post-dispersal seed mortality in *A. thaliana*.

Does reduction of the number of seeds affect plant densities in subsequent stages? Our results showed that seedling recruitment is to some extent seed-limited since plots with more seeds contained more seedlings. This indicates that the presence of the weevils can reduce seedling recruitment.

In addition, recruitment can be limited by availability of suitable sites for germination, growth and reproduction (Harper, 1977; Bergelson, 1990a,b; Houle, 1996). Microsite limitation may be due to seed germination requirements (e.g. light, moisture, etc.) and/or to competitive exclusion of seedlings by existing vegetation (Juenger and Bergelson, 2000). We tested these two possibilities experimentally by adding water and by providing two kinds of disturbances (scratching and compacting soil). The results showed that water addition had a significant effect on seed germination but scratching, which resulted in removal of some vegetation, and making the soil more compact, did not. This suggests that germination was not inhibited by existing vegetation.

Seeds that did not germinate due to microsite limitation can potentially be incorporated into the seed bank. Thompson and Grime (1976) studied the composition and dynamics of the seed bank in a range of vegetation types in northern England. They indicated that *A. thaliana* belongs to the group of plants with many seeds that ger-

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minate in the year of release and with few seeds that become incorporated into a persistent seed bank.

The survivorship curve for *Arabidopsis thaliana* is similar to that found for a variety of other autumn-germinating annuals (Beatley, 1967; Mack, 1976). This pattern of survivorship has also been found in populations of plants that grow in open but predictable habitats (Silvertown, 1982). It is however difficult to generalise about survivorship for particular species (Watkinson and Davy, 1985) as mortality may vary between cohorts (Watkinson, 1981; Keddy, 1982; Jefferies et al., 1983), between sites (Jefferies et al., 1981) and may depend on density (Keddy, 1981). Density is of particular importance since it has important consequences for the natural regulation of *A. thaliana* populations. In consistence with the above-mentioned studies, the manipulation of factors like water, nutrient and two kinds of disturbance in experimental demography showed the importance of these factors on survivorship curve.

Is fecundity density-dependent?

Myerscough and Marshall (1973) showed in a laboratory study that increased density negatively affected overall plant performance. Growth, mortality, and fecundity of *A. thaliana* (strain Estland) were all negatively affected by density. We also found negative density dependence in fecundity, between the number of flowering plants and the number of fruits per plant. Similarly, Watkinson and Davy (1985) found a negative density-dependent relationship between reproductive output and the density of surviving plants from three habitats. Also for *Cakile edentula* (Keddy, 1981, 1982), *Salicornia europaea* (Jefferies et al., 1981), *Vulpia fasciculata* (Watkinson, 1978c) and *Vulpia ciliata* ssp. *ambigua* (Carey et al., 1995) plant performance was always higher amongst the sparse vegetation of the pioneer zone than in more densely vegetated areas. We did not find density-dependent mortality but instead our results indicate competitive interactions between *A. thaliana* and other plant species since disturbance (scratching the soil) significantly increased survival of rosette and flowering plants and production of seed.

Which factors cause mortality in different life stages?

Survival from seedling to flowering plant was significantly correlated with soil water content. Water was especially important during late

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March and April, at the time of stem elongation. Whether moisture in the soil at this time is adequate to meet the demands of the winter annual population depends mainly upon precipitation. Comparison of average rainfall for February, March and April in 2002 (119.9, 35.1 and 55.7 mm) and for the same months in 2003 (21.0, 9.7 and 39.5 mm) with fruit production and seed production (Table 1) indicates the positive correlation between these traits and precipitation. Beatley (1967), similar to our results, also found in a study of survival of 53 taxa of winter annuals that mortality happened in March, the period of stem elongation, and was strongly correlated with precipitation.

Our results showed that *Ceutorhynchus atomus* and *C. contractus* (Curculionidae), as natural herbivores of *Arabidopsis thaliana*, reduce significantly the production of viable seeds. These inflorescence feeders had a strong detrimental effect on seed production. The effect of predation on population density is probably buffered by a persistent seed bank (Baskin and Baskin, 1983; Thompson and Grime, 1976) and density-dependent fecundity.

Do specialist herbivores act in a plant size-dependent manner?

At the individual plant level, we showed that herbivores act in a plant size-dependent manner. This indicates that variation in individual fecundity results in differential seed predation among *A. thaliana* plants. The potential consequences of seed predation at the individual level are more interesting but less widely explored. Particularly when seeds are immature and still retained on the parent, adult characters such as fecundity, timing of seed production, and spatial location may influence the severity of predispersal predation on different individuals (Moore, 1978a). If herbivory acts in a density or plant size-dependent manner then it will contribute to the regulation of the plant population and influence the size distribution within population (Ehrlén, 1995). Changes in plant size can alter the competitive relationships between individuals where competition is intense (Harper, 1977).

Many of the secondary metabolites in plants acts as defense against herbivores and it is often postulated that these compounds have evolved under selective pressure from insect herbivory (Ehrlén and Raven, 1964). Some studies have shown that total glucosinolate concentration in *A. thaliana* is under active selection pressure by insects (Mauricio and Rausher, 1997). Mauricio and Rausher measured rosette damage. We observed the damage of several thousands

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of plants during this experiment and during two subsequent growing seasons. We found that rosette damage was always low (2-5%) and the cause of damage was unknown. This indicates that *Arabidopsis thaliana* in this area does not have important specialist or generalist leaf-eating herbivores. Instead, the weevils have a strong effect on seed production of *A. thaliana* and are therefore probably the most important agents of selection for the evolution of defense mechanisms in the Dutch sand dunes.

To sum up, density of *A. thaliana* in our area depends on many factors. Mortality in the seed stage through predation is probably not so important for total mortality. Mortality of rosettes is the key factor: low mortality in this stage corresponds most closely with low mortality over the entire life of the plant. When more plants survive to reproduce, this results in reduced seed production per plant, but this effect is only slight. Also large plants suffer slightly more from seed herbivory. Climate factors in early spring, especially water, have considerable impact at all stages and cause two fold differences per capita seed production between years.

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Herbivory and local adaptation in natural populations of *Arabidopsis thaliana*



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In a transplant experiment *Arabidopsis thaliana* plants from a dune and inland habitat were studied with respect to morphological traits, survival, flowering time, seed production and susceptibility to the specialist beetles *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) that feed on flowers and fruits. We investigated 1) whether differences in these plant characters were environmentally or genetically determined, 2) whether plants were adapted to their local environment, and 3) the importance of specialist herbivores for plant adaptation.

There was a significant site effect in all traits, such that all plants performed better in the inland site with respect to number of stems, plant height, rosette size and seed production. Fruit damage was less in the inland site as well. Genetic differences between populations were observed for several life history and morphological characters and for percentage of fruit damage. In particular, inland origins flowered 10 days earlier, grew better and produced 20% more fruits, but experienced 36% more total fruit damage by weevils. No significant site $\times$ origin interaction was found for rosette size and for percentage of damaged fruits by weevil larvae. Instead, for number of stems, fruit number before herbivory occurs, percentage of damaged fruits by adult weevils, stem height and number of intact fruits an interaction did exist. At least one population at its home site had the highest fitness, defined as, the probability of surviving until reproduction multiplied by unit of intact fruits after herbivory had taken place, which demonstrates adaptation to local environment. In this process the ability of dune genotypes to restrict herbivory by specialist beetles and high survival of inland genotypes to own habitat appears to be an important mechanism responsible for greater relative fitness of *A. thaliana* plants on their home site.

Plants not only stand still and wait to be counted (Harper, 1977) but they are also ideal for transplant experiments to study local adaptation experimentally. Transplant experiments as pioneered by Clausen et al. (1940, 1948) provided evidence of a home site advantage for local genotypes against alien genotypes, illustrating the role of adaptation and genetic differentiation (Antonovics, 1971). Adaptation has been demonstrated as a result of differences in many

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environmental characteristics, including elevation (Clausen et al., 1940; Gurevitch et al., 1986), latitude (Mooney and Billings, 1961), heavy metals (McNeilly and Antonovics, 1968), water availability (Farris, 1987), soil type (Snaydon, 1970), salinity (Antlfinger, 1981) and competition (Turkinson and Harper, 1979; Schoen et al., 1986). The role of herbivores in local adaptation, however, is largely unknown (but see Prins, 1989). Physical and biotic components of the environment could well differ from each other in the patterns of differentiation they generate (Linhart and Grant, 1996).

Arabidopsis thaliana (L.) Heynh. (Cruciferae) is a small annual plant originating from Europe and is now widely distributed in many parts of the northern-temperate zones of the world (Ratcliffe, 1961). *Arabidopsis thaliana* has a rather wide climate amplitude, which makes it suitable for analyzing variation in adaptive traits (Koornneef et al., 2004). While genetic variation within populations is quite low (Bergelson et al., 1998), ecotypes from different locations have been shown to differ from one another in important life history traits. Several studies have demonstrated variation in resistance traits (Mauricio, 1998), flowering time and in morphological and physiological characters (e.g., Griffing and Scholl, 1991; Pigliucci, 1998; Nordborg and Bergelson, 1999; Mitchell-Olds, 2001; Pigliucci and Marlow, 2001; Kover and Schaal, 2002; Koornneef et al., 2004). In *A. thaliana*, however, it is unknown whether these variations are the result of genetic adaptation and lead to a home site advantage for local genotypes. Griffith et al. (2004) tested life-history variation and adaptation in *A. thaliana* plants in North America. They did not find significant differences in performance in a common garden, for plants from different populations or regional groups. However, the common garden and greenhouse experiments do not directly address the potential adaptive nature of genetic variation (Rice and Mack, 1991). Antonovics and Primack (1982) argued that field transplants lead to a more realistic assessment of genetic and environmental effects because they also include biotic factors that might be important for the development of local adaptation.

In this study, we are interested in whether selection by insect herbivores and/or by abiotic factors can be sufficiently strong to cause local adaptation in populations of *A. thaliana*. We will test for adaptation in a transplant experiment. We found *A. thaliana* populations in two different habitats, dune and inland. These habitats differ

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in abiotic conditions (Table 1). In the dune habitat, *A. thaliana* grows in small patches and produces significantly smaller rosettes, smaller stems and fewer fruits compared to plants in the inland. *A. thaliana* in the dune also experienced more than 40% fruit damage by the weevils *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) (Mosleh Arany et al., 2005), whereas no fruit damage was observed on plants in the inland habitat. These observations suggest that biotic and abiotic differences in these two habitats may have a significant effect on the expression of the phenotypes in *A. thaliana*.

This paper addresses the following questions: 1) Are life history traits and susceptibility to herbivores environmentally and/or genetically based? 2) Do local populations perform better than introduced populations? 3) What is the role of naturally occurring herbivores in adaptation?

MATERIAL AND METHODS

Habitat description

One ecosystem where *A. thaliana* can be found in the Netherlands is formed by the coastal sand dunes. Our study area is Meijendel, north of The Hague (latitude 52° 08' N, longitude 4° 22' E). In these dunes *A. thaliana* grows naturally in two habitat types. It is locally common along roads on the old dune system that was formed between 3000 and 5000 years ago and that is still visible in the landscape as long stretches of sand that run parallel to the coast (we will refer to this as inland). It also occurs locally on the more calcareous new dunes that were formed partly on top of the old soil profile c. 800 years ago (we will refer to this as dune).

Offspring of plants from three populations in the dunes (Meijendel) and two in the inland were used for a transplant experiment. All populations in the dunes were found within 20 m from woody vegetation with trees like *Populus nigra*, *P. alba*, *Betula pubescens* and *Crataegus monogyna*. The distance between population 1 and 2 is only 5 m and the third one is separated from the other two by 500 m. Humus content and water content of the top 15 cm of the soil in population 3 were relatively low but in population 2 are high compared to the others (Table 1). The sandy surface at these sites is covered with moss, grasses and small herbs with about 10 percent bare soil. Accompanying species included among others *Erophila verna*,

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TABLE 1. Soil parameters for dune and inland habitat (mean values per population $\pm$ SE). The values in each treatment, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$).

Soil parameters	Dune Habitat			Inland habitat	
	Population (1)	Population (2)	Population (3)	Population (1)	Population (2)
Humus content of the top 10 cm (%) $n = 4$	0.51 ± 0.11 a	0.96 ± 0.24 ab	0.45 ± 0.24 a	1.18 ± 0.68 ab	1.62 ± 0.27 b
Water content of the top 15 cm (%) $n = 3$	4.30 ± 0.09 ab	8.13 ± 2.10 bc	3.04 ± 0.27 a	13.01 ± 0.09 d	12.55 ± 0.14 cd

Cardamine hirsuta, *Rubus caesius*, *Calamagrostis epigejos* with small *Hippophae rhamnoides* shrubs nearby.

Population (1) in the inland is located in Leiden, 3 m from a paved road and the second one, population (2) grows near a stream in Noordwijk. Both sites were covered with *Lolium* sp. with about one percent bare soil. Humus content and water content of the top 15 cm of the soil in both populations were almost the same (Table 1). Accompanying species included amongst others *Erophila verna*, *Cardamine hirsuta*, *Plantago lanceolata*. The distance between the two

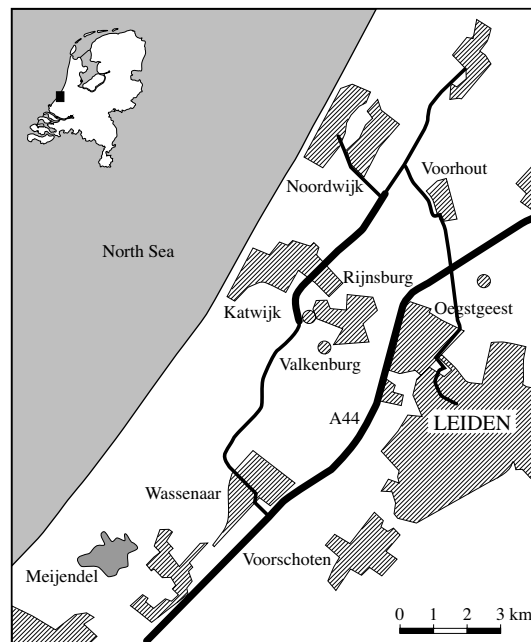


FIGURE 1. Map shows the location of study areas.

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inland populations is about 8 km and the minimal distance between the dune and the inland habitat is about 16 km (Fig. 1).

Transplant experiment

Seeds of ten randomly chosen plants were collected from each of three populations in the dune and in two populations in the inland sites in July 2002. Plants were grown for one generation under controlled conditions in a growth chamber (20°C, 18-h light, 70% humidity and 2 months in a cold room at 4°C at the rosette stage) to reduce maternal effects due to variable field environment. Selfed seeds from these plants were then kept at room temperature until October 2003 when they were germinated in a growth chamber. When rosettes were also the same size and age as *A. thaliana* rosettes in the field, they were transplanted into an enclosure of 16 m² in the dunes close to population 3 and another one in the common garden at Leiden, near population 1. We were not allowed to set up an enclosure at the original inland site but consider the chosen garden site to be very similar to the two inland sites (*A. thaliana* naturally is growing in the garden as well). Rosettes of 10 families in 8 replicates from each of the 5 populations were transplanted into a randomized complete block design in each of the 2 enclosures. Hence a total of 800 plants were transplanted. Within each enclosure rosettes were positioned at 10-cm intervals. The rosettes were transplanted into small holes with minimal disturbance of the surrounding vegetation and the position of each plant was mapped. Survival, flowering time, fecundity and morphological characteristics such as rosette size, stem number and plant height were measured before harvesting the plants. Fruit damage by adult weevils and their larvae was measured after harvesting the plants. Damage by weevil larvae was measured by opening the fruits and looking for seed damage. Weevil larvae damaged about 80% of the seeds in a damaged fruit (Mosleh Arany et al., 2005). In this study we measured damage only at the fruit level and in further calculations assume that the seed damage per damaged fruit was equal for all genotypes. Fitness was estimated as the probability of surviving until reproduction multiplied by units of intact fruits after herbivory had taken place. Each damaged fruit counts as 0.2 unit of intact fruit since on average it contains only 20% of the seeds in an intact fruit. In equation: fitness = survival $\times$ (intact fruit + damaged fruit $\times$ 0.2).

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Data were analyzed with SPSS (SPSS Inc., Chicago, USA). Differences in all measured traits within and between genotypes in each site, were tested with SPSS 10 (Nonparametric Test, K-independent Sample, test criterion Kruskal-Wallis H). To analyze the interaction between origin and site, it is convenient to use an ANOVA. For this purpose data were log-transformed and we tested whether the residuals of the full ANOVA were normally distributed. The residuals for fruit number, damage by weevils and intact fruit did not deviate significantly from a normal distribution. The other variables (number of stems, stem height and rosette size) were near the normal distribution, but due to the high number of data points (696) significant differences from normality did exist. Specifically the distribution of the residuals of log stem number was slightly skewed to the left (skewness = -0.695, kurtosis = 1.643), the distribution of log height was slightly skewed to the left as well (skewness = -1.802, kurtosis = 9.801) and the log rosette size was slightly skewed to the right (skewness = 0.870, kurtosis = 11.909). Because these deviations from normality were small we did analyze all data for the origin $\times$ site interaction with ANOVA (SPSS 10, General Linear Model, Univariate, type III Sums of Squares). To make the analysis simple as straightforward we, first check whether within the same population the different mother plants from which seeds were used were significantly different. Because in no case these differences were significant we pooled all data of seedling originating from different mother plant within the same population.

RESULTS

There was both environmental and genetic variation in the observed phenotypes in two habitats. For all traits there was a significant site effect (Table 2). The number of stems, stem height, rosette size and the number of fruits before and after herbivory was higher when plants grew in the inland site. Percentage of damage by adult weevils and their larvae was lower in the inland site.

A Kruskal-Wallis H test showed significant genetic differentiation between the dune and the inland origins for all traits measured in the dune site and for number of stems, number of fruits and damage by weevil larvae in the garden site. There were within the dunes differences for all traits except for damage by adult weevils and by their

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TABLE 2. Analysis of variance for morphological and life history traits (SPSS 10, General Linear Model, Univariate, type III Sums of Squares).

Trait	Source	df	F value	P
No. stems	Origin	4	16.18	$P < 0.001$
	Site	1	329.23	$P < 0.001$
	Site $\times$ Origin	4	2.57	$P = 0.037$
Stem height	Origin	4	4.20	$P < 0.05$
	Site	1	1447.37	$P < 0.001$
	Site $\times$ Origin	4	4.13	$P < 0.003$
Rosette size	Origin	4	13.36	$P < 0.001$
	Site	1	46.88	$P < 0.001$
	Site $\times$ Origin	4	1.24	ns
Fruit number	Origin	4	8.66	$P < 0.001$
	Site	1	1488.10	$P = 0.001$
	Site $\times$ Origin	4	2.76	$P < 0.027$
Damage by weevil larvae (%)	Origin	4	23.28	$P < 0.001$
	Site	1	256.22	$P < 0.001$
	Site $\times$ Origin	4	1.00	ns
Damage by weevil adults (%)	Origin	4	14.07	$P < 0.001$
	Site	1	608.50	$P < 0.001$
	Site $\times$ Origin	4	7.96	$P < 0.001$
Intact fruits	Origin	4	5.80	$P < 0.001$
	Site	1	1892.08	$P < 0.001$
	Site $\times$ Origin	4	9.19	$P < 0.001$

larvae in the dune site and for the number of fruits before herbivory occurs and intact fruits in the garden site. There also were differences within inland for number of stems, stem height and number of fruits in the dune site and for stem height, rosette size, number of fruits and for damage by weevil larvae in the garden site (Fig. 2, Table 3).

In the dune site, plants from inland origins started flowering on 30 March (population 1) and on 3 April (population 2). The dune origins (all 3 populations) started flowering on 12 April. In the garden site all genotypes behaved similar as they did in the dune site but with approximately 4 days delay.

The survival of plants was recorded from rosette to seed production. The comparison of survival of origins within sites, using a chi-square analysis, showed no significant differences in the garden site. There were significant differences in survival between the different origins in the dune site. The dune origins survived better than the inland origins in the dune site (Fig. 2 H).

There was an origin $\times$ site interaction for the number of stems, stem height, fruit number, for damage by adult weevils and most

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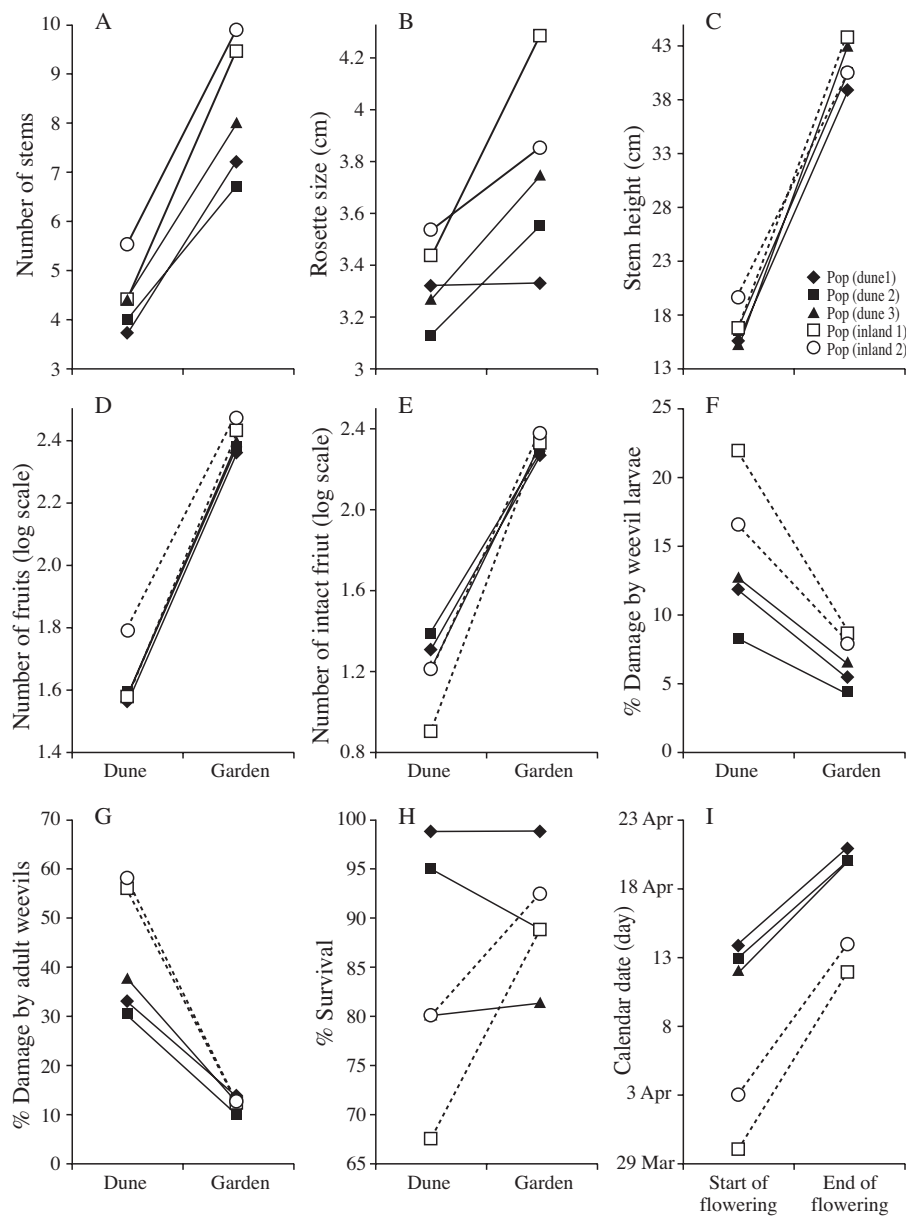


FIGURE 2. Mean values for (A) number of stems, (B) rosette size (cm), (C) stem height (cm), (D) number of fruits, (E) intact fruits, (F) % fruit damage by weevil larvae (G) % fruit damage by adult weevils, (H) % survival, for plants from 5 populations (3 in dunes, 2 in inland) are given for two habitats (the dune and the inland). Mean values for (I) flowering time is given for the dune habitat.

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TABLE 3. The differences within and between the dune and the inland genotypes in damage by adult and weevil larvae, morphological and life history traits (SPSS 10, Non-parametric Test, K-independent Sample, test criterion Kruskal-Wallis H). D = within the three dune genotype, I = within the two Inland genotype, D-I = between the dune and the inland genotypes.

Trait	Source	The dune site	The inland site
Number of stems	D	$P < 0.003$	$P < 0.036$
	I	$P < 0.008$	ns
	D-I	$P < 0.001$	$P < 0.001$
Stem height	D	ns	$P < 0.022$
	I	$P < 0.027$	$P < 0.016$
	D-I	$P = 0.001$	ns
Rosette size	D	$P < 0.002$	$P < 0.020$
	I	ns	$P < 0.022$
	D-I	$P < 0.001$	$P < 0.001$
Number of fruits	D	$P < 0.046$	ns
	I	$P < 0.003$	ns
	D-I	$P < 0.025$	$P < 0.006$
Damage by weevil larvae (%)	D	ns	$P = 0.001$
	I	ns	$P < 0.046$
	D-I	$P < 0.001$	$P < 0.001$
Damage by weevil adults (%)	D	ns	$P < 0.002$
	I	ns	ns
	D-I	$P < 0.001$	ns
Intact fruits	D	$P = 0.001$	ns
	I	ns	ns
	D-I	$P < 0.001$	ns

importantly for the number of intact fruits produced (Table 2). There were a significantly higher number of intact fruits for dune origins, populations 1 and 2, at the dune site and for inland origins, population 2, at the garden site.

Results demonstrate that native genotypes, population 2 of dune origins in dune site and population 2 of inland origins in the garden site had a home site advantage for fitness compared to introduced genotypes (Fig. 3).

DISCUSSION

Our transplant experiment revealed that there were both environment and genetic components linked to the observed phenotypic differences between the natural populations of *A. thaliana* found in dune and in the inland habitats. Plants grown at the dune site produced

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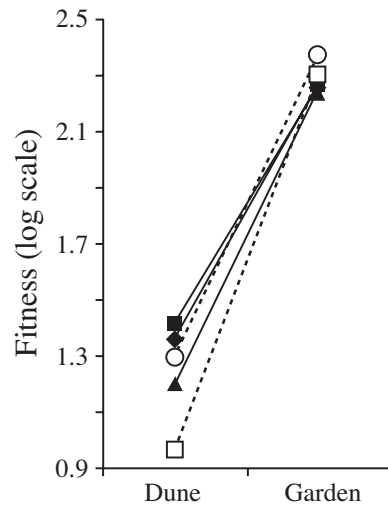


FIGURE 3. Mean values for fitness for 5 populations (3 in dunes, 2 in inland) are given for two habitats (the dune and the inland).

smaller rosettes, shorter and fewer stems, fewer fruits and experienced more damage than plants in the garden. Differences in the abiotic factors of two habitats that may account for this plasticity include water and humus content of the soil (Table 1). To include stress imposed by intraspecific competition as well as herbivory, plants were transplanted to the two sites with minor disturbance of the substrate. Herbivore pressure differed between two sites. Only *C. contractus* occurred with low frequency in the garden site and at the dunes *C. atomus* and *C. contractus* occurred in high frequency. The herbivory differences between two habitats may also account for the plasticity in herbivory damage between two genotypes. Our study is consistent with other studies of phenotypic plasticity in *A. thaliana* (e.g. Clauss and Aarssen, 1994; Pigliucci and Schlichting, 1995, 1998).

We found a significant effect of origin between genotypes of two habitats. The inland origins (population 2) performed better for number of fruits and number of stems in both sites. Origin effect was found for another inland population (population 1) for damage by weevil larvae. This population experienced more fruit damage in both sites. We also found the inland origins produced flowers sooner than dune origins. Several other studies have demonstrated similar results for flowering time as well as for important morphological and physiological characteristics (Griffing and Scholl, 1991; Pigliucci, 1998;

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Nordborg and Bergelson, 1999; Mitchell-Olds, 2001; Pigliucci and Marlow, 2001; Kover and Schaal, 2002; Koornneef et al., 2004).

Three dune populations performed almost similarly for susceptibility to weevil herbivory in the dune site but not in the garden site. This similarity may reflect a similarity in the past natural selection that was experienced by ancestors of all the dune plants in the dune habitat. Selection may act in similar directions in different populations because the habitat of *A. thaliana* may be similar in the dune site. The biotic factor such as the main herbivores of *A. thaliana* in this area, *Ceutorhynchus atomus*, *C. contractus*, and also intraspecific competition are almost the same for all populations at the dune site (Mosleh Arany et al., 2005). However, the dune populations differed for the other studied traits. The differences in abiotic (edaphic, climate) factors between populations in the dune probably are sufficiently strong to cause differences between populations. These were almost true for inland populations as well.

In general inland origins flowered sooner than the dune origins. Earlier flowering was associated with the highest fruit production, as has been observed in other studies in *Arabidopsis* (Clauss and Aarssen, 1994; Dorn et al., 2000). Early flowering permits fruit maturation before the summer drought. The inland origins that had these favourable phenotypic characteristics had unfavourable ones for others, such as susceptibility to larva and adult weevil herbivory. The opposite was true for dune origins. The number of intact fruits that is a result of a combination of all characters appears to be important determinant of performance in these genotypes.

The inland origins survived better in the garden compared to the dune site. Rosette mortality between February and the beginning of flowering in April seems most important for the success of a cohort and are dependent on the rainfall. Mortality during this stage is the key factor and has a major affect on total population density (Mosleh Arany et al., 2005). The rainfall between February and April at the time of experiment (2004) was sufficient for the rosettes to survive and to flower (Feb, 72.3 mm; Mar, 48.9; Apr, 40.9). Van Groenendael (1985) questioned whether in the long run is the test really reciprocal, after all the hazards of life have been encountered. In the short term there might be a severe imbalance in the reciprocity. Differences in survival of rosette to seed set between the dune and the inland origins could be demonstrated better in environmentally severe years such if the rainfall was as in 2003 (Feb, 21 mm; Mar, 9.7; Apr, 39.5).

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It has been discussed that in transplant experiments, the various phases of the life cycle should be investigated as completely as possible (van Groenendael, 1985). We only tested the growth and survival of *Arabidopsis* rosette. The survival of seeds in the soil, the germination and subsequent survival of seeds and seedlings as well as pollen production deserve future consideration.

Adaptive genetic differentiation between the dune and the inland genotypes is demonstrated by interaction between site and origins, which suggests local adaptation (van Groenendael, 1985). This interaction was significant for all studied traits. The origin $\times$ site interaction illustrates that not only are the dune and the inland genotypes genetically distinct, but that selection has acted differently at these two sites and we can speak of distinct ecotypes. Metabolomic analysis performed by NMR spectroscopy and multivariate data analysis of these two genotypes also gave clear discrimination based on metabolites in the seeds and leaves (chapter 4, 5). Plants at their home site had the highest fitness, which demonstrates adaptation to local environment. The components of fitness here, variation in survival of rosette to flowering and seed set (caused by abiotic factors of environments) and variation in number of intact fruits (caused by biotic factors), each one separately showed home site advantage for local genotypes (Fig. 2 H, E). This showed both abiotic as well as biotic parameters of environment are important for the development of localized adaptation. These results also showed physical and biotic components of the environment differ from each other in the patterns of differentiation they generate. Therefore, both abiotic and biotic factors appear to be important mechanisms responsible for greater relative plant fitness.

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Differences in chemical composition of *Arabidopsis thaliana* seeds and implications for plant-herbivore interactions



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Plants of *Arabidopsis thaliana* that originated from dune or inland populations transplanted to the same habitat (dune or inland) were affected differently by the specialist weevils, *Ceutorhynchus atomus* and *Ceutorhynchus contractus* (Curculionidae) which feed on flowers and fruits. To confirm the possible genetic differences in defense we collected seeds of two plant types in the field, grew them in the growth room for one generation and performed a metabolomic analysis on the new seeds produced using NMR spectroscopy and multivariate data analysis. Major differences in chemical composition were found in the water-methanol fractions: more thioglucosinolates and sucrose in dune and more sinapoylmalate in inland populations.

Quantitative analysis of glucosinolates was done with HPLC-UV, using the same seed batches. Glucosinolate composition and concentration differed between individual plants, populations and sites.

Fruit damage by adult weevils and their larvae was not correlated with field concentrations of individual glucosinolates, glucosinolate groups and total concentration of glucosinolates in seeds.

We conclude that, given the range of glucosinolate concentrations in dune and inland plants of *Arabidopsis thaliana*, other factors might also be involved in defense against herbivory by the weevils.

Many specialist insect herbivores on Cruciferae use glucosinolates as oviposition and feeding stimulants (Chew, 1988; Nielsen, 1988; and others). Some specialists are also able to discriminate between different members of the Cruciferae family (Nielsen, 1978; Nielsen et al., 1979, 1989; Larsen et al., 1985; Moyes and Raybould, 2001; Kalischuk and Dosdall, 2004), as well as among plants of the same species (Louda and Rodman, 1983; Moyes, 1998; Bossdorf et al., 2004). Qualitative and quantitative variations in glucosinolates content might explain variations in host plant selection by specialized insects (Nielsen et al., 2001).

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Different responses of specialist herbivores to glucosinolates might be explained by the fact that more than one single chemical factor affects selection and performance. Variation in nitrogen and sugars (Blau et al., 1978; van der Meijden et al., 1989) may additionally affect oviposition and larval growth of specialist herbivores. The presence of other feeding and oviposition stimulants in cruciferous host plants has been demonstrated by Nielsen et al. (1979), Schoni et al. (1987), Nielsen (1991) and Griffiths et al. (2001) and some of these compounds have been identified.

In our study on natural herbivores in *A. thaliana* populations we found that plants experienced more than 40% fruit damage by the specialist weevils *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) in coastal sand dune (Mosleh Arany et al., 2005), whereas hardly any fruit damage was observed on plants in inland habitat. To test whether these differences are due to environmental influences or to plant genotype, we set up a transplant experiment. To examine the cause of differences in plant defense we compared herbivory on fruits with data on glucosinolate concentration of seeds, collected in the field. We also aimed to look in more detail at differences in glucosinolates and often chemical composition in the seeds of plant originating from dune and inland populations of *A. thaliana*. For this reason we used two analytical techniques. NMR spectroscopy is a technique that produces a wide spectrum chemical analysis, which is rapid, reproducible, stable in time and gives information about a range of chemicals. HPLC-UV in general offers good selectivity and sensitivity and provides detailed data on the quantity of target compounds (Summer et al., 2003). Therefore we used NMR spectroscopy to find the differences between plants with respect to a large number of chemical compositions and we used HPLC-UV to look in more detail at a single group of target compounds, the glucosinolates. Therefore, in this way, we use only HPLC-UV for the compounds that were found to differ in NMR spectroscopy, we did not have to look at all chemical compositions. We analysed *A. thaliana* plants grown under identical conditions in the growth room with NMR spectroscopy, and plants from growth room and field with HPLC-UV. This paper addresses the following questions. Is there a difference in herbivory on *A. thaliana* from dune and inland when plants are grown under the same conditions? What are the differences in chemical composition of the seeds of *A. thaliana* from dune and inland? Are differences in chemical com-

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position environmentally or genetically based? Do glucosinolate concentrations affect the suitability of *A. thaliana* as food for the specialist herbivores *Ceutorhynchus atomus* and *C. contractus* (Curculionidae)?

MATERIAL AND METHODS

Habitat description

The coastal sand dunes form one of the ecosystems where *A. thaliana* is found in the Netherlands. In these dunes *A. thaliana* grows naturally in two habitat types. It occurs locally on the more calcareous new dunes that were formed on top of the old soil profile c. 800 years ago (called dune hereafter). In South and North Holland, two Dutch coastal provinces, it is also locally common in road verges of roads on top of the old dune system that was formed between 3000-5000 years ago and that is still visible in the landscape as long stretches of sand that run parallel to the coast (called inland hereafter). Populations may have grown here for a long time or seeds came from elsewhere, the age of the populations we study here is not known.

Offspring of plants from three populations in the dunes, Meijendel, north of The Hague (latitude 52° 08' N, longitude 4° 22' E) and two in the inland were used for a transplant experiment. In this paper we studied only two of these three dune populations (called dune 2 and dune 3 hereafter). All populations in the dunes were found within 20 m from woody vegetation with trees like *Populus nigra*, *P. alba*, *Betula pubescens* and *Crataegus monogyna*. The distance between population 2 and 3 is 500 m. Humus content and water content of top 15 cm of the soil in population 3 are relatively low but in population 2 are high compared to others (Table 1). The sandy surface at these sites is covered with moss, grasses and small herbs with about 10 percent bare soil. Accompanying species included amongst others *Erophila verna*, *Cardamine hirsuta*, *Rubus caesius*, *Calamagrostis epigejos* with small *Hippophae rhamnoides* shrubs nearby. Population (1) in the inland is located in Leiden, 3 m from a paved road and the second one, population (2) growing near a canal in Noordwijk. Both sites were covered with *Lolium* sp. with about one percent bare soil. Humus content and water content of top of 15 cm of the soil in both inland populations are almost similar (Table 1). Accompanying species included amongst others *Erophila verna*, *Cardamine hirsuta* and *Plantago lanceolata*. The distance between the two inland populations (called inland

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TABLE 1. Soil parameters for dune and inland habitats (mean values per population (+ SE). The values in each treatment, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$).

Soil parameters	Dune Habitat		Inland habitat	
	Dune 2	Dune 3	Inland 1	Inland 2
Humus content of top 10 cm (%) $n = 4$	0.96 (0.24) ab	0.45 (0.24) a	1.18 (0.68) ab	1.62 (0.27) b
Water content of top 15 cm (%) $n = 3$	8.13 (2.10) b	3.04 (0.27) a	13.01 (0.09) c	12.55 (0.14) bc

1 and inland 2 hereafter) is about 8 km and the minimal distance between the dune and the inland habitat is about 6 km.

Transplant experiment

Seeds of ten randomly chosen plants were collected from each of the dune and inland populations in July 2002. To reduce maternal effects, plants were grown for one generation under controlled conditions in a growth chamber (20°C, 18-h light, 70% humidity and, to induce bolting, 2.5 months in a cold room at 4°C at the rosette stage). Selfed seeds from these plants were then kept at room temperature until October 2003 when they were germinated in a growth chamber. When rosettes had approximately reached the same size as *A. thaliana* rosettes in the field, they were transplanted into an enclosure of 16 m² in the dunes close to dune 3 and into another one in the common garden at Leiden, near inland 1. We were not allowed to set up enclosures at the original inland site but consider the garden site to be similar to the two inland sites (*A. thaliana* was growing naturally in the garden as well). Rosettes of 10 families in 8 replicates from each of the populations were transplanted into a randomized complete block design in each of the two enclosures. Within each enclosure rosettes were positioned at 10-cm intervals. The rosettes were transplanted into small holes with minimal disturbance of the surrounding vegetation, after which the position of each plant was mapped. Fruit damage by adult weevils and their larvae was measured one month after harvesting the plants. Damage by weevil larvae was measured by opening all fruits and looking for seed damage. Weevil larvae consumed about 80% of the seeds in an attacked fruit (Mosleh Arany et al., 2005). In this study we measured damage only at the fruit level and in further calculations assume that the number of

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intact seeds per damaged fruit was equal for dune and inland type plants.

Chemical analysis

NMR spectroscopy

The same seed batches used for setting up the transplant experiment were used for NMR spectroscopy. Seeds of 8 dune and 8 inland plants were grown in a growth chamber and 30 mg of the new seeds produced were used for metabolome analysis. Extraction and measurement of compounds followed the procedure of Choi et al. (2004).

All spectra were recorded on a Bruker AV-400 NMR spectrometer operating at a proton NMR frequency of 400.13 MHz. After measurements, the ^1H -NMR spectra were automatically reduced to ASCII files using AMIX (Analysis of MIXtures software v. 3.8, Bruker Biospin). Spectral intensities were scaled to HMDSO (hexamethyl disilane) and trimethyl silane propionic acid sodium salt (TSP- d_4) for chloroform and water-methanol fractions, respectively, and reduced to integrated regions, called 'buckets', of equal width (0.02 ppm) corresponding to the region of δ 10.0 to -0.1. The generated ASCII file was imported into Microsoft Excel for the addition of labels and then imported into SIMCA-P 10.0 (Umetrics, Umeå, Sweden) for PCA analysis.

HPLC-UV and glucosinolates assay

Seed batches of nine individual plants, from each dune and inland population, grown at the two sites (dune and garden) in the transplant experiment and from the growth room were used for HPLC-UV analysis. Sample sizes decreased to 7 seed batches for dune 3, 6 for inland 1 and 5 for inland 2 as not enough seeds were available for analysis, due to heavy herbivory by weevils. During the experiment we accidentally lost two samples, which reduced the sample size of the inland 1 to 8 for seeds collected from garden site and growth room. For HPLC-UV analysis 30 mg seeds was used. Extraction, purification and glucosinolate measurement followed the protocol used by van Dam et al. (2003). Glucosinolates were extracted with 70% methanol solution, desulphatased with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column and separated on a reversed phase C-18 column on HPLC with an acetonitril-water

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gradient. The elution program was a linear gradient starting at 0% acetonitrile (ACN) that increase to 35% ACN in water over 30 minutes. Detection was performed with a single wavelength detector set to 229 nm. Glucosinolates that could not be identified were clarified based on their UV absorption spectrum.

Statistical analysis

Data were analysed with SPSS 10 (SPSS Inc., Chicago, USA). Normality of the data was checked by posthoc analysis of the residuals using the Kolmogorov-Smirnov test of normality. Differences in damage by adult weevils and their larva and differences in glucosinolate concentration between dune and inland populations were tested with ANOVA (General Linear Model, Univariate, type III Sums of Squares). The correlation between glucosinolates and damage by adult weevils and their larva was analysed with a Pearson test. To make the analysis simple and straightforward, we first checked whether for one population the mother plants from which 8 seedling analysed were significantly different. In no case this difference was significant and therefore we pooled within each population all data of seedlings delivered from different mother plants.

Data analysed with a principal component analysis. Principal component analysis (PCA) is a clustering method requiring no knowledge of the data set, which acts to reduce the dimensionality of multivariate data, while preserving most of the variance within the data (Goodacre et al., 2000). The principal components can be displayed in a graphical fashion as a 'scores' plot. This plot is useful for observing any grouping in the data set. PCA models were constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Eriksson et al., 2001). Thus for NMR spectroscopy data, loading plots can be used to detect the spectral areas responsible for the separation in the data.

RESULTS

Herbivory assessment

In the dune site *A. thaliana* plants that originated from the inland populations, experienced significantly more fruit damage by both adults and larvae of *Ceutorhynchus atomus* and *C. contractus* (Curculionidae)

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TABLE 2. The mean (+ SE) fruit damage per plant by adults and larvae of *Ceutorhynchus atomus* and *Ceutorhynchus contractus* (Curculionidae) on dune and inland type plants in two sites. Herbivore in garden site is *Ceutorhynchus contractus*. (*n*, denotes replicates, i.e. the number of plants within cell).

		Transplant site			
		Dune site		Garden site	
		Fruit damage (%) by weevil			
Origin plants	Populations	adults	larvae	adults	larvae
Dune plants	Dune 2	30.07 (2.48) a*	8.24 (0.80) ab	9.67 (0.61) a	4.17 (0.47) a
		<i>n</i> = 76	<i>n</i> = 76	<i>n</i> = 72	<i>n</i> = 72
	Dune 3	37.98 (2.69) a	12.73 (1.51) a	12.37 (0.84) ab	6.49 (0.48) b
		<i>n</i> = 64	<i>n</i> = 64	<i>n</i> = 65	<i>n</i> = 65
Inland plants	Inland 1	55.77 (4.21) b	21.94 (3.06) c	12.31 (0.69) ab	8.80 (0.56) bc
		<i>n</i> = 51	<i>n</i> = 51	<i>n</i> = 69	<i>n</i> = 69
	Inland 2	58.11 (3.14) b	16.59 (1.35) bc	12.45 (0.84) b	7.70 (0.70) b
		<i>n</i> = 67	<i>n</i> = 67	<i>n</i> = 74	<i>n</i> = 74

*Within a column the values in each treatment, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$).

compared to plants from the dune populations in the same site (Table 2). In the garden site, the inland plants suffered more damage by the larvae of *C. contractus* compared to dune 2. In the garden site, inland 2 suffered more damage by the adult weevils compared to dune 2.

Chemical analysis

Chemical composition of seeds detected by NMR spectroscopy

Examination of the score plot of the chloroform fraction demonstrated that all four populations are separated by PC1 and PC2 (Fig. 1A) and the separation between dune and inland is mainly linked to PC1. The loading plots (Fig. 1B) of PC1 show that this separation is due to the signals at δ 5.34, 4.30, 4.14, 1.28 and 0.88, which belong to fatty acids and lipids. More fatty acids and lipids were present in the inland plants.

The score plot of the water-methanol fraction is shown in Fig. 2A. PC1 and PC2 give a clear separation of populations and the separation between dune and inland populations is mainly linked to PC1. The loading plots of PC1 show that this separation is mostly due to signals of thioglucosinolates (δ 5.00, 2.70, 2.10, 1.72), sinapoylmalate (δ 6.94) and sucrose (δ 5.42). Thioglucosinolates and sucrose signals show a negative value, and sinapoylmalate signals show a positive

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TABLE 3. Glucosinolate composition of seeds of dune and inland populations after growing plants in dune, inland and growth room. Mean concentration ($\pm$ SE) (μ moles/g) per glucosinolate is given.

	Dune site			Garden site			Growth room					
	D 2*	D 3	I 1	I 2	D 2	D 3	I 1	I 2	D 2	D 3	I 1	I 2
Glucosinolates (type)	n = 9	n = 7	n = 6	n = 5	n = 9	n = 9	n = 8	n = 9	n = 7	n = 7	n = 8	n = 8
Unknown	0.76 (0.06)	0.87 (0.22)	1.02 (0.18)	0.1	0.66 (0.08)	0.89 (0.07)	0.86 (0.07)	0.07 (0.03)	0.76 (0.03)	0.56 (0.03)	0.97 (0.15)	0.02 (0.02)
alkenyl GLS (D)												
3 OH propyl	2.06 (0.25)	1.14 (0.26)	4.24 (0.99)	0.29 (0.03)	3.19 (0.37)	3.97 (0.28)	3.86 (0.34)	0.55 (0.09)	3.63 (0.14)	2.87 (0.15)	4.30 (0.64)	0.35 (0.06)
GLS (E)												
Unknown sulfur	0.82 (0.25)	0.48 (0.12)	0.35 (0.29)	0.32 (0.03)	0.29 (0.06)	0.49 (0.09)	0.12 (0.09)	0.14 (0.04)	0	0	0.12 (0.11)	0.23 (0.07)
containing GLS (A)												
Progoitrin (D)	0.03 (0.01)	0.03 (0.03)	0.03 (0.01)	0	0	0	0	0	0	0	0	0
Epiprogoitrin (D)	0.13 (0.04)	0.07 (0.03)	0.15 (0.07)	0.23 (0.07)	0.07 (0.03)	0.16 (0.03)	0	0	0.29 (0.01)	0.29 (0.01)	0	0.08 (0.04)
Sinigrin (D)	7.43 (1.43)	4.77 (0.38)	2.25 (0.23)	7.33 (2.24)	2.33 (0.27)	2.73 (0.33)	0.17 (0.17)	1.27 (0.40)	3.84 (0.08)	3.12 (0.34)	0	1.08 (0.31)
Unknown thio	2.68 (0.73)	1.44 (0.32)	1.89 (1.55)	1.70 (0.09)	3.43 (0.45)	3.90 (0.39)	1.65 (1.22)	1.79 (0.39)	0.29 (0.02)	0.95 (0.20)	1.18 (1.15)	2.69 (0.68)
GLS (D)												
4 hydroxy- glucobrassicin (I)	0	0	0	0	0	0	0	0	0.04 (0.01)	0	0	0
Unknown sulfur	0.42 (0.09)	0.29 (0.02)	0.28 (0.02)	0.41 (0.02)	0.38 (0.06)	0.44 (0.07)	0.19 (0.01)	0.20 (0.04)	0.55 (0.03)	0.45 (0.03)	0.12 (0.04)	0.27 (0.04)
containing GLS (A)												
Glucorucin (A)	0.12 (0.09)	0	0	0	0	0	0	0	0	0	0	0
Glucobrassicin (I)	0.18 (0.04)	0.07 (0.01)	0.12 (0.02)	0.10 (0.01)	0.12 (0.01)	0.09 (0.01)	0.09 (0.02)	0.06 (0.01)	0.85 (0.04)	0.27 (0.02)	0.21 (0.02)	0.17 (0.02)
Glucohirsutin (A)	4.98 (0.94)	3.58 (0.20)	3.82 (0.28)	5.7 (0.29)	5.62 (0.58)	5.98 (0.66)	3.33 (0.18)	5.11 (0.37)	8.46 (0.42)	6.53 (0.31)	3.72 (0.37)	5.99 (0.78)
Unknown thio	2.67 (0.56)	1.68 (0.11)	1.99 (0.20)	2.09 (0.12)	2.54 (0.21)	2.87 (0.23)	2.04 (0.13)	2.01 (0.15)	4.03 (0.23)	3.90 (0.12)	1.91 (0.07)	2.19 (0.22)
GLS (D)												

*D 2 and 3 refer to two populations originating from the dunes and I 1 and 2 refer to populations originating from Leiden and Noordwijk. (D) = aliphatic glucosinolates with straight and branched chains (olefins); (A) = glucosinolates with sulfur-containing side chains; (E) = glucosinolates with alcohol side chains; (I) = indol glucosinolates. 1 (0) means below detection limits.

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value in PC1. This indicates that contents of thioglucosinolates and sucrose in dune populations (negative position in PC1) are higher than those of inland ones. On the other hand, sinapoylmalate content is higher in inland plants.

Glucosinolate of seeds in HPLC-UV

Thirteen principal glucosinolates were found in the *A. thaliana* seeds (Table 3). They could be classified into four structural types according to Fahey et al. (2001): indol glucosinolates (I), aliphatic glucosino-

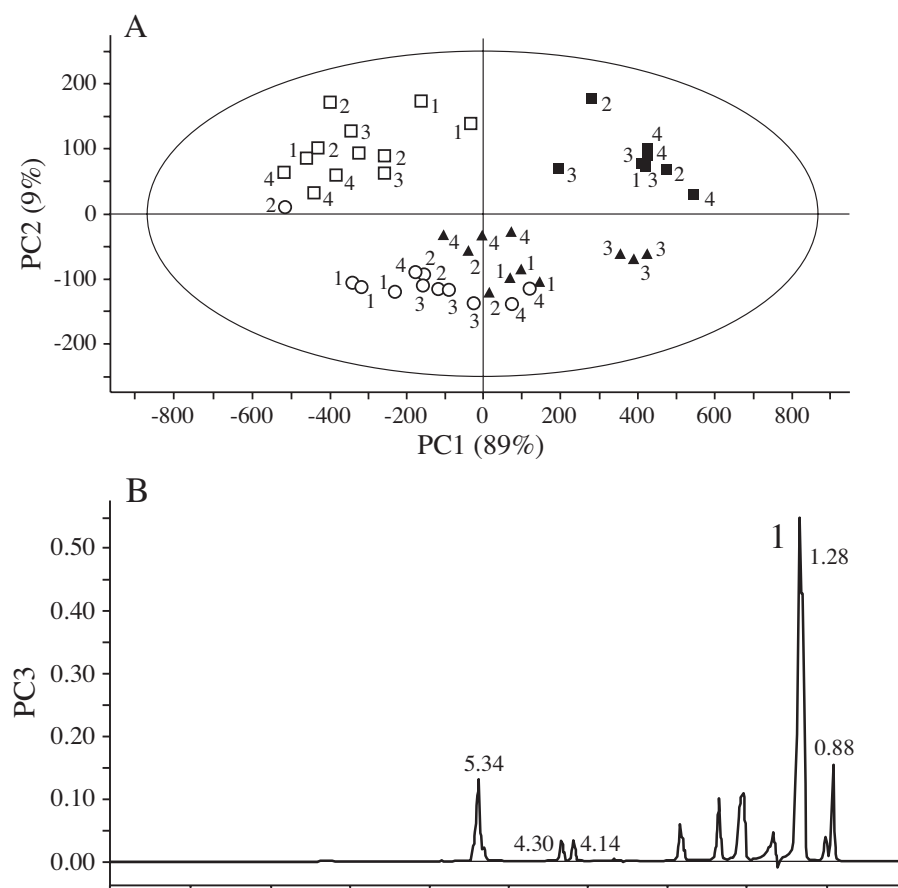


FIGURE 1. Score (A) plot and loading (B) plot of principal component analysis of the chloroform fraction of *Arabidopsis thaliana* seed extracts. Black squares: inland 1; black triangles: inland 2; white squares: dune 2; white circles: dune 3. The ellipse represents the Hotelling T^2 with 95% confidence in score plots. The experiments were based on the 2-3 replicated samples from 8 dune and 8 inland plants. (1) The number over a peak in the loading plot refers to the chemical shift (δ) in the NMR spectrum.

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lates with straight and branched chains glucosinolates or olefins (D), glucosinolates with alcohol side chain (E), and glucosinolates with sulfur-containing side chains (A) (Table 4). Glucosinolates differed in their quantities and abundance between individual plants, populations and between the three sites. At site level (dune, garden and growth room), glucoerucin and progoitrin were found only in seeds collected from the dune and 4-hydroxyglucobrassicin only in seeds collected from growth room (Table 3). The concentration of olefin glucosinolates was significantly higher in seeds collected from dune site com-

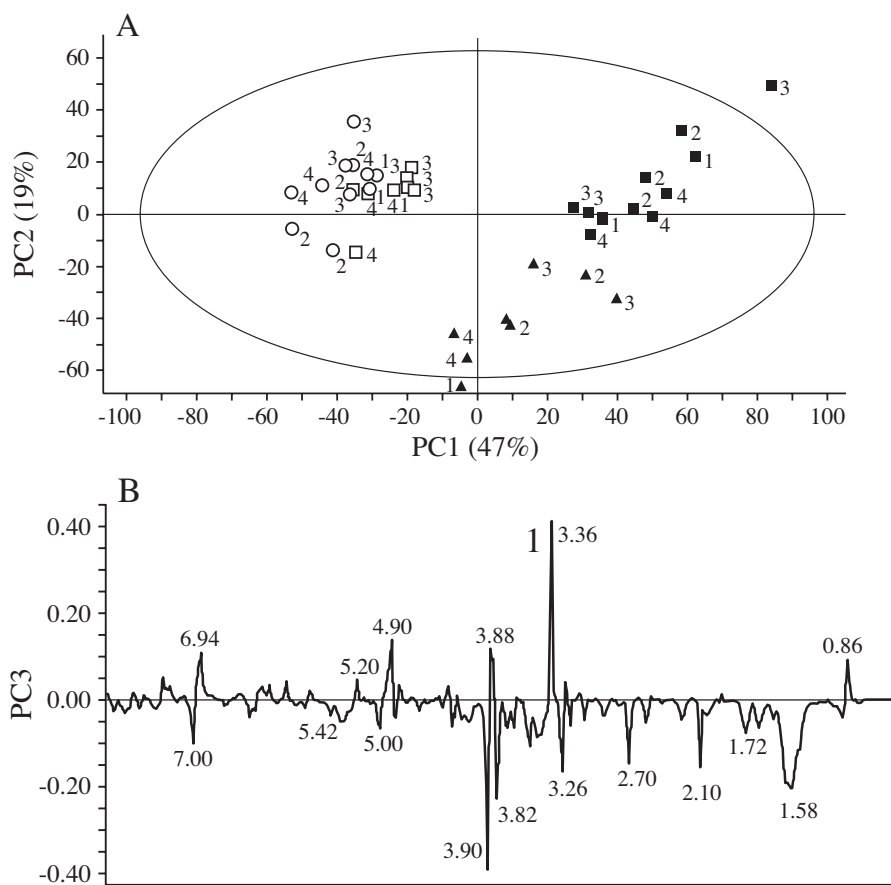


FIGURE 2. Score (A) plot and loading (B) plot of principal component analysis of aqueous fraction of *Arabidopsis thaliana* seed extracts. Black squares: inland 1; black triangles: inland 2; white squares: dune 2; white circles: dune 3. The ellipse represents the Hotelling T^2 with 95% confidence in score plots. The experiments were based on the 2-3 replicated samples from 8 dune and 8 inland plants. (1) The number over a peak in the loading plot refers to the chemical shift (δ) in the NMR spectrum.

CHEMICAL COMPOSITION OF *A. THALIANA* SEEDS AND HERBIVORYTABLE 4. Glucosinolate type of seeds from plants originating from dune and inland populations, which were grown in three sites. Mean concentration (+ SE) ($\mu\text{moles/g}$) for each type is given.

GLS type	Dune site			
	D 2* <i>n</i> = 9	D 3 <i>n</i> = 7	I 1 <i>n</i> = 6	I 2 <i>n</i> = 5
I	0.18 a (0.04)	0.06 a (0.01)	0.11 a (0.01)	0.10 a (0.01)
D	13.66 a (2.64)	8.83 a (0.63)	7.32 a (1.28)	11.47 a (2.55)
E	2.05 a (0.25)	1.14 a (0.26)	5.24 b (0.99)	0.29 a (0.02)
A	6.33 a (1.30)	4.34 a (0.28)	4.45 a (0.28)	6.52 a (0.34)
Total GLS	22.06 a (4.11)	14.31 a (0.93)	17.02 a (1.03)	18.29 a (2.86)
	Garden site			
	D 2 <i>n</i> = 9	D 3 <i>n</i> = 9	I 1 <i>n</i> = 8	I 2 <i>n</i> = 9
I	0.12 a (0.04)	0.09ab (0.04)	0.09 ab (0.04)	0.07b (0.03)
D	9.04 a (0.79)	10.55a (0.79)	4.72 b (1.17)	5.14 b (0.67)
E	3.03 a (0.42)	3.97a (0.28)	3.86 a (0.34)	0.55 b (0.09)
A	6.28 a (0.65)	6.91 (0.80)	3.64 b (0.21)	5.45 ab (0.39)
Total GLS	18.36 a (1.45)	21.44a (1.61)	12.22 b (1.20)	11.16 b (0.95)
	Growth room			
	D 2 <i>n</i> = 7	D 3 <i>n</i> = 7	I 1 <i>n</i> = 8	I 2 <i>n</i> = 8
I	0.88 a (0.05)	0.27 b (0.02)	0.21 b (0.02)	0.18 b (0.03)
D	9.22 a (0.27)	8.84 ab (0.38)	4.06 c (1.16)	6.05 bc (0.64)
E	3.63 ab (0.14)	2.87 b (0.15)	4.30 a (0.64)	0.35 c (0.06)
A	9.01 a (0.44)	6.98 ab (0.34)	3.96 c (0.49)	6.50 b (0.86)
Total GLS	22.76 a (0.80)	18.96 a (0.72)	12.54 b (1.87)	13.09 b (1.21)

*D 2 and 3 refer to two populations originating from the dunes and I 1 and 2 refer to populations originating from Leiden and Noordwijk. (I) = indol glucosinolates; (D) = aliphatic glucosinolates with straight and branched chains (olefins); (E) = glucosinolates with alcohol side chains; (A) = glucosinolates with sulfur-containing side chains. The values in each type, followed by a different character are significantly different from other values in the same row (ANOVA, Tukey test, $P < 0.05$).

pared to other sites (Tables 4 and 5). The concentration of indol glucosinolates was significantly higher in seeds collected from the growth room. Total glucosinolate concentration was higher in the dune-collected seeds but differences were not significant (Tables 4 and 5).

There were differences in quantities and abundance of glucosinolates at the population level as well. In the dunes transplant site (Table 3), glucoerucin was found only in plants from dune 2, progoitrin and an unknown alkenyl glucosinolate were not found in inland 2. In garden transplant site, epiprogoitrin was found only in dune populations. In growth room, 4-hydroxyglucobrassicin was

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TABLE 5. Analysis of variance for glucosinolate types of seeds for plants of four origins (dune 2, dune 3, inland 1, inland 2) grown at three sites (dune, inland and growth room).

GLS concentration*	Source	df	F value	P	% variance
I	Pop	3	90.08	$P < 0.001$	28.7
	Site	2	174.70	$P < 0.001$	37.2
	Site $\times$ Population	6	51.54	$P < 0.001$	32.9
D	Pop	3	9.54	$P < 0.001$	20.5
	Site	2	7.12	$P = 0.001$	10.2
	Site $\times$ Population	6	1.83	$P = 0.103$	7.9
E	Pop	3	54.18	$P < 0.001$	56.5
	Site	2	3.49	$P = 0.035$	2.4
	Site $\times$ Population	6	5.81	$P < 0.001$	12.1
A	Pop	3	11.14	$P < 0.001$	25.1
	Site	2	3.46	$P = 0.036$	5.1
	Site $\times$ Population	6	2.35	$P = 0.038$	10.5
Total GLS	Pop	3	9.50	$P < 0.001$	21.4
	Site	2	1.19	$P = 0.310$	1.8
	Site $\times$ Population	6	2.98	$P = 0.011$	13.4

* (I) = indol glucosinolates; (D) = aliphatic glucosinolates with straight and branched chains (olefins); (E) = glucosinolates with alcohol side chains; (A) = glucosinolates with sulfur-containing side chains.

found only in dune 2 and an unknown sulfur containing glucosinolate in inland populations. Epiprogoitrin and sinigrin were not found in inland 1 in the growth room. In the growth room and garden site (Table 4), total glucosinolate concentration was different between populations, with higher concentration in plants with a dune origin ($P < 0.001$). However, at the dune site no significant differences existed between dune and inland type plants (Table 4).

ANOVA (Table 5) showed that the population in which seeds were collected explained most variance in concentration of total glucosinolate concentration and in all glucosinolate types. Apart from that the site at which the plants were grown had a significant effect, there always was a significant interaction between population and site.

Herbivory and glucosinolates and other chemical composition

We examined the relation between damage by weevils and glucosinolates separately for dune and inland plants. In the dune site, fruit damage by adult weevils and their larvae was not significantly correlated (two-sided, $P > 0.05$) with total glucosinolate concentration (Fig. 3), or with individual glucosinolates and glucosinolate groups of seeds

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as measured in the field. Similarly in seeds of the garden site plants no significant correlation was found between damage by adult weevils or their larvae and total glucosinolate concentration (Fig. 3), nor with individual glucosinolates or glucosinolate groups. There is a tendency for a negative correlation between damage by larvae and aliphatic glucosinolates ($r = -0.63$, $P = 0.009$) in dune site and for damage by adult weevils and aliphatic glucosinolates in the garden site ($r = -0.41$, $P = 0.045$). However, these are not significant after Bonferroni correction (if we calculate 34 correlations the α -level is reduced from 0.05 to 0.0015, (<http://home.clara.net/sisa/bonhlp.htm>)).

DISCUSSION

Chemical composition in dune and inland type plants

Thioglucosinolates was the main glucosinolate in the seeds of dune plants that was either absent or occurred at concentrations too low in

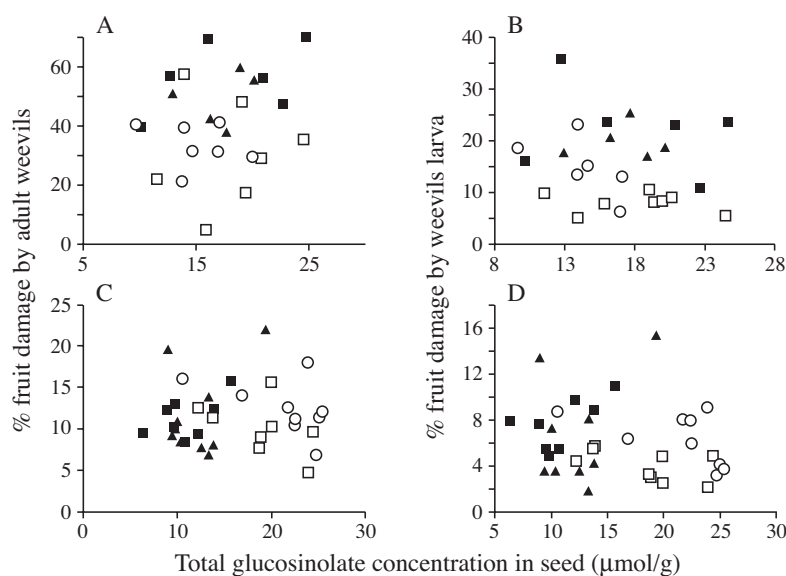


FIGURE 3. Patterns between fruit damaged by adult (A) and larvae (B) of the weevil, *Ceutorhynchus atomus* and *Ceutorhynchus contractus* (Curculionidae) and total glucosinolate concentration of the seeds measured in the dune site for *Arabidopsis thaliana*. Patterns between fruit damaged by adult (C) and larvae (D) of weevils *Ceutorhynchus contractus* and total glucosinolate concentration of the seeds measured in the garden site. Black squares: inland 1; black triangles: inland 2; white squares: dune 2; white circles: dune 3. None of the correlations was significant ($P > 0.05$).

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seeds of inland plants to be detected by NMR spectroscopy (Fig. 2 B). When analyzed using HPLC-UV, thioglucosinolate was again at high concentration in the dune plants. These results confirmed that these two analytical methods not only can provide informative multidimensional data (Bailey et al., 2000) they also can provide detailed data on suitable target compounds.

By far the largest part of the variation in both composition and concentration of glucosinolates among populations and transplant sites is due to the genetic background of the *A. thaliana* plants (Table 5). Population effects are reflected in a generally higher total glucosinolate concentration and in higher concentrations of the different types of glucosinolates in the two dune populations (Table 4). Glucosinolates are known to defend plants against herbivores (Giamoustaris and Mithen, 1995). We demonstrated that their main effect lies in defense against generalist herbivores (chapter 5). The differences in concentration and composition of glucosinolates between dune and inland plants may be related to selection pressures by the different guilds of herbivores present in these habitats. To test this idea a thorough knowledge of the potential generalist herbivores will be necessary. Right now information on generalist herbivory in the field is fragmentary. Additional protective functions of the glucosinolates relate to pathogen infections and UV-B resistance (see Kliebenstein 2004 for a review).

The environmental conditions for growth are considerably poorer in these dune populations (Table 1). This results in significantly smaller size and a significantly lower fruit production when plants grow in dunes, as compared to inland (chapter 3). The patterns found in the glucosinolates and the patterns in plant production are in accordance with the predictions of Coley's (1985) paper on optimal defense in plants: plants adapted to poor dunes environments invested more in defense than plants of rich environments. However, from Coley's (1985) theory you would also predict that the same genotype would plastically lower its glucosinolate concentration when conditions become more favourable for growth. This is found in the inland types, but not in the dune types: Plants from the dunes (dune 3) produce even more glucosinolates under the favourable garden and growth room conditions, as compared to the dune conditions. This would be relevant if this information is extended to the glucosinolate concentration of the leaves. That the *Arabidopsis* plants are indeed

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adapted to their local environment was affirmed by our experiments reported in chapter 3. Contrary to the predictions of the above-mentioned theory, we did not find inherent differences in growth rate.

Next to these genetic differences, there are considerable environmentally induced differences. We found both differences among sites and site $\times$ population interactions (Table 5). Especially the concentration of indole glucosinolates was affected by site. The detailed information on the separate glucosinolates in Table 3 revealed that, apart from differences in concentration, some substances are only produced under specific growth conditions. With our present knowledge, we cannot explain the differences. Induction of glucosinolates has been reported on, both by abiotic (Louda and Rodman, 1983; Mithen et al., 1995; Wolfson, 1982) as well as biotic factors, like damage by aphids (Kim and Jander, 2003). The contribution of this paper is that induction is genotype dependent and that genotype $\times$ environment interactions are important.

In a study of 39 different ecotypes of *A. thaliana*, Kliebenstein et al. (2001) stated that polymorphism at only five loci was sufficient to generate 14 qualitatively different leaf glucosinolate profiles. They also concluded that changes in herbivory or other selective pressures might generate new glucosinolates. However, our results showed not only environmental and genetic components but the interaction between these two also was linked to the observed glucosinolate variation between dune and inland plants (Table 5).

Differences in weevil herbivory in dune and inland populations of A. thaliana and their correlation with glucosinolates

The present study revealed that plants of *A. thaliana* were affected differently by specialist weevils, *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) (Table 2). In both dune and garden sites fruit damage by adult weevils and their larvae were not correlated with individual glucosinolates, glucosinolate groups and with total glucosinolate concentration within each group (Fig. 3).

Mauricio and Rausher (1997) found in their study on *Arabidopsis* in the field that the glucosinolate concentration was negatively correlated with a combination of herbivores and plant pathogens. Clearly some of these organisms were negatively affected by glucosinolate profiles. The species behind this effect were not identified, whether they were specialist or generalist herbivores. Generalist herbivores are more

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likely to be negatively influenced by the glucosinolates (Chew, 1988).

In specialist herbivores, Kliebenstein et al. (2002) found a positive correlation between glucosinolate concentration in *A. thaliana* and *Plutella xylostella* suggesting that glucosinolates were acting as feeding stimulants. Nielsen et al. (2001) who used transgenic *A. thaliana* plants with a four- fold increase in total glucosinolate levels, did not find any effect on the suitability of *A. thaliana* for two specialist flea beetle species, *Phyllotreta nemorum* and *P. cruciferae*. The flea beetles did not discriminate between transgenic and wild type plants.

Studies on the interaction between specialist herbivores and other members of Cruciferae were consistent with our results. A survey of the literature (Nielsen et al., 2001) showed that the majority of experiments demonstrated no effect by glucosinolates on the suitability of plants by Cruciferae specialist herbivores. Only a few experiments showed positive or negative correlation between glucosinolates and suitability. Part of this discrepancy might be due to the methods that have been used to obtain different levels of glucosinolate in plants (Nielsen et al., 2001). For example, sulfur fertilization and jasmonic acid treatment might not only influence the glucosinolate, but might also cause other changes in the plant (Bodnaryk, 1994; Bodnaryk and Palaniswamy, 1990). Topical application of glucosinolate on surfaces might prove to have some other drawbacks since it does not mimic the natural situation where glucosinolates are stored mainly inside plant tissue.

Another explanation for the different responses of specialist herbivores to glucosinolates might also be related to other correlated chemical compounds. Nielsen et al. (1989) showed in a host plant recognition experiment that sinigrin extracted from leaves of *Alliaria petiolata* was not a feeding stimulant to *Ceutorhynchus constrictus* Marsh. (Coleoptera: Curculionidae) when presented alone. However, a clear synergistic effect was found when it was combined with flavone glycosides.

We conclude that, given the range of glucosinolate concentration in dune and inland plants, other factors might also be involved in herbivory by the weevils in *A. thaliana* in our study sites.

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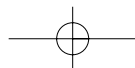
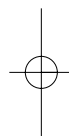
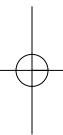
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Glucosinolates and some other chemical compounds in leaves from natural populations of *Arabidopsis thaliana* and their effects on generalist and specialist herbivores



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Many of the secondary metabolites in plants act as defense against herbivores and it is often postulated that these compounds have evolved under selective pressure of different insect herbivores. We tested this hypothesis with *Arabidopsis thaliana* plants of dune and inland areas.

We analyzed *Arabidopsis thaliana* leaves using NMR spectroscopy and multivariate data analysis. The major differences of chemical composition were found in water-methanol fractions and were due to higher concentrations of sinigrin and fumaric acid in the dune plants. Inland plants showed lower levels of glucose. Quantitative analysis of each glucosinolate was done with HPLC-UV. Glucosinolates were different in their quantities and abundance between individual plants and populations. In growth chamber experiments, the generalist herbivore, *Spodoptera exigua* grew significantly better on the inland plants, while the specialist herbivore *Plutella xylostella* fed equally on both types. To test if the diversity of metabolites in *Arabidopsis thaliana* is the result of different selection pressures by different herbivores, we looked if damage by these herbivores depended on different chemical compounds. No significant correlations were found between larval weight of *P. xylostella* and glucosinolates in the leaves but glucosinolates negatively correlated with larval weight of *S. exigua*.

We suggest that the evolution of chemical compounds might be driven by different selection pressures from different herbivores with a major role for generalist herbivores.

Plants synthesize a surprisingly large number of different secondary metabolites and within single species a staggering variety of structurally related compounds can be found. The evolution and maintenance of this diversity in related compounds is still poorly understood. Because many secondary metabolites act as defense

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against herbivores, it has been postulated that insect herbivores play a dominant role in the evolution of these compounds (Ehrlich and Raven, 1964; Rhoades and Cates, 1976). Recently it has been shown that plant chemistry can indeed be under selection by insect herbivores (Shonle and Bergelson, 2000). One explanation for the variation in both the concentration as well as the diversity of structurally related compounds within a single plant species is selection by different herbivores (Simms, 1990, 1992; Mithen et al., 1995; van der Meijden, 1996). While secondary metabolites may provide effective defence against generalist herbivores, specialist herbivores, may use specific plant chemicals as a cue to find and even identify their food plants. It is therefore expected that these contrasting selection pressures will affect the metabolite composition.

The glucosinolates in *A. thaliana* are now often used as a model system to study the evolution of secondary metabolites. Glucosinolates are secondary metabolites, which occur in the Capparales and a few other taxa. At least 120 different glucosinolates have been identified in these plants (Fahey et al., 2001). Glucosinolates and/or their breakdown products have long been known for reducing palatability of leaf tissue to generalist herbivores (Chew, 1988; Giamoustaris and Mithen, 1995). Specialist insect herbivores however, do not respond unequivocally to glucosinolates levels (Nielsen et al., 2001).

It is known that the effect of a single chemical factor on the performance of herbivores often depends on its interaction with other chemical factors. Variation in nitrogen and sugars (Blau et al., 1978; van der Meijden et al., 1989) may affect oviposition and larval growth of specialist herbivores. It is therefore important to study the effect of different glucosinolates in the context of morphological traits and other chemical compounds. We used two analytical techniques. NMR spectroscopy produces a wide spectrum chemical analysis, which is rapid, reproducible, stable in time and can meet our requirements of a range of chemical compounds. However, NMR spectroscopy offers low sensitivity and is especially good for compounds that are present in high concentration (Summer et al., 2003). HPLC-UV in general offers good selectivity and sensitivity and can provide detailed data on single compounds. In this study we used NMR spectroscopy to find differences between plants with respect to a large number of chemical compounds and we used HPLC-UV to look in more detail at relevant single compounds (glucosinolates). With these two analytical tech-

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niques we looked in more detail at differences in glucosinolate composition and other chemical compounds of the leaves of plants originating from different natural populations of *A. thaliana*. Therefore, in this way, we use only HPLC-UV for the compounds that showed the main differences in NMR spectroscopy. In our study on natural herbivores in *A. thaliana* populations we compared two different habitats, dune and inland populations in the Netherlands. *Arabidopsis thaliana* experienced more than 40% fruit damage by the specialist weevils *Ceutorhynchus atomus* and *C. contractus* (Curculinoidae) in the dune habitat (Mosleh Arany et al., 2005), but hardly any fruit damage by these weevils was observed on plants in the inland habitat. We observed aphid infection and a small amount of leaf herbivory by unknown herbivores in the inland populations. If generalist and specialist insect herbivores do indeed exert a contrasting selection pressure on *A. thaliana*, they must show a different preference for the plants based on their chemical compounds. To test this, we looked if the specialist herbivore *Plutella xylostella* and the generalist herbivore *Spodoptera exigua* were differently affected by chemical compounds in the leaves.

This paper addresses the following questions: What are the differences in chemical compounds in the leaves of plants originating from dune and inland populations of *A. thaliana* and grown for one generation in the lab? Do variation in glucosinolate profile influences the suitability of *A. thaliana* for generalist and specialist insect herbivores?

MATERIAL AND METHODS

Plants and insects

In coastal sand dunes in the Netherlands *A. thaliana* grows naturally in two habitat types. It occurs locally on the more calcareous new dunes that were formed on top of the old soil profile c. 800 years ago (called dune hereafter). In South and North-Holland, two Dutch coastal provinces, it is also locally common in road verges of roads on top of the old dune system that was formed between 3000-5000 years ago and that is still visible in the landscape as long stretches of sand that run parallel to the coast (called inland hereafter). Populations may have grown here for a long time or seeds came from elsewhere following road construction works, the age of the populations we study here is not known.

Seeds of ten plants from three populations in the dunes of Meijendel, north of The Hague (latitude 52° 08' N, longitude 4° 22'

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E) and two in the inland were collected in July 2002. All populations in the dunes were found within 20 m from woody vegetation with trees like *Populus nigra*, *P. alba*, *Betula pubescens* and *Crataegus monogyna*. The sandy surface at these sites is covered with moss, grasses and small herbs with about 10 percent bare soil. Accompanying species included amongst others *Erophila verna*, *Cardamine hirsuta*, *Rubus caesius*, *Calamagrostis epigejos* with small *Hippophae rhamnoides* shrubs nearby. Seeds from two of these dune populations studied by Mosleh Arany et al. (2005) were used for this study (called dune 2 and dune 3 hereafter, the numbering corresponds to Mosleh Arany et al. (2005).

Population (1) in the inland is located in Leiden, 3 m from a paved road and the second one, population (2), was growing near a canal in Noordwijk. Both sites were covered with *Lolium* sp. with about one percent bare soil. Accompanying species included amongst others *Erophila verna*, *Cardamine hirsuta* and *Plantago lanceolata*. The distance between the two inland populations is about 8 km and the minimal distance between the dune and the inland habitat is about 6 km.

Caterpillars from *Spodoptera exigua* were obtained from a lab culture, reared on an artificial diet in a growth chamber at 25°C, 16h/8h L/D photoperiod, 70% RH. Caterpillars from *Plutella xylostella* were obtained from a lab culture reared on *Brassica oleracea* in a growth chamber at 25°C, 16h/8h L/D photoperiod, 40-50% RH.

Metabolomic analysis

Chemical analysis (HPLC-UV and NMR spectroscopy)

Plants were grown under controlled conditions in a growth chamber (20°C, 18-h light, 70% humidity). Seeds produced by these plants were germinated and the resulting two-months-old rosettes were used to make extracts for HPLC-UV and NMR spectroscopy. One hundred mg dry mass of leaves of 5 rosettes of each population was used for HPLC-UV and five hundred mg of 9 samples for each type of plants (6 samples for dune 2 and inland 1 and 3 samples for dune 3 and inland 2) for NMR spectroscopy. Extraction, purification and glucosinolate measurement follows the procedure used by van Dam et al. (2003) with sinigrin as the external standard. Glucosinolates were extracted with 70% methanol solution, desulphatased with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column and separated on a reversed phase C-18 column on HPLC with an acetonitril-water gradient. The elution program was a linear gra-

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dient starting at 0% acetonitrile (ACN) that increases to 35% ACN in water over 30 minutes. Detection was performed with a single wavelength detector set to 229 nm. Glucosinolates that could not be identified were clarified based on their UV absorption spectrum.

For NMR spectroscopy extracts we followed the procedure of Choi et al. (2004). All spectra were recorded on a Bruker AV-400 NMR spectrometer operating at a proton NMR frequency of 400.13 MHz. After measurements, the ^1H -NMR spectra were automatically reduced to ASCII files using AMIX (Analysis of MIXtures software v. 3.8, Bruker Biospin). Spectral intensities were scaled to HMDSO (hexamethyl disilane) and trimethyl silane propionic acid sodium salt (TSP- d_4) for chloroform and water-methanol fractions, respectively, and reduced to integrated regions, called 'buckets', of equal width (0.02 ppm) corresponding to the region of δ 10.0 to -0.1. The generated ASCII file was imported into Microsoft Excel for the addition of labels and then imported into SIMCA-P 10.0 (Umetrics, Umeå, Sweden) for PCA analysis.

Experiment with generalist and specialist herbivores

Rosettes of 5 plants in 5 replicates from the same rosettes as used for HPLC-UV analysis were used for this experiment. Two second instar caterpillars from *Spodoptera exigua* and two second instar caterpillars from *Plutella xylostella* were placed on each of the rosettes. Larval weight of both herbivores was measured after 5 days.

Statistical analysis

Data were analyzed with SPSS 10 (SPSS Inc., Chicago, USA). Normality of the data was checked by post-hoc analysis of the residuals using the Kolmogorov-Smirnov test of normality. Differences in larval weight of herbivores and differences in glucosinolate concentration between dune and inland populations were tested with ANOVA (General Linear Model, Univariate, type III Sums of Squares). The correlation between glucosinolates and larval weight of herbivores was analyzed with a Pearson test. To make the analysis simple as straightforward as possible we first checked whether within the same origin the mother plants from which seedlings were used were significantly different. In no case this differences was significant and therefore we pooled all data on seedling of different mother plant within the same population.

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Data were analyzed with a principal component analysis. Principal component analysis (PCA) is a clustering method requiring no knowledge of the data set, which acts to reduce the dimensionality of multivariate data while preserving most of the variance within the data (Goodacre et al., 2000). The principal components can be displayed in a graphical fashion as a 'scores' plot. This plot is useful for observing any grouping in the data set. PCA models were constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Eriksson et al., 2001). Thus for NMR spectroscopy data, loading plots can be used to detect the spectral areas responsible for the separation in the data.

RESULTS

Herbivory assessment

The larval weight of *P. xylostella* was not significantly different between populations of the two types of plants (Table 1). The larval weight of *S. exigua* fed on the dune plants was significantly lower compared to inland plants ($P = 0.001$), but no differences were found among the two dune populations and among the two inland populations ($P = 0.65$, $P = 0.88$, respectively) (Table 1).

Metabolomic analysis

NMR spectroscopy analysis

The chloroform fractions of leaves were not clearly separated in the PC1-PC2 score plot of the dune and inland populations (Fig. 1). Data for the water-methanol fraction of the two type plants were clearly separated from each other by PC3 (Fig. 2). The loading plot of PC3 show that this separation was mainly due to the signals of sinigrin (δ 6.02, 5.30, 5.04, 3.90, 3.52), the signal of fumaric acid (δ 6.52) and the signal of glucose at δ 4.64. Visual examination of the original NMR spectra, confirmed these results, as none of inland populations showed sinigrin signals. However, one of the leaf extracts from dune plants did not contain sinigrin and this one point is thus located among the inland populations in the score plot. In addition, small pos-

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TABLE 1. The mean ($\pm$ SE) of larval weight (mg) of *Spodoptera exigua* and *Plutella xylostella*, fed on dune and inland populations. The values in each row, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$). $n = 25$.

Herbivore	Dune 2	Dune 3	Inland 1	Inland 2
Larval weight of <i>S. exigua</i>	15.7 $\pm$ 3.56 a	19.9 $\pm$ 4.02 a	128.5 $\pm$ 21.96 b	122.2 $\pm$ 5.16 b
Larval weight of <i>P. xylostella</i>	7.20 $\pm$ 0.20 a	6.66 $\pm$ 0.18 a	7.16 $\pm$ 0.19 a	7.11 $\pm$ 0.24 a

itive PC3 values are detected in the range of δ 6.5 - δ 10.0. It means that phenolic compounds are more abundant in the dune populations than in the inland populations.

Glucosinolate differences in HPLC-UV analysis

Twelve principal glucosinolates were found in the leaves of plants grown in the growth room (Table 2). They could be classified into four structural types according to Fahey et al. (2001): indol glucosinolates (I), aliphatic with straight and branched chains glucosinolates or olefins (D), alcohols side chains glucosinolates (E) and sulfur-containing side chains glucosinolates (A) (Table 3). Individual plants and populations differed in glucosinolate composition. Epiprogoitrin, 4-

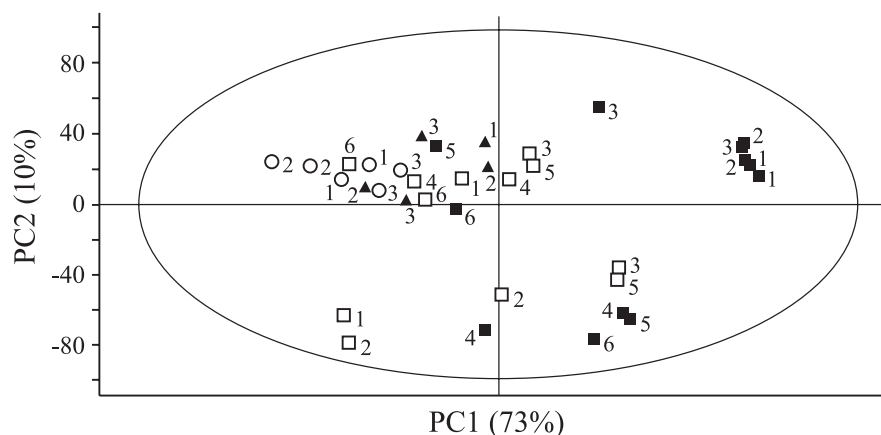


FIGURE 1. Score plot (PC1 vs. PC2) of principal component analysis of the chloroform fractions of *Arabidopsis thaliana* leaf extracts. Black squares: inland 1; black triangles: inland 2; white squares: dune 2; white circles: dune 3 plants. The ellipse represents the Hotelling T^2 with 95% confidence in score plots. The experiments were based on the 2 replicated samples from 9 dune and 9 inland plants. Sometimes there is no replicate, which shares the same symbol and number.

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TABLE 2. Glucosinolate composition of leaves of plants originating from two different sites in dune and inland after growing plants on a growth room. Mean concentration (SE) ($\mu\text{moles/g}$ dry weight) per glucosinolate is given. Dune 2 and 3 refer to dune and Inland 1 and 2 refer to inland populations. (I) = indol glucosinolates; (D) = aliphatic with straight and branched chains (olefins); (E) = alcohol side chains and (A) = sulfur-containing side chains.

Glucosinolate type	GLS type	Dune 2 <i>n</i> = 5	Dune 3 <i>n</i> = 5	Inland 1 <i>n</i> = 5	Inland 2 <i>n</i> = 5
Unknown alkenyl GLS	D	0	0	2.06 (0.38)	0
3 OH propyl GLS	E	0	0	4.37 (0.79)	0
Unknown sulfur containing GLS	A	0	0.17 (0.02)	0.18 (0.05)	0.08 (0.03)
Epiprogoitrin	D	0	0.01 (0.01)	0	0
Sinigrin	D	13.89 (2.38)	25.47 (1.84)	0.04 (0.04)	8.35 (0.79)
Gluconapin	D	0.17 (0.03)	0.33 (0.02)	0	0
4 Hydroxy-glucobrassicin	I	0	0.01 (0.01)	0	0
Unknown sulfur containing GLS	A	0	0.03 (0.01)	0	0
Glucobrassicin	I	0.63 (0.10)	0.77 (0.08)	0.78 (0.14)	0.67 (0.06)
Glucohirsutin	A	0.59 (0.09)	1.10 (0.07)	0.83 (0.13)	1.22 (0.07)
4 Methoxy-glucobrassicin	I	0.26 (0.04)	0.38 (0.01)	0.19 (0.04)	0.18 (0.02)
Neo-glucobrassicin	I	0.11 (0.03)	0.09 (0.01)	0.06 (0.03)	0.12 (0.01)

TABLE 3. Glucosinolate type of leaves for dune and inland plants grown in growth room. Mean concentration ($\pm$ SE) ($\mu\text{moles/g}$ dry weight) for each type is given, *n* = 5. (I) = indol glucosinolates; (D) = aliphatic glucosinolates with straight and branched chains (olefins); (E) = glucosinolates with alcohol side chains; (A) = glucosinolates with sulfur-containing side chains. The values in each row, followed by a different character are significantly different (ANOVA, Tukey test, $P < 0.05$).

Structural type	Dune 2	Dune 3	Inland 1	Inland 2
I	1.01 $\pm$ 0.16 a	1.26 $\pm$ 0.09 a	1.04 $\pm$ 0.19 a	0.97 $\pm$ 0.07 a
D	14.06 $\pm$ 2.41 b	25.80 $\pm$ 1.85 a	2.14 $\pm$ 0.39 c	8.35 $\pm$ 0.79 bc
E	0	0	4.37 $\pm$ 0.79	0
A	0.59 $\pm$ 0.09 b	1.31 $\pm$ 0.07 a	1.01 $\pm$ 0.17 ab	1.31 $\pm$ 0.07 a
Total glucosinolate concentration	15.66 $\pm$ 2.67 b	28.37 $\pm$ 1.99 a	8.56 $\pm$ 1.53 b	10.63 $\pm$ 0.99 b

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hydroxyglucobrassicin and an unknown sulfur-containing glucosinolate were found only in dune 1 and gluconapin was found only in dune plants. 3-OH propylglucosinolate and an unknown alkenyl glucosinolate were found only in inland 1 (Table 1). Furthermore, glucosinolate concentration was different between populations. The plants from dune 1 had a significantly higher concentration of total glucosinolates as compared to inland plants (Table 3). Concentration in dune 2 was also higher but this was not significant.

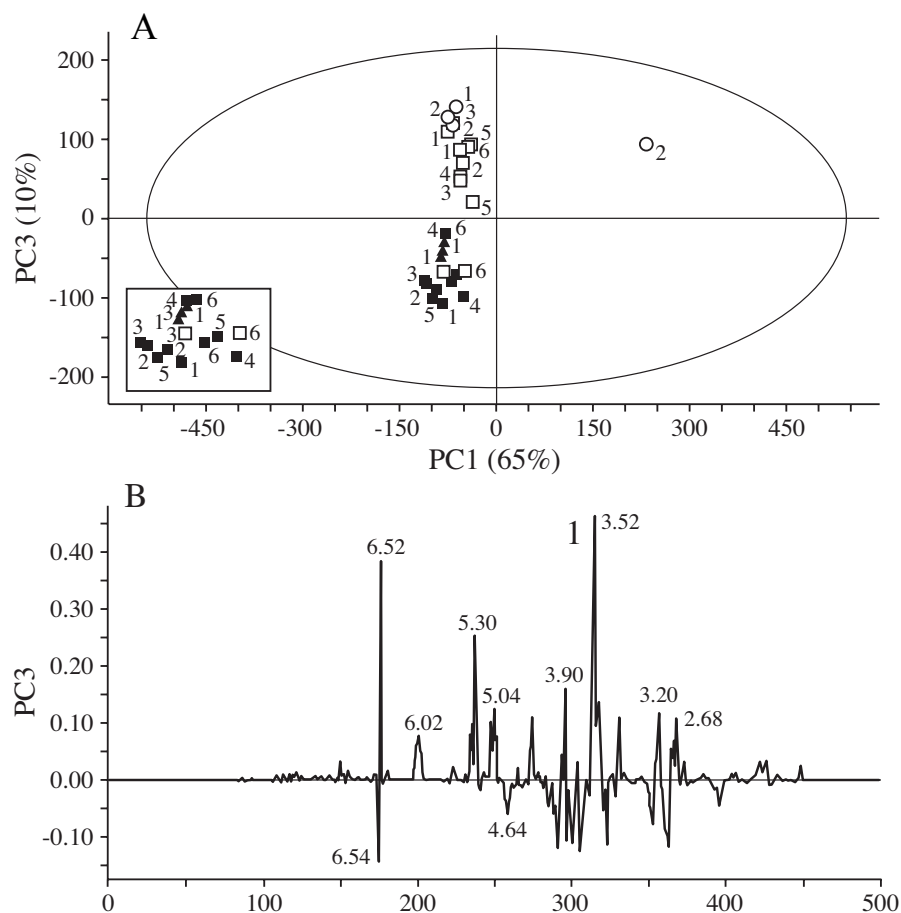


FIGURE 2. Score (A) plot and loading (B) plot of principal component analysis of the water-methanol fraction of *Arabidopsis thaliana* leaf extracts. Origin: inland 1 (black squares); inland 2 (black triangles); dune 2 (white squares); dune 3 (white circles). The ellipse represents the Hotelling T^2 with 95% confidence in the score plot. The experiments were based on the 2 replicated (1-2 plants in 1 replicate) samples from 9 dune and 9 inland plants. (1) The number over a peak in the loading plot refers to the chemical shift (δ) in the NMR spectrum.

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Herbivory in relation to glucosinolates and other chemical compounds

In the feeding trial in the growth room, larval weight of the specialist herbivore *P. xylostella* was not significantly correlated with total glucosinolate concentration or with individual glucosinolate groups (Table 4) of the leaves and with individual glucosinolates. The larval weight of generalist herbivore *S. exigua* was negatively correlated with total glucosinolate concentration (Fig. 3) and with the olefin group in the leaves (Table 4). The larval weight of the generalist herbivore *S. exigua* was also negatively correlated with gluconapin, sinigrin and 4-methoxyglucobrassicin ($r = -0.71$, $P < 0.001$; $r = -0.65$, $P = 0.002$; $r = -0.59$, $P = 0.006$, respectively). These correlations were still significant (except for 4-methoxyglucobrassicin) after conservative Bonferroni correction (16 correlations, $\alpha = 0.003$).

TABLE 4. Pearson correlation coefficient (r) between larval weight of *P. xylostella* and *S. exigua* with (I), indol glucosinolates; (D), aliphatic glucosinolates with straight and branched chains (olefins); (E), glucosinolates with alcohol side chains; (A), glucosinolates with sulfur-containing side chains, total glucosinolate concentration, fumaric acid and glucose (**: $P < 0.01$).

Larval weight	I	D	E	A	Total GLS	Fumaric acid	Glucose
<i>P. xylostella</i> $n = 20$	$r = 0.14$	$r = -0.39$	$r = 0.25$	$r = -0.196$	$r = -0.36$	$r = -0.27$	$r = -0.12$
<i>S. exigua</i> $n = 20$	$r = -0.32$	$r = -0.67^{**}$	$r = 0.39$	$r = 0.14$	$r = -0.64^{**}$	$r = 0.14$	$r = -0.47$

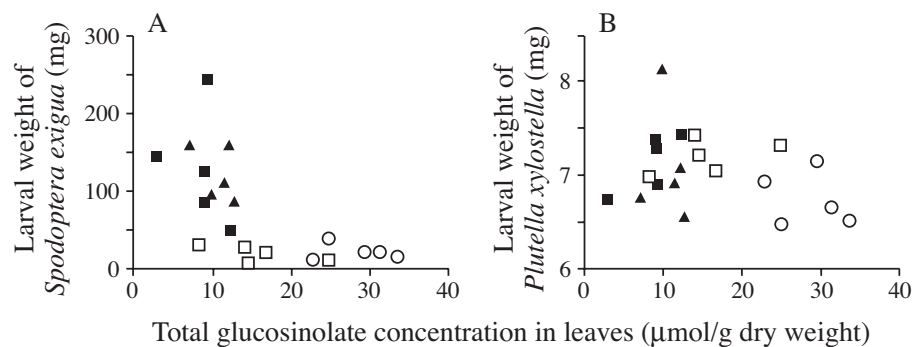


FIGURE 3. Pattern between larval weight of *Spodoptera exigua* (a) and *Plutella xylostella* (b) and total glucosinolate concentration of the leaves of dune and inland populations of *A. thaliana* grown in growth room. Black squares: inland 1; black triangles: inland 2; white squares: dune 2; white circles: dune 3.

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Within each population, this correlation was not significant. However, individual population samples only consisted of five plants and covered only a relative small range of glucosinolate concentrations. Because 14 out of 16 (85%) of these latter correlations, between larval weight of *S. exigua* and glucosinolate groups, were negative (Binomial test, $P = 0.001$), our data suggest that glucosinolates reduce *S. exigua* growth.

Larval masses were not significantly correlated with glucose or fumaric acid levels, which were the main additional chemical differences between the two plant types in the NMR spectroscopy.

DISCUSSION

Sinigrin was the main glucosinolate in the leaves of dune plants that was either absent or occurred at concentrations too low to be detected by NMR spectroscopy (Fig. 2). When analysed using HPLC-UV, sinigrin was again at high concentration in the dune plants. These results confirmed that these two analytical methods not only can provide informative multidimensional data (Bailey et al., 2000) they also can provide detailed data on suitable target compounds.

We found differences in types and quantities of glucosinolates between individual plants that were grown together in a controlled environment and analyzed with HPLC-UV. These observations show that there are genetic components linked to the observed glucosinolate variation within and between *A. thaliana* populations found in dune and in inland populations. The results from the NMR spectroscopy confirmed that dune and inland plants are chemically different.

Other studies on *A. thaliana* yielded similar results. Glucosinolates, accumulation and hydrolysis products are highly variable in wild-collected accessions (Mithen et al., 1995; Kliebenstein et al., 2001). Both are controlled by genetic variation for the presence/absence of a number of enzymes that function in a sequential pathway (Kliebenstein et al., 2001; Lambrix et al., 2001). Thus a mutation in one of the enzymes or a cross-pollination event could lead to a rapid glucosinolate profile shift from one generation to the next (Kliebenstein, 2004).

We found a negative correlation between glucosinolates and *S. exigua* weight. However, this correlation does not necessarily mean that glucosinolates directly reduce feeding. In Fig. 3 several genotypes are included and it may be this genotypes are also different for

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other unknown factors that covary with glucosinolates. From a chemical prospective, there was no significant correlation with other main chemical compounds that were different between two types of plants. The morphological differences between dune and inland plants may also be involved in the suitability of *S. exigua*.

Glucosinolate composition negatively impacted the generalist herbivore *Trichoplusia ni* (Kliebenstein et al., 2002). In addition to glucosinolate composition, glucosinolate concentration also negatively impacted generalist herbivory for both *Trichoplusia ni* and *Spodoptera exigua* (Kliebenstein et al., 2002; Kroymann et al., 2003). Plants with higher glucosinolate levels were more resistant to herbivory than were plant lines with lower glucosinolates. Mauricio and Rausher (1997) found in the field that glucosinolate concentration was negatively correlated with insect herbivory and damage by plant pathogens. Clearly some of these organisms were negatively affected by glucosinolate profiles. The herbivores in their study were not identified, so that it is not clear whether they were specialist or generalist herbivores. Generalist herbivores are more likely to be negatively influenced by glucosinolates (Chew, 1988). Only total glucosinolate concentration was measured and not the glucosinolate type or hydrolysis products. However, experiments with lines specifically varying for the different glucosinolate composition could lead to a better understanding of the biological basis for glucosinolate variation maintenance in *A. thaliana* (Kliebenstein, 2004).

Kliebenstein et al. (2002) found a positive correlation between glucosinolates in *A. thaliana* and herbivory by the specialist *Plutella xylostella* suggesting that glucosinolates acted as feeding stimulants. Nielsen et al. (2001) who used transgenic *A. thaliana* plants with a four-fold increase in total glucosinolate levels, did not find any effect on the suitability of *A. thaliana* for two specialist flea beetle species, *Phyllotreta nemorum* and *P. cruciferae*. The flea beetles did not discriminate between transgenic and wild type plants. Studies on the interaction between specialist herbivores and other members of the Cruciferae were consistent with our results. A survey of the literature (Nielsen et al., 2001) shows that the majority of experiments demonstrate no effect of glucosinolates on specialist herbivores.

Van der Meijden (1996) theoretically predicted that, when attacked by generalist herbivores, plants with a lower concentration of chemical compounds will be more affected. Herbivory by specialist herbivores, in contrary, would show a positive correlation with sec-

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ondary compounds at relatively low concentrations and no relation with secondary compounds at high concentration. So for generalist herbivores a negative and for specialist herbivores a positive correlation or no effect were predicted. We found a negative correlation between larval weight of generalist herbivore, *Spodoptera exigua*, and glucosinolate levels and no correlation for the specialist herbivore *Plutella xylostella*. We also found no significant correlation between larval weight of these herbivores and glucose, fumaric acid that are the main differences compound in the two type plants.

These herbivores do not feed naturally on *A. thaliana* populations in our study site. The main herbivores in the dune site were *Ceutorhynchus atomus* and *C. contractus* (Curculionidae). Because the dune type is less affected by the weevils than inland type, it is suggested that these common specialist herbivores exerted a selection pressure on the plants growing in the dunes. However, in a field experiment we did not find any correlation between the glucosinolate concentration in seeds and herbivory damage by these two specialist weevils (chapter 4).

We suggest that the evolution of glucosinolate level may be driven by different selection pressures from different herbivores with a more important role for generalist herbivores

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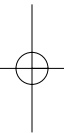
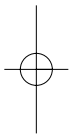
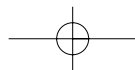
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Summary



As a first step in understanding the evolutionary ecology of *Arabidopsis thaliana*, with an emphasis on the role of herbivores, I studied the population dynamics of this species at the coastal dunes of the Netherlands (chapter 2). To elucidate factors controlling population dynamics and the relative importance of factors determining final population density, demography was studied in permanent plots and in experimental plots several treatments were applied.

In the permanent plots, plants were mapped to obtain data on survival and reproductive performance of each individual, with special attention to herbivore damage. Mortality was low during autumn and early winter and high at the time of stem elongation, between February and April. A key-factor analysis showed a the highest correlation between mortality from February to April and total mortality.

The specialist weevils *Ceutorhynchus atomus* and *C. contractus* (Curculionidae) were identified as major insect herbivores on *A. thaliana*, reducing seed production by more than 40% (range 30-66.5%). These herbivores acted in a density-dependent manner, attacking a greater fraction of the fruits on large plants. While mortality rates were not affected by density, fecundity decreased with density, although the effect was small.

In the experimental plots, we studied how addition of water, addition of nutrients, artificial disturbance and herbivores affected survival and growth. Adding water reduced mortality in rosette and flowering plant stage. Soil disturbance did not increase seed germination, but did have a significant positive effect on survival of rosettes and flowering plants.

Seed production of *A. thaliana* populations varied greatly between years, leading to population fluctuations, with a small role for density-dependent fecundity and density-dependent herbivory.

Next, *A. thaliana* populations growing in the dune habitat were compared to inland populations (chapter 3). Whereas plants in the dunes suffered greatly from seed predation, there was hardly any seed predation observed in the inland populations. We hypothesized that herbivore pressure is higher in the dunes and also plants have adapted to reduce this pressure. To show whether the differences in her-

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bivory were environmentally or genetically based, we set up a reciprocal transplant experiment with plants originating from dune and inland populations. We transplanted plants between dunes and inland and recorded morphological traits, survival, flowering time, seed production and susceptibility to herbivory by the specialist weevils that feed on flowers and fruits.

There was a significant site effect in all traits, such that all plants performed better in the inland site with respect to number of stems, plant height, rosette size and seed production. The percentage fruit damage was less in the inland site as well. Also genetic differences between plants of different origin were observed for several life history and morphological characters and for percentage fruit damage. In particular, plants of inland origin flowered 10 days earlier, grew better and produced 20% more fruits. This gives the inland plants an edge in their own environments. However, plants from the inland experienced 36% more fruit damage by weevils in the dune habitat. For number of stems, fruit number before herbivory occurs, percentage of damaged fruits by adult weevils, stem height and number of intact fruits, interactions existed between site and population. When fitness of an individual plant is defined as the probability of surviving until reproduction, multiplied by the estimated number of seeds unit of intact fruits after herbivory had taken place, the plants from the dune and inland populations scored higher in their own dune habitat. This demonstrates adaptation to the local environment. In this process the ability of dune genotypes to restrict herbivory by specialist beetles and high survival of inland genotypes in own habitat, appears to be an important mechanism responsible for greater relative fitness of *A. thaliana* dune plants on their home site.

Because plants of *A. thaliana* from dune and inland habitats were affected differently by specialist weevils at the transplant sites, we studied genetic differences in defense level. Specifically we studied if glucosinolate levels affect herbivory in the field by the weevils (chapter 4). Seeds were collected from two plant types in the field and were grown in the growth room for one generation. We performed a metabolomic analysis on the new seeds produced using NMR spectroscopy and multivariate data analysis. Major differences in chemistry were found in water fractions and were due to more thioglucosinolates and sucrose in dune and more sinapoylmalate in inland populations.

SUMMARY

Quantitative analysis of glucosinolates was made with an HPLC-UV analysis, using the same seed batches. Glucosinolates were different in their quantities and abundance between individual plants, populations and sites. The results showed that genetic components most strongly affected glucosinolate variation between dune and inland plants. On top of that, there was an effect of environment and also a significant population by site interaction. This interaction shows that genotypes that have the highest concentration of glucosinolates in the dunes do not necessarily have the highest concentration when grown in the inland site.

Fruit damage by adult weevils and their larvae was not significantly correlated with field concentrations of individual glucosinolates, glucosinolate groups or total glucosinolates in seeds. Therefore, it is concluded that differences in weevil herbivory on plants from dune and inland population must be due to other factors in which the plants differ.

Finally, to examine the idea that different glucosinolate level in *A. thaliana* can be the result of natural selection driven by herbivores (chapter 5), we studied if generalist and specialist insect herbivores respond differently to variation in glucosinolate levels. If these herbivores exert a contrasting selection pressure on glucosinolate concentration in *A. thaliana*, they must show a different preference for plants based on their glucosinolates.

When analyzed using NMR spectroscopy and multivariate data analysis, the major differences in leaf chemistry of the dune and inland plants were found in water fractions and were due to higher concentration of sinigrin and fumaric acid in the dune and glucose in the inland plants. Quantitative analysis of glucosinolates was made with a HPLC-UV analysis, using the same leaves. Glucosinolates were different in their quantities and abundance between individual plants and populations.

In growth chamber experiments, the generalist herbivore *Spodoptera exigua* grew significantly better on the inland plants, while the specialist herbivore *Plutella xylostella* fed equally on both types. No significant correlation was found between larval weight of the specialist *P. xylostella* and glucosinolates in the leaves. Larval weight of the generalist *S. exigua* was negatively correlated with glucosinolates over all plants from all populations. Within each population, this correlation was not significant. However, individual population samples

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only contained of five plants and covered only a relative small range of glucosinolate concentrations. Because more than 85% (14 out of 16) of these latter correlations between larval weight of *S. exigua* and glucosinolates groups were significantly negative, the conclusion holds that glucosinolates restrict *S. exigua* herbivory. Of course, it is possible that other factors (likely leaf thickness) than glucosinolates are also involved in the suitability of dune and inland plants for *S. exigua*. In the evolution of chemical compounds, the selection pressure exerted by generalist herbivores might be most important. Because the common weevils cause less damage on dune type than on inland type, it is likely that these specialist herbivores drove selection for improved defense in the dunes. However glucosinolates appear not to affect these specialists and the selection pressure exerted by uncommon generalist herbivores might be most important for the observed differences in glucosinolate level between plant types.

Nederlandse samenvatting

De zandraket is een winterannuel. De planten kiemen in het najaar en bloeien in april. Ze groeien op zandige, voedselrijke, verstoorde plekken, onder meer in de duinen. Vanwege zijn geringe formaat is de zandraket, zelfs tijdens de bloei, een weinig opvallende plant. Toch is de zandraket voor biologen bijzonder interessant omdat zijn genoom is ontrafeld en de plant daarmee is uitgegroeid tot modelplant voor moleculair-genetisch onderzoek. Op een recent congres dat ik bezocht in Madison (USA), werden meer dan 600 posters over deze plantensoort gepresenteerd! Minder dan een handvol van deze posters behandelde de ecologie van de soort of de herbivoren die er van eten. Dit gebrek aan aandacht was een reden om te beginnen om de ecologie van de zandraket, in het duingebied Meijendel (bij den Haag) en op vergelijkbare zandige plakken in de buurt, eens goed te bestuderen. Welke factoren zijn belangrijk voor de overleving en zaadzetting? Welke biotische factoren zijn, naast de abiotische factoren zoals een droog voorjaar of zomer, van belang voor het uiteindelijke succes van de zandraket? Meijendel is een interessant studiegebied omdat de soort hier waarschijnlijk al lang groeit. Daarom kan onderzocht worden of de duinpopulaties van de zandraket zich over een lange periode erfelijk aan het duinmilieu hebben aangepast, iets wat problematischer is voor bijvoorbeeld de zandraket-plantjes die we als pioniers op pas opgespoten zandterreinen vinden.

Ik heb eerst populaties van de zandraket gezocht om wekelijks, na de zaadkieming in oktober, de aantallen kiemplanten, rozetten en bloeiende planten te tellen. Uit dit onderzoek bleek dat vooral de periode voor de bloei en zaadzetting in april-mei kritisch is voor de aantallen planten die overleven en tot reproductie komen. Veel regen in maart en april betekent dus een goed jaar qua zaadproductie voor de zandraket. Biotische factoren zijn echter ook van groot belang. In Meijendel eten twee kevertjes, *Ceutorhynchus atomus* en *C. contractus* (allebei horend tot de Curculionidae), van de bloemen. De kevers leggen ook eitjes in de zich ontwikkelende vruchten en de larven consumeren daar de meeste zaden. *C. atomus* staat bekend als een specialist op *A. thaliana* en *C. contractus* als een specialist op Cruciferen. Ik heb de kevers inderdaad ook wel op ander cruciferen waargenomen. Gemiddeld wordt van een plant in Meijendel meer dan de helft van de

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zaden geconsumeerd. In binnenlandpopulaties, ik koos een populatie op de rand van Leiden en een in Noordwijk, was de schade aan vruchten elk jaar dat ik keek verwaarloosbaar. Nu kunnen er natuurlijk meerdere redenen zijn waarom duinen en binnenland verschillen. Misschien zijn duinplanten minder goed verdedigd tegen herbivoren en is er daarom meer schade in het duin. Als uitgangspunt van het onderzoek koos ik echter de hypothese dat de herbivorendruk in het duin hoger is en dat als gevolg hiervan in het duin selectie heeft plaats gevonden op planteneigenschappen die vraat tegen gaan. Planten in het duin zouden dan juist beter verdedigd zijn tegen herbivoren. We toetsten de hypothese door een reciprook transplantatie-experiment op te zetten. Binnen een aantal omheiningen bij de Kijfhoef in Meijndel werden genotypen uit duin en binnenland netjes gemengd. Om er zeker van te zijn dat er geen andere plantverschillen waren kweekten we eerst nog een generatie onder gecontroleerde omstandigheden in Leiden. Jonge plantjes werden uitgezet in het duin en vlak voordat het zaad rijp was werden alle planten geoogst en afgevoerd. De planten uit het binnenland groeiden beter en maakten 20% meer vruchten. Echter, een veel hoger percentage (36% meer) van deze vruchten werd aangetast door de kevers en als we uitrekenen welk type uiteindelijk in het duin de meeste intacte zaden maakte, bleek dat het duintype te zijn. Het duintype verdedigde zich beter tegen vraat door het specialistische kevertje en is daarmee het beste aangepast aan zijn eigen duinmilieu. In een proeftuin in het binnenland was er nauwelijks vraat aan de planten en maakte het binnenland genotype meer zaden. Ook het binnenlandtype is dus het beste aangepast aan zijn eigen binnenlandmilieu.

We hebben de planten ook onder gecontroleerde omstandigheden in het lab aangeboden aan de generalist *Spodoptera exigua*, de rupsen van dit vlindertje aten minder van de duinplanten. We waren enthousiast geraakt door deze duidelijke verschillen in verdediging van de plant tussen genotypen die binnen 10 km van elkaar groeien en hebben bladeren en zaden van de planten chemisch vergeleken. De genotypen bleken in veel aspecten van elkaar te verschillen, maar uit een NMR analyse bleek dat planten vooral verschillen wat betreft de mosterdolieglucosiden, een familie van stoffen die vooral in de kruisbloemigen (o.a. in koolsoorten) voorkomen. Met een andere analyse methode (HPLC) werden de mosterdolieglucosiden daarom nog eens apart onder de loep genomen. De duinplanten bevatten deels andere

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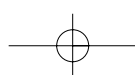
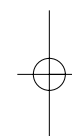
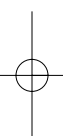
mosterdolieglucosiden en meestal, deels afhankelijk van de milieumstandigheden, ook een hogere concentratie van deze stoffen. Vooral de hoeveelheid sinigrine was hoger in het duin type.

Vraat door de twee *Ceutorhynchus*-soorten bleek niet gecorreleerd te zijn met de concentraties van de individuele glucosinolaten, glucosinolaat-groepen of the totale concentratie aan glucosinolaten in de zaden. Andere factoren zijn dus verantwoordelijk voor de verschillen in vraat tussen duin- en binnenlandplanten. Het lijkt waarschijnlijk dat deze specialisten een rol hebben gespeeld bij de selectie op een hogere afweer van de duinplanten.

De afwezigheid van (negatieve) correlaties tussen afweerstoffen van een plant en vraat door specialistische herbivoren is vaker gevonden. Specialisten blijken veel vaker ongevoelig voor het afweersysteem van planten. In vervolgetperimenten testten we een andere crucifeerspecialist (*Plutella xylostella*) en een generalistische herbivoor (*Spodoptera exigua*). De larven van de specialistische bladeter *Plutella* groeiden even goed duin- en binnenlandplanten. De larven van de generalist *Spodoptera* groeiden significant beter op de binnenlandplanten met de lagere concentratie aan mosterdolieglucosiden in de bladeren. Deze afweerstoffen lijken dus een goede bescherming op te leveren tegen generalisten (hoewel niet geheel uit te sluiten valt dat deze correlatie niet veroorzaakt wordt door de concentratie glucosiden, maar door factoren die met die stoffen geassocieerd zijn). De verschillen in glucosinolaatgehalte tussen duin- en binnenlandplanten zijn dus wellicht ontstaan door selectiedruk van generalistische herbivoren.

De oorzaak van de verschillen in vraatniveau door de specialistische zaadherbivoren is dus nog niet ontrafeld. Een andere factor dan glucosidenconcentratie of samenstelling is hiervoor verantwoordelijk. Nu bloeit het duingenotype zo'n tien dagen later dan het binnenlandgenotype en dit kan ook de vraat beïnvloeden.

Overigens ben ik van mening dat dit ecologische onderzoek in veld en lab, met aandacht voor het afweersysteem van *Arabidopsis*, duidelijk laat zien dat planten in hun strijd tegen herbivoren voor een evolutionair dilemma staan. Het systeem biedt daarmee een unieke kans om dat evolutionaire dilemma tegelijkertijd te bestuderen op het niveau van de ecologie, het metaboolom en het genoom. Ik ben er van overtuigd dat het synergistische effect van een dergelijke aanpak de theorievorming over de afweer van planten tegen vraat een grote stap vooruit kan brengen.



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Curriculum vitae



I was born on 22 December 1964 in Kashan, Iran. I started my undergraduate study at Shahid Beheshti University in Tehran and got my B.Sc. in botanical sciences in February 1988. I did my Master of Science in plant systematics at Tarbiat Moalem University in Tehran, and graduated in March 1992. My M.Sc. thesis was called 'Vegetation mapping of plants in the Kashan area based on a floristic method'. I was appointed at a permanent position as an academic member of the Faculty of Natural Resources and Desert Studies in Yazd University in 1994. During my work in Yazd University I taught botany and identification of Iranian pasture plants (level 1 and level 2). In 1995 I was involved in a project on collection and identification of desert plants in the center of Iran, that resulted in the foundation of the Herbarium of desert plants in Yazd University. This project was finished in 1998. In 2000 I was awarded a scholarship by the Ministry of Science, Research and Technology of Iran for a Ph.D. study abroad. In September 2001 I came to the Netherlands to start my Ph.D. study at the section of Plant Ecology at the Institute of Biology Leiden at Leiden University. My study was completed in 2005, resulting in this thesis.

List of publications

Scientific publications

- Mosleh Arany, A., de Jong, T.J. and van der Meijden, E. (2005) Herbivory and abiotic factors affect population dynamics of *Arabidopsis thaliana* in a sand-dune area. *Plant Biology* 7, 549-556.
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- Mosleh Arany, A. (1992) Vegetation mapping of plants in desert area based on floristic method. In: International symposium of problems in Kavir and desert areas. Tehran University publisher: Tehran, pp. 766-772.
- Mosleh Arany, A. (1998) Flora of Bafgh, Yazd. Yazd University: Yazd.
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