

Fitness effects of *Alternaria dauci* on wild carrot in The Netherlands

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In a field experiment with a susceptible population of annual wild carrot (*Daucus carota*) from Iran, artificial inoculations with the fungal pathogen *A. dauci* led to a strong and very significant increase of the diseased leaf area. The pathogen caused a very significant decrease in fecundity and seed survival of the host. This considerable fitness reduction by *A. dauci* would suggest that introgression of disease resistance from cultivated (transgenic) carrot cultivars into wild carrot populations could strongly increase the fitness of wild carrot. In spite of the potential ability of *A. dauci* to lower the fitness of the host considerably, wild carrot is very common in The Netherlands. Disease levels of wild carrot were estimated in 26 natural populations in 1998. No *A. dauci* could be detected on the leaves, and only 0.4% of the seeds were contaminated with *A. dauci*, in spite of the conducive weather for *A. dauci*. Resistance tests showed that all 26 monitored populations were highly resistant to *A. dauci* strains from The Netherlands. It is probable that the strong potential fitness reduction by *A. dauci* led to a high selection pressure towards resistance in The Netherlands. In conclusion, our results suggest that transgenic resistance to *A. dauci* would not be beneficial to wild carrot populations in The Netherlands because they are already resistant to this pathogen.

Key words: biosafety, carrot leaf blight, *Daucus carota*, fecundity, fitness, natural vegetation, resistance, seed survival, transgene.

INTRODUCTION

If a transgenic crop is grown near sexually compatible wild relatives, the transgene might introgress into natural populations, *e.g.* via pollen flow (Ellstrand et al., 1999; Linder et al., 1998). If the transgene confers resistance to a pathogen, the progeny that carries the transgene will become resistant to that pathogen. Such progeny has acquired a selective advantage (Bartsch et al., 1999), which may provoke expansion of the wild relative at the cost of other plant species (Parker and Kareiva, 1996; Schouten, 1999). This selective advantage depends strongly on the fitness reduction by the pathogen before introgression of the transgene. If the pathogen strongly reduced the fitness of the wild relative, then introgression of the transgene may significantly increase the fitness. However, if the pathogen did not significantly reduce the fitness of the wild relative, then addition of resistance will have hardly any effect upon fitness.

We investigated the effect of carrot leaf blight (*Alternaria dauci* (Kühn) Groves and Skolko) on the fitness of wild carrot (*Daucus carota* (L.)). We did not work with transgenic carrot, rather we focused on potential effects of (transgenic) resistance on fitness. For judgement of real fitness effects of a transgene, *via* resistance or *via* other mechanisms, an experiment with transgenic carrot is required.

Wild carrot is a common plant in Western Europe and elsewhere (Mitich, 1985). Wild carrot and cultivated carrot belong to the same species, and are cross-fertilized (Mitich, 1985). Gene flow between wild carrot and cultivated carrot occurs in areas with flowering cultivated carrot (Wijnheijmer et al., 1989). If transgenic carrot cultivars were to be grown widely, then gene flow to wild carrot cannot be prevented. As carrot leaf blight is a serious disease of cultivated carrot, resistance to this

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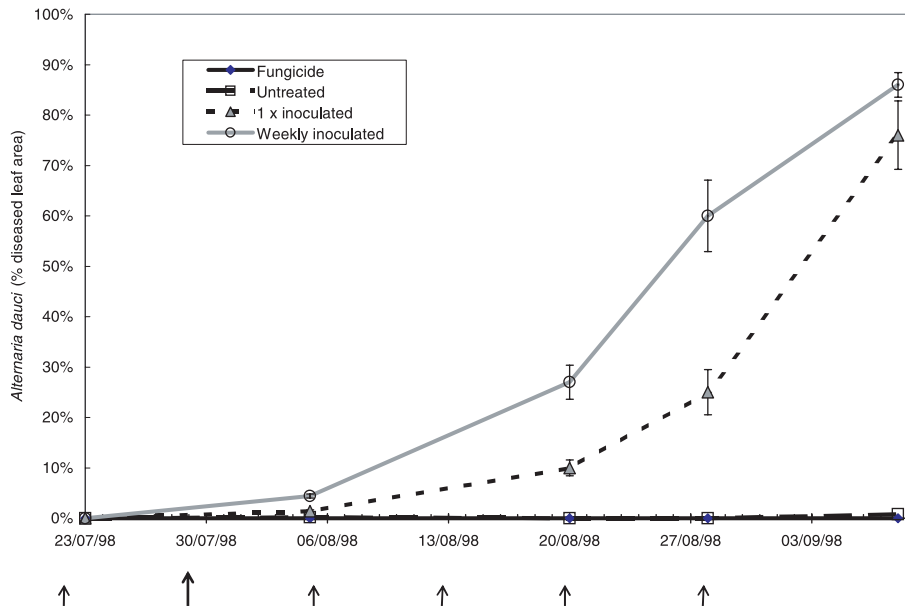


Figure 1. Development of leaf blight (*Alternaria dauci*) in a field experiment with susceptible annual wild carrot from Iran. The arrows indicate the timing of artificial inoculation with spores of *A. dauci*. The plots that were inoculated on one occasion only were sprayed with spores on 30 July (large arrow). Means of five replications and the corresponding error bars are shown.

disease has a high priority in breeding programs. Both conventional breeding methods and genetic transformation are applied to obtain resistant carrots (Boiteux et al., 1993; Strandberg et al., 1972; Takaichi and Oeda, 2000).

A. dauci causes leaf necrosis. Under conditions favorable to the disease, a severe and early infection may lead to complete loss of leaves (Maude et al., 1985). This may lower seed production, and therefore the fecundity of carrot. The fungus may also infect the seeds of the carrot, resulting in a lowered seed survival or damping-off of seedlings. Reductions of fecundity and seed survival imply reduction of fitness.

The research aimed at the following objectives: (1) Determination of the relationship between the level of leaf blight and the fitness of wild carrot. This was performed in a field experiment using a susceptible wild population from Iran. (2) An inventory of disease levels of wild carrot in natural habitats in The Netherlands. (3) Determination of levels of resistance to *A. dauci* of these Dutch wild carrot populations.

Combination of the results has provided insight into the reduction of fitness of wild carrot by *A. dauci* in vegetations in The Netherlands. Indirectly this also provides insight into potential effects of the introgression of transgenic resistance into wild carrot.

RESULTS AND DISCUSSION

Field test

Disease progress

Figure 1 shows strong differences in disease progress in the experimental plots. The plots sprayed with the fungicide showed no carrot leaf blight, whereas the untreated plots showed a very low level of blight during the last monitoring only (0.8% on average). The wide isolation buffers of hemp were apparently very effective for prevention of inter-plot interference by wind borne spores of *A. dauci*. In the plots that were inoculated once only, a polycyclic epidemic started. Close observations of the leaves revealed abundant sporulation. This single inoculation was enough to give rise to a very serious epidemic. Probably the extremely humid growing season of 1998 contributed to this. Leaf blight developed even more strongly in the plots that were artificially inoculated six times. This stronger development is not necessarily caused by the higher frequency of inoculation, but may be caused by the earlier first inoculation. Soon after the first inoculation, the number of spores produced in the plots probably was much higher than the number of added spores during subsequent inoculations.

Fitness effects of *Alternaria* on wild carrot

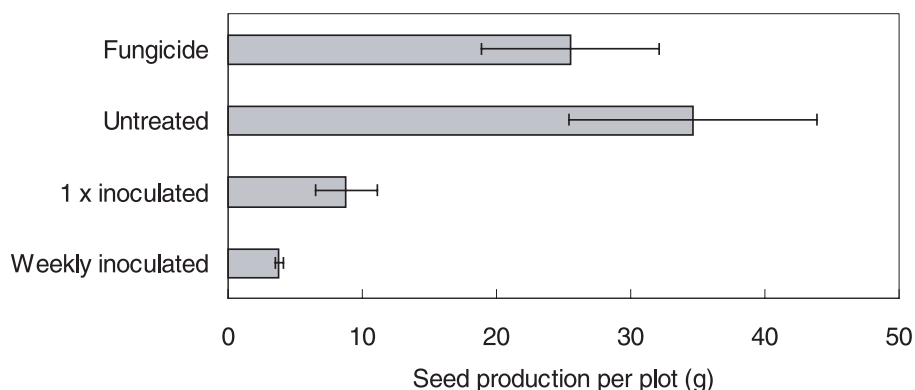


Figure 2. Seed production in the field experiment with wild carrot at different levels of inoculum pressure by *A. dauci*. Means of five replications and the corresponding error bars are shown.

During the last monitoring, the inoculated plots were almost all dead, but the non-inoculated plots were still green and the majority of the plants were completely healthy. All four treatments were significantly different from one another ($P < 0.05$) with respect to the amount of leaf blight. There was no significant block effect.

Effects of *A. dauci* on fecundity of wild carrot

The plots that were inoculated once or inoculated weekly produced only 30% and 13% respectively of the seed weight of the healthy plots (Fig. 2). The effect of inoculation on seed weight was significant ($P < 0.05$), according to t-tests for pairwise differences. Apparently, the fecundity of wild carrot was lowered significantly by *A. dauci*.

The plots sprayed with fungicide did not differ significantly from the non-treated plots ($P > 0.05$). Neither did the once inoculated plots differ significantly from the weekly-inoculated plots. Probably this is due to the relatively high standard variation for seed weight within a treatment, as is indicated by the error bars in Figure 2. There was no significant block effect.

Effects of *A. dauci* on survival of wild carrot seeds

Inoculation reduced the germination capacity of the seeds significantly, according to Student's t-tests ($P < 0.05$; Fig. 3). There was no significant block effect. Apparently, *A. dauci* not only reduced the seed weight (Fig. 2), but also the capacity of the remaining seeds to produce seedlings (Fig. 3). In other words, *A. dauci* reduced both the fecundity and the seed survival. Via

both mechanisms *A. dauci* reduced the fitness of wild carrot. However, inoculation did not significantly effect the percentage of diseased seedling ($P > 0.05$).

Effect of *A. dauci* on fitness of its host

For survival of a generatively reproducing plant population, it is necessary that one mother seed gives rise to at least one germinative daughter seed (Crawley et al., 1993). For the field experiment, this is evaluated in Figure 4. These charts are obtained through multiplication of the seed weight per plot (Fig. 2) by the number of seedlings per g seed (Fig. 3), and dividing by the number of mother seeds sown per plot (approximately 2175). In case of the non-inoculated plots the number of germinative seeds per mother seed exceeded 1, but this was not the case in the inoculated plots. This implies that at a high inoculum pressure, *A. dauci* would be able to reduce the wild carrot population, provided that the circumstances remained similar.

It should be realized, however, that the seed production of even the healthy plots was low in spite of abundant flowering. This may have been caused by a low pollen production because of the rainy season, by a low level of pollination by the lack of insects due to the high hemp buffers used, by inbreeding depression, or by a combination of these factors. Besides, in the inoculated plots the monoculture of the host will have contributed to the epidemic, whereas in nature a greater variety of plant species are present. The microclimate was also very conducive for *A. dauci* with plenty of rain, and the protection of the plants against wind by the hemp borders resulted in long periods of wetness. Moreover, the population chosen to study was previously known to be

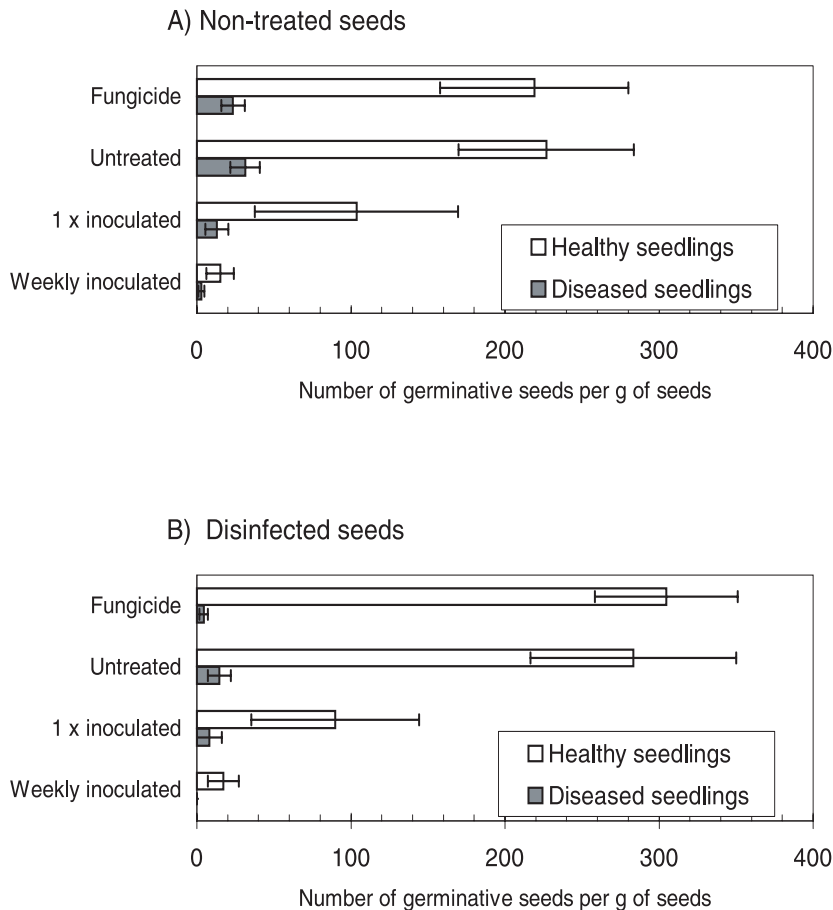


Figure 3. Germination of seeds harvested from the field experiment. The germination capacity was determined in soil under controlled glasshouse condition. The diseased seedlings were infected by *A. dauci*. In A, seeds were not treated prior to sowing, while in B they were disinfected. Means of five replications and the corresponding error bars are shown.

very susceptible to *A. dauci*. Under these circumstances *A. dauci* was apparently destructive to its host.

An optimum disease level for survival of the pathogen

From Figure 4 it appears that the nearly healthy plots provided more diseased seedlings than the most diseased plots. Carry over of the fungus from one season to the next was more likely at a moderate disease level than at a high disease level. So, a moderate disease level probably would have enhance survival of *A. dauci* relative to a high disease level. If the disease level were extremely low, then the pathogen would not produce enough spores for survival, and would disappear. If the disease level were very high, the pathogen would destroy its host, and therefore itself. A moderate disease level would be most beneficial for the pathogen. This disease level can be called the “optimum disease level”.

Natural populations

General observations

Wild carrot in The Netherlands was frequently found on road verges, on inclines of dikes and approach roads, as well as in fields, but it also occurred in dunes along the North Sea (Fig. 5). Wild carrot usually competed with grasses. If carrot was found on dike slopes, it occurred on the southern sides of the dikes, not on the northern sides. Wild carrot was frequently found in calcareous dunes, but not in acid dunes that are located above Bergen. If wild carrot was detected in calcareous dunes, it appeared particularly in sites that were frequently disturbed by people. In fenced off dunes wild carrot did not appear. In The Netherlands, the majority of the populations are annual (personal observation), whereas populations in other regions can be biennial (Mitich, 1985).

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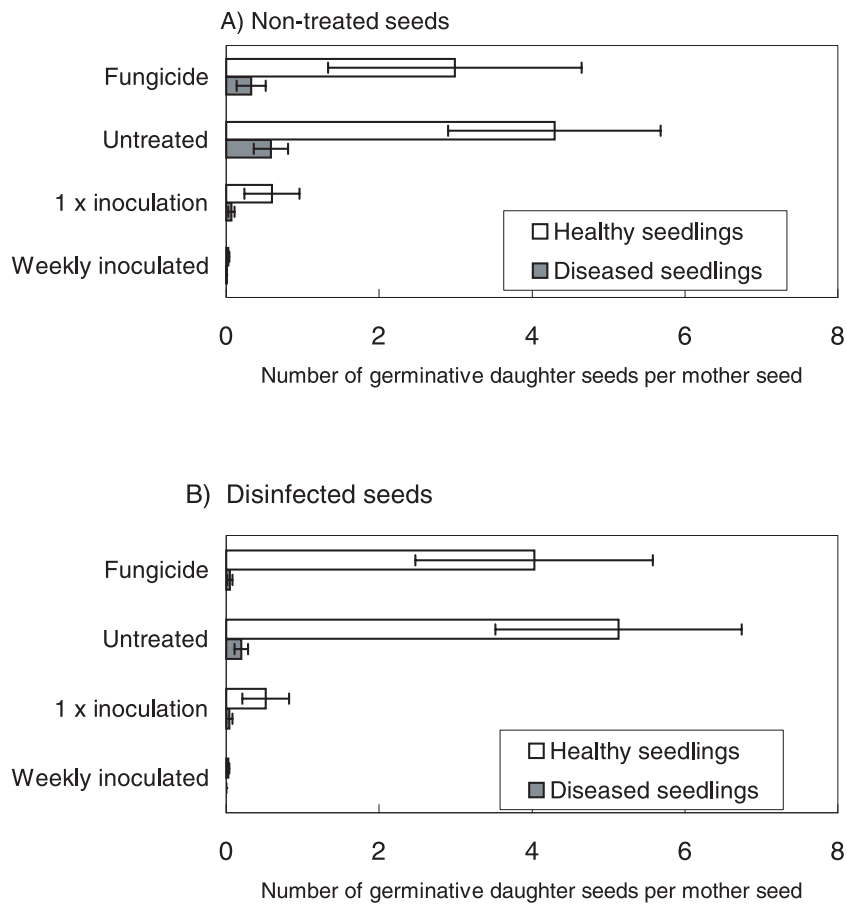


Figure 4. Fitness of wild carrot from Iran at the four different levels of inoculum pressure by *A. dauci*. The values represent the number of germinative seeds produced per seed sown in the field trial. In A, progeny seeds were not treated prior to sowing, while in B they were disinfected. Means of five replications and the corresponding error bars are shown.

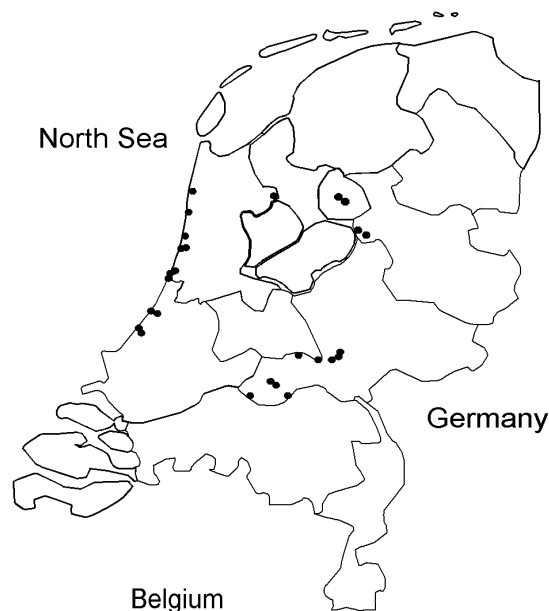


Figure 5. Distribution of the 26 sampled populations of wild carrot in The Netherlands.

Diseases

During the weeks 30 and 33 in 1998 only a maximum of 2% of the leaf area of the 26 wild populations showed necrosis or chlorosis. Examination in the laboratory of these leaf parts did not reveal presence of *A. dauci*. The weather was very conducive for *A. dauci* during the growing season. A low level of *A. radicina* was detected on three populations, *i.e.* on 1–2% of the leaf area of those populations. Reddening of up to 20% of the leaf area was frequently observed. Carrot red leaf virus probably caused this reddening.

The seeds collected from the natural populations showed a very low level of contamination by *A. dauci*, *i.e.* 0.4% (Fig. 6). When the seeds were sown, approximately the same percentage of seedlings was diseased by *A. dauci*. Apparently, the seeds that were contaminated with *A. dauci* were able to act as source of inoculum during a next growing season.

Apart from *A. dauci*, other *Alternaria* species were detected, *i.e.* *A. radicina* and *A. alternata*. These other *Alternaria* spp. are closely related to *A. dauci*, and are

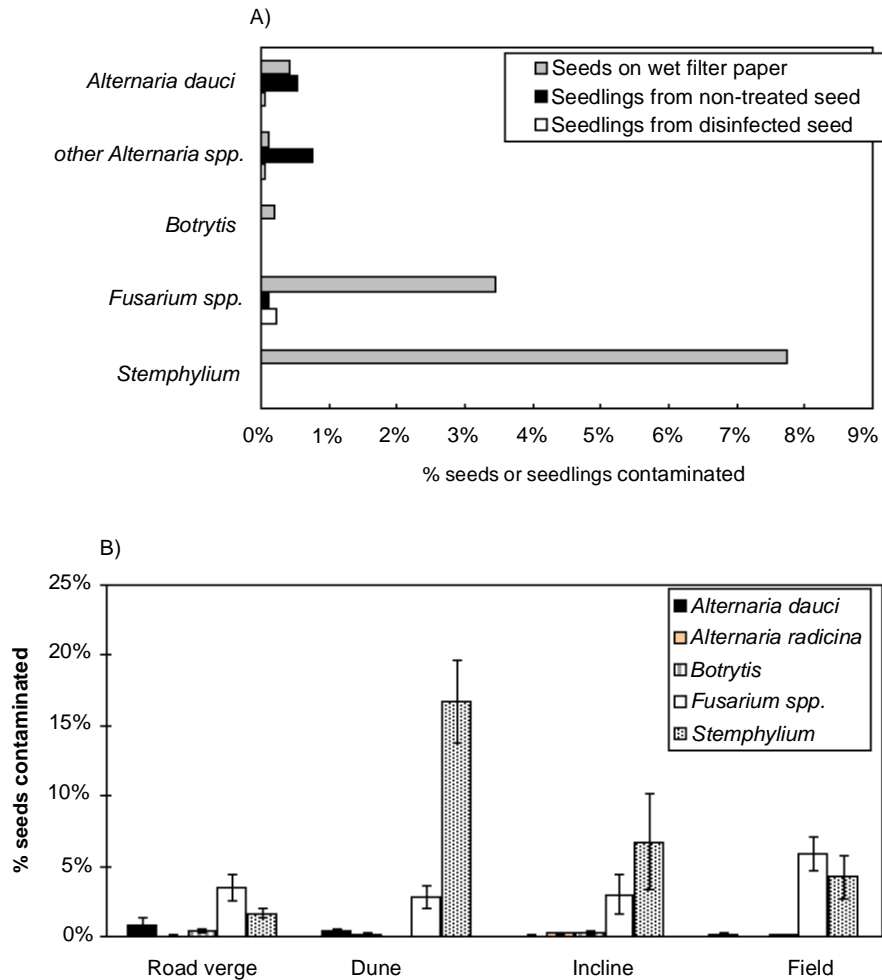


Figure 6. Contamination of seeds collected from the 26 wild carrot populations. In A, both the harvested seeds and also the young seedlings that arose from the seeds after sowing in a glasshouse were tested for the presence of different fungi. B shows the presence of different fungi in the populations, classified by sites.

also pathogenic to carrot. However, they occurred at very low levels (Fig. 6). *Botrytis* was nearly absent, but there were higher incidences of *Fusarium* and *Stemphylium* on the seeds (Fig. 6). *Botrytis* usually are saprophytic to carrot, but they can behave as pathogens of carrot. *Stemphylium* usually colonizes plant parts that are weak or dead, and was particularly prevalent on wild carrot found in dunes. The upper plant parts of wild carrot in the dunes were usually damaged by the strong wind from the sea. Due to this damage, *Stemphylium* was able to colonize the dead parts. *Stemphylium* was not detected on the young seedlings (Fig. 6).

Levels of resistance

Harvested seeds from the 26 wild carrot populations were sown in 1999 in a large field for resistance tests to *A. dauci*. In addition to these 26 populations, the field contained approximately 2500 hybrids and inbred lines of carrot, many of which were susceptible to *A. dauci*. The field was inoculated weekly during two months. This led to an average disease score of 2.06 for the hybrids and inbred lines on a scale of 0 to 8 (0 = all leaf area diseased; 8 = completely healthy). The disease score of the susceptible population used in the field experiment in 1998 equaled 1. In contrast, the disease scores of the

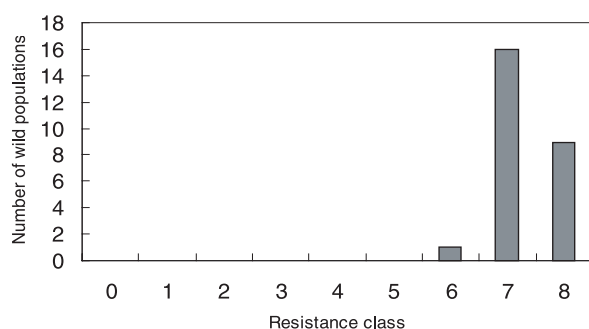


Figure 7. Levels of resistance of 26 wild carrot populations from The Netherlands. 0 = very susceptible; 8 = completely resistant.

26 wild populations averaged 7 to 8, as shown in Figure 7. All wild populations appeared to have a high level of resistance to *A. dauci*.

These high levels of resistance of the 26 populations will have contributed strongly to the low incidence of *A. dauci* in the natural populations. Other factors, like the mixture of different plant species, and low inoculum pressure, may also have contributed to the low incidence.

CONCLUSIONS

Effect of *A. dauci* on Dutch wild carrot populations

From the field experiment with the susceptible wild carrot population from Iran, it appeared that *A. dauci* was able to develop as a strong epidemic on the leaves. The fungus lowered significantly and strongly the fecundity and the seed survival of the susceptible host, and therewith the host's fitness. However, during the same year, *A. dauci* was hardly present in wild carrot in the natural habitats in The Netherlands. The resistance tests showed that all 26 sampled Dutch wild carrot populations had a high level of resistance.

Probably, the strong potential fitness effect of the pathogen has led to a selective advantage of resistant plants compared to susceptible plants in the natural populations. This may have led to high levels of resistance in the natural populations in The Netherlands.

Potential effect of a transgene for resistance to *A. dauci*

If gene flow occurs from a transgenic carrot crop to a wild carrot population in The Netherlands, and the transgene provides resistance to *A. dauci*, then the effect of this

transgenic resistance on the competitiveness of wild carrot is expected to be negligible. The natural populations tested in this study were already resistant, and would probably gain no fitness increase through an extra resistance gene.

MATERIALS AND METHODS

Field tests

Sowing

From previous tests, an annual wild carrot population was selected that was susceptible to *A. dauci*. The population was originally collected in Iran in the region Khuzestan, and stored in Wellesbourne, record number 7170. Seeds from this population were sown in The Netherlands for seed multiplication. Twenty-four flowering plants from these seeds were put together under one cover, to prevent pollination from other carrot plants. Under the cover, flies pollinated the flowers. Seeds from this population were sown on 23 April 1998 in 20 plots in Wageningen, The Netherlands. The field size was $45 \times 58 \text{ m}^2$, and the plot size $4.5 \times 5 \text{ m}^2$. The seeds were sown in 10 rows of 5 m per plot. In total, 58 g of seed was sown, which equaled approximately 43 500 seeds. A 1.5 m wide path surrounded the plots. Between the plots there was a 3 m wide buffer of hemp, in order to minimize exchange of spores between the plots (Langenberg et al., 1977; Strandberg, 1977).

The pathogen

The inoculum was prepared from a mixture of three *A. dauci* isolates from carrot fields in Noord-Holland (The Netherlands), Friesland (The Netherlands), and Osnabrück (Germany). Before inoculum production, the pathogenicity of these isolates was confirmed. The three isolates were grown separately by weekly inoculation of hundreds of Potato Dextrose Agar plates. The plates were placed for seven days at room temperature in the laboratory, and subsequently for 14 days at UV light at 20°C . Water was poured on the plates, and the spores were suspended by scraping the agar. The suspensions were filtered, and the spore concentrations measured by using a haemocytometer. The three different isolates were mixed, providing approximately equal numbers of spores. The inoculum was diluted to 10^5 spores per ml. For each inoculation, 100 ml inoculum was used per plot. The plots were inoculated during the evenings, to obtain long periods of leaf wetness.

There were four levels of inoculum pressure of *A. dauci*:

1. Plots with regular fungicide applications, *i.e.* on 20 and 27 July, 3, 11 and 17 August, and on 1 September. We sprayed 4 ml Iprodion Rovral Aquaflow in 1 L water per plot.
2. Untreated plots.
3. Plots that were inoculated once with a spore suspension of *A. dauci* just before flowering on 30 July 1998.
4. Plots that were inoculated six times, *i.e.* on 23 and 30 July, 6, 13, 20 and on 28 August.

Five replicates were used in a block design. The disease progress of the foliage was monitored five times by non-destructive visual estimation of the percentage of diseased leaf area of 30 randomly chosen plants per plot.

Plant development and seed harvest

On 5 August 1998, all flower buds were still closed. On 20 August many main umbella were in full bloom, by 25 August the first ripe seed clusters were present, but on 8 September the majority of the main umbella were still flowering. The seeds from the plots were collected manually by cutting off the seed clusters, leaving the remaining plant parts undamaged. Because of strong differences in flowering time of the umbella and ripening of the seed clusters, the seeds were collected on five different dates, *i.e.* on 25 August, 11, 14 and 30 September, and on 15 October. The seeds were dried for one month with forced air at 20 °C, threshed, cleaned, and weighed per plot.

Germination capacity

For seed fungicide treatment, 1 g Aatiram (50% thiram) and 2.5 g Rovral (50% iprodione) were mixed, and applied to part of the harvested seeds (14 mg.g⁻¹ seed). Per plot, 140 treated and 140 untreated seeds were sown in small pots, one seed per pot. During germination the pots were kept moist in a plastic tunnel. After germination this cover was removed. The temperature regime was 20 °C at daytime and 15 °C at night, and relative humidity was 70%. After emergence, the plants were screened weekly for disease symptoms. Diseased plants were removed and incubated on wet filter paper in a growth cabinet with nUV illumination at 20 °C for confirmation of the causal pathogen.

Statistical analysis

The disease levels on the leaves in the field experiment (Fig. 1) were integrated over time by calculation of the Areas Under Disease Progress Curves according to Shaner and Finney (Shaner and Finney, 1977). The variances of the integration data were made more homogeneous by applying the ¹⁰log-transformation. This transformation was also applied to the data for seed production and germination capacity (Figs. 2–4). Then, analyses of variance and Student's t-tests for pairwise differences were performed. We used the statistical software package GenStat, release 4.22.

Natural populations

Determination of diseases on leaves and seeds

26 populations of wild carrot (Tab. 1) in The Netherlands were examined for leaf blight in 1998. As appears from literature (Langenberg et al., 1977) and our own experience, the severity of leaf blight in carrot crops is usually low during the first half of the growing season. Later, when plants flower and when seeds develop, *A. dauci* usually develops more strongly on the leaves (Langenberg et al., 1977). Therefore, we decided to evaluate the leaves of the wild carrot populations relatively late during the growing season, *i.e.* during the weeks 30 and 33 of 1998. At least 30 plants per population were evaluated visually. Yellow and brown leaves were collected and put on wet filter paper. After incubation for 10 days at 20 °C and alternating cycles of nUV light (12 hours light, 12 hours dark) the leaf parts were examined for determination of pathogens, using a microscope.

During the weeks 38 and 39 we collected seeds from at least 30 plants per populations. After drying, threshing, and cleaning, the presence of seed-borne fungi was determined with the deep-freeze blotter test. For this purpose, 400 seeds per population were put on wet filter

Table 1. Number of examined populations of wild carrot, classified for site and mowing.

Site	Mowing		Total
	No	Yes	
Dune	8	0	8
Field	1	2	3
Incline	3	3	6
Road verge	0	9	9
Total	12	14	26

paper and incubated for 3 days at 20 °C in the dark. Then the seeds were frozen for 24 hours at -20 °C, and subsequently incubated at 20 °C and alternating nUV for six days. Parasitic and saprophytic fungi were identified, using a microscope.

In addition, seed germination and health of the seedlings was determined for 140 untreated seeds per population and 140 treated seeds per population, as described for the germination capacity tests of the seeds from the field test.

Resistance testing

Seeds collected from the 26 populations were sown in 1999 in a field of a Dutch seed company for evaluation of the levels of resistance. Apart from these 26 populations, in the same field approximately 2500 inbred lines and hybrids of carrot were evaluated for resistance for breeding purposes. Because many of these lines were susceptible to *A. dauci*, they provided a control group for the resistance tests (*i.e.*, they demonstrate that the inoculum was effective). Per natural population two rows of 3 m were sown, and artificially inoculated with *A. dauci* (10^5 spores per ml) from 28 July till 15 September at weekly intervals. The inoculum was produced as described for the field test. The disease levels were evaluated on 21 September, using a disease index from 0 (all leaves completely blighted) to 8 (completely healthy).

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