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# The effect of mature plant resistance in sugar beet (*Beta vulgaris spp. vulgaris*) on survival, fecundity and behaviour of green peach aphids (*Myzus persicae*)

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**Research Paper**

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## Abstract

Several studies have shown the negative effects of mature plant resistance (MPR) on aphids in sugar beet, which is correlated to the formation of black deposits in their stomach. However, the underlying mechanism of MPR still needs to be elucidated, by understanding the toxicity effects of MPR on aphids and the role of the plant phenological stage and the environment. Here, we report that MPR in sugar beet does not only affect *Myzus persicae* mortality rate and the formation of a black deposit in the aphid stomach, but also aphid fecundity and behaviour. In addition, experiments in climate-controlled and field settings showed quantitative variation in MPR to *M. persicae* between six genotypes of sugar beet. Our results indicate that environmental effects, such as temperature, play a major role in MPR and underscore the importance of proper climate-controlled experiments for investigating MPR. In climate-controlled experiments, 83.3% of aphids on old leaves developed a black deposit, in contrast to only 16.8% of aphids on young leaves. This shows that not only plant age, but also leaf age plays a major role in the intensity of MPR. Further research will be needed to identify the underlying mechanism, before MPR can be used as a viable and sustainable solution to aphid pests in sugar beet.

## Introduction

Mature plant resistance (MPR) is used as a generic term for developmental resistance mechanisms induced by plant ageing. MPR is described for multiple crops and under different names: age-dependent resistance or adult plant resistance. It is described to be present in potato (*Solanum tuberosom*) against viruses and in maize (*Zea mays*) against northern leaf spot caused by the fungus *Cochliobolus carbonum* (Venekamp and Beemster, 1980*a*; Marla *et al.*, 2018). In addition, MPR has been investigated in *Arabidopsis thaliana* against insects (Mao *et al.*, 2017). The underlying mechanisms are poorly understood and differ per plant and pest species. In *A. thaliana*, MPR to herbivorous insects has been linked to the accumulation of glucosinolates (Mao *et al.*, 2017). However, in potato, MPR was reported to be linked to the lower RNA and ribosome content in older plants, which could result in lower transcription rates of the virus (Venekamp and Beemster, 1980*b*), while in maize, MPR is linked to the partial loss of functional alleles of the *Hm1* gene (Marla *et al.*, 2018). In sugar beet (*Beta vulgaris spp. vulgaris*), MPR has been described to the fungus *Rhizoctonia solani* (Liu *et al.*, 2019) and to aphids (Kift *et al.*, 1998*a*) but the underlying mechanisms are still unknown. To summarize, MPR is a broad-scale phenomenon that is observed in many plant species and implicated in a number of pathways to resist pests and diseases.

MPR in sugar beet to aphids has been observed since decades. Thornhill and Heathcote (1987) were the first to publish data in which the decline in aphid numbers in field observations from 1978 until 1981 is clearly shown. When plants reach the 10th–12th leaf stage, aphid field mortality increases and less insecticides are needed to control aphid infestation (Kift *et al.*, 1998*a*). Although MPR to aphids is exploited routinely by using damage thresholds, only limited research has been performed on understanding MPR in sugar beet (Williams, 1995; Kift *et al.*, 1996). Nowadays, more insights in the mechanism of MPR will be valuable, as the availability of insecticides decreases and with fewer active ingredients the frequency of resistance to the remaining active ingredients will increase. This is already evidenced in populations of the green peach aphid (*Myzus persicae*; *Mp*) which demonstrate resistance to several groups of insecticides due to the intensive usage in multiple crops (Bass *et al.*, 2014). Due to the lack of effective control of aphid in sugar beet in the EU (Foster and Dewar, 2013; Dewar and Qi, 2021), breeding for plant resistance by increased MPR may be a viable and sustainable solution for the virus yellows for which certain aphids are the vector.



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Yellowing viruses, mainly transmitted by *M. persicae*, are a major threat to sugar beet production. The major aphid-transmitted viruses in sugar beet are *Beet yellows virus* (BYV), *Beet mild yellowing virus*, *Beet western yellowing virus*, *Beet chlorosis virus* and *Beet mosaic virus*. They all cause Virus Yellows (VY) disease in sugar beet. Symptoms related to VY are yellowish discoloration of the leaves and necrosis, which reduces photosynthetic capacity of the plant and thus has a major impact on sugar yield. Virus infection can lead to yield decreases up to 50%, depending on the virus, moment of infection and amount of virus transmitting aphids (Van der Werf *et al.*, 1992; Stevens *et al.*, 2004; Hossain *et al.*, 2021).

Between 2014 and 2016, 59–100% of the farmers used neonicotinoids, which were introduced in 1991 to avoid aphid infestation in the field and which strongly reduced virus incidence (Qi *et al.*, 2004; Hauer *et al.*, 2017; Dewar and Qi, 2021). Since the restrictions in the European Union (EU) on the outside use of neonicotinoids in 2018, first implemented in 2019, an increase in the number of fields with VY damage has been observed. The area with VY damage has increased from 0% up to 82% in certain regions of the Netherlands in 2020 (Cosun Beet Company, 2021). In the UK, the national incidence of VY at the end of August in 2020 was 38.1%, while since 1996 the national virus incidence was kept below 5% (Dewar and Qi, 2021).

As VY was properly controlled since 1991, there was no pressure to further investigate MPR against aphids in sugar beet but this has changed due to recent restrictions on the use of neonicotinoids. The existing studies on MPR to aphids indicate it is active against multiple aphid species including *M. persicae*, *Aphis fabae* and *Macrosiphum euphorbiae* (Akers, 1988; Kift *et al.*, 1996). MPR is characterized by the appearance of black deposits in the aphid stomach and their subsequent death (Kift *et al.*, 1996). In searching for the underlying mechanism of MPR, Kift *et al.* (1998*b*) analysed the molecular structure of the black deposit. The black deposit appeared to be a complex of multiple compounds and therefore it could not be traced to the underlying pathway involved in MPR. MPR is not only affected by the age of the plant, but differences in the intensity of MPR were also observed between leaves irrespectively of plant age (Kift *et al.*, 1998*a*). Differences in MPR were also observed between field trials performed in subsequent years, which indicated that MPR may be strongly influenced by additional external factors like weather conditions (Kift *et al.*, 1998*a*).

MPR is a potential alternative to insecticides. However, first a better understanding of the mechanism of MPR in sugar beet in relation to aphids is needed and what roles the phenological plant stage and the environment play. Such information is crucial for further exploitation of this trait. In addition, the interaction of aphids with MPR may help define how and when MPR is triggered and can best be employed.

In this study, we have investigated whether there is variability in timing and intensity of MPR on *M. persicae* between different genotypes of sugar beet, both under indoor climate-controlled and field conditions. In addition, the toxicity of MPR on aphids was closely monitored as well as its effect on aphid fecundity and behaviour in relation to MPR.

## Materials and methods

### Plants and aphids

To measure the toxicity effects and aphid behaviour in relation to MPR, climate-controlled experiments were performed. In addition, two climate-controlled experiments were performed to compare timing and intensity of MPR between different genotypes of sugar beet. All indoor climate-controlled experiments were conducted at the Dutch Institute for Sugar Beet Research (IRS, Dinteloord, the Netherlands). Trial conditions were at a 16 h:8 h light:dark period (LED 119 mmol $m^{-2}$ $s^{-1}$, RAZRx PLUS, Fluence Bioengineering, Austin, Texas, USA) with 23°C during the day and 16°C during the night. Unless mentioned otherwise, 6-week-old sugar beet plants (8th–10th leaf stage) of a commercial variety of Strube (D&S GmbH, Söllingen, Germany) were used. Seeds were sown on sterilized river sand to which Dolokal (0.3% (v/v)) and Osmocote Exact Mini (ICL speciality fertilizer; 0.1% (v/v)) were added. Dolokal contains calcium carbonate, magnesium carbonate and magnesium oxide, preventing a low pH. Osmocote is a controlled release fertilizer, consisting of a resin-coated nutrient core containing nitrogen, phosphorus and potassium. After one week, plants were transferred to 700 ml pots containing 50:50 river sand:potting soil (Primasta, zaai-en stekgrond, Primasta, Asten, The Netherlands). For all experiments, the oldest fully expanded leaf without symptoms of senescence was used. In practice, this was always a leaf from the second-oldest leaf pair as during the experiments the two oldest leaves started to display senescence symptoms such as yellowing.

In all experiments, apterous nymphs of the green peach aphid (*M. persicae*) were used and reared on 6–10 weeks old sugar beet plants. For infestation of plants in the climate-controlled experiments, aphids were synchronized by placing fully grown apterous aphids on 6-week-old sugar beet plants. After 2–3 days the adults were removed and 3 days after removal, the 5–6 days old nymphs were used for infestation. For climate-controlled and field experiments, onset and intensity of MPR was monitored by visually assessing the presence of the black deposit in the stomach in individual aphids. If aphids died without a black deposit, these aphids were not included in the analysis, because no distinction could be made with aphids which died from other causes (e.g. not feeding or broken stylet).

### Toxicity effects of MPR in young plants on aphids

To compare the toxicity effects of young and old sugar beet leaves on *M. persicae*, climate-controlled experiments were performed, in which mortality, number of black deposits and fecundity were investigated. Aphid development on old and young leaves was compared by counting the aphid numbers and appearance of the black deposit in the stomach after 3, 7, 10 and 14 days. For each replicate, ten aphids were confined on the inner leaf of the plant by a small tube made of a fine mesh (250 µ) (fig. S1A), or on the oldest fully expanded leaf (without clear senescence symptoms, such as yellowing) by a self-made box, made of a Petri dish, with a rectangular cut-out of 8 × 8 cm, covered with a fine mesh. Around the plant stem, foam was placed to close the hole and prevent aphids from escaping (fig. S1B).

### Aphid preference assay

To explore the effects of MPR on aphid behaviour, the aphid preference for older or younger sugar beet leaves was investigated under climate-controlled conditions by placing 30 nymphs (5–6 days old) on a 6 × 3 cm paper on top of a 6-week-old sugar beet plant. The paper was positioned in such a way that it was in contact with the youngest and oldest leaves, allowing the aphids to move freely over the plants. Five days post infestation (DPI), the locations of the aphids were monitored.

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### Electrical Penetration Graph recording

To further explore the effects of MPR on aphid behaviour, Electrical Penetration Graph (EPG) recordings (McLean and Kinsey, 1964; Tjallingii, 1988) were performed, according to an adapted methodology by ten Broeke *et al.* (2013). In short, an 18 µm-thin gold wire with a length of 2 ± 0.5 cm was gently attached to the back of the aphid using water-based silver glue. Subsequently, aphids were either placed on the adaxial side of a young heart leaf, or on the adaxial side of an oldest fully expanded leaf (without clear senescence symptoms such as yellowing) of a 6-week-old sugar beet plant. Aphid behaviour was monitored with a Direct Current Giga-8 system (http://www.epgsystems.eu) for a total duration of 8 h with the program EPG Stylet + d. Annotation of the waveforms was done in EPG Stylet + a (www.epgsystems.eu). During each recording, a total of ten aphids could be monitored; one per plant. During each recording, five aphids were placed on young leaves and five on old leaves in an alternating position within a Faraday cage. In total, seven EPG recordings, of each ten aphids, were performed. For the analysis, 29 EPG annotation files from aphids on young heart leaves, were compared to 34 EPG annotation files from aphids on older leaves and further processed in R (R-Core-Team, 2020). Aphid feeding behaviour was compared for 22 feeding characteristics (table S1) by performing Mann–Whitney *U* tests (sometimes with continuity correction), and in case of proportional variables, Pearson's $\chi^2$ tests with Yates' continuity correction. Waveforms that did not occur were considered as missing data for total duration, mean duration and latency variables. Feeding events that were interrupted by the end of the recording, and thus continued after the 8 h of recording, were included in all calculations.

### Variation in mature plant resistance between plant genotypes

To investigate the variation in the intensity and timing in MPR, two climate-controlled experiments and one field experiment were performed. For this, two hybrids and three recombinant inbred parent genotypes were used, provided by SESVanderHave (Tienen, Belgium) (table 1). One commercial variety originally obtained from Strube (see above) was used as a control. The seeds were treated with standard amounts of the fungicide hymexazol (g ai/unit) for Aphanomyces and the pyrethroid contact insecticide tefluthrin, except for genotype 2 which was only treated with hymexazol. Tefluthrin is a soil pesticide with no systemic activity that might affect aphids. The seeds from the cultivar from Strube were also treated with the fungicides sedaxane, fludioxonyl and metalaxyl-M (fig. S2).

To test for differences in MPR under climate-controlled conditions, two climate room experiments were performed in which the six sugar beet genotypes were compared in a randomized block design with ten replicates. Ten aphids were confined on the second oldest leaf pair of 6-week-old plants and 3, 7, 10 and 14 DPI aphid numbers (alive and dead) and the presence of the black deposit were registered.

In addition, to compare climate-controlled conditions with outdoor trials, a field experiment was performed at Oude Molen (Noord-Brabant, The Netherlands). Seeds from the six different sugar beet genotypes were sown in a complete randomized block design with four blocks. The seeds were sown to final stand with a specialised seed drill on 27 March 2020. Tillage, seed bed preparation and applications of fertilizers and pesticides (except for insecticides) were done according to the best local practice. Over the growing season, plants were infested with *M. persicae* at four different time points after sowing. For the confinement, ten aphids were placed on a leaf surrounded by a bag made of an aphid proof mesh (Polyester, 250 µ). Foam was used to properly and non-intrusively close the bag around the stem. After placing the aphids in the bag, the bag was closed at the top with a fine wire. For all infestations, bags of one size were used (20 × 39 cm) (fig. S1C). The dates of the infestations were: 7 May (6 weeks), 25 May (9 weeks), 22 June (13 weeks) and 13 July (16 weeks). During the infestations, plants were in their 4th, 8th–10th, 16th–22nd and 24th–30th leaf stage, respectively. In total, 40 plants per sugar beet genotype (without VY symptoms) were selected for infestation, ten plants for each time point of infestation. At 4 DPI, the bags with the leaves were collected from 20 plants per genotype and taken to the laboratory, where aphid numbers were counted and the presence of the black deposit was noted. The infested leaves from the remaining 20 plants were collected and counted 8 DPI.

**Table 1.** Specifications of the sugar beet genotypes provided by SESVanderHave and Strube used in the experiments

| Number | Breeding specifications | Breeding company |
|---|---|---|
| Genotype 1 | Commercial variety | Strube |
| Genotype 2 | Parental line | SESvanderHave |
| Genotype 3 | Parental line | SESvanderHave |
| Genotype 4 | Parental line | SESvanderHave |
| Genotype 5 | Hybrid | SESvanderHave |
| Genotype 6 | Hybrid | SESvanderHave |

### Statistical analysis

To model the probability that a single aphid will accumulate a black deposit in its stomach, generalized linear models with a log link function were used to ensure positive fitted values.

To determine the effect of leaf age on the probability that an aphid will become black in the climate-controlled experiment, the following model was applied:

$$\frac{p_{\text{black}_i}}{1-p_{\text{black}_i}}\Big| u = \exp\left(\mu_0 + \beta_1 \text{Age Leaf}_i + \beta_2 \text{DPI}_i + u\,\text{Plant}_i + \varepsilon_i\right)$$
$$\varepsilon_i \sim N(0,\ \sigma^2) \quad (1)$$

Here, $p_{\text{black}_i}$ is the probability that aphid $i$ will become black. By rewriting the probability into odds $\frac{p_{\text{black}_i}}{1-p_{\text{black}_i}}$, it was possible to apply a linear regression model as per definition of the logit model. In equation 1, $\beta_1$ and $\beta_2$ represent the estimated coefficients of the parameters Age Leaf and DPI (days post infestation), respectively. Age Leaf is a binary independent variable which represents (1) the young inner heart leaves, or (2) the oldest leaves (without clear senescence symptoms such as yellowing). The factor DPI has four levels, as measurements were taken at 3, 7, 10 and 14 DPI. In this experiment, groups of aphids were confined for an extended time on separate plants. As a result, the observed aphids with black deposits on each plant for a separate time interval were not an independent measurement. Aphids that were observed to have a black deposit on a specific plant ($\text{Plant}_i$) would also be observed to have a black deposit in sequential

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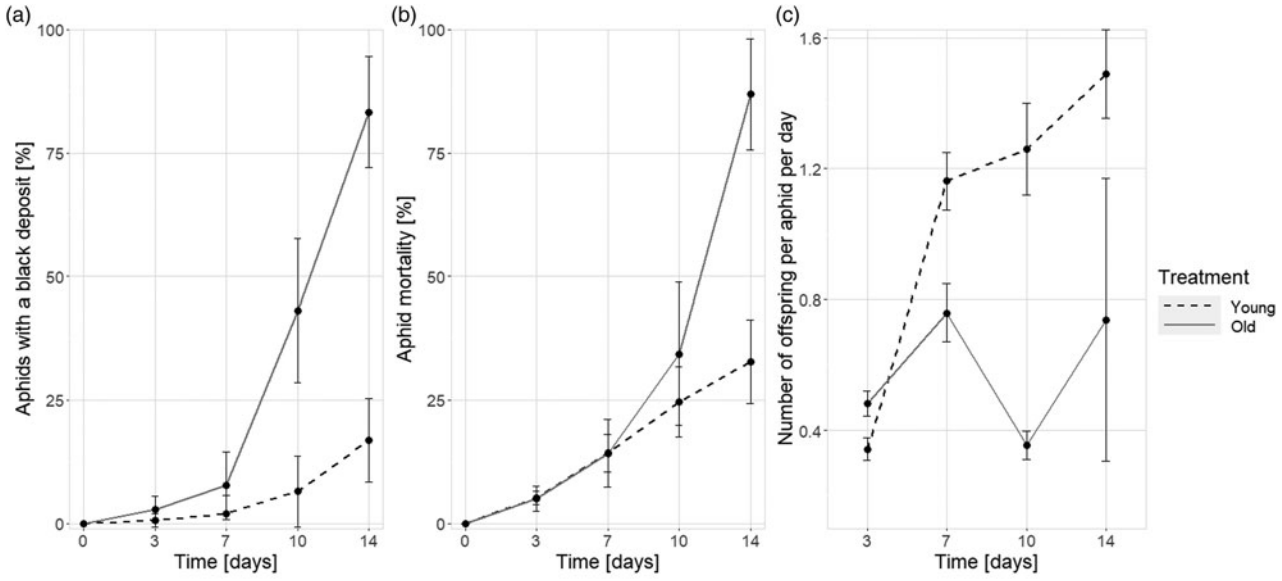


**Figure 1.** Effect of mature plant resistance on *Myzus persicae* aphids on young (dotted line) and old (solid line) leaves of 6-week-old sugar beet plants in a climate-controlled experiment. (a) Percentage of aphids with a black deposit on young and old leaves. (b) Percentage of aphids that died on young and old leaves. (c) Aphid fecundity on young and old sugar beet leaves per aphid per day. Error bars represent 95% confidence intervals, $n = 15$.

time steps, creating a pooled dataset. This dependence was corrected for by using a random effects model in which the probability $p_{\text{black}_i}$ was modelled, given the estimated random effect ($u$) of each plant. $\epsilon_i$ represents the error term.

For the climate chamber experiment in which multiple sugar beet genotypes were compared, the following model was applied:

$$\frac{p_{\text{black}_i}}{1 - p_{\text{black}_i}} \Big| u = \exp\left(\mu_0 + \beta_1 \text{Genotype}_i + \beta_2 \text{DPI}_i + u\,\text{Plant}_i + \varepsilon_i\right)$$
$$\varepsilon_i \sim N(0,\ \sigma^2) \tag{2}$$

Here, Genotype represents the six sugar beet genotypes. The factor DPI and the random effect $u$ for each Plant represent the same as in equation 1.

For the field trial, the model was as follows:

$$\frac{p_{\text{black}_i}}{1 - p_{\text{black}_i}} \Big| u = \exp(\mu_0 + \beta_1 \text{Genotype}_i + \beta_2 \text{DPI}_i + \beta_3 \text{Date}_i + \beta_{1\times2} \text{Genotype}_i \times \text{DPI}_i + \beta_{1\times3}\ \text{Genotype}_i \times \text{Date}_i + u\,\text{Plant}_i + \varepsilon_i)$$
$$\varepsilon_i \sim N(0,\ \sigma_\varepsilon^2) \tag{3}$$

Here, Genotype represents the individual sugar beet genotypes. The factor Date represents the four infestation times. In this experiment, DPI is a binary variable and modelled whether aphids were confined for 4 or 8 days on the leaf. In this experiment, ten aphids were confined on the same leaf of an individual plant. As a result, the number of observed aphids with black deposits on each plant is not an independent measurement and the random effect $u$ for each Plant had to be included. For both Date and DPI, we investigated if there was a different impact for each genotype on the probability of aphids turning black by adding the interaction terms Genotype × Date and Genotype × DPI. Other interactions did not have significant effects on the formation of black deposits and were therefore taken out of the model.

For the analysis, the function *glmer* of the R package '*stats*' (R-Core-Team, 2020) was used to fit the model (equations 1–3). The package *ggplot2* (Wickham, 2016) was used to make graphs. The dataset met all requirements for a logit regression. Overdispersion was not an issue with a binary response variable. Statistical analyses of the EPG data are described in the paragraph '*Electrical Penetration Graph recording*'.

## Results

### *Toxicity effects of MPR in young plants on aphids*

In earlier work, the effects of MPR in the field on aphid numbers were observed when plants had reached the 10th–12th leaf stage (Kift *et al.*, 1996). However, in preliminary experiments using the older leaves of 6-week-old plants (6th–8th leaf stage), we also observed aphids with black deposits. Therefore, we hypothesized that MPR is already present in the older leaves of 6-week-old sugar beet plants. To investigate this, aphid mortality, formation of the black deposit and aphid fecundity were measured when aphids were confined on the young inner heart leaves or the second-oldest leaves of 6-week-old sugar beet plants. Both formation of black deposits and mortality were higher for aphids on old leaves, compared to aphids on young leaves ($P$ value $< 2 \times 10^{-16}$, generalized linear model, $n = 150$, table S2). After 14 days of confinement, 83.3 and 16.8% of the total amount of aphids formed a black deposit on old and young leaves, respectively (fig. 1a). The percentage of aphids that had died within 14 days was 86.8 and 32.8% on old and young leaves, respectively (fig. 1b). In addition, aphid mortality and the presence of a black deposit were significantly correlated ($P$ value $< 2.2 \times 10^{-16}$, $r = 0.8$, Pearson correlation test, $n = 111$). The difference in the number of aphids that formed a black deposit on young and old leaves was significant, as demonstrated by the positive value (2.835) of the estimate (table S1). Aphid fecundity was in general higher for aphids confined on young inner heart leaves compared to aphids confined on the old leaves (fig. 1c, all $P$ values $< 0.01$, Mann–Whitney

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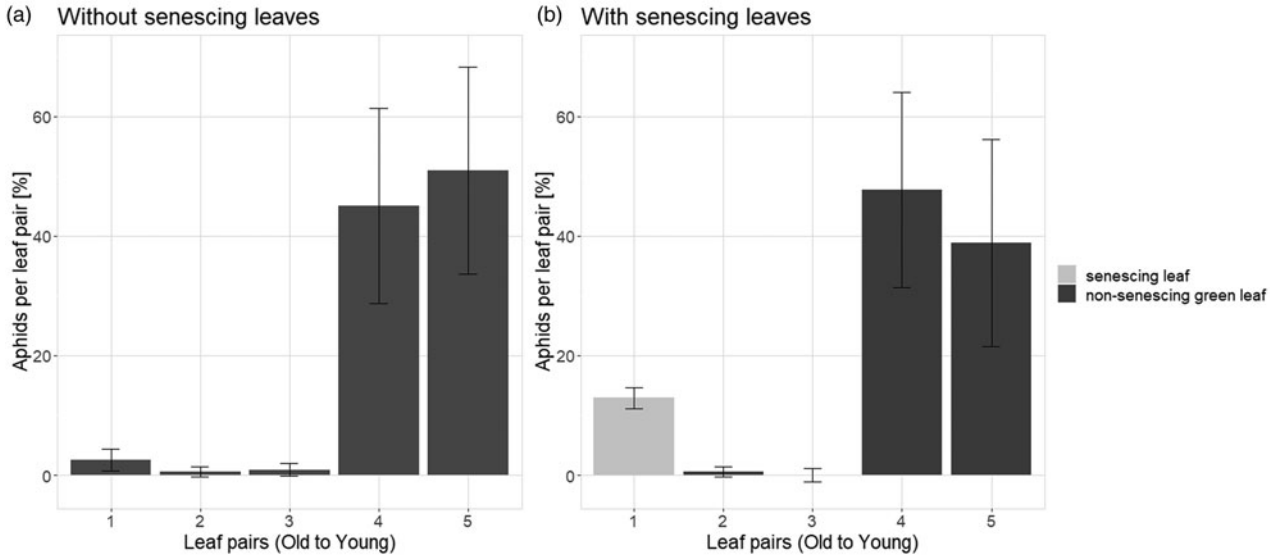


**Figure 2.** Preference of the aphids for the different leaf pairs on 6-week-old sugar beet plants without senescing leaves (a) and with senescing older leaves (b) in the climate room. Error bars represent the 95% confidence interval, $n = 10$.

*U* test, $n = 15$). Overall, this experiment showed that leaf age plays a major role in MPR as on old leaves aphids developed more often a black deposit in their stomach, had a higher mortality rate and lower fecundity than on young leaves of 6-week-old sugar beet plants.

### *Effects of MPR on aphid behaviour*

Subsequently, we investigated whether aphid host preference was affected by MPR and whether aphids choose to avoid the older leaves. Even though plants were of the same age, some showed senescence-related symptoms such as yellowing of the oldest leaf pair. Therefore, the preference of aphids on the senescing and non-senescing plants was compared, because senescence-related symptoms such as yellowing could influence aphid behaviour (Holopainen *et al.*, 2009). We observed that on non-senescing plants, the aphids were most attracted to the two youngest leaves (fourth and fifth leaf pair), while roughly 4% was located on the older leaves (first, second and third leaf pair) (fig. 2a, $n = 10$, $P$ value $< 0.01$, $\chi^2$ test). However, on plants of which the oldest (first) leaf pair displayed clear senescence symptoms, roughly 11% of the aphids migrated to those senescing leaves (fig. 2b). This was significantly more than on the non-senescing plants ($P$ value $< 0.01$, $n = 10$ and 5 for non-senescing and senescing plants, respectively, $\chi^2$ test).

### *Aphid feeding behaviour*

To investigate whether the formation of black deposits, increased mortality and decreased fecundity were caused by differences in the aphid's feeding pattern on young and old leaves, feeding behaviour was monitored by EPG recordings. Significant differences were found between aphids feeding on old and young leaves. The number of ultra-short probes (< 0.5 min) was significantly higher for young leaves, with $5.2 \pm 1$ and $9.9 \pm 1.3$ for old and young leaves, respectively (mean ± standard error) ($P$ value = 0.0015, Mann–Whitney *U* test, $n = 34$ and 29 for old and young leaves respectively, table S1). In addition, it was found that the mean duration of probing in the epidermis or mesophyll cells was significantly higher on the young leaves, compared to the old leaves ($P$ value = 0.0458, $n = 34$ and 29 for old and young leaves respectively, table S3). The latency in time to first sustained phloem feeding was also longer on young leaves compared to old leaves with $154.7 \pm 20.1$ min and $213.7 \pm 20.1$ min for old and young leaves, respectively ($P$ value = 0.0229, $n = 30$ and 27 for old and young leaves respectively, table S3). Together, this behaviour illustrates that *M. persicae* aphids have more problems reaching the phloem on young leaves, but do not reveal any effect of MPR in the older sugar beet leaves.

### *Variation in mature plant resistance between plant genotypes*

The variation in MPR between different sugar beet genotypes was investigated, as this information could be useful to help unravel the underlying mechanism of MPR in the future. First, two climate-controlled experiments were performed with six sugar beet genotypes (table 1), followed by a field experiment.

Both climate-controlled and field experiments showed that different sugar beet genotypes had significantly different effects on the formation of black deposits (tables S3–S5). However, in the climate-controlled experiments, less variation between the genotypes was observed compared to the field experiment. Aphids on genotype 4 suffered from the highest proportion of black deposits (92%), while for genotype 2 only 58% of the aphids had formed a black deposit 14 DPI (fig. 3). The GLM results confirmed that aphids on plants of genotype 4 had a significantly higher probability to form a black deposit compared to aphids on genotype 2 ($P$ value $< 0.01$, $n = 70$, table S3). In the repetition, the model predicted the same trend, whereby aphids on plants of genotype 4 had a significantly higher chance to form a black deposit, compared to genotype 2 ($P$ value $< 0.01$, $n = 70$, table S4), while aphids confined on plants of genotype 2 had again the lowest probability (fig. S3). However, in the repetition, genotype 6 had an even stronger positive effect on the formation of black deposits relative to genotype 4, shown by the higher estimate (table S4).

The variation in MPR between the sugar beet genotypes was further investigated in a field trial in which different sugar beet

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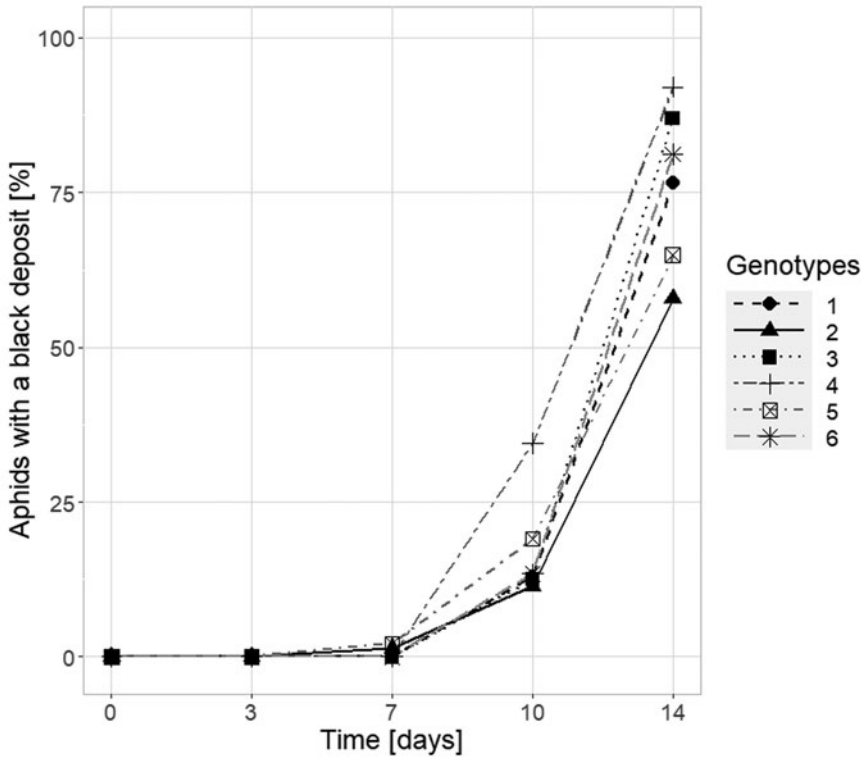


**Figure 3.** Percentage of aphids with black deposits found after 3, 7, 10 or 14 days of confinement on different genotypes of sugar beet in a climate-controlled experiments, $n = 70$.

plants were infested with green peach aphids at four different time points in the course of the growing season. The percentage of aphids that developed a black deposit after confinement for 4 or 8 days differed per genotype and over the four different time points of infestation (fig. 4). In accordance with the climate-controlled experiments, we observed the longer the aphids were confined on the leaves, the more aphids developed a black deposit. In addition, for all sugar beet genotypes, an increase in aphid mortality was found over time until 22 June (day 59), as at 13 July (day 87) lower aphid mortality was observed compared to June 22nd (fig. 4 and table S5). In general, the GLM analyses showed that genotype 5 resulted in highest formation of black deposits ($P$ value $< 0.001$, $n = 400$, generalized linear model). Lowest formation of black deposits was found for genotype 1 (table S5). However, many interaction effects between the genotypes and infestation times were found. This implies that the increase or decrease in MPR in time is relatively smaller or larger for specific sugar beet genotypes. For example, genotype 1 resulted in the lowest number of black deposits during the first infestation at 7 May, but during infestation 2 and 3 (25 May and 22 June) relatively more black deposits were formed, compared to the other genotypes.

## Discussion

Although sugar beet growers use MPR in practice already for decades in integrated pest management by using a higher aphid threshold for spraying insecticides after plants have reached their 10th–12th leaf stage, the underlying mechanisms of MPR are not yet fully understood. In addition, there was only limited knowledge available on the precise toxicity effects of MPR on *M. persicae* and its developmental and behavioural responses to MPR. Therefore, we have investigated how aphid mortality and fecundity is affected by MPR in sugar beet. In addition, preference assays and EPG recordings were performed to test if MPR affects aphid behaviour.

We observed that aphids die within 36 h of the formation of a black deposit in their stomachs, even when transferred to other plants species on which normally no black deposits are formed such as Chinese cabbage (data not shown). Thus, formation of the black deposit appears irreversible and directly linked to the death of the aphids. This was also supported by the strong correlation between aphid mortality and formation of black deposits (fig. 1a, b).

As fecundity and survival rate were both negatively affected on older leaves of sugar beet because of MPR, we investigated if aphids avoid older leaves, as this would indicate that aphids are able to sense MPR. The preference assay showed us that aphids prefer young inner heart leaves and avoid the older green leaves. However, when the oldest leaves were already showing senescence symptoms such as yellowing, 11% of the aphids settled on those leaves (fig. 2). The preference for senescing leaves by aphids has been previously observed and is suggested to be related to the availability of nutrients in the phloem sap for translocation to other plant organs (Kennedy and Booth, 1961; Holopainen *et al.*, 2009). Nevertheless, a certain degree of MPR, though only 16.8% of the aphids developed black stomach deposits, was also observed for young leaves. This could indicate two things: (1) the pathway related to MPR must also be active in young leaves, or (2) the compounds related to MPR are produced in the older leaves but (partly) relocated to the phloem of the younger leaves, where they are taken up by the aphids.

Surprisingly, if aphids were given no choice, they ingested phloem from older leaves for at least 8 h as was shown by the EPG recordings. Alvarez *et al.* (2006) investigated the effects of different resistance factors in tuber-bearing *Solanum* species on

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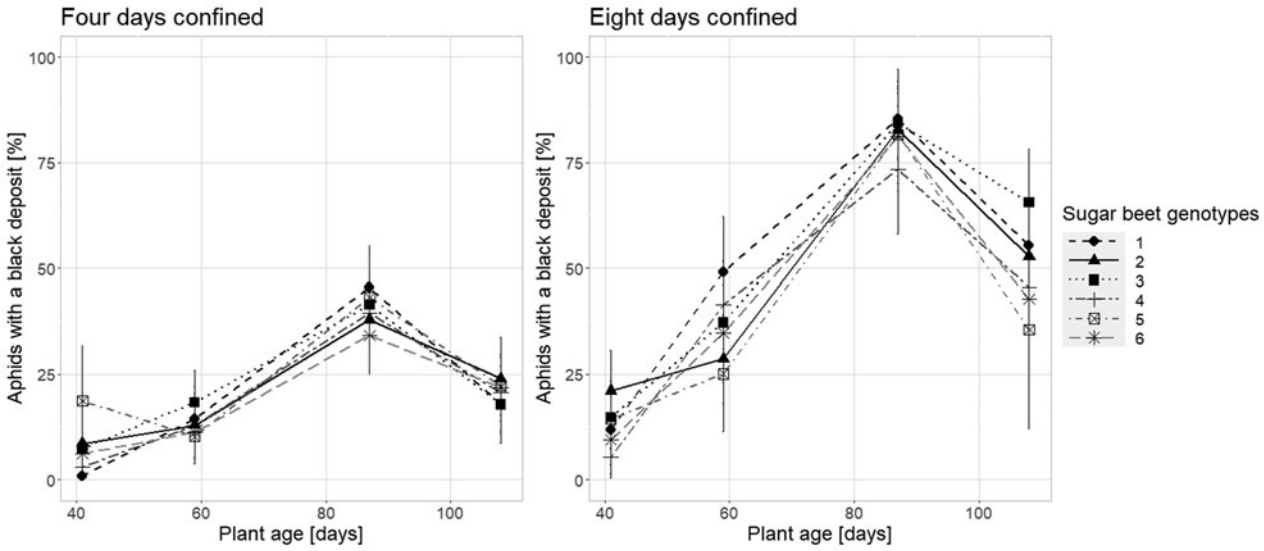


**Figure 4.** Percentage of aphids with a black deposit over time per sugar beet genotype, when confined for 4 or 8 days on a sugar beet leaf in the field experiment in Oude Molen (2020). Error bars represent 95% confidence intervals, $n = 40$.

the probing behaviour of *M. persicae*. Longer or more non-probing events and less phloem feeding immediately after colonization of the plants were linked to resistance against aphids. However, based on the results of our experiment, aphids on old leaves did not show compromised feeding compared to aphids on young leaves during the 8 h recording. This indicates that either the effect of MPR requires more time to accumulate in the aphid stomach before it becomes detrimental, or that MPR is an induced mechanism that requires more than 8 h or higher aphid infestation levels before it is manifested. On the other hand, significant differences were found between feeding behaviour of aphids on young and old leaves; on the younger leaves more ultra-short probes were observed, longer probing duration in the epidermis or mesophyll cells and longer time until first sustained phloem feeding. These differences indicate feeding difficulties on the young inner heart leaves. However, based on the preference assay, the aphids still prefer to feed on those young leaves despite having the feeding difficulties (fig. 2). Nevertheless, the EPG recordings, which were restricted to 8 h, do not show how much phloem sap is ingested and whether feeding behaviour is affected after a longer time period.

Next to studying the toxicity effects of MPR, we have also investigated variation in MPR between six sugar beet genotypes. We observed significant differences in the field trial between the six sugar beet genotypes and between the four infestation moments (table S5) as well as significant differences between the sugar beet genotypes in climate-controlled experiments. The differences in MPR observed between the genotypes in field and climate-controlled experiments were however not fully consistent. For example, genotype 5 resulted in a relatively high level of black deposits under field conditions, while in the climate chambers it resulted in one of the lowest levels of black deposits (tables S3 and S4). In the field many interaction effects between the different sugar beet genotypes and moments of infestation were found. This implies that although in general the same trend could be visualized for all sugar beet genotypes, whereby an increase in the number of black deposits was found over time until the third infestation, for some genotypes the increase over time was relatively higher or lower compared to other sugar beet genotypes (fig. 4).

Based on the differences between the results of the field trial, the climate-controlled experiments and the decline in number of aphids with black deposits at the fourth infestation, we suggest there is genetic variation for MPR but environmental factors, such as high temperatures, have a larger effect on MPR. The decline in levels of black deposit at the fourth infestation coincided with changes in weather conditions. During the third infestation (22 June), the mean daily temperature was 26.3°C, with a maximum of 32.0°C. This was much higher compared to infestation 1, 2 and 4, with mean daily temperatures of 18.2, 23.7 and 21.6°C, respectively (data Dutch weather service KNMI (Gilze-Rijen)). The total precipitation in the 2 weeks prior to, during and 1 week after infestation was 11.3 mm (infestation 1), 3.1 mm (infestation 2), 62.3 mm (infestation 3) and 54.8 mm (infestation 4), respectively (KNMI).

Plant physiology was not monitored during this experiment, but could potentially play an important role in MPR and explain the interaction between the sugar beet genotypes and moment of infestation. Next to that, aphid survival and behaviour could have been directly affected by the weather conditions. However, as we used the same *M. persicae* clone in this experiment for all treatments, we expect that the aphids' behaviour does not differ between plant genotypes due to environmental stimuli. The different levels of MPR between the six sugar beet genotypes observed in our field experiment could be the result of genetic responses to stimuli such as drought, temperature and light stress.

The negative effect of VY on MPR has already been described in the past by Kift *et al.* (1996). In our field experiment, only 18 out of the 960 plants tested positive for VY infection by RT-PCR when tested for BYV and the presence of a *polerovirus* (results not shown). None of the plants were BYV positive and when investigated further, the partial sequences of the 18 polerovirus isolates were very similar to *Turnip yellows virus*. It is probable there were no significant VY effects on MPR in our experiment due to the low number of infections; however, given the small sample size of infected plants in this experiment, this is an area that should be investigated further.

Currently, we are further investigating which environmental factors lead to higher MPR, including understanding how plant physiology is modified by certain environmental or biological

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factors which lead to higher MPR. Moreover, further research is underway to identify the chemical nature of the black deposit and the underlying mechanism involved in MPR in sugar beet.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0007485322000128.

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**Conflict of interest.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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