Unravelling the resistance mechanism of lettuce against *Nasonovia ribisnigri*

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Abstract

Aphids are serious pests of crop plant species, and host plant resistance is often the most effective and environmentally friendly control strategy to control these pests. One of these aphid pests is the black currant - lettuce aphid, *Nasonovia ribisnigri* (Mosely), an economically important pest of cultivated lettuce, *Lactuca sativa* L. Host plant resistance has been used since 1982 to control this aphid species and is mediated by the *Nr*-gene, originating from wild lettuce *Lactuca virosa* L. However, this resistance is not effective anymore, since *N. ribisnigri* aphids virulent to the *Nr*-resistance have been reported since 2007. The aim of this thesis was to unravel the mechanism of resistance mediated by the *Nr*-gene against *N. ribisnigri*, by behavioural studies on the aphids on both resistant and susceptible lettuce, to allow lettuce breeders to accelerate their resistance breeding programmes. Although the exact mechanism of *Nr*-mediated resistance remains unknown, the data in this thesis provide insight into this mechanism. The active site of the *Nr*-mediated resistance is mainly located in the phloem and some resistance might be encountered by the aphids along the pathway to the phloem. The inability of the avirulent aphids to feed from the resistant plant could be caused by the failure of aphids to suppress the wound response of the sieve element. The resistance factor(s) are only produced in the shoot, because grafts with resistant shoots and susceptible roots were resistant, whereas grafts with susceptible shoots and resistant roots remained susceptible. An intact vascular system is needed for full resistance, because both detached leaves and leaf disks of resistant lettuce plants were less resistant.

Variation in virulence was observed among populations of different geographical origin. Aphids from a highly virulent population performed equally well on both resistant and susceptible lettuce plants, whereas semi-virulent aphids performed better on susceptible lettuce plants. Both short-term and long-term virulence loss were observed for virulent aphid populations differing in virulence level, which indicates this virulence is associated with fitness costs. A possible mechanism underlying virulence in *N. ribisnigri* to the *Nr*-resistance is the presence of an effector protein in the salivary secretion of the aphids suppressing resistance. Virulent aphids seemed to actively suppress the resistance in lettuce against the avirulent aphids.

The original donor or the *Nr*-resistance, *L. virosa* accession IVT 280, was tested as possible source of new resistance against the virulent biotypes of *N. ribisnigri* and was found fully resistant against virulent aphids, and can be exploited as a source of resistance in breeding for new resistance in cultivated lettuce.

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Chapter 1

General Introduction

Cindy J.M. ten Broeke

Insect-plant interactions

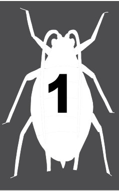
The Insecta is the most speciose class of organisms and its members can be found in all terrestrial ecosystems around the world due to adaptations to a wide range of environmental conditions (Schowalter, 2006). They play an important role in several ecological functions such as pollination, decomposition, and maintenance of wildlife species and ecosystem services such as natural pest control (Losey & Vaughan, 2006). Plants constitute the second most speciose group of organisms and are the biggest contributors to terrestrial biomass. Of all insect species described, almost half are herbivorous *i.e.* they feed on living plant tissues (Schoonhoven et al., 2005). Plants and insects interact in various ways, ranging from mutualism to parasitism, a major characteristic being the high degree of specialism that has evolved in the large majority of these interactions. Plants provide shelter, food and oviposition sites for insects (Panda & Khush, 1995). Insect - plant interactions are of economic importance. On the one hand herbivorous insects are important pests in agriculture by damaging food crops and causing significant economic losses (Schoonhoven et al., 2005). On the other hand pollinating insects are essential for reproduction of many crops (Losey & Vaughan, 2006).

Aphid-plant interactions

Aphid biology

Aphids (Aphididae) belong to the family Aphidoidea within the order of Sternorrhyncha (plant sucking bugs). About 4700 species have been described world-wide (Blackman & Eastop, 2007), a relatively low number of species (Dixon, 1998). About 450 aphid species have been recorded from crop plants, but only ~100 species are of economic importance, by successfully exploiting the agricultural environment (Blackman & Eastop, 2000). Aphids are distributed world-wide, but are predominant in the temperate regions of the world (Dixon, 1998; Goggin, 2007). The abundance of aphids in the tropics is much lower compared to the temperate regions. This is probably caused by the very high diversity of plants in the tropics and as a consequence there are very few plant species that show an abundance that is high enough to support an aphid species (Dixon et al., 1987).

Aphids are a major concern for farmers and gardeners. They are among the world's most serious pests of crop plants, causing major economic damage that is estimated to amount up to hundreds of millions of dollars each year (Dreyer & Campbell, 1987; Rabasse & Van Steenis, 1999; Tagu et al., 2008). Aphids can cause a reduction in yield and vigour of plants, they transmit plant-pathogenic viruses and can cause plant mortality when infestation is heavy (Irwin et al., 2007; Reid & Cuthbert, 1977). Almost every terrestrial plant species is attacked by aphids, both below- or above-ground, and almost every major crop is a host for at least one aphid species (Blackman & Eastop, 2000; Guerrieri & Digilio, 2008).
The combination of specific feeding and reproductive habits make aphids one of the most



economically important pests in agriculture (Guerrieri & Digilio, 2008). Aphids have high reproductive rates and short life cycles, making them successful in colonizing plants in a short time (Dreyer & Campbell, 1987). The high reproduction rates are achieved by clonal reproduction (parthenogenesis) and occur combined with vivipary, giving birth to nymphs that immediately start to feed (Dixon, 1998; Guerrieri & Digilio, 2008). Another feature of aphid biology, contributing to their success as pest species, is polyphenism, the occurrence of different forms and morphs, winged (alatae) and wingless (apterae) within a species (Dixon, 1998; Goggin, 2007) (Figure 1).

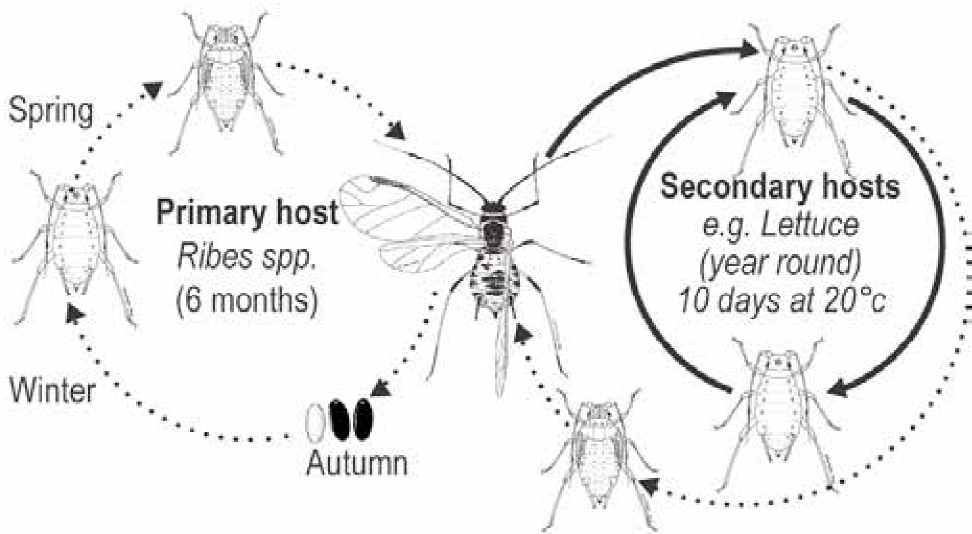


Figure 1: Polyphenism and host-plant alternation of *Nasonovia ribisnigri* (McDougall & Creek, 2007).

In apparent contrast, aphids also fulfil important functions in ecosystems. Honeydew, the sugar-rich faecal secretion produced by aphids, for example, is consumed by more than 250 different insect species, and forms a key nutritional substrate for many micro-organisms (Stadler et al., 1998). Additionally, aphids are prey for natural enemies, like ladybird beetles (Obrycki & Kring, 1998), carabids (Sunderland & Vickerman, 1980), lacewing larvae (Völkl et al., 2007), syrphid fly larvae (Smith & Chaney, 2007) and parasitoid wasps (Müller et al., 1999). Furthermore, many aphid species have evolved a mutualistic relationship with honeydew-collecting ants (Völkl et al., 2007).

Aphid feeding

Aphids feed on phloem sap of plants, by inserting their stylets into sieve elements (Figure 2). In this way withdrawing nutrients from the plant and thereby weakening it (Dixon, 1998). Some aphids also ingest xylem sap to maintain their water balance (Spiller et al., 1990; Will & Van Bel, 2006). The stylet paths toward the phloem are intercellular and multi-branched (Kimmins, 1988; Tjallingii & Hogen Esch, 1993). During the pathway phase aphids briefly puncture nearly all mesophyll cells along their path to the phloem, to "taste" their contents, to orient the stylet tip toward a sieve element, and to locate possible plant resistance factors in the tissues encountered from the epidermis to the phloem (Niemeyer, 1990; Prado & Tjallingii, 1994; Tjallingii & Hogen Esch, 1993). Furthermore, cell puncturing plays an important role in the transmission of plant viruses (Powell, 1991; Prado & Tjallingii, 1994).

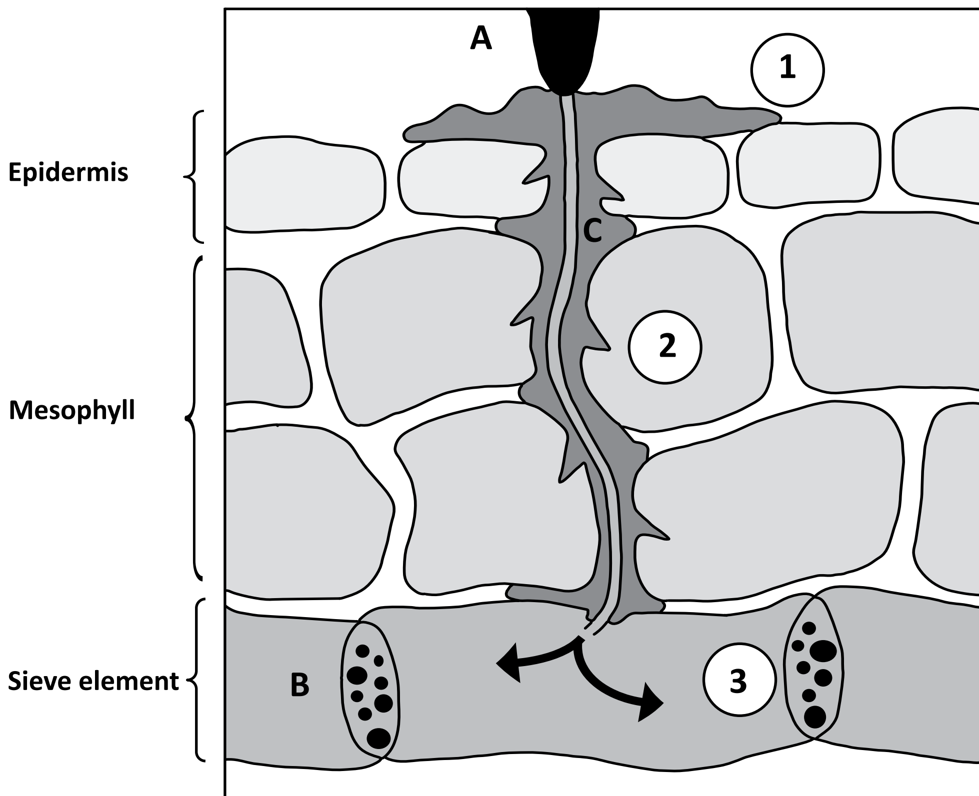
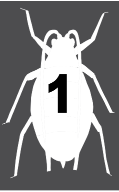


Figure 2: Aphid feeding. 1) The aphid starts salivating gelling saliva and penetrates the epidermis with its stylets. The aphid will continue to secrete gelling saliva on its way to the phloem. 2) The aphid punctures the membrane of every cell along its way to the phloem. 3) When the stylets reach the phloem, secretion of watery saliva starts (black arrows) into the sieve element. Dark grey: gelling saliva; proboscis (A); sieve plates between sieve elements (B); stylet bundle (C).



It takes an aphid on average about 15 minutes to penetrate a leaf from the epidermis to the phloem (Tjallingii, 1994). Before entering the plant, aphids secrete a small amount of gelling saliva on the plants surface, and will continue doing so during the pathway to the phloem (Tjallingii, 2006). This gelling/sheath saliva gives rigidity to the flexible stylets and enables aphids to control the direction of the probe (Pollard, 1977). Furthermore, the gelling/sheath saliva seals the puncture sites on the track to the sieve element and the puncture site in the sieve element itself, thereby reducing the influx of extra cellular calcium and reducing the loss of phloem sap (Walling, 2008; Will et al., 2007).

The phloem phase always starts with injecting watery saliva into the sieve element (Prado & Tjallingii, 1994). This saliva plays an important role in the feeding process (Dreyer & Campbell, 1987; Miles, 1999). Aphids are able to ingest phloem sap for hours or even days from a single sieve element, and to allow sustained feeding aphids keep sieve elements alive and open during feeding (Tjallingii, 1995; 2006). To prevent the loss of nutrient-rich phloem sap, plants evolved mechanisms to prevent damage to the sieve tubes (Figure 3). As a response to sieve element wounding, the calcium concentration in sieve elements is elevated (Ding & Pickard, 1993; Will & Van Bel, 2006). This increase in calcium (Ca^{2+}) concentration is believed to stimulate further wound responses like sieve plate occlusion by phloem (P)-proteins and callose (Will & Van Bel, 2006). Additionally, P-proteins detach from the sieve element plasma membrane by the surge caused by pressure loss upon sieve element damage, forming a plug on the sieve plate (Knoblauch & Van Bel, 1998; Sjölund, 1997; Taiz & Zeiger, 2002). These closing agents can also block the aphid's stylets (Walling, 2008). Aphids effectively inactivate the occlusion of the sieve elements, by secreting watery saliva into the sieve elements (Knoblauch & Van Bel, 1998). This saliva contains Ca^{2+} binding proteins that decrease Ca^{2+} concentration in order to prevent the triggering of the occlusion mechanisms (Furch et al., 2007). In addition, gelling saliva is excreted by the aphid to seal the stylet puncture sites, to minimise the influx of extracellular Ca^{2+} (Pettersson et al., 2007; Walling, 2008; Will et al., 2007; Will & Van Bel, 2006). Aphids also prevent blocking of the food canal of their stylets by ingesting watery saliva together with the phloem sap (Prado & Tjallingii, 1994; Tjallingii & Hogen Esch, 1993). Aphid saliva may act as an elicitor of plant reactions and might cause damage (Cherqui & Tjallingii, 2000).

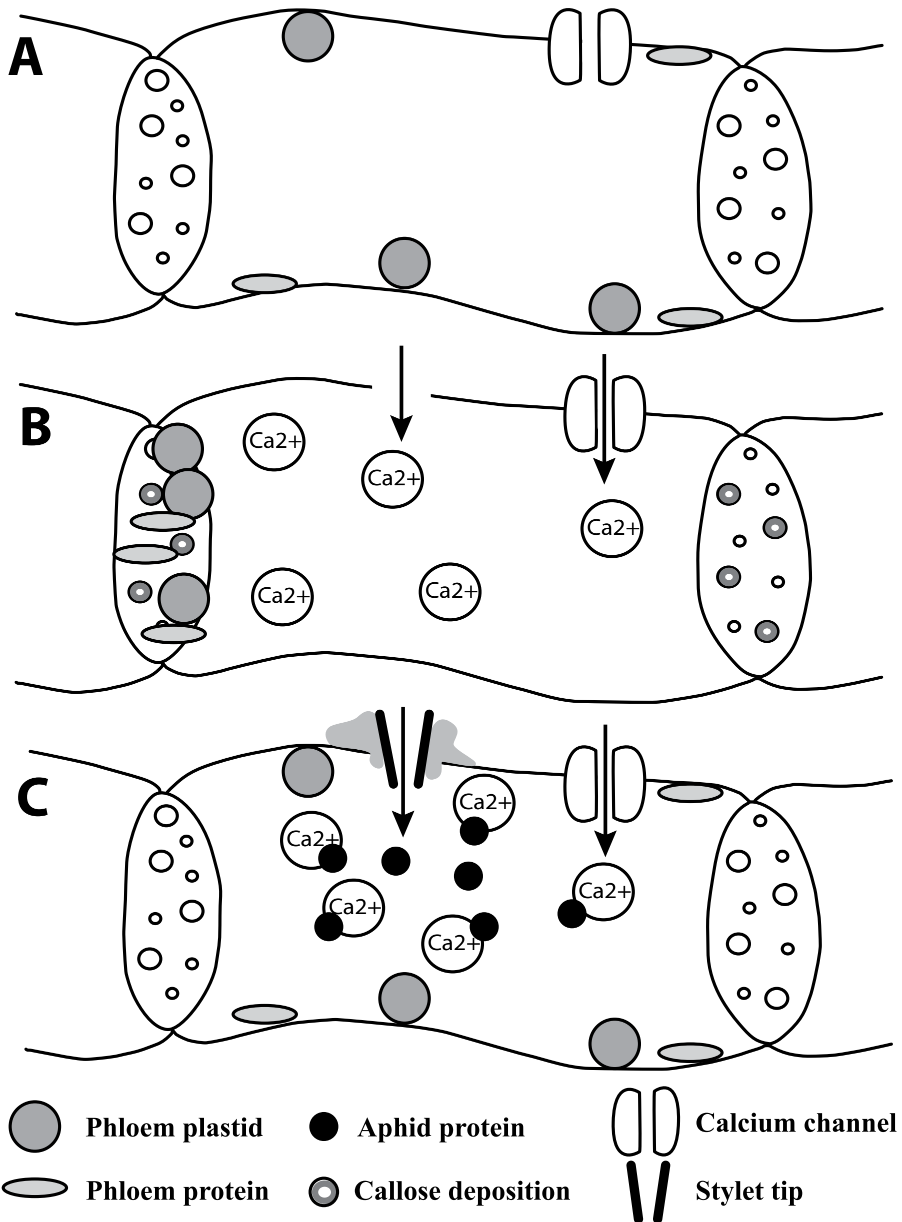
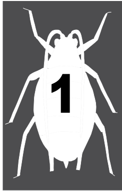


Figure 3: Sieve element wound response. A. An undamaged sieve element. B. A damaged sieve element. An influx of Ca^{2+} ions takes place resulting in blockage of the sieve plates by phloem proteins and plastids, and callose deposition. C. A sieve element punctured by aphid stylets. After Ca^{2+} influx, proteins from the watery saliva (containing aphid proteins) bind to the Ca^{2+} , and consequently the wound response is inhibited.



Recording stylet penetration behaviour: the electrical penetration graph technique

The feeding behaviour of chewing insects can easily be observed visually, however, the feeding behaviour of piercing insects is much more difficult to observe (Walker, 2000). The development of the electrical penetration graph (EPG) technique made it possible to electronically record the feeding behaviour of Hemiptera/ Sternorrhyncha and other piercing-sucking insects (McLean & Kinsey, 1964; Tjallingii, 1978; 1985b; 1988) like whiteflies (Janssen et al., 1989), plant hoppers (Kimmins, 1989), psyllids (Bonani et al., 2009), leafhoppers (Backus et al., 2005) and mealybugs (Calatayud et al., 1994).

In the EPG set-up, a plant and a piercing insect are made part of an electrical circuit, by inserting an electrode in the soil of the plant and attaching a thin metal wire (2.5-25 μm diameter) to the insect (Figure 4). Generally, a thin gold wire is used (Tjallingii, 1988). The wire is attached to the dorsum or abdomen of the insect with an electrically conductive adhesive, like silver glue (Van Helden & Tjallingii, 2000). This thin wire is flexible, allowing some insect mobility (Walker, 2000). As soon as the insect's stylets penetrate the plant, the electrical circuit is completed and a fluctuating voltage, called the EPG signal, is amplified and recorded (Tjallingii, 1988; 1990). The fluctuating voltages in the EPG signal represent two signal components: voltages generated in the insect-plant interaction (electromotive force components, due to plant membrane potentials recorded during stylet punctures and streaming potentials) and fluctuations in electrical resistance of the insect itself, due to activity of valves in the food and salivary canals within the stylets (Pettersson et al., 2007; Tjallingii, 1985a; Walker, 2000).

The fluctuating voltages in the EPG signal occur in distinct patterns, so called waveforms, that correlate with different aphid activities and tissue locations of stylet tips (Kimmins & Tjallingii, 1985; Prado & Tjallingii, 1997; Tjallingii, 1978; 1985b; 1988; 2003; Tjallingii & Hogen Esch, 1993). The pathway phase is referred to as pattern C, and the intracellular punctures during the pathway are reflected in the signal as potential drops, sudden drops in electrical potential caused by the stylets puncturing the cell membrane (Tjallingii, 1985b, 2003; Walker, 2000). Waveform E1 occurs at the same voltage level as the potential drops and corresponds to the secretion of watery saliva in the sieve elements by a muscular pump (Prado & Tjallingii, 1994; Tjallingii, 1994). Waveform E2 reflects passive food uptake from a sieve element (Tjallingii, 1990; 1994), while waveform F corresponds with derailed stylets or penetration difficulties (Tjallingii, 1990). Tjallingii (1994) reported xylem ingestion as waveform G.

The EPG technique has been used in numerous studies on homopteran-plant interactions, like the transmission of plant viruses and identification and localisation of the tissues containing resistance factors (Tjallingii, 1995; Walker, 2000). However, important parameters of aphid-plant interactions, like aphid performance, i.e. growth and reproduction, and host plant acceptance, need to be studied on freely moving aphids since the EPG technique restricts aphid movement, the so-called 'tether effects'(Tjallingii, 1995; Van Helden & Tjallingii, 2000).

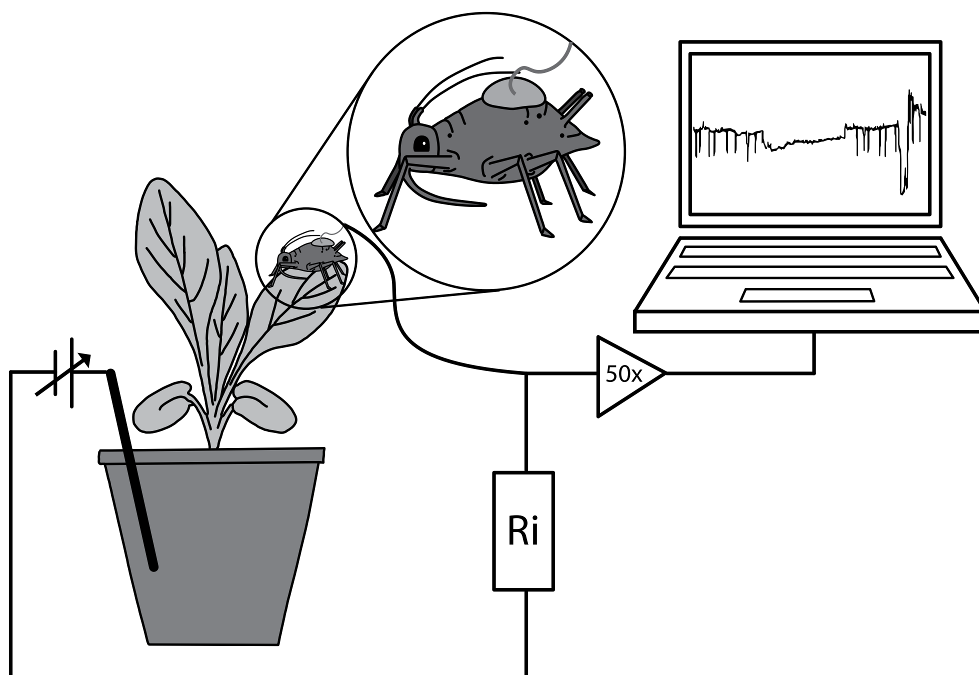
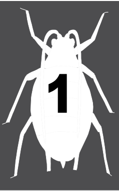


Figure 4: The EPG set up. One electrode is connected to the back of a piercing-sucking insect, and the other electrode is inserted in the soil of the test plant. When the aphid penetrates the plant cuticle, the electrical circuit is completed and the EPG signal is amplified and recorded real-time on a personal computer.

Protection of plants against aphids

Chemical control

When genetic plant resistance against aphids in not available in a crop species, the use of insecticides is an option to control aphid pests. The use of insecticides differs between crops, as does the efficacy, which varies between 20-90% (Tagu et al., 2008). Insecticides can be effective on a short term, but there are important economic and environmental costs associated with their use (Pimentel et al., 1992). The application of insecticides involves direct costs of the insecticide itself, its handling, application equipment, and labour (Dreyer & Campbell, 1987). Examples of indirect costs are negative impacts on human health, poisoning of domestic animals, mortality of natural enemies and pollinators, reducing crop pollination rates, and crop losses, groundwater and surface water contamination, mortality of fish, wildlife and microorganisms, and governmental expenditures to reduce the environmental and social costs of insecticide use (Pimentel et al., 1992). Additionally,



insecticides often lose their efficacy after a while due to resistance development in the pest species (Rabasse & Van Steenis, 1999). For example, the green peach aphid (*Myzus persicae*) has evolved resistance to more insecticides than any other insect (Anstead et al., 2005).

Biological control

The last decades the demand for biological control of pest insects has been increasing. Aphids have several natural enemies that can be used in controlling their numbers and infestation rates (Atkinson & Dennis, 1984; Powell & Pell, 2007; Reid & Cuthbert, 1977). Hymenopterous parasitoids (Aphidiinae and Aphelinidae) are widely used in the biological control of aphids, both in greenhouses and in the field and have achieved the most success as control agents (Müller et al., 1999; Powell & Pell, 2007). Predatory gall midges (*Aphidoletes aphidimyza*) (Rondani) are effective in the control of several aphid species in the field and greenhouses (Meadow et al., 1985; Powell & Pell, 2007; Růžička & Havelka, 1998). Both adult and larval ladybirds feed on aphids and are, therefore, considered to be valuable natural enemies of aphids (Obrycki & Kring, 1998; Powell & Pell, 2007). The larvae of hoverflies (Diptera: Syrphidae) and some lacewing species (Neuroptera: Chrysopidae) feed on aphids and have been reported to act as biological control agents (Smith & Chaney, 2007; Turquet et al., 2009; Völkl et al., 2007). Other potential biological control agents for the future are entomopathogenic fungi of which the majority is able to infect aphids directly, not requiring ingestion (Powell & Pell, 2007). Plant growth-promoting rhizobacteria can be used for biological control, because they are known to enhance the ability of a plant to defend itself against insects and pathogens by eliciting defensive responses, known as induced systemic resistance (Kloepper et al., 2004). However, plant growth-promoting rhizobacteria can also induce susceptibility to aphids as was shown by Pineda et al. (2012).

Cultural practice

Cultural practice of aphid pests is based on the management of the biological and physical environment of the crop plants during growth or establishment, to reduce the damage by aphids (Wratten et al., 2007). Ground cover is used to improved crop yield, by deterring alate aphids by highly reflecting materials, like aluminium foil and white plastic (Adlerz & Everett, 1968). Particle films sprayed on plants can also be used as reflecting material repelling aphids (Wratten et al., 2007). Straw mulches can increase predator populations, like spiders and ground beetles, and thereby decrease aphid densities (Schmidt et al., 2004). Row covers (webs or sheaths of spun-bonded polyester or polyethylene) which are designed to float on top of the crop plants can deter alate aphids and prevent aphids from inserting their stylets into crop plants (Wratten et al., 2007). Other methods that influence aphid colonisation and population densities are sowing, planting date, plant densities, pruning, irrigation, fertilizer application, intercropping (growing multiple crops in a field), living mulches (cover crops) and trap crops (Wratten et al., 2007).

Host plant resistance

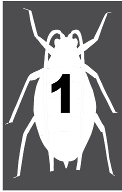
The use of host plant resistance is an effective strategy to control aphid pests. For many crop species, cultivars have been bred for resistance against aphids (Dreyer & Campbell, 1987; Tagu et al., 2008). Three mechanisms of host plant resistance against insects are distinguished: antixenosis, antibiosis and tolerance, that can occur singly or combined in a plant genotype (Casteel et al., 2006; Van Emden, 2007). Antixenosis is a form of resistance affecting behaviour and acts before or during settling, and affects the colonisation of aphids by repelling or deterring them (Goggin, 2007; Harrewijn, 1990; Van Emden, 2007). It determines the acceptability of a plant to serve as host for an aphid, influenced by both chemical and morphological plant traits inciting aphids to select an alternative host plant (Smith et al., 1994). Antibiosis-based resistance expresses itself after acceptance for feeding and affects the aphid's rate of population increase by, for instance, reducing development rate, survival and fecundity (Harrewijn, 1990; Smith et al., 1994; Van Emden, 2007). Both chemical and morphological features can mediate antibiosis, the effects on insects ranging from mild to lethal (Smith et al., 1994). Tolerance is the ability of a resistant host plant to grow and reproduce or to repair injury in spite of supporting a population of insects that would severely damage or kill susceptible plant hosts, without yield loss, and only involves plant characteristics (Smith et al., 1994).
Host plant resistance is often based on major resistance (*R*) genes and their homologues, and are mostly species-specific (Tjallingii, 2006). Unfortunately, the resistance mechanism against aphids is unknown for many crop species.

The *Nasonovia* – lettuce interaction

The *Nasonovia* – lettuce problem

The black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely), is an economically important pest of lettuce, *Lactuca sativa* L. Lettuce can get seriously damaged by high numbers of this aphid species, causing deformation of the head, changing leaf colour and reducing seedling vigour (McCreight, 2008; Stufkens & Teulon, 2003). *Nasonovia ribisnigri* is capable of transmitting viruses to lettuce, including Cucumber mosaic virus, Lettuce mosaic virus and Lettuce necrotic yellow virus (Blancard et al., 2005; Davis et al., 1997). Although small numbers of aphids have no effect on yield, the presence of living aphids is a cosmetic problem, making the lettuce unmarketable (Diaz & Fereres, 2005; Liu, 2004; McCreight, 2008).

Nasonovia ribisnigri is able to colonise lettuce in all developmental stages (Liu, 2004; Stufkens & Teulon, 2003). It starts colonizing the young leaves inside the lettuce head (Liu, 2004; McCreight, 2008). Due to an increasing population density and the resulting crowding, the aphid population distributes to the frame leaves of the lettuce plant (Liu, 2004). The preference for young heart leaves over older frame leaves by the aphids is



potentially caused by differences in nutritional quality and/or microclimate between leaves of different ages (Liu, 2004). Growing leaves often maintain phloem sap with higher levels of soluble nitrogen, making these leaves more suitable for the aphids (Dixon, 1998). Additionally, the microclimate within lettuce heads and under wrapped leaves may be more humid and stable, and less accessible for natural enemies, compared to the frame leaves (Liu, 2004). *Nasonovia ribisnigri* is rarely observed to be parasitised, possibly due to the inaccessibility of the aphids (Smith et al., 2004).

Biological characteristics of *Nasonovia ribisnigri*

Nasonovia ribisnigri shows host alternation by which oviparous sexual individuals move to the primary host, *Ribes* spp. (currants and gooseberries; Rosaceae), in autumn (Figure 1). To survive the cold winter period, the aphids lay eggs on their primary host plants (McDougall & Creek, 2007). The eggs are laid around the buds of the plant, and hatch in the spring. The new-born aphids multiply on the primary host for a few generations, causing leaf curling and decreased stem elongation. The aphids then fly to secondary hosts, mainly liguliforous Compositae (e.g. lettuce, chicory, hawksbeard), some Scrophulariaceae (e.g. speedwell) and Solanaceae (e.g. tobacco, petunia; Blackman & Eastop, 2000).

Nasonovia ribisnigri has both winged (alatae) and wingless (apterae) morphs, apterae measuring up to 2.7 mm in length and alatae 1.5 - 2.5 mm in length. The aphids are coloured red or yellow to green with brown spots on the dorsum of the adult. The alatae engage in dispersion (McDougall & Creek, 2007). Apterous aphids in general colonise new habitats by interplant walking, and alate aphids by either walking or flying, although, *N. ribisnigri* has limited flying abilities (Diaz et al., 2012; Nebreda et al., 2004).

Lettuce

Cultivated lettuce probably originated from the Mediterranean basin, possibly from Egypt (Davis et al., 1997). Nowadays, lettuce is commercially grown for consumption all around the world. The USA is the biggest commercial producer of lettuce, and lettuce is one of the most commonly consumed of all vegetables and fruits in America (Johnston et al., 2000). There are different types of lettuce, varying in popularity between different countries.

The most common types are crisphead, romaine, leaf/cutting and butterhead lettuce. Less common are stem and oil-seed lettuces (Davis et al., 1997). Lettuce leaves are commonly consumed in salads or sandwiches in the USA, Europe, and Australia. The stems of lettuce are consumed in Egypt (raw) and in China (cooked) (Ryder, 1999).

Overall, lettuce is an important vegetable source of vitamins and minerals in the human diet. It contains nitrate, phosphor, sodium, potassium, vitamins A and C, iron, calcium, water and fibre (Reinink, 1991; Ryder, 1999). The concentrations of these components vary between lettuce types (Ryder, 1999).

Significant limiting factors in commercial lettuce production are plant pathogens. Diseases in lettuce are caused by various organisms, that affect either the plant by damaging and/or killing, or reducing the quality of the lettuce (Ryder, 1999). Diseases can be caused by fungi, bacteria, nematodes, viruses and phytoplasmas. Vectors of lettuce diseases are aphids, leafhoppers and whiteflies. These vectors can also cause direct damage to the lettuce (Davis et al., 1997).

***Nasonovia ribisnigri* distribution**

Nasonovia ribisnigri originally occurred in Europe and has spread to Asia, the Middle East, and North and South America largely due to transportation by humans (Blackman & Eastop, 2000) (Figure 5). Since 1970, *N. ribisnigri* has been a major problem in lettuce production in Europe (Reinink & Dieleman, 1993). More than 90% of all aphids found in Europe on lettuce are *N. ribisnigri* (Nunnenmacher, 1998; Van der Arend & Schijndel, 1999). In 1981 the lettuce aphid arrived in Canada, and has since been the most damaging lettuce pest there (Forbes & Mackenzie, 1982). The lettuce aphid was further spread to the USA, where it caused major problems for lettuce growers when it appeared in the Salinas Valley, in 1998, and has since been found in all of the lettuce producing districts of Arizona and California (Palumbo, 1999; 2000). In March 2002 the aphid appeared in New Zealand, and spread throughout the whole country within a year (Stufkens & Teulon, 2003; Stufkens et al., 2002). It thereafter appeared in Tasmania in 2004, probably originating from the *N. ribisnigri* populations in New Zealand, since aphids from both countries shared identical genetic sequences for cytochrome b, cytochrome oxidase I and a microsatellite locus. It is hypothesised they arrived as adults by air currents and as eggs in plant material (Stufkens et al., 2004).

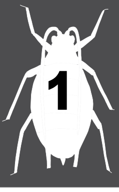


Figure 5: Distribution and occurrence of *Nasonovia ribisnigri* according to the database of www.plantwise.org. Black arrows: The aphids originated from Europe and have spread to other continents.

Protection of lettuce against *Nasonovia ribisnigri*

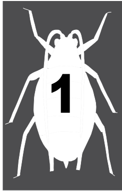
Chemical control

The use of contact insecticides to control *N. ribisnigri* is not effective, because *N. ribisnigri* infests, and prefers to feed on young, inner leaves in the 'heart' of lettuce plants that are not reached by insecticide spraying during maturation of the lettuce head (Liu, 2004). To control this aphid effectively, frequent application of pesticides is needed, due to the risk of a repeated secondary infestation by winged aphids (Dieleman & Eenink, 1980). Commonly used insecticides against *N. ribisnigri* are triazamate, endosulfan, imidacloprid, pymetrozine, thiamethoxam, acetamiprid and pirimicarb (Fagan et al., 2010; McDougall & Creek, 2007; Palumbo, 1999; 2000; Palumbo et al., 1999; Rufingier et al., 1997). Additionally, *N. ribisnigri* has developed resistance to several insecticides, first in Europe (Barber et al., 1999; Rufingier et al., 1997) and later in New Zealand (Kift et al., 2004; Stufkens & Wallace, 2004; Workman et al., 2004). Insecticides might negatively affect lettuce production. Haile et al. (2000) studied the impact of insecticides and surfactants on photosynthesis in lettuce. A reduction in photosynthesis due to applying insecticides and surfactants at seedling stage was documented. Application on older lettuce plants, however, did not affect photosynthesis.

Postharvest control of *N. ribisnigri* by controlled atmosphere can be used for export of lettuce to overseas markets, like Japan and Taiwan, where *N. ribisnigri* is a quarantine pest (Liu, 2003; 2012). In these counties the presence of living insects on lettuce leads to rejection or fumigation with methyl bromide or hydrogen cyanide, which damages lettuce and alternative, non-harmful post-harvest measures are desired (Liu, 2005). Additionally, methyl bromide has been banned in developed countries from 2005 onwards, and will be banned in developing countries from 2015 (Goodhue et al., 2005). Controlled atmosphere treatments often use elevated CO_2 to kill insects on crops, which damages lettuce (Stewart & Uota, 1976). Ultralow oxygen treatment (< 1% O_2) and vacuum treatment in controlled atmosphere chambers, were shown to be effective in control of *N. ribisnigri* and did not damage lettuce (Liu, 2003; 2005).

Behavioural disruption by semiochemicals

Plant volatiles are known to affect aphid colonisation (Tatchell, 2007). *Ribes nigrum* (the black currant), the primary (winter) host of *N. ribisnigri*, is attractive to the autumn morphs of *N. ribisnigri,* and repellent to the spring and summer morphs of this species, which are attracted to the secondary host *L. sativa*. From *R. nigrum* flowers a volatile has been isolated, (*Z*)-jasmone, the release of which is induced by plant damage, and has been shown to be repellent for the summer morph of *N. ribisnigri*, in the presence of lettuce (Birkett et al., 2000). This volatile could be used to prevent the colonisation of lettuce by summer morphs of *N. ribisnigri*. Zapata et al. (2010) studied the effectiveness of both polygodial and the n-hexane-extract from bark of *Drimys winteri* in interfering with the settling of *N. ribisnigri*.



The compounds negatively affected settling, probing and feeding behaviour.

Biological control

Larvae of syrphid flies are important for the biological control of *N. ribisnigri* in organically grown lettuce (*L. sativa)* on the Central Coast of California. Actually, the organic lettuce producers in this region almost exclusively rely on these larvae to remove aphids from the lettuce before harvesting (Smith & Chaney, 2007). Smith et al. (2008) illustrated that syrphid larvae are indeed primarily responsible for the suppression of aphid infestations in organic lettuce on the Central coast of California. Although the bio-control of *N. ribisnigri* by syrphids is largely successful, the farmers still lose 5% or more of their harvest due to infestation by this aphid (Smith et al., 2008). To enhance the number of syrphid flies, farmers intercrop lettuce with flowering plants, that provide floral resources for the syrphids to feed on (Bugg, 2008; Smith & Chaney, 2007). Thirteen species of syrphid larvae were found that were involved in the suppression of *N. ribisnigri* (Smith & Chaney, 2007). Hopper et al. (2011) tested the capacity of four syrphid species to control *N. ribisnigri*. The two larger species *Allograpta obliqua* (Say) and *Eupeodes fumipennis* (Thomson) had a greater control capacity as compared to *Sphaerophoria sulphuripes* (Thomson) and *Toxomerus marginatus* (Say). This could be taken into account when planting floral resources that attract adults of the two larger syrphid species. In New Zealand lacewings and spiders play a major role in suppressing aphids (Fagan et al., 2010). Fagan et al. (2010) showed that maintaining a predator to aphid ratio of 1 : 10 resulted in an 'aphid-free' crop at harvest without the use of insecticides. Another potential biological control agent is the parasitoid *Aphidius hieraciorum* (Tatchell, 2007). However, certain lettuce cultivars, like Romaine lettuce can enclose the aphids during its growth, protecting the aphids from natural enemies (Bugg, 2008). It is therefore important that the aphids are controlled early in the growth cycle of lettuce to prevent this. Unfortunately, the use of biological control agents has complications, due to 'zero tolerance' of consumers to the occurrence of arthropods on lettuce when marketed (McCreight, 2008; Tatchell, 2007).

The use of fungal entomopathogens might be applied for the biological control of *N. ribisnigri* as well. Fournier & Brodeur (2000) studied the effect of the hyphomycete *Verticillium lecanii*, and reported a reduction in lettuce aphid population densities. Aphids were still found on lettuce, implying that the application of the fungus alone is not sufficient. The same happened with the fungus *Pandora neoaphidis* that was studied as potential control agent against *N. ribisnigri* on lettuce in Argentina. Aphids were successfully infected, however, aphids killed by the fungus were tightly held to the leaf surface by the fungal rhidoids (Scorsetti et al., 2010). A strain of the fungus *Lecanicillium lecanii* (ICAL6) was proven to be a potentially effective control measure against *N. ribisnigri* (Diaz et al., 2009).
Knowledge about the genetic structure of *N. ribisnigri* populations, their mode of colonising plants, and interplant movement can contribute to developing an effective strategy to

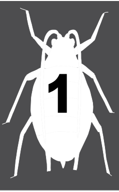
control this aphid pest (Diaz et al., 2012; Kim et al., 2008). Early detection of *N. ribisnigri* on lettuce is important for the effectiveness of the control measures taken (Diaz et al., 2007). However, monitoring methods such as water traps and yellow sticky traps are not effective enough to detect early infestation (Liu, 2004). By studying the influence of temperature on survival, fecundity and population growth, models to forecast aphid outbreaks could be constructed (Diaz & Fereres, 2005; Diaz et al., 2007). *Nasonovia ribisnigri* has a maximum population increase at temperatures between 20-24 ^{o}C, however, it can also reproduce at lower temperatures (Diaz & Fereres, 2005). Therefore, it is able to establish and develop its population early in the growing season (Diaz et al., 2007; Palumbo, 1999). Diaz et al. (2012) found that the population sizes on lettuce of *N. ribinisgri* in Spain were larger in the growing season in autumn compared to spring. However, the percentage of lettuce plants colonised was higher in spring compared to autumn, indicating dispersal is not dependent on the population size.

Host plant resistance in lettuce

The most desirable control measure against the lettuce aphid is considered to be genetic host plant resistance, for economic and environmental reasons (McCreight, 2008).

Complete and partial resistance to *N. ribisnigri* was found in *Lactuca virosa*, a distant wild relative of the cultivated lettuce. The near-complete resistance was transferred to *Lactuca sativa* by interspecific crosses (Dieleman & Eenink, 1980; Eenink et al., 1982). One dominant gene, the *Nr*-gene (*Nasonovia* resistance gene), seemed to be responsible for the near-complete resistance in the resistant *L. sativa*, although minor genes (*nr*-genes) also contributed some level of resistance (Eenink & Dieleman, 1983; Eenink et al., 1982; Reinink & Dieleman, 1989). The resistance to aphids is mostly very species-specific (Tjallingii, 2006). However, Reinink & Dieleman (1989) and Montllor & Tjallingii (1989) studied the effect of the *Nr*-gene on other aphids infesting lettuce, and partial resistance was observed against *M. persicae* but no resistance against the potato aphid *M. euphorbiae* was found (Reinink & Dieleman, 1989).

The tissue location of the *Nr*-mediated resistance was studied using the electrical penetration graph (EPG) technique, and both studies found a reduction in time spent on phloem ingestion (Montllor & Tjallingii, 1989; Van Helden & Tjallingii, 1993). This reduction in feeding indicates a resistance factor in the phloem, encountered during phloem sap ingestion from the sieve elements. No difference was observed in the duration of the first non-penetration period between susceptible and resistant plant, suggesting the absence of a resistance mechanism encountered by the aphid before penetration (Van Helden & Tjallingii, 1993).Van Helden & Tjallingii (1993) also studied developmental parameters of *N. ribisnigri* on susceptible and resistant lettuce plants. The performance of aphids on resistant plants was significantly lower than the performance on susceptible plants, resulting in higher mortality, lower mean relative growth rate, unsuccessful development from larvae



to adulthood and absence of reproduction. Similar results were found by Liu & McCreight (2006). The total absence of honeydew production and gain of weight after being transferred from a susceptible plant to a resistant plant, suggests that the aphids were not feeding from phloem or xylem sap (van Helden et al., 1993). A hypothetical toxic component in the food as resistance mechanism is unlikely, for there is no intake of food by the aphids. Additionally, aphid mortality on resistant plants is comparable to the mortality of aphids kept without food. Moreover, transfer of the aphids after two days on resistant plants to susceptible plants showed no sign of intoxication (Van Helden et al., 1993).

Since the phloem is suggested to be the tissue expressing the resistance mechanism, several attempts have been performed to analyse the phloem sap to find differences between susceptible and resistant lettuce. Different methods to collect phloem sap have been used, like stylectomy, honeydew collection and EDTA extracts, however, in these studies no clear difference was found between the phloem sap of susceptible and resistant lettuce (Van Helden, 1993; Van Helden et al., 1994a; b). Van Helden et al. (1995) performed artificial diet choice experiments, in which *N. ribisnigri* could choose between phloem sap collected from susceptible and resistant lettuce. In these experiments, *N. ribisnigri* preferred phloem sap of susceptible plants over resistant plants, suggesting that resistance is based on a feeding deterrent or a compound blocking activity of the phloem sap in the resistant plants.

Appearance of a virulent biotype

Van der Arend (2003) raised concern about the development of insensitive biotypes of *N. ribisinigri* for the resistance provided by the *Nr*-gene, if the *Nr*-gene would not be ‘protected’ by combining its use with other means of control. Unfortunately, since 2007 reports appeared of *N. ribisnigri* populations infesting resistant lettuce varieties in several parts of Europe, meaning that a new biotype had emerged that was insensitive to the resistance provided by the *Nr*-gene (Thabuis et al., 2011). This is a grim prospect in view of the absence of any other form of genetic resistance in lettuce to this aphid.

Objectives of this thesis

The *Nr*-gene provides resistance against *N. ribisnigri* in lettuce. Despite its importance in controlling *N. ribisnigri*, the gene has not yet been cloned, and the mechanism causing resistance at the molecular level is not known.

The objective of the TTI-GG **(**Technological Top Institute Green Genetics**)** *Nasonovia* project was to unravel the resistance mechanism based on the *Nr*-gene in lettuce by a combined metabolomics / proteomics / transcriptomics approach of phloem composition studies. Studies on host-plant selection behaviour and performance of the lettuce aphid were performed to investigate the resistance mechanism. Identification of the molecular basis of host-plant resistance would help plant breeders to accelerate their breeding

programmes.
A second objective was to screen for or develop novel sources of resistance against *N. ribisnigri,* including the *Nr*-insensitive virulent populations, based on understanding of the resistance mechanism.

Thesis Outline

Chapter 2 focusses on the performance and feeding behaviour of two biotypes of *N. ribisnigri*, i.e. an avirulent and virulent aphid biotype. Seven near-isogenic lines of *L. sativa* were used in this study, among which two susceptible and five resistant lines.

In Chapter 3, five populations of *N. ribisnigri* were studied, originating from different locations in Europe. The performance and feeding behaviour of one avirulent and four virulent aphid biotypes were studied on a susceptible and a resistant near-isogenic line and on a susceptible and a resistant cultivar of *L. sativa*.

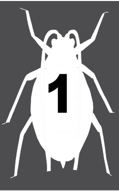
In Chapter 4 the original donor of the *Nr*-gene, *L. virosa,* was studied to check if alternative resistance against an avirulent biotype of *N. ribisnigri* was available. The performance and feeding behaviour of one avirulent and four virulent biotypes of *N. ribisnigri* was studied on the original *Nr*-donor and on two susceptible *L. virosa* accessions.

In Chapter 5 the effect of the presence on the same plant of other aphids on the behaviour of *N. ribisnigri* was studied. One avirulent and one virulent biotype of *N. ribisnigri* were studied combined with either the same biotype or the other biotype on susceptible and resistant lettuce. The effect of the presence of *M. persicae* and *M. euphorbiae* was also tested.

Chapter 6 discusses the effect of rearing plant on aphid behaviour. Two virulent populations of *N. ribisnigri* were reared on resistant and susceptible lettuce for two months to test for possible differences in behaviour and performance that might have occurred.

Chapter 7 focusses on the mobility of the *Nasonovia* resistance factor in lettuce. To study this, grafts between resistant and susceptible lettuce, cuttings, cut leaves and leaf discs were tested for their level of resistance against an avirulent population of *N. ribisnigri*.

In Chapter 8 the results are summarised and discussed, and suggestions for further research are given. In addition, preliminary transcriptomic data of the salivary glands and guts of an avirulent and virulent biotype of *N. ribisnigri* are presented.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Chapter 2

Performance and feeding behaviour of two biotypes of the black currant-lettuce aphid, *Nasonovia ribisnigri*, on resistant and susceptible *Lactuca sativa* near-isogenic lines

Cindy J.M. ten Broeke, Marcel Dicke & Joop J.A. van Loon

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Abstract

The black currant-lettuce aphid, *Nasonovia ribisnigri*, is an important pest of cultivated lettuce, *Lactuca sativa.* Since 1982, the control of this aphid on lettuce is largely based on host plant resistance, conferred by the *Nr*-gene, introgressed from *Lactuca virosa*. The resistance mechanism remains to be identified. *Nasonovia ribisnigri* populations virulent on the *Nr*-based resistance in lettuce have emerged in several locations in Europe since 2007.

The objective of this study was to investigate the resistance mechanism mediated by the *Nr*-gene in lettuce by detailed studies of aphid feeding behaviour and performance. Both a avirulent (Nr:0) and virulent (Nr:1) biotypes of *N. ribisnigri* were studied on five resistant and two susceptible near isogenic lines (NILs). Additionally, survival and colony development were quantified.

Nr:0 aphids showed a strong decrease in sieve element ingestion and took longer to accept a sieve element on resistant NILs compared to susceptible NILs, and no aphids survived on the resistant NIL. Nr:1 aphids fed and performed equally well on the resistant and susceptible NILs.

The resistance mechanism against Nr:0 aphids encoded by the *Nr*-gene seems to be located in the phloem, although we also observed differences in feeding behaviour during the pathway phase to the phloem. Nr:1 aphids were highly virulent to the resistance conferred by the *Nr*-gene. The consequences of the appearance of Nr:1 aphids for the control of *N. ribisnigri* are discussed.

Introduction

The black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely), is an economically important pest of cultivated lettuce, *Lactuca sativa* L. High densities of this aphid inflict serious damage to lettuce, causing deformation of the head, change in leaf colour and reduced vigour in seedlings (McCreight, 2008). Although low densities do not affect yield, the presence of living aphids is a cosmetic problem, making lettuce unmarketable (Liu, 2004; McCreight, 2008). Additionally, *N. ribisnigri* is capable of transmitting viruses to lettuce, including Cucumber mosaic virus and Lettuce mosaic virus (Davis, 1997).

Nasonovia ribisnigri prefers to feed on young, inner leaves in the heart of lettuce plants, making it difficult to control the aphids with contact insecticides (Liu, 2004). To control this aphid effectively with insecticides, frequent applications are needed, due to a repeated secondary infestation by winged aphids (Dieleman & Eenink, 1980). Moreover, resistance to several insecticides, including systemic ones, has been reported for this aphid species (Barber et al., 1999; Kift et al., 2004; Stufkens & Wallace, 2004). Furthermore, application of insecticides causes reduced photosynthesis (Haile et al., 2000).

The most effective control measure for *N. ribisnigri* is host plant resistance, for both economic and environmental reasons (McCreight, 2008). Near-complete and partial resistance against *N. ribisnigri* was found in *Lactuca virosa* L., a distant wild relative of cultivated lettuce, and transferred to *L. sativa* by interspecific crosses (Dieleman & Eenink, 1980). One dominant gene, the *Nr*-gene (*Nasonovia* resistance gene), appeared to be responsible for near-complete resistance in resistant *L. sativa* i.e., only few aphids survive on lettuce lines containing the *Nr*-gene during bio tests. Additionally, partial resistance is conferred by recessive *nr*-genes, located at the same locus as the dominant *Nr*-gene. Partial resistance reduces aphid population growth during bio tests (Reinink & Dieleman, 1989).

Increased insecticide resistance and crop damage by aphids have led to the development and cultivation of many aphid-resistant crop varieties. These resistances are often based on dominant resistance genes in the plant, so called *R*-genes, and are mostly specific to a certain aphid species (Dogimont et al., 2010). To identify the tissue(s) where resistance to *N. ribisnigri* is expressed to and investigate how the resistance affects the aphids, the behaviour of *N. ribisnigri* on both susceptible and resistant lettuce has been studied extensively (Mentink et al., 1984; Montllor & Tjallingii, 1989; Van Helden & Tjallingii, 1993; Van Helden et al., 1993; Van Helden et al., 1994b). These studies employed the electrical penetration graph (EPG) technique (Montllor & Tjallingii, 1989; Van Helden & Tjallingii, 1993). Both studies found that aphids spent less time on the ingestion of sieve-element contents on resistant lettuce compared to susceptible lettuce. This reduction in feeding indicates a resistance factor in the phloem, encountered during phloem sap ingestion from the sieve elements. Aphids also produced less honeydew on resistant compared to susceptible lettuce (Mentink et al., 1984), and nymphs were not able to survive on resistant lettuce (Van Helden et al., 1993).

The formation of virulent biotypes may occur when monogenic resistant plants are cultivated over large areas. Especially in agro-eco-systems, pests are exposed to strong human-imposed selective pressures. Virulent biotypes arise from genotypic variation in insects, expressed as differences in behavioural traits (Lombaert et al., 2009; Van der Arend, 2003). Van der Arend (2003) raised concern about the development of biotypes of *N. ribisnigri* that are virulent to the resistance conferred by the *Nr*-gene, if the *Nr*-gene would not be protected by combining its use with other means of control. Since 2007 reports have appeared of *N. ribisnigri* populations infesting resistant lettuce varieties in several locations in Europe, indicating that at least one new biotype (Nr:1) had emerged that is able to colonise resistant lettuce (Thabuis et al., 2011). This is a grim prospect, considering the absence of any other form of genetic resistance in cultivated lettuce to this aphid.

The aim of the present study is to locate the resistance to both biotypes of *N. ribisnigri* in other lettuce material than previously studied (Van Helden & Tjallingii, 1993; Van Helden et al., 1993) and obtain information about the possible resistance mechanism by behavioural studies on the aphids using the electrical penetration graph (EPG) technique and other behavioural experiments. This study also investigates a newly emerged *N. ribisnigri* biotype (Nr:1), that was able in the field to colonise resistant lettuce, to test the degree of virulence of this biotype. Seven lettuce lines that were near isogenic for the *Nr*-locus were tested against an avirulent (Nr:0) and possibly virulent biotype (Nr:1) of *N. ribisnigri*. To our knowledge this is the first study that uses EPG to describe the feeding behaviour of the Nr:1 biotype of *N. ribisnigri*.

Materials and Methods

Plants and Aphids

The plants used for the EPG recordings were seven near isogenic lines (NILs) of *L. sativa* (*L. sativa* cv Salinas x *L. sativa Nr*-resistant cross) that are listed in Table 1. The lettuce material studied differs from the material reported on by Van Helden & Tjallingii (1993). The susceptible parent into which the *Nr*-gene was introgressed in the present study was a different iceberg/crisp head cultivar.

Plants were grown in a greenhouse at Keygene N.V. (Wageningen, The Netherlands) at a temperature of 20°C during the day and 18°C during the night, 60% RH and L14/D10 photoperiod.

Nasonovia ribisnigri biotype Nr:0, originally collected in the Netherlands in 2001 (Dr.K. Posthuma, pers. comm.) was reared on Nr:0-susceptible *L. sativa* cultivar Fatima and Nr:1, originally collected in Germany in 2007, on Nr:0-resistant *L. sativa* cultivar Corbana (Enza Zaden), in a climate chamber at 23°C during the day and 19°C during the night, 60% RH and L14/D10 photoperiod. In the field, *N. ribisnigri* shows host alternation in which sexual individuals that produce overwintering eggs move to the primary host, *Ribes* spp. (currants

and gooseberries), in autumn (McDougall & Creek, 2007). Newborn aphids multiply on primary hosts for a few generations, and subsequently alatae migrate to secondary hosts, mainly liguliforous Compositae (e.g. lettuce)(Blackman & Eastop, 2000). In our laboratory colony aphids only reproduce asexually, and produce both winged (alatae) and wingless morphs (apterae). Winged adults mediate for the dispersal of the population. In the rearing they probably develop due to crowding, rather than due to unsuitability of the rearing plant.

Table 1: The near isogenic lines (NILs) used in this study, provided by Enza Zaden (Enkhuizen, The Netherlands). Susceptibility and resistance relate to aphid Nr:0 biotype. *Nr* refers to the resistance allele and *nr* refers to the susceptibility allele.

Abbreviation	Seed code	Generation	Zygosity	Susceptible/ resistant
S1	2006.01027	F3	*nr:nr*	susceptible
S2	2006.01041	F4	*nr:nr*	susceptible
R1	2006.01052	F3	*Nr:Nr*	resistant
R2	2007.60837	F4	*Nr:Nr*	resistant
R3	2007.60838	F4	*Nr:Nr*	resistant
R4	2007.60839	F4	*Nr:Nr*	resistant
R5	2006.01028	F3	*Nr:Nr*	resistant

Performance test

Performance of *N. ribisnigri* biotypes Nr:0 and Nr:1 was quantified by assessing nymph survival and development time from nymph to the adult stage on one susceptible NIL (S1) and one resistant NIL (R2), randomly selected. About 200 adult alatae of *N. ribisnigri* were kept in clip cages (10 per clip cage) on susceptible *L. sativa* cultivar Fatima, for 24 h. After 24 h, the adult aphids were removed and nymphs were transferred into clip cages on 3-week-old lettuce plants. One clip cage, containing five nymphs, was placed on a single plant. Mortality and development time were recorded daily until aphids reached the adult stage. Experiments were conducted in a greenhouse compartment at a temperature of 18-20°C, 60% RH and L14/D10 photoperiod. For every NIL x biotype combination, 20 plants were tested.

Population development

Population development of *N. ribisnigri* biotypes Nr:0 and Nr:1 was studied on one susceptible and one resistant NIL, i.e. S1 and R2. Five adults that had moulted within the preceding 24 h were transferred to 4-week-old lettuce plants. A gauze bag was placed over the plant to prevent the aphids from escaping. After 14 days the numbers of nymphs, alatae and apterous adults were counted. The experiment was conducted in the same

greenhouse compartment where the performance test was carried out, under the same environmental conditions. Six plants per NIL x biotype combination were tested.

EPG recording

Studying the behaviour of aphids feeding on resistant and susceptible crops can provide information about the possible tissue location and mechanism of resistance. The feeding behaviour of piercing-sucking insects cannot be observed directly (Walker, 2000). The electrical penetration graph technique (EPG) allows the electronic recording of the feeding behaviour of aphids and other piercing-sucking insects. EPG parameters, that correlate with different aphid activities and tissue locations of the stylet tips, can be used to identify and localise tissues containing resistance factors (Tjallingii, 1995), and this has been applied in several studies (Alvarez et al., 2006; Kaloshian et al., 2000; Van Helden & Tjallingii, 1993).

In an EPG set-up a plant and a piercing insect are made part of an electrical circuit, by inserting an electrode in the soil of the plant and attaching a thin gold wire to the insect (Tjallingii, 1985; 1988). For details on the EPG methods used, see Van Helden & Tjallingii (2000). Alate aphids of unknown age were collected from the rearing cage and attached to the electrode. Alate aphids were used because they are the dispersing morph and are, therefore, performing host-plant selection. Pompon & Pelletier (2012) showed that the age of aphids influences their behaviour in terms of EPG parameters on resistant and susceptible plants. The use of random ages of aphids should therefore give a clear view of the behaviour of adult aphids of all ages within a population, instead of selecting one age of aphids, not knowing how the other aphids might respond. Aphids were placed on the abaxial side of 3-week-old lettuce plants. The DC-EPG device (Giga-8, EPG-Systems, Wageningen, The Netherlands) was used to monitor probing behaviour of aphids during eight hours. All plants were watered before recording, because humid soil provides better electrical contact (Walker, 2000). Directly after wiring of the aphids recordings were started under constant laboratory conditions at 20 ± 2°C under continuous artificial illumination (HF fluorescent tubes, 1700 Lux at plant level). Signals of sixteen aphids, each on a separate plant, were simultaneously acquired during eight hours in two EPG setups and recorded on a PC hard disc. Data acquisition rate was 100 Hz and waveform analysis was done by PROBE 3.0 software (EPG Systems). Table 2 presents the waveforms which can be distinguished in an EPG recording of aphids. Tjallingii (1995) estimated a duration of 10 minutes of phloem ingestion to be sustained phloem ingestion. For every NIL 25 replicates (individual aphids on separate plants) were recorded. Failed recordings were excluded from the data set.

Table 2: The different waveforms distinguishable in an EPG recording (van Helden & Tjallingii, 1993) .

Waveform	Abbreviation
Stylet pathway	C
Cell penetration (potential drop)	pd
Non-penetration period	NP
Phloem salivation	E1
Phloem ingestion	E2
Phloem phase	E
Xylem ingestion	G
Penetration difficulties	F

Statistics

EPG parameters were calculated individually for every aphid using the EPG analysis worksheet created by Sarria et al. (2009). The Kruskal-Wallis test was used to test for overall differences among aphids on different resistant NILs. For the EPG parameters that differed significantly among the NILs, the Mann-Whitney U test was used for pairwise comparisons.

A Bonferroni correction was used to adjust α for multiple-comparisons.

The Mann-Whitney U test was applied to analyse differences in aphid parameters between NIL S1 and NIL R2. Fisher's exact test was performed to analyse differences in the percentage of aphids that showed a certain EPG parameter.

For the performance test every plant represented one block. Results were first calculated per plant, and means and standard error were calculated over all plants. The Mann-Whitney U test was used to test for significant differences in aphid mortality, time until mortality and development time. This test was also used to test for differences in the population development test for the total number of aphids, nymphs, alate adults and apterous adults.

All statistical tests were performed with IBM SPSS Statistics version 19.

Results

Performance data

Approximately 80% of the aphids of either biotype survived on NIL S1 (Table 3). On NIL R2, a significant difference in survival was observed between the two biotypes. None of the Nr:0 aphids survived, whereas 80% of Nr:1 aphids survived ($P < 0.0001$). Development time of Nr:0 aphids, from 24 h-old nymph to the adult stage, on the NIL S1 was seven days while Nr:1 developed in eight days on both NILs.

Population development

From the initial five adult aphids (all moulted within 24 h) placed on the plant, no Nr:0 aphids were found after 14 days on NIL R2, whereas on NIL S1 127 aphids were counted after 14 days (P = 0.002), of which most (113) were nymphs. Only 16 adults were found: nine alate and seven apterous individuals (Table 3).

For Nr:1 aphids an average of 220 and 149 aphids were found on NIL S1 and NIL R2 respectively. This difference was not significant (P = 0.20). Of the 220 aphids on NIL S1, 187 were nymphs and 33 adults, with 25 alatae and eight apterae. On NIL R2 135 nymphs and 14 adults were counted, of which eight were alatae and six apterae. Significantly more alatae were found on NIL S1 than on NIL R2 (P = 0.030).

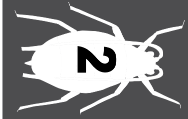


Table 3: Performance parameters (mean ± SEM) for both biotypes (Nr:0 and Nr:1) *N. ribisnigri* . The Mann-Whitney U test was used test to differences between resistant and susceptible lettuce, * = P < 0.05; ** = P < 0.005; *** = P < 0.001.

	Biotype Nr:0			**Biotype Nr:1**		
	NIL S1 (susceptible)	**NIL R2 (resistant)**		**NIL S1 (susceptible)**	**NIL R2 (resistant)**	
Survival (%)	81%	0%	***	77%	82%	
Days to death	2.63 ± 0.36	2.52 ± 0.19		3.26 ± 0.30	3.33 ± 0.33	
Development time (days)	7.30 ± 0.11			8.44 ± 0.18	8.77 ± 0.20	
Nr. aphids	129 ± 28	0	**	220 ± 49	149 ± 50	
Nr. nymphs	113 ± 25	0	**	186 ± 45	135 ± 47	
Nr. alatae	9 ± 2.77	0	**	24.67 ± 6.95	7.83 ± 1.66	*
Nr. apterae	6.83 ± 2.15	0	**	8.5 ± 0.96	6.5 ± 3.6	
Alatae/total	0.07 ± 0.01			0.13 ± 0.03	0.07 ± 0.01	
Alatae/aptarae	2.68 ± 1.06			3.56 ± 1.48	1.99 ± 0.75	

Table 4: EPG parameters (mean ± SEM) of N. ribisnigri biotype Nr:0 on susceptible and resistant NILs. EPG parameters are presented for which significant differences between lettuce lines were found based on the Kruskal-Wallis test for the resistant NILs the Mann-Whitney U test for the two susceptible NILs. All pair-wise combinations between the resistant NILs were tested with the Mann-Whitney U test. A Bonferroni correction was applied to account for the 10 comparisons made by setting α = 0.005. Means within a column having no letters in common are significantly different.

NILs	Time to first E from first probe (seconds)	Total duration of the phloem phase (seconds)	Number of sustained E2	Total duration of single E1 (seconds)	N*
susceptible					
S1	5141 ± 919 a	17538 ± 1529 a	2.05 ± 0.22 a	386 ± 98 a	20
S2	6048 ± 1377 a	15469 ± 2112 a	1.30 ± 0.21 b	491 ± 222 a	23
resistant					
R1	8816 ± 1698 ab	1859 ± 615 ab	0.28 ± 0.12 ab	669 ± 205 ab	25
R2	12792 ± 1891 ab	1182 ± 474 ab	0.18 ± 0.13 ab	562 ± 154 ab	17
R3	9269 ± 1900 ab	1119 ± 528 ab	0.13 ± 0.09 ab	436 ± 87 ab	16
R4	13569 ± 2193 a	629 ± 210 a	0.06 ± 0.06 a	254 ± 86 a	18
R5	6240 ± 1331 b	3947 ± 1313 b	0.80 ± 0.25 b	864 ± 158 b	23

N* = total number of replicates.

EPG analysis of biotype Nr:0 on different NILs

EPG recordings were performed on two susceptible and five resistant NILs. Comparisons were made between the two susceptible NILs and between the five resistant NILs. When comparing aphids on the susceptible NILs, only one significant difference was found. Aphids on NIL S1 displayed a larger number of sustained phloem ingestion events (≥10 minutes), compared to NIL S2 ($P = 0.022$) (Table 4). However, the total duration of phloem ingestion over eight hours did not differ (Figure 1A).

When comparing aphids on resistant NILs, there was a trend that aphids on NIL R5 had less problems accepting the phloem compared with aphids on NIL R4 (Table 4). Aphids on NIL R5 accepted the phloem quicker compared with aphids on resistant NIL R4 ($P = 0.005$). Total duration of phloem phase was significantly longer for aphids on NIL R5 compared with aphids on resistant NIL R4 ($P = 0.002$). Number of sustained phloem ingestion periods was higher for aphids on NIL R5 compared with aphids on resistant NIL R4 ($P = 0.003$). However, aphids on NIL R4 had a lower number of single phloem salivations (no ingestion before or after) compared with aphids on NIL R5 ($P = 0.004$). There were no significant differences in total duration of phloem ingestion between the different resistant NILs (Figure 1A).

EPG analysis of biotype Nr:1 on different NILs

EPG recordings were performed on two susceptible and five resistant NILs. Comparisons were made between the two susceptible NILs and between the five resistant NILs. When comparing aphids on the susceptible NILs, only one EPG parameter significantly differed (Table 5). The mean duration of penetration difficulties (F) was more than twice as long for Nr:1 aphids on susceptible NIL S2 ($P = 0.011$) compared with susceptible NIL S1.

When comparing the resistant NILs, aphids on NIL R5 showed a higher number of sustained phloem ingestions compared with aphids on NIL R4 ($P = 0.004$), however, the duration of single phloem salivation (without being followed by ingestion) was longer for aphids on NIL R5 compared with NIL R4 ($P < 0.0001$) (Table 5). The total duration of phloem ingestion (E2) did not significantly differ between the resistant lines (Figure 1B).

EPG analysis for biotype Nr:0 on NIL S1 and NIL R2

EPG parameters that significantly differed among aphids on the two susceptible NILs and among the aphids on the five resistant NILs were qualified as less discriminative for differences between susceptible and resistant plants. We randomly selected two NILs to test for differences between susceptible and resistant lettuce, susceptible NIL S1 and resistant NIL R2 (Table 6).

Time until the first stylet probe did not differ significantly between aphids on the resistant and susceptible NIL ($P = 0.522$). Differences between the two NILs were found in the pathway phase and in the phloem phase (Table 6). Time until first phloem acceptance was twice as long for aphids on NIL R2 compared with aphids on NIL S1 ($P = 0.016$).

Additionally, aphids feeding on the resistant NIL spent more time in pathway (P < 0.0001) and non-penetration (P = 0.012) than aphids on NIL S1. Total number of probes and number of probes before the first phloem event were twice as high for aphids on NIL R2 (P < 0.0001 and P = 0.003, respectively) compared to aphids on NIL S1. More cell penetrations were made by aphids feeding on NIL R2 (P = 0.012).

Although the aphids spent similar amount of time in phloem salivation on both NILs, aphids on NIL R2 did often not proceed to feeding (Table 6). The percentage of phloem salivation followed by ingestion was four times lower (P < 0.0001), and the contribution of phloem salivation to the phloem phase was much larger for aphids on NIL R2 compared with the NIL S1 (P < 0.0001). Additionally, number of phloem ingestions was six times as low (P < 0.0001) and less time was spent on phloem ingestion (P < 0.0001) by aphids on NIL R1 compared with aphids on NIL S1 (Figure 1A). The overall duration of the phloem phase was shorter on NIL R2 (P < 0.0001). No significant differences were found in xylem ingestion and penetration difficulties between aphids on both NILs.

EPG analysis for biotype Nr:1 on NIL S1 and NIL R2

No significant differences were found in EPG parameters for Nr:1 aphids feeding on NIL S1 and R2 (Table 6 and Figure 1B).

EPG analysis for biotype Nr:0 and biotype Nr:1

Minor differences appeared between the aphid biotypes feeding on NIL S1 (Table 6). Biotype Nr:0 had a higher number of phloem ingestions compared to biotype Nr:1 (P = 0.042). Biotype Nr:1 had more probes in total (P = 0.029), and more probes before the first phloem event compared to biotype Nr:0 (P = 0.038).

Nr:0 aphids were less successful in feeding on NIL R2 compared with Nr:1 aphids (Table 6 and Figure 1). It took longer for Nr:0 aphids to reach the first phloem ingestion (P = 0.021), less time was spent in phloem ingestion (P < 0.004), the number of phloem ingestions was lower (P < 0.0001) and the total duration of the phloem phase was shorter (P = 0.001). Phloem salivation of Nr:0 aphids contributed more to the phloem phase (P < 0.0001) and a smaller percentage was followed by ingestion (P = 0.003) compared with Nr:1 aphids.

Table 5: EPG parameters (mean ± SEM) of *N. ribisnigri* biotype Nr:1 on susceptible and resistant NILs. EPG parameters are presented for which significant differences between lettuce lines were found based on the Kruskal-Wallis test for the resistant NILs or the Mann-Whitney U test for the two susceptible NILs. All pair-wise comparisons between the resistant NILs were tested with the Mann-Whitney U test. A Bonferroni correction was applied to account for the 10 comparisons made by setting α = 0.005. Means within a column having no letters in common are significantly different.

NILs	Mean duration F (seconds)				Number of sustained E2				Total duration of single E1 (seconds)				N*
susceptible													
S1	1719	±	301	a	1.71	±	0.52	a	496	±	157	a	14
S2	4936	±	1540	b	1.80	±	0.24	a	153	±	25	a	15
resistant													
R1	3227	±	1183	a	1.13	±	0.34	ab	693	±	269	ab	15
R2	2058	±	553	a	1.64	±	0.29	ab	341	±	64	ab	14
R3	2520	±	384	a	1.21	±	0.24	ab	211	±	40	ab	14
R4	2826	±	1091	a	1.00	±	0.14	a	313	±	71	a	15
R5	1278	±	524	a	1.93	±	0.27	b	485	±	79	b	15

N* = total number of replicates.

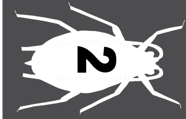
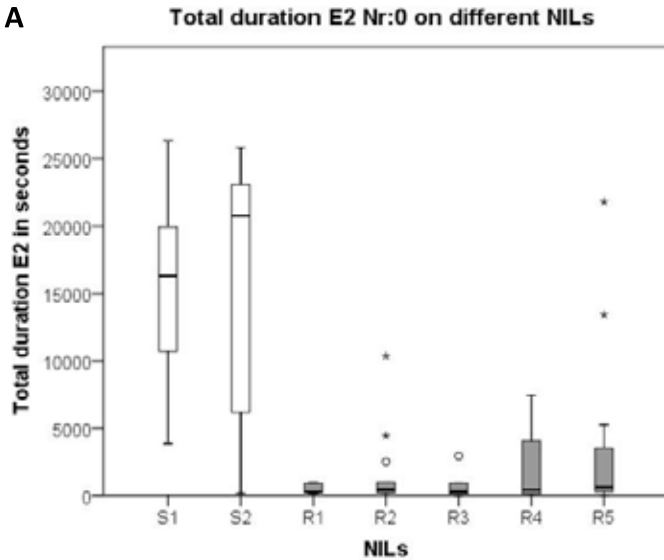


Table 6: EPG parameters (mean ± SEM) for both biotypes (Nr:0 and Nr:1) of *N. ribisnigri*. Time and duration values are in seconds. The Mann-Whitney U test was used to test for differences between susceptible and resistant lettuce, * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$. Significance of differences in percentage of aphids showing E2 was calculated with Fisher's exact test.

	Biotype Nr:0			Biotype Nr:1	
	NIL S1 (susceptible)	NIL R2 (resistant)		NIL S1 (susceptible)	NIL R2 (resistant)
Duration first NP	461 ± 168	613 ± 302		206 ± 45.91	316 ± 98.29
Duration of NP before the 1st E	1756 ± 394	2696 ± 824		2015 ± 343	2159 ± 439
Time to first visivle E#	5141 ± 919	12792 ± 1891	**	6807 ± 1005	8140 ± 1310
Time to first E2#	6674 ± 1311	16897 ± 3136	*	9352 ± 1760	7840 ± 1347
Time to first sustained E2#	7428 ± 1331	16648 ± 3126	*	9590 ± 1971	7890 ± 1024
Total duration E1	1580 ± 758	837 ± 255		1019 ± 241	686 ± 103
% E1 followed by E2	66.39 ± 6.58	15.13 ± 7.88	***	71.52 ± 8.93	56.18 ± 9.24
Total duration single E1	386 ± 98	562 ± 154		496 ± 157	341 ± 64
Contribution of E1 to E	9.68 ± 3.10	88.21 ± 5.58	***	13.86 ± 7.32	17.87 ± 8.14
Total duration E2	15949 ± 1547	827 ± 550	***	15570 ± 1904	11550 ± 1375
Nr. E2	3.20 ± 0.44	0.53 ± 0.26	***	2.14 ± 0.57	2.14 ± 0.33
Nr. sustained E2 (> 8 minutes)	2.05 ± 0.22	0.18 ± 0.13	***	1.71 ± 0.52	1.64 ± 0.29
% aphids showing E2	95	41.67	***	93	86
Total duration of E	17538 ± 1529	1182 ± 474	***	15391 ± 2105	11411 ± 1533
Nr. probes to the 1st E	8.05 ± 1.48	20.00 ± 3.41	**	12.15 ± 1.38	15.00 ± 3.93
Nr. probes after 1st E	5.68 ± 1.60	18.67 ± 4.45	**	10.14 ± 2.79	14.00 ± 2.90
Total duration NP	2881 ± 582	5511 ± 877	*	3510 ± 588	4415 ± 646
Total duration C	8721 ± 1201	19981 ± 1068	***	9735 ± 1593	11264 ± 1170
Total duration F	1728 ± 917	5598 ± 1887		3978 ± 1363	3122 ± 922
Total duration G	3735	1343 ± 698		13566	2091
Nr. probes	14.90 ± 2.79	33.71 ± 3.70	***	24.43 ± 3.30	29.64 ± 5.73
C/pd	63.83 ± 6.59	98.45 ± 14.96	*	86.34 ± 12.50	125.30 ± 52.23

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

A



B

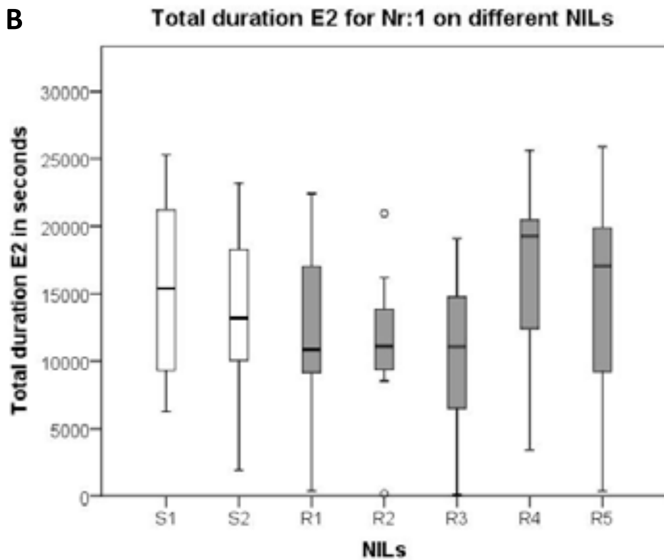


Figure 1: The total duration of E2 for different NIL x biotype combinations. A shows the total duration (median, first and third quartiles) of E2 for Nr:0 aphids on different NILs. No significant differences were found among the two susceptible NILs or among the five resistant NILs, however, susceptible and resistant NILs differed significantly from each other. B shows the total duration of E2 for Nr:1 aphids on different NILs. No significant differences were found among the two susceptible NILs or among the five resistant NILs and these two groups did not differ from each other either. The outliers are shown as circles or asterisks.

Discussion

Differences among NILs

The EPG parameters indicate that the two susceptible NILs are equally susceptible to both biotypes of *N. ribisnigri* during the eight hours of EPG recording, because both biotypes feed equally well on the two lines.

When feeding behaviour of Nr:0 aphids on resistant NILs is compared, the plants seem equally resistant, although for some EPG parameters aphids on NIL R5 differ from the other NILs, in particular NIL R4, indicating NIL R5 is less resistant.

Only minor significant differences appear in EPG parameters for Nr:1 aphids on the resistant NILs, between NIL R5 and NIL R4. The number of sustained phloem ingestions and the total duration of single phloem salivation patterns (not followed by phloem ingestion) is longer on NIL R5, compared to NIL R4. These differences were also found for the Nr:0 aphids feeding on these lines. Total duration of phloem ingestion does not differ among the resistant NILs, indicating that the aphids can feed equally well on the resistant NILs. However, we cannot rule out that a possible resistance against Nr:1 aphids works in a delayed manner, and is therefore not measured during the eight hour EPG recording (Sauge et al., 2012). To test this performance data needs to be collected for Nr:1 aphids on all NILs.

Differences between biotypes

Both biotypes behaved similarly on the susceptible NILs. The ability of Nr:1 and inability of Nr:0 to feed on the resistant lettuce lines, mainly displayed in duration of phloem ingestion, is the major difference in behaviour between these two biotypes. This can also be concluded from the performance and population development of both biotypes on NIL R2. Whereas Nr:1 aphids survive and reproduce on NIL R2, Nr:0 aphids suffer 100% mortality due to their inability to feed. Differences in behaviour between the two biotypes might have been caused by different rearing history, the Nr:0 aphids were reared on a susceptible cultivar, whereas the Nr:1 aphids were reared on a resistant cultivar.

Plant surface effects

Duration of the first non-penetration period in an EPG provides information on possible resistance factors encountered by aphids on the plant's surface (colour, volatiles, waxes etc.), although pre-treatment of aphids, like extensive handling the aphids before an EPG recording, can also influence duration of the first non-penetration period (Van Helden & Tjallingii, 1993). In wild tomato (*Solanum pennellii*), for example, glandular trichomes have shown to provide resistance against the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Goffreda et al., 1988). Resistance in *Pisum sativum* to certain clones of the pea aphid, *Acyrthosiphon pisum* (Harris) was concluded to be mediated by olfactory cues before the aphid penetrates the plant, because the capacity of aphids to gain access to, or to feed from

sieve elements was not altered (Wilkinson & Douglas, 1998).

In this study, however, there is no difference in duration of the first non-penetration period between aphids feeding on susceptible and resistant NILs, suggesting the absence of a resistance mechanism encountered by the aphid before penetration.

Pathway phase

Nr:0 aphids on resistant NIL R2 take twice as long before showing the first phloem event compared with Nr:0 aphids on the susceptible NIL S1. This can indicate that Nr:0 aphids on the resistant NIL encounter resistance factors on their way to the phloem, which was not found by Van Helden & Tjallingii (1993). A possible explanation can be that in this study genetically different lettuce plants have been investigated, compared to the plants used by Van Helden & Tjallingii (1993). The resistance factors found on the way to the phloem can be located in the epidermis or mesophyll, but also in the phloem itself. It takes an aphid about 15 minutes to penetrate a leaf from the epidermis to the phloem and during this pathway phase they penetrate many cells on the way before reaching the phloem. However, after reaching the phloem, salivation or ingestion of phloem sap does not occur directly. Transmission electron microscopy (TEM) combined with electrical penetration graphs (EPG) showed that a sieve element is not directly accepted by an aphid after reaching it with its stylets (Tjallingii, 1994). Montllor & Tjallingii (1989) also found indications for resistance factors that acted before accepting the phloem, in their EPG data for *N. ribisnigri* on a resistant lettuce. The number of probes and total time spent in pathway was higher were phloem ingestion was shorter when feeding on the resistant lettuce compared to a susceptible lettuce variety. An increase in total number of probes and number of probes before the first phloem event was also found in this study. Chen et al. (1997) also found that *Aphis gossypii* (Glover) feeding on resistant melon took longer to reach the first phloem salivation than on susceptible melon. Similar results were found by Crompton & Ode (2010) for the soybean aphid (*Aphis glycines* (Matsamura)) on resistant soybean (*Glycine max* (L) Merr.).

Phloem phase

Nr:0 aphids have difficulties accepting the phloem of the resistant NIL R2. The phloem phase consists mainly of phloem salivation. Although total time spent on phloem salivation does not differ between aphids feeding on the resistant NIL R2 and susceptible NIL S1, 42% of the aphids on the resistant NIL versus 95% of the aphids on susceptible NIL, show phloem ingestion, however, on the resistant NIL phloem ingestion lasted only for a short time. The number of phloem ingestion events was also low on the resistant NIL. These results indicate that the resistance mechanism encoded by the *Nr*-gene is probably located in the phloem, as has been found previously (Montllor & Tjallingii, 1989; Van Helden & Tjallingii, 1993). Additional evidence was found by Van Helden et al. (1995) by performing artificial diet choice experiments, in which *N. ribisnigri* could choose between phloem sap from susceptible and

resistant lettuce. In these experiments, *N. ribisnigri* preferred phloem sap of susceptible plants over phloem sap of resistant plants, suggesting that resistance could be based on feeding deterrent activity of the phloem sap of resistant plants.

The Nr:0 aphids did not feed on the phloem of the resistant NIL. They spent more time on other activities like non-penetration and pathway, with a higher number of cell penetrations, as was found also by Montllor & Tjallingii (1989) and Van Helden & Tjallingii (1993) for non-virulent *N. ribisnigri* on resistant lettuce. In the EPG setup the aphids were restricted to a certain plant for eight hours. Although the resistant plant is not suitable to feed on, the aphid will try to feed and find a suitable sieve element, to prevent starvation. This can explain why ca. 40% of the Nr:0 aphids do show phloem ingestion, but only for short durations. This can lead to an underestimation of the resistance (Tjallingii, 1986).

Several *R*-gene-related resistances in plants against aphids have been proven to be phloem-based. The mechanism of the *Vat*-gene in melon (*Cucumis melo* L.) that provides resistance to the melon aphid (*A. gossypii*), is probably also located in the phloem. EPG data showed that resistance caused reduced duration of phloem ingestion on resistant plants, although the frequency of initiation of feeding was not altered (Chen et al., 1997; Klingler et al., 1998). Reduced duration of phloem ingestion was also found by Caillaud et al. (1995) on resistant lines of *Triticum monococcum* L. (Tm44 and Tm46) for the aphid *Sitobion avenae* F. Similar results were found when the potato aphid was tested on resistant potato expressing the *Mi*-1.2 gene (Kaloshian et al., 2000). Phloem-based resistance was also found for several other aphid-plant interactions, like in cowpea (*Vigna unguiculata* L. Walp.) against the cowpea aphid (*A. craccivora*) (Annan et al., 2000), lettuce (*L. sativa*) against the lettuce root aphid (*Pemphigus bursarius* L.) and soybean (*Glycine max*) against the soybean aphid (*A. glycines*)(Crompton & Ode, 2010). In a recent study QTLs for resistance against *M. persicae* in wild peach were linked to EPG parameters, including the reduction of phloem intake in resistant peach (Sauge et al., 2012). This is a promising new step in studying host plant resistance against aphids, making it possible to further analyse the underling resistance mechanisms.

Resistance mechanism

Nr:0 aphids insert their stylets into the sieve element of the resistant NIL, and did show phloem salivation, but did not ingest contents from the sieve elements. This finding might suggest that the stylet canal is blocked, thereby disabling the aphid to feed on the plant. Van Helden et al. (1994b) and Mentink et al. (1984) also found that Nr:0 reached the phloem on resistant lettuce, because after cutting the stylets of the aphids that were feeding on resistant plants, the cut stylets exude sap.

Mechanical blocking of stylets in the sieve element was suggested as the resistance mechanism in wild *Brassica* species to the cabbage aphid (*Brevicoryne brassicae* L.)(Cole, 1994). Caillaud & Niemeyer (1996) proposed a blocking mechanism, the phloem sealing

system, to be responsible for the rejection of resistant lines of *T. monococcum* (Tm44 and Tm46) by the aphid *S. avenae,* because amputated stylets on the resistant plants did not or only shortly exude phloem sap. This short exudation of phloem sap out of stylets of aphids was also found by van Helden et al. (1994b) for *N. ribisnigri* on resistant lettuce.

In the performance tests, Nr:0 nymphs were not able to survive on the resistant NIL, and no aphids were found back on the plants after two weeks in the colony development test. Van Helden & Tjallingii (1993) also found a reduced performance of *N. ribisnigri* on resistant lettuce, compared with susceptible lettuce, observed as higher mortality, lower mean relative growth rate, unsuccessful development from larvae to adulthood and absence of reproduction. Similar results for *N. ribisnigri* on resistant lettuce were found by Liu & McCreight (2006). Kaloshian et al. (1997) reported a reduced longevity and fecundity of potato aphids tested on resistant potato.

Nr:0 nymphs survived for only three days on the resistant NIL, likely due to starvation, because EPG results showed that Nr:0 aphids ingested far less phloem sap on the resistant NIL compared with the susceptible NIL, and about 60% the aphids did not show phloem ingestion during the eight hour recording period. Total absence of honeydew production and gain of weight after being transferred from a susceptible plant to a resistant plants was found by Van Helden et al. (1993), suggesting there was neither ingestion of phloem nor xylem sap by *N. ribisnigri* on resistant lettuce. They also suggested that a hypothetical toxic component in the food as resistance mechanism is probably absent, for there was almost no intake of food by the aphids. Additionally, mortality of the aphids on resistant plants was comparable to mortality of aphids in a Petri dish without food, moreover, transfer of aphids after two days on resistant plants to susceptible plants showed no sign of intoxication (Van Helden et al., 1993). Therefore the resistance component seems to be an inhibiting factor blocking the feeding of the aphids on resistant lettuce.

Virulent biotype Nr:1

The resistance mechanism in the resistant NIL that acts against Nr:0 is ineffective against biotype Nr:1, because Nr:1 aphids feed and survive equally well on both the resistant NIL and the susceptible NIL, and suggest that this aphid is highly virulent against the resistance conferred by the *Nr*-gene. No differences were observed for EPG parameters between Nr:1 aphids feeding on the resistant and susceptible NIL. In contrast with Nr:0 aphids, Nr:1 aphids were able to ingest phloem sap from the resistant NIL and to survive on this plant. Also the duration from the first probe until the first acceptance of a sieve element did not differ between Nr:1 aphids on resistant and susceptible NILs. More alate aphids were produced on NIL S1 during the population development test, compared to NIL R1. This could be an effect of crowding, as there tended to be more aphids on NIL S1, although the difference was not significant. NIL S1 could also be less preferred by the Nr:1 aphids, and therefore alatae may have been produced to move to other plants. This might also explain the high

number of failed EPG recordings we observed for the Nr:1-NIL S1 combination (11 of 25 failed). There might be a negative trade-off for the Nr:1 aphids in performance on resistant versus susceptible lettuce lines.

The occurrence of virulent biotypes is a growing problem in several aphid-plant interactions. For example, *Vat*-gene virulent populations of *Aphis gossypii* (Glover) have been reported (Lombaert et al., 2009). For wheat several biotypes of *D. noxia* have emerged that are virulent to several of the resistance sources and others to all (Tolmay et al., 2007). *Aphis glycines* (Matsumura) biotypes have emerged, that are able to colonise resistant soybean plants (Kim et al., 2008). *M. euphorbieae* aphid biotypes differ in virulence on resistant tomato (Pallipparambil et al., 2010).

Genetic basis of plant resistance and aphid virulence

In agro-ecosystems, aphids are exposed to strong human-imposed selection pressures and might therefore evolve virulence to host plant resistance (Mitchell et al., 2009). However, the mechanisms behind this phenomenon remain unknown. The interaction between aphids and their host plants are often hypothesised to function in a gene-for-gene manner. According to Flor (1955) in the gene-for-gene model a single dominant resistance gene (*R*-gene) in the plant codes for a defence response that is triggered by the product of a single avirulence (*Avr*) gene in the pest species. Jones & Dangl (2006) proposed the zig-zag model, in which a plant responds to an attacker, through a two-branched immune system in the plant, in which the first branch responds to commonly occurring molecules associated with pathogens or insects, and in the second branch the plant reacts to an effector specific for the attacker, via either direct or indirect recognition. The ability of the newly emerged biotypes to overcome *R*-gene resistances, might be attributed to evading recognition by the plant and/or suppressing these plant defences, by loss or modification of avirulence gene products (Hogenhout & Bos, 2011; Parker & Gilbert, 2004). Another model to describe the interaction between aphids and their host plants is Ehrlich and Raven's (1964) model of chemical coevolution. In this model plants accumulate defence compounds that negatively affect the herbivores, and herbivores might evolve behavioural or biochemical strategies to avoid these plant compounds. Sauge et al. (2011) studied whether the tendency of *M. persicae* aphids to leave *RM2*-resistant peach (induced resistance), matches one of the above-mentioned models involved in *R*-gene-mediated resistance. The induced resistance matched the gene-for-gene model, because the level of resistance was independent of the aphid density and time since inoculation used to induce the plants. However, there was a significant quantitative variation for avirulent aphids in the tendency to leave the plant, indicating virulence in these aphids may also match the chemical coevolution model. *R*-genes involved in plant–aphid interactions may function in a combination of the gene-for-gene and chemical coevolution model. In a study by Thomas et al. (2012) the relation between the genotype and the phenotype of *A. gossypii* on 33 melon genotypes was studied

by microsatellite markers and testing plant acceptance, colonizing ability and resistance to virus inoculation on several melon accessions. Plant acceptance and resistance to virus inoculation were dependent on the genotype of the aphids, matching the gene-for-gene model. However, for the ability to colonise a plant, phenotypic variability was found for aphids of the same genotype, suggesting polygenic control of this trait.

In the interaction of *Meloidogyne javanica* with the *Mi*-gene in tomato, Tzortzakakis et al. (1998) found a dosage effect of the *Mi*-gene. More eggs were produced by nematodes on heterozygous compared to homozygous plants. This dosage effect was also studied by Jacquet et al. (2005) for several lines of *Meloidogyne incognita* infecting a number of *L. esculentum* genotypes. Here also the reproduction of the nematodes was often significantly higher on heterozygous than on homozygous tomato genotypes.

Both the *Vat*-gene in melon and the *Mi-1.2* gene in tomato belong to the NBS-LRR family of *R*-genes (Milligan et al., 1998; Pauquet et al., 2004). Proteins of NBS-LRR genes are involved in the recognition of pathogens by the plant, and might also be involved in the recognition of aphids by plants (McHale et al., 2006). Although the *Mi-1.2* gene and *Vat*-gene are both NBS-LRR genes, they differ in species specificity. The *Vat*-gene was shown to be species-specific as it was effective against *A. gossypii*, but not against *B. tabaci* biotype B (Boissot et al., 2010). In contrast, the *Mi-1.2*-gene confers resistance against multiple unrelated attacker species: *Macrosiphum euphorbiae*, several nematode species, whiteflies and psyllids (Casteel et al., 2006). An important difference between the aphid species discussed above and *N. ribisnigri*, is that the former are generalists whereas *N. ribisnigri* is a specialist.

Methodological limitations

Although EPG is a very suitable technique to study the penetration behaviour of piercing-sucking insect herbivores, it has some limitations. A major disadvantage of the EPG technique is that the penetration behaviour cannot be recorded from freely moving aphids. Although the gold wire allows some movement, the insects are still fixed to a limited part of the plant they feed on (Tjallingii, 1986). Side effects may occur and can be described as 'tether-effects'. Tjallingii (1986) studied the tethering effect for the aphid species *B. brassicae* and *A. pisum*. Smaller differences in terms of longevity, fecundity, settling ratio, penetration time, and the number of penetrations were found than were normally found between aphids on host and non-host plants. Minor tether-effects were found in several studies (Caillaud et al., 1995; Montllor & Tjallingii, 1989; Tjallingii, 1986; Van Helden & Tjallingii, 1993), which suggests that the extent of the tether-effects differ among species. Therefore, additional experiments with free-moving aphids are necessary, to study the influence of the EPG recordings technique on differences between host and non-host plants, or between resistant and susceptible cultivars that can differ pet plant-aphids species combination (Van Helden & Tjallingii, 2000).

Conclusion

Our studies indicate that the resistance mechanism of the *Nr*-gene against *N. ribisnigri,* is mainly located in the phloem. However, we also found that aphids encounter difficulties on resistant lettuce already during the pathway phase. This was not found in previous research and is probably caused by the use of different lettuce and aphid material. A hypothetical stylet blocking mechanism could be responsible for the inability to feed on resistant lettuce. The newly emerged Nr:1 biotype of *N. ribisnigri* is strongly virulent to the resistance mechanism acting on Nr:0 aphids, conferred by the *Nr*-gene. Nr:1 aphids feed and perform the same on both susceptible and resistant lettuce. This aphid biotype is a threat for the lettuce growing industry since currently there is no other source of genetic resistance available to this aphid biotype.

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2

Chapter 3

Feeding behaviour and performance of different populations of the black currant-lettuce aphid, *Nasonovia ribisnigri*, on resistant and susceptible lettuce

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Abstract

When crops are bred for resistance to herbivores, these herbivores are under strong selection pressure to overcome this resistance, which may result in the emergence of virulent biotypes. This is a growing problem for several crop species attacked by aphids. The *Nr*-gene in lettuce confers near-complete resistance against the black currant-lettuce aphid*, Nasonovia ribisnigri* (Mosely)(Homoptera: Aphididae). Since 2007 populations of *N. ribisnigri* have been reported in several locations in Europe to infest resistant lettuce varieties that possess the *Nr*-gene.

The objective of this study was to analyse the behaviour and level of virulence of several *N. ribisnigri* populations observed to have colonised *Nr*-locus-containing lettuce lines. We analysed the stylet penetration and feeding behaviour and the performance of these *N. ribisnigri* populations on resistant and susceptible lettuce lines.

Large variation in the degree of virulence to the *Nr*-locus-containing lettuce lines was found between different populations of the Nr:1 biotype. The German population was highly virulent on the *Nr*-containing resistant lettuce lines, and showed similar feeding behaviour and performance on both the susceptible and resistant lettuces. The French population from Paris was the second most virulent, though reproduction on the resistant lines was reduced. The French population from Perpignan and a population from Belgium, however, showed a reduced performance and feeding rate on the resistant lettuces compared to the susceptible lettuces.

The lettuce background in which the *Nr*-gene is expressed, influences the level of resistance to the different Nr:1 aphid populations, because the performance and feeding behaviour differed between the aphids on the cultivars (romaine lettuce) compared to the near isogenic lines (butterhead/iceberg lettuce). This study also shows that being able to feed on a plant, does not automatically imply that a population can successfully develop on that plant, because aphids showed phloem ingestion during the eight hour recording period on resistant lettuce, but were not able to survive and reproduce on the same lettuce line.

Introduction

One of the most economically important pests of lettuce is the black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely), which can cause serious damage in lettuce by deforming the head, changing leaf colour, reducing seedling vigour (McCreight, 2008; Stufkens & Teulon, 2003) and virus transmission (Davis, 1997). In Switzerland, yield losses up to 70% of lettuce were caused by aphids, of which *N. ribisnigri* is the most common (Kesper, 2002; Sauer-Kesper, 2011). To control this aphid, host-plant resistance is the most desirable control measure (McCreight, 2008). In lettuce the *Nr*-gene transferred from *Lactuca virosa* L. (a distant wild relative of cultivated lettuce) to *Lactuca sativa* L. by interspecific crosses, provides near-complete resistance against *N. ribisnigri* (Eenink & Dieleman, 1983; Eenink et al., 1982; Reinink & Dieleman, 1989). Despite its importance in providing resistance to *N. ribisnigri*, this gene has not yet been cloned, and the molecular mechanism causing resistance is still unknown. Previous studies located the resistance in the phloem (Van Helden & Tjallingii, 1993). *Nr*-based resistance in different lettuce genotypes resulted in differences in stylet penetration behaviour during pathway phase as well as in phloem ingestion between aphids on susceptible and resistant lettuce (Chapter 2). Van der Arend (2003) predicted the emergence of virulent biotypes of *N. ribisinigri* for the resistance provided by the *Nr*-gene, because alternative crop protection measures, like use of chemicals and crop rotation, that could serve to prolong the durability of the effectiveness of the *Nr*-gene, were abandoned. Since 2007, populations of *N. ribisnigri* have indeed been reported infesting resistant lettuce varieties in several locations in Europe. These populations were identified as biotype Nr:1 by The Netherlands Inspection Service (Naktuinbouw) and characterised by their ability to multiply on resistant lettuce varieties. The populations of aphids that the *Nr*-resistance is still effective against are referred to as biotype Nr:0. Currently, no alternative genetic resistance in lettuce to this aphid is available. Also in other aphid-plant interactions virulent aphid biotypes have emerged since the introduction of resistance genes. Examples are the *Macrosiphum euphorbiae* (Thomas) - tomato interaction (*Mi*-gene) (Cooper et al., 2004; Goggin et al., 2001; Pallipparambil et al., 2010), *Diuraphis noxia* (Mordvilko) - wheat interaction (*Dn4*-gene) (Haley et al., 2004), *Aphis gossypii* (Glover) - melon interaction (*Vat*-gene) (Lombaert et al., 2009), and *Aphis glycines* (Matsumura) - soybean interaction (*Rag1*-gene)(Kim et al., 2008).

Virulent biotype formation occurs in specialist plant-feeding insects that are under strong selection pressure, and enables insects to extend their host range and occupy new and/or empty niches (Dreyer & Campbell, 1987; Van der Arend, 2003). Especially in agro-ecosystems, aphids are exposed to strong human-imposed selection pressures, on the one hand by the application of insecticides, on the other hand by large scale use of resistant cultivars (Lombaert et al., 2009). The number of genes involved in plant resistance influences the durability of resistant cultivars. The use of cultivars with a single resistance gene, can cause development of virulent biotypes of aphids (Smith, 1989; Van der Arend, 2003).

Plant resistance mechanisms against insects have been categorised in: antixenosis, antibiosis and tolerance, which occur singly or in combination (Casteel et al., 2006; Van Emden, 2007). Antibiosis-based resistance expresses itself after a prolonged stay of a feeding insect on a resistant plant, affecting the life-history traits, reducing growth, survival and fecundity mediated by both chemical and morphological features of the plant (Lombaert et al., 2009; Smith et al., 1994; Van Emden, 2007). Antixenosis affects insect settling behaviour, through for example, repellence or deterrence (Goggin, 2007; Lombaert et al., 2009; Van Emden, 2007). Tolerance is a plant trait involving reduced damage to the plant upon insect feeding (Van Emden, 2007). Resistance through antibiosis imposes strong selection pressure on herbivores leading to biotype development, whereas antixenosis exerts less selection pressure, because antibiosis affects the life-history traits of insects, while antixenosis affects only the settling behaviour (Van der Arend, 2003). For example, antibiosis was more frequently overcome than antixenosis by *A. gossypii* in response to the *Vat*-gene in melon, although antibiosis and antixenosis might in some cases share a common mechanism (Lombaert et al., 2009). Tolerance is also predicted to put less selection pressure on pest insects, compared to antibiosis (Porter et al., 1997).

Several techniques are available to study the behaviour of aphids on both resistant and susceptible plants. To study the stylet penetration and feeding behaviour of aphids the electrical penetration graph technique (EPG) has proven to be very useful, because it is impossible to observe stylet penetration and feeding directly (Tjallingii, 1995). Performance studies, like survival and reproduction experiments, on free-moving aphids can provide additional data on the behaviour of aphids on resistant and susceptible plants.

The objective of this study was to get a better understanding of the behaviour and level of virulence of four *N. ribisnigri* populations observed to have colonised Nr:0-resistant lettuce in different geographical locations in Europe (Nr:1 biotype), where resistant lettuces are almost exclusively grown. We, therefore, studied the stylet penetration and feeding behaviour of the aphid populations on resistant and susceptible lettuces, using the electrical penetration graph technique. Additionally, the performance of the aphid populations on resistant and susceptible lettuces has been analysed in terms of survival and reproduction. As a control, one *N. ribisnigri* population known to be unable to colonise *Nr*-containing lettuce (Nr:0 biotype) was used.

Materials and Methods

Plants

The plants used in the experiments were two *L. sativa* cultivars, Corbana (Nr:0 resistant) and Pinokkio (Nr:0 susceptible), and two *L. sativa* lines derived from a mapping population and which are near isogenic for the *Nr*-gene (NILs), NIL S (2006.01027, F3, homozygous, Nr:0 susceptible) and NIL R (2007.60837, F4, homozygous, Nr:0 resistant). The cultivars

were included in the experiments because they are considered to be more isogenic for the *Nr*-gene than the two NILs, and because they differ in genetic background from the NILs (Romaine vs Butterhead/Iceberg, respectively). Plants were grown in a greenhouse at 18-20°C, 60% RH and L14/D10 photoperiod.

Aphids

Five different populations of *N. ribisnigri* were used in the experiments (Table 1). After collection from the field in five European locations, the populations had been maintained on different cultivars (Table 1). Upon receipt in our laboratory, all five *N. ribisnigri* populations were subsequently reared on *L. sativa* cultivar Fatima (Nr:0 susceptible) to standardise the rearing plant, five weeks prior to the experiments. The five aphid colonies (Table 1) were reared in a greenhouse, in separate rearing cages, at 18-20°C, 60% RH and L14/D10 photoperiod. The Nr:0 population was reared in a separate greenhouse compartment from the Nr:1 populations, to prevent mixing of Nr:0 and Nr:1 aphids. Plants were treated with ENTONEM (a formulation of the entomopathogenic nematode, *Steinernema feltiae* from Koppert (www.koppert.com) once a week, to control the larvae of sciarid flies.

Table 1: Populations of *N. ribisnigri* studied and their origin. Nr:0 aphids cannot colonise resistant lettuces. Nr:1 aphids are able to colonise Nr:0-resistant lettuce plants in the field.

Aphid biotype	Origin of colony	Year of collection in the field	Lettuce cultivar on which the biotype had been maintained	Number of founder individuals
Nr:0	The Netherlands	2001	Fatima (Nr:0 susceptible)	1 individual
Nr:1	Germany (Ge)	2007	Dynamite (Nr:0 resistant)	5-10 individuals
Nr:1	Belgium (Be)	2007	Dynamite (Nr:0 resistant)	5-10 individuals
Nr:1	France (Perpignan) (Pe)	2007	Quenty (Nr:0 resistant)	5-10 individuals
Nr:1	France (Paris) (Pa)	2007	Quenty (Nr:0 resistant)	5-10 individuals

EPG recording

To study the behaviour of *N. ribisnigri* feeding on resistant and susceptible lettuce we used the electrical penetration graph technique (EPG), which allows the electrical recording of the feeding behaviour of aphids and other piercing-sucking insects. In an EPG set-up, a plant and a piercing insect are components of an electrical circuit, by inserting an electrode in the soil of the plant and attaching a thin gold wire to the insect's dorsum. For details on the EPG methods used see (Chapter 2). We distinguished seven EPG patterns; stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F). Recordings were made during eight hours from winged adult aphids (alatae) of unknown age. Alatae of unknown age were used

to get a broad view on the behaviour of alatae in general within a population. For every aphid population x lettuce line combination between 16 and 21 replicates (individual aphids on separate plants) were recorded. Every aphid and plant individual was only used for one recording.

Performance test

Performance of the five *N. ribisnigri* populations on the two NILs and the two cultivars was measured in terms of nymphal survival and development time from nymph to adult. About 200 alatae of *N. ribisnigri* of every population were kept for 24 h in Petri dishes on leaves from the susceptible *L. sativa* cultivar Fatima leaves. The next day the newly produced nymphs were collected. Five nymphs were contained in one clip cage that was then placed on a 3-week-old lettuce plant. The number of aphids alive and their developmental stage were recorded daily, until the aphids had reached the adult stage. Experiments were conducted in a greenhouse at a temperature of 18-20°C, 60% RH and L14/D10 photoperiod. For every aphid population x lettuce line combination, 20 replicates (*i.e.* 5 x 20 individual aphids in 20 clipcages) were performed.

Reproduction test

Reproductive success of aphids from the five *N. ribisnigri* populations was measured by counting the number of offspring over a time span of eight days and the average development time from nymph to adult (Chapter 2). Aphids of the same age were obtained by relocating final stage nymphs to a new rearing cage on a clean plant two days before the experiment. Alate aphids that had moulted within 48 h were placed individually in clip cages on 3-week-old lettuce plants. Number of offspring was counted every second day, for eight days or until the aphid died. Offspring was removed after every counting event to prevent crowding.

Statistics

EPG parameters were calculated for every aphid using the EPG analysis worksheet created by Sarria et al. (2009). The Kruskal-Wallis test was used to test for overall differences among the aphid populations on the cultivars and NILs. For the EPG parameters that differed significantly among the NILs, the Mann-Whitney U test was used for pairwise comparisons applying the Bonferroni correction for multiple comparisons. Fisher's exact test was performed to analyse differences in the percentage of aphids that showed phloem intake.
For the performance and reproduction test every plant represented one replicate. Results were first calculated per plant, and means and standard error were calculated over all plants. The Mann-Whitney U test was used to test for significant differences in aphid mortality (in the survival experiment), time until mortality, development time and nymphs produced. A Chi-square test was used to test for significant differences in aphid mortality, during the

reproduction test, between Nr:0 and the Nr:1 populations.

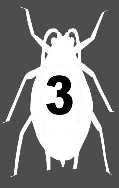
All statistical tests were performed with IBM SPSS Statistics version 19.

Results

Aphid populations on the cultivars

EPG parameters

When EPG parameters of the different aphid populations on the susceptible cultivar Pinokkio were compared, only few differences were found (Table 2 and Appendix 1), an increased time until first phloem ingestion (P = 0.003) and first sustained phloem ingestion (P = 0.001) for Nr:0 compared to Nr:1 Be aphids.



In contrast, several EPG parameters for the different aphid populations on the resistant cultivar Corbana differed (Table 2 and Appendix 1). Of the 20 parameters quantified, eight differed significantly between the Nr:0 population and the Nr:1 Ge population (Table 2, Appendix 1 and Figure 1A). Only 35% of the Nr:0 aphids showed phloem ingestion during the EPG recording compared to 75% of the Nr:1 Ge aphids (P = 0.028). The 35% that showed phloem ingestion only did so for a short duration compared to the 75% of the Nr:1 Ge aphids (P = 0.003) (Figure 1A). Since Nr:0 aphids spent less time on phloem ingestion, they spent more time in the pathway phase (pattern C) compared to Nr:1 Ge aphids (P < 0.001). Total number of probes was twice as high for Nr:0 aphids compared to Nr:1 Ge aphids (P = 0.003), especially the number of probes before the first phloem event were higher for Nr:0 aphids compared to Nr:1 Ge aphids (P = 0.003). Salivation contributed more to the phloem phase for Nr:0 aphids compared to Nr:1 Ge aphids (P = 0.005).

On Corbana, EPG parameters of the Nr:1 Pa population were statistically similar to those for the Nr:1 Ge population except for a higher number of probes performed by the Nr:1 Pa population. Compared with the Nr:0 population, the total duration of the pathway phase (C) was significantly shorter and total duration of phloem phase (E) was significantly longer, of which total duration of phloem ingestion was marginally significantly higher for Nr:1 Pa aphids (P = 0.006).

Penetration behaviour of Nr:1 Be and Nr:1 Pe was overall similar to that of the Nr:0 population on the resistant cultivar Corbana. Of the 20 parameters, none differed between Nr:1 Be and Nr:0, and only one between Nr:1 Pe and Nr:0. Significant differences were observed between the former two Nr:1 populations and the Nr:1 Pa and Nr:1 Ge aphids. The Nr:1 Pe aphids showed a higher number of probes after the first phloem event compared to the Nr:1 Ge (P = 0.004) and Nr:1 Pa population (P < 0.001). The number of phloem ingestions was lower for Nr:1 Be aphids compared to Nr:1 Ge aphids (P = 0.004).

Performance and reproduction

Survival of all five *N. ribisnigri* populations on susceptible cultivar Pinokkio ranged between 89 and 96% (Table 3). The average development time from 24-h-old nymph to adult, ranged from 7.7 to 8.8 days on Pinokkio. Reproduction of Nr:1 Ge aphids on Pinokkio was significantly lower than for Nr:1 Be aphids (P = 0.002) and Nr:1 Pe (P = 0.004).

On the resistant cultivar Corbana only 4% of the Nr:0 aphids survived, a significantly lower proportion than for the other four populations (P < 0.0001) (Table 3). Of the Nr:1 Ge population, 85% of the aphids survived on Corbana, a significantly higher proportion than for the Nr:1 Pe population (P = 0.001) of which 56% of the aphids survived. The average age at which aphids died was between five and six days. The development time of Nr:1 Ge aphids (10.3 days) was significantly shorter than for the other populations, with development times ranging from 12.3 to 13.8 days (Nr:1 Be, Pa, Pe (P< 0.0001)) and Nr:0 (P = 0.004) on Corbana. Nr:1 Ge aphids produced by far the highest number of offspring on Corbana, i.e. 13 nymphs in eight days compared to the other populations, 0.4, 0.5 and 1.9 nymphs in eight days (P< 0.0001). However, Nr:1 Ge aphids took two days longer to develop into adults on Corbana compared to Terlana.

Table 2: EPG parameters; non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2) of N. ribisnigri populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on susceptible lettuce cultivar Pinokkio and resistant lettuce cultivar Corbana (for explanation of population abbreviations, see Table 1). The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the 10 comparisons made by setting α = 0.005. Means having no letters in common within a row are significantly different. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds.

	Nr:0		Nr:1 Ge		Nr:1 Pa		Nr:1 Pe		Nr:1 Be	
Pinokkio	(n=19)		(n=20)		(n=19)		(n=20)		(n=16)	
Duration first NP	274 ± 64	a	288 ± 110	a	299 ± 87	a	201 ± 67	a	149 ± 36	a
Time to 1st visible E[#]	1919 ± 350	a	1516 ± 225	a	1664 ± 305	a	1564 ± 324	a	1228 ± 326	a
Contribution E1 to E (%)	15.38 ± 4.91	a	17.06 ± 6.85	a	12.52 ± 3.84	a	13.02 ± 3.97	a	10.02 ± 5.71	a
% aphids showing E2	100	a	90.48	a	100	a	100	a	100	a
Nr. probes	17.53 ± 2.22	a	15.43 ± 2.54	a	17.89 ± 3.11	a	14.9 ± 2.19	a	10.5 ± 2.3	a
Corbana	(n=20)		(n=20)		(n=18)		(n=19)		(n=20)	
Duration first NP	204 ± 61	ab	467 ± 110	b	243 ± 65	ab	249 ± 46	ab	208 ± 72	a
Time to 1st visible E[#]	1589 ± 284	a	1472 ± 236	a	1651 ± 426	a	1311 ± 362	a	1456 ± 288	a
Contribution E1 to E(%)	78.65 ± 8.09	a	36.93 ± 9.51	b	32 ± 9.74	ab	62.01 ± 9.53	ab	70.05 ± 10.25	ab
% aphids showing E2	35	a	75	b	72.22	b	52.63	ab	35	a
Nr. probes	32.1 ± 3.14	a	16.55 ± 2.92	b	20.67 ± 2.87	a	31.63 ± 3.69	a	30.3 ± 3.62	a

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe

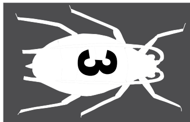
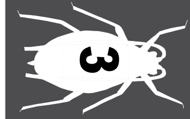


Table 3: Performance and reproduction parameters (mean ± SEM) for the N. ribisnigri populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on the susceptible cultivar Pinokkio and resistant lettuce cultivar Corbana (for explanation of population abbreviations, see Table 1) . The Mann-Whitney U test was used test to analyse differences between resistant and susceptible lettuce, * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$. Means having no letters in common within a column are significantly different.

Pinokkio	**Nr:0**	**Nr:1 Ge**	**Nr:1 Pa**	**Nr:1 Pe**	**Nr:1 Be**
Survival (%)	96 ± 1.84 a	91 ± 3.4 a	89 ± 3.4 a	89 ± 3.4 a	91 ± 3.4 a
Age at death (days)	2.33 ± 0.33 a	3.63 ± 0.73 a	3.17 ± 0.65 a	2.86 ± 0.4 a	2.2 ± 0.2 b
Development time (days)	8.82 ± 0.38 a	8.51 ± 0.21 a	7.67 ± 0.34 a	8.35 ± 0.15 a	8.76 ± 0.25 a
Reproduction (nymphs/ female)	15.37 ± 0.92 ab	12.7 ± 1.19 a	13.83 ± 1.28 ab	13.53 ± 1.16 a	18.87 ± 1.25 b
Females dead during reproduction (%)	17 a	17 a	33 a	30 a	33 a
Corbana	**Nr:0**	**Nr:1 Ge**	**Nr:1 Pa**	**Nr:1 Pe**	**Nr:1 Be**
Survival (%)	4 ± 2.34 a	85 ± 5 b	66 ± 5.82 ac	56 ± 6.47 c	60 ± 7.95 ac
Age at death (days)	5.47 ± 0.33 a	5.75 ± 0.38 a	5.31 ± 0.53 a	5.25 ± 0.54 a	5.07 ± 0.58 b
Development time (days)	13.67 ± 0.33 a	10.27 ± 0.16 b	13.81 ± 0.74 a	12.31 ± 0.71 a	12.8 ± 0.76 a
Reproduction (nymphs/ female)	0.53 ± 0.23 a	13.13 ± 1.16 b	0.47 ± 0.15 a	1.87 ± 0.61 a	0.43 ± 0.16 a
Females dead during reproduction (%)	100 a	40 b	100 a	100 a	100 a

Figure 1: The total duration of phloem ingestion (E2) for different cultivar and NIL x aphid combinations. A shows the total duration of E2 for Nr:0, Nr:1 Pa, Nr:1 Pe , Nr:1 Be and Nr:1 Ge aphids on the susceptible (S) and resistant NIL (R). B s hows the total duration (median, first and third quartiles) of E2 for Nr:0 (0), Nr:1 Pa (Pa), Nr:1 Pe (Pe), Nr:1 Be (Be) and Nr:1 Ge (Ge) aphids on Pinokkio (P) and Corbana (C). The small letters above the bars show the significant differences between the aphid population on the resistant lines. No significant differences were observed for the aphid populations on both Terlana and NIL S. For explanation population abbreviations, see Table 1.



Aphid populations on the NILs

EPG parameters

The only difference in EPG parameters between the aphid populations on the susceptible NIL was the higher number of probes for Nr:1 Pa compared to Nr:1 Be (P = 0.004)(Table 4 and Appendix 2). Comparable with the findings for the cultivars, on the resistant NIL, eight out of 20 EPG parameters for Nr:0 aphids differed from the Nr:1 Ge aphids. Only 31% of the Nr:0 aphids, compared to 84% of the Nr:1 Ge aphids, showed phloem ingestion (P = 0.001, Table 4). The duration of the first phloem event was longer for Nr:1 Ge aphids compared to Nr:0 aphids (P = 0.001) on the resistant NIL. Also the number of phloem ingestion events and sustained phloem ingestion events, was significantly higher for Nr:1 Ge aphids than for Nr:0 aphids (P = 0.002 and P < 0.0001 respectively). Number of probes was higher for Nr:0 aphids compared to Nr:1 Ge aphids (P = 0.001). Additionally, Nr:0 aphids spent more time salivating than Nr:1 Ge aphids (P = 0.001) on the resistant NIL.

On the resistant NIL, the EPG parameters for the Nr:1 Pa population again did not differ significantly from those for the Nr:1 Ge aphids. The Nr:1 Be and Nr:1 Pe aphids did not differ from the Nr:0 aphids. The latter two Nr:1 populations significantly differed in five of 20 EPG parameters from the Nr:1 Ge aphids. Of both the Nr:1 Be and Nr:1 Pe aphids only between 25% and 33% of the aphids showed phloem ingestion compared to 85% of Nr:1 Ge aphids (P = 0.002). The total duration of phloem events was shorter and total duration of the pathway phase longer for both the Nr:1 Pe (both P < 0.001), and Nr:1 Be aphids (P = 0.002 and P < 0.001) compared to the Nr:1 Ge aphids. Both the Nr:1 Pe and Nr:1 Be aphids spent more time salivating during the phloem phase (both P = 0.001) compared to the Nr:1 Ge aphids.

Performance and reproduction

Of all five populations, about 95% of the aphids survived on NIL S (Table 5). The development time, from 24 h old nymph to adult, was significantly higher for Nr:1 Pe and Be aphids compared to Nr:1 Ge and Pa aphids (Be (P = 0.003) and Pe (P = 0.001)) on NIL S. Number of offspring was significant lower for Nr:1 Be aphids compared to Nr:0 (P < 0.0001), Nr:1 Ge (P < 0.0001), and Nr:1 Pe (P = 0.002). Nr:1 Pa aphids had also significant lower number of offspring than Nr:0 (P = 0.002) and Nr:1 Ge (P = 0.005).

Almost no aphids of Nr:1 Pa, Nr:1 Pe and Nr:1 Be populations survived on NIL R and there were no surviving aphids of the Nr:0 population. For the Nr:1 Ge population, 73% of the aphids survived, a significantly higher proportion than for the others (P < 0.0001) (Table 5). Aphids of population Nr:1 Pe died on average after 4.5 days, which was significantly earlier than Nr:1 Be aphids, that died after 5.9 days (P = 0.002), and Nr:1 Ge aphids, 27% of which died on average after 7.1 days (P = 0.001) on NIL R. One Nr:1 Pe aphid survived during the performance experiment, and developed into an adult in 19 days on NIL R. The

Nr:1 Ge and Nr:1 Be aphids developed into adults in respectively 10 and 11 days. Nr:1 Ge aphids produced significantly more offspring, on average, about six nymphs in eight days, compared to the other populations, that produced on average less than one nymph ($P < 0.0001$) on NIL R in the same period.

Table 4: EPG parameters; non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2) (mean ± SEM) of *N. ribisnigri* populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on the susceptible NIL S and resistant NIL R (for explanation of population abbreviations, see Table 1). The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed, all pair-wise between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the 10 comparisons made by setting α = 0.005. Means having no letters in common within a row are significantly different. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds.

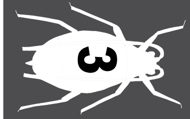
	Nr:0				Nr:1 Ge				Nr:1 Pa				Nr:1 Pe				Nr:1 Be			
NIL S	(n=18)				(n=19)				(n=20)				(n=20)				(n=16)			
Duration first NP	557	±	223	a	702	±	164	a	268	±	83	a	201	±	61	a	324	±	111	a
Time to 1st E [#]	1501	±	227	a	1691	±	296	a	1559	±	164	a	1486	±	236	a	1724	±	229	a
Contribution E1 to E (%)	24.05	±	9.12	a	3.65	±	1.32	a	13.14	±	4.97	a	6.87	±	4.85	a	2.44	±	0.64	a
% aphids showing E2	77.78			a	100			a	95			a	95			a	100			a
Nr. probes	23	±	4.01	ab	11.79	±	2.42	ab	18.25	±	2.21	a	17.75	±	3.2	ab	10.75	±	1.5	b
NIL R	(n=19)				(n=19)				(n=18)				(n=20)				(n=18)			
Duration first NP	163	±	30	a	469	±	140	a	264	±	64	a	389	±	138	a	389	±	138	a
Time to 1st visible E[#]	2265	±	391	a	1751	±	335	a	1544	±	277	a	1629	±	254	a	1629	±	254	a
Contribution E1 to E (%)	71.31	±	10.15	a	20.71	±	8.28	b	31.69	±	9.77	ab	67.43	±	11.14	a	67.43	±	11.14	a
% aphids showing E2	31.58			a	84.21			b	72.22			ab	25			a	33.33			a
Nr. probes	41.32	±	4.59	a	19.84	±	3.62	b	27.39	±	3.04	ab	34.28	±	5.13	ab	34.28	±	5.13	ab

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Table 5: Performance and reproduction parameters (mean ± SEM) for the *N. ribisnigri* populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on the susceptible NIL S and resistant NIL R (for explanation of population abbreviations, see Table 1) . The Mann-Whitney U test was used to analyse differences between resistant and susceptible lettuce, * = P < 0.05; ** = P < 0.005; *** = P < 0.001. Means having no letters in common within a column are significantly different.

NIL S	**Nr:0**				**Nr:1 Ge**				**Nr:1 Pa**				**Nr:1 Pe**				**Nr:1 Be**			
Survival (%)	93	±	2.19	a	97	±	1.64	a	94	±	2.55	a	93	±	2.19	a	94	±	2.55	a
Age at death (days)	4	±	0.95	a	2.67	±	0.67	a	3.5	±	0.67	a	4.14	±	1.3	a	6.17	±	0.72	b
Development time (days)	10.53	±	0.21	ab	9.66	±	0.21	a	9.6	±	0.2	a	10.73	±	0.24	b	3.2	±	1.2	a
Reproduction[#]	11.53	±	0.85	a	11.13	±	0.95	a	7.4	±	1.03	b	11.03	±	1.09	a	11.27	±	0.49	b
Females died during reproduction (%)	23				30				46				30				40			
NIL R	**Nr:0**				**Nr:1 Ge**				**Nr:1 Pa**				**Nr:1 Pe**				**Nr:1 Be**			
Survival (%)	0			a	73	±	4.17	b	1			a	0			a	4	±	1.84	a
Age at death (days)	4.88	±	0.55	ab	7.09	±	0.68	b	5	±	0.42	ab	4.53	±	0.58	a	5.9	±	0.65	b
Development time (days)					9.97	±	0.41	a	19			b					11.25	±	0.75	a
Reproduction[#]	0.17	±	0.14	a	5.93	±	1.07	b	0.17	±	0.08	a	0.43	±	0.22	a	0.23	±	0.09	a
Females died during reproduction (%)	100				50				100				97				100			

[#] Reproduction rate is expressed as number of nymphs per female over the duration of the experiment



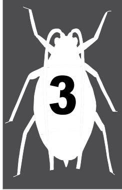
Discussion

Differences in virulence between populations on resistant lines

Nr:0 population is characterised by high sensitivity to the *Nr*-resistance in both the resistant cultivar and the resistant NIL. Most aphids do not reach phloem ingestion during the eight hour-EPG recording on resistant lettuce, and if they do, only for a short duration. This correlates well with the very low survival and reproduction found for the free-living aphids in the performance tests.

According to our results, the Nr:1 populations differ in their virulence on lettuce plants carrying the *Nr*-gene. The Nr:1 Ge population shows the highest level of virulence on *Nr*-resistant plants, judged by the longest phloem intake, highest survival and reproductive success. Nr:1 Pa aphids are second most virulent based on EPG parameters but their reproduction is significantly lower than that of Nr:1 Ge on both the resistant cultivar and the resistant NIL. The Nr:1 Be and Nr:1 Pe populations did not significantly differ from Nr:0 in terms of EPG parameters and reproduction. They had, however, significantly higher survival on Corbana (60%) compared to Nr:0 (4%). On NIL R, in contrast, almost no aphids survived of the Nr:1 Be and Nr:1 Pe population. Differences in virulence between aphid biotypes were also found for *Mi-1.2*-mediated resistance in tomato against two biotypes of *M. euphorbiae*, WU11 and WU12. These biotypes were previously designated as being avirulent based on a significantly faster population growth on susceptible plants compared to resistant plants (Goggin et al., 2001; Rossi et al., 1998). However, Cooper et al. (2004) showed that WU11 aphids were more strongly affected by the resistance than the WU12 aphids, suggesting that virulence in these two aphid biotypes is rather a quantitative trait than a qualitative one.
Although duration of phloem ingestion during the EPG experiments did not differ between the Nr:1 Pa and Nr:1 Ge population, development time of Nr:1 Ge was significantly shorter and its reproduction significantly higher. Nr:1 Pa aphids took almost four days longer on Corbana and nine days longer on NIL R to develop into adults compared to Nr:1 Ge aphids; moreover, whereas reproduction was almost zero for Nr:1 Pa aphids, Nr:1 Ge aphids larviposited 13 nymphs on Corbana and six nymphs on NIL R during eight days. This demonstrates that phloem ingestion during EPG recordings and a relatively high survival rate do not automatically result in successful population development. A possible explanation for this could be that these aphids might be able to ingest just enough phloem sap to survive on Corbana, but lack sufficient nutrients to produce offspring. Another explanation could be that the *Nr*-resistance exerts an antibiotic effect on free living aphids. No evidence was found in the EPG experiments for antixenotic resistance in the resistant cultivar and resistant NIL, however, a possible antibiosis-type resistance could be present but work in a delayed manner, and would therefore not be detected in the short-term EPG experiments as was hypothesised by Sauge et al. (2012).

Alvarez et al. (2006) found similar results for *M. persicae* on *Solanum cardiophyllum*. Although EPG parameters indicated that the feeding and probing behaviour was not affected, the colony-development test showed that aphids performed poorly on this plant. Here, antibiosis resistance was proposed as explanation for this result. This was also suggested by Herbert et al. (2007) who studied two biotypes of *M. euphorbiae*, WU11 and WU12, on resistant tomato, by analysing the honeydew production and population development of these aphids. WU12 aphids were less deterred from feeding on *Mi*-resistant tomato compared to WU11 aphids, and appeared to be virulent on *Mi*-resistant tomato. However, population growth for WU12 aphids was significantly lower on resistant compared to susceptible tomato. According to this study this could indicate that *Mi*-resistance has both antixenotic and antibiotic effects on the aphids. The opposite was found by Seo et al. (2010) who recorded that the survival of virulent biotypes of brown plant hopper, *Nilaparvata lugens* (Stål) was high on resistant rice; however, EPG results showed that these biotypes fed less on phloem of resistant plants.



Differences between resistant lettuce cultivar and NIL

A striking difference appears when comparing survival of several populations between resistant cultivar Corbana and the resistant NIL. Phloem ingestion is comparable for the Nr:1 Pa, Pe and Be populations on both resistant lines (Figure 1B). However, survival is nearly 0% on NIL R compared to about 60% on Corbana (Table 5). We assume that this effect is the result of differences in the genetic background between the used elite cultivars and the NILs derived from a single cross between a resistant and susceptible *L. sativa*. The NILs are butterhead/iceberg lettuce types while Corbana and Pinokkio are romaine lettuce types. In tomato it was also shown that the genetic background had a big influence on the effectivity of the *Mi*-gene, which confers resistance against *M. euphorbiae*, *Bemisia tabaci,* tomato psyllid and root knot nematodes. Jacquet et al. (2005) discovered that the reproduction of the root knot nematode, *Meloidogyne incognita,* differed on *Mi*-resistant tomato plants, depending on the genetic backgrounds of these plants.

Development of virulence

Little is known about the mechanism underlying the emergence of aphid biotypes insensitive for host-plant resistance. In agro-ecosystems, pest insects are exposed to strong human-imposed selection pressures (Mitchell et al., 2009). Several factors may contribute to emergence of virulent biotypes.

Virulent biotypes may arise from genotypic variation in pest insects, expressed as differences in behavioural traits (Lombaert et al., 2009; Van der Arend, 2003; Van Emden, 2007). In gene-for-gene interactions in pathogens, the ability to overcome host-plant resistance often evolves because of the loss or modification of avirulence gene products, to evade the detection by the plant (Parker & Gilbert, 2004). In the case of partial/semi virulence

the recognition of the product of an avirulence gene by the plant might be reduced but not completely absent (Hebert et al., 2007). In some aphid species large genetic variation for virulence is already present in the field. As mentioned before, in studies by Porter et al. (1997) considerable genetic variation was found in green bugs, *Schizaphis graminum* (Rondani), in the field, for virulence against resistance in wheat and sorghum. Additionally, Michel et al. (2010) shows that laboratory populations of soybean aphids did not clearly represent the field populations, since there was more genetic variation in the field. Therefore, the extent of adaptation against host-plant resistance might be underestimated and the effectiveness of the host-plant resistance strategy overestimated. Finally, biotypes might intermingle in the field as was suggested by Goggin et al. (2001) for *M. euphorbiae*, because two populations were found that were heterogeneous for the virulence on resistant tomato.

Secretion of factors by aphids that suppress plant defences can also contribute to development of virulence. Dreyer & Campbell (1987) reported an enhanced pectin methylesterase activity in the saliva of *S. graminum,* allowing for the virulent biotype to feed on resistant sorghum accessions, for which the resistance is based on increased methylation of middle lamellar pectin.

Ruggle & Gutierrez (1995) suggested symbionts to be responsible for the resistance breaking of *Therioaphis trifolii* f. *maculata* (Monell) of several lucerne varieties. A possible involvement of symbionts in overcoming host-plant resistance was also suggested by Herbert et al. (2007) and Francis et al. (2010) for *M. euphorbiae* in *Mi*-mediated resistance. More than 30% of the differences in protein profiles between avirulent and virulent aphids originated from endosymbionts, mainly originating from the primary symbiont *Buchnera aphidicola* and partly from a secondary *Rickettsia*-like symbiont.

Adaptation to host-plant resistance might also be caused by phenotypic plasticity. Lombaert et al. (2009) studied 49 *A. gossypii* populations on melon with and without the *Vat*-gene. Although genetic variation was very low between populations, phenotypic variability, measured in behaviour and life history traits was high. This resulted in populations ranging from being avirulent to completely virulent on the *Vat*-carrying melon.

For *N. ribisnigri* it is unknown which genetic differences made it possible for biotype Nr:1 aphids to feed, survive and reproduce on resistant lettuce. It will be interesting to investigate the genetic differences between biotypes, to get more insight in the virulence development of *N. ribisnigri* and the resistance mechanism conferred by the *Nr*-gene.

Loss of virulence

Although the Nr:1 Be, Nr:1 Pe and Nr:1 Pa were all collected in the field on *Nr*-carrying lettuce plants and supposedly virulent, none of these populations was able to successfully reproduce and develop a population on the cultivar containing the *Nr*-gene. A possible explanation could be that Nr:1 Be, Nr:1 Pe and Nr:1 Pa aphids partially lost their virulence since collection, and/or ability to utilise the resistant plants during their five week rearing

period on the susceptible cultivar Fatima. The laboratory populations of all Nr:1 populations were started with several individuals collected in the field. Therefore, genetic variation for virulence might have been present in our populations. If genetic variation for virulence would not have been absent, the loss of virulence might have been a plastic response of the aphids. In a study by Michel et al. (2010) on *A. glycines* biotypes feeding on resistant and susceptible soybean plants, several individuals of both biotypes did not behave as expected, in being more or less virulent than expected. Possible causes mentioned here were that (a) certain individuals in a population might have gained or lost their virulence during the maintenance of the colony, (b) there was already variation for virulence present within a population, and (c) contamination of the colonies had occurred. In our study, contamination of Nr:1 populations with Nr:0 aphids is very unlikely, because the populations were reared in seperater greenhouse compartments.

According to Goggin et al. (2001) virulence of biotypes of *M. euphorbiae* for *Mi*-resistance is a persistent trait within these aphid populations. This might also be true for Nr:1 Ge aphids, which did not lose their ability to feed and reproduce on the resistant lettuces. Compared to previous EPG results of Nr:1 Ge reared on Corbana and tested on NIL R, the percentage of aphids showing phloem sap ingestion during the recordings was the same (Chapter 2). The total duration of phloem sap intake was longer for the aphids in the current experiment than in the previous experiment (Chapter 2; 294 ± 29 versus 193 ± 23 minutes per eight hours, respectively).

Insensitivity to insecticides is known to be associated with fitness costs. For example, for *M. persicae* populations that are resistant against certain insecticides, a fitness cost was increased susceptibility to parasitoids and reduced reproductive rate (Foster et al., 2003, 2010). Similarly, virulence might incur fitness costs for aphids. Pallipparambil et al. (2010) found some evidence for possible costs of virulence of *M. euphorbiae* on resistant tomato. WU11 (avirulent) aphids performed better on susceptible tomato compared to the WU12 (virulent) aphids. Time untill first sustained phloem ingestion was shorter for the WU11 aphids compared to the WU12 aphids. These findings indicate potential costs for being virulent on *Mi*-resistant plants. Nr:1 populations might have lost their virulence when they were reared on susceptible lettuce, due to genetic variation of phenotypic plasticity for virulence. However, when the performance of Nr:0 is compared with the Nr:1 populations on susceptible lettuce in terms of phloem sap ingestion, development time and reproduction no evidence of fitness costs is observed. However population Nr:1 Ge attains a lower reproduction on a resistant lettuce compared to a susceptible lettuce line, pointing to a fitness cost.

Conclusion

In this study we have shown that there is large variation in virulence on *Nr*-based resistant lettuce plants among different populations of *N. ribisnigri*. However, it is unclear what has caused this variation in virulence among the aphid populations. It would be interesting to further study the persistence of virulence of *N. ribisnigri* to the *Nr*-resistance in lettuce.

The present study also shows that being able to feed on a plant does not necessarily imply a reproductive success, which might indicate the presence of antibiosis next to the antixenosis resistance in *Nr*-resistant lettuces against several of the tested aphid populations.

It would be interesting to further study the mechanism underlying virulence of *N. ribisnigri* populations to the *Nr*- resistance, which will contribute to a better understanding of the *Nr*-resistance mechanism in lettuce.

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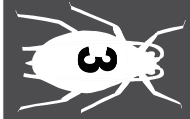
Appendix 1: EPG parameters; stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F) (mean ± SEM) of *N. ribisnigri* populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on susceptible lettuce cultivar Pinokkio and resistant lettuce cultivar Corbana (for explanation of population abbreviations, see Table 1). The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the 10 comparisons made by setting α = 0.005. Means having no letters in common within a row are significantly different. Time parameters are in seconds.

	Nr:0				Nr:1 Ge				Nr:1 Pa				Nr:1 Pe				Nr:1 Be			
Pinokkio	(n=19)				(n=20)				(n=19)				(n=20)				(n=16)			
Duration of NP before 1st E	2813	±	626	a	1697	±	410	a	1897	±	627	a	2123	±	828	a	884	±	159	a
Time to 1st E2#	2207	±	313	a	1753	±	263	ab	2527	±	470	ab	2202	±	473	ab	1323	±	324	b
Time to 1st sustained E2#	2186	±	337	a	1756	±	256	ab	2497	±	475	ab	1933	±	493	ab	1537	±	385	b
Total duration of E1	1739	±	462	a	544	±	119	a	1162	±	304	a	1378	±	459	a	736	±	252	a
Total duration of single E1	778	±	401	a	304	±	105	a	550	±	139	a	510	±	131	a	544	±	136	a
Nr. E2	3.05	±	0.47	a	2.1	±	0.29	a	2.21	±	0.26	a	2.05	±	0.33	a	2.19	±	0.44	a
Nr. sustained E2	2.05	±	0.29	a	1.71	±	0.23	a	2	±	0.24	a	1.5	±	0.17	a	1.56	±	0.26	a
Total duration of E	14351	±	1355	a	15451	±	1869	a	15832	±	1756	a	15471	±	1884	a	19894	±	1829	a
Nr. probes to 1st E	12.05	±	2.15	a	8.95	±	1.84	a	6.95	±	1.29	a	8.75	±	1.76	a	4.81	±	0.62	a
Total duration NP	4096	±	594	a	3062	±	590	a	4656	±	892	a	3723	±	933	a	2037	±	495	a
Total duration C	9039	±	946	a	8534	±	1138	a	6515	±	894	a	8503	±	1071	a	6029	±	1332	a
Total duration F	4858				2370				9480											
Total duration G	2009	±	337	a	2870	±	498	a	2741	±	511	a	2515	±	433	a	2689	±	396	a

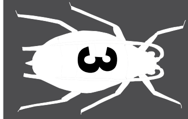


Corbana	(n=20)				(n=20)				(n=18)				(n=19)				(n=20)			
Duration of NP before 1st E	3681	±	920	a	2166	±	706	a	4793	±	1572	a	2626	±	882	a	4008	±	1007	a
Time to 1st E2#	1962	±	565	a	1753	±	283	a	2006	±	545	a	1020	±	115	a	1776	±	661	a
Time to 1st sustained E2	2925	±	1009	a	2457	±	408	a	2233	±	708	a	1161	±	125	a	3207	±	1011	a
Total duration of E1	1412	±	567	a	2084	±	772	a	1821	±	759	a	897	±	243	a	880	±	140	a
Total duration of single E1	522	±	187	a	765	±	189	a	1456	±	874	a	600	±	223	a	697	±	112	a
Nr. E2	1.15	±	0.54	ab	1.95	±	0.37	b	1.78	±	0.36	ab	1	±	0.29	ab	0.6	±	0.21	a
Nr. sustained E2	0.45	±	0.26	ab	1.15	±	0.24	c	1.33	±	0.29	bc	0.58	±	0.22	abc	0.25	±	0.1	a
Total duration of E	2696	±	939	a	14464	±	2348	b	11037	±	1813	b	5064	±	1738	ab	4974	±	1895	ab
Nr. probes to 1st E	13.18	±	1.98	a	7	±	1.96	b	13	±	2.97	ab	8.39	±	1.39	ab	11.75	±	2.87	ab
Total duration NP	7603	±	877	a	4846	±	1123	a	6308	±	1539	a	7882	±	880	a	7293	±	888	a
Total duration C	16010	±	975	a	9028	±	1337	bc	9575	±	874	b	13583	±	1040	ac	14786	±	1224	a
Total duration F	1058	±	542	a					3793	±	825	a	3967				1159	±	267	a
Total duration G	4880	±	1800	a	2652	±	413	a	2824	±	380	a	2950	±	426	a	3282	±	466	a

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Appendix 2: EPG parameters; stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F) (mean ± SEM) of *N. ribisnigri* populations Nr:0, Nr:1 Pa, Nr:1 Pe, Nr:1 Be and Nr:1 Ge on the susceptible NIL S and resistant NIL R (for explanation of population abbreviations, see Table 1). The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the 10 comparisons made by setting α = 0.005. Means having no letters in common within a row are significantly different. Time parameters are in seconds.

NIL S	Nr:0 (n=18)		Nr:1 Ge (n=19)		Nr:1 Pa (n=20)		Nr:1 Pe (n=20)		Nr:1 Be (n=16)	
Duration of NP before 1st E	2786 ± 752	a	2739 ± 556	a	1794 ± 264	a	2266 ± 571	a	1379 ± 281	a
Time to 1st E2#	1777 ± 368	a	1474 ± 215	a	2235 ± 420	a	1726 ± 240	a	2039 ± 352	a
Time to 1st sustained E2#	1805 ± 362	a	1641 ± 233	a	2059 ± 243	a	1977 ± 318	a	2076 ± 348	a
Total duration of E1	1128 ± 348	a	519 ± 202	a	1057 ± 277	a	433 ± 112	a	464 ± 129	a
Total duration of single E1	908 ± 470	a	135 ± 38	a	515 ± 242	a	406 ± 184	a	280 ± 127	a
Nr. E2	1.83 ± 0.47	a	1.84 ± 0.31	a	2.2 ± 0.35	a	1.5 ± 0.28	a	1.63 ± 0.24	a
Nr. sustained E2	1.61 ± 0.39	a	1.53 ± 0.21	a	1.65 ± 0.2	a	1.25 ± 0.23	a	1.38 ± 0.15	a
Total duration of E	15707 ± 2422	a	18903 ± 1686	a	16058 ± 1617	a	19310 ± 1477	a	20407 ± 1185	a
Nr. probes to 1st E	9.94 ± 1.8	a	9.84 ± 2.48	a	10.85 ± 1.45	a	10.42 ± 2.03	a	7.38 ± 1.13	a
Total duration NP	3426 ± 1324	a	3308 ± 603	a	4142 ± 875	a	4169 ± 933	a	2050 ± 402	a
Total duration C	8028 ± 1149	a	5746 ± 990	a	6987 ± 927	a	5542 ± 779	a	5393 ± 830	a
Total duration F	1743		3425 ± 43	a						
Total duration G	5579 ± 1552	a	1835 ± 369	a	3178 ± 603	a	2126 ± 419	a	3040 ± 434	a



NIL R	(n=19)	(n=19)	(n=18)	(n=20)	(n=18)
Duration of NP before 1st E	5170 ± 1072 a	3202 ± 664 a	3296 ± 837 a	4243 ± 948 a	4243 ± 948 a
Time to 1st E2#	3107 ± 893 a	1829 ± 336 a	2152 ± 346 a	2274 ± 453 a	2274 ± 453 a
Time to 1st sustained E2#	3336 ± 1298 a	1822 ± 338 a	2394 ± 410 a	2668 ± 464 a	2668 ± 464 a
Total duration of E1	758 ± 257 a	1683 ± 1026 a	1192 ± 283 a	837 ± 272 a	837 ± 272 a
Total duration of single E1	501 ± 195 a	2670 ± 1920 a	706 ± 269 a	342 ± 74 a	342 ± 74 a
Nr. E2	0.63 ± 0.27 a	1.95 ± 0.39 b	1.5 ± 0.28 ab	0.72 ± 0.29 ab	0.72 ± 0.29 ab
Nr. sustained E2	0.37 ± 0.22 a	1.37 ± 0.21 c	1.11 ± 0.2 bc	0.5 ± 0.25 ab	0.5 ± 0.25 ab
Total duration of E	3309 ± 1721 a	16540 ± 1864 c	10654 ± 1918 bc	5539 ± 2403 ab	5539 ± 2403 ab
Nr. probes to 1st E	17.8 ± 3.88 a	13.26 ± 2.88 a	12.81 ± 2.25 a	14.36 ± 2.62 a	14.36 ± 2.62 a
Total duration NP	7975 ± 941 a	4680 ± 960 a	6899 ± 891 a	6653 ± 952 a	6653 ± 952 a
Total duration C	16024 ± 1199 ab	7192 ± 1219 c	10095 ± 1119 bc	15148 ± 1358 a	15148 ± 1358 a
Total duration F	4730 ± 383 a	1666 a			
Total duration G	4590 ± 802 a	1428 ± 365 a	4204 ± 710 a	3403 ± 533 a	3403 ± 533 a

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

3

Chapter 4

Resistance to a new biotype of the lettuce aphid *Nasonovia ribisnigri* in a *Lactuca virosa* accession

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Abstract

Host plant resistance is an effective strategy to control aphids in many crops. However, the evolution of insensitive aphid biotypes necessitates the search for new resistance sources. Wild relatives of crop plants can be important sources for resistance genes to be introgressed into new cultivars. *Nasonovia ribisnigri* (Mosely) (Homoptera: Aphididae) is an important pest of cultivated lettuce, *Lactuca sativa*. Since 1982, resistance introduced into lettuce cultivars has relied on the *Nr*-gene, originating from a wild relative, *Lactuca virosa*. In 2007 first reports appeared that *Nr*-based resistance had become ineffective against certain populations of *N. ribisnigri*.

The objective of this study was to establish if the original donor of the *Nr*-gene *L. virosa* accession, IVT 280, is resistant against recently emerged virulent (Nr:1) *N. ribisnigri* biotypes. To this end we investigated feeding and penetration behaviour of virulent and avirulent (Nr:0) aphids on the resistant *L. virosa*, IVT 280, and two susceptible *L. virosa* accessions, using the electrical penetration graph (EPG) method. Additionally, aphid performance was analysed in terms of survival, development time and reproduction on these accessions.

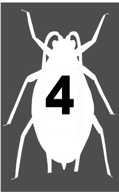
Lactuca virosa accession IVT 280 was resistant against all populations of *N. ribisnigri* tested. The ingestion of phloem was strongly reduced on the resistant accession compared to the susceptible *L. virosa* accessions. Additionally, none of the aphids survived on the resistant accession that, therefore, constitutes a good source of resistance in lettuce against both biotypes of *N. ribisnigri*.

Introduction

Aphids are among the world's most serious crop pests, especially in temperate zones, causing major damage to plants of almost every main crop (Blackman & Eastop, 2000; Dreyer & Campbell, 1987; Tagu et al., 2008). Hundreds of millions of dollars are lost each year due to damage caused by these insects (Tagu et al., 2008). Host plant resistance has proven to be an effective control measure in many crop species (Dreyer & Campbell, 1987). However, the large-scale use of resistant cultivars results in a strong selection pressure on the aphids, leading to the emergence of virulent biotypes (Lombaert et al., 2009).

When host plant resistance is no longer effective against an aphid pest species, new host plant resistance needs to be identified. Alternatively, if no effective plant resistance can be found, the use of insecticides remains the cheapest and easiest way to control aphid pests but bears considerable detrimental environmental consequences (Tagu et al., 2008).

For the identification of host plant resistance, landraces or wild relatives in many cases offer the only resources available (Dogimont et al., 2010). Wild relatives of crop plants have been important sources of resistance genes that have been introgressed into new cultivars in modern agriculture (Hajjar & Hodgkin, 2007). The use of these wild crop relatives in plant breeding has increased since the last decade and will probably increase even more due to the development of new interspecific hybridisation techniques, increases in the number of collected wild accessions, and improvements in molecular and genetic technologies. Pest and disease resistances are the most exploited traits in crop breeding programs, having huge economic impacts (Dwivedi et al., 2008). The use of wild crop relatives for other breeding goals such as abiotic stress tolerance, improved yield and quality traits is increasing, but still less frequently used compared to pest and disease resistance (Hajjar & Hodgkin, 2007).



In lettuce, a related wild species has been used for resistance breeding against downey mildew (*Bremia lactucae*) (Crute, 1992), Beet western yellows virus (Maisonneuve et al., 1991), big-vein disease (Bos & Huijberts, 1990) and the black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely) (Eenink et al., 1982). This aphid species seriously damages lettuce plants, making them unmarketable (McCreight, 2008; Stufkens & Teulon, 2003). A resistance gene, the *Nr*-gene, originating from *Lactuca virosa* L., a distant wild relative of cultivated lettuce was introgressed into cultivated lettuce, *Lactuca sativa* (Dieleman & Eenink, 1980; Eenink et al., 1982; McCreight, 2008). The use of resistance conferred by the *Nr*-gene is the most used control strategy against *N. ribisnigri* in lettuce. Lettuce lines bearing the *Nr*-gene cannot be colonised and impair feeding by *N. ribisnigri* aphids biotype Nr:0 (Chapter 2) (Van Helden & Tjallingii, 1993). However, in 2007 the emergence of a virulent biotype (Nr:1) was reported in several locations in Europe. This biotype was able to infest resistant lettuce plants, making the *Nr*-resistance ineffective (Thabuis et al., 2011). No behavioural or feeding differences were found for a German population of Nr:1 aphids between susceptible and resistant lettuce lines in a recent study (Chapter 2), in contrast to

a study by Sauer-Kesper (2011) who found reduced performance of another German Nr:1 population on resistant lettuces.

The objective of this study was to investigate if host plant resistance against *N. ribisnigri* Nr:1 aphids is available in the same accession of *L. virosa*, that has been exploited as the original donor of the *Nr*-gene, by comparative studies of the feeding and penetration behaviour of Nr:0 and Nr:1 aphids on the resistant *L. virosa* accession and on two *L. virosa* accessions that are susceptible to Nr:0. Additionally, the performance of the aphids was analysed in terms of survival and reproduction on these accessions.

Material and Methods

Plants and Aphids

The plants used for the experiments were three *L. virosa* lines, two Nr:0-susceptible accessions CGN16204 referred to as Vs1, and PI26165 referred to as Vs2, and an Nr:0-resistant accession IVT280 (CGN04683) referred to as Vr. Seeds were sown and kept at 4 ^{o}C, L14/D10 photoperiod for a week to germinate in a climate cabinet. Afterwards, they were transferred to a greenhouse compartment at 18-20 ^{o}C, 60% RH and L14/D10 photoperiod. The plants were used for experiments after growing for three weeks in the greenhouse compartment. Plants were treated with ENTONEM (a formulation of the entomopathogenic nematode *Steinernema feltiae*, www.koppert.com) once a week, to control the larvae of sciarid flies.

Two biotypes of *N. ribisnigri*, Nr:0 and Nr:1 were used in the experiments. All aphids were reared on Nr:0-susceptible *L. sativa* cultivar Fatima, in a greenhouse compartment at 18-20 ^{o}C, 60% RH and L14/D10 photoperiod. Populations of Nr:1 aphids originating from four locations were used: Germany (Nr:1 Ge), Paris (France) (Nr:1 Pa), Perpignan (France) (Nr:1 Pe) and Belgium (Nr:1 Be). For details about the origin of the populations see Chapter 3. In the aphid rearing both winged and wingless adults were present.

EPG recording

The electrical penetration graph technique (EPG) was used to study the penetration and feeding behaviour of the two *N. ribisnigri* biotypes on resistant and susceptible *L. virosa* accessions. This method allows the electrical recording of the penetration and feeding behaviour of aphids and other piercing-sucking insects, by making a plant and a piercing insect part of an electrical circuit. This is realised by inserting an electrode in the soil near the plant and attaching a thin gold wire to the insect's dorsum (Tjallingii, 1988). For details on the EPG methods used see Chapter 2. Seven EPG patterns were distinguished in this study: stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F). The first phloem phase that appears in the EPG recording will be referred to as the first visible phloem event.

Contact with the phloem before the first visible phloem event cannot be ruled out, because the EPG signal does not allow identification of the cell type in which cell punctures, visible as potential drops in the signal, take place.

Eight hour recordings were performed with winged adult aphids (alatae), randomly collected from the rearing, to get a general view on the behaviour of alatae within a population. For every aphid biotype x *L. virosa* accession combination 24 replicates (individual aphids on separate plants) were recorded. Every aphid and plant individual was only used for one recording. Incomplete recordings, due to death or escape of aphids, were excluded from the analyses, leaving between 16 and 21 replicates for every aphid biotype x *L. virosa* accession combination.

Performance

Performance of the populations of *N. ribisnigri* biotypes Nr:0 and Nr:1 on the *L. virosa* accessions was measured by assessing nymph survival and development time from nymph to the adult stage. About 150 *N. ribisnigri* alatae per biotype population were kept in Petri dishes on leaves of *L. sativa* cultivar Fatima for 24 h. The next day new born nymphs were transferred into clip cages on 3-week-old *L. virosa* plants, five nymphs per cage, one cage per plant. Number of aphids alive and their developmental stage were recorded daily until the aphids had reached the adult stage. Experiments were conducted in a greenhouse compartment at a temperature of 18-20 ^{o}C, 60% RH and L14/D10 photoperiod. Twenty plants were tested per population x *L. virosa* accession combination.

Reproduction test

Reproductive success of aphids from two populations, biotype Nr:0 and biotype Nr:1 Ge of *N. ribisnigri* was quantified by the number of offspring produced over a time span of eight days, the average development time from nymph to adult (Chapter 2). Aphids of the same age were obtained by relocating final stage nymphs to a new rearing cage on a clean plant two days before the experiment. Alate aphids that had moulted within 48 h were placed individually in clip cages on 3-week-old *L. virosa* plants. Number of offspring was counted and removed every second day, for eight days or until the aphid died. The experiment was conducted in the same greenhouse compartment, under the same environmental conditions as the performance tests. Thirty replicates were done per biotype x *L. virosa* accession combination.

Statistics

EPG parameters were calculated per aphid using the EPG analysis worksheet created by Sarria et al. (2009). The Kruskal-Wallis test was used to test for overall differences among the biotypes on the *L. virosa* accessions. For the EPG parameters that differed significantly among the *L. virosa* accessions, the Mann-Whitney U test was used for pairwise comparisons,

applying the Bonferroni correction for multiple comparisons. Fisher's exact test was used to analyse differences in the percentage of aphids that showed phloem intake. For aphid performance and reproduction every plant was considered one replicate. Results were first calculated per plant, and means and standard error were calculated over all plants. The Mann-Whitney U test was used to test for significant differences in aphid mortality (in the performance experiment), time until mortality, development time and reproduction. The Chi-square test was used to test for significant differences in aphid mortality during the reproduction test.

All statistical tests were performed with IBM SPSS Statistics version 19.

Results

In the following the results from EPG recordings and performance will be presented for each aphid population separately.

Nr:0

When comparing the EPG parameters for Nr:0 aphids on Vs1 and Vs2 plants no significant differences were found (Table 1 and Appendix 1).

When comparing the EPG data for aphids on Vr and Vs1 plants, it took longer for the first visible phloem event to occur on Vr compared to Vs2 ($P = 0.007$). The phloem phase lasted shorter ($P < 0.0001$) and consisted almost entirely (92%) of salivation for aphids on Vr compared to 19% salivation on Vs1 ($P < 0.0001$) and 4% on accession Vs2 ($P < 0.0001$). Only 16% of the aphids displayed phloem ingestion on Vr, compared to 90% on Vs1 and 95% Vs2 (both $P < 0.0001$). The aphids that did show phloem ingestion on Vr, did so for a shorter duration compared to the aphids on Vs1 ($P = 0.004$) and Vs2 ($P = 0.014$) (Figure 1A). Also the total number of probes was higher on Vr compared to Vs1 and Vs2 plants ($P < 0.0001$).

In the performance experiment, none of the Nr:0 nymphs survived on Vr (Table 2). Significantly more nymphs survived on Vs2 compared to Vs1 ($P < 0.0001$). Average nymph age at death during the performance experiment was significantly higher for aphids on Vs1, compared to Vr ($P < 0.0001$) and Vs2 ($P < 0.0001$). The development time of the aphids was longer for nymphs on accession Vs1 compared to Vs2 ($P = 0.002$). Reproduction was significantly lower on Vr compared to Vs1 ($P = 0.006$) and Vs2 ($P < 0.0001$). All adult aphids died on the accessions Vr and Vs1 during the reproduction experiment that lasted for eight days and 80 % died on Vs2.

Nr:1 Germany

In the EPG experiments, Nr:1 Ge aphids on Vs2 spent more time on phloem events compared to aphids on Vs1 ($P = 0.026$) (Table 3).

When comparing the Nr:1 Ge aphids on Vr with those on Vs1 and Vs2, the total duration of phloem events was shorter for aphids on Vr compared to the aphids on Vs1 (P

= 0.002) and Vs1 (P < 0.0001), of which 63 % of the time was spent on salivation by aphids on Vr compared to 9% on Vs2 (P < 0.0001) and 12 % on Vs1 (P = 0.002). Only half of the aphids on Vr displayed phloem ingestion, compared to 90% of Vs1 (P = 0.007) and 95% of Vs2 (P = 0.002). The total duration of phloem ingestion was also shorter for the aphids on Vr compared to the aphids on Vs2 (P = 0.004) (Figure 1B). Additionally, the number of probes was higher for aphids on Vr compared to those on Vs1 and Vs2 (both P < 0.0001).

In the performance test, none of the Nr:1 Ge nymphs survived on Vr. Only half of the nymphs survived on Vs1 compared to 90% on Vs2 (P < 0.0001; Table 4). The average age at which the nymphs died during the performance experiment was lower on Vr (P < 0.0001) and Vs2 (P = 0.008) compared to Vs1. The development time was equal for nymphs on Vs1 and Vs2, as was their reproduction rate. Significantly fewer nymphs were produced on Vr compared with the Vs1 and Vs2 (both P < 0.0001). All adult aphids died on Vr before the end of the reproduction experiment, whereas 77% and 67% of the adults died on Vs1 and Vs2 respectively (P < 0.0001).

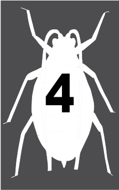


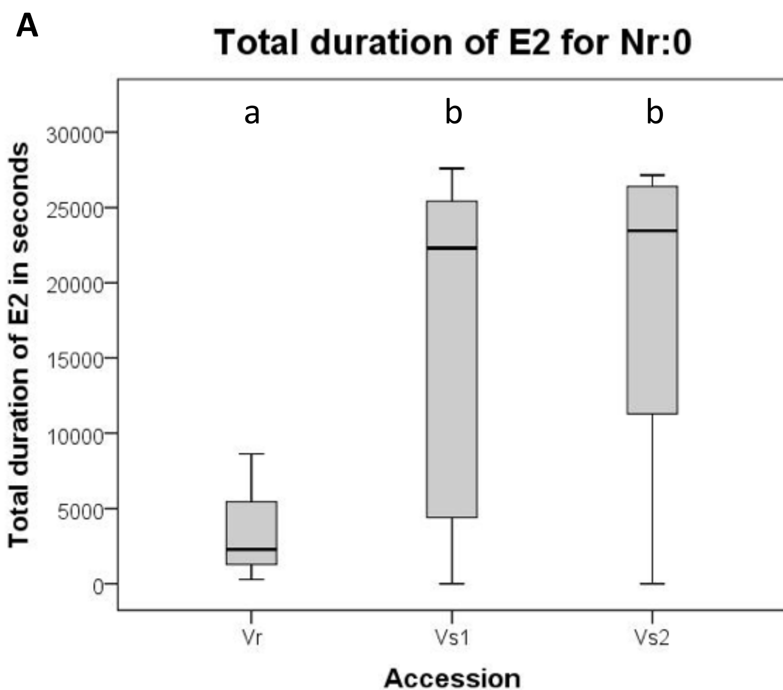
Table 1: EPG parameters (mean ± SEM) of *N. ribisnigri* population Nr:0 on *L. virosa* accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.017. Means within a row having no letters in common are significantly different. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, E = phloem event, E1 = phloem salivation and E2 = phloem ingestion.

Nr:0	Vr (n=19)				Vs1 (n=21)				Vs2 (n=20)			
Duration first NP	201	±	56	a	219	±	52	a	141	±	31	a
Time to 1st visible E#	10615	±	1601	a	7130	±	1512	ab	5315	±	1264	a
Contribution E1 to E(%)	92	±	6	a	19	±	7	b	4	±	1	b
% aphids showing E2	15.79			a	90.48			b	95			b
Nr. probes	31.89	±	2.80	a	11.95	±	2.00	b	8.62	±	1.57	b

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Table 2: : Performance and reproduction parameters (mean ± SEM) for the *N. ribisnigri* population Nr:0 on *L. virosa* accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for parameters that differed, all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.017. Significance of differences in the percentage mortality during reproduction was analysed by a Chi-Square test. Means within a row having no letters in common are significantly different.

Nr:0	Vr				Vs1				Vs2			
Survival (%) (n=20)	0			a	26	±	5.25	b	95	±	1.99	c
Age at death	2.90	±	0.10	a	5.74	±	0.34	b	2.80	±	0.40	a
Development time					11.45	±	0.30	a	10.09	±	0.21	b
Reproduction (nr. nymphs per female)	0.07	±	0.05	a	0.86	±	0.24	b	4.28	±	1.09	b
Adult mortality (%) (n=30)	100			a	100			a	80			b



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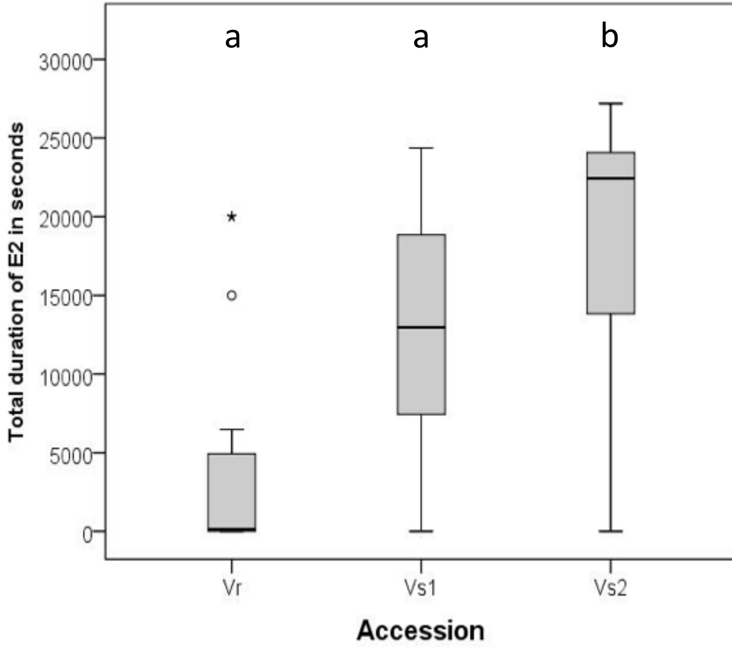

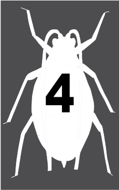

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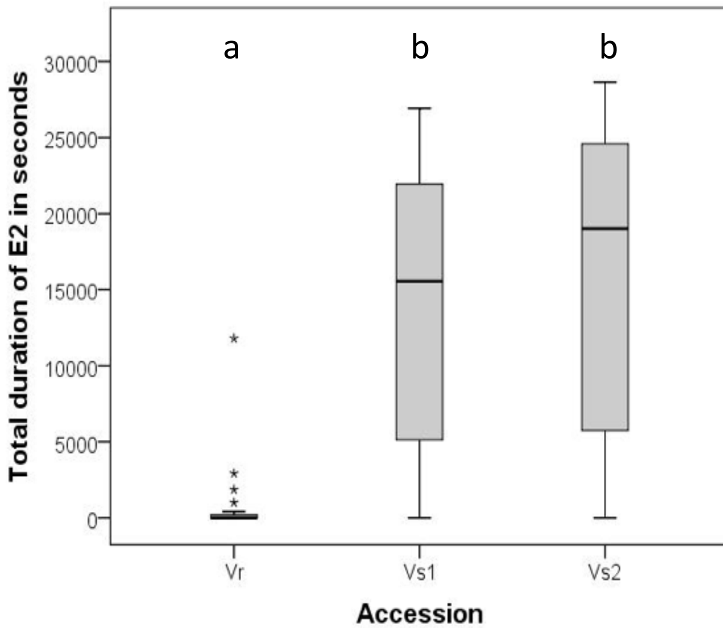


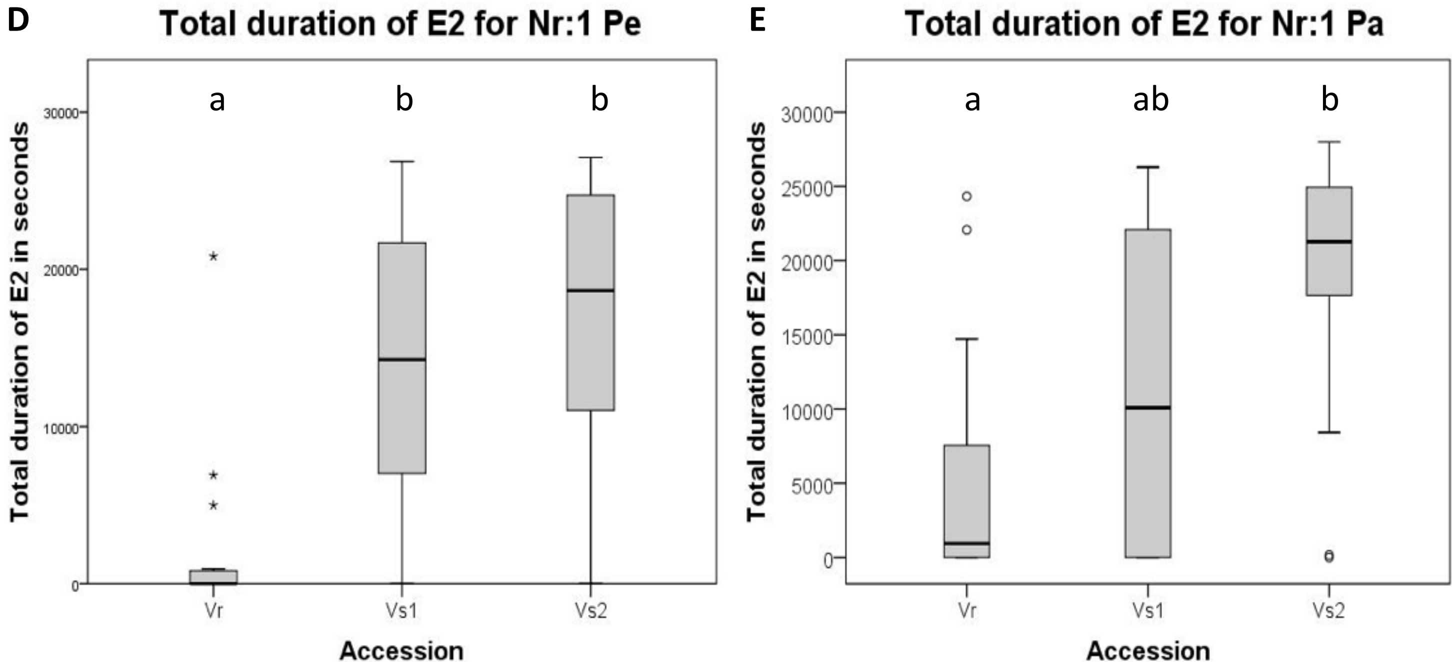
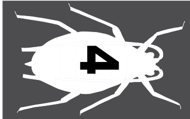


Figure 1: The total duration of EPG parameter E2 (phloem ingestion) (median, first and third quartiles and range) for *N. ribnisnigri* Nr:0 (A), Nr:1 Ge (B), Nr:1 Be (C), Nr:1 Pe (D) and Nr:1 Pa (E) aphids on *L. virosa* accessions. Bars having no letters in common differ significantly. Outliers are plotted as asterisks and circles Vs1 = Nr:0-susceptible accessions CGN16204, Vs2 = Nr:0-susceptible accessions PI26165 and Vr = Nr:0-resistant accession IVT280 (CGN04683).

Table 3: EPG parameters (mean ± SEM) of N. ribisnigri Nr:1 populations; Nr:1 Ge, Nr:1 Be, Nr:1 Pe and Nr:1 Pa, on L. virosa accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.017. Means within a row having no letters in common are significantly different. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, E = phloem event, E1 = phloem salivation and E2 = phloem ingestion.

	Vr				Vs1				Vs2			
Nr:1 Ge	(n=18)				(n=19)				(n=21)			
Duration first NP	203	±	52	a	333	±	104	a	254	±	65	a
Time to 1st visible E#	8268	±	1769	a	7967	±	1264	a	5616	±	1062	a
Contribution E1 to E(%)	63	±	10	a	9	±	5	b	12	±	6	b
% aphids showing E2	50.00			a	89.47			b	95.24			b
Nr. probes	27.83	±	2.77	a	11.58	±	1.36	b	9.20	±	1.52	b
Nr:1 Be	(n=19)				(n=22)				(n=22)			
Duration first NP	149	±	36	a	199	±	53	a	379	±	129	a
Time to 1st visible E#	8895	±	1295	a	8477	±	1287	a	5015	±	898	b
Contribution E1 to E(%)	79.28	±	8.27	a	22.10	±	7.66	b	14.04	±	6.98	b
% aphids showing E2	26.32			a	86.36			b	90.91			b
Nr. probes	31.21	±	2.33	a	9.27	±	1.21	b	11.68	±	2.10	b
Nr:1 Pe	(n=20)				(n=19)				(n=19)			
Duration first NP	182	±	46	a	190	±	59	a	255	±	105	a
Time to 1st visible E#	10559	±	1758	a	6194	±	1333	ab	4163	±	824	b
Contribution E1 to E(%)	78.81	±	7.91	a	7.33	±	2.05	b	7.89	±	5.52	b
% aphids showing E2	35.00			a	84.21			b	87.50			b
Nr. probes	33.90	±	2.80	a	10.47	±	1.47	b	11.11	±	1.64	b



Nr:1 Pa	(n=16)				(n=20)				(n=21)			
Duration first NP	337	±	83	a	199	±	88	b	167	±	36	ab
Time to 1st visible E#	6552	±	1781	a	6528	±	1368	a	4121	±	688	a
Contribution E1 to E(%)	49.13	±	11.26	a	17.82	±	8.82	b	9.72	±	5.53	b
% aphids showing E2	62.50			a	70.00			a	89.47			a
Nr. probes	28.56	±	4.43	a	18.05	±	2.81	a	7.90	±	1.07	b

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Table 4: Performance and reproduction parameters (mean ± SEM) for the *N. ribisnigri* population Nr:1 Germany on *L. virosa* accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.017. Significance of differences in the percentage mortality during reproduction was analysed by a Chi-Square test. Means within a row having no letters in common are significantly different.

Nr:1 Germany	**Vr**				**Vs1**				**Vs2**			
Survival (%) (n=20)	0.00			a	51.25	±	7.96	b	91.75	±	10.59	c
Age at death	3.81	±	0.16	a	6.52	±	0.51	b	3.27	±	0.37	a
Development time					10.77	±	0.27	a	10.59	±	0.217	a
Reproduction (nr. nymphs per female)	0.14	±	0.06	a	5.17	±	1.06	b	5.03	±	0.97	b
Adult mortality (%) (n=30)	100			a	76.67			b	66.67			b

Nr:1 Belgium

In the EPG experiment, the time to the first visible phloem event and phloem ingestion was significantly shorter on Vs2 compared to Vs1 for Nr:1 Be aphids (P = 0.015 and P = 0.013) (Table 3). For Nr:1 Be aphids on Vr the time to reach the first visible phloem event was longer than for aphids on Vs1 and Vs2 (P = 0.013). The time that aphids on Vr spent on phloem events was short compared to the aphids on Vs1 and Vs2 (both P < 0.0001) and on Vr most of this time was spent on salivating (both P < 0.0001). Only 26% of the aphids on Vr showed phloem ingestion compared to 86% and 91% of the aphids on Vs1 and Vs2 respectively (both P < 0.0001). Additionally, the total duration of phloem ingestion was also longer for aphids on Vs1 and Vs2 compared to the ones on Vr (both P < 0.008; Figure 1C). Also the number of probes was higher for aphids on Vr compared to the aphids on Vs1 and Vs2 (both P < 0.0001).

In the performance test, no nymphs survived on Vr (Table 5). A significantly higher proportion of nymphs survived on Vs2 compared to Vs1 (P < 0.0001). Development time was longer for nymphs on Vs2 compared to Vs1 (P < 0.0001).

Nr:1 Perpignan

No significant differences were found in EPG parameters between Nr:1 Pe aphids on Vs1 and Vs2 plants (Table 3 and Appendix 1).

For Nr:1 on Vr the time to reach the first visible phloem event was longer than for aphids on Vs2 (P = 0.006). The total duration of phloem events was very short for the aphids on Vr compared to the aphids on Vs1 and Vs2 (both P < 0.0001), of which almost 80% was spent on salivating by aphids on Vr compared to 7% and 8% by aphids on Vs1 and Vs2 respectively (both P < 0.0001). Only 35% of the aphids showed phloem ingestion on Vr, compared to 84% and 87% of the aphids on Vs1 (P = 0.002) and Vs2 (P < 0.0001) respectively. Of the aphids that did ingest phloem on Vr, the total duration of phloem ingestion was very short compared to the aphids on Vs1 (P = 0.004) and Vs2 (P = 0.002) (Figure 1D). The number of probes was also higher for aphids on Vr compared to the aphids on Vs1 and Vs2 (both P < 0.0001).

In the performance experiment, no nymphs survived on Vr (Table 5). More nymphs survived on Vs2 compared to Vs1 (P = 0.014). Nymphs lived longer on Vs1 compared to Vr and Vs2 (both P < 0.0001). Nymphs took longer to develop on Vs1 compared to nymphs on Vs2 (P < 0.0001).

Nr:1 Paris

In the EPG experiment, the total number of probes was higher for Nr:1 Pa aphids on Vs1 plants compared to aphids on Vs2 plants (P = 0.003) (Table 3).

When the aphids on Vr were compared with aphid on Vs1 and Vs2, they took longer on Vr to start probing the plant compared to aphids on Vs1 (P = 0.014). Aphids on Vr

had a shorter duration of phloem events compared to the aphids on Vs2 (P = 0.002), which consisted almost 50% of salivating for aphids on Vr versus 18% for the aphids on Vs1 (P = 0.011) and 10% for the aphids on Vs2 (P = 0.001). Aphids on Vr spent significantly less time ingesting phloem compared to aphids on Vs2 (P = 0.003), although, the percentage of aphids showing phloem ingestion did not differ (Figure 1E). The number of probes was higher for aphids on Vr compared to the aphids on Vs2 (both P < 0.0001).

In the performance experiment, no nymphs survived on Vr. Significantly more nymphs survived on Vs2 compared to Vs1 (P = 0.005) (Table 5). Nymph longevity was higher on Vs1 compared to Vr and Vs2 (both P < 0.0001). The development time was significantly longer for nymphs on Vs2 compared to Vs1 (P < 0.0001).

Table 5: Performance parameters (mean ± SEM) for the *N. ribisnigri* population Nr:1 Be, Nr:1 Pe and Nr:1 Pa on *L. virosa* accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.017. Means within a row having no letters in common are significantly different.

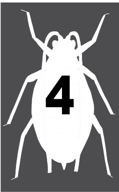
Nr:1 Be	**Vr**				**Vs1**				**Vs2**			
Survival (%) (n=20)	0.00	±	0.00	a	56.00	±	7.76	b	94.00	±	2.10	c
Age at death	3.13	±	0.14	a	4.62	±	0.33	b	4.50	±	1.06	a
Development time					10.00	±	0.37	a	10.88	±	0.15	b
Nr:1 Pe	**Vr**				**Vs1**				**Vs2**			
Survival (%) (n=20)	0.00	±	0.00	a	40.00	±	6.81	b	97.00	±	1.64	c
Age at death	3.12	±	0.15	a	5.99	±	0.53	b	3.00	±	1.00	a
Development time					13.06	±	0.91	a	10.59	±	0.18	b
Nr:1 Pa	**Vr**				**Vs1**				**Vs2**			
Survival (%) (n=20)	0.00	±	0.00	a	39.00	±	7.03	b	94.00	±	2.55	c
Age at death	2.83	±	0.13	a	5.22	±	0.42	b	4.20	±	1.02	b
Development time					10.74	±	0.71	a	10.28	±	0.12	b

Discussion

Lettuce aphid behaviour and performance on susceptible *L. virosa* accessions

The behaviour of five *N. ribisnigri* populations on accession Vs1 differs in several ways from

that on Vs2. Neither the Nr:0 nor the Nr:1 Pe population shows significant differences in the EPG parameters between the two susceptible *L. virosa* accessions: however, the other populations do show minor differences. For example, the Nr:1 Ge aphids display longer phloem events on Vs2 than on Vs1 plants. However, the total duration of phloem ingestion, the EPG parameter representative for total feeding duration, does not differ between the two susceptible accessions for any of the aphid populations. Also, no differences were found in reproduction by Nr:0 and Nr:1 Ge on Vs1 and Vs2, suggesting the aphids perform equally well on both accessions. However, the survival data provide a different view. All tested populations show a reduced survival on Vs1, compared to Vs2. Additionally, all populations, except for the Nr:1 Ge population, display a longer development time on Vs2 compared to Vs1. This result shows that Vs1 is a less suitable host plant for the tested *N. ribisnigri* populations than Vs2. Vs1 is partially resistant to both the Nr:0 and Nr:1 populations, because mortality is not 100%. Partial resistance was also found in *Lactuca serriola* (PI 491903) against Nr:0 aphids by McCreight & Liu (2012). The partial resistance in this study might be explained by the induction of resistance after an infestation duration longer than eight hours, which is the time span of the EPG experiments. This remains to be addressed. Another explanation could be that antibiosis is the resistance mechanism. Antibiosis resistance cannot be measured in the EPG set-up, which mainly measures antixenosis resistance. In the case of antibiosis, the phloem sap of Vs1 might be nutritionally unbalanced or toxic, a finding also suggested for resistance of on wild tomato (*Solanum pinnatisectum*) to *M. euphorbiae* (Pompon et al., 2010).



New resistance source against Nr:1

Both the EPG data and performance data show that Vr, *L. virosa* IVT280, is resistant to all tested populations of *N. ribisnigri.* During the survival test all aphids died on Vr. Additionally, the EPG data show that the aphids experience difficulties when feeding on Vr compared to Vs1 and Vs2. All aphid populations displayed a shorter phloem ingestion period on Vr, compared to Vs1 and Vs2, therefore more time was spent on non-penetration and pathway activities, probably in response to the inability to feed on this plant (Appendix 1). Additionally, on Vr plants the phloem phase consisted mainly of phloem salivation. The aphids were able to reach the phloem with their stylets, and salivated into the phloem; however, this was not followed by phloem ingestion. Finally, the number of probes was higher on Vr, probably caused by the aphids trying over and over again to find a suitable phloem vessel to feed on. In a choice-set up these aphids probably would soon have left the plant, but the wiring prevented this in the EPG setup. Similar EPG results were also found for *B. brassicae* on a wild cabbage, *B. fruticolosa,* compared to aphids feeding on *Brassica oleracea* (Cole, 1994). Contrasting results were found by Cid et al. (2012) who studied Spanish Nr:1 *N. ribisnigri* population on several accessions of *L. virosa*. Although their EPG results also indicated a reduction or absence of feeding, the survival data in contrast showed a survival percentage

ranging from 75% to 100% on selected "resistant" *L. virosa* accessions; however, *L. virosa* Vr, IVT 280 was not included in their study. Another explanation for this difference could be that the Nr:1 populations differ in virulence, because they originate from distinct geographical regions. In a previous study by (Chapter 3), the virulence of the five Nr:1 populations was quantified. The Nr:1 Ge population was highly virulent and the Nr:1 Pa was the second most virulent. The Nr:1 Pe and Be populations were the least virulent; yet, survival on resistant lettuce cultivar was higher compared to Nr:0.

The percentage of aphids displaying phloem ingestion on Vr plants was lower compared to Vs1 and Vs2 plants except for the Nr:1 Pa population that showed no decrease in this parameter. About 60% of the Nr:1 Pa aphids on Vr displayed phloem ingestion, but only for short periods. This was not sufficient to survive because all aphids died during the survival test. The total duration of the phloem phase was shorter on Vr compared to Vs2 and Vs1 for all populations. No significant differences were found in xylem feeding between the aphids of the five populations on the resistant and susceptible accessions, indicating that the aphids had no restrictions in staying hydrated. Both the Nr:1 Be and Pe population took more time to display the first visible phloem event and phloem ingestion on Vr compared to Vs1 and Vs2 plants, indicating that these aphids encountered deterrent compounds in the epidermis or mesophyll. They could also be deterred by the phloem, during a brief cell puncture in a phloem cell. The EPG signal does not allow identification of the cell type in which the puncture takes place. Thus, there might have already been contact with the phloem before the first visible phloem event.

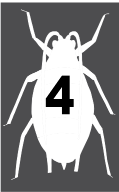
Wild relatives of crops have been successfully used in breeding for aphid resistance. In potato, for example, there is considerable variation in aphid resistance among wild potato species and traits of resistant potato species can be used to breed for aphid resistance (Pompon et al., 2011; Radcliffe, 1982). Accessions of two wild *Solanum* species, *Solanum chomatophilum* and *Solanum stoloniferum*, were proven to be potential candidates for breeding for resistance against *Myzus periscae* (Sulzer) and *Macrosiphum euphorbiae* (Thomas) in potato (Alvarez et al., 2006; Le Roux et al., 2007). In a follow-up study, different genotypes of one *S. chomatophilum* accession differed in their resistance against *M. euporbiae* (Pompon et al., 2011). This should be taken into account when screening for resistance against aphids. Resistance against aphids was also found in wild *Brassica* species. *Brassica fruticulosa*, bears resistance against the specialist aphids *Brevicoryne brassicae* L. and *Liphapis erysimi* (Kaltenbach) and could be used to introgress resistance genes into cultivated *Brassica's* (Cole, 1994; Kumar et al., 2011). In both studies, high lectin concentrations seemed to be the underlying resistance mechanism.

Utilising resistance against the Nr:1 biotype

Although there seems to be an alternative resistance available in *L. virosa* accession IVT280 against both the Nr:0 and the Nr:1 biotypes of *N. ribisnigri*, implementing this resistance

into cultivated lettuce might be a long and difficult process, as was the introgression of the *Nr*- gene from *L. virosa* to *L. sativa* (Eenink et al., 1982). *Lactuca serriola* is more closely related to *L. sativa*, and therefore alternative resistance in *L. serriola* might be more easily to introgressed in *L. sativa*, compared to resistance in *L. virosa* (Eenink et al., 1982).

Analysis of the mechanism of resistance can be used to identify the gene(s) (and vice versa) associated with the new resistance (Murugan et al., 2010), and is necessary to determine the response of an aphid population to the new resistance, that might give rise to insensitivity. For example, strong antibiosis exerts a strong selection pressure on the aphid population, which could result in emergence of virulent biotypes. Antixenosis exerts less selection pressure on the aphid, but is not strong enough to completely eliminate aphids from a crop. Additionally, antixenosis might have limited durability, because there is no negative effect for the herbivore accepting the host. Tolerance is not an option as it allows unrestrained multiplication of the aphid population that makes the lettuce unmarketable. Combinations of different categories of resistance are more beneficial in controlling a pest, compared to a single category. This also makes it more difficult for the pest to evolve virulent biotypes (Smith, 1989). Especially for *Diuraphis noxia* (Kurdjumov) in wheat, control measures resulting in lower selection pressure would be useful, because it has eveolved insensitivity to several resistances (Murugan et al., 2010). The resistance in IVT280 seems to be based on both antixenosis and antibiosis. The EPG results show reduced acceptance of the phloem, indicating antixenosis resistance, however, antixenosis resistance is underestimated in the EPG experiment, because aphids are not able to move from the plant.



Mapping new resistance genes will provide molecular tools useful in breeding for resistance programs (Lambert & Pascal, 2011). In soybean, for example, recently a new resistance gene against the soybean aphid was mapped, and molecular markers were identified closely linked to the gene which can be used in marker-assisted breeding (Jun et al., 2012).

Conclusion

Lactuca virosa IVT 280 is resistant against both the Nr:0 and the Nr:1 biotypes of *N. ribisnigri* that we tested, and may, therefore, be exploited as a source of resistance to be introduced into cultivated lettuce against both biotypes of these aphids. The *L. virosa* Vs1 accession is partially resistant against the tested *N. ribisnigri* populations. None of the four Nr:1 populations that we tested, were able to survive on this accession. More information about the genetic basis of this resistance is needed. Furthermore, the development of molecular markers for this resistance is essential for efficient breeding programs.

Acknowledgements

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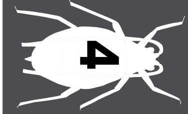
Appendix 1: EPG parameters (mean ± SEM) of *N. ribisnigri* populations Nr:0, Nr:1 Ge, Nr:1 Be, Nr:1 Pe and Nr:1 Pa on *L. virosa* accessions, Vr, Vs1 and Vs2. The Kruskal-Wallis test was used to test for differences between the populations, and for EPG parameters that differed all pair-wise differences between populations were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the 3 comparisons made by setting α = 0.017. Means within a row having no letters in common are significantly different. Time parameters are in seconds. NP = non-penetration, C= pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties.

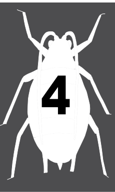
	Vr		Vs1		Vs2	
Nr:0	**(n=19)**		**(n=21)**		**(n=20)**	
Duration of NP before 1st visible E	3107 ± 690	a	1292 ± 288	b	1328 ± 356	b
Time to 1st E2#	5931 ± 1996	a	7768 ± 1718	a	6635 ± 1524	a
Time to 1st sustained E2#	2622 ± 2086	a	1195 ± 934	a	1321 ± 696	a
Total duration of E1	1327 ± 535	a	648 ± 199	a	558 ± 144	a
Total duration of single E1	939 ± 350	a	491 ± 258	a	576 ± 206	a
Nr. of E2 periods	0.32 ± 0.19	a	1.19 ± 0.18	b	1.38 ± 0.22	b
Nr. of sustained E2 periods	0.11 ± 0.07	a	1.14 ± 0.17	b	1.38 ± 0.22	b
Nr. of probes to the 1st E1	13.19 ± 2.18	a	8.14 ± 1.24	ab	6.65 ± 1.15	b
Total duration NP	6477 ± 670	a	2257 ± 545	b	1522 ± 372	b
Total duration C	18048 ± 691	a	8798 ± 1714	b	6548 ± 1251	b
Total duration F	1128					
Total duration G	3973 ± 751	a	2429 ± 564	a	3289 ± 1018	a
Nr. short probes (C < 3 min)	16.42 ± 2.08	a	5.33 ± 1.07	b	4.24 ± 1.09	b
Nr:1 Ge	**(n=18)**		**(n=19)**		**(n=21)**	
Duration of NP before 1st visible E	2925 ± 749	a	2621 ± 671	a	1407 ± 525	a
Time to 1st E2#	12831 ± 3096	a	8983 ± 1285	a	7039 ± 1452	a
Time to 1st sustained E2#	9483 ± 4052	a	2068 ± 1256	a	2310 ± 1022	a
Total duration of E1	972 ± 196	a	327 ± 61	b	766 ± 319	b

Total duration of single E1	684	±	157	a	273	±	97	b	641	±	290	b
Nr. of E2 periods	1.17	±	0.38	a	1.68	±	0.25	a	1.40	±	0.23	a
Nr. of sustained E2 periods	0.56	±	0.20	a	1.53	±	0.22	b	0.95	±	0.05	c
Nr. of probes to the 1st E1	10.94	±	2.05	a	6.06	±	0.96	a	6.35	±	1.05	a
Total duration NP	7540	±	1127	a	4399	±	662	b	2294	±	689	c
Total duration C	14698	±	1347	a	9658	±	1175	b	6655	±	1104	b
Total duration G	2673	±	382	a	3119	±	404	a	4088	±	1537	b
Nr. of short probes (C < 3 min)	13.11	±	2.03	a	4.26	±	0.72	b	3.90	±	0.90	b
Nr:1 Be		**(n=19)**				**(n=22)**				**(n=22)**		
Duration of NP before 1st visible E	2426	±	417	a	1396	±	317	a	1551	±	370	a
Time to 1st E2#	9780	±	2479	a	10055	±	1486	b	5465	±	1147	c
Nr. of probes to the 1st E1	12.88	±	1.66	a	6.57	±	0.82	b	6.57	±	1.04	b
Time to 1st sustained E2#	11253	±	2925	ab	10701	±	1673	a	5459	±	1209	b
Total duration of E1	564	±	119	ab	1696	±	703	a	654	±	384	b
Total duration of single E1	464	±	110	a	2888	±	1538	b	379	±	45	ab
Nr. of E2 periods	0.47	±	0.21	a	1.36	±	0.19	b	1.14	±	0.14	b
Nr. of sustained E2 periods	0.26	±	0.13	a	1.23	±	0.19	b	1.09	±	0.15	b
Total duration NP	7424	±	673	a	2624	±	773	b	3797	±	827	b
Total duration C	16627	±	741	a	9636	±	1329	b	7204	±	1326	b
Total duration F					6851							
Total duration G	3916	±	1076	a	2101	±	210	a	3084	±	595	a
Nr. of short probes (C < 3 min)	17.05	±	1.83	a	3.68	±	0.79	b	5.14	±	1.09	b
Nr:1 Pe		**(n=20)**				**(n=19)**				**(n=19)**		
Duration of NP before 1st visible E	3604	±	909	a	1030	±	200	b	1393	±	336	b
Time to 1st E2#	14738	±	2557	a	7404	±	1491	b	6720	±	1612	b
Nr. of probes to the 1st E1	13.05	±	2.27	a	6.56	±	1.35	b	6.39	±	0.92	b



Time to 1st sustained E2[#]	19786	±	2842	a	8354	±	1588	b	6720	±	1612	b
Total duration of E1	792	±	235	a	991	±	281	a	405	±	167	a
Total duration of single E1	466	±	81	a	498	±	152	b	720	±	445	b
Nr. of E2 periods	0.50	±	0.17	a	1.37	±	0.22	b	1.21	±	0.16	b
Nr. sustained E2 periods	0.30	±	0.11	a	1.16	±	0.18	b	1.21	±	0.16	b
Total duration NP	8362	±	934	a	2166	±	390	b	3659	±	732	b
Total duration C	15964	±	1111	a	10656	±	1742	ab	6677	±	1273	b
Total duration F	3711			a	4387			a				
Total duration G	2351	±	292	a	2301	±	321	a	5623	±	2686	a
Nr. of short probes (C < 3 min)	19.85	±	2.56	a	3.84	±	0.73	b	5.42	±	1.05	b
Nr:1 Pa		**(n=16)**				**(n=20)**				**(n=21)**		
Duration of NP before 1st visible E	1941	±	606	a	1601	±	483	ab	703	±	154	b
Time to 1st E2[#]	8801	±	2626	a	8849	±	2509	a	5320	±	1189	a
Nr. of probes to the 1st E1	9.00	±	1.81	ab	10.40	±	2.01	a	4.38	±	0.69	b
Time to 1st sustained E2[#]	14547	±	3451	a	9149	±	2614	a	5362	±	1185	a
Total duration of E1	1095	±	276	a	720	±	437	b	356	±	82	b
Total duration of single E1	850	±	267	a	271	±	75	ab	267	±	71	b
Nr. of E2 periods	1.44	±	0.35	a	1.00	±	0.22	a	1.48	±	0.21	a
Nr. of sustained E2 periods	0.63	±	0.18	a	0.85	±	0.21	ab	1.38	±	0.22	b
Total duration NP	6759	±	1194	a	4218	±	870	a	1879	±	468	b
Total duration C	12820	±	1310	a	10771	±	1624	ab	5828	±	1144	b
Total duration F	2510	±	1010	a	3115			a	786			a
Total duration G	4006.54	±	967.55	a	4606.49	±	1007.29	a	3901.96	±	678.50	a
Nr. of short probes (C < 3 min)	16.81	±	3.63	a	10.55	±	2.38	a	3.62	±	0.62	b

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe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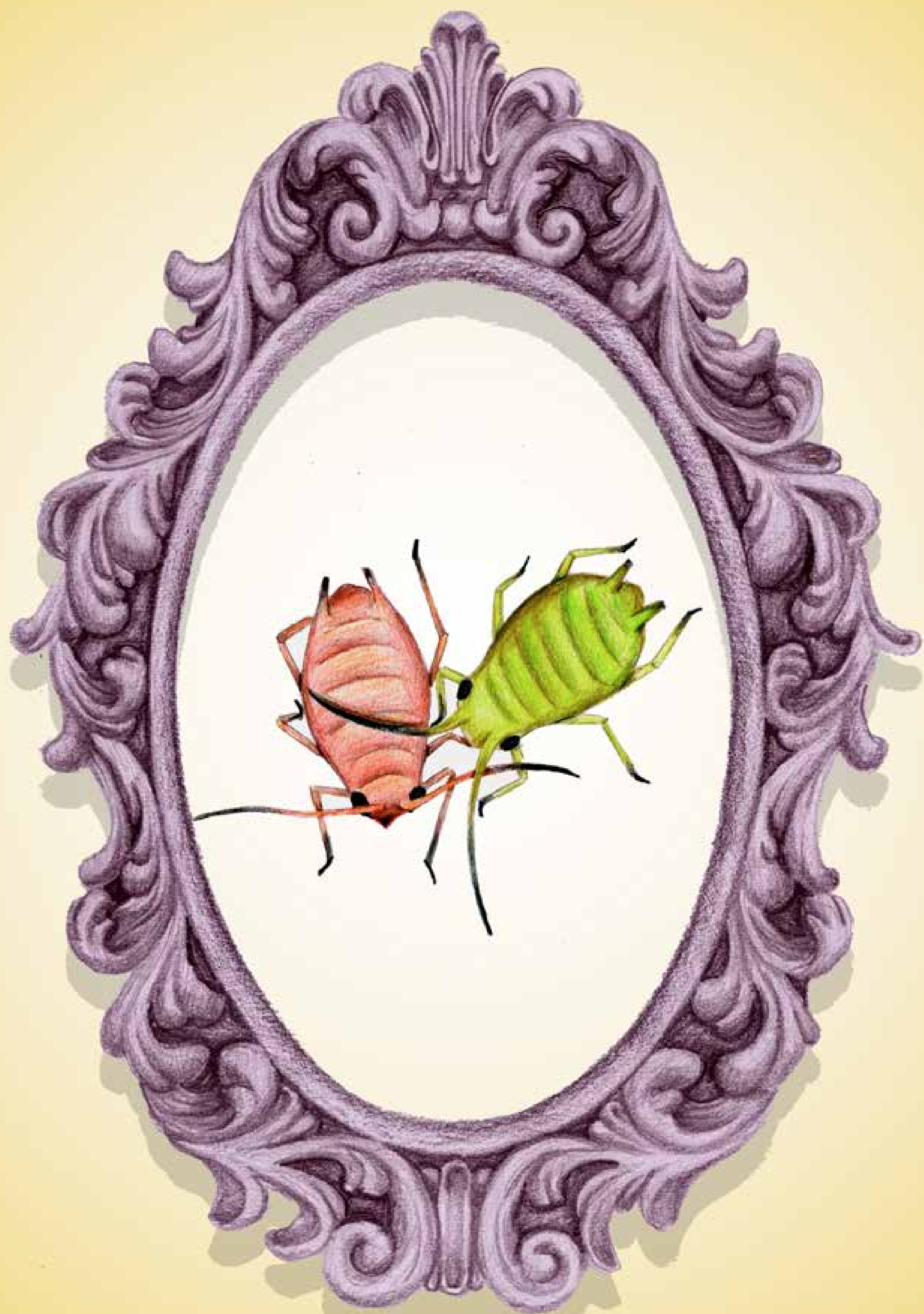


Chapter 5

The effect of co-infestation by conspecific and heterospecific aphids on the feeding behaviour of *Nasonovia ribisnigri* on resistant and susceptible lettuce cultivars

Cindy J.M. ten Broeke, Marcel Dicke and Joop J.A van Loon

Abstract

Aphid saliva can suppress the blocking of sieve elements, a reaction that plants employ to inhibit aphid feeding, but aphid saliva can also elicit plant reactions that can lead to damage to plants. Pre-infestation of plants by aphids can, therefore, be hypothesised to interact with the behaviour and/or performance of other aphids. Such interactions might occur both intraspecifically, *e.g.* among different biotypes, or among different species.

In this study we focus on the effect of feeding of aphids on the behaviour of other aphids. The objectives of our study were to investigate if feeding behaviour and performance of two biotypes of the lettuce aphids *Nasonovia ribinsigri* is affected by (1) feeding by the other biotype and (2) feeding by the green peach aphid *Myzus persicae* or the potato aphid *Macrosiphum euphorbiae*. As an control the effect of feeding by the same biotype was studied.

All experiments were performed on both a *N. ribisnigri* Nr:0-resistant and a susceptible lettuce cultivar. Additionally, the performance of both *M. persicae* and *M. euphorbiae* on both the susceptible and the resistant lettuce cultivar was evaluated to check if the aphids performed equally well on both cultivars. *Nasonovia ribisnigri* biotypes were differently affected by the presence of conspecific and heterospecific aphids. Both *N. ribisnigri* virulent biotype Nr:1 and *M. euphorbiae* are able to suppress the resistance in lettuce against Nr:0 in the resistant lettuce cultivar Corbana. All aphids, except Nr:1, induced defences against Nr:1 in Corbana.

Introduction

Nasonovia ribisnigri (Mosely), the black currant-lettuce aphid, is an important pest of lettuce, causing significant economic losses in the lettuce industry (McCreight, 2008). Control of this aphid was based on host plant resistance conferred by the *Nr*-gene, originating from *Lactuca virosa* L. (a distant wild relative of cultivated lettuce), which provided near-complete resistance against this aphid (Reinink & Dieleman, 1989). This resistance, however, is no longer effective, because virulent populations of *N. ribisnigri*, designated as biotype Nr:1, have been reported infesting resistant lettuce since 2007 (Thabuis et al., 2011).

The exact resistance mechanism encoded by the *Nr*-gene is still unknown. However, resistance components were shown to operate during the pathway to the phloem and in the phloem itself (Chapter 2), and inhibit and block sustained feeding of avirulent *N. ribisnigri,* biotype Nr:0. Nr:0 aphids are not able to ingest phloem and do not survive on resistant lettuce. In contrast, Nr:1 aphids were able to survive and ingest phloem of resistant lettuce, performing equally well on resistant and susceptible lettuce cultivars (Chapter 2). The mechanism underlying the virulence of Nr:1 aphids is unknown. It is hypothesised that the ability of virulent aphid biotypes to overcome *R*-gene based resistance could be attributed to the loss or modification of avirulence gene products, thereby suppressing plant defences and/or evading recognition by the plant (Hogenhout & Bos, 2011; Parker & Gilbert, 2004).

Aphid saliva plays an important role in suppressing the blocking of sieve elements and the food channel in the aphid's stylet (Will et al., 2007). In contrast, the saliva of aphids may also act as an elicitor of plant reactions that result in damage to the plant (Cherqui & Tjallingii, 2000). Because aphids are able to induce plant defences, pre-infestation of plants by aphids might affect the behaviour and performance of other aphids. The effect of previous infestation on aphids that subsequently attack the plant depends on the aphid species. For example, intraspecific pre-infestation of *Vicia faba* (L.) had beneficial effects for *Aphis fabae* (Scopoli), however, *Rhopalosiphum padi* (Linnaeus) did not seem to benefit from intraspecific pre-infestation of *Triticum aestivum* L. (Prado & Tjallingii, 1997). Increase in susceptibility after pre-infestation was found by Civolani et al. (2010) for *Myzus persicae* (Sulzer) on susceptible tomato, *Lycopersicon esculentum* L. The effect of pre-infestation might also differ between different parts of the same plant. Prado & Tjallingii (2007) showed that pre-infestation had a negative impact on the feeding behaviour of *Brevicoryne brassicae* (L.) on systemic leaves of resistant *Brassica oleracea* (L.) However, aphid feeding was enhanced on local infested leaves, suggesting a local suppression of resistance.

Different biotypes of aphids may influence each other's performance on resistant and susceptible plants. For example pre-infestation of *T. aestivum* by a virulent biotype of *Schizaphis graminum* (Rondani) increased the susceptibility of the plant to an avirulent biotype of this species (Dorschner et al., 1987). In contrast, Herbert et al. (2007) found that a virulent isolate of *Macrosiphum euphorbiae* (Thomas) did not enhance the performance of avirulent *M. euphorbiae*. The first objective of our study was to investigate the effect of

feeding by another biotype of *N. ribisnigri* on the feeding behaviour and performance of biotype Nr:0 and *vice versa*, studied on both a susceptible and resistant lettuce cultivar. Although many previous studies focus on the effect of pre-infestation on aphid behaviour or performance, we investigated the effect of pre-infestation and simultaneous feeding and presence of aphids on the behaviour of other aphids, and therefore, the aphids initially inoculated were left on the plants during the experiments. We refer to the effect of presence of aphids, because in the treatment Nr:0 aphids on Corbana, feeding was probably absent. As an control, we also investigated the effect of feeding by the same biotype on Nr:0 and Nr:1 aphids on the two lettuce cultivars.

Both *M. persicae* and *M. euphorbiae* are found on lettuce. The *Nr*-gene only confers partial resistance against certain *M. persicae* isolates and has no effect on *M. euphorbiae* (Reinink & Dieleman, 1989). The third objective of our study was to investigate the effect of feeding by *M. persicae* or *M. euphorbiae* on the feeding behaviour and performance of Nr:0 and Nr:1 biotypes of *N. ribisnigri* on a susceptible and resistant lettuce cultivar. The performance of both *M. persicae* and *M. euphorbiae* on both the susceptible and the resistant lettuce cultivar was evaluated to check if the aphids performed equally well on both cultivars.

Materials and Methods

Plants and Aphids

The plants used in the experiments were two near-isogenic *L. sativa* cultivars, *i.e.* Corbana, carrying the *Nr*-gene and resistant to *N. ribisnigri* Nr:0, and Terlana, susceptible to Nr:0 aphids; both supplied by Enza Zaden, Enkhuizen, The Netherlands. Plants were grown in a greenhouse compartment at 18-20°C, 60% RH and L14/D10 photoperiod. The potting soil was treated with ENTONEM (a formulation of the entomopathogenic nematode, *Steinernema feltiae*, www.koppert.com) once a week, to control soil infestation by larvae of sciarid flies. Three-week-old plants were used for the experiments.

Two biotypes of *N. ribisnigri*, Nr:0 and Nr:1 were used in the experiments. Nr:0 aphids (collected in The Netherlands in 2001) were reared on Terlana, and Nr:1 aphids (collected in Germany in 2007) were reared on Corbana, in a greenhouse compartment at 18-20°C, 60% RH and L14/D10 photoperiod. The two other aphid species, *M. euphorbiae* and *M. persicae* were reared on Corbana, under the same climatic condition as the *N. ribisnigri* populations. In the colonies of *N. ribisnigri* and *M. euphorbiae* both alate and apterous adults were present. In the rearing of *M. persicae* only apterous adults were present.

EPG recording

To study the effect of other aphids on the behaviour of *N. ribisnigri,* the electrical penetration graph technique (EPG) was used. This technique allows the electrical recording of the feeding behaviour of aphids and other piercing-sucking insects. In the EPG set-up, a plant and a piercing insect are made part of an electrical circuit, which is realised by attaching a thin gold wire to the insect's dorsum and inserting an electrode in the soil of the plant. For details on the EPG methods used see Chapter 2. Recordings were made during eight hours from winged adult aphids (alatae) of unknown age, for the *N. ribisnigri* and *M. euphorbiae* aphids. For *M. persicae*, wingless adults were used due to the absence of winged aphids in the rearing. The EPG patterns that we distinguished in the analysis were; stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F). The first contact of the aphids with the phloem is referred to as the first visible phloem contact. Aphids make cell-punctures on their way to the phloem. These punctures can be distinguished in the EPG signal, but identification of the cell type of the plant in which the punctures take place is not possible. Therefore brief phloem contact before the first visible phloem event cannot be excluded.

The two *N. ribisnigri* biotypes, *M. euphorbiae* and *M. persicae* were all four tested separately on Corbana and Terlana plants, which is common practice in EPG studies. Additionally, each biotype of *N. ribisnigri* was also tested in a group of 10 aphids, *e.g.* other biotype of *N. ribisnigri*, the same biotype of *N. ribisnigri*, *M. euphorbiae* and *M. persicae* on both Corbana and Terlana plants (Table 1). Two days prior to EPG recordings 10 third/ fourth instar nymphs were placed on a lettuce leaf, contained in a Perspex clip cage closed by metal gauze. The clip cage was removed before recording, and a ring was placed around the aphids to contain them on the leaf during the EPG recording. The test aphid was placed on the leaf such that it was in the middle of the 10 nymphs. For every treatment (Table 1) 24 replicates were recorded. Every aphid and plant individual was only used for one recording. Incomplete recordings were excluded from the analyses, leaving between 17 and 23 replicates per treatment.

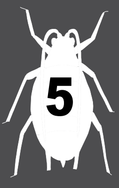


Table 1: Experimental treatments investigated, in terms of test plant, test aphids and aphid biotype or species co-infested.

Test plant	Test aphid	Treatment
Corbana	Nr:0	-
Corbana	Nr:0	Nr:0
Corbana	Nr:0	Nr:1
Corbana	Nr:0	*Macrosiphum euphorbiae*
Corbana	Nr:0	*Myzus persicae*
Terlana	Nr:0	-
Terlana	Nr:0	Nr:0
Terlana	Nr:0	Nr:1
Terlana	Nr:0	*Macrosiphum euphorbiae*
Terlana	Nr:0	*Myzus persicae*
Corbana	Nr:1	-
Corbana	Nr:1	Nr:1
Corbana	Nr:1	Nr:0
Corbana	Nr:1	*Macrosiphum euphorbiae*
Corbana	Nr:1	*Myzus persicae*
Terlana	Nr:1	-
Terlana	Nr:1	Nr:1
Terlana	Nr:1	Nr:0
Terlana	Nr:1	*Macrosiphum euphorbiae*
Terlana	Nr:1	*Myzus persicae*

Performance test

Performance of the two biotypes of *N. ribisnigri,* in all treatments (see Table 1) on Corbana and Terlana was measured by assessing nymphal survival and development time from nymph to the adult stage.

For the experiments on *N. ribisnigri* biotypes and the two other aphid species put separately on a plant, five 24 h-old nymphs were transferred to a lettuce leaf and contained in a clip cage. The mortality and the developmental stage of the aphids were recorded daily until they reached the adult stage. To obtain 24 h-old nymphs, about 150 adult aphids were kept in Petri dishes on leaves of cultivar Terlana (Nr:0) or Corbana (Nr:1, *M. euphorbiae* and

M. persicae), and the next day the new-born nymphs were used for the experiments.

For the combination treatments, ten 76 h-old nymphs of the treatment aphid species or biotype were transferred to a lettuce leaf and contained in a clip cage. After one day five 24 h-old nymphs of the test aphid species or biotype were transferred into the clip cages with the treatment aphids. Treatment aphids that turned into adults during the experiments were replaced by nymphs, to prevent an increase in density of treatment aphids by reproduction. For the combinations, Nr:0/Nr:0 and Nr:1/Nr:1, the difference in age between the test and treatment aphids allowed distinction between them. Test aphid mortality and development were recorded daily. Experiments were conducted in a greenhouse compartment at a temperature of 18-20°C, 60% RH and L14/D10 photoperiod. For every treatment, 20 plants were tested.

Statistics

All statistical tests were performed with IBM SPSS Statistics version 19. EPG parameters were calculated for every aphid tested using the EPG analysis worksheet created by Sarria et al. (2009). The Mann-Whitney U test was used for pairwise comparisons between different treatments applying the Bonferroni correction for multiple comparisons. Fisher's exact test was performed to analyse differences in the percentage of aphids that showed phloem ingestion (E2). For aphid performance every plant was considered one replicate. Results were first calculated per plant, and means and standard error were calculated over all plants. The Mann-Whitney U test was used to test for significant differences in aphid mortality (in the performance experiment), time until mortality and development time as an effect of treatment.

Results

Effects of feeding in an intraspecific group

No significant difference in EPG parameters was found between Nr:0 aphids feeding in a group and single aphids on cultivar Terlana (Table 2, Figure 1B, Appendix 2). Nr:0 aphids in a group on Corbana displayed a higher number of probes (P = 0.001) compared to single aphids. No significant differences were found in performance (Table 3).

Nr:1 aphids in a group on Terlana displayed a higher number of probes (P = 0.001) compared to single aphids on Terlana (Table 2, Figure 1D). No significant differences were found in performance (Table 3). On Corbana Nr:1 aphids in a group did not differ significantly from single Nr:1 aphids.

Effects of Nr:1 on Nr:0 aphids

EPG recordings of the feeding behaviour of single Nr:0 aphids placed in a group of Nr:1 aphids on Terlana showed that less time was spent in the phloem phase (P = 0.003), of which 14% on salivation, compared to 2% for single Nr:0 aphids (P = 0.001) (Table 2 and Appendix 2). Significantly less time was spent on phloem ingestion (P = 0.006) (Figure 1B) by Nr:0 aphids in an Nr:1 group compared to individual aphids. Development time was longer in an Nr:1 group compared to single aphids (P < 0.0001) (Table 3).

The percentage of Nr:0 aphids displaying phloem ingestion was higher in an Nr:1 group compared to single Nr:0 aphids (P = 0.013; Table 2, Appendix 2, Figure 1A). None of the single Nr:0 aphids survived the performance test on Corbana; however, 15 % survived in a group of Nr:1 aphids (P = 0.001) (Table 3). Aphids died when on a plant singly compared to the ones in a group of Nr:1 aphids (P = 0.003).

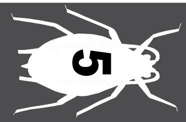
Effects of Nr:0 on Nr:1 aphids

EPG recordings of the feeding behaviour of single Nr:1 aphids placed in a group of Nr:0 aphids on Terlana showed that less time was spent on phloem events (P = 0.005) compared to single Nr:1 aphids (Table 2). The phloem phase consisted for 24% out of salivation for Nr:1 aphids in an Nr:0 group compared to 5% for individual aphids (P = 0.011). Development time was shorter for Nr:1 aphids in an Nr:0 group compared to single Nr:1 aphids (P = 0.005) (Table 3).

On Corbana, Nr:1 aphids in an Nr:0 group made a higher number of probes (P < 0.0001), spent less time in the phloem phase (P < 0.0001) and on phloem ingestion (P = 0.007), and took longer to reach the first visible phloem contact (P < 0.0001) compared to single Nr:1 aphids (Table 2 and Figure 1C). The phloem phase consisted for 42% out of salivation for the Nr:1 aphids in the Nr:0 group versus 5% for single Nr:1 aphids (P = 0.0001). Additionally, the percentage of Nr:1 aphids displaying phloem ingestion was lower in an Nr:0 group compared to single Nr:1 aphids (P = 0.004). No significant differences were found in performance parameters (Table 3).

Table 2: EPG parameters (mean ± SEM) of *N. ribisnigri* population Nr:0 and Nr:1 on *L. sativa* cultivars, Corbana (C) and Terlana (T) as single aphids or in a group of Nr:0, Nr:1, *M. euphorbiae* (Macro) and *M. persicae* (Myzus). Pairwise comparisons were made for EPG parameters between plants without other aphids and plants with other aphids, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the four comparisons made by setting α = 0.013. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, E = phloem event, E1 = phloem salivation and E2 = phloem ingestion. * = P < 0.013; ** = P < 0.005; *** = P < 0.001.

Test aphid:	**C**	**C(Nr:0)**		**C(Nr:1)**		**C(Macro)**		**C(Myzus)**	
Nr:0	**(n=19)**	**(n=23)**		**(n=18)**		**(n=21)**		**(n=23)**	
Duration first NP	417 ± 142	315 ± 140		339 ± 95		120 ± 22		485 ± 110	
Time to 1st visible E#	7643 ± 1350	12010 ± 1894		8755 ± 1250		10275 ± 1287		8915 ± 1415	
Contribution E1 to E(%)	98.66 ± 0.99	97.52 ± 2.48		74.78 ± 8.43		89.96 ± 4.39		87.67 ± 7.12	
% aphids showing E2	10	6		42	*	24		13	
Nr. of probes	27.05 ± 2.11	43.67 ± 3.79	***	34.16 ± 3.34		46.67 ± 2.87	***	41.78 ± 3.18	***
Test aphid:	**T**	**T(Nr:0)**		**T(Nr:1)**		**T(Macro)**		**T(Myzus)**	
Nr:0	**(n=20)**	**(n=18)**		**(n=23)**		**(n=21)**		**(n=22)**	
Duration first NP	366 ± 115	154 ± 41		519 ± 274		423 ± 95		337 ± 64	
Time to 1st visible E#	5879 ± 1007	5239 ± 901		7485 ± 1237		5814 ± 8120		4706 ± 553	
Contribution E1 to E(%)	1.64 ± 0.55	7.24 ± 4.29		12.91 ± 6.05	**	5.73 ± 3.90		21.37 ± 7.50	*

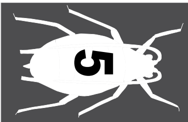


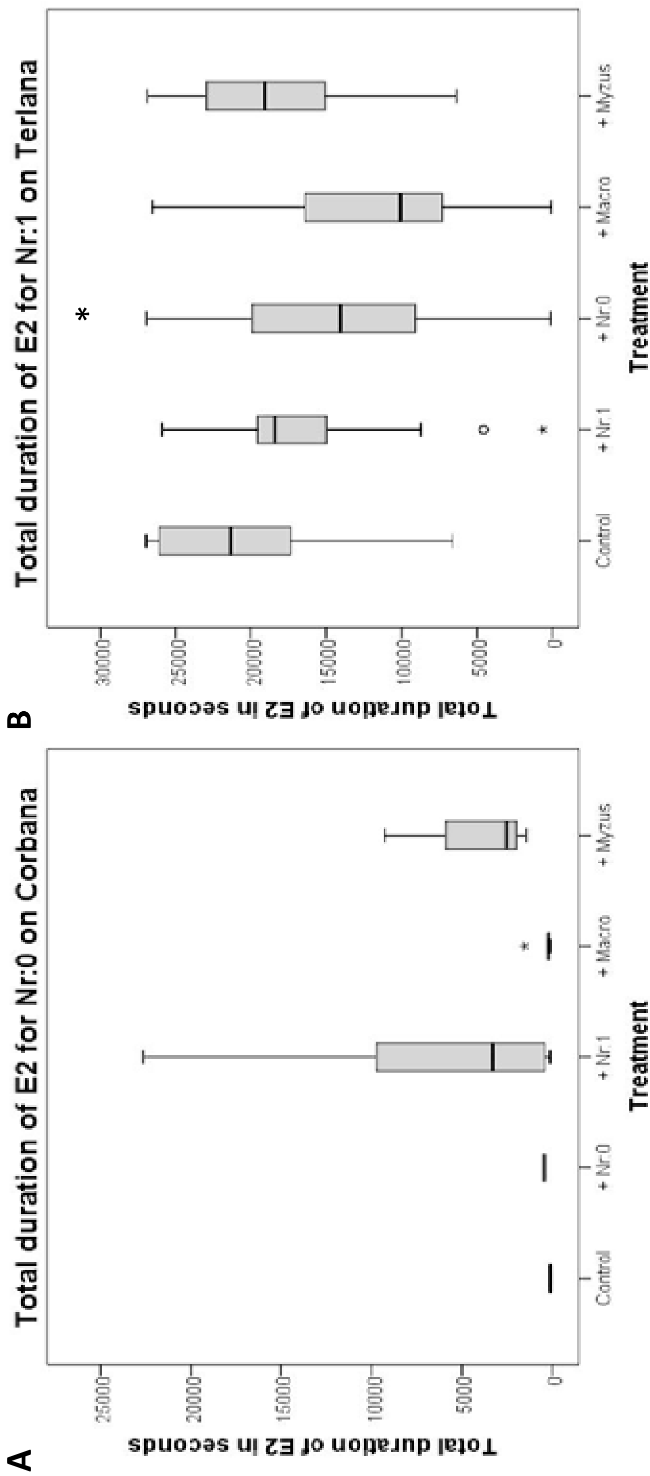
% aphids showing E2	100		86		87		100		86	
Nr. of probes	8.45 ± 1.53		12.29 ± 2.31		11.78 ± 1.64		11.00 ± 2.20		16 ± 2.60	*
Test aphid:	**C**		**C(Nr:1)**		**C(Nr:0)**		**C(Macro)**		**C(Myzus)**	
Nr:1	**(n=20)**		**(n=18)**		**(n=20)**		**(n=18)**		**(n=19)**	
Duration first NP	464 ± 119		368 ± 130		519 ± 181		647 ± 201		402 ± 77	
Time to 1st visible E#	4181 ± 694		5881 ± 1236		10232 ± 1511	***	6900 ± 1154		6347 ± 832	
Contribution E1 to E(%)	5.09 ± 1.55		15.76 ± 6.74		40.94 ± 10.03	***	47.39 ± 9.61	***	5.70 ± 1.90	
% aphids showing E2	100		94		65	**	72	*	95	
Nr. of probes	7.30 ± 1.55		16.94 ± 3.62		22.30 ± 3.39	***	30.39 ± 3.54	***	18.05 ± 2.27	***
Test aphid:	**T**		**T(Nr:1)**		**T(Nr:0)**		**T(Macro)**		**T(Myzus)**	
Nr:1	**(n=19)**		**(n=22)**		**(n=22)**		**(n=20)**		**(n=17)**	
Duration first NP	400 ± 112		199 ± 69		217 ± 35		719 ± 220		166 ± 74	
Time to 1st visible E#	3959 ± 716		4089 ± 576		7242 ± 1171		5499 ± 1131		5315 ± 1288	
Contribution E1 to E(%)	4.69 ± 1.95		7.92 ± 2.88		22.10 ± 7.89	*	19.97 ± 6.20	**	17.89 ± 8.04	
% aphids showing E2	100		100		82		90		88	
Nr. of probes	9.05 ± 1.74		15.18 ± 1.75	*	13.95 ± 1.72		22.25 ± 3.51	**	16.53 ± 3.64	

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Table 3: Performance parameters (mean ± SEM) for the *N. ribisnigri* population Nr:0 and Nr:1 on *L. sativa* cultivars, Corbana (C) and Terlana (T) as single aphids or in a group of Nr:0, Nr:1, *M. euphorbiae* (Macro) or *M. persicae* (Myzus). Pairwise comparisons were made for performance parameters between plants without other aphids and plants with other aphids, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the four comparisons made by setting α = 0.013. * = P < 0.013; ** = P < 0.005; *** = P < 0.001.

Test aphid: Nr:0	**C**	**C(Nr:0)**	**C(Nr:1)**		**C(Macro)**		**C(Myzus)**	
Survival (%)	0	0	15 ± 4.34	**	7 ± 3.00		4 ± 2.34	
Age at death	3.17 ± 0.12	3.36 ± 0.15	4.26 ± 0.32	**	3.88 ± 0.19	*	4.21 ± 0.23	**
Development time			13.13 ± 0.52		12.00 ± 0.56		12.00 ± 0.00	
Test aphids: Nr:0	**T**	**T(Nr:0)**	**T(Nr:1)**		**T(Macro)**		**T(Myzus)**	
Survival (%)	90 ± 3.08	94 ± 2.20	88 ± 3.09		91 ± 2.70		93 ± 3.00	
Age at death	3.50 ± 0.42	3.83 ± 0.79	4.23 ± 0.46		3.06 ± 0.43		3.30 ± 0.49	
Development time	8.63 ± 0.10	8.24 ± 0.20	9.97 ± 0.13	***	9.25 ± 0.12	***	8.79 ± 0.23	
Test aphid: Nr:1	**C**	**C(Nr:1)**	**C(Nr:0)**		**C(Macro)**		**C(Myzus)**	
Survival (%)	84 ± 3.46	78 ± 3.45	87 ± 3.33		87 ± 3.94		89 ± 3.12	
Age at death	3.38 ± 0.32	3.27 ± 0.39	4.45 ± 0.57		3.98 ± 0.37		3.61 ± 0.33	
Development time	9.32 ± 0.14	9.28 ± 0.14	9.32 ± 0.12		10.03 ± 0.16	*	10.25 ± 0.25	*
Test aphid: Nr:1	**T**	**T(Nr:1)**	**T(Nr:0)**		**T(Macro)**		**T(Myzus)**	
Survival (%)	88 ± 3.37	80 ± 4.34	87 ± 3.67		92 ± 2.25		91 ± 2.70	
Age at death	3.38 ± 0.59	3.38 ± 0.30	3.89 ± 0.49		5.00 ± 0.94		4.44 ± 0.64	
Development time	9.88 ± 0.19	9.28 ± 0.21	8.70 ± 0.13	***	10.31 ± 0.23		9.87 ± 0.13	




A
Total duration of E2 for Nr:0 on Corbana
Total duration of E2 in seconds
25000
20000
15000
10000
5000
0
Control
+ Nr:0
+ Nr:1
+ Macro
+ Myzus
Treatment
B
Total duration of E2 for Nr:1 on Terlana
Total duration of E2 in seconds
30000
25000
20000
15000
10000
5000
0
Control
+ Nr:1
+ Nr:0
+ Macro
+ Myzus
Treatment

C Total duration of E2 of Nr:1 on Corbana

Total duration of E2 in seconds

0 5000 10000 15000 20000 25000 30000

Control + Nr:0 + Nr:1 + Macro + Myzus

Treatment

D Total duration of E2 for Nr:0 on Terlana

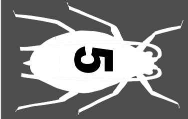
Total duration of E2 in seconds

0 5000 10000 15000 20000 25000 30000

Control + Nr:0 +Nr:1 + Macro + Myzus

Treatment

Figure 1: The total duration of EPG parameter E2 (phloem ingestion) (median, first and third quartiles and range) for *N. ribnisnigri* Nr:0 on Corbana (A), Nr:0 on Terlana (B), Nr:1 on Corbana (C) and Nr:1 on Terlana (D) as single aphids (Control) or in a group of Nr:0, Nr:1, *M. euphorbiae* (Macro) and *M. persicae* (Myzus). Pairwise comparisons were made for total duration of E2 between plants without other aphids and plants with other aphids, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the four comparisons made by setting $\alpha = 0.013$. Bars with bold asterisks differ significantly from the control plants. Outliers are plotted as asterisks and circles. * = $P < 0.013$; ** = $P < 0.005$; *** = $P < 0.001$.



Effects of *Macrosiphum euphorbiae*

No significant differences in EPG parameters were found between Nr:0 aphids feeding in a group of *M. euphorbiae* and single Nr:0 aphids on Terlana (Table 2, Appendix 2 and Figure 1B). Development time was higher in a group of *M. euphorbiae* compared to single Nr:0 aphids (P = 0.001) (Table 3). On Corbana Nr:0 aphids in a group of *M. euphorbiae* showed a higher number of probes compared to single Nr:0 aphids (P < 0.0001). In the performance test the age at death was higher in an *M. euphorbiae* group compared to single Nr:0 aphids on Corbana (P = 0.006).

Nr:1 aphids in a group of *M. euphorbiae* on Terlana, spent less time on phloem phase (P = 0.001), a higher percentage of the phloem phase was spent on salivation (P = 0.003) and the number of probes was higher (P = 0.003) compared to single Nr:1 aphids (Table 2 and Figure 1D). Additionally, the percentage of aphids displaying phloem ingestion was lower in an *M. euphorbiae* group compared to single aphids (P = 0.010). No significant difference was found in performance (Table 3). On Corbana Nr:1 aphids in an *M. euphorbiae* group displayed a higher number of probes (P < 0.0001) and less time was spent on phloem events (P < 0.0001), compared to single Nr:1 aphids. The percentage of time spent on salivation in the phloem phase was 5% for single Nr:1 aphids versus 47% for Nr:1 aphids in a *M. euphorbiae* group (P < 0.0001). The development time was longer in an *M. euphorbiae* group compared to single Nr:1 aphids Corbana (P = 0.007) (Table 3).

Effects of *Myzus persicae*

The number of probes was higher when Nr:0 aphids were feeding in a group of *M. persicae* on both cultivar Terlana (P = 0.008) and Corbana (P =0.001) compared to single Nr:0 aphids (Table 2). The age at death was higher in a group of *M. persicae* on Corbana compared to single Nr:0 aphids (P = 0.004) (Table 3). For Nr:0 on Terlana no significant differences were found between aphids in an *M. persicae* and single Nr:0 aphids.

No significant differences in EPG parameters were found between Nr:1 aphids on Terlana in an *M. persicae* group and single Nr:1 aphids. On Corbana Nr:1 aphids within an *M. persicae* group displayed a higher number of probes (P < 0.0001), took longer to display the first phloem ingestion (P = 0.012) and spent less time on xylem feeding (P = 0.006) compared to single Nr:1 aphids. Development time was longer for Nr:1 aphids in an *M. persicae* group compared to single Nr:1 aphids on Corbana (P = 0.009).

Discussion

Living in a group

Some aphid species are known to benefit from living in groups. *Aphis fabae* and *B. brassicae* are also known to benefit from living in a colony (Dixon & Wratten, 1971; Hayamizu, 1984). *Brevicoryne brassicae* showed enhanced growth and increased fecundity when living in a group, compared to solitary individuals and enhanced nutritional food quality is suggested as the underlying mechanism (Hayamizu, 1984). Sandström et al. (2000) showed that aphids are indeed able to enhance the nutritional quality of the phloem sap by increasing the concentration of amino acids and essential amino acids. However, this ability was shown to be species-specific; *S. graminum* and *Diuraphis noxia* Mordvilko were able to increase the nutritional quality of the phloem sap, while *R. padi* was not. Enhanced susceptibility was also found for a susceptible tomato cultivar after pre-infestation of *M. persicae,* the total duration of phloem ingestion was longer on the pre-infested plants, and saliva of aphids enhancing phloem ingestion or preventing wound responses of the plants were suggested as possible mechanisms (Civolani et al., 2010). Increased susceptibility was also observed for *M. persicae* on a susceptible potato cultivar and a susceptible peach cultivar after conspecific pre-infestation of *M. persicae* or of *M. euphorbiae* (Dugravot et al., 2007; Sauge et al., 2002). However, on potato plants these effects were only observed on previously infested leaves, systemic leaves showed a decrease in susceptibility, suggesting a systemic induced resistance triggered by pre-infestation (Dugravot et al., 2007). This difference in effect between local and systemic leaves was also found by Prado & Tjallingii (2007) for *B. brassicae* on local and systemic pre-infested leaves of broccoli. Weng et al. (2005) showed that the duration of pre-infestation can determine the effect on the feeding behaviour. Infestation of the first true leaf of wheat by *S. graminum* led to induced susceptibility after two days; however, after these two days induced resistance was observed.

In our study, there did not seem to be an effect of living in a group for Nr:1 and Nr:0 feeding in a group of the same biotype on Corbana and Terlana. Both Nr:0 on Corbana and Nr:1 on Terlana in a group of the same biotype, displayed a higher number of probes compared to single aphids, which might be caused by disturbance of other aphids in the group. However, the duration of phloem ingestion and performance did not differ.

Defence suppression in lettuce

Interestingly, on Corbana Nr:0 aphids benefit from the pre-infestation by Nr:1 aphids. No Nr:0 aphids survived the performance test on Corbana in the absence of Nr:1 aphids, whereas 15% survived and developed into adults in a group of feeding Nr:1 aphids. Additionally, a significantly higher proportion of Nr:0 aphids in the Nr:1 group displayed phloem ingestion. We hypothesise that Nr:1 aphids suppress the defence mechanism in Corbana, making it possible for part of the Nr:0 aphids to survive and develop on an Nr:0-resistant lettuce

plant. The Nr:1 aphids might accomplish this by the secretion into the phloem of salivary components that suppress the *Nr*-based resistance. The observation that only 15% of the aphids was positively affected, could result from suppression of the defence of only the sieve tube that the Nr:1 aphids were actually feeding on. We tested only a 1:10 ratio between Nr:0 and Nr:1 aphids. Investigation of other ratios could lead to a better understanding of the mechanisms operating.

Increased susceptibility of a resistant plant as a result of infestation by a virulent biotype was also found for *S. graminum* on winter wheat. Pre-infestation by a virulent biotype of this species led to increase population growth of an avirulent biotype on resistant winter wheat (Dorschner et al., 1987). In contrast, a virulent biotype of the same aphid species on barley did not alter the feeding behaviour of an avirulent biotype (Hays et al., 1999), which was also found for the combination of a virulent and avirulent biotype of *M. euphorbiae* on resistant and susceptible tomato (Hebert et al., 2007). The studies above only focussed on performance of aphids, no EPG data were collected.

The interaction of piercing/sucking insects with their host plant has analogies with pathogen-plant interactions, both triggering mainly the salicylic acid-dependent pathways and in some cases the JA and ethylene-dependent pathways (Walling, 2000). Additionally, aphids were shown to induce the expression of genes involved in defences against pathogens (De Vos et al., 2005). For pathogens the ability to overcome *R*-gene-based resistance is often attributed to the loss or modification of avirulence gene products, to evade the detection by the plant and/or suppressing plant defences through the injection of effectors, which is also hypothesised to be the underlying mechanism of aphid virulence (Hogenhout & Bos, 2011; Parker & Gilbert, 2004). It is unknown what mechanism is responsible for the virulence in Nr:1 aphids. Our data suggest that Nr:1 aphids are able to suppress the *Nr*-based defence mechanism in resistant lettuce that is effective against Nr:0 aphids. This suppression, however, seems to be local and not spreading systemically, because only 15% of the Nr:0 aphids was benefitting. Nr:1 aphids might, for example, only be able to suppress the defence of one sieve tube, or one bundle of sieve tubes. It is of interest to identify the effector in the saliva of Nr:1 aphids that is responsible for the virulence and suppression of resistance against Nr:0. Only a few aphid effectors triggering plant responses are known. De Vos & Jander (2009) identified proteinaceous elicitors with sizes ranging between 3 and 10 kD from *M. persicae* that is involved in inducing defences in *Arabidopsis thaliana*. Two candidate effectors, sharing features with known pathogen effectors, were identified by Bos et al. (2010), and induced defences in *Nicotiana benthamiana* resulting in decreased aphid fecundity. Another effector was shown to enhance *M. persicae* fecundity, therefore being advantageous for the colonisation of this aphids species (Bos et al., 2010; Pitino & Hogenhout, 2013). Orthologs of this effector produced by *Acyrthosiphon pisum* (Harr.) had no effect on *M. persicae* (Pitino & Hogenhout, 2013). Atamian et al. (2013) identified two *M. euphorbiae* effectors responsible for enhanced *M. persicae* fecundity in *N. benthamiana*.

Only one of these was able to increase the fecundity of *M. euphorbiae* in tomato.

Induced defence in lettuce

The effect of the feeding activities of Nr:1 aphids on Nr:0 aphids seem to be resistance dependent. As shown before, on Corbana Nr:0 aphids benefit from the simultaneous feeding by Nr:1 aphids, whereas on Terlana, in contrast, behaviour and performance of Nr:0 aphids is negatively affected by Nr:1 aphids. On Terlana duration of phloem ingestion was shorter and, probably as a consequence, the development time longer in the presence of Nr:1. About 10% of the Nr:0 aphids did not even start phloem ingestion in the presence of Nr:1, during the eight hours of EPG recording. Additionally, the Nr:0 aphids in an Nr:1 group salivated more, indicating difficulties to overcome the wound response of lettuce by producing more watery saliva. Similar results were found for Nr:1 aphids feeding on Terlana together with Nr:0 aphids; more salivation, longer development time and 18% of the aphids did not even start phloem ingestion. Both the virulent Nr:1 and avirulent Nr:0 aphids seem to increase defence in Terlana against the other biotype, which was not observed when feeding in a group of the same biotype. However, survival did not differ. Resistance induced as a result of pre-infestation by the same biotype was found in *Medicago truncatula* Gaertn. pre-infested by *Acyrthosiphon kondoi* Shinji (Klingler et al., 2005) and peach cv. Rubira pre-infested by *M. persicae* (Sauge et al., 2002), both decreasing the duration of phloem ingestion.

Nr:0 also increased resistance against Nr:1 on Corbana. Again, the phloem ingestion was shorter and the aphids salivated more. However, on the longer term, the survival and development time were not affected by feeding by Nr:0 aphids. We cannot prove that Nr:0 was actually feeding from Corbana, although stylet penetration should have taken place. The feeding behaviour of virulent *S. graminum* on resistant barley was also negatively affected by pre-infestation by an avirulent biotype; phloem ingestion was shorter and a larger number of probes was observed (Hays et al., 1999).

The effect of heterospecific aphid species on *N. ribisnigri*

Above we have discussed the effect of conspecific biotypes on each other. However, heterospecific aphid species might differently affect Nr:0 and Nr:1 aphids. Here, we tested the effect of *M. euphorbiae* and *M. persicae* on the behaviour and performance of *N. ribisnigri.* First, we tested the feeding behaviour and performance of *M. euphorbiae* and *M. persicae* on both Corbana and Terlana. Only minor differences were found in EPG parameters and performance for *M. euphorbiae* and no differences for *M. persicae* (Appendix 1). It is known that the *Nr*-gene confers partial resistance against certain *M. persicae* isolates and has no effect on *M. euphorbiae* (Reinink & Dieleman, 1989). The *M. persicae* biotype in our study was shown to be virulent on Corbana. For Nr:0 and Nr:1 aphids in combination with *M. euphorbiae* and *M. persicae*, differences were found in the

effect of the co-infesting aphid species, and also in the reaction of both biotypes to feeding by these species.

For Nr:1 aphids no positive effect was found when *M. euphorbiae* or *M. persicae* were feeding on the leaf as well. *Macrosiphum euphorbiae* seemed to increase defence in both Terlana and Corbana against Nr:1; the percentage of time spent on phloem salivation and number of probes was higher. On Terlana *M. euphorbiae* also caused a reduction in phloem ingestion. The increased defence in Terlana seems to be of short duration, because the development time and survival were not affected by *M. euphorbiae*. On Corbana, the development time was negatively affected by *M. euphorbiae*, indicating a long term increased defence by *M. euphorbiae*. For Nr:0 on Terlana *M. euphorbiae* also increased the development time, but no differences were found in EPG parameters, indicating a slower induction of defence against Nr:0 by *M. euphorbiae.* On Corbana only the number of probes were affected by *M. euphorbiae.* Higher numbers of probes were also caused by feeding by *M. persicae* on Terlana and Corbana. Dugravot et al. (2007) found a reduction in the number of probes for *M. persicae* on potato after previous infestation by *M. euphorbiae.*

Nr:1 was not affected by *M. persicae* on Terlana, whereas on Corbana the number of probes was higher and the development time longer due to feeding by *M. persicae*, indicating induced defence. For Nr:0 only the number of probes was influenced by feeding of *M. persicae* on both Corbana and Terlana.

As shown above, Nr:0 and Nr:1 react differently to the presence of *M. euphorbiae* and *M. persicae,* and *M. euphorbiae* and *M. persicae* might elicit different plant responses, therefore these interactions can be considered asymmetrical (Moran & Whitham, 1990). On the one hand, plant responses to aphids are aphid-species-specific (Messina et al., 2002), on the other hand, the response of an aphid species to aphid-feeding induced plant responses might also differ between aphid species (Smith & Boyko, 2007). Therefore, the alteration in plant responses upon aphid feeding, may affect the fitness of other aphids negatively, positively or not at all (Petersen & Sandström, 2001). This could be caused by the differences in composition of saliva between aphid species which might elicit different plant responses (Prado & Tjallingii, 1997). Asymmetrical interactions between aphids was also found for *D. noxia* and *R. padi* on winter wheat (Messina et al., 2002), *Monellia caryella* Fitch and *Melanocallis caryaefoliae* Davis on pecan (Petersen & Sandström, 2001) and for root aphid *Pemphigus betae* (Doane) and galling aphid *Hayhurstia atriplicis* (L.) on *Chenopodium album* plants ((Moran & Whitham, 1990).

Species of different taxonomical groups can also influence the feeding behaviour of aphids, for example by induction of susceptibility by plant growth-promoting rhizobacterium (Pineda et al., 2012) and induced/increased resistance by endophytes (Siegel et al., 1990), nematodes (Ogallo & McClure, 1996; Wurst & van der Putten, 2007) and whiteflies (Xue et al., 2010).

Conclusions

Nasonovia ribisnigri biotypes are differently affected in their behaviour on resistant and susceptible lettuce by the presence of conspecific and heterospecific aphids. Virulent biotype Nr:1 is able to suppress the absolute resistance in lettuce against Nr:0. It would be interesting to further study this resistance suppression, for example, by studying the attraction of Nr:0 aphids to plants infested by Nr:1 aphids and identify the possible component in the salivary secretion of virulent *N. ribisnigri* responsible for the resistance suppression. On the susceptible lettuce cultivar Terlana, the behaviour and performance of both Nr:0 and Nr:1 aphids was negatively affected by the other biotype. On resistant cultivar Corbana, Nr:1 was negatively affected by feeding/stylet penetration of the Nr:0 biotype and either of the two heterospecific aphid species, but not by aphids of Nr:1 biotype itself, indicating the induction of defence by the other aphids against Nr:1 aphids. The level of these induced defences was such that it affected feeding behaviour and in some cases development time, but not survival. More information is needed about the effectors in the saliva of the aphids tested here, that are likely responsible for the suppression and induction of defence in lettuce, to better understand the mechanism of virulence in *N. ribisnigri*.

Acknowledgements

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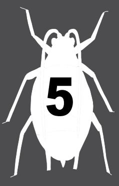


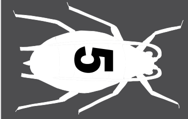
Appendix 1: EPG parameters (mean ± SEM) of *M. euphorbiae* (Macro) and *M. persicae* (Myzus) on *L. sativa* cultivars, Corbana (C) and Terlana (T). Pairwise comparisons were analysed by the Mann-Whitney U test. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, C= pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties. * = P < 0.05; ** = P < 0.005; *** = P < 0.001.

	Macrosiphum euphorbiae							***Myzus persicae***					
	Corbana (n=21)			**Terlana (n =19)**				**Corbana (n=22)**			**Terlana (n=19)**		
Duration first NP	294	±	156	200	±	125	*	131	±	44	150	±	39
Duration of NP before 1st visible E	1510	±	380	1681	±	296		1114	±	454	645	±	124
Nr. of probes to the 1st E1	4.90	±	0.53	5.68	±	0.76		7.09	±	1.24	5.05	±	0.63
Time to 1st visible E#	3775	±	609	4593	±	1244		4636	±	861	4288	±	569
Total duration of E	16267	±	1500	16252	±	1919		14456	±	1647	11496	±	1877
Total duration of E1	1580	±	304	1404	±	322		1654	±	415	1870	±	364
Contribution E1 to E(%)	13	±	3	15	±	5		25	±	7	29	±	7
Total duration of single E1	724	±	121	938	±	192		1378	±	433	1331	±	337
% aphids showing E2	100			100				91			95		
Total duration of E2	14687	±	1624	14847	±	2028		14083	±	1837	10161	±	2014
Time to 1st E2#	6126		1434	8592	±	1900		8936	±	1859	9811	±	1660
Time to 1st sustained E2#	9437	±	1697	9538	±	1904		7382	±	1429	11118	±	1656
Nr. of E2 periods	2.90	±	0.34	1.95	±	0.26		1.82	±	0.27	2.16	±	0.34
Nr. of sustained E2 periods	2.05	±	0.20	1.58	±	0.21	*	1.41	±	0.20	1.37	±	0.23
Total duration of NP	3457	±	645	3736	±	613		2051	±	538	2074	±	394
Total duration of C	7775	±	985	7718	±	1255		11117	±	1335	14446	±	1651
Total duration of F								1653	±	283	2973	±	1149
Total duration of G	2483	±	478	2079	±	230		2325	±	488	1993	±	249
Nr. of probes	9.86	±	1.38	10.32	±	1.60		16.82	±	2.54	14.68	±	1.97
Nr. of short probes (C < 3 minutes)	4.76	±	1.15	5.32	±	1.05		8.05	±	1.87	5.05	±	1.05

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

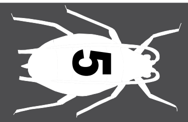
Appendix 2: EPG parameters (mean ± SEM) of *N. ribisnigri* population Nr:0 and Nr:1 on *L. sativa* cultivars, Corbana (C) and Terlana (T) as single aphids or in a group of Nr:0, Nr:1, *M. euphorbiae* (Macro) and *M. persicae* (Myzus). Pairwise comparisons were made for EPG parameters between plants without other aphids and plants with other aphids, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the four comparisons made by setting α = 0.013. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, C= pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties. * = P < 0.013; ** = P < 0.005; *** = P < 0.001.

Test aphid: Nr:0	C	C+Nr:0	C+Nr:1	C+Macro	C+Myzus
Duration of NP before 1st visible E	2786 ± 688	5671 ± 1049	2728 ± 685	3614 ± 721	5699 ± 864 **
Time to 1st E2[#]	13640 ± 5614	11701	12103 ± 2611	12087 ± 2365	8164 4349
Time to 1st sustained E2[#]			12451 ± 3648	7055	14532 4696
Total duration of E	434 ± 97	393 ± 97	3580 ± 1467	372 ± 115	1594 ± 718
Total duration of E1	424 ± 96	361 ± 78	821 ± 209	254 ± 47	814 ± 270
Total duration of single E1	403 ± 94	348 ± 73	405 ± 106	182 ± 33	490 ± 130
Nr. of E2 periods	0.11 ± 0.07	0.06 ± 0.06	0.68 ± 0.24	0.29 ± 0.12	0.43 ± 0.28
Nr. of sustained E2 periods	0.00 0.00	0.00 0.00	0.32 ± 0.13	0.05 ± 0.05	0.22 ± 0.13
Nr. of probes to the 1st E1	7.83 ± 1.16	17.86 ± 2.16 ***	12.67 ± 1.84	18.74 ± 2.55 **	15.59 ± 2.10 *
Total duration of NP	7095 ± 944	9572 ± 820	6282 ± 822	8635 ± 893	9531 ± 765
Total duration of C	19283 ± 769	16957 ± 707	16932 ± 1179	17543 ± 664	16091 ± 748 *



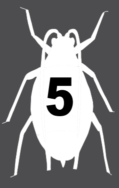
	T	T+Nr:0		T+Nr:1		T+Macro		T+Myzus	
Total duration of F		3896				890 ± 240		4990	
Total duration of G	2881 ± 556	3150 ± 813		3476 ± 759		3082 ± 765		2408 ± 372	
Nr. of short probes (C < 3 min)	11.58 ± 1.84	25.89 ± 3.51	**	17.84 ± 2.78		26.90 ± 2.74	***	24.78 ± 2.97	***
Test aphid: Nr:0	**T**	**T+Nr:0**		**T+Nr:1**		**T+Macro**		**T+Myzus**	
Duration of NP before 1st visible E	1619 ± 255	2281 ± 609		1884 ± 358		2222 ± 491		2107 ± 402	
Time to 1st E2#	6414 ± 1070	7022 ± 1242		8718 ± 1484		6519 ± 1026		6438 ± 837	
Time to 1st sustained E2#	6414 ± 1070	7419 ± 1366		8984 ± 1498		7229 ± 1079		6063 ± 711	
Total duration of E	21933 ± 1083	19954 ± 1738		15513 ± 1662	**	20280 ± 1429		17254 ± 2050	
Total duration of E1	294 ± 78	723 ± 249		664 ± 98	**	382 ± 140		857 ± 245	
Total duration of single E1	197 ± 58	267 ± 62		427 ± 120		184 ± 31		339 ± 90	
Nr. of E2 periods	1.20 ± 0.12	1.52 ± 0.37		1.30 ± 0.20		1.38 ± 0.13		1.45 ± 0.26	
Nr. of sustained E2 periods	1.15 ± 0.08	1.00 ± 0.14		1.04 ± 0.12		1.05 ± 0.08		1.14 ± 0.21	
Nr. of probes to the 1st E1	8.15 ± 1.51	8.11 ± 1.76		7.23 ± 1.00		7.67 ± 1.17		8.67 ± 0.96	
Total duration of NP	1753 ± 329	2786 ± 654		2611 ± 465		2834 ± 659		3692 ± 765	
Total duration of C	4923 ± 848	7466 ± 1344		8818 ± 1150	*	5151 ± 873		7758 ± 1275	
Total duration of F		3049 ± 462				3858		1984 ± 1656	
Total duration of G	1912 ± 211	4532 ± 2020		3882 ± 856		2462 ± 497		2569 ± 298	
Nr. of short probes (C < 3 min)	4.85 ± 1.19	6.10 ± 1.46		6.30 ± 1.08		6.76 ± 1.48		8.91 ± 1.96	

Test aphid: Nr:1	C	C+Nr:1	C+Nr:0		C+Macro		C+Myzus	
Duration of NP before 1st visible E	993 ± 168	2454 ± 636	3678 ± 666	***	2630 ± 489	**	2980 ± 576	**
Time to 1st E2#	5563 ± 1015	6194 ± 1360	12842 ± 1783	**	9829 ± 1886		8652 ± 1229	*
Time to 1st sustained E2#	6673 ± 1122	9687 ± 1903	12744 ± 2138		13111 ± 2737		8652 ± 1229	
Total duration of E	20634 ± 1249	15530 ± 2158	9760 ± 2145	***	6303 ± 1872	***	17039 ± 1185	
Total duration of E1	773 ± 186	617 ± 147	1055 ± 189		637 ± 118		701 ± 155	
Total duration of single E1	667 ± 235	461 ± 136	576 ± 142		447 ± 120		401 ± 106	
Nr. of E2 periods	4.20 ± 0.34	7.06 ± 1.23	13.71 ± 2.79	*	10.28 ± 1.47	***	11.61 ± 1.91	**
Nr. of sustained E2 periods	1.75 ± 0.20	1.72 ± 0.24	0.90 ± 0.18	**	1.50 ± 0.35		1.37 ± 0.14	
Nr. of probes to the 1st E1	1.50 ± 0.15	1.17 ± 0.17	0.65 ± 0.15	***	0.56 ± 0.15	***	1.37 ± 0.14	
Total duration of NP	1788 ± 360	4790 ± 1082	5260 ± 721	***	7135 ± 974	***	3815 ± 559	**
Total duration of C	4984 ± 743	7588 ± 1191	12589 ± 1335	***	13566 ± 1262	***	7596 ± 1041	
Total duration of F	2697	1925	3620		3403 ± 1181			
Total duration of G	4198 ± 1178	2341 ± 703	4497 ± 912		3100 ± 1502		1579 ± 123	*
Nr. of short probes (C<3 min)	3.85 ± 1.11	9.61 ± 2.47	11.10 ± 2.28	*	16.94 ± 3.07	***	10.74 ± 1.63	***



Test aphid: Nr:1	T			T+Nr:1			T+Nr:0				T+Macro				T+Myzus		
Duration of NP before 1st visible E	1391	±	271	1825	±	332	1701	±	383		3413	±	915		1986	±	576
Time to 1st E2#	5373	±	1002	7684	±	1030	8533	±	1420		6337	±	1022		6167	±	1390
Time to 1st sustained E2#	5925	±	1094	9395	±	1317	9180	±	1495		7713	±	1187		6433	±	1350
Total duration of E	21405	±	1205	17307	±	1367	13370	±	1959	**	11799	±	1975	**	17523	±	1907
Total duration of E1	767	±	262	739	±	119	662	±	125		1015	±	348		1025	±	380
Total duration of single E1	250	±	40	356	±	69	372	±	69		714	±	348		624	±	196
Nr. of E2 periods	6.95	±	1.45	6.36	±	0.93	6.40	±	0.83		8.16	±	1.37		9.53	±	2.41
Nr. of sustained E2 periods	1.47	±	0.14	1.86	±	0.20	1.09	±	0.16		1.65	±	0.27		1.65	±	0.28
Nr. of probes to the 1st E1	1.37	±	0.11	1.27	±	0.12	0.86	±	0.14	*	1.25	±	0.22		1.41	±	0.21
Total duration of NP	1952	±	393	3507	±	395	3264	±	524		6574	±	1106	***	3162	±	649
Total duration of C	4563	±	801	6978	±	811	11156	±	1428	***	10295	±	1104	***	7484	±	1368
Total duration of F	2610	±	1471								2178	±	772				
Total duration of G	2877	±	820	3045	±	611	4894	±	1304		2519	±	375		1786	±	396
Nr. of short probes (C < 3 min)	5.16	±	1.20	9.00	±	1.20	6.00	±	0.87		12.80	±	2.44	*	9.47	±	2.49

Time to first E, E2 and first sustained E2 was measured from the start of the first probe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Chapter 6

The effect of rearing history on behaviour and performance of two virulent *Nasonovia ribisnigri* populations on two lettuce cultivars

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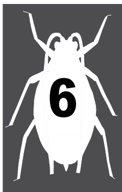
Abstract

Many aphid species have become virulent to host plant resistance, which limits the sustainability of insect resistance breeding. When this adaptation to resistant plants is associated with fitness costs for the aphids, however, virulence can be lost in the absence of resistant plants. For two populations of the lettuce aphid *Nasonovia ribisnigri* (Mosely) (Homoptera: Aphididae) virulent to *Nr*-gene based resistance we evaluated whether virulence was lost on a susceptible lettuce, *Lactuca sativa* L., and assessed possible costs of virulence. The feeding behaviour and performance of these aphid populations, reared and tested on susceptible and resistant lettuce, was investigated. The rearing plant affected feeding behaviour and performance of the aphids. Both temporary reduction and long-term loss of virulence was found. The total duration of phloem intake was shorter after being reared on susceptible lettuce and tested on resistant lettuce. Additionally, one population had a lower survival on resistant lettuce after being reared on susceptible lettuce. There were also indications of fitness costs of the virulence in both populations.

Introduction

Aphids are one of the most economically important groups of agricultural pests, attributed to the combination of their specific feeding and reproductive traits (Guerrieri & Digilio, 2008). The major cause of the pest status of aphids is their high reproductive rate and short generation time (Dreyer & Campbell, 1987). The evolution of virulence to plant resistance by aphids is a growing problem in agriculture. The rate of evolution of virulence to plant resistance in herbivores and pathogens can be driven by the level and mechanism of the resistance, determining the strength of selection, genetic variation, inheritance and life history traits of the herbivores/pathogens, the ecological setting and the linkage between herbivore/pathogen fitness and virulence (Parker & Gilbert, 2004; Wilhoit et al., 1992). Many aphid species have become virulent to host plant resistance, and often need only a few years to adapt to resistance, which causes problems for breeding of resistant plants (Haley et al., 2004; Hill et al., 2012; Shufran, 2011; Wilhoit et al., 1992). Aphids are able to reproduce both clonally (parthenogenesis) and sexually (Dixon, 1990). On the one hand the spread of favourable mutations is facilitated by parthenogenesis, and on the other hand genetic variation is facilitated by recombination through sexual reproduction (Wilhoit et al., 1992). Additionally, the high fecundity and short generation times enhance the probability for new genotypes to emerge (Wilhoit et al., 1992).

Virulence enables insects to colonise initially resistant plants. However, when the adaptation to these resistant plants is associated with fitness costs, virulence might be lost in the absence of exposure to resistant plants (Zhang et al., 2011). Van der Plank (1984) postulated that on susceptible plants there will be selection against unnecessary virulence, because of fitness costs associated with the virulence. Fitness costs of adaptation to resistant plant varieties were found in several plant attackers, such as bacteria, nematodes and insects (Castagnone-Sereno et al., 2007; Pallipparambil et al., 2010; Vera Cruz et al., 2000; Zhang et al., 2011). In the case of effector-triggered immunity in plant-pathogen interactions, an avirulence protein of a pathogen that is recognised by a product of an *R* gene in a plant also promotes colonisation by the pathogen (Hogenhout et al., 2009; Jones & Dangl, 2006). Therefore, there is a trade-off for the pathogen when losing an avirulence protein: there is no/reduced recognition by the plant, but at the same time the pathogen will have a reduced colonisation ability (Zhang et al., 2011). If the adaptation to certain *R*-genes has fitness costs for the plant attacker, this will influence whether the attacker will adapt to the resistance and if it adapts, fitness costs will affect the rate at which the adaptation spreads through the attacker population (Zhang et al., 2011). The fitness costs of virulence can, therefore, help to predict the durability of *R*-gene-based resistance in plants (Leach et al., 2001).



The black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely) is a specialist herbivore on lettuce. This aphid causes dramatic economic losses in the lettuce industry, and is therefore an important pest of lettuce (McCreight, 2008). Host-plant resistance conferred by the *Nr*-gene is the best measure available to control this aphid species, which provides

near-complete resistance in terms of reduction or absence of phloem ingestion and high mortality of the aphids (Chapter 2)((Reinink & Dieleman, 1989; Van Helden & Tjallingii, 1993). Since 2007, virulent biotypes of *N. ribisnigri* (Nr:1 biotypes) have been reported infesting resistant lettuce, making the *Nr*-resistance ineffective (Thabuis et al., 2011). An Nr:1 population from Germany performed equally well on resistant and susceptible lettuce and did not seem to be affected by the *Nr*-resistance (Chapter 2). In a previous study it was shown that there is large variation in virulence among different populations of *N. ribisnigri*, however, it is unclear what caused this variation (Chapter 3). A possible cause mentioned was the loss of virulence of certain populations during the rearing period on susceptible lettuce plants that lack the *Nr*-gene.

The objective of this study is to assess whether virulence was lost on a susceptible lettuce and to evaluate possible costs of virulence to the *Nr*-resistance in lettuce. We investigated two virulent (Nr:1) *N. ribisnigri* populations, an Nr:1 population originating from Germany (Nr:1 Ge), previously shown to be highly virulent, and an Nr:1 population originating from Belgium (Nr:1 Be), previously shown to be semi-virulent (Chapter 3). The loss of virulence was tested by studying the effect of rearing virulent *N. ribisnigri* populations on susceptible lettuce. The behaviour of both aphid populations reared and tested on both susceptible and resistant lettuce was evaluated, by studying the feeding behaviour and stylet penetration on resistant and susceptible lettuce cultivars, using the electrical penetration graph (EPG) technique, to assess whether there was a reduction in virulence. Additionally, the performance of the aphid populations on resistant and susceptible lettuce cultivars was analysed in terms of survival and development time until adulthood. The cost of virulence was evaluated by comparing the collected EPG and performance data of both Nr:1 Ge and Nr:1 Be reared on resistant lettuce, and tested on resistant and susceptible lettuce. Additionally, the performance data of both Nr:1 Ge and Nr:1 Be was compared to the performance data of Nr:0.

Materials and Methods

Plants and Aphids

The plants used in the experiments were two *Lactuca sativa* cultivars, Corbana (Nr:0- resistant) and Terlana (Nr:0-susceptible). The plants were grown in a greenhouse compartment at 18-20°C, 60% humidity and L14/D10 photoperiod, and treated with ENTONEM (a formulation of the entomopathogenic nematode, *Steinernema feltiae*, www.koppert.com) once a week, to control larvae of sciarid flies. Three-week-old plants were used for the experiments.

Two different populations of *N. ribisnigri* biotype Nr:1 were used in the experiments; Nr:1 aphids originating from Germany (Ge) and Nr:1 aphids originating from Belgium (Be), all reared in a greenhouse compartment at 18-20 °C, 60% RH and L14/D10 photoperiod. For details about the origin of these populations see Chapter 3. The Nr:1 populations were

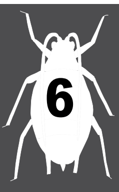
reared on both Nr:0-susceptible *L. sativa* cultivar Terlana and Nr:0-resistant *L. sativa* cultivar Corbana (Enza Zaden), for two months prior to the experiment (5-6 generations), creating two separate colonies per Nr:1 population reared on different lettuce cultivars. In the colonies both winged and wingless adults were present.

An Nr:0 population, reared on Nr:0-susceptible *L. sativa* cultivar Terlana in the same greenhouse compartment as the Nr:1 populations, was used as control in the performance test.

EPG recording

The electrical penetration graph technique (EPG) was used to study the effect of the rearing plant on the behaviour of the two *N. ribisnigri* Nr:1 populations on *L. sativa* cultivars Corbana and Terlana during eight hours. The EPG technique allows the electrical recording of the penetration and feeding behaviour of aphids and other piercing-sucking insects, by making a plant and a piercing insect part of an electrical circuit, realised by inserting an electrode in the soil near the plant and attaching a thin gold wire to the insect's dorsum (Tjallingii, 1988). For details on the EPG methods used, see Chapter 2. Winged adult aphids (alatae) were randomly collected from the rearing, to obtain a general view on the behaviour of alatae within a population. The EPG patterns distinguished in this study were: stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F). The first phloem phase appearing in the EPG recording will be referred to as the first visible phloem event.

For every *N. ribisnigri* colony (four in total) x *L. sativa* cultivar (Corbana and Terlana) combination 20 replicates (individual aphids on separate plants) were recorded. Plants and aphids were only used once. Incomplete recordings, due to death or escape of aphids, were excluded from the analyses, leaving between 17 and 20 replicates for every aphid colony x cultivar combination.



Performance test

Performance of the *N. ribisnigri* Nr:1 Ge and Be populations on Corbana and Terlana was measured by assessing nymph survival and development time from nymph to the adult stage. About 150 *N. ribisnigri* alatae per population were kept in Petri dishes on leaves of *L. sativa* cultivar Corbana or Terlana, depending on the rearing origin (either Corbana or Terlana) of the aphids. The next day new born nymphs were transferred into clip cages on 3-week-old plants, five nymphs per cage, two cages per plant, on separate leaves (third and fourth fully expanded leaf). Mortality and development time were recorded daily. Experiments were conducted in a greenhouse compartment at 18-20°C, 60% humidity and L14/D10 photoperiod. Four aphid colonies were tested; Nr:1 Ge reared on Corbana, Nr:1 Ge reared on Terlana, Nr:1 Be reared on Corbana and Nr:1 Ge reared on Corbana. Per aphid colony, 10 plants per cultivar were tested, with in total 20 clip-cages and 100 aphids.

Data on performance of Nr:0 on Terlana was collected and used for comparisons with performance data of both Nr:1 Ge and Nr:1 Be on Terlana.

Statistics

EPG parameters were calculated per aphid using the EPG analysis worksheet created by Sarria et al. (2009). The Mann-Whitney U test was used for pairwise comparisons between the different aphid colonies within one aphid population on one cultivar. Fisher's exact test was used to analyse differences in the proportion of aphids that showed phloem intake.
For aphid performance a clip-cage was considered one replicate and the results were first calculated per clip-cage. The means and standard error were calculated over all clip-cages within one treatment. The Mann-Whitney U test was used to test for significant differences in aphid mortality (in the performance experiment), time until mortality and development time. All statistical tests were performed with IBM SPSS Statistics version 19.

Results

Nr:1 Ge colonies on Corbana

Several differences in EPG parameters appeared when comparing the Nr:1 Ge aphids reared on either Corbana or Terlana, tested on Corbana plants (Table 1). Aphids reared on Terlana salivated more ($P = 0.007$) and the phloem phase, which was shorter ($P = 0.021$), consisted for a higher percentage of salivation ($P < 0.0001$) compared to the aphids that had been reared on Corbana. The duration of phloem ingestion was shorter for the aphids reared on Terlana, compared to the ones reared on Corbana ($P = 0.039$) (Figure 1A). Additionally, the total duration of non-penetration was longer ($P < 0.0001$) and the number of probes higher ($P = 0.001$) for the aphids reared on Terlana, compared to the aphids reared on Corbana.
No significant differences were found in the performance parameters between Nr:1 Ge aphids reared on either Corbana or Terlana, tested on Corbana plants (Table 2).

Nr:1 Ge colonies on Terlana

Several differences in EPG parameters were found when comparing the Nr:1 Ge aphids reared on either Corbana or Terlana, tested on Terlana plants (Table 1). Aphids reared on Corbana took longer to initiate the first probe compared to aphids reared on Terlana ($P = 0.009$). The total duration of phloem events and phloem ingestion was shorter for aphids reared on Terlana compared to aphids reared on Corbana (both $P = 0.034$) (Figure 1A). Aphids reared on Terlana spent less time on xylem ingestion compared to aphids reared on Corbana ($P = 0.039$).

The development time until adult on Terlana was two days longer for aphids reared on Corbana compared to those reared on Terlana ($P < 0.0001$) (Table 2).

Table 1: EPG parameters (mean ± SEM) of *N. ribisnigri* aphids from population Nr:1 Ge reared on resistant lettuce Corbana (Nr:1 Ge-Corbana) and susceptible lettuce cultivar Terlana (Nr:1 Ge-Terlana) tested on Corbana and Terlana. Abbreviations of parameters: C - stylet pathway; NP - non-penetration period; E - phloem phase; E1 - phloem salivation; E2 - phloem ingestion; G - xylem ingestion; F - penetration difficulties. The Mann-Whitney U test was used to analyse differences between populations with different rearing history, and between Nr:1 Ge-Corbana on Corbana and Terlana, * = P < 0.05; ** = P < 0.005; *** = P < 0.001. Significance of differences in proportion (%) of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds.

	Test plant Corbana			Test plant Terlana			Terlana vs Corbana
	Nr:1 Ge-Corbana	Nr:1 Ge-Terlana		Nr:1 Ge-Corbana	Nr:1 Ge-Terlana		
Duration first NP	231 ± 48	928 ± 234		195 ± 70	687 ± 161	**	*
Duration of NP before 1st visible E	1783 ± 589	3225 ± 834	*	1397 ± 396	1668 ± 327		
Nr. of probes to the 1st E1	6.06 ± 1.19	7.22 ± 1.67		5.35 ± 1.12	5.53 ± 1.04		
Time to 1st visible E#	8812 ± 1666	5999 ± 1322		3979 ± 832	5184 ± 1036		*
Total duration of E	17562 ± 1484	11084 ± 1910	*	20115 ± 1438	15387 ± 1585	*	
Total duration of E1	342 ± 73	1086 ± 328	**	481 ± 108	1107 ± 342		
Contribution E1 to E (%)	2 ± 0	32 ± 9	***	3 ± 1	14 ± 6		
Total duration of single E1	170 ± 45	632 ± 119	**	366 ± 91	476 ± 126		
% aphids showing E2	100	78		100	94		
Time to 1st E2#	9108 ± 1655	7723 ± 1831		4557 ± 1042	6567 ± 1232		*
Time to 1st sustained E2#	9260 ± 1689	11491 ± 2575		5549 ± 1102	6700 ± 1204		
Nr. of E2 periods	1.94 ± 0.18	1.68 ± 0.43		2.12 ± 0.21	2.18 ± 0.29		
Nr. of sustained E2 periods	1.59 ± 0.17	1.21 ± 0.3		1.59 ± 0.17	1.88 ± 0.21		
Total duration of NP	2001 ± 583	6276 ± 1152	***	2269 ± 656	3417 ± 683		
Total duration of C	8309 ± 1212	10771 ± 1129		6502 ± 982	9068 ± 1440		
Total duration of F		1179 ± 917		1096	3602 ± 1290		
Total duration of G	2631 ± 428	1947 ± 259		2822 ± 499	1405 ± 309	*	
Nr. of probes	7.06 ± 1.28	18.16 ± 2.28	***	7.65 ± 1.41	9.82 ± 1.32		
Nr. of short probes (C < 3 min)	2.94 ± 1.04	8.47 ± 1.45	***	3.29 ± 0.91	3.82 ± 0.64		

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe

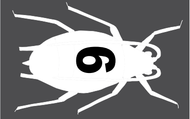
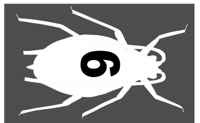


Table 2: Performance (mean ± SEM) of *N. ribisnigri* population Nr:1 Ge reared on resistant lettuce Corbana (Nr:1 Ge-Corbana) and susceptible lettuce cultivar Terlana (Nr:1 Ge-Terlana) tested on Corbana and Terlana. The Mann-Whitney U test was used to analyse differences between populations with different rearing history, Nr:1 Ge-Corbana on Corbana and Terlana, and Nr:1 Ge-Corbana and Nr:0 on Terlana, * = P < 0.05; ** = P < 0.005; *** = P < 0.001.

	Test plant Corbana		**Test plant Terlana**		**Terlana vs Corbana**	**Nr:1 Ge Terlana vs Nr:0 Terlana**
	Nr:1 Ge Corbana	**Nr:1 Ge Terlana**	**Nr:1 Ge Corbana**	**Nr:1 Ge Terlana**		
Survival (%)	92 ± 0.03	86 ± 0.03	94 ± 0.02	94 ± 0.03		
Age at death (days)	4 ± 0.58	4.18 ± 0.54	5.17 ± 1.13	2.7 ± 0.3		
Development time (days)	10.28 ± 0.17	10.48 ± 0.18	11.06 ± 0.12	9.2 ± 0.15	***	***

Figure 1: The total duration of EPG parameter E2 (phloem ingestion) (median, first and third quartiles and range) for *N. ribnisnigri* Nr:1 Ge (A) and Nr:1 Be (B) reared on Corbana (Nr:1-C) and Terlana (Nr:1-T) tested on Corbana and Terlana. The Mann-Whitney U test was used to analyse differences in total duration of E2 between aphids reared on Corbana and Terlana. Asterisks indicate significant differences between two groups reared on either Terlana or Corbana and tested on the same cultivar. Outliers are plotted as circles. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$.



Nr:1 Be colonies on Corbana

Only one significant difference in EPG parameters was found when comparing Nr:1 Be aphids either reared on Corbana or Terlana, tested on Corbana (Table 3). The total duration of phloem intake was shorter for aphids reared on Terlana compared to aphids reared on Corbana (P = 0.032) (Figure 1B).

In the performance test survival was higher for aphids reared on Corbana compared to aphids reared on Terlana (P < 0.0001; Table 4). The age at death was higher for aphids reared on Terlana compared to Corbana (P < 0.0001).

Nr:1 Be colonies on Terlana

Several differences in EPG parameters showed up when comparing the Nr:1 Be aphids reared on either Corbana or Terlana, tested on Terlana plants (Table 3). Aphids reared on Terlana started later with the first phloem event (P = 0.019), first phloem ingestion (P =0.007) and first sustained phloem ingestion (P = 0.004) compared to aphids reared on Corbana. The total time spent on phloem phase (P =0.006) and phloem ingestion (P = 0.002) was shorter for aphids reared on Terlana compared to aphids reared on Corbana (Figure 1B). Additionally, aphids reared on Terlana spent more time on pathway activities compared to aphids reared on Corbana (P = 0.023).

No significant differences were found in the performance parameters between Nr:1 Be aphids reared on either Corbana or Terlana, tested on Terlana plants (Table 4).

Table 3: EPG parameters (mean ± SEM) of *N. ribisnigri* aphids from population Nr:1 Be reared on resistant lettuce Corbana (Nr:1 Be-Corbana) and susceptible lettuce cultivar Terlana (Nr:1 Be-Terlana) tested on Corbana and Terlana. Abbreviations of parameters: C - stylet pathway; NP - non-penetration period; E - phloem phase; E1 - phloem salivation; E2 - phloem ingestion; G - xylem ingestion; F - penetration difficulties. The Mann-Whitney U test was used to analyse differences between populations with different rearing history, and between Nr:1 Ge-Corbana on Corbana and Terlana, * = P < 0.05; ** = P < 0.005; *** = P < 0.001. Significance of differences in proportion (%) of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds.

	Test plant Corbana		Test plant Terlana			Terlana vs Corbana
	Nr:1 Be-Corbana	Nr:1 Be-Terlana	Nr:1 Be-Corbana	Nr:1 Be-Terlana		
Duration first NP	344 ± 111	703 ± 270	629 ± 167	308 ± 76		
Duration of NP before 1st visible E	1140 ± 215	2898 ± 956	1441 ± 331	1952 ± 642		
Nr. of probes to the 1st E1	6.47 ± 1.28	5.79 ± 1.27	4.95 ± 0.78	5.88 ± 1.14		
Time to 1st visible E#	5557 ± 1333	5754 ± 1600	3873 ± 679	7997 ± 1384	*	
Total duration of E	12602 ± 2539	9828 ± 2018	21953 ± 1292	15993 ± 1692	**	*
Total duration of E1	1784 ± 721	1704 ± 465	1064 ± 440	2350 ± 1617		
Contribution E1 to E (%)	46 ± 11	31 ± 7	7 ± 4	13 ± 6		*
Total duration of single E1	882 ± 236	433 ± 79	817 ± 607	443 ± 106		
% aphids showing E2	65	85	100	94		**
Time to 1st E2#	8605 ± 2604	9002 ± 2076	4929 ± 921	11273 ± 1737	**	
Time to 1st sustained E2#	8807 ± 2569	11016 ± 2262	5920 ± 1384	12366 ± 1958	**	
Nr. of E2 periods	1.29 ± 0.34	2.35 ± 0.50	1.74 ± 0.24	1.18 ± 0.20	*	
Nr. of sustained E2 periods	1.12 ± 0.30	1.50 ± 0.29	1.42 ± 0.14	1.12 ± 0.21		
Total duration of NP	4014 ± 1037	6408 ± 1161	2024 ± 433	2664 ± 662		
Total duration of C	8361 ± 1370	12119 ± 1192	4548 ± 896	7945 ± 1296	*	*
Total duration of G	3573 ± 971	2341 ± 477	2056 ± 327	4103 ± 609		
Nr. of probes	15.12 ± 2.86	19.50 ± 2.56	7.37 ± 1.37	9.82 ± 1.66		
Nr. of short probes (C < 3 min)	8.65 ± 1.91	9.90 ± 1.90	3.58 ± 1.10	4.24 ± 0.74		

Time to first E, E2 and first sustained E2 was measured from the start of the first prob

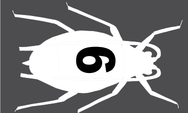


Table 4: Performance (mean ± SEM) of *N. ribisnigri* population Nr:1 Be reared on resistant lettuce Corbana (Nr:1 Be-Corbana) and susceptible lettuce cultivar Terlana (Nr:1 Be-Terlana) tested on Corbana and Terlana. The Mann-Whitney U test was used to analyse differences between populations with different rearing history, Nr:1 Be-Corbana on Corbana and Terlana, and Nr:1 Be-Corbana and Nr:0 on Terlana, * = P < 0.05; ** = P < 0.005; *** = P < 0.001.

	Test plant Corbana			Test plant Terlana		Terlana	Nr:1 Be Terlana
	Nr:1 Be	Nr:1 Be		Nr:1 Be	Nr:1 Be	vs	vs
	Corbana	Terlana		Corbana	Terlana	Corbana	Nr:0 Terlana
Survival (%)	88 ± 3.72	55 ± 5.24	***	92 ± 2.75	94 ± 2.65		
Age at death (days)	2.5 ± 0.25	4.14 ± 0.39	***	3.64 ± 0.66	5 ± 1.14		
Development time (days)	9.07 ± 0.09	9.52 ± 0.17		9.51 ± 0.12	9.42 ± 0.12	*	***

Corbana versus Terlana

The behavioural data recorded for the Nr:1 populations tested on Terlana and Corbana were compared only for Nr:1 populations reared on Corbana to rule out the possible effect of virulence lost during the rearing period on Terlana.

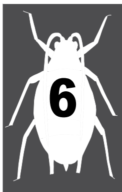
Minor differences were found in EPG parameters comparing Nr:1 Ge reared on Corbana and tested on Terlana and Corbana (Table 1). Aphids on Corbana took longer to start the first probe (P = 0.044), the first visible phloem contact (P = 0.012) and first phloem ingestion (P = 0.018), compared to Terlana. No significant differences were found in the performance of Nr:1 Ge reared on Corbana and tested on Corbana or Terlana (Table 2).
For Nr:1 Be reared on Corbana and tested on Corbana, the duration of the phloem phase was shorter (P = 0.007) and consisted of a higher percentage of phloem salivation (P = 0.007) compared to the aphids on Terlana (Table 3). Additionally, more time was spent on pathway activities by aphids on Corbana compared to the aphids on Terlana (P = 0.028). The percentage of aphids showing phloem ingestion was significantly lower for aphids on Corbana compared to Terlana (P = 0.004). In the performance experiment aphids on Terlana took longer to develop into an adult compared to the aphids on Corbana (P = 0.005).

Nr:0 versus Nr:1 on Terlana

When the performance data of Nr:1 Ge were compared to the performance data of Nr:0 on Terlana, the development is significantly longer for Nr:1 Ge (P < 0.0001; Table 2 and Appendix 1). The development time was also longer for Nr:1 Be compared to Nr:0 (P < 0.0001; Table 4 and Appendix 1).

Discussion

When fitness costs are associated with overcoming an *R*-gene-mediated resistance in plants, one would expect that insensitivity to this resistance would be lost in the absence of resistant cultivars, as selection for virulence is absent.



In terms of penetration behaviour, Nr:1 Ge aphids reared on Terlana had become less virulent, compared to when being reared on Corbana. On the resistant cultivar Corbana, less time was spent on phloem ingestion and more on phloem salivation by the aphids reared on Terlana. This indicates that aphids had to salivate more to overcome the wound response reaction in the sieve elements before being able to feed. More time was spent on non-penetration and a higher number of probes was observed for aphids reared on Terlana, indicating difficulties accepting the phloem.

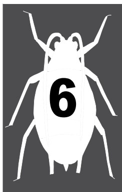
Although differences in feeding behaviour as derived from EPG parameters suggest a reduction in virulence for Nr:1 Ge after being reared on a susceptible cultivar, the performance data did not differ between the aphids reared on Corbana or Terlana, when tested on the resistant cultivar Corbana. This suggests that the reduction in virulence was temporary. The EPG recordings lasted eight hours after transferring the aphids from the

rearing to the test plant, whereas the performance experiment lasted for 10-11 days after transfer. In the performance test the aphids reared on the susceptible lettuce probably were able to adapt again to the resistant lettuce, and to perform equally well compared to the aphids reared on the resistant lettuce. The virulence of *Macrosiphum euphorbiae* (Thomas) to the *Mi*-resistance in tomato was also shown to be persistent within the virulent populations, after a rearing period on susceptible tomato (Goggin et al., 2001). In a choice bioassay, equal numbers of aphids were found on both resistant and susceptible plants after a 10-14 days exposure time. In nature the virulence of *M. euphorbiae* was also persistent, because virulent aphids were collected from New Jersey, where *Mi*-resistant varieties are rarely grown and after overwintering on alternate hosts no decrease in virulence was found (Goggin et al., 2001). No loss of virulence was found by Kim et al. (2008) for a *Rag1*-virulent biotype of *Aphis glycines* (Matsumura) reared on a susceptible soybean cultivar. No significant differences in population development were found for aphids tested on susceptible and *Rag1*-resistant soybean, indicating the virulent aphids had not lost their virulence to the *Rag1*-resistance.

Nr:1 Be aphids, similar to the Nr:1 Ge, reared on Terlana also showed a reduction in time spent on phloem ingestion compared to aphids reared on Corbana, although no elevated phloem salivation was observed. In contrast to Nr:1 Ge, Nr:1 Be aphids had a reduced survival on Corbana, after being reared on Terlana. This suggests, together with the shorter phloem ingestion, a reduction in virulence caused by being reared on Terlana. This reduction in virulence for Nr:1 Be seems to be more persistent than that observed for Nr:1 Ge, because it was also found in the performance experiment. Chapter 3 also suggested loss of virulence for Nr:1 Be after being reared on the susceptible cultivar Fatima, after which survival was significantly lower on a resistant lettuce cultivar compared to a susceptible cultivar. Loss of virulence was also stated by Michel et al. (2010) as one of the possible causes of finding individuals of virulent *A. glycines* behaving less virulently on resistant sorghum than expected. However, contamination and virulence variation in the colony could not be ruled out. For the nematode *Meloidogyne incognita* (Kofoid & White), contrasting results were found for an artificially selected virulent laboratory population and a natural virulent population in the loss of virulence after a rearing period on susceptible tomato. The laboratory population showed no signs of virulence loss and reproduced equally well on resistant tomato after being reared on resistant or susceptible tomato. The virulent field population, however, showed a decrease in ability to reproduce on resistant tomato after being reared on susceptible tomato (Castagnone-Sereno et al., 1993).

Cost of virulence

Fitness costs might occur for aphids to overcome *R*-gene-mediated resistance in plants. Fitness costs are especially large when the virulence is due to loss of an effector that mediates an important biological function for the aphid (Vera Cruz et al. 2000). For both Nr:1 populations tested here, indications of fitness costs associated with the virulence were found in the performance experiment. When the development time of both Nr:1 populations on Terlana is compared to the development time of Nr:0 on Terlana, Nr:0 aphids develop significantly faster into adults than both Nr:1Ge and Nr:1 Be. A fitness cost in terms of reproduction was found for Nr:1 compared to Nr:0 in Chapter 3. A possible cost of virulence was also found by Defibaugh-Chavez (2007) comparing virulent and avirulent *M. euphorbiae* on *Mi-1.2*-resistant and susceptible tomato. Juvenile development was faster for the avirulent aphids compared to the virulent aphids on susceptible tomato. Additionally, in EPG experiments, avirulent aphids took less time to initiate sustained phloem ingestion and the total duration of phloem ingestion was longer compared to virulent aphids (Pallipparambil et al., 2010). The Nr:1 Ge aphids reared on Corbana in our study needed more time to initiate probing and feeding on Corbana compared to Terlana. However, no differences in total phloem ingestion was found. The aphids might need some time to overcome the resistance component in Corbana before feeding. For Nr:1 Be also no differences were found in duration of phloem ingestion; however, about 35% of the aphids on Corbana were not able to ingest phloem, whereas all aphids showed phloem ingestion on Terlana. This indicates that some aphids were not able to adapt to the resistant plant. Additionally, aphids on Corbana spent almost five times longer in phloem events on salivation compared to the aphids on Terlana to overcome the resistance in Corbana before being able to feed. In Chapter 3 we did not observe differences in development time between Nr:1 Ge and Nr:0 on two other near isogenic susceptible lettuce lines, however, these were different lettuce lines compared to the ones used in this study. A different lettuce background was shown to influence the behaviour of *N. ribinisgri* on resistant and susceptible lettuce (Chapter 3).



Costs of virulence were also reported for other plant attackers. For example, virulence of the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (Xoo), in rice (by loss of avirulence gene function) was shown to have fitness costs in terms of pathogen aggressiveness and persistence (Vera Cruz et al., 2000). Virulent strains of *M. incognita* suffered from fitness costs, in terms of reproduction index, on susceptible tomato (Castagnone-Sereno et al., 2007). A reproductive fitness cost was also found for Hessian flies overcoming *H*-resistance in wheat (Zhang et al., 2011).

Effect of different rearing plants

Aphids might get adapted to the plant they are reared on, and therefore the plant on which a colony is reared might affect the behaviour of the aphids on other plants. For example, *Diuraphis noxia* (Mordvilko) was shown to be affected by the rearing plant in terms of reproduction (Schotzko & Smith, 1991). Aphids were reared on either susceptible wheat or oats with antibiosis resistance. More nymphs were produced on both the susceptible wheat and resistant oats by aphids that were reared on susceptible wheat compared to the ones reared on resistant oats. Although no effects of plant of origin was found for *Aphis gossypii* (Glover) colonies collected from the field and tested on resistant and susceptible melon cultivars, an effect of laboratory rearing on cucumber was found; the longer the colony was reared on cucumber, the higher aphid fecundity on *Vat*-resistant melon and the poorer on susceptible melon (Lombaert et al., 2009).

For both Nr:1 Ge and Nr:1 Be reared on Corbana and tested on Terlana, an increase in time spent on phloem ingestion was observed compared to being reared on Terlana. More time spent on phloem ingestion could result in higher survival and faster development, however, this was not found in our data. In the EPG setup we can only measure the total duration of phloem intake but not the amount of phloem ingested. On the one hand, Nr:1 Ge and Be reared on Corbana might ingest less phloem sap per unit of time from Terlana than from Corbana, thus needing to feed longer on Terlana to ingest the same amount of phloem sap. On the other hand, the quality of the phloem sap of Corbana might be better than that of Terlana, resulting in a longer feeding period on Corbana.

Individuals of polyphagous aphids can be specialised, in terms of preference for and performance on certain host-plant species, even though at species level they have a wide range of host-plant species they could exploit (Via, 1991). This specialisation within a single polyphagous aphid species might result from differences in experience on host-plants and/or from genetic differences among aphid individuals (Via, 1991). De Barro et al. (1995) showed, for example, that *Sitobion avenae* performed better on the host plant they originated from, either wheat or cocksfoot, compared to alternative host plants. Host-plant specialisation in *Myzus persicae* (Sulzer) on either pepper or tobacco was found for both performance and preference (Nikolakakis et al., 2003). In *Schizaphis graminum* (Rondani), only preference was influenced by the rearing plant, no effects on the growth and reproduction were found (Schweissing & Wilde, 1979). For *Acyrthosiphon pisum* (Harris) clones which were either specialised on alfalfa or red clover, specialisation was not caused by experience with the host-plant species. Even after prolonged stay on the alternative host, aphids did not perform better, suggesting a genetic basis causing the observed intraspecific specialisation (Via, 1991). Leonardo & Muiru (2003) found evidence that the specialisation of these *A. pisum* clones for either alfalfa or red clover is based on facultative symbionts.

Conclusion

In this study we have shown that the lettuce plant on which an *N. ribisnigri* colony is reared, can influence the behaviour and performance of these aphids when tested on lettuce plants differing in *Nr*-resistance. For Nr:1 Ge a short-term reduction of virulence was observed after being reared on susceptible lettuce, indicating this aphid population needed some time to adapt to the plant it was feeding on. For Nr:1 Be long term loss of virulence was found. The virulence in both Nr:1 populations seems to have fitness costs, which could eventually lead to the loss of virulence when being reared on lettuce in the absence of *Nr*-resistance. The rearing period used in this study was not long enough to result in a complete loss of virulence. A longer period without exposure to *Nr*–resistance might cause a stronger or complete loss of virulence.

Acknowledgements

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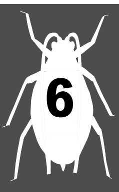


Chapter 7

Feeding behaviour and performance of *Nasonovia ribisnigri* on grafts, detached leaves and leaf disks of resistant and susceptible lettuce

Cindy J.M. ten Broeke, Marcel Dicke and Joop J.A van Loon

Abstract

Aphids are dependent on the phloem sap of plants as their only source of nutrients. Host plant resistance in lettuce mediated by the *Nr*-gene is used to control the lettuce aphid *Nasonovia ribisnigri*. The resistance is located in the phloem, however, the exact mechanism of the resistance is unknown. In this study, the location of synthesis, either the root or the soot, of the resistance factor(s) of the *Nr*-gene was investigated. Another objective was to measure the persistence of resistance in excised lettuce tissue.

The feeding behaviour and performance of avirulent *N. ribisnigri* was studied on grafts of resistant and susceptible lettuce to study whether the resistance component is produced in the roots or the shoots and is translocated to the feeding site. Additionally, the feeding behaviour and performance of *N. ribisnigri* were studied on detached leaves and leaf disks of resistant lettuce to study whether the resistance was lost or retained in the lettuce tissue.

In the grafting experiment, a grafted resistant shoot keeps its resistance regardless of the root origin. Partial loss of resistance was observed after detachment of leaves and leaf parts from resistant plants. Screening for *N. ribisnigri* resistant lettuce should focus on intact plants, instead of excised plant parts.

Introduction

Aphids are phloem feeders and tap from sieve elements with their stylets, withdrawing nutrients and by doing so weakening the plant (Dixon, 1998). Some aphids also ingest xylem sap to maintain their water balance (Spiller et al., 1990; Will & Van Bel, 2006). Together, phloem and xylem form the long-distance transport conducts in the vascular bundles of angiosperms (Sjölund, 1997). Xylem consists of dead tubes, and is responsible for the transport of minerals and water from the roots to the aerial parts of the plants, driven by transpiration (Taiz & Zeiger, 2002). Phloem consists of sieve tubes that translocate photosynthesis-derived assimilates from mature leaves to the growing parts and storage organs of the plant, but also redistribute water and various other compounds through the plant (Van Bel et al., 2002; Will & Van Bel, 2006). Viruses can also travel via the phloem, enabling quick dispersal through the plant (Gosalvez-Bernal et al., 2008). Phloem plays an important role in defence against plant attackers by transporting secondary metabolites and phyto-hormones to distantly located plant organs (Brudenell et al., 1999; Jorgensen, 2002). Additionally, the transport function of phloem is also used in crop protection, because the phloem is able to transport insecticides, fungicides and herbicides to remote parts of the plant (Lichtner, 2000). Especially insecticides against phloem sucking insects are very effective when transported by the phloem (Lichtner, 2000).

The sieve elements and companion cells are the functional units of the sieve tubes in the phloem (Van Bel, 2003). Upon wounding, sieve plates are plugged by the plant to prevent loss of phloem sap. As a wound response to sieve element damage, the calcium concentration in sieve elements is elevated which stimulates further wound responses like sieve plate occlusion by phloem (P)-proteins and callose (Will & Van Bel, 2006). P-proteins play an important role in these wound and defence responses, and are synthesised in the companion cells and can be transported throughout the plant (Golecki et al., 1999; Kehr, 2006). P-proteins can plug sieve plates as a wound response (Knoblauch & Van Bel, 1998). Additionally, some P-proteins are known to act as protease inhibitors and lectins (Schobert et al., 1998; Walz et al., 2004). When aphids are feeding from the phloem they pierce into the sieve elements with their stylets. Proteins in aphid saliva play an important role in the feeding process, countering the occlusion of sieve plates and for some species also countering resistance factors in plants (Hogenhout et al., 2009; Will & Van Bel, 2006). In contrast, aphid saliva might also contain proteins which elicit plant defences (Hogenhout et al., 2009). Pest management of several aphid species relies on phloem-based host plant resistance. For example the *Vat*-gene- mediated resistance in melon against the melon aphid *Aphis gossypii* (Glover) (Chen et al., 1996), the *Mi*-1.2-gene-mediated resistance in tomato against the potato aphid *Macrosiphum euphorbiae* (Thomas)(Kaloshian et al., 2000) and the *Nr*-gene-mediated resistance in lettuce against the black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely) (Chapter 2).

Nasonovia ribisnigri is an important pest of cultivated lettuce and is controlled by host plant resistance conferred by the *Nr*-gene which provides near-complete resistance against this aphid species (Reinink & Dieleman, 1989; Van Helden & Tjallingii, 1993) (Chapter 2). This resistance, however, is no longer effective because since 2007, virulent biotypes of *N. ribisnigri* (Nr:1 biotype) have been reported infesting resistant lettuce (Thabuis et al., 2011). The mechanism of *Nr*-resistance in lettuce is unknown, although it has been shown by previous studies that the resistance is located in the phloem, because avirulent *N. ribisnigri* displayed a reduction or absence of phloem ingestion and 100% mortality occurred on resistant lettuce plants (Reinink & Dieleman, 1989; Van Helden & Tjallingii, 1993)(Chapter 2).

In many crop plants, like solanaceous and cucurbitaceous crops, grafting is an important strategy in integrated pest management to control mainly soil-borne pests and less commonly also foliar pests (Louws et al., 2010). In tomato, for example, grafting cultivated tomato onto wild *Solanum* rootstocks was an effective measure to reduce the incidence of several tomato pests like the sweet potato whitefly (*Bemisia tabaci* Genn.), potato psyllid (*Bactericera cockerelli* Sulc.) and melon aphid (*A. gossypii*) (Álvarez-Hernández et al., 2009). Grafting can also be used to determine whether insect resistance factors are synthesised in the roots or in the shoots of plants (Edelstein et al., 2000; Pelletier & Clark, 2004). The first objective of this study is to investigate the location of synthesis, either the root or the shoot, of the resistance factors of the *Nr*-gene in a resistant lettuce cultivar against *N. ribisnigri.* As a second objective we studied the persistence of resistance of excised resistant lettuce tissues, by testing the feeding behaviour of *N. ribisnigri* on detached leaves and leaf disks.

Materials and Methods

Plants and Aphids

Two *Lactuca sativa* cultivars were used in the experiments, Nr:0-resistant Corbana and Nr:0-susceptible Terlana. These plants were grown in a greenhouse compartment at 18-20 °C, 60 % humidity and L14/D10 photoperiod. To control the larvae of sciarid flies, the plants were treated with ENTONEM (a formulation of the entomopathogenic nematode, *Steinernema feltiae*, www.koppert.com) once a week. Three-week-old plants were used for the experiments.

An avirulent population of *N. ribisnigri*, biotype Nr:0 (originating from The Netherlands) was used in the experiments, and was reared on Nr:0-susceptible *L. sativa* cultivar Terlana in a greenhouse compartment at 18-20 °C, 60% RH and L14/D10 photoperiod. In the aphid colonies both winged and wingless adults were present.

Plant treatments

Leaves of three-week old Corbana and Terlana plants were excised using a carbon steel sterile surgical blade size 15 and their petioles were placed into tap water at 0, 24, 48 and 76 h before the experiments. Leaf disks with a diameter of 2 cm were prepared from leaves of three-week-old Corbana and Terlana plants.

Grafts were made using the protocol of Brosnan et al. (2007), with some adjustments (Dr. G.C. Angenent, pers. comm.) (Figure 1). The lettuce seeds were sterilised in 50 ml plastic tubes in a fumehood. The seeds were first washed for 30s in 70% ethanol, before they were kept in a sodium hypochlorite solution for 20 min, then washed three times for 10 min with MilliQ. The seeds were sown directly after sterilizing on plates containing 50ml 0.5X Murashige and Skoog (MS) medium with 1% agar and 0.5% sucrose (van de Mortel et al., 2012). Grafting plates (50 ml 0.5X MS medium with 1% agar) were created by cutting small strips of medium out of the plates, in which the graft will be placed so they could not move during the fusion process. Three-to-four-day old seedlings were grafted. The seedlings were cut in half on the medium with a sterile surgical blade (size 15). The shoots and roots were immediately transferred to the grafting plate and were aligned in the slits. Thin strips of agar were placed on top of the grafting surface. The grafts were grown in a climate cell at 21 ± 1°C, 60 ± 10 % humidity and L8/D16 photoperiod (TL-D36W/840, Philips, The Netherlands), plates in upright position to allow the grafts to grow to the light. After 10 days, successful grafts were transplanted into soil and grown for another 7-10 days in a greenhouse compartment at 18-20 ^{o}C, 60% RH and L14/D10 photoperiod . At this stage, the size and number of leaves of the plants was comparable with three-week-old intact plants. Four different grafts were produced; Corbana shoot on Terlana root (CT), Terlana shoot on Corbana root (TC), Corbana shoot on Corbana root (CC) and Terlana shoot on Terlana root (TT).

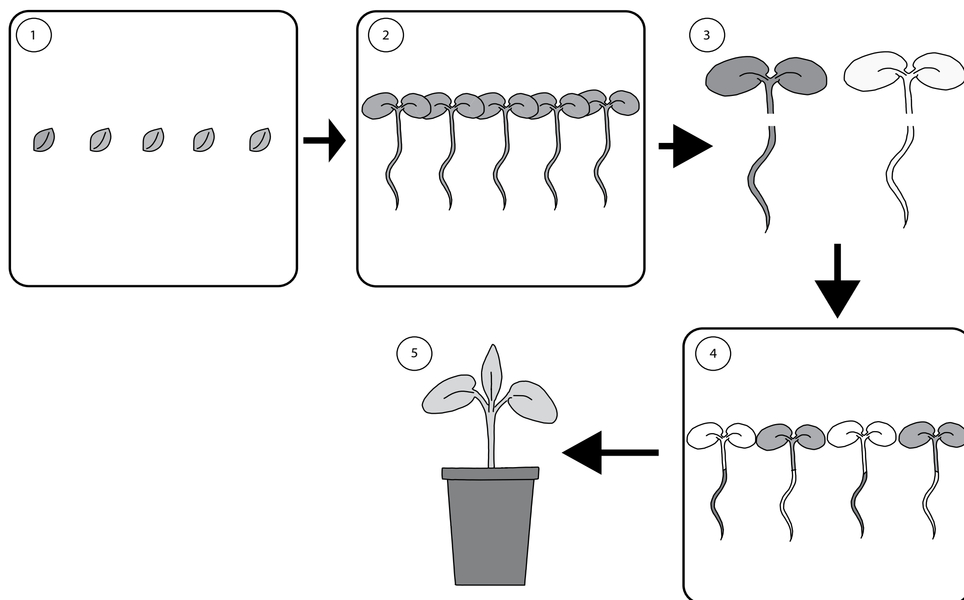


Figure 1: Grafting procedure lettuce. 1) Sterilised lettuce seeds were sown on agar plates. 2) Seeds were grown for three - four days on agar. 3) Seedlings were cut in two parts. 4) Roots and shoots of seedlings were combined and placed on agar. 5) After 17-20 days the successful grafts were transplanted to soil.

EPG recording

The electrical penetration graph technique (EPG) was used to study the effect of the plant treatments (intact plant, cutting, detached leaves, leaf disk and graft) on the stylet penetration and feeding behaviour of *N. ribisnigri* biotype Nr:0 on *L. sativa* cultivars Corbana and Terlana. The EPG technique allows the electrical recording of the penetration and feeding behaviour of aphids and other piercing-sucking insects, by making a plant and a piercing insect part of an electrical circuit (Tjallingii, 1988). A thin gold wire is attached to the insect's dorsum. For details on the EPG methods used, see Chapter 2. In the case of the intact plants and grafts an electrode is inserted in the soil near the plant. For the detached leaves, 100 ml glass pots with tap water were used to put the leaf petioles and electrodes in. For the leaf disks, 3-cm square blocks of 1% agar were used to put the leaf disk on and to insert the electrode in. The agar blocks with leaf disks were positioned in a small Petri dish filled with tap water.

The recordings lasted for eight hours and were performed on winged adult aphids (alatae), randomly collected from the rearing, to get a representative view on the behaviour of alatae within a population. The EPG patterns distinguished in this study were: stylet pathway (C), non-penetration period (NP), phloem phase (E), phloem salivation (E1), phloem ingestion (E2), xylem ingestion (G) and penetration difficulties (F). The first phloem

phase appearing in the EPG recording will be referred to as the first visible phloem event. For more details on the EPG patterns, see Chapter 2.

For every *N. ribisnigri* Nr:0 biotype x cultivar treatment combination 22 replicates (individual aphids on separate plants) were recorded. Plants and aphids were only used once. Incomplete recordings, due to death or escape of aphids, were excluded from the analyses, leaving between 17 and 22 replicates for every aphid x plant treatment combination. The EPG data for Nr:0 on intact Corbana and Terlana plants was collected in Chapter 6 and compared to the EPG data collected in this study.

Performance test

Performance of *N. ribisnigri* Nr:0 biotype on Corbana and Terlana grafts was measured by assessing nymph survival and development time from nymph to the adult stage. About 200 *N. ribisnigri* alatae were kept in Petri dishes on leaves of *L. sativa* cultivar Terlana. The next day new-born nymphs were transferred into clip cages on the plants, five nymphs per cage, two cages per plant. Both the mortality and development time were recorded daily, and the experiments were conducted in a greenhouse compartment at 18-20 ^{o}C, 60% humidity and L14/D10 photoperiod. The performance data of Nr:0 on Corbana and Terlana intact plants, collected in Chapter 6, was compared to the performance data of Nr:0 on Corbana and Terlana cuttings and grafts.

Statistics

EPG parameters were calculated per aphid using the EPG analysis worksheet created by Sarria et al. (2009). The Mann-Whitney U test was used for pairwise comparisons between the intact plants of one cultivar with the different plant treatments of one cultivar (cuttings, detached leaves, leaf disks and grafts). Additionally a pairwise comparison was made between the CT graft and the TC graft, the CT graft and intact Corbana plant, and the TC graft and the intact Terlana plant. The Kruskal-Wallis test was used to test for overall differences among the detached leaf treatments that had been kept for 0, 24, 48 and 76 hours in tap water. The EPG parameters that differed significantly were compared using the Mann-Whitney U test applying the Bonferroni correction for multiple comparisons. Fisher's exact test was used to analyse differences in the percentage of aphids that showed phloem intake. Due to the small sample size of aphids showing phloem intake for Nr:0 on Corbana intact plants, the Mann-Whitney U test (one tailed) was calculated for pairwise comparisons of the duration of phloem ingestions between the Corbana treatments.

For aphid performance, every clip cage was considered one replicate and the results were first calculated per clip cage. The means and standard error were calculated over all clip cages within one treatment. The Mann-Whitney U test was used to test for significant differences in aphid mortality (in the performance experiment), time until mortality and development time. All statistical tests were performed with IBM SPSS Statistics version 19.

Results

Grafts

When the EPG data of aphids on intact Corbana plants and CC grafts are compared, a few EPG parameters differed (Table 1, Figure 2A and Appendix 1). The number of probes (P = 0.003) and short probes (P = 0.002) was higher on the CC graft compared to the intact Corbana plant. No significant differences were found in performance parameters between aphids on intact and grafts of Corbana (Table 3). There were also no significant differences found in the EPG data between aphids on intact Terlana plants and TT grafts (Table 1, Figure 2B and Appendix 1). The development time till adulthood was shorter on intact Terlana plants compared to TT grafts (P < 0.0001) (Table 3).

When the EPG data for aphids on the combination grafts, Corbana shoot on Terlana root (CT) and Terlana shoot on Corbana root (TC), were compared, several EPG parameters differed (Table 2, Figure 2C and Appendix 2). Aphids on the TC graft spent more time on phloem events and a lower percentage of this time on phloem salivation compared to the CT graft (both P < 0.0001). The percentage of aphids displaying phloem ingestion was higher (P < 0.0001), the total duration of phloem ingestion longer (P = 0.004) and number of phloem ingestions higher (P < 0.0001) on the TC graft compared to the CT graft. The aphids on the TC graft had a lower number of probes and spent less time on pathway activities and non-penetration compared to the CT graft (all P < 0.0001). In the performance experiment no aphids survived on the CT graft compared to 82% on the TC graft (P < 0.0001; Table 3).
When the EPG data of aphids on the CT grafts and the intact Corbana plants were compared, several EPG parameters differed (Table 2, Figure 2C and Appendix 2). Aphids on the CT graft spent more time on phloem events (P = 0.019) and the number of probes was higher (P = 0.005) compared to intact plants. In the performance experiment the age at death of the aphids was half a day higher for aphids on the CT graft compared to the intact Corbana plant (P = 0.016; Table 3). No significant differences were found in the EPG data and performance data between aphids on the TC grafts and intact Terlana plants (Table 2 and 3, Figure 2C and Appendix 2).

Table 1: EPG parameters (mean ± SEM) of *N. ribisnigri* Nr:0 on *L. sativa* cultivars Corbana and Terlana: intact plant (plant), detached leaf (leaf), leaf disk and grafts (Corbana shoot on Corbana root and Terlana shoot on Terlana root). Pairwise comparisons were made for EPG parameters between intact plants and the other treatments (leaf, leaf disk, graft), and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.0167. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Times are in seconds. NP = non-penetration, E = phloem event, E1 = phloem salivation and E2 = phloem ingestion. * = P < 0.017; ** = P < 0.005; *** = P < 0.001.

	plant	leaf		leaf disk		graft	
Corbana	**(n=20)**	**(n=22)**		**(n=17)**		**(n=18)**	
Duration first NP	417 ± 142	489 ± 124		327 ± 97		347 ± 118	
Time to 1st visible E#	7644 ± 1351	7187 ± 986		6533 ± 1244		9228 ± 1340	
Contribution E1 to E (%)	98.66 ± 0.99	42 ± 9.50	***	14 ± 5.43	***	84 ± 7.51	
% aphids showing E2	10.53	70	***	100	***	33.33 ±	
Nr. of probes	27 ± 2.11	24 ± 2.87		20 ± 3.95	*	42 ± 3.58	**
Terlana	**(n=20)**	**(n=21)**		**(n=17)**		**(n=17)**	
Duration first NP	366 ± 115	248 ± 54		240 ± 63		348 ± 132	
Time to 1st visible E#	5880 ± 1007	4058 ± 754		3707 ± 426		4376 ± 1032	
Contribution E1 to E (%)	1.64 ± 0.55	3.73 ± 1.35		1.73 ± 0.48		3.68 ± 1.93	
% aphids showing E2	100	100		100		100	
Nr. of probes	8.45 ± 1.53	5.19 ± 0.79		10.06 ± 1.37		10.65 ± 1.42	

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

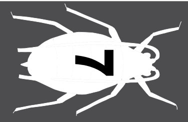


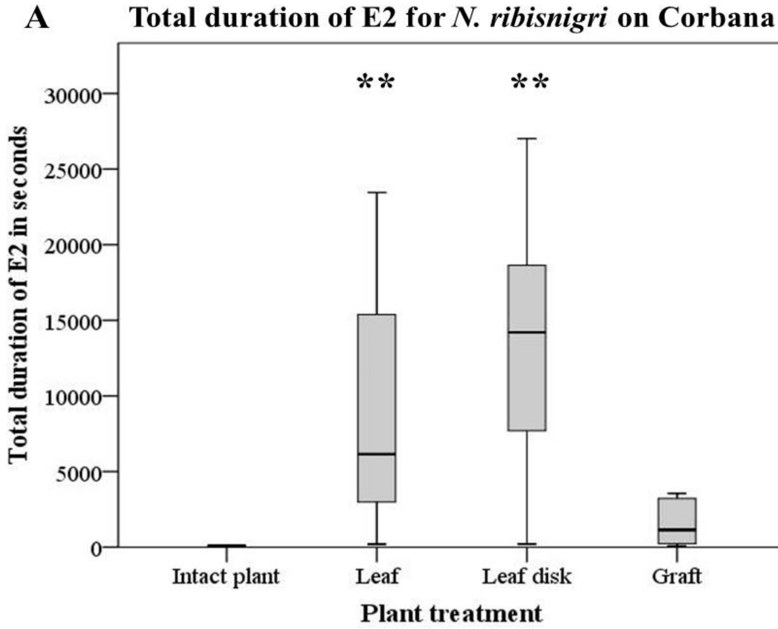
Table 2: EPG parameters (mean ± SEM) of *N. ribisnigri* Nr:0 on *L. sativa* grafts with a Corbana shoot and Terlana root (CT) and a Terlana shoot and Corbana root (TC). Pairwise comparisons were made for EPG parameters between the CT and TC graft, between intact Corbana plant and CT graft and between intact Terlana plant and TC graft, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the two comparisons made by setting α = 0.025. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Times are in seconds. NP = non-penetration, E = phloem event, E1 = phloem salivation and E2 = phloem ingestion. * = P < 0.025; ** = P < 0.005; *** = P < 0.001.

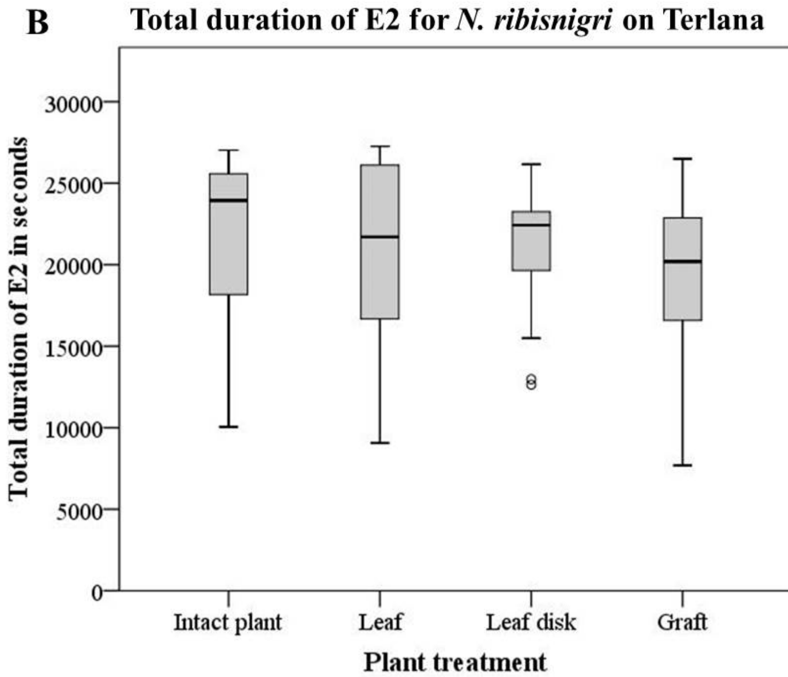
	graft CT (n=20)			graft TC (n=19)				C-CT	T-TC
Duration first NP	269	±	73	342	±	91			
Time to 1st visible E[#]	8564	±	1404	5981	±	1144			
Contribution E1 to E (%)	92	±	4.91	6	±	2.37	***		
% aphids showing E2	20			100			***		
Nr. of probes	40	±	3.37	8.47	±	1.79	***	**	

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Table 3: Performance parameters (mean ± SEM) for *N. ribisnigri* Nr:0 on *L. sativa* cultivars Corbana and Terlana: intact plant (plant), graft of Corbana shoot on Corbana root (CC) and of Terlana shoot on Terlana root (TT), and graft of Corbana shoot on Terlana root (CT) and Terlana shoot on Corbana root (TC). Pairwise comparisons were made for performance parameters between intact plants and the grafts and the grafts CT and TC were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the two comparisons made setting α = 0.025. * = P < 0.025; ** = P < 0.005; *** = P < 0.001.

Corbana	**Plant**			**Graft (CC)**				**Graft (CT)**				**CT-TC**
Survival (%)	0			0				0				***
Age at death (days)	3.14	±	0.12	3.13	±	0.14		3.76	±	0.18	*	
Development time (days)												
Terlana	**Plant**			**Graft (TT)**				**Graft (TC)**				
Survival (%)	90	±	3.08	86	±	2.94		82	±	3.52		
Age at death (days)	3.5	±	0.42	3.25	±	0.38		3.86	±	0.47		
Development time (days)	8.63	±	0.1	9.6	±	0.11	***	9.41	±	0.11	***	


A
Total duration of E2 for N. ribisnigri on Corbana
Total duration of E2 in seconds
30000
25000
20000
15000
10000
5000
0
**
**
Intact plant
Leaf
Leaf disk
Graft
Plant treatment


B
Total duration of E2 for N. ribisnigri on Terlana
Total duration of E2 in seconds
30000
25000
20000
15000
10000
5000
0
Intact plant
Leaf
Leaf disk
Graft
Plant treatment

7

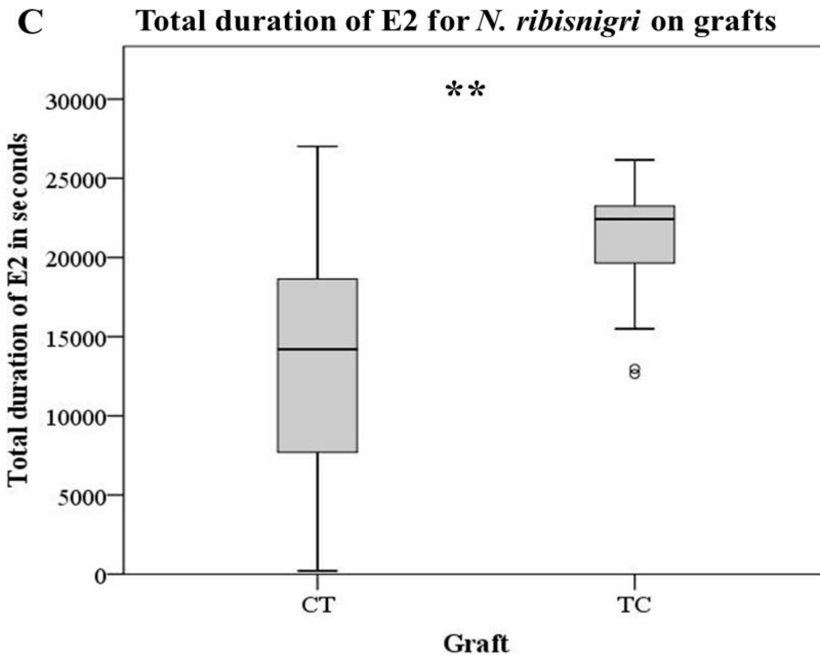


Figure 2: The total duration of EPG parameter E2 (phloem ingestion) (median, first and third quartiles and range) for *N. ribnisnigri* Nr:0 on (A) Corbana: intact plant (plant), detached leaf (leaf), leaf disk and graft (Corbana shoot on Corbana root)(B), Terlana: intact plant (plant), detached leaf (leaf), leaf disk and graft (Terlana shoot on Terlana root) and (C) grafts of a Corbana shoot on a Terlana root (CT) and a Terlana shoot on a Corbana root (TC). Pairwise comparisons were made for total duration of E2 between plants without other aphids and plants with other aphids, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction for A and B to account for the three comparisons made by setting $\alpha = 0.0167$. Bars with bold asterisks differ significantly from the control plants. Outliers are plotted as circles. * = $P < 0.017$(A+B) and 0.05 (C); ** = $P < 0.005$; *** = $P < 0.001$.

Detached leaves

Several EPG parameters differed between aphids on intact plants and on detached leaves of Corbana (Table 1, Figure 2A and Appendix 1). On detached leaves, aphids spent more time on phloem events and a smaller percentage of this time was spent on phloem salivation, although, in total more time was spent on phloem salivation compared to aphids on intact plants (all $P < 0.0001$). A higher percentage of aphids displayed phloem ingestion ($P < 0.0001$), more time was spent on phloem ingestion ($P = 0.01$) and less time on pathway activities ($P < 0.0001$) on detached leaves compared to intact plants.

When the EPG data of aphids on intact plants and detached leaves of Terlana were compared, more time was spent on non-penetration before the first visible phloem event (P

= 0.015). (Table 1, Figure 2B and Appendix 1).

No significant differences were found in EPG parameters between the aphids on detached Corbana leaves kept for different times (0, 24, 48 or 76 hours) in tap water before the EPG recording; Appendix 3).

Leaf disks

Several EPG parameters differed between aphids on intact plants and on leaf disks of Corbana (Table 1, Figure 2A and Appendix 1). Aphids on leaf disks spent more time on phloem events and a lower percentage of this time was spent on phloem salivation (both P < 0.0001). More time was spent on phloem ingestion (P = 0.010), and a higher percentage of aphids displayed phloem ingestion (P < 0.001) on leaf disks compared to intact plants. Additionally, the number of phloem ingestions and sustained phloem ingestions was larger on leaf disks (both P < 0.0001). On leaf disks the number of probes was lower (P = 0.005) and less time was spent on pathway activities (P < 0.0001) and non-penetration (P = 0.005). No significant difference was found in the EPG data between aphids on intact plants and leaf disks of cultivar Terlana (Table 1, Figure 2B and Appendix 1).

Discussion

Grafts

Our data shows that the grafting method has minor effects on the behaviour and performance of the aphids. On CC grafts, aphids probed more, indicating they might need more test probes to accept the plant, but feeding duration and survival did not differ. For TT grafts the development time was 12% longer. The duration of phloem ingestion was the same on both the TT graft and the intact Terlana plants; however, EPG data provide no information on the amount of phloem that is ingested. Aphids on the Terlana graft might have ingested less phloem during the same time span, or phloem of a lower nutritional quality, compared to the intact plant.

Grafts can be used to study if plant compounds responsible for resistance against aphids originate from the root system. The components responsible for the *Nr*-based resistance are likely produced in the shoot. Grafts with susceptible shoots and resistant roots (TC graft) were not resistant against *N. ribisnigri,* whereas grafts with resistant shoots and susceptible roots (CT graft) were still resistant. Aphids on the CT graft showed an reduction in feeding activity compared to the TC graft. Probably, as a consequence of this reduction, aphids on the CT graft spent more time on non-penetration and pathway activities. Aphids on the CT graft salivated more compared to the ones on the TC graft, suggesting more saliva was needed to overcome the wound response in the plant. CT grafts and intact Corbana plants seem to be equally resistant. Although more time was spent on phloem events by aphids on the CT graft compared to the intact Corbana plant, no differences were observed

in duration of phloem ingestion and survival. Aphids on the intact Corbana plant tended to die faster compared to the ones on the CT graft; however, this was only half a day faster. The TC graft was equally susceptible to the intact plant of Terlana; no differences in aphid feeding behaviour and performance were observed.

Other examples of resistance factors that are synthesised in the shoots are found in tobacco against *M. persicae* (Jackson et al., 1985), in alfalfa against the spotted alfalfa aphid (Harvey & Hackerott, 1958) and in resistant melon AR5 against *A. gossypii* (Kennedy & Kishaba, 1977). In contrast to our grafting method, de Ilarduya et al. (2003) made grafts of tomato, above the second fully expanded leaf and *M. euphorbiae* were tested on leaves above and below the graft union to determine whether the resistance mediated by the *Mi-1*-gene in tomato against *M. euphorbiae* was systemically translocated. No translocation of resistance components through the graft union was observed, and it was hypothesised that *Mi-1*–mediated resistance is cell autonomous and interaction between aphid elicitor and *Mi-1* is required for resistance. De Jager et al. (1995) observed translocation of resistance from the scion to leaves of the rootstock of chrysanthemum grafts against western flower thrips, *Frankliniella occidentalis* (Pergande); the susceptible rootstock with the first leaves became resistant when a resistant scion was grafted on top. The resistance factor seemed to be produced in the top of the plant, because the leaves of the scion were more resistant than the ones of the rootstock. In our study we used seedling grafting. To test whether the resistance components in lettuce are transported from the top of the shoot to the lower leaves, grafts should be made between upper part of shoots, and rootstock with the lower part of the shoot. This might be difficult, however, because lettuce has very short internodes. The roots of lettuce could also be presented to *N. ribisnigri*, to test the transmission of resistance to the roots, however, in previous experiments *N. ribisnigri* was unable to feed from lettuce roots (Ten Broeke, unpublished results).

Pelletier & Clark (2004) observed that resistance against different insects in one plant might be produced in different plant parts. Resistance against *M. euphorbiae* was not transported from the roots to the shoots in grafts of susceptible potato and resistant wild *Solanum* species. In contrast, the Colorado potato beetle had an inferior performance on the grafted foliage on resistant roots compared to susceptible plants.

Excised leaves and leaf disks

The susceptibility of the susceptible lettuce cultivar Terlana did not seem to be influenced by detachment of leaves or excision of leaf disks. Slightly more time was spent on non-penetration before the first visible phloem event, when aphids were feeding on detached leaves compared to the intact plant, however, no difference was found in phloem ingestion or total duration of non-penetration. The susceptibility in sorghum for *Schizaphis graminum* (Rondani) was also not affected by detachment of leaves (Montllor et al., 1990).

For resistant lettuce, our data show that detached leaves and leaf disks partly

lose their resistance. The *Nr*-resistance is known to be active in the phloem and causes a reduction in or lack of phloem ingestion in *N. ribisnigri*. On both the detached leaves and leaf disks of Corbana, more time was spent on phloem ingestion, and less time on pathway activities compared to intact plants, probably as a consequence of spending more time on phloem events. *Schizaphis graminum* also spent more time on phloem ingestion on detached leaves compared to intact plants of resistant sorghum varieties (Montllor et al., 1990). On resistant lettuce, the percentage of aphids displaying phloem ingestion was also higher on both the detached leaves and leaf disks. Here, leaf disks seem to be even more susceptible than detached leaves, because all aphids displayed phloem feeding compared to 70% on detached leaves. A smaller percentage of the total time spent on phloem events was spent on salivation by aphids on leaf disks compared to aphids on intact plants, indicating less salivation was needed to overcome the resistance component. This was also found for aphids on detached leaves; however, the total duration of phloem salivation was higher, which could be the consequence of spending more time on phloem events. Liu & McCreight (2006) also studied *N. ribisnigri* on leaf disks of resistant and susceptible lettuce cultivars, by performing survival and reproduction experiments. No differences in adult survival were found between susceptible and resistant leaf disks, and differences in reproduction were smaller on leaf disks of resistant and susceptible lettuce compared to intact plants, also indicating loss of resistance. The overall survival, however, was very low on the leaf disks, probably caused by the difficulty maintaining the turgor of leaf disks over a prolonged period.

Loss of phloem-based resistance after excision of plant parts was also found in *Medicago truncatula* against *Acyrthosiphon kondoi* (Shinji) and *Acyrthosiphon pisum* (Harris) in excised shoots of resistant plants (Gao et al., 2008; Klingler et al., 2005). In soybean resistance against *Aphis glycines* (Matsumura) both loss of resistance and retention of resistance were found in different soybean varieties (Michel et al., 2010). Detached leaves of PI 567301B (antixenosis resistance) had no resistance against the soybean aphid, whereas the detached leaves of PI 243540 and PI 200538 (antibiosis resistance) maintained their aphid resistance in the same study (Jun et al., 2012; Michel et al., 2010). Leaf disks of Brussels sprouts lost their resistance against *Brevicoryne brassicae* but retained their resistance against *M. persicae* (Van Emden & Bashford, 1976). Retention of resistance was also found for *A. gossypii* resistance in excised leaves of melon (Kennedy & Kishaba, 1977). In the case of retention of resistance in detached leaves, the resistance may be either constitutively expressed or induced locally at the feeding site of the aphid (Michel et al., 2010).

As mentioned before, the resistance seems to be partly lost in leaf disks and detached leaves of resistant lettuce cultivar Corbana. The duration of phloem ingestion is an important parameter for resistance in lettuce. The total duration of phloem ingestion on detached leaves and leaf disks on Corbana is shorter compared to Terlana, although, the resistance seems to be partially lost. The nutritional quality of detached leaves and leaf disks

might be altered compared to intact plants, which could have affected the results. Changes in metabolism of the plant tissue due to excision were suggested by Edelstein et al. (2000) and Gao et al. (2008). Cutting a leaf or preparing leaf disks triggers many physiological changes that can affect resistance (Kessler & Baldwin, 2002). Montllor et al. (1990) observed an increase in free amino acids in detached leaves of both susceptible and resistant sorghum compared to intact sorghum plants, however, no differences were observed in aphid growth and feeding behaviour on intact and detached leaves of susceptible plants, indicating the nutrition was not the determining factor for the suitability of detached versus intact leaves. For detached lettuce leaves, the susceptibility was not increased after periods of 0, 24, 48 or 76 hours in tap water. In sorghum, however, one of two resistant varieties did show an increase in susceptibility in terms of phloem ingestion after 24 h since detachment, for the other resistant variety, however, phloem ingestion was not increased after a 24 h detachment (Montllor et al., 1990).

Resistance factor

The *Nr*-resistance factor seems to be produced in the shoot and transport from the roots was ruled out. Additionally, the resistance is partly lost when excising leaves or preparing leaf disks, which suggests that the lettuce plant needs to be intact to express full resistance against *N. ribisnigri.* The resistance factor itself or signalling molecules might be phloem-mobile and might need to be translocated to the site of aphid feeding, instead of being produced at the site of feeding. Systemic translocation of resistance factors could be tested by making grafts of the top of shoots and the lower part of shoots. The partial resistance that we observed in detached leaves and leaf disks might be caused by the resistance factors that were already constitutively present in the phloem, but could not be transported to the feeding site anymore because of the disruption of the vascular system. Excision of plant parts cause loss of root pressure and as a consequence turgor loss. On intact plants aphids take up phloem sap passively due to the high turgor pressure in the sieve tubes (Tjallingii, 1995). On detached leaves and leaf disks, the turgor pressure is very low and aphids have to ingest phloem sap actively using muscular pumps. In a previous study by Will et al. (2008), the feeding and penetration behaviour of aphids was shown to be influenced by the pressure of the artificial diet they fed on. Loss of turgor might also have affected the feeding and penetration behaviour of *N. ribisnigri* in our study.

Some aphid species are able to increase the nutritional quality of their food, by manipulating the resource allocation within plants, increasing the import of resources from other sites of the plant to the feeding site (Goggin, 2007). This was observed for the pea aphid, *A. pisum* on alfalfa (*Medicago sativa*), which was able to mobilise and translocate nitrogen from the growth zone of alfalfa to the feeding site (Girousse et al., 2005). Aphids might also cause translocation of resistance factors to the feeding site. Antibiotic components like lectins and protease inhibitors as well as secondary metabolites can accumulate

in the phloem sap and be translocated to the aphid feeding site (Brudenell et al., 1999; Kehr, 2006). Phytohormones are also transported via the phloem, for example jasmonic acid, which is involved in the systemic defence response against herbivores (Schilmiller & Howe, 2005). Salicylic acid (SA), which is also transported through the phloem, is required as signalling molecule for systemic acquired resistance, a mechanism of induced resistance against pathogens and often related to *R*-gene mediated resistance (Durrant & Dong, 2004; Westwood et al., 2010). The SA signal-transduction pathway is also commonly reported to mediate plant-aphid interactions, but not for chewing insects (Zhu-Salzman et al., 2004). The *Mi-1*- gene mediated resistance in tomato, was shown to be dependent upon the SA signalling pathway for the resistance of tomato against potato aphids (*M. euphorbiae)* (Li et al., 2006). The octadecanoid (jasmonic acid) pathway is important for the resistance in *Medicago truncatula* against the blue green aphid (*A. kondoi*) (Gao et al., 2007). The *Nr*-gene mediated resistance of lettuce might also be dependent upon phytohormones, however, this remains to be studied.

Whether resistance components are soluble components of the phloem sap can be tested in artificial diets containing phloem sap exudates. For example, Chen et al. (1996) showed in a choice test using artificial diet containing phloem sap from resistant and susceptible melon that sap from susceptible melon was preferred. Reproduction of *Myzus persicae* (Sulz.) was negatively affected on artificial diets containing petiole exudates of resistant *Arabidopsis thaliana* (Louis et al., 2010). Phloem sap of susceptible lettuce was also preferred by *N. ribisnigri* compared to phloem sap of resistant lettuce, indicating a resistance factor in the phloem sap (Van Helden et al., 1995).

Conclusion

The resistance factors in lettuce against *N. ribisnigri* are synthesised in the shoot, and not transported from the root the shoot. The partial loss of resistance after detachment of leaves and leaf parts that we observed, suggests that an intact vascular system is needed for a full resistance and that the resistance component itself or signalling molecules need to be translocated to the site of aphid feeding. More studies are needed to explain the exact mechanism involved in causing partial loss of resistance. From a breeder’s perspective, screening for resistance in lettuce against *N. ribisnigri* should focus on intact plants, instead of excised plant parts.

Acknowledgements

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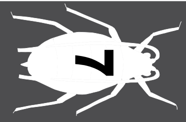
Appendix 1: EPG parameters (mean ± SEM) of N. ribisnigri Nr:0 on L. sativa cultivars Corbana and Terlana: intact plant (plant), detached leaf (leaf), leaf disk and graft (Corbana shoot on Corbana root and Terlana shoot on Terlana root). Pairwise comparisons were made for EPG parameters between intact plants and the other treatments (leaf, leaf disk, graft), and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the three comparisons made by setting α = 0.0167. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Time parameters are in seconds. NP = non-penetration, C = pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties. * = P < 0.017; ** = P < 0.005; *** = P < 0.001.

Corbana	plant (n=20)			leaf (n=22)				leaf disk (n=17)				graft (n=18)			
Duration of NP before 1st visible E	2786	±	688	3478	±	597		1884	±	486		3111	±	735	
Time to 1st E2#	13640	±	5614	11547	±	1839		8793	±	1573		14962	±	3771	
Time to 1st sustained E2#				13520	±	2235		10207	±	1890		16661	±	4562	
Total duration of E	434	±	97	9230	±	1809	***	14588	±	1871	***	1190	±	380	
Total duration of E1	424	±	96	1971	±	436	***	891	±	152	*	638	±	195	
Total duration of single E1	403	±	94	1047	±	313		613	±	164		369	±	112	
Nr. of E2 periods	0.11	±	0.07	1.40	±	0.29	***	2.06	±	0.30	***	0.44	±	0.17	
Nr. of sustained E2 periods	0.00	±	0.00	0.90	±	0.22	***	1.44	±	0.20	***	0.17	±	0.09	
Nr. of probes to the 1st E1	7.83	±	1.16	10.47	±	1.54		9.00	±	1.56		19	±	2.99	**
Total duration of NP	7095	±	944	6279	±	710		3583	±	550	*	7291	±	900	
Total duration of C	19283	±	769	12679	±	1185	***	10188	±	1505	***	18611	±	750	
Total duration of G	2881	±	556	3319	±	1162		2345	±	664		2272	±	395	
Nr. of short probes (C < 3 minutes)	11.58	±	1.84	11.75	±	1.96		10	±	2.99		26	±	3.16	**

Terlana	(n=20)			(n=21)				(n=17)			(n=17)		
Duration of NP before 1st visible E	1619	±	255	867	±	178	*	982	±	201	1134	±	281
Time to 1st E2[#]	6414	±	1070	5009	±	849		3883	±	444	4505	±	1024
Time to 1st sustained E2[#]	6414	±	1070	5928	±	948		3883	±	444	5361	±	950
Total duration of E	21933	±	1083	21799	±	1083		21382	±	929	20114	±	953
Total duration of E1	294	±	78	768	±	298		323	±	86	561	±	231
Total duration of single E1	197	±	58	214	±	66		98	±	17	554	±	426
Nr. of E2 periods	1.20	±	0.12	1.43	±	0.15		1.71	±	0.21	2.29	±	0.43
Nr. of sustained E2 periods	1.15	±	0.08	1.24	±	0.12		1.59	±	0.17	1.59	±	0.21
Nr. of probes to the 1st E1	8.15	±	1.51	4.67	±	0.78		5.88	±	0.74	5.71	±	1.24
Total duration of NP	1753	±	329	1161	±	239		1893	±	376	1940	±	270
Total duration of C	4923	±	848	4868	±	930		5135	±	615	5866	±	853
Total duration of F	1358										4325		
Total duration of G	1912	±	211	2549	±	448		1660	±	255	1773	±	38
Nr. of short probes (C < 3 min)	4.85	±	1.19	2.43	±	0.55		5.06	±	1.02	5.94	±	1.23

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe



Appendix 2: EPG parameters (mean ± SEM) of *N. ribisnigri* Nr:0 on *L. sativa* grafts of a Corbana shoot on a Terlana root (CT) and a Terlana shoot on a Corbana root (TC). Pairwise comparisons were made for EPG parameters between the CT and TC graft, between intact Corbana plant and CT graft and between intact Terlana plant and TC graft, and were analysed by the Mann-Whitney U test, applying the Bonferroni correction to account for the two comparisons made by setting α = 0.025. Significance of differences in percentage of aphids showing E2 was analysed by Fisher's exact test. Times are in seconds. NP = non-penetration, C = pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties. * = P < 0.025; ** = P < 0.005; *** = P < 0.001.

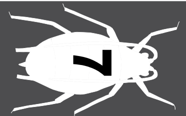
	graft C-T			graft T-C				C-CT	T-TC
		(n=20)			(n=19)				
Duration of NP before 1st visible E	2670	±	631	1731	±	422			
Time to 1st E2#	6631	±	1783	7256	±	1279			
Time to 1st sustained E2#	6200	±		6603	±	1113			
Total duration of E	1498	±	475	20677	±	1565	***	*	
Total duration of E1	1159	±	381	789	±	305		*	
Total duration of single E1	862	±	326	379	±	109			
Nr. of E2 periods	0.45	±	0.22	1.47	±	0.21	***		
Nr. of sustained E2 periods	0.10	±	0.10	1.21	±	0.14	***		
Nr. of probes to the 1st E1	13.25	±	1.86	7.37	±	1.42	*		
Total duration of NP	7496	±	725	2040	±	509	***		
Total duration of C	18479	±	551	5548	±	1076	***		
Total duration of F	3665			2892					
Total duration of G	2079	±	308	1815	±	478			
Nr. of short probes (C < 3 min)	24	±	2.98	4.68	±	1.01	***	**	

Time to first E, E2 and first sustained E2 was measured from the start of the first probe

Appendix 3: EPG parameters (mean ± SEM) of *N. ribisnigri* Nr:0 on *L. sativa* Corbana leaves, directly (0 h), 24 h, 48 h and 76 h after detachment. The Kruskal-Wallis test was used to test for differences in EPG parameters between the treatments. Time parameters are in seconds. NP = non-penetration, C = pathway, E = phloem event, E1 = phloem salivation, E2 = phloem ingestion, G = xylem ingestion, F = penetration difficulties. No significant differences were found.

	0h (n=22)	24h (n=17)	48h (n=20)	72h (n=21)
Duration first NP	489 ± 124	463 ± 158	291 ± 85	314 ± 82
Time to 1st visible E[#]	7187 ± 986	9756 ± 1321	7865 ± 1732	8351 ± 1270
Contribution E1 to E (%)	42 ± 9.50	68 ± 9.54	52 ± 9.44	64 ± 9.59
% aphids showing E2	70.00	47.06	60.00	47.62
Nr. of probes	24 ± 2.87	32 ± 3.91	25.00 ± 1.90	26 ± 2.55
Total duration of E2	8821 ± 1934	6852 ± 2694	7406 ± 2082	10163 ± 3045
Duration of NP before 1st visible E	3478 ± 597	4235 ± 596	3093 ± 791	2526 ± 291
Time to 1st E2[#]	11547 ± 1839	9644 ± 2022	13564 ± 2945	9686 ± 1787
Time to 1st sustained E2[#]	13520 ± 2235	10266 ± 2403	12731 ± 2973	10014 ± 1873
Total duration of E	9230 ± 1809	4685 ± 1583	6080 ± 1580	6064 ± 1845
Total duration of E1	1971 ± 436	1326 ± 434	1143 ± 208	983 ± 250
Total duration of single E1	1047 ± 313	698 ± 117	764 ± 185	915 ± 242
Nr. of E2 periods	1.40 ± 0.29	0.94 ± 0.30	1.05 ± 0.25	0.71 ± 0.22
Nr. of sustained E2 periods	0.90 ± 0.22	0.65 ± 0.24	0.60 ± 0.13	0.57 ± 0.16
Nr. of probes to the 1st E1	10.47 ± 1.54	16 ± 2.74	9.33 ± 1.67	10.15 ± 1.25
Total duration of NP	6279 ± 710	6444 ± 695	6097 ± 866	6036 ± 676
Total duration of C	12679 ± 1185	15359 ± 1208	14806 ± 1037	14982 ± 1252
Total duration of F		2259 ± 893		535
Total duration of G	3319 ± 1162	3579 ± 1132	3234 ± 604	2433 ± 407
Nr. of short probes (C < 3 min)	11.75 ± 1.96	19 ± 3.16	12.30 ± 1.57	13.00 ± 1.87

[#] Time to first E, E2 and first sustained E2 was measured from the start of the first probe



Chapter 8

General discussion

Cindy J.M. ten Broeke

Introduction: Host plant resistance against aphids

Aphids are serious pests of crops, causing economic losses by reducing plant growth and vigour, and transmitting plant viruses (Tagu et al., 2008; Westwood et al., 2010). They damage many important crops, like oilseed rape, cereals, fruits and vegetables, but also medically important plants and timber trees (Dedryver et al., 2010). During the last decades the demand for aphid control strategies, including integrated pest management (IPM) strategies, has been increasing. Aphid control by insecticides results in environmental and economic costs and many pest species have developed insecticide resistance (Anstead et al., 2005; Pimentel et al., 1992; Westwood et al., 2010). Since 1999, several insecticides have been banned by law in countries all around the world and farmers are now lacking efficient treatment for several insect pests, like against the woolly apple aphid *Eriosoma lanigerum* (Hausemann) in France (Dedryver et al., 2010). Some recently banned insecticides that are used to control aphids are endosulfan (USA in 2010) and dieldrin (Lubick, 2010) (EPA). Some neonicotinoids have been proposed to be banned in Europe (Grimm et al., 2012). Recently, three of them; clothianidin, imidacloprid, and thiamethoxam, have been banned from use on flowering crops (Erickson, 2013). Host plant resistance is often the most effective and environmentally sound control strategy to control aphid pests, and many cultivars have been bred for resistance against aphids (Dreyer & Campbell, 1987; Tagu et al., 2008). These resistances are often based on major resistance (*R*) genes, and are mostly species-specific (Tjallingii, 2006).

The *R*-genes are part of a molecular surveillance system that allows certain genotypes of plants to specifically recognise and resist pathogens and insect pests (Goggin, 2007). They can be involved in classical gene-for-gene interactions, in which the product of an avirulence gene of the attacker is directly or indirectly recognised by the product of an *R*-gene in the plant, triggering a defence response against the attacker (Flor, 1955). A more complex interaction is described by the zig-zag model, in which plants recognise and respond to attackers in a two-branched system, the first branch recognising common molecules of attackers and the second recognizing specific effectors of attackers (needed for effector-triggered susceptibility) by direct or indirect interactions with the *R*-gene product, resulting in effector-triggered immunity (Jones & Dangl, 2006) (Figure 1). These *R*-gene mediated resistances were first only associated with plant defence against pathogens, but are now also known to be involved in resistance against insects, including aphids (Kaloshian, 2004).

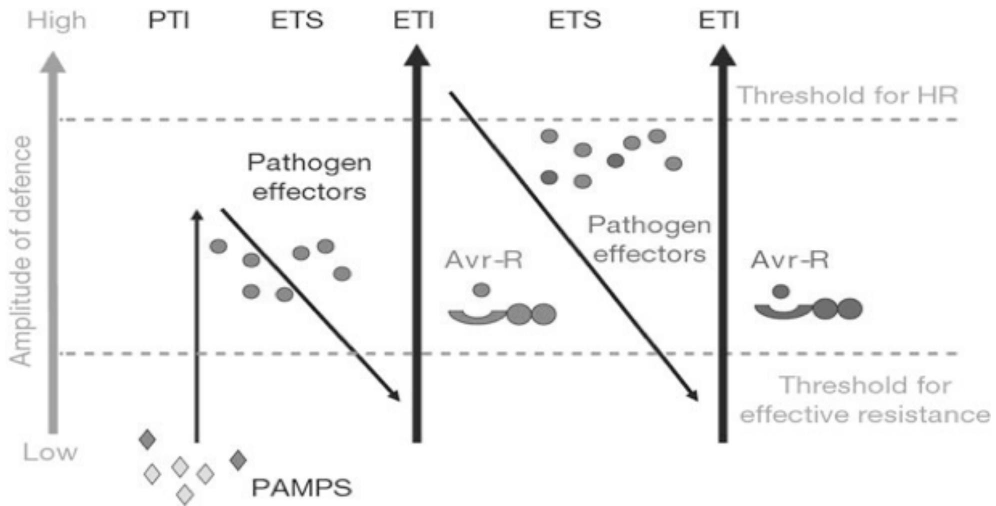
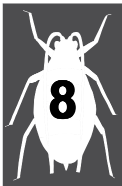


Figure 1: The Zig-zag model according to Jones & Dangl (2006). PAMPS: pathogen-associated molecular patterns; PTI: PAMP-triggered immunity: ETS: effector-triggered susceptibility; ETI: effector triggered immunity; Avr: avirulence; R: *R*-gene.

The *Nr*-gene

Host-plant resistance mediated by the *Nr*-gene in lettuce is an important control strategy to control an economically important pest in lettuce, the black currant-lettuce aphid, i.e. *Nasonovia ribisnigri* (Mosley). Despite its importance in controlling *N. ribisnigri*, the *Nr*-gene has not been cloned, and the mechanism causing resistance at the molecular level is unknown. The project reported in this PhD thesis was one of three projects in the TTI-GG (Technological Top Institute Green Genetics) *Nasonovia* programme. The aim of this programme was to unravel the mechanism of resistance mediated by the *Nr*-gene in lettuce against the lettuce aphid *N. ribisnigri*, in order to accelerate breeding for host-plant resistance. A multidisciplinary approach was used, by combining studies of the host-plant selection behaviour and performance of *N. ribisnigri* (this thesis) with metabolomic, proteomic and transcriptomic analyses of the phloem composition of susceptible and resistant lettuce. Although the *Nr*-gene was not cloned and the compound(s) (proteins or metabolites) responsible or involved in the resistance were not identified in the time span of the programme, information about the possible resistance mechanism was gathered in this thesis by behavioural studies on *N. ribisnigri*, both virulent (Nr:1) and avirulent (Nr:0) populations, on resistant and susceptible lettuce lines (Chapters 2, 3 and 4).

The electrical penetration graph method (EPG) made it possible to identify the plant tissue in which the *Nr*-mediated resistance was encountered by the *N. ribisnigri* aphids. This resistance is located in the phloem, because the phloem ingestion of the Nr:0 aphids is negatively affected (Chapter 2), a conclusion previously drawn by Mentink et al.



(1984) and van Helden and Tjallingii (1993). Nr:0 aphids on resistant lettuce were able to reach the phloem and salivate in it, but this was mostly not followed by phloem ingestion, or phloem ingestion took only place for a short while, resulting in 100% mortality on resistant lettuce during performance experiments. Indications for resistance within the pathway to the phloem were also found, because aphids on resistant lettuce took longer to display the first visible phloem event. This, however, can also be caused by resistance components in the phloem which are encountered during brief punctures into the phloem (Chapter 2). Van Helden and Tjallingii (1993) did not observe a delay in the first phloem contact on resistant lettuce, which could be explained by the use of different lettuce and aphid material in the present study. A delay in first phloem contact on resistant plants was also found by Caillaud et al. (1995) for the cereal aphid *Sitobion avenae* F. on wheat, by Chen et al. (1997) for the melon aphid *Aphis gossypii* (Glover) on melon, by Alvarez et al. (2006) for the green peach aphid *Myzus persicae* (Sulzer) on *Solanum*, by Crompton and Ode (2010) for the soybean aphid *Aphis glycines* (Matsumura) on soybean and by Fartek et al. (2012) for the sugarcane aphid *Melanaphis sacchari* (Zhentner) on sugarcane. Next to the antixenosis resistance conferred by the *Nr*-gene, observed as a severe reduction or complete absence of feeding, antibiosis resistance might be involved. Evidence for antibiosis was found in Chapter 3 when several Nr:1 populations were able to feed on resistant lettuce but reproduction was absent. Successful colonisation of plants by aphids often depends on the ability of aphids to overcome the sieve element wounding response (Westwood et al., 2010). Phloem sap loss after wounding of the sieve elements is prevented by a sieve element sealing system, the calcium (Ca^{2+})-triggered plugging of the sieve plates by co-agulated phloem proteins and callose (Will & Van Bel, 2006). Aphids are known to prevent this wound response by the secretion of watery saliva, which contains Ca^{2+} - binding proteins, the sheath saliva which seals the wound in the sieve element, and the small diameter of the stylet which both prevent the influx of Ca^{2+} (Will et al., 2007; Will & Van Bel, 2006). When aphids fail to prevent the wound response in plants, they might be unable to feed from the phloem. This might also be the case for the *Nr*- gene mediated resistance in lettuce, because aphids show a strong reduction in feeding on resistant plants (Chapter 2). Both the sieve plates and the aphid stylets might be blocked making phloem ingestion impossible, and starvation would then be the cause of mortality, rather than the presence of a toxic component in the phloem. Nr:1 aphids might be able to successfully prevent this wound response, also locally, probably in the same sieve element, for Nr:0 aphids feeding on the same plant (Chapter 5). Other resistances like the one in wild *Brassica* species against the cabbage aphid (*Brevicoryne brassicae* L.) (Cole, 1994) and resistant lines of wheat, *Triticum monococcum* L. (Tm44 and Tm46) against *S. avenae* (Caillaud et al., 1995) were also hypothesised to be caused by blocking of the stylets and sieve plates. Table 1 gives an overview of aphid resistance genes and resistant cultivars and their mode of action and location of resistance.

The *Nr*-resistance factor is likely produced in the shoot and not in the root of lettuce, because grafts with a resistant shoot and susceptible root remained resistant whereas grafts with a susceptible shoot and resistant root were susceptible (Chapter 7). Additionally, to get complete resistance against *N. ribisnigri* an intact vascular system is needed, because the resistance was partly lost in detached leaves and leaf disks of resistant lettuce, which might be caused by the loss of turgor (Chapter 7). This might suggest that the *Nr*-resistance component or signalling molecules need to be translocated to the site of aphid feeding, instead of being present or produced at the feeding site itself. The *Nr*-resistance factor in lettuce was shown in a previous study by van Helden et al (1995) to be present in phloem sap, because aphids preferred to feed on artificial diets which included phloem sap of susceptible lettuce instead of resistant lettuce.

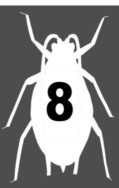
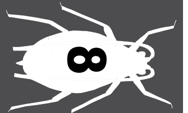


Table 1: Resistance in plants against aphids.

Plant species	**Aphid species**	***R*-gene(s) or resistant cultivars**	**Mechanism and location of resistance**	**Virulent biotype(s)**	**References**
Lettuce (*Lactuca sativa)*	*Nasonovia ribisnigri* *Pemphigus bursarius*	*Nr*-gene *Lra*-gene	Antixenosis; phloem-based Antibiosis	Nr:1	(Van Helden & Tjallingii, 1993) Chapter 2,3 (Ellis et al., 2002)
Tomato (*Solanum lycopersicum*)	*Macrosiphum euphorbiae*	*Mi*-gene (NBS-LRR)	Phloem-based	Semi-virulent WU12	(Kaloshian et al., 1997; Kaloshian et al., 2000; Pallipparambil et al., 2010; Rossi et al., 1998)
Melon (*Cucumis melo)*	*Aphis gossypii*	*Vat*-gene (NBS-LRR)	Antibiosis and antixenosis; phloem-based	Virulent colonies	(Chen et al., 1996; Lombaert et al., 2009)
Soybean (*Glycine max*)	*Aphis glycines*	*Rag1, Rag2, rag 4, rag1c, Rag3, Rag5*	Antibiosis; phloem-based	Biotypes 1,2,3	Summarised by (Hill et al., 2012)
Barley (*Hordeum vulgare*) / Wheat (*Triticum aestivum)*	*Diuraphis noxia*	*Dn1, Dn2, Dn3, Dn4, Dn7, Dn8, Dn9, Dny*		Biotype 1,2,3,4,5 RWASA2, RWASA3, USARWA	(Burd et al., 2006; Haley et al., 2004; Jankielsohn, 2011; Tolmay et al., 2012)
Wheat (*Triticum aestivum*)/ Sorghum (*Sorghum bicolor*)	*Schizaphis graminum*	*Gb1,Gb2, Gb6, Gb5, Gbx1, Gba, Gbb, Gbc, Gbd, Gbz, Gb3 and Gbx2*		Virulent biotypes against Gb2 and Gb3	(Azhaguvel et al., 2012; Porter et al., 1997; Puterka & Peters, 1989; Puterka & Peters, 1995)

Triticum durum	*Sitobion avenae*	*RA-1* (cultivar C273)			(Liu et al., 2012)
Triticum monococcum		cv. Tm44, Tm46	Phloem-based		(Caillaud & Niemeyer, 1996; Caillaud et al., 1995)
Red Raspberry (*Rubus idaeus)*	*Amphorophora agathonica*	*Ag1,Ag2, Ag3*	Phloem-based	6 virulent biotypes	(Dossett & Kempler, 2012; Lightle et al., 2012)
Nectarine (*Prunus persicae*)	*Myzus persicae*	Cv August Red, July Red-NS92	Pre-phloem-based		(Verdugo et al., 2012)
Alfalfa (*Medicago truncatula)*	*Acyrthosiphon kondoi*	AKR (candidate NBS-LRR)	Antixenosis, antibiosis; phloem-based	Virulent biotype	(Gao et al., 2008; Humphries et al., 2013; Klingler et al., 2005)
	Acyrthosiphon pisum	*RAP1* gene		Virulent biotype Australia	(Humphries et al., 2013; Stewart et al., 2009)
	Aphis craccivora	cv. SA30199	Antibiosis; phloem-based		Kamphuis et al., 2012)
Sugarcane (*Saccharum spec*)	*Melanaphis sacchari*	cv. R365	Antibiosis and antixenosis; phloem-based		(Fartek et al., 2012)
Cowpea (*Vigna unguiculata*)	*Aphis craccivora*	cv. ICV-12	Phloem-based		(Annan et al., 2000)



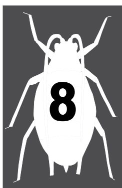
Wild *Solanum* species: *Solanum* spp., *Solanum tuberosum* and *S. tuberosum*	*Myzus persicae*		leaf surface, epidermis, mesophyll and phloem		(Alvarez et al., 2006)
Brassica fruticolosa	*Brevicoryne brassicae*		Phloem-based		(Cole, 1994)
Poplar (*Populus x canadensis*)	*Phoeomyzus passerninii*	cv. Brenta	Antixenosis and antibiosis; surface- and parenchyma-based		(Pointeau et al., 2013)
Hop (*Humulus lupulus*)	*Phorodon humuli*	Genotype INT 101	Phloem-based		(Paul et al., 1996)
Apple (*Malus domestica*)	*Dysaphis plantaginea*	cv. Florina	Surface-, pathway- and phloem-based		(Marchetti et al., 2009)

Signalling pathways

Upon aphid feeding, plants perceive several signals that trigger defence pathways; local tissue damage, proteins released from aphid saliva (effectors or elicitors), electrical and/or hydraulic signals, and phloem sap loss (Walling, 2008). Several studies have been performed on gene expression during plant defence responses against aphids, to identify the signalling cascades that are activated by resistance genes (Goggin, 2007; Thompson & Goggin, 2006). Defence responses can involve phytohormones, kinases, pathogenesis-related (PR) genes, proteins, and reactive oxygen species (Gao et al., 2007; Li et al., 2006; Walling, 2000).

Chewing insects like caterpillars have a different mode of feeding compared to phloem feeding insects, like aphids. These insects with different feeding strategies also induce different signalling pathways in plants. Chewing insects mainly induce jasmonic acid (JA) and ethylene (ET) pathways, while piercing/sucking insects mainly induce salicylic acid (SA)-mediated pathways, resulting in differences in gene expression specific to the feeding mode of the attacker (Broekgaarden et al., 2011; De Vos et al., 2005; Walling, 2000). Cross- talk between these two pathways takes place and might affect the plant response to insects with different feeding modes. *Pieris brassicae* (L.), for example, was positively affected by pre-infestation of *B. brassicae* on *Brassica oleracea* (L.), but not the other way around (Soler et al., 2012). Effectors suppressing JA might be present in the saliva of aphids, as was suggested by De Vos et al. (2005; 2007).

Although aphids mainly induce the SA pathway, different phytohormones can be upregulated in *R*-gene mediated resistance responses in different crop species to aphids. For example, SA is involved in the interaction between the *Mi*-gene in tomato and the potato aphid *Macrosiphum euphorbiae* Thomas (Li et al., 2006), JA is involved in the alfalfa (*Medicago truncatula* Gaertn.) - *Acyrthosiphon kondoi* (Shinji) interaction (Gao et al., 2007) and ethylene is involved in the *Vat*-gene mediated resistance against *A. gossypii* (Anstead et al., 2009). One *R*-gene can also be involved in several signalling pathways, for example, the *Mi*-gene, which mediates aphid and nematode resistance in tomato, was involved in different pathways against these two pests (Goggin, 2007). Some aphid species are known to suppress plant defences by inducing the SA pathway, thereby suppressing the JA pathway (Walling, 2008). Alvarez et al. (2013) observed that a large number of regulatory and metabolism-related genes were expressed in *Solanum stoloniferum* L. by *M. euphorbiae*, a compatible interaction in which the aphid is able to feed from the plant, indicating this aphid might benefit from the induction of these genes. Studham and MacIntosh (2013) also found a large number of transcripts when *A. glycines* was feeding on susceptible soybean. In contrast, only one transcript was found upregulated on aphid- induced resistant soybean. A possible explanation is that the resistance-related transcripts are constitutively expressed. Recently, the role of microRNAs (miRNAs) in the *VAT*-gene mediated resistance in melon against *A. gossypii* were studied (Sattar et al., 2012). Differences in accumulation of miRNAs in aphids feeding on susceptible and resistant melon were observed, suggesting a role in



regulating aphid developmental processes in the *VAT*-gene mediated resistance (Sattar et al., 2012). It is not known what signalling cascades are involved in the *Nr*-resistance in lettuce against *N. ribisnigri;* however, intact plants were shown to be necessary for a full defence response against this aphid pest (Chapter 7).

NBS-LRR genes

Mammals have a somatic adaptive immune system and mobile defender cells; however, plants lack these and rely for their defence response on the innate defence of each cell and on systemic signals from infection sites, which can be mediated by *R*-genes (Jones & Dangl, 2006). Many *R*-genes are known to confer resistance against aphids but only two of these have been cloned, the *Mi*-gene in tomato and the *VAT*-gene in melon (Milligan et al., 1998; Pauquet et al., 2004). Both genes are members of the family of the nucleotide-binding and leucine-rich repeat (NBS- LRR) genes. Additionally, the *AKR*-gene in *M. truncatula* conferring resistance against *A. kondoi* was mapped to a cluster of NBS-LRR associated sequences (Klingler et al., 2005). Candidate genes conferring resistance to *A. glycines, Rag1* (Kim et al., 2010b) and *Rag2* (Kim et al., 2010a), and the gene *Raso1* conferring resistance to *Aulacorthum solani* Kalt. (foxglove aphid) in soybean were also members of the NBS-LRR gene family (Ohnishi et al., 2012).

Lettuce is also known to contain a cluster of NBS-LRR genes, called the *Resistance Gene Candidate 2* (*RGC2*) family (Wroblewski et al., 2007). At least 15 genes (*Dm1, Dm2, Dm3, Dm4, Dm6, Dm7, Dm8, Dm10, Dm11, Dm12, Dm13, Dm14, Dm15, Dm16, Dm18*) in this cluster have been associated with resistance to isolates of the oomycete pathogen *Bremia lactucae* (Regel); for example, *Dm3* is responsible for the resistance to isolates of *B. lactucae* that carry the avirulence gene *Avr3* (Okubara et al., 1994). The known *Dm*-genes and isolates of *B. lactucae* against which they provide resistance are summarised by Okubara et al. (1994). The *LRa*-gene, which is linked to the *Dm16*-gene, mediates resistance against the lettuce root aphid (*Pemphigus bursarius* L.; (Ellis et al., 2002). Whether the *Nr*-gene is also an NBS-LRR gene within the *RGC2*-family is not known yet.

In pathogen-plant interactions, many *R*-genes in plants encode for NBS-LRR proteins, and so far these proteins are only known to function in *R*-mediated resistance (Dangl & Jones, 2001). The nucleotide-binding site is known to be crucial for ATP and GTP binding in other proteins, while the LRR domain is also found in other proteins and functions as site for protein-protein interactions, peptide-ligand binding and protein-carbohydrate interaction (Dangl & Jones, 2001; Jones et al., 1997). Most of the NBS-LRR proteins are intracellular and are receptors for specific proteins from a pathogen, or function in a protein complex that is the functional receptor inside the cell (Dangl & Jones, 2001). The recognition of such proteins is followed by signalling cascades that can rapidly activate plant defences against the attacker (Goggin, 2007). The above-mentioned pathogen protein recognition

and signalling system might also work for the recognition of aphid-specific proteins by plants.

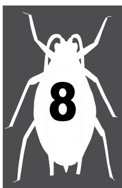
Factors influencing resistance

Screening for resistance should take into account that the expression of certain host plant resistances might be affected by other factors. In Chapter 3, the genetic background of lettuce was shown to have a significant effect on the level of resistance provided by the *Nr*-gene, and thereby affected the behaviour of the aphids. Many factors can affect the behaviour of aphids. Vermicompost application, for example, decreased the development and reproduction of *A. gossypii* on cucumber cultivars (Razmjou et al., 2011). Compost also had a negative effect on the colonisation of the strawberry aphid (*Chaetosiphon fragefolii* Cockerell) on strawberry (Bobev et al., 2012). The rhizobacterium *Pseudomonas fluorescens* had a positive effect on the performance of *M. persicae* on *Arabidopsis*, while no effect was observed for *B. brassicae* (Pineda et al., 2012). An effect of temperature on the expression of resistance in soybean against *A. glycines* was observed by Richardson (2012). Elevated CO_2 reduced the resistance in red raspberry against the large raspberry aphid, *Amphorophora idaei* (Born), which could become a problem considering the elevated CO_2 levels caused by global climate change (Martin & Johnson, 2011). Drought stress reduced the resistance in a hybrid of poplar (*Populus trichocarpa* · *Populus maximowiczii* Henry) against *Chaitophorus leucomelas* Koch, whereas tolerance was increased (Ramírez & Verdugo, 2009).

When large plant collections need to be screened for resistance, excised plant tissue or leaf disks are sometimes used for practical reasons. However, for aphids this might not always be applicable. Chapter 7 shows that the *Nr*-mediated resistance was partly lost when leaves were cut off the plant and in leaf disks of resistant lettuce. Phloem-based resistance was also lost after excision in *M. truncatula* against *A. kondoi* and *A. pisum* (Harris) (Gao et al., 2008; Klingler et al., 2005), and soybean resistance against *A. glycines* (Michel et al., 2010). Screening for resistance in lettuce against *N. ribisnigri* should therefore focus on intact plants, instead of excised plant parts.

Virulence in aphids

The emergence of virulent aphid biotypes is a growing problem in sustainable crop protection. Virulence formation enables aphids to extend their host range and occupy new niches, *i.e.* resistant cultivars in the case of host plant resistance (Van der Arend, 2003). The high human-imposed selection pressure upon aphid pests in agricultural systems can cause the emergence of virulent aphid biotypes (Mitchell et al., 2009). Especially in crop systems where only cultivars are used that have one and the same aphid resistance gene, the selection and rapid spread of virulent biotopes is promoted. Together with the genetic variation in aphids, expressed also in differences in behavioural traits, this could give rise to more virulent aphid biotypes (Lombaert et al., 2009).



Virulent populations of *N. ribisnigri*, that were able to colonise resistant lettuce cultivars in the field, have been reported since 2007 (Thabuis et al., 2011). Aphids of a virulent *N. ribisnigri* population collected in Germany performed equally well and showed the same feeding/penetration behaviour on resistant and susceptible isogenic lettuce (Chapter 2). Variation in virulence to the *Nr*-based resistance was found between several populations of *N. ribisnigri* originating from different regions in Europe (Chapter 3). Semi virulence was found in terms of feeding behaviour and performance of *N. ribisnigri* populations on resistant lettuce. Semi virulence was also reported for *M. euphorbiae* biotype WU12 (Pallipparambil et al., 2010). Semi virulence was hypothesised by Herbert et al. (2007) to be caused by a reduction, instead of complete absence, of recognition of a virulence product of the aphid by the plant.

Some other crops for which virulent aphid biotypes have been reported are summarised in Table 1. The underlying mechanism of virulence in aphids is not fully understood yet, and several factors may contribute to this phenomenon. For pathogens the ability to overcome *R*-gene resistances is often attributed to the loss or modification of elicitors, products of avirulence genes, to evade the detection by the plant and/or suppressing plant defences, which is also hypothesised to be the underlying mechanism of aphid virulence (Hogenhout & Bos, 2011; Parker & Gilbert, 2004).

Aphid effectors

Aphid effectors are important mediators in aphid-plant interactions. In this thesis, effectors are defined as proteins or small molecules of the pathogen/insect, that alter processes in the plant that are beneficial to the pathogen/insect, and elicitors are defined as proteins or small molecules produced by the pathogen/insect that elicit defence responses in the plant. In summary, aphid effectors can suppress plant defence, modulate plant processes beneficial for aphid colonisation, and elicit effector-triggered resistance, by recognition of effectors by receptors involved in plant resistance (Hogenhout& Bos, 2011). In the last case, the effector becomes an elicitor of plant defence, when plants specifically recognise target effectors of the aphid to elicit a defence response. Two recent reviews have summarised the latest developments in aphid effector biology (Hogenhout & Bos, 2011; Rodriguez & Bos, 2013).

Knowledge about the mechanism(s) by which products of *R*-genes provide resistance can be gathered by studying the difference between resistant and susceptible plants. Moreover, the proteins from aphids that trigger or suppress the resistance in plants can be used as a tool to study the *R*-gene related processes in plants. In plant-microbe interactions, pathogen effector biology is already an important field, and in aphid-plant interactions aphid effector biology is receiving more attention. The development of new molecular and genetic techniques/tools, and the full genome sequence of aphids, like that of the pea aphid, *A. pisum* (International Aphid Genome Consortium, 2010) will make it

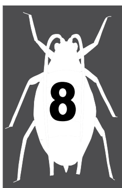
possible to further study the role of effectors in aphid-plant interactions.

To identify components such as effectors in aphid saliva, saliva needs to be collected. One way to collect saliva from aphids is through the use of defined artificial diets, on which aphids feed and salivate in between two layers of Parafilm (Cherqui & Tjallingii, 2000). Both watery and gelling saliva can be collected with this method. This technique has led to the identification of several proteins in the saliva of aphid species, such as *M. persicae* (Harmel et al., 2008), *A. pisum* (Carolan et al., 2009) and *D. noxia* (Nicholson et al., 2012) , summarised by Rodriguez and Bos (2013). However, the composition of aphid saliva that they inject into artificial diets might not reflect the composition secreted into plants. Will et al. (2012) observed differences in salivary secretion depending on the stylet milieu. The hardening of the gelling saliva was dependent on oxygen, and was absent in diets containing dithiothreitol. Additionally watery saliva with soluble protein fractions was mostly secreted in diets mimicking sieve element sap and watery saliva with non-soluble protein fractions in diets mimicking cell-wall fluids.

An alternative way to study the proteins in saliva of aphids is by transcriptomic or proteomic analysis of aphid salivary gland tissue (Carolan et al., 2011). Several proteins in saliva of aphids have been identified this way. Mutti et al. (2008) identified an *A. pisum* protein called C002, which was proven to be crucial for successful feeding on *Vicia faba* L. Silencing of C002 in *A. pisum*, by microinjection of siDNA, was shown to be lethal when the aphids were feeding from their host plant (Mutti et al., 2006). A homologue of this protein in *M. persicae* saliva, MpC002, was shown to enhance aphid fecundity when over-expressed in *Nicotiana benthamiana* (Bos et al., 2010) and *Arabidopsis thaliana* (Pitino & Hogenhout, 2013). This effector and Mp10 and Mp42 were identified as effectors affecting the virulence of *M. persicae* (Bos et al., 2010). Differences were also found in proteomes of secreted saliva of *D. noxia* biotypes, differing in virulence (Nicholson et al., 2012).

The *A. pisum* C002 effector was not able to enhance the fecundity of *M. persicae* on *A. thaliana*, which indicates that only *M. persicae* effectors modulate *A. thaliana* responses beneficial for *M. persicae*, whereas the *A. pisum* effectors cannot, probably related to the fact that *A. thaliana* is a host for *M. persicae* and not for *A. pisum* (Pitino & Hogenhout, 2013). In Chapter 5, Nr:1 aphids were able to suppress resistance for Nr:0 aphids, but *M. euphorbiae* and *M. persicae* were not. Gong et al. (2012) studied differences in DNA methylation of four salivary gland genes of two *D. noxia* biotypes differing in virulence. Differences were found in methylation levels between the biotypes which might be related to virulence, although further studies are needed to confirm this. Salivary proteins identified by Carolan et al. (2011) in aphids were previously characterised in nematodes, indicating similarities in saliva composition and host-plant manipulation between aphids and nematodes.

Many of the proteins identified in saliva of aphids have as yet unknown functions, and functional analyses should be performed to link identity of proteins with their function. Silencing the gene expression of certain genes in aphids by RNA interference, can be a tool



to identify function of genes and proteins, by either direct injection of double stranded DNA into the aphid or indirectly via plants (Jaubert-Possamai et al., 2007; Mutti et al., 2006; Pitino et al., 2011). Additionally, genes encoding certain proteins might be overexpressed in plants to study their function (Bos et al., 2010).

Virulence in *Nasonovia ribisnigri*

It is unknown what mechanism is responsible for the virulence in *N. ribisnigri* Nr:1. Our data suggest that *N. ribisnigri* Nr:1 aphids are able to suppress the defence in resistant lettuce against Nr:0 aphids (Chapter 5). This suppression, however, seems to occur locally and not systemically, because only a small proportion of the Nr:0 aphids was affected. They might, for example, only be able to suppress the defence of one sieve tube, or one bundle of sieve tubes. Alvarez et al. (2013) also stated that the manipulation of plant processes by aphids seems to occur at the feeding site in local leaves, not in systemic tissues. *Macrosiphum euphorbiae* and *M. persicae* were not able to suppress the resistance in lettuce for Nr:0 aphids (Chapter 5) suggesting specificity of suppression.

Effectors in the salivary secretion of Nr:1 aphids might be responsible for the virulence to the *Nr*-gene mediated resistance, however, possible additional involvement of detoxifying genes in the gut of the Nr:1 aphids cannot be ruled out. In the presence of multiple individuals of Nr:0 in the same feeding area on a resistant lettuce plant, Nr:1 aphids seem to have difficulties in successfully preventing the wound response (Chapter 5). The elicitors of Nr:0 aphids inducing the resistance, might neutralise the resistance suppression of Nr:1 aphids. Mutual induced resistance against Nr:1 by Nr:0 aphids and *vice versa* was found on susceptible lettuce, indicating that these biotypes might indirectly affect each other through differences in effectors and elicitors in the saliva.

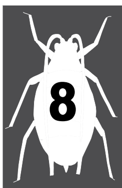
Although all tested virulent populations in Chapter 3 were collected from resistant lettuce in the field, some of these populations failed in reproducing and developing on resistant lettuce in the laboratory. These populations might have lost their virulence during the rearing period in the laboratory, during which the colonies were kept on a susceptible lettuce cultivar. Loss of virulence after being reared on a susceptible host was studied in Chapter 6 for a virulent and partially-virulent Nr:1 population (Chapter 3). Long-term loss of virulence was observed for the partially-virulent population, and temporal loss of virulence for the virulent population, after being reared on susceptible lettuce for 2 months. Loss of virulence in the absence of resistant plants, takes place when the adaptation of the insect is associated with fitness costs (Van der Plank, 1984; Zhang et al., 2011). For example, effectors that are recognised by the plant, often are needed for successful colonisation (Rodriguez & Bos, 2013), and losing or modifying such an effector would on the one hand eliminate the recognition by the plant but on the other hand reduce the colonisation ability of the aphid. The loss of virulence (short- as well as long-term) observed for the Nr:1 populations after being reared on susceptible lettuce, indicates this virulence is associated with fitness costs.

The loss of virulence observed for the virulent Nr:1 population after being reared on susceptible lettuce was temporary, and only visible in the short-term EPG experiment and not in the long-term performance experiment (Chapter 6). The EPG data indicated that the aphids had difficulties countering the wound response in the sieve element. The active suppression of the resistance against Nr:0 aphids by Nr:1 aphids might, among other possibilities, suggest that the virulence is mediated by an effector suppressing the resistance (Chapter 5). The virulent Nr:1 aphids might be able to down-regulate the transcription of the effector protein responsible for the virulence when feeding on susceptible lettuce, and up-regulate it again after a prolonged stay on resistant lettuce. For the partially virulent Nr:1 aphids both the EPG and performance data indicated virulence loss, however, the virulence was not completely lost (Chapter 6). These aphids might also have down-regulated the transcription of the effector gene responsible for the virulence, but were not able to up-regulate it anymore to the same level. Costs of virulence were also observed by Pallipparambil et al. (2010) for *M. euphorbiae* on *Mi*-resistant tomato, although Goggin et al. (2001) reported that this virulence was a persistent trait.

Transcriptomics of gut and salivary gland tissue of *Nasonovia ribisnigri*

In an attempt to identify the factors underlying the virulence in Nr:1 aphids, transcriptomic analyses were performed on gut and salivary gland tissue of both Nr:0 and Nr:1 aphids that had fed for 48 hours on either Terlana (susceptible) or Corbana (resistant) cultivar (Figure 2).

Additionally, Nr:1 aphids that were reared for 2 months prior to the transcriptomic experiment on either Corbana or Terlana were also collected and analysed. The aphids were dissected and both the guts and the salivary glands were collected, and analysed separately for their transcripts by using RNA-Seq (Ten Broeke et al., unpublished data). Table 2 below shows the preliminary results of the transcriptomic analyses. Detailed bioinformatics analyses are in progress. Significant differences in gut transcriptomes were found when comparing the Nr:0 and Nr:1 pooled data, but not for the other samples. For the salivary gland data, the abundance of many sequences differed between the Nr:0 and Nr:1 pooled data. No significant differences were found in sequences between Nr:0 and Nr:1 reared and tested on Terlana, or reared on Terlana and tested on Corbana. This is unexpected, because Chapter 5 suggests an effector, suppressing resistance in the salivary secretion of Nr:1 aphids to be responsible for its virulence. Variability between the replicates might cause the absence of significant differences. Significant differences were found when Nr:1 aphids were compared that were reared either on Corbana or Terlana and tested on Corbana and Terlana. This is in line with the data of Chapter 6 in which a temporal loss of virulence was found for the same Nr:1 population after being reared on Terlana. Nr:1 aphids might down-regulate the expression of the virulence factor on Terlana, because it is associated with fitness costs on susceptible lettuce. The aphids might need time to adapt again to Corbana and up-regulate the expression of this factor, which could have caused the differences



observed in the salivary gland data. This might also work the other way around, that aphids have to adapt to Terlana by down-regulating the expression of the virulence factor. The data of both the gut and salivary gland mRNAs should be analysed further to identify candidate proteins that differ between Nr:0 aphids and Nr:1 aphids, and between Nr:1 populations reared on different plants prior to the bioassay.

Figure 2: Dissection of gland and gut tissue of *N. ribisnigri.* A: location of gut and salivary glands inside aphids; B: gut; C: salivary glands (shaded in grey).

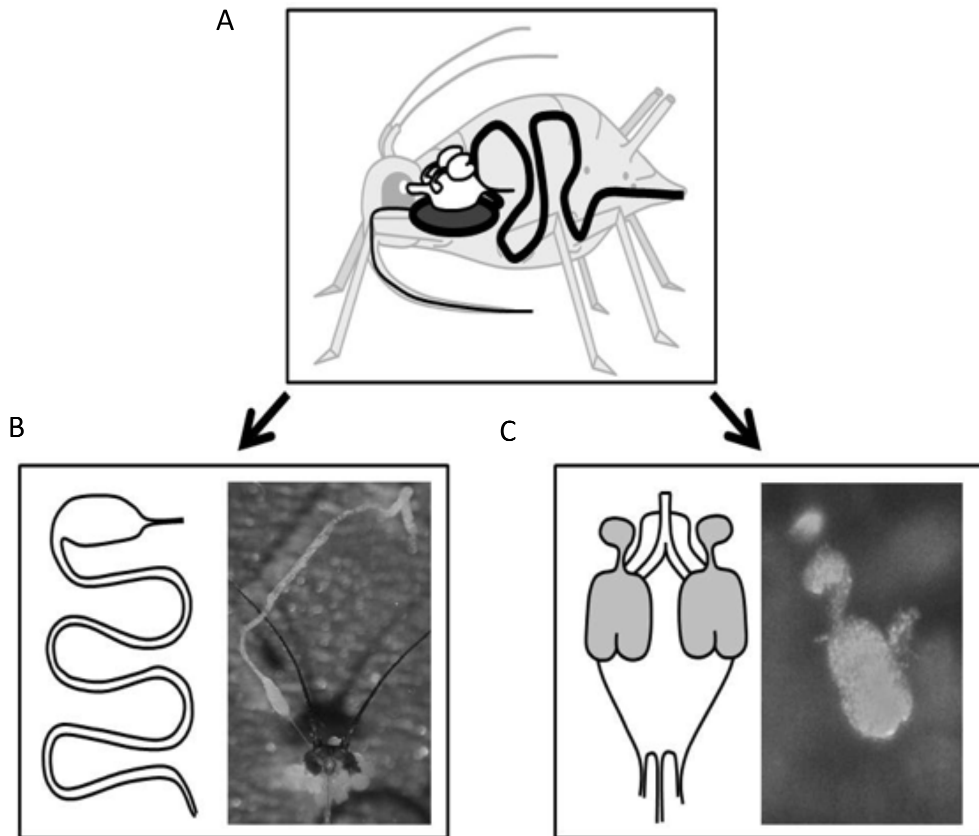


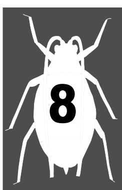
Table 2: Preliminary results of transcriptomic analyses of aphid guts and salivary glands of both Nr:0 and Nr:1 aphids; all = all data of the respective biotype pooled, T-T = reared on Terlana, tested on Terlana, T-C = reared on Terlana, tested on Corbana, C-C = reared on Corbana, tested on Corbana and C-T = reared on Corbana, tested on Terlana.

Tissue	mRNA data compared			Number of differentially expressed genes (P < 0.05)
Gut	Nr:0 all	vs	Nr:1 all	1241
	Nr:0 T-C	vs	Nr:0 C-T	0
	Nr:0 T-T	vs	Nr:1 T-T	0
	Nr:1 T-C	vs	Nr:1 C-C	0
	Nr:1 T-T	vs	Nr:1 C-T	0
Salivary gland	Nr:0 all	vs	Nr:1 all	2583
	Nr:0 T-C	vs	Nr:1 T-C	0
	Nr:0 T-T	vs	Nr:1 T-T	0
	Nr:1 T-C	vs	Nr:1 C-C	3066
	Nr:1 T-T	vs	Nr:1 C-T	1149

Symbionts

The endosymbionts of aphids are important for their survival, because they provide the aphid with the essential nutrients lacking in the phloem sap (Douglas & van Emden, 2007). The majority of aphid species contain the primary symbiont species *Buchnera aphidicola* and some aphid species have additional secondary symbionts (Douglas & van Emden, 2007). Aphid symbionts have been shown to have a variety of effects on their host. Symbionts are, for example, shown to be involved in aphid resistance against parasitoids (Schmid et al., 2012). These interactions are genotype-specific and this specificity is probably mediated by the symbionts (Rouchet & Vorburger, 2012). Symbionts can also provide protection against pathogenic fungi (Łukasik et al., 2013) and heat shock (Montllor et al., 2002), affect aphid reproduction (Simon et al., 2011), aphid body colour (Tsuchida et al., 2010) and host plant specialisation (Leonardo & Muiru, 2003), summarised by Frago er al (2012).

It has been hypothesised that symbionts of aphids might also play a role in virulence of aphids to *R*-gene mediated resistance (Francis et al., 2010; Hebert et al., 2007). Symbionts were suggested to be responsible for the virulence of *Therioaphis trifolii* f. *maculata* (Monell) to several lucerne varieties (Ruggle & Gutierrez, 1995). They are possibly also involved in virulence of *M. euphorbiae* to *Mi*-based resistance (Francis et al., 2010; Hebert et al., 2007). The bacterial symbiont *Serratia symbiotica,* that provides heat tolerance to *A. pisum,* had a substantial effect of the aphid's metabolome, however, this is likely a result of metabolism



of the symbiont itself, because only small changes in gene expression of the aphid were observed after *S. symbiotica* infection (Burke & Moran, 2011).

Resistance against pesticides

Similar to virulence to host plant resistance, resistance to insecticides occurs in many aphid species. The green peach aphid (*M. persicae*) is the front runner, and has evolved resistances to more insecticides than any other insect (Anstead et al., 2005). *Nasonovia ribisnigri* also developed resistance to several insecticides (Barber et al., 1999; Kift et al., 2004; McDougall & Creek, 2007; Rufingier et al., 1997; Stufkens & Wallace, 2004; Workman et al., 2004). The mechanism of resistance to insecticides can be categorised into three main mechanisms: reduction of insecticide uptake (reduction in permeability of insect cuticle), enzymatic detoxification and insensitivity (point mutations in genes encoding for the target site protein(s)) (Silva et al., 2012).

Esterases in cotton aphids were suggested to play an important role in neonicotinoid resistance in this aphid species (Gerami, 2012). A transcriptomic study by Silva et al. (2012) of several genotypes of *M. persicae* differing in insecticide resistance, showed that insecticide resistance mechanisms are variable and complex. Similar to virulence to *R*-gene based resistance, resistance to insecticides is often associated with fitness costs. For example, insecticide resistant *M. persicae* became more susceptible to parasitoids (Foster et al., 2010).

Alternative resistance

The occurrence of virulent aphid biotypes is a growing problem in crop protection, and when host plant resistance is no longer effective, new sources of resistance need to be identified. Wild relatives and landraces can be used to identify new resistance genes (Dogimont et al., 2010). Wild relatives of crop plants have been important sources of pest and disease resistance genes in modern agriculture, and have also been used for other breeding purposes like abiotic stress tolerance, improved yield and quality traits (Hajjar & Hodgkin, 2007).

New host plant resistance in *Lactuca virosa*

The *Nr*-gene in lettuce is no longer effective in controlling *N. ribisnigri,* because of the emergence of the virulent Nr:1 biotype, that is able to infest resistant lettuce in the field (Thabuis et al., 2011) and feeds equally well on resistant and susceptible lettuce cultivars (Chapter 2). The *Nr*-gene originates from *Lactuca virosa* L., a distant wild relative of cultivated lettuce (Dieleman & Eenink, 1980; Eenink et al., 1982). In Chapter 4, the same *L. virosa* accession, IVT 280, was investigated as potential source of resistance against the Nr:1 biotype of *N. ribisnigri.* Complete resistance was indeed found in accession IVT 280 against

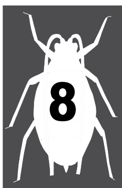
Nr:1 aphids, and partial resistance in another *L. virosa* accession (CGN16204). The complete resistance against Nr:1 and Nr:0 aphids in IVT280 had a similar mode of action as the *Nr*-mediated resistance against Nr:0 aphids in *L. sativa*, i.e. phloem-based resistance causing a reduction of feeding followed by mortality (Chapters 2 and 4). *Lactuca virosa* IVT 280 is, therefore, a good source of resistance against Nr:1 aphids. Wild relatives of crops have also been used for aphid resistance in potato against *M. euphorbiae* (Pompon et al., 2011), in *Solanum* against *M. persicae* (Alvarez et al., 2006) and in *Brassica'*s against *B. brassicae* (Kumar et al., 2011).

Host plant resistance is an environmentally benign strategy to control aphid pests but is not always available and the variation in degree of aphid resistance among crossable plant lines is often small. Additionally, breeding programs to develop insect- or pathogen-resistant crops can be time-consuming and costly (Dreyer & Campbell, 1987). Furthermore, host plant resistance might sometimes not be effective because of fitness costs to plants, accelerated transmission of viruses, negative effects on non-target organisms such as natural enemies (Van Emden, 2007). It is, therefore, important to explore alternatives when effective host-plant resistance is not available in crossable material.

Transgenic plants

One alternative is the exploitation of the available transgenic technologies to insert the desirable resistance into plants. Transgenic plants can be developed that for example express insecticidal proteins of plant origin. Lectins, which bind to the insect gut, are toxic for aphids and several were shown to successfully control aphids in transgenic plants, like snowdrop lectin (Hilder et al., 1995) and bulb lectin (from *Pinellia pedatisecta*) (Wu et al., 2012). Other insecticidal proteins originating from plants and effective in controlling aphids are protease inhibitors (Carrillo et al., 2011). However, these compounds affect non-target organisms because of their lack of specificity. Transgenic plants can also express compounds with a non-toxic mode of action, for example, (*E*)-β-farnesene, an alarm pheromone for aphids (Beale et al., 2006; Yu et al., 2012).

Like with conventional host-plant resistance, the indirect effects of an inserted resistance gene on other organisms should be taken into account. For example, in transgenic wheat resistance against powdery mildew led to an increase in susceptibility to aphids (von Burg et al., 2012). Additionally, transgenically expressed insecticides may also affect non-target organisms, like snowdrop lectin excreted in the honeydew of *Rhopalosiphum padi* L. negatively affected the performance of the aphid parasitoid wasp *Aphidius ervi* Haliday (Hogervorst et al., 2009). Cotton plants transgenically expressing a bacterial protein toxic to Lepidoptera were more susceptible to the non-target aphid species *A. gossypii* (Xue et al., 2009).



Another possible alternative is the use of RNA interference (RNAi). RNAi is a biological response inhibiting expression of specific genes by sequence-specific silencing, degrading

target mRNA by small interference RNAs (siRNA) from double-stranded RNA (dsRNA), and is common in both plants and animals (Ding et al., 2004). It plays an important role in the protection of hosts against foreign genes and is also exploited as tool to experimentally manipulate gene expression (Ding et al., 2004). RNAi can be used to inhibit target genes in insects and might be used in breeding insect-resistant crops, by producing transgenic plants that produce dsRNA directly targeted against a specific insect pest (Price & Gatehouse, 2008). In aphids, RNAi was proven to be successful in targeting specific genes, for example the knock down of salivary transcript C002 in *M. persicae* leading to aphid mortality (Mutti et al., 2006) and knock down of genes in several body tissues of *A. pisum* (Jaubert-Possamai et al., 2007). However, the delivery of dsRNA needed for the siRNA causing knock down of genes in aphids in these studies was done by micro-injection. Pitino et al. (2011) developed a method to deliver dsRNA in the aphid via the plant, with leaf disks producing dsRNA to knock out genes in aphids. A recent study by Bhatia et al. (2012) showed that RNAi of the aphid-specific serine protease (important in digestion) was successfully induced by feeding on transgenic plants, indicating the successful uptake of dsRNA from transgenic plants.

In lettuce, the use of RNAi to control *N. ribisnigri* could also be an alternative strategy when effective host plant resistance is no longer available. If salivary effectors of *N. ribisnigri* Nr:1 would be responsible for the virulence to *Nr*-gene mediated resistance, identification of these factors could be useful for developing transgenic, resistant plants. Transgenic lettuce plants could be developed targeting the specific effector(s) needed by the Nr:1 aphids to feed on resistant lettuce. The selection pressure on loss or mutation of this effector is low, because the aphid needs this effector for the colonisation of resistant lettuce. Additionally, RNAi is likely to possess a low risk for non-target organisms. Pollinators, for example, could ingest siRNA via the nectar; however, it was shown that the sequence of serine protease in *M. persicae* does not overlap with corresponding genes of pollinators (Bhatia et al., 2012).

Combining the field of aphid-effector biology with the recent developments in RNAi techniques in aphids, could yield a promising new approach for the control of major aphid pests.

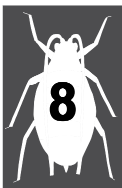
How to extend the durability of *R*-genes?

Lactuca virosa contains effective resistance against Nr:1 *N. ribisnigri* aphids (Chapter 4); however, the release of new commercial cultivars into which this resistance has been introgressed will take several years. Meanwhile the *Nr*-mediated resistance in lettuce is no longer effective against Nr:1 aphids. It is, therefore, important to look at the possibilities to extend the durability of existing and future resistance genes. McCreight & Liu (2012) describe the potential of partial resistance in the control of *N. ribisnigri* in the absence of the implementation of complete resistance. Partial resistance has the potential to delay or prevent the emergence of virulent *N. ribisnigri* biotypes, either on its own or implementing

it as a component of IPM. In pathogen-plant systems, resistance conferred by a single *R*-gene is more durable when this *R*-gene is combined with quantitative trait loci (QTL) associated with partial resistance (Palloix et al., 2009). Guo et al. (2012) identified a QTL in *M. truncatula* against *A. pisum* and *A. kondoi* which can be combined with known *R*-genes. Combining both antibiosis and antixenosis in commercial cultivars might also increase the durability of a resistant cultivar (Lambert & Pascal, 2011). Stacking or pyramiding genes was also suggested by Kim et al. (2010b) to slow down or delay insensitive biotype formation in soybean against the soybean aphid, *A. glycines*. The combination of *Rag1* and *Rag2* resistance genes in soybean were shown to be beneficial for the resistance against *A. glycines*, compared to cultivars with only one of the two resistance genes (Wiarda et al., 2012). The effect of the combination of two resistance genes on plant traits important for consumption should be studied (Brace & Fehr, 2012). Mornhinweg et al. (2011) also state that releasing multiple barley cultivars differing in sources of genetic resistance against *D. noxia*, will increase the durability of the resistance. Recently, genes conferring tolerance against *D. noxia* in barley were mapped which could be used in combination with other genes, to pyramide genes in barley to enlarge the genetic base of defence against this aphid species (Tocho et al., 2012). For soybean, multiple resistance genes against the soybean aphid may be needed to effectively use host-plant resistance, because virulence patterns of the soybean aphids were variable and dynamic over the years in which the study was conducted (Hesler et al., 2012). For lettuce, however, no QTLs conferring resistance or new *R*-genes have been identified which could be combined with the *Nr*-gene. *Lactuca virosa* contains resistance against Nr:1 aphids which was not introgressed into *L. sativa* with the *Nr*-gene (Chapter 4). Further research is needed to identify the gene(s) involved, either a dominant *R*-gene or QTLs, and introgress it into cultivated lettuce.

Prior to the actual emergence of an insensitive biotype of *N. ribisnigri*, Van der Arend (2003) proposed a strategy to prevent formation of insensitive biotypes. The strategy involves combining resistance with chemical control, continuous monitoring of resistant crops for aphids and searching for alternative resistance. Getting entomologists involved in breeding for resistance is also proposed by Dogimont et al. (2010). Knowledge about the genetic structure of aphid populations, their mode of reproduction, and ability to attain virulence could contribute to durable resistance breeding. It is therefore essential to monitor aphid populations to be able to identify newly emerged virulent biotypes (Kim et al., 2008). Occurrence of virulence can be monitored by testing the existence of virulent biotypes before a new resistant cultivar will become commercially available (Dogimont et al., 2010).

Another strategy is creating so-called refuges. These refuges are fields or parts of fields planted with susceptible material to decrease the selective pressure on pest species (Fenton et al., 2010). In some crops, the use of natural enemies next to resistant cultivars, could delay virulent biotype formation, whereas in others it would do the opposite when



virulent or avirulent aphids are more favoured by the natural enemies. The impact that natural enemies will have on the durability of resistance is strongly dependent on biological characteristics of both natural enemy and pest species and the resistance mechanism of the plant (Bottrell et al., 1998). Compatibility between host-plant resistance and biological control is not ensured, for example in soybean, host plant resistance had a negative effect on the foraging behaviour of *Aphidius colemani* (Viereck), a biological control agent of *A. glycines* (Ode & Crompton, 2013). In contrast, compatibility between natural enemies occurring in the field and host-plant resistance against the same aphid species was shown to be present by McCarville and O'Neal (2012). Several studies showed the potential of syrphid fly larvae and lacewing larvae as biological control agents of *N. ribisnigri* on lettuce (Fagan et al., 2010; Hopper et al., 2011; Smith & Chaney, 2007; Smith et al., 2008). For lettuce, however, the use of natural enemies might be problematic since the presence of any insect, including aphid natural enemies, makes lettuce unmarketable (Diaz & Fereres, 2005; Liu, 2004; McCreight, 2008). It would be beneficial for the lettuce industry if the attitude of consumers towards a low number of insects on their lettuce would change from zero tolerance to some tolerance. Host-plant resistance does not have to be absolute, and plant tolerance might also be used as strategy in lettuce. Additionally, the presence of some aphids on lettuce can be used as indicator that no insecticides were used.

Conclusion

Although plant molecular studies have not yet identified the exact mechanism of *Nr*-mediated resistance in lettuce against *N. ribisnigri*, the data in this thesis provide important insights into this mechanism. The resistance operates in the phloem and an intact vascular system is needed for its full expression (Chapters 2 and 7). The resistance component is probably produced in the shoot and needs to be translocated to the feeding site of the aphids and/or signalling molecules need to be translocated to the feeding site (Chapter 7). The *Nr*-gene has similar modes of action as the *Mi*-gene in tomato and *VAT*-gene in melon (NBS-LRR genes), phloem-based resistance that causes a reduction in phloem ingestion. Lettuce is known to contain a large cluster of NBS-LRR genes against downey mildew, *B. lactucae* and lettuce root aphid, *P. bursarius* (Wroblewski et al., 2007).

Proteins coded for by NBS-LRR genes are receptors that recognise specific effectors from their attacker, in this case the aphid feeding on the plant. Salivary effectors of avirulent Nr:0 aphids might be recognised by similar proteins triggering a resistance response (Figure 9). The salivary effectors of virulent Nr:1 aphids seem to actively suppress *Nr*-mediated resistance, also against Nr:0 aphids (Chapter 5)(Figure 3). Variation in virulence was observed between different Nr:1 populations (Chapter 3). The virulence of Nr:1 seems to be associated with fitness costs. One of the tested Nr:1 populations showed a long-term reduction in virulence after being reared on susceptible lettuce for two months (Chapter 6). However, another Nr:1 population only showed a short-term reduction in virulence (Chapter

6). This population might down-regulate its virulence on susceptible lettuce, for example by reducing the synthesis of the effector, and up-regulate it again when feeding on resistant lettuce. This might also be the cause of the differences observed in mRNA sequences of the salivary gland of the same Nr:1 population reared on resistant or susceptible lettuce, tested on resistant or susceptible lettuce. For the other Nr:1 population this down-regulation might be permanent.

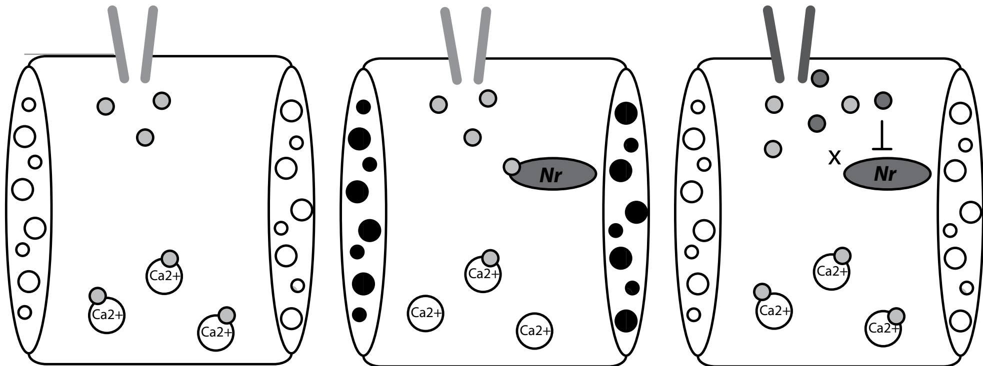
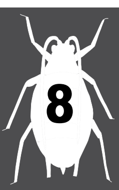


Figure 3: Model of resistance mechanism in lettuce and interference by *Nasonovia ribisnigri* A. Nr:0 aphid feeding on a sieve element of susceptible lettuce. Effectors in the watery saliva of the aphid (grey dots) prevent sieve plate plugging by binding Ca^{2+}. B. Nr:0 aphid feeding on a sieve element of resistant lettuce. Effectors in the watery saliva of the aphid are recognized by products of the *Nr*-gene and plugging of the sieve plates is not prevented. C. Nr:1 aphid feeding on a sieve element of resistant lettuce. Effectors in the watery saliva of the aphid (grey and dark-grey dots) suppress the activation of products of the *Nr*-gene. The sieve plates stay unplugged. Alternatively, new effectors of Nr:1 aphids might not be recognized anymore.

Lactuca virosa accession IVT 280, the original donor of the *Nr*-gene was fully resistant against both Nr:0 and Nr:1 aphids (Chapter 4). This lettuce accession, therefore, is a source of additional resistance factors effective against the virulent *N. ribisnigri* biotype and can be used to breed for new resistance in cultivated lettuce. Stacking genes can slow down virulence development in aphids, and the *Nr*-gene could be combined with the new resistance gene into one cultivar. However, combinations of different categories of resistance are more beneficial in controlling a pest, and the *Nr*-gene mediated resistance seems to be similar to the new resistance.



Future perspectives

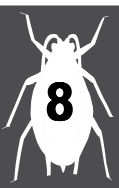
Although information was gathered in this thesis about the resistance mechanism of the *Nr*-gene in lettuce against *N. ribisnigri*, further identification of the resistance is needed to unravel the exact mechanism. More research needs to be done to clone the *Nr*-gene and to identify the components responsible for resistance. In Chapter 7, evidence was found that the whole lettuce plant is needed for a full defence response, and that the resistance factor is synthesised in the shoot. The possible involvement of signalling molecules, such as phyto-hormones, in the *Nr*-mediated resistance could be studied to get more insight in the molecular processes related to the resistance.

Aphid effector biology is a relatively new research field, and full genome sequences of aphids, like that of the pea aphid, *A. pisum* (International Aphid Genome Consortium, 2010) and plants allow detailed mechanistic studies of aphid-plant interactions. It can therefore also be used to gain insight in the processes underlying the interactions between aphids (avirulent and virulent) and *R*-genes in plants. For *N. ribisnigri* both avirulent (Nr:0) and virulent (Nr:1) biotypes occur in the field. The virulent biotype poses a significant threat for the lettuce industry, because no resistant cultivar against this biotype is available at the moment. The virulence factor of the Nr:1 aphids seems to be an effector in the salivary secretion that can actively suppress *Nr*-based resistance, and seems to have fitness costs for the aphids (Chapters 5 and 6). Transcriptomic data of the salivary glands of both Nr:0 and Nr:1 aphids were gathered and the number of differential sequences between treatments was analysed. These data should be further analysed to identify candidate effectors that are responsible for the virulence of Nr:1 aphids. Transcriptomic data were also obtained from the guts of both Nr:0 and Nr:1 aphids, to check the presence of *e.g.* detoxifying gut enzymes that might be involved in the virulence of Nr:1 aphids. Alternatively, the data in Chapter 5 suggests a salivary compound to be responsible for the virulence in Nr:1. In Chapter 6 short- and long-term loss of virulence were observed for two Nr:1 populations, indicating that fitness costs are associated with the virulence. The Nr:1 population that showed temporal loss of virulence, is the same as the one that was used for the transcriptomic experiments. The genes that are up- or down-regulated could be identified in the gut and salivary gland data of Nr:1 aphids reared on different lettuce cultivars.

After the identification of candidate effectors causing virulence, RNA-interference can be used to knock out the responsible genes to further study their function in the aphid. Direct injection of dsRNA into the aphid (Mutti et al., 2006) or plant-mediated RNAi, by transformation of plants, might be used to silence these genes (Hogenhout & Bos, 2011). Identification of the effector responsible for the virulence in *N. ribisnigri* could be used for new control strategies, like using RNAi to target the specific effector(s) needed by the Nr:1 aphids to feed on resistant lettuce.

Another control strategy against the virulent biotype of *N. ribisnigri* is the use of a new resistance gene in lettuce. In Chapter 4, *L. virosa* accession IVT 280 (donor of *Nr*-gene)

was proven to be resistant against Nr:1 aphids. This resistance could be introgressed into cultivated lettuce to create new resistant cultivars, after extensive studying the resistance itself (number of factors involved, effect on non-target organisms, etc.) Further studies should focus on mapping this new resistance gene and implement it into *L. sativa.* The introgression of the *Nr*-gene was a long and difficult process (Eenink et al., 1982), however, state-of-the-art marker-assisted breeding techniques might speed up this process.
Insecticide use results in environmental and economic costs, and more and more active ingredients are getting banned worldwide. As an alternative, host plant resistance should be used, which is an environmentally benign aphid control strategy and therefore an important part in IPM-strategies to control aphid pests. Host plant resistance was, is, and will remain one of the most important control measures against many aphid pest species.



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Summary

Aphids are among the world's most serious pests of crop plant species, causing major economic losses by reducing plant growth and vigour, and by transmitting plant viruses. Host plant resistance is an effective and environmentally friendly strategy to control aphid pests, and for many crops, cultivars have been bred for resistance against aphids. The black currant-lettuce aphid, *Nasonovia ribisnigri* (Mosely), is an economically important pest of cultivated lettuce, *Lactuca sativa* L. (Asteraceae). Host plant resistance mediated by the *Nr*-gene, which originates from a wild lettuce species, *Lactuca virosa* L., has been used since 1982 to control this aphid. However, despite its importance in controlling *N. ribisnigri*, the *Nr*-gene has not been cloned, and the mechanism causing resistance at the molecular level is unknown. Unfortunately, this resistance is not effective anymore, since *N. ribisnigri* aphids colonising *Nr*-resistant lettuce have been reported since 2007.

The aim of this PhD-thesis (part of the Technological Top Institute Green Genetics-*Nasonovia* programme) was to unravel the mechanism of resistance mediated by the *Nr*-gene in lettuce against *N. ribisnigri*, by studying the aphid's host-plant selection behaviour and performance. Additionally, new resistance sources against the virulent biotype of *N. ribisnigri* needed to be identified.

In Chapter 1, relevant literature is summarised about common aphid-plant interactions and the different strategies to control aphids pests and background information about the *N. ribisnigri*-lettuce problem is presented.

To identify the location and possible mechanism of resistance against *N. ribisnigri* in lettuce, the performance and feeding behaviour of an avirulent and a virulent biotype of *N. ribisnigri* were tested on susceptible and resistant near-isogenic lines of *L. sativa* (Chapter 2). The resistance is mainly located in the phloem and some resistance might be encountered by the aphids on their way to the phloem inside the plant. The inability of the avirulent aphids to sustain feeding from the resistant near isogenic lines (NILs) resulted in zero survival. This inability to feed could be caused by blocking of the stylets, as a consequence of the failure of aphids to suppress the wound response of the sieve element. The virulent aphids fed and performed equally well on the resistant and susceptible NILs and pose a threat for the lettuce industry in the absence of alternative host plant resistance.

To obtain a better understanding of the behaviour and performance of virulent *N. ribisnigri* populations, four *N. ribisnigri* populations observed to have colonised resistant lettuce in different geographical locations in Europe were studied on susceptible and resistant NILs and cultivars, and compared with an avirulent *N. ribisnigri* population (Chapter 3). Large variation was observed for the virulence to the *Nr*-mediated resistance among the different aphid populations tested. Aphids from a highly virulent population performed equally well on both the resistant and susceptible lettuce plants, whereas aphids from the other three populations were characterised as partial-virulent and performed better on the

susceptible lettuce plants. The lettuce background, butterhead/iceberg lettuce for the NILs and romaine lettuce for the cultivars, was shown to affect the level of resistance to the different *N. ribisnigri* populations, i.e. performance on resistant NIL was lower than on the resistant cultivar.

The performance and feeding behaviour of one avirulent and four virulent populations *N. ribisnigri* were studied on *L. virosa* accession IVT 280, the original donor of the *Nr*-gene, to check its resistance against the virulent biotype (Chapter 4). *Lactuca virosa* accession IVT 280 was fully resistant against all tested populations of *N.ribisnigri*. Phloem ingestion was strongly reduced and survival was zero on accession IVT 280. This accession can, therefore, be exploited as a source of resistance against the virulent biotype in cultivated lettuce.

To assess whether virulence in *N. ribisnigri* was lost on susceptible lettuce and to evaluate possible costs of this virulence, the effect of rearing plants on aphid behaviour was tested (Chapter 6). A highly virulent and a partial-virulent population of *N. ribisnigri* were reared on a resistant and a susceptible lettuce cultivar for a certain period and performance and feeding behaviour were recorded on resistant and susceptible lettuce cultivars. Short-term virulence loss was observed for the highly virulent aphid population indicating that the aphids needed time to adapt to the plant they were feeding on. Long-term virulence loss was observed for the partial-virulent aphid population. This indicates that virulence in *N. ribisnigri* is associated with fitness costs.

The effect of the presence of other aphids on the behaviour of *N. ribisnigri* was assessed by studying the feeding behaviour and performance of individual avirulent or virulent *N. ribisnigri* aphids combined with a group of either the same biotype or the other biotype on susceptible and resistant lettuce cultivars (Chapter 6). Additionally, the effect of the presence of *Myzus persicae* and *Macrosiphum euphorbiae* on penetration behaviour and performance of both biotypes *N. ribisnigri* was tested. The virulent aphids were able to locally suppress the resistance in lettuce against avirulent aphids, however, both *M. persicae* and *M. euphorbiae* were not. A possible mechanism behind the virulence in *N. ribisnigri* to the *Nr*-resistance is the presence of an effector protein that is able to suppress resistance in the salivary secretion of the aphids. Virulent aphids were either negatively affected or not affected by the presence of other aphids.

The location of synthesis of the resistance factors of the *Nr*-gene in lettuce were studied by producing grafts between resistant and susceptible lettuce cultivars and studying the feeding behaviour and performance of avirulent *N. ribisnigri* on these grafts (Chapter 7). Additionally the persistence of resistance was assessed on excised tissue of resistant lettuce *i.e.* detached leaves and leaf disks, by studying feeding behaviour of avirulent aphids. The resistance factor(s) were produced in the lettuce shoot, not in the root, because grafts with resistant shoots and susceptible roots were resistant against the aphids, whereas grafts with susceptible shoots and resistant roots remained susceptible. An intact plant with an intact

vascular system is needed for full resistance, because both detached leaves and leaf disks of resistant lettuce plants partly lost their resistance against the aphids.

The results presented in Chapters 2-7 give insight in the resistance mechanism underlying the *Nr*-mediated resistance as well as the mechanism responsible for the virulence in *N. ribisnigri.* These results are discussed in the context of recent literature in Chapter 8. In an attempt to identify the factor responsible for the virulence in *N. ribisnigri*, transcriptomic data on salivary glands and guts of an avirulent and virulent biotype of *N. ribisnigri* were collected (Chapter 8). Further analysis of these data are needed to identify the factor(s) responsible for the virulence.

Nederlandse Samenvatting

Bladluizen behoren tot 's werelds meest ernstige plagen van gewassen en zorgen voor grote economische verliezen doordat ze de groei van planten reduceren en planten-virussen overdragen. Waardplant-resistentie is een effectieve en milieu-vriendelijke strategie om aantasting door bladluizen te voorkomen, en voor veel gewassen zijn dan ook via veredeling cultivars beschikbaar die resistent zijn tegen bladluizen. De zwarte bes-slabladluis, *Nasonovia ribisnigri* (Mosely), is een economisch belangrijke plaag van gecultiveerde sla, *Lactuca sativa* L. (Asteraceae). Waardplant-resistentie gebaseerd op het *Nr*-gen, afkomstig van een wilde sla-soort, *Lactuca virosa* L., wordt sinds 1982 gebruikt om deze bladluissoort te bestrijden. Ondanks het belang van dit gen voor de bestrijding van *N. ribisnigri*, is het *Nr*-gen nog niet gekloneerd en is het resistentie-mechanisme op moleculair niveau nog onbekend. Helaas is de resistentie niet meer effectief omdat er sinds 2007 populaties van een virulent *N. ribisnigri* biotype gerapporteerd zijn die resistente sla kunnen koloniseren.
Het doel van het onderzoeksproject, dat deel uit maakt van het *Nasonovia*-programma van het Technologisch Top Instituut Groene Genetica en dat tot dit proefschrift heeft geleid, was het ontrafelen van het resistentie-mechanisme gebaseerd op het *Nr*-gen door bestudering van het waardplantselectiegedrag en de overleving en reproductie van de bladluizen. Tevens is gezocht naar nieuwe resistentie-bronnen tegen de virulente biotypen van *N. ribisnigri*.

In Hoofdstuk 1 wordt de literatuur samengevat over bladluis-plant interacties en de verschillende bestrijdingsstrategieën die er bestaan om bladluis-plagen te voorkomen. Tevens wordt achtergrondinformatie over de *N. ribisnigri* – sla interactie gegeven.
Om de locatie van het resistentie-mechanisme in sla tegen *N. ribisnigri* te identificeren zijn het voedingsgedrag, overleving en reproductie van avirulente en virulente biotypen van *N. ribisnigri* getest op vatbare en resistentie bijna-isogene lijnen, NILs, van *L. sativa* (Hoofdstuk 2).

De resistentie is voornamelijk gelokaliseerd in het floëem en enige mate van resistentie tijdens de stiletpenetratie onderweg naar het floëem is aangetroffen. Het onvermogen van de avirulente bladluizen om zich te voeden op een resistente NIL, leidde tot 100% mortaliteit. Dit onvermogen om te voeden kan veroorzaakt zijn door het blokkeren van de stiletten, mogelijk een gevolg van het onvermogen de wond-reactie van de plant te onderdrukken. De virulente bladluizen voeden en overleven even goed op de resistente en vatbare NILs, en vormen daardoor een bedreiging voor de slaproductie-industrie in afwezigheid van alternatieve waardplantresistentie.

Om meer over het gedrag, de groei en reproductie van virulente populaties van *N. ribisnigri* te weten te komen, zijn vier populaties bestudeerd afkomstig van uiteenlopende geografische locaties in Europa. Deze populaties werden bestudeerd op vatbare en resistente NILs en bijna-isogene cultivars, en vervolgens ook vergeleken met een avirulente *N. ribisnigri* populatie (Hoofdstuk 3). Grote variatie in virulentie voor de *Nr*-gemedieerde

resistentie werd waargenomen tussen de verschillende bladluis-populaties. Overleving en reproductie van bladluizen van een zeer virulente populatie waren gelijk op resistente en vatbare sla, terwijl de bladluizen van de andere drie populaties gekarakteriseerd werden als semi-virulent, omdat overleving en groei hoger was op vatbare slaplanten dan op slaplanten met het *Nr*-gen. De achtergrond van de sla, in het geval van de NILs boter/ijsberg sla en voor de cultivars bindsla, had een effect op het niveau van resistentie voor de verschillende *N. ribisnigri* populaties. Overleving en reproductie op de resistente NIL waren lager dan op de resistente cultivar.

Voedingsgedrag, overleving en groei van één avirulente en vier virulente *N. ribisnigri* populaties werden bestudeerd op *L. virosa*-accessie IVT 280, de originele donor van het *Nr*-gen, om te kijken of deze accessie resistent was tegen het virulente biotype (Hoofdstuk 4). *Lactuca virosa* accessie IVT 280 was volledig resistent tegen alle onderzochte populaties van *N. ribisnigri.* De floëem-opname was sterk gereduceerd en de bladluizen konden niet overleven op accessie IVT 280. Deze accessie kan daarom benut worden als bron van resistentie tegen het virulente biotype in gecultiveerde sla.

Om te beoordelen of de virulentie in *N. ribisnigri* verloren gaat op vatbare sla en de mogelijke kosten van deze virulentie te evalueren, werd het effect van kweekplant op het gedrag van de bladluizen bestudeerd (Hoofdstuk 6). Een zeer virulente en een semi-virulente populatie van *N. ribisnigri* werden gekweekt op resistente en vatbare sla cultivars voor een bepaalde periode en voedingsgedrag, overleving en groei werden bestudeerd op zowel resistente als vatbare sla-cultivars. Kortdurend verlies van virulentie werd waargenomen voor de zeer virulente bladluispopulatie, wat een indicatie kan zijn dat de bladluizen een adaptatie-periode nodig hebben op de waardplant. Langdurig verlies van virulentie werd waargenomen voor de semi-virulente bladluis-populatie. Dit is een indicatie dat er fitness-kosten geassocieerd zijn met de virulentie in *N. ribisnigri*.

Het effect van de aanwezigheid van andere bladluizen op het gedrag van *N. ribisnigri* werd bepaald door het bestuderen van voedingsgedrag, overleving en groei van individuele avirulente of virulente *N. ribisnigri* bladluizen in combinatie met een groep van hetzij hetzelfde biotype of het andere biotype op vatbare en resistente sla-cultivars (Hoofdstuk 6). Daarbij werd er tevens gekeken naar het effect van de aanwezigheid van de bladluissoorten *Myzus persicae* en *Macrosiphum euphorbiae* op voedingsgedrag, overleving en groei van beide biotypen van *N. ribisnigri*. Het virulente biotype was in staat om lokaal de resistentie in sla tegen het avirulente biotype te onderdrukken, echter *M. persicae* and *M. euphorbiae* gaven geen onderdrukking te zien. Een mogelijk mechanisme achter deze virulentie in *N. ribisnigri* tegen de *Nr*-resistentie kan de aanwezigheid van een effector-eiwit in het speeksel van de bladluis zijn, dat in staat is de resistentie te onderdrukken. Bladluizen van het virulente biotype werden ofwel negatief of niet beïnvloed door de aanwezigheid van andere bladluizen.

De locatie waar het *Nr*-gen product in sla tot expressie komt werd bestudeerd door middel van enten tussen resistente en vatbare sla-cultivars en studies aan voedingsgedrag, overleving en groei van avirulente *N. ribisnigri* (Hoofdstuk 8). Daarnaast werd de persistentie van de resistentie beoordeeld op afgesneden weefsel van resistente sla, in dit geval afgesneden bladeren en bladponsen, waarop het voedingsgedrag van avirulente bladluizen bestudeerd werd. De resistentiefactor(en) werd(en) geproduceerd in de spruit van de slaplant, niet in de wortels, omdat de enten met een resistente spruit en vatbare wortels resistent bleven, terwijl de enten tussen vatbare spruit en resistente wortels vatbaar bleven. Een intacte plant met een intact vasculair systeem is nodig voor een volledige resistentie, omdat zowel afgesneden bladeren als bladponsen van resistente sla deels hun resistentie tegen de bladluizen verloren.

De resultaten uit de hoofdstukken 2 - 7 geven inzicht in het resistentie-mechanisme van sla met het *Nr*-gen en in het mechanisme dat verantwoordelijk is voor de virulentie in *N. ribisnigri.* Deze resultaten worden bediscussieerd in de context van de recente literatuur in hoofdstuk 8. In een poging om de factoren te identificeren die verantwoordelijk zijn voor de virulentie in *N. ribisnigri*, werden transcriptoom-data van speekselklieren en darmen van avirulente en virulente biotypen van *N. ribisnigri* verzameld (Hoofdstuk 8). Verdere analyses van deze data zijn nodig voor de identificatie van de factoren die verantwoordelijk zijn voor de virulentie.

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During my Master thesis I already found out that the Laboratory of Entomology is a sociable and very diverse group where everyone is welcome. I would like to thank my colleagues for the great time I had during my PhD-project. Especially Katja, Marjolein, Karen, Ana, Joop W, Foteini, Dani, Camille and Alex, and ex-colleagues Gabriella, Martine, Fedor, Remco, Marit, Tullu and Tjeerd. I would also like to thank everyone for their love and support for me and my family during a difficult period.

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I was also a member of the party committee of Ento for almost four years and had a great time. I would like to thank all the party committee members, it was always nice to organise drinks and parties with you all. We had a lot of fun during the preparations, especially with Sinterklaas, the multiculti parties and hiding Easter eggs.

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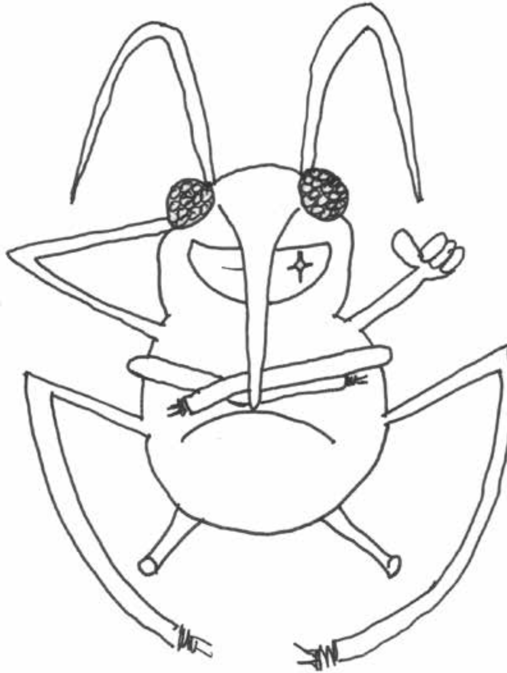

Curriculum Vitae

Cindy J.M. ten Broeke was born on December 3, 1984 in Eibergen, The Netherlands. After finishing secondary school, she started her study Biology in 2003 at Radboud University in Nijmegen, The Netherlands. During her BSc she developed an interest in both ecology and entomology. During her BSc-thesis project at Stichting Bargerveen she worked on the distribution of carabid beetles in sand dunes in The Netherlands. This project involved collecting insects and identifying Coleoptera, Orthoptera and Dermaptera. During her MSc she specialized in ecology and entomolgy.

Her first MSc-thesis project was conducted at Stichting Bargerveen on the distribution of macrofauna in shallow peat lakes, which involved collecting water macro-invertebrates and water samples, and identifying Trichoptera, Zygoptera and Asellidae. Her second MSc-thesis project was carried out at the Laboratory of Entomology of Wageningen University. This thesis focused on the effects of herbivory by *Pieris brassicae* caterpillars on pollinator behaviour and nectar production in Brassica nigra. Behavioural studies with *Episyrphus balteatus, Pieris rapae* and *Apis mellifera* were conducted, and nectar was extracted and analysed. Additionally the pollination effectiveness of *Pieris rapae* on *Brassica nigra* was evaluated. During her MSc she was a teaching assistant twice (in 2007 and 2008) for the Biodiversity course (Radboud University) in which she lectured, supervised field practicals and mini- projects and taught insect identification. In 2008 Cindy obtained her MSc-degree and started a job as research assistant at the Laboratory of Entomology, Wageningen University. For three months she worked on the transmission of *Tomato Yellow Leaf Curl Virus* (TYLCV) by *Bemisia tabaci* to different plant species.

In 2009 she continued to work at the Laboratory of Entomology, Wageningen University and started a PhD-project on the resistance mechanism in lettuce against the black currant-lettuce aphid, *Nasonovia ribisnigri*, of which the results are presented in this thesis. This PhD project was part of the *Nasonovia* – lettuce project funded by the Top Technological Institute Green Genetics, in which a multidisciplinary approach involving transcriptomics, proteomics, metabolomics, behaviour and performance was employed to gain a better understanding about the resistance mechanism in lettuce against this aphid species. Cindy participated in the training and supervision programme of the national Graduate School Experimental Plant Sciences (EPS), and was a member of the EPS PhD Council for three

years. Cindy was involved in the organization of the 5th workshop Plant-Insect Interactions for young scientists (Wageningen University, 2010), the EPS Expectations career day for PhD students (Wageningen University, 2010), the InsectenExperience festival (Wageningen, 2011) and the EPS company excursion for PhDs at KeyGene (2012).

In September 2012 Cindy started her own company, Cindy's Art, for commissioned pencil drawings (portrait of humans and animals, cars, plants, etc.), airbrush paintings, insect stuffed dolls (HugBugs), sculptures and jewelry. She also continued to work at the Laboratory of Entomology after her PhD as field assistant.

Publications

Published:

Yildizhan S, Van Loon J, Sramkova A, Ayasse M, Arsene C, **Ten Broeke C** & Schulz S (2009) Aphrodisiac pheromones from the wings of the Small Cabbage White and Large Cabbage White butterflies, *Pieris rapae* and *Pieris brassicae.* ChemBioChem 10: 1666-1677.

Ten Broeke CJM, Dicke M & Van Loon JJA (2013) Performance and feeding behaviour of two biotypes of the black currant-lettuce aphid, *Nasonovia ribisnigri,* on resistant and susceptible *Lactuca sativa* near-isogenic lines. Bulletin of entomological Research (in press).

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Submitted:

Bruinsma M, Lucas-Barbosa D, **Ten Broeke CJM**, Van Dam NM, Van Beek TA, Van Loon JJA & Dicke M (2013) Folivory affects floral traits and pollinator behaviour.

In preparation:

Ten Broeke CJM, Dicke M & Van Loon JJA (2013) Feeding behaviour and performance of *Nasonovia ribisnigri* on grafts, detached leaves and leaf disks of resistant and susceptible lettuce.

Kloth KJ, **Ten Broeke CJM**, Thoen MPM, Hanhart-van den Brink M, Wiegers GL, Krips OE, Dicke M & Jongsma MA (2013) Automated video tracking of aphid probing behaviour: a high-throughput phenotyping system for plant resistance.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Cindy ten Broeke
Date: 5 September 2013
Group: Entomology, Wageningen University & Research Centre

1) Start-up phase		*date*
►	**First presentation of your project**	
	Resistance mechanisms in lettuce, Lactuca sativa L., to the lettuce aphid Nasonovia ribesnigri M.	Jun 23, 2009
►	**Writing or rewriting a project proposal**	
	Resistance mechanisms in lettuce, Lactuca sativa L., to the lettuce aphid Nasonovia ribesnigri M.	March-May 2009
►	**Writing a review or book chapter**	
►	**MSc courses**	
►	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure		*date*
►	**EPS PhD student days**	
	EPS PhD student day, Wageningen University	May 19, 2011
	EPS PhD student day, University of Amsterdam	Nov 30, 2012
	EPS Expectations Carreer day 2010, Wageningen University	Nov 19, 2010
	EPS Expectations Carreer day 2011, Wageningen University	Nov 18, 2011
	EPS Expectations Carreer day 2013, Wageningen University	Dec 01, 2013
	International PhD retreat in Plant Sciences 2012, Norwich (UK)	Aug 15-17, 2012
►	**EPS Theme Symposia**	
	EPS Theme 2 Symposium & Willie Commelin Scholten Day, 'Interactions between Plants and Biotic Agents, Wageningen University	Jan 15, 2010
	EPS Theme 2 Symposium & Willie Commelin Scholten Day, 'Interactions between Plants and Biotic Agents, University of Amsterdam	Feb 03, 2011
	EPS Theme 2 Symposium & Willie Commelin Scholten Day, 'Interactions between Plants and Biotic Agents, Utrecht University	Feb 10, 2012
	EPS Theme 2 Symposium & Willie Commelin Scholten Day, 'Interactions between Plants and Biotic Agents, Utrecht University	Jan 24, 2013
►	**NWO Lunteren days and other National Platforms**	
	Annual Meeting of the Netherlands Entomological Society	Dec 2009, 2010, 2011 & 2012
	Netherlands Annual Ecology Meeting	Feb 2010 & 2012
	ALW Symposium 'Experimental Plant Sciences' 2010	Apr 2010, 2011, 2012 & 2013
	TTI-GG Networking Event	Sep 2011 & 2012
	PE&RC Day 'Extreme Life'	Nov 01, 2012
	Silent Spring Symposium (Naturalis)	Nov 17, 2012

	Plantum/KNPV/EPS Symposium ‘Intraspecific Pathogen Variations’	Jan 22, 2013
►	**Seminars (series), workshops and symposia**	
	Local monthly seminars at the Laboratory of Entomology	2009, 2010, 2011 & 2012
	Yearly Entomology Research Exchange Meeting	2009, 2010, 2011 & 2012
	Yearly TTI-GG Nasonovia Meeting	2009, 2010, 2011 & 2012
	EPS symposium “Ecology and Experimental Plant Sciences 2”	Sep 22, 2009
	EPS/PE&RCWorkshop Plant-Insect Interactions (4th, 5th, 6th and 7th)	Nov 2009, 2010, 2011 & Sep 2011
►	**Seminar plus**	
►	**International symposia and congresses**	
	International Symposium on Insect-Plant Relationships, Wageningen, NL	Aug 13-18, 2011
	Hemipteran-Plant Interactions Symposium, BRAZIL	Jul 11-14, 2011
►	**Presentations**	
	Netherlands Annual Ecology Meeting (poster)	Feb 09-10, 2010
	TTI GG Nasonovia meeting (oral)	Mar 03, 2010
	ALW Symposium ‘Experimental Plant Sciences’ 2010 (poster)	Apr 19, 2010
	TTI GG networking event 2010 (poster)	Sep 22, 2010
	TTI GG Nasonovia meeting (oral)	Oct 07, 2010
	5th workshop Plant-Insect Interatctions (oral)	Nov 11, 2010
	EPS Expectations Carreer day 2010 (poster)	Nov 19, 2010
	Annual Meeting of the Netherlands Entomological Society 2010 (oral)	Dec 17, 2010
	TTI GG Nasonovia meeting (oral)	Mar 01, 2011
	ALW Symposium ‘Experimental Plant Sciences’ (oral)	Apr 05, 2011
	EPS PhD student day (poster)	May 19, 2011
	Hemipteran-Plant Interactions Symposium, Brazil (oral)	Jul 11-14, 2011
	International Symposium on Insect-Plant Relationships, Wageningen (poster)	Aug 15, 2011
	TTI GG networking event 2011 (poster)	Sep 21, 2011
	TTI GG networking event 2011 (oral)	Sep 21, 2011
	TTI GG Nasonovia meeting (oral)	Oct 04, 2011
	Seminar University of Giessen (oral)	Oct 06-10, 2011
	6th workshop Plant-Insect Interactions (oral)	Nov 22, 2011
	Annual Meeting of the Netherlands Entomological Society (oral)	Dec 16, 2011
	Netherlands Annual Ecology Meeting 2012 (poster)	Feb 08, 2012
	TTI GG Nasonovia meeting (oral)	Feb 29, 2012
	ALW Symposium ‘Experimental Plant Sciences’(oral)	Apr 03, 2012
	TTI GG Nasonovia meeting (oral)	Oct 02, 2012
	Annual Meeting of the Netherlands Entomological Society (oral)	Dec 14, 12
	EPS Theme 2 Symposium & Willie Commelin Scholten day: ‘Interactions between Plants and Biotic Agents (poster)	Jan 24, 2012
	ALW Symposium ‘Experimental Plant Sciences’ 2013 (poster)	Apr 22, 2013
►	**IAB interview**	Feb 18, 2011
►	**Excursions**	
	EPS excursion Monsanto	Jan 27, 2011
	EPS excursion Keygene	Jan 26, 2012
	Subtotal Scientific Exposure	*38.4 credits**

3) In-Depth Studies		date
►	**EPS courses or other PhD courses**	
	Basic Statistics	May-Jun 2010
►	**Journal club**	
	PhD journal club Entomology	2009, 2010, 2011 & 2012
	Insect-Plant Interactions discussion group at Entomology	2009, 2010, 2011 & 2012
►	**Individual research training**	
	EPG instructions by Freddy Tjanllingii	Apr 06-09, 2009
	Stylectomy at Univerity of Giessen	Oct 05-07, 2011
	Subtotal In-Depth Studies	*6.6 credits**

4) Personal development		date
►	**Skill training courses (highly recommended)**	
	PhD Competence Assessment	Jan 19, 2010
	Techniques for Writing and Presenting a Scientific Paper	Aug 31-Sep 03, 2010
	Advanced course Guide to Scientific Artwork	Nov 07-08, 2011
	Last Stretch of your PhD	Apr 19, 2013
	Carreer Orientation	Sep 07,14, 21 & 28, 2012
►	**Organisation of PhD students day, course or conference**	
	EPS PhD Party	2009 & 2010
	Organising 5th Workshop Plant-Insect Interactions	2010
	Expectations 2010	Nov 2010
	Insectenexperience	May 2011
►	**EPS excursion Keygene**	Jan 2012
►	**Membership of Board, Committee or PhD council**	
	EPS PhD-council	2009-2011
	Subtotal Personal Development	*8.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	60.7

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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