Induced plant responses in willow to a gall-forming insect

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Abstract

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The interaction between the basket willow *Salix viminalis,* and the gall midge *Dasineura marginemtorquens,* includes responses known to enhance as well as reduce herbivore performance i.e., gall formation, and hypersensitive response (HR), respectively. There is large genotypic variation in resistance in *S.viminalis* against *D. marginemtorquens*. In the first of two studies in this thesis we report, variation in larval survival and induced plant responses within a full-sib *S. viminalis* family. Resistance (larval mortality) was associated with HR, but to different degrees among willow genotypes. Hydrogen peroxide, used as a marker for HR, was induced in genotypes expressing HR but not in resistant genotypes without symptoms. These data suggest that production of hydrogen peroxide, and accompanying cell death, cannot explain larval mortality in the symptomless reaction. Another, yet unknown, mechanism of resistance may therefore be present. If so, then it is possible that this unknown mechanism also contributes to resistance in plants displaying HR.

Resistant willows usually have a very few galled leaves, whereas on the great majority of leaves all larvae die before any galls are formed. This observation, of high but not complete resistance, formed the basis for the second study. We found support for rapid induced susceptibility i.e., factors acting on the target leaf immediately before and during gall formation, but no support for delayed induced susceptibility, i.e., responses induced in the shoot the previous year and expressed when leaves develop the next season. In a combined greenhouse and field experiment the possibilities that susceptibility was induced by abiotic factors at the time of gall initiation were tested. Although resistance remained strong, significantly more leaves with surviving larvae, and galls were found on potted plants moved to the field, compared with identical plants that remained in the greenhouse. These findings suggest that plasticity in plant resistance may be linked to ambient light conditions.

This system is a semi-natural one, where no intentional breeding for insect resistance has taken place. The interaction between *S. viminalis* and *D.marginemtorquens* includes responses and phenomena important for understanding plant-herbivore interactions in general. Thus, results from this research contribute to general models of plant resistance as well as to breeding programs in connection with short-rotation forestry based on willow.

Key words: *Salix viminalis, Dasineura marginemtorquens,* the hypersensitive response, HR, symptomless response, phenotypic plasticity, plant-insect interaction, induced susceptibility

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Contents

Appendix

Papers I-II

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Höglund, S., Larsson, S. & Wingsle, G. 2005. Both hypersensitive and non-hypersensitive responses are associated with resistance in *Salix viminalis* against the gall midge *Dasineura marginemtorquens*. *Journal of Experimental Botany* vol 56 no 422, 3215-3222.

II. Höglund, S. & Larsson, S. 2005. Abiotic induction of susceptibility in insect-resistant willow. *Entomologia Experimentalis et Applicata* 115, 89-96.

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Introduction

Induced plant responses in the basket willow *Salix viminalis* L. are associated with susceptibility as well as resistance against the gall midge *Dasineura marginemtorquens* Bremi (Diptera, Cecidomyiidae). The interaction is complex and includes induced symptoms known to enhance (gall formation) as well as reduce (the hypersensitive response, HR) insect performance. The resistance is expressed as rapid neonate larval mortality at the time of gall initiation. Thus, larval mortality may be due to either poor food quality poisoning the larva, or starvation due to the absence of a functioning gall. The interaction between *S. viminalis* and *D. marginemtorquens* includes phenomena that, in general, are central for the understanding of resistance and susceptibility in plants to insects and pathogens.

Background

The concept of plant resistance/susceptibility – comparing plant or herbivore fitness?

Variation in population density within and among insect species can be explained by their capability to survive, grow, reproduce or disperse. The potential of these properties within a single individual is explained by heritable characters, however, its realization depends on environmental conditions (Slansky, 1982).

The study of plant-insect interactions may involve measuring performance properties of the plant as well as the insect. In order to describe the outcome of the interaction the concepts of plant resistance and plant susceptibility are often used. The common definition of plant resistance is a plant species, or genotype within a species, with superior growth and production in comparison with others in the presence of insects, whereas susceptibility is used for reduced plant fitness (Painter, 1951). Plant resistance/susceptibility, however, has concurrently been used to represent plant traits that reduce, or enhance, insect performance (survival, fecundity, growth) (Hanks et al., 1991; Karban & Baldwin, 1997).

The former definition, focusing on plant fitness, is most often used by plant breeders and plant ecologists, where the mechanism behind plant resistance/susceptibility is mainly explained by plant traits affecting plant damage caused by herbivores (Painter, 1951; Kennedy & Barbour, 1992; Schoonhoven *et al.,* 2005). Insect ecologists that are interested in how plant traits influence insect population dynamics more often use the latter definition. In this perspective a resistant/susceptible plant is one that reduces or enhance insect performance (Karban & Baldwin, 1997; Larsson, 2002), often evaluated as differences in insect abundance (Fritz, 1999).

As illustrated, the concept of resistance/susceptibility used to describe the outcome of plant-insect interactions is ambiguous and depends on the context in which it is used. For this reason it is important that the terms are defined when applied. Regardless of whether plant or herbivore fitness is compared, it is a relative measurement concerning plant characteristics.

In this thesis, resistance/susceptibility will refer to plant traits that affect insect fitness. The first aim is to explain associations between induced plant responses in *Salix viminalis* and neonate larval mortality of the gall midge *Dasineura marginemtorquens.* The second aim is to suggest explanations to observed intra-plant heterogeneity in larval mortality on resistant genotypes.

Plant traits associated with herbivore performance

Plant traits affect herbivorous insects in several ways. The traits can influence behaviour by changing preference for feeding (Finch & Collier, 2005) and oviposition (Hopkins & van Loon, 2001) leading to alteration in insect distribution among plants. The behaviour of natural enemies (often arthropod predators and parasitoids) can also be affected by plant traits leading to indirect effects on herbivores, i.e., interactions over three trophic levels (Price *et al.,* 1980; Dicke & van Loon, 2000).

Plant traits can furthermore directly interfere with the physiology of the herbivore once it has accepted to feed on the plant, resulting in changes in performance affecting growth (Schriber & Slansky, 1981), fecundity (Awmark *et al.,* 2002), or survival (Höglund *et al*., 2005).

Nutrional requirements in herbivorous insects are similar to other animals, although the needs can differ greatly among insect species and developmental stage (Schoonhoven *et al*., 2005). In natural systems, the food resource (plant material) is seldom found to limit herbivore population growth (Schoonhoven *et al.,* 2005). The food availability can nevertheless be restricted because of physical barriers or plant phenology. Physical factors believed to restrict food availability are e.g., leaf thrichomes (Southwood, 1986), leaf toughness (Coley, 1983), and hypersensitive responses (HR) (Fernandes & Negreiros, 2001). Plant phenology can be a problem for insects that depend on specific plant tissue types that are not available over an extended time period, like buds or developing leaves. Asynchrony between such periodic plant events, like bud break, and insect hatching is known to influence insect survival (Yukawa, 2000).

Plant traits affecting food quality are primary and secondary metabolites. Primary metabolites such as nitrogen and carbohydrates (Schoonhoven *et al*., 2005) are crucial for insect growth as is water (Henriksson *et al*., 2003). The nutritional composition of the plant material is considered to be suboptimal for insect growth and reproduction. For example, the nitrogen content of insects is 8-14% (dw) whereas plants usually contain 2-4% (Mattson, 1980). Consequently, performance indices of many insects are strongly correlated with the nitrogen concentration of the plant while the energy value is less restricting (Schoonhoven *et al*., 2005). Depending on species and instar, herbivorous insects cope with nutritionally poor food by having the capacity to select and ingest large amounts of plant material (Berner *et al.,* 2005).

Secondary metabolites, such as phenolics, terpenoids, and alkaloids, affect insects in several ways. To insects with special adaptations for feeding on a certain type of plant, they guide the insect to its host plant and act as feeding stimuli (Chapman, 2003). Many secondary compounds, however, are more or less toxic depending on the insect's capability to digest and secrete these products, leading to reduced growth and survival (Rosenthal & Berenbaum, 1992). The effect from secondary compounds on food quality may be modified by the composition and concentration of primary metabolites (Duffey & Stout, 1996; Haukioja *et al*., 2002).

Constitutive vs. induced (plastic) traits

Plant traits known to influence insect performance are often defined as either constitutive or induced (Gatehouse, 2002). The biochemical processes in constitutive and induced plant responses are fundamentally the same, and involve expression of the same gene products (Gatehouse, 2002). The two responses differ only insofar as in the constitutive case, gene expression occurs as a result of normal plant development whereas in the induced case expression is altered by a signal caused by external stimuli (Gatehouse, 2002).

Plant chemistry, physiology, phenology and morphology can be changed in response to a wide variety of environmental conditions of abiotic as well as of biotic origin. Abiotic conditions known to affect plant traits considered to be important for insect performance are light (Larsson *et al.,* 1986; Mole *et al*., 1988), temperature (Veteli *et* *al*., 2002), access to water (Koricheva *et al*., 1998a), nutrient supply (Koricheva *et al.,* 1998b), and presence of pollutants (Hughes, 1988). Plant competition (Kurashiege & Agrawal, 2005), herbivory (Karban & Baldwin, 1997), and plant disease (Stout *et al*., 2006) are biotic factors that can induce changes in the plant that directly affect insect performance.

Induced resistance, i.e., reduced insect performance, often follows after damage of leaf tissue (Karban & Baldwin, 1997). Induction of resistance is also believed, however, to correlate with nutrient availability of the plant, where shortage of nutrients increases resistance (Bryant *et al*., 1993; Koricheva *et al.,* 1998b).

Induction of plant susceptibility to insects most often refers to abiotic factors, e.g., drought stress inducing biochemical changes in the plant that result in a subsequent increase in insect performance (White, 1974; but see Larsson, 1989; Koricheva *et al*., 1998a). In recent years, it has become clear that induction of susceptibility also is common in plants following biotic stress, such as herbivore damage (Nykänen & Koricheva, 2004).

Phenotypic plasticity – genotype by environment interactions

The presence of induced traits indicates that plants are highly plastic (Sultan, 2000). Apart from inter-specific variation, plant characteristics also differ among populations and genotypes of the same species (Agrawal *et al.,* 2002) as well as within the same plant individual (Orians & Jones, 2001; de Kroon *et al*., 2004). Phenotypic plasticity is defined as the ability of an organism to express different phenotypes depending on the biotic or abiotic environment (Bradshaw, 1965). In addition, the degree of phenotypic plasticity among genotypes of the same plant species may differ. Genotypes can respond to the same environmental cue unequally i.e., genotype by environment interaction (Schlichting, 1986; Callaway, 2003). High plasticity implies that the genotype is very sensitive to changes in the environment (Pigliucci, 2001). The trait(s) responsible for the sensitivity is coded in all cells and tissues within a plant. Due to variation in the external environment, however, the response may not be manifested in all plant parts simultaneously leading to within-plant heterogeneity (Oborny, 2004). Thus, variation among plant individuals in intra-plant distribution of herbivores may be explained by heterogeneous expression of a trait affecting herbivore performance (Suomela, 1996; Höglund & Larsson, 2005).

The effects of induction last for varying time periods. Induced responses triggered by herbivores can affect the same insect individual, or the same generation of insects, that caused the induction (see review by Karban & Baldwin, 1997), sometimes referred to as rapid induced response (RIR) (Haukioja & Hanhimäki, 1985). In contrast, some induced responses last for several years, and thus can influence the performance of several generations of insects (Karban & Baldwin, 1997), a phenomenon termed delayed induced response (DIR) (Haukioja & Hanhimäki, 1985).

Induced plant responses that deviate from normal plant growth

Some locally induced plant responses deviate from normal plant development. The formation of galls, by insect, fungi, or bacteria, is one example of such a response where the plant produces an abnormal tissue growth in response to stimuli from the invading organism. The gall is generally considered to be controlled by and to support the gall maker (Stone & Schönrogge, 2003).

Another response diverging from a plant's normal reaction is the hypersensitive response (HR) where dead plant cells appear in connection with restriction of pathogen growth (Heath, 2000a). The intruder elicits the HR but in this case it is to the benefit of the plant because it restricts further development of the inducing agent, although the consequence for the plant is that a few of its own cells are killed (Richael & Gilchrist, 1999).

Gall formation can in this perspective be considered as an induced susceptible reaction enhancing insect performance while HR is an induced resistance response, reducing insect performance.

Gall formation

Galls are close associations between arthropods (usually insects) and plants. The adaptive value of the gall for the galler may be to enhance food availability and food quality, as well as protect from harsh abiotic conditions and natural enemies (Price *et al.*, 1987). The nutrition hypothesis states that galls provide enhanced food resources compared to other feeding modes, but also that the food quality is improved (enhanced nitrogen content and reduced concentration of secondary metabolites) in comparison with unmodified plant tissue (Price *et al.*, 1987). The food resources can be enhanced in gall tissue because the galls act as strong sinks for photosynthtate (Larson & Whitham, 1997). Strong evidence supporting the hypothesis that the galls improve food resources is, for example, the situation where more than 100 000 aphids occupy the same gall (Wool, 2005).

It is not clear whether or not the food quality, in general, is enhanced in the gall tissue. Reports regarding nitrogen content in galls are inconsistent. Hartley *et al.* (1998) found the nitrogen content generally to be lower in galls compared with ungalled tissue when examining 20 species of gall-formers. There is also evidence, however, that the gall tissue can be superior to unmodified tissue (Abrahamson & Weis, 1987).

The same conflicting result is found regarding the content of secondary compounds. When galled and ungalled tissue were compared among 33 galling systems, the total phenolic content was found to be higher in the galls in three (3), lower in eight (8) and to show no difference in the rest (Hartley, 1998). The content of phenolic compounds in galls from chestnut leaves was reduced in the tissue nearest the larvae compared with gall cortex (Allison & Schultz, 2005).

Difficulties in making bioassays with galling insects make it hard to properly compare food quality in galling and free-living insects (Hartley, 1998; Stone & Schönrogge, 2003). Studies regarding the effect of galls on galling insects consider the situation where the galls are already formed; much less is known about the crucial event when the gall is initiated and the gall is still invisible (Stone & Schönrogge, 2003; Schoonhoven *et al.*, 2005). Therefore, larval mortality occurring before the gall is functioning may be explained either by larva not being able to induce a gall, and therefore starve to death, or the presence of toxic substances killing the larva before the gall is initiated.

The hypersensitive response

A specific induced response associated with resistance is the hypersensitive response (HR), which is generally recognized by the presence of brown, dead cells and believed to isolate the intruder from the surrounding area and starve it to death (Agrios, 1997; Heath, 2000a). The cause-effect relationship, however, between cell death and restriction of pathogen growth is not clear. A number of biochemical changes coincide with the process of cell death; thus, it is possible that HR could be the consequence of a process that is actually killing both host and microbe cells (Dangl *et al.*, 1996). Rapid death of host cells associated with resistance has been described in response to microorganisms (Heath, 1998) as well as insects (reviewed by Fernandes, 1990; Ollerstam *et al.*, 2002).

Few studies have unequivocally documented HR as a response to feeding by piercing/sucking insects (Walling, 2000; Kaloshian, 2004; but cf. Ollerstam *et al.*, 2002) although insects that cause limited tissue damage are believed to induce pathways commonly activated by pathogens (Walling, 2000). An intimate relationship, such as in the case of gall-inducing insects and its host plant, seems also to promote HR (Fernandes, 1990, 2000; Ollerstam *et al*., 2002). Morphological and biochemical responses of a HR type have been observed to be induced by economically important gall midge species; *Mayetiola destructor*, the Hessian fly, *Oresolia orzyae*, the rice gall midge, and *Sitodiplosis mosellana*, the wheat gall midge (Harris *et al*., 2003, and references therein). However, whether or not theses responses actually cause larval death, or occur after some other mechanism actually have killed the insects, is not known (Harris *et. al*., 2003).

In the case of HR, as in the case of unsuccessful gall initiation, there may be at least two possible mechanisms explaining neonate larval mortality. The HR can either limit food resources by reducing transport of assimilate to the larva (the starvation hypothesis), or alternatively, that toxic compounds induced simultaneously with the cell death (Richael & Gilchrist, 1999) may lead to intoxication of the larvae (the food quality hypothesis).

To separate the starvation hypothesis from the food quality hypothesis as explanation of neonate larval mortality in the system of *S.viminalis/D.marginemtorquens* have been a major issue through years in our research group.

Where to look for causal explanations - In genetics or in the environment?

The two plant responses – HR and gall formation – have much in common despite the opposite effect on the eliciting organism i.e., death vs. survival. Hereditary characteristics are emphasized concerning factors important for the outcome of both responses i.e., the “gene for gene” concept. The interaction is assumed to contain inherited properties of both the plant (presence or absence of resistance genes) and the intruder (presence or absence of virulence genes) (Agrios, 1997). The gene for gene model was first developed based on studies of rust (*Melamsora lini*) against flax (*Linium usitatissimum*) in the early 1940s (Flor, 1971), and is now widely accepted as an explanation of variation in the outcome of plant/pathogen interactions (Agrios, 1997; Levine, 2004). The gene for gene concept is also used to explain gall formation in several plant/gall midge systems, especially systems linked to breeding programs such as wheat (*Triticum aestivum*) and the Hessian fly (Harris *et al.,* 2003; Kaloshian, 2004). In this system, focus is on the plant response, i.e., a stunted (galled) or a healthy (ungalled) plant, whereas the effect on larval survival is less emphasized. Biotypes of the Hessian fly are defined based on their ability to stunt (being virulent) or not to stunt (being avirulent) a particular combination of plant varieties, i.e., seemingly a problematic argument because the response that is meant to be explained is part of the definition. The composition of most occurring Hessian fly populations is found to be a mixture of different biotypes (Williams *et al*., 2003; Subramanyam *et al*., 2005). Surprisingly, the mechanisms behind gall initiation (Harris *et al*., 2003), or HR (Heath, 2000b), are not known in detail despite extensive investigations. Even though the wheat/Hessian fly system is very similar to the *S.viminalis/D.marginemtorquens* system there is a major difference in how virulence is perceived. In the former, virulence is considered an inherited trait while in our system we consider virulence to be influenced by environment conditions.

Phenotypic plasticity (the genotype by environment concept) is rarely used to explain the outcomes of interactions between plants and pathogens or plants and galling insects. However, observed intra-plant heterogeneity regarding resistance, where an initial pathogen attack protects the plant from further infection, may be perceived as an example of plant plasticity, i.e., systemic acquired resistance (SAR) (Karban & Kuc, 1999). There are a few examples where phenotypic plasticity has been proposed to explain the outcome of plant/gall midge interactions. In these cases the environmental conditions linked to solar radiation, light quality or sunshine hours are proposed to intervene with the expression of resistance (Sharma *et al.,* 2003; Höglund & Larsson, 2005).

Aims of the present study

The basket willow *Salix viminalis* shows great genotypic variation in resistance against the gall midge *Dasineura marginemtorquens*. In a common garden experiment, Strong *et al.* (1993) found certain genotypes to host only a few galled leaves, whereas other genotypes had 20-30 galled leaves per shoot. Later, it was found that the lack of galls is not explained by midges discriminating against certain genotypes; instead it is correlated with neonate larval mortality (Larsson & Strong, 1992). On resistant genotypes, necrotic lesions, interpreted as a hypersensitive response (HR) are induced by the larvae (Ollerstam *et al*., 2002). On other resistant genotypes, however no visible plant responses occur (Ollerstam *et al*., 2002).

The aim of the first paper in this thesis is to better document preliminary observations of a range of resistance responses in *S. viminalis* against *D. marginemtorquens*. Within a full-sib family (72 individuals) of *S. viminalis* the distribution of living and dead larvae, as well as induced plant responses, was documented by means of biotests (Höglund *et al.,* 2005). The hypothesis that resistance against *D. marginemtorquens* can be expressed without symptoms associated with HR was then tested (Höglund *et al.,* 2005). Hydrogen peroxide was used as a marker for HR (Höglund *et al.,* 2005). Finally, it was investigated whether or not there were any differences in the rate by which resistance is expressed (Höglund *et al.,* 2005). It was expected that gall midge larvae would respond by losing size and dying more quickly on genotypes with HR, compared with resistant genotypes lacking HR symptoms, i.e., without lesion formation and production of hydrogen peroxide.

Strong et al. (1993) reported the outcome of an outbreak of the gall midge on *S. viminalis* planted in a common garden experiment. Although there was great genotypic variation in number of galled leaves, most of the resistant individuals had a few leaves harboring a few galls (Strong *et al*., 1993; Höglund & Larsson, 2005). Thus, resistant genotypes were not immune. This observation, of high but not complete resistance, formed the basis for the second paper in this thesis. Specifically, we aimed to understand what factors that are behind the putative plasticity in leaf suitability for gall formation on resistant genotypes. A first experiment aimed at testing the hypothesis that random events (biotic or abiotic) induce susceptibility in willow shoots that is expressed at a later stage, e.g., when leaves develop from the shoot in the next season (Höglund & Larsson, 2005). In a second study a combined field and greenhouse experiment was conducted, which tested the possibility that susceptibility is induced by abiotic factors acting on the target leaves immediately before and during gall formation, i.e., a rapidly induced susceptibility (Höglund & Larsson, 2005).

Study organisms

The gall midge *Dasineura marginemtorquens* is monophagous on *Salix viminalis*. The midge is an ephemeral 2-3 mm insect whose larva induces a gall on *S. viminalis* leaves. The life cycle takes about three weeks and the midge has 3-4 generations per year in Sweden (Glynn & Larsson, 1994). The female disperses 20-50 eggs along the midrib of unfurled leaves in the leaf bud. About nine young leaves per shoot are accepted as oviposition sites each day (Larsson & Strong, 1992). Several females can lay eggs on the same leaf at the same time. After 3-5 days, depending on the temperature, the eggs hatch and the first instar larvae crawl into the scrolls of the unfurled leaves where the larva induces a gall in the young leaf tissue. The mature gall is a 5-10 mm pocket (Mani, 1964) along the leaf margin and consists of enlarged plant cells. Each leaf can harbor up to 60 galls, and at high densities the leaf becomes twisted resulting in reduced photosynthetic leaf area. In the field, each midge generation leaves a band of galled leaves with interspaces of ungalled leaves on the willow shoot.

Salix viminalis grows along streams and lakes in southern Sweden. In recent years *S. viminalis* has been used in short rotation forestry for biomass production (Ledin, 1992). Although introduced into Sweden from east Europe and central Asia (Jonsell, 2000), it has spread and become part of the natural vegetation. The first record of *S. viminalis* in Sweden is from the eighteenth century (Leche, 1774; referenced in Hylander, 1971). *Salix viminalis* is a rapid grower and produces new leaves throughout the growing season. Genotypes are easily propagated from stem cuttings. When cultivated for biomass production, 15 cm long cuttings are planted in rows at a density of one cutting per m^2. Each rooted cutting produces several shoots and this plant unit is referred to as a stool.

Methods

The basic method used to study the interaction between *Salix viminalis* and *Dasineura marginemtorquens* was by means of biotests i.e., the plant quality is evaluated from its impact on the target insect in this case number of living and dead larva. Through the years, our research group at the Department of Entomology, SLU has developed a protocol in order to carry out the biotests. The protocol includes methods to grow plants, find fields with natural occurrence of galls, collecting galled leaves and handling midges, and how and when to collect data about larval performance and induced plant responses.

Plant material: The plant material used by Strong *et al.* (1993) originated from crosses between 15 collected wild plants from Sweden and one from Holland. The number of galled leaves was reported from 40 full-sib *S. viminalis* families. Six offspring from each family were tested. In the current study, a new crossing was conducted in order to determine the level of variation in larval performance and induced responses for a higher number of siblings within a family. The crossing was between genotype 78-0-195 as the mother and genotype 81-0-084 as the father (equivalent to family no. 46, a family with some resistant siblings, in Strong *et al.*, 1993). The crossing was made at the Department of Plant breeding and Forest Genetics, SLU. The original plants were harvested and three cuttings per genotype were used to establish a combined plant archive and experimental field at Pustnäs, 1 km southeast of Uppsala.

Handling of plants: When used in greenhouse experiments, cuttings were planted in 2l pots filled with soil ("Hasselfors Garden Special") and given a supply of optimal nutrient solution (Wallco 51-10-43+ micronutrients, 100 mg N l^{-1}), the technique of nutrient supplying being adapted to maximum growth rate (Ingestad, 1987). Plants were watered and fertilized daily until they were about 50 cm high.

Handling of midges: Midges used to conduct biotests were collected in the field, as pupae or third instar larvae in galled leaves, from outbreak populations around Uppsala. Due to variation in gall midge population densities among fields and years, galled leaves were collected at different field sites at different years. In the laboratory, galled leaves were put in Petri dishes with moist filter paper. After emerging, the adults were caught with an aspirator, and transported to experimental cages covered with muslin where female midges were allowed to oviposit for 24 hours.

Documenting larval survival and induced plant responses among genotypes: Number of living and dead larvae, as well as necrotic lesions, was counted under a stereomicroscope on a sample of leaves per plant, five days after egg hatch. The resistant genotypes, i.e. genotypes without surviving larvae, were classified according to the presence of necrotic lesions. Genotypes were designated 'Resistant Few Lesions' (RFL) genotypes when there were fewer lesions than larvae (in many cases no lesions at all), and 'Resistant Many Lesions' (RML) genotypes when there were more lesions than larvae. The consistency in expression of responses over time was investigated by repeating the biotest another two years on a subsample of genotypes.

Documenting performance of young larvae: It was investigated whether or not there were any differences in the rate by which resistance is expressed among genotypes with dissimilar responses. The effect of different mechanism of resistance was evaluated by comparing body size of 30-h-old larvae. Larvae reared on RML plants were compared with larvae on RFL plants. Larvae on susceptible plants as well as newly hatched larvae were used as controls. The length and width of individual larvae were measured by means of a stereomicroscope. Body volume (approximated to be a cylinder) was calculated as $v = l \times (w/2)^2 \times \pi$ where v is the volume, l is the length, and w is the width.

Documenting presence of hydrogen peroxide, a marker for the hypersensitive response: The connection between formation of necrotic lesions and production of hydrogen peroxide was tested. A fluorescent probe (DCFH-DA, 2',7-dichlorofluorescein diacetate) was employed to detect the presence of hydrogen peroxide (Wolfe *et al.*, 2000). The number of greenish spots, visible in fluorescent light, were counted on a transect (about 5 mm in length) from the leaf edge to the midrib in a combined fluorescence and light microscope

Documenting genotype by environment interactions: An experiment was conducted during three consecutive years in order to investigate variation in *D. marginemtorquens* larval survival among individual plants of the same resistant willow genotype. We hypothesized that observed variation in larval survival within resistant genotypes can be caused by random environmental events (biotic or abiotic) inducing changes in the stem that are later expressed as increased susceptibility in the developing shoot. Plants were obtained from an experimental willow plantation situated at Pustnäs, Uppsala that was established in 1988. At Pustnäs, individuals from the same crossings as reported by Strong *et al.* (1993) were planted in eight 23 x 23m blocks (Rönnberg-Wästljung & Gullberg, 1999).

Stem cuttings used to produce test plants for the present experiment were harvested in the winters of 1999/2000, 2000/2001 and 2001/2002. Stem segments were taken from each resistant genotype in each block, giving eight replicates per resistant genotype (but from different stools). Each stem segment was cut into two cuttings, thus giving replicates of individual stools.

To exclude the possibility that the observed plasticity is only observed when there is an intact connection between stem and an established root system, which is not the case if the experiment is conducted from cuttings in the greenhouse, we performed a field experiment at Pustnäs in 2001. The experimental plantation at Pustnäs was harvested during the winter of 2000/2001. Specifically for this experiment, one stem each on every stool was left intact so that we could compare the old, five-year old shoot, with current-year shoots on the same stool. Such experimental units were created in each of the eight blocks. The biotest was done in the same way as described earlier.

In addition, a combined greenhouse and field experiment was conducted in 2002 in order to test for induction of susceptibility by abiotic factors at the time of gall induction. Resistant and susceptible genotypes originating from the new crossing earlier described were used. Potted plants in the greenhouse (PG), potted plants moved to the field (PF), and one-year-old stools growing in the field (SF) were inoculated with midges, and larval survival was compared.

Treatment effects on plant characteristics possibly of importance for gall formation were evaluated by examining two traits: leaf trichome density and leaf toughness. Trichome density was determined by counting the number of trichomes along a 1.2 mm straight line. Leaf toughness was measured by determining the amount of force needed to penetrate through the leaf by using a calibrated penetrometer.

Results and discussion

Intraspecific variation in larval survival and induced plant responses (Paper 1)

The new crossing revealed great variation in neonate larval survival among sibling plants. Out of 72 sibling plants, 50% had no surviving larvae, and are therefore referred to as resistant genotypes. In 11 genotypes (15%), all larvae survived, and are referred to as susceptible. In the 25 (35%) remaining genotypes there were living and dead larvae on the same leaf, these genotypes are therefore referred to as variable genotypes. Different genotypes displayed dissimilar responses to larval gall initiation attempts. Within the group of resistant genotypes, there was variation in the number of necrotic lesions formed, despite the fact that all larvae died. There was a strong consistency in plant responses over time. No living larvae and no galls were ever found on the resistant genotypes, in contrast to susceptible genotypes where all larvae were alive, galls were formed, and no necrotic lesions were visible.

Resistant genotypes with and without lesions were used to test the correlation between production of hydrogen peroxide, a marker for the hypersensitive response (HR), and neonate larval mortality. It was found that production of hydrogen peroxide was only associated with plant cell death and only occurred on resistant genotypes with lesions (RML). Symptomless resistant genotypes (RFL) as well as susceptible genotypes showed no production of hydrogen peroxide.

Ollerstam *et al.* (2002) found that the young larva react by loosing size and die after 48 h on resistant genotypes, contrary to susceptible genotypes where larva grow exponentially. In this paper, the two kinds of induced response, associated with resistance, (HR) as well as symptomless responses, were compared regarding their influence on larval growth reduction. Contrary to prediction, the larvae on genotypes with a symptomless response lost weight quicker than larvae reared on genotypes with HR, although at the end larvae died on both kinds of resistant genotypes.

Data from this experiment suggest that the production of hydrogen peroxide, and the accompanying cell death, cannot explain larval mortality in the symptomless reaction. Another, as yet unknown, mechanism of resistance has to be considered in these plants. If correct, it is possible that this unknown mechanism also contributes to resistance in plants showing HR, and that the association between larval mortality, induction of hydrogen peroxide, and necrotic cells is spurious. The presence of a powerful symptomless mechanism of resistance against a sessile insect in a plant species that has not been intentionally bred for resistance may suggest that this is a common phenomenon in natural plant populations. There are very few other observations on locally induced symptomless responses reported (Yu *et al*., 1998), none of which come from non-agricultural plants. Such a phenomenon, however, would be very difficult to observe unless, like in the present study, a plant population with enough variation in resistance traits is studied under controlled conditions.

Mechanisms explaining plasticity among resistant genotypes (Paper 2)

In the second study we investigated why certain *Salix viminalis* genotypes that show an unusually strong resistance against *Dasineura marginemtorquens* occasionally harbour a few leaves with galls, whereas on the great majority of leaves all larvae die before any galls are formed. We first tested in what time frames the induction of susceptibility might occur. When tested in the greenhouse environment we did not find support for delayed induced susceptibility i.e., phenotypic plasticity triggered by some environmental conditions acting on the stool the year prior to harvest and maintained in the cutting the year after. When the biotest, however, was conducted on shoots of different age in the field some galls were found on resistant genotypes, however, with no relationship to shoot age. Because of the inconsistencies in earlier results an experiment was performed in order to separate the two possible explanations behind induction of susceptibility, i.e., the outdoor environment *per se* vs. shoots being connected with roots. In the combined field and greenhouse experiment the possibility that susceptibility is induced in otherwise resistant genotypes by abiotic factors at the time of gall induction (within days), i.e., rapid induced susceptibility was tested.

We found support for rapid induced susceptibility, although resistance remained strong. Significantly more leaves with surviving larvae, and galls, were found on potted plants moved to the field compared with identical plants that remained in the greenhouse.

What kind of abiotic conditions could have been associated with induction of susceptibility in this case? Potted field plants were exposed to field conditions during a few days in connection with gall initiation. Thus, we can exclude soil moisture, nutrient availability, and history of the plant as factors explaining induction of susceptibility. In addition, we found that leaf toughness and trichome density were less expressed in the greenhouse than in the outdoor environment, contrary to what would be expected if these traits were part of the mechanisms of resistance.

One of the big differences between the greenhouse environment and the field site is the light quality. We tentatively suggest that radiation interferes with expression of resistance. Our findings suggest that plasticity in plant resistance may be linked to ambient light conditions.

The genotypic variation in *S. viminalis* resistance against *D. marginemtorquens* is unusually high (Strong *et al*., 1993). It is uncommon to find highly resistant (with close to zero survival) plant genotypes that can be considered host plants for herbivorous insects (Mattson *et al*., 1988). In most cases, females discriminate against such plants at oviposition (e.g., Thompson, 1988), and thus are not part of the host range. However, sometimes females, for a variety of reasons (Larsson & Ekbom, 1995), accept plants where there is low offspring survival. Such plants, by definition (Larsson, 2002), are classified as resistant. The degree to which insect resistance in natural plant populations is plastic is not well understood. Truly resistant plant individuals will, most of the time, harbor very few, if any, insects to observe, and thus are difficult to study. Therefore, data on resource heterogeneity exclusively concern susceptible plants, and refer primarily to variation caused by a decrease in suitability (Schultz, 1983; Agrawal & Karban, 1999; Orians & Jones, 2001). We show in this paper that highly resistant plants are also plastic and that under certain environmental conditions susceptibility can be induced, even though in this case resistance is still very high. It seems likely that similar plastic responses occur in other resistant plants. If so, then induction of susceptibility could be an important, but as yet underestimated, factor explaining some of the variation in resistance that occur in plant populations.

Concluding remarks and suggestions for future research

The interaction between *Salix viminalis* and *Dasineura marginemtorquens* is very complex. It contains symptoms known to enhance herbivore performance (gall formation) as well as reduce performance (the HR and the symptomless response). In the following section I will summarize my views on this interaction, and suggest key questions in need of additional research.

The system *S. viminalis/D. marginemtorquens* has several advantages as a model for studying plant-herbivore interactions. Plant genotypes are easily propagated simply by making cuttings. The effect of the resistance trait is unusually clear; either there is a living larva with gall formation or a dead larva with or without HR symptoms. A natural outbreak of *D. marginemtorquens* in a large common garden experiment, designed for the analysis of wood productivity in *S. viminalis*, was the start for the research of resistance in this system. Inherited plant properties largely explain

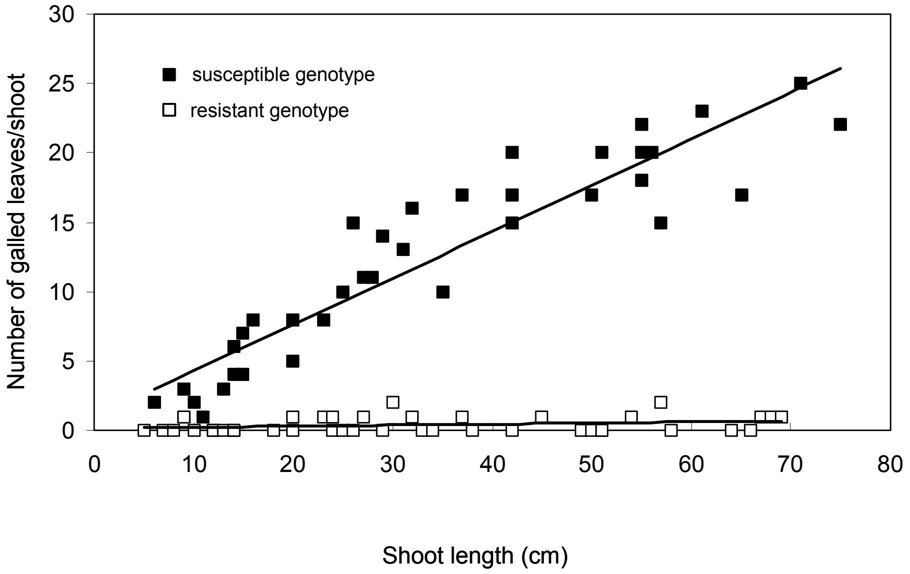

Fig 1. Number of leaves galled by *Dasineura marginemtorquens* per shoot in relation to shoot length of one field-grown resistant (78-195) and one susceptible (78-021) genotype of *Salix viminalis*. (y (res) = 0.0077x + 0.1444, r^2 = 0.06, $p>0.05$; y (sus) = 0.3349x + 0.9198, r^2 = 0.86, $p< 0.0001$) (n= 10 stools per genotype, 4 shoots per stool) (S. Larsson & S. Höglund, unpublished data)

the great differences in number of galled leaves among genotypes, most evident on vigorously growing shoots (Fig 1). The difference in number of galled leaves is due to neonate larval mortality. Observed intra-plant heterogeneity among resistant plants seemingly is the result of phenotypic plasticity (Fig 2).

A major question over the years has been: Why do larvae die on resistant genotypes? Two hypotheses have been developed 1) the food quality hypothesis and 2) the starvation hypotheses. **The food quality hypothesis** states that the food that the larva gains is toxic. This hypothesis is supported by data from other systems; plant quality is often the main factor explaining variable herbivore performance (Schoonhoven *et al.,* 2005). However, most data are from systems with free-living caterpillars. Our research, with a galling insect compared larval performance on resistant willow genotypes showing hypersensitive response (HR) with those showing a symptomless response; the results did not support the food quality hypotheses because larvae were bigger on genotypes with rapid cell death (HR),

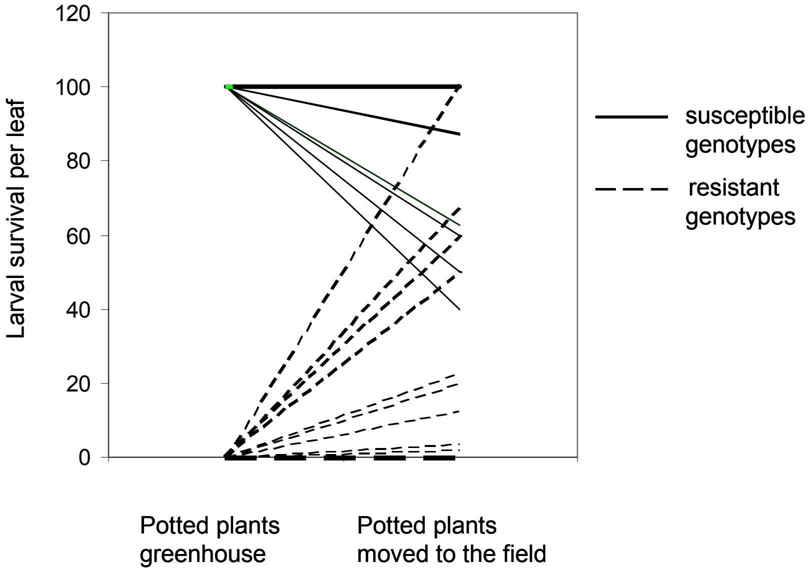

Fig 2. Phenotypic plasticity (genotype by environment interaction) among *Salix viminalis* genotypes resistant and susceptible to the gall midge *Dasineura marginemtorquens*. The plants were exposed to greenhouse and field environments in connection with gall initiation. (n(res)= 12; n(sus)= 6 genotypes)(data from paper 2, Höglund & Larsson, 2005).

a process known to be induced simultaneously with production of toxic substances, such as hydrogen peroxide, phytoalexins, phenolic compounds and proteinase inhibitors (Richael & Gilchrist, 1999). In addition, the fact that the outdoor environment induced susceptibility further contradicts the food quality hypotheses. In general the content of secondary metabolites, which are associated with reduced food quality, is higher in leaves exposed to sunshine than those exposed to shade, such as the environment in our greenhouse experiment.

The starvation hypothesis suggests that the larva starves to death because it is unable to initiate gall formation and thus will not have access to the nutritive tissue in the gall. Much less is known, compared to the conventional view of food quality, about how nutritive tissue is induced and its effect on herbivore performance. It is accepted, however, that the gall acts as a strong sink and draws resources from other parts of the plant. For example galls from gall-making aphids compete with the plants own sinks (like buds), and by experimentally removing sinks it is possible to enhance aphid survival (Larson & Whitham, 1997). If the *S.viminalis/D.marginemtorquens* system functions in a similar way, then the larva may die because no sink relation is established. The neonate larvae attack, irrespective of genotypes and plant responses, the epidermis cells on the young unfurled leaves (Höglund *et al.*, 2005). The cells in the case of HR respond by collapsing and dying, in contrast to gall initiation where the cells become enlarged and nutritious (Ollerstam *et al.*, 2002). It is possible that the plant response triggered by larval activity changes the cell-wall permeability in both cases but with different result. In this perspective the HR can be seen as an unsuccessful gall initiation. This view is supported by the fact that in our experiment the larvae were bigger on genotypes with HR compared with those with no symptoms. Perhaps the abiotic environment, by inducing susceptibility, may then modify the cell response from collapsing towards cell enlargement and sink establishment. If this is correct, then the mechanism of resistance would be related to getting access to food resources rather than food quality *per se*. By comparing early events in the HR and gall initiation, it may be possible to confirm or reject these possibilities.

The concept of virulence is central in interpreting many plant-pathogen interactions, and is also used to explain patterns of plant damage to crops caused by different gall midge species. The virulence concept states that the outcome of an interaction is due to inherited properties of participating organisms. I believe, however, that our findings may support phenotypic plasticity related to the plant as an alternative explanation. To what degree, however, phenotypic plasticity can replace the gene for gene concept, as a phenomenon explaining variation within this system is unknown. In order to separate these two explanations for plant heterogeneity concerning larval survival we have to find, and control for, the abiotic conditions that trigger plasticity in the plant and then test if there is variation among gall midge populations and gall midge individuals regarding capacity to induce galls.

The **induction of susceptibility** that we have found may be linked to production of secondary metabolites because the induction seems to be triggered by factor(s) in the abiotic environment, tentatively high level of radiation (photosynthetic active radiation (PAR) or/and UV). Secondary metabolites in the plant (e.g. phenolic compounds) are believed to protect the plant against herbivores. In addition, however, secondary metabolites seem to be important in protecting plant cells from photodamage through scavenging of reactive oxygen species (ROS). Therefore, the induced susceptibility in willow to the gall midge may be linked to the increased capacity of leaves to take care of ROS generated by the larva when attempting to initiate the gall. This hypothesis would be possible to test by studying resistant plants with different capacity to scavenge ROS, and compare the effect on larval survival and plant cell death. A correlation between ROS protection and herbivore performance would imply that light protection under certain circumstances can explain variation in resistance against galling insects.

The system that we are studying is a semi-natural one, where no intentional breeding for insect resistance has taken place. The interaction between *S. viminalis* and *D.marginemtorquens* include responses and phenomena central for understanding plant-herbivore interactions in general. Thus, results from this research contribute to general models of plant resistance to insects as well as to breeding programs in connection with short-rotation forestry based on willows.

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