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Βιοτεχνολογικοί τρόποι καταπολέμησης του εντόμου εχθρού καλλιεργειών *Nezara viridula*

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Thesis

Biotechnological ways to manage the pest *Nezara viridula*

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Περίληψη

Το έντομο *Nezara viridula* αποτελεί ένα σημαντικό εχθρό αγροτικών και κηπευτικών καλλιεργειών. Οι απώλειες καλλιεργειών που αποδίδονται σε αυτό το έντομο εκτιμήθηκαν σε 60 εκατομμύρια US$ το 2014. Τα έντομα αυτά μπορούν να μειώσουν την παραγωγή προκαλώντας ζημιές σε νεαρά στάδια των φυτών, αλλά και μεταφέροντας παθογόνα βακτήρια. Υπάρχει μεγάλη ανησυχία ότι η *Nezara viridula* θα δημιουργήσει μεγαλύτερο πρόβλημα στα επόμενα χρόνια, εξαιτίας της εύκολης προσαρμογής και της γρήγορης εξάπλωσης του εντόμου, σε συνδιασμό με τις αλλαγές στις αγροτικές πρακτικές.

Σε αυτή την μελέτη εξετάσαμε την ανάπτυξη ανθεκτικότητας σε δυο πληθυσμούς *N. viridula,* ελέγχοντας για πιθανές μεταλλαγές σε γονίδια στόχους εντομοκτόνων (οργανοφωσφορικά, κετοενόλες, διαμίδια και πυρεθροειδή). Συγκεκριμένα στο γονίδιο του τασεοευαίσθητου καναλιού νατρίου, στο γονίδιο της ακετυλοχολινεστεράσης, του ακετυλο-συνένζυμου α και του υποδοχέα ρυανοδίνης. Παράλληλα, προτείνουμε μία νέα προσέγγιση για την αντιμετώπιση της *N. viridula,* μέσω του προσδιορισμού πρωτεϊνών-effectors στη σίελο των εντόμων, οι οποίες ευθύνονται για τις ζημιές στα φυτά. Με αυτό τον τρόπο θα μπορούσαμε να ανακαλύψουμε τα μονοπάτια μέσω των οποίων δρουν αυτές οι πρωτεΐνες και έτσι να δημιουργήσουμε φυτά που δεν θα προσβάλλονται από το συγκεκριμένο έντομο.

Abstract

Nezara viridula is a polyphagous insect of significant economic importance to global agricultural and horticultural crops. Crop losses attributed to stink bugs in cotton were estimated at $60 million in 2014 across the Cotton Belt. Stink bug injury can reduce yield through aborted young bolls, stained lint, and reduced lint yield. *N. viridula* bugs have also been shown to be a vector of bacterial pathogens affecting boll development. There is a growing concern that due to its high vagility, polyphagous feeding habits and its ability to move to alternative hosts, combined with global warming and changes in agricultural practices, *N. viridula*, as well as other stink bugs, will become even more important pests worldwide.

In this study we examined target site mutations in two strains of *N. viridula* that are able to confer resistance. We checked for mutations of the voltage-gated sodium channel, the acetylcholinesterase (AchE) gene, the acetyl-coenzyme A carboxylases (ACCs) and the ryanodine receptor (RyR). Mutations of these genes are associated with resistance to ketoenols, pyrethroid, organophosphate and diamide insecticides. In parallel we propose a new way of managing this pest, through the identification of effectors in the watery saliva of the insects intending to unravel the plant pathways that are deteriously being affected and create plants that will not be affected by this insect.

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1.General introduction

1.1 A review of pertinent literature on *Nezara viridula*

1.1.1 Origin & Spread

Nezara viridula is a polyphagous insect and a worldwide pest of numerous crops and cropping systems. The species is adept at exploiting a wide range of noncultivated plants in the absence of preferred food resources and during the overwintering period and this may contribute to its broad distribution. This insect possesses the broadest distribution range of the Pentatomids (Panizzi and Slansky 1985).

Nezara viridula has been referred to in the literature and throughout the warmer regions of the world by various common names: "chinche verde" (Peña M. and Sifuentes 1972, Rodríguez Vélez 1974), "cosmopolitan stink bug" (Hokkanen 1986), "cotton green bug" (Kamal 1937), "green bug" (Hubbard 1885), "green-bug" (Drake 1920), "green-bug of India" (Atkinson 1889), "green plant-bug" (Drake 1920), "green soldier bug" (Turner 1918, Watson 1918), "green soldier-bug" (Hubbard 1885), "green stink bug" (CABI Invasive Species Compendium 2015), "green vegetable bug" (Gu and Walter 1989), "plant-bugs" (Riley and Howard 1893b), "pumpkin bug" (Watson 1918, 1919a), "southern green plant-bug" (Jones 1918), "southern green stink-bug" (Drake 1920), "southern green stinkbug" (Wolfenbarger 1947), "southern stinkbug" (Demaree 1922), "southern stink bug" (Nishida 1966), "stink-bugs" (Jones 1918), and "tomato and bean bug" (Froggatt 1916).

The exact, or even approximate, date of the invasion of *Nezara viridula* into the United States is unknown, but the species was recorded in Texas in 1880 (Distant 1880). Subsequently, Hubbard (1885) first reported *N. viridula* as a pest of citrus in Florida.

The species was first described by Linnaeus in 1758 as Cimex viridulus from material collected 'in Indiis' (Freeman, 1940). Based on the distribution pattern of distinct colour morphs, it was first assumed to have originated from the Indo-Malayan Region (Yukawa & Kiritani, 1965). However, in later studies, which also considered the distribution of other species of the genus and examined the distribution of specialist parasitoids, it was proposed that the area of origin of N. viridula was most probably the Ethiopian region (Hokkanen, 1986). In the 18th century it was already present in Africa and Mediterranean region, in Madeira, in tropical parts of Asia and in the West Indies (Hokkanen, 1986). It was first recorded in Japan in 1879 (Jones, 1988), in Australia in 1916 (Clarke, 1992), in New Zealand in 1944 (Cumber, 1949), in Hawaii in 1961 (Jones, 1995) and in California in the mid 1980s (Jones, 1988). It was not reported from Brazil prior to 1923 (A.R. Panizzi, personal communication). It is still an active invading species and newly established populations have recently been reported from the Galapagos (Henry & Wilson, 2004) and England (Barclay, 2004). It is assumed that current spread into temperate regions is made possible by global warming (Musolin & Numata, 2003, 2004).

Distribution of *Nezara viridula* often is referred to as worldwide or cosmopolitan as the species is known to occur throughout tropical, subtropical, and warm temperate regions of Eurasia, Africa, Australia, and the Americas approximately between latitudes 45°N and 45°S (McPherson and McPherson 2000; Panizzi et al. 2000; Yukawa et al. 2007, 2009; Musolin 2007, 2012; Tougou et al. 2009, Panizzi and Lucini 2016). The species is constantly expanding its range (Yukawa et al. 2007, 2009; Musolin 2007, 2012; Rabitsch 2008, 2010), both in the Northern and Southern Hemispheres,

by natural dispersal (Tougou et al. 2009) and human-assisted translocation, which complicate a strict and comprehensive determination of its boundaries.

1.1.2 Biology

1.1.2.1 Life history (Eggs, Nymphs, Adults)

The developmental life stages of *Nezara viridula* have been firstly described by Jones (1918) and Drake (1920). Characteristics and descriptions of the eggs, nymphs of different stadia/stages, and adults have changed minimally since those original descriptions.

Female *Nezara viridula* oviposit clusters of eggs (= egg masses; McLain and Mallard 1991). The numbers of eggs per cluster usually range from 60 to 90 (extreme recorded values are 1 to 184) (Musolin et al. 2007). As eggs are oviposited, each is covered (or "smeared") with a viscous liquid or glue-like material that adheres the eggs to the oviposition substrate (e.g., leaf surface) and each other (Drake 1920) (Figure 1). Prado et al. (2006) indicate this "smearing" process also deposits beneficial endosymbionts on the surface of the egg cluster. Ovipositing females have been observed using their hind tarsi to position eggs within these clusters (McLain and Mallard 1991, Panizzi 2006). Drake (1920) described changes in the appearance of the egg before hatch as well as the method used by the first instar to exit the egg through the operculum, and will be briefly addressed here.

Figure 1. Egg masses from *Nezara viridula* (Bayer strain)

Freshly deposited eggs are cream in colour, darkening slightly after one day. The eyes of the developing nymph are red in colour and at some point become visible through the operculum (Todd 1989). After almost 3 days, the eggs hatch and the newly enclosed first instars cluster atop the egg choria.

First instars are red in colour immediately after hatching and turn red by the second day of the stadium (Figure 2). They remain clustered atop the egg choria, probing the egg surfaces to obtain beneficial endosymbionts (Prado et al. 2006). Dispersal from the hatched eggs to feeding sites typically begins at or near emergence of the second instars and continues through subsequent instars.

Figure 2. First instars of *Nezara viridula* (Bayer strain)

Coloration in second through fourth instars of the common green adult form is fairly uniform black dorsal coloration with white spots (Rojas and Morales-Ramos 2014). However, the fourth and fifth instars can also be in a "pale form" or "dark form" (Jones 1918), represented by "green" and "dark brown, nearly black" dorsal coloration, respectively. Morrill (1910) also noted the occurrence of "light" and "dark" forms for fifth instars. Light and dark forms also are present in the orange adult form of *Nezara viridula* (Follett et al. 2007). The fifth instar possesses the distinct and characteristic white spots on the dorsal surface (Figure 3). The characters for determining sex are easily visible at the fifth instar (Esquivel and Ward 2014), becoming more pronounced as the stadium nears enclosion to adulthood.

Behaviourally, newly emerged first instars were long thought to not feed (Morrill 1910). However, first instars of *Nezara viridula* provisioned with a green bean were observed inserting their stylets into the food source. Upon microscopic inspection of this behaviour, the protraction and retraction of stylets were visible through the outer surface of the green bean. These observations raised the question of whether the commonly accepted belief of nonfeeding by first instars was accurate. Using sections of green bean infected with a marker pathogen, Esquivel and Medrano (2014) demonstrated that first instars indeed feed as defined by the ingestion of the marker pathogen that, subsequently, was found within the insects. Skeptics of these findings argued that the first instars were not "feeding" but "drinking" instead. However, "feeding" by *N. viridula* is accomplished through uptake of liquefied food resources (i.e., "drinking"). Thus, "drinking" would also be defined as an aspect of "feeding," regardless.

Figure 3. Fifth instar of *Nezara viridula* (dorsal and abdominal view, Tomato strain)

Developmental time from egg to adulthood is approximately 30 days but will greatly vary based on rearing temperatures and food source. Nondiapausing adults began mating as early as 5 days for females and 6 days for males, indicating females reach sexual maturity earlier than males (Musolin and Numata 2003a). Mated females deposited viable eggs within 7–8 days after mating. This premating period (= precopulation period) differs from that reported by Fortes (2010). However, the time frame for oviposition corresponds with Fortes (2010) and Fortes et al. (2011) who observed chorionated eggs ready for fertilization at 10 days of age.

Longevity of adults is variable and greatly depends on whether a particular adult reproduces directly or enters winter diapause and reproduces after overwintering. Thus, under summer field conditions in central Japan, nondiapausing (i.e., actively reproducing) adults lived on average less than 50 days, whereas those adults that emerged later in the season (e.g., in September) entered diapause and survived until the next summer (most of this time in diapause), began reproducing in April–May, and died in July–August (Musolin et al. 2010).

In the laboratory, at 25°C, postdiapause females lived up to 351 days (including a long diapause period) (Musolin et al. 2007). Fecundity of females also is variable. Under laboratory conditions at 25°C, fecundity of reproductive females ranged from 18 to 1,496 eggs, and 1 to 19 egg clusters, per female (Musolin and Numata 2003a, Musolin et al. 2007).

As noted earlier, adults exhibit several colour morphs including gold (or orange), blue, green, and black forms. Although the green and orange forms have been known for some time (Vivan and Panizzi 2002, Golden and Follett 2006), a blue form was observed in a field collection in Brazil in 2015 and, most recently, a black form was reported in a laboratory colony in Texas (Esquivel et al. 2015).

1.1.2.2 Host plant associations

Nezara viridula is highly polyphagous. Although this species prefers leguminous plant taxa (i.e., members of the Fabaceae; Table 1), noncultivated herbaceous plants, other cultivated crops (including fruit and nut trees), ornamentals, and noncultivated trees can be exploited, or utilized, throughout the year. However, all exploited plant species may not meet the definition of a "host plant" (Smaniotto and Panizzi 2015). That is, Smaniotto and Panizzi (2015) proposed the term "associated plant" in lieu of "host plant" to recognize plant species utilized by *N. viridula*, rationalizing that "host plant" *sensu stricto* is defined as a host where an insect species can feed, reproduce, and seek shelter within. They concluded that "...the ideal host plant, the one to fulfil the three features [i.e., feeding, reproduction, and shelter within], is seldom encountered by pentatomids." Thus, as further clarified below, "associated plant" (or appropriate variant) is a more accurate term, and the term will be incorporated henceforth.

Table 1.Plant families associated with *Nezara viridula* (Babin et al., 2018)

Plant Family (n = 43)	No. Total Taxa	Percent of Total	No. of Plant Taxa Identified to: Genus[1]	Species[1]	Cumulative Percent of Total
Fabaceae	49	24.87%	9	40	24.87%
Asteraceae	21	10.66%	2	19	35.53%
Brassicaceae	15	7.61%	2	13	43.15%
Poaceae	14	7.11%	4	10	50.25%
Solanaceae	10	5.08%	2	8	55.33%
Cucurbitaceae	9	4.57%	1	8	59.90%
Malvaceae	9	4.57%	1	8	64.47%
Amaranthaceae	6	3.05%	1	5	67.51%
Chenopodiaceae	6	3.05%	1	5	70.56%
Rutaceae	6	3.05%	1	5	73.60%
Rosaceae	5	2.54%	2	3	76.14%
Capparaceae	4	2.03%	0	4	78.17%
Lamiaceae	3	1.52%	1	2	79.70%
Moraceae	3	1.52%	1	2	81.22%
Polygonaceae	3	1.52%	2	1	82.74%
Rubiaceae	3	1.52%	1	2	84.26%
Cannabaceae	2	1.02%	1	1	85.28%
Convolvulaceae	2	1.02%	0	2	86.29%
Cupressaceae	2	1.02%	2	0	87.31%
Vitaceae	2	1.02%	1	1	88.32%
Acanthaceae	1	0.51%	0	1	88.83%
Anacardiaceae	1	0.51%	0	1	89.34%
Apiaceae	1	0.51%	0	1	89.85%
Boraginaceae	1	0.51%	1	0	90.36%
Bromeliaceae	1	0.51%	0	1	90.86%
Cyperaceae	1	0.51%	0	1	91.37%
Euphorbiaceae	1	0.51%	0	1	91.88%
Juglandaceae	1	0.51%	0	1	92.39%
Liliaceae	1	0.51%	1	0	92.89%
Myrtaceae	1	0.51%	0	1	93.40%
Nyctaginaceae	1	0.51%	0	1	93.91%
Onagraceae	1	0.51%	0	1	94.42%
Orchidaceae	1	0.51%	1	0	94.92%
Passifloraceae	1	0.51%	0	1	95.43%
Pedaliaceae	1	0.51%	0	1	95.94%
Phytolaccaceae	1	0.51%	0	1	96.45%
Pinaceae	1	0.51%	0	1	96.95%
Portulacaceae	1	0.51%	0	1	97.46%
Proteaceae	1	0.51%	0	1	97.97%
Scrophulariaceae	1	0.51%	0	1	98.48%
Sterculiaceae	1	0.51%	0	1	98.98%
Ulmaceae	1	0.51%	0	1	99.49%
Verbenaceae	1	0.51%	1	0	100.00%
Total taxa	197	100.00%	39	158	

[1] Number of taxa identified to genus or species within respective plant family.

These data clearly reflect the polyphagous nature of *N. viridula*. Although *Nezara viridula* feeds on a wide range of host plants, it prefers to utilize hosts that are actively producing seeds or fruits. Because stink bugs are active throughout the warmer parts of the year, they must rely on a variety of overlapping host plants that occur in a dynamic ecosystem. As soon as the host begins to senesce or is mowed, the bugs will disperse to a new host that is just starting to produce seeds or fruit. Bundy and McPherson (2000b) demonstrated this host plant movement for stink bugs, primarily *N. viridula*, in a soybean-cotton ecosystem. The bugs were first attracted to the early-maturing soybean cultivar and remained there until the plants began to mature. Then, they moved into the later-maturing cultivar as pods were filling with seeds and finally into conventional and transgenic cotton (i.e., Bt-cotton = cotton containing the toxin *Bacillus thuringiensis* [Bt] *kurstaki*) when flowers and bolls were forming. Additionally, the planting date of a particular agronomic crop has a profound impact on its host suitability to stink bugs. For example, Tillman (2010) observed greater populations of *Euschistus servus* (Say) (brown stink bug) and *N. viridula* in late-planted com- pared to early-planted corn. Understanding these movements and being able to predict when the population is likely to disperse and colonize new habitats is important for formulating management strategies.

Direct empirical evidence on stink bug dispersal is limited. However, stink bugs are widely regarded as strong fliers based on their ability to find and colonize susceptible agronomic crops that are isolated spatially in the landscape. Tillman et al. (2009) showed that an individual stink bug could be recaptured as far as 120 meters from the release site. Huang (2012) showed that marked stink bugs were much more likely to be recovered only a few meters from where they were marked in agronomic crops. A tethered stink bug, *Graphosoma rubrolineatum* (Westwood), was estimated to fly a distance of over 27 kilometers in a single 7-hour flight (Cui and Cai 2008), indicating that stink bugs are capable of long-distance flights. However, focusing on the individual as opposed to populations may oversimplify the importance of local dispersal, largely because increased dispersal distance tends to dilute the population in a particular space.

1.1.3 Feeding behaviour

Nezara viridula possesses a needle-like rostrum comprised of the labrum and labium (Snodgrass, 1993); the latter is comprised of three articulating segments. A stylet fascicle, or bundle, comprises the piercing and sucking mechanism of the insect. The stylet bundle is formed by two pairs of stylets. Two apposing maxillary stylets at the centre of the bundle intricately interlink to form separate food and salivary canals. Two mandibular stylets encase the maxillary stylets. As the stylet bundle exits the head dorso-anteriorly, the stylet bundle is immediately housed within the labrum. At rest, the labrum is held against the ventral surface of the head with the distal end of the labrum lying between paired bucculae, the latter being extensions of the ventral surface of the head. The remaining length of the stylet bundle is held within a groove or sheathed along the length of the segments of the labium.

In general, while exploring the surface of the host tissue, the insect egests and then ingests saliva through the maxillary stylets to assess host suitability. An acceptable feeding site solicits stylet probing, and Backus (1988, citing Sogawa, 1973) describes two additional phases of probing: test probing and exploratory probing. Test probing involves shallow stylet insertion to assess internal resources of the host, and exploratory probing involves deeper penetration of stylets during the feeding process. At the conclusion of feeding, the insect retracts all the stylets, and the rostrum returns to a non-feeding position.

When at rest, the rostrum lies on the ventral surface of the insect body, virtually parallel to the length of the body. The proximal end of segment 2 can articulate at the juncture with the bucculae. Direct observations of probing and feeding behaviour indicate that the distal end of segment 2 swings away from the insect body, thereby forming an angle anteriorly between the bucculae and the proximal end of segment 2 (Figure 4A). When probing (Figure 4A), the distal end of the labrum continues to lie between the paired bucculae, and the stylet bundle continues to be held within the groove of segments 2–4 (Figure 5A). Proximally, segment 3 articulates with the distal end of segment 2, aligning itself with segment 4, and both segments 3 and 4 are held virtually perpendicular to the host surface while probing and feeding. Thus, the stylet bundle remains lying within segments 2–4 when the insect assesses host suitability. When the proximal end of segment 2 articulates at the bucculae, an unknown span (i.e., distance) results between the apex of the head (where stylet bundle exits) and the proximal end of segment 3 (Figure 1B). A critical note here is that the changes in position of segments 2, 3, and 4 during probing do not necessarily involve the protraction of the stylet bundle

from the distal apex of the labium, although Backus (1988) indicates test probing does result in shallow penetration of the host surface.

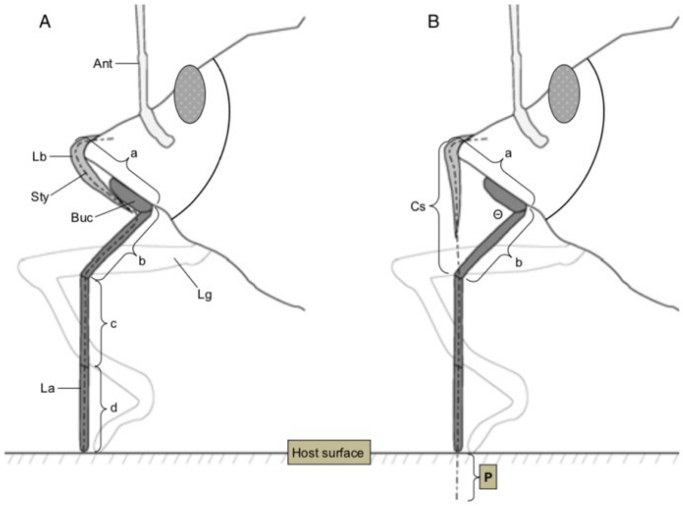

Figure 4. Schematic of stink bug head with rostrum (A) in probing and (B) feeding postures (not to scale). A-d,length of segments1-4, respectively; Ant,antenna; Buc,bucculae; Cs,calculated span; La,labium; Lb, labrum; Lg, leg; P, penetration depth into host tissue; Sty, stylet bundle; Θ,angle at segment 2 and bucuulae (Esquivel 2011).

Upon establishing a feeding site, segments 2–4 remain in position as described earlier, but a key change occurs in that the distal end of the labrum is swung away from the bucculae, and the stylet bundle is extracted from the groove in segment 2 (Figures 4B and 5B). These actions cause the labrum to span the unknown distance from the apex of the head to the proximal end of segment 3 (Figure 4B). Because the stylets of N. viridula are a fixed length, any change from the rostrum position while probing (Figure 4A) to the feeding position (Figures 4B and 2C) causes a shortening of the overall distance from the apex of the head to the distal end of the labium. This results in the stylet bundle exiting the distal end of the labium, thereby causing penetration into the host tissue.

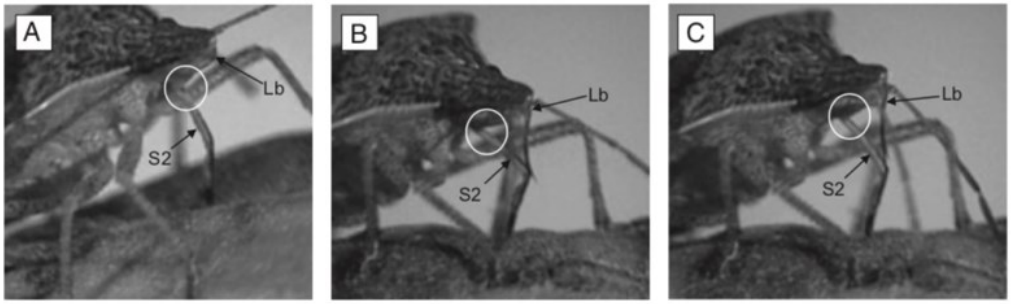

Figure 5. Stink bug rostrum (and stylet bundle) depicted in different sequential positions (a) Probing with labrum (Lb) held against ventral surface of the head and stylet bundle contained within segment 2 (S2), with segment 2 virtually perpendicular (encircled) to the ventral surface of the insect. (B) Feeding position with labrum pulled away from the ventral surface of the head and stylet bundle extracted from segment 2 with subsequent alignment with proximal end of segment 3 – note relatively acute angle (encircled) at the juncture of the bucculae and proximal end of segment 2, as well as distal tip of labrum pulling away from stylet bundle. (C) Feeding position following that shown in B – note change in head position and less acute angle (encircled) at the juncture of the bucculae and proximal end of segment 2 (Esquivel 2011).

1.2 Insect – plant interactions

1.2.1 Plant-herbivore interactions

There are almost a million insect species on this planet and nearly half of them feed on plants. This ongoing battle between plants and insects has lasted over 350 million years. During their coevolution with plants, insects have evolved to be able to locate their host plants for feeding and oviposition using physical or chemical cues from host plants. Generalist herbivorous insects feed on many plant species spanning different families, whereas specialists attack only one or a few plant species within the same family (Wu and Baldwin 2010).

Accordingly, plants have evolved elaborate defense systems to resist insect herbivores. They equip themselves with physical barriers, such as thorns, trichomes, and cuticles. Moreover, many of the secondary metabolites in plants are powerful chemical weapons. There are estimated to be more than 500,000 secondary metabolites in plants. Plants' direct defenses, which include glucosinolates, cyanogenic glucosides, alkaloids, phenolics, and proteinase inhibitors (PIs), function as toxins, repellents, or antidigestives. Plants' indirect defenses—green leaf volatiles, volatile organic compounds, and extrafloral nectars—attract the natural enemies (such as parasitoids) of herbivores. These two powerful defense systems, acquired by plants during the long arms race with herbivores, have enabled plants to survive. Inducible defenses consist of three components: a perception component, which triggers a downstream regulatory network, which mediates the biosynthesis of metabolites that function as defenses (Wu and Baldwin 2009).

Insect species have to find specific plant species on which they can feed and reproduce (host plants) among plant species that do not support feeding and/or reproduction of the insects (non-host plants). Insect responses to host plant cues from their external environment can be very quick because they have a sophisticated system for sensing their external environment and processing the sensory input. Plants not only respond to insect feeding damage but they have also been shown to be responsive to insect egg laying, the very earliest stage of insect attack (Bruce 2015).

Oral secretions are likely to be a major factor in limiting the host range of herbivorous insect species and biotypes (Elzinga and Jander, 2013). Insect oral secretions include salivary enzymes such as glucose oxidase and β-glucosidase, peptides like inceptin, and fatty acid conjugates (FACs) like volicitin that can trigger plant defense responses (Wu and Baldwin, 2009) but also suppress defense (Consales et al., 2012) depending on whether the plant or the insect is ahead in the evolutionary game.

When plants perceive herbivore-derived physical and chemical cues, such as elicitors in insects' oral secretions and compounds in oviposition fluids, plants dramatically reshape their transcriptomes, proteomes, and metabolomes. All these herbivory-induced changes are mediated by elaborate signaling networks, which include receptors/ sensors, Ca_2^+ influxes, kinase cascades, reactive oxygen species, and phytohormone signaling pathways. Furthermore, herbivory induces defense responses not only in the wounded regions but also in undamaged regions in the attacked leaves and in distal intact (systemic) leaves.

1.2.2 Effectors

Effectors can be viewed as "parasite genes having phenotypic expression in host bodies and behavior". They are products of genes that reside in pathogen genomes but that actually function at the interface with the host plant or even inside plant cells (Dawkins 1999). Effectors include all pathogen/pest proteins and small molecules that alter host-cell structure and function. These alterations may trigger defense responses induced by avirulence factors, elicitors, microbial/pathogen/herbivore-associated molecular patterns (MAMPs, PAMPs of HAMPs) or promote infection (mediated by virulence factors or toxins) or both (Hogenhout and Bos 2011).

The inducible plant defense response to effectors is a multilayered process consisting of several phases. Currently, plant pathologists recognize two related categories of immunological mechanisms: basal and R-gene mediated immunity. Basal immunity uses transmembrane pattern recognition receptors (PRRs) that elicit pathogen-triggered immunity (PTI) when they detect pathogen-associated molecular patterns (PAMPs) or herbivory-associated molecular patterns (HAMPs). PAMPs are essential, highly conserved, slowly evolving molecules that exist across a broad range of taxa and HAMPs are compounds from herbivorous insects (Figure 6) (Hogenhout and Bos 2011).

It is becoming increasingly clear that molecular recognition via ligand–receptor binding phenomena plays important roles in plants and that this plays a role in insect–plant interactions. PRRs are therefore capable of protecting the plant from most nonadapted infective organisms (phase 1). An evolutionary arms race ensues when invasive organisms become host adapted. To defeat PTI, plant pathogens use effectors (phase 2). To defend themselves against these host-adapted parasites, the plant uses R gene-mediated immunity (phase 3). R-gene encoded proteins act as sentries for effectors and elicit a resistance response, called effector triggered immunity (ETI), when effectors are perceived. The arms race continues with the evolution of effectors that evade detection (phase 4) and persists as plant R-gene-encoded proteins evolve that are capable of perceiving new and modified effectors (Stuart 2015).

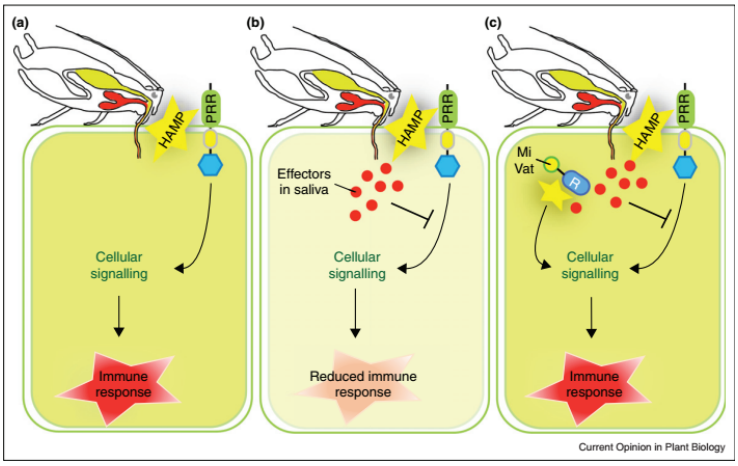

Figure 6: Model of the multi-layered plant defense response to aphid herbivory. (a) Plant cells perceive aphid herbivore-associated molecular patterns (HAMPs) leading to HAMP-triggered immunity (HTI). When the aphid is unable to secrete effectors that suppress HTI, this effective defense response deters the aphid from further feeding. (b) Although plants perceive the aphid HAMPs, the defense response is effectively suppressed by aphid effectors leading to aphid colonization. (c) The aphid species produces effectors that effectively suppress HAMP-triggered immunity responses, but in certain clones of this aphid species one or more effectors are being recognized by R genes leading to a plant effective immune response and plant resistance to the aphid clone (Hogenhaut and Bos 2011).

1.2.3 Emerging concepts in effector biology

Many effectors are delivered into host cells. Plant pathogenic bacteria, fungi, oomycetes, and nematodes have evolved the capacity to deliver effector proteins inside host cells through a diversity of mechanisms. Gram-negative bacteria use specialized secretion systems, such as T3SS, to deliver proteins inside host cells (Zhou and Chai 2008). Biotrophic fungi and oomycetes have evolved haustoria for this purpose. Haustoria are specialized structures that form within plant cells but remain encased in a modified plant cell membrane, known as the extrahaustorial membrane (Panstruga 2003). Haustoria were initially thought to primarily function in nutrient uptake, but more recently, evidence emerged that haustoria take part in the secretion of particular classes of host-translocated fungal and oomycete effectors (Whisson et al. 2007). Plant parasitic nematodes utilize a specialized feeding organ known as the stylet, to inject their effector proteins inside a parasitized plant vascular cell (Davis et al. 2008).

Other effectors act in the apoplast. Some effectors act in the extracellular space at the plant-microbe interface, where they interfere with apoplastic plant defences (Misas- Villamil and van der Hoorn 2008). Examples include the secreted protein effectors of the tomato fungal pathogen *Cladosporium fulvum*. This fungus is an extracellular parasite of tomato that grows exclusively in the apoplast and does not form haustoria or haustoria-like structures (Thomma et al. 2005). All known *C. fulvum* effectors, such as Avr2, Avr9, Avr4, and ECP2, are small cysteine-rich proteins that are thought to function exclusively in the apoplast (Thomma et al. 2005). Oomycetes, such as *Phytophthora infestans*, are also known to secrete apoplastic effectors in addition to host translocated (cytoplasmic) effectors (Tian et al. 2007).

One common activity ascribed to many apoplastic effectors of *C. fulvum* and other fungal and oomycete pathogens is their ability to inhibit and protect against plant hydrolytic enzymes, such as proteases, glucanases, and chitinases (reviewed by (Misas-Villamil and van der Hoorn 2008).

One effector—many host targets. Plant pathogen effectors frequently have more than one host target (Figure 7). *Pseudomonas syringae* AvrRpt2 is a T3SS effector with proteolytic activity against at

least five *Arabidopsis* proteins, including the negative defense regulator RIN4 (Takemoto and Jones 2005). AvrPto, another *Pseudomonas syringae* T3SS effector, is a kinase inhibitor that binds and inhibits the tomato kinase Pto (Xing et al. 2007). In addition, AvrPto inhibits the kinase domains of FLS2 and EFR, which are two pathogen recognition receptors, as well as the kinase domain of their signaling partner BAK1 (Xiang et al. 2008). These transmembrane receptor-like kinase proteins participate in the recognition of conserved pathogen molecules, and their inhibition by AvrPto presumably acts to suppress the innate immune response mediated by these receptors. Other examples of multiple targets include the protease inhibitors Avr2 and EPIC2B, which, as discussed above, inhibit several tomato apoplastic proteases (Tian et al. 2007).

Each interaction of an effector and a host protein can be either beneficial for the pathogen, have negative consequences, or have neutral effects on the interaction between the pathogen and plant. In light of these ideas, Van der Hoorn and Kamoun (2008) defined operative targets as those host targets that, when manipulated by effectors, result in an altered state of defence or susceptibility. It therefore becomes important to distinguish operative targets from other types of host targets. These thoughts led to the concept that some host targets are decoys, proteins that are not operative targets but that, when perturbed by effectors, trigger host recognition by cognate R proteins (van der Hoorn and Kamoun 2008).

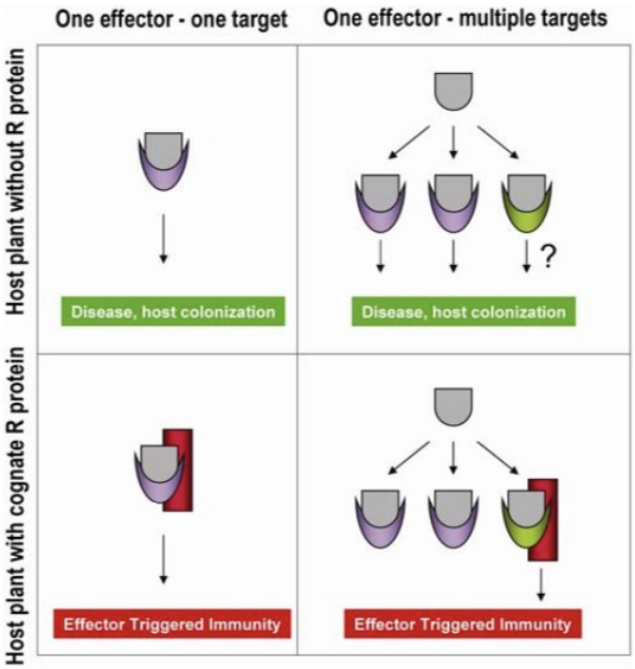

Figure 7. One effector many effector targets. The cartoons compare the traditional one pathogen effector-one host effector target model (left panels) to the emerging view that effectors frequently have more than one host target (right panels). These effector targets can be components of the plant defence response that are being inactivated by pathogen effectors, and in such cases have been

termed operative effector targets (OT). In susceptible plants, the interaction between effectors and effector targets results in molecular events that facilitate colonization, such as suppression of defence responses, enhanced disease susceptibility and elicitation of disease symptoms. In resistant plants, plant resistance R proteins recognize effector-virulence target complex, resulting in the activation of the hypersensitive response. Recognition of the effectors by R proteins is often indirect, via perception of manipulated effector target. These recognised effector targets may contribute to host defence or susceptibility (guarded effector

targets) or may not function in defence or susceptibility, thus acting as decoys that trap the effector. Effectors are depicted by grey

half circles, OT by purple crescents, guarded effector targets or decoys by green crescents, and R proteins by red squares (Hogenhout et al., 2009).

Many effectors suppress plant immunity. Suppression of plant innate immunity has emerged as the primary function of effectors, particularly of T3SS effectors of plant pathogenic bacteria (Zhou and Chai 2008). Several T3SS effectors contribute to virulence by suppressing basal defences induced by conserved pathogen epitopes named PAMPs (M. G. Kim et al. 2005). Other T3SS effectors suppress hypersensitive cell death elicited by various Avr proteins, explaining, in some cases, earlier observations of epistatic interactions among *Avr* genes (Tsiamis et al. 2000). T3SS effectors probably interfere with host immunity via a diversity of mechanisms, but the effectors studied so far are known to target three plant processes that are key to innate immunity, namely protein turnover, RNA homeostasis, and phosphorylation pathways (Block et al. 2008).

Some effectors alter plant behaviour and development. As the previous section illustrates, it is now well established that many effectors interfere with host innate immunity. Nonetheless, there are instances of effectors that have activities other than suppression of innate immunity. Some effectors alter host plant behaviour and morphology. One elegant example is coronatine, which was shown by Melotto and co-authors (2006) to trigger stomatal reopening in *Arabidopsis* and thereby facilitate bacterial entry inside the plant apoplast. *Xanthomonas* effectors of the AvrBs3 family of transcriptional activators are known to induce cellular division and enlargement in susceptible host plants (Kay et al. 2007). Expression of *Xanthomonas citri pthA* in citrus cells is sufficient to cause macroscopic hyperplastic lesions analogous to the canker symptoms caused by the pathogen (Duan et al. 1999). These canker lesions are thought to facilitate bacterial release from infected tissue and to enhance bacterial dissemination. *X. vesicatoria* AvrBs3 is also known to cause cell hypertrophy, although the impact of such a symptom on bacterial fitness is less clear (Kay et al. 2007).

Many other plant-associated organisms are known to alter the morphology of their host plant, resulting in malformations that either create a protective niche or enhance dispersal. Classic examples include rhizobial nodules (Oldroyd and Downie 2008), galls induced by *Agrobacterium* spp. and other bacteria (Chalupowicz et al. 2006), and Witches' broom and other developmental alterations caused by several pathogens such as phytoplasmas (Hogenhout et al. 2008).

In summary, some phytopathogen effectors appear to have activities other than suppression of immunity. It is reasonable to expect that natural selection would favour effectors with any type of phenotypic expression that improves pathogen fitness, and researchers in the field should keep an open mind to effector activities that do not involve the host immune response.

Molecular mimicry by effectors. Although effectors are encoded by pathogen genes, they function in a plant cellular environment and, therefore, could have been selected to mimic plant molecules. Strikingly, many effectors produce analogs and mimics of plant hormones. One example is coronatine, a toxin secreted by several pathovars of *Pseudomonas syringae* that is a structural and functional mimic of the plant hormone jasmonoyl-isoleucine (Weiler et al. 1994). Coronatine has many effects that enhance bacterial colonization of plants. These include impacting phytohormone pathways, such as jamming the induction of the salicylic acid– mediated resistance response and increasing the opening of plant stomates. Other classic examples of phytohormone mimicry in plant pathogens include auxins and cytokinins produced by various bacteria, including agrobacterium (Costacurta and Vanderleyden 1995), and gibberellins produced by several fungi such as *Gibberrella fujikuroi*, which causes the foolish seedling disease of rice (Tudzynski 1999).

Fascinating examples of plant mimics include secreted nematode proteins with similarity to expansins, components of the plant proteasome, and CLAVATA3 signalling peptides. Remarkably, the CLAVATA3- like 4G12 gene of the soybean cyst nematode *Heterodera glycines* complements the *Arabidopsis clv3-1* mutant and, similarly to CLAVATA3, negatively regulates the expression of the *Arabidopsis WUSCHEL* gene (Wang et al. 2005). How these CLAVATA3-mimicking peptides contribute to parasitism is un- known but could involve interfering with plant-cell growth and development (Mitchum et al. 2008).

Effector genes evolve at highly accelerated rates relative to the core genome. Genes that encode effector proteins are expected to be direct targets of the evolutionary forces that drive coevolution between host and pathogen (McCann and Guttman 2008). Effector alleles that increase the reproductive success of the pathogen will be immediately favoured by natural selection and positively selected. Indeed, many effector genes have evolved at accelerated rates compared with the pathogen core genome and often display extreme levels of positive selection with significantly higher rates of nonsynonymous to synonymous nucleotide substitutions (Win et al. 2007).

Besides acting on nucleotide polymorphisms, natural selection is known to act on copy number polymorphisms of effector genes (presence or absence polymorphisms and variation in gene copy number). Effector genes of filamentous pathogens often localize in loci with high genome plasticity including transposon-rich and telomeric regions (Orbach et al. 2000). The *P. infestans Avr3b- Avr10-Avr11* locus exhibits remarkable copy number variation resulting in amplification of up to 25 truncated copies of the candidate *Avr* gene *pi3.4* (Jiang et al. 2006). The association of effector genes with plastic genomic loci could confer a mechanism of adaptation to host resistance, perhaps by increasing genetic and epigenetic variation and enabling accelerated evolution.

Some effector targets evolved to evade manipulation by effectors. Since it is becoming evident that effectors enhance disease susceptibility, it can be expected that host target alleles would evolve to elude those effectors. The recessive rice mutations in *xa13* render the promoter of this gene insensitive to transcription-activating effectors of *Xanthomonas oryzae* pv. *oryzae*, thus resulting in resistance to bacterial blight disease (Yang et al. 2006). Another recessive rice blast resistance gene, *xa5*, is caused by mutations in transcription factor IIA, which presumably prevents actions by the cognate effector (Iyer-Pascuzzi and McCouch 2007). Furthermore, mutations in elongation factor elF4E are known to evade interactions with potyvirus effector VPg (Charron et al. 2008). More recently, an allele of the tomato cysteine protease Rcr3 was identified to carry a mutation that renders the protein insensitive to inhibition by *C. fulvum* Avr2 (Shabab et al. 2008).

1.2.4 Salivary glands

N. viridula adults have two salivary glands with two major lobes, the principal salivary gland (PSG) and the accessary salivary gland (ASG), along with a salivary duct connected at the junction of the PSG and ASG (Fig.8). The salivary glands were flanked by and attached to the first section of the midgut.

In general, there are two types of saliva produced by most phytophagous Hemiptera. First, the watery saliva is involved with digestion of plant food and contains digestive enzymes among other protein components. Watery saliva is thought to be produced by the accessory salivary glands. Second,

‘‘gel’’ saliva is the basis for the formation of the salivary sheath. The salivary sheath forms a hardened lining around the feeding stylets and the plant tissues. The sheath is necessary to prevent loss of plant juices during feeding by allowing the insect to form a seal around the stylets and the plant tissue. The sheath saliva is released through the salivary canal and rapidly hardens once it is secreted. The sheath adheres to plant surfaces but not to the surface of the stylets. When the insect is finished with a feeding bout, the sheath remains in the plant tissues when the insect withdraws its feeding stylets. A new sheath is formed during each successive feeding bout. Although the salivary sheaths of piercing-sucking insects have been studied for over 60 years, there is still limited progress on the identification of the salivary components responsible for the formation and hardening or gelling of the sheath. The salivary sheath is believed to be a product of the principal salivary glands, whereas the watery saliva is a product of the accessory salivary glands (Peiffer and Felton 2014).

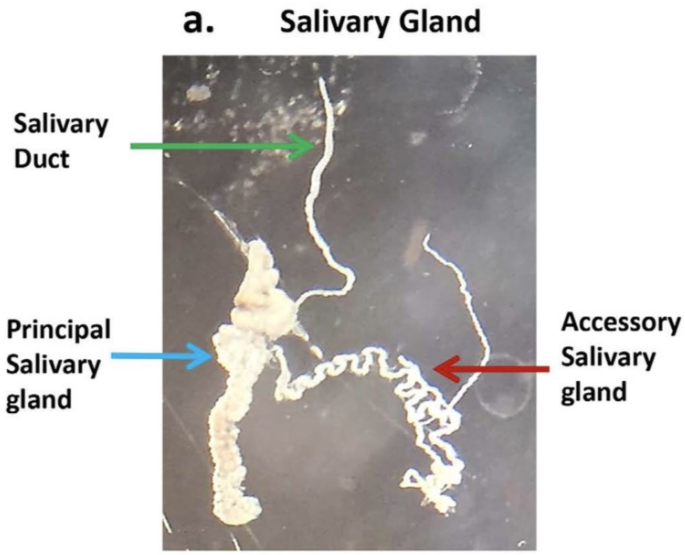

Figure 8. Structure of the salivary gland of *Nezara viridula*. Salivary duct, principal and accessory salivary glands are indicated (Lomate and Boning 2016).

Proteomic analysis to identify proteases and nucleases from *N. viridula* resulted in identifcation of 631 proteins from the salivary gland. Nucleases (DNase, RNase, dsRNase) are abundant in the saliva and salivary gland. Approximately 26% of the salivary gland enzymes were involved in energy metabolism whereas 23% of the enzymes function in protein metabolism. Significant proportions of the enzymes identified function in detoxification and antioxidation (8% salivary gland), and in carbohydrate metabolism (15%). Proteases comprised 4.25% of enzymes from the salivary gland (Figure 9). The higher number of proteases and nucleases detected in the salivary gland supports the higher activity levels for some protease and nuclease groups (e.g. serine proteases and dsRNases) in salivary gland extracts. Eight proteases and one nuclease were identified from salivary gland. Aminopeptidases and metalloproteases with cathepsin D, cysteine protease and a nuclease were also present. Sequence alignment showed that although salivary gland proteases belong to similar mechanistic classes, their sequences difer (Lomate. and Bonning 2016).

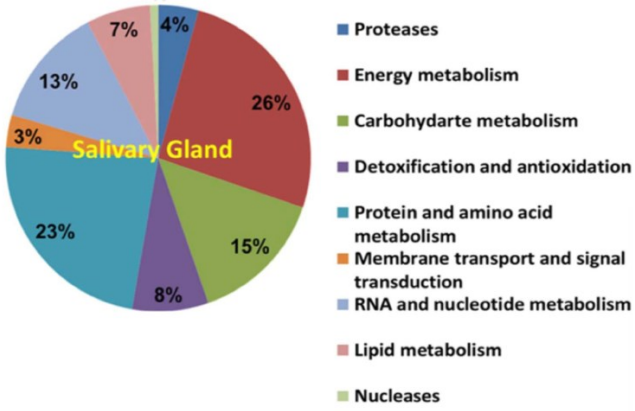

Figure 9. Functional annotation of enzymes identifed from the *Nezara viridula* salivary gland proteome (Lomate and Bonning 2016). The abundance of proteases and nucleases in the salivary gland of *N. viridula* is similar to enzyme abundance in other insect species, and the divergence in sequences between salivary proteases supports the presence of enzymes with distinct properties in these two tissues.

The high level of dsRNase activity in saliva compared to the very low level of activity detected in the salivary gland suggests that dsRNases are produced as zymogens that are activated on release into the saliva. This in turn suggests that the presence of active dsRNases in the salivary gland may be harmful to the stink bug. In addition to dsRNase activity, high levels of specifc DNase and RNase activity were detected in the salivary gland and saliva of *N. viridula*.
Nucleases in saliva directly or indirectly protect insects from infection by viruses present in the host plant, be they insect pathogens, or insect vectored viruses of plants. Nucleases therefore are likely to provide defense against pathogens, in addition to mediating digestion of host plant-derived nucleic acids (Lomate and Bonning 2016).

1.3 Importance of pest control

Nezara viridula is a highly polyphagous species, attacking both monocot and dicots. Oho and Kiritani (1960) listed as many as 145 plant species belonging to 32 families as its host plants. Todd (1989) describes the *N. viridula* host range as "over 30 families of dicotyledonous plants and a number of monocots". In North America, 43 families with a total of 197 plant taxa (i.e., 158 identified to species and 39 identified to genera) have been associated with *N. viridula*. Taking into consideration that the species can breed, or at least feed, during some stages of its development on numerous cultivated and wild subspecies, cultivars or forms, it seems impossible to compile a comprehensive list of its host plants. However, it always is mentioned that the species demonstrates a strong preference for leguminous plants (Panizzi et al. 2000).

It is often mentioned that during the vegetative stages of host plants throughout the season, different generations of *Nezara viridula* breed upon or utilize different plant species (Singh 1973). Most of the damage comes from the feeding of nymphs on developing pods, seeds, or fruits and results in significant reductions in yield, quality, and germination of seeds.

In Japan, *Nezara viridula* is a pest of rice, soybean, fruits, and many other agricultural crops. For

some of them (such as rice, soybean, and tomatoes), it is a major pest, for others, it is minor, one of a large complex of heteropteran pests. This variable pest status is the reason why statistics estimating damage to agriculture caused by this pest are lacking. Also, damage to rice caused by stink bugs is mostly not the yield loss but loss of grain quality (i.e., pecky grains) as a result of feeding by the bugs. If pecky grains are present at a rate over 1/1000 grains, then the quality grade of rice goes down to second class with a substantial reduction in price.

Damage caused by *Nezara viridula* to cultivated crops and ornamentals is documented in the native Mediterranean range in Europe, although damage levels usually are considerably lower than in subtropical and tropical regions. Soybeans are attacked in central Italy, with mean seed weight loss (18%), altered seed composition (reduced oil content), and reduced germination rate (up to 95% in susceptible strains) as the most important effects (Colazza et al. 1986). Damage has also been reported in France (Le Page 1996). In field experiments with castor (*Ricinus communis* L.) in France and Italy, reduced seed yield was observed (Conti et al. 1997).

Reports of damage in the introduced European range are scarce and often anecdotal. Simov et al. (2012) mention preliminary evidence that, in 2007–2008, damage (chlorosis) was observed in Bulgaria on tomato fruits, and Grozea et al. (2012) observed damage to tomatoes in Romania. Salisbury et al. (2009) listed 26 observed host plants in the United Kingdom, with runner bean (*Phaseolus coccineus* L.) as the most important. Most records were from late summer and autumn when harvesting was more or less completed, so there was little if any damage. They concluded that, for the time being, *Nezara viridula* is not a major pest in the country, but it could become problematic if populations increase and start feeding earlier in the season. This statement can be extended to the European nonnative range.

In field crops of the southern United States, stink bugs are considered primary pests requiring monitoring and control tactics annually. Nationwide crop losses plus management costs attributed to stink bugs in cotton exceeded $106 million (M) in 2005, but have moderated to $82.5 M in 2013 and $67.9 M in 2014 (Williams 2006, 2014, 2015). In corn and soybean, stink bugs are considered major pests, with *Nezara viridula* leading in importance, particularly in the southern areas of the region. When Bt soybeans are commercialized in North America, stink bugs will become the most prominent insect pests of the crop requiring foliar insecticide control in the crop, nearly identical to the situation with cotton in the southeastern United States. Stink bugs are considered major pests of corn, but there are limitations to the effectiveness of the insecticide approach for the pest group because of crop structure interfering with effective delivery of foliar-applied active ingredients and a lack of any labelled organophosphate insecticides.

In addition to physical damage caused by stink bug feeding, these pests also transmit common plant pathogens that further decimate cash crop value. Turner (1918) discovered that when he caged *Nezara viridula* on pecan nuts, it resulted in kernel spot and black pit; both conditions are reflections of reduced nut quality. More recent work shows that pathogens causing boll rot in cotton are physically present on stink bug mouthparts, and the pathogens can be transmitted when feeding. For example, feeding on developing cotton bolls can result in the loss of individual locks or the entire boll (Medrano et al. 2009a,b). Young bolls (1–2 weeks postanthesis) inoculated with these pathogens will be consumed completely by the pathogen, whereas older bolls (≈3 weeks postanthesis) likely

will show only a localized infection. *N. viridula* can acquire and transmit at least two boll rot pathogen species that have been isolated from diseased cotton bolls in the Southeast. These pathogens include the bacterium *Pantoea agglomerans* and the fungal pathogen *Nematospora coryli* (Medrano et al. 2009a). Quick field assays to detect boll rot pathogens in bolls or stink bugs currently are not available.

1.3.1 Biological control

There are many known natural enemies of *Nezara viridula*. Parasitoids are the most important. Jones (1988) listed 57 species among two families of Diptera and five families of Hymenoptera, with 41 of these species parasitizing the egg stage. Only a few species appeared to be well-adapted or of significant importance. Although no hyperparasitoids were reported, Clarke and Seymour (1992) subsequently reported two species of *Acroclisoides* (Hymenoptera: Pteromalidae) attacking *N. viridula* eggs previously attacked by *Trissolcus basalis* (Wollaston) (Hymenoptera: Platygastridae) in Australia. Recent studies showed that *Ooencyrtus telenomicida* Vassiliev (Hymenoptera: Encyrtidae) is a facultative hyperparasitod of *T. basalis* attacking *N. viridula* in Europe (Cusumano et al. 2013). Waterhouse (1998) presented another comprehensive list of parasitoids, adding a few more species found attacking *N. viridula*.

Nezara viridula has been the target of many classical biological control programs around the world. Parasitoids have been imported and released against this pest nearly everywhere it is found. Targeted areas include New Zealand (Cumber 1951), Australia (Coombs and Sands 2000), southern Africa (Farinelli et al. 1994), the continental United States (Jones et al. 1995), Taiwan (Su and Tseng 1984), Brazil (Kobayashi and Cosenza 1987), and several Pacific islands (Rao et al. 1971). The egg parasitoid *Trissolcus basalis* has been the most often imported species to control *N. viridula* followed by the New World tachinids, *Trichopoda* spp. (Farinelli et al. 1994, Jones et al. 1995, van den Berg and Greenland 1996).

The egg parasitoid *Telenomus turesis* Walker [as *Telenomus chloropus* (Thomson)] from Japan was released in Mississippi and Louisiana in the United States during 1981–1982 (Jones et al. 1983). However, it did not become established, apparently due to its inability to successfully emerge at lower humidities (Orr et al. 1985). Three species of Japanese egg parasitoids were released in Brazil, but there have been no reports of their establishment (Kobayashi and Cosenza 1987). One of the Japanese species released, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Platygastridae), has been less able to successfully parasitize and emerge from *N. viridula* eggs than the well-established *Trissolcus basalis*. *T. basalis* was absent from the Asian range of *N. viridula* until it was released in Taiwan in 1983 (Su and Tseng 1984). It recently was discovered in Central Honshu and Kyushu, Japan (Mita et al. 2015).

Trichopoda pennipes L. (Diptera: Tachinidae), a native of the New World, was discovered attacking *Nezara viridula* in Italy in 1988 (Colazza et al. 1996) and has since spread through much of the western Palearctic (Tschorsnig et al. 2012) following the establishment and spread of its primary

host (Rabitsch 2010).

1.3.2 Attract-and-kill strategies

It is well established that stink bugs prefer to colonize seed- and fruit-producing hosts. The attract-and-kill strategy involves taking advantage of this behaviour by recruiting *Nezara viridula* through use of a highly attractive crop (or lure) and then, subsequently, controlling the pests through site-specific insecticide applications and/or beneficial insects. The attractive crop (often termed a trap crop) typically is planted early on a small amount of land for the sole purpose of luring the pest away from the principle cash crop. Trap crops for *N. viridula* are well studied in many agronomic production systems including, but not limited to, cotton (Tillman 2006, Tillman et al. 2015), pecan (Smith et al. 1996), sweet corn (Rea et al. 2002), and soybean (Smith et al. 2009). Results are variable, but this practice generally is successful at reducing stink bug populations as long as the bugs are eliminated before the trap crop matures or the nymphs complete development.

In addition to recruiting populations of pest insects, trap crops can aid in recruitment of beneficial insects and lead to reduced number of insecticide applications to the main commercial crop (Tillman 2006, Tillman and Carpenter 2014). Also, trap crops such as sorghum can act as a physical barrier in the landscape for pest insects moving into commercial cotton production (Tillman 2014).

1.3.3 Chemical control

Chemical control of insects in agriculture involves the use of synthetic or naturally derived insecticides to reduce populations before they cause economic damage equal to insect management costs. Chemical control is the most successful and widely used control strategy for stink bugs including *Nezara viridula*. Unfortunately, there are currently no selective insecticide chemistries for managing stink bugs, so growers rely on broad spectrum materials such as organophosphates (IRAC [Insecticide Resistance Action Committee] group 1B), pyrethroids (IRAC group 3A), and neonicotinoids (IRAC group 4A). Numerous studies have evaluated the efficacy of various chemical control options for stink bugs in agricultural settings (e.g., Willrich et al. 2004a,b), and some research trials have provided information regarding insecticide performance specific to *N. viridula* (Tillman 2006). Generally speaking, *N. viridula* is more susceptible than *Euschistus* spp. to pyrethroid insecticides including cyfluthrin, cypermethrin, and lamda-cyhalothrin (Willrich et al. 2003).

1.4. Pesticide resistance and mechanisms

1.4.1 Resistance mechanisms

Resistance is the inherited characteristic of an organism that lessens the effect of an adverse environmental factor, such as pathogen or parasite, a biocide (e.g. herbicide, insecticide, antibiotic). Insects, particularly, are able to develop resistance against toxic agents, like insecticides. Public health and agriculture are being severely affected by the insecticide resistance problem. Insect pests cause chronic and often severe crop loss and, when insecticides fail, there are serious economic losses and consequences for food security. Integrated pest management (IPM) reduces reliance on insecticides by drawing on a much wider range of control measures, including biological control, cultural practices, host plant resistance, semiochemicals, surveillance, and monitoring. Consequently, IPM reduces selection for insecticide resistance and serves as one of the main strategies for resistance management (although it is noteworthy that insecticide resistance is not the only driver for the development and uptake of IPM in agriculture) [Kogan, 1998]). The evolutionary forces and mechanisms of resistance both in agricultural pests and in vector human diseases are often the same. Thus, we might expect there to be a common ground and perhaps common solutions for insecticide resistance in public health and agriculture.

In general, according to IRAC four different mechanisms of resistance against insecticides can be employed: a) metabolic resistance, b) behavioural resistance, c) penetration resistance and d) target site resistance.

1.4.1.1 Metabolic resistance

A major mechanism of resistance against insecticides is the increased metabolic - detoxifying activity performed by specific enzymes. The enzymes involved are normally esterases, cytochrome P450s (CYPs), and glutathione S-transferase (GSTs) of many types, varying with species and strains and difficult to predict in their sites of metabolic attack and contribution to resistance (Casida, 2017).

The two main mechanisms by which those enzymes mediate resistance in the insects are: a) gene overexpression for their overproduction in order to sequester the toxic agents they are exposed to, b) single aminoacid alteration which increases the affinity of the detoxifying enzyme with its substrate.

1.4.1.2 Behavioural resistance

Behavioural avoidance is defined as the ability of an insect to escape from an insecticide-treated area, often without lethal consequences. This response can be further divided into direct contact excitation (sometimes referred to as 'irritancy') and non-contact spatial repellency (Feyereisen 1999). The term 'contact irritancy' involves an insect leaving an insecticide treated area

only after making physical (tarsal) contact with the chemical, whereas 'spatial repellency' is when insects move away from the insecticide-treated area without making direct contact (Furlong et al., 1989). Lastly, some chemicals, such as DEET, can elicit a fourth action by effectively masking/jamming the presence of a host through the inhibition of odor-activated receptors (Ogita 1989).

1.4.1.3 Penetration resistance

Tarsal contact of the insects and cuticle penetration are required for the insecticide to reach its target. However, in some cases like in *A. gambiae* (Balabanidou et. Al., 2016) insects develop a physicochemical alteration of the cuticle, making difficult for the insecticide to reach its target. Tis mechanism of resistance, also known as penetration resistance, protects the insects from a wide range of insecticides, specifically when it acts in synergism with other mechanisms such as metabolic resistance.

1.4.1.4 Target-site resistance

Every insecticide has a specific target, which could be a specific aminoacid residue of the protein target. Any modification at this specific aminoacid can potentially reduce the binding affinity of the insecticide, resulting to resistance. Target site alterations can provide high levels of resistance, but this tends to be specific to a particular chemical class of insecticides (Panini et al., 2016). According to Dang and coworkers (2017) there are four main types of target site insensitivity mechanisms in resistant insects: a) kdr (knock-down resistance) which involves specific alterations in the sodium channel sequence conferring resistance to pyrethroids and DTT, b) altered AChEs, c) rdl-mutations which are correlated with alterations in the sequence of the GABA receptor and d) altered nAChRs conferring resistance to neonicotinoids.

1.4.1.4.1 kdr

VGSCs are essential for normal transmission of nerve impulses. DDT and pyrethroids act on or bind to the VGSC proteins to disrupt the process, which is followed by knockdown, paralysis, and eventually death of the insect. Many insect pests have developed levels of resistance to DDT and pyrethroids by reducing target site sensitivity (so-called kdr).

The most important kdr mutation is the convertion of leucine to phenylalanine in the residue site 1014 (using *M. domestica* numbering), in the S6 segment of the II domain of VGSC, which has been originally found in *M.domestica*. Since then, many different species of agricultural pests and diseases vectors have been found to carry this mutation alone or in combination with other alterations in the VGSC sequence. Apart from L1014F mutations, several divergent mutations in other residue sites have been mapped and correlated with pyrethroid resistance. M918T mutation is a mutation that when it occurred alone, it did not have great effect in resistance, but when it is together with L1014F it causes the super-kdr phenotype by leading to greater reduction of sodium channel sensitivity against pyrethroids. The contribution of those mutations to resistance against pyrethroids have been

verified by heterologous expression of sodium channel cDNA in *Xenopus* oocytes in combination with electrophysiological experiments (Dong et al., 2014).

Recently, a novel mutation, I936F (isoleucine 936 to phenylalanine), was identified in the VGSC gene of one field-collected C. lectularius strain (from Adelaide in Australia) that was linked to low levels of resistance to d-allethrin (Dang et al., 2015).

Another recent study has identified four novel mutations in the VGSC genes of C. hemipterus collected from multiple countries, including Australia: L899V (leucine 899 to valine), M918I (methionine 918 to isoleucine), D953G (aspartic acid 953 to glycine) and L1014F (leucine 1014 to phenylalanine) (Dang et al., 2017). M918I and L1014F) were associated with high resistance to pyrethroids in C. hemipterus (Dang et al., 2015); however the presence of other resistance mechanisms were not excluded. These two sites are known to confer pyrethroid resistance in a wide range of insect pests.

1.4.1.4.2 ACHEs_Ace

The biochemical characterization of acetylcholinesterase (AchE) activity with decreased sensitivity to inhibition by organophosphate or carbamate insecticides is relatively straightforward, but the molecular basis of this insensitivity is made somewhat complicated because most insects have two AchE genes (*Ace* 1 and 2), whereas higher Diptera have only one (now designated as *Ace* 2).

Field strains revealed multiple alleles with many mutations, that indicate multiple origins of resistance. In the house fly, at least three origins of the Phe290Tyr; Gly227Ala/Val alleles were shown, each then complemented with the Val150Leu or Ala201Ser mutations. In *Drosophila*, the four most frequent mutations, Ile129Val, Gly227Ala, Phe290Tyr and Gly328Ala can be found independently or in various combinations. In another study, several origins of the I120V mutation were then seen complemented with the Gly227Ala and Phe290Tyr mutations (Feyereisen et al., 2015).

1.4.1.4.3 ACCase

Acetyl-coenzyme A carboxylases (ACCs) have crucial roles in the metabolism of fatty acids and they catalyze the carboxylation of acetyl-CoA to produce malonyl-CoA. In mammals, ACC1 is present in the cytosol of liver and adipose tissues and controls the committed step in the biosynthesis of long-chain fatty acids (Wakil et al., 1983). In comparison, ACC2 is associated with the outer membrane of mitochondria in the heart and muscle. Its malonyl- CoA product is a potent inhibitor of carnitine palmitoyl- transferase I, which facilitates the transport of long- chain acyl-CoAs into the mitochondria for oxidation (Ramsay et al., 2001).

Spiromesifen and spirotetramat are novel insecticides belonging to the new chemical class of tetronic and tetramic acid derivatives, also known as ketoenols, developed by Bayer CropScience. They are listed in group 23 as inhibitors of acetyl CoA carboxylase in the IRAC mode of action classification. These insecticides have a new mode of action, as they interfere to the lipid biosynthesis and act both against juvenile stages and adult fecundity. Spiromecifen supresses effectively whiteflies and B. tabaci, when applied at an appropriate stage, and shows excellent efficacy against tetranychid mites.

Spirotetramat is also effective against whiteflies, thrips and sucking pests, such as aphids, mealy bugs and armoured scales (Bielza et al., 2018).

1.4.1.4.4 Ryr

Ryanodine receptor (RyR) is a large (homo)tetrameric calcium channel located in thesarco- and endoplasmic reticulum in neuromuscular tissues (Sattelle et al., 2008). Activation of this receptor is mediated by calcium influx, driven by voltage-gated calcium channels upon depolarization of the cell membrane (Lummen, 2013). Diamide insecticides act as conformation sensitive activators of the RyR. Diamides poisoning includes muscle contraction, paralysis and eventually death (Cordova et al., 2006). Insects encode a single RyR gene with an open reading frame (ORF) of >15,000 nucleotides translated into a RyR promoter with a molecular weight of more than 5000 kDa, as first described for Drosophila melanogaster (Takeshima et al., 1994). Each receptor is composed of six helical transmembrane spanning domains at the C-terminal end containing the calcium ion-conducting pore and a large N-terminal cytosolic domain (Lummen, 2013).

Scope of the study

In this study we tried to detect novel biotechnological ways to fight the polyphagous and cosmopolitan pest *Nezara viridula,* which has the ability to attack cultivated and ornamental plants causing severe crop reduction and substantial economic loss.
At first, we tried to identify putative saliva effectors that interfere with host plants and cause plant damage, aiming to create plants that will be resistant to these effectors. We also tried to identify any mutations that could cause resistance to several insecticides in three strains of *N. viridula* (Bayer, Bean and Tomato strain) intending to point out the most effective insecticide.

2. Effectors

2.1. Materials & Methods

2.1.1. Nezara colony maintenance

Nezara viridula (Bayer strain) eggs were imported from Bayer (Crop Science department) and maintained in the laboratory according to Bayer's rearing protocol. Moreover, adults were collected from fields in Heraklion (Beans strain) and Chania (Tomato strain) and established in the laboratory. The three colonies were kept in a growth chamber under controlled conditions, at 25 °C with a 16:8 light dark cycle. Insects were provided with water, organic beans, organic carrots purchased from a grocery store as well as sunflower seeds (Figure 10). Adults typically laid eggs on the papers and nymphs were reared in the same manner.

Figure 10. *N. vididula* growing champer

2.1.2 Watery saliva collection

To collect watery saliva from *Nezara viridula*, adult insects were chilled on ice for about 4 minutes, then placed ventral side up and observed with a dissecting stereoscope. As bugs returned to room temperature, the watery saliva was secreted from the spinneret of the beak (Figure 11). This saliva was collected into a gel loading pipet tip containing 2 μl of buffer [50mM EDTA in 50mM Tris-HCl (ph:8)] or glycerol. After collection, the buffer and saliva were expelled into a 1.5 ml tube and stored at -80 °C.

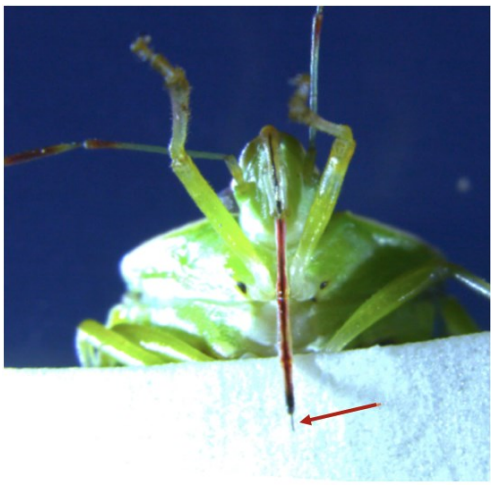

Figure 11. The spinneret of the beak of an adult N. viridula from abdominal side

2.1.3 Silver staining of proteins in polyacrylamide gel

To analyse watery saliva with gel electrophoresis, SDS sample buffer was added directly to saliva collected in glycerol. Samples were boiled for 5 min, centrifuged at 15000 rpm for 5 min and then loaded onto a 12% resolving gel and electrophoresed at 100V. Protein bands were visualized by silver staining (Beier and Gross 1987, Rabilloud 1990). For size determination a protein ladder (BlueStar Prestained Protein Marker) was also run.

2.2 Results

In order to identify the putative saliva effectors, we first had to check the morphology of the salivary glands from *Nezara viridula*. We dissected adult insects under a dissecting stereoscope and we confirmed the existence of one pair of salivary glands in the prothoracic region (Figure 12).

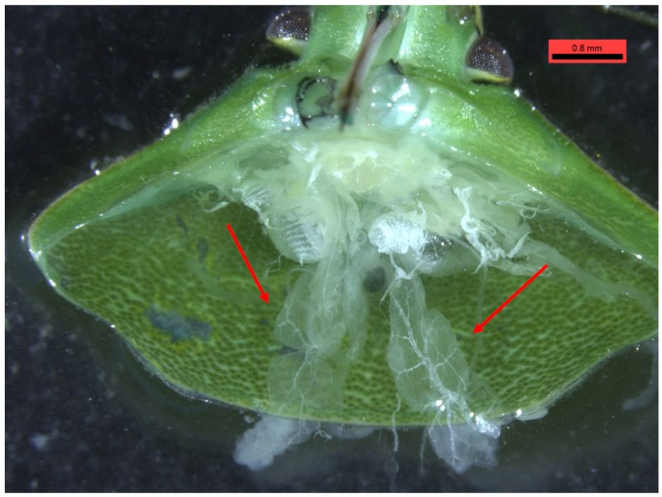

Figure 12. A pair of salivary glands from an adult *N. viridula*

To see more the details of the morphology of the glands, we further examined the glands under a scanning electron microscope (SEM) (Figure 13). Our results revealed that every gland consisted of two lobes, the principal salivary gland (PSG) and the accessary salivary gland (ASG), along with a salivary duct.

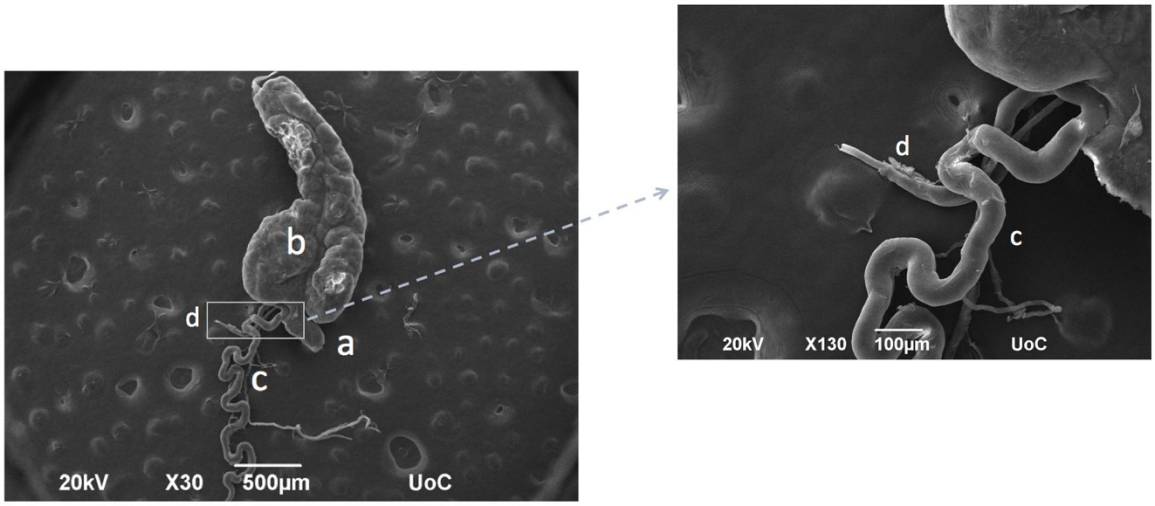

Figure 13. Structure of *N.viridula* (Bayer strain) salivary glands. (a) anterior lobe of principal gland; (b) posterior lobe of principal gland; (c) accessory gland; (d) salivary duct

Subsequently, we examined the existence of two saliva types (watery and "gel" saliva). For this reason, we provided adults insects with organic tomatoes and after 24 hours we observed the tomatoes under a stereoscope. The first thing to notice was the discoloration at the surface of the tomatoes and then the salivary sheaths, indicating that "gel" saliva is being secreted from Nezara's stylet. (Figure 14)

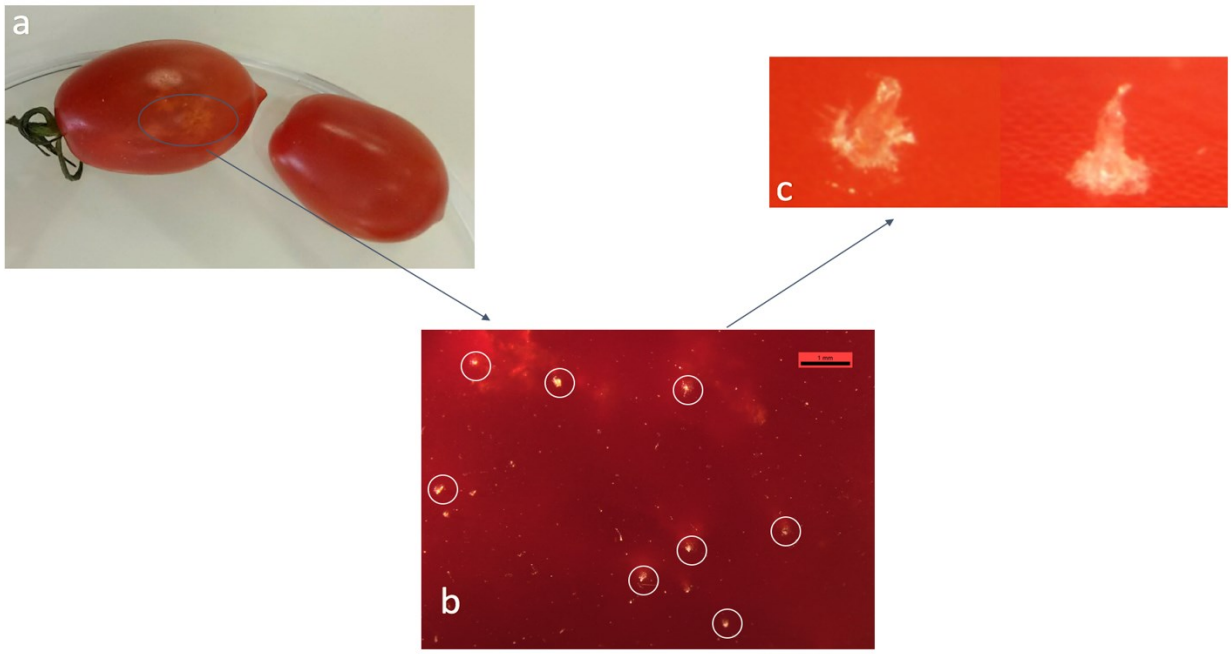

Figure 14. Salivary sheaths on grape tomatoes.

Finally, we collected only the watery saliva (see 2.1.2) from the tip of the stylet. We used adults from Bayer (N.v_B) and Beans strains (N.v._K). After gel electrophoresis and silver staining and we got the following picture (Figure 15).

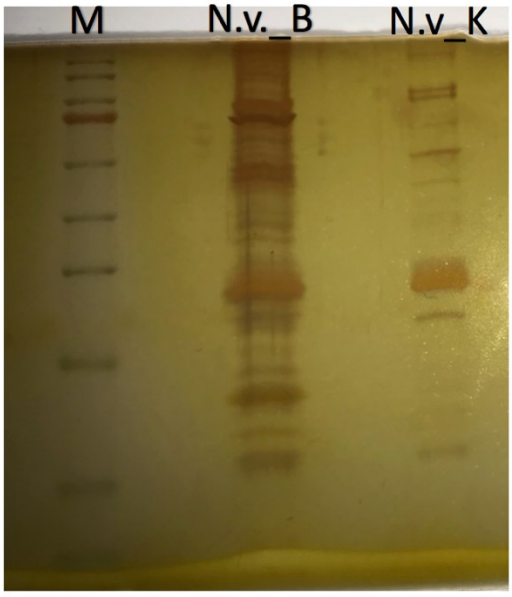

Figure 15. Polyacrylamide gel electrophoresis (PAGE) of watery saliva from Bayer strain (N.v._B) and Bean strain (N.v._K).

After confirming the existence of peptides in the samples collected, we sent 3 replicates of watery

saliva from each strain (Bayer and Bean) for proteomic analysis at the Proteomic Facility of the Gent University (Belgium).

3. Resistance

3.1 Materials & Methods

3.1.1 Nezara populations

At this point we worked with three different populations of *Nezara viridula*. We had a Bayer population (N.v._B), a population from Heraklion (Chersonisos) that infested bean plants (N.v._K), and a population from Chania (Platanias) that that was collected from tomato plants (N.v._T).

3.1.2 RNA Extraction

RNA was extracted by crushing 10-15 insects of each strain from N2 and N3 developmental stage with a plastic pestle in a 1.5 mL micro centrifuge tube, using Gene JET RNA Purification Kit according to the instructions of the manufacturer.

3.1.3 cDNA Synthesis

An aliquot of approximately 4 ng total RNA from each strain served as template for cDNA synthesis with Reverse Transcriptase (Minotech) using oligo-dT, according to the manufacturer's instructions.

Fragments containing the desired mutations were amplified using the appropriate primers (Table 2). PCR reaction (50 μL) contained 1 μL of cDNA, 0,6 μL primers, 1 μL dNTPS, 1x Buffer and 1U Kapa Taq DNA polymerase (Kapa Biosystems). Mutations of these fragments are associated with resistance to spiromecifen, pyrethroid, organophosphate and diamide insectides.

Table 2. List of primers used in this study

No.	Primer name	Primer sequence (5'->3')
1	Nv_ACC_F1	GGAGAGGATTTTCTGGAGGAAT
2	Nv_ACC_R1	GGCGTAAGGTATCTCTCCCT
3	Nv_ace_F1	CGAACCTGTCCGAGGACTGC
4	Nv_ace_R1	ACACTGGTTTCCCTGTAAGCG
5	Nv_ace_F2	TATCGCCGGTGACGAGAGGG
6	Nv_ace_R2	CGTTGCAGCCGCAGTCCTCC
7	Nv_kdr_F1	GAAGAGGATGAGGAGCCGAC
8	Nv_kdr_R1	TCTTGATCCAACCAGTGAACCG
9	Nv_RyR_F1	GATGTGAGTGGCGGAGGAAG
10	Nv_RyR_R1	ACCAATACCACCACCAGCTC

3.1.4 Sequencing Analysis

PCR products were purified using NucleoSpin Extract II (Macherey Nagel) and directly sequenced with the original PCR primers (Table 2). Sequencing reactions were performed at CeMIA sequencing facility (Larisa, Greece).

3.2 Results

After RNA extraction, cDNA synthesis and PCR with the appropriate set of primers for the kdr mutations, we got the expected bands (535 bp) (Figure 16) from the three insects strains that we used and subsequently we moved on to sequence analysis of these fragments.

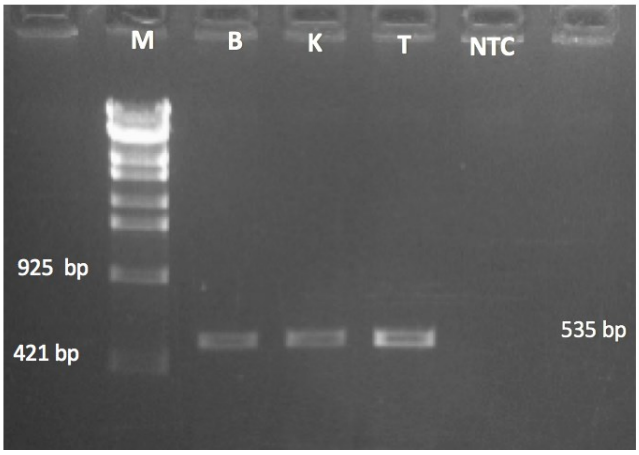

Figure 16. PCR with kdr primers yielding 535bp product running in 1,5% agarose gel. M indicates the ladder, B indicates the Bayer strain, K indicates the Bean strain, T indicates the tomato strain, NTC is the blank in which distilled water replaces the cDNA template.

Sequence analysis revealed that these strains did not develop any mutations associated with the voltage-gated sodium channels. (Figure 17)

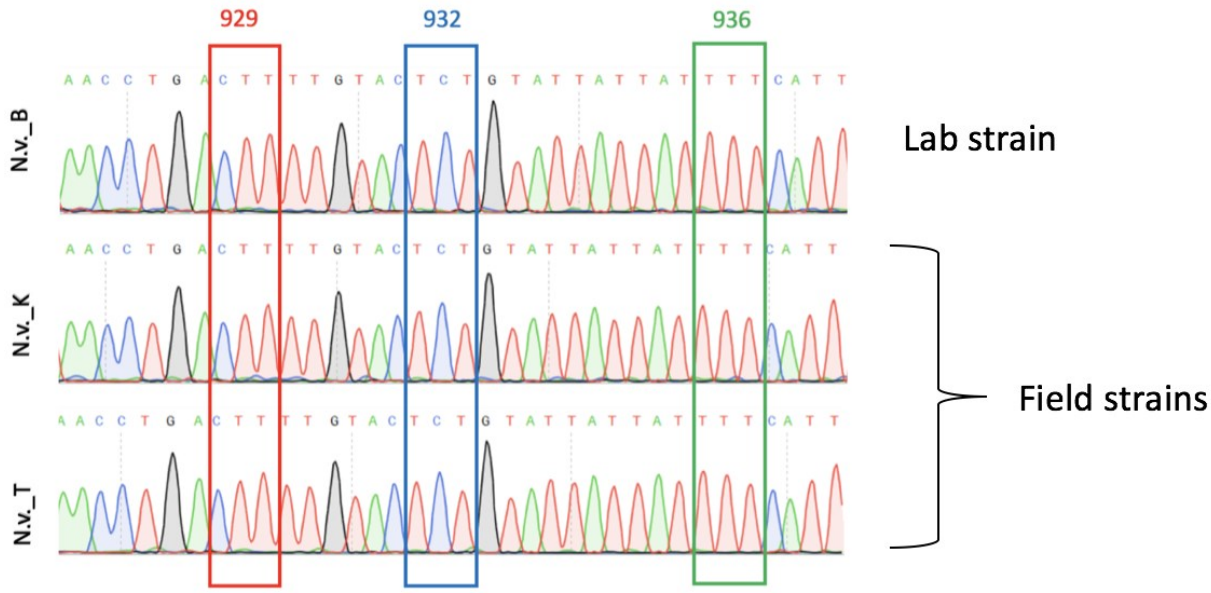

Figure 17. Chromatographs of the sequencing results of the three strains. Positions of expected target site *kdr* mutations 929, 932 and 936 are depicted in red, blue and green boxes respectively.

After performing PCR for the gene of acetylcholinesterase (ace), the acetyl-coenzyme A carboxylases (ACCase) and the ryanodine receptor (RyR) we got the following bands (Figure 18), which were then sent for sequence analysis.

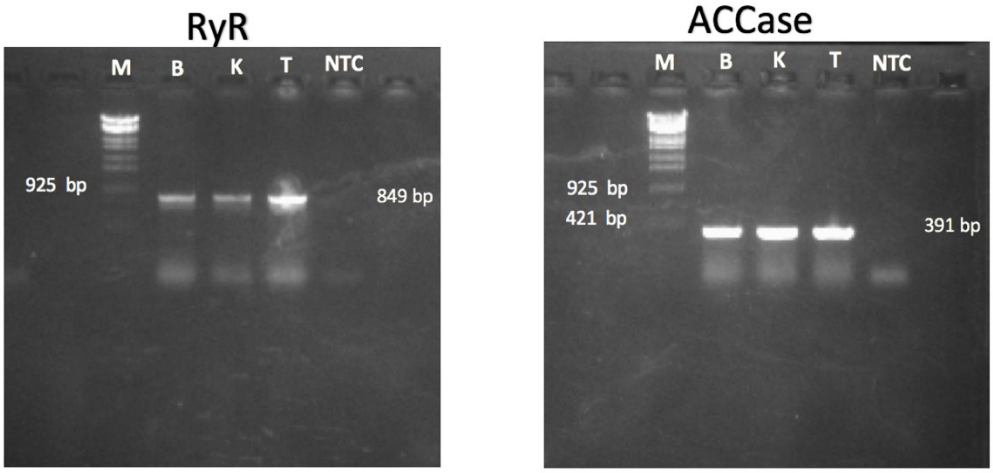

Figure 18. PCR with RyR and ACCase primers yielding 849bp and 391bp products, running in 1,5% agarose gel. M indicates the ladder, B indicates the Bayer strain, K indicates the Bean strain, T indicates the tomato strain, NTC is the blank in which distilled water replaces the cDNA template.

According to the sequence analysis the three strains did not bear any mutations in the genes that we checked. (Figure 19,20, 21)

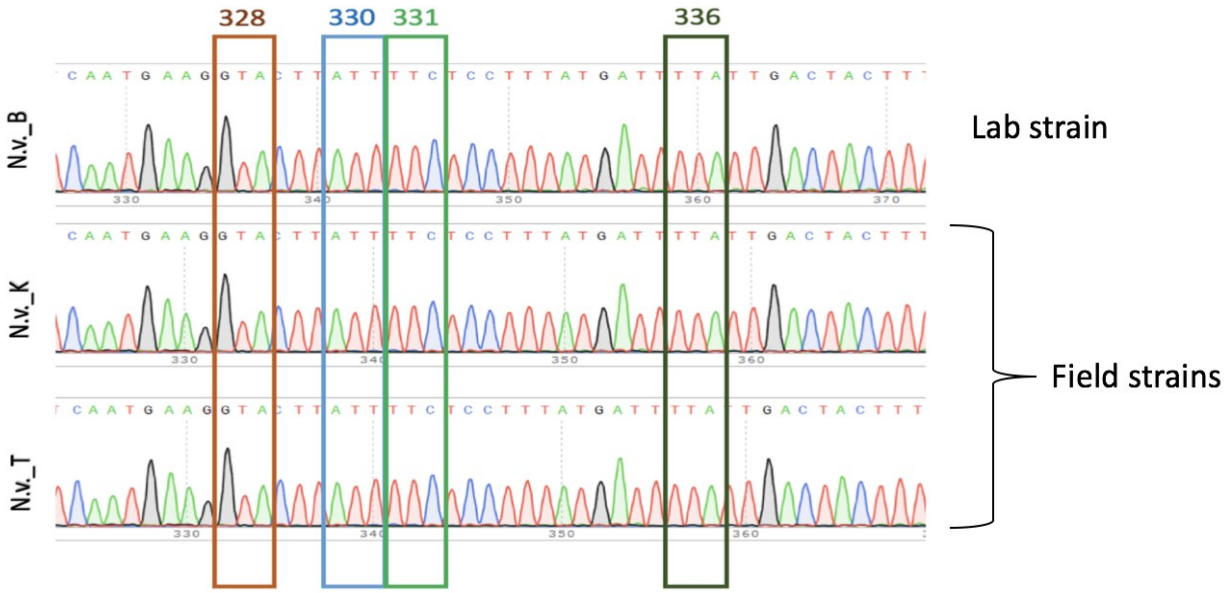

Figure 19. Chromatographs of the sequencing results of the three strains. The boxes indicate the positions of the mutations 328, 330, 331 and 336 at the *acetylcholinesterase* (*ace*) gene.

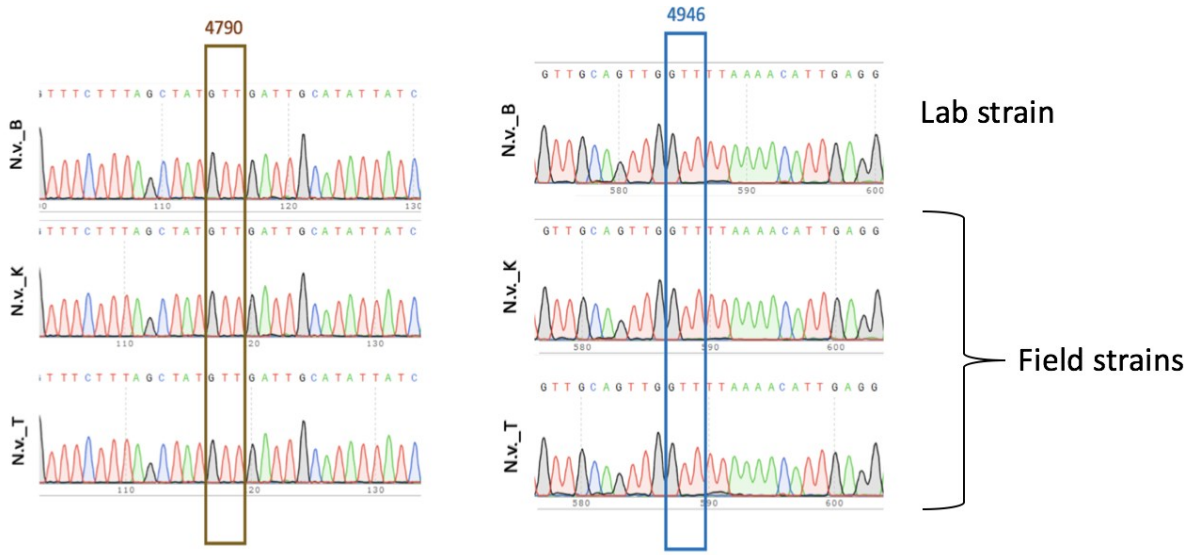

Figure 20. Chromatographs of the sequencing results of the three strains. The boxes indicate the positions of the expected mutations 4790 and 4946 of the *ryanodine receptor* (*RyR*) gene.

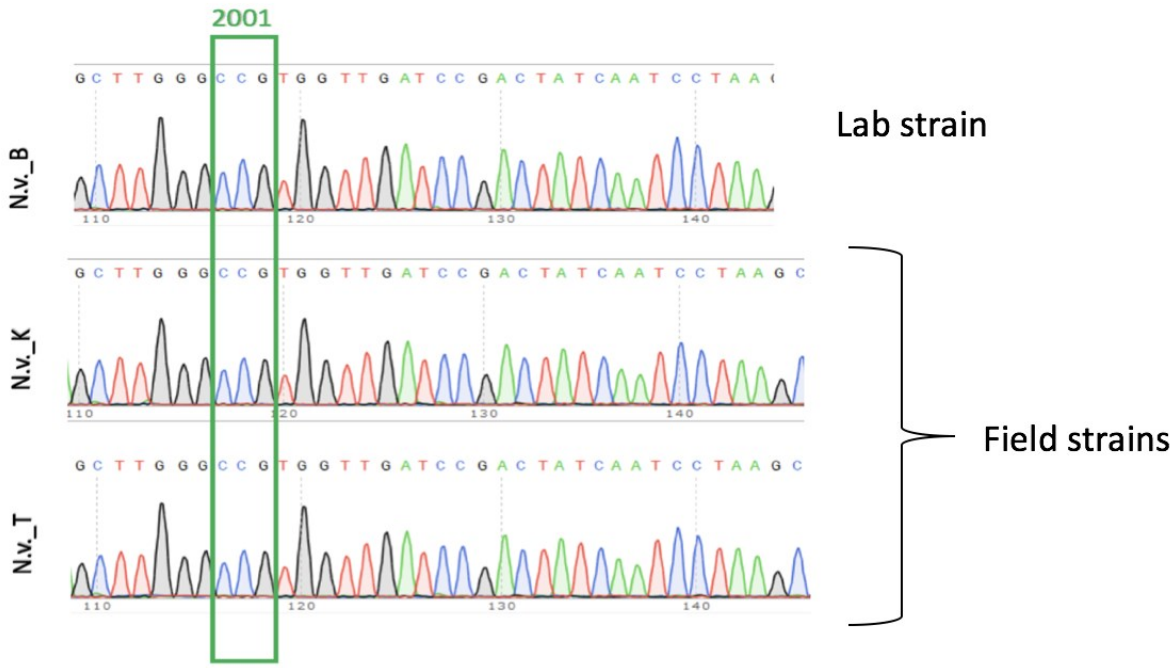

Figure 21. Chromatographs of the sequencing results of the three strains. The box indicates the position of the mutation 2001 at the gene of the *acetyl-coenzyme A carboxylase* (*ACCase*).

4. General discussion

Insecticide resistance is agreed to be an evolutionary phenomenon and the best proof of the effectiveness of natural selection yet obtained. The traditional classification of resistance distinguishes toxicodynamic and toxicokinetic changes in the physiology and biochemistry of resistant strains, and often also includes behavioural changes. It is possible to describe resistance in two dimensions, a molecular genetic dimension, and a biochemical/physiological dimension (the classical way). In the molecular dimension, alterations of the target site are toxicodynamic changes that would be better dealt with by a pesticide with a different mode of action, thus designing resistance management strategies.

Different classes of mutations affecting a gene implicated in resistance can be distinguished, and fall broadly in three categories. The first are mutations affecting the coding sequence of the gene and thereby structurally alter the gene product. The second are mutations causing an increase in gene dosage or expression. The third are mutations causing a decrease in gene dosage or expression. The latter two classes can be further distinguished in mutations affecting the whole gene (such as duplication and amplification, or disruption and loss) or just the *trans* regulation or the *cis* regulatory elements of the gene (Feyereisen et al., 2015). At this study we focused on mutations that belong in the first category (i.e. affect the coding sequence of the gene). We

checked for eventual mutations in the coding sequence of four genes that are directly associated with high levels of insensitivity, the the voltage sensitive sodium channel (VGSC) gene, the acetylcholinesterase gene, the gene of the acetyl-coenzyme A carboxylase and the ryanodine receptor gene.

The voltage-gated sodium channel is the target of DDT and pyrethroid insecticides. Sixty-one different mutation positions or combinations of up to five individual mutations have been reported in 51 different species. Two mutations form the "core" of the VGSC structural changes, the kdr mutation at Leu1014 and the super-kdr mutation at Met918, with 81 reported cases of one or both mutations (Dong et al., 2014). Organophosphate or carbamate insecticides are correlated with the acetylcholinesterase gene, but the molecular basis of this insensitivity is made somewhat complicated because most insects have two AchE genes (*Ace* 1 and 2), whereas higher Diptera have only one (Feyereisen et al., 2015). In *Nezara viridula* we found one acetylcholinesterase gene. The the acetyl-coenzyme A carboxylase is being inhibited by spiromecifen and spirotetramat insecticides in *Bemisia tabaci* and *Tetranichus urticae* (Bielza et al., 2018). The ryanodine receptors genes are activated by diamide insecticides (widely used in lepidopteran pests) but mutations G4966E/V and I4790M are directly correlated with resistant phenotypes (Douris et al., 2017).

Sequence of the abovementioned genes in three strains of *Nezara viridula* (a Bayer strain and two field strains from Heraklion and Chania) revealed that there are no mutations that affect the coding sequence of these genes, thus indicating that there is no sign of insensitivity for the moment being, in contrast to other major pests. However, insecticide resistance can easily be developed thus occasional monitoring is required.

In this study we also focused on another promising way of managing insect pests, which is the identification of salivary effectors that could help us deal with insects, especially polyphagous, that cause considerable economic loss to several crops. Three biological replicates (100 ul each) of the Bayer and the Heraklion strain were collected and sent for proteomic analysis in Belgium, and we eagerly wait for the results. Identification of novel insect effectors will ultimately help us to engineer crops that can suppress the expression of these effectors, thereby providing novel approaches to control insect pests of agricultural crops.

Louis and co-workers studied the saliva components of the European corn borer (ECB) that either induce or suppress defences in Solanum lycopersicum (tomato) and Zea mays (maize). Their results demonstrate that the components present in the ECB saliva induce defence-related proteinase inhibitors in both tomato (*PIN2*) and maize (*MPI*). Presence of glucose oxidase in the ECB saliva induced defences in tomato, but not in maize. According to van Bel and Will, who worked with aphids, salivary polyphenoloxidases, peroxidases and oxidoreductases were suggested to detoxify, e.g., plant phenols and act in the suppression (*C002* or *MIF* cytokine) or the induction (e.g., *Mp10* or *Mp42*) of plant defense. Moreover, they identified the protein GroEL that originates from *Buchnera aphidicola*, the obligate symbiont of aphids and probably reflects an excretory product that induces plant defense responses.

Investigations in the Asian rice gall midge (RGM, *Orseolia orhyzae*) in the rice (*Oriza sativa*) indicated that the gall-inducing substances include effector proteins and resulted in mapping 11 RGM R genes (*Gm1–Gm11*) (Stuart 2015). Modern approaches focused on rice and Brown

planthoper (BHP, *Nilaparvata lugens*) interactions revealed at least 20 BHP *R* genes on rice chromosome, showing that *Bph14* is an NB-LRR protein. The *Hessian* fly (HF, *Mayetiola destructor*) is a gall-forming parasite of wheat and genetic analysis identified at least 35 different HF *R* genes, named *H1* – *H34* and *Hdic* (Hao et al., 2013).

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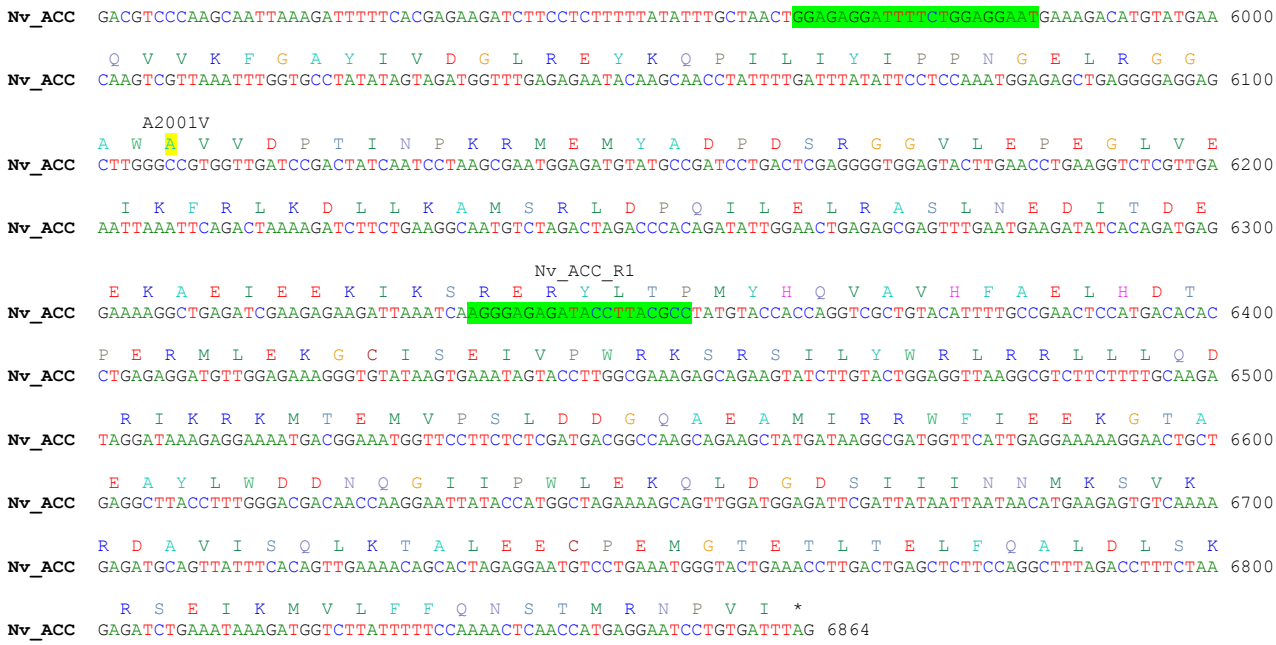
Supplementary

Design of ACCase primers



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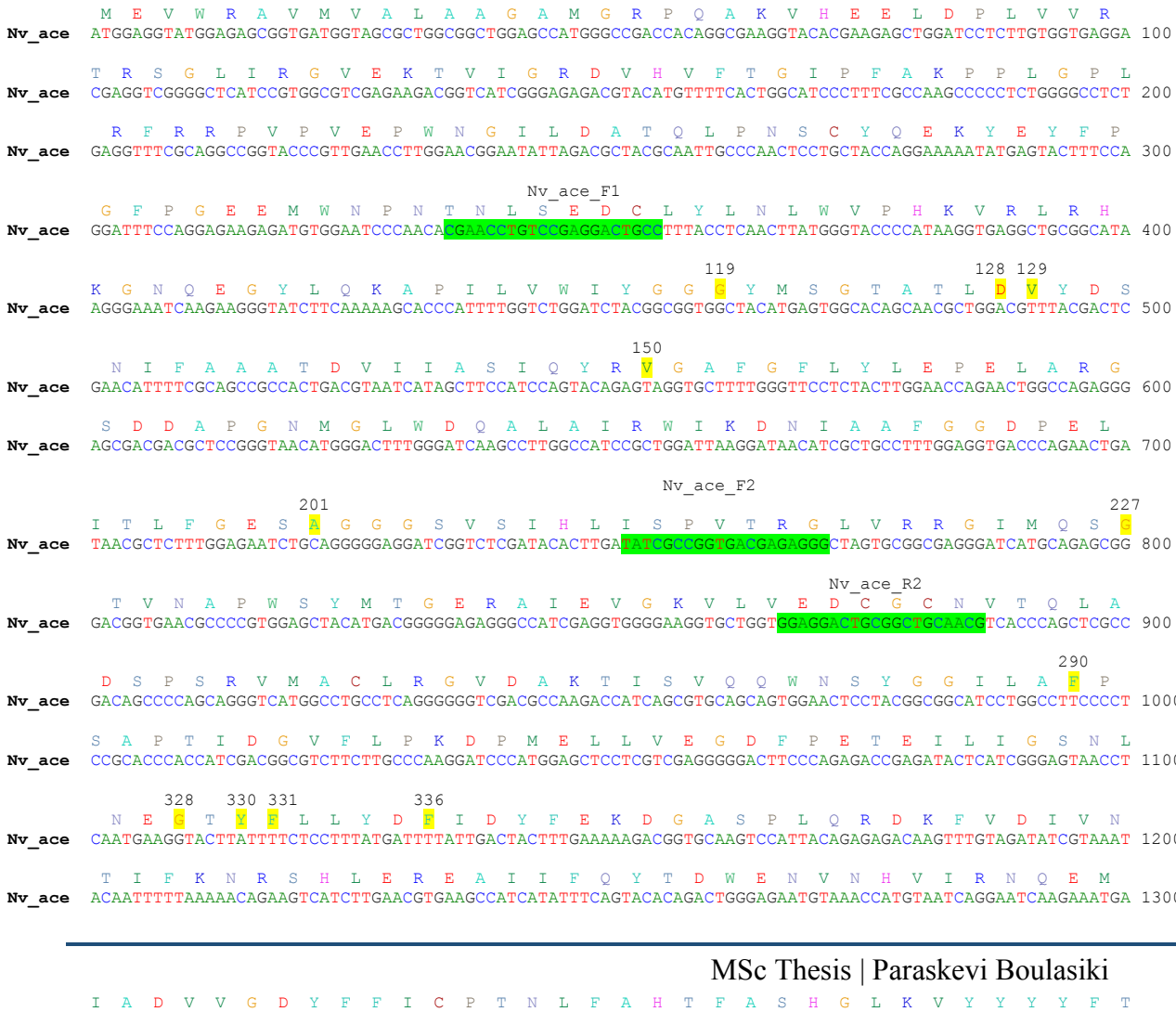

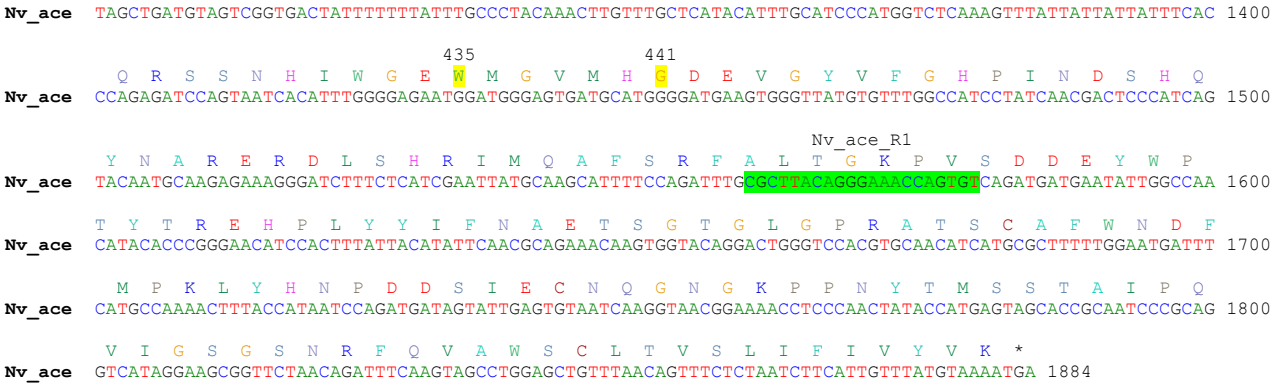

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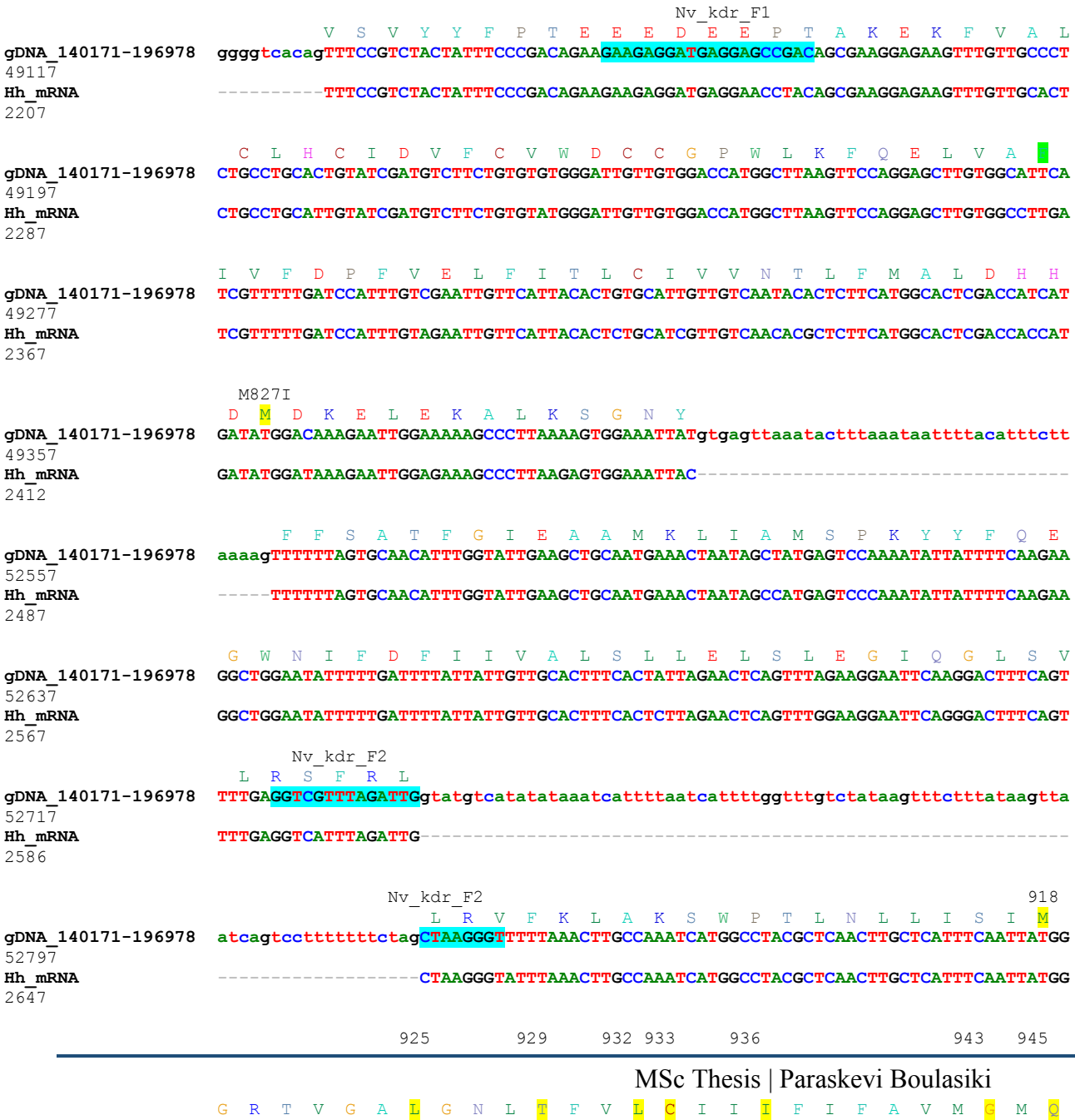

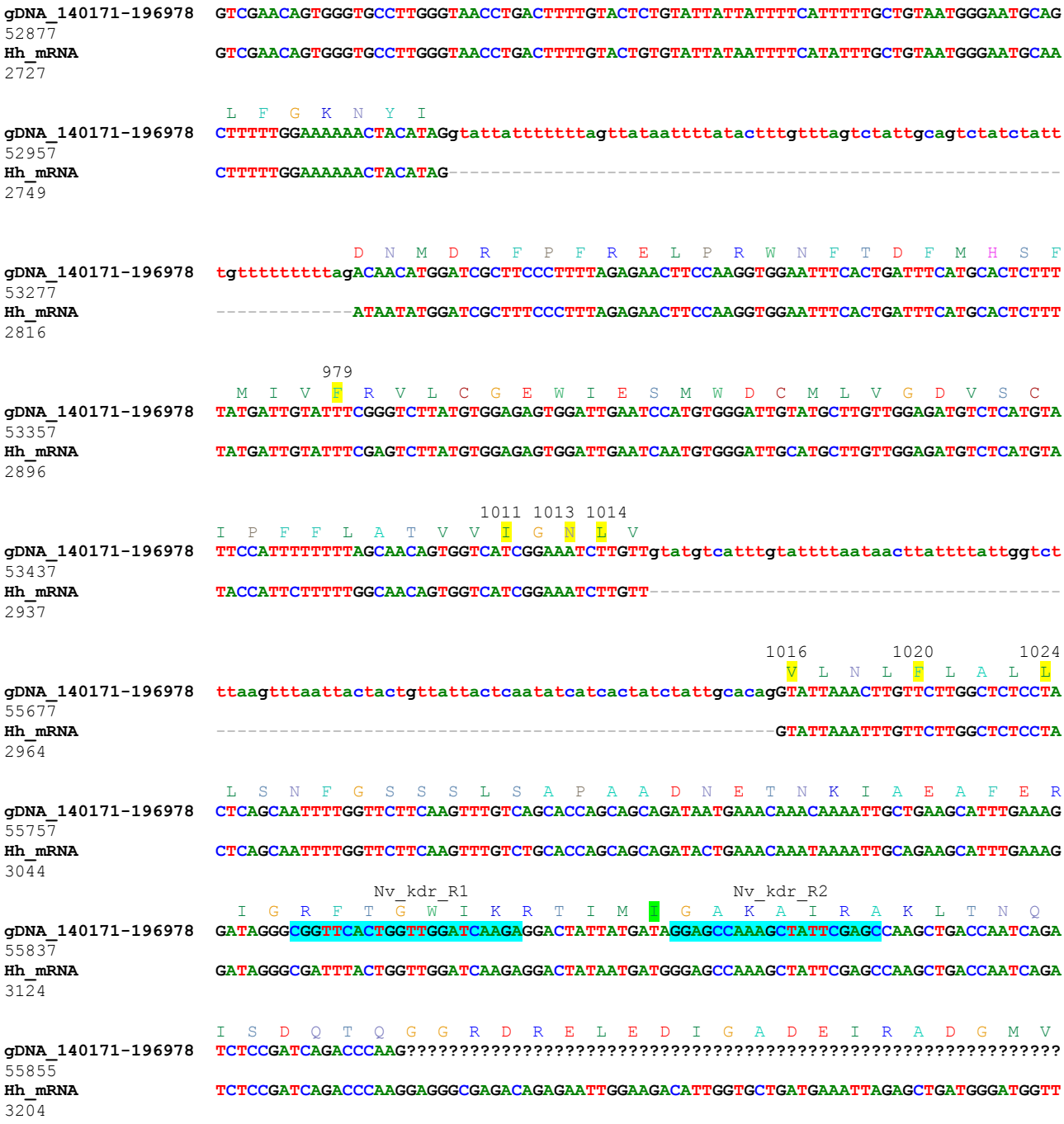

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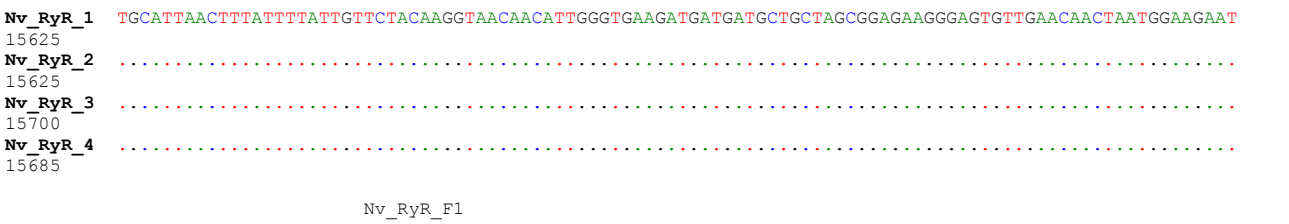


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