COLLECTION AND ANALYSIS OF VOLATILES OF VARIOUS CULTIVATED SUNFLOWER, *HELIANTHUS ANNUUS*, (ASTERACEAE) GERMPLASM AND INVESTIGATION OF SOME ASPECTS OF HOST SELECTION IN ADULT RED SUNFLOWER SEED WEEVIL , *SMICRONYX FULVUS L.*, (COLEOPTERA: CURCULIONIDAE)

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MASTER OF SCIENCE

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ABSTRACT

Plants of sunflower germplasm putatively resistant or susceptible to the red sunflower seed weevil, *Smicronyx fulvus* (Coleoptera: Curculionidae) were used to test the hypothesis that the volatile composition of these two types are different and may influence acceptance/rejection of sunflower germpalsm by *S. fulvus*. At least 13 volatile terpenoids were released by the different plant lines, with some varying in concentration according to plant line, head maturity, and time of day. Comparison between resistant and susceptible plant lines showed differences in concentrations of less abundant compounds (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, β-selinene and germacrene-D, indicating that, one or more of these compounds may be useful markers for resistance/susceptibility to *S. fulvus*. In behavioral binary choice bioassays, *S. fulvus* adults were attracted to sunflower heads, preferring R5.5 over R4 and R6 heads, and susceptible to resistant plants. Video recordings indicated both volatile and contact chemicals may be involved in host acceptance/rejection.

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DEDICATION

To my loving parents, Ajantha and Athula.

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Literature Review

Introduction

The major component of animal biodiversity on the planet is insects (Schoonhoven *et al.* 2005). Of the roughly one million known species of insects, around 46% are phytophagous, with the balance feeding on a variety of other organisms (Strong *et al.* 1984). In natural ecosystems, insects and plants interact extensively with each other, with plants deriving important benefits from insects, notably defense and pollination, and insects deriving a wide range of benefits from plants, including food, shelter, camouflage, and oviposition sites (Schoonhoven *et al.* 2005).

Insects use their visual and chemosensory modalities to locate plants. But, plants are highly variable in color and shape, thus, visual senses are unlikely to provide specific recognition cues to locate a plant from a distance. In contrast, insects have an extraordinary ability to smell and taste plant chemicals (i.e. volatile and non-volatile secondary chemical compounds) and use them as plant recognition cues to locate their host plants (Chapman 2009).Of the plant chemicals, those that are volatile in nature are especially useful to locate distant potential hosts efficiently, since they can be detected at some distance from the source (Schoonhoven *et al.* 2005).

Plant volatiles are carried by air flow and formed into a plume with a fine structure (Murlis *etal*.1992). An insect can reliably locate the source (i.e., the plant) of these chemicals using plume contact by flying or walking upwind (Miller and Strickler 1984; Murlis *et al*. 1992). Plants usually produce a chemical or a blend of chemicals that is highly specific to a taxon, stage, or structure (e.g., flower) and are used by insects to detect and recognize their correct host plant (Schoonhoven *et al.* 2005). However, there are limiting factors for using odors to locate plants. A main limitation is that odors are directional, moving with the wind direction, giving a

better chance to an insect flying downwind to detect and find a host plant than an insect flying upwind of a host plant (Miller and Strickler 1984; Murlis *et al*. 1992). Another limiting factor is that, in the environment, there are many volatile chemicals released by different plants and microbes, thus, an insect has to detect its own host plant odor from a mixture of odors. In order to solve this problem, insects have developed highly efficient nervous systems that can filter and detect useful background signals. Having a peripheral detection system (usually on the antenna with olfactory neurons) that can identify a limited number of compounds and a central nervous system that can process these specific compounds help them to accomplish this task (Reisenman and Riffell 2015).

Having located a host plant, an herbivorous insect has to verify its suitability before feeding or ovipositing. Harrison (1987) suggested that the contact chemicals provide an insect with most reliable information about host suitability, usually with a typical behavior pattern before acceptance or rejection of a host. This includes antennating, palpating, test biting, and test feeding. Bernays and Chapman (1994) also have emphasized the role of biting and chewing in determining plant suitability among many insects. It is well-established that some plant varieties contain characteristic secondary metabolites that are used by insects for assessing suitability of a host plant. For example, the mustard plant family (Brassicaceae) contain allylglucosinilates that stimulate the beetle *Ceutorhynchus inaffectatus* (Coleoptera: Curculionidae) to feed (Larsen *et al.* 1992), while cucurbitacin in the Cucurbitaceae stimulates feeding of some specialist insects (Metcalf *et al.* 1980; Tallamy and Krischik 1989). Most of the checkerspot butterfly species which are oligophagous or monophagous, feed on plant species that produce iridoid glycosides (IGs) (Wahlberg 2001; Murphy *et al*. 2004; Talsma *et al*. 2008).

Moreover, IGs aucubin and catalpol are known to act as oviposition cues for the butterfly *Junonia coenia* (Lepidoptera: Nymphalidae) (Pereyra and Bowers 1988).

Plant volatiles

The volatiles in plants are usually a mixture of many different volatile chemical compounds. They may be characteristic to a stage of maturity, structure or state (e.g., damaged or undamaged) of a plant (Dudareva *et al*. 2004). They are the chemicals with sufficient vapor pressure at ambient temperature and pressure (i.e., natural conditions) that can be detected at a distance from the site of release. The variety of volatile compounds produced by plants is very high, with thousands of different chemical structures known (Dicke and Loreto 2010).

The most important plant volatiles can be classified into three major groups, according to their biosynthetic route. The largest group of plant volatile compounds is the terpenoids, sometimes called isoprenoids. These are biosynthesized from acetyl-CoA or glycolytic intermediates, using one of two pathways, the mevalonic acid or HMG-CoA reductase pathway or the 2-methyl-D-erythritol-4-phosphate-pathway (Dubey *et al.* 2003). Terpenoids are classified by the number of pairs of isoprene units they contain; ten-carbon terpenoids are called monoterpenoids because they contain one pair of condensed isoprene units, 15-carbon terpenoids are sesquiterpenoids, and 20-carbon terpenoids are diterpenoids (Engelberth 2006). Only smaller terpenoids, specifically, hemiterpenoids (C5), monoterpenoids, sesquiterpenoids, homoterpenoids (C11 and C16) and some diterpenes have sufficiently high vapor pressure to be effectively volatile (Osbourne and Lanzotti 2009). Of these, mono- and sesquiterpenoids comprise the largest proportion of the volatile compounds released by plants (Dudareva *et al.* 2004). There are thousands of known plant terpenoids and many play a role in plant attraction, plant defense by acting as repellents, anti-feedants, toxins or as modifiers of insect development

(Aharoni *et al.* 2005). Terpenoids frequently give characteristic odors to plant varieties; e.g., limonene is the dominant odor of plants of the citrus family (Bourgou *et al.* 2012), pinenes and bicyclic terpenes are characteristic odors of coniferous trees (pines), turpentine, lavender and rosemary (da Silva *et al*. 2012).

The next largest group of plant volatiles is the phenyl propanoids (e.g.,methyl salicylate, methyleugenol). They are characterized by an aromatic ring derived from the amino acid phenylalanine. The biosynthesis of this volatile group involves a sequence of central enzyme-regulated reactions (termed the general phenylpropanoid pathway), from which branch pathways arise to produce different aromatic end products (Zhang and Liu 2015). These compounds provide specific plant odors to many plants; e.g., the scent of lilies (*Lilium* sp.) is attributed to the phenyl propanoids methyl benzoate and iso-eugenol (Morinaga *et al.* 2009; Oyama-Okubo *et al.* 2011), while the floral scent of an orchid variety called *Vanda* Mimi Palmer is attributed to the phenyl propanoids methyl benzoate, benzyl acetate, phenyl ethanol, and phenyl ethyl acetate (Rahim *et al.* 2010).

The third major group of plant volatiles is fatty acid derivatives including short-chain aldehydes, alcohols and esters. The most common sub-group being green leaf volatiles (GLV), which give the characteristic smell of mown grass and crushed green leaves (Visser 1986; Wu and Baldwin 2009). These GLVs (Fig.1) are primarily C6–aldehydes, C6-alcohols, and their acetates. They are biosynthesized via the lipoxygenase or hydroperoxidelyase (HPL) pathway (Matsui 2006).

Volatile terpenoids mediating insect-plant interactions

Volatile terpenoids, the most abundant and structurally diverse group of plant secondary metabolites, play an important role in plant-insect, plant-plant and plant-pathogen

interactions (Dudareva *et al.* 2004; Paschold *et al.* 2006). Commonly occurring in higher plants, several thousand terpenoids have been identified (Dudareva *et al*. 2004) (Fig.2). These compounds either promote or deter plant-herbivore interactions (Pare and Tumlinson 1999).

Volatile plant terpenoids as attractants for herbivores and pollinators

There are numerous examples of volatile terpenoids facilitating the attraction of insect herbivores to plants (Hick *et al.* 1999). Various tree monoterpenes (e.g., α-pinene, myrcene, terpinolene, β-pinene) are attractive to many bark beetle species of the genus *Ips,* (Coleoptera: Scolytidae) and *Dendroctonus* (Coleoptera: Scolytidae) (Byers 1995). Initial colonizers are attracted to these host terpenes and subsequently these colonizing beetles biosynthesize further terpenes which attract other beetle species leading to aggregate and colonize the host tree. As a result, the host tree defenses are broken down leading to the death of the tree (Byers 1995). The European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), uses a range of terpenoids, including α-humulene, α-farnesene, β-farnesene and β-caryophellene oxide produced and released by its host plant maize, *Zea mays*, to select suitable oviposition substrates (Binder and Robbins 1997). Pollinating honeybees, *Apis mellifera* (Hymenoptera: Apidae) seem to employ a diverse range of compounds including the terpenoids, α-pinene, *p*-cymene, α-terpinene, linalool, (1*S*)-Δ3-carene and (*E*,*E*)-α-farnesene in plants for locating food (Blight *et al.* 1997).

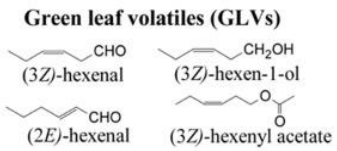

Figure 1**.** Molecular structures of selected plant green leaf volatiles (Source: Niinemets *et al.* 2013).

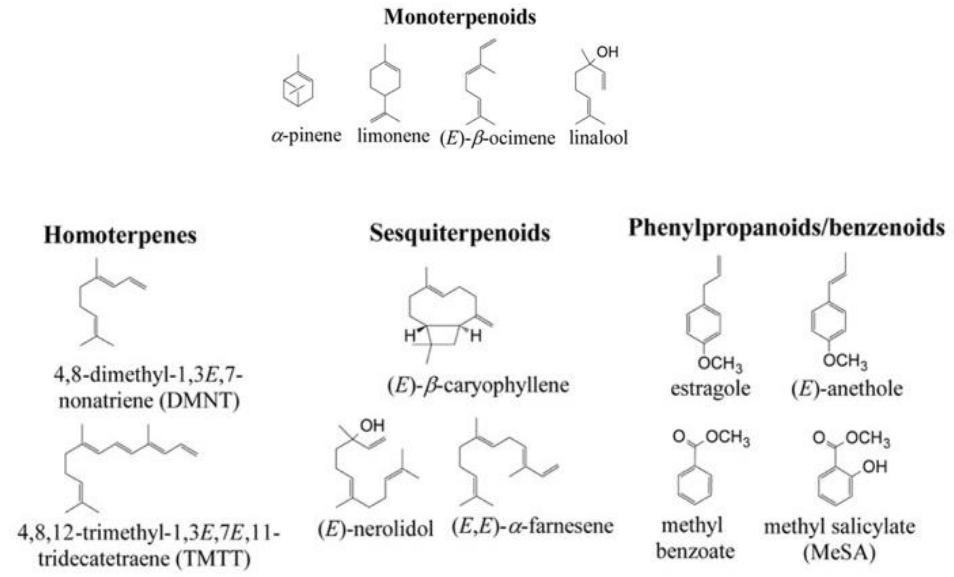

Figure 2. Molecular structures of selected plant volatile terpenoids (Source: Niinemets *et al.* 2013).

Volatile plant terpenoids in direct defense against herbivorous insects

Another function of terpenoids produced in plants is direct defense against herbivores and pathogenic microorganisms (Tholl 2015). It is well-established that terpenes are toxic to many insects. Essential oils, for example, are mostly, mixtures of volatile monoterpenes and sesquiterpenes (Furstenberg-Hagg *et al.* 2013), and are used for control of both medical and agricultural pests (Pare and Tumlinson 1999). Many studies have established the repellent and toxic effects of plant-produced volatile terpenes on herbivorous insects. The monoterpene limonene, produced by citrus plants, repels *Atta cephalotes,* a leafcutter ant (Hymenoptera: Formicidae), (Cherrett 1972), mealybugs (Hemiptera: Pseudococcidae), and scale insects (Hemiptera: Coccoidea) (Hollingsworth 2005). Monoterpenes that are produced in large quantities by conifers like fir and pine are toxic or repel a variety of colonizing bark beetle species (Coleoptera: Curculionidae) (Trapp and Croteau 2001; Byers 1995;Bordasch and Berryman, 1977). Smith (1961, 1965) has observed that limonene was the most toxic

monoterpene to bark beetles, followed by (+)-3-carene, myrcene, (-)-P-pinene and α-pinene. Gollob (1980)observed that resistant loblolly pines, *Pinus taeda,* in an epidemic area, which survived attack by southern pine beetle *Dendroctonus frontalis* (Coleoptera: Curculionidae) had a much higher content of myrcene in their oleoresin compared to other trees that were killed by the beetle. The role played by volatile plant terpenoids as direct defense against herbivorous insects in general can be considered extremely important for the survival of plant species.

Volatile plant terpenoids in indirect defense against herbivorous insects

Studies have shown that changes in terpenoid production had an indirect defense response (Hilker *et al*. 2002; Mumm *et al*. 2003). For example, (*E*)-β-farnesene and (*E*)-α-bergamotene were released from *Zea mays* when attacked by caterpillars of *Spodoptera littoralis,* (Lepidoptera:Noctuidae) and the females of the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae) were attracted to these induced terpenoids and oviposited when they found a host larva (i.e., *Spodoptera littoralis* caterpillars) reducing the damage caused by the caterpillar (Schnee *et al.*2006). Similarly, when roots of *Z. mays* were attacked by *Diabrotica virgifera* (Coleoptera:Chrysomelidae) larvae, it induced the plant to release the sesquiterpene (*E*)-β-caryophyllene, which in turn attracted *Heterorhabditis megidis* nematodes to the plant roots, where the nematode fed on *D.virgifera* larvae (Rasmann *et al.* 2005).

Use of plant terpenoids in integrated pest management

Integrated pest management (IPM) requires employing sound, economic and ecological pest control measures, among other things. Usually several effective control strategies such as cultural and biological control, use of resistant cultivars and use of insecticides at economic injury levels are combined together in an IPM program (Charlet *et al* 1997). In the context of insecticides, insecticidal allelochemicals extracted from plants appear to be a promising

alternative strategy for better environmental protection (Regnault-Roger 1997). Of these, terpenoids may play several roles such as fumigants and topical toxin, as well as anti-feedants and repellents. They are usually toxic to adults while they also inhibit reproduction. Therefore, terpenoids as a new class of ecological products have potential for controlling insect pests (Regnault-Roger 1997).

Terpenoids in sunflower, *Helianthus annuus*

The secondary metabolite chemistry of sunflower also has been researched and is characterized by the production of a wide range of terpenoids, both volatile and non-volatile. Etievant *et al.* (1984) isolated 84 compounds, of which 57 have been identified. There were 20 terpene hydrocarbons, 9 alcohols, 3 phenols, 6 esters, and 19 oxygenated compounds (Table 1). Flath *et al.* (1985) reported14 monoterpene hydrocarbons, 25 oxygenated monoterpenes, and several sesquiterpene hydrocarbons from different parts of cut sunflower heads (Table 1). Constituents with higher molecular weight such as sesquiterpene lactones (Melek *et al*. 1985,Alfatafta and Mullin 1992,Prasifka *et al.* 2015) and diterpenes (Melek *et al*. 1985) have been isolated from different parts of the sunflower plant.

Terpenoids in sunflower have shown to facilitate host attraction, oviposition, feeding and resistance to various insects of sunflowers. A sub-class of non-volatile sesquiterpenoids found in capitate glandular trichomes of sunflower florets, have drawn attention for their putative role in defense. It has been shown that these sesquiterpene lactones act against floret feeding insects (Rossiter *et al.* 1986; Rogers *et al*. 1987; Alfatafta and Mullin 1992; Chou and Mullin 1993; Prasifka *et al*. 2015). Prasifka *et al*. (2015) reported that the sesquiterpene lactones on the florets' glandular trichomes act against floret feeding insects due to their very high density on disc florets.

Table 1. Chemical compositions detected in sunflower heads in some previous studies.

Chemical composition of the different sunflower extracts isolated from batches A (consisted of bulk sample of flower heads from cultivars H9P2, US894,Mariane and Mirasol) and B (consisted of H9P1known to be poorly visited by insects) (only identified chemicals are given) + -present (Etievant et al. 1984)				Chemical components identified in sunflower heads of hybrid 894 (only identified chemicals are given) (Flath *et al.* 1985)
	A	B		1-hexanol
Acetic acid, ethyl ester	+	+		3-methylbutyl benzoate
2-methylpropanal	+	+		3-methylbutyl salicylate
3-methylbutanal		+		α –copaene
2-methylbutanal		+		α -humulene
1-pentanol		+		α –phellandrene
3-hydroxy-2-butanone	+	+		α –terpinene
Hexanal	+	+		α –pinene
2-butenoic acid, 3-methyl-, methyl ester		+		α –terpinoeol
2-hexenal (trans)	+	+		α –thujene
1-hexanol		+		β – elemene
2-pentanone		+		β –phellandrene
bicyclo [3.1.0] hex-2-ene, 2-methyl-5-(1-methylethyl) (α thujene)		+		β –pinene
bicyclo [3.1.1]hept-2-ene,2,6,6-trimethyl- ***(a*** pinene)		+		β-gurjunene,
bicyclo[2.2.l]heptane,2,2-dimethyl-3-methylene- (camphene)		+		Borneol
bicyclo [3.1.01 hexane, 4-methylene-1-(1-methylethyl)- (sabinene)		+		bornyl acetate
bicyclo [3.1.1]heptane,2-methylene-6,6-dimethyl- β-pinene)		+		Camphene
1,6-octadiene, 7-methyl-3-methylene- (myrcene)		+		campholene aldehyde
1,3-cyclohexadiene, 2-methyl-5(1-methylethyl) ***(a*** phellandrene)		+		caryophyllene
1,3-cyclohexadiene, 1- methyl-4-(1-methylethyl) ***(a*** terpinene)		+		cis-3-hexene-1-ol
benzene, 1-methylethy-4-(1-methylethyl)- p-cymene)		+		Crysanthenol
cyclohexene- 1-methyl- 4(1-methyletheny1)-(limonene)		+		Crysanthenone
2-oxabicyclo[2.2.2]octane,1,3,3-trimethyl- (1,8-cineole or eucalyptol)	+	+		gamma terpinene
bicyclo[4.1.0] heptene,4,7,7-trimethyl- (4-carene)		+		Limonene
Phenylacetaldehyde	+	+		Myrcene
2,4, 6-octatriene1, 2,6-dimethyl- (alloocimene)		+		Myrtenal
1,7-octadien-3-one, **2**-methyl-6-methylene-		+		para cymene
3-cyclopentene-lacetaldehyde,2,2,3-trimethyl- (campholenal)	+	+		Perillene
bicyclo [3.1.0] hexan-3-o1,4-methylene-l-(1-methylethyl) (sabinol)	+	+		Pinocamphone
bicyclo[2.2.l]heptan-2-o1,1,7,7-trimethyl-, endo-(borneol)		+		Pinocarveol
3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) (1-terpinen-4-o1)	+	+		Pinocarvone
benzenemethanol-4-(1-methylethyl) (cumic alcohol)	+	+		Sabinene
3-cyclohexene-methanol,α,α,4-trimethyl- ***(***α-terpineol)	+	+		sabinene hydrate
bicyclo [3.1.1] hept-2-ene-2-carboxaldehyde, 6,6-dimethyl- (myrtenal)	+	+		terpinen-4-ol
bicyclo[3.1.1] hept-3-one, 2,6,6-trimethyl-(isopinocamphone or cis-3-pinanone)		+		terpinolene
bicyclo[3.1.1] hept-3-en-2 one, 4,6,6-trimethyl-(verbenone)	+	+		Tricyclene
2-cyclohexen-1-o1, 2-methyl-5-(**1**-methylethenyl)., trans-(trans-carveol)	+	+		Verbenone
2,3-dioxabicyclo[2.2.2]-oct-5-ene, 1-methyl-4-(1 -methyle thy1)-(ascaridole)	+			
bicyclo[2.2.1] heptan-2-01,1,7,7 -trimethyl-, acetate(bornyl acetate)	+	+		
1-cyclohexene-1-me thanol,44 1-methylethenyl).,acetate (perillyl acetate)	+	+		
α 2,5-decadienal	+			
tricyclo[4.4.0.0] dec-3-ene,1,3-dimethyl-8-(1-methylethenyl- ***(a***-copaene)		+		
cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1- methyletheny1)-*(β*-elemene)		+		
bicycle [7.2.0] undec-4-ene, 4,11,11-trimethyl-8-methylene- *(β* –caryophyllene)		+		
bicyclo[3.3.11 heptane,6-methyl-2-me thylene-6- (4-methyl-3-penteny1)-		+		
1H cyclopropa [α] naphthalene, 1a,2,3,4,5,6,7,7a,7b-octahydro-l,1**,**7,7a-tetramethyl- *(p* -gurjunene)		+		
naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methyletheny1)-*(β* -selinene)		+		
1 H-cyclopropeazulene ,decahydro-1,1,7-trimethyl-4-methylene-(aromadendrene)		+		
lH-cyclopenta-l,3-cyclopropa-l,2-benzene,octahydro-7-methyl-3-methylene-4-(1-methylethy1)-		+		
phenol, 2-methoxy-4-(2-propenyl) (eugenol)	+			
benzaldehyde, 4-hydroxy-3-methoxy- (vanillin)	+			
decanoic acid, methyl ester(methyl caprate)	+			
pentyl benzoate	+			
5,9-undecadien-2-one,6,l0-dimethyl-(geranylacetone)	+			
2,6,10,10- tetramethyl-1-oxaspiro[4.5] dec-2-en-8-one (8,9-dehydro-4,5-(dihydrotheaspirone)	+			
2-tridecanone	+			
1-naphthalenol, 1,2,3,4,4a,- 7,8,8a-octahydro-1,6-dimethyl-4-(**1**-methylethy1)- (δ -cadinol)	+			
1-propanone, 1-(4-hydroxy-3-methoxypheny1)-(propiovanillone)	+			

Alfatafta and Mullin (1992) studying the feeding and toxic effects of a range of terpenoids in cultivated sunflower, including floral sesquiterpene lactones, diterpenes and phenolics on adult western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) observed 15 active chemicals, of which the most potent were two sesquiterpene lactone angelates.

Roseland *et al*.(1992) carried out field trials on red sunflower seed weevil attraction to a combination of five monoterpenoids and other volatile chemicals of sunflower heads that resembled two lines of sunflower, a USDA standard line and a French line which was considered as 'poorly visited by insects'. There was a marked difference in attraction and the chemical ratio resembling that of the USDA standard line was more attractive. The mixture contained α-pinene, β-pinene, limonene, camphene and bornyl acetate. This study emphasizes the possibility of volatile application in controlling one of the major insect pest in sunflower, the red sunflower seed weevil (RSSW), *Smicronyx fulvus* (Coleoptera: Curculionidae).

Present Study

It is clear that sunflower pest control is moving towards a more integrated approach with less use of toxic chemicals. Studies show that there is potential for using terpenoids in sunflower pest control. However, specific tools for monitoring and/or controlling the various insect pests of sunflower are only available for a few species [e.g., sunflower moth, *Homoeosoma electellum* (Pyralidae) and banded sunflower moth, *Cochylis hospes* (Tortricidae); (Prasifka and Hulke 2012]. Moreover, the actual mechanisms by which many sunflower insects find and accept suitable or non-suitable hosts is not known. Thus, there is a need to determine these mechanisms and to identify chemicals that could be used for monitoring or management of the pests of sunflower crops. I chose to focus on one of the major pests of sunflower, the red sunflower seed

weevil (RSSW), *Smicronyx fulvus*. The red sunflower seed weevil occurs from the Appalachian Mountains westward through the Great Plains to the Pacific Northwest (Anderson 1962). It is considered a consistent, economic pest of sunflower in the Dakotas and Minnesota. Of the two sunflower seed weevil species [i.e., RSSW and gray sunflower seed weevil, *Smicronyx sordidus* LeConte (Coleoptera: Curculionidae)], RSSW is more common in the northern latitudes (Charlet and Brewer 2009).

This insect was selected because it is difficult to control due to its cryptic feeding damage (inside seeds), thus needs more pragmatic control measures. Roseland *et al*. (1992) suggested that volatile chemicals released by sunflower might be involved in host finding or acceptance by this insect, but further study has been limited because adults cannot be reared continuously in the laboratory (due to no diet and an obligate diapause) and are only available for a short time in the field. If chemicals involved in host finding or acceptance could be identified, they could be used in an IPM-compatible program to monitor or manage this pest.

For this study:

1) I chose a comparative approach of studying volatiles from putative RSSW-preferred (susceptible) and RSSW-non-preferred (resistant) sunflower germplasm to determine if they differed both qualitatively and quantitatively from each other, and combined the volatile data to see if any compound(s) or amount of compound(s) correlated with the resistant/susceptible categories. My hypothesis was that the volatile composition of putative RSSW-susceptible and RSSW-resistant sunflower plants are different both qualitatively and quantitatively and this may determine the acceptance/rejection of a sunflower plant by RSSW.

2) To complement the chemical study, I devised bioassays using adult RSSW emerged from field-collected larvae, to determine whether adult RSSW could distinguish between two sunflower head maturity stages and between putatively susceptible and resistant sunflower lines. My hypothesis was that RSSW can distinguish between two plant lines based, at least in part, on the concentrations and types of volatile chemicals

Such information should be useful for developing hybrid varieties with RSSW resistance and also for using synthetic volatile blends as attractants or repellents to help manage this important pest.

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CHAPTER 2. COLLECTION AND ANALYSIS OF VOLATILES OF VARIOUS SUNFLOWER, *HELIANTHUS ANNUUS*, (ASTERACEAE) GERMPLASM

Introduction

Sunflower *Helianthus annuus* L. (Family: Asteraceae), is an endemic (to North America) flowering plant that is utilized around the world as a crop, both for oilseed and edible seed production (Schneiter 1997). The long evolutionary history of the genus *Helianthus* in the Americas has led to the co-evolution of a large number of insect pests that utilize species in the genus, with over 150 phytophagous insects occurring on both cultivated and native sunflower in the United States (Charlet and Brewer 2009). Of the species found on the genus, most are in the orders Coleoptera, Lepidoptera, and Homoptera (Rogers 1988). A number of species in these orders are economic pests of sunflower, with the most significant being, the sunflower moth, *Homeosoma electellum* (Lepidoptera: Pyralidae), banded sunflower moth, *Cochylis hospes* (Lepidoptera: Tortricidae), sunflower stem weevil, *Cylindrocopturus adspersus* (Coleoptera: Curculionidae), red sunflower seed weevil (RSSW) *Smicronyx fulvus* (Coleoptera: Curculionidae), and the gray sunflower seed weevil, *Smicronyx sordidus* (Coleoptera: Curculionidae) (Rogers 1988; Charlet *et al.* 1997).

Controlling this diverse range of insect pests in a relatively low value crop can be difficult, since use of broad spectrum insecticides can be both economically and environmentally problematic. Thus, the general practice for control of sunflower pests has been the combined application of modified cultural methods (e.g., adjusting planting dates, fall or winter tillage, removal of uncultivated areas and other oviposition sites of pests etc.) along with judicious use of insecticides (Charlet *et al.*1997). With severe outbreaks of pests, such as sunflower moth, *Homoeosoma electellum* and banded sunflower moth, *Cochylis hospes*, being relatively common

in North America (Prasifka and Hulke 2012), there is a need to develop low-cost and environmentally benign control methods for these sunflower pests. One particularly useful method compatible within an overall integrated pest management (IPM) strategy is host plant resistance developed through interspecific hybrids or accessions of sunflower (Brewer and Charlet 1995; Charlet *et al.* 2008; Charlet *et al.* 2010; Prasifka and Hulke 2012). Effective and durable host plant resistance needs to be developed through knowledge of the mechanisms involved in host plant selection (Dent 2000). If we know what factors attract/repel or influence insects to or on plants, then these factors can be used as the basis for host plant resistance or as augmentative control methods.

One of the major mechanisms involved in host selection by most herbivorous insects is the use of chemicals, both volatile and non-volatile, emanating from or on the plant and of these, terpenoids play a very vital role (Feeny *et al.* 1989; Pare and Tumlinson 1999; Dudareva *et al.* 2004; Paschold *et al.* 2006). The chemistry of sunflower is characterized by the production of a wide range of volatile and non-volatile terpenoids (Etievant *et al.* 1984; Flath *et al.* 1985; Rossiter *et al.* 1986; Rogers *et al*. 1987; Alfatafta and Mullin 1992; Chou and Mullin 1993; Prasifka *et al*. 2015) and the involvement of these sunflower chemicals that stimulate finding, repellence, oviposition and feeding has been well documented for a number of insect species (Rossiter *et al*. 1986; Roger *et al*. 1987; Alfatafta and Mullin 1992; Chou and Mullin 1993; Mphosi and Foster 2012; Prasifka *et al*. 2015). However, such information is sparse with respect to the red sunflower seed weevil (RSSW) which is considered a consistent economic pest of sunflower in the Dakotas and Minnesota, where the majority of the US crop is cultivated (Anderson 1962).

Roseland *et al*. (1990) studied the role played by male pheromones of RSSW in host finding and suggested that there is a combined action of both pheromones and plant volatiles in host finding. Roseland *et al.* (1992) also found that a terpenoid mixture containing α-pinene, β-pinene, limonene, camphene and bornyl acetate that resembled the volatile ratios of one of the two sunflower plant lines they studied attracted more RSSWs. Their study emphasized two important aspects; the possibility of using mixtures of volatiles in controlling RSSW in sunflower and possibility of developing effective and durable resistant genotypes of sunflower. For this, there is a need to study volatile composition of different host plant resistant genotypes that have been introduced and more in-depth studies on the role of sunflower volatiles on host choice of RSSW.

As a first step to this understanding, I analyzed volatiles released by putatively RSSW-resistant and RSSW-susceptible sunflower genotypes that have previously been identified as resistant or susceptible based on the severity of damage caused by RSSW [Gao and Brewer (1998); Charlet *et al*. (2010); Prasifka (unpublished data)]. I chose a range of plants that included inbreds, cultivated lines, male sterile (pollen free) plant lines, different crosses etc. which have been previously identified as RSSW-resistant and RSSW-susceptible, in order to compare and contrast the differences in volatile compositions among them. I have analyzed, quantitatively and qualitatively, the volatiles from each plant line and then statistically compared the volatile profiles to see if any compound(s) or amount of compound(s) correlated with the resistant/susceptible categories.

Materials and Methods

Selection of plant lines and stages

Details of plant lines studied are given in Table 2.

Table 2. Details of *Smicronyx fulvus* susceptible and resistant plant lines used in experiment 1 and 2.

Plant Variety	Plant Line Identifier	Description
RSSW Susceptible	HA 441	Inbred check with higher weevil damage. Parent to best 10 GH RSSW selections
	HA 445	Inbred check with higher weevil damage
	cmsHA 441	Male sterile (pollen-free) version of HA 441
	HA 467	Parent in mapping population
	HA 89	Cultivated line showing RSSW damage
	cmsHA 467	Male sterile (pollen-free) version of HA 467
	HA 445 x RHA 377	Public line testcross with fairly high RSSW damage
RSSW Resistant	PI 431542	RSSW resistance source
	PI 431545	RSSW resistance source, secondary
	Mycogen 8H449	Commercial hybrid which has had low/very low RSSW damage
	11 630-6	Resistant inbred progeny (PI 431542 x HA 441) with low damage
	12GH 1220x1221	Sterile analog of 11 630-6
	11 630-6 tester	Testcross to examine transmission of blends into hybrid
	PI 170411	Showing resistance to RSSW (Gao and Brewer 1998)

Source: Prasifka, J.R. USDA ARS (personal communication)

Abbreviations: **RSSW**- red sunflower seed weevil (*Smicronyx fulvus*); **HA** -female heterotic group; **RHA**-male heterotic group; **Cms-** Cytoplasmic Male Sterility(e.g. HA 441 is used to create cms HA 441, and the two should have near identical nuclear DNA but different cytoplasm); **PI** - Plant Introduction (information on the entry and the seed itself can be accessed through the USDA GRIN database (now GRIN Global); but a PI can be a wild plant, an inbred line or a hybrid. The resistant PI materials were originally identified in Charlet *et al*. (2010)
The information on susceptibility of the other lines (e.g., HA 441) from Prasifka (unpublished data)

For the collection of volatiles, I used plants when the head maturity had reached stage R4, R5.5 or R6 (Table 3; see Schneiter and Miller1981). These head maturity stages were chosen because it is roughly over these developmental stages that the red sunflower seed weevils start and complete feeding on sunflower (Peng and Brewer 1994). Therefore, I hypothesized that volatiles released by sunflowers at these head maturity stages are likely to be important during

host finding (see also Mphosi and Foster 2012) and that differences in volatile production and release by the heads may contribute to discrimination of suitable head stage by RSSW.

Planting

The plant lines were sampled in two different experiments at two different time periods. The first set of plants (experiment 1) using 5 plant lines (Table 4) were grown from October 27th to December 30th , 2013 and the second set of plants (experiment 2) using 9 plant lines (Table 5) were grown from September 15th to October 24th, 2014.

I planted 10 pots, each of the different plant lines per week, every two weeks apart. Plants were grown using a special mixture (Metro Mix 902, Hummert International) and were kept in a growth chamber at the USDA/ARS greenhouse for the first 2 months of each experiment. This was done to minimize damage by thrips (Thysanoptera) and whiteflies (Hemiptera) during this initial period, which would have resulted if the plants had been grown in the greenhouse. However, as a precautionary measure, the beneficial nematode Nemasys, *Steinernema feltiae* (Evergreen Growers Supply, LLC, 15822 SE 114th Ave., Clackamas, OR 97015), was used as a weekly foliar spray for the first month. Granular fertilizer (Multicote 4 controlled release fertilizer, NPK Pro 14- 14-16 + Minors, Haifa Group) was applied when the plants were 3 cm high and each week an application of nitrogen solute fertilizer (Jack's professional water soluble fertilizer, 20-20-20 General purpose) was carried out, in order to obtain vigorous plant growth.

After plants reached the required height (~60cm; after ca. 60 days), they were moved to the greenhouse, where they were maintained until sampling of volatiles.

Plant volatile collection

A "push-pull" system, capable of sampling three individual plants simultaneously was constructed to collect volatile chemicals from plants (Fig. 3 a, b). Air was 'pushed' into the

system at 29 ml/min by an air pump (Air Cadet diaphragm pump, Cole-Parmer, Vernon Hills, IL) after first being passed through activated charcoal to purify the air. Air was then passed over three sunflower heads of three plants enclosed separately by large nylon oven bags (KNF Flexpak Corp., Tamaqua, PA) with Teflon tubes inserted on either side of each bag to connect the three bags to the system. After exiting each bag, 3 tubes carrying effluent were passed through 3 rotameters (to maintain an air flow of 5ml/min in each tube) to three adsorbent tubes, each constructed of a glass Pasteur pipet filled with adsorbent powder Tenax-GR(TM) (60/80 mesh, Scientific Instrument Services1027 Old York Rd, Ringoes, NJ 08551-1054). The ends of each tube were packed with clean glass wool to prevent the adsorbent from spilling out. Air was pulled through the adsorbent tubes at 23 ml/min by a vacuum pump (Model 300, Rocker Corp., New Taipei City, Taiwan) (see Fig. 3 a, b). Before use, the absorbent tubes were conditioned at a 200°C for ~2 hours with oxygen-free nitrogen flowing through the tube. Conditioned tubes were wrapped in foil and stored at ambient temperature until used for volatile collections but were never stored for more than 1 day.

Once a set of 3 plants were used for volatile collection, a new set of nylon oven bags were used for the new set of plants to minimize contamination and the activated charcoal was thoroughly cleaned after each run and also they were frequently replaced with new activated charcoal. Caution was also taken not to use any additional materials that released volatile chemicals in the system in order to keep the volatile background as low as possible. All collections were carried out in the USDA/ARS green house facility at ambient temperatures ranging from ~10°C–21°C.

Table 3. Sunflower head maturity stages used and description.

Head maturity stage	Description	Figure
R4	The inflorescence begins to open. When viewed from directly above, immature ray flowers are visible. Average days from planting: 71.	
R5 (decimal) (e.g., R5.1, R5.2, R5.3, R5.4, R5.5etc.)	This stage is the beginning of flowering. The stage can be divided into sub-stages depending on the percent of the head area (disk flowers) that has completed or is in flowering. Ex. R5.3 (30%), R5.8 (80%), etc. Average days from planting: 77.	
R6	Flowering is complete and the ray flowers are wilting. Average days from planting: 84.	
Source : Schneiter, A. A. and J. F Miller 1981. Description of Sunflower Growth Stages. *Crop Sci.* 11: 635-638.		

Table 4. *Smicronyx fulvus* susceptible and resistant plant lines and number of plants used in experiment 1.

Plant variety	Plant line	Head maturity stage								
		R4			R5.5			R6		
		Number of plants			Number of plants			Number of plants		
		Morning	Noon	Over Night	Morning	Noon	Over night	Morning	Noon	Over Night
RSSW Susceptible	HA441	9	9	9	7	7	7	9	9	9
	HA445	7	7	7	6	6	6	9	9	9
	cmsHA441	9	9	9	6	6	6	8	8	8
RSSW Resistant	PI431542	8	8	8	7	7	7	6	9	9
	PI431545	9	9	9	6	6	6	7	7	7
										Total tested plants =345

Source:Prasifka,J.R. USDA ARS (personal communication)

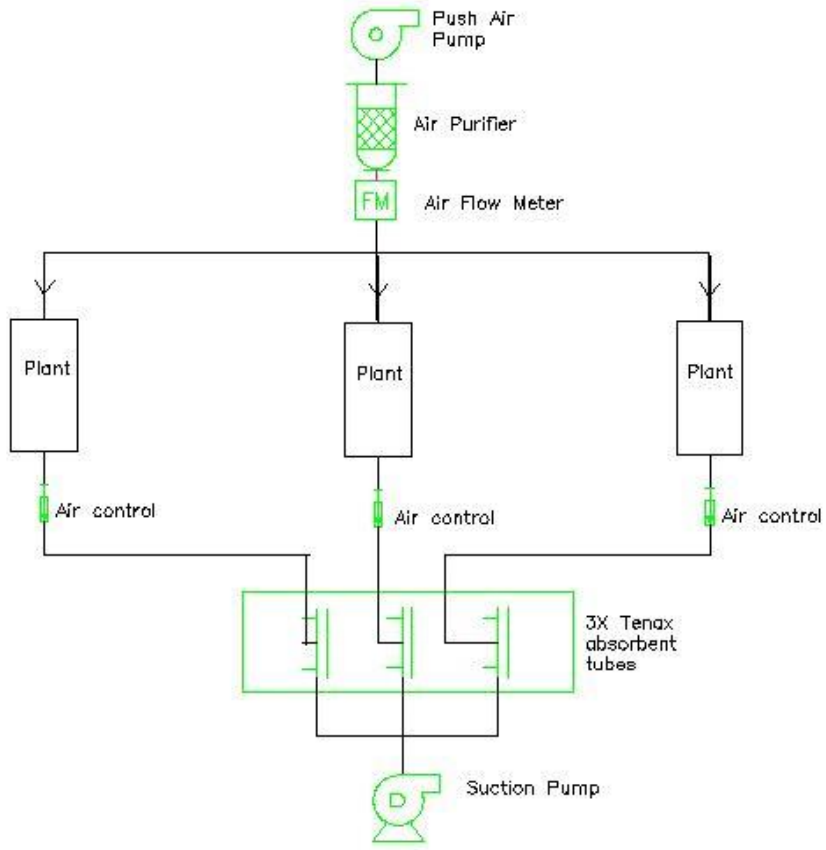
Abbreviations: **RSSW**- red sunflower seed weevil (*Smicronyx fulvus*); **HA** -female heterotic group; **RHA**-male heterotic group; **Cms**- Cytoplasmic Male Sterility(e.g. HA 441 is used to create cms HA 441, and the two should have near identical nuclear DNA but different cytoplasm); **PI** - Plant Introduction (information on the entry and the seed itself can be accessed through the USDA GRIN database (now GRIN Global); but a PI can be a wild plant, an inbred line or a hybrid. The resistant PI materials were originally identified in Charlet *et al.* (2010). The information on susceptibility of the other lines (e.g., HA 441) from Prasifka (unpublished data)

Table 5. *Smicronyx fulvus* susceptible and resistant plant lines and number of plants used in experiment 2.

Plant Variety	Plant Line	R5.5/noon run Number of plants
RSSW Susceptible	HA 467	8
	HA 89	8
	cmsHA 467	8
	HA 445x RHA377	8
RSSW Resistant	Mycogen 8H449	8
	11 630-6	6
	12 GH 1220x1221	8
	11630-6xtester	8
	PI 170411	8
	Total tested plants	**70**

Source:Prasifka,J.R. USDA ARS (personal communication)

Abbreviations: **RSSW**- red sunflower seed weevil (*Smicronyx fulvus*); **HA** -female heterotic group; **RHA**-male heterotic group; **Cms**- Cytoplasmic Male Sterility(e.g. HA 441 is used to create cms HA 441, and the two should have near identical nuclear DNA but different cytoplasm); **PI** - Plant Introduction (information on the entry and the seed itself can be accessed through the USDA GRIN database (now GRIN Global); but a PI can be a wild plant, an inbred line or a hybrid. The resistant PI materials were originally identified in Charlet *et al.* (2010). The information on susceptibility of the other lines (e.g., HA 441) from Prasifka (unpublished data)



a. Schematic diagram showing the “push-pull” pump sampling method.

b. “Push-pull” set up used in the study.

Figure 3. “Push-pull” pump sampling method used for the volatile collections.

Experimental design

The plant lines were tested in two different experiments (according to the time plants were grown). In experiments 1 and 2, 5 and 9 plant lines, respectively, were tested (Table 4, Table 5). The whole idea of using many plant lines as much as possible was to see if there was any chemical/s that determines susceptibility or resistance of a plant for RSSW. In experiment 1, four independent variables; plant line (individual plant lines), susceptible plant lines versus resistant plant lines (pooled data), time of day and sunflower head stage, were tested using the dependent variable of individual volatile concentration. The same plants of each of the 5 plant lines were used for volatile collection at different head stages (R4, R5.5 and R6) and three different times of the day [08.00h-12.00h (morning), 12.00h-16.00h (afternoon) and 20.00h-8.00h the next day (overnight)]. For plant line comparison, pooled data of volatiles collected from each plant line of the three different stages and three different times of the day were combined and used. Each plant was identified individually so that the effect of individual plant could be accounted for in the model. Only healthy/undamaged plants were used for collections, with 7–9 different plants tested per independent variable, giving a total of 345 samples in experiment 1 (Table 4).

In experiment 2, the independent variables plant line (individual plant lines), susceptible plant lines versus resistant plant lines (pooled data) were tested for volatile concentrations. For this, R5.5 stage heads of 9 plant lines were used and the volatile collections carried out from 10.00h – 14.00h. Eight plants (except in the case of 11 630-6 for which only 6 plants were tested) of each plant line were tested, giving a total of 70 samples (Table 5).

Chemical extraction

After each collection, the absorbent tubes were brought to the laboratory for extraction. Five microliters of an internal standard of 1-pentadecene (2.5µg/µl; 12.5 µg) was placed on the glass wool plug and the volatiles on the Tenax were desorbed with 1.5 ml of pentane; the resultant eluent was collected in a 2 ml glass vial. A gentle stream of nitrogen was used to concentrate the extract, making sure that solvent was never completely evaporated (and hence risking potential loss of the volatiles). Extracts were stored in a freezer (-15^{o}C) until analysis by gas chromatography /mass spectrometry (GC/MS).

Gas chromatography/mass spectrometry analysis

Two microliters of a concentrated extract of sunflower volatiles was injected into a Hewlett-Packard 6890/5972 GC/MS for analysis. The GC/MS was fitted with a Zebron ZBWax column (30 m long X 0.25 mm i.d., x 250 mm coating; Phenomenex Inc, Torance, CA) and a split/split-less injector operated in the split-less mode. The carrier gas was helium, operated at a constant flow of 1.3 ml/min. Peaks were identified tentatively by using the NIST GC/MS library and confirmed by comparison of mass spectral and retention time data with those of authentic samples (all purchased from Sigma-Aldrich, St Louis, MO). Peak areas of a mass chromatogram were integrated manually using ChemStation software and amounts of compounds calculated relative to the internal standard.

Statistical analysis

Factorial ANOVA Generalized Linear Model (GLM) at a significance level of 5% (α = 0:05) was conducted using SAS/STAT(R) 9.4 software to compare the mean effects of plant line (i.e. each plant line separately and between susceptible and resistant plant lines), time of day, and sunflower head stage on volatile concentrations. After reviewing the results for each variable, the

data seemed reasonably unimodal and, as the sample sizes were similar across the treatment levels and the inferences seemed reasonable, no transformation of data was done before conducting the GLM. The compounds that showed significant effects with these independent variables were subjected to further analysis of means in ANOVA using Duncan's Multiple Range Test.

Results

Experiment 1: Identification of compounds in sunflower volatile collections

GC/MS analysis of the collected sunflower volatiles of 3 RSSW-susceptible (HA 441, HA 445, cmsHA 441) and 2 RSSW-resistant (PI 431542 and PI 431545) sunflower lines identified 13 peaks, representing at least 13 compounds (some peaks may have contained multiple compounds, due to overlapping retention times and the similarity of mass spectra) that were consistently found in most or all of the samples analyzed (Fig. 4). Some peaks were omitted after concluding them as contaminants. The compounds identified, consisted of monoterpenes, sesquiterpenes and a C_{11}-homoterpene, and were identified on the basis of the correspondence of their retention times and mass spectra to those of authentic samples (Table 6).

Variation of volatile concentrations with respect to different sunflower lines

GLM factorial analysis of different concentrations (µg/h) of different volatiles found in 3 RSSW-susceptible (HA 441, HA 445, cmsHA 441) and 2 RSSW-resistant (PI 431542 and PI 431545) plant lines showed an effect ($p<0.05$) of sunflower line on concentration of each of the 13 peaks, except for the peaks corresponding to the monoterpenes β-myrcene and camphene/γ-carene/β-pinene (Table 7). In general, across all the lines, the mean concentrations of the monoterpenes α-pinene, sabinene/β-phellandrene and limonene were greater than the

concentrations of the other compounds, which were often found in small amounts (Fig. A1; Table 8).

The 11 compounds that showed significant effects with plant line were subjected to further analysis of means using Duncan's Multiple Range Test. While the patterns of concentrations of individual compounds are complex, there was a clear pattern of the susceptible line HA445 producing the greatest amounts of α-pinene, limonene, γ-terpinene /α-terpinene, calarene/β-gurjunene and germacrene-D, whereas the resistant line PI431545 had the greatest concentrations of the other six peaks (i.e.,sabinene/β-phellandrene, (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene and β-selinene) (Table 8).

If the plant lines are considered only as either resistant or susceptible, and compared using GLM factorial analysis, then there were differences in mean concentrations of α-pinene, calarene/β-gurjunene, germacrene–D, γ-terpinene/α-terpinene, (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, and β-selinene. Of these, mean concentrations of α-pinene, calarene/β-gurjunene and germacrene–D were greater in susceptible plant lines while γ-terpinene/α-terpinene, (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, and β-selinene were greater in resistant plant lines (Table 9).

Volatile concentrations with respect to sunflower head maturity

Generalized Linear Model (GLM) factorial analysis of the different volatiles released by the three different flower head maturity stages (R4, R5.5 and R6) showed an effect ($p<0.05$) for nine of the 13 peaks (Table 7). In general, across all the maturity stages, the mean concentrations of the monoterpenes α- pinene, sabinene/β-phellandrene and limonene were greater than for the other compounds (Fig. A2; Table10).

The nine compounds that showed significant effects with head maturity stages were subjected to further analysis of means using Duncan's Multiple Range Test. The patterns of concentrations of individual compounds were seemed to go either up or down as the heads matured. There was a clear pattern of an increasing trend in the mean concentrations of α-pinene, sabinene/β-phellandrene and limonene with the increasing head maturity. R6 head also seems producing the greatest amounts of γ-terpinene/α-terpinene, bornyl acetate and calarene/β-gurjunene, whereas the R4 head had the greatest concentrations of the other three peaks (β-elemene, β-selinene and (3*E*)-4,8- dimethyl-1-3-7-nonatriene; Table 10).

Volatile concentrations with respect to period of day

Generalized Linear Model (GLM) factorial analysis of the different concentrations (µg/h) of the different volatiles collected during morning, evening or overnight showed an effect ($p<0.05$) of time of the day for 11 of the 13 peaks (camphene/γ- carene/β-pineneand (3*E*)-4-8- dimethyl-1-3-7-nonatriene showed no effect; Table 7). In general, across all 3 different times of the day, the mean concentrations of the monoterpenes α- pinene, sabinene/ β-phellandrene and limonene were greater than for the other compounds (Fig. A3; Table 11).

The 11 compounds that showed significant effects with time of the day were subjected to further analysis of means using Duncan's Multiple Range Test. While the patterns of concentrations of individual compounds are complex, there is a clear pattern of the morning samples producing the greatest amounts of all compounds, whereas the overnight samples had the least amounts of all volatiles (Table 11).

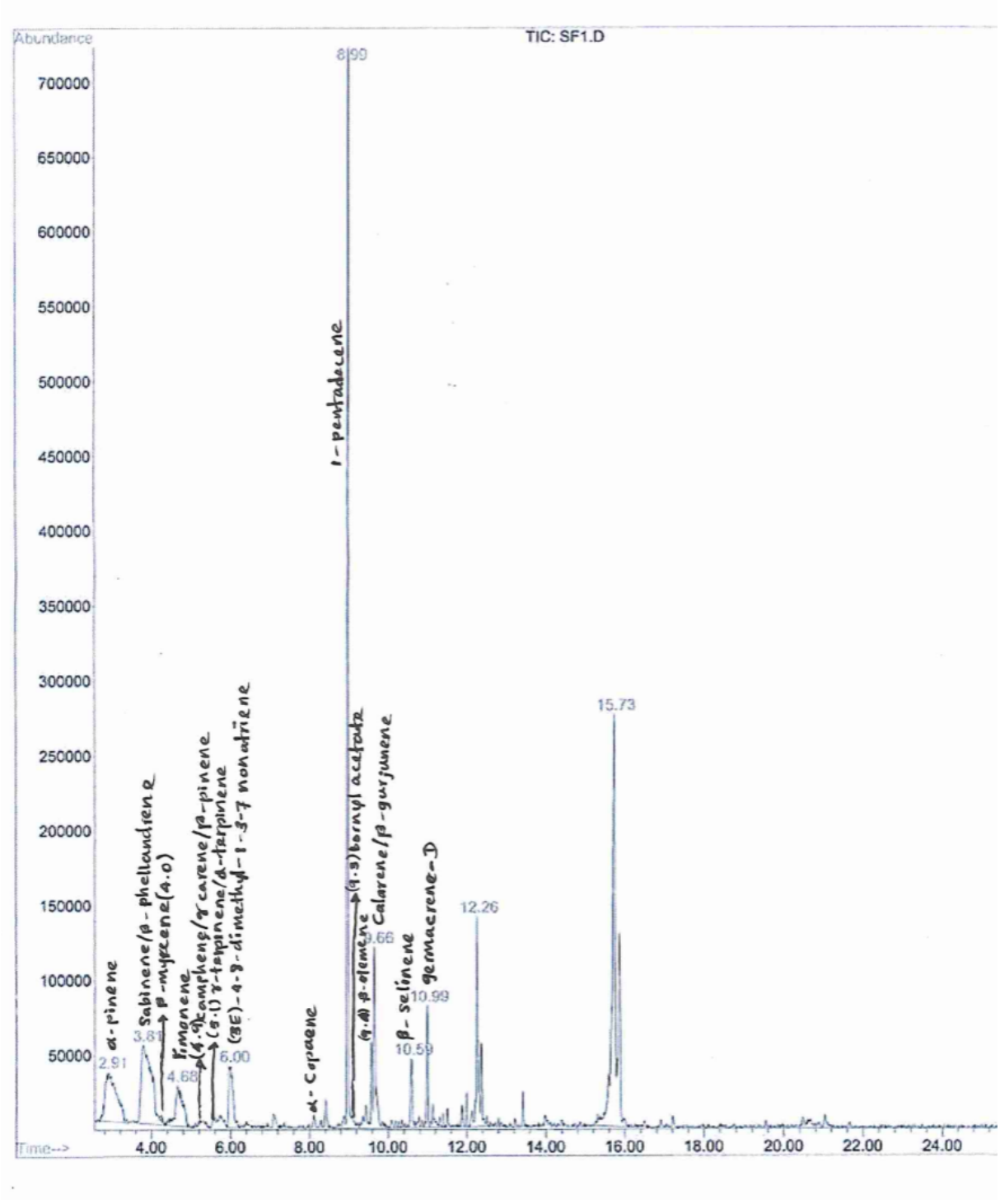


Figure 4. A typical total ion mass chromatogram of volatiles collected from R5.5 flower heads of Mycogen 8H449 sunflower plant line.

Table 6. Volatile compounds (and retention times in min.) identified in sunflower plant lines in experiment 1 and 2 (Multiple compounds given for a peak indicate compounds with similar retention times that could not be identified unequivocally on the basis of mass spectral data).

Monoterpenes	**Sesquiterpenes**	**C-11-homoterpene**
Peak 1 (2.91) α-pinene **Peak 2** (3.81) sabinene /β-phellandrene **Peak 3** (4.0) β-myrcene **Peak 4** (4.68) limonene **Peak 5** (4.9) camphne /γ-carene / β- pinene **Peak 6** (5.1) γ-terpinene/ α-terpinene **Peak 10** (9.3) bornyl acetate * **Standard Peak 9** (8.9±0.1) 1-pentadecene	**Peak 8** (8.0) α-copaene **Peak 11** (9.4) β-elemene **Peak 12** (9.66) calarene/ β-gurjunene **Peak 13** (10.59) β-selinene **Peak 14** (10.99) germacrene-D	**Peak 7** (6.0) (3*E*)-4,8-dimethyl-1-3-7-nonatriene

Table 7. Statistical analysis of mean concentrations of volatile compounds (µg/h) with respect to head maturity, time of the day, and plant line in experiment 1, using Generalized Linear Model Analysis at $\alpha = 0.05$.

Parameter	α-pinene	sabinene/ β-phellandrene	β-myrcene	limonene	camphene/γ- carene/ β-pinene	γ-terpinene/α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	α-copaene	bornyl acetate	β-elemene	calarene/ β-gurjunene	β-selinene	germacrene-D
Head Maturity	DF=2 F=4.68 p=0.01	DF=2 F=4.77 p=0.01	DF=2 F=0.72 p=0.48	DF=2 F=8.92 p=0.001	DF=2 F=0.34 p=0.70	DF=2 F=9.35 p=0.001	DF=2 F=14.42 p<0.001	DF=2 F=0.33 p=0.71	DF=2 F=8.49 p=0.003	DF=2 F=11.45 p=0.001	DF=2 F=8.42 p=0.003	DF=2 F=17.33 p<0.001	DF=2 F=1.41 p=0.24
Time of day	DF=2 F=35.27 p<0.001	DF=2 F=22.64 p<0.001	DF=2 F=10.47 p<0.001	DF=2 F=3.46 p=0.03	DF=2 F=2.19 p=0.11	DF=2 F=6.84 p=0.001	DF=2 F=2.01 p=0.13	DF=2 F=22.52 p<0.001	DF=2 F=22.83 p<0.001	DF=2 F=5.72 p=0.003	DF=2 F=11.36 p<0.001	DF=2 F=7.30 p=0.008	DF=2 F=10.92 p<0.001
Line	DF=4 F=9.02 p<0.001	DF=4 F=4.95 p=0.007	DF=4 F=1.17 p=0.32	DF=4 F=12.2 p<0.001	DF=4 F=1.71 p=0.14	DF=4 F=11.83 p<0.001	DF=4 F=72.40 p<0.001	DF=4 F=8.52 p<0.001	DF=4 F=7.30 p<0.001	DF=4 F=20.96 p<0.001	DF=4 F=9.94 p<0.001	DF=4 F=20.08 p<0.001	DF=4 F=9.14 p<0.001

Head Maturity: R4, R5.5, R6; Time: Evening, Morning, Overnight Line:HA 441, HA 445, PI 431542, PI 431545, cmsHA 441
Number of observations used- 321; Highlights show the significantly different chemicals for resistant and susceptible plant lines

Table 8. Comparison of mean concentrations of individual volatile compounds released by different sunflower plant lines in experiment 1, using Duncan's Multiple Range Test at $\alpha = 0.05$.

	Mean volatile concentration (µg/h)(Number of observations in parentheses)										
	α-pinene	sabinene/ β-phellandrene	Limonene	γ-terpinene/ α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	α-copaene	bornyl acetate	β-elemene	calarene/ β-gurjunene	β-selinene	germacrene -D
HA445 (S)	2.06[a] (58)	1.93[b] (58)	2.20[a] (58)	0.30[a] (58)	0.82[b] (58)	0.06[b] (58)	0.21[b] (58)	0.41[b] (58)	0.82[a] (58)	0.28[c] (58)	0.57[a] (58)
HA 441 (S)	1.32[b] (74)	1.78[b] (74)	0.87[bc] (74)	0.15[bc] (74)	0.44[c] (74)	0.07[b] (74)	0.16[b] (74)	0.48[b] (74)	0.79[a] (74)	0.23[c] (74)	0.35[b] (74)
cmsHA 441 (S)	1.29[b] (63)	2.14[b] (63)	0.44[c] (63)	0.09[c] (63)	0.32[c] (63)	0.05[b] (63)	0.16[b] (63)	0.44[b] (63)	0.74[a] (63)	0.48[b] (63)	0.21[b] (63)
PI 431545 (R)	1.20[b] (57)	2.67[a] (57)	1.06[b] (57)	0.17[b] (57)	2.50[a] (57)	0.11[a] (57)	0.28[a] (57)	1.29[a] (57)	0.37[b] (57)	0.75[a] (57)	0.22[b] (57)
PI 431542 (R)	0.95[b] (69)	1.67[b] (69)	1.70[a] (69)	0.30[a] (69)	0.98[b] (69)	0.05[b] (69)	0.17[b] (69)	0.20[b] (69)	0.14[b] (69)	0.24[c] (69)	0.28[b] (69)

Different superscript letters(a, b, c)/ colors in the same column represent differences among plant lines for a given chemical.
S-RSSW susceptible; R-RSSW resistant

Table 9. Comparison of mean concentrations of individual volatile compounds (pooled data) released between *Smicronyx fulvus* -susceptible (HA 441, HA 445, cmsHA 441) and *Smicronyx fulvus* -resistant (PI 431542 and PI 431545) plant lines in experiment 1, using factorial ANOVA Generalized Linear Model Analysis at $\alpha = 0.05$.

Parameter	Mean volatile concentration (µg/h)(Number of observations in parentheses)												
	α-pinene	sabinene/β-phellandrene	β-myrcene	limonene	camphene/γ-carene/β-pinene	γ-terpinene /α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	α-copaene	bornyl acetate	β-elemene	calarene/β-gurjunene	β-selinene	germacrene-D
Mean concentrations (µg/h) of volatiles: RSSW-susceptible	1.53	1.94	0.12	1.13	0.003	0.17	0.52	0.06	0.17	0.45	0.78	0.32	0.37
Mean concentrations (µg/h) of volatiles: RSSW-resistant	1.06	2.12	0.11	1.40	0.01	0.24	1.67	0.08	0.22	0.69	0.24	0.47	0.26
Statistical analysis-Mean concentrations (µg/h) of volatiles RSSW Susceptible vs Resistant	DF=1 F=13.0 p=0.001	DF=1 F=1.6 p=0.2	DF=1 F=0.05 p=0.82	DF=1 F=1.4 p=0.2	DF=1 F=0.56 p=0.45	DF=1 F=5.04 p=0.02	DF=1 F=163.5 p<0.001	DF=1 F=4.58 p=0.03	DF=1 F=6.1 p=0.01	DF=1 F=13.6 p=0.001	DF=1 F=31.2 p<0.001	DF=1 F=13.4 p=0.001	DF=1 F=7.91 p=0.01
Number of observations: Resistant 126; Susceptible 195 Abbreviation: RSSW - red sunflower seed weevil *Smicronyx fulvus* Highlights show the significantly different chemicals for RSSW-resistant andRSSW-susceptible plant lines													

Table 10. Comparison of mean concentrations of individual volatile compounds released by different head maturity stages of sunflower lines in experiment 1, using Duncan's Multiple Range Test at $\alpha = 0.05$.

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Head maturity stage	Mean volatile concentration (µg/h)(Number of observations in parentheses)								
	α-pinene	sabinene/ β-phellandrene	limonene	γ-terpinene/α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	bornyl acetate	β-elemene	calarene/ β-gurjunene	β-selinene
R4	1.15[b] (125)	1.78[b] (125)	0.83[b] (125)	0.13[b] (125)	1.27[a] (125)	0.17[b] (125)	0.80[a] (125)	0.41[b] (125)	0.54[a] (125)
R5.5	1.30[b] (85)	1.99[ab] (85)	1.18[b] (85)	0.22[a] (85)	0.87[b] (85)	0.17[b] (85)	0.36[b] (85)	0.51[b] (85)	0.29[b] (85)
R6	1.59[a] (111)	2.29[a] (111)	1.74[a] (111)	0.26[a] (111)	0.70[b] (111)	0.24[a] (111)	0.39[b] (111)	0.81[a] (111)	0.27[b] (111)
Different superscript letters(a, b, c)/colors in the same column represent differences among head maturity stages for a given chemical Head Maturity: R4, R5.5, R6 (see Table 2.2)									

Table 11. Comparison of mean volatile concentrations released at different times of the day by sunflower lines in experiment 1, using Duncan's Multiple Range Test at $\alpha = 0.05$.

	Mean volatile concentration (µg/h)(Number of observations in parentheses)										
Time of the day	α-pinene	sabinene/ β-phellandrene	β-myrcene	limonene	γ-terpinene /α-terpinene	α-copaene	bornyl acetate	β-elemene	calarene/β-gurjunene	β-selinene	germacrene-D
Morning	1.77 [a] (101)	2.53 [a] (101)	0.20 [a] (101)	1.53 [a] (101)	0.26 [a] (101)	0.10 [a] (101)	0.25 [a] (101)	0.68 [a] (101)	0.78 [a] (101)	0.45 [a] (101)	0.44 [a] (101)
Evening	1.62 [a] (114)	2.24 [a] (114)	0.08 [b] (114)	1.22 [ab] (114)	0.18 [b] (114)	0.08 [b] (114)	0.21 [a] (114)	0.59 [a] (114)	0.65 [a] (114)	0.43 [a] (114)	0.34 [a] (114)
Overnight	0.66 [b] (106)	1.28 [b] (106)	0.08 [b] (106)	0.98 [b] (106)	0.16 [b] (106)	0.03 [c] (106)	0.11 [b] (106)	0.36 [b] (106)	0.29 [b] (106)	0.26 [b] (106)	0.20 [b] (106)

Different superscript letters (a, b, c)/ colors in the same column represent statistical differences among time of the day and chemical compounds.
Time of the day: 08.00h-12.00h (Morning), 12.00h-16.00h (Evening) and 20.00h-8.00h the next day (Overnight)

Experiment 2: Volatile concentrations with respect to more sunflower lines

GC/MS analysis of the volatiles of 4 RSSW-susceptible (HA 467, HA 89, cmsHA467, HA445xRHA337) and 5 RSSW-resistant (Mycogen 8H449, 11630-6, 12 GH 1220x1221, 11630-6x tester, PI 170411) sunflower lines identified the same 13 peaks as found in experiment 1 (Fig.4).

However, the mean concentrations observed for all chemicals in experiment 2 were much higher than for the (different) plant lines tested in experiment 1. The reason for this disparity is unclear. It is unlikely that differences in lines explain it. Because the experiments were conducted at different times, it is possible that differences in environmental factors, such as temperature and pest and pathogen pressure (Schoonhoven *et al.* 2005), affected the release rates of plant volatiles in the two experiments.

Generalized Linear Model (GLM) factorial analysis of concentrations (µg/h) of the different volatiles showed an effect ($p<0.05$) of sunflower line for the five peaks corresponding to the monoterpenes α-pinene, bornyl acetate and sabinene/β-phellandrene and the sesquiterpenes β-selinene and germacrene-D (Table 12). In general, across all the lines, the mean concentrations of the monoterpenes α- pinene, sabinene/β-phellandrene and limonene were greater than the other compounds (Fig. A4; Table 13).

The five compounds that showed significant effects with plant line were subjected to further analysis of means using Duncan's Multiple Range Test. The susceptible line HA 445x RHA 377 produced the greatest amounts of β-selinene, germacrene-D and bornyl acetate, the susceptible line HA 89 produced the greatest amount of α-pinene and the resistant line PI170411 produced the greatest amount of sabinene/β-phellandrene (Table 13).

Combining the data as either resistant or susceptible lines and comparing them using Generalized Linear Model (GLM) factorial analysis, then the susceptible lines have produced greater amounts of α-copaene, β-elemene, β-selinene, germacrene-D, (3*E*)-4,8-dimethyl-1-3-7-nonatriene and bornyl acetate than did the resistant lines (Table 14)**.**

Table 12. Statistical analysis of mean concentrations (µg/h) of individual volatile compounds from R5.5 flower head stage from 10am- 2.00pm with respect to plant lines in experiment 2, using ANOVA Generalized Linear Model (GLM) Analysis at α = 0.05.

Parameter	α-pinene	sabinene/ β-phellandrene	β-myrcene	limonene	camphene/ γ- carene/β-pinene	γ-terpinene / α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	α-copaene	bornyl acetate	β-elemene	calarene/ β-gurjunene	β-selinene	germacrene -D
Plant line	DF=8 F=2.40 p=0.02	DF=8 F=2.26 p=0.03	DF=8 F=0.59 p=0.78	DF=8 F=1.13 p=0.35	DF=8 F=0.97 p=0.46	DF=8 F=1.04 p=0.41	DF=8 F=1.39 p=0.21	DF=8 F=1.2 p=0.31	DF=8 F=2.87 p=0.01	DF=8 F=1.91 p=0.07	DF=8 F=1.05 p=0.41	DF=8 F=2.23 p=0.03	DF=8 F=2.17 p=0.04
Head Maturity: R5.5 Number of observations used- 72 Time: from 10am- 2.00pm Highlights show the significantly different chemical for head maturity R5.5													

Table 13. Comparison of mean concentrations of volatiles released by R5.5 head stage of different sunflower plant lines from 10am - 2.00pm in experiment 2, using Duncan's Multiple Range Test at $\alpha = 0.05$.

Plant line	Mean volatile concentration (µg/h)(Number of observations in parentheses)				
	α-pinene	sabinene/ β-phellandrene	bornyl acetate	β-selinene	germacrene -D
HA 445x RHA 377 (S)	122.85[a] (8)	221.94[ab] (8)	49.52[a] (8)	29.32[a] (8)	13.43[a] (8)
cmsHA 467 (S)	59.07[b] (8)	86.90[c] (8)	28.29[abc] (8)	11.76[bc] (8)	10.62[ab] (8)
HA 467 (S)	100.56[b] (9)	180.03[abc] (9)	22.02[bc] (9)	8.19[ab] (9)	6.58[bc] (9)
HA 89 (S)	238.39[a] (8)	192.64[abc] (8)	19.39[bc] (8)	17.89[abc] (8)	8.99[abc] (8)
PI170411 (R)	136.93[ab] (8)	280.37[a] (8)	26.08[abc] (8)	17.89[abc] (8)	8.94[abc] (8)
Mycogen8H449 (R)	56.61[b] (8)	135.76[bc] (8)	37.27[ab] (8)	14.77[bc] (8)	6.18[bc] (8)
12 GH1220x1221 (R)	137.61[ab] (8)	195.43[abc] (8)	7.51[c] (8)	12.61[bc] (8)	6.34[bc] (8)
11 630-6x tester (R)	61.66[b] (8)	152.80[bc] (8)	12.72[bc] (8)	13.58[bc] (8)	8.15[abc] (8)
11 630-6 (R)	85.77[b] (8)	114.41[bc] (8)	4.99[c] (8)	4.45[c] (8)	4.38[c] (8)

Different superscript letters (a,b,c)/ colors in the same column represent differences among plant lines for a given chemical.
S-RSSW susceptible; R-RSSW resistant

Table 14. Comparison of mean concentrations of volatiles released by R5.5 heads of *Smicronyx fulvus*-susceptible and *Smicronyx fulvus*-resistant lines (pooled data) in experiment 2, using ANOVA Generalized Linear Model (GLM) Analysis.

Parameter	α-pinene	sabinene/ β-phellandrene	β-myrcene	limonene	camphene/ γ- carene/β-pinene	γ-terpinene / α-terpinene	(3*E*)-4,8-dimethyl-1-3-7-nonatriene	α-copaene	bornyl acetate	β-elemene	calarene/ β-gurjunene	β-selinene	germacrene -D
Mean concentrations (µg/h) of volatiles: RSSW-susceptible	129.32	170.67	13.87	225.01	0.71	3.56	26.29	9.21	29.57	10.19	94.64	19.63	9.81
Mean concentrations (µg/h) of volatiles: RSSW-resistant	95.71	175.75	14.81	39.44	1.57	3.13	14.02	4.91	17.71	6.21	38.54	12.66	6.80
Statistical analysis- Mean concentrations (µg/h) of volatiles – RSSW Susceptible vs Resistant	DF=1 F=1.94 p=0.17	DF=1 F=0.04 p=0.84	DF=1 F=0.04 P=0.85	DF=1 F=1.39 p=0.24	DF=1 F=2.71 p=0.10	DF=1 F=0.14 p=0.71	DF=1 F=4.01 p=0.05	DF=1 F=5.39 p=0.02	DF=1 F=4.66 p=0.03	DF=1 F=4.21 p=0.04	DF=1 F=1.64 p=0.21	DF=1 F=5.52 p=0.02	DF=1 F=6.37 p=0.01
Number of observations: Resistant =40, Susceptible = 33 Abbreviation: RSSW - red sunflower seed weevil(*Smicronyx fulvus*) Highlights show the significantly different chemical between RSSW-resistant and RSSW- susceptible plant lines													

Discussion

I analyzed the volatiles released by heads of various sunflower lines, previously found to be either susceptible or resistant to damage by RSSW [Charlet *et al*. (2010); Prasifka (unpublished data)], with an aim of attempting to identify whether differences in presence/absence of particular compounds or their concentrations were indicative of susceptibility or resistance to RSSW. All the sunflower lines tested showed a consistent pattern of thirteen peaks, corresponding to at least 13 terpenoids (some peaks may have contained multiple compounds, due to overlapping retention times and the similarity of mass spectra), with no apparent consistent qualitative differences among lines. The compounds in the peaks were the monoterpenes α-pinene, β-myrcene, camphene/γ-carene/β-pinene, bornyl acetate, γ-terpinene/α-terpinene, limonene and sabinene/β-phellandrene, the sesquiterpenes α-copaene, β-elemene, β-selinene, calarene/ β-gurjunene and germacrene-D, and the C_{11} homomonoterpene; (3*E*)-4,8- dimethyl-1-3-7-nonatriene. All these compounds have been identified previously in sunflower plants (Etievant *et al*. 1984; Flath *et al*. 1985). Etievant *et al*. (1984) identified 84 volatile components in sunflower extracts, among which were 20 terpene hydrocarbons, accounting for more than 93% of the mass of the volatile blend, 9 alcohols, 3 phenols, 6 esters, and 19 oxygenated compounds. The six most abundant terpene hydrocarbons in their study were α-pinene, β-pinene, camphene, limonene, *p*-cymene, and α-terpinene, all of which, except *p*-cymene, were found in the sunflower plant lines in my work. Etievant *et al*. (1984) also reported 17 other monoterpenes, including sabinene and 11 sesquiterpenes among which were the compounds recorded in my study. Flath *et al*. (1985) studying the volatile constituents of oilseed sunflower heads found monoterpenes, including all the monoterpenes found in my study, to predominate, constituting approximately 95% of the total mass of volatiles. The major

components they found were α-pinene and camphene. Flath *et al*. (1985) also identified several sesquiterpene hydrocarbons including α-copaene, β-elemene and β-gurjunene, also identified in my study.

The qualitative and quantitative differences in volatiles between my study and those of Etievant *et al*. (1984) and Flath *et al*. (1985) are likely a consequence of the methodologies employed, and perhaps also due to difference in the sunflower plant lines analyzed. The volatile isolation method I used in my study was modified head space collection using a "push-pull" system, capable of sampling three individual plants simultaneously. In this method, I could sample a single flower head from each of the three while the flower heads were still intact on the plant, which is similar to what an insect is likely to perceive in the natural environment. However, many compounds might have been below detection threshold of my collection method. In contrast, Flath *et al.* (1985) used 1.69 kg of trimmed 9-12mm thick disks of sunflower heads and extracted this with hexane using vacuum distillation/solvent extraction to yield 2 g of sunflower volatiles over a 3.5 hour extraction period. When Etievant *et al.* (1984) isolated and identified volatiles in sunflower cultivars, they used flower heads removed from the stems combined with head space and solvent extraction to isolate the volatiles. Such disparities in volatile isolation methods would likely have led to significant quantitative differences in volatile collection, with my method not only collecting the smallest amount but also not extracting volatiles from within the plant tissue and possibly not released by intact plants.

In my study I analyzed the volatile compounds released by 7 sunflower lines, consisting of female heterotic in-breds (i.e., HA 441, HA445, HA467, HA 89), male sterile versions of in-breds (i.e., cms HA 441, cms HA 467) and a test cross (i.e., HA445 x RHA377) that have shown to be susceptible to attack by RSSW and 7 sunflower lines, consisting of introduced plant lines

(i.e., PI 431542, PI 431545, PI 170411), a resistant inbred progeny (11 630-6,an inbred of PI 431542 x HA441), a sterile analog (i.e. 12GH1220 x 1221 –a sterile analog of 11 630-6), a commercial hybrid (i.e. Mycogen 8H449) and a test cross (11 630-6x tester) that have shown to be resistant to attack by RSSW [Charlet *et al*. (2008); Prasifka (unpublished data)]. Etievant *et al.* (1984) analyzed 5 different sunflower cultivars, namely, H_9P_2, US894, Mariane and Mirasol and H_9P_1, whereas, Flath *et al.* (1985) analyzed the volatile constituents of sunflower Hybrid 894. Such differences in germplasm tested are also likely to lead to differences in volatiles collected.

In general, across all the plant lines investigated, the mean concentrations of the monoterpenes α-pinene, sabinene/β-phellandrene and limonene were greater than for the other compounds, which were often found in very small amounts. Higher concentrations of these three compounds were also reported in the studies of different sunflower cultivars by Etievant *et al.*(1984) and Flath *et al.* (1985). Etievant *et al.* (1984) reported that more than 93% of their extracts were terpene hyrdocarbons, with α-pinene, sabinene and limonene being found in greater concentrations than the other compounds. The sunflower hybrid analyzed by Flath *et al.* (1985) consisted of 77% α- pinene and 13.7% sabinene and only 0.8% of limonene, along with traces of other compounds. Bertoli *et al.* (2011) investigated the pollen aroma fingerprints of two sunflower genotypes and found α-pinene, sabinene and limonene to be the main monoterpene hydrocarbons in the headspace. All these observations demonstrate that the monoterpenes α-pinene, sabinene/β-phellandrene and limonene are the main volatile compounds released by sunflower.

When plant lines were considered only as either resistant or susceptible and compared, there were no differences among the two of the three most abundant volatiles, sabinene/ β-

phellandrene and limonene. However, there were differences in α-pinene and among the less abundant compounds. In experiment 1, comparing 3 RSSW-susceptible and 2 RSSW-resistant plant lines, mean concentrations of α-pinene, calarene/ β-gurjunene and germacrene-D were greater in susceptible plant lines, while γ-terpinene /α-terpinene, (3E)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, and β-selinene were present in greater concentrations in resistant plant lines. In experiment 2, comparing 4 RSSW-susceptible and 5 RSSW-resistant plant lines, mean concentrations of α-copaene, β-elemene, β-selinene, germacrene -D, (3E)-4,8-dimethyl-1-3-7- nonatriene and bornyl acetate were greater in susceptible lines than in resistant lines. In both experiments, there were differences among the less abundant compounds, including (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, β-selinene and genrmacrene-D. Thus, concentrations of one or more of these compounds may be useful markers for resistance/susceptibility to *S. fulvus*, although any role they have in host selection requires further work to determine. In particular, germacrene-D, which was consistently present in greater concentrations in the susceptible plant lines than in the resistant plant lines, draws special attention. One interpretation of these results is that germacrene-D is an important compound in host selection by adult RSSW, with adults being more attracted to plants that produce greater quantities of this compound. Alternatively, specific individual compounds could occupy a role in host selection or repellence for each of the different susceptible/resistance lines, thereby giving no discernible pattern across all the lines tested.

A role for germacrene-D as an attractant for insects has been reported previously (Rostelien *et al*. 2000; Mozuraitis*et al*. 2002). Rostelien *et al.* (2000) and Mozuraitis (2002) showed that females of the tobacco budworm moth, *Heliothis virescens* (Lepidoptera: Noctuidae) are attracted to germacrene-D. Germacrene-D also has found to be the main volatile constituent

of walnut and fig tree leaves, along with α-pinene and limonene. These compounds have been suggested to be general insect attractants for these trees (Buttery *et al.* 1986).

Mean individual volatile concentrations of 2 plant lines, RSSW-susceptible HA445 and RSSW-resistant PI431545 showed some noteworthy results in experiment 1, that is HA445 producing the greatest amounts of α-pinene, limonene, γ-terpinene /α-terpinene, calarene/ β-gurjunene and germacrene-D, whereas the resistant line PI431545 had the greatest concentrations of six peaks (i.e., sabinene/ β-phellandrene , (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene , bornyl acetate, β-elemene and β-selinene). This observation demonstrates that different sunflower plant lines are capable of releasing greater concentrations of different blends of volatiles. These kinds of blends have proven to play a role on host selection (Roseland *et al.* 1992; Byers 1995; Binder and Robbins 1997; Blight *et al.* 1997).

Plant volatiles are thought to have an important role in host selection across a wide range of insects (Cherrett 1972; Feeny *et al*. 1989; Byers 1995; Binder and Robbins 1997; Blight *et al.* 1997; Hick *et al.*1999; Pare and Tumlinson 1999; Dattilo *et al* 2009; Pan *et al* 2015). They may play a role in host finding/selection in a specific insect-plant system either through a single highly specific volatile, characteristic of the plant, influencing the behavior of an insect (Cherrett 1972; Visser 1986; Mozuraitis *et al.* 2002) or through a unique mixture (blend) of volatiles being characteristic of the plant (Fein *et al*.1982; Visser 1986; Barker1997; Morris *et al.* 2009**;** Mphosi and Foster 2012). Roseland *et al.* (1992) have shown that the latter is likely true for the attraction of RSSW to sunflower volatiles. In their study, they combined five monoterpenoids and other volatile chemicals that resembled the volatile concentrations of a RSSW-susceptible USDA standard cultivar (Hybrid 894) and a French cultivar (H_9P_1) "which is poorly visited" by insects. They prepared two mixtures, termed M1 and M3 resembling the volatile mixture of

French cultivar H_9P_1. M1composed of α-pinene, β-pinene, limonene, camphene and hexanal and in M3, hexanal was replaced with bornyl acetate. A third mixture termed M2 resembling the volatile mixture of USDA standard cultivar (Hybrid 894) was identical to M3 but differed mainly in α-pinene content; 92% in M2 and 43% in M3.They found that the weevils were more attracted to M2 mixture that contained the ratio resembling the RSSW susceptible Hybrid 894 and the differences in α-pinene ratio appears to have been distinguished by RSSW. In my study, all 5 chemical compounds found to be more attractive to RSSW (i.e. α-pinene, β-pinene, limonene, camphene and bornyl acetate; Roseland *et al.* 1992) were present in all plant lines, and, of these, α-pinene and limonene recorded higher concentration than the others. However, there was no marked difference of these compounds between RSSW susceptible and RSSW resistant plant lines. More studies such as ratios in compound blends of susceptible and resistant plant lines are needed to identify the best volatile blend for RSSW attraction.

In general, across all three head maturity stages (R4 pre-anthesis, R5.5 anthesis and R6 post-anthesis) investigated, the mean concentrations of the monoterpenes α- pinene, sabinene/ β-phellandrene and limonene were greater than the concentrations of the other compounds. The release rates of these three compounds were different across the three head stages, with a trend of an increasing concentration of each of these three compounds with the increased maturity of head. Similar observations have been made with regards to the flower maturity in other plant varieties (Mactavish and Menary 1997; Azam *et al.* 2013). Mactavish and Menary (1997) investigating the effect of flower maturity and harvest timing on floral extract from *Boronia megastigma* (Nees), found that the concentrations of volatiles in extracts increased as buds mature and the highest concentrations of volatiles were found in open flowers. A comparative study of flower volatiles of nine citrus species at three blooming stages detected 110 volatiles,

with the greatest amounts of volatiles released by fully opened flowers of most species (Azam *et al.* 2013). A study on spatial distribution of RSSW (Peng and Brewer 1994) found that there was a significant effect of sunflower head stage on the density of adult weevils, with flowering plants (> R5 stage) attracting more adult weevils than plants in the bud stages (R2, R3 and R4).

This kind of differences in volatile concentrations found in sunflower head stages have shown to be related to the host selection behavior of insect pests of sunflower. Mphosi and Foster (2012) demonstrated that females of *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae) showed strong preferences for ovipositing on R5 sunflower heads over R2 sunflower heads. The females were able to differentiate between the two head stages through differences in volatile and contact chemicals.

While the increased release of α-pinene, sabinene/ β-phellandrene and limonene with increasing head maturity correlates with the preferences of adults for mature and open heads, more research on the attraction of RSSW to different blends and quantities of volatiles is needed to establish the role of volatile chemicals in the head stage-specific attraction of RSSW to sunflower.

At all three periods of the day (morning, evening and overnight), I also found that the mean concentrations of the monoterpenes α-pinene, sabinene/ β-phellandrene and limonene were greater than the concentrations of the other compounds. The greatest concentrations of these chemicals were found in the morning with the lowest concentration being at night. The role of the differences in these three chemicals, at different times of the day along with others identified in my study, in host-finding by adult RSSW was not determined in my study. Clearly, both further chemical and behavioral studies are needed to determine this aspect. However, in previous studies, the difference in chemical compositions and their role in insect attraction have

been identified. An investigation on patterns of daily floral scent production in three *Lithophragma* species (Saxifragaceae) showed that floral scents were emitted in higher amounts during the day, when their major pollinator, the floral parasitic day flying moths *Greya politella* (Prodoxidae) are active (Friberg *et al*. 2014) relating the release of more floral scents as an attractant to the pollinator. Pare and Tumlinson (1999) related the variation of the amount of volatiles released by individual plants to a plant's physiology that is influenced by environmental conditions. Species such as corn, cotton, and lima bean have shown a decline in the release of herbivore-induced volatiles under reduced light intensity (e.g. lower light intensity or shorter day length)(Pare and Tumlinson 1999).

The aim of my study was to identify whether the presence/absence and concentrations of particular compounds varied in sunflower plant lines and whether these variances were potentially indicative of susceptibility or resistance to RSSW. I showed that qualitatively, there were at least 13 compounds released by all plant lines in detectable quantities (with regard to my collection system) and that, certain of these volatiles varied according to plant line, albeit in a highly variable pattern. The most abundant of these chemicals, irrespective of plant line, head maturity stage or time of day, were (at least) three monoterpenes, namely, α- pinene, sabinene/β-phellandrene and limonene. Although these three compounds can be considered as the main volatiles released by the heads of the sunflower lines I tested, and may be involved in attraction of RSWW to sunflowers, however, they do not appear to indicate susceptibility or resistance to RSSW, as they were found in high concentrations in both RSSW susceptible and resistant plant lines. If indeed plant volatiles mediate host acceptance/rejection of sunflower plants by adult RSSW, then this suggests that other detected or undetected compounds may be involved in determining susceptibility or resistance to RSSW. For example, the occurrence of germacrene-

D in higher concentrations in all RSSW susceptible plant lines in my study suggest that this compound may be involved in mediating attraction of RSSW to acceptable/non-acceptable plants.

Clearly, though, my work is preliminary in terms of determining a role of plant chemicals in suitability of sunflower plants for RSSW, future work should look at isolating individual compounds and blends of these chemicals, by techniques such as coupled gas chromatograph-electroantennographic detector (GC-EAD) and test these chemicals in suitable bioassays for attracting RSSW. This device facilitates the identification of the chemical/s an insect can smell, as it is capable of identifying those that stimulate the olfactory sensilla of an insect, from a complex mixture (as observed in the plant lines I have studied).This device also can use odors derived directly from natural sources (Sullivan 2007). Identification of chemicals that determine the preference of different sunflower germplasm can help in the development of improved RSSW resistant sunflower hybrids. Such work would be facilitated by the development of a rearing method that would make adult weevils more readily available for study in the laboratory.

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CHAPTER 3. BEHAVIORAL BIOASSAYS

Introduction

Sunflower, *Helianthus annuus* L., (Family: Asteraceae) is an important crop in the United States, with the seed being used for both oil and consumption. The genus *Helianthus* (Family: Asteraceae) is endemic to the Americas, and consequently there are a large number of insect pests that have coevolved with the genus and hence adapted to this crop (Charlet and Brewer 2009; Rogers 1988). The red sunflower seed weevil (RSSW), *Smicronyx fulvus* (Coleoptera: Curculionidae), is one of the most significant economic pests of sunflower in the United States, occurring where much of the crop is cultivated, from the Appalachian Mountains, west through the Great Plains up to the Pacific Northwest. In particular, it is considered a consistent economic pest of sunflower in the Dakotas and Minnesota, where the majority of the US crop is cultivated (Anderson 1962).

Adult RSSW emerge following a larval diapause over the winter. The larvae live at a depth of about 15 cm in the soil to overwinter. Larval pupation takes place the following year, in mid to late June, and the pupal period lasts about one week. Newly emerged adults (2.5–3 mm long with reddish-orange coloration) start feeding on stems and the leaf petioles of sunflower plant until the sunflower heads are developed, and then move to the developing heads of the plant to feed (Rana and Charlet 1997). When plants are developed to about 50% anthesis, weevil populations usually reach their peak (Peng *et al.* 1997). When pollen becomes available on the developing flower, weevils supplement their diet with the pollen, which helps induce female egg maturation (Rana and Charlet 1997). Once anthesis is completed in the plant on which they are feeding, adults move to new plants to continue feeding on pollen (Peng *et al.* 1997). Adults live for about 53 days. Inside the pericarp of developing sunflower seeds, a female usually lays white

eggs (about 0.3 × 0.7 mm), one egg per seed, as many as in approximately 27 seeds (Peng and Brewer 1995). Larvae are cream colored, legless, and about 1–2 mm long and consume the kernel of the developing sunflower seeds, causing reductions in seed weight and oil content (Oseto and Braness 1979). In late August, mature larvae (fifth instars) chew through the seed, make holes and drop to the ground directly beneath the sunflower head, in order to enter diapause to overwinter (Oseto and Braness 1979; Charlet and Brewer 2009).

The general approach for insect pest control on sunflower has been the use of modified cultural methods (e.g., adjusting planting dates, fall or winter tillage, removal of uncultivated areas and other oviposition sites of pests etc.) combined with the timely application of insecticides (Charlet *et al* 1997). However, the continued use of insecticides in agricultural production is problematic, due to adverse environmental and health impacts, and the development of insecticide resistance (Damalas and Eleftherohorinos 2011). Thus, there is an urgent need to develop new, sustainable methods that will complement existing cultural methods. One potential approach is to utilize the mechanisms that insects use to find, accept or reject host plants, either directly (e.g., use attractants or repellents that insects utilize during host selection) or indirectly through targeted host plant resistance breeding (Prasifka and Hulke 2012).

Although the RSSW is a major pest of sunflower production in the United States, relatively little is known about many aspects of its biology and, in particular, what factors influence its host selection behavior. Nevertheless, a study by Roseland *et al*. (1992) found that a mixture of chemical volatiles acted well to attract RSSW. Thus, it appears that chemicals may be used in host selection by adults of this insect, although the specific mechanisms (e.g., use of volatile chemicals for attraction, or contact chemicals for host acceptance) and the precise chemicals involved are yet to be determined.

One of the problems in studying this insect is the inability, to date, to rear continuous laboratory cultures. An artificial diet has not been developed for this insect and its diapause is obligate. The usual practice of researchers, who work with this insect in the laboratory, is to collect wild diapause larvae from soil samples. The problem arises in maintaining the larvae because larvae brought into the laboratory from the field often die from desiccation and infection by microorganisms (Barker *et al.* 1991). This is further complicated by the relatively short period that diapause larvae can be collected from the field. Moreover, the adult insects are rather small, hence it is difficult to observe its behavior in the field. Thus, it is a challenge to maintain live specimens and to conduct research on this insect. In this chapter, I investigate some aspects of host selection in adult RSSW, collected as diapausing larvae, using the comparative approach of host selection of RSSW to preferred (i.e. susceptible) and -non-preferred (i.e. resistant) sunflower plants. The hypothesis underlying this work is that the differences in chemistry of flower heads between the two plant lines are responsible for the differences in host selection by adult RSSW.

Materials and Methods

Insects

RSSW Larvae were collected from R6 sunflower heads obtained from NDSU Agronomy Seed Farm in Casselton, North Dakota and from research field near Pierre, South Dakota. Sunflower heads were brought into the laboratory at the USDA-ARS, Fargo, ND. Larvae were made to surface by disturbing the disk florets of sunflower head with a pair of soft forceps and the surfacing larvae were collected using the same and were placed in soil tubs containing unsterilized moist soil. These tubs were placed in cold storage at -5±5°C for 90 days to give larvae sufficient time in diapause. After the cold storage period of 90 days, the soil tubs were

removed and kept at ambient laboratory temperature (~21°C) for 60-80 days to break the diapause.

Emerged adults were collected using a pair of soft forceps daily and placed in plastic containers with a meshed lid, along with a sterilized sucrose solution (30% w/v) to feed ad libitum and maintained under moist conditions through use of wet cotton swabs at ambient temperature (~21°C) until used in bioassays when 7–14 d old.

Plants

Two sunflower lines, namely, the RSSW-susceptible HA 441-(female heterotic group inbred with higher RSSW damage) (Prasifka, USDA/ARS -unpublished data) and RSSW-resistant PI 431542, originally identified as resistant in Charlet *et al*. (2010), were used in the bioassays.

Planting of sunflower seeds, provided by Dr. Jarrad R. Prasifka (USDA-ARS, Fargo, ND), for the bioassays was carried out in the laboratory at the USDA-ARS, Fargo, ND in March 2014, August 2014 and September 2014. Sunflower plants take up to 2 months to reach anthesis. Seeds were planted in pots using a special mixture (Metro Mix 902 ,Hummert International) prepared for potted plants. Two seeds were planted in each pot and, 2 weeks after planting, the healthier plant in each pot was selected and the other plant removed and discarded. Selected plants were placed in a small, enclosed growth chamber at USDA/ARS greenhouses for the first 2 months of the experiment (temperature 10-20 °C, under artificial light) to limit infestation and damage by thrips and whitefly. When necessary, they were controlled by using spot spray application of beneficial nematode Nemasys, *Steinernema feltiae* (Evergreen Growers Supply, LLC, 15822 SE 114th Ave., Clackamas, OR 97015) weekly for one month. Granular fertilizer (Multicote 4 controlled release fertilizer, NPK Pro 14- 14-16 + Minors , Haifa Group)

application when the plants were 3 cm high and weekly application of nitrogen solute fertilizer (Jack's professional water soluble fertilizer, 20-20-20 General purpose) was carried out in order to obtain healthy flowering and plant growth. After plants reached the required height (~60cm; approximately after 60days) and anthesis stage, they were moved to the greenhouse (temperature, 15±5°C, natural light) until used in the binary-choice bioassays.

Binary-choice plant bioassays

The binary-choice plant bioassays were carried out in the greenhouse at the USDA/ARS, Fargo, ND in popup rearing and observation cages (2 x 2 x 2 m; BioQuip Products 2321 Gladwick Street Rancho Dominguez, CA 90220, USA) (Fig. 5). In preliminary trials, I found that adult RSSW were most responsive to plants at nighttime and that more of the weevils in a cage were eventually found on the head, rather than on the vegetative tissue at the conclusion of the experiment. Therefore, I ran the bioassay proper, starting just prior to the dark period and ending just after daybreak (i.e., 18.00 h – 09.00 h).

Plants were placed opposite each other in the cage, but not touching each other or the wall of the cage (Fig. 6).Twenty weevils were introduced into a cage just prior to the start of the experiment in a mesh-covered dish. The dish was placed in the center of the floor of the cage, equidistant between the two plants being tested. Then, the lid was removed carefully, so as not to disturb the weevils, the cage sealed, and the experiment started.

Figure 5. A popup rearing and observation cage used in the binary choice experiments.

Figure 6. Arrangement of two plants and the weevil container in the cage.

Three binary choice experiments were designed as a series of experiments.

Experiment 1- Preference of adult RSSW for R4, R5.5, R6 head stages of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

The first experiment was performed from May to June 2014. The aim was to observe whether there were any differences in host choice of adult RSSW between the two plant lines (RSSW susceptible and resistant) at the same head stage and whether there were differences in host choice at different head stages (i.e., R4, R5.5, R6; Schneiter and Miller 1981). For this experiment, I used three cages each night so that I could simultaneously test the three head stages. In the first part of the experiment, I tested plants of the same head stage of the two lines in each cage; i.e., R4 of HA 441 and R4 of PI 43542 in one cage, R5.5 of HA 441 and R5.5 of PI 43542 in the second cage, etc. In the second part of the experiment, I tested the same plant line at different head stages, comparing the other two head stages against the R5.5 stage in binary-choice tests (i.e., R4 Vs R5.5 and R5.5 Vs R6 of HA 441 and R4 Vs R5.5 of PI4354 etc.,).

At the conclusion of each replicate of the experiment, the numbers and positions of the weevils in the cage were recorded as follows: on the head, on other parts of the plant, on the cage walls/floor or on the container. In total, 8 replicates were performed for both parts of the experiment.

Experiment 2 – Preference of adult RSSW for bagged R4, R5.5 and R6 head stages of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

The second experiment was conducted from October to November 2014. The aim in this experiment was to observe whether precluding contact with the plant heads influenced the host preferences of RSSW between HA 441 and PI 431542,observed in the first experiment. Precluding contact with the plant heads should remove weevil contact with cues they can only

perceive in direct contact with the head (e.g., non-volatile chemicals, tactile cues, pollen). In other words, it should implicate whether cues perceived over distance, particularly volatile chemicals emanating from the plant are involved. For this, heads of the two plant lines were covered with 18"x 24" fine meshed cloth bags and placed in a cage. I tested the plants of the two lines (HA 441 and PI 431542) against each other at the same stage (i.e., at R5.5) and same plant line at different head stages, comparing the other two head stages against the R5.5 stage in binary-choice tests (i.e., R4 Vs R5.5 and R5.5 Vs R6 of HA 441 and R4 Vs R5.5 of PI4354 etc.,). Eight replicates, each using 20 weevils, of the treatments were performed.

Experiment 3 – Observations of landing of adult RSSW on bagged R5.5 stage heads of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

The third experiment was conducted in November 2014. The aim of this experiment was to quantify the landing of adult RSSW on bagged heads of the resistant and susceptible plant lines. That is, to determine whether differences in volatile chemicals between the two lines, result in differing attraction (as measured by landings). The experimental setup was the same as in the previous experiment, except that only plants with heads of R5.5 maturity were used. However, rather than recording the positions of the weevils at the conclusion of the experiment, two digital video recording camcorders (Canon Vixia HF M50 and Canon FS200) were mounted on tripods with their field of view focusing on the two bagged heads. To obtain reasonable images during the night, two incandescent red lamps of low light intensity (ca. 30 lux) were used to illuminate the heads. Following the conclusion of a recording night, the movies were replayed and the number and time of landings on the two heads recorded. Eight separate recordings were carried out, with each using 20 weevils released into the cage.

Pollen preference of adult RSSW

The fourth experiment was conducted in December 2014. Since my previous experiments could not discount a role for pollen in host selection by adult RSSW, I decided to carry out an experiment, testing the preference of RSSW to pollen of the susceptible and resistant lines. Unfortunately, insufficient pollen was collected from the plant lines I used in previous experiments. However, large quantities of pollen, collected by Dr. J. Prasifka, from HA 89 (female heterotic cultivated line showing RSSW damage- Ref. Prasifka personal communication) and PI 170411 (RSSW-resistant variety; Gao and Brewer 1998) were available for testing.

For the pollen study, Petri dishes (15X100 mm diameter) were used. At two places on the dish, a 10 mm diameter circle of 5 mg of pollen was placed, one from HA 89 and the other from PI 170411. The circles of pollen were about 40mm from each other and at least 10 mm from the perimeter of the Petri dish (Fig. 7). Four such dishes were prepared at a time, covered and placed on a table lined with a white paper in the laboratory. At 17.00 h, one adult RSSW (7-14 d after emergence) was put in the center (between the two circles of pollen) of each Petri dish. A digital video camera (Canon Vixia HF M50) was used to record the behavior of the weevils. An incandescent red lamp was used to provide low level illumination (ca 30 lux) for the night recordings. During playback, I recorded the time each weevil made contact with a pollen circle as well as the duration of the contact and which circle was contacted, and their hourly landing counts (i.e. number of times a weevil lands on pollen of the two plant lines) for a period of 15 hours (17.00–08.00 h). Four Petri dishes were run in a night, with the experiment carried out over 10 nights (i.e., a total of 40 replicates).

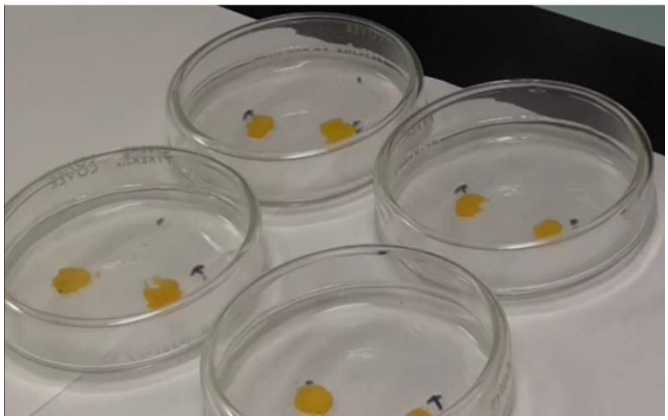

Figure 7. Arrangement of Petri dishes for pollen preference test.

Statistical analysis

In the first two experiments (with plants), the counts of weevils on the plant parts or cage in each replicate were converted to proportions of total weevils introduced. The differences in proportions on the two plants tested were compared by non-parametric Kruskall-Wallis tests, since the proportional data were not normally distributed. In the video recording experiments (plants and pollen), the numbers of contacts of insects on each plant were compared also by non-parametric Kruskal-Wallis tests as, again, the data were not normally distributed.

Results

The proportions were lower than expected for all experiments. This could be because the weevils were stressed in an artificial environment, or they were distracted due to greenhouse illumination or due to other chemicals that were prevalent in the greenhouse. One or more of these factors could have impacted on their poor visitations to flower heads and pollen.

Experiment 1- Preference of adult RSSW for R4, R5.5, R6 head stages of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

Weevils were found on all three head stages of both susceptible and resistant plants (Fig. 8). Heads of the susceptible plant had a greater proportion of weevils than did heads of the resistant plant for both the R5.5 ($p=0.001$; Chi-Square =10.99) and R6 ($p=0.001$; Chi-Square =10.96) stages. There was no difference ($p = 0.14$; Chi-Square =2.16) in the proportions of weevils on heads of the susceptible and resistant plants at the R4 stage (Fig.8).

Within-line comparisons of different head stages showed weevils preferred R5.5 over R4 ($p = 0.001$, Chi-Square =11.29 for susceptible; $p = 0.001$, Chi-Square =11.31 for resistant; Fig. 9) and R5.5 over R6 ($p < 0.001$, Chi-Square =11.31 for susceptible; $p < 0.001$, Chi-Square =11.37 for resistant) heads for both susceptible and resistant lines (Fig. 9).

Experiment 2 – Preference of adult RSSW for bagged R4, R5.5 and R6 head stages of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

Weevils were found on bagged R5.5 heads of both susceptible and resistant plants (Fig. 10). Heads of the susceptible plant had greater proportions of weevils than did heads of the resistant plant ($p=0.03$, Chi-Square 4.5475) (Fig. 10).

In the within-line comparisons of bagged heads, weevils preferred R5.5 over R6 ($p=0.01$, Chi-Square = 6.63; Fig. 11) but there was no difference in preference of weevils between R5.5 heads and R4 ($p=0.35$ Chi-Square = 0.87; Fig. 11) for the susceptible plant. For the resistant plant, there was no difference in preference of weevils between R5.5 heads and R4 [$p=0.08$, Chi-Square = 3.15; Fig. 11) or R6 ($p=0.23$, Chi-Square = 1.46; Fig. 11) heads.

Experiment 3 – Observations of landing of adult RSSW on bagged R5.5 stage heads of RSSW susceptible (HA 441) or RSSW resistant (PI 431542) plant lines

The temporal pattern of landing data (Fig. 12) shows that the greatest number of landings on susceptible heads occurred between 6.00–00.00 h; i.e., in the early night. These data were strongly influenced by the high number of landings on the susceptible plant head. Overall, there was no significant difference between the two plant lines in the early night; i.e. 6.00–00.00 h ($p=0.11$, Chi square =2.58) or late night h; i.e., 00.00–9.00h ($p=0.11$, Chi square =0.02).

Pollen preference of adult RSSW

Adult RSSW showed a clear preference ($p < 0.0001$, Chi square=55.87) for pollen of the susceptible HA89 plant line compared to pollen from the resistant (PI 170411) plant line (Fig. 13). On both susceptible and resistant plant pollen, weevils appeared to be active throughout the night.

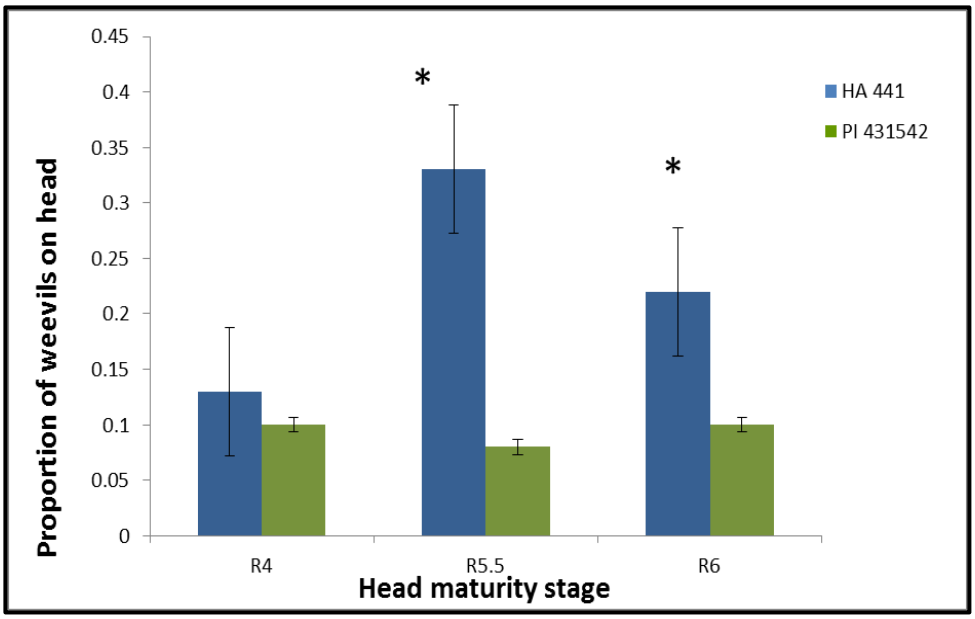

Figure 8. Proportions of adult *Smicronyx fulvus* on three head stages R4, R5.5, R6 of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines (*Indicates a difference, $p<0.05$, in proportions for a given head stage between the two lines).

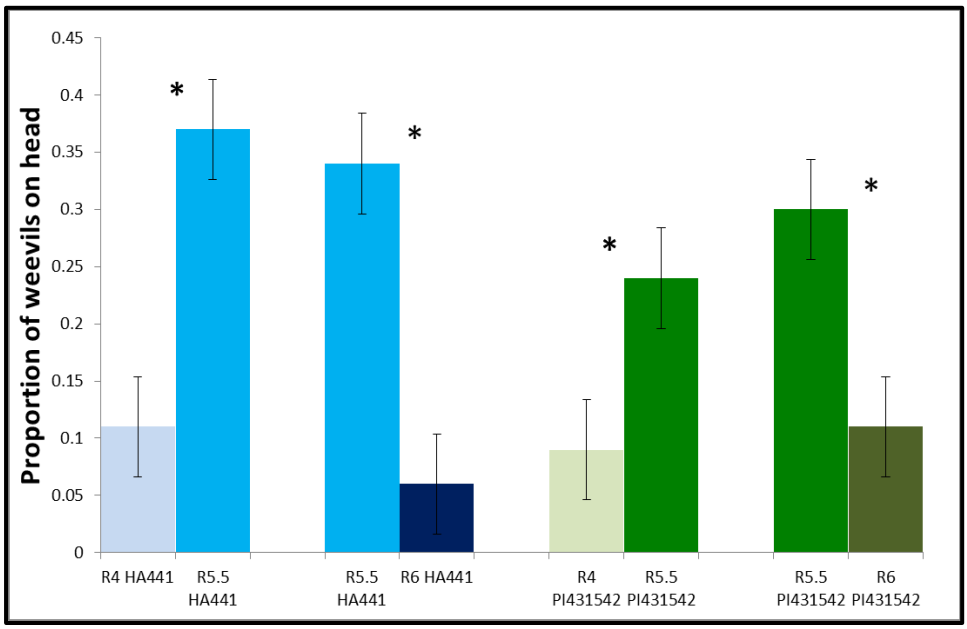

Figure 9. Comparison of proportions of adult *Smicronyx fulvus* on R5.5 and R4 heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines; R5.5 and R6 heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines (*Indicates a difference, $p<0.05$, in proportions for a given head stage between given two plant lines).

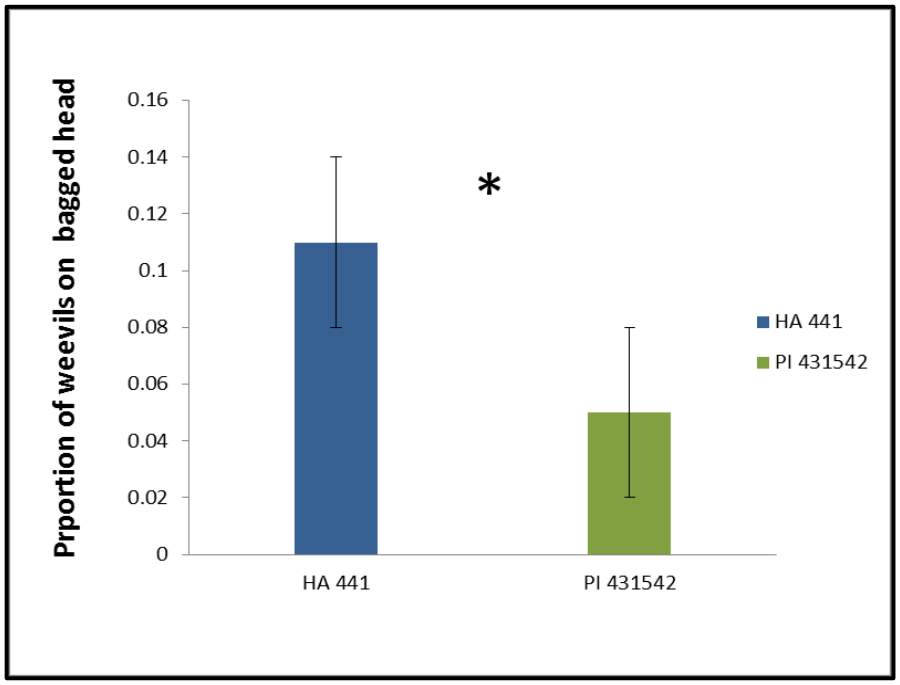

Figure 10. Proportions of adult *Smicronyx fulvus* on bagged R5.5 heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines (*Indicates the difference, $p<0.05$, in proportions for R5.5 head stage between the two lines).

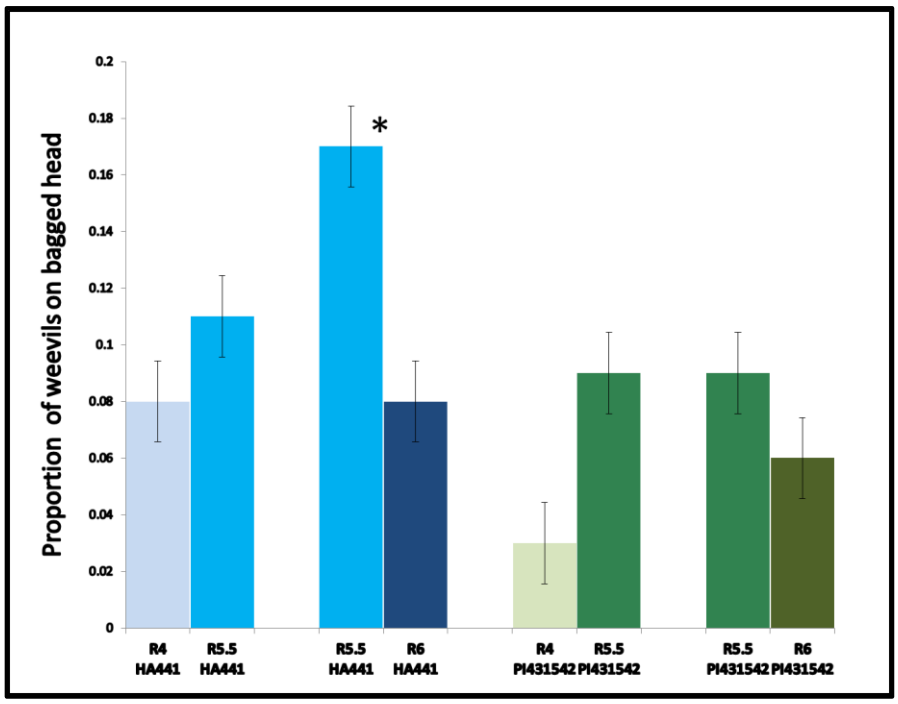

Figure 11. Comparison of proportions of adult *Smicronyx fulvus* on R5.5 and R4 bagged heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines; R5.5 and R6 bagged heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines (*Indicates a difference, $p<0.05$, in proportions for a given head stage between two lines).

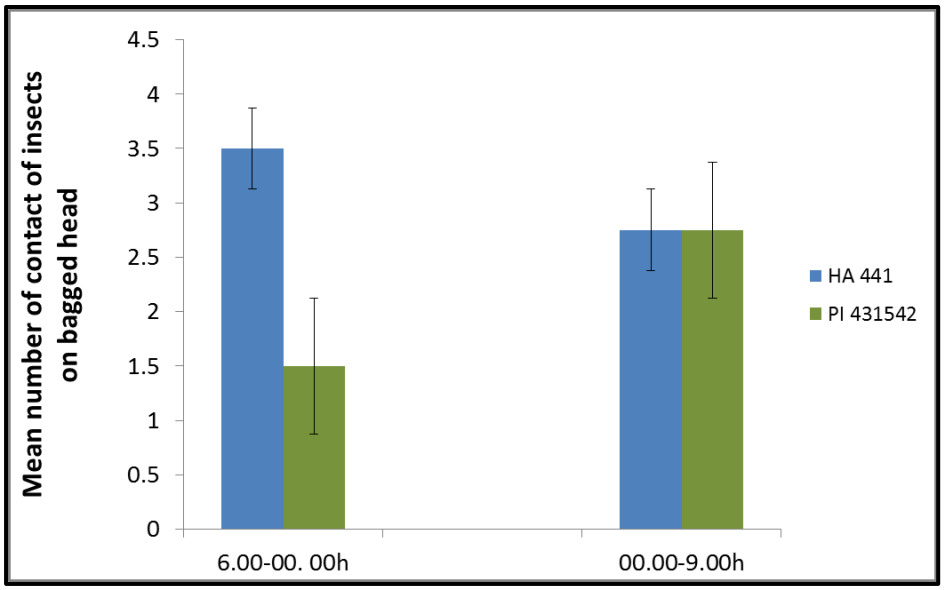

Figure 12. Mean number of contact of adult *Smicronyx fulvus* on bagged R5.5 heads of *Smicronyx fulvus* susceptible (HA 441) or resistant (PI 431542) plant lines under video observations over a 15h period.

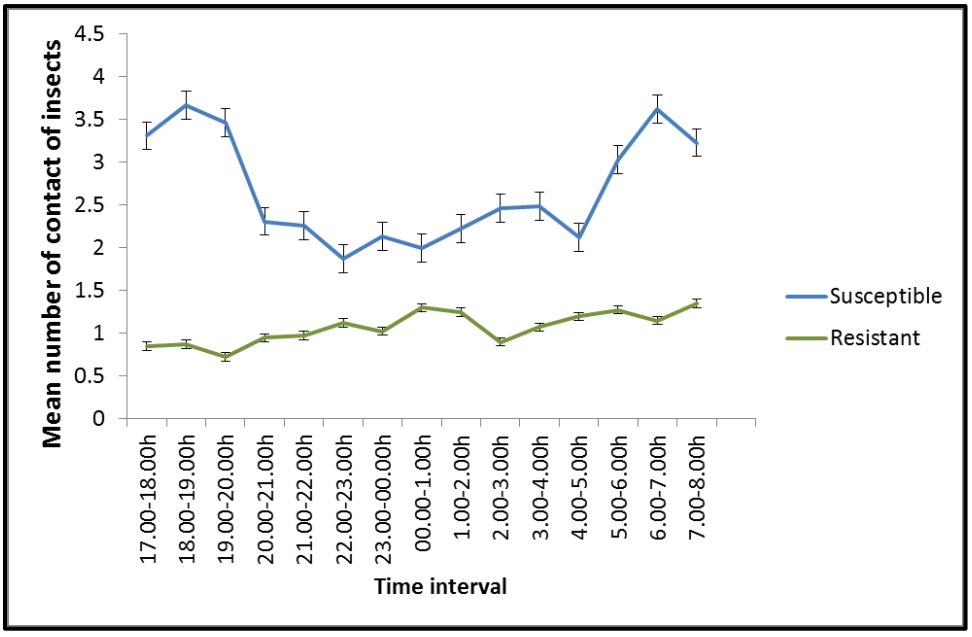

Figure 13. Mean number of contact of adult *Smicronyx fulvus* on pollen of a *Smicronyx fulvus* susceptible (HA89) or a resistant (PI 170411) plant line under video observations over a 15h period.

Discussion

In my bioassay, consisting of placing two sunflower plants in a cage, releasing adult weevils at dusk, and counting the numbers of weevils on each of the plants in the morning, I was able to demonstrate that adult RSSW are attracted to sunflower heads. I used these bioassays to test what stage of head adults were most attracted to and whether adults were preferentially attracted to purportedly susceptible plant heads over purportedly resistant plant heads. The experiment clearly showed that weevils preferred R5.5 heads over both R4 and R6 heads, and that weevils preferred both R5.5 and R6 heads of susceptible plants to those of resistant plants. Thus, given the similar sizes and appearance of the susceptible and resistant plants, it appears likely that either volatile chemicals or contact cues, either chemical or tactile, are involved in host finding and host discrimination by adult weevils.

However, on the basis of this experiment alone, I could not conclude that the distribution of weevils on the plants at the end of the experiment was due to more weevils being attracted to preferred plants using host volatiles. The distributions of weevils could be explained, for example, by random finding of a plant and then weevils being influenced by contact cues, either non-volatile chemicals or tactile cues, or indeed by plant feeding (itself likely influenced by chemicals in the plant tissue). To test whether volatile chemicals were involved in the discrimination, I enclosed the heads with netting that allowed volatile chemicals to escape but precluded contact of the weevils with the head, thus precluding contacting chemical/tactile cues or even feeding on head material. This showed conclusively that volatile chemicals were involved in the host discrimination as more RSSW were found on bagged susceptible plant heads than on bagged resistant plant heads, and on bagged R5.5 over bagged R6 heads of the susceptible line. I further confirmed this effect by video recording of landings on the bagged

heads and found that weevils landed more frequently on bagged susceptible heads during the early part of the night than they did on bagged resistant heads.

To further test whether pollen was involved in this discrimination, I video recorded weevil responses to pollen from susceptible and resistant heads in a choice test overnight. This experiment showed that adult weevils clearly contacted susceptible over resistant pollen. The higher frequency of contact with the susceptible plant pollen suggests that volatile chemicals may influence the contact, although given the closeness of the two pollen piles in the Petri dish and the relatively low resolution of the video recordings in low light, I cannot rule out that weevils contacted pollen with their antennae but this was not observed and recorded. In that case, brief antennal contact with preferred pollen could result in more weevils moving onto the pollen, with only the more apparent contact being recorded when I reviewed the videos.

Altogether, my results demonstrate that adult RSSW use plant chemicals during host selection, and that differences in chemicals are likely responsible for the preferences of weevils for different stage of plant development, and between resistant and susceptible sunflower plants. While my results suggest that both volatile and contact chemicals may be involved during host selection and for determining the preferences, how these chemicals function, by either stimulating or repelling, cannot be determined and needs further study. For instance, the preferences toward R5.5 over other heads, or the preference of the susceptible over the resistant line, might be due to increased production of stimulatory ("attractants") volatiles by the R5.5 heads, or susceptible line, or by production of increased quantities of repellent chemicals, or of repellent blends of chemicals by the R4 and R6 heads and the resistant line, respectively, or even a combination of both. Similarly, R5.5 (or the susceptible line) heads might contain greater

amounts of chemicals that stimulate feeding or staying (e.g., for oviposition) or lesser amounts of inhibitory compounds than do R4/R6 heads or resistant plants.

Role of chemicals in stimulatory/inhibitory roles have been demonstrated in previous studies where different chemical combinations have been used and where the chemical compositions of plants have been examined. For example, Nordlander (1990) investigated the effect of several combinations of two host monoterpenes (i.e. α-pinene and limonene) on field responses of *Hylobius abietis* and *H. pinastri* (Coleoptera: Curculionidae) and found that both species were attracted to α-pinene, but limonene, released even at very low concentrations (ca. 1/50 that of α-pinene) completely inhibited the attraction of these species to α-pinene. In another study, the effect of volatiles on the host choice in RSSW was well demonstrated (Roseland *et al.*1992). They combined five monoterpenoids (i.e. α-pinene, β-pinene, limonene, camphene and bornyl acetate) along with other volatile chemicals to resemble a RSSW-susceptible USDA standard cultivar (Hybrid 894) or a French cultivar (H_9P_1), "which is poorly visited" by insects. They found that weevils were more attracted to a mixture of volatiles that contained α-pinene, β-pinene, limonene, camphene and bornyl acetate in a ratio resembling the RSSW-susceptible Hybrid 894 rather than to a mixture of volatiles that contained α-pinene, β-pinene, limonene, camphene and bornyl acetate in a ratio resembling the RSSW-resistant H_9P_1. Further, Roseland *et al.* (1992) also observed that when sabinene, another prominent volatile compound in sunflower, was substituted for one of the five compounds (i.e., α-pinene, β-pinene, limonene, camphene and bornyl acetate), there was a tendency for decreased attraction of RSSW. They suggested that sabinene might be acting as a deterrent to RSSW.

Wilkinson (1980) studying recurrent white-pine weevil, *Pissodes strobe* attacks on eastern white pines (*Pinus strobus*), found that trees with both low limonene and high α-pinene

concentrations were the least susceptible to white-pine weevil attacks. A study by Vuts *et al.* (2015) on responses of the two-spotted oak buprestid, *Agrilus biguttatus* to host tree volatiles of Oak isolated γ-terpinene from bark and (E)-4,8-dimethyl-1,3,7-nonatriene from foliage as two important components among 13 substances identified. For virgin females and males, (*E*)-4,8-dimethyl-1,3,7-nonatriene was among the most active compounds for attraction while for mated females, γ-terpinene was among the most active compounds for attraction. Agrawal *et al*. (2002), who studied the attractiveness of bitter cucumber plants to natural enemies of herbivores, suggested that (3***E***)-4,8-dimethyl-1,3,7-nonatriene could be an attractant for potential predators of herbivorous insects.

My chemical analysis (see Chapter 2) of sunflower cultivars, including the two used in this bioassay, identified, α- pinene, sabinene and limonene as the principle volatile compounds emitted by sunflower. The role of these chemicals, along with others identified in my study, in host-finding by adult RSSW was not determined in my study. Clearly, both further chemical and behavioral studies are needed to determine the volatile and contact chemicals emanating from both pollen and head tissue of sunflowers that contribute to host selection and/or host resistance to RSSW. Such studies have already been done on other important insect pests of sunflower. For example Morris *et al*. (2005) reported that two diterpenoids, entkauran-16α-ol and ent-atisan-16α-ol from sunflowers stimulate oviposition by female banded sunflower moth, *Cochylis hospes* (Lepidoptera: Tortricidae). Subsequently, Morris et al. (2009) isolated three more oviposition stimulant Diterpenoid Acids for this insect; grandifloric acid, 15β-hydroxy-ent-trachyloban-19-oic acid, and 17- hydroxy-16α-ent-kauran-19-oic acid. They suggested that an alcohol functional group on ring D (at positions 15, 16, or 17) in all these 5 compounds may be responsible for stimulating oviposition in the insect.

With reference to insect attraction to contact stimuli, non-volatile terpenoids sesquiterpene lactones that are found in capitate glandular trichomes of sunflower leaves and florets have drawn attention for their role in defense against insects (Alfatafta and Mullin 1992;Chou and Mullin 1993; Prasifka *et al*. 2015). Alfatafta and Mullin (1992) examined the epicuticular floral chemistry of the cultivated sunflower isolating 5 new sesquiterpene lactones; 3-*O*-methylniveusin A and 1,10-*O*-dimethyl-3-dehydroargophyllin B diol, the eudesmanoic acid eudesma-1,3,11(13)-trien-12-oic acid, the diterpene 7-oxo-trachyloban-15α, 19-diol and the new 5-hydroxy-4,6,4′-trimethoxyaurone. They have shown to be strong antifeedant to adult western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae).Chou and Mullin (1993) identified seven antifeedant sesquiterpene lactones (STLs) in 3 sunflower cultivars and the antifeedant activity for Western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)showed that, there was a positive relationship with sesquiterpene lactone content, particularly with argophyllin A and its isomer argophyllin B. Antifeedant argophyllins were particularly high in newly grown leaf and floral tissues, strongly suggesting a chemical defense of the chemical against insect herbivory. Prasifka *et al*. (2015) point out that sesquiterpene lactones on the floret glandular trichomes act against floret feeding insects due to their very high density on disc florets and, bioassays with larvae of sunflower moth, *Homeosoma electellum* (Lepidoptera: Pyralidae) showed that the larval mass decreased by more than 30% when the argophyllin B concentrations used were higher than that of florets. They also found that the mixtures of sesquiterpene lactones extracted from cultivated sunflower florets were more effective causing 40% larval mortality. The role of glandular trichome sesquiterpene lactones from other *Helianthus* species as defense against sunflower moth, *H. electellum,*also has beendemonstrated in previous studies (Rossiter *et al*. 1986; Roger *et al*. 1987).

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CHAPTER 4. CONCLUSIONS

Plants of sunflower germplasm putatively resistant or susceptible to the red sunflower seed weevil, *Smicronyx fulvus* (Coleoptera: Curculionidae) were used to test the hypothesis that the volatile composition of these two types are different and may the acceptance/ rejection of sunflower germpalsm by *S. fulvus*. My results showed that, at least 13 volatile terpenoids were released by the different plant lines. These compounds included monoterpenes α-pinene, β-myrcene, camphene/γ-carene/β-pinene, bornyl acetate, γ-terpinene/α-terpinene, limonene and sabinene/β-phellandrene, the sesquiterpenes α-copaene, β-elemene, β-selinene, calarene/ β-gurjunene and germacrene-D, and the C_{11} homomonoterpene; (3*E*)-4,8- dimethyl-1-3-7-nonatriene. In general, across all the plant lines investigated, the mean concentrations of the monoterpenes α- pinene, sabinene/ β-phellandrene and limonene were greater than for the other compounds, which were often found in very small amounts. Some of the compounds varied in concentration according to plant line, head maturity, and time of day. When volatiles released by resistant lines were compared against those released by susceptible lines, there were no differences among the most abundant volatiles, α- pinene, sabinene/ β-phellandrene and limonene. However, there were differences among the less abundant compounds, including (3*E*)-4,8-dimethyl-1-3-7-nonatriene, α-copaene, bornyl acetate, β-elemene, β-selineneand germacrene-D. Thus, concentrations of one or more of these compounds may be useful markers for resistance/susceptibility to *S. fulvus*, although any role they have in host selection requires further work to determine. In behavioral binary choice bioassays, *S. fulvus* were attracted to sunflower heads, preferring R5.5 over R4 and R6 heads, and susceptible to resistant plants. Video recordings indicated both volatile and contact chemicals may be involved in host acceptance/ rejection by adult weevils.

APPENDIX. FIGURES

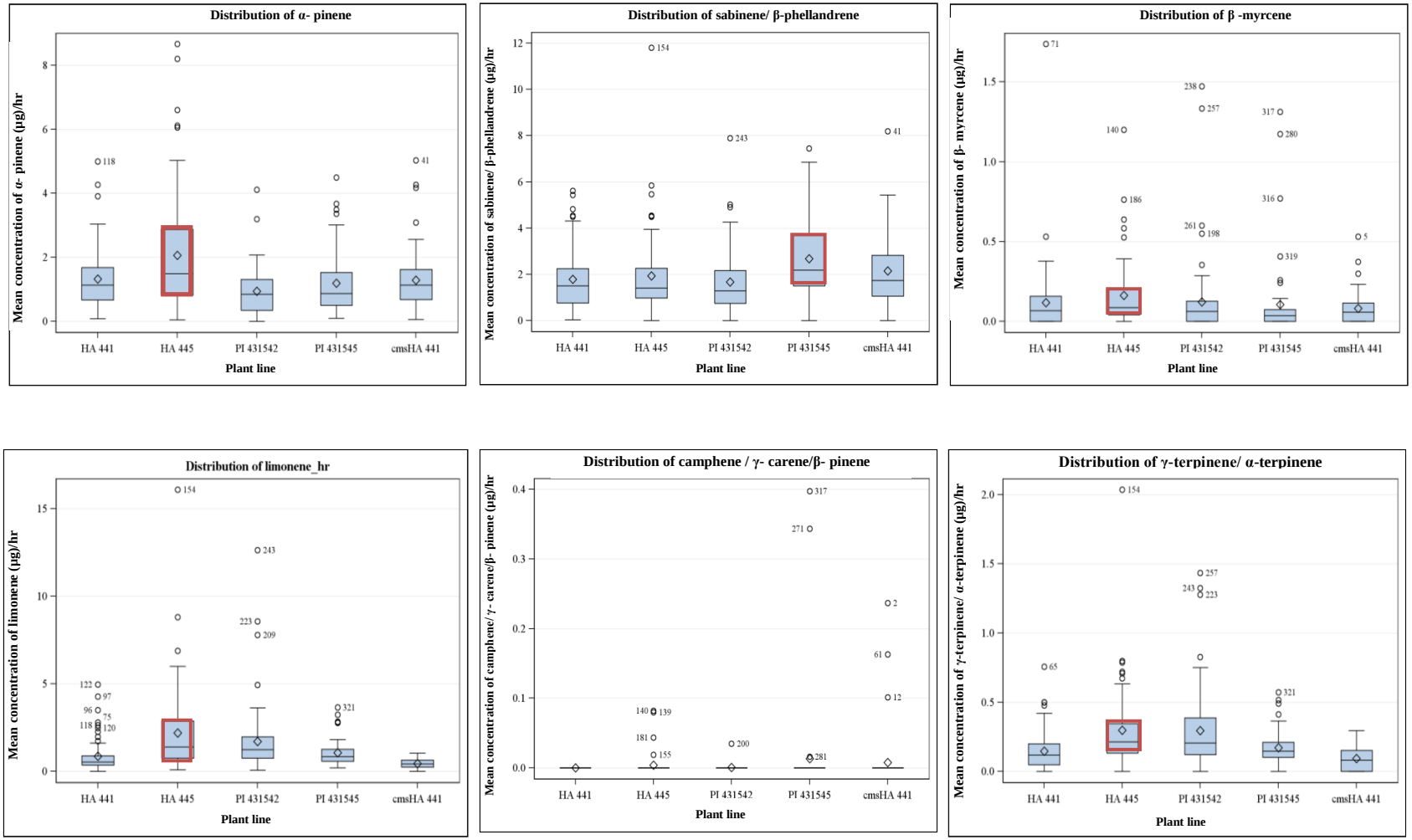

Figure A1. Distribution of each volatile compound for different sunflower lines in experiment 1 (The box plots show the distributions of the dependent variable for each plant line. The diamond inside the box is the mean, the box shows the 75th, 50th /median and 25th percentiles. Separate dots show points that are more than 1.5 IQ ranges above the upper quartile – thus they may be considered potential outliers). The red box plots are those significantly different from others.

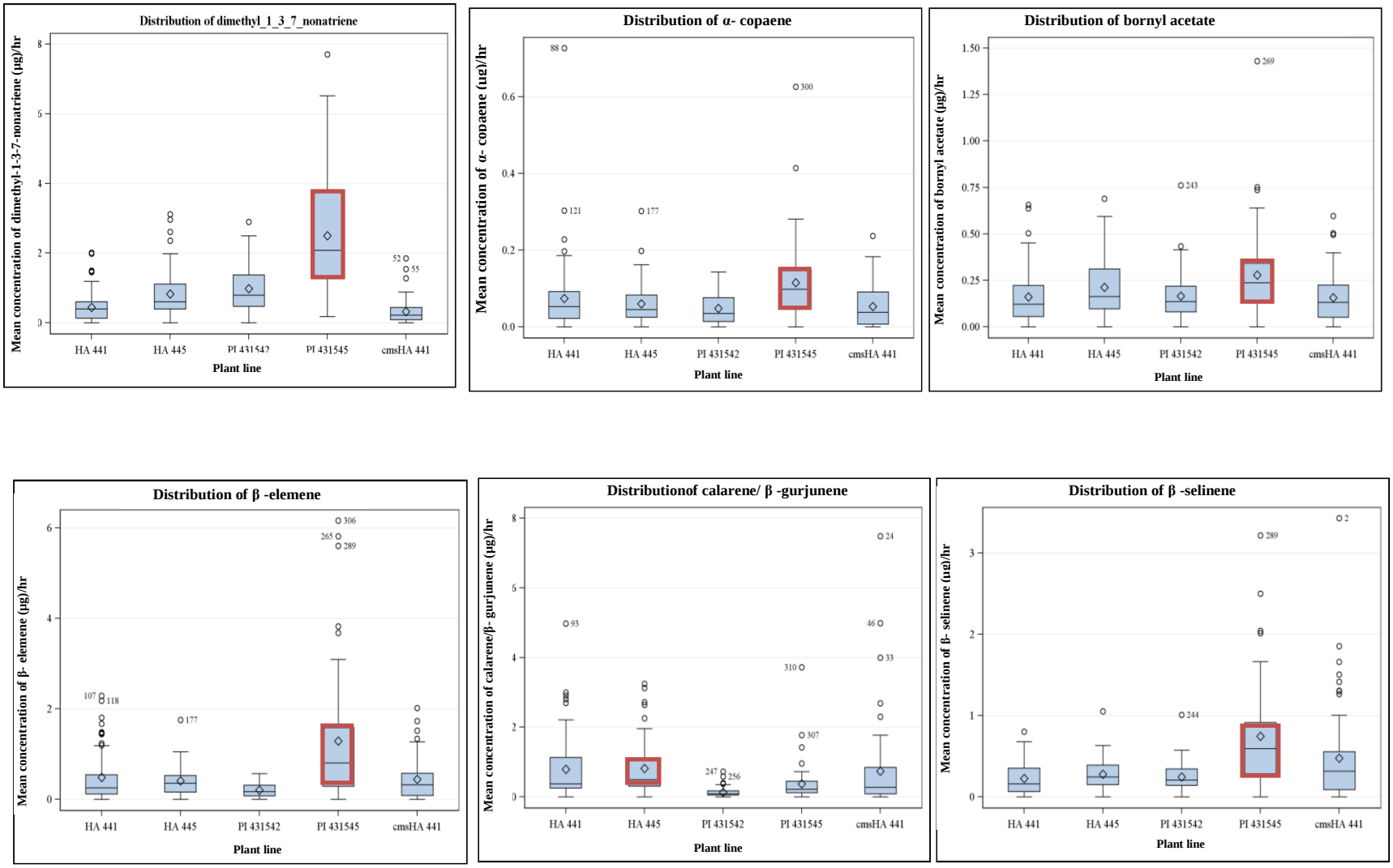

Figure A1. Distribution of each volatile compound for different sunflower lines in experiment 1 (continued).

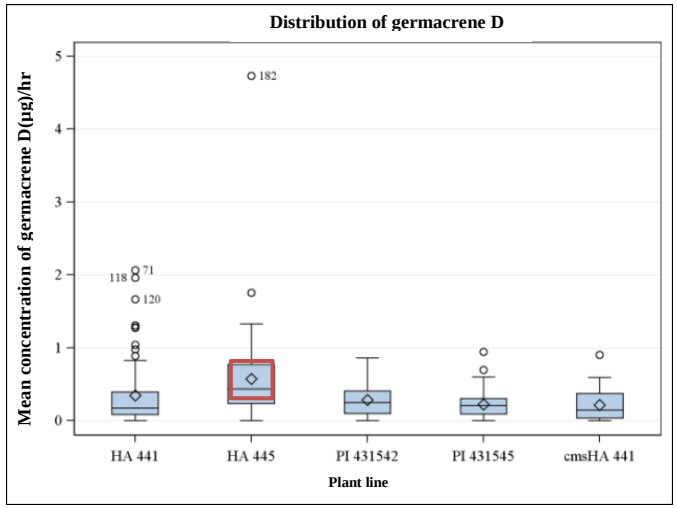

Figure A1. Distribution of each volatile compound for different sunflower lines in experiment 1 (continued).

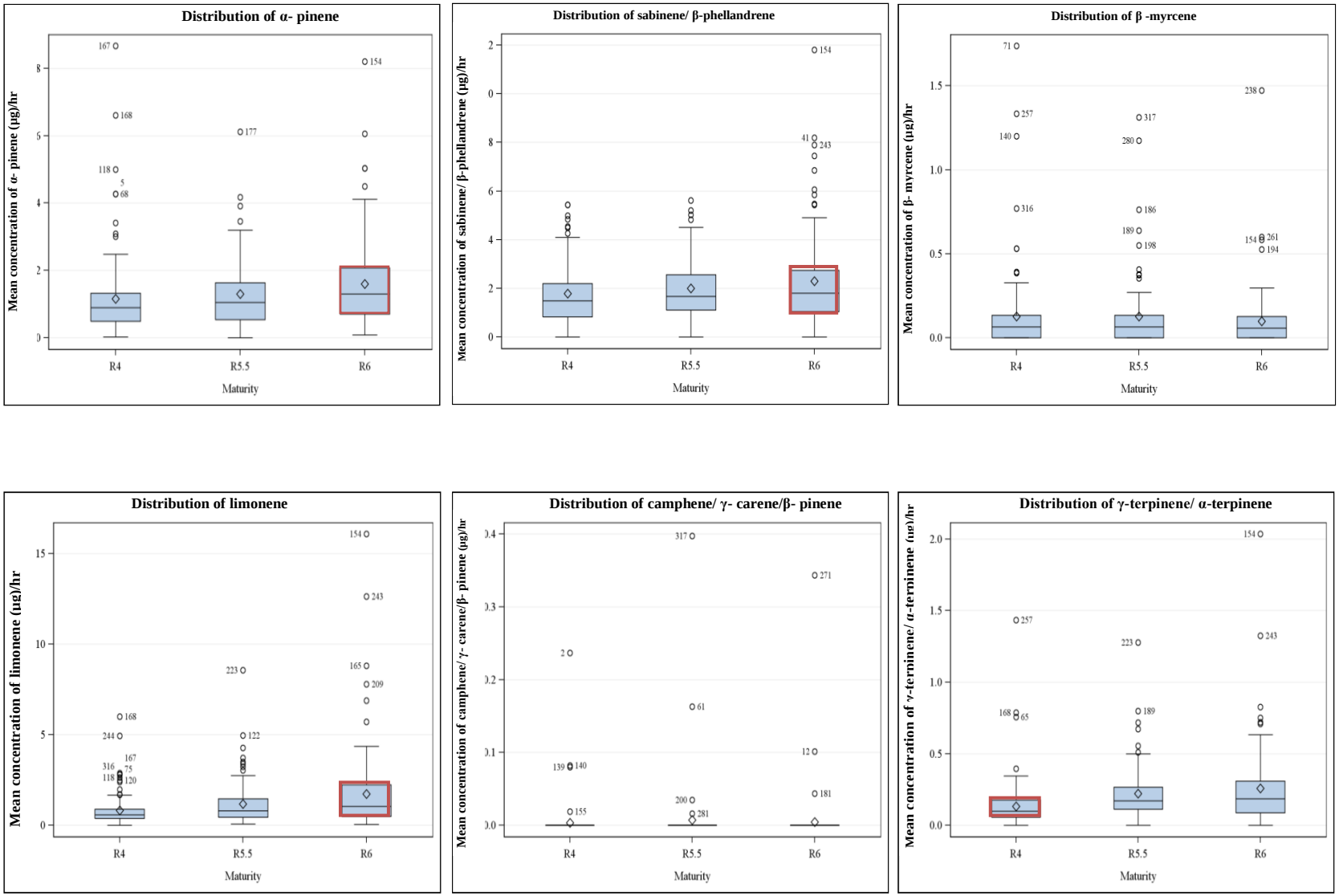

Figure A2. Distribution of each volatile compound for different sunflower head stages in experiment 1 (The box plots show the distributions of the dependent variable for each head stage. The diamond inside the box is the mean, the box shows the 75th, 50th/median and 25th percentiles. Separate dots show points that are more than 1.5 IQ ranges above the upper quartile – thus they may be considered potential outliers). The red box plots are those significantly different from others.

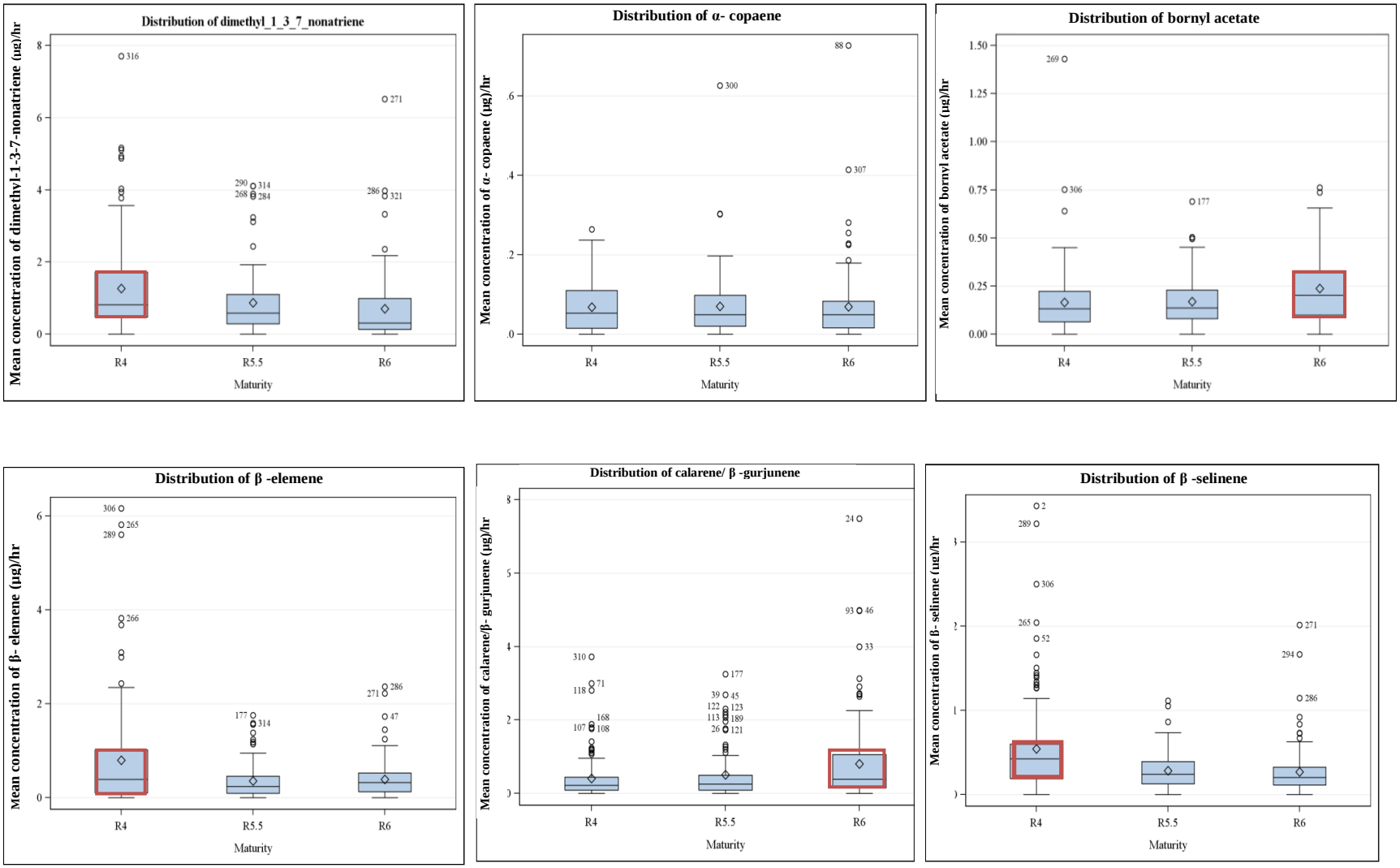

Figure A2. Distribution of each volatile compound for different sunflower head stages in experiment 1 (continued).

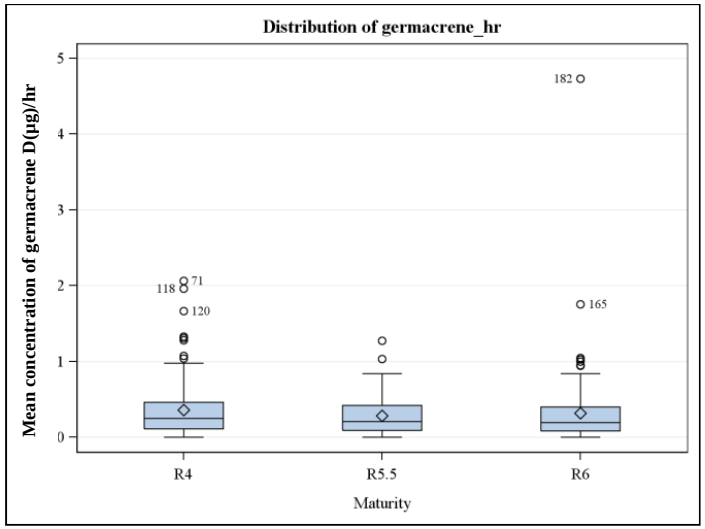

Figure A2. Distribution of each volatile compound for different sunflower head stages in experiment 1 (continued).

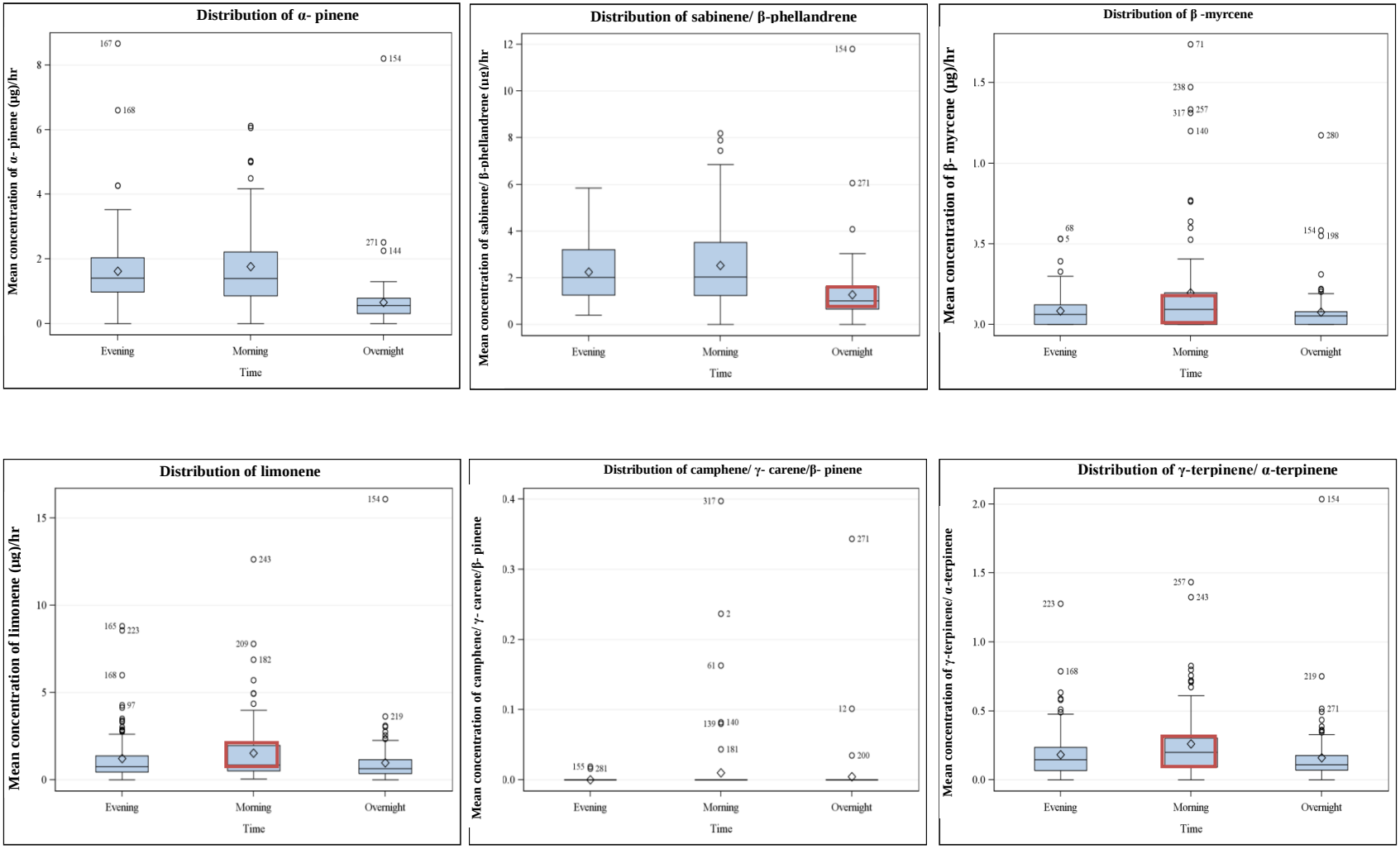

Figure A3. Distribution of each volatile compound for different time periods in experiment 1 (The box plots show the distributions of the dependent variable for each time period. The diamond inside the box is the mean, the box shows the 75th, 50th /median and 25th percentiles. Separate dots show points that are more than 1.5 IQ ranges above the upper quartile – thus they may be considered potential outliers). The red box plots are those significantly different from others.

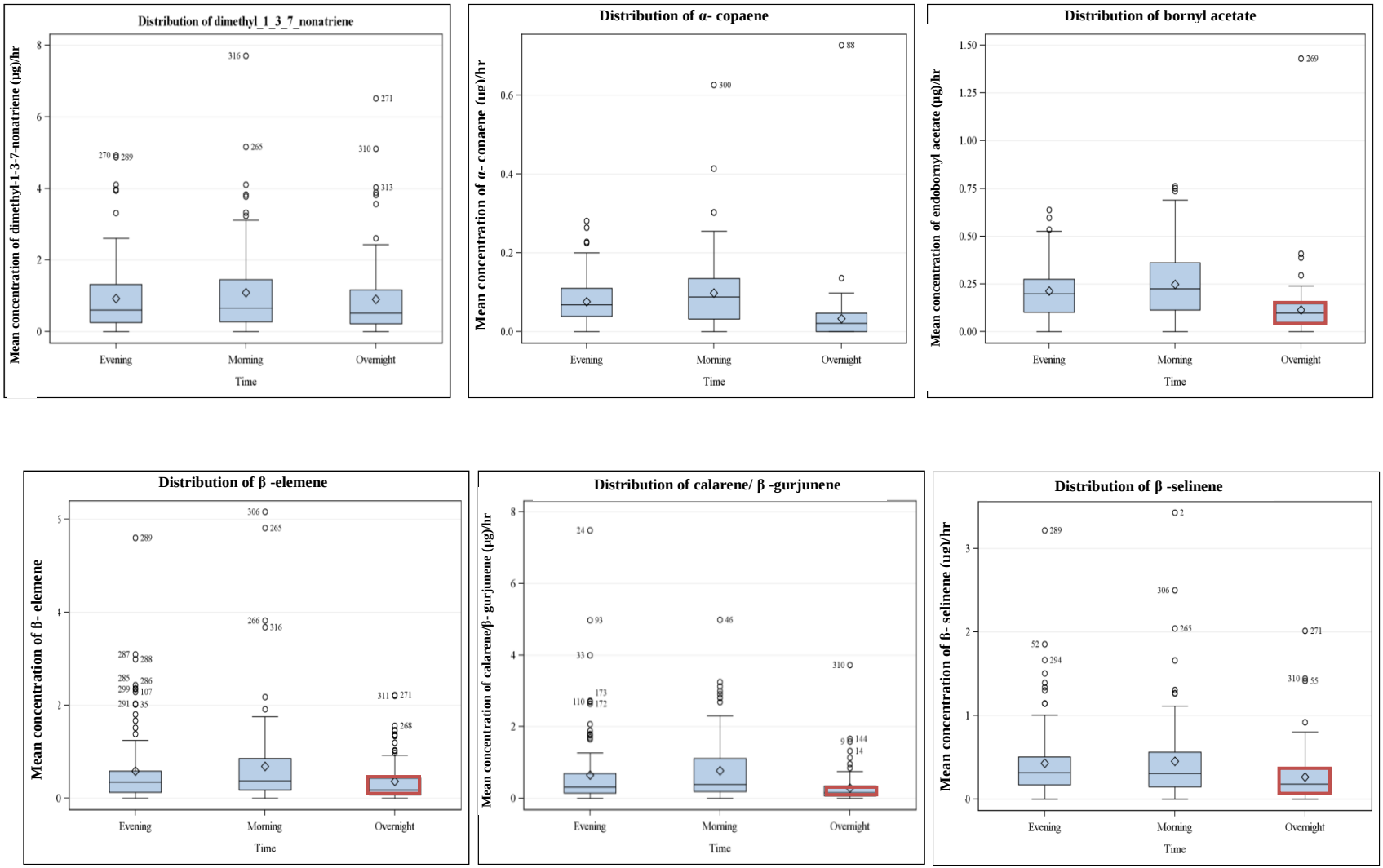

Figure A3. Distribution of each volatile compound for different time periods in experiment 1(continued).

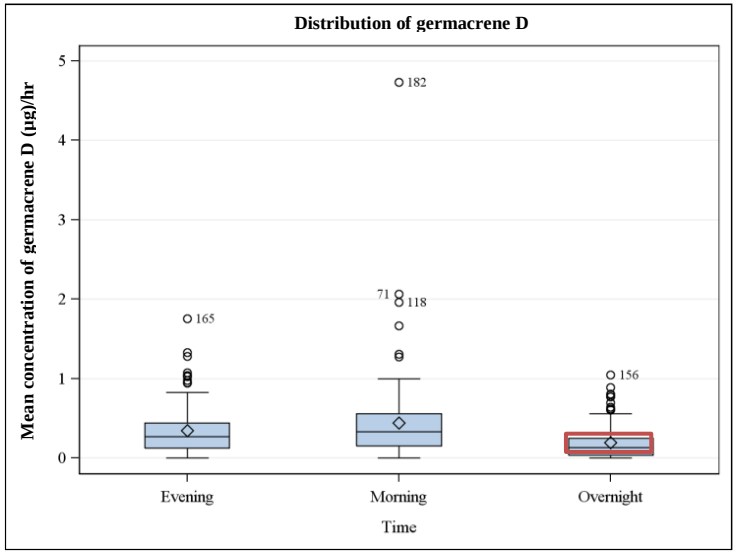

Figure A3. Distribution of each volatile compound for different time periods in experiment 1(continued).

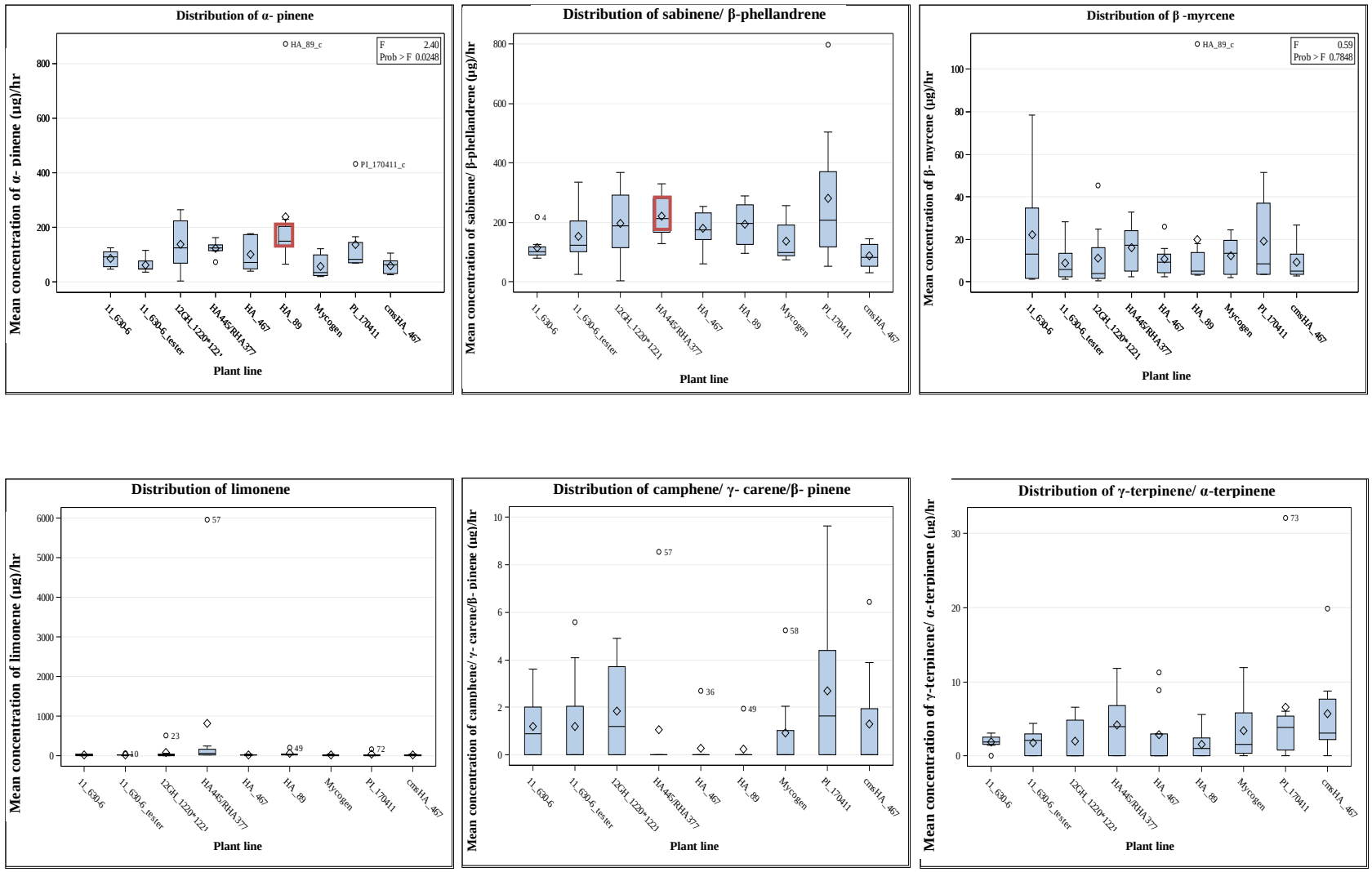

Figure A4. Distribution of each volatile compound for different sunflower lines in experiment 2 (The box plots show the distributions of the dependent variable for each plant line. The diamond inside the box is the mean, the box shows the 75th, 50th/median and 25th percentiles. Separate dots show points that are more than 1.5 IQ ranges above the upper quartile – thus they may be considered potential outliers). The red box plots are those significantly different from others.

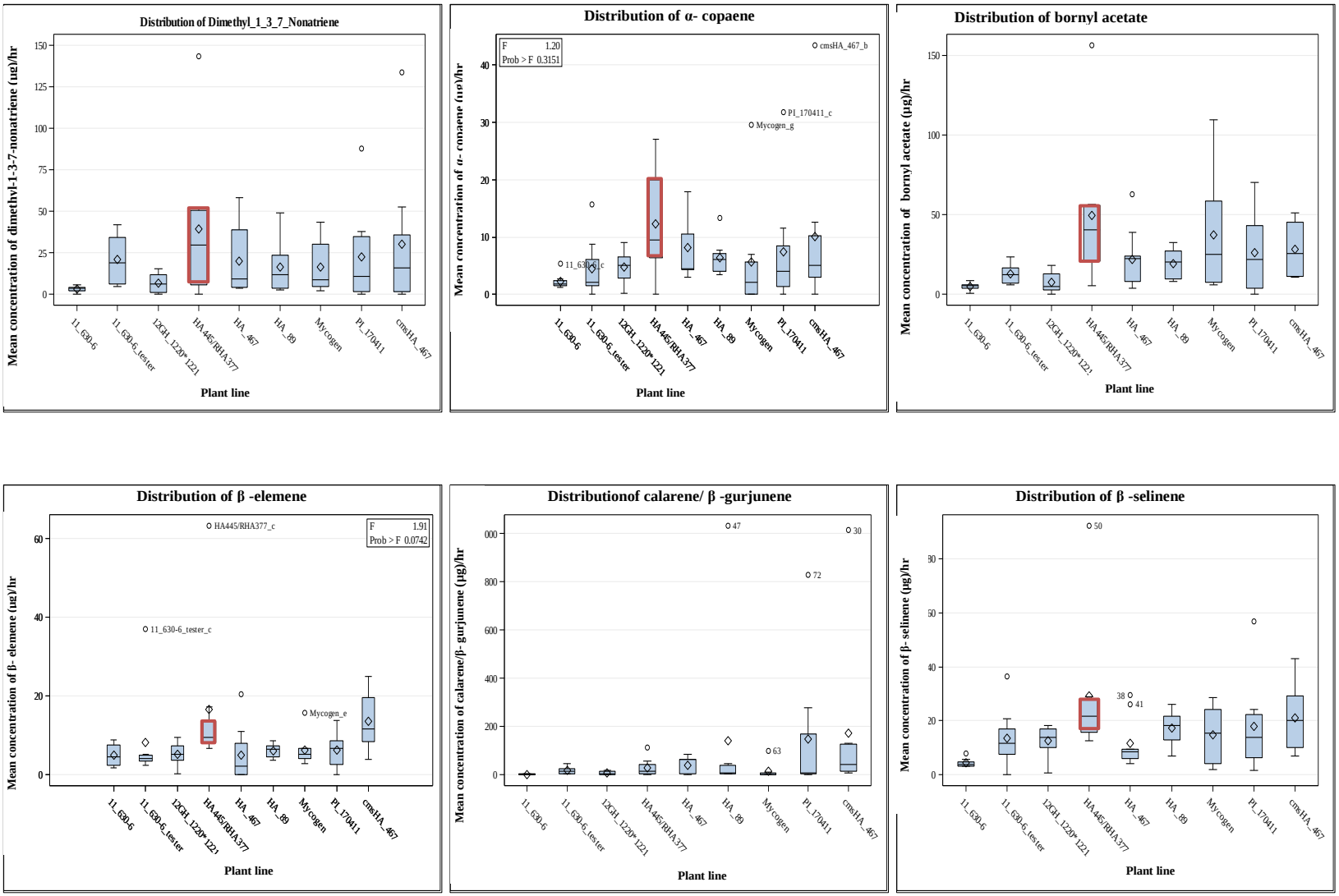

Figure A4. Distribution of each volatile compound for different sunflower lines in experiment 2 (continued).

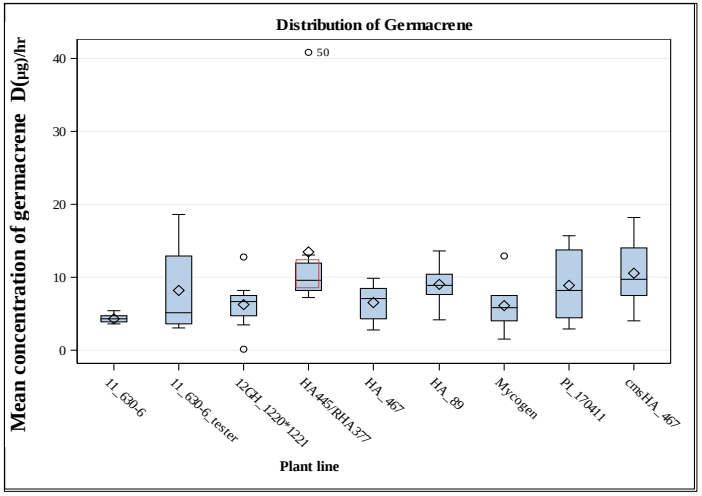

Figure A4. Distribution of each volatile compound for different sunflower lines in experiment 2 (continued).