COMMUNAUTE FRANCAISE DE BELGIQUE

UNIVERSITE DE LIEGE – GEMBLOUX AGRO-BIO TECH

Volatile Organic Chemicals in the Rhizosphere of Barley, and their Role on the Foraging Behavior of Wireworms (Coleoptera: Elateridae)

Fanny BARSICS

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs : Dr François J. Verheggen

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Barsics Fanny (2015). Volatile Organic Chemicals in the Rhizosphere of Barley, and their Role on the Foraging Behavior of Wireworms (Coleoptera: Elateridae). (PhD Thesis) Gembloux, Belgique, University of Liège, Gembloux Agro-Bio Tech, 182 p., 8 tabl., 14 fig.

Abstract – Many species of wireworms, the larvae of click beetles (Coleoptera: Elateridae), are known as worldwide belowground pests of a large range of crops including cereals. Pesticide based agricultural practices seem to have allowed significant population reduction in the past, but there is an increasing need for alternative control methods. In the first Chapter of this work, we review the current knowledge concerning 1) Integrated Pest Management (IPM) of wireworms and 2) their chemical ecology. These reviews shed light on promising approaches to develop new management strategies, as well as gaps of knowledge to be filled in that purpose. One of them is investigated here.

The research aim of this PhD thesis (detailed in Chapter II) is the identification of the volatile organic compounds (VOC) released by the roots and used by wireworms to locate their food source. To achieve this goal, we defined three objectives: 1) developing a behavioral assay suitable for the study of wireworm orientation behavior under exposure to root-produced VOC; 2) profiling VOC released in the rhizosphere, according to different growth conditions; and 3) evaluating the role of the identified VOC on wireworm foraging behavior. Our experimental model involved *Agriotes sordidus* Illiger wireworms and *Hordeum distichon* L. (spring barley).

In Chapter III, we report on the developmental steps of an adequate olfactometric method for wireworms. This includes an initial setup, the "Y-shaped" olfactometer, and dual-choice pipes gathering the necessary upgrades to efficiently observe the behavior of wireworms exposed to a VOC source. By resorting to live roots in a variety of features, we attempted to highlight appropriate abiotic and biotic experimental parameters, as well as the limits to the use of our olfactometric devices. The bioassays confirmed the attraction of wireworms towards VOC emitted by live roots. With a first sampling method on excised roots, Solid – Phase Micro – Extraction (SPME – semi-quantitative), coupled to Gas Chromatography – Mass Spectrometry (GC-MS), we detected a high number of molecules that might act as semiochemicals on wireworms, notably in our bioassays.

In the fourth Chapter, the bioassays were performed with selected VOC, detected during the chemical profiling. Firstly, we assessed if wireworms were attracted towards 2-pentylfuran, the main VOC highlighted with SPME. The molecule was submitted to the larvae by encapsulation in alginate beads. An adequate formulation allowed reaching an emission rate matching quantification estimates. In the aim to improve the VOC profiling, we also used Dynamic Head-Space Sampling (DHS), on roots ground in liquid nitrogen. It highlighted four volatile aldehydes. Their biological activity on wireworms was evaluated thanks to glass wool/triacetin-based dispensers, inserted in the dual-choice pipes.

Through this work, we show that VOC produced by barley roots carry information useable by foraging wireworms. The two sampling methods indicate two different modalities of root-VOC production and are encouraging for further understanding of belowground VOC production and emission. The developed olfactometric method and related results open new perspectives to increase knowledge on wireworm ecology. Combined to other developments in research on their management, they could lead to interesting, innovative and ecological management practices.

Barsics Fanny (2015). Le Rôle des Composés organiques Volatils de la Rhizosphère de l'Orge sur le Comportement des Larves de Taupins (Coleoptera : Elateridae). (Thèse de doctorat) Gembloux, Belgique, Université de Liège, Gembloux Agro-Bio Tech, 182 p., 8 tabl., 14 fig.

Résumé – Les « vers fils-de-fer » sont les larves des taupins (Coléoptères, Elateridae). Beaucoup d'espèces se développent dans le sol et sont reconnues comme ravageurs de multiples cultures dont des céréales. Les pesticides semblent avoir mené à la réduction des populations par le passé, mais il existe un besoin croissant de méthodes de lutte alternatives. Dans le Chapitre I de ce travail, nous révisons les connaissances actuelles 1) en matière de Lutte Intégrée contre les Ravageurs (LIR) appliquée aux larves de taupins, et 2) au sujet de leur écologie chimique. Nous dévoilons les approches prometteuses pour le développement de nouvelles stratégies de gestion, et les manques de connaissance à endiguer pour y parvenir. Nous investiguons l'une de ces voies dans cette thèse.

Notre objectif de recherche (détaillé au Chapitre II) est l'identification des composés organiques volatils (COV) émis par les racines, et utilisés par les larves pour localiser leur source de nourriture. Cela se décline en trois sous-objectifs : 1) développer un système d'observation du comportement d'orientation des larves de taupins soumises à des COV racinaires ; 2) définir les COV émis par des racines sous différentes conditions de croissance ; et 3) évaluer leur rôle sur le comportement des larves. Le modèle expérimental implique le taupin *Agriotes sordidus* Illiger et l'orge de printemps, *Hordeum distichon* L.

Dans le Chapitre III, nous développons une méthode olfactométrique adaptée aux vers fils-de-fer. Ceci inclut l'utilisation d'olfactomètres « en Y », et de tubes à choix-double pourvus des améliorations nécessaires pour observer efficacement l'orientation de larves exposées à des COV. Par recours à des racines vivantes produites sous diverses conditions expérimentales, nous identifions les paramètres expérimentaux biotiques et abiotiques adéquats, ainsi que les limites d'utilisation des tubes. Nous montrons que les larves sont attirées par les COV issus de racines vivantes. Une méthode d'échantillonnage, la Micro-Extraction sur Phase Solide (SPME – méthode semi-quantitative), couplée à la Chromatographie en Phase Gazeuse – Spectrométrie de Masse (GC-MS), permet la détection de nombreuses molécules à caractère potentiellement sémiochimique, notamment dans nos tests biologiques.

Dans le Chapitre IV, les tests biologiques sont réalisés avec des COV détectés durant le profilage chimique des racines. Nous déterminons si les larves sont attirées par le 2-pentylfurane, un composé détecté par SPME. Il est diffusé dans les tubes à choix double grâce à une encapsulation en billes d'alginate. Une formulation adéquate permet un taux d'émission proche des quantités estimées émises lors des prélèvements par SPME. Pour améliorer le profilage de COV, nous utilisons l'Échantillonnage Dynamique en Espace de tête (DHS), sur des racines broyées en azote liquide. Cela montre quatre aldéhydes volatils. Leur activité biologique sur les taupins est testée grâce à des diffuseurs basés sur un mélange en triacétine intégré dans de la laine de verre.

Ce travail démontre que les COV racinaires sont porteurs d'informations utilisées par les larves de taupins. Les méthodes d'échantillonnage de COV montrent des aspects différents de leur production et sont encourageantes pour la compréhension future de l'émission de COV souterrains. Notre méthode olfactométrique et les résultats y afférent ouvrent de nouvelles perspectives d'amélioration des connaissances écologiques sur les larves de taupins. Combinées à d'autres développements dans la recherche sur leur gestion, cela pourrait mener à des méthodes de lutte intéressantes, innovantes et écologiques.

There are many people whom I wish to thank for their constant support, sharing of ideas, and most importantly for helping me keeping it up day after day during recent years.

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To conclude this acknowledgement section on a funny tone, here are some amazing things I've learned about my coworkers… The Chief knows an extraordinary amount of Walloon sayings. Curiously, they are often entomology related. The best one is "*On n'fé nin des mohes a deûs cous*" (i.e. "One can't create flies with two butts"). The Former Chief does not like when chairs are stolen, and is unstoppable when involved in crazy bets. My advisor, a.k.a "The First", "Optimus Prime", or "Primy", has terrific diabolo skills. In the Unit, everybody has excellent culinary abilities and likes barbecues and whatever drinks come along with the meat. Many coworkers are excellent river swimmers or tiger sharks. A few people are addicted to a strange worldwide treasure hunt. I got caught in the game easily. In their passion for entomology, they even help some peculiar bugs travelling. One of these cachers works in my office. She is unbreakable, although she tries to prove that wrong. The French buddies like Belgium and are amazed by our version of the French language. One of them works with the "zaidenôseuh". I still have no idea what this is.

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General Introduction

The current worldwide agricultural challenges involve a convolution of disciplines. The biggest concern consists in providing present and future generations food supplies with equivalent, if not better, nutritional quality than has been produced up to now (Gilland, 2002, El-Hage Scialabba, 2007, Niggli et al., 2007, Lairon, 2010). This means producing more, since populations are growing, while maintaining the integrity of ecosystems and their services (Pimentel et al., 1997, Daily et al., 1998, Foley et al., 2005, Tilman et al., 2011). As a consequence, we need to shift from intensive, health and environment threatening crop production methods, to safer and sustainable strategies. This implies the revision and optimization of agricultural systems. This idea has been reinforced in the last twenty-five years, as witness the many scientific reviews discussing promising biological methods (Vet and Dicke, 1992, Agelopoulos et al., 1999, Cook et al., 2007, Wenke et al., 2010, Witzgall et al., 2010). They are essentially based on understanding interactions between organisms of the environment (Duffy, 2002). Once these mechanisms are depicted, it is possible to manipulate them. Developing biological methods therefore necessitates an increase of ecological knowledge concerning organisms belonging to agricultural landscapes. Applied studies linking theoretical concepts, laboratory and field observations are essential in that approach (Vet and Dicke, 1992).

Among the factors determining the ecology and behavior of insects, one can find an abundant list of natural chemical compounds, which they produce or perceive from their environment. These semiochemicals carry information related to available resources, notably host physiological stage and suitability (Vet and Dicke, 1992, Kessler and Baldwin, 2001), and the presence of conspecifics (Carroll et al., 2006, Witzgall et al., 2010), competitors (Verheggen et al., 2013), preys (Svensson et al., 2004, Verheggen et al., 2008) or natural enemies (Cook et al., 2007). They govern many inter- and intra-specific interactions. Chemical ecology is the discipline describing these trophic interactions, considering the identities, roles and cause of emission of compounds in a given interactive model. Semiochemicals are of great interest for Integrated Pest Management (IPM) approaches, as they can be used to control agricultural pests, leading, in the most successful cases, to the reduction of noxious impact of intensive agriculture (Vet and Dicke, 1992, Agelopoulos et al., 1999, Cook et al., 2007). With the current health and environment concerns, global legislations progressively tend to favor control strategies involving natural control agents (i.e.

active substances, biocontrol agents), rather than synthetic chemicals (EC, 2009). A direct consequence is the progressive withdrawal from markets of synthetic compounds previously considered as the best treatments for crop threatening diseases and pests. The need for alternative strategies is therefore no longer an option, but is driven by Regulations (Jehle, 2011). This concerns many pests across the Class of Insects.

Wireworms are the larval stage of click beetles (Coleoptera: Elateridae) and many species are important pests of a wide range of cultivated crops. These species develop in the soil during several years before pupating and inflict damages hardly manageable once their populations are established. In Europe and North America, yield losses are often attributed to species of the genus *Agriotes*. Most of the recent research on their integrated management concerns tools to forecast wireworm swarming, and identify combinations of factors leading to it (Barsics et al., 2013). Investigating wireworm chemical ecology represents an interesting approach for completing currently developed management methods. The knowledge in this area is indeed characterized with gaps and could benefit from methodological and technological updates in several fields of bioengineering.

This introductive chapter is divided into two review papers, mostly addressing *Agriotes* wireworms, and shedding light on 1) recent advances in management strategies and promising approaches to improve them (Barsics et al., 2013), and 2) the available data regarding the chemical ecology of wireworm pest species, and the methods and techniques that should be combined in order to provide new usable information (Barsics et al., 2014b).

Finally, it should be clarified that wireworm species cited in this work are pests found in agricultural land, and information regarding other species, such as saproxylic species (e.g *Elater* and *Ampedus* spp.) was not included in the reported literature. The conclusions at the second section of this introduction can however be applied to these species, since data concerning their chemical ecology has already proven useful in conservation biology, notably involving the click beetle *Elater ferrugineus* (Svensson et al., 2004, Svensson and Larsson, 2008, Larsson and Svensson, 2009).

Chapter I.1 – Wireworms' Management: An Overview of the Existing Methods, with Particular Regards to *Agriotes* spp. (Coleoptera: Elateridae)

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Abstract - Wireworms (Coleoptera: Elateridae) are important soil dwelling pests causing worldwide yield losses in many crops. The progressive restrictions in the matter of efficient synthetic chemicals for health and environmental care brought out the need for alternative management techniques. This paper summarizes the main potential tools that have been studied up to now and that could be applied together in integrated pest management systems, and suggests guidelines for future research.

Keywords: wireworms; click beetles; *Agriotes*; integrated pest management

1. Introduction

Wireworms are the larvae of click beetles (Coleoptera: Elateridae). They consist of more than 9,000 species distributed worldwide, (Jeuniaux, 1996) and some are important pests of a wide variety of crops, such as potato, cereals, carrot, sugar beet, sugarcane and soft fruits (Miles, 1942, Hall, 1990, Blot et al., 1999, Vernon et al., 2000, Johnson et al., 2008). In Europe, damages due to wireworm infestation are mainly attributed to the genus *Agriotes* Eschscholtz, as witnessed by the numerous studies aiming at their management. It comprises more than 200 species worldwide, including more than 100 in the Palearctic region (Jeuniaux, 1996). Different recent sampling plans set in all of Europe (pheromone traps, molecular identification of collected larvae) allowed a reliable species mapping, showing that *Agriotes* communities were widely distributed across the continent (Kudryavtsev et al., 1993, Furlan et

al., 2001b, Furlan et al., 2006, Pic et al., 2008, Burghause and Schmitt, 2011, Kozina et al., 2012, Mulercikas et al., 2012). Their distribution in North America is also well described (Lafrance and Cartier, 1964, Vernon et al., 2001). The genus *Melanotus* Eschscholtz is mostly studied in Japan (*Melanotus okinawensis* Ohira) and in the USA (*Melanotus communis* (Gyll.)) ((Kuhar and Alvarez, 2008, Lindroth and Clark, 2009, Arakaki et al., 2010, Cherry and Nuessly, 2010); the species *Limonius canus* Leconte, is responsible, with other of the genus, for crop damage alongside the western coast of North America (Williams et al., 1993, Pantoja et al., 2010, van Herk et al., 2010). Other genera strongly impacting agriculture are *Athous* Eschscholtz, *Conoderus* Eschscholtz, *Ctenicera* Latreille and *Hypolithus* Eschscholtz (Lafrance and Cartier, 1964, Doane et al., 1975, Chalfant et al., 1992, Seal et al., 1992, Seal and Chalfant, 1994, Cherry and Alvarez, 1995, Hemerik et al., 2003, Jedlicka and Frouz, 2007, van Herk and Vernon, 2007b, Kuhar and Alvarez, 2008, Vernon et al., 2008, Lindroth and Clark, 2009, Willis et al., 2010). The main effects of wireworm feeding on stem bases and belowground plant organs are seedling mortality (Balachowsky and Mesnil, 1936) and all implied yield losses. Damage to potato tubers largely consists of small holes, narrow tunnels, a couple or more millimetres deep, and scarring to the periderm, which can significantly reduce tuber quality (d'Aguilar et al., 1980, Johnson et al., 2008).

Despite the numerous studies conducted to improve their management, wireworms remain important pests. In the UK, lindane and aldrin remained the mainstay of wireworm control in potato crops, until well into the 1990s. Their apparent effectiveness even stifled research on alternative control strategies for thirty years (1960 to 1990) (Parker, 2005). Their withdrawal from registration for environmental and health concerns gave rise to the need for alternative control methods (Parker, 2005). Soil-incorporated organophosphorous and carbamate insecticides replaced organochlorines, but consequent control levels were overall variable (Edwards and Thompson, 1971, Parker et al., 1990). Due to similar adverse secondary effects, including long persistence in the environment (with dramatic effects on non-target species as shown recently for fonofos (Elliott et al., 2008)) and human health concerns, they were progressively withdrawn from the markets, and those remaining may soon be phased out, even in North America (Waliwitiya et al., 2005, Kuhar and Alvarez, 2008). Multiple alternatives to broadcast application of pesticides have been examined worldwide. Their implementation in Integrated Pest Management (IPM) systems should provide good results, but demonstrations of such synergistic interactions are still rare. This paper summarizes the key data that should be considered and the IPM strategies that could be

used against wireworms, with particular regards to *Agriotes* spp. in Europe, and suggests guidelines for future research.

2. Species Identification, Lifecycle and Key Moments for Crop Protection

The key moments for predicting the implementation of IPM strategies are, firstly, adult population emergence (before oviposition and/or field cultivation), in order to prevent population settling or reinforcements and to enhance eggs and first instars mortality. Secondly, the periods during which the larvae actively feed are important, in regard to the timing of the cultivation of sensitive crops (Furlan, 2004). It is crucial for crop protection to know the species distribution and the features of their lifecycles that help spotting the right timing for control measures.

Adults of all agronomically important species have been well described (Jagemann, 1955, Leseigneur, 1972, Lohse, 1979, Zeising, 1984, Platia, 1994, Jeuniaux, 1996, Cate and Platia, 1997, Laibner, 2000). Although many tools are available for the identification of *Agriotes* wireworms, based on morphology (Emden, 1945, Dolin, 1964, Rudolph, 1974, Dolin, 1978, Klausnitzer, 1994, Cocquempot et al., 1999, Pic et al., 2008) or on genetic footprints (Pic et al., 2008, Ellis et al., 2009, Staudacher et al., 2011a), *in situ* direct identification remains an issue. In most cases, determining if individuals belong to a species is more difficult for larvae than for click beetles, sometimes suggesting doubts as to their actual separation (Furlan, 2005). Nonetheless, molecular advances have rendered possible community description at field scale (Ellis et al., 2009). For both click beetles and wireworms, an identification key (morphological, including molecular validation, as was done by Pic *et al.* (2008) for France) that is valid for all regions concerned (mainly Europe) should be created, that is suitable for use by a large group of interested people (Furlan, 2005). Very recent molecular research suggested the possibility of cryptic wireworm species or misidentifications on *Hypnoidus bicolor* Eschscholtz populations in Canada, highlighting the necessity to consider that approach in identification (Benefer et al., 2013).

Reliable information concerning the biology of the different species can be obtained by studying concurrently their entire cycle under laboratory conditions (rearing chambers, constant conditions), in rearing cages close to natural conditions and in open fields (Furlan, 2005), as reported in several studies (Furlan, 1996, Furlan, 1998, Furlan, 2004). Their study across different regions is interesting, but a better overall understanding can result from the comparison of development cycles in different environments (Furlan et al., 2004b). Good

biological information is available for the following species: *A. ustulatus* Schäller (Hinkin, 1983, Furlan, 1994, Furlan, 1996, Furlan, 1998), *A. sordidus* Illiger (Furlan, 2004), *A. brevis* Candeze (Rusek, 1972, Masler, 1982) and *A. litigiosus* (information only available for *tauricus* (Kosmacevskij, 1955)). Insufficient information is available for *A. obscurus* L. (Roberts, 1919, Roberts, 1921, Roberts, 1922, Regnier, 1928, Roberts, 1928, Subklew, 1934, Balachowsky and Mesnil, 1936, Cohen, 1942, Evans and Gough, 1942, Gough and Evans, 1942, Miles, 1942, Langenbuch, 1972), *A. sputator* L. (Roberts, 1919, Roberts, 1922, Gough and Evans, 1942, Kosmacevskij, 1955) *and A. lineatus* L. (Roberts, 1919, Subklew, 1934, Langenbuch, 1972). Information about *A. proximus* Schwarz and *A. rufipalpis* Brullé is almost inexistent and should be completed following the methods used for species whose biology has already been described (Furlan, 2005).

Click beetles are characterized by a multi-year lifecycle that differs dramatically between species. They can be divided into two main groups. The first one is constituted of species with adults that do not overwinter, live a few days and lay eggs a few days after swarming (*A. ustulatus*, *A. litigiosus*). The second one assembles species with adults that overwinter and live for months. These lay eggs for a long period after adult hardening (*A. sordidus*, *A. brevis*, *A. lineatus*, *A. sputator*, *A. obscurus*, *A. rufipalpis* and *A. proximus*) (Furlan, 2005). Adults emerge from the soil in spring. *A. sordidus* usually appears from late March to early April (Furlan, 2004). Females of *A. obscurus L., A. sputator L.* and *A. sordidus* lay their eggs in May or June, singly or in clusters, below the soil surface (Evans and Gough, 1942, Miles, 1942, Furlan, 2004), while the oviposition peak of *A. ustulatus* occurs in July and early August (Furlan, 1996). Oviposition would be reduced on arable land compared with grass (Gough and Evans, 1942), where comparatively more wireworms survive the early instars (Jary, 1942), because the eggs benefit from the protection against desiccation. Conditions in grasslands are so stable that wireworms may remain in the top 12 cm of soil during their whole development (Miles, 1942). The monitoring of wireworm survival in different rotations (continuous, very short interruption between different crops, discontinuous, long periods with bare soil between crops, meadow) confirms that soil with continuous vegetation cover allows more wireworms to survive (Furlan et al., 2009). The embryonic development is inversely related to temperature (Furlan, 1998, Furlan, 2004): *A. ustulatus* eggs hatch in 13 days when maintained at 29 °C and in 45 days when maintained at 15 °C (Furlan, 1998), while in the U.K, under agronomic conditions, *Agriotes* spp. eggs only hatch after 46 weeks (Miles, 1942, Parker and Howard, 2001). Temperature also influences wireworm movement: *A. obscurus*-

lineatus speed increases linearly from 8 to 19 °C in lab conditions, an important factor for behavioral experiments (van Herk and Vernon, 2013a). *Agriotes* larvae rarely disperse between crops or move far in the soil, putatively as long as local food supply is sufficient (Hemerik et al., 2003, Schallhart et al., 2011). Resistance to starvation increases with the age of wireworms: the last instars of *A. sordidus* can survive up to one year without food at 20 °C (Furlan, 2004). *Agriotes* wireworms are predominantly phytophagous, with an influence of the type of vegetable food on growth rates (Evans and Gough, 1942). The youngest larvae need live vegetable material to grow (Evans and Gough, 1942, Furlan, 1998, Furlan, 2004). Besides crops and weeds, *Agriotes* spp. also feed on animal prey. Traugott et al. (2008), using naturally occurring stable isotope signatures, highlighted intraspecific trophic plasticity by showing that 10% of a population of *A. obscurus* fed primarily on animal prey. They may even feed on congeners: cannibalistic interactions have been reported, where larval density is high and food is scarce (Langenbuch, 1972, Furlan, 1998). Some studies report on soil organic matter as a possible alternative food source (Schaerffenberg, 1942, Langenbuch, 1972, Hemerik and de Fluiter, 1999), but there is no proof that wireworms can survive or grow solely on non-live vegetable tissues. Recent work proved that high instars of *A. obscurus* predominantly fed on plant material (only 10% on soil organic matter, 95% confidence limits: 0.20%) (Traugott et al., 2008). In a study based on the isotopic signatures of organisms according to their trophic levels, wireworms kept 128 days without food in moist garden soil only negligible fed on soil organic matter (Traugott et al., 2007), which confirms observations of Evans and Gough (1942). With PCR-based detection of plant DNA in soil-living insects, such as *Agriotes* larvae (Staudacher et al., 2011b), it is possible to examine whether general patterns exist as to their dietary choices. Although it is not yet possible to determine the amount of plant tissue per plant species consumed, it was shown that they seem to depreciate decayed plants, while no significant consumption of soil organic matter has been detected with this technique up to now (Wallinger et al., 2013). Living plant material would thus be by far the main food source of *Agriotes* wireworms all along their development, and soil organic matter cannot replace it.

According to the species, latitude, temperature and food availability during larval development, wireworms may pass through a variable number of instars. *A. sordidus* goes through 8 to 13 instars, depending essentially on the centigrade degree day accumulation (CDDA) since egg hatching. It also influences the number of years needed to complete the cycle and is strongly related to geographical location (Furlan, 1998, Furlan, 2004, Blot et al.,

2008). *Agriotes* wireworms in the U.K. (mainly *A. obscurus*, *A. lineatus* and *A. sputator*) develop up to four or five years before pupating (Miles, 1942). In contrast, *A. sordidus* is able to complete its whole lifecycle in 24 months, over two calendar years, provided that food is available and that 4,000 DD are accumulated during the first year of larval development. The cycle can last longer if no food is available for long periods. For some individuals maintained at 29 °C, three to four months can be enough to complete development from egg to adult (Furlan, 2004). Each larval instar passes through three phases: mandible hardening and darkening, feeding and pre-molting. The intense feeding phase associated with plant damage can last less than 20% (*A.ustulatus*), 25% (*A.sordidus*) or 29% (*A. obscurus*) of the whole development time (Evans and Gough, 1942, Furlan, 1998, Furlan, 2004). Wireworms generally have two intense activity periods that may result in significant crop damage over one calendar year, according to suitable temperature and moisture conditions in the different soil layers. They occur from March to May and from September to October (Balachowsky and Mesnil, 1936). The depth at which *A. ustulatus* larvae can be found depends mostly on late autumn, winter and early spring temperatures and on soil moisture during the rest of the year. It can be as deep as 60 cm in the winter. In spring, provided that soil temperatures are above 10 °C and there is suitable soil moisture content, most of the larvae are in the upper 20 cm of the soil (Furlan, 1998). In between sensitive periods, wireworms perform vertical migration related to soil temperature, soil moisture and soil type (Balachowsky and Mesnil, 1936, Furlan, 1998, Jung et al., 2012), the preferred range of soil moisture being related to soil type, especially to the permanent wilting point and the soil moisture tension (Jung et al., 2012). Evaluating the soil conditions during the critical phases of the cycle is of importance, as it shows the suitability of young larvae. High moisture and live roots in the upper layer of the soil in late May-June may cause severe attacks by *A. sordidus* the following autumn and subsequent years if nothing is undertaken to control the population (Furlan, 2004). The first crop threatening instars occur in the root layer sooner or later according to the duration of the lifecycle. *A. obscurus* starts damaging during their first spring, a year after eggs have hatched. They get increasingly harmful season after season (Balachowsky and Mesnil, 1936). *A. sordidus* wireworms develop faster and reach a damageable size in only three to four months. They may damage any sensitive crop sown in late summer or early autumn (Furlan, 2004). In some regions, especially with mild climates, impacts on crops may carry on all year long, even in the winter (Balachowsky and Mesnil, 1936). The length of 10 mm associated with yield losses is reached between the fifth and sixth instar for both *A. sordidus* and *A. lineatus*

wireworms, whilst the latter need two years to acquire it (two instars per year) (Balachowsky and Mesnil, 1936, Furlan, 2004). Most of the last instar larvae transform into pupae and adults in the summer. They pupate in the upper 30 cm of the soil and remain inside cells until the subsequent spring. Soil tillage can however induce cell breakage and adult emergence and exposure before spring (Furlan, 2004).

3. Available Data on the Chemical Ecology of Wireworms

The number of studies reporting on the orientation of soil insects in response to chemical cues released by roots has gradually increased, and the location of the latter by random insect movement is now seldom reported (Johnson and Gregory, 2006). To our knowledge, the first evidence of such response in wireworms was reported by Thorpe *et al.* (1946). The authors demonstrated elicitation of the biting response when wireworms (*A. lineatus, A. obscurus and A. sputator*) encountered plant juices or solutions containing either one or more of a number of carbohydrate, fatty or protein substances and certain common plant sugars (glucose, fructose, sucrose, galactose, maltose and stachyose). They all induced a biting response threshold progressively lowered with starvation, up to seven days. Substances eliciting orientation were also highlighted (aspartic acid, asparagine, malic acid, succinic acid, glutamine and glutamic acid). The sensitivity for the orientation response tends to vary with the nutritional state, as well as with season and time in relation to molt, as it does for biting (Thorpe et al., 1946). The perception of natural chemicals associated with the host plant will vary accordingly to physiological particularities of the plant, which has an impact on the host-plant choice at the individual scale. For example, *A. obscurus* wireworms are able to avoid high glycoalkaloid (mainly -solanine and -chaconine) and low sugar concentrations and also solanine and chaconine, compounds found in potatoes (Jonasson and Olsson, 1994), even if these are not the only ones responsible for varietal susceptibility (Johnson et al., 2008). Wireworms are able to orient towards carbon dioxide by klinotaxis (Klingler, 1957). *Ctenicera destructor* responds to concentration differences of 0.002 mmol mol^{-1} (Doane et al., 1975). Carbon dioxide is perceived by clusters of sensilla on the labial and maxillary palps (observed on *Limonius californicus*, *A. obscurus* and *A. lineatus*) (Doane and Klingler, 1978). Other species probably perceive CO_2 this way, as hypothesized for *Limonius canus* (Horton and Landolt, 2002). It remains unclear whether other volatile chemicals emitted by food baits or germinating seeds affect wireworm movements (Doane et al., 1975), and this question

obviously merits additional research efforts (Horton and Landolt, 2002), keeping in mind that physiological differences occur between species (Parker and Seeney, 1997).

Johnson and Gregory (2006) put forward a model of attraction/location of root feeding insects, including wireworms, towards root systems and based on three important phases (Johnson and Gregory, 2006). Insects first move at random, any orientation being under the influence of heterogeneity in soil architecture: they follow the lines of least resistance (Thorpe et al., 1946). When they perceive general (non-specific) semiochemical(s), such as CO_2, the random movement is biased. The stronger vertical gradients (between the air and the upper soil) and the high density of roots question CO_2 efficiency as a local host-locator, particularly for specialized herbivores in mixed plant communities (Johnson and Gregory, 2006). The rhizosphere is favorable for volatile-mediated communication, since volatiles are more likely to accumulate and reach their activity threshold than in the wind-exposed above-ground environment (Bailly and Weisskopf, 2012). The perception of host specific semiochemical(s), such as volatiles, would therefore elicit recognition of different host species (Johnson and Gregory, 2006). This step might explain the preference of *A. obscurus* and *A. haemorrhoidalis* for two nutrient-rich grassland plant species next to two nutrient-poor ones, observed by Hemerik *et al.* (2003). In the belowground interactions, it should be noted that there is a trend for low molecular weight compounds (e.g., alcohols, esters, aldehydes) to have 'attractant' properties, while hydrocarbons tend to be 'repellent' (Johnson and Nielsen, 2012). The final orientation step involves chemosensory cues at root surface, inducing acceptance or rejection. Root-associated microorganisms and co-occurring herbivores impact on these processes, equally affected by soil properties, whether they act on semiochemical diffusion or insect motility. Holistic approaches in realistic soil conditions should help determine the relative roles of CO_2 and other volatiles as host location cues and how these relate to the host-plant range (Johnson and Gregory, 2006, Johnson and Nielsen, 2012).

The use of natural, plant-based chemistry is gaining greater attention, due both to increases in analytical capability and the enhanced ability to dissect complex biochemical systems (Waliwitiya et al., 2005). The soil volatiles are valuable infochemicals and will have to be considered to understand the entire integrity of the ecosystems. They constitute a new source of tools for belowground pest management (Agelopoulos et al., 1999, Wenke et al., 2010). Quantification of specific herbivore-induced released plant compounds and assessing their role in the increase or decrease of the attractiveness of a plant host to another type of herbivore will perhaps enhance our capability to manage populations of soil-dwelling

herbivore pests and, thus, secure optimal outputs. Filling the current gap of knowledge concerning the role of belowground host plant location cues should enhance the improvement of IPM tools against subterranean insect pests (Johnson and Nielsen, 2012), tools that would be very useful for wireworm control. The available data on wireworm chemical ecology will be deeper addressed in Chapter I.2, by reviewing 1) the compounds known to impact foraging and 2) the degree of knowledge related to the perception of these compounds.

4. From Risk Assessment to Mass Trapping

Wireworms do not cause systematic damage during all the critical cropping phases. The first step in their management consists in assessing the risk of crop damage, *i.e.*, the level of populations in place, preferably in relation to crop sensitivity thresholds. For example, if early spring soil samplings indicate the presence of high levels of *A. sordidus* last instars, damage on sensitive crops sown during the same spring can be forecast; if high populations of younger larvae are found in June-July, severe attacks may occur in sensitive crops sown in late autumn or the following spring (Furlan, 2004). Crop rotation, availability of food resources through the season, climatic-agronomic features and soil characteristics are the main known factors influencing the composition of species communities and larval population density (Furlan, 2005). At the local scale, site-specific variables show a positive association with the level of infestation, like grass duration, surrounding grassy margins and weedy spots (Miles, 1942, Parker and Seeney, 1997, Kovacs et al., 2006, Hermann et al., 2012, Mulercikas et al., 2012). The presence of meadows and double cropping within the rotation cycles would result in a population increase of species overwintering as adults (Furlan, 2005). However, for *A. ustulatus*, a negative correlation has been observed between spatial distribution of larval population and grass cover (Toepfer et al., 2007). Another positive correlation is generally found with soil bulk density (Parker and Seeney, 1997, Kovacs et al., 2006, Hermann et al., 2012, Mulercikas et al., 2012). Correlations between soil humidity and infestation rate have also been reported (Lefko et al., 1998, Toepfer et al., 2007).

But, the real difficulty lies in mapping the zones where wireworm scouting should be performed preferentially because infestation is expected, combining time, space, agronomic and climatic variables (Lefko et al., 1998). The scale at which correlations are investigated is very important to define. In Hungary, when studied in small-scale maize fields and in a flat relief environment, *A. ustulatus* wireworm populations appeared clustered in 75% of cases

(Toepfer et al., 2007). A prediction model for wireworm activity and appearance in the damage zone, in relation to soil moisture, temperature and type, has been developed in Rhineland-Palatinate (Germany), with determination coefficients of 0.81 to 0.89 (Jung et al., 2012). That very promising tool in risk assessment deserves to be tested in other regions. Multiscale correlations can be sought between landscape factors and damage levels on particular crops (which implies prior definition of such levels). The distribution of wireworm damage in potato crops has been associated to landscape structure at a scale of 25 ha in Austria, grassy field margins being potentially the key landscape variable, sand content in the soil coming in second place (72% of the total variability explained by the two factors) (Hermann et al., 2012). Differences between *Agriotes* species distributions are closely linked to the scale and to species-specific factors. Predation, competition or social aggregation might intervene; the consideration of other pest/non-pest species in place is therefore essential (Toepfer et al., 2007, Benefer et al., 2010), as when studying wireworm chemical ecology. Highlighting association/dissociation between species could help define indicator species useful for management. The impact of field or site characteristics may differ drastically between species. Also, the association between certain species might be linked to differences in soil pH, grass duration and the amount of organic matter content between fields. For so many reasons, there is a strong necessity to separate taxa into species when assessing the pest-complex (Benefer et al., 2010). Disregarding the distribution of the species forming the community where integrated wireworm management is planned could serve for its useless modification, without even affecting its size or impact on belowground stages of the pest. Developed below as risk assessment or mass trapping tools, the sampling methods we mention in this paper also relate to that necessity.

4.1 Wireworm Sampling: From Prevention to Trap Crops

Several sampling methods for assessing infestation rate (number of wireworms per surface unit) have been developed over time. The infestation thresholds above which treatments are recommended depend on the species, the proportion of voracious instars in the catches, the sampling method and the crops concerned (Furlan, 2005). Also, the limit of detection of traps must be smaller than the damaging population threshold (Parker and Howard, 2001). Soil core sampling and counting of caught individuals lacks interest for growers and agronomists (Parker and Howard, 2001), because they are time-consuming (Lafrance and Tremblay, 1964, Furlan, 2005) and subject to significant sampling errors,

essentially due to the sampling effort, to the non-random aggregated distribution of the larvae (Salt and Hollick, 1946, Furlan and Burgio, 1999) and particularly important in the case of low, yet damageable wireworm populations (Yates and Finney, 1942). It is also important to consider that small larvae are more likely to be markedly aggregated, while medium-sized ones are more dispersed and large ones closer to random (Salt and Hollick, 1946). These patterns are involved in the interpretation of trap catches. Many bait-based sampling techniques have proven to be at least as effective as soil core for detecting wireworms (Parker, 1994, Parker, 1996, Horton and Landolt, 2002). Bait efficiency notably depends on the period of bait exposure (to be adapted according to case-specific parameters, such as trap handling or sowing period of the future crop) and the time of the year (Cherry and Alvarez, 1995, Parker, 1996, Arakaki et al., 2009, Landl et al., 2011). It decreases when alternative food sources are present, which occurs in freshly ploughed grass fields (Doane et al., 1975, Chabert and Blot, 1992, Parker and Howard, 2001). Germinating cereal seed baits, whose efficiency seems enhanced by daily irrigation (Arakaki et al., 2009), is the most efficient sampling technique (including cost and accuracy concerns) for determining wireworm populations in worldwide agricultural habitats and in grass fields, intended for arable production (Parker, 1994, Simmons et al., 1998, Horton and Landolt, 2002, Vernon et al., 2003, Brunner et al., 2005, Arakaki et al., 2009). Chabert and Blot (1992) used a modified version of traps designed originally against *Melanotus* spp. populations in the USA (Kirfman et al., 1986). Briefly, they consisted of 650 ml plastic pots filled with vermiculite and a mix of 30 ml of maize and 30 ml of wheat as baits, entirely watered and buried in the ground, covered with soil and a plastic lid, under a second soil layer. Traps were manually checked after 10 to 15 days. For an equivalent number of samplings, baited traps will allow the retrieval of two- to four-times more wireworms and save up to two thirds of the sampling time compared to simple soil core sampling (Chabert and Blot, 1992, Parker, 1994). On maize, the two techniques had comparable accuracies for high populations. When these decreased, accuracy remained acceptable with baited traps, while turning disastrous with soil cores. Such traps allowed linking the percentage of deceased plants to the wireworm infestation rate in several situations. For example, in maize, sowing before May 1st in fields infested by five to 10 larvae per square meter caused the destruction of 30% of the plants (Chabert and Blot, 1992). Bait traps are also better presence/absence indicators of wireworm infestation than soil cores alone, especially where populations are below the limit of detection of soil cores (in potato 62,500 wireworms/ha with 20 soil cores), even if the effective

sampling area of a trap may be influenced by many factors (Parker, 1994, Parker, 1996). In potato crops, there is a trend towards a higher level of damage in untreated plots with higher wireworm abundance, but even at densities of less than 100,000 larvae/ha, damage can range between 20% and 80% of tubers attacked (Parker et al., 1994), which is hardly useable in prediction models. Similar wide ranges were found in Romania: wireworm populations (mainly *Agriotes*) ranging from 510 larvae/m^2 to 70100 larvae/sq.m will induce up to 31.6% of attacks on wheat, 22.5% on barley, 42.8% on corn seeds, 54.0% on corn at stem base, 64% on sunflower seed and 53.0% on sunflower at stem base (Popov et al., 2001). This emphasizes the difficulty to define infestation thresholds above which treatment has to be applied and is partly attributable to sampling accuracy. A remarkable example of efficient sampling plan to assess wireworm occurrence and control measure necessity was reported by Cherry *et al.* (2011). A sequential core-sampling plan at sugarcane planting was conducted to target areas needing treatment. If nine or more wireworms (*Melanotus communis* and *Conoderus* spp.) were retrieved from a total of 25 samples evenly spaced in a diagonal field transect, the latter were treated. By showing no significant differences in further yield and wireworm numbers between adjacent paired fields (identical agronomic conditions, one treated, the other untreated), they showed that both the sampling method and the used economic injury level allowed $100(US)/ha savings in unnecessary soil insecticide application (Cherry et al., 2013). In Italy, data collected over 15 years showed significant correlations between infestation rate or the average number of larvae per bait trap and damage in maize plants by *A. brevis*, *sordidus* and *ustulatus* (Furlan, 2005). Significant relationships between pre-planting catches of wireworms by baited traps and damage to maize plants were found for all three species, but symptoms of damage varied. When the population is greater than five larvae/trap, *A. ustulatus* larvae may significantly affect plant stands by damaging seeds. *Agriotes brevis* was found to be the most harmful, with catches above one per trap causing considerable plant damage that may result in a yield reduction. For the same damage level in maize fields, five-times more *A. ustulatus* larvae are needed. *Agriotes sordidus* has an intermediate damage potential (wireworm densities above two larvae/trap may cause a yield reduction). These thresholds are reliable for: 1) bare soil in which there are no alternative food sources, also after meadow (e.g., alfalfa and ryegrass) if this has previously been cultivated (in this case, the field has to have been ploughed at least three months before baited trap placement); 2) the average soil temperature at 10 cm depth is over 8 °C (for more than 10 days); and 3) the soil humidity is high (near to field water capacity) (Lorenzo Furlan -

personal communication). These examples highlight the accuracy needed as to the conditions in which thresholds are described.

It is generally admitted that further improvements in bait traps or other sampling methods will be needed to accurately estimate the density of wireworms. Considering the factors involved, the outcomes in terms of treatment decision baseline may be as various as existing agronomic situations, spatial distribution (large-scale patterns) and crop-wireworm species combinations. The combined use of soil-core sampling and bait trapping may give a more accurate representation of potentially damaging species present, because it reflects more properly the wireworm species and distribution within the soil (Benefer et al., 2012). The closer to reality the estimation of the population is, the more precisely damaging thresholds can be defined, therefore reducing unnecessary treatment cases.

In-soil baits are useful in wireworm mass trapping, trap crops being the most efficient in that purpose, providing a very attractive alternative food source. By planting wheat rows eight days in advance of intercropped rows of strawberries, the seedling mortality due to *Agriotes* feeding can be reduced from 43% in unprotected plots to 5.3% in plots with an intercrop of wheat (Vernon et al., 2000). Maize protection can also be achieved and enhanced as the plant species diversity in the trap rows increases. Molecular analysis of gut content screening for plant DNA (Staudacher et al., 2011b) outlines that wireworms are lured away from the target crop and also preferentially consume the intercrops (Thalinger et al., 2011), e.g. *Pisum sativum* cv. Valverde are more attractive to wireworms than potatoes (Landl and Glauninger, 2013). These efficient techniques, difficult to set up at field scale, can only be developed with an economically interesting approach.

4.2 Pheromone Trapping: Mass Trapping and Monitoring

Three main elements account for the fascination of insect sex pheromones and their feasibility for insect management: 1) they are species-specific (even when synthetic and incomplete, and blends usually affect only the target, with the possible exception of taxonomically closely related species); 2) they are active in very small amounts; and 3) the vast majority are not known to be toxic to animals (Witzgall et al., 2010). The identification of click beetles sex-pheromone main compounds in the late 1980s provided the qualitative bases of sampling techniques and mating disruption/mass trapping according to species.

Geranyl and (*E,E*)-farnesyl esters are major components of *Agriotes* natural sex pheromones (Borg-Karlson et al., 1988). Some species of click beetles may contain up to 24

substances in their pheromone glands (Yatsynin et al., 1996). Tóth (2012) summarized the identification status of pheromones and attractants described in a variety of species among which 22 (73.3%) were *Agriotes* (Toth, 2013). Click beetles show a variety of responses according to changes taking place in the structure of the pheromone component, especially in its acyl group or allylic fragment (Siirde et al., 1993). The sex attractant pheromone of one species may act as a sex inhibitor with respect to the other (Siirde et al., 1993) through one of the compounds of the blend (Toth et al., 2008). The same component can capture different target species, e.g., geranyl hexanoate alone actively attracts both *A. sordidus* and *A. rufipalis* Brullé (Toth et al., 2002a). Geranyl butyrate (GB) and (*E*,*E*)-farnesyl butyrate (FB) were identified in the pheromone gland extract of females *A. brevis* as the major sex pheromone components. Used as field baits (Italy, Hungary, Bulgaria) where both *A.sputator* and *A. brevis* were present, they selectively caught only *A. brevis,* whereas GB is also the main pheromone component of *A. sputator,* suggesting that FB has a role in reproductive isolation (Toth et al., 2002b). On the other hand, one sex-pheromone can attract individuals of species other than the target one, depending on geographic location (Toth et al., 2003, Furlan et al., 2006, Toth et al., 2008, Burghause and Schmitt, 2011). For *A. lineatus* and *A. proximus*, the comparative study between pheromone profile and the mitochondrial cytochrome c oxidase subunit I gene even led to the consideration of a necessary taxonomic revision (Vuts et al., 2012).

The pheromone gland extract composition does not necessarily follow the proportions obtained by volatile collection when available, e.g., for *A. lineatus* and *A. proximus* (Vuts et al., 2012), while the optimal ratio of concerned compounds in pheromone lures is also different from the two latter extracts (Toth et al., 2008). In the future, if the pheromones of problematic species are to be analyzed by volatile collection (an unavailable method when most *Agriotes* gland extracts were studied in the 1980s), then more accurate pheromone elucidations may result. Besides, it has been noticed in some cases that females could be attracted towards blends of synthetic pheromonal compounds, revealing potential aggregation traits of the latter (Toth, 2013). The same analytical improvements could provide information to understand these observations.

Very recently, a binary combination of two synthetic floral compounds, (*E*)-anethol and (*E*)-cinnamaldehyde, was optimized as a female-targeted lure for female *Agriotes ustulatus* Schwartz click beetles, opening a new perspective for adult click beetle mass trapping (Toth et al., 2011, Toth, 2013), but most of the applied cases of adult monitoring have focused on

male click beetles thanks to pheromone traps. Guidelines for the synthesis of sex-pheromone, highly effective for baits, are available (Odinokov et al., 1991, Toth et al., 2003).

Several sex pheromone traps have been developed for monitoring all the most important *Agriotes* species (Oleschenko et al., 1987, Kudryavtsev et al., 1993, Furlan et al., 2001c, Vernon, 2004, Milonas et al., 2010). Among them, bottle traps (homemade funnel traps) and VARb funnel traps, an adaptation of CSALOMON Var funnel traps (Plant Protection Institute of Budapest, Hungary), were adapted for species privileging flying over crawling. TAL traps (adapted pitfall traps) and YATLOR traps, similar to the Estron trap described by Oleschenko (1987) and Kudryavtsev et al. (1993) were designed for species privileging crawling over flying. A combination of the bottom part of a YATLOR trap and the upper part resembling the bottle trap gave birth to a high capacity trap, the YATLORfunnel, or YATLORf trap, effective in monitoring all the target species during all the swarming season (Furlan et al., 2001c). It allowed detecting wireworm population levels in different European countries (Furlan et al., 2001b) and establishing distribution maps of the predominant species, even at low population densities (Furlan et al., 2006). Trap design matters: during the early part of the trapping season, traps into which both crawling and flying beetles can enter may capture significantly more individuals than designs which insects can only enter by flying. This has been observed for *A. brevis* and disappears later in the season. It indicates the need for traps suitable for use throughout the whole season (Toth et al., 2002b).

A ground-based pheromone trap highly efficient for monitoring *A. lineatus* and *A. obscurus* has also been developed in Canada (the "Vernon Beetle Trap", Phero Tech Inc., Delta, British Columbia) (Vernon, 2004), and attempts for their monitoring highlighted that pheromone trapping should be the choice method for their surveying in North America (Vernon and Tóth, 2007).

Blackshaw and Vernon (2006) demonstrated the usefulness of pheromone trapping as a sampling method in addressing ecological questions at a landscape scale, by studying the spatial stability in non-farmed habitats of male *A. lineatus* and *A. obscurus* (Blackshaw and Vernon, 2006). Click beetle species have been monitored thanks to large-scale pheromone trapping in Croatia. The abundance and dominance of *A. lineatus* decreased compared to average data from 1961-1990, abundance being negatively correlated with average air temperature. This result was postulated to be a potential effect of climate change (Kozina et al., 2012). In Rhineland-Palatinate (Germany), pheromone trap catches performed from 2008 to 2010, with the aim to define click beetle communities, highlighted the unexpected presence

of *A. sputator*, possibly related to the increased potato damage observed during the previous seven years (Burghause and Schmitt, 2011). Such studies perfectly illustrate large-scale direct uses of pheromone trapping. Interpreting adult male trap counts for quantitative predictions of population size is complex and limited, unless the observed differences between species can be explained by dissimilar movement rates of the species and interferences between traps (Blackshaw and Vernon, 2008), hence the necessity to acquire accurate knowledge of the pheromone traps' ranges of attraction (Sufyan et al., 2011). It is essential, although not necessarily always possible yet, to: 1) define the biological significance of the pheromone trap catches; 2) determine the actual range of attractiveness (related to the movement rates – male *A. obscurus* are able to migrate at least 80 m and *A. sputator* up to 27 m, distances linked to site-specific factors (Gough and Evans, 1942, Schallhart et al., 2009)); 3) study the relationship between males captured and the level of the female population; and 4) establish a reliable correlation between adult trap catches and subsequent larval populations for all the species and varieties in different climatic and agronomic conditions (mainly rotation) (Furlan et al., 2001c). In Italy, sex pheromone traps proved to be much more sensitive in detecting species than the tools used to monitor larval populations, and the trap catches were correlated with estimated larval populations (Furlan et al., 2001a). A mark-release-recapture study performed in South Devon, U.K., in a topographically flat and wind obstacle-free area, helped in getting species-specific migration distance data. Inventories led to the definition of maximal sampling ranges and effective sampling areas of *A. lineatus*, *A. obscurus* and *A. sputator* traps. Recapture rates were significantly different between species and recapture distances (ranging from 4 to 32 m). With such results, an estimation of the minimum cost of mass trapping programs to prevent males from mating was possible: 165€/ha/year (*A.lineatus*), 247.5€/ha/year (*A.obscurus*) and 2343€/ha/year (*A.sputator*) (Hicks and Blackshaw, 2008), illustrating the importance of separating wireworms into species. Sufyan *et al.* (2011) worked on the same problem in Germany and obtained slight differences, mainly due to the employed regression method. They took account of the attractive potential, *i.e.*, the maximum sampling area of pheromone traps in the equation. On the basis of the estimated probabilities of recapture, a maximum distance of 20 m between individual traps would be needed to ensure substantial mass trapping, *i.e.*, 25 traps/ha to theoretically reduce the male click beetle populations by more than half (Sufyan et al., 2011).

The potential of using pheromone trapping for male click beetles with subsequent wireworm reduction remains unclear, but will definitely require a dense network of traps

(Sufyan et al., 2011). The spatial relationship between aboveground adults and belowground larval distributions may not always be straightforward, unlike for other pests, which is important in cases where the monitoring and/or management is carried out on a different life stage to that causing damage (Benefer et al., 2012, Blackshaw and Hicks, 2013). Unfortunately, the spatial relationships between adult male catches and the distribution of their larvae is such that pheromone traps alone will not reliably indicate where wireworms occur in a field or an area (Blackshaw and Vernon, 2008).

The use of sex-pheromones to monitor and trap *Agriotes* click beetles illustrates the issues splitting the understanding of an ecological phenomenon at the individual scale from its application in IPM strategies. Behavioral and ecological differences between species need to be acknowledged when researching the pest complex at the field and larger scale (Benefer et al., 2010, Blackshaw and Hicks, 2013, Staudacher et al., 2013a). Separating wireworms to species instead of grouping as a complex reveals more as to the relationship between adult and wireworm distributions (Benefer et al., 2012). Also, the usefulness of pheromone traps in assessing *Agriotes* spp. populations could be restricted to larger scales than those at which pest management is normally undertaken. Distribution maps should be defined using sex pheromone traps within zones defined according to prevalent crop rotation organic matter content, soil type and precipitation (Furlan, 2005), as illustrated above. In fragmented rural landscapes, the monitoring of Elateridae should take into account the heterogeneous pattern of the adult population (stratified sampling) (Burgio et al., 2012). Combining a geostatistic analysis of agronomic characteristics of soil and climatic conditions of the investigated area with adult distribution pattern, it should be possible to draw risk-areas on a predetermined scale of investigation (such as a province) and to provide more precise monitoring and management, provided that further studies show more details of how key variables influence the distribution (Burgio et al., 2012).

5. Management practices

Beside avoidance of sensitive crops in highly infested areas (Balachowsky and Mesnil, 1936, Furlan, 2005), cultural manners efficient against wireworms can be summarized as follows: 1) avoidance of grass in the crop rotation, since it promotes oviposition, egg and larval survival and is generally correlated with further crop damage (Balachowsky and Mesnil, 1936, Gough and Evans, 1942, Jary, 1942, Miles, 1942, Furlan et al., 2009, Keiser et al., 2012); 2) drainage of slow soaking fields suitable for wireworm development, at the end

of spring; 3) appropriate crop choice in regard to the infestation rate (see above) with prolonged rotations with hooded crops in May to suppress suitable conditions for egg laying; and 4) soil tillage in late spring and late summer when larvae or eggs are in the upper soil layers to enhance their death by desiccation, mechanical injuries or predator exposure (Balachowsky and Mesnil, 1936, Furlan, 2005). Agrobiocenozes characterized with intensive tillage may also affect click beetle abundance (Mulercikas et al., 2012), potentially with population reduction year by year after grass cover (Miles, 1942). Field flooding can be envisaged in infested fields, likely providing more effective control of wireworm populations in fall or summer (high temperatures), with an efficiency decreasing as soil salinity increases (van Herk and Vernon, 2006), but it requires significant field modifications (Waliwitiya et al., 2005).

In areas where the cropping history shows systematic infestation, a forward planning is required to allow the implementation of risk assessment methods well in advance of final decisions being made on field choice (Parker and Howard, 2001). Preventing wireworm damage starts with avoiding growing particularly sensitive crops where and when infestation rates are significant (such as lettuce or maize highly sensitive to *A. sordidus* (Furlan, 2004)). While non- or low-sensitive crops can be planted in infested fields, the remaining cultivated soil can be planted with any other crop (Furlan, 2005). Jary (1942) produced interesting guidelines to follow as to what crop should be preferred according to the level of infestation observed. The crops' sensitivity to attack is highly variable (Furlan and Toffanin, 1996) and the single seedlings sensitivity should be distinguished from that of the crop itself (agronomic sensitivity) (Furlan, 2005). The identification of crops and varieties less sensitive to wireworms than others is essential. It has been undertaken notably for potato varieties (Jonasson and Olsson, 1994, Parker and Howard, 2000, Johnson et al., 2008), sugarcane clones (Hall, 1990) or for different crops attacked by *A. ustulatus* (Furlan and Toffanin, 1996). Damage on potatoes would be more important in organic farming systems (Keiser et al., 2012). Thresholds therefore have to be defined for every existing situation (crop-wireworm species, method used to assess the infestation rate – see above), especially since *Agriotes* species show different responses to bait traps. For example, *A. ustulatus* can be three- to four-times more damageable to maize than *A. brevis*, as to the number of damaged plants, which has an impact on the infestation threshold considered. The sowing period has to be considered equally. In controlled conditions, it is possible to define a precise scale of sensitivity. For example, two lettuce plants can be completely destroyed by 10 to 20

A. ustulatus wireworms, while there would be no plant loss for cabbage or capsicum in the same conditions (Furlan, 2005). Cultural manners could be combined with trap crop strategies undertaken preferably before any other treatment. Whenever impossible, the whole integrity of the systems should be revised, considering the economic issues involved, and point towards systems where they could be followed.

6. From Broadcast Application of Synthetic Insecticides to Environmentally Friendly Alternatives

Extensive studies conducted in northern Italy outlined that less than 5% of fields planted with maize and sugar beet needed soil insecticides (Furlan, 1989, Furlan, 1990, Furlan et al., 1992) or seed treatments (Furlan et al., 2002). Decisions to treat or not should rely on the threshold of infestation and not only on presence/absence indications (Furlan et al., 2002). Resorting to soil insecticide or insecticide-treated seeds to control wireworm populations is often unnecessary, but information to implement IPM strategies is missing or unknown to farmers (Furlan, 2005). When treatment has been shown unavoidable, efficient chemical alternatives to broadcast application of synthetic chemicals should be preferred. In this section, we discuss the alternatives and the importance of behavioral information brought to light by recent studies.

6.1 Seed Coatings with Synthetic Chemicals

Efficient active substances should be available in soil during critical cropping phases. Wireworms temporarily repelled or deterred from feeding on germinating seeds and seedlings will subsequently feed on older and less vulnerable tissue, which could be tolerated (Williams et al., 1993). In any case, the timing of sowing in relation to upward wireworm movements in the root layer is a key factor to consider (Furlan and Campagna, 2002). When corn is protected from damage during the first three weeks of growth, economic impacts can be minimized (Waliwitiya et al., 2005). With seed coatings, the area treated with chemicals is considerably reduced and they appear just as effective as full-soil treatments. Moreover, they may well be combined with fungicide seed treatments, therefore providing easy handling by farmers together with adequate wireworm control, notably on wheat and barley (Popov et al., 2001, Huiting and Ester, 2009). Good candidates for seed coatings should unite four characteristics. They must 1) be efficient through wireworm feeding, notably for low integumental penetration rate insecticides, unfortunately usually inefficient on wireworms

(Chaton et al., 2003, Chaton et al., 2008), 2) be integrated in a solid state in the seed-coating mixture in order to avoid atmospheric dispersion during mechanical sowing, 3) have a low water solubility and a high affinity for soil particles and 4) remain active against wireworms after sowing (approximately one month for corn), during the whole seedling establishment/strong wireworm feeding phase (Chaton et al., 2008). Neonicotinoid insecticides as a treatment applied to potato seed at planting can enhance a significant reduction of attacked tubers between sowing and harvest (imidacloprid - 70 g/ton seed, thiamethoxam - 50 g/ton seed). Compared with broadcast organophosphorous soil treatments, the amount of needed insecticide can be reduced by over 90% depending on the amount of potato seeds per hectare. Such numbers, altogether with the lack of knowns adverse effect on potato yield, stand for an adequate alternative to soil treatments with organophosphorous compounds (Huiting and Ester, 2009).

Seed treatments cannot automatically be linked with wireworm population mortality. On wheat, neonicotinoid (imidacloprid, clothianidin and thiamethoxam), pyrethroid (tefluthrin) and a combination of the latter and thiamethoxam applied as seed treatments can provide excellent stand protection, likely through prolonged wireworm intoxication, but the reductions of neonate and in place wireworm populations are not significant. To the contrary, fipronil seed treatment provides an excellent protection with population reduction, which makes it as efficient as lindane (Vernon et al., 2009). If blended with thiamethoxam, wheat stand and yield are improved over the individual chemicals applied alone. They lead to high mortality of resident and neonate wireworms, which implies this treatment should only be applied once every three years during which time susceptible crops could be grown, with decreased rates (50 times lower) compared to the formerly used Vitavax Dual containing lindane (Vernon et al., 2013). Furthermore, if using seed treatment on only a fraction of the seeds, the proportion of treated seeds could even be adjusted to the infestation level: decreased proportions of treated seeds are needed when higher levels of larval infestation are observed, reducing even more the used quantity of active ingredients (0.095 g/ha). Such mixtures of untreated and blend-treated seeds could also be used in push-pull strategies as trap crops (Vernon et al., 2013). This rate of application is as far as we know the lowest ever envisaged for wireworm control.

6.2 Natural Plant-Derived Chemicals

Control with naturally occurring active substances is possible. Both mortality and morbidity depend on the target species, and the specific effects should be taken into account (van Herk et al., 2007). Besides, if a biopesticide, intended as a plant-derived component, has a beneficial effect on the control of a pest species, potential phytotoxic effects on specific crops must be assessed before any field trial (Waliwitiya et al., 2005).

The bioactive hydrolysis products of glucosinolates (GLs - contained in several *Brassica* species), particularly isothiocyanates (ITC), can be used to control soil pests and weeds through in-soil incorporation of GLs-containing plant material, a practice known as biofumigation. The relatively rapid sorption and degradation of ITC in the days after incorporation minimizes its persistence and leaching risks, which makes it a promising technique in the aim to reduce reliance on synthetic pesticides (Gimsing and Kirkegaard, 2009). Allyl-isothiocyanate (AITC) has been tested on several important wireworm species. To our knowledge, Tattersfield and Roberts (1920) were the first to show its high respiratory-toxic effect on click beetle larvae, among a wide range of other substances (Tattersfield and Roberts, 1920). Laboratory assays led to the assessment of the AITC LC50 for *Limonius californicus* and showed its lethal and sublethal effects, which in the meantime highlighted potential for other wireworm species control (Williams et al., 1993). The key factor of efficiency is the GL richness of the plant tissue or derived meal used for control (Elberson et al., 1996). Biocidal plant root systems (*Brassica juncea* var. ISCI 99) do not necessarily cause significant mortality, while the incorporation of aboveground material at a dosage of 55 t/ha of fresh matter (corresponding to about 290 μmoles of GLs/l of soil), can significantly reduce wireworm populations (Furlan et al., 2009). In both pot assays and field trials for *Agriotes brevis*, *A. sordidus* and *A. ustulatus* management, the AITC tested sources were chopped *Brassica juncea* (L.) Czern fresh plants and biofumigant meals derived from defatted seeds of *Brassica carinata* A. Braun. In pot assays, a clear rate effect was demonstrated, with sufficient seed meal to supply approximately 160 μmoles of GLs/l of soil, resulting in significant wireworm mortality. The effect of the chopped *B. juncea* plants was less consistent. Accurate incorporation of defatted seed meals resulted in an efficacy level sufficient to protect susceptible crops from damage. Globally, the level of crop protection was comparable to that of conventional insecticide treatments and no evidence of phytotoxicity was noticed (Furlan et al., 2010). Spotting the appropriate timing for broadcast application is the critical factor here, because the active volatile substances remain available for only 24 -

36 hours (Furlan et al., 2004a). Successful practical results depend on simultaneously united conditions: 1) suitable GL dosage (the target dose is related to infestation rate and degradation parameters); 2) homogeneous broadcast of biofumigant defatted seed meals, 3) effective and prompt soil incorporation, 4) suitable soil temperature (10.5-16 °C, available in spring and autumn in most European countries) and sufficient soil water content (enzymatic hydrolysis of glucosinolates) and 5) the occurrence of wireworms in the upper soil layers (Furlan et al., 2004a, Furlan et al., 2010). To ensure this, 10-15 days with a soil temperature over 18-20 °C after cold or dry conditions should pass so that the larvae in the little mobile stage in the deeper soil layers can turn into the feeding stage and move upwards looking for food (Furlan et al., 2004a). It may also be possible to improve population management by applying biofumigant defatted seed meals when the first instar larvae are present, which would induce significant reduction in the following years population (Furlan et al., 2004a, Furlan et al., 2010).

A few other plant-derived active substances affect wireworms. Cinnamaldehyde can significantly reduce damage to mother tubers when applied as a drench at 150 g a.i./ton tubers (Ester and Huiting, 2007). The most effective active ingredient in the neem tree *Azadirachta indica* A. Juss insecticides is azadirachtin. Its repellent effect on the sugarcane wireworm *Melanotus communis* decreases overtime. Future research determining whether repellency at different rates may be useful in reducing wireworm damage in both sugarcane and other agroecosystems and on other wireworm species (Cherry and Nuessly, 2010). Naturally occurring monoterpenoid essential oil constituents thymol, citronellal, eugenol and rosemary essential oil have been tested for their acute toxicity (LD50 and LC50) against late instars of *A. obscurus.* Their phytotoxicity was evaluated on corn germination and seedling establishment. Thymol had the greatest contact toxicity, while rosemary oil did not show any. Citronellal had the greatest volatile toxicity, before rosemary oil, thymol and eugenol. Thymol, eugenol and citronellal had significant phytotoxic effects (inhibition of corn seed germination and seedling establishment) and rosemary had only minimal ones. Acceptable qualities of a biopesticide include direct toxicity combined to repellent effects on the pest. Also, for economic reasons, the best way to use these compounds would be according to their repellency and not their toxicity, thus in push-pull strategies (Waliwitiya et al., 2005).

The efficacy of tefluthrin in wheat seed treatments is likely due to a combination of repulsion and short-term morbidity events (Vernon et al., 2009), but although repellency to an insecticide may provide temporary protection to plants and allow stand establishment,

wireworms initially repelled may damage crops later in the season. Consequently, it is important to observe the direct effects of novel insecticides on them. Chemicals identified as eliciting repellency should be tested to determine whether the attractive cues of host plants dominate repellent stimuli (van Herk et al., 2008a). Moreover, the ability of wireworms to recover from an active substance-induced morbidity may seriously limit the efficacy of the substance, as it has been shown for *L. canus* and tefluthrin-treated seeds (van Herk and Vernon, 2007a). Their sensitivity to repellent compounds may decrease when repeatedly made moribund. They may even be capable of associative learning (van Herk et al., 2010). Whenever they are conducted, toxicity trials should include observations on morbidity, and the trials should last until morbidity symptoms cease. Long-term morbidity (lethal, sublethal and behavioral effects) and potential recovery or death of wireworms exposed to certain insecticides have implications on how laboratory and field studies should be designed and interpreted (Vernon et al., 2008, Vernon et al., 2009). The on-going work on alternative active substances that affect wireworms can benefit lessons from previous research on synthetic chemicals. Table I-1 shows insecticides tested against *Agriotes* wireworms since 1990. Some studies in which identification to the genus was not stated were cited for their impact on research against wireworms. We distinguish between studies with behavioral observations on the larvae in regard to the insecticidal effect and those with field efficiency and crop protection considerations alone. Most of the behavioral effects (repellency, morbidity, sublethal effect, *etc*.,) concern *A. obscurus* and should be conducted on other species. Wireworm mobility categories proposed by van Herk and Vernon (2011) should allow comparing active substance efficiencies and species resistance (van Herk and Vernon, 2013b). Side effects on non-target organisms and acute or chronic effects on human health will always have to be investigated (Waliwitiya et al., 2005) (Furlan et al., 2004a). For any substance, field-realistic doses should be tested on potentially armed organisms, such as studied on bees for imidacloprid and clothianidin (Schneider et al., 2012). Globally, considering the overall apparent recovery and avoidance abilities of wireworms, push-pull strategies could be an interesting approach, with the advantage of limiting accelerated natural selection (Waliwitiya et al., 2005, Cook et al., 2007).

Table I-1. Insecticides tested against *Agriotes* since 1990, with sources, mode of application, protected crop, *Agriotes* species (whenever possible) and specific behavioral observations (sublethal effects, such as morbidity, lowered activity and recovery periods or repellency).

Active substance	Source	Application mode	Target crop	*Agriotes* species	Behavioural observations
Organochlorines					
Lindane	(Pique et al., 1998)	Granular, Furrow treatment	Corn	*A. lineatus*	
	(Eizaguirre et al., 2005)	Furrow treatment	Corn	*A. lineatus, A. segetum*	
	(Popov et al., 2001)	Seed dressing (+ fungicide)	Wheat, Barley	*Agriotes* spp.	
	(Vernon, 2005)	Seed dressing	Wheat	*A. obscurus*	
	(van Herk and Vernon, 2007b)	Seed dressing	Wheat	*A. obscurus*	x
	(van Herk et al., 2008b), (van Herk et al., 2008a), (Vernon et al., 2008)	Soil amendment/ Topical application, Soil less bioassay, Dermal exposure		*A. obscurus*	x,x,x
	(Vernon et al., 2009)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
Metoxychlor	(Vernon, 2005)	Seed dressing	Wheat	*A. obscurus*	
Aldrin	(Parker et al., 1990)	Broadcast	Potato	*Agriotes* spp.	
Organophosphates					
Chlorpyrifos	(van Herk et al., 2007)	Soil amendment/ Topical application		*A. obscurus, A. sputator*	x
	(Ester and Huiting, 2007)	Furrow treatment	Potato	*Agriotes* spp.	
	(Huiting and Ester, 2009)	Furrow treatment	Potato	*A. lineatus, A. obscurus*	
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(van Herk et al., 2008a)	Soil less bioassay		*A. obscurus*	x
	(Eizaguirre et al., 2005)	Furrow treatment, Granular	Corn	*A. lineatus, A. segetum*	
	(Vernon et al., 2008)	Dermal exposure		*A. obscurus, A. sputator*	x
Diazinon	(Vernon, 2005)	Seed dressing	Wheat	*A. obscurus*	
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
Ethoprophos	(Parker et al., 1990),	Broadcast	Potato	*Agriotes* spp.	
	(Huiting and Ester, 2009)	Broadcast	Potato	*A. lineatus, A. obscurus*	
	(Ester and Huiting, 2007)	Granular, Broadcast	Potato	*Agriotes* spp.	
	(Pavlista, 1997) in (Parker and Howard, 2001)		Potato		
Fonofos	(Parker et al., 1990)	Broadcast	Potato	*Agriotes* spp.	
Carbamates					
Aldicarb	(Parker et al., 1990)	Broadcast	Potato	*Agriotes* spp.	
Carbofuran	(Parker et al., 1990)	Broadcast	Potato	*Agriotes* spp.	
	(Eizaguirre et al., 2005)	Furrow treatment	Maïze	*A. lineatus, A. segetum*	
	(Pique et al., 1998)	Granular, Furrow treatment	Maïze	*A. lineatus*	
	(Popov et al., 2001)	Seed treatment	Sunflower	*Agriotes* spp.	

Table I-1. *Cont.*

Active substance	Source	Application mode	Target crop	*Agriotes* species	Behavioural observations
Carbodan (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methyl carbamate)	(Popov et al., 2001)	Seed treatment	Sunflower	*Agriotes* spp.	
Thiofanox	(Parker et al., 1990)	Broadcast	Potato	*Agriotes* spp.	
Neonicotinoids					
Imidacloprid	(Pons and Albajes, 2002)	Seed dressing	Corn	*A. lineatus*	
	(Bucurean and Gheorghe, 2011)	Seed dressing	Corn	*Agriotes* spp.	
	(Rose and Oades, 2001)	Seed dressing	Wheat	*Agriotes* spp.	
	(Furlan and Campagna, 2002)	Seed dressing	Sugar beet	*A. brevis, A. ustulatus*	
	(Meredith and Morris, 2003)	Seed dressing	Sugar beet	*Agriotes* spp.	
	(Ester and Huiting, 2007)	Seed dressing	Potato	*Agriotes* spp.	
	(Huiting and Ester, 2009)	Seed dressing	Potato	*A. lineatus, A. obscurus*	
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(van Herk et al., 2008a)	Soil less bioassay		*A. obscurus*	x
	(Vernon et al., 2008)	Dermal exposure		*A. obscurus*	x
	(Vernon et al., 2009)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
	(Popov et al., 2001)	Seed dressing	Sunflower	*Agriotes* spp.	
	(Furlan and Toffanin, 1998) in (Parker and Howard, 2001)				
Acetamiprid	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(Popov et al., 2001)	Seed dressing		*Agriotes* spp.	
Clothianidin	(van Herk et al., 2007)	Soil amendment/ Topical application		*A. obscurus, A. sputator*	x
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(van Herk et al., 2008a)	Soil less bioassay		*A. obscurus*	x
	(Vernon et al., 2008)	Dermal exposure		*A. obscurus, A. sputator*	x
	(Vernon et al., 2009)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
Thiacloprid	(Huiting and Ester, 2009)	Seed dressing	Potato	*A. lineatus, A. obscurus*	
Thiamethoxam	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(Popov et al., 2001)	Seed dressing	Sunflower	*Agriotes* spp.	
	(Vernon et al., 2009), (Vernon et al., 2013)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
	(Huiting and Ester, 2009)	Seed dressing	Potato	*A. lineatus, A. obscurus*	
	(Bazok et al., 2012)	Seed dressing	Tobacco	*A. ustulatus*	
	(Bucurean and Gheorghe, 2011) (Senn et al., 1998) in (Parker and Howard, 2001)	Seed dressing	Corn	*Agriotes* spp.	

Table I-1. *Cont.*

Active substance	Source	Application mode	Target crop	*Agriotes* species	Behavioural observations
Pyrethroids					
Bifenthrin	(van Herk et al., 2013), (van Herk and Vernon, 2013b)	Soil amendment/ Topical application	Potato	*A. obscurus*	x,x
	(Popov et al., 2001)	Seed dressing	Sunflower	*Agriotes* spp.	
Cyfluthrin	(Pavlista, 1997) in (Parker and Howard, 2001)		Potato		
Tefluthrin	(van Herk and Vernon, 2007b)	Seed dressing	Wheat	*A. obscurus*	x
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x,x
	(van Herk et al., 2008a)	Soil less bioassay		*A. obscurus*	x
	(Vernon et al., 2008)	Dermal exposure		*A. obscurus*	x
	(Popov et al., 2001)	Seed dressing	Sunflower	*Agriotes* spp.	
	(Vernon et al., 2009)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
	(Bazok et al., 2012)	Seed dressing	Tobacco	*A. ustulatus*	
	(Bucurean and Gheorghe, 2011)	Seed dressing	Corn	*Agriotes* spp.	
Phenyl pyrazole					
Fipronil	(Chaton et al., 2003), (Chaton et al., 2008)	Feeding assay, Seed dressing	Corn	*Agriotes* spp.	x,x
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
	(Vernon et al., 2008)	Dermal exposure		*A. obscurus*	x
	(Vernon et al., 2009)	Seed dressing	Wheat	*A. lineatus, A. obscurus*	
	(Vernon et al., 2013)	Seed dressing	Wheat	*A. obscurus*	
	(Furlan and Campagna, 2002)	Seed dressing	Sugar beet	*A. brevis, A. ustulatus*	
	(Furlan et al., 2010)	Seed dressing	Corn, Lettuce	*A. sordidus, A. brevis, A. ustulatus*	
	(Popov et al., 2001)	Seed dressing	Sunflower	*Agriotes* spp.	
	(Shamiyeh et al., 1999) in (Parker and Howard, 2001)		Potato		
Spinosyn insecticides					
Spinosad	(Ericsson et al., 2007)	Soil amendment/ Topical application		*A. lineatus, A. obscurus*	x
	(van Herk et al., 2008b)	Soil amendment/ Topical application		*A. obscurus*	x
Nematicides					
1,3-dichloropropene	(Grove et al., 2000)	Soil amendment/ Topical application		*Agriotes* spp.	x
Fosthiazate	(Grove et al., 2000)	Granular		*Agriotes* spp.	x
Fungicides					
Difenoconazole and Mefenoxam (Dividend XLRTA)	(van Herk and Vernon, 2007b)	Seed dressing	Wheat	*A. obscurus*	x

7. Biological Control Using Micro- and Macro-organisms

7.1 Entomopathogenic Fungi

The efforts to develop the use of entomopathogenic fungi (EPF) against wireworms are very recent, although these enemies were known since early in the 20th century. To our knowledge, the first report of *Metarhizium anisopliae* Sorokin as able to infect wireworms in nature was made in 1932 (Thomas, 1932). It can be applied in the field and infect and kill wireworms (Kabaluk et al., 2007). Laboratory assays allowed identification of virulent strains of EPF against larvae of *A. lineatus*, namely V1002 and LRC181A, respectively able to cause 90 and 100 % mortality three weeks post-inoculation (Ansari et al., 2009). Applied before planting in an area with wireworms (95 % *A. obscurus*) as a corn seed treatment alone or in combination with clothianidin or spinosad, the strain F52 can induce significant increases in stand density and stock and foliage area fresh weight yield. Not only does *M. anisopliae* strain F52 growth observed on retrieved wireworm cadavers link the positive effects on corn with the wireworm control, but it also suggests a synergistic interaction between EPF and insecticides (Kabaluk and Ericsson, 2007b), like when the strain is combined with spinosad: *A. obscurus* and *A. lineatus* wireworm mortality is higher when treatments are combined than for each treatment applied alone (Ericsson et al., 2007). The efficacy of *M. anisopliae* can also be enhanced by incorporation of neem seed cakes, as shown for the control of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) (Shah et al., 2008). Moreover, because *M. anisopliae* gradually degrades in the soil, the inclusion of spinosad or other insecticide treatments could extend the total control period provided by one application (Ericsson et al., 2007).

Beauveria bassiana (Bals.-Criv.) Vuill. is also efficient, even more with low doses of imidacloprid (Ester and Huiting, 2007). Presumably, the low rate (bio)insecticides stress the target or alter their behavior and immune mechanisms in such a way as to make them more susceptible to infection (Ericsson et al., 2007, Shah et al., 2007, Butt and Ansari, 2011). Some insecticides may increase pest mobility and increase acquisition of EPF conidia and, since mortality is dose-related, increase mortality (Butt and Ansari, 2011). Low levels of reduced-risk pesticides can be combined with biological agents without environmentally harmful traditional pesticide strategies (Ansari et al., 2009, Butt and Ansari, 2011), which clearly represents potential savings for growers (Butt and Ansari, 2011).

Further investigations concerning virulent strains of EPF should be undertaken taking into account factors including temperature, duration of exposure to the studied strain, soil

concentration of conidia and food availability. These will affect the levels of mortality and the rate of emigration of wireworms repelled by the fungi (Kabaluk and Ericsson, 2007a). The use of fungus against click beetles is less developed, though *Zoophthora elateridiphaga* has been found to be an important mortality factor of the click beetle *A. sputator* in Swiss meadows (Keller, 1994) and strains efficient against larvae have been suggested as potentially equally efficient against click beetles (Ansari et al., 2009).

7.2 Entomopathogenic Nematodes

Entomopathogenic nematodes (EPN) are also efficient against wireworms. *Steinernema feltiae* and *S. glaseri* caused a high mortality on the sugar beet wireworm *Limonius californicus*, however, field experimentations did not show very efficient and economically interesting results. The timing of application in this regard is a very important factor (Toba et al., 1983). On *Agriotes* wireworms, *S. feltiae* furrow treatments have been tested, but were not efficient (Ester and Huiting, 2007). The strain UWS1 of *Heterorhabditis bacteriophora* Poinar has proven very efficient against *A. lineatus* (67% mortality three weeks post-inoculation) (Ansari et al., 2009). *Hexamermis* spp. is also a known parasite of *A. obscurus* (Doane et al., 1973). Field damage to maize by wireworm can significantly be reduced with applications of *H. bacteriophora* and *Steinernema* species (Kovacs et al., 1980). The most efficient strains among those screened by Ansari *et al.* (2009) were local and originated directly from wireworm cadavers (Ansari et al., 2009). Where IPM is considered, native strains that co-evoluted with a target pest, and therefore adapted to local conditions, should be screened for their efficiency. More than identifying perfectly adapted strains, less risky as to their impact on non-target organisms, this helps outlining their optimal development conditions. This was undertaken for Spanish strains of *S. feltiae,* even if, in this case, the most efficient one only led to a 7% larval mortality on *A. sordidus* (Campos-Herrera and Gutiérrez, 2009). Any potential remains interesting because of possible synergies (Butt and Ansari, 2011), such as observed with *M. anisopliae* in the control of the pest *Hoplia philanthus* Fuessly (Coleoptera: Scarabaeidae) and the black vine weevil *O. sulcatus* (Ansari et al., 2004, Ansari et al., 2008). Synergies could equally work against wireworms and offer an organic or chemical-free approach in the control of *A. lineatus* (Ansari et al., 2009) and potentially other *Agriotes* species.

The mechanisms underpinning synergy between agents remain unclear. Furthermore, not all interactions are synergistic. Antagonistic or barely additive effects have been observed in a

wide range of situations involving other pests, strains and pesticides. The outcomes appear to be dependent on several factors including EPF strain (a virulent strain is vitally important), dose and the synergist type or efficacy enhancing agent used, which overall underlines the need to identify and optimize synergies (Butt and Ansari, 2011). Differences in wireworm species susceptibility to EPN exist and may be due to a range of physical barriers, including morphological barriers or behavioral responses to attacks (Eidt and Thurston, 1995). Once more, the necessity to consider the overall pest complex, case by case, is pointed out. Incidentally, comparisons between existing management options is necessary, whether they concern different treatments or consideration of potential control agents in place. Their may be drastic differences between agronomic situations. For example, natural infestations of *Metarhizium* spp., *Beauveria* spp. and nematodes should be evaluated when studying any treatment, as was done for biocidal seed meals in a field trial with rearing cages (*A. sordidus* and *A. ustulatus*) in different crop rotation systems. In this case, their efficiency was overall low and inferior to that of GL-containing plant material and did not differ between rotations (Furlan et al., 2009), but one can imagine the biases caused by omitting their roles in the results interpretation.

7.3 Other Organisms

By screening among dead wireworms collected in Germany in the aim to find new entomopathogenic taxa, an intracellular bacterium was recently identified: a pathogen belonging to the genus *Rickettsiella* (Leclerque et al., 2011, Kleespies et al., 2013). tested yet. The insecticidal potential of the bacterial flora of *A. lineatus* and other hosts was also studied. Of the isolated strains, five induced 100% mortality on third instar *A. lineatus* larvae 10 days after treatment, three of them belonging to the wireworm flora, highlighting their microbial control agents potential (Danismazoglu et al., 2012). Among potentially interesting predators of wireworms, some Carabidae and Staphylinidae (Coleoptera) are known to feed on *A. sputator* (Fox and MacLellan, 1956), but their use as control agents is inexistent. Conversely, recent observations on *Thereva nobilitata* (Fabricius) (Diptera: Therevidae), highlighted its potential as a top-down regulator of *Agriotes* larval populations in the field (van Herk et al., 2014). Other predators or parasitic pathogens should be researched, for example, in systems where everything points to wireworm infestations without occurrence. Then again, such natural control is possible in very diversified agricultural systems, where there is room for species other than crops and their pests. As stated above, grassy landscape margins would be

key factors explaining wireworm potato damage. Their role for the maintenance of natural pest control should be investigated (Hermann et al., 2012), especially since weed spots are key landscape elements for oviposition (Kovacs et al., 2006).

8. Conclusions

According to Furlan (2005), a rational IPM strategy against wireworms should be based on 1) locating high risk areas, notably with sex pheromone traps, 2) planting sensitive crops in areas with very low or no risk and 3) locating areas with actual *Agriotes* populations over thresholds. If no larval population is present, sensitive crops may be sown without any treatment. Where economically relevant populations have been found, sensitive crops should be sown late in the season or the next year, after application of an adequate treatment (biological or insecticide, period, rate) to control larval stages present, and tillage should be undertaken in the most suitable period to ensure high mortality (Furlan, 2005). In infested fields, damage may not occur every year and can be forecast by detecting wireworms at those periods, to prevent from further damage (Furlan, 2004), provided that the lifecycles of the species in place are well known and that mortality is ensured by the technique resorted to in the problematic areas.

As outlined throughout this paper, rational IPM strategies are increasingly available, and the treatment options or monitoring tools in this regard are in good development. Whenever considered, any element of IPM strategies should be planned, taking into account the encountered wireworm species, because of the differences in biology, ecology and behavior that can occur between them. Species for which gaps of knowledge remain should be studied following a similar methodology to those already well described (Furlan, 2005). Although not conceivable for growers, the identification of the main *Agriotes* pest species is possible, thanks to morphological characteristics or genetic footprints (Klausnitzer, 1994, Cocquempot et al., 1999, Pic et al., 2008, Ellis et al., 2009), a tool that will probably, in the near future, outline the diversity of pest populations in place (Benefer et al., 2013). Wireworm sampling is time-consuming and not always reliable. While there are examples of methods efficient for infestation/attack rate assessment (maize, France and Italy) (Chabert and Blot, 1992, Furlan, 2005) or treatment decisions (sugarcane, Florida) (Cherry et al., 2013), counter-examples exist, especially in potato (Yates and Finney, 1942, Parker et al., 1994, Parker, 1996, Parker and Howard, 2001). When the infestation level can't be related to adult trapping, the latter could be used as a cotrol tool, since the components of the main *Agriotes* species sex

pheromones have been identified and proportions optimized for efficient baits (Borg-Karlson et al., 1988, Siirde et al., 1993, Toth et al., 2003, Toth, 2013). Their potential in wireworm reduction over the years following male click beetle mass trapping remains unclear and may require a dense network of traps (Sufyan et al., 2011). More research should focus on the comparison of agricultural systems: those where attacks are nearly systematic and devastating compared to those where attacks are rare or still not intense enough to significantly reduce yields. Enough data should be acquired on both types of situations so that large-scale analysis can help pointing out subtle factor combinations that remain invisible for now (Parker and Seeney, 1997). The impact of field or site characteristics may differ drastically between species, hence the strong necessity to separate taxa into species when assessing the pest-complex (Benefer et al., 2010). This could highlight more of what should be considered a priority when managing wireworms in a preventive way. Chances are that the most efficient IPM strategies will take place in the most diversified agricultural systems. A preventive systemic approach is always the most interesting to consider on the long-term. Moreover, it gives time for treatment options to be developed and repeatedly improved.

The resort to insecticides can be much reduced, notably by privileging seed treatment, which can ensure seedling establishment where and when it is the most needed (Huiting and Ester, 2009, Vernon et al., 2009, Vernon et al., 2013). Besides, when insecticides are combined to other control agents, such as entomopathogenic fungi or nematodes, synergies may be observed (Ericsson et al., 2007, Ansari et al., 2009, Butt and Ansari, 2011). Promising studies highlighted the effects of natural substances-derived chemicals on wireworms and their potential as synergists as well (e.g., (Ericsson et al., 2007, Kabaluk and Ericsson, 2007b, Shah et al., 2008, Cherry and Nuessly, 2010, Furlan et al., 2010, Butt and Ansari, 2011)). They should be preferred to insecticides whenever possible, especially regarding the latest European regulations in the matter of plant protection and their implications (Jehle, 2011). Among such substances, those ensuring mortality have effects lasting longer than stand establishment, which is economically more relevant, but considering the overall apparent recovery and avoidance abilities of wireworms, push-pull strategies could be an interesting approach, with the advantage of limiting natural selection of the pest (Waliwitiya et al., 2005, Cook et al., 2007). In order to compare and identify what synergies are the most reliable on wireworms, potential synergists and other available tools should be reunited in common trials and their combinations tested on several, if not all, of the most important *Agriotes* species, through a standard methodology developed for different regions. Given the concerns of

environmental effects, such as animal and human health, any product destined to control wireworm populations should always be studied as to its potential side effects with field realistic doses (to be established in every case), such as investigated for the effect of neonicotinoid seed treatment residues on honey bees (Schneider et al., 2012).

Regardless of treatment efficacy, there are differences in the susceptibility for wireworm damage between crops, cultivars and farming systems (Jary, 1942, Hall, 1990, Jonasson and Olsson, 1994, Furlan and Toffanin, 1996, Parker and Howard, 2000, Furlan, 2004, Furlan, 2005, Johnson et al., 2008, Keiser et al., 2012). The production of more resistant cultivars thanks to breeding may rely on the understanding of mechanisms underpinning these differences. Improvements of current knowledge on wireworm chemical ecology could help in achieving that purpose. Johnson and Gregory (2006) suggested that plant-produced products other than CO_2 have a role in the feeding choice of the larvae, notably root-emitted volatiles (Johnson and Gregory, 2006). The identification of such semiochemicals might help explaining the diverse sensitivity among varieties. Studies on specificity and variability of belowground responses should be included in efforts to exploit tritrophic interactions to improve biological control practices (Rasmann and Turlings, 2008). Such advances could lead to both varietal selection and new integrated wireworm management options.

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Chapter I.2 – The Foraging Behavior and Sensory Apparatus of Wireworms

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Abstract: Integrated management of wireworms (Coleoptera: Elateridae) depends upon approaches applied both above- and belowground, and over several spatial scales. While foraging, these soil pests use biotic and abiotic signals to orientate towards target plant organs. Development of efficient techniques for implementation in integrated strategies relies upon improved knowledge of this process. In particular, an important stage consists in elucidating the role of volatile organic compounds (VOC), emitted by belowground plant organs, in wireworm chemical ecology. This would have a positive impact on push-pull strategies and varietal selection developed against these insects. In this work, we summarized the available data regarding wireworm foraging behavior as well as variables that should be considered when studying the potential role of plant-produced volatile semiochemicals. This includes CO_2 gradients and other host-related cues, temperature, relative humidity and soil moisture, and wireworm physiological stage. We also review what is known of the sensory apparatus of wireworms, since this is involved in every step of the foraging process. Some baseline data for studying VOC related wireworm foraging behavior exists. Using it as a tool in applied entomology should result in discovery of the semiochemicals that underpin trophic interactions involving these pests. However, most of the key pest species are not fully described with regards to the parameters detailed here. Obtaining accurate information to fill the current knowledge gaps will be needed in order to devise new integrated management strategies.

Key words: *Agriotes*, chemical ecology, volatile compounds, rhizosphere, integrated pest management, semiochemicals, trophic levels.

1. Introduction

Multi-trophic interactions involving herbivorous insects and their host plants are mediated by semiochemicals; compounds conveying information, including, notably, the nature or the physiological state of the host (Vet and Dicke, 1992, Kessler and Baldwin, 2001). Many insect pests have been studied in this regard in order to develop IPM strategies, leading to efficient crop protection without resorting to synthetic pesticides (Cook et al., 2007). To date, the majority of scientific papers on this subject concern aboveground interactions. Indeed, on the Scopus search engine (www.scopus.com, consulted on 24.07.2014), insect-plant interactions are addressed in 10365 documents, among which 454 discuss semiochemicals (4.4%). Of these, only 143 concern roots (32%), and 115 root pests (25%). However, the latter are attracting increasing attention from the scientific community, and approaches based on semiochemical volatile organic compounds (VOC) in aboveground interactions are now also being applied to soil pests. A recent review outlined the role of VOC for soil insects, and reported compounds with semiochemical properties for a wide variety of pest species (Johnson and Nielsen, 2012). The model "maize (*Zea mays* L.) - *Diabrotica virgifera virgifera* (LeConte) larvae (Coleoptera: Chysomelidae)" constitutes a very good example of how belowground interactions can be as complex and multi-trophic as aboveground ones (Rasmann et al., 2005, Hiltpold and Turlings, 2008, Robert et al., 2012). Many studies also report on the impact of root pests in aboveground interactions with other pests, as reviewed in Erb et al. (2008).

The chemical ecology of belowground pests, including the roles of root-emitted VOC in pest-host plant interactions, is still relatively unknown. In particular, data concerning certain wireworm species, belowground crop pests and larval stages of click beetles (Coleoptera: Elateridae) is scarce (Johnson and Nielsen, 2012). Large-scale approaches to achieve wireworm control could benefit from new insights at the individual scale. Since new alternative management strategies against these insects are needed, research on their chemical ecology and foraging behavior, notably under the influence of belowground plant VOC, is also needed (Barsics et al., 2013). This paper summarizes knowledge on i) the foraging steps, known compounds, and abiotic parameters involved in the activity of wireworm pest species; and ii) the functions and anatomical locations of their sensory appendages. The information already available constitutes a solid base to investigate the role of VOC in wireworm behavior, but important and accurate information is still missing.

2. The Foraging Behavior of Wireworms

2.1 First Step: the Role of Carbon Dioxide

The orientation of wireworms and other belowground pests towards host plants is seen as a three-step sequence influenced by soil nature and structure, abiotic parameters and other soil organisms (Johnson and Nielsen, 2012). Figure I.1 shows this sequence. In wireworms, the feeding phase of each instar only lasts a short time, i.e. 20-29% of the whole development, with the rest being devoted to the molting metabolism (Evans and Gough, 1942, Furlan, 1998, Furlan, 2004). Independent of chemical stimulations, wireworms follow lines of least resistance (Thorpe et al., 1946). This behavior changes in response to encountered gradients of CO_2, a very efficient general semiochemical indicating food location (Johnson and Nielsen, 2012). Several species in the genus *Agriotes* Eschscholtz, can locate excised plant roots or CO_2 sources (Klingler, 1957, Doane et al., 1975). The most efficient baits for these species are germinating grains such as wheat, corn and barley (Parker, 1996, Simmons et al., 1998). These larvae are also strongly attracted to seeds of *Medicago sativa* L. (alfalfa), *Brassica napus* L. (rape), *Melilotus alba* Desv. (sweet clover) and *Helianthus annuus* L. (sunflower) (Doane et al., 1975). Other wireworm species equally respond to germinating grains: wheat and barley strongly attract *Limonius canus* (LeConte, 1853) (Horton and Landolt, 2002), while rice attracts *Melanotus okinawensis* (Ôhira, 1982) (Arakaki et al., 2009). In terms of sensitivity, the response threshold of *Ctenicera destructor* (Brown, 1935) wireworms to CO_2 gradients has been reported to be as fine as 1 to 2 ppm over the distance involved in one deflection of the head during klinotactic orientation (Doane et al., 1975), demonstrating the importance of such a signal. Attraction occurs at concentrations between 360 ppm and 15000 ppm but depends on the physiological stage: it decreases with proximity to ecdysis (Doane et al., 1975, Doane and Klingler, 1978). In laboratory conditions at between 22 and 25°C, *Agriotes* larvae may take up to 70 minutes to find a CO_2 source located 20 cm away (Doane and Klingler, 1978). Repellency may also occur under gradient steepness or overexposure (Doane et al., 1975, Doane and Klingler, 1978).

2.2 Second Step: Attraction towards Host-Specific Semiochemicals

The next foraging step for belowground herbivorous insects involves plant-originating substances, notably VOC, allowing host-specific recognition (Johnson and Nielsen, 2012). Foraging behavior is impacted by the nature and gradient of concentration of the semiochemical blends wireworms are exposed to (Thorpe et al., 1946, Crombie and Darrah,

1947). A preference for grass species from nutrient-rich grasslands (*Lolium perenne* L. and *Holcus lanatus* L.) over species from nutrient-poor grasslands (*Festuca rubra* L. and *Anthoxanthum odoratum* L.) has been shown for *Agriotes obscurus* (L.), with the hypothesis that specific cues other than CO_2 underpin this discrimination (Hemerik et al., 2003).

To classify compounds likely to induce a response in wireworms, one can use two measures: the compound threshold of response and its activity. The first is the lowest concentration needed for a response to occur. The second is defined as minus the logarithm of the threshold, e.g. 9 is the activity of a compound with a threshold of response of 10^{-9} g/ml (Doane et al., 1975, Doane and Klingler, 1978). A second way to discriminate between compounds is related to induced responses. There is a distinction between compounds eliciting solely orientation responses, mainly involved in the second foraging step, and those inducing both biting and orientation. In almost all cases, compounds eliciting biting responses have high activities (i.e. with thresholds between 10^{-9} and 10^{-11} g/ml), while compounds eliciting only orientation are less active (i.e. with thresholds close to 10^{-3} g/ml) (Crombie and Darrah, 1947). These thresholds are 1000 fold higher than that of CO_2 (see above), and suggests that such compounds need to be emitted or produced in higher amounts than biting-eliciting compounds to be used by wireworms, since orientation occurs before biting in the foraging process (Thorpe et al., 1946). It can also indicate at what scale, or from what distance from the target organs, these compounds may influence foraging, provided that their diffusing properties are known.

To our knowledge, the first molecules highlighted as inducing orientation in wireworms consist of a group of sugars, peptone, triolein and tannic acid each of activity 2-3, and of a group of acids and amides widely distributed in plants, with higher activities (Thorpe et al., 1946). All amides tested by Crombie and Darrah (1947), as well as urea and guanidine, can attract wireworms. The response is stimulated by chemical structures including $-CONH_2$ or $-CNHNH_2$, but not $-CNH_2$ (indicated by the inactivity of glycine and alanine). Asparagine and glutamine for example, are ubiquitous in plants, reaching high concentrations in certain seedlings as secondary products of seed protein breakdown. Arginine is found in considerable amounts in some seedlings, and is widely distributed at lower concentrations in mature plants (Crombie and Darrah, 1947). This is consistent with constituents of wireworm preferential target plant-organs: mesocotyls, seeds, and seedling (Chaton et al., 2008). Some dicarboxylic acids are also active, notably citric, succinic and malic acids, compounds involved in oxidative respiration in plants. There isn't, however, a clear rule for predicting that a given

dicarboxylic acid will be active (Crombie and Darrah, 1947). In *Agriotes* species, there is only one report of VOC inducing wireworm orientation, attributed to 2-pentylfuran (Barsics et al., 2012), a compound listed in the volatile profile of barley roots (Fiers et al., 2013, Gfeller et al., 2013). Finally, regardless of the semiochemical, the sensitivity for orientation increases with starvation duration for seven days (Thorpe et al., 1946). As wireworms approach target organs, the third foraging step starts.

2.3 Final step: Biting and Retention in the Root System

Most of the compounds capable of inducing biting are members of the three major food groups; carbohydrates, fats and proteins (Thorpe et al., 1946). The most active ones are commonly found in plant sugars, plant juices (e.g. potato, sugar-beet), or, such as starch, in seeds (Thorpe et al., 1946, Chaton et al., 2008). In *Agriotes lineatus* (L.) and *Agriotes obscurus*, appetence is equivalent for seed flours of corn, wheat, walnut and sunflower (Chaton et al., 2003). In sugars, the polyhydric alcohol groupings were found to be responsible for induced activity (Thorpe et al., 1946). Triolein is the only triglyceride that induces biting responses even if sodium salts of certain fatty acids are active as well (Thorpe et al., 1946). Active proteins are of animal origin; tested plant proteins are inactive. Partially broken proteins may be active although the parent proteins are not, but no amino acids or mixtures thereof have shown activity (Thorpe et al., 1946). As for orientation, wireworm sensitivity to biting compounds is progressively increased with starvation up to seven days (Thorpe et al., 1946). These properties may extend to other *Agriotes* species or other genera.

The biting response is strongly involved in the final step of the foraging process. Unlike compounds causing only orientation, those inducing biting (and therefore also orientation: see above) seem to retain wireworms in the area in which they are detected, with some exceptions. Indeed, although asparagine and related substances are described as orientation compounds, wireworms are extremely sensitive to these substances. They increase the target nature of a root system by causing wireworms to remain in the vicinity of fine roots (Thorpe et al., 1946).

It was shown that, although inactive individually at certain concentrations, some compounds can induce a response when exposed simultaneously. For example, solutions of 0.5% glucose and 0.126% sucrose are inactive individually, but are active when mixed (Crombie and Darrah, 1947). The following compounds are synergistic: glucose and sucrose for biting and orientation; glucose and peptone, glucose and triolein, peptone and triolein,

glucose and tannin for biting (Crombie and Darrah, 1947). It is therefore important to consider as complete and accurate a blend as possible when assessing the effect of substances emitted by plants in the soil on wireworm behavior (De Bruyne and Baker, 2008). As we have seen above, chemosensory cues encountered before the vicinity of roots and at the root surface can induce acceptance. Conversely, some compounds inhibit activity or repel insects (Johnson and Nielsen, 2012), which emphasizes the need to consider the entire chemical environment. For example, the glucose-induced biting response can be inhibited by lead acetate, quinine, allyl-isothiocyanate, and common salt, as well as by acid and alkaline solutions (Crombie and Darrah, 1947). Common salt also inhibits the glucose-induced orientation, but not the asparagine-induced one (Crombie and Darrah, 1947). Known natural deterrents are chalconine, solanine and glycoalkaloids in potato (Jonasson and Olsson, 1994). However, field experimentations have proved that glycoalkaloid content alone could not be the sole mechanism underpinning wireworm preference among different potato varieties (Johnson et al., 2008). Finally, repulsion due to insecticides has been reported, either with synthetic compounds such as bifenthrin (van Herk et al., 2013) or in soils treated with the plant derived biopesticide azadirachtin (Cherry and Nuessly, 2010), and their effects seem to extend beyond the foraging behavior.

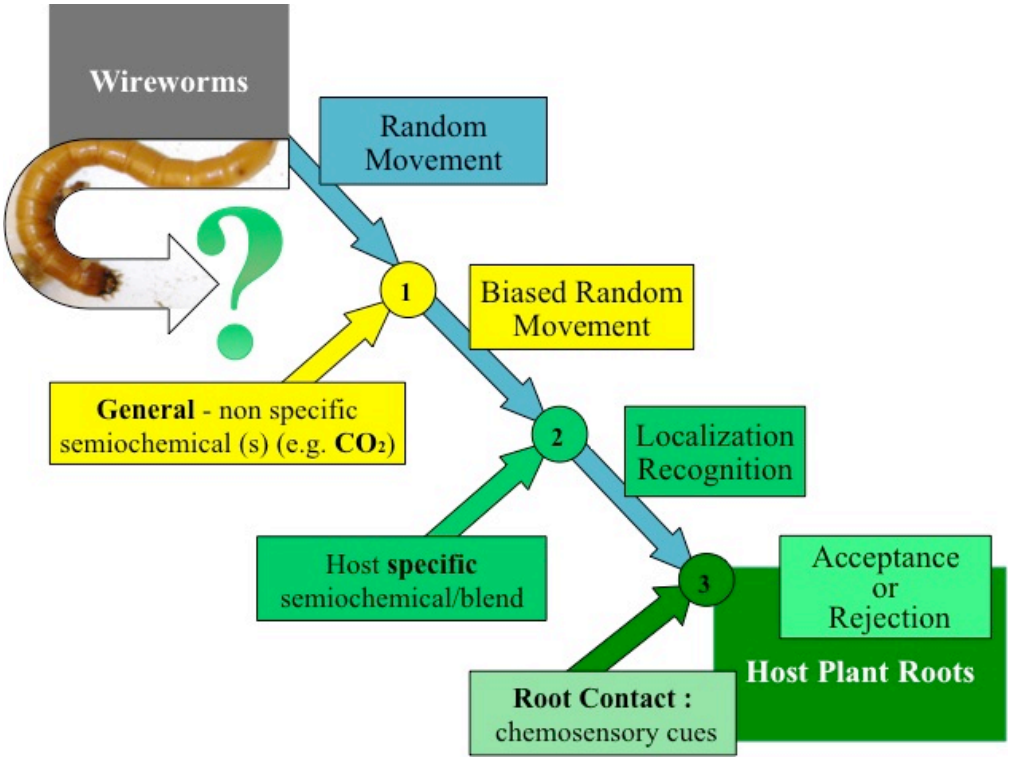

Figure I.1. The foraging behavior of wireworms, redrawn and adapted from Johnson and Gregory (2006). The experimentations detailed in this work concern the role of VOC in the second foraging step, such as indicated by the red arrow.

2.4 Factors affecting Wireworm Movement and Activity

In the sections above, we depicted what is known of the chemical background for foraging. Factors other than the compounds themselves will impact the foraging process by affecting wireworm movement or activity. Although not assessed for all of the important wireworm pest species, speed of movement may vary between them. In similar conditions, some species tend to be found further from a given release point than others, as observed for *Athous haemorrhoidalis* (Fabricius, 1801) over *A. obscurus* (Hemerik et al., 2003). For certain species, in situ dispersal abilities have been assessed. For *Melanotus okinawensis*, mark-release-recapture methods allowed estimating the lifetime natural mean dispersal distance to 105.6 ± 20.1 cm (Arakaki et al., 2010). Another *in situ* method, based on stable isotope signatures of different crops, has revealed that the larvae of *Agriotes obscurus* will not move laterally in the ground as long as their food supply is sufficient (Schallhart et al., 2011). It has been suggested that horizontal migration will only occur if necessary, i.e. in case of food depletion, because it represents an important energy loss (Sonnemann et al., 2014). Moving rates depend on factors such as temperature (Campbell, 1937, van Herk and Vernon, 2013a), physiological stage, as reported for *A. obscurus* (van Herk and Vernon, 2013a), and relative humidity, soil moisture and pH (Lees, 1943b, Lees, 1943a, Thorpe et al., 1946). In addition, individual behavior may be modified under high densities of individuals or when food is scarce, which sometimes leads to cannibalism (Furlan, 1998, Hemerik et al., 2003, Traugott et al., 2008).

Relative humidity (RH) and real water content have slightly different effects on wireworm activity. The optimal RH level for *Agriotes* wireworms is air saturation; lower RH areas are avoided (Lees, 1943a). In fact, dry air is more intensely avoided when alternatives are closer to saturation. A difference of 7.5% RH (17°C) suffices to ensure total avoidance of lower RH areas (Lees, 1943a). Moving air or humid air currents have been shown to be attractive to wireworms, although they are not as efficient as carbon dioxide (Doane and Klingler, 1978). Similarly, wireworms migrate rapidly out of dry soil and aggregate in areas with sufficient moisture (Lees, 1943b). From field observations, it is known that wireworms kept in dry soil rapidly die of desiccation. In sandy-loam, the optimal water content ranges from 8 % to 20 % with an apparent preference for 12-16 % (Campbell, 1937). Conversely, water-saturated soil can induce complete discontinuance of activity and sometimes death (Campbell, 1937). Moisture inhibits all muscular activity, including that of mouthparts. Incidentally, the feeding activity of *Agriotes* larvae is greater at low moistures compared to

higher ones (tested ranges: 10-80%), which is linked to both inactivity in high moistures and the consequent failure to reach food (Lees, 1943b).

Temperature influences wireworm activity, behavior, and distribution. As for many insects, higher temperatures lead to increased activity and lower ones result in inactivity in wireworms. Extremes lead to death, and larval development rate is strongly related to intermediate temperatures (Campbell, 1937, Furlan, 2004). Throughout their multiyear development cycle, field activity of wireworms is particularly high in spring and fall. With sufficient soil moisture and food, it continues throughout the summer, and decreases in the winter with a temperature-related intensity (Campbell, 1937, Furlan, 2004). One given species will complete its cycle with a different development rate according to latitude. In the winter in Italy, at 41°N compared to 45°N, *Agriotes sordidus* (Illiger, 1807) larvae continue their development in the food-rich top soil layers instead of burrowing deep into the soil in search of more suitable temperatures (Furlan, 2004). In Southern California (latitudes 34 to 36°N), *Limonius californicus* (Mannerheim) wireworms never cease their activity entirely (Campbell, 1937). Apparently, in this species, adjustment to the most suitable temperatures will not occur until larvae have been subjected to the changing (lower or higher) temperatures for a month or more (Campbell, 1937). It was shown in rearing conditions that *A. sordidus* does not grow below 9°C. In contrast, a constant temperature of 29°C allows it to complete its lifecycle in four months, starting from the eggs (Furlan, 2004). Developmental rate will also depend on whether or not wireworms previously underwent a period of cold during the winter (Furlan, 2004). Finally, temperature influences the appetence of wireworms; *A. obscurus* will destroy a higher number of seedlings at 22°C compared to lower temperatures in similar experimental conditions (van Herk and Vernon, 2013a). As temperature influences wireworms in so many ways, it is one of the key parameters to control during rearing and behavioral assays.

Gravity has been suggested to exert some influence: when stimulated by an unfavorable, dry environment, the natural movement trend of wireworms is downward, as suggested for *L. californicus* (Campbell, 1937). However, burrowing *Agriotes* wireworms do not respond to gravity (Lees, 1943b). Wireworms only occasionally walk on the soil surface, but it does not seem possible to detect any response to odors under such conditions (Thorpe et al., 1946). Physicochemical properties of the substrate are also major factors. The direction of movement direction is determined by soil structure (Thorpe et al., 1946) and soil pH can affect wireworm feeding, through changing the activity of compounds, regardless of the nature of

the induced response. For example, the pH activity curve for glucose ranges between 6 and 8, while most plant juices have a pH between 5 and 7 (Thorpe et al., 1946). It may also influence the chemical background of the soil, as some reactions will give different products according to the soil pH. Such chemicals have already been shown active on wireworms (Chen and Andreasson, 2001).

2.5 Recommendations for Behavioral Bioassays

This section details optimal conditions for behavioral assays on wireworms, especially when evaluating the impact of plant VOC.

Odorous stimuli contain three elements of information: identity, intensity, and temporal variation of the latter. Moreover, if an odor is a mixture, its entire identity can change with the slightest change in the mixture (De Bruyne and Baker, 2008). In behavioral experimentations on wireworms, in a static environment, concentrations of tested blends, as well as ratios between compounds, should therefore be realistic. It requires prior quantification of root VOC emission and/or content and adequate VOC releasing formulations. Also, the devices and substrate must be strictly controlled with regards to known active substances, and insecticide-free plants should be used whenever live material is needed. Considering the importance of carbon dioxide in foraging, its concentration must be at least controlled, and should match soil concentrations. Using these conditions, results should highlight VOC soil concentrations that can impact behavior. In addition, selection of individuals in the feeding phase is possible with baiting techniques, e.g. carbon dioxide sources themselves (Doane and Klingler, 1978).

In the literature cited, experimentations were performed in a substrate and/or under reduced light conditions. In novel experimentations, these two elements should be consistently combined. As for any other insect, all abiotic parameters should be standardized, both spatially and temporally. But in the particular case of wireworms, which have limited feeding phases, abiotic factors could be more important than tested volatile cues. Olfactometric devices should be designed in a horizontally balanced way. It is not clear whether gravity impacts wireworm movement, but it can modify soil moisture distribution and so indirectly affect behavior. Moreover, moisture should represent less than 20% of the substrate and dry air and soil should be avoided. Temperature regulates wireworm activity in the short and the long term. Although laboratory conditions are often constant, temperature-related life history traits have to be taken into account as well, especially when planning

experimentations with wireworms collected from the field. Rearing conditions should match temperatures needed for activity and remain relevant to field reality. In addition, when assessing the role of VOC, there will be a compromise between wireworm activity and optimum temperatures for chemical diffusion. If gradients disappear due to fast diffusion, attractant or repellent effects may be erased, which would make any conclusions unreliable.

Uniformity is also important among tested individuals. As much as possible, they should belong to one instar or to a known range of instars, and be in a feeding stage. The cropping history of fields in which the individuals were collected should be known, and areas with no or non-recent history of pesticide use should be preferred for collection. Species-specific foraging speed and interactions with other organisms in the soil community must be accounted for. While foraging speed will determine the experimental conditions suitable to observe behavioral effects, especially timing and temperature, species interactions will shed light on impacts of congeners or conspecifics on foraging behavior. These factors are important for designing management strategies. To our knowledge, nothing is known of the direct or indirect (e.g. through the host plant) effects of other soil organisms on the behavior of wireworms. However, there is evidence for specific aggregation patterns, notably at the field scale, as well as recurrent proximity between given taxa at different spatial scales, such as observed for *A. obscurus* and non-*Agriotes* wireworms (Benefer et al., 2010). A further consideration is that of the impact aboveground organisms may have on belowground wireworms, by mediation through host-plants. However, providing accurate explanations concerning mechanisms controlling such multi-trophic systems involving wireworms necessitates prior investigations, as described.

3. Wireworm Chemoreceptors

Not all substances released by a plant extract necessarily activate chemoreceptors and further induce a specific behavior (Agelopoulos et al., 1999). Electrophysiological assays, including electroantennography (EAG) and single cell recordings, allow discrimination between active compounds and others. This requires accurate description and location of all sensory receptors (Zacharuk and Shields, 1991). Once determined, relevant blends of active compounds can be tested as to their impact on insect behavior (Agelopoulos et al., 1999). In larvae, nerve impulses induced by signals from the environment can be recorded notably from under-developed antennae, labial or maxillary palps. Some studies link sensilla to feeding-related responses after organ ablation, as shown for carbon dioxide detection in wireworms

(Doane and Klingler, 1978). Research increasingly correlates feeding behavior with electrophysiological responses, particularly of sensilla involved in gustation. Similar studies on larval olfaction are more limited (Zacharuk and Shields, 1991), though a recent study on olfaction in belowground larvae of *Melolontha melolontha* (Coleoptera, Melolonthidae) combined morphological descriptions from SEM (scanning electron microscopy) and electrophysiological recordings (Eilers et al., 2012); techniques that would be useful for similar investigations in wireworms.

Larval sensilla are often difficult to categorize. In Table I-2, we provide descriptions and functions of sensilla types commonly found on immature insects to contextualize the following discussion on knowledge related to wireworm chemosensory receptors. This table is based on sensilla classification followed by Zacharuk and Shields (1991) for immature insects. Table I-3 gives anatomical locations and functions of sensilla found on wireworms. It is organized based on the work of Zacharuk (1962), who provided details for twelve wireworm species found in different habitats, including agricultural lands, sandy soils and decaying wood.

Table I-2. Sensilla commonly found on immature insects, based on Zacharuk and Shields (1971).

Category, number of pores (AP: aporous; UP, uniporous; MP, multiporous), functional type (MS: mechanosensilla; T-HS: thermo-hygrosensilla; GCS: gustatory chemosensilla; GC-MS: gustatory- chemo-mechano sensilla; OCS: olfactory chemosensilla) and structural description.

Category	Porous type	Functional type	Structural description
sensilla squamiformia	Not found on immature insects		
s. chaetica	AP UP	MS (tactile) GC-MS	heavy, thick-walled bristles or spines
s. trichodea	AP UP MP	MS, T-HS, rarely OCS GC-MS (commonly) OCS	hairs
s. basiconica	UP	GC-MS - osmo-sensors	peg-like
s. coeloconica	AP MP	T-HS OCS	pegs in shallow pits
s. ampullacea	close to *coelonica*		pegs in deep pits
s. campaniformia	AP	MS	domes
s. placodea	UP MP	GCS OCS	plates generally level with the cuticle surface
s. styloconica	UP AP	GCS Mainly MS	pegs set on an elongated style
s. scolopalia	AP	MS	largely subcuticular, dentritic insertion in or attachment to the cuticle through an accessory cell, generally not surface cuticular

Table I-3. Anatomical locations and functions of sensilla found on wireworms.
Based on the work of Zacharuk (1962), who provided details for twelve wireworm species found in different habitats.

Type of sensilla	Anatomic location	Function	References
Thick-walled setae			
Sub-type: long (situated on exposed surfaces, usually come into contact with material before the cuticle does)	Cephalic sclerites Antennae Mandibles Maxillary stipes and palps Labial palps Tip of galeae Lacinia Pre- and postmentum Ligula	Primarily tactile, protective mechanism for the head or other shorter sensilla, or static organs involved in orientation and movement	(Zacharuk, 1962)
Sub-type: short (project slightly above the surface of the cuticle)	Cephalic sclerites Epicranial plates : medioventral margin, posterodorsal surface Base of the prementum (ventral aspect) Base of the nasale (ventral aspect)	Primarily tactile, tactile proprioceptors involved mainly during feeding	
Sub-type: minute (with tips approximately level with the surface of the cuticle)	Cephalic sclerites Frontoclypeus Epicranial plate Maxillary stipes Postmentum	Primarily tactile, static organs, possibly involved in temperature or moisture sensing	
Campaniform			
dome-shaped	Cephalic sclerites Antennae Mandibles Maxillary stipes Maxillary and labial palps Frontoclypeus Epicranial plates Post and pre-mentum Base of the nasale (dorsal aspect) Galeae Lacinia	Mechanical propioceptors, exocuticular layers	(Zacharuk, 1962)

Zacharuk (1962) gathered morphological observations on 12 species, namely *Athous haemorrhoidalis* (F.), *Ctenicera aena* (L.), *Ctenicera destructor*, *Limonius minutus* (L.), *Hypolithus riparius* (F.), *Dalopius marginatus* (L.), *Agriotes obscurus* (L.), *Agriotes lineatus* (L.), *Lepturoides linearis* (L.), *Melanotus rufipes* (Herbst), *Ampedus nigrinus* (Herbst), *Adelocera murinus* (L.). *Agriotes lineatus* and *Agriotes obscurus* were also studied by Crombie et al. (1947), Thorpe et al. (1946) who also described *A. sputator* L., and Doane et al. (1978), who also referred to *Limonius californicus*. All other references in the table refer to *C. destructor*.

Table I-3. *Cont.*

Type of sensilla	Anatomic location	Function	References
plate-shaped	Frontoclypeus	Endocuticular layers - mechanical propioceptors - close to muscles attached to the cuticle	(Zacharuk, 1962)
	Epicranial plates		
	Maxillary stipes		
	Postmentum		
cone-shaped	Membranous ligula (anterodorsal aspect)	Propioceptive receptors, changes in body fluid pressure	
peg-shaped	Similar to campaniform cone-shaped		
Pore canal organs	Tips of the mandibles, resemble classical placodea sensilla	Contact chemoreceptors, substances in solution, more likely gustation than orientation	(Zacharuk, 1962)
Scolopophorous (a variant of *scolopalia*)	Maxillary and labial palps	Mechanical stimuli, vibratory receptors	(Zacharuk, 1962)
	Mandibles	Proprioceptive mechanoreceptors	(Zacharuk and Albert, 1978)
Peg or thin-walled hair	Tips of the antennae	Primarily contact chemoreceptors with orientation to substances in solution	(Zacharuk, 1962)
	Antennae, small multiporous sensilla	-	(Scott, 1969)
	Distal segment of labial palp, digitiform sensilla	CO_2 receptors in *A. lineatus-obscurus* contrarily to *L. californicus*	(Doane and Klingler, 1978)
	Basal margins of annular sclerites of the 2nd and 3rd maxillary and the 2nd labial segment, more related to thick–walled than thin	Primarily chemosensory, primarily contact chemoreceptors with orientation to substances in solution	(Zacharuk, 1962)
	Labial palp, digitiform sensilla	CO_2 receptors, olfactory pegs	(Doane and Klingler, 1978)
	Galeae	Contact chemoreceptors, stimulation leads to both orientation and biting	(Crombie and Darrah, 1947)
		Tactile mechanoreceptor	(Bellamy, 1973) (Bellamy and Zacharuk, 1976)
		Primarily tactile, changes in pressures of body fluids in ventral mouthparts	(Zacharuk et al., 1977)
	Antennae, multiporous sensilla	Observations on *C. destructor* suggest that they respond strictly to mechanostimulation, despite the subapical pore	(Zacharuk, 1962)
	Labial and maxillary palps	Primarily chemosensory, olfactory orientation and/or gustation; or contact chemoreceptor, primarily gustation	(Zacharuk et al., 1977)
	Maxillary palps and labial palps to a lesser	CO_2 receptive-sensilla	(Zacharuk, 1962)
	Labial palps, maxillary palps, *Agriotes*	Contact chemoreceptors	(Doane and Klingler, 1978)
	Tip of labial palp, basiconic pegs	-	(Doane and Klingler, 1978)

Zacharuk (1962) gathered morphological observations on 12 species, namely *Athous haemorrhoidalis* (F.), *Ctenicera aena* (L.), *Ctenicera destructor*, *Limonius minutus* (L.), *Hypolithus riparius* (F.), *Dalopius marginatus* (L.), *Agriotes obscurus* (L.), *Agriotes lineatus* (L.), *Lepturoides linearis* (L.), *Melanotus rufipes* (Herbst), *Ampedus nigrinus* (Herbst), *Adelocera murinus* (L.). *Agriotes lineatus* and *Agriotes obscurus* were also studied by Crombie et al. (1947), Thorpe et al. (1946) who also described *A. sputator* L., and Doane et al. (1978), who also referred to *Limonius californicus*. All other references in the table refer to *C. destructor*.

Table I-3. *Cont.*

Type of sensilla	Anatomic location	Function	References
Sensory plate organs	Tips of the antennae (third segment; ventral wall of the annular sclerite)	Suitably positioned to function for orientation stimuli, but would require very low threshold of response since not numerous, or very high sensitivity	(Zacharuk, 1962)
	Distal segment of each antenna, cup-shaped sensilla	Chemoreceptors, stimulation leads to orientation only	(Crombie and Darrah, 1947)
	Pre-oral cavity, dorsal lining (opening to the pharynx)	Primarily involved in gustation, olfactory less likely (or stimuli that initiate extra-oral digestive process)	(Zacharuk, 1962)
Large sensory appendix on antennae	On the second segment, largest of sensilla found on wireworms, the most variable in numbers and sizes among species. Basiconicum (cone-shaped), multiporous and multiple celled	Not classifiable in the general classes of sensilla found in Arthropods, could be olfactory	(Zacharuk, 1962)
		Chemoreceptors with orientation only	(Crombie and Darrah, 1947)
		In *Agriotes,* no response to air-borne odours, contact chemoreceptors	(Thorpe et al., 1946)
			(Scott and Zacharuk, 1971a) (Zacharuk, 1971)

Zacharuk (1962) gathered morphological observations on 12 species, namely *Athous haemorrhoidalis* (F.), *Ctenicera aena* (L.), *Ctenicera destructor*, *Limonius minutus* (L.), *Hypolithus riparius* (F.), *Dalopius marginatus* (L.), *Agriotes obscurus* (L.), *Agriotes lineatus* (L.), *Lepturoides linearis* (L.), *Melanotus rufipes* (Herbst), *Ampedus nigrinus* (Herbst), *Adelocera murinus* (L.). *Agriotes lineatus* and *Agriotes obscurus* were also studied by Crombie et al. (1947), Thorpe et al. (1946) who also described *A. sputator* L., and Doane et al. (1978), who also referred to *Limonius californicus*. All other references in the table refer to *C. destructor*.

Multiporous (MP) olfactory sensilla in larvae are not as numerous as in mature insects, but still play an important role (Visser, 1986). Many larvae have large composite MP sensilla on their antennae, generally made of a distinct scape and pedicel, and a one-segmented flagellum (Zacharuk and Shields, 1991), such as observed on wireworms (Zacharuk, 1962). *Ctenicera destructor* (Brown) antennae, has two types of MP sensilla. One is a composite basiconicum sensilla, abundantly perforated by a slit-tubule system (Scott and Zacharuk, 1971b), made of 36 neurons grouped by bundles of three. Each of them is ensheathed by an individual inner sheath cell; 12 intermediate and 12 outer sheath cells subtend and enclose a common outer sinus around the neuronal bundles (Scott and Zacharuk, 1971b). The second one is an individual peg-like MP sensilla made of two neurons (Scott, 1969, Scott and Zacharuk, 1971b). Three peg-like sensilla are also found on wireworm labial palps, which contain a total of nine neurons (Bellamy, 1973).

Many uniporous (UP) peg and hair sensilla have one neuron whose dendrite ends in a tubular body at the base of the sensory cuticle. Those found on antennae are considered to be dually chemo-mechanosensory (Zacharuk, 1985). *C. destructor* wears four of these sensilla, with a total of 17 chemosensory and three mechanosensory neurons (Zacharuk, 1971).

Electrophysiological assays applied on digitiform sensilla (pegs) of *C. destructor* labial palps suggest that they respond strictly to mechanostimulation, despite the subapical pore (Zacharuk et al., 1977). However, according to Bellamy (1973), the labium possesses a total of 20 such sensilla, provided with 39 chemosensory and 19 mechanosensory neurons. Electrophysiological assays performed on these organs are therefore candidates for mechanical biases. In *Agriotes* wireworms, known contact chemoreceptors are of two kinds: peg organs, located on labial, maxillary palps and galea, whose stimulation leads to both orientation and biting; and a cup-shaped sensilla on the distal segment of each antenna, whose stimulation leads to orientation only (Campbell, 1937). Finally, antennae, maxillary palps, and labials palps notably have the ability to detect variations of relative humidity (Lees, 1943a).

Only a few compounds are known to stimulate these described sensilla. MP sensilla on the antennae of *L. californicus* do not appear to be CO_2 receptive. CO_2 receptors, mainly olfactory pegs, are located on the maxillary palps and on labial palps and to a lesser extent, on labial palps. However, on *A. lineatus* and *A. obscurus* larvae, CO_2 receptive-sensilla are not restricted to any one of the antennae, maxillary palps, labial palps, or galea, because amputation of these alone does not suppress the orientation response. Contact chemoreceptors

found on *Agriotes* wireworms are stimulated by asparagine and glucose (Campbell, 1937). Stimulating molecules reaching receptive chemo-sensilla typically diffuse to the dendritic terminations through a single terminal pore (UP sensilla) or the many pores (MP sensilla) of the chemical conduction system. The form and function of the sidewall pore canal system is modified into a pore tubule system in many MP sensilla (Zacharuk, 1985), clearly distinct in an elaterid larva (Zacharuk, 1971). The sensory tubules are lipoidal; they ensure moisture conservation in the sensillum and channel stimulating molecules through the cuticle to the lymph in the receptor cavity. Dendrites seem to gain contact with signal molecules through the receptor lymph (Zacharuk, 1971). Nothing in the literature deals with the nature of stimulant receptor sites, ion constituents, channels and pumps, or probable enzymes and second messengers in sensilla of wireworms (Zacharuk and Shields, 1991).

To conclude, most of the descriptions cited here concern *C. destructor* wireworms (Zacharuk, 1962, Scott and Zacharuk, 1971b, Zacharuk, 1971, Bellamy, 1973, Zacharuk et al., 1977, Zacharuk, 1985), while Table I-3 refers to at least 12 species from different habitats, including species that develop in decaying wood. According to Zacharuk (1962), there are only a few anatomical differences across them, with variation in number and size being the most important for the large antennal sensory appendix. We have seen above that some of the functions may not be conserved from one appendix to another, or across species, as for CO_2 receptive sensilla. It seems useful to proceed to complete and specific descriptions, starting with morphological aspects. The *C. destructor* background could be upgraded with SEM tools, or transmission electron microscopy (TEM), as suggested by Zacharuk and Shields (1991), in order to provide complete sensory maps. This could be done following the morphological approach of Eilers et al. (2012). Where detailed morphology exists, function should be assessed by directly testing rather than by comparison to structure and putative function of similar sensilla in the literature. More collaborative or corroborative studies by morphologists and physiologists would alleviate this deficiency (Zacharuk and Shields, 1991).

Furthermore, all aspects of the sensory channel should be studied, starting with the role of each of the neurons in sensilla. Concerning olfaction, the scope and accuracy with which odors can be identified is determined by how many olfactory receptor neurons (ORNs) there are and how they are tuned to different chemicals (De Bruyne and Baker, 2008). Identifying genes coding for odorant binding proteins in wireworms, through studying complete genomes of close and distant species, would allow the search for common sensory abilities between

them. The electrophysiological approach, although far from being complete currently, is complementary to behavioral bioassays, as it indicates where to search for behavioral responses, and reinforces the relevancy of such experimentations. It has been acknowledged that the convergence of genomic, physiological, and ecological data will elucidate the physiological and genetic bases of odor-mediated behaviors (De Bruyne and Baker, 2008).

4. Discussion

The application of semiochemicals in pest management strategies has received increasing interest in recent years (Agelopoulos et al., 1999, Cook et al., 2007). For several belowground pests, including wireworms (Barsics et al., 2013), such approaches still necessitate discovery of compounds involved in their chemical ecology (Johnson and Nielsen, 2012).

For the wireworm species detailed here, existing data on parameters influencing foraging steps, or morphological description and location of their sensilla, are not sufficiently complete or up to date. A model species could be used for complete description. There are sufficient methods in the literature that allow definition of threshold temperatures influencing activity or movement rates, as well as morphological descriptions. It seems that focusing research on the foraging process itself, at the individual scale, necessitates pooling such protocols together to provide researchers with a solid and holistic reference. Consequently, we suggest that the impact of temperature, which seems to have the most variable effect across species and populations from different geographical locations, should be assessed following the work of Furlan (2004) and van Herk and Vernon (2013a). Concerning the complete morphological descriptions of the sensory arsenal on head appendages, the work of Eilers et al. (2012) constitutes a perfect example of what should be applied to wireworms. Starting with a morphological approach, the achievement of electrophysiological experimentations would be more accurate, and allow a faster highlight of compounds detected by the larvae. There is a wide range of available chemical analysis for profiling plant-produced compounds, which allow investigation of plant-insect interactions, and the number and diversity of those applied belowground is increasing, with an equally increasing list of species subjected to behavioral and electrophysiological experimentations.

We also insist on the relevancy of assessing behavioral effects induced by blends as realistic as possible. It seems that most phytophagous insects target specific ratios among compounds (Visser, 1986). We have seen here that although inactive individually, some of the compounds induce a behavioral response in wireworms when paired (Crombie and

Darrah, 1947), which confirms that assumption in our case. Since most behavioral assays available in the literature were performed with only one compound, the realistic semiochemically-induced responses in wireworms have not yet been identified.

Finally, advances in the chemical ecology of wireworm pest species, with the long-term purpose of this information being applied in integrated management, rely on both behavioral assays and electrophysiological approaches. These are more than complementary; they are synergistic. Considering the scope of the work to be done, multidisciplinary teams of researchers are necessary. Large-scale population studies, very well investigated (Barsics et al., 2013), combined to approaches at the individual scale, could provide very concrete control measures.

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Chapter II – Objectives

In Chapter I, we highlighted that several wireworm management approaches are currently being developed. Chemical ecology is a field that has provided practical outcomes in terms of pest management alternatives. As an example, we have cited the soil pest *Diabrotica virgifera virgifera*, against which strategies, based on the attraction of entomopathogenic nematodes by ß-caryophyllene[1], have been developed (Hiltpold et al., 2013). Before implementing semiochemicals in IPM programs against wireworms, they need to be identified, and their roles in wireworm ecology need to be fully understood.

The main purpose of the present PhD work is to identify volatile organic molecules (VOC) that are used by wireworms to forage for their host plants. Wireworms are generalist pests, attacking many plant species, including several cereals. The contribution of plants to the semiochemical background of the rhizosphere probably differs from one species to the next, as it does for aboveground semiochemistry (Cook et al., 2007, van Dam et al., 2009). This diversity combined to the challenge to detect, identify and quantify belowground plant volatiles in conditions as realistic as possible, favors the study of interactions between wireworms and root-emitted VOC by starting with only one plant species rather than several. The present PhD work started in the context of the Rhizovol project, a multidisciplinary research focused on the root-emitted VOC of barley and their impact on the rhizosphere organisms. Our study model therefore involves spring barley (*Hordeum distichon* L.). *Agriotes sordidus* was selected as wireworm model species in this research because of its short life cycle compared to other species of the same genus. Here, we aim at identifying the volatile organic chemicals produced by barley roots, released in the rhizosphere, and evaluating their biological activity on *A. sordidus* foraging behavior.

The content of the two next chapters (chapter III and chapter IV) is built around three specific objectives directly related to the perception of VOC by wireworms during the foraging process (see Figure I.1, p.41):

1) Developing an olfactometer suitable for behavioral experimentations on wireworms exposed to root-produced VOC;
2) Identifying and quantifying the VOC produced by barley roots with different sampling methods in controlled growth conditions;

[1] A defensive semiochemical whose emission by maize roots increases upon *D. virgifera virgifera* attacks.

3) Evaluating the biological activity of the identified VOC on wireworms foraging for their host plant.

These objectives, translated in hypothesis, can be presented as follows:

A) Dual-choice olfactometers are suitable tools for observing that behavior, as long as soil experimental conditions are close enough to wireworm ecological optimums;
B) The qualitative and quantitative chemical composition of VOC sampled from barley roots varies according to the sampling conditions and methods;
C) Wireworms are able to orientate according to a blend of VOC emanating from roots or belowground plant organs. All compounds of the blend are therefore potential semiochemicals influencing their behavior;
D) VOC produced by barley roots can be included in new integrated strategies against wireworms.

In **Error! Reference source not found.**, we compare the adequacy and efficiency of two olfactometric designs in showing the behavioral responses of wireworms under the influence of barley-produced VOC. Y-shaped and cylindrical olfactometers are presented in Chapter III.1 and Chapter III.2 respectively. All behavioral assays were performed with barley roots as stimulus. A preliminary VOC profiling method is also evaluated in Chapter III.2 (SPME – solid phase micro-extraction) on roots of different growth stages.

In Chapter IV, based on the volatiles collected and identified, we have selected some VOC to perform behavioral experimentations. This chapter also reports on a recently developed VOC sampling method, DHS – Dynamic Head-Space Sampling, performed on roots ground in liquid nitrogen. This method allowed refining the chemical profile, and highlighted the VOC root content. It led us to evaluate the biological activity of a group of organic molecules using an optimized behavioral assay.

In Chapter V we discuss our results and propose perspective of future work on the understanding of wireworms' (chemical) ecology and prospects for IPM strategies. We also insist on upgrades that are needed for further behavioral experimentations on wireworms and their understanding in realistic conditions.

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Chapter III – Barley – Wireworm Interactions

Introduction to Chapter III

The main purpose of the present PhD work is to identify the volatile organic molecules (VOC) that are used by wireworms to forage for their host plants. We therefore needed a bioassay that would allow studying the behavior of crawling belowground insects. Such devices have already been developed in previous work on various animal models including earthworms ("Y-shaped" olfactometers, Zirbes et al. 2012), or nematodes (6-arm olfactometer, Rasmann et al. 2005). However, available devices are not always suitable for use with other insect models. Therefore our first objective consisted in developing an olfactometer suitable for behavioral experimentations on wireworms exposed to root-produced VOC. The system had to 1) gather a maximum of the natural conditions wireworms are exposed to; 2) allow easy and rapid non-destructive observations; and 3) allow the inclusion and diffusion of volatile molecules as stimulus.

We have started methodological developments with a Y-shaped system because this type of dual-choice device is classically used in insect behavioral experimentations, especially when they imply walking or crawling insects (Koschier et al., 2000, Colazza et al., 2004, Carroll et al., 2006, Wäschke et al., 2014). They also allow robust statistical data analysis. In (Barsics et al., 2011), we describe a Y-shaped olfactometer and associated methodology applied to study the ability of wireworms to orientate according to a blend of volatiles released by chopped barley roots (Barsics et al., 2011). In a subsequent paper (Gfeller et al., 2013), we describe a second olfactometer, which has been conceived to encounter the main issues related to the Y-shaped olfactometer described in Barsics et al. (2011). We have built dual-choice devices consisting in glass cylinders, in the ends of which were placed the stimuli: barley roots (Gfeller et al., 2013, Barsics et al., 2014a). Simultaneously, we have provided a first identification of the volatiles released in barley by Solid-Phase Micro-Extraction, or SPME. It is a rapid, cheap and convenient technique that allows collecting and identifying volatile chemicals released by a sample. It consists in a polymer-coated fiber that adsorbs molecules, whether they are present on the sample's surface or in its "headspace", when it is confined in a vial. In this preliminary assay, we have inserted SPME fibers in hermetically sealed vials containing barley roots. The analysis of the adsorbed volatile blend was performed by Gas Chromatography coupled to Mass-Spectrometry (GC-MS).

After having confirmed that the olfactometer described in (Gfeller et al., 2013) was appropriate for observing the behavior of wireworm exposed to a given plant root system, we have evaluated in the third section of this chapter (Barsics et al., 2014a) the impact of infection of barley roots with a phytopathogenic fungus on wireworms' behavioral choices. This short communication also reports on the limitations to the use of our olfactometric cylinders, when using a variety of living baits. They are discussed in terms of suitability of both the analytic and behavioral methods, keeping in mind that our experimental conditions greatly differ from realistic soil conditions. In this section, we introduce the next chapter, where experimentations are performed with selected volatiles, incorporated in synthetic baits.

Chapter III.1 - A Simple Device to Investigate the Role of Root-emitted Volatile Organic Compounds on Wireworm Orientation

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Adapted from the reference - Barsics, F., Verheggen, F. J. & Haubruge, E. Attraction of wireworms to root-emitted volatile organic compounds of barley. *In:* Ehlers, R.-U., Crickmore, N., Enkerli, J., Glazer, I., Kirchmair, M., Lopez-Ferber, M., Neuhauser, S., Strasser, H., Tkaczuk, C. & Traugott, M., eds. IOBC/WPRS Working Group "Insect Pathogens and Insect Entomopathogenic Nematodes" 13th European Meeting :"Biological Control in IPM Systems" 2011 Innsbruck (Austria) 19-23 June 2011. *IOBC/wprs Bulletin* **66**, 475-478.

Abstract: The ability of wireworms (*Agriotes sordidus*) to orientate towards a blend of volatiles emitted by chopped roots of barley was tested. During individual tests, larvae chose between the two sides of a Y-shaped olfactometer. One side was connected to a chamber containing chopped roots and the other was connected to an empty chamber. Wireworms chose almost significantly more often the side of the olfactometer providing the blend of root volatiles. This result underlines the importance of the identification of these compounds and the assessment of their effect on wireworms, alone or combined. Such compounds could be used in IPM strategies. This work also highlights important considerations for the improvement on behavioral assays undertaken on wireworms.

Key words: wireworms, barley roots, volatile organic compounds, integrated pest management.

1. Introduction

Wireworms, the soil dwelling larvae of click beetles (Coleoptera: Elateridae), remain unmanaged crop pests in many countries. Since the decrease of organochlorine pesticides use in the middle of the 1990s, considered alternatives against wireworms are essentially parts of integrated pest management (IPM) strategies, such as risk assessment by site characteristics parameters (Parker and Seeney, 1997), bait trapping and pheromone trapping both as monitoring and control methods (Parker, 1996, Hicks and Blackshaw, 2008), reduced treatment by seed protection (Vernon et al., 2009), biopesticides (Cherry and Nuessly, 2010), and biocontrol (Ansari et al., 2009). A gap persists concerning wireworms' chemical ecology while filling it would provide new angles of attack to combine to already existing ones and reinforce click beetles confinement.

If the general plant-insect relations are well documented for emerged parts of plants (Powell et al., 1998, Meiners and Hilker, 2000, Verheggen et al., 2008, Webster et al., 2008, Mendesil et al., 2009), such information at root level is less frequent but tend to increase. Rasmann et al. (2011) underlined the suppression of root herbivores of milkweed (*Asclepias syriaca* L.) mediated directly by cardenolides and indirectly by the attraction of nematodes. The identification of root-emitted volatile organic compounds that impact wireworm behavior would lead to new trails in matter of click-beetles control.

The work presented here is involved in a project aiming at identifying the nature and role of root-emitted VOC of barley (*Hordeum distichon* L.). Several actors of the rhizosphere are taken into account and among them, wireworms (*A. sordidus*) have been chosen to stand for belowground herbivory. As a first step, we observed the individual orientation of wireworms exposed to a blend of VOC emitted by chopped roots of barley, in Y-shaped olfactometers.

2. Material and Methods

2.1 Wireworms

The larvae of *A. sordidus* were collected in early October 2010 in Montardon, South France. All individuals were taken from a plot previously cropped with wheat (from November 2009, after maize harvest, until July 2010). To trap wireworms, we used potato baits buried a few centimeters deep and covered with opaque plastic jars allowing humidity conservation. New wheat shoots were removed from the ground in a 20 cm radius and their roots were used as complementary baits to potato slices. Baited wireworms were kept

individually in laboratory conditions, in 6 x 4 x 5 cm polystyrene crystal vials filled with humidified leaf mould. Before use, the substrate was heated 3 hours under 95°C to kill any potential wireworm enemy such as entomopathogenic fungi. The larvae were regularly fed with thin tuber slices. Between field extraction and behavioral assays, wireworms were kept in rearings for no more than three months.

Before they were used in experimentations, *A. sordidus* wireworms were identified using morphological characters (Cocquempot et al., 1999, Pic et al., 2008). The larvae were selected according to their length, as 10 mm and longer individuals are considered the most damaging instars (Furlan, 2004, Blot et al., 2008). The time separating the extraction of the larvae out of its vial and its introduction in the experimental device is favourable to stress induction, which is not recommendable in orientation tests. Working on at least 10 mm long larvae allowed the reduction of stress induction risks, because they were easier to manipulate. Every larva was isolated from all organic matter 6 days before it was tested, in order to enhance their ability to detect attractive blends. They were put in similar vials than rearing ones, but filled with humidified and compacted vermiculite. A total of 60 larvae were used in these tests.

2.2 Experimental Setup

Orientation of wireworms was observed in Y-shaped polypropylene olfactometers (Figure III.1). Wireworms were introduced individually in the main arm of the olfactometer. The two other arms were connected to baits chambers. One of them was filled with 3 g of chopped barley roots; the other was empty. We used roots produced by 7-day-old barley seedlings grown in 2 l jars sown with 50 caryopses. Before introduction in the olfactometers, roots were gently extracted from the substrate, and cleaned with tap water. Root baits were used for no more than three hours in a row and then replaced for subsequent individual tests. An air flux of 0.2 l/min was divided into the two olfactometric chambers, and carried the volatile blend into the olfactometer. To suppress the influence of relative humidity in both air currents, the air flux was carried towards distilled water before flux division, to ensure saturation. Five identical olfactometers were used to allow adequate cleaning between every test. The three arms of each Y measured 49 mm long and 12 mm in diameter. They were filled, in their inferior half, with moist and compressed vermiculite. This eased the progression of wireworms and putatively allowed the formation of volatile trails by adsorption on the substrate. We observed the first choice made by each larva and considered

it was done after they were no longer visible in the entry arm. If one wireworm remained in the main arm for more than 5 min, and showed a reduced activity compared to others, with no evident mobility, it was considered as inactive and the test was interrupted. The available paths were covered with a translucent red envelope to darken the entire olfactometer. Tests were performed over three days, with a room temperature varying between 18.3° and 20.3°C.

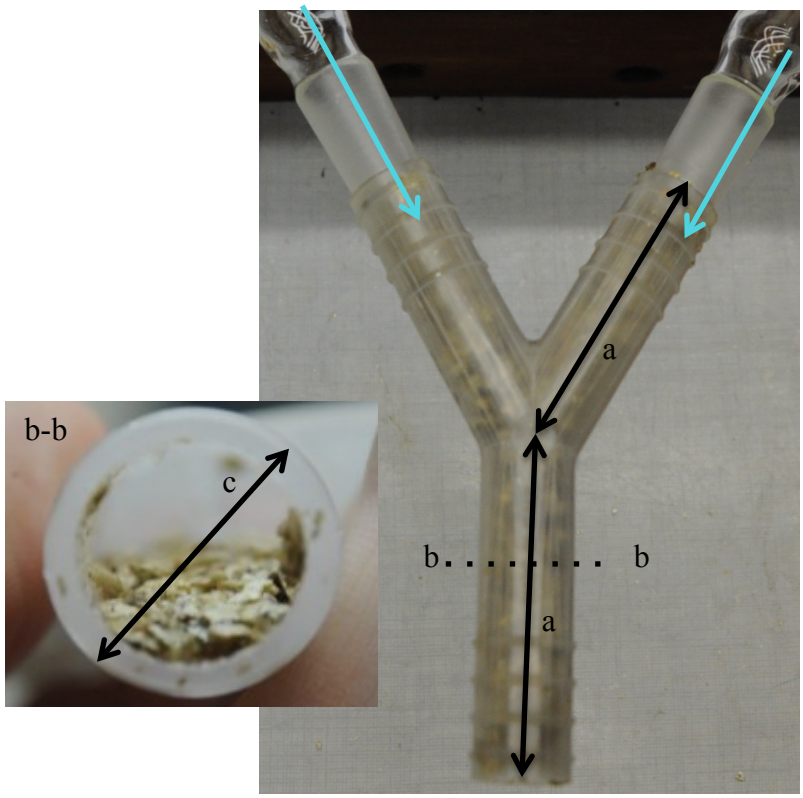

Figure III.1. Y-shaped olfactometers.
a = 49 mm, b-b is a transverse section showing substrate distribution, c = 12 mm.
Blue arrows indicate a 0.2 l/min air flow.

3. Results and Discussion

Out of the 60 larvae, only 7 (11,6%) were inactive and remained in the main arm for more than five minutes. The neutral or inactive behaviors consisted in a very reduced activity, almost always ending with attempts to burrow in the vermiculite, sometimes very quickly after they were introduced in the olfactometer. Among the 53 remaining wireworms, 34 orientated towards barley. A two-sided binomial test showed that wireworms had an almost statistically significant preference for the side of the olfactometer containing barley roots (P-value = 0.053). As such, our experimentation only tended to show the ability of the larvae of *A. sordidus* to orientate towards a blend of VOCs coming from barley roots. It doesn't point out what compounds might be responsible for their ability to orientate in the

bait direction. Carbon dioxide has been shown to attract wireworms (Doane et al., 1975), and its presence in the blend must have played a role here. The amount of CO_2 produced by the chopped roots was not regulated in our experimental setups or measured, but other compounds of the volatile blend be involved, notably by reinforcing the "search trigger" information (Johnson and Gregory, 2006) carried by CO_2. Considering the results, it seems that a number of improvements are necessary to ensure good interpretation of results and statistically significant outcomes. Firstly, and this is in line with relatively old observations (Thorpe et al., 1946), it seems obligatory to perform behavioral assays in a substrate rather than by having wireworms to walk on a substrate's surface, because they do not display natural orientation in these conditions. Secondly, wireworms dwell in the soil and only encounter air-flows such as developed in the assays on rare occasions, if ever. Subsequent tests should therefore be undertaken solely in static conditions, resorting to VOC diffusion through the substrate. Finally, wireworms should be selected more thoroughly. Their length does not ensure they will be in a feeding phase, i.e. they could not be influenced by food originating cues because they would be irrelevant to them. Indeed, *A. sordidus* wireworms only feed during 25 % of their larval development (Furlan, 2004). Ideally, further behavioral assays should take that into account and involve a method to discriminate among wireworms actively feeding from those in pre- or post-molting phases.

Concerning the bait features, one valuable hypothesis is that decaying roots might not be a suitable food source for wireworms, as it has recently been pointed out (Wallinger et al., 2013). Even though we resorted to chopped roots to ensure exposure of wireworms to a rich blend, the VOC might have carried repulsive information for the larvae, leading to a minimal repulsion effect. This should be verified once the necessary upgrades are incorporated in a new olfactometric device. It will also be necessary to find analytic methods to identify and quantify the VOC composing a blend emitted by roots, in order to point out semiochemical candidates other than CO_2.

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Chapter III.2 - Characterization of Volatile Organic Compounds emitted by Barley (*Hordeum distichon* L.) Roots and their Attractiveness to Wireworms

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Abstract: Root volatile organic compounds (VOC), their chemistry and ecological functions have garnered less attention than aboveground-emitted plant VOC. We report here on the identification of VOC emitted by barley roots (*Hordeum vulgare* L.). Twenty-nine VOC were identified from isolated 21-d-old roots. The detection was dependent on the medium used for root cultivation. We identified 24 VOC from 7-d-old roots when plants were cultivated on sterile Hoagland gelified medium, 33 when grown on sterile vermiculite, and 34 on non-sterile vermiculite. The major VOC were fatty acid derived compounds, including hexanal, methyl hexanoate, (*E*)-hex-2-enal, 2-pentylfuran, pentan-1-ol, (*Z*)-2-(pentenyl)-furan, (*Z*)-pent-2-en-1-ol, hexan-1-ol, (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol, oct-1-en-3-ol, 2-ethylhexan-1-ol (likely a contaminant), (*E*)-non-2-enal, octan-1-ol, (2*E*,6*Z*)-nona-2,6-dienal, methyl (*E*)-non-2-enoate, nonan-1-ol, (*Z*)-non-3-en-1-ol, (*E*)-non-2-en-1-ol, nona-3,6-dien-1-ol, and nona-2,6-dien-1-ol. In an olfactometer assay, wireworms (larvae of *A. sordidus*) were attracted to cues emanating from barley

seedlings. We discuss the role of individual root volatiles or a blend of the root volatiles detected here and their interaction with CO_2 for wireworm attraction.

Key words: volatile organic compound, barley, roots, solid-phase-micro-extraction, *Agriotes sordidus*, root – insect interactions

1. Introduction

Plants emit complex blends of volatile organic compounds (VOC), ranging from fatty acid derivatives, terpenoids, and sulfur compounds to phenylpropanoids (Qualley and Dudareva, 2009). The emission can be constitutive and/or induced by environmental or physiological stresses (Maffei, 2010). Depending on the stress type (wounding, herbivory, pathogen attack, dehydration, (UV) light, heat, etc.), the composition and amounts of released VOC can vary (Ferry et al., 2004, Kuhn et al., 2004, Filella et al., 2009, Jansen et al., 2011). Various organs emit VOC (seeds, flowers, leaves, stems, and roots). The soil provides a nutrient-rich environment for many organisms, as shoots export up to 50 % of the photosynthetically fixed carbon to roots (Lambers, 1987). In barley, up to 40 % of the photosynthetic intake is "rhizodeposited" in the soil, through respiration or exudates, with values generally approaching 25 to 30 % (Barber and Martin, 1976, Whipps and Lynch, 1983, Whipps, 1984, Gregory and Atwell, 1991, Johansson, 1992, Jensen, 1993, Zagal, 1994, Swinnen et al., 1995). Vertebrates, invertebrates, plants, fungi, and bacteria all share the same underground space in which VOC-mediated interactions take place and even affect aboveground plant insect interactions (Johnson et al., 2009, Wenke et al., 2010, Effmert et al., 2012, Soler et al., 2012). However, the belowground VOC potentially responsible for such interactions have to date been partially neglected, due to technical limitations. The release of root VOC can mediate various interactions: direct or indirect defense of roots against pests (Rasmann et al., 2005, Ali et al., 2011, Rasmann et al., 2012a) plant – plant competition (Viles and Reese, 1996, Ens et al., 2009, Jassbi et al., 2010), resistance against pathogens (Cobb F.W et al., 1968, Kalemba et al., 2002, Vilela et al., 2009), and symbiotic interactions (Paavolainen et al., 1998, Asensio et al., 2012). The emission of repellent compounds tends to decrease in unattacked conditions (Piesik et al., 2011b), thus limiting the energy costs incurred by their synthesis (Herms and Mattson, 1992, Rasmann et al., 2012a, Rasmann et al., 2012b). Root-derived compounds can also cause herbivore attraction (Wenke et al., 2010). Carbon dioxide gradients are ubiquitous belowground herbivore attractants, but other volatile and non-volatile semiochemicals are involved in directing herbivores towards

roots (Reinecke et al., 2008, Johnson and Nielsen, 2012, Weissteiner et al., 2012). Several studies have shown the attractive role of root-emitted VOC towards Arthropods. For example, di- and trisulfides produced by *Allium cepa* are potent attractants of the larvae of the fly *Delia antiqua* (Matsumoto, 1970); VOC released by damaged oak roots are perceived by the larvae of the forest cockchafer *Melalontha hippocastani* and attract the larvae in natural soil (Weissteiner et al., 2012); volatiles of ryegrass roots attract the larvae of *Costelytra zealandica* (Sutherland, 1972). Arthropods can differentiate between released root VOC that differ with respect to physical or physiological traits (Witcosky et al., 1987, Aratchige et al., 2004, Tapia et al., 2007); they also can differentiate between root VOC released by different varieties of plants (Guerin and Ryan, 1984).

In this study, we characterized VOC emitted by isolated barley roots. Root emissions from barley cultivated under sterile and non-sterile conditions were compared in order to characterize emission in the absence of microorganisms. Moreover, the development of an orientation test with the larvae of the click beetle *A. sordidus* led us to investigate the potential semiochemical role of the VOC blend emitted by isolated barley roots. The working questions were: 1) How complex is the volatile blend of isolated barley roots? 2) What is the impact of microorganisms on the volatile blend released by barley roots? 3) How attractive is this volatile blend of barley roots to wireworms?

Larvae of *Agriotes* wireworms are polyphagous and feed on belowground organs of a variety of crops, including cereals (Johnson et al., 2009, van Herk and Vernon, 2013a). Baits based on germinating wheat and barley seeds have been proven to be efficient (Parker, 1996). However, few studies of wireworm - barley interactions are available, although that crop is the second most important in Europe (production, 2010, FAOstat). As for most of the root herbivores, the CO_2 gradient is a general search trigger for wireworms (Doane et al., 1975, Johnson and Nielsen, 2012). Plant-derived VOC can affect the behavior of root-feeding insects, while their identity and role in wireworm chemical ecology still has to be revealed (Johnson and Nielsen, 2012, Barsics et al., 2013). As a first step towards the elucidation of compounds and blends thereof with such properties, we focused on the release of VOC from barley roots and tested the attraction of wireworms towards the complete blend emanating from barley roots.

2. Methods and Materials

2.1 Plant Material

Growth conditions

All barley plants (var. Quench, Jorion, Belgium) were grown in a controlled chamber, at 22°C under LED light (95 µmol m^{-2} sec^{-1}) with a 20/4 h L/D photoperiod and 65 % RH.

Cultivation of 21 days-old plants

Caryopses were sown at a density of 10 plants per pot (7 l) in vermiculite (Sibli, Belgium). Plants were watered daily and fertilized three times per week with aqueous Hoagland's solution (Hoagland's NO.2 basal salt mixture, Sigma, Belgium).

Aseptic cultivation of seven days-old plants

Barley caryopses (28 g) were sterilized as described by (Lanoue et al., 2010). They were incubated in 50 ml H_2SO_4 (50 % v/v) for 1 h and washed five times in 150 ml sterile bidistilled water. Caryopses were then shaken for 20 min in 80 ml $AgNO_3$ (1 % w/v) and washed successively with 150 ml sterile NaCl (1 %, w/v), 150 ml sterile bidistilled water, 150 ml sterile NaCl (1 %, w/v) and × 5 with 150 ml sterile bidistilled water, before sowing: (**1**) on 124 cm^2 Petri dishes filled with Hoagland medium (Hoagland's NO.2 basal salt mixture, Sigma, Belgium), solidified with 0.8 % agar (w/v; Plant agar, Duchefa Biochemie, Belgium) or (**2**) on vermiculite with Hoagland solution (Hoagland's NO.2 basal salt mixture, Sigma, Belgium). (**1**) Sterile caryopses were placed on Hoagland's medium with the ventral furrow underneath and left to grow for seven days. (**2**) Sterile caryopses were sown in 2 l jars (le Parfait, Villeurbanne, France), filled with 600 ml sterile vermiculite humidified with 300 ml sterile Hoagland solution. Jars were closed and sealed with plastic film and left closed for seven days. On the sampling day, vermiculite isolated in the vicinity of the roots was incubated on tryptic soy agar (Fluka, Belgium) for 1 week at 37 °C to check sterility. All glass, media and jars were sterilized. As controls, non-sterile plants were grown for seven days in 600 ml vermiculite humidified with 300 ml Hoagland solution in 2 l open jars.

2.2 Analyses of Volatile Organic Compounds

Head-Space Solid-Phase-Microextraction (HS-SPME)

Roots were isolated from the substrate by shaking the plant gently and were separated from the upper part of the plant by cutting just below the caryopsis. Then 3 g ± 0.1 g of fresh

entire roots were placed in 20 ml SPME vials (Filter Service, Belgium) fitted with a sealed cap (white silicone/blue PTFE, Filter Service). Samplings were operated directly after treatment of the roots, with the same conditions for all samples. An internal standard (1 µl of a methanolic solution of butyl benzene (≥ 99 %, Sigma-Aldrich (S.-A.), Belgium) at 0.86 mg/l) was carefully added on the surface of the vial by avoiding any contact with the roots. The method was optimized on the basis of observations on four compounds emitted by barley roots, selected for the diversity of their molecular weight, function and polarity (2-pentylfuran, hexan-1-ol, 6-methylhept-5-en-2-one and (*E*)-non-2-enal). Chromatographic performance and the abundance of detected peaks also formed part of the decision criteria. Different operating conditions were examined with the main objective of maximizing the number of peaks and their abundance in a single run. We tested four fiber coatings (polyacrylate - 85 µm; PA), polydimethylsiloxane (100 µm PDMS), carboxen / polydimethylsiloxane (85 µm; CAR / PDMS) and divinylbenzene / carboxen / polydimethylsiloxane (DVB / CAR / PDMS - 50/30 µm). As illustrated on (Figure III.4), the monophasic fibers allowed the capture of a limited number of VOC, while the mixed coated fibers extracted the widest range. The CAR / PDMS fiber allowed the detection of a lower number of peaks than the DVB / CAR / PDMS fiber, which was further used in our study.

The fiber was the same for all repetitions of the same experiment. It was conditioned before first use at 270 °C for one hour. Samplings performed at 30°C resulted in a peak area increase of approximately 14, 1.5 and 11 times for 2-pentylfuran, 6-methylhept-5-en-2-one and hexan-1-ol, respectively compared to sampling carried out at 23°C. and allowed the detection of other VOCs such as (*E*)-2-hexenal and (*Z*)-2-(2-pentenyl)furan. Higher temperatures were not tested. After equilibration of the vial for 15 min at 30 °C, the fiber was inserted into the headspace for 30 min at the same temperature. The effects of different equilibration (5, 10, 15, 20 min) and exposure (15, 30, 60 min) duration were also evaluated. The volatile content was relatively constant after 15 min of equilibration, although different VOC did not show the same behavior with respect to equilibration time. While the peak area of 2-pentylfuran increased with equilibration time, peak areas of (*E*)-non-2-enal and 6-methylhept-5-en-2-one decreased. Increasing exposure time from 15 to 30 min led to a rise of the overall peak area and a greater number of components in the headspace. Further increase of exposure time to 60 min did not achieve any additional improvement. In summary, the following optimized parameters were selected: 15 min of sample equilibration; 30 min sampling time both at 30°C.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

After extraction, the VOC were desorbed in pulsed splitless mode for 10 min at 250 °C (which ensured complete desorption). GC-MS analyses were performed on an Agilent Technologies 7890A GC System coupled to an Agilent Technologies 5975C Mass Spectrometer equipped with Wax factor four (Agilent technologies USA; 30 m x 0.250 mm I.D, 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The inlet temperature was 250 °C. Pulsed splitless injection mode was used in a 1.5 mm HS-liner (injection pulse pressure of 30 psi for 1 min). The following temperature program was used: 40 °C for 4 min; 15 °C/min to 160 °C; 20 °C/ min to 250 °C; and 250 °C for 5 min; 30 °C/min to 300 °C; and final hold at 300 °C for 15 min. The MS was carried out in EI mode at 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; scanned mass range: from 20 to 350 amu, threshold of 150 amu; scan speed, 4.27 scans/s.

Chemical identification

Components were identified, by comparing recorded mass spectra, with the NIST and Wiley spectral databases. Further identification was carried out by calculating non-isothermal Kovats retention indices by injecting saturated n-alkane standard solution (C_7-C_{30} 1.000 µg/ml in hexane, Supelco, Belgium) under the same chromatographic conditions, using the definition of van Den Dool and Dec. Kratz (1963). Whenever possible, identifications were confirmed by injection of available commercial standards. As the same chromatographic conditions with the same column were used for the analyses of the standards, identification of the detected compounds in the headspace of barley roots was confirmed by comparing their retention data and mass spectra with those of the commercially available reference compounds.

Peaks showing a signal/noise ratio of three compared to the blank controls were identified and integrated manually with the Agilent MSD Chemstation. The relative area of a target compound was calculated by dividing the peak area of this compound by the total peak area of the sample. Statistical analysis was performed on the relative area with a two-tailed paired t-test after checking that the data were normally distributed with Kolmogorov – Smirnov test.

Concentration estimates

Samples were extracted using an autosampler (MPS2, Gerstel) equipped with a sample tray holder and a needle heater for heating the vials. Gerstel Maestro software was used for autosampler control. Standards and dilutions were always handled with a Hamilton syringe with a volume of 1 µl. The molecules identified by GC-MS were grouped into the following classes: alkanes, aldehydes, alcohols, esters, sulfur, and furan compounds. In each class, a representative compound was selected as the basis of the calibration curves: n-tetradecane (S.-A.; 99 %), (*E*)-non-2-enal (SAFC; ≥ 93 %), (*E*)-non-2-en-1-ol (SAFC; ≥ 96 %), methyl benzoate (Fluka; ≥ 99.5 %), dimethyl sulfoxide (S.-A.; ≥ 99.9 %), and 2-pentylfuran (SAFC; 97 %). The calibration curves included at least four points and were performed in triplicate (Table III-1). For each measurement, the experiment was carried out according to the following process. A stock solution was diluted in methanol by using volumetric glassware. One microliter of each dilution then was placed in a vial (20 ml) with 1 µl of the internal standard (butylbenzene 0.86 mgl^{-1} in methanol). After an equilibration period of 15 min at 30 °C, the fiber was exposed for 30 min (at 30 °C) before analysis as described above.

Table III-1. Equations of the calibration curves.

Chemical class	Equation	R^2	Quantity range (ng)
Alkanes - tetradecane	y = 1.2841x – 0.1845	0.9981	0.3 – 1.5
Alcohols - (*E*)-non-2-en-1-ol	y = 0.3323x – 0.0271	0.9988	0.8 – 16.8
Aldehydes - (*E*)-non-2-en-1-al	y = 0.0272x + 0.08	0.9991	0.8 – 33.8
Esters - Methylbenzoate	y = 0.5355x + 0.0348	0.9953	2.2 – 21.7
Furan compounds - 2-Pentylfuran	y = 0.9568x -1.0604	0.9957	1.7 – 17.2
Sulfur compounds - Dimethyl sulfoxide	y = 0.0731x + 0.2472	0.9975	1.5 – 22.0

2.3 Wireworms Orientation Bioassay

Wireworms were collected in November 2011 in Montardon (Pau, France), from grass-covered soil edging a fallow plot and an untreated wheat field. Morphological criteria described in the keys of Cocquempot et al. (1999) and Pic et al. (2008) allowed *A. sordidus* individuals to be identified. Each larva was kept individually in an 80 ml capped vial, with a mix of leaf mold and vermiculite (1/1 v/v, 16.5 % water) and meadow seeds (0.130 – 0.160 g, Prelac Bio, SCAR, Belgium). All vials were kept in the dark at 21.2 ± 0.7 °C. Seven days prior to the test, wireworms greater than 10 mm in length were individually isolated in vermiculite (16.5 % water). Wireworms were selected for testing from the isolated individuals according to their apparent activity: those visibly in the pre-molting or post-

molting phases were excluded from the tests. In total, 60 larvae were submitted to the olfactometry bioassay.

The bioassay set up (Figure III.2) consisted of a glass pipe (32 cm long, 3.6 cm internal diameter), with both extremities closeable with GL45 caps (Duran, Belgium), which allowed filling and emptying the set-up with substrate. Two GL14 holes (Duran, Belgium) pierced 3 cm from both extremities allowed the introduction of stimuli in both sides, in the bait areas. The larvae entry point was provided by a third lateral hole in the middle of the pipe, diametrically opposite to both lateral connections.

The substrate used was vermiculite. It was removed to a depth of 4 cm at the two ends of the equipment to leave room for the bait and control compartments (final vermiculite content: 64.4 ± 0.9 g, 53.0 ± 0.4 % water). In each of the pipes, the bait consisted of the roots of 10 developed barley seedlings gently removed from growth medium before introduction in the bait zone. They were positioned through one of the lateral perforations (Figure III.2-a).

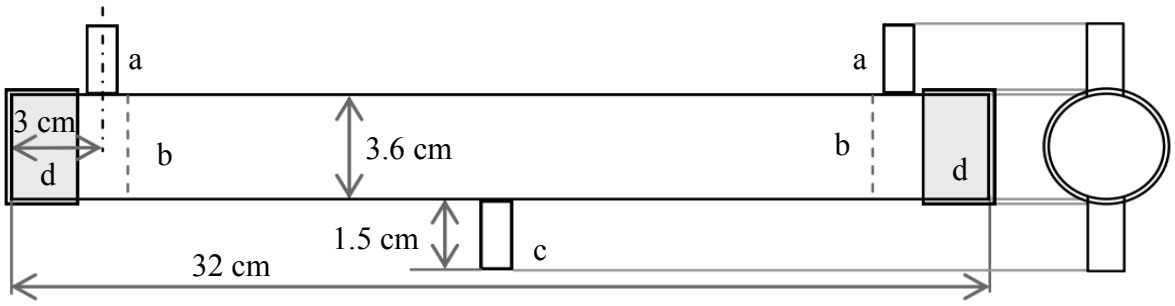

Figure III.2. Dual-choice olfactometer used for the orientation experimentation of *A. sordidus* towards the blend emitted by 7-d-old barley roots.
(a) lateral connection for root insertion, (b) stainless gauze separating the roots from the substrate, (c) wireworm entry point, (d) standard GL45 screwed ends and caps.

Plants were held in position with both aluminum foil and PTFE tape (EGEDA, Belgium). Roots were the only plant material exposed to wireworms, as the rest of the plant was isolated out of the bioassay. Blank culture medium (240 mg – average amount of medium remaining upon the roots after extraction) was introduced into the opposite side of the bait compartment. To prevent any contact between a wireworm and the roots, a thin piece of fabric (3.6 cm diam., stainless steel; width: 0.042 mm; mesh: 0.036 mm; Haver, Belgium) was used to separate the substrate from the bait and control compartments. Tests were performed in batches of 10 olfactometers at a room temperature of 21.9 ± 0.5 °C (Figure III.3).

Bioassays were performed in the dark. Each wireworm was introduced individually 40 min after setting the bait in the system, and a red plastic sheet was placed on each bioassay during the test in order to suppress light biases. The position (left or right) of the baits on the

laboratory bench was randomly assigned and recorded, as was the bait position with respect to the side of the olfactometers by which the substrate had been introduced.

The position of the wireworms was recorded after 60 min. Wireworms located within 3 cm from the olfactometer entry were considered as non-responding. We performed replicates until recording 50 effective responses. All material was cleaned with water and norvanol (VWR, Belgium) between each test. The number of successes was tested with a two-tailed binomial test using R software, version 2.14.1 (2011-12-22; Development Core Team 2008). Potential sources of bias (bait position on the laboratory bench and with regard to the entry side of the substrate) were tested with Fisher's exact test for count data (one factor, four modalities). The orientation bioassay also was performed 'blank-to-blank', i.e. without baits and controls, in order to assess the nature of wireworm behavior in a stimulus-free environment. They were also left orientating during 60 min. The results were treated with a binomial test applied on the side of wireworm retrieval. As to the potential impact of the entry side of the substrate on the results, it was tested with a χ^2 test for independence.

Figure III.3. Ten olfactometers with 7-day-old barley seedlings as baits for wireworms.
In experimental conditions, they are covered with a red plastic film during moments when light is not yet switched off – before all wireworms are inserted in their respective olfactometers, or while retrieving the first individuals while the others still have time orientating.

3. Results

3.1 Identification of Volatile Organic Compounds in 21-d-old Barley Roots

We detected 29 volatile compounds in the headspace of isolated 21 days old barley roots (Table III-2, N = 5 replicates). Confirmation of the occurrence of 24 VOC was possible by comparison with the NIST 08 and Wiley 275 k databases, the library retention index, and standards. The retention index (RI) of dimethyl sulfide could not be precisely calculated as it was eluted in the very first minutes of the chromatogram. Relative experimental RI deviations from the database's RI ranked from -1.4 to 1.7 %. Relative quantities were determined by calculating the area of the compound peak relative to the total peaks area. In order to estimate the amounts of the 29 compounds listed in Table III-2, response curves were calculated for major chemical families of the profile by using one representative compound for each. This approach involved performing six calibration curves linear in the ranges of concentration tested, as evidenced by the values of correlation coefficients, always > 0.99 (Table III-2).

In Figure III.4, the development of the SPME-GC-MS method is described. An optimized protocol for SPME analysis of root volatiles was developed. An important point was the use of the DVB/CAR/PDMS fiber with a 50/30 µm coating, and a fixed equilibration and sampling time. Exactly 3 g roots were used, and a wax column proved to be suitable for separation of the volatiles. Figure III.5 shows a total ion current chromatogram of a headspace sample of 21-day-old isolated barley roots.

3.2 Barley Root VOCs after Seven Days of Culture on Hoagland Gelified Medium

Table III-3 provides a list of the compounds that were emitted by 7-day-old barley roots cultivated on Hoagland gelified medium. Confirmation of the occurrence of 16 VOC was possible by the injection of standards, and 4 VOC could be tentatively identified by their theoretical RI. Estimation of the amount of VOCs released was performed in the same way as described for the data shown in (Table III-2).

Barley root VOCs after seven days of culture in sterile/non-sterile conditions

The potential contribution of micro – organisms present in the environment of the roots in the degradation or emission of the VOCs was assayed by cultivating plants for seven days in sterile vermiculite (ST) and in non – aseptic (NS) vermiculite fertilized with Hoagland

solution. Thirty-three VOC were identified from the roots of ST plants, 34 were detected as emitted from the roots of NS plants (Table III-3).

Statistical analysis of the relative area of VOC emitted by ST and NS roots showed that six VOC (pentan-3-one, pent-1-en-3-ol, 2-ethylhexan-1-ol, dodecan-1-ol, dihydro-5-pentyl-2(3H)-furanone, hexadecanal) were differentially detected (two-tailed paired t-test; $P<0.05$) in NS compared to ST conditions (Table III-4), whereas (*E*)-pent-2-en-1-ol and methyl-dodecanoate were found only in the headspace of roots of NS plants. Commercial standards used for identification were: dimethyl sulfide (S.-A.; ≥ 99%); hexanal (S.-A.; 98 %); methyl hexanoate (Fluka; 99.8%); (*E*)-2-hexenal (S.-A.; ≥ 95%); 2-pentylfuran (SAFC; 97%); pentan-1-ol (S.-A.; ≥ 99%); (*Z*)-pent-2-en-1-ol (SAFC; ≥ 96%); 6-methylhept-5-en-2-one (S.-A.; 99%); hexanal (S.-A.; 98%); hexan-1-ol (Fluka; 98%); (*Z*)-hex-3-en-1-ol (SAFC; ≥98%); (*E*)-hex-2-en-1-ol (SAFC; ≥ 96%); heptan-1-ol (S.-A.; 98%); tetradecane (S.-A.; 99%); oct-1-en-3-ol (A.; 98%); 6-methyl-hept-5-en-2-ol (SAFC; ≥ 99%); 2-ethyl hexan-1-ol (Fluka; ≥ 99%); (*E*)-non-2-enal (SAFC; ≥ 93%); dimethyl sulfoxide (S.-A.; ≥ 99.9%); octan-1-ol (S.-A.; 99%); (*2E*,*6Z*)-nona-2,6-dienal (SAFC; ≥ 95%); methyl (*E*)-non-2-enoate (SAFC; ≥ 97%); methyl benzoate (Fluka; ≥ 99.5%); nonan-1-ol (Fluka; 100%); (*Z*)-non-3-en-1-ol (A.; 95%); (*E*)-non-2-en-1-ol (SAFC; ≥ 96%); tetradecanal (TCI; > 95%); dodecan-1-ol (S.-A.; ≥ 98%); dihydro-5-pentyl-2(3H)-furanone (A.; 97%), butan-1-ol (Janssen Chimica; 99.5 %), 2-phenylethanol (S.-A.; ≥ 99%).

Table III-2. Volatile organic compounds emitted by excised barley roots aged of 21 days, analysed by a HS-SPME GC-MS method

CAS Number [1]	IUPAC Name	Identification [2]	Retention Index		A	B
			Measured [3]	Reference [4]	Relative area (%) ± SD, n=5	Estimation (ng/g RFW ± SD, n=5) [5]
75-18-3	Dimethyl sulfide	STD	N.D.	844[c]	25.8 ± 6.3	38.8 ± 9.1
66-25-1	Hexanal	STD	1075	1074[b]	2.2 ± 0.4	9.9 ± 1.0
106-70-7	Methyl hexanoate	STD	1179	1185[b]	1.1 ± 0.9	0.13 ± 0.07
6728-26-3	(*E*)-Hex-2-enal	STD	1206	1207[a]	0.59 ± 0.32	1.7 ± 0.7
3777-69-3	2-Pentylfuran	STD	1213	1229[a]	27.9 ± 5.0	4.2 ± 0.5
71-41-0	Pentan-1-ol	STD	1246	1244[d]	1.23 ± 0.48	0.52 ± 0.11
70424-13-4	2-(Pentenyl)furan[#]	MS	1287	-	1.01 ± 0.22	0.46 ± 0.03
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1311	1313[d]	0.33 ± 0.06	0.15 ± 0.02
110-93-0	6-Methyl-5-hepten-2-one	STD	1325	1319[e]	0.55 ± 0.44	-
111-27-3	Hexan-1-ol	STD	1345	1351[f]	9.27 ± 1.94	3.8 ± 0.5
928-96-1	(*Z*)-Hex-3-en-1-ol	STD	1374	1351[a]	0.55 ± 0.14	0.24 ± 0.02
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1389	1400[f]	2.26 ± 0.75	0.9 ± 0.2
3391-86-4	Oct-1-en-3-ol	STD	1438	1420[a]	0.22 ± 0.04	0.09 ± 0.02
104-76-7	2-Ethyl-1-hexanol	STD	1483	1504[g]	2.59 ± 1.71	1.0 ± 0.3
18829-56-6	(*E*)-Non-2-enal	STD	1524	1540[a]	1.71 ± 0.93	7.6 ± 2.0
67-68-5	Dimethyl sulfoxide	STD	1551	1553[h]	0.91 ± 0.58	1.4 ± 0.5
111-87-5	Octan-1-ol	STD	1552	1557[i]	0.51 ± 0.25	0.22 ± 0.04
557-48-2	(2*E*,6*Z*)-Nona-2,6-dienal	STD	1575	1597[j]	0.66 ± 0.20	2.4 ± 0.4
111-79-5	Methyl (*E*)-non-2-enoate	STD	1602	-	0.41 ± 0.16	0.09 ± 0.02
93-58-3	Methyl benzoate	STD	1614	1600[a]	3.75 ± 2.12	0.92 ± 0.27
143-08-8	Nonan-1-ol	STD	1656	1678[g]	0.58 ± 0.08	0.26 ± 0.03
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1680	1682[k]	4.44 ± 2.98	1.8 ± 0.5
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1710	1722[l]	3.29 ± 2.61	1.3 ± 0.5
76649-25-7	Nona-3.6-dien-1-ol[#]	MS, RI	1733	1759[k]	0.79 ± 0.46	0.33 ± 0.07
7786-44-9	Nona-2,6-dien-1-ol[#]	MS, RI	1764	1776[l]	1.18 ± 0.61	0.49 ± 0.10
124-25-4	Tetradecanal	STD	1968	1940[m]	0.62 ± 0.16	2.2 ± 0.5
112-53-8	Dodecan-1-ol	STD	1971	1970[n]	0.30 ± 0.07	0.14 ± 0.02
104-61-0	Dihydro-5-pentyl-2(3H)-furanone	STD	2022	2024[j]	0.71 ± 0.38	0.16 ± 0.05
629-80-1	Hexadecanal[#]	MS, RI	2052	2020[o]	1.94 ± 1.09	8.3 ± 2.3

[1] CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); [2] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section [4]); STD, comparison of retention time and mass spectra of available standards; [3] Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; [4] Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Sanchez-Ortiz et al. 2012; the others are taken from Pherobase : [c] Varming et al. 2004, [d] Umano et al. 2002, [e] Chung et al. 1993, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Chisholm et al. 2003, [n] De Marques et al. 2000; [o] Kohara et al. 2006, [5] Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always >0.99) # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOC.

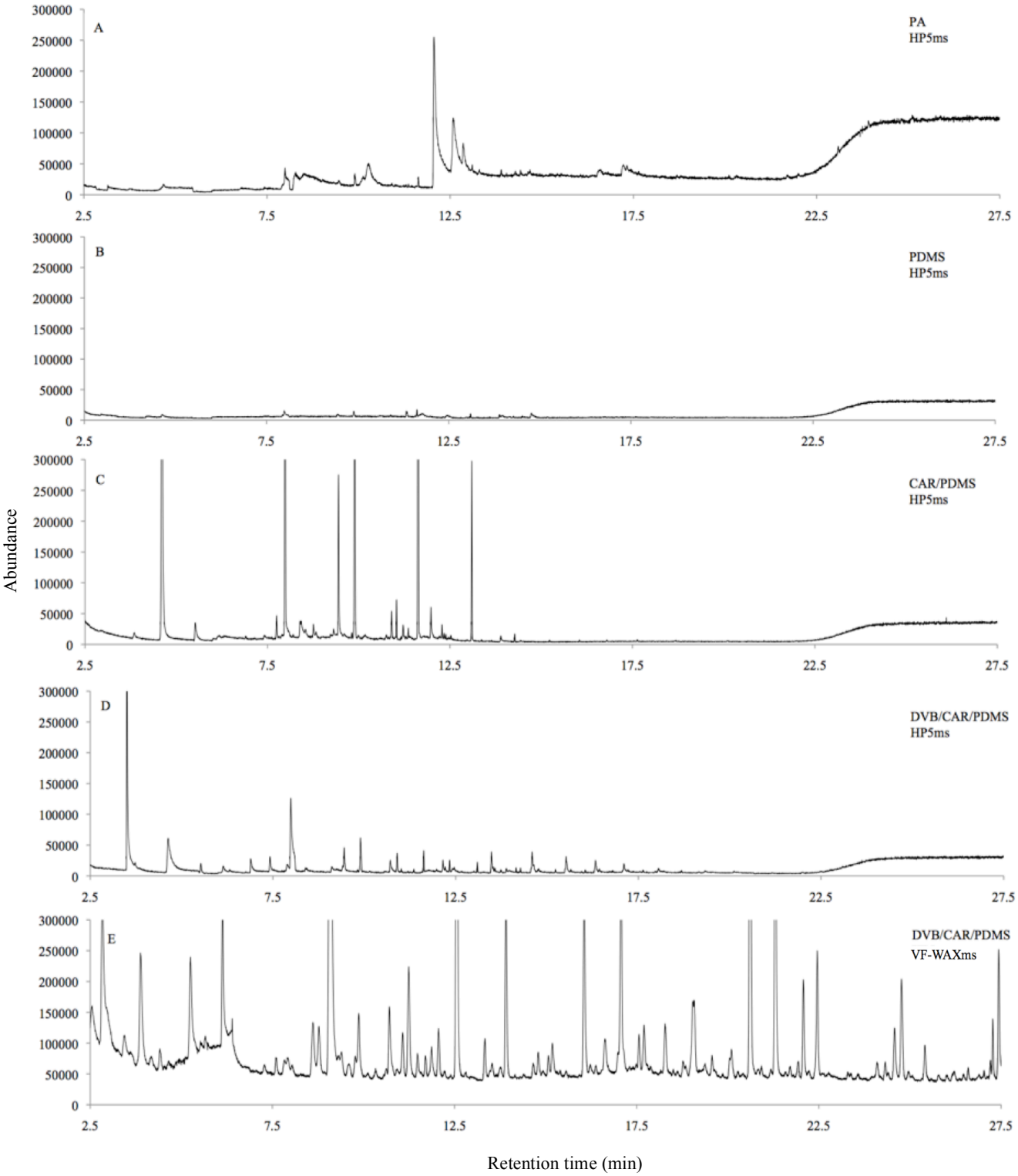

Figure III.4. Optimization of the HS-SPME-GC-MS method.
Comparison of VOC profiles released by roots of barley and trapped on four types of fibre: A = polyacrylate (PA), B = polydimethylsiloxane (PDMS), C = carboxen / polydimethylsiloxane (CAR/PDMS), and D, E = divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS).
(A) to (D) column HP5ms, (E) column VF-WAXms.

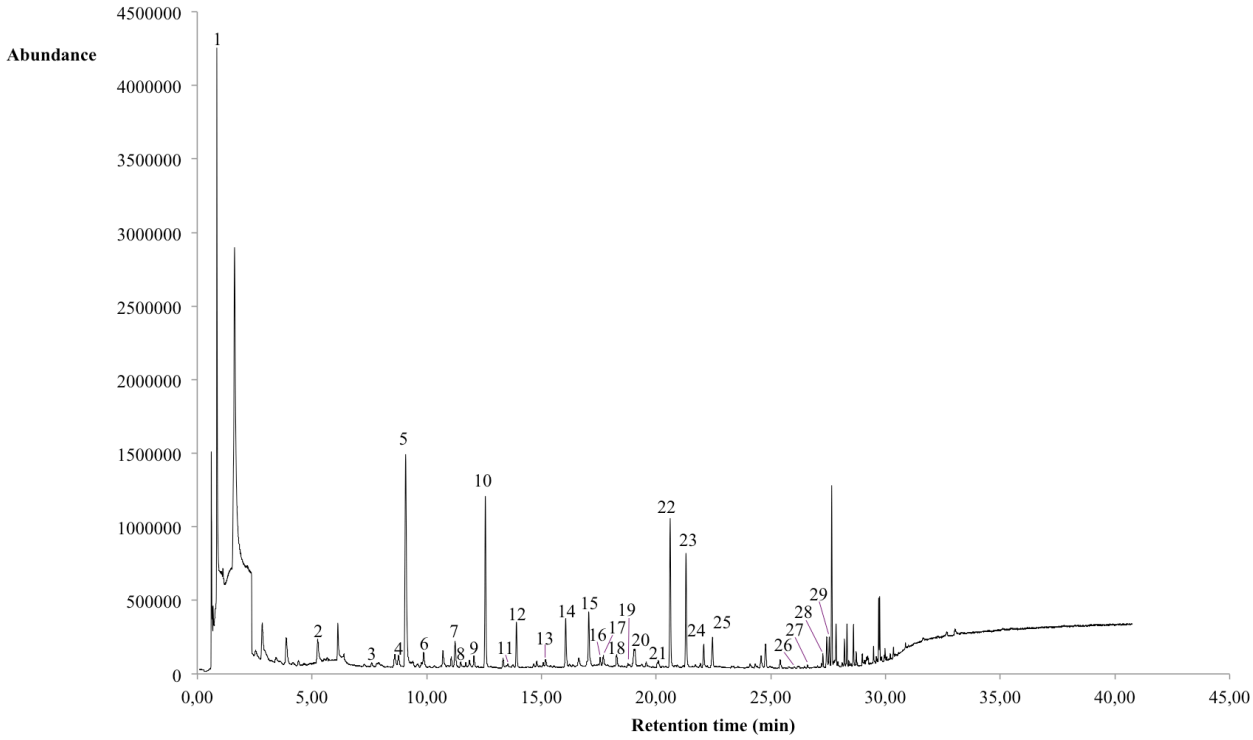

Figure III.5. Typical GC–MS chromatogram of the SPME analysis of VOC emitted by excised 21-day-old barley roots.
1, Dimethyl sulfide; **2**, Hexanal; **3**, Methyl hexanoate; **4**, (*E*)-Hex-2-enal; **5**, 2-Pentylfuran; **6**, Pentan-1-ol; **7**, 2-(Pentenyl)-furan; **8**, (*Z*)-Pent-2-en-1-ol; **9**, 6-Methyl-hept-5-en-2-one; **10**, Hexan-1-ol; **11**, (*Z*)-Hex-3-en-1-ol; **12**, (*E*)-Hex-2-en-1-ol; **13**, Oct-1-en-3-ol; **14**, 2-Ethylhexan-1-ol; **15**, (*E*)-Non-2-enal; **16**, Dimethyl sulfoxide; **17**, Octan-1-ol; **18**, (2*E*,6*Z*)-Nona-2,6-dienal; **19**, Methyl (*E*)-non-2-enoate; **20**, Methyl benzoate; **21**, Nonan-1-ol; **22**, (*Z*)-Non-3-en-1-ol; **23**, (*E*)-Non-2-en-1-ol; **24**, Nona-3,6-dien-1-ol; **25**, Nona-2,6-dien-1-ol; **26**, Tetradecanal; **27**, Dodecan-1-ol; **28**, Dihydro-5-pentyl-2(3H)-furanone; **29**, Hexadecanal.

Table III-3. SPME analysis of VOC emitted by excised 7-d-old barley roots cultivated on Hoagland gelified medium

CAS Number (1)	IUPAC Name	Identification (2)	Retention Index		A	B
			Sampled (3)	Reference (4)	Hoagland gelified medium Relative area (% ± SD, n=5)	Hoagland gelified medium Estimation (ng/g RFW ± SD, n=5)
75-18-3	Dimethyl sulfide	STD	712	844[c]	5.03 ± 1.03	13.55 ± 4.51
3777-69-3	2-Pentylfuran	STD	1212	1229[a]	24.33 ± 2.89	7.39 ± 2.61
100-42-5	Ethenylbenzene#	MS,RI	1237	1273m	12.86 ± 0.88	-
70424-13-4	2-(Pentenyl)furan[#]	MS	1284	-	0.98 ± 0.30	1.22 ± 0.16
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1305	1313[d]	0.66 ± 0.10	0.56 ± 0.16
-	Oct-6-en-2-one#	MS	1313	-	0.98 ± 0.25	-
111-27-3	Hexan-1-ol	STD	1339	1351[f]	4.81 ± 0.25	3.68 ± 1.27
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1391	1400[f]	0.93 ± 0.03	0.77 ± 0.25
3391-86-4	Oct-1-en-3-ol	STD	1437	1420[a]	1.86 ± 0.52	1.42 ± 0.42
1569-60-4	6-Methyl-hept-5-en-2-ol	MS	1451	-	1.68 ± 0.43	1.28 ± 0.36
104-76-7	2-Ethyl hexan-1-ol	STD	1447	1504[g]	1.16 ± 0.17	0.92 ± 0.24
1731-84-6	Methyl nonanoate#	MS, RI	1479	1487b	1.62 ± 0.37	0.66 ± 0.16
18829-56-6	(*E*)-Non-2-enal	STD	1519	1540[a]	2.86 ± 0.36	23.35 ± 7.53
67-68-5	Dimethyl sulfoxide	STD	1543	1553[h]	0.45 ± 0.15	-
111-87-5	Octan-1-ol	STD	1547	1557[i]	0.94 ± 0.15	0.77 ± 0.26
557-48-2	(2*E*,6*Z*)-Nona-2,6-dienal	STD	1550	1597j	1.05 ± 0.21	7.55 ± 0.26
111-79-5	Methyl (*E*)-non-2-enoate	STD	1592	-	4.50 ± 0.92	1.95 ± 0.47
93-58-3	Methyl benzoate	STD	1603	1600[a]	2.93 ± 1.01	1.19 ± 0.24
143-08-8	Nonan-1-ol	STD	1651	1678[g]	1.94 ± 0.27	1.55 ± 0.55
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1673	1682[k]	1.91 ± 0.40	1.57 ± 0.73
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1704	1722[l]	11.81 ± 3.84	9.68 ± 5.86
7786-44-9	Nona-2,6-dien-1-ol[#]	MS, RI	1758	1776[l]	3.27 ± 0.86	2.67 ± 1.36
124-10-7	Methyl tetradecanoate	STD	1974	2034e	2.05 ± 0.22	0.92 ± 0.92
104-61-0	Dihydro-5-pentyl-2(3H)-furanone	STD	2010	2024[j]	0.90 ± 0.33	0.32 ± 0.05

(1) CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); (2) Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section (4)); STD, comparison of retention time and mass spectra of available standards; (3) Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; (4) Retention indices taken from a Jennings and Shibamoto 1980; [b] Tressl et al. 1978; [c] Varming et al. 2004, [d] Umano et al. 2002, [e] Choi 2003, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Sanz et al. 2001, (5) Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99); N.D.: not determined; # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOCs

Table III-4. SPME analysis of VOCs emitted by excised 7-d-old barley roots cultivated in vermiculite.

CAS Number [1]	IUPAC Name	Identification [2]	Retention Index		A		B	
			Measured [3]	Reference [4]	Sterile Relative area (% ± SD, n=5)	Non Sterile Relative area (% ± SD, n=5)	Sterile Estimation (ng/g RFW ± SE), n=5 [5]	Non Sterile Estimation (ng/g RFW ± SE), n=5 [5]
75-18-3	Dimethyl sulfide	STD	712	844[c]	14.93 ± 7.99	14.20 ± 7.79	20.34 ± 13.82	24.83 ± 11.89
96-22-0	Pentan-3-one	STD	965	971[b]	0.61 ± 0.10	0.95 ± 0.20	-	-
66-25-1	Hexanal	STD	1068	1074[b]	1.81 ± 0.39	-	5.59 ± 1.52	-
71-36-3	Butan-1-ol	STD	1140	-	0.31 ± 0.10	0.39 ± 0.13	0.18 ± 0.03	0.24 ± 0.03
616-25-1	Pent-1-en-3-ol#	MS	1153	-	0.77 ± 0.09	0.60 ± 0.13	0.37 ± 0.11	0.35 ± 0.08
6728-26-3	(*E*)-Hex-2-enal	STD	1199	1207[a]	0.57 ± 0.24	0.42 ± 0.16	-	-
3777-69-3	2-Pentylfuran	STD	1212	1229[a]	28.18 ± 8.11	27.72 ± 6.23	4.71 ± 1.66	5.32 ± 1.22
71-41-0	Pentan-1-ol	STD	1240	1244[d]	2.82 ± 0.51	2.05 ± 0.57	1.14 ± 0.29	1.00 ± 0.30
70424-13-4	*Z*-2-(Pentenyl)furan#	MS	1284	-	0.79 ± 0.26	0.98 ± 0.15	1.06 ± 0.04	1.11 ± 0.04
1576-96-1	(*E*)-Pent-2-en-1-ol	MS, RI	1297	1307[b]	-	0.54 ± 0.08	-	0.32 ± 0.05
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1305	1313[d]	0.79 ± 0.17	0.50 ± 0.30	0.37 ± 0.09	0.29 ± 0.13
-	Oct-6-en-2-one#	MS	1313	-	0.69 ± 0.30	0.71 ± 0.28	-	-
111-27-3	Hexan-1-ol	STD	1339	1351[f]	5.23 ± 0.46	4.79 ± 0.38	2.08 ± 0.57	2.27 ± 0.68
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1391	1400[f]	1.86 ± 0.21	1.73 ± 0.28	0.80 ± 0.27	0.87 ± 0.29
3391-86-4	Oct-1-en-3-ol	STD	1437	1420[a]	0.87 ± 0.33	0.67 ± 0.06	0.40 ± 0.13	0.38 ± 0.08
111-27-3	Heptan-1-ol	STD	1442	-	0.54 ± 0.47	0.33 ± 0.09	0.25 ± 0.11	0.22 ± 0.04
1569-60-4	6-Methyl-hept-5-en-2-ol#	STD	1451	-	1.46 ± 1.27	0.60 ± 0.35	0.57 ± 0.40	0.32 ± 0.12
104-76-7	2-Ethylhexan-1-ol	STD	1447	1504[g]	2.26 ± 1.08	4.17 ± 1.42	0.99 ± 0.51	1.91 ± 0.49

[1] CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); [2] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section [4]); STD, comparison of retention time and mass spectra of available standards; [3] Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; [4] Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Binder et al. 1990,[c] Varming et al. 2004, [d] Umano et al. 2002, [e] Choi 2003, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Sanchez-Ortiz et al. 2012, [n] De Marques et al. 2000, [o] Kohara et al. 2006; [5] Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99) tr: trace; N.D.: not determined; # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOC

Table III-4 *Cont.*

CAS Number [1]	IUPAC Name	Identification [2]	Retention Index		A		B	
			Measured [3]	Reference [4]	Sterile Relative area (% ± SD, n=5)	Non Sterile Relative area (% ± SD, n=5)	Sterile Estimation (ng/g RFW ± SE), n=5 [5]	Non Sterile Estimation (ng/g RFW ± SE), n=5 [5]
18829-56-6	(*E*)-Non-2-enal	STD	1519	1540[a]	3.03 ± 0.44	2.64 ± 0.91	11.78 ± 4.65	12.67 ± 7.95
67-68-5	Dimethyl sulfoxide	STD	1543	1553[h]	1.31 ± 1.46	0.90 ± 0.83	-	-
111-87-5	Octan-1-ol	STD	1547	1557[i]	2.68 ± 1.96	1.02 ± 0.33	1.18 ± 0.97	0.53 ± 0.15
557-48-2	(2*E*,6*Z*)-Nona-2,6-enal	STD	1550	1597[j]	1.03 ± 1.46	1.14 ± 0.17	2.30 ± 1.48	3.97 ± 2.30
111-79-5	Methyl (*E*)-non-2-enoate	STD	1592	-	1.00 ± 0.25	1.06 ± 0.20	0.18 ± 0.08	0.24 ± 0.06
93-58-3	Methyl benzoate	STD	1603	1600[a]	1.97 ± 0.34	1.90 ± 0.52	0.40 ± 0.11	0.48 ± 0.17
143-08-8	Nonan-1-ol	STD	1651	1678g	1.88 ± 0.37	1.53 ± 0.12	0.81 ± 0.30	0.76 ± 0.15
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1673	1682k	3.29 ± 0.30	3.93 ± 0.68	1.33 ± 0.34	1.92 ± 0.79
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1704	1722[l]	12.94 ± 5.27	15.01 ± 7.99	5.41 ± 3.51	7.41 ± 5.45
56805-23-3	Nona-3,6-dien-1-ol[#]	MS, RI	1741	1759[k]	1.10 ± 1.12	0.63 ± 0.12	0.49 ± 0.46	0.37 ± 0.15
7786-44-9	Nona-2,6-dien-1-ol[#]	MS, RI	1758	1776[l]	3.52 ± 2.04	3.98 ± 2.03	1.49 ± 1.14	2.03 ± 1.48
111-82-0	Methyl dodecanoate[#]	MS, RI	1794	1813[e]	-	0.34 ± 0.13	-	0.03 ± 0.03
60-12-8	2-Phenylethanol	STD	1893	1931[j]	0.37 ± 0.20	0.21 ± 0.08	0.21 ± 0.07	0.17 ± 0.04
112-53-8	Dodecan-1-ol	STD	1967	1970[n]	0.36 ± 0.17	0.87 ± 0.27	0.22 ± 0.10	0.46 ± 0.11
124-10-7	Methyl tetradecanoate	STD	1974	2034[e]	0.24 ± 0.12	0.73 ± 0.24	0.05 ± 0.09	0.15 ± 0.06
104-61-0	Dihydro-5-pentyl-2(3H)-furanone	STD	2010	2024[j]	0.35 ± 0.12	0.55 ± 0.11	0.02 ± 0.01	0.10 ± 0.04
629-80-1	Hexadecanal[#]	MS, RI	2023	2020[o]	0.39 ± 0.19	1.03 ± 0.34	-	-

[1] CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); [2] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section [4]); STD, comparison of retention time and mass spectra of available standards; [3] Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; [4] Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Binder et al. 1990,[c] Varming et al. 2004, [d] Umano et al. 2002, [e] Choi 2003, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Sanchez-Ortiz et al. 2012, [n] De Marques et al. 2000, [o] Kohara et al. 2006; [5] Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99) tr: trace; N.D.: not determined; # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOCs

3.3 Wireworms Olfactory Orientation Bioassay

Seven-day-old barley roots grown in axenic conditions were used as VOC sources (10 plantlets/olfactometric test) for an orientation bioassay of the belowground pest insect *A. sordidus*. We tested 60 larvae, 10 of which did not respond (16.5 %). 35 oriented towards barley roots, and 15 towards the control (P = 0.007**). The bait position with regard to both the substrate entry side and the left or right position of the roots on the laboratory bench did not significantly affect the response of the wireworms (Fisher's test of exact count, P = 0.861). The blank-to-blank experimentation confirmed the absence of biases in the experimental set-up. Fifteen wireworms orientated to the left, eleven to the right (P = 0.572), and nine did not respond (26 % of the individuals) (Figure III.6). In the same experiment, the χ^2 test of independence showed that the entry side of the substrate, i.e. its potential lack of uniform density, had no impact on the results ($\chi^2 = 1.418$; 1 df; P = 0.214).

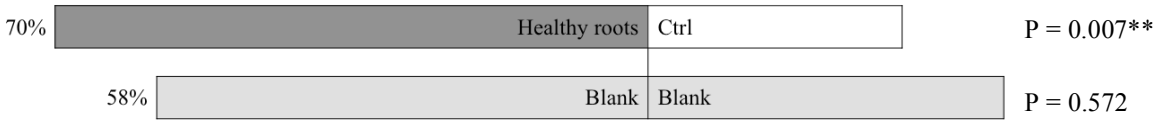

Figure III.6. Attraction of wireworms towards VOC emitted by healthy 7-day-old roots.
Grown on agar-agar medium (first line, n = 60, 10 non-responding). In the absence of stimuli, there was no position effect of the olfactometers, i.e. the blank-to-blank dual-choice experimentation did not reveal left or right bias (second line, n = 35, 9 non-responding).

4. Discussion

We developed an SPME method that enabled us to detect a wide range of VOC released by barley roots. Furthermore, our method allowed the estimation of the amounts of VOC emitted from roots. The physicochemical properties of a VOC depend significantly on its chemical family. The sorption of a volatile compound on the SPME fiber depends on the functional groups, vapor pressure, and constitution of the headspace of the sample. This needs to be taken into account when considering the quantitative values presented in Table III-2, Table III-3, and Table III-4 (column B). These quantitative values are only estimations based on calibration curves obtained from the analyses of compounds that are considered representative for the respective detected root volatile. Exact quantification of VOC in a mixture of approximately thirty compounds is quite difficult, as many VOC have to be quantified simultaneously in the volatile blend.

The major volatiles emitted by barley aerial parts were described by Bukovinszky et al. (2005), Piesik et al. (2010, 2011a), and Wenda-Piesik (2010), whereas VOC emitted by barley roots have not been reported previously in the literature, nor has any description of

their biological activity been published. In the research reported herein, a total of 29 compounds were identified from excised roots after 21 days of growth. Estimation of the concentration of barley VOC on a fresh weight basis revealed a similar range of emission when compared to that reported for barley leaves under controlled conditions or for β-caryophyllene in maize roots (Piesik et al., 2010, Hiltpold et al., 2011). In our study, VOC profiling was performed on roots separated from the aerial parts of the plant. In order to validate the working conditions, roots were wounded manually and changes in the profile of VOC were analyzed. This additional wounding of roots resulted in a dramatic increase in the amount of VOC emitted with no major changes in the qualitative VOC profile (data not shown).

VOC derived from polyunsaturated fatty acids constituted the largest number of VOC of the barley root blend. These comprise hexanal, methyl hexanoate, (*E*)-hex-2-enal, 2-pentylfuran, pentan-1-ol, (*Z*)-2-(pentenyl)-furan, (*Z*)-pent-2-en-1-ol, hexan-1-ol, (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol, oct-1-en-3-ol, 2-ethylhexan-1-ol, (*E*)-non-2-enal, octan-1-ol, (2*E*,6*Z*)-nona-2,6-dien-al, methyl (*E*)-non-2-enoate, nonan-1-ol, (*Z*)-non-3-en-1-ol, (*E*)-non-2-en-1-ol, nona-3,6-dien-1-ol, nona-2,6-dien-1-ol, and dihydro-5-pentyl-2(3H)-furanone (Min et al., 2003, Shiojiri et al., 2006). Most of these compounds have been largely described in leaves as direct or indirect defense molecules, produced in response to herbivory or wounding (Arimura et al., 2000). Similarly to our study, barley aerial volatiles emitted under unwounded conditions were mainly C18 fatty acid derived volatile compounds, such as (*E*)-2-hexenal and (*Z*)-hex-3-en-1-ol (Piesik et al., 2010, Wenda-Piesik et al., 2010). Fatty acid derived VOC might have a basal level of emission under unattacked conditions. With respect to root VOC, quite similar compounds (hexanal, (*E*)-2-hexenal, 2-pentylfuran, 2-ethylhexan-1-ol, octan-1-ol, (*E*)-non-2-enal) were identified in grapevine ground roots (Lawo et al., 2011). As these VOC have been described in the wound response, they are interesting candidates in the study of root - wireworm interactions. As 2-ethylhexan-1-ol has never been clearly demonstrated to be of plant origin, this compound might be regarded as a plastifying contaminant (Yi et al., 2009).

Apart from fatty acid derived VOC, two sulfur-containing volatile molecules (dimethyl sulfide and dimethyl sulfoxide) were constituents of the volatile blend. These two compounds have not previously been described as emitted by barley. Dimethyl sulfide is released from wounded citrus and guava leaves (Rouseff et al., 2008). Sulfur compounds have been shown to be attractants of the fly *Delia antiqua* (Matsumoto, 1970).

Surprisingly, we did not identify any terpenes, such as β-caryophyllene. Nevertheless, we detected exogenously applied monoterpenes and sesquiterpenes (data not shown). This means either that barley roots do not emit terpenes or emit them below detection limits. This result is in agreement with the measurement of VOC emitted by barley leaves; no terpene was detected in the headspace of non-wounded barley leaves; however, terpenes were detected after wounding (Piesik et al., 2010, Wenda-Piesik et al., 2010). The impact of microorganisms in the measured volatile blend of barley roots was low since 32 VOC out 34 were the same between the NS and ST roots (Table III-4). Similarly, 28 VOC were present in similar amounts in the headspace of roots kept under the two conditions.

The orientation bioassay showed that wireworms exploit the emission of VOC from 7-day-old barley roots and use it for location of host roots. The percentages of non-responding individuals in the blank-to-blank bioassay and in the root-baited bioassay tended to show a slightly increased activity of wireworms in the presence of a stimulus. As roots were still breathing, CO_2 obviously formed part of the blend. Its involvement in the attraction of *Ctenicera destructor* (Brown) and other wireworms has been demonstrated (Doane et al., 1975). However, CO_2 emission from roots is probably not a reliable cue for host root location by rhizophagous insects, since CO_2 is emitted from numerous sources in the soil and thus, lacks specificity. Nevertheless, it probably acts as a general signal or a search trigger. Moreover, chemically-mediated orientation due to volatile or non-volatile compounds of the rhizosphere often is proposed whenever root location by subterranean insects is investigated (Reinecke et al., 2008, Wenke et al., 2010, Johnson and Nielsen, 2012, Weissteiner et al., 2012). Such cues could be considered within an integrated management perspective, which is not necessarily conceivable for CO_2, present in all soils. Future studies need to elucidate whether each of the barley root volatiles detected in our study serves as an attractant to pest of the rhizosphere. The VOC identified using the protocol described for 7-day-old sterile roots (Table III-3) need to be tested alone and in combination in the olfactometers, in order to assess both their potential attractive properties and their possible synergistic interactions with each other and with CO_2. This can be achieved by using slow release systems such as alginate beads (Heuskin et al., 2011), with the advantage of suppressing carbon dioxide gradients.

The assessment of the role of volatile compounds in the chemical ecology of wireworms is promising, especially regarding the developed bioassay. Further experiments involving the natural enemies of wireworms, such as entomopathogenic nematodes, could lead to a higher trophic level and could also provide useful information. Such tri-trophic interactions have

already been studied between the root pest *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), maize (Zea mays, L. Poaceae), and the entomopathogenic nematode *Heterorhabditis megidis* (Hiltpold et al., 2011), and their outcomes have shown promising trails of applied control measures.

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Chapter III.3 - Exploring the Potential of Dual-Choice Devices with Live-Baits: Opportunities and Limits

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Abstract: The different steps of the foraging process of wireworms (Coleoptera: Elateridae) would be better understood if accurate and holistic information regarding the role of plant-produced chemicals constituting their environment were available. Volatile organic compounds (VOC) play important roles in the interactions between plants and insects in many ecosystems, whether they take place aboveground or belowground. Their roles on foraging wireworms are still relatively unknown. Here, we performed three experimentations with barley roots as baits, mainly to determine the experimental limits of olfactometers designed for the observation of wireworms' behavior. In the first two, we assessed the effect of chopped roots and fungus infected roots on the orientation of wireworms. In the third experiment, the larvae were confronted to both healthy and fungus infected roots. Wireworms were significantly attracted towards chopped roots and slightly towards fungus infected ones. They could not discriminate between healthy and infected roots in the last experiment. We discuss the results in terms of suitability of the olfactometers we designed for the investigation of VOC preception in wireworms, and we provide suggestions to improve their use.

Key words: chemical ecology, belowground pests, insect-plant interactions, olfactometry, integrated pest management.

1. Introduction

Pest management strategies arise from the increasing knowledge concerning mechanisms underpinning interactions between organisms (Agelopoulos et al., 1999, Cook et al., 2007). Soils are complex matrixes sheltering many organisms that can interact with each other, with plants, and aboveground organisms (Johnson and Rasmann, 2015). While foraging in the dark, belowground phytophagous insects must locate and identify suitable resources. They rely on plant chemical signals which potential in pest control is admitted (Johnson and Nielsen, 2012, Hiltpold et al., 2013).

The semiochemical roles of VOC in aboveground interactions have been studied on various models for many years, but the recent advances on multi-trophic models, notably focusing on the Western Corn Rootworm (*Diabrotica virgifera virgifera* Leconte) (Rasmann et al., 2005, Erb et al., 2008, Hiltpold et al., 2011, Robert et al., 2012), highlighted equally complex interactions in the soil (Johnson and Rasmann, 2015). Many other pests develop underground, but the VOC impact on their chemical ecology is far less advanced, while increasing that knowledge could lead to new alternatives for their management (Johnson and Nielsen, 2012, Hiltpold et al., 2013). Wireworms, the larvae of click beetles (Coleoptera: Elateridae), represent a typical example thereof. Many species are crop pests and develop belowground for many years. They feed on a variety of plants to reach maturity (cereals, potatoes, sugarcane, lettuce, strawberries…), inducing considerable economic losses in wide areas of the Palearctic Region. As other pest species, they require efficient management, with decreasing resort to synthetic pesticides. Investigating the role of olfaction in their foraging behavior and chemical ecology constitutes an important step that could lead to interesting management alternatives (Johnson and Nielsen, 2012, Barsics et al., 2013).

Behavioral experimentations with VOC require the use of olfactometers in which insects are confronted with a cue originating from plants themselves or reconstituted synthetic blends. These devices allow highlighting the attractive or repellent effect of volatile compounds. Olfactometry requires conditions as close to reality as possible. For soil-dwelling insects, assays should occur in a substrate. Defining experimental parameters that are suitable to observe significant behavioral responses is a key step to that approach. In this work, we report three different experimentations performed in dual-choice olfactometers on *Agriotes sordidus* wireworms, with baits composed of roots. These bioassays are complementary to those reported in Barsics et al. (2012), Gfeller et al. (2013), where experimental parameters

were set in both presence and absence of CO_2, a known key semiochemical in wireworm foraging (Doane et al., 1975).

In our assays, we aim at showing differences of attraction towards different root baits, and to verify whether it is possible or not to highlight preferences when two live baits are suggested simultaneously to wireworms. Firstly, we exposed wireworms to chopped barley roots (*Hordeum distichon* L.), in order to determine the effect of cues from decaying plant material. Secondly, to verify if they could display orientation towards unhealthy roots, we exposed wireworms to roots infected with a phytopathogenic fungus, *Cochliobolus sativus* (Ito and Kuribayashi) Drechsler ex. Dastur, 1942. Finally, we investigated the ability of wireworms to discriminate between healthy and fungus-infected roots. We discuss these results and previously reported ones, in relation to the scale at which we work and in terms of suitability of designed olfactometers.

2. Materials and Methods

2.1 Wireworms

Wireworms were collected in experimental plots in Montardon (Arvalis Institute, France), between 2010 and 2012. The collection area was chosen in control plots, free of pesticide treatments. Wireworms were transferred into rearings and separated from each other in order to prevent cannibalistic events. Each individual was kept in 80 cm^3 vials filled with a mix of compost and vermiculite (1/1, v/v, 16.5% water), and kept in the dark at room temperature (22 ± 1°C). Food was provided with meadow seeds from organic production (0.130 – 0.160 g, Prelac Bio, SCAR, Belgium). *Agriotes sordidus* wireworms were selected with reference to morphological criteria reported by Cocquempot et al. (1999) and Pic et al. (2008). Those having a length of at least 10 mm were selected for experimentation, as they represent critical instars for crops (Furlan, 2004). Seven days prior to testing, they were isolated from organic matter (either from food or their rearing substrate), by being transferred into separate vials filled with moist vermiculite (16.5%). On the experimentation day, individuals that were visibly inadequate for testing were excluded. These include larvae that just molted (not darkened mouth parts, light colored cuticle) or that are close to a subsequent molt (lowered activity and swollen, compared to their conspecifics). The remaining individuals (50 - 70 % of isolated ones) were closer to the feeding phase, which lasts less than 25 % of the whole development time in *A. sordidus* (Furlan, 2004).

2.2 Plant Material

Barley plants used in these experiments were all seven days old. They were produced with different methods. Chopped plants were simply grown in compost, by groups of ten, in one-litre jars. They were watered daily. Other plants were produced with the same protocol as that detailed in (Gfeller et al., 2013) for sterile plants grown on agar-agar medium. To infect plants with *C. sativus*, the caryopses were pre-germinated for two days and then sprayed with 1 mL of a solution containing 10^{-6} conidia/mL. Plants were stored in a growth chamber until experimentation, with 22°C, 65 % RH, 20/4 L/D photoperiod and under LED light (95 µmol m^{-2} sec^{-1}). For each modality, plants were extracted from their substrate in the minutes preceding the beginning of the behavioral assays.

2.3 Behavioral Assays

Olfactometers

Individual behavioral experimentations on wireworms were performed in dual-choice glass pipes (Figure III.2, Figure III.3) filled with moist vermiculite (grade 2, Sibli, Andenne, Belgium). Their suitability for assessing wireworm behavior towards plant-emitted VOC was already demonstrated in previous studies (Barsics et al., 2012, Gfeller et al., 2013). The cylinders (32 cm long, 3.6 cm internal diameter) are provided with one central tubular aperture (3 cm long, 1 cm diameter), allowing wireworm entry. Vermiculite is introduced through both extremities, which are closable with screwed caps. Two other tubular apertures are located 3 cm away from each extremity and are diametrically opposite to the one used for the introduction of wireworms. Pipes are therefore connectable to external odorant systems. These apertures also serve to introduce roots as baits, without cutting them off the rest of the plant, which is maintained out of the system.

Protocol for dual-choice bioassays

After filling pipes with moist vermiculite (30%), 4 cm of substrate is removed from each side, leaving room for baits. A stainless-steel boundary (3.6 cm diameter, 0.04 mm thickness and mesh-size) is inserted against the remaining substrate to avoid further contact between burrowing wireworms and roots, ensuring that the final choice is solely related to the diffusion of volatile cues rather than to physically encountered plant material. In the three different experiments, the control baits were introduced first. They consisted of 240 mg of leaf mold when chopped roots were tested, 240 mg of blank agar-agar medium when fungus

infested roots were tested, and healthy roots in the experiments opposing healthy to fungus infected roots. The plants were systematically extracted from their substrate within 5 minutes before each test, and grouped by bundles of ten. When chopped, the roots were firstly cleaned with tap water to remove as much substrate as possible. There systematically were residues, explaining the use of compost as a control. When entire plants were inserted, they were gently removed from the growth medium, and introduced by pulling the leaves through the cylinder and then through the lateral tubular aperture, until nothing else but the roots occupied the bait zone. As soon as baits were inserted, the pipes were closed with the screwed caps. Then, PTFE and aluminium tape were immediately wrapped around the stem bases and the lateral apertures to prevent any leak of volatiles out of the device. The left of right position of the different baits inside olfactometers were assessed randomly before experimentation.

Experimentations were run by batches of ten. Time was noted down to the minute when each olfactometer was completely loaded. Exactly 40 min later, one of the selected wireworms was allowed to penetrate the glass-pipe through the middle entry. Each larvae was given 80 min to explore and orientate towards the olfactometer, at 21 ± 0.5°C in a room without light. The latter was turned back on to retrieve wireworms and to record their final position. The zone comprised within 3 cm from the entry point was considered as the neutral zone, so that wireworms found at that level were ranked as non-choosing individuals. Tests were performed with pre-selected individuals until fifty effective choices were obtained. Results were treated with a two-tailed binomial test.

3. Results

The selection of individuals used in the bioassays, according to their apparent feeding activity, gave the results reported in Figure III.7. Chopped roots highly attracted wireworms ($P < 0.001$***). Fungus infected roots were not significantly attractive although the probability outlined by the binomial test is close to statistical significance ($P = 0.065$). The larvae did not show any preference between healthy and infected roots in these experimental conditions ($P = 0.659$). With chopped roots, only 18% of the tests resulted in neutral responses, whereas a third of the replicates were inconclusive when live roots were used (32.4 and 34.3%). In each case, wireworms were easily retrieved from the vermiculite since they form tunnels against the glass while burrowing. When healthy and fungus infected roots

were used simultaneously, only 46 replicates were performed due to a reduced number of available wireworms.

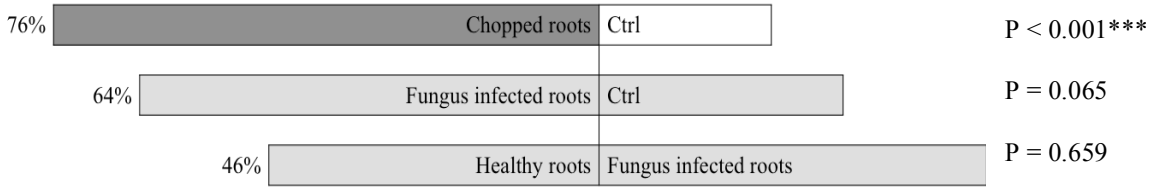

Figure III.7. Attraction of wireworms towards chopped roots and fungus infected roots.
Proportions of wireworms retrieved on in each side of the olfactometers for the three behavioral tests, and associated P-values. Ctrl: Control.

4. Discussion

The first assay showed that wireworms starved during seven days are highly attracted towards freshly chopped roots. Although there is evidence that they seem to depreciate decaying plant material (Wallinger et al., 2013), there might be situations under which cues emanating from highly damaged roots will lead larvae towards such a plant system. This should be verified in more realistic conditions. Wireworms were also attracted towards fungus-infected roots, at a level close to that observed when they are exposed to VOC emitted by healthy roots (Gfeller et al., 2013). Thanks to a recent report on VOC emitted by the same fungus strain (Fiers et al., 2013) it could be possible to highlight what compounds are common to both blends, and to find putatively attractive semiochemicals. However, when comparing the healthy and fungus infected roots, wireworms in our experimental failed to reveal a preferential target. Besides from the possibility that wireworms do not need to differentiate a healthy plant from a fungus-infected one, there are two hypotheses as to why they did not discriminate between the two at this scale. Firstly, the experimental set up may have been saturated with volatile cues, annihilating guiding gradients that should have played a role in the orientation. The comparison of two live baits could be performed with adjusted experimental parameters. For example, by reducing the diffusing time before wireworm release, or by using fewer amounts of root material in the baits. Secondly, wireworms were unable to reach the roots because of the steel boundaries between the baits and the substrate. Yet, after orientation during the foraging process, root contact seems necessary to eventually confirm the suitability of the host (Johnson and Nielsen, 2012). Without the possibility to access the roots, the larvae may have orientated according to other cues, if available, such as when live baits were compared. In the experimentations with chopped or fungus-infected roots, this step may have been necessary to reject or accept the unhealthy host.

Complementary experiments in which boundaries are removed between the baits and the substrate would reveal whether wireworms remain in the roots. Since the position of wireworms was accurately recorded when they were retrieved from the pipes, it would be easy to compare these positions between cases with and without boundaries. Taking account of the visible tracks they leave against the inside wall of the glass pipes while burrowing (small tunnels in the vermiculite) may reveal more of their foraging behavior as well.

The amounts of carbon dioxide released by roots during the assays were not measured. CO_2 is however known to be involved in wireworm foraging (Doane et al., 1975). It is possible that CO_2 saturation in the olfactometers might have represented an overriding cue for wireworms, leading to their inability to detect a preferable food source. To assess the semiochemical roles of other compounds of the cue than CO_2, behavioral tests could be performed in a CO_2-controlled environment. The olfactometers used here are suitable to assess the effect of selected VOC, such as 2-pentylfuran (Barsics et al., 2012), a compound listed in blends sampled from barley roots in Fiers et al. (2013) and Gfeller et al. (2013). But many compounds of one blend may be needed to induce a particular response. Exposing wireworms to these compounds would require using a synthetic blend as bait. Expressing results taking account of the relative amount of CO_2 is possible when its concentrations are measured. This could be done in experimentations with groups of individuals, for which results would be presented as ratios of wireworms retrieved on each side of the olfactometer, in relation to ratios of CO_2 produced by each type of bait (Robert et al., 2012). In this scenario, the impact of congeners on the foraging process would need to be investigated.

These olfactometers are useful tools to investigate the role of volatile organic compounds on the foraging behavior of wireworms. Further experimentations revealing interesting semiochemicals should provide new perspectives for wireworm management.

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Chapter IV – Identification, Quantification, and Biological Activity of Volatile Organic Chemicals produced by Barley Roots

Introduction to Chapter IV

After having developed an olfactometer that allows the study of wireworm behavior, the second objective of our work consisted in identifying and quantifying, as accurately as possible with available techniques, the blend of volatiles characterizing a plant's rhizosphere. Firstly, we used SPME to investigate the volatile content of barley roots. Because SPME has limitations in terms of quantification and sensitivity, we have adopted a second method to collect root VOC. This method is called Dynamic Head-Space Sampling (i.e. a pumping through the vial headspace), and is directly followed by adsorption of the sampled volatiles on a high affinity phase. As we will see in the second section of this chapter, the number of identified compounds will be lowered to only four VOC with this method. They represent the VOC produced by barley roots ground in liquid nitrogen, according to a method developed by Delory et al. (2015).

The third objective of the thesis was to evaluate the biological activity of VOC that could guide wireworms toward their host plant. We suggest that our olfactometric method is suitable to that purpose. With the hypothesis that VOC intervene somewhere from the random movement in the soil to the final host plant choice (Johnson and Nielsen, 2012), the following sections of this thesis will report on behavioral assays performed with molecules included as stimuli in baits. To understand the two experimental steps taken in the next publications, it is important to remind that semiochemicals have to be considered as blends (De Bruyne and Baker, 2008). One compound carrying information may carry totally different information when it belongs to a blend, or in our case, in the semiochemical background characterizing the rhizosphere in which wireworms locate their food. Therefore:

- the proof that some VOC induces orientation in olfactometric assays does not ensure that the same VOC will produce the same response in the field;
- ideally, VOC should not be tested alone but rather in blends representing what really occurs in the soil.

This is why the approach developed here aims at increasing the complexity of synthetic blends exposed to larvae. We managed to release volatile molecules in our olfactometric assays using two emitting devices/formulations. Firstly, we used alginate beads, i.e. slow-release beads constituted of alginate, sunflower oil, tocopherol and a given odorant stimulus

(Heuskin et al., 2011). With that method, we exposed wireworms to 2-pentylfuran, one of the main compounds sampled by SPME (Gfeller et al., 2013). Secondly, we used volatile dispensers containing a blend of volatiles, at different doses, stabilized in triacetin (von Mérey et al., 2012), in an attempt to outline dose-related behavioral shifts, such as observed in many insects. Here, the selected VOC were four aldehydes identified and quantified thanks to the DHS method (Barsics et al., 2015). Doing so, we improved the bioassays by exposing wireworms to a more complete blend than 2-pentylfuran alone. Moreover, due to the nature of the method used to sample VOC, that blend is closer in relevancy to blends potentially existing in the soil or in the root vicinity. As we will explain in the discussion, many improvements are still necessary in order to approach field reality as well as developing field approaches with laboratory acquired semiochemical data.

Chapter IV.1 - Taking it Down to the VOC Level

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Abstract: In the aim to develop alternative management strategies against a high number of pests, entomological research is notably set to unveil knowledge related to pest chemical ecology. In this work, we investigate whether wireworms, crops pests and larvae of click beetles (Coleoptera: Elateridae), are able to display orientation towards a root-emitted volatile organic compound. In related research, 2-pentylfuran was sampled by Solid-Phase Micro-Extraction in the head-space of vials containing excised barley roots. We report here on 1) the formulation of alginate beads releasing 2-pentylfuran at ca. 1 ng/h and the system allowing monitoring of their emission kinetics; and 2) the outcomes of behavioral assays in dual-choice olfactometers where alginate beads are used as 2-pentylfuran dispensers to evaluate wireworms' ability to orientate according to a volatile cue diffusing through a substrate.

Key words: *Agriotes sordidus*, root pest, chemical ecology, volatile organic compounds, dual-choice olfactometers.

1. Introduction

Wireworms are the larvae of click beetles (Coleoptera: Elateridae). Their worldwide importance as belowground crop pests is well documented. Resort to insecticides for their management has considerably decreased and alternatives to synthetic chemicals have emerged. Many IPM approaches have already proven to be useful at field scale while some need improving. More efficient integrated management could be undertaken if gaps of knowledge in wireworm biology and ecology were filled (Furlan, 2005).

Research could beneficiate from new insights in wireworms' chemical ecology, especially regarding their foraging behaviour (Barsics et al., 2014b). Wireworms and other soil pests likely orientate towards food sources along a three-step mechanism (Johnson and Gregory, 2006, Johnson and Nielsen, 2012). First, CO_2 gradients originating from roots or any other belowground plant organ biases their random movement (Doane et al., 1975). The second step involves sensing specific semiochemicals, including volatile organic compounds (VOC) or root exudates and residues. Finally, chemosensory cues at root surface act as factors of acceptance or rejection of the host plant (Johnson and Gregory, 2006, Johnson and Nielsen, 2012). Several plant-produced substances, reviewed in (Barsics et al., 2014b), have been shown to elicit an orientation or a biting response, but the role of root-emitted VOC remains unclear.

Here, we report on VOC-induced orientation of *A. sordidus* wireworms. We selected a compound resulting from SPME sampling of barley roots (*Hordeum distichon* L.), 2-pentylfuran (2-PF), in dual-choice orientation bioassays. This molecule was loaded in alginate beads which formulation was adapted to simulate a range of emission close to one nanogram per hour, a dose in line with estimated quantities reported earlier in this thesis (Gfeller et al., 2013). Alginate beads were then used as bait in olfactometric devices.

2. Materials and Methods

2.1 Volatile Organic Compound Selection

Root-emitted VOC were identified for 7-day-old barley roots grown on culture medium. Identifications were performed with Solid Phase-Micro Extraction (SPME) and Gas Chromatography coupled to Mass Spectrometry (GC-MS). 2-pentylfuran was one of the first identified volatiles (mass spectrum comparison, internal standard, and non-isothermal Kovats index) during the methodological developments. In terms of relative abundance, the first estimations lead to a peak area representing 6.9 % of the overall chromatogram area. The

estimated released dose of 2-pentylfuran during 45 min of SPME fibre exposure (after 15 min of head-space equilibrium) was of 2.4 ± 0.3 ng/g of roots (early estimations, unpublished data). Further work slightly reviewed these value upwards, i.e. from 4.2 ± 0.5 to 7.66 ± 1.22 ng/g of roots, according to plant age and growth conditions (Gfeller et al., 2013).

2.2 Formulation of Alginate Beads

Alginate beads were formulated with 2-pentylfuran (Sigma Aldrich, Belgium (S.-A.): 97 %) and their emission kinetics was evaluated with protocols adequate for trapping of VOC dispensed by alginate beads (Heuskin et al., 2010, Heuskin et al., 2011). The formulation was adapted to reach an emission range of 1 to 10 ng/h. A 2 % (w/v) alginic acid solution was prepared with distilled water (Alginic acid Sodium salt, low viscosity, S.-A.). To 16 ml of this solution, 4 ml of 2-pentylfuran-loaded sunflower oil (0.1 µl/ml) were added. 150 mg of α-tocopherol (S.-A.) were weighed in the mixture. The solution was then blended with an ultraturrax system (IKA T18 Basic, QLab, Belgium) at 24000 rpm until obtaining a homogeneous emulsion. Subsequently, the emulsion was extruted through a syringe needle (0.4 mm internal diameter, Terumo, Belgium) thanks to a peristaltic pump, which produced droplets delivered in a 0.2 M $CaCl_2$ solution under magnetic agitation (300 rpm). A distance of 20 cm between syringe head and the $CaCl_2$ solution surface was maintained to ensure the formation of fully spherical beads. They were conserved as such 48 h, a step necessary to ensure stabilization. Beads were then sifted and dried under active-carbon filtered compressed air for 2 h. They were finally weighed and stored in a glass vial before subsequent analysis. They were conservation at 4°C in case of delayed behavioral experimentation.

2.3 Assessment of the Emission Rate

The release kinetics of the beads was monitored with a release-recapture system (Figure IV.1). Five hermetically sealed PTFE tanks (h: 12 cm, id: 8 cm, ISOFLON, France) containing 2 g of 2-pentylfuran-loaded beads were each connected to a suction pump (Escort Elf Pump, S.-A.). To simulate olfactometer-like conditions, the beads were placed under 10 cm of vermiculite. Two perforations in the tank screwtop allowed air movement. The in-flow was first cleaned with active-carbon filters. The 2-pentylfuran-loaded air was filtered with an adsorbing cartridge filter (Hayesep Q phase, 50 mg, S.-A.), connected between the tank exit

and the pumping system. A subsequent filter was used to check for potential 2-PF excess. Pumps were set to 0.5 ± 0.05 l/min. Connections between elements were ensured with PTFE pipes (V.W.R., Belgium) and PTFE tape sealing the junctions. The emission kinetics was performed in a thermostatic chamber (20.2 ± 0.2°C; HR: 54.3 ± 6.7 %). The monitoring was performed during 6 hours, with filters being replaced every hour.

Compounds were extracted from the filters by eluting 2 x 250 µl of hexane (≥ 97,0 % (GC), S.-A.) in a single vial. In each sample, 10 µl of a 5 µg/µl 2,3-dihydrobenzofuran (DHBF, ≥ 97,0 % (GC), Fluka) solution were added, as internal standard. The extract was analyzed with a Thermo Trace GC Ultrafast (Thermo Electron Corporation) equipped with a flame ionization detector (FID at 250°C) (300 Hz) and a Ph5 column (5 m x 0.1 mm x 0.1 µm). A calibration curve of 2-PF by DHBF was built prior to the release kinetic analysis for further quantification.

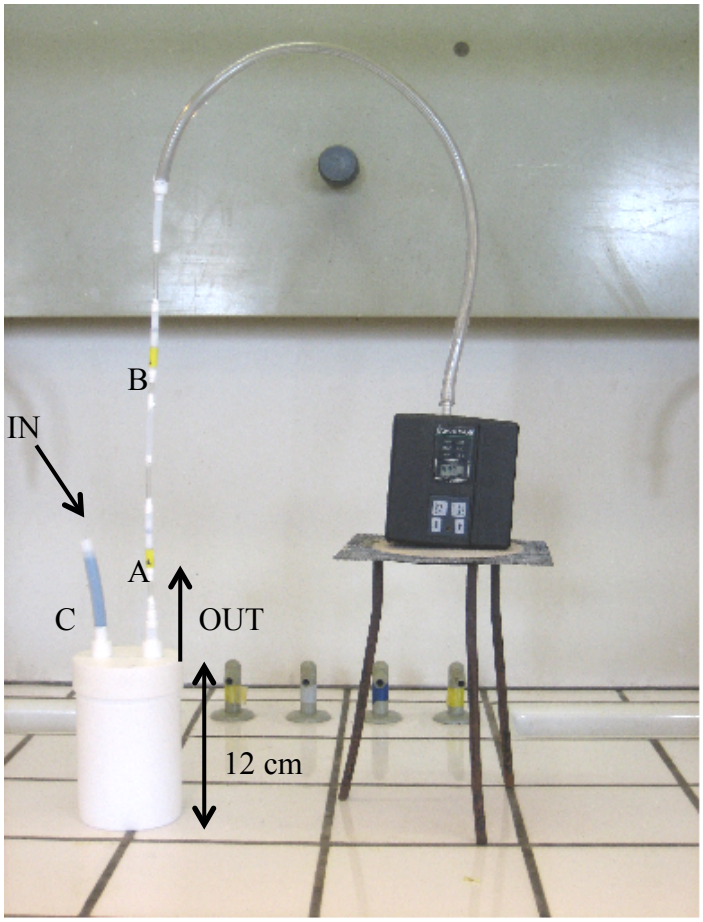

Figure IV.1. Trapping of 2-pentylfuran emitted by alginate beads.
IN: air entry point; OUT: air exit point;
A: first filter; B: second filter (breakthrough filter); C: active carbon filter.

2.4 Orientation Bioassay towards Alginate Beads loaded with 2-pentylfuran

Wireworms

Wireworms were collected in November 2011 in Montardon (Pau, France) from an untreated wheat field. *A. sordidus* individuals were identified with morphological keys (Cocquempot et al., 1999, Pic et al., 2008). Each larva was kept individually in 80 ml capped vials with a mix of leaf mould and vermiculite (1/1 v/v, 16.5 % water, Sibli, Belgium) and a mix of meadow seeds (0.130–0.160 g, BIO Saatgut, Feldsaaten Freundenberger, Germany). Vials were stored in the dark at 21.2 ± 0.7°C. Wireworms of more than 10 mm were individually isolated in vermiculite (16.5% water (v)) seven days prior to the orientation tests. Larvae visibly in pre/post-moulting phases were excluded from the tests.

Bioassay

The set up consisted of a glass pipe (32 cm long, 3.6 cm internal diameter). It was filled with wet vermiculite removed to a depth of 4cm at the two ends of the equipment, to leave room for bait and control compartments (standardized final vermiculite content: 64.4 ± 0.9g, 53.0 ± 0.4% water). Both extremities were closed with GL45 caps through which bait and control were inserted. The larvae entry point consisted of a tubular connection in the middle of the pipe. Baits were alginate beads formulated accordingly to an adequate 2-pentylfuran release. Controls consisted in alginate beads formulated without any volatile, i.e. blank beads. A 3.6 cm diameter wire-cloth (stainless steel; width: 0.042 mm; mesh: 0.036 mm; Haver, Belgium) separated the substrate from the bait and control compartments. Tests were performed by batches of 10 olfactometers at a room temperature of 21.9 ± 0.5°C. Each wireworm was introduced 15 min after bait setting. A red plastic sheet was placed on each bioassay for the entire duration of the tests, preventing from light-induced biases while manipulating. All the material was cleaned with water and norvanol (VWR, Belgium) between each test.

Wireworm positions were recorded after 120 min. When located within 3 cm from the entry point, they were considered non-responding. New tests were repeated with new individuals until 50 choices were obtained. Observed frequencies relating to the choices of the larvae in dual choice bioassays were compared to corresponding theoretical frequencies by using a binomial test, using Minitab® release 14.2 (Coventry, U.K.). The orientation bioassay was also compared to the test performed without baits and controls.

3. Results and Discussion

3.1 Alginate Beads Formulation and Release Kinetics

The emission rate of the 2 g of alginate beads passed from 34 ± 14 ng/h to 114 ± 2 ng/h between the first sample (1h) and the last one (6h) (Figure IV.2). This formulation approaching the scale of one ng/h, it was used in the olfactometers, with 50 mg doses of beads for each bait. They were used as baits during the two hours following $CaCl_2$ stabilization and subsequent drying.

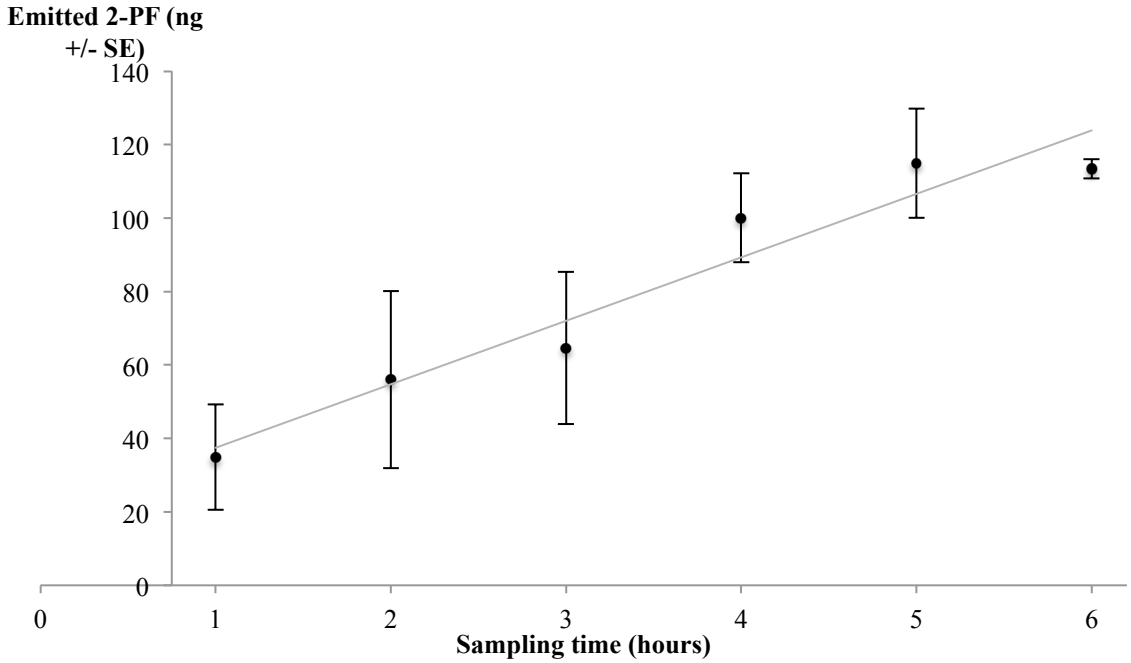

Figure IV.2. Emission rate of 2 g of alginate beads loaded with 2-pentylfuran

3.2 Orientation Bioassay

Figure IV.3 summarizes the results of the two orientation bioassays. The results of the blank-to-blank experimentation show that no biases result from the positioning of the olfactometers on the lab bench (left or right). Wireworms were significantly attracted towards 2-pentylfuran (P = 0.007**).

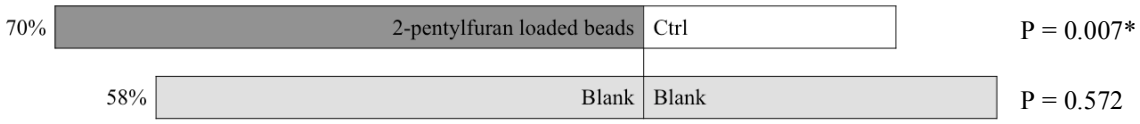

Figure IV.3. Wireworms are attracted towards 2-pentylfuran (first line).
The second line represents the blank-to-blank reference experimentation, as a comparison – see Chapter III.2.

Provided that 2-pentylfuran release remains homogeneous with 50 mg of beads, they could release 0.87 ± 0.36 ng during the first hour and 1.4 ± 0.6 ng during the second hour of testing, in pumping conditions. The emission rate might have been lowered in diffusion-only conditions, such as occurring in the bioassays. The results show that diffusion was sufficient to create a gradient, according to which wireworms could orientate. Non-responding behaviors were higher in the test with alginate beads than in the blank-to-blank experimentation (35.1 % against 26.7%). This may be explained by several hypotheses related to some wireworms' physiological stage; e.g. feeding/non feeding phase or handling induced stress. It may also be attributed to a late perception of the 2-pentylfuran cue. In that case, a wireworm engaged in the blank side of the olfactometer would move backwards to follow the gradient and be retrieved in the neutral area. The time devoted to diffusion should be increased (i.e. match durations used in tests with live baits, i.e. 40 min). The same experimentation in presence of carbon dioxide could point out its role of triggering signal. Other volatiles should be tested to detect their interaction potential.

4. Conclusion

Agriotes sordidus wireworms and potentially all *Agriotes* species can use cues complementary to CO_2 to orientate in soil while foraging. Such cues should be studied and used to develop new efficient baits and develop varietal selection of sensitive crops. Alginate beads could be adapted to in-soil emission conditions and take part to integrated management of wireworms and other soil pests.

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Chapter IV.2 - Attraction of Wireworms towards Root-produced Volatile Aldehydes

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Abstract: Whether belowground arthropod pests use volatile organic cues to locate their host plant is still poorly understood. Here, we assessed the ability of *Agriotes sordidus* wireworms (Coleoptera, Elateridae) to orientate towards the volatile organic compounds (VOC) emanating from barley roots, using dual choice bioassays. Furthermore, we collected the VOC produced by the roots by Dynamic Head-Space Sampling (DHS), quantified and identified them using Gas Chromatography – Mass-Spectrometry (GC-MS). The odorant blend is made of four aldehydes, namely the hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal, (*E*,*Z*)-nona-2,6-dienal. A synthetic blend of these four compounds was exposed to wireworms in a second series of orientation bioassays, which showed their attractive potential at doses matching realistic quantities, and a tendency to repulsion at higher doses. Our work provides evidence that foraging wireworms rely on volatile signals to locate their host plants, and that sensory perception in these insects follows patterns found in others, i.e. repulsion due to overexposure.

Key-words: *Agriotes*, integrated pest management, volatile compounds, chemical ecology, dynamic head-space sampling

1. Introduction

The development of integrated pest management (IPM) approaches largely relies on a broad knowledge of the biology of target pests and their broad ecological interactions. Semiochemicals, i.e. compounds carrying information from one organism to another, play important roles in these interactions (Agelopoulos et al., 1999, Cook et al., 2007, Witzgall et al., 2010), and are increasingly included in various IPM programs (Cook et al., 2007, Weddle et al., 2009, Rodriguez-Saona et al., 2014). Very few models have however been developed against belowground arthropod pests (Barsics et al., 2014b), even if many semiochemicals have been highlighted for soil dwelling organisms (Johnson and Nielsen, 2012). Nonetheless, a high-applied potential has been demonstrated in the particular case of the Western Corn Rootworm (Erb et al., 2010, Hiltpold et al., 2010, Rasmann et al., 2011). Soil pests are known to search for a putative host plant first orientating toward gradients of CO_2, secondly using host plant specific chemicals. The latter usually indicate the suitability of a food source (Johnson and Nielsen, 2012). The cryptic nature of soil pests renders their monitoring and control difficult. New outcomes in the understanding of mechanisms leading to population establishment and swarming are key elements to the development of new integrated approaches against many soil pests (Johnson and Nielsen, 2012, Hiltpold et al., 2013).

Wireworms, the belowground larval stages of click beetles (Coleoptera, Elateridae) are worldwide generalist pests. The knowledge on their chemical ecology needs to be improved (Johnson and Nielsen, 2012, Barsics et al., 2013), in particular regarding their foraging behavior and the role of volatile organic compounds released by their hosts in the rhizosphere (Barsics et al., 2014b). Identifying chemicals naturally involved in these insect-plant interactions and that have potential in IPM methods requires that 1) the behavioral activity of natural blends are understood, and 2) the identification of the chemicals constituting these blends is complete. In this paper, we provide a method suitable for studying the impact of semiochemicals on wireworm behavior. We evaluate the behavioral response of *Agriotes sordidus* to VOC either naturally produced by 39-day-old barley roots (*Hordeum distichon* L.), or formulated in an experimental blend based on the VOC profile of roots the same growth-stage. The qualitative and quantitative VOC profile was defined by Dynamic Head-Space Sampling (DHS) coupled to gas-chromatography mass-spectrometry (GC-MS).

2. Material and Methods

2.1 Wireworms

Wireworms were collected in South France (Pau), in Spring 2013. Dichotomous keys reported in Pic et al. (2008) and Cocquempot et al. (1999) were employed to sort out *A. sordidus* larvae. Each was isolated in 80 ml vials. The rearing substrate consisted in a mixture of vermiculite and leaf mould (1/1, v/v, humidified at 16.5% vol). Germinating meadow seeds were provided in excess on a fortnight basis (0.130 – 0.160 g, Prelac Bio, SCAR, Belgium). All vials were kept in the dark at room temperature (21-22°C). Prior to behavioral assays, wireworms measuring at least 10 mm in length were selected (to ensure they belonged to crop-threatening stages (Furlan, 2004)). Wireworms were transferred in 80 ml vials filled with moist vermiculite (16.5% vol), seven days prior to testing. Because the active feeding phase of *A. sordidus* is estimated at 25% of the whole multi-year development time (Furlan, 2004), a second round of larvae selection is needed. It consists in rejecting individuals either (1) visibly about to enter a molting phase, showing reduced activity and reactivity to pliers stimulation, and displaying a pudgy aspect compared to others, or 2) those larvae that just underwent a molting phase, displaying a light-colored cuticle and unhardened, non-darkened mouth-appendices. Unsuitable larvae are stored back in the rearing conditions.

2.2 Plant Growth Conditions

Barley plants were produced in a growth chamber, at 22°C and 65 % RH, equipped with LED light (95 µmol m^{-2} sec^{-1}) with a 20/4 L/D photoperiod. Caryopses weighing between 38.5 and 42.6 mg were pre-germinated 24 hours before sowing. Grains were soaked between tap water loaded filter papers in closed Petri dishes, covered with aluminium foil. The growing substrate consisted in a mix of clean sand and leaf-mould (4/1, v/v). For further eased handling of root systems, the substrate was poured in PVC pipes (height: 29 cm; diam.: 8.5 cm) fitted with thin bottom-pierced plastic bags and held in the pipes thanks to rubber bands.

Plants were watered with 15 ml every two days up to the eighth growth day, from which they received alternatively either standard Hoagland nutritive solution or tap water, in equal amounts. On the harvest day, they were first extracted from the PVC pipes, then out of the plastic bags by incising on the length. Roots were than gently separated from the substrate by pouring tap water all over the system. Before further handling relative to behavioral assays and chemical analyses, they were rinsed in tap water.

2.3 Dual-choice Bioassays

2.3.1 Olfactometers

The olfactometers were previously described in (Barsics et al., 2012, Gfeller et al., 2013). In brief, they consist of glass pipes (32 cm long, 3.6 cm internal diam.), closeable at both extremities with GL45 caps (Duran, Belgium). These openings serve to fill and empty the devices with a substrate. Two GL14 openings (Duran, Belgium) pierced 3 cm from both extremities allow the introduction of an entire plant root system, leaving stem and leaves outside the olfactometer. The larvae entry point is provided by a third GL14 lateral opening, placed at the middle of the pipe, diametrically opposite to the two other lateral connections. The olfactometer is filled with vermiculite, except the last 4 cm at both ends, to leave room for the bait and control compartments. A round piece of stainless steel fabric (3.6 cm diam.; width: 0.042 mm; mesh: 0.036 mm; Haver, Belgium) separates the substrate from the bait and control compartments, thus preventing contact between wireworms and the baits. Several olfactometers were built up and allowed conducting several replicates simultaneously.

2.3.2 Baits

Behavioral assays were first performed with entire 39-day-old barley root systems (860 DD, calculated on a 0°C daily basis), and in a second step with VOC dispensers loaded with several doses of reconstituted VOC blend, based on their identification and quantification (see section 2.4).

The root system was inserted in one of the two sides of each olfactometer. Plants were attached with aluminum foil, and roots were the only plant material exposed to wireworms, as the rest of the plant was isolated out of the bioassay. Blank culture medium was introduced into the opposite side of the olfactometer. While testing reconstituted volatile blends, the dispensers were made according to von Mérey et al. (2012). They consisted in 2 ml chromatography amber glass vials filled with 100 mg of glass wool, in which were added 200 µl of reconstituted blend diluted in triacetin (Sigma-Aldrich, Belgium). The dispensers were closed with screwed caps, pierced with 2 µl capillary tubes. The blank consisted in the same dispensers loaded with 200 µl of pure triacetin.

2.3.3 Experimental Parameters and Statistical Treatments

Ten olfactometers were used simultaneously, with one wireworm and one plant per device, at a room temperature of 22.0 ± 0.6 °C. Each test lasted 2 hours from bait insertion to

wireworm retrieval. While testing roots, wireworms were inserted in the olfactometers 40 min after root insertion, as previously done in Gfeller et al. (2013). In tests with reconstituted blends, they were inserted in the olfactometers only 15 min after, a timing in line with tests performed in Barsics et al. (2012), were 2-pentylfuran loaded beads were used as baits. 120 min after bait insertion; the position of wireworms was recorded in each olfactometer. The results of dual-choice bioassays, i.e. the proportion of wireworms managing to reach the side of the olfactometer connected to the bait compartment, were treated with a two-tailed exact binomial test applied on the number of orientating wireworms, for each modality. Wireworms retrieved in the central 6 cm of the olfactometers were considered as neutral or non-responding. The three experiments with the reconstituted blend were also treated with a χ^2 test-of-independence assessing the potential dose effect. Since blank-to-blank assays were performed with the same apparatus, in similar conditions and reported in a previous study (Gfeller et al., 2013), they were not repeated here.

2.4 Chemical Analyses

2.4.1 Sample Preparation

Barley roots grown for volatile identification and quantification were flash-frozen in liquid nitrogen in the minute after extraction from their substrate, including rinsing. Each root system was stored individually at -80°C, then reduced in powder by crushing in liquid nitrogen. Half a gram of root powder was inserted in a 20 ml SPME vial fitted with a sealed cap (white silicone/blue PTFE, Filter Service, Belgium). Root powders, the vials and all the tools used during this step were kept in liquid nitrogen. Therefore, during weighing, the root powders were not directly exposed to room temperature. Weighed samples were put straight back in liquid nitrogen, to prevent from all biochemical activity. Cold weight was recorded for each sample for further quantification and dry weight calculation. Each vial was closed immediately to avoid condensation of water on the frozen material inside the vials. Still maintained in liquid nitrogen, each of the samples was then exposed to a N_2 flow to limit biological activity while temperature increases, during the beginning of the chemical analysis.

2.4.2 Chemical Identification and Quantification

The VOC analyses were performed by using a Dynamic Head-Space sampler (DHS, Gerstel, Germany) coupled to a Thermo-Desorption Unit (TDU, Gerstel, Germany), a Gas

Chromatograph (Agilent Technologies 7890A) and a mass spectrometer (Agilent Technologies 5975C). Each sample was conserved in liquid nitrogen until the beginning of the analysis. One µl of standard solution of methanolic 3,5,5-trimethylhexanal (130 ng/µl, Sigma-Aldrich, Belgium) was injected before treatment in the DHS unit. The vials were then conditioned at 25°C for 15 min, with cyclic stirring (5 s on – 5 s off). The head-space sampling was performed on Gerstel TDU desorption tubes (OD 6.00 mm, filled with Tenax TA, Gerstel, Germany), during 10 min, at 20 ml/min. Desorption was then held during 10 min at 280°C. Sampled compounds were trapped with a Cooled Injection System (CIS, Gerstel, Germany), from -150°C, rising by 12°C/s up to 260°C, and held during 5 min. Separation of compounds was performed on a Wax factor four column (Agilent technologies USA; 30 m x 0.250 mm I.D, 0.25 µm film thickness), with helium as carrier gas at a flow rate of 1.5 ml/min. The temperature program was: 0.5 min of equilibration time; 35°C for 2 min; 5°C/min to 155°C; 20°C/min to 250°C; 10 min at 250°C. The MS was carried out in EI mode at 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; scanned mass range: from 20 to 350 amu, threshold of 150 amu; scan speed, 4.27 scans/s. Data was simultaneously acquired in SIM mode, in order to target the compounds of interest during their respective elution time range. Identification of detected compounds was performed by comparison with reference mass-spectrum libraries (NIST08 and Wiley275), by injection of commercially available standards, and calculation of Kovats retention indices according to (van Den Dool and Dec. Kratz, 1963).

3. Results

3.1 Orientation of Wireworms towards 39-day-old Barley Roots

Wireworms were significantly attracted towards the blend emitted by 39-day-old barley roots. Among the 36 responding larvae, 27 orientated toward the roots. (P = 0.004**, Figure IV.5a).

3.2 Identification and Quantification of Root Contained VOC

Four compounds were retrieved in the ground root samples: hexanal, (E)-hex-2-enal, (E)-non-2-enal and (E,Z)-nona-2,6-dienal. SIM acquisition parameters were set as follows, in order of elution, with one or two trace ions: from 6.00 min upward: ion 56; from 8.00 upward: ions 69 and 109; from 10.50 min upward: ion 60; and from 17.00 min upward: ion 70. We provide data in both SCAN (Figure IV.4a) and SIM mode (Figure IV.4b), resulting from these settings, as well as calibration curves allowing their quantification (Table IV – 1).

3.3 Orientation of Wireworms towards the Reconstituted VOC Blend

A representative synthetic blend of the four aldehydes was made with the following proportions: 9% hexanal, 8% (*E*)-hex-2-enal, 52% (*E*)-non-2-enal, 31% (*E*,*Z*)-nona-2,6-dienal. This blend was added to an equivalent volume of triacetin, and was then diluted 100 and 10,000 fold. The three dilutions were then tested as baits in the olfactometers. Wireworms were attracted towards the two lower doses (10 µg and 1 mg, P-value = 0.065 and 0.032* respectively), and showed a tendency to repulsion with the highest dose (100 mg), with more individuals found on the olfactometer side opposite to the bait (P-value = 0.349). The results are shown in Figure IV.5b. The χ^2 test-of-independence confirmed the dose effect (2 df; $\chi^2 = 6.66$; P-value = 0.036*).

Table IV-1. Compounds identified in the liquid-nitrogen ground root samples. Retention indexes, calibration equations and quantification results.

Compound	CAS Number	Retention index – calculated/theoretical	Calibration		Quantification	
			Equation	R^2	Estimated amount - µg/g fresh root (st. dev)	Proportion - % (st. dev)
Hexanal	66-25-1	1101 / 1093[a]	y = 2.1307x + 0.0105	0.9545	1.041 (± 0.362)	10.5 (± 3.6)
(*E*)-Hex-2-enal	6728-26-3	1228 / 1201[b]	$y = 1.5363x^{0,8265}$	0.9312	1.075 (± 0.436)	10.8 (± 4.4)
(*E*)-Non-2-enal	18829-56-6	1531 / 1538[c]	y = 0.6812x – 0.1392	0.8456	4.700 (± 1.116)	47.3 (± 11.2)
(*E*,*Z*)-Nona-2,6-dienal	2277-19-2	1582 / 1597[d]	y = 2.0515x – 0.4676	0.9154	3.125 (± 0.756)	31.4 (± 7.6)
				Total amount	9.942 (± 2.534)	

References for retention indexes: a (Högnadóttir and Rouseff, 2003); b (Ruther, 2000); c (Valim et al., 2003); d (Ferreira et al., 2001).

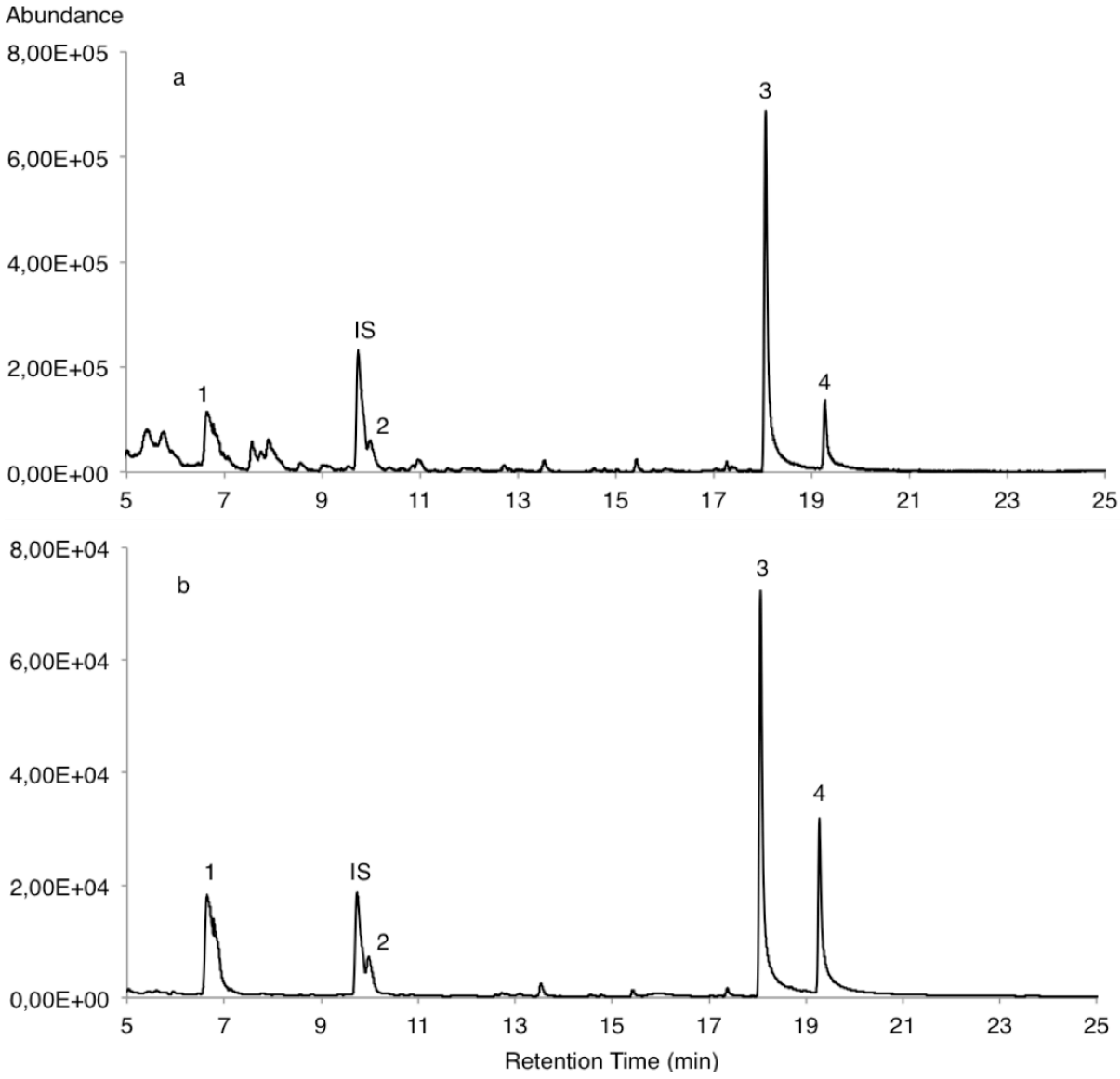

Figure IV.4. Chromatograms scaled on the four aldehydes.
a, SCAN acquisition; **b**, SIM acquisition. Peaks: **1**, hexanal; **2**, (*E*)-hex-2-enal; **3**, (*E*)-non-2-enal; **4**, (*E*,*Z*)-nona-2,6-dienal; **IS**, internal standard.

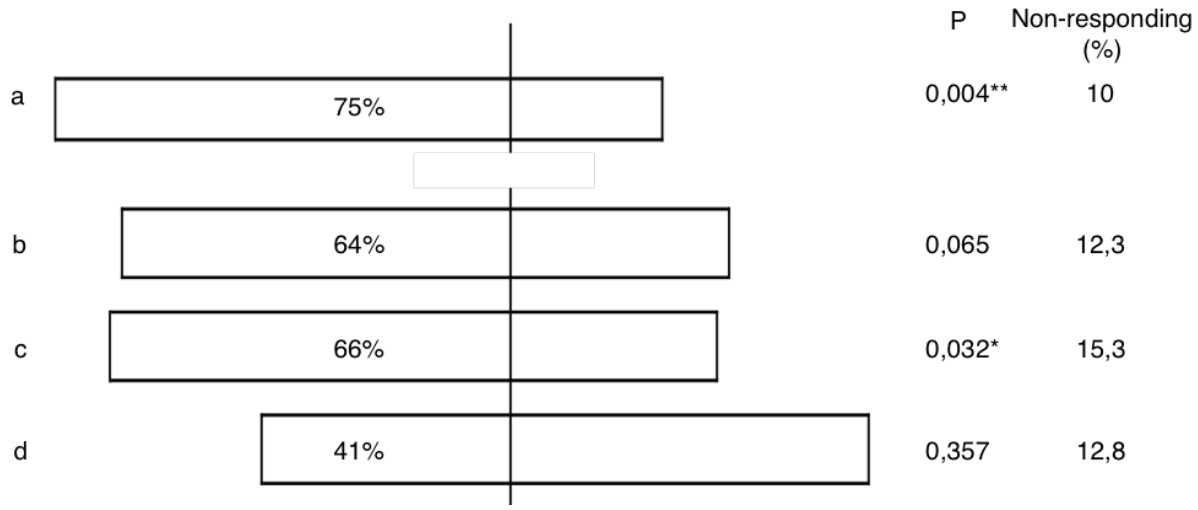

Figure IV.5. Proportion of wireworms succeeding in reaching one side of the olfactometer.
Baits are: **a**, 39-day-old live roots; **b**, a dispenser loaded with a 10µg dose of the experimental VOC blend, diluted in triacetin; **c**, same as b with 1mg; **d**, same as b with 100mg of experimental blend.

4. Discussion

In this study, we have collected, separated and identified the volatile chemicals released by 39-day-old barley roots using a DHS-GC-MS approach. Moreover, we have shown that wireworms are able to locate the rhizosphere of their host plant using the four aldehydes produced by the roots in the DHS experimental conditions. In the foraging process, belowground herbivorous insects rely on a sequence of semiochemical occurrence and their gradients, the first of which is CO_2. Specific compounds translating the nature or health state of the host come secondly. Thirdly, semiochemicals encountered at root surface indicate their degree of suitability of the host (Johnson and Nielsen, 2012). The role of VOC has often been associated to the second step of the foraging process, and has been experimentally suggested for wireworms in another study (Barsics et al., 2012).

Our results confirm that wireworms display orientation behaviors upon response to volatile stimulation. When CO_2 lacks from the stimulus, foraging larvae manage to orientate towards baits composed of four aldehydes. Moreover, they are repelled by high doses of stimulus, which is in line with their repulsion from CO_2 sources in case of overexposure and gradient steepness (Doane et al., 1975, Doane and Klingler, 1978). In order to determine the exact contribution of each compound to wireworm orientation, their foraging behavior should be compared in CO_2-controlled conditions. This would alleviate the relative roles of CO_2, VOC, and their putative synergistic interaction, giving more accuracy to result interpretation. The amount of compounds to which wireworms can be exposed in soils is hardly estimable. Whether VOC or not, the perception mechanisms (olfaction, taste) of these many compounds

yet to identify will remain unknown until their diffusion properties in the different soil-phases (air or water) are unveiled. Future behavioral bioassays should be performed complementary to electrophysiological recordings indicating what compounds induce measurable neurotransmission (Barsics et al., 2014b). The experimental blend used here results from an estimate of the VOC root-content. It does not match their in-soil emission rate, in both quantity and quality. It does not account for their putative transformation and the nature of the latter. The actual chemical environment that wireworms encounter in the rhizosphere is situated between a potential emission of the Lox-derived compounds reported here (Lipoxygenase pathway, a plant metabolic process allowing oxidation of linolenic acid) (Dudareva et al., 2013, Delory et al., 2015), and the volatile profiles showing volatile degradation resulting from SPME sampling (Gfeller et al., 2013). Moreover, in the work of Delory et al. (2015), the proportions of the four aldehydes, clearly varies according to the root growth stage. The information carried by the different resulting emitted blends may also impact wireworm behavior differentially, although it is not yet possible to assess how, and whether these proportions are really emitted in the soil.

The volatile collection and bioassay methods show good potential for further experimentation on the interactions between belowground insect pests and associated rhizosphere. As suggested in (Barsics et al., 2014a), the timeframe devoted to volatile diffusion, prior to wireworm entry in the olfactometer, should be increased. The ability of the insects to use the signal they perceive, could be better-expressed.

It seems imperative, due to the wide variety of soil pests for which semiochemical data has been acquired (Johnson and Nielsen, 2012), to develop analytic methods allowing fast and accurate profiling of plant semiochemical in soils. Wireworms and other soil pests can be confronted to a wide variety of cues according to the cropping situation they are subjected to. Any of the encountered blends, once described, should be tested for their potential in acting on the behavior of the insect. For insects with a long life cycle and several years of development in the ground, such as wireworms, the resulting data will be useful in 1) understanding the in-soil insect ecology at different scales, 2) consequently, adapting crop rotations to favour repulsion of wireworms before sawing sensitive crops, and 3) adapting these results in innovative push-pull strategies involving traps and combinations with other elements of integrated management strategies. The most directly related tools are the molecular methods allowing description of wireworm feeding choices and in-soil movements by screening the plant diversity in their gut content (Staudacher et al., 2013b, Wallinger et al.,

2014). By mutual confrontation of data acquired through different methods focusing on wireworm feeding ecology, there will be opportunity to adjust wireworm management, at the individual and population scales.

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Chapter V – Conclusions and Perspectives

1. Development of an Olfactometer Suitable for Behavioral Experimentations on Wireworms exposed to Root-Produced VOC

Behavioral studies on insects exposed to chemical stimulation necessitate devices in which behavioral traits under focus are easily observable. The first objective of this work consisted in developing a device suitable to study the behavioral responses of foraging wireworms, exposed to volatile cues characterizing roots. When working on a new organism, it is not necessarily straightforward whether existing methods will be adjustable or not. Moreover, some species are less prone than others to display natural behaviors in conditions differing from reality. Thus, the elaboration of an adequate olfactometric method is a crucial step.

In this work, we started olfactometric bioassays with classical horizontal Y-shaped olfactometers (Koschier et al., 2000, Colazza et al., 2004, Carroll et al., 2006, Wäschke et al., 2014), containing a thin vermiculite layer. In the literature, such olfactometers are notably used to elucidate earthworms' behavior, without necessitating a substrate (Zirbes et al., 2012). This evident advantage, allowing direct observation of the orientation (and optimal use of research time) explains our first choice. Although this method tended to show the ability of wireworms to detect and orientate towards a blend emanating from chopped barley roots, the weak nature of the statistical results brought about the need for a finer adjustment of experimental conditions (Barsics et al., 2011). The first unsuitable condition we identified is the air-flow (necessary to carry the VOC blend through the olfactometers). In the ground, wireworms are unlikely to encounter air movements as high as produced in this experiment. Secondly, the quantity of substrate was insufficient. This was highlighted by the burrowing behavior of several tested individuals. It confirms Thorpe et al. (1946)'s statement on the impossibility to observe response to odors with wireworms walking on the soil surface.

The literature contains many examples of olfactometers or systems allowing study of soil insects (Hemerik et al., 2003, Turlings et al., 2004). However, most of them report on experimentations with groups of insects. Two elements have limited their use in the present work: the number of available wireworms and their tendency to display cannibalistic behaviors, especially in foraging conditions. We managed to maintain a sufficient number of testable individuals, through repeated collection events, in collaboration with Arvalis (Pau, France). We also attempted to multiply individuals by using the optimal rearing parameters published by Furlan (2004). Our attempts failed, probably due to the life history traits of wireworms, mainly the temperature variations they had already encountered in natural

conditions. In fact, the optimal rearing method only seems applicable starting from egg hatching (Furlan, 2004). Incidentally, several personal communications from searchers working on wireworms confirm the need for improved rearing techniques (Ir. Laurent Maunas and Ir. Jean-Baptiste Thibord, Arvalis) or abnormally high investments in collection events, almost necessitating human resources entirely devoted to that task (Dr. Robert Vernon, University of British Columbia). Concerning cannibalism-derived disadvantages (loss of individuals and uncompleted experimentations), they were highly important since all our experimentations treat of wireworms in a foraging stage. Yet cannibalism mostly occurs when food is scarce (Langenbuch, 1972, Furlan, 1998) or in case of extended food deprivation, such as necessitated in our protocol (hence increasing probabilities to observe orientation towards food originating stimuli).

These limitations narrowed our choices to classical dual-choice olfactometers that could contain a substrate as uniform as possible, and in which individuals are tested one by one. The cylindrical shape of our olfactometric pipes fitted these criterions and allowed quick filling, emptying and cleaning. The devices were designed with dimensions in line with previous observations on the speed of movement of *A. sordidus*, during the first months of research. The substrate and the absence of an air-flow (diffusion based olfactometry) were the two main upgrades. We have clearly seen that they are good tools for checking the ability of wireworms to detect a food source thanks to volatile cues (Gfeller et al., 2013, Barsics et al., 2014a). The cylinders were therefore reliable for showing attraction towards a blend containing carbon dioxide, the main attractant cue for wireworms (Doane et al., 1975, Johnson and Nielsen, 2012). However, there are some limitations to their use. As outlined in Barsics et al. (2014a), it seems hardly possible to highlight preferences between two living baits, such as two root systems differing by their health traits. Furthermore, if it were possible to detect preferences, accurate interpretation would depend on CO_2 calibration across all repetitions (Robert et al., 2012). Setting up such calibrations is difficult with the current design.

For future use of our olfactometers on wireworms, we recommend the following approach. One should start with performing assays with living roots as baits, to assess 1) the duration parameters (volatile diffusion, orientation timeframe) characterizing the plant-wireworm species model under study, and 2) the impact of the blend emanating from the plant under study. In the aim to reduce the root damage implied by our methods (root transplant in the olfactometers before wireworm insertion), a complementary system could be designed, in

which modular plant jars could be connected to our olfactometers. Consequently, it would involve differing diffusion timing prior to olfactometric tests. We insist on the necessity to adapt the timing devoted to orientation to the speed of movement of the wireworm species under study. The overall parameters used in this thesis might not be adapted to other models, even in the *Agriotes* genus.

Once the nature of an interaction is known (induced attraction, repulsion or even inactivity) the third step can be undertaken. It will necessitate elucidation of all candidate molecules to semiochemical activity, i.e. identification and quantification of volatile compounds constituting the blend. The discussion concerning the advances in that matter is addressed in the next section (2). Provided that these compounds are incorporated in synthetic dispensers, adapted to emission during olfactometric tests, it is possible to assess whether they induce a response similar to that observed when wireworms are exposed to the plant blend itself. We have shown that in the last experimental chapter. In our case, however, we hypothesize that the time allocated to VOC diffusion prior to wireworm insertion, should be increased (from 15 min to 40 min for example), to improve the chances for wireworms to effectively perceive the exposed blend. Doing so, they would necessitate less time to orientate towards the bait. It could result in an increase of the accuracy of the overall behavioral answers, less biased by the inter-individual variations from one test to another. To reduce even more the biases related to inter-individual variations, we suggest another method to select wireworms adapted for testing. Rather than simply isolating active wireworms seven days prior to tests, an individual follow up should be performed, and isolation from all organic matter should be undertaken as soon as the first signs of molting. Doing so, one could monitor the wireworms until they are ready for feeding, in the next larval stage, and the starvation degree would be more uniform across individuals.

Finally, we remind that the entire behavioral approach should be complemented, or even preceded, by electrophysiological investigations pointing out compounds and blends relevant to wireworms. This implies a thorough description of their sensory anatomy and could be achieved on the basis of knowledge reported in Barsics et al. (2014b).

2. Identification and Quantification of the VOC produced by Barley Roots according to Different Growth Conditions

The two methods we used to investigate the VOC profiles produced, emitted by, or contained in the roots, give information at several levels. The DHS-derived profile indicates the nature and an estimate of quantities of the VOC resulting from the liquid nitrogen crushed

root powder, conditioned at 25°C in the DHS module, after being stored at -80°C (Barsics et al., 2015). The quantities of hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal, have been shown to vary according to the growth stage at which barley roots are analysed (Delory et al., 2015). The semiochemical meaning of the aldehyde blend may therefore vary similarly. These compounds consist of fatty acid derivatives (linoleic and other polyunsaturated fatty acids), undergoing the lipoxygenase pathway (Delory et al., 2015), involved in the secondary metabolism of many plants (Dudareva et al., 2013). In the presence of two enzymes - the alcohol dehydrogenase and the alcohol acyltransferase - they can be converted into alcohols (e.g. hexan-1-ol), then into esters (e.g. (*E*)-2-hexenyl esters) (Dudareva et al., 2013). The VOC profiles detected with SPME (Gfeller et al., 2013), include not only the four aldehydes also detected by DHS sampling, but also alcohols and esters produced by these enzymes: e.g. (*E*)-hex-2-en-1-ol, methyl hexanoate, hexan-1-ol, (*E*)-non-2-en-1-ol, nona-2,6-dien-1-ol. In fact, most of the VOC sampled by SPME are polyunsaturated fatty acids derivatives (Min et al., 2003, Shiojiri et al., 2006). 2-pentylfuran was among the most abundant in SPME samplings. It is associated to different transformation undergone by lipids, and is found in plant (essential) oils (Min et al., 2003, Beltrán et al., 2011, Radulovič and Dordevič, 2014) or in food products after heating or cooking processes (Fromberg et al., 2014, Toci and Farah, 2014). It is associated to lipid photooxidation (Min et al., 2003), and peroxidation (Dong et al., 2013). The two VOC sampling methods we used allow observing transformation of root lipids. They both show different information, none of which actually describes the real VOC profile emitted by the roots in the soil. Moreover, the established profiles do not translate what wireworms perceive or the physiological mechanisms involved. To describe realistic in-soil VOC-wireworm interactions was probably the most difficult objective of this thesis. It is due to the difficulty to unveil the compounds that are actually emitted in the ground. Nonetheless, in the next discussing section, we interpret the results of behavioral experimentations performed across this work with concerns as realistic as possible.

Some compounds identified here (hexanal, (*E*)-2-hexenal, 2-pentylfuran, 2-ethylhexan-1-ol, octan-1-ol, (*E*)-non-2-enal) were also identified in grapevine ground roots (Lawo et al., 2011). Furthermore, many of the identified polyunsaturated fatty acids derivatives, have been described in direct or indirect responses to herbivory wounding (Arimura et al., 2000). Altogether, this highlights interesting candidates in the study of root-wireworm interactions. Fittingly, in the frame of this work, behavioral experimentations

were undertaken with 2-pentylfuran as single stimulus; and with hexanal, (*E*)-2-hexenal and (*E*)-non-2-enal completed by (2*E*,6*Z*)-nona-2,6-dienal. All other identified compounds conserve a status of semiochemical candidate, whether or not they have been previously shown as involved in mechanical or pest-induced wounding.

In terms of improvements, it would be necessary to develop a method allowing detection of compounds emitted in the ground, notably by pumping air through layers of ground containing roots, or by in-soil SPME. However, the attempts to do so during this project did not show many encouraging results. We know from personal communication (Ir. Benjamin Delory), that out of the four aldehydes sampled by DHS, the only detected compounds with a pumping method are the (*E*)-non-2-enal and the (*E*2,*Z*6)-nona-2,6-dienal. Compounds of lower molecular weight cannot be sampled. This may be due to the water solubility of aldehydes, which decreases as the carbon chain length and presence of double bonds increases[2]. None of the other compounds deriving from polyunsaturated fatty acids degradation are detectable, with currently available analytic methods. By lowering limits of detection of available techniques, it would be possible to verify whether other compounds are actually diffused in the ground.

Developing more sensitive methods, applied to the detection of VOC in the soil itself, seems a beneficial approach:

- Firstly, it would allow defining precisely how far from a root system a plant-originating compound could be found. Incidentally, it would indicate better-adjusted candidates to use in IPM strategies against wireworms. Indeed, among the highlighted semiochemicals, the more suitable to use are those intervening early in the foraging process, i.e. far from the food source, because they would allow a higher distance of action in push-pull systems. This could be undertaken to detect compounds that diffuse through the soil as solute elements. One compound's polarity will determine its affinity with the soil water phase and therefore its propensity to diffuse in the soil (Peñuelas et al., 2014). Whether a given compound has affinity with the water phase of the soil or not will also impact the perception mechanism at stake on wireworm sensory appendages. This should be accounted for when designing electrophysiological assays.
- Secondly, it would allow screening a higher amount of plants and compounds, volatile or not, they can release in the ground. This advantage is important in the case of wireworms, which are generalist pests, and can therefore be found in the vicinity of

[2] Data consulted on http://www.chemicalbook.com, 2/2/2015.

many plant species. It would also benefit to research on interactions with other soil organisms, including bacteria. For example, some microorganisms have been shown to alter the volatile bouquet in the vicinity of a root system (Del Giudice et al., 2008).

- Thirdly, it would decrease the timeframe devoted to analytical development needed to detect volatiles emitted, contained, or produced by different plant species.

3. Evaluation of the Biological Activity of the Identified VOC on the Foraging Behavior of Wireworms

Our third objective consisted in verifying if identified VOC had an effect on wireworm orientation. Since preliminary bioassays highlighted attraction towards barley roots in several growth and physiological features, we hypothesized that most of the identified VOC, as a blend or alone, would also induce attraction. However, this hypothesis was jeopardized by the long-known and demonstrated attractant role of CO_2 (Klingler, 1957, Doane et al., 1975). When CO_2 is no longer part of the bait, such as when we replace living roots with a synthetic VOC dispenser, the outcome of the behavioral experimentation is unpredictable; i.e. it is not straightforward that without CO_2, the bait will remain as attractive as with CO_2. Moreover, the synthetic mixture of VOC itself may be irrelevant to wireworms or induce repulsion. The choices regarding the compounds we have tested depended upon the number of available individuals. Since it is known that wireworms may be capable of associative learning, notably on exposure to insecticides (van Herk et al., 2010), we organized behavioral assays so that each available wireworm only gave one effective response (not a neutral response) during a given series of foraging experimentations. These important restrictions led to the design of two different sets of assays: one evaluating the role of 2-pentylfuran, and another evaluating the role of a blend of hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal.

To expose wireworms to 2-pentylfuran, we resorted to alginate beads (Barsics et al., 2012). These formulations have the property to release low amounts of encapsulated VOC on a long period of time (Heuskin et al., 2011). We developed a formulation adjusted to an emission range of 1-10 ng/hour, a quantity estimated thanks to SPME on different growth stages of barley (Gfeller et al., 2013). Using these beads in the olfactometers, we showed that wireworms significantly orientated towards the 2-pentylfuran source. This constituted the first evidence that wireworms use VOC cues other than CO_2 in the foraging process. Concerning the ecological role of 2-pentylfuran, several things must be discussed:

- It is not necessarily straightforward, that the presence or an increase of this compound in a given root system will render the latter more attractive to wireworms. It will depend on the entire occurring semiochemical background;
- Consequently, the best blend that should be tested consists in the natural blend of a given root system minus CO_2, or taking account of its effect thanks to a calibration of the results according to its concentration (Robert et al., 2012);
- The presence of 2-pentylfuran in the soil, as a result of plant metabolism, whether or not it depends on transformation that some compounds might undergo in stress conditions or outside the roots, remains to be proven;
- The semiochemical activity of this compound should be verified in more realistic conditions, i.e. in real soil in semi-controlled field conditions;
- Due to the statistical strength of this result, we conserved timing parameters and temperature used here for further tests with synthetic VOC dispensers.

As a second step to submit identified VOC to wireworms, we increased the number of compounds exposed during one olfactometric assay (which is in line with the second discussion point related to 2-pentylfuran experimentations). In order to select among the compounds identified with SPME, and to approach more accurate quantities, we performed the analysis in DHS reported in Barsics et al. (2015). Allowing real quantification compared to semi-quantification provided by SPME, these analyses translated an estimation of volatile produced by ground roots, rather than emitted quantities. Nevertheless, it narrowed down the number of compounds to four (see above, discussion section 1.2). In terms of feasibility, this allowed the resort to simple blending methods while avoiding VOC loss during formulation. The proportions of compounds we used represent an example of a blend that can be produced in 39-day-old barley roots ground in liquid nitrogen and conditioned at 25°C during 15 min. The aldehydes were blended together in triacetin and several dilutions were tested in the olfactometers (resulting in doses of 10ng, 1µg, and 100µg). Wireworms were attracted towards volatile emissions of the two first doses. Overall, we indicated a dose effect on wireworm behavior, with a tendency to repulsion with the higher one. The following elements must be remembered:

- The observed attraction towards the blend seems less important than it was towards 2-pentylfuran. However, a higher amount of tested individuals, and entirely equivalent experimental parameters would be necessary to confirm this apparent difference ;

- Between the quantities contained in the dispensers, and the doses actually emitted in the olfactometers, there is a difference, and we did not assess releasing kinetics of our dispensers ;
- Again, it remains to be proven that the blend we have used during these testings can actually exist in the soil, and how emission properties and degradation outside the root system modifies the proportions in the blend.

Overall, we have identified five VOC that have a semiochemical activity on wireworms. In the next paragraph, we discuss more deeply the differences between the effects we have observed through this work and what happens at field scale, since the main perspective of this work is to deliver new trails for wireworm management.

When semiochemical effects are observed in olfactometers, many of the experimental parameters may differ from reality. The main parameters we need to discuss here are distance and substrate. We hypothesize that they have the biggest biasing effect, probably in combination, on the ecological interpretation we could draw out of the results. The porosity of vermiculite, its capacity to absorb water and to be malleable, make it a highly interesting substrate for wireworm movement. Furthermore, it allows fast VOC diffusion. In the field, the soil is denser and non homogeneous. The VOC effects we have observed here, would certainly be translated in nature, on a much shorter distance than observed in the olfactometers (15 to 30 cm).

Another important olfactometer bias consists in the presence of steel fabric boundaries employed to prevent wireworms from reaching the bait. As a reminder, these were useful to ensure that the presence of a wireworm in one arm of the olfactometer was solely due to VOC cues and not derived from a possible contact during random burrowing. However, during the foraging process, one necessary step consists in confirming or infirming the suitable nature of a food source by contact with the roots and their surface semiochemicals (Johnson and Nielsen, 2012). It means that the semiochemical information has to add up overtime, until final confirmation, otherwise wireworms will attempt to find another food source. In Figure I.1 (p.41), the suggested pattern for belowground herbivory shows that VOC might occur as second triggering signal, after general semiochemicals such as CO_2. We suggest an improved pattern in Figure V.1. The second and third steps should not be separated as if they occurred sequentially. Considering the literature on the subject, notably detailed for wireworms (Barsics et al., 2014b), these steps occur simultaneously, and all

substances encountered on the way to the food source add up to the overall information perceived by the wireworms. The blend is progressively enriched and the perception may change very quickly if one particular compound or a change of concentration signifies inadequacy of the food source, whether by contact with the roots or not.

The actual distance, over which the addition of chemical information occurs, is unknown to us. It will depend on the diffusing properties of the compounds, themselves relying on the physicochemical properties of the soil. This will determine their "resistance" to diffusion, i.e at what distance from the plant they could be perceived by wireworms. Derived compounds, such as alcohols and esters notably revealed by SPME (Gfeller et al., 2013), may also have a semiochemical action, although we could not test in this work. Without bringing light on all these questions, we can however postulate that, in crop conditions, the sawing density will determine at what distance from two plants wireworms would be able to discriminate between the two, whether they are two different species or incarnate two different physiological stages of a same one. This concern is of high importance, especially if chemical ecology findings are implemented in IPM under the form of trap crops.

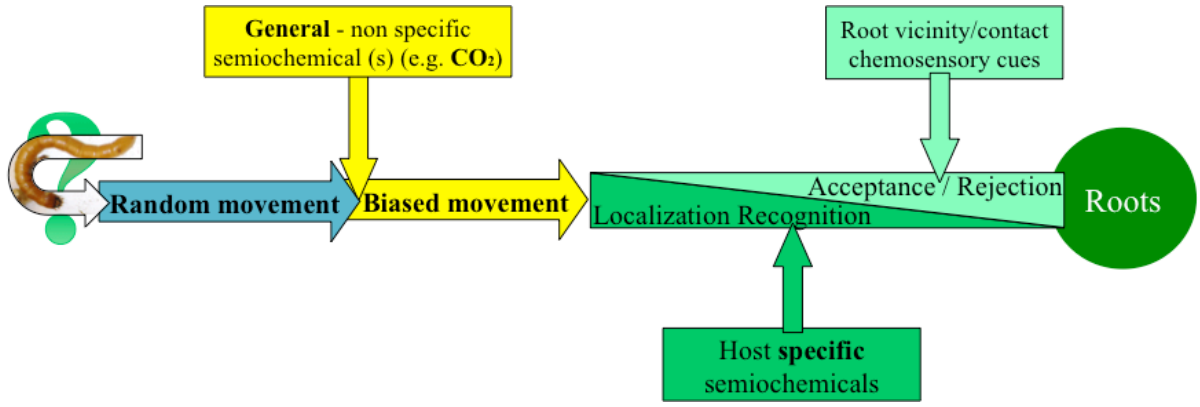

Figure V.1. The foraging behavior of wireworms, where the localization/recognition step is gradually overlapped by acceptance/rejection cues.

To conclude on the ecological role of the investigated VOC in the foraging behavior of wireworms, one important element has to be reminded. Several sources indicate that the preferred wireworm food source is the kernel itself, or tubers, rather than the roots, because they contain appropriate nutrients to their development (Hemerik et al., 2003, van Herk and Vernon, 2007b, Chaton et al., 2008). There is also a general consensus to develop stand protection especially during the first weeks of growth on sensitive crops, because further damage in the season is acceptable for the attacked plants (van Herk et al., 2008a). During the early growth stages, the plants depend upon the storage components contained in the grain, unfortunately also targeted by wireworms (in the particular case of cereals). With barley, we

can therefore hypothesize that the aldehydes with semiochemical activity highlighted here, serve as guiding compounds towards the core of interest that is the grain, and even the stem base of the plant. They might act so through root exudates. We suggest that the response observed in bioassays with the two lower doses might represent that behavior in soil conditions. Contrarily, in an environment rather poor in suitable nutrients, wireworm will attempt to feed on the roots. Doing so, and considering only the mechanical wounding entailed by the biting of one root (leaving aside the enzymatic reactions that can occur on the plant through wireworm saliva), we can also logically assume that the four aldehydes and lipoxygenase pathway derived compounds literally burst in the wireworm's "headspace". At that precise moment, the semiochemical information carried by the VOC would be interpreted as a repellent signal, indicating that the roots themselves are not suitable. Incidentally, we attempted infestations of barley root systems with famished wireworms (data not shown). During these, we could observe wireworms attempting to feed on the roots, but the cuts were never completed and the armed roots remained apparently functional although wounded. We suggest that the resulting behavior is illustrated by the olfactometric bioassays conducted at the highest dose of aldehyde mix, i.e. repellency. These hypotheses underline the necessity to assess the responses of wireworms when exposed to different aldehyde blends, specifically corresponding to the blends that are really emitted (not produced) by barley, at different growth stages, since their proportions vary according to that parameter (Delory et al., 2015). This may reveal a "built-in" defence mechanism of barley, against attacks from wireworms, during the early growth stages that need to be protected because the storage compounds still hidden in the kernel are vital to the plant growth.

Aside from the suggestions on the subject during the previous paragraph, in this work, we did not provide information related to a potentially triggered defence mechanisms, in barley roots, in response to wireworm attack. Compounds classically involved in that being ideal candidates for integration in IPM, we led several attempts to observe physiological responses, but could not detect qualitative or quantitative modifications. This can be due to the high variability that is observable with barley samplings, in terms of detected quantities of VOC. Moreover, our attempts to produce wireworm attacks never reached more than 30% of success (data not shown), and were largely distributed overtime after infestation (36 to 48 hours). The real limitation in these attempts was the number of available wireworms to repeat the infestations. Since olfactometric assays were considered a priority, we could not spend a

large quantity of individuals on manipulations exposing them to barley root VOC, without risking induced biases on further behavioral bioassays with the same individuals.

In purpose to implement our findings in IPM, it is necessary to evaluate if the movement of conspecifics impacts a single wireworm's behavior, especially when the community is foraging. If the attraction of one wireworm towards a food source can lead other wireworms in the same direction, then semiochemicals may have a broader impact. These behavioral traits are notably known in earthworms (Zirbes et al., 2012), and could imply different ways to design, spatially and temporally, the use of semiochemicals and plants producing them in management strategies.

Finally, it seems that a combination of several attractants and repellents should be adopted in order to develop efficient push-pull strategies to control wireworms. Incidentally, many other wireworm-plant models would be relevant to study. One compound alone is not efficient enough to keep wireworms away from their target plant. For example, CO_2 gradients developed by in-soil inclusion of yeast-loaded alginate beads, can lure wireworms away from a target plant, but with an efficiency that does not reach a duration ensuring protection of neighbouring food source (Mävers et al., 2014). Contrarily, when wireworms are rewarded with an actual food source after they were diverted from a given crop, by intercropping for example, they seem to remain in the vicinity of the lure (Thalinger et al., 2011). From another point of view, considering only the solanine, chalconine and glycoalkaloid contents of potato varieties is not sufficient to predict their level of protection against wireworms in field conditions (Johnson et al., 2008). If we want to design efficient and alternative control methods against wireworms, with information related to their biology and (chemical) ecology (Barsics et al., 2013, Traugott et al., 2015), we must multiply the angles of attack along the different gradual steps of the foraging process. The push-pull strategies will necessitate many different steps mimicking natural repulsion followed by natural attraction away from the crop to be protected. With the hypotheses that this might be economically irrelevant to set complex chemical lures at field scales, it will probably be more suitable to design IPM strategies involving trap crops (Vernon et al., 2000, Landl and Glauninger, 2013, Staudacher et al., 2013b), while accounting for the impact of their "chemical arsenal" on wireworm behavior. Thanks to molecular ecology, it is possible to explore the nature of wireworm movement in the soil, in relation to plant diversity and feeding choices (Staudacher et al., 2013b, Wallinger et al., 2014). This, by combination with behavioral observations in

olfactometers, could provide concrete data to apply IPM approaches against wireworms in the field. Luring wireworms away from crops that will be further installed and really need protection could be achieved by these associated approaches. Moreover, they are compatible with the use of entomopathogenic organisms, fungi or nematodes that have already proven efficient against wireworms.

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Chapter VI – Scientific Communications

1. Publications

Barsics, F., Delory, B. M., Delaplace, P., Francis, C., Fauconnier, M. L., Haubruge, E. & Verheggen, F. J. 2015. Attraction of wireworms towards root-produced volatile aldehydes. *J. Pest Sci.,* Ready for submission.

Barsics, F., Haubruge, E., Francis, F. & Verheggen, F. J. 2014. The role of olfaction in wireworms: a review on their foraging behavior and sensory apparatus. *Biotechnol. Agron. Soc. Environ.,* 18(4), pp. 524-535.

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2. Communications

Orales

Barsics, F., Delory, B., Delaplace, P., Fauconnier, M.-L., Haubruge, E., Francis, F., & Verheggen, F. (2014, December13). *Belowground Chemical Ecology: The Case of Wireworms*. Paper presented at 21st Benelux Congress of Zoology - Zoology 2014, Liège, Belgique.

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Barsics, F., Gfeller, A., Fauconnier, M.-L., Delory, B., Fiers, M., Hirtt, L., Laloux, M., Camerman, M., Destain, M.-F., Lepoivre, P., Verheggen, F., Haubruge, E., Lognay, G., Wathelet, J.-P., Delaplace, P., & du Jardin, P. (2011, October 13). *Les volatils racinaires de l'orge : un langage souterrain ?* Paper presented at Journée de la Recherche, Gembloux, Belgique.

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Barsics, F., Haubruge, E., & Verheggen, F. (2011, May 24). *Attraction of wireworms to root-emitted volatileorganic compounds of barley*. Paper presented at 63d International Symposium on Crop Protection, Gand, Belgium.

Écrites (posters)

Vandereycken, A., Fassotte, B., Barsics, F., Durieux, D., Joie, E., Francis, F., Haubruge, E., & Verheggen, F. (2014, October). Study of sex ratio and morphotypes of the Multicoloured Asian Ladybird, *Harmonia axyridis* Pallas in Belgian maize. Poster session presented at Premières rencontres nationales des Coccinellistes, Angers, France.

Barsics, F., Delory, B., Delaplace, P., Fauconnier, M.-L., Fiers, M., Jijakli, H., Francis, F., Haubruge, E., & Verheggen, F. (2014, June). *Belowground Chemical Ecology: The Case of Wireworms*. Poster session presented at ICE 2014 - Insect Chemical Ecology 2014 - Doctoral Course, State College, Pennsylvania, USA.

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Barsics, F., Haubruge, E., & Verheggen, F. (2013, October 19). *Le taupin : un ravageur souterrain préoccupant*. Poster session presented at 11ème Journée entomologique de Gembloux, Gembloux, Belgique.

Barsics, F., Malaisse, F., Razafimanantsoa, T., Minet, J., Lognay, G., Wathelet, B., Haubruge, E., & Verheggen, F. (2013, October 19). *Les Ressources Sauvages des Bois de Tapia (Uapaca bojeri) à Madagascar*. Poster session presented at 11ème Journée entomologique de Gembloux, Gembloux, Belgique.

Barsics, F., Fiers, M., Francis, F., Haubruge, E., & Verheggen, F. (2012, August). *Volatile organic compounds released by barley roots attract wireworms*. Poster session presented at XXIV International Congress of Entomology, Daegu, Korea.

Barsics, F., Fiers, M., Haubruge, E., Verheggen, F., & Latine, R. (2012, February 10). *Volatile organic compounds released by barley roots attract wireworms*. Poster session presented at 17th Symposium on Applied Biological Sciences, Leuven, Belgique.

Barsics, F., Latine, R., Haubruge, E., Verheggen, F., & Kati, D.E. (2011, August). *Attraction of wireworms towards root-emitted volatile organic compounds of barley*. Poster session presented at 14th Symposium on Insect-Plant Interactions, Wageningen, Netherlands.

Barsics,F., Latine, R., Haubruge, E., Verheggen, F., & Kati, D.E. (2011, July). *Wireworms use barley root volatile chemicals to locate their host plant in soil*. Poster session presented at International Society of Chemical Ecology 27th Annual Meeting, British Columbia, Canada, Burnaby, British Columbia, Canada.

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