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LABORATORY OF ENTOMOLOGY

Host searching behavior of 1st instar *Aleochara bipustulata* larvae

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LIST OF ABBREVIATION

Ø	diameter
Cm	centimeter
h	hour
HIPVs	Herbivore Induced Plant Volatiles
hpi	hours post inoculation
L_2, L_3	2^{nd} and 3^{rd} instar larva
mm	millimeter
ml	milliliter

THANKS

After nearly two years at Wageningen, this thesis was the occasion to work with a new team and to discover research universe in France. I thank Anne Marie Cortesero for receiving me in her department, and Marcel Dicke who supervised this project from Wageningen.

I spent four remarkable months in the laboratory of ecobiology of parasitoid insects in Rennes 1 University. I acquired new skills and this experience will remain an important part in my studies.

The team I worked with forms a wonderful group of friends and I hope to be part of it.

Romain

ABSTRACT

The study of biological interactions mainly focuses on aboveground communities. Root herbivory can cause major damages if not controlled by the third trophic level. Plant-mediated interaction transfers this impact aboveground and over several trophic levels. Thus belowground interactions study is a key notion for developing biological control tools.

The cabbage root fly *D. radicum* is a major pest damaging seedling in Brassicacea. The rove beetle *Aleochara bipustulata* is a candidate to control *D. radicum* outbreaks. Indeed imago feed on eggs and larvae, whether the 1st instar *A. bipustulata* larvae parasitize *D. radicum* pupae.

This study aims to understand how the larva finds a host belowground, from the place it hatches to the pupa. Females carefully select their oviposition site. They locate infested plants and perceive if *D. radicum* pupae are parasitized or not. But it is the 1st instar larva which locates and invades its host pupa belowground presenting kin-recognition. Two hypothesis guided this project: i) The female already selected the host plant thus *A. bipustulata* larvae locates its host thanks to cues from *D. radicum* only; ii) Selection presses *D. radicum* pupae to remain unnoticed. Thus the host plant also provides cues to 1st instar *A. bipustulata.*

Aleochara bipustulata larvae reply significantly to volatile cues from infested turnips at long distance. The plant seems to shape similarly belowground and aboveground communities. Investigations concerning cues from *D. radicum* gave very few responses, but the impact of gallery odors laid by *D. radicum* maggot before pupating can be rejected.

This study allowed observation of the host searching behavior of 1st instar rove beetles for the first time. It opens a wide range of research from speciation to biological control.

HOST SEARCHING BEHAVIOR OF 1ST INSTAR *ALEOCHARA BIPUSTULATA* LARVAE

INTRODUCTION

The consumable part of some vegetables as turnips, rutabaga or radish grows belowground. Herbivorous insects as the cabbage root fly *Delia radicum* evolved abilities to use this resource even if the nutritional quality of roots is lower than shoots. They became a pest causing severe losses (Hunter, 2001). Health and environmental concern demands agriculture to reduce pesticides. To understand biological interactions which take place in the agro-ecosystem is essential to provide new tools to farmers so that they may protect their productions in a sustainable way. The first step is to observe the behavior of organisms. Parasitoid insects are interesting candidates to control pests. The manner they forage for their hosts is an unavoidable step to then exploit their talent.

Several factors condition searching behavior in insects. The resource the organism requires and when, the periodic nature of its occurrence and which alternative strategies animals develop when the resource decreases in quality or quality (tolerance level). Bell (1990) remarkably summarizes searching pattern in insects by giving key points to consider as information for locating resource, scanning, timing of search bouts or resource assessment. Such parameters require *de visu* observations which might explain why belowground interactions are less acknowledged than in aboveground systems. Indeed part of the knowledge collected on belowground interactions comes from the timeframe where organisms are observable, thus aboveground. Flight for instance inform about the relation between an host and its parasitoid, or relations between the plant and its attacker (Marazzi and Stadler, 2005, Andreassen et al., 2010).However to acknowledge belowground multitrophic interactions remains a key source of improvement for integrated pest management (Hunter, 2001, Ferry et al., 2009).

This project aims to develop a method to observe the host searching behavior of the parasitoid larvae *Aleochara bipustulata*, a great candidate for controlling *Delia radicum* outbreaks. After shortly describing research on belowground interactions, the biology of both species will be presented to justify our research hypotheses.

A. MULTITROPHIC BELOWGROUND INTERACTIONS

Plants are the central element orchestrating biological interactions in the ecosystem (Ohgushi, 2005, Bukovinszky et al., 2008). The root system has a great impact on soil's physical (aeration) and chemical (pH, exudates, volatiles) properties which orientate biological interactions within communities associated to the plant (Bertin et al., 2003). Studies on root herbivore-Plant interactions including the 3rd trophic level are too rare compared to aboveground investigations. In addition damages resulting from root herbivory are difficult to quantify when they do not kill the plant or do not affect the economic part directly (Hunter, 2001). The soil created a barrier for researchers' minds which separated belowground and aboveground interactions. This is not the case anymore and organisms sharing the same host plant do indeed affect each others. The ABBE model includes the impact of herbivores, parasitoids, hyper parasitoids and decomposers on shoot and roots performances. It revealed that Insect-Plant interactions do cross belowground-aboveground levels. Interestingly belowground parasitoids were responsible for a decrease in plant mortality (-43%). When root feeders were not controlled, the empirical approach confirming the model registered 100% death and thus made aboveground organisms starving until death. This study highlights the importance of belowground interactions on plant performance and their potential impact on the associated biocoenose (Meyer et al., 2009). In addition observations showed that plant defense strategy involved both roots and shoots upon attack which can explain plant-mediated above-below ground interactions in both directions (van Dam et al., 2005, Erb et al., 2008) and among several trophic levels (Soler et al., 2007). In conclusion belowground interaction studies appear relevant when investigating for biological control agents improving ecosystem functioning.

B. THE PARASITOID *ALEOCHARA BIPUSTULATA* AND ITS PHYTOPHAGOUS HOST *DELIA RADICUM*

1) The cabbage root fly *Delia radicum* L. (Diptera : Anthomyiidae)

Figure 1 *Delia radicum* male. Picture made by B. Chaubet (Fournet, 2000)

Delia radicum (figure 1) is a phytophagous fly sprayed over the temperate part of the Holarctic area (35-60°N; figure 2). Oligophagous specialist of Brassicacea seedlings, *D. radicum* is a major pest in all cultivated crops of this family (Finch, 1989). Damages vary according to the plant and its ontological stage. The highest economic losses occur when *D. radicum* develops on cultivars dedicated to root production (Fournet, 2000).

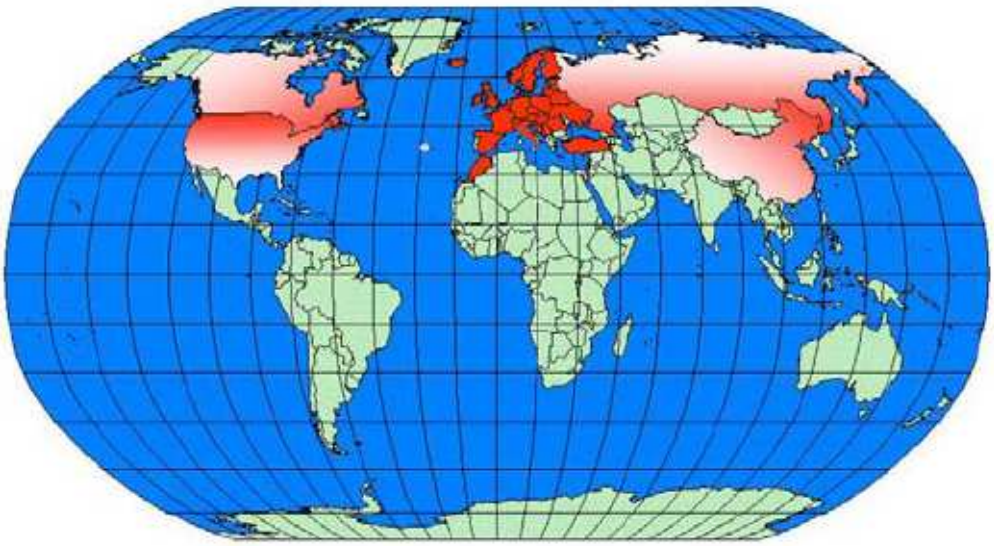

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Figure 2 *Delia radicum* repartition (Fournet, 2000).

There are three to four generations per year, from April till October (HYPPZ). Mated females chose their oviposition sites in a remarkable sequence of behaviors. As an example, females appear to estimate root system quality by walking around the stem (Zohren, 1968, Schoonhoven et al., 2005).

Oviposition occurs on the annulus, the transition between roots and shoots. Eggs hatch in 2 to 14 days depending mostly on humidity level. Three larval stages feed on the roots (≈25days). The amount of damage can be considerable (figure 3). Then 3rd instars leave the root and bury the soil in order to pupate between 8-10cm deep (Fournet, 2000). Interestingly these pupae seems to aggregate in patches near the root of infested plants (Mukerji and Harcourt, 1970). The nymph develops during 20 days (HYPPZ), or can also diapauses to overwinter.

Figure 3 A turnip infested by *D. radicum*.

2) The rove beetle *Aleochara bipustulata* L. (Coleoptera: Staphylinidae).

Aleochara bipustulata is present in the Palaearctic ecozone (figure 4). Within the Staphylinidae family, only the genus *Aleochara* contains parasitoid species. *A. bipustulata* can exploit resources from nearly all stages (egg, larva and pupa) of *D. radicum*. Moreover it parasitizes height families within the true flies, thus it is an interesting candidate as biological control agent (table 1) (Fournet, 2000).

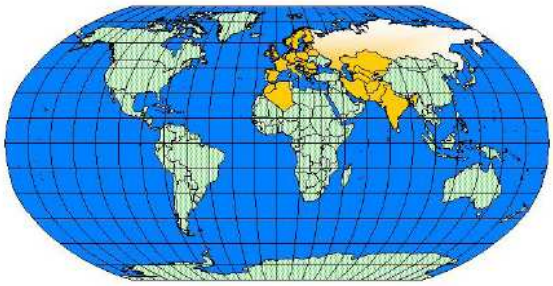

Figure 4 *Aleochara bipustulata* repartition (Fournet, 2000)

Family	Host species	Diet	Host plant
Anthomyiidae	*Delia radicum* L.	P	Wild and cultivated Brassicacea
	D. platura Meig.	P	Seedlings, highly polyphagous
	D. antiqua Meig.	P	Onion, shallot and leak
	D. floralis Fall.	P	Brassicacea
	Pegomya betae Curtis	P	Amaranthaceae
Muscidae	*Musca domestica* L.	S	
Calliphoridae	*Lucilia sericata* Meig.	N, Co	
Sarcophagidae	*Helicophagella gorodkovi*	Co	
	H. rohdendorfi	Co	
	Ravinia pernix	Co	

Table 1 List of *Aleochara bipustulata* hosts (parasitization observed in field). P: phytophagous; S: saprophyte; Co: coprophagous. (Fournet, 2000, HYPPZ)

Table 1 presents the variety of potential hosts for *A. bipustulata*. On one hand this diversity in host plants and food diet increases the possibility to maintain the beneficial organism in situ when the crop and its pest are not present. On the other hand the use of *A. bipustulata* as biological control agent may be discussed regarding to its selectivity.

Figure 5 Mating in *A. bilineata*. Picture from B. Chaubet (Fournet, 2000)

Adults emerge in spring, just after the first *D. radicum* emerged. The length of imago is 5.3 ± 0.1 mm according to Fuldner (1960) but seems to vary (Fournet, 2000). A lot of pictures and observations have been realized on *A. bilineata* which is a near cousin of *A. bipustulata* (figure 5).

Mating occurs after birth and before the first food uptake (figure 5). Then the female will locate a host plant attacked by *D. radicum.* It will be motivated by two needs: finding food source and a suitable oviposition site (Fournet, 2000, Fuldner, 1960). Regarding to the predation behavior of both males and females in Y-tube olfactometer, adults prefer a plant infested by *D. radicum* than a healthy plant. Then when offering the choice between an infested plant from which *D. radicum* maggot were removed and 3rd instars maggot only, *A. bilineata* were strongly attracted by larvae. The priority for food uptake compared to oviposition may come from the protocol of the experiment. Indeed both male and female were tested without possibilities to discriminate them. Most of all the adult starved prior to this experiment (Royer and Boivin, 1999). Interestingly an infested plant provides orientation cues for *A. bilineata* when herbivores had been removed, suggesting HIPVs emitted from the plant. Fournet (2001) find a similar attraction of *A. bilineata* females for infested rather than healthy plants. Interestingly females sense the parasitization status of pupae. Indeed *A. bilineata* laid more eggs near an infested plant associated with healthy *D. radicum* pupae than in the plot containing infested plant with parasitized pupae. This reinforces the idea that the female participate to provide an optimal host for their progeny. If we do not know where oviposition occurs exactly, these studies supposed a subtle belowground prospection of female prior oviposition.

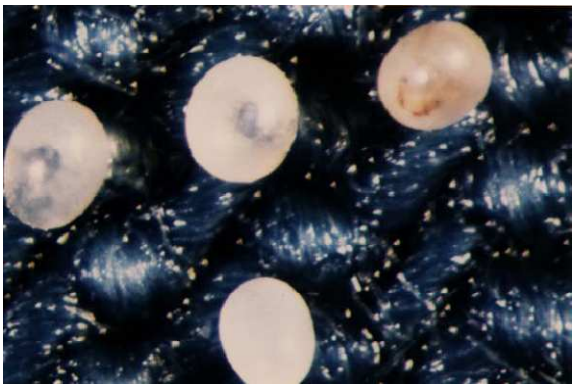

Figure 6 Rove beetle eggs (Ø≈0.4mm) at different development stages. At the top right the mandibles of a future larvae can be distinguished (Fournet, 2000)

Eggs (figure 6) have an elliptic shape and their diameter is around 0.4 mm. White eggs turn brown when the larvae develop and the darkest anatomical parts as mandibles are visible (Fuldner, 1960). They are sensitive to humidity and temperature (Fournet, 2000).

Hatching depends on humidity level and varies a lot. In standard conditions the egg develops within a week. Then the neonate larva has to locate a host. There are three larval stages, only 1st instars are mobile. Their first meal is possible only when a suitable host is parasitized. In between survival of larvae depends on their reserves : 96h maximum according to Fournet (Fournet, 2000), confirming Fuldner (1960).

Figure 7 *A. bipustulata* larvae entering the puparium of a *D. radicum* pupa (Fournet, 2000)

The host searching behavior of the larvae is poorly acknowledged. At long distance, first instar larvae seem to search at random, parasitization occurring only if a pupa happens to be on the way (Fuldner, 1960). If little is known concerning how larvae find their hosts belowground, what is happening after contact is well documented. The larva digs the pupa wall (figure 7). The entrance in the puparium is preferentially located on the dorsal side of the pupae, in median position, where the wall is thinner (Fuldner, 1960, Fournet, 2000, Royer et al., 1998). Then the parasitoid larva secretes a liquid (with feces generated from reserves and its first meal) which close the entrance to the puparium (Fuldner, 1960). Interestingly this plug is an important site for interspecific and intraspecific recognition. Indeed from its smell an *Aleochara* larva arriving on a parasitized pupa can discriminate the species already inside

it. Remarkably, the larva also perceives from these cues if a sibling is occupying the fly pupa. This recognition involves Short Term Memory and affects the behavior of *A. bilineata*. Indeed a larva prefers not to parasitize a pupa containing a sibling and start looking for a new host. *Aleochara bilineata* is a case of non social insect presenting kin selection (Lize et al., 2010a, Lize et al., 2010b, Lize et al., 2006). There is no doubt about the importance of olfaction in host foraging for *A. bipustulata*, as antennae nearly disappear after invading the host pupa (Fuldner, 1960).

Aleochara bipustulata larva then completes its second and third larval stages in the pupa feeding on the *D. radicum* nymph. The parasitoid exploits remarkably the resource available (figure 8). After transformation the nymph of *A. bipustulata* remains in the puparium for 14 days and the imago emerges. Hence the complete cycle from egg to imago takes around 35 days. Roves beetles overwinter as 1st instar larva in their host pupa (Fournet, 2000, Fuldner, 1960).

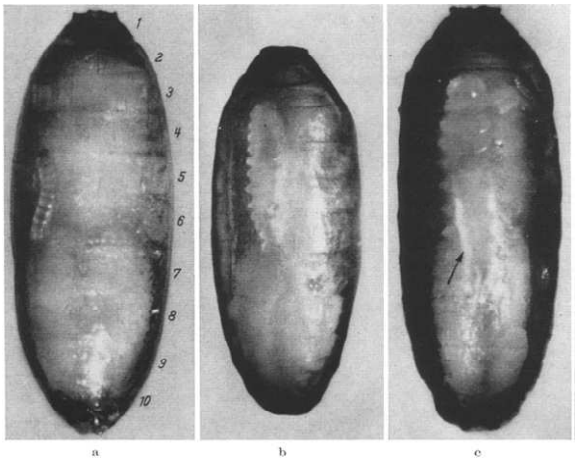

Figure 8 Development of an *A. bipustulata* larva parasitizing a *D. radicum* pupa. a: the first instar ha just invade its host; b: L_2; c: L_3 (Fuldner, 1960).

C. RESEARCH HYPOTHESES

In our system both the female and the 1st instar larva participate in host location. Indeed females prefer infested than healthy plant for oviposition and after contact with a pupa, parasitoid larva demonstrates remarkable recognitions of host quality and parasitism status (Fuldner, 1960, Fournet, 2000, Lize et al., 2006). Thus we may wonder if the parasitoid larva uses cues from the plant to locate *D. radicum* pupae. Indeed the female seems to locate suitable host plant in such an efficient manner that its progeny may just have to prospect directly for a host. According to the search at random hypotheses expressed by Fuldner (1960) and the remarkable kin-recognition observed in larvae this will be the first hypothesis treated in this work: *i) Host searching behavior in 1st instar A. bipustulata larvae is conditioned by D. radicum cues*.

Nevertheless we cannot reject the role of the host plant in this interaction. Indeed Fuldner (1960) did not integrate plant volatiles in his long distance search at random hypothesis. Let's assume *A. bipustulata* locates their host only from *D. radicum* cues. Then it generates a high selection pressure on maggots and pupae to remain unnoticed in the ground. Thus *A. bipustulata* larvae have to choose between reliable cues from their host, present in low concentration, and abundant but less reliable cues emitted from the host plant. This system has been described in aboveground interactions and is common (Vet and Dicke, 1992). Furthermore entomopathogenic nematodes also present this pattern. Juvenile nematodes have to find a caterpillar host *D. virgifera,* helped by symbiotic bacteria. *D. virgifera* is an important root herbivore in maize. Entomopathogenic nematodes rely on volatiles emitted by infested maize, whereas their host itself remained as attractive as sand (Rasmann et al., 2005). This study clearly shows the role of plant-induced indirect defense in belowground interactions. Hence the second hypotheses presented will be: ii) *Host searching behavior in 1st instar A. bipustulata larvae is conditioned by plant cues*.

This second hypothesis concerns mainly long distance research. But what does "far" mean belowground? In a study evaluating host plant recognition by the root feeding clover weevil *Sitona Lepidus,* Johnson et al. (2004) selected 25mm as a meso-scale distance and macro-scale for a distance superior to 60mm. Entomopathogenic nematodes can locate a host in a six arms olfactometer crossing 20 cm of sand (Turlings et al., 2004). Thus we separated long distance (≈20cm) from medium distance (≈10cm) and short distance (≈4cm) in our observations.

PART A HOST SEARCHING BEHAVIOR IN *A. BIPUSTULATA* LARVAE USING *D.* RADICUM CUES

MATERIAL AND METHOD (PART A)

Part A aimed to design olfactometer tests to observe the host searching behavior of *A. Bipustulata*. This first part investigated the hypothesis on host location according to host volatile cues. This hypothesis assumed that *A. bipustulata* females had already chosen the optimal host plant for oviposition, thus the larvae is going to select a suitable host directly without considering the plant anymore.

A. INSECTS

The rearing of the Laboratory of Ecobiology of parasitoid insects in Rennes 1 University provided all the living material. A population of *A. bipustulata* from Le Rheu (Brittany) is maintained and renewed with fresh individuals each year. A population of *A. bipustulata*'s hosts, the cabbage root fly *D. radicum*, is maintained similarly and originates from the same location.

Aleochara bipustulata couples were isolated in cups, with food and water ad libitum. A piece of cloth served as oviposition site. Each single egg was collected using a brush and transferred to an individual cup. This isolation excluded the hypothetical effect of intraspecific competition on the searching behavior of parasitoid larvae. Hatching was monitored on a daily basis.

B. OLFACTOMETERS DESIGN

1) The four arms olfactometer

The team of Prof. Ted Turlings kindly lent to us the 6 arms olfactometer designed to study the behavior of entomopathogenic nematodes (Turlings et al., 2004). Travel conditions broke two arms; hence the shift to 4 arms olfactometer. This olfactometer was a good candidate material because it already worked on entomopathogenic nematodes. In addition it is made of glass with is more respectful with odors than PVC. Nevertheless it is a unique piece, thus another olfactometer had to be designed.

2) Tube olfactometers

This model of dual choice olfactometer consisted of 20ml tubes, inspired from a study on belowground herbivory (Johnson et al., 2004). The length of olfactometers and their middle parts were measured using the graduations on their side. The middle part was a 3ml section of tube. This section was glued on two plugs so that we might link a tube on each side. The speed of movement of *A. bipustulata* larvae in the soil is unknown. Thus the extremity of a Pasteur pipette had been incorporated on both side of the central part of the olfactometer (figure 9). This should avoid the larvae to go back after making a choice. The hole in tubes' plugs was the same width as the Pasteur pipette (0.6 cm).

The pastor pipette was removed after few repetitions. Thus larvae had to pass by the 0.6 cm Ø hole made in the plugs to travel from the middle section to the tubes. Finally the opening of the plugs had been completely enlarged. The opening was as wide as the tubes to put off all relief between sections. This was the final version of the model (figure 12).

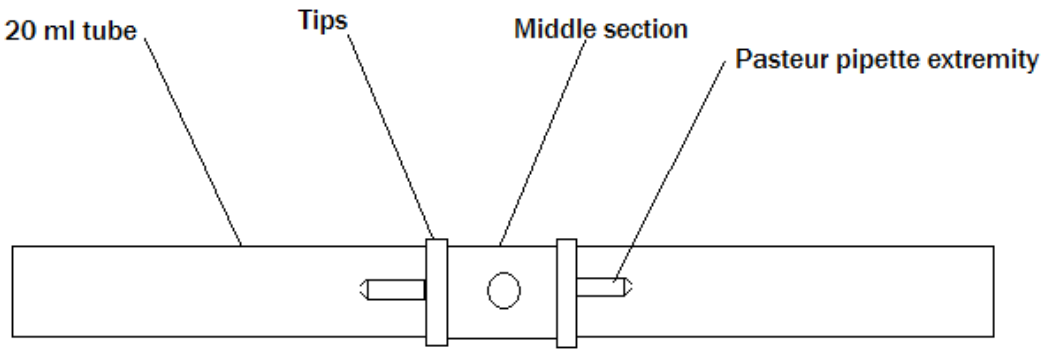

Figure 9 Dual choice olfactometer made from two 20ml tube separated by a middle section of 3 ml. The extremity of a Pasteur pipette prevented the larvae to go back after its first choice.

3) Petri dish olfactometers

Petri dish olfactometers were created to confirm results from the 4 arms olfactometer. A carton board separated a Petri dish in two equal sides. The separation forms a rectangle in the centre where a single *A. bipustulata* larva was released (figure 10).

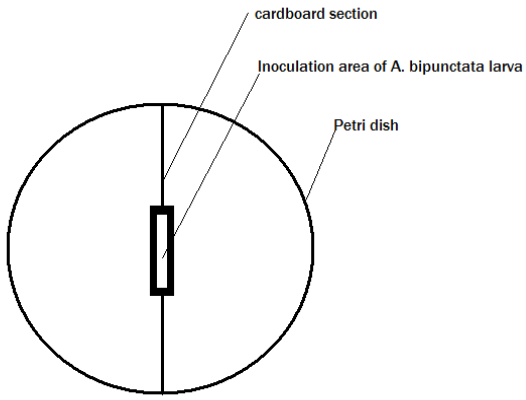

Figure 10 Petri dish olfactometer. A cardboard separated a Petri dish in two with a square at the middle formed our second home made olfactometer. The larva of *A. bipustulata* was inoculated in the middle rectangle before removing the cardboard.

4) The substrate

Big vermiculite was the optimal substrate. Indeed a mix of soil with sand was too hard to manipulate without creating compaction zones in olfactometers' arms. Vermiculite acts like a sponge and conserves water long enough to allow 4 day choice experiments. The humidification was 2.5 volume of water for 10 volume of vermiculite.

C. RESPONSE OF *A. BIPUSTULATA* LARVAE TO *D.* RADICUM PUPAE IN THE 4 ARMS OLFACTOMETER: LONG DISTANCE HOST LOCATION

For the two first attempts, each glass arm end received two *D. radicum* pupae. Height *A. bipustulata* larvae were released in the central part of the olfactometer. Larvae had 96hpi to make a choice and to parasitize a host pupa. Then parasitism was monitored. Observation of pierced pupae without parasitoid larvae in it happened. This was scored as a parasitization because there is very low chance that a larva locates a potential host, makes a complete entrance to the puparium and then decides to abandon it for another pupa. Even if larvae would do so, they would not survive long enough to locate a new host and dig a hole again.

The number of parasitoid tested (n=15) and their host (n=4 per arms) increased proportionally to fasten the accumulation of data.

An extension of this experiment assessed whether *A. bipustulata* larvae detect the smell emitted by *D. radicum* galleries when maggots burry the soil in order to pupate (figure 11). A portion of 25ml of vermiculite received 3th instars *D. radicum* larvae (n=50) during 3h. Then the maggots were removed. This vermiculite was the substrate in two arms out of four. In all four arms two *D. radicum* pupae were placed and parasitization scored 96hpi.

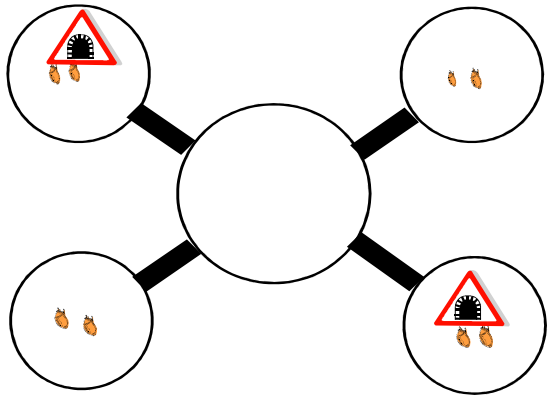

Figure 11 Experimental scheme investigating the role of galleries smell generated by *D. radicum* larvae when burying the soil to pupate. Orange oval shape represents a pupae and tunnel sign represents vermiculite pre-infested by 3th instars *D. radicum* larvae (n=50, 3h)

D. RESPONSE OF *A. BIPUSTULATA* LARVAE TO *D. RADICUM* PUPAE IN TUBE OLFACTOMETERS: MEDIUM DISTANCE HOST LOCATION BY *A. BIPUSTULATA*

One olfactometer consisted in two tubes linked by a 3ml section where the parasitoid larva was to be released. Each tube was filled with humid vermiculite till the middle, a *D. radicum* pupa added in it, and then the tube was completely filled. The middle section contained humid vermiculite as well, forming a complete olfactometer (figure 12). *A. bipustulata* larva was inoculated immediately and the little opening belonging to the middle section closed with a piece of tape. Parasitism was monitored paying attention on which side parasitism occurred to detect any pitfalls that may affect larval choice.

Figure 12 Tube olfactometer. A: olfactometer arm; B: central part. This picture presents the final model. Pastor pipettes had been removed and the diameter of separations between A and B (blue caps) had been enlarged at the maximum.

1) Response of two *A. bipustulata* larvae in a tube olfactometer

Two *A. bipustulata* larvae had to make a choice between the right and the left arm of the olfactometer. Parasitization was scored 96hpi.

2) Response of a single *A. bipustulata* larva in a tube olfactometer

Similarly, a single parasitoid larva had 24-, 48- or 96hpi the make a decision for a side of our olfactometers. In a different test run the substrate varied, comparing olfactometers filled with humidified vermiculite (n=15) and sand (n=15). In this case larvae had 48hpi to find a host.

3) Impact of the smell of *D. radicum* gallery on the host searching behavior of *A. bipustulata*

Until now both arms released the same odor of *D. radicum* fresh pupa. This section supposed that 3th instars *D. radicum* larvae might deposit an odor source when burying the soil in order to pupate. 3th instar *D. radicum* larvae (n=50) buried ten volumes of vermiculite during 2-3h. After collecting maggots back, this vermiculite was used in one arm of a tube olfactometer. Then two methods were employed. In the first test runs the pupae was added after the decision, when stopping olfactometers. In this case a single *A. bipustulata* larva had 24hpi to choose between a substrate with or without gallery smell. Then each olfactometer arm received a *D. radicum* pupa near the opening, both tubes were closed and parasitism scored 24hpi. This protocol had the advantage to exclude the factor pupae in larva's decision.

But taking into account how difficult it is to manipulate this living material, we put the host pupae in the middle of the olfactometer tube since the beginning of the experiment. The control was a tube filled with non infested vermiculite with a *D. radicum* pupa in the centre (figure 13).

Figure 13 Tube olfactometer used in the "gallery odor" experiment. The tunnel traffic sign represents vermiculite emitting gallery odors from *D. radicum* larvae. Orange ovoides are *D.radicum* pupae.

4) Does *A. bipustulata* larvae prospect better when installing olfactometers vertically?

The last attempted aimed to figure it out if the response rate could be higher when olfactometers were vertical. Maybe horizontal research did not suit parasitoid larvae in this material. The same experiment as 2) was repeated but this time the position of olfactometers was vertical.

E. DOES *D. RADICUM* GALLERY SMELL ATTRACT *A. BIPUSTULATA* LARVAE AT SHORT DISTANCE?

This test investigated the "Gallery" effect in Petri dishes olfactometer which gave better responses in past experiments concerning parasitism. 3rd instars *D. radicum* larvae buried a volume of humid vermiculite (20cl) during 3h and 24h. Then maggots were removed and the infested vermiculite placed in one side of the plate with a *D. radicum* pupa. On the other side of the cardboard, the control treatment consisted in clean humid vermiculite with a *D. radicum* pupa. A single *A. bipustulata* larva was released in center of the cardboard which contained clean vermiculite as well. Just after inoculating the parasitoid larva the cardboard was removed (figure 14). Petri dishes were randomly placed with 45° rotation each time on a horizontal table. Parasitism was scored 48hpi.

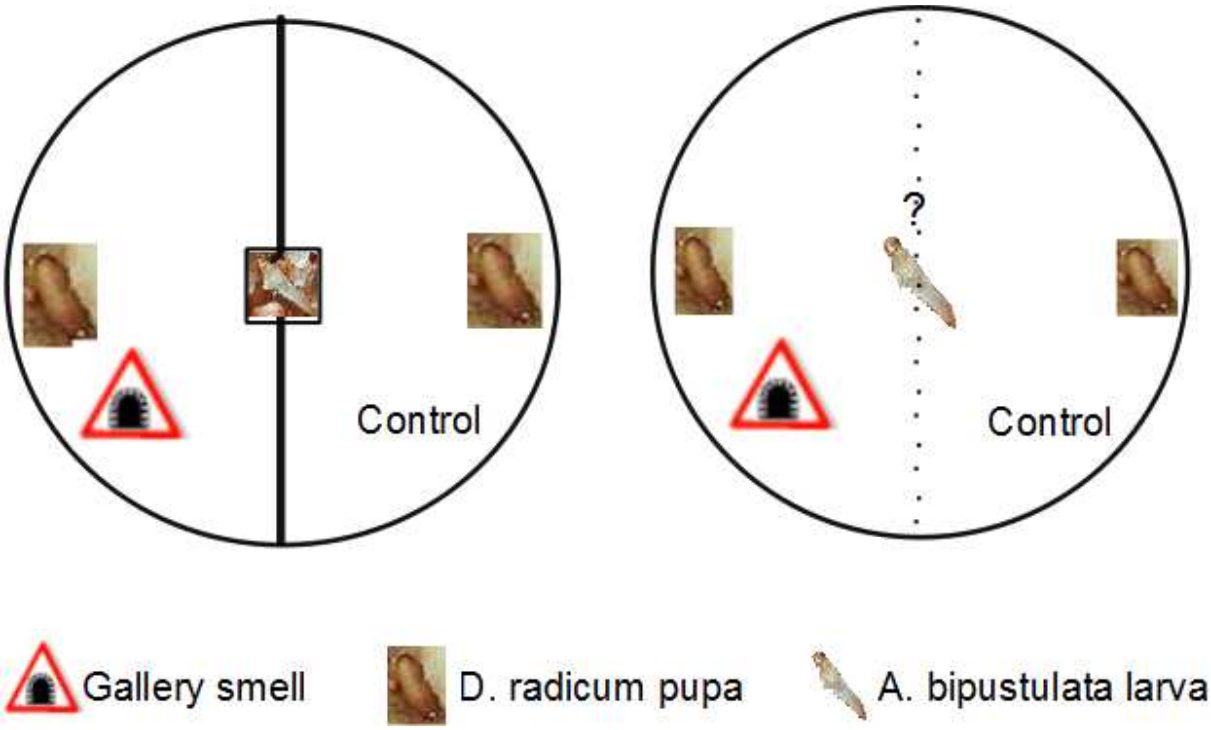

Figure 14 Petri dish experiment investigating the effect of *D. radicum* galleries on *A. bipustulata* host searching behavior.

F. STATISTICAL ANALYSIS

Count of parasitizations follows Poisson's distribution. We performed a generalized linear model (GzLM) with Poisson's distribution with log as link function in SPSS®. If observations were fewer than n=30, U Mann and Whitney's test replaced the GzLM.

RESULTS (PART A)

A. RESPONSE OF *A. BIPUSTULATA* LARVAE TO *D. RADICUM* PUPAE: LONG DISTANCE HOST LOCATION

15 *A. bipustulata* larvae responded over the 31 individuals tested. Thus 48% of parasitoid larvae succeed in searching a host pupae placed at the extremity of an olfactometer arm. There were no difference in parasitization among the different olfactometer arms (H=3.667; P=0.402). Each arm has the same probability to be the place of a parasitization.

The gallery smell hypothesis has been tested one single time and gave a lower response than the preliminary tests for parasitization. We found back 2 larvae out of 8, the parasitization rate dropping at 25%. Combined with observations realized in the tube olfactometer we did not perform other repetitions.

B. MEDIUM DISTANCE HOST SEARCHING BEHAVIOR OF *A. BIPUSTULATA* LARVAE

1) Response of two *A. bipustulata* larvae in a tube olfactometer

We investigated the response of *A. bipustulata* larvae when perceiving *D. radicum pupae* at medium distance (≈10cm). For the three first dates n=2 *A. bipustulata* larvae have been tested per olfactometer during 96h. Tables 2 and 3 present the data generated.

Count of Parasitization	DATE	
Olfactometer	17/12/2010	05/01/2011
A	0	0
B	0	1
C	0	1
D	1	2
E	0	0
F	0	1
G	1	1
H	0	1
I	0	
J	1	
K	0	
L	0	
M	0	
Response rate	3/26	7/16

Table 2 Count of parasitizations observed for dates 17/12 and 05/01. Note that 2 *A. bipustulata* larvae were released per olfactometer. As an example the ratio 3/26 means 3 *A. bipustulata* larvae, out of 26 tested, have been found parasitizing a *D. radicum* pupae.

Count of Parasitization	DATE
Olfactometer	06/01/2011
1	1
2	1
3	1
4	1
5	1
6	2
7	1
8	2
9	1
10	1
11	1
12	1
13	1
14	1
15	2
16	2
17	2
18	2
19	1
20	1
21	1
22	1
23	2
24	1
26	0
27	2
28	1
29	2
30	1
31	1
32	2
Response rate	40/62

Table 3 Count of parasitizations observed in tube olfactometers for the date 06/01.

Over the three dates, the parasitization rate was 48%. The good level of response on the 06/01/2011 improved it a lot. This result confirmed that there is no difference among the right or the left side in this experiment (table 4). The date had an important impact. On the last day, the 06/01, the level of responses increased dramatically to 64%. There is a slight improvement between date 17/12 and 05/01 which corresponds to a modification of olfactometer design. Pastor pipettes fixed on each side of the central part had been removed.

We could not estimate the difference among olfactometers because we changed their identification during the experiment. That is why the source of variation “olfactometer identity” does not appear in the analysis. Still each olfactometer got a new proper identity so that we may check for a right/left pitfall.

Source	df	H	Pvalue
Count parasitization	51	51	0.474

Table 4 Data analysis of count of parasitization referring to results B-1).

2) Response of a single *A. bipustulata* larva in a tube olfactometer

Only one parasitoid larva was tested per olfactometer. The first run was launched the 13/01/2011 and stopped 24hpi. Twelve *A. bipustulata* responded over 36 larvae tested (33%). Then two runs where *A. bipustulata* larvae had 96hpi to parasitize a host pupa revealed that a longer time frame did not improve parasitization rate (table 5). Days 13/01 and 26-27/01 have a different foraging time, but also olfactometers were modified. The section between the arms and the central section was enlarged at the maximum. Thus a longer time to forage coupled with an easier access to the resource did not improve the parasitism.

Date and decision time	Number of *A. bipustulata* tested	Count of parasitizations observed	Response rate
13/01/2011 24hpi	36	12	0.33
26/01/2011 96hpi	22	9	0.40
27/01/2011 96hpi	19	4	0.21

Table 5 Count of parasitizations of *D. radicum* pupae by *A. bipustulata.* Parasitoids larvae had 24 or 96hpi to find a pupa.

Changing the substrate for sand instead of vermiculite did not improve the parasitization rate. Four *A. bipustulata* larvae found a host pupa out of 15 individuals tested in both case (table 6).

Date	Substrate	*A. bipustulata* tested	Parasitizations observed	Parasitization rate
10/01/2011 48 hpi	Vermiculite	15	4	0.26
	Sand	15	4	0.26

Table 6 Parasitization rates of *A. bipustulata* according to two different substrates vermiculite and sand.

3) Impact of the smell of *D. radicum* gallery on the host searching behavior of *A. bipustulata*

Date	Olfa. ID	Arm	Parasitization
17/01	1	g	0
	1	d	0
	5	g	0
	5	d	1
	6	g	0
	6	d	0
	17	g	0
	17	d	0
	18	g	0
	18	d	1
	21	g	0
	21	d	0
	19	g	0
	19	d	1
	9	g	0
	9	d	1
	30	g	0
	30	d	0
	31	g	0
	31	d	0

Table 7 Parasitization when investigating for gallery smell effect on host location behavior in *A. bipustulata* larvae. 0: healthy *D. radicum* pupae; 1: parasitized pupae; g: left side containing control vermiculite; d: right side containing pre-infested vermiculite (gallery odors). Olfa. ID means olfactometer identity.

Date	Olfa. ID	Arm	Parasitization
18/01	40	d	0
	40	g	0
	39	d	1
	39	g	0
	36	d	0
	36	g	0
	35	g	0
	35	d	0
	34	d	0
	34	g	0
	33	d	no pupae
	33	g	no pupae
	32	d	0
	32	g	0
	29	g	0
	29	d	0
	28	g	0
	28	d	1
	27	d	0
	27	g	0
	26	g	0
	26	d	0
	24	g	0
	24	d	0
	23	d	0
	23	g	0
	22	g	0
	22	d	1
	20	d	0
	20	g	0
	16	d	0
	16	g	0
	15	g	0
	15	d	0
	14	d	0
	14	g	0
	13	d	0
	13	g	0
	12	d	1
	12	g	0
	11	d	0
	11	g	0
	10	d	0
	10	g	0
	8	g	0
	8	d	1
	7	d	0
	7	g	0
	4	d	0
	4	g	0
	3	g	0
	3	d	0
	2	g	0
	2	d	0

Table 8 Parasitization when investigating for gallery smell effect on host location behavior in *A. bipustulata* larvae. 0: healthy *D. radicum* pupae; 1: parasitized pupae; g: left side containing control vermiculite; d: right side containing pre-infested vermiculite (gallery odors). In olfactometer 33 did not contained host pupae by mistake.

When adding host pupae 24hpi, four *A. bipustulata* larvae succeeded in parasitizing a *D. radicum* pupa out of 10 individuals tested (table 7). All of them went in the right arm containing gallery smell. A single test of 10 individuals cannot provide significant results.

When adding host pupae since the beginning of the run, five *A. bipustulata* larvae succeeded in parasitizing a *D. radicum* host pupa out of 27 individuals tested (table 8). All of them went to the right side containing gallery smell. No conclusion can be taken out off 5 responses. Only 18% of the larvae tested responded.

4) Do *A. bipustulata* larvae perform better in vertical tube olfactometers?

Date	Olf.	Arm	Parasitization
28/01	3	up	1
	3	down	0
	7	up	0
	7	down	0
	8	up	0
	8	down	0
	10	up	1
	10	down	1
	11	up	0
	11	down	1
	17	up	0
	17	down	0
	18	up	0
	18	down	0
	24	up	1
	24	down	0

Table 9 Parasitization of *D. radicum* pupae by *A. bipustulata* in olfactometer put vertically. 0: healthy *D. radicum* pupae; 1: parasitized pupae.

Five *A. bipustulata* larvae parasitized a host pupa out of 8 individuals tested (table 9). Three parasitizations occurred in the upper arm and two in the lower arm of olfactometers. *A. bipustulata* larvae located a host pupa by moving upper in the substrate in this experiment. Only one run had been tested on the date 28/01/2011.

C. DOES *D. RADICUM* GALLERY SMELL ATTRACT *A. BIPUSTULATA* LARVAE AT SHORT DISTANCE?

The potential "Gallery" effect had been tested in Petri dish olfactometers. Results are separated in two parts according to the inoculation time of the substrate employed (table 10).

Date	Vermiculite		*A. bipustulata* tested
	Clean	Infested 3h	
09/02/2011	15	9	28
10/02/2011	6	3	20

Table 10 *D. radicum* galleries effect in Petri dish olfactometer: parasitization scores in infested (3h) or clean vermiculite.

33 *A. bipustulata* larvae found a host out of 48 individuals tested (68%). Over two dates 21 found their host in the side containing clean vermiculite and 12 in the side filled with infested vermiculite. Parasitization rate was not different in clean vermiculite than infested vermiculite (Wald's X^2=2.391; P=0.122). Thus the odor *D. radicum* larvae might let when burring the soil during 3h does not affect the decision of *A. bipustulata* larvae.

Date	Vermiculite		*A. bipustulata* tested
	Clean	Infested 24h	
15/02/2011	4	6	19
16/02/2011	7	3	17

Table 11 *D. radicum* galleries effect in Petri dish olfactometer: parasitization scores in infested (24h) or clean vermiculite

20 *A. bipustulata* larvae parasitized a pupa out of 36 individuals tested (55%). 11 parasitoids found their host in the side containing clean vermiculite and 9 in vermiculite infested during 24h. Parasitization rate was the same in clean vermiculite than in infested vermiculite (U=1; P=0.667). Allowing *D. radicum* maggots to bury vermiculite during 24h instead of 3h did not change the choice of *A. bipustulata* larvae.

DISCUSSION (PART A)

Generally the response of *A. bipustulata* larvae was too low according to manipulations realized in previous projects. They are clearly two important factors to explain these disappointing results: the competency of the material and the decision process of larvae itself. Inadequacy between the material and living material is a plausible a prudent explanation, thus it will be treated first.

A. AN UNSUITABLE MATERIAL?

The four arms olfactometer gave a higher response rate than the tube olfactometer. As both were performing moderately well at the beginning, we focused on improving tube olfactometers to test a larger quantity of larvae in a smaller time frame. The response rate was lower than 50% while our team expected 90% at the minimum. Unfortunately all attempts to create suitable conditions for host location studies failed (substrates, removal of Pasteur pipette, plugs enlargement). The efficiency of *A. bipustulata* to move in the soil might have been overestimated, an especially regarding to vermiculite. At first sight vermiculite looks like a light substrate able to keep a good humidity level and a nice aeration in the olfactometer. But the texture might be too complex for such a tiny parasitoid larva. Indeed the vermiculite has an “accordion” shape and the larva seem to try to get in between each anfractuosities of each grain to make its way (Pierron, personal observations). Unfortunately changing vermiculite for sand did not improve parasitism. Humid sand was too difficult to incorporate into olfactometer arms. Finally Petri dish olfactometers performed well, but allowed only short distance observations which are already documented (Fuldner, 1960).

B. ARE *A. BIPUSTULATA* LARVAE NOT RESPONDING TO *D. RADICUM* VOLATILE CUES?

Apart from the material this result may also reveals that *A. bipustulata* larva does not respond to *D. radicum* cues in order to locate its host. The smell of *D. radicum* gallery is clearly rejected in this experiment. Responses were low at long and medium distance, and the analysis of short distance data from Petri dish olfactometer did not reveal any effect of the presence of galleries. However the higher response rate indicates the presence of cues from *D. radicum* pupae exploited by *A. bipustulata* at short distance.

If host-foraging groups several sequences of behavior, the first one may not involve *D. radicum* cues. It does not mean these cues are not use at all among the whole

detection process. Thus *A. bipustulata* may not initiate its searching for a host by using *D. radicum* cues. This hypothesis would suit observations realized on above ground hymenopteran parasitoids. The selection pressure develops host abilities to remain undetectable. Thus on one hand *A. bipustulata* could use host cues which are diluted in a large environment but reliable because emanating directly from the host. And on the other hand plant volatile cues appear to be less reliable but produced in large amounts, so rapidly and easily detected. Evolution encouraged such phenotypes which confer indirect defense to the plant. The compromise between reliability and detection is proposed by Vet et al. (1992). It has already been observed in belowground interactions with entomopathogenic nematodes (Rasmann et al., 2005).

CONCLUSION (PART A)

The general response level was too low according to our expectations. Nevertheless the role of galleries made by *D. radicum* in the soil can be rejected. Few observations let enough hopes to persist in investigating the behavior of 1st instar *A. bipustulata* larvae. The first hypotheses concerning host location according to host cues only do not seems to be verifiable thanks to the material involved in this experiment. In addition, according to the literature, there is a strong chance that parasitoid larvae respond to plant cues which are less reliable but much easier to detect at long distance. The second part of the project will focus on this hypothesis.

PART B INFLUENCE OF PLANT VOLATILE CUES ON THE BELOWGROUND PARASITOID LARVA *A. BIPUSTULATA*.

MATERIAL AND METHOD (PART B)

The second part of the project used the same living material and olfactometers described in the material and method in part A. Only the four arms and Petri dish olfactometers were used to investigate the role of the plant *Brassica rapa* in the host searching behavior of 1st instar *A. bipustulata.*

A. PLANTS

Turnips *Brassica rapa* were used in our experiments so that we may link our results to a complementary work related to the oviposition behavior of *A. bipustulata* females. Furthermore the population of *D. radicum* is reared on this plant species. We used regrown plants from piece of turnip roots.

B. DOES *BRASSICA RAPA* ATTRACT *A. BIPUSTULATA* LARVAE?

1) Exploitation of plant cues by *A. bipustulata* larvae at long distance

This part of the project used a different approach by decomposing the environment of an *A. bipustulata* larva. The four arm olfactometer offered this opportunity. Each arm received a different treatment (figure 15):

- Arm a: an infested plant with infested vermiculite and *D. radicum* pupae in excess compared to the larvae tested.
- Arm b: an infested plant with clean vermiculite and *D. radicum* pupae in excess;
- Arm c: infested vermiculite and *D. radicum* pupae in excess;
- Arm d: clean vermiculite and *D. radicum* pupae in excess.

A. bipustulata larvae had 48-60h to locate and parasitized their host pupa. Parasitism rate in each arm informed about larval decision. The quantity of *A. bipustulata* larvae was not as high as expected when the experiment begun. Thus individuals tested vary from n=8 to 36

larvae released in the centre of the olfactometer. The protocol was then standardized to n=30 larvae tested for the three last test runs.

Plant infestation:

The olfactometer consisted in 4 arms. Two contained an infested plant. Turnips uses reserves store in the root to develop a compensation growth. Only one turnip regrowth sliced in two was used per repetition. This avoided the variation due to the plant. 1st instar *D. radicum* larvae (n=30) developed on the plant until they reached 3rd instar. The plant was then transferred in the olfactometer.

Infested vermiculite:

3rd instar *D. radicum* larvae (n=50) buried during 3h in a 20cl of vermiculite able to fit in the olfactometer arms (≈ 15cl). Maggots were the removed and the infested vermiculite added to the olfactometer arm.

Figure 15 The four arm olfactometer running with Chinese cabbages.

2) Confirmation of the plant effect in Petri dish olfactometers

Observation with the 4 arm olfactometer might help to clarify interactions within this tritrophic system. Nevertheless a second experiment separating each factor is required to fully investigate their role in the decision process of *A. bipustulata.*

A piece of infested turnip root from the rearing of *D. radicum* was placed in one side of the plate and then covered with humidify vermiculite. The opposite side only contained humid vermiculite and the central part of the cardboard as well. A single *A. bipustulata* larva was released in the middle of the plate just before removing the cardboard. The cardboard was placed back 16hpi and *D. radicum* host pupae (n=2) were added on each side. Petri dishes were placed in different positions on a horizontal table to randomize any uncontrolled effects. Parasitism rate was score 48hpi (figure 16).

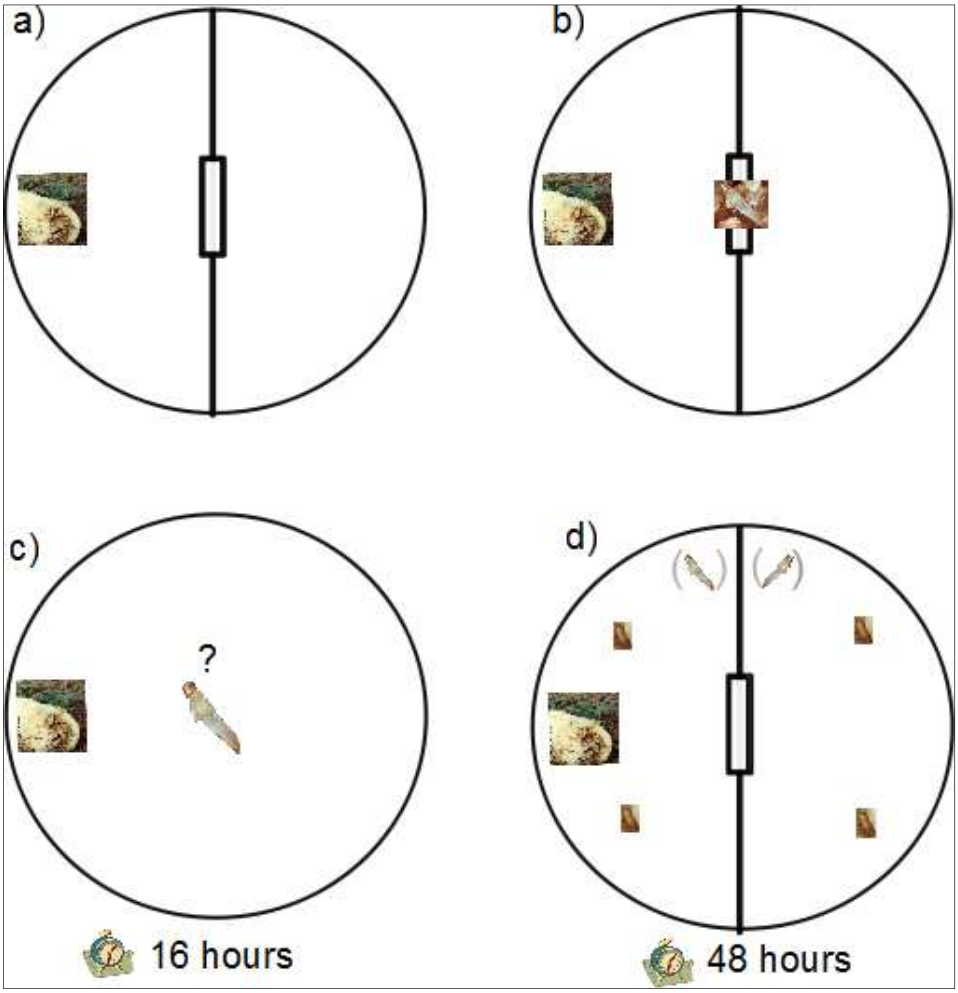

Figure 16 Experimental scheme of Petri dishes olfactometers investigating for the plant effect on *A. bipustulata* foraging for a *D. radicum* pupae. a) a piece of infested turnip root was placed in humid vermiculite, the control treatment contained humid vermiculite; b) A single *A. bipustulata* larva was released in the centre of the cardboard which was then removed; c) The larvae had 16h to migrate in the plate before placing the cardboard back into the olfactometer; d) *D. radicum* pupae (n=2) were added to each side of the plate. Then the parasitoid had 48h to locate a host before our team scored for parasitization.

C. STATISTICAL ANALYSIS:

Count of parasitism follows Poisson's distribution. Data analysis used a generalized linear model with link Log and Poisson's law in SPSS®.

RESULTS (PART B)

A. DOES *BRASSICA RAPA* ATTRACT *A. BIPUSTULATA* LARVAE?

85 *A. bipustulata* larvae succeed in parasitizing a *D. radicum* pupa out of 157 individuals tested (54%). Table 13 and figure 17 illustrate results obtained in the four arm olfactometer. 41 parasitizations occurred in the arms corresponding to the full combination (PGP), 21 in the treatment "Plant + pupae" (PP), 12 in the treatment "gallery+pupae" (GP) and 11 in the treatment containing "Pupae" only (P). Thus *A. bipustulata* larvae were attracted preferentially by the plant than *D. radicum* galleries (X^2=14.281; P<0.001). Note that the infested plant consisted in both shoot and root compartments. There was no interaction between factors "Plant" and "Gallery" in this experiment (table 12).

Source	Wald's X^2	P
Plant	14.281	<0.001
Gallery	2.321	0.128
Plant*Gallery	1.376	0.241

Table 12 Statistical analysis investigating the impact of plant volatiles and *D. radicum* gallery on *A. bipustulata.*

Date	TTT	Para	Nb Aleo	Nb Host
02/02/2011	PGP	1	8	5
	GP	0		5
	P	0		5
	PP	1		5
08/02/2011	PGP	4	15	16
	GP	1		16
	P	1		16
	PP	1		16
11/02/2011	PGP	4	28	20
	GP	1		20
	P	4		20
	PP	4		20
14/02/2011	PGP	11	36	30
	GP	2		30
	P	1		30
	PP	6		30
17/02/2011	PGP	8	32	30
	GP	3		30
	P	3		30
	PP	7		30
21/02/2011	PGP	13	38	30
	GP	5		30
	P	2		30
	PP	2		30
TOTAL	PGP	41	157	
	GP	12		
	PP	21		
	P	11		

Table 13 Parasitization of *D. radicum* pupae by *A. bipustulata* according to different odor combinations (TTT). PGP: plant+gallery+pupae; PP: plant+pupae; GP: gallery+pupae; P: pupae. Para refers as the number of parasitized pupae observed in the corresponding treatment. Nb Aleo reminds the quantity of larvae tested with the quantity of host (Nb Host) associated.

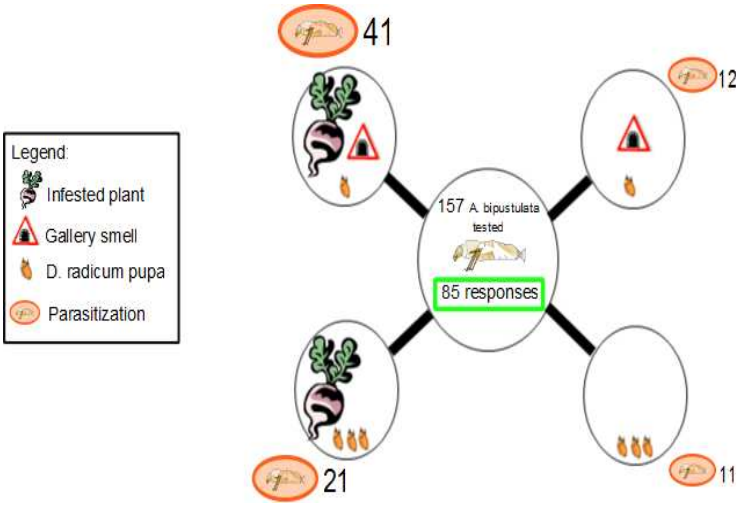

Figure 17 Sum of parasitization observed according to different combination of odor sources. Infested plant + gallery+pupae (PGP); Infested plant+pupae (PP); Gallery+pupae (GP); Pupae (P).

B. CONFIRMATION OF THE PLANT EFFECT IN PETRI DISH OLFACTOMETERS

The data concerning this final part was not available. Nevertheless first results showed a clear effect of the plant in the decision of *A. bipustulata* larvae.

DISCUSSION

This experiment clearly shows that the plant attracts *A. bipustulata* larvae. The model *B. rapa – D. radicum – A. bipustulata* seems to follow the biological interaction described by Vet et al. (Vet and Dicke, 1992).The influence of plant cues was demonstrated in long distance search. Experiments in Petri dish olfactometers allow separating each odor source individually. *A. bipustulata* seems to be highly attracted by a piece of infested root, even if more observations need to be done to confirm this trend. One important treatment is missing in this work: the healthy plant. That is why, unfortunately, it is not possible to distinguish the role of volatiles constitutively produced by turnips than volatiles induced by *D. radicum* damages. This is a critical point to acknowledge.

Indeed the recruitment of *A. bipustulata* larvae by the plant is questionable. The parasitoid is looking for a pupa, thus damages have already been done. If there is no increase in fitness for the plant, then this type of indirect defense is unlikely to evolve. Unlikely, excepted if we consider other beneficials associated with Brassicacea. The parasitoid wasp *Trybliographa rapae* followed plant volatiles and forage for *D. radicum* young larvae to parasitize. Thus *A. bipustulata* larvae may use dimethyl disulfid, a molecule involved in interaction between *T. rapae* and the plant. Dimethyl disulfid also attracts *A. bipustulata* adults which eat *D. radicum* eggs and larvae (Ferry et al., 2009, Ferry et al., 2007). The attraction of several predators and parasitoids with a molecule associated with tissue degradation increases the selection pressure on this plant phenotype. Dimethyl disulfide is an interesting candidate to test on 1st instar *A. bipustulata.*

The host plant appears to be omnipresent in the interaction between *A. bipustulata* and *D. radicum.* In this parasitoid species both the female and its progeny are implicated in host selection (Fournet, 2000). The female increases the fitness of its progeny by carefully selecting host plants. Thus females reply to herbivore-induced plant volatiles (Fournet et al., 2001, Royer and Boivin, 1999). Our work showed that the plant is also influencing the larvae in a considerable manner. So the belowground parasitoid *A. bipustulata* seems to rely on plant volatiles cues for two major decision processes that condition host location. The impact of plant on arthropod diversity is clearly described through the impact of induced defense on plant-mediated interactions (Ohgushi, 2005, Poelman et al., 2008, Utsumi et al., 2010). This impact may even be stronger in *A. bipustulata* fitness. Indeed a modification in plant trait

would affect both female and larva decisions. This hypothesis is highly speculative but opens a new aspect of the strength of plant-mediated interactions.

In part A we rejected the potential impact on galleries made by *D. radicum* when maggots bury the soil to pupate. The second part of the project confirmed this deduction. *A. bipustulata* larvae do not rely on gallery odors to locate their host.

The R^2 of the model generated to highlight a Plant effect in the four arm olfactometer was around 0.3. Thus the variable "Plant" explains few of the variation observed. Other parameters as the variation of humidity or temperature inside the olfactometer might explain it. There could also be others parameters that we did not test. The parameter humidity is difficult to maintain stable over treatments and time. Over time we can assume that evaporation in constant over treatments. The plant is an extra source of humidity. Treatment which had not plant received some more water but it was subjective (the aspect of vermiculite). This effect can be considered as minor. Indeed only the very top of the olfactometer contained a plant. The central part and the junctions (≈20cm) were identical for every treatment. So if the variation in humidity affects the behavior of the larvae (Fuldner, 1960), it will be after it made a decision. Finally it will be important to clarify the impact of releasing 30 *A. bipustulata* larvae in one olfactometer on their behavior. Competition may affect our observations.

CONCLUSION

This work showed that the behavior of 1st instar *Aleochara sp.* is observable in olfactometer experiment. In the mean time it provides some clues on the interaction between the parasitoids *A. bipustulata* and its host *D. radicum*. The host plant appears to be more influent than assumed. If the female uses plant volatiles to select oviposition site, host searching behavior in the 1st instar larva was unknown. After rejecting the detection of cues from *D. radicum* galleries, olfactometers clearly highlighted a plant effect. *A. bipustulata* does locate their host using volatiles emitted by infested plants.

This finding reinforces the position of the plant in orchestrating arthropod communities. Furthermore a large range of studies is now conceivable. As an example studies on intra- and inter-specific competition between *A. bipustulata* and *A. bilineata* can be done with a new angle because adding parameters on parasitization success is directly linked to the fitness of the progeny. Understanding the behavior of larvae will also be helpful to design biological control techniques to increase parasitism rate. Finally the implication of the host plant also opens the field of plant breeding for indirect defense in this system.

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