CRA
CONSIGLIO PER LA RICERCA
E LA SPERIMENTAZIONE
IN AGRICOLTURA

CRA-API
UNITÀ DI RICERCA
DI APICOLTURA E BACHICOLTURA

"Effects of coated maize seed on honey bees"

Report based on results obtained from the second year (2010) activity of the APENET project

Compared to the Italian version, this report contains updates of results made available during winter 2010/2011, concerning chapters 3, 4 and 6.

All times cited in this report are CET.

A list of the symbols of the Italian provinces can be found here:
http://www.tuttocamere.it/files/varie/Province_Sigle.pdf

1. The monitoring network

In recent years numerous and sometimes severe bee die-offs have been reported in Europe and in other countries. The investigations conducted to date have shown that the most likely risk factors include bee diseases, pesticide treatment, bee management practices and climatic trends. Pesticide treatments play a particularly critical and influential role, above all if carried out in the spring-summer season in areas devoted to intensive cultivation. The majority of the active ingredients utilized in pesticides exert some degree of toxicity on bees, and the effects can be immediate and extremely evident if the bees are directly affected. But in the case of products used in seed coating (eg. neonicotinoids), microencapsulated formulations and growth regulators (IGR), the effects may be more insidious and difficult to link specifically to the cause.

Action designed to monitor hive depopulation and bee losses has been or is being undertaken in various European countries. In Italy, after the fairly isolated phenomena that began to be described as from 2000, the need for a monitoring and die-off reporting system became particularly urgent in 2008, when the severity of events that occurred in the spring of 2008 was highlighted. This prompted awareness of the importance of creating a nation-wide monitoring system in order to acquire knowledge on hive health and to determine the extent and possible causes of bee losses.

In the framework of the Italian APENET Project, a national monitoring network has been set up in Italy, composed of surveillance modules, with at least one module for each Region and Autonomous Province. Every module consists of 5 stations (apiaries), each of which is in turn made up of 10 hives, located in representative geographic areas of each Region. To date, the network is composed of 20 modules, 94 apiaries and 940 hives. The function of the monitoring network is to gather information on the health status of the bee families contained within the modules, by means of periodic surveys and subsequent laboratory analyses performed on the different matrices collected (dead bees, live bees, brood, wax, pollen). In addition to routine analyses at the pre-established dates, the programme also specifies that special surveys, sample collection and analyses should be carried out at any time if abnormal mortality is reported.

Periodically (4 times a year: at the end of winter, in late spring, during the summer, before winter) the hives of each station are inspected by an operator, with careful recording of all data concerning bee health status (presence of parasites and pathogens), nutritional status (abundance of pollen and honey) and family status (number of bees and brood number, age of the queen, etc.). To record the data gathered during these periodic inspections, the operators fill in purpose-designed report forms; subsequently, the data are entered on a web site (www.izsvenezie.it/apenet) equipped with a dedicated software program for collection and management of the monitoring network data. The website can be accessed by the persons in charge of the surveillance modules, in order to obtain real time knowledge of the status of the hives under observation.

During the inspections, samples of bee matrices (dead bees, live bees, brood, honey, wax or pollen) are collected for laboratory (chemical, pathology and palinologic) tests. In order to compare the data obtained from the different modules and avoid subjective evaluation of the findings, the operators in charge of the modules have attended a training course designed to ensure that proper procedures are observed in data gathering, filling in the forms, utilising the IT platform, sampling and sample management (cataloguing, storage and shipping).

The activity of the national network is further supported by the work of other local monitoring activities, some of which are already active, while others are currently being activated, including:

- the monitoring network of protected nature areas, funded by the Ministry of the Environment and managed by ISPRA, activated in 4 areas (Veneto, Emilia Romagna, Tuscany, Lazio);
- the regional monitoring networks active in Lombardy, Tuscany, Friuli Venezia Giulia and Piedmont, together with further regional modules (eg. Umbria, Calabria) which will supplement those already in existence. The region of Veneto supports its own module with direct funding.

During the first year one module per region was activated. Each of these modules was composed of 5 apiaries of 10 hives each, with the exception of the following regions, which activated a monitoring programme of their own: Lombardy (50 apiaries), Friuli Venezia Giulia (10 apiaries) and Piedmont (10 apiaries).

In 2009 no cases of bee die-off or losses were reported, except for station CLB 2 of Rossano Calabro, which borders on the citrus-growing area of the Plane of Sibari. At this station an elevated die-off of bees was recorded concomitantly with citrus tree flowering. For winter 2009-2010, the mortality percentage recorded by the APENET monitoring network was 17.6% (113 dead hives out of 753). This figure is comparable with the national data obtained by the use of specific questionnaires, which gave a mortality percentage of 19.5% (2.437 dead hives out of 12.933) for the same period.

Analyses conducted to identify any pathogenic agents concentrated on *Nosema apis, Nosema ceranae* and viruses. Results showed endemic spread of the fungus (Microsporidia) *Nosema ceranae* throughout all Italian regions. This fungus has almost completely replaced the species previously present (*Nosema apis)*, with the exception of one apiary in the province of Bolzano, where both species were detected. Thus the investigation, which is still on-going, confirmed the first reports that date back to 2007 indicating the presence of *N. ceranae* in Italy as well. Findings obtained so far have allowed a clearer picture of the spread of this pest over the different areas of Italy.

Among viruses, the presence of Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV), Sacbrood Virus (SBV), Chronic Bee Paralysis Virus (CBPV) and Acute Bee Paralysis Virus (ABPV), either individually or in varying combinations, was confirmed by the analyses referring to the 2009 samples. In none of the hives on which analyses were performed was the presence of Apis Iridescent Virus (AIV), Kashmir Bee Virus (KBV) or Israeli Acute Paralysis Virus (IAPV) detected. Our findings show that the main bee viruses are present in Italy, similarly to their presence throughout Europe, but the presence of DWV and BQCV is particularly marked in Italy. It is important to note that this is the first nation-wide investigation based on biomolecular techniques undertaken in Italy to examine the presence of bee viruses. Previous studies, which date back to a considerable number of years ago, were not only limited to just a few regions, but were also based on electron microscope and serologic methods, which at that time were the only techniques available to test for the presence of these pathogens. The new knowledge acquired on bee virus distribution is of considerable interest and represents a valid starting point for further research.

The data obtained from the winter 2009-2010 inspections were used as the basis for chemical analyses on bee and wax samples, to test for residues of organophosphate, organochlorurate, carbamate and neonicotinoid pesticides, but no significant presence of these substances was detected. These findings indicate a favourable situation for the bee-keeping sector, apart from the acute die-off phenomena that occurred in specific individual situations, and which were brought to attention through the reporting system.

1.1 The reporting system

The monitoring network is further supported by the important tool of the reporting system, which makes it possible to notify the authorities of abnormal events occurring in hives even if the hives in question do not form part of the network. By means of the reporting system, bee-keepers send a notification of any abnormal mortality to the Veterinary Service of the Health District that exercises authority for their area. The Veterinary Service is then responsible for conducting a site inspection, collecting samples, ensuring appropriate storage (-20°C) and shipping the samples to the laboratory of the *Istituto Zooprofilattico Sperimentale delle Venezie* (IZSVe), where analyses will be performed in cooperation with the APENET network.

In the past, above all in the spring of 2008, the reports on population losses or hive mortality sent in by bee-keepers were of fundamental importance for identification and quantification of bee die-

offs attributable to coated maize seed sowing. In the spring of 2008 all 185 of the reports proved to have been concomitant with maize sowing, and of the 132 samples gathered and analyzed, 57.5% tested positive for the neonicotinoids used in maize seed coating. In 2009 three cases were reported, two of which were official and were submitted to the Veterinary Service during the maize sowing period, while the third was not submitted by the official route but reported directly to CRA-API. All three of these cases were found to be linked to non-authorized utilization of coated maize seed.

During the spring of 2009, in connection with reports that were not associated with maize sowing, another 7 samples were submitted to the Veterinary Services. Of these seven, 5 tested positive for neonicotinoids, but in contrast to the previous cases these events proved to have been caused by improper use of neonicotinoid sprays in orchards. For the other 2 samples the analyses did not detect the presence of residues.

With regard to the spring of 2010, reports (table 1) did not involve maize-growing areas. It should also be noted that in 16 out of the 21 cases reported, the Veterinary Services of the Local Health district (ASL) in charge of the given local area took action.

Table 1 – Number of reports submitted to the veterinary services in the spring of 2008, 2009 and 2010 in maize-growing and non maize-growing areas (Source IZSVe, in the Veneto regions).

Region	N. of reports in maize-growing areas			Reports in non maize-growing areas	
	Spring 2008	Spring 2009	Spring **2010**	Spring 2009	Spring **2010**
Lombardy	40	1	-		nd
Piedmont	8		-	2	nd
Emilia-Romagna	7	1 + 1*	-		2** + 3*
Veneto and Trentino	20		-	3	8***
Bolzano			-		2
Friuli Venezia Giulia	110		-	1	1
Calabria	0		-	1	1 + 2*
Basilicata			-		1
Sardinia					1
TOTAL	**185**	**2 + 1***	**0**	**7**	**16 + 5***

* Unofficial reports

** One of the 2 reports concerned a station of the Apenet network

*** In two cases, we detected presence as follows: 1. Bees: Thiametoxam, Penconazole; 2. Bees: Acetamiprid; Leaves: Acetamiprid, Iprodione, Tebuconazole; the analyses of the other samples are in progress.

nd = following direct contacts (2/7/10) with IZSPLV (Asti) and IZSLER (Brescia) no official communications of die-off were submitted.

1.2 Conclusions

The results outlined above, which highlight the doubling of the number of reports in 2010 as compared with 2009, indicate that the integrated monitoring-reporting system, configured according to the description presented here, is capable of providing adequate information on the health status of bee hives present in Italy, with particular reference to specific disease agents and residues of pesticides that may cause hive mortality. The surveys conducted so far have shown that in the absence of pesticide residues, the presence of *nosema* and bee viruses did not cause bee population decline or die-off of bees and hives. By contrast, reports of acute bee die-offs were associated with marked presence of pesticide residues.

2. Dust dispersal during coated maize seed sowing and estimated effects on bees

This research line focuses on:
- dustiness of maize seed coated with the 4 active ingredients investigated;
- quantification of dust and active ingredient deposited at ground level and dispersed in the air during sowing with the modified and unmodified pneumatic seeder;
- evaluation of the effects induced in bees by dust emitted during sowing;
- assessment of the productive and agronomic utility of maize seed coating;
- evaluation of persistence of active ingredients in soil and their translocation into plant parts.

Seed dustiness was evaluated by subjecting seed samples to the Heubach test.
Quantification of abrasion-induced dust dispersed during sowing was performed both at fixed points and during field tests, utilizing a pneumatic seeder equipped with a deflector pipe system.
Observations on bees were carried out during field sowing trials, by placing test cages around each trial field.
These activities are described in detail below.
The results on assessment of the productive and agronomic utility of seed coating, soil persistence of active ingredients and translocation of these ingredients into plant tissues are reported in chapter 4.

2.1 Seed dustiness test

According to the Heubach test Protocol (*Description of the Heubach method for the determination of the fine dust quantity of corn seeds treated with insecticides, Author: JKI Institute for Plant Protection in Agriculture and Grassland, Braunschweig, December 2008*), coated seed must be sampled at the source, from the direct flow up to packaging, and subsequently conditioned for at least 48h at constant temperature and relative humidity. The aim of the test is not to check whether the coated seed corresponds to the specifications declared by the manufacturer, but to provide data on the evolution of the product during its normal route from the manufacturing plant to the seed merchant. Such data are of use to the manufacturing industry itself.

In this context the 2010 dustiness test was repeated following the procedure adopted in 2009, on hybrid maize seed PR32G44 supplied by Pioneer Hi-Breed, coated with the same four active ingredients as used in 2009. Seed was delivered to CRA-ING on 3rd March 2010. The seed samples to be subjected to the Heubach test were immediately taken out of the package and stored according to the instructions given in the above Protocol. The test was performed on 17th March 2010. The results are shown in Table 2, where a comparison with the manufacturer's specifications is also shown.

The fine dust quantity (Table 2, column with bold lettering) , i.e. the dust retained by the Heubach cylinder filter and on which the evaluation was performed, was found to be lower than that declared by the manufacturer. The coarse dust fraction was also quantified. This fraction, not trapped by the filter, falls to the bottom of the Heubach cylinder glass filter, and it represents roughly 90% of the total dust extracted.

As a rough estimate, by associating quantity of fine dust and doses/seed (multiplying the corresponding values in Table 2), a ranking scale is obtained according to which the greatest quantity of abrasion dust-dispersed active ingredient is observed for clothianidin (with which a greater amount of dust is produced and a higher dose is applied), followed by imidacloprid, thiamethoxam and fipronil, in this order. Although these observations refer to laboratory conditions, which are likely to differ from operating conditions, they proved to be helpful in subsequent evaluation of the results obtained in the sowing trials.

Table 2 – Dustiness of seed coated with the 4 active ingredients, as measured with the Heubach cylinder.

Name of the commercial product (active ingredient)	Data supplied by manufacturer		Data detected by CRA-ING		
	Fine dust (Heubach filter) g/q	Acive ingredient dose. mg/seed	**Fine dust. (Heubach filter) g/q**	Coarse dust. g/q	Total dust. g/q
Gaucho (imidacloprid) + Celest	**1.100**	1.000	**0.875**	10.83	11.71
Poncho (clothianidin) + Celest	**2.430**	1.250	**1.833**	19.16	20.99
Cruiser (thiamethoxam) + Celest	**1.200**	0.600	**0.950**	5.00	5.95
Regent (fipronil) + Celest	**1.780**	0.500	**0.723**	9.08	9.81

Finally, the Heubach test was also carried out on a sample of trial seed from the 2009 trials, coated with clothianidin and taken from an intact package. The test gave a dustiness value of 1.248 g/q, analogous to that obtained in the test performed by CRA-ING during the 2009 trials (May 2009). This testifies to the stability of the seed coating over time, as was already noted in the conclusions to the report on 2009 activity. The same seed batch from which the above described 2009 sample was taken was also used for a "fixed point" sowing test, which allowed the seed to be collected in vessels placed beneath the seeding elements; the seed was then submitted again to the Heubach cylinder. In this case the result was 3.998 g/q. In a possible future study on this issue, the difference between the two values could represent a parameter to estimate both the type of "ill-treatment" the seed suffers as it passes through the seeder and coated seed resistance to such damage.

2.2 Fixed point Tests

The first experiences with fixed point seeders began to be carried out by CRA-ING at the beginning of 2008, on the assumption that these experiments would provide greater knowledge on the behaviour of the seeders and the above described type of damage inflicted on seed. Thus development of a fixed point system and evaluation of machines and devices designed to abate dispersal of abrasion dust constitutes part of the activities specified in the APENET research project. During the first year of the project, this activity could not be given top priority on account of the emergency conditions in which the network found itself operating. However, some procedures were developed for subsequent use in the second year of the project.

2.2.1 Aims

The main aim is to devise a system capable of setting up controllable and repeatable test conditions for conducting observations on the behaviour of pneumatic seeders equipped with or lacking modifications designed to abate the quantity of abrasion dust dispersed in the environment. This test system pursues the following goals:

- Determination of the concentration of the different active ingredients at ground level and in the air at various distances from the seeder, testing the machine both in standard conditions and also with any modifications that have been applied;

- Objective comparison among different machines, different modifications, etc., as the premise for a possible certification system concerning such machines.

2.2.2 Materials

A Gaspardo Magica six-row precision pneumatic seeder (planting layout 0.75m x 0.18m, 75000 seed /ha) (Figure 1-A), was used. The seeder was equipped with a system of 4 deflector pipes which, coupled two-by-two, channel the air expelled from the ventilator posteriorly to the two central coulters (Figures 1-B and C). Here the dust, screened by the two moldboards of the coulters, is channelled into the furrow and partially covered by soil after passage of the machine. This system was supplied together with the machine by the manufacturer. In contrast to a universal machine, this is a dedicated type. It is important to note that the very cumbersome dual pipe deflector supplied by Syngenta in 2009 could not be mounted on the Gaspardo Magica seeder, due to the particular structure and shape of the latter. The seeder was transported to the trial area, raised off the ground and coupled to a tractor, in order to drive the depressor by means of power take-off. The power take-off regime was regulated in such a manner as to obtain a depression of 45 mbar.

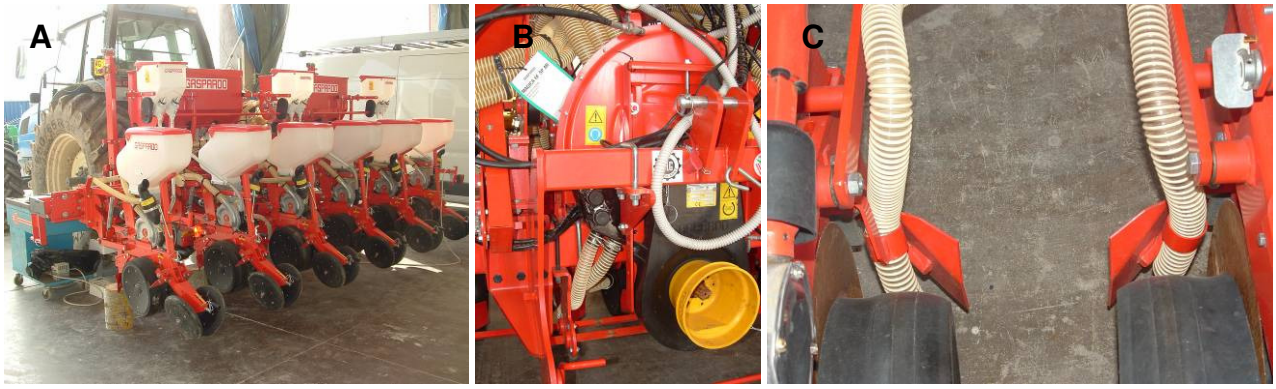


Figure 1 - A) Seeder utilized in the trials; B) detail of ventilator with air vent oriented downwards, on which the modification is applied; two of the four deflector pipes visible on the modification; C) two of the four deflector pipes that terminate behind the coulters.

Coated maize seed as described in point 1 was utilized. Prior to use, seed was subjected to the Heubach dustiness test. To power the seed distribution system from stationary, simulating field seeding speed, an electric engine coupled to the drive shaft that transmits motion from the drive wheel to the distribution organs was used (Figure 2-A). The speed of rotation of the drive wheel can be regulated as desired, by means of an inverter, in order to obtain the required peripheral speed (corresponding to working speed). The speed of 6 km/h was adopted. A trial surface made of concrete was used. For tests carried out with the modification, the screening effect of the coulter and the field soil conditions were simulated by placing plastic vessels, lined with wet jute sacks, immediately beneath the two central coulters in order to trap any dust dispersed by possible turbulence (Figure 2-B).

The trial area, represented diagrammatically in Figure 3, necessarily had to be protected. A long wide portico situated within the CRA-ING complex was used for this purpose, after carefully placing drapes to close the external side. Figure 4 shows a modified atomiser (in the lower left-hand corner) used to create controlled air speed conditions, with the seeder pointing downwind. Petri dishes and air samplers were placed at varying distances in the monitored area. Wind, temperature and humidity conditions were sampled continuously by specific instruments placed in the trial area.

Figure 2 – A) Electric motor to start up and drive the seeding device from stationary with the machine raised off the ground; B) a container lined with a wet jute sack was placed beneath the coulter in order to protect the dust against turbulence, simulating real field conditions.

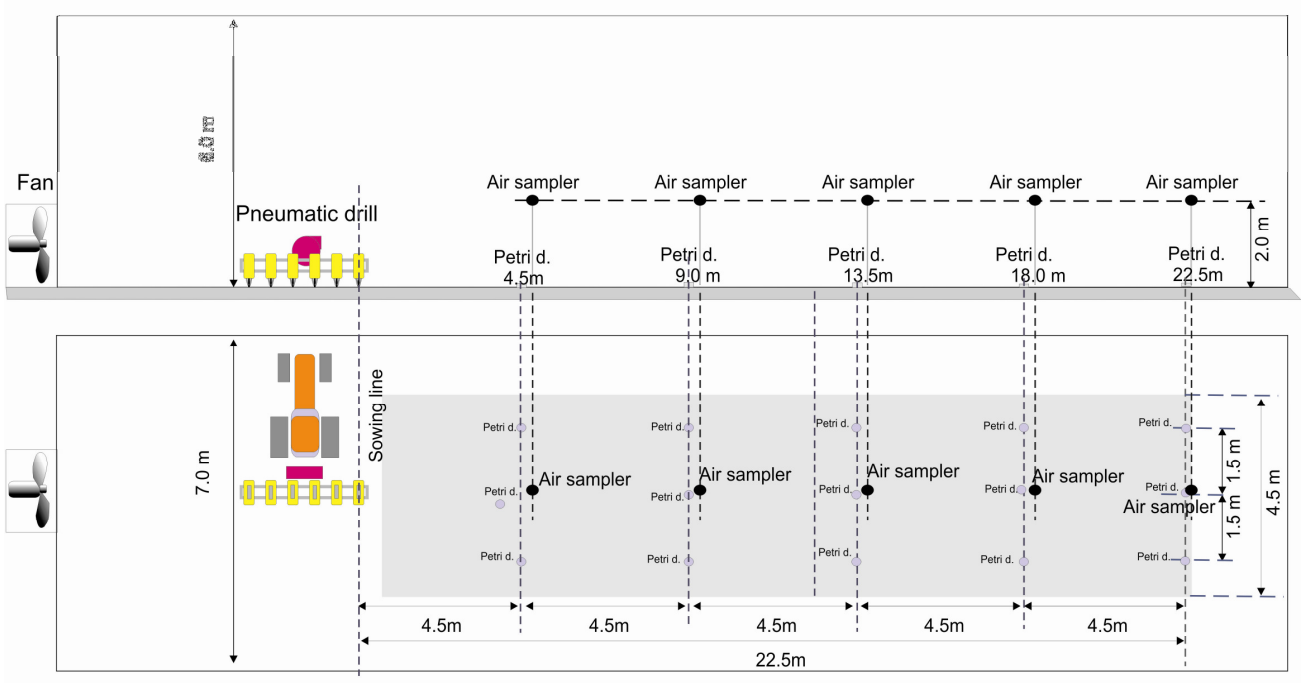


Figure 3 – Schematic of the layout of the fixed point trials. The Petri dishes were placed to the right of the seeder (pneumatic drill), at distances representing multiples of the sowing width (4.5 m, 9 m, 13.5 m, 18 m 22.5 m) along three rows spaced 1.5 m apart. The three air samplers were positioned along the central row at 5 m, 10 m and 20 m. The yellow area indicates the sampling zone. Its width of 4.5 m was determined by the distance of 1.5 m between the three rows. These measurements are important for processing the data. Ventilatore= ventilator; Seminatrice= seeder.

Figure 4 – Fixed point test zone. A) visualization of the seeder raised off the ground , and in the lower left-hand corner the atomizer utilized for wind generation; B) View from the opposite end of the portico, showing the Petri dishes placed on the ground and the air samplers at the various distances.

2.2.3 Methods

Preliminary tests were conducted in order to:

- Identify the distance between the ventilator and the seeder that can most satisfactorily guarantee uniformity of air speed throughout the trial area.
- Define the most suitable sampling distances from the seeder.
- Carry out an air speed mapping (gradient) in the trial area.
- Check repeatability of trial conditions.

Tests conducted

Seeder with and without air deflectors – In order to conduct observations on abrasion dust drift in the two conditions (seeder with and without air deflectors), for the controlled environment we adopted a procedure paralleling the framework proposed for field trials both in the work carried out by APENET (2009 Report) and also by Syngenta and Bayer (the latter makes reference to the document: *BBA Drift Guideline, Part VII, 2-1.1, 1992, "Measuring direct drift when applying liquid plant protection products outdoors*). However, we introduced some modifications concerning the controlled conditions. Thus in addition to a series of three Petri dishes, containing a solution of acetonitrile and water, placed at each distance (for determination of active ingredient concentration at ground level in the area downwind from the seeder), a series of three air samplers was placed near the Petri dishes, along the central row. The purpose of the air samplers was to determine active ingredient concentration in the air (ppb) at various distances. The details are shown in Figure 3. The sampling distances adopted for the Petri dishes were multiples of the seeder's working width. The rationale for this choice was to simplify data elaboration, by using a form of computation capable of estimating the pattern of active ingredient concentration that would be obtained in the field under conditions (working speed, wind speed and direction) corresponding to those of the fixed point simulation.

Utilization of the air samplers – The purpose of the air samplers is to provide information on dust drift in the air. There is no specific reference methodology for this type of sampling in an agricultural context as such tests are generally intended for air quality assessment in other work environments, in particular industrial environments. In the 2009 field tests, the capsules with the filters were placed at a sampling height of 1.7 m above the ground: this height was chosen on the basis of the specification *UNI CEN TR 15547:2007,* corresponding to the average air intake height of an operator breathing in this kind of environment. In 2010, to achieve greater correspondence with the typical conditions of bee flight, sampling height was raised to 2 m. Air filtration was carried out by means of 0.2 µm PTFE Millipore filter discs, with 37 mm diameter. For the sampling procedure, the three instruments were set at an air flow of 5 l/min.

The tests were conducted on the four active ingredients imidacloprid, clothianidin, thiamethoxam and fipronil. The seeder was tested with and without the dual pipe deflector modification. Furthermore, as stated in 2.2.2, the seeder was raised off the ground in order to allow the drive wheel to rotate at the speed of 6 km/h. Three repetitions were carried out for each test condition. For each repetition, the quantity of seed distributed was a dose (25000 seeds) corresponding to 3333 sq m. Sampling began at the start of sowing and continued for an additional 5 minutes after the end of sowing in order to allow dispersal/deposition of dust present in the air (in the field trials this additional time was 15 minutes). After each repetition, residues of dust and active ingredients were carefully cleaned away from the trial area by aspiration.

Sample treatment and analysis – The Petri dish samples were treated according to the instructions provided by the industry at the beginning of the APENET project. The acetonitrile solutions containing the dust deposited after the sowing trial were preserved in the freezer and away from light. Samples were analyzed at CRA-PAV of Rome according to the HPLC MS/MS method, applying the methodologies devised by the manufacturing companies for Imidacloprid and for the other active ingredients at the time of product registration. An analogous method was adopted for analysis of the air sampling filters.

Elaboration of the test results - The results of the analyses provide data on a theoretical seeded area measuring one-third of a hectare; the data are concentrated in a sampling area 4.5 m wide and 22.5 m long. Thus the expected quantities of active ingredient are very elevated. This partially explains why no observations on bees were set up during these trials, as the situation does not correspond to real exposure conditions.

Analyses were also conducted on the Petri dishes to determine the quantity of active ingredient contained in each dish (µg/dish). The quantity was measured firstly in relation to surface unit (µg/m2). Active ingredient concentration curves were then plotted on the basis of the means obtained for each sampling distance, showing the concentration trend with increasing distance from the sowing line, in the two trial conditions.

Through calculations based on (simulated) working speed, width of the sampling zone (yellow area in Figure 3) and trial duration, the theoretical number of runs performed by the seeder in front of the sampling area can be determined. Dividing the concentration per surface unit by this value, we obtain the quantity of active ingredient per sq m and per run (µg/m2 run), at the various distances that are multiples of 4.5 m. Since at each subsequent run the seeder moves a further 4.5 m away from the sampling area, the theoretical distribution in the field obtained after a certain number of runs can be reconstructed. Accordingly, sampling distances that were multiples of 4.5 m were adopted. For example, after three runs the expected total quantity of active ingredient at 4.5 m from the initial sowing line will be obtained from the sum of three values, referring respectively to concentration calculated at 4.4 m (first run), at 9 m (second run) and at 13.5 m (third run). In the present case, the number of runs (8) corresponding to a 36 m wide plot was taken as the reference. (It is worth noting that the *BBA Drift Guideline, Part VII, 2-1.1, 1992, "Measuring direct drift when applying liquid plant protection products outdoors"* prescribes that trials should be conducted on 3600 m plots having a width of 36 m. On the basis of this method, if only five sampling distances are available (up to 22.5 m), it is possible to reconstruct the distribution for no more than five runs. However, given the availability of the series of five mean values which describe the variation along the sampling area, utilizing the regression functions of each series, it becomes possible to calculate the probable concentrations referring to runs subsequent to the fifth, in order to obtain a picture of active ingredient distribution over a width of 50 m downwind from the sowing area, deriving from a total of 8 runs. Theoretically, this procedure can be applied to any number of runs and any distance from the sowing line.

The values obtained from this type of calculation were compared with those of the 2009 field trials in which wind conditions (direction and speed) were comparable to those of the fixed point tests.

Analyses were also performed on the air samplers, in order to determine the quantity of active ingredient intercepted by the filter (μg/filter). Taking into account the duration of sampling as well as air flow and density, active ingredient concentration in the air can be calculated in μg/kg (= ppb). The data from the three sampling points can be used to plot curves showing the pattern of air concentration with and without seeder modification. In this case, application of a method similar to the one just described for estimation of concentration at ground level in field conditions was not considered to be feasible.

2.2.4 Results

The arrangements adopted in setting up the trial system gave satisfactory results both as regards the facilities and the equipment utilized. Protection of the external side of the portico with movable drapes in order to block out the wind proved to be functional. Use of the atomizer modified in such a manner as to direct air exclusively towards the right-hand side made it possible to create constant and repeatable conditions of wind speed and direction. The only precaution required was a few minutes' wait prior to the simulated sowing test, thus allowing the air flow to become stabilized throughout the length of the tunnel.

Direction of the air flow was found to be consistently perpendicular to the direction of sowing. Table 3 shows the wind speed values recorded at various heights in various points of the tunnel.

Downstream from the seeder it was observed that wind speed at 2 m above the ground initially had a value of 2.5 m/s fairly close to the seeder, subsequently stabilizing at around 1.7 m/s along the whole sampling area. Greater variability was observed in the vicinity of the Petri dishes, close to the ground, due to the presence of the seeder itself and, above all, of the rear wheels of the tractor which, in particular, screened the dishes placed at 4.5 m, 9 m and 13.5 m of row nr. 3 . Wind speed at ground level was roughly 1.5 m/s. However, the value indicated was recurrent in the 2009 field trials.

Table 3 – Wind speed at various points and heights of the tunnel.

Position at which data were acquired [right-hand side of the seeder assumed as origin (=0) (m)]	**Wind speed at 1 m from the ground (m/s)**	**notes**	**Wind speed at ground level (m/s)**	**notes**
-4.5	3.75	On the left-hand side of the seeder towards the ventilator	-	
0	2.9	On the right-hand side of the seeder towards the trial area	1	Near the extremity of the right-hand deflector pipe

Position at which data were acquired [right-hand side of the seeder assumed as origin (=0) (m)]	**Wind speed at 2 m from the ground (m/s)**	**notes**	**Wind speed at ground level near the Petri dishes (m/s)**			**notes**
			row 1	row 2	row 3	

4.5	2.5	At the centre of the trial area		Row 2 is central and corresponds to the position of the seeding organs. Row 3 is located in a position corresponding to the rear wheels, by which it is screened: at the lower distances wind is absent and this affects the quantity of active ingredients recorded.
9	1.75			
13.5	1.75			
18	1.55			
22.5	1.7			

Results of the analyses –
The results deriving from analysis of the contents of the Petri dishes are given in Table 4, in terms of µg/ Petri dish, showing the percentage of abatement of concentration following use of the modification. Air concentration was analyzed through a similar procedure. Mean values of active ingredient content in the air were decidedly lower when the modification was used, except for the case of fipronil, in which an increase was detected at the distance of 20 m from the sowing line.

Table 4 – Comparison of the quantities of the different active ingredients intercepted by the Petri dishes using the modified and unmodified seeder (mean ± standard error, n = 9).

Distance from the sowing line (m)	Concentration of **imidacloprid** at ground level (µg/ Petri dish) (mean ± standard error)		Reduction in concentration at ground level (%)
	Unmodified seeder	Modified seeder	
4.5	2.82 ± 0.56	0.99 ± 0.15	65%
9	1.88 ± 0.34	0.35 ± 0.05	82%
13.5	1.06 ± 0.13	0.25 ± 0.06	76%
18	0.85 ± 0.15	0.31 ± 0.07	63%
22.5	0.72 ± 0.12	0.09 ± 0.01	87%

Distance from the sowing line (m)	Concentration of **clothianidin** at ground level (µg/ Petri dish) (mean ± standard error)		Reduction in concentration at ground level (%)
	Unmodified seeder	Modified seeder	
4.5	5.74 ± 1.00	2.74 ± 0.63	52%
9	3.20 ± 0.30	1.10 ± 0.15	66%
13.5	2.19 ± 0.22	0.96 ± 0.16	56%
18	1.45 ± 0.07	0.86 ± 0.08	41%
22.5	1.00 ± 0.07	0.81 ± 0.06	20%

Distance from the sowing line (m)	Concentration of **thiamethoxam** at ground level (µg/ Petri dish)	Reduction in concentration

	(mean ± standard error)		at ground level (%)
	Unmodified seeder	Modified seeder	
4.5	2.75 ± 0.57	1.95 ± 0.55	29%
9	2.27 ± 0.26	1.28 ± 0.12	44%
13.5	1.20 ± 0.26	0.53 ± 0.10	56%
18	1.56 ± 0.14	0.52 ± 0.04	66%
22.5	0.41 ± 0.06	0.25 ± 0.03	39%

Distance from the sowing line (m)	Concentration of **fipronil** at ground level (µg/ Petri dish) (mean ± standard error)		Reduction in concentration at ground level (%)
	Unmodified seeder	Modified seeder	
4.5	4.30 ± 0.68	1.54 ± 0.32	64%
9	2.56 ± 0.25	0.99 ± 0.12	61%
13.5	1.28 ± 0.11	0.67 ± 0.05	48%
18	0.48 ± 0.03	0.27 ± 0.04	44%
22.5	0.28 ± 0.03	0.14 ± 0.02	50%

The data used in compiling Table 4 were submitted to factorial analysis of variance for each sampling distance. Factors of variation included position (row 1. 2.3) of the Petri dishes at each distance (Table 3). presence or absence of modification of the seeder and the interaction between these two factors. The results of the analyses are shown in Table 5.

Table 5 – Results of the analysis of variance carried out on the data acquired at ground level in the fixed point tests. Translation of text within table: *Analisi della varianza – Grado di significatività alle varie distanze* = Analysis of variance – Degree of significance at the various distances; *cause variazione* = causes of variation; *F tavole* = tabular value of F; *Ris*. = Result; *Ripetizioni* = Repetitions; *Posizioni* = Positions; *Sem M* = Modified seeder; *Sem NM* = Non modified seeder; *Interaz. Sem (Macch) / Pos* = Interaction Seeder / Position. Pink: not significant; Light green: significant at p=0.05; Dark green: significant at p=0.01. Commas indicate decimal separation.

cause variazione		Analisi della varianza - Grado di significatività alle varie distanze																			
		4,5 m				9 m				13,5 m				18 m				22,5 m			
		F calc	F tavole		Ris.	F calc	F tavole		Ris.	F calc	F tavole		Ris.	F calc	F tavole		Ris.	F calc	F tavole		Ris.
			p = 0,05	p = 0,01			p = 0,05	p = 0,01			p = 0,05	p = 0,01			p = 0,05	p = 0,01			p = 0,05	p = 0,01	
Clothianidin	Ripetizioni (3)	0,24	19,39	99,40		2,76	19,39	99,40		2,46	19,39	99,40		2,93	19,39	99,40		1,19	19,39	99,40	
	Posizioni (3)	9,64	4,10	7,56		22,09	4,10	7,56		9,03	4,10	7,56		0,96	4,10	7,56		0,83	4,10	7,56	
	Sem M - Sem NM	13,82	4,96	9,65		171,59	4,96	9,65		59,92	4,96	9,65		36,63	4,96	9,65		4,51	4,96	9,65	
	Interaz. Sem. / Pos.	2,19	4,10	7,56		6,39	4,10	7,56		7,15	4,10	7,56		0,20	4,10	7,56		0,14	4,10	7,56	
imidacloprid	ripetizioni	0,24	19,39	99,40		2,76	19,39	99,40		2,46	19,39	99,40		2,93	19,39	99,40		1,19	19,39	99,40	
	posizioni	9,64	4,10	7,56		22,09	4,10	7,56		9,03	4,10	7,56		0,96	4,10	7,56		0,83	4,10	7,56	
	MM / MNM	13,82	4,96	9,65		171,59	4,96	9,65		59,92	4,96	9,65		36,63	4,96	9,65		4,51	4,96	9,65	
	int. Macch./Pos.	2,19	4,10	7,56		6,39	4,10	7,56		7,15	4,10	7,56		0,20	4,10	7,56		0,14	4,10	7,56	
thiametoxam	ripetizioni	1,18	19,39	99,40		1,31	19,39	99,40		0,87	19,39	99,40		0,20	19,39	99,40		2,48	19,39	99,40	
	posizioni	8,14	4,10	7,56		7,85	4,10	7,56		17,28	4,10	7,56		1,78	4,10	7,56		0,26	4,10	7,56	
	MM / MNM	2,88	4,96	9,65		27,26	4,96	9,65		15,43	4,96	9,65		50,68	4,96	9,65		6,37	4,96	9,65	
	int. Macch./Pos.	0,56	4,10	7,56		4,76	4,10	7,56		5,69	4,10	7,56		0,85	4,10	7,56		0,09	4,10	7,56	
fipronil	ripetizioni	2,14	19,39	99,40		1,17	19,39	99,40		0,88	19,39	99,40		0,42	19,39	99,40		0,92	19,39	99,40	
	posizioni	12,90	4,10	7,56		6,86	4,10	7,56		3,97	4,10	7,56		0,55	4,10	7,56		0,25	4,10	7,56	
	MM / MNM	44,04	4,96	9,65		56,01	4,96	9,65		34,96	4,96	9,65		14,31	4,96	9,65		11,65	4,96	9,65	
	int. Macch./Pos.	2,10	4,10	7,56		0,67	4,10	7,56		0,16	4,10	7,56		0,22	4,10	7,56		0,72	4,10	7,56	

Legenda
non significativo
significativo per p=0,05
significativo per p=0,01

The results shown in Table 5 have the following implications.

Firstly, variability linked to the repetitions was never significant, thereby confirming the robustness of the test as regards the actual test conditions;

Secondly, the position of the Petri dishes was an important factor of variability (often being responsible for differences that were 99% significant). The weight of this factor tended to decrease with increasing distance, confirming the description given above on the screening action of the seeder itself and above all of the tractor.

Finally, the differences attributable to the presence of the modification were almost always highly significant. The exception was the case of thiamethoxam at 4.5 m (in which very elevated variability was observed, linked to position, which proved to be only scantily affected by the modification). With increasing sampling distance, the differences gradually faded, and thus were non significant for clothianidin at 22.5 m, although they were 95% significant for thiamethoxam, again at 22.5 m.

It can therefore be concluded that from a statistical point of view as well, the modification applied to the seeder proved to be a significant factor influencing active ingredient concentration levels on the ground.

Air concentration data were processed according to the same procedure as previously adopted for concentration on the ground. Results are given in Tables 6 and 7. In virtually all cases the quantities of active ingredients in the air were found to be decidedly lower when the modified seeder was used, except for fipronil, which, at 20 m from the sowing line, showed an increase (Table 6). From the statistical point of view, differences were significant (analysis of variance, Table 7) only in the case of clothianidin (5 m and m) and thiamethoxam (10 m), probably on account of the low number of values available.

Table 6 –Comparison of the quantities of the different active ingredients intercepted by the air samplers with the modified and unmodified seeder (mean ± standard error, n = 3).

Distance from the sowing line (m)	Air concentration of **imidacloprid** (ppb) (mean ± standard error)		Abatement percentage
	Unmodified seeder	Modified seeder	
5	2.31 ± 0.65	1.58 ± 0.28	32%
10	1.39 ± 0.08	1.00 ± 0.35	28%
20	1.25 ± 0.30	0.99 ± 0.30	21%

Distance from the sowing line (m)	Air concentration of **clothianidin** (ppb) (mean ± standard error)		Abatement percentage
	Unmodified seeder	Modified seederr	
5	5.17 ± 0.27	1.16 ± 0.44	78%
10	3.18 ± 0.39	1.03 ± 0.05	68%
20	1.63 ± 0.12	1.12 ± 0.27	32%

Distance from the sowing line (m)	Air concentration of **thiamethoxam** (ppb) (mean ± standard error)		Abatement percentage
	Unmodified seeder	Modified seederr	
5	2.94 ± 0.45	2.12 ± 0.29	28%
10	2.67 ± 0.55	1.80 ± 0.57	33%
20	1.92 ± 0.29	1.35 ± 0.20	30%

Distance from the sowing line (m)	Air concentration of **fipronil** (ppb) (mean ± standard error)		Abatement percentage
	Unmodified seeder	Modified seeder	
5	6.80 ± 0.40	5.96 ± 0.08	12%
10	6.45 ± 0.12	6.34 ± 0.87	2%
20	5.03 ± 0.33	5.88 ± 1.10	-17%

Table 7 – Results of analysis of variance performed on air sampling filter data in the fixed point tests. Translation of text within table: *Analisi della varianza – Grado di significatività alle varie distanze* = Analysis of variance – Degree of significance at the various distances; *cause variazione* = cause of variation; *F tavole* = tabular value of F; *Ris.* = Result; *Ripetizioni* = Repetitions; *MM* = Modified seeder; *MNM* = Non modified seeder. Pink: not significant; Light green: significant at p=0.05; Dark green: significant at p=0.01. Commas indicate decimal separation.

Analisi della varianza - Grado di significatività alle varie distanze												
cause variazione		5 m				10 m				20 m			
		F calc	F tavole p = 0,05	F tavole p = 0,01	Ris.	F calc	F tavole p = 0,05	F tavole p = 0,01	Ris.	F calc	F tavole p = 0,05	F tavole p = 0,01	Ris.
Clothianidin	ripetizioni	17,56	200,00	4999,00		1,52	200,00	4999,00		1,11	200,00	4999,00	
	MM / MNM	554,30	18,51	98,49		38,29	18,51	98,49		3,22	18,51	98,49	
Imidacloprid	ripetizioni	0,18	200,00	4999,00		1,82	200,00	4999,00		1,00	200,00	4999,00	
	MM / MNM	0,63	18,51	98,49		1,61	18,51	98,49		10,65	18,51	98,49	
Thiametoxam	ripetizioni	0,05	200,00	4999,00		27,33	200,00	4999,00		3,32	200,00	4999,00	
	MM / MNM	1,23	18,51	98,49		39,51	18,51	98,49		16,71	18,51	98,49	
Fipronil	ripetizioni	0,70	200,00	4999,00		0,57	200,00	4999,00		1,75	200,00	4999,00	
	MM / MNM	3,71	18,51	98,49		0,01	18,51	98,49		0,74	18,51	98,49	

Legenda
non significativo
significativo per p=0,05
significativo per p=0,01

The values shown in Tables 4 and 6 were computed in order to plot the diagrams describing ground level and air concentration trends in the different test conditions.

In Figure 5, for all the active ingredients investigated, with or without utilization of the seeder modification, the content of the Petri dishes was examined in relation to surface unit (μg/m^2). As stated earlier (section 2.2.3), values refer to a seed quantity corresponding to 1/3 hectare, with seeding concentrated in the sampling area. Consequently, active ingredient values were very elevated, a result that serves to highlight the outcome in the area downwind from the seeder, in our test conditions. In particular, the differences between the quantities observed with and without the seeder modification were clearly noticeable. The % reduction in active ingredient concentration observed along the entire sampling distance was on average 50% with the seeder modification (>50 % for imidacloprid and fipronil; <50% for thiamethoxam). Details of the values are as shown in Table 4.

The precision curves of ground level active ingredient distribution in field conditions were plotted on the basis of the data of Figure 5, and subsequently also of the analysis of variance as described in the previous section (2.2.4). According to our data elaboration, Figure 6 shows a good representation of the results that would be expected on plots similar to those described in the Agrofarma trials (surface area: 3600 m^2; side: 36 m; number of runs: 8) at the same wind and working speed.

In all cases, the pattern of concentration calculated proved to be more regular than appears from Figure 5, as the various runs are overlaid on one another in the figure. The reduction in concentration with use of the seeder modification was clearly noticeable, maintaining around 50%, as indicated in Fig. 5. Comparing the values observed for each active ingredient, the dose/seed and dustiness values reflect the trends shown in Table 2. These two parameters were higher for clothianidin, followed by imidacloprid and then by the other two active ingredients, which presented values similar to each other. The same ranking was found for calculation of concentration values (Figure 6).

With regard to the reliability of expected concentration values, the results were clearly of the same order of magnitude as the 2009 values, although they were generally higher above all for

imidacloprid (5.8 µg/m^2 as against 4.2 µg/m^2 in 2009 at 5 m) and clothianidin (9.2 µg/m^2 as against 4.5 µg/m^2 at 5 m in 2009).

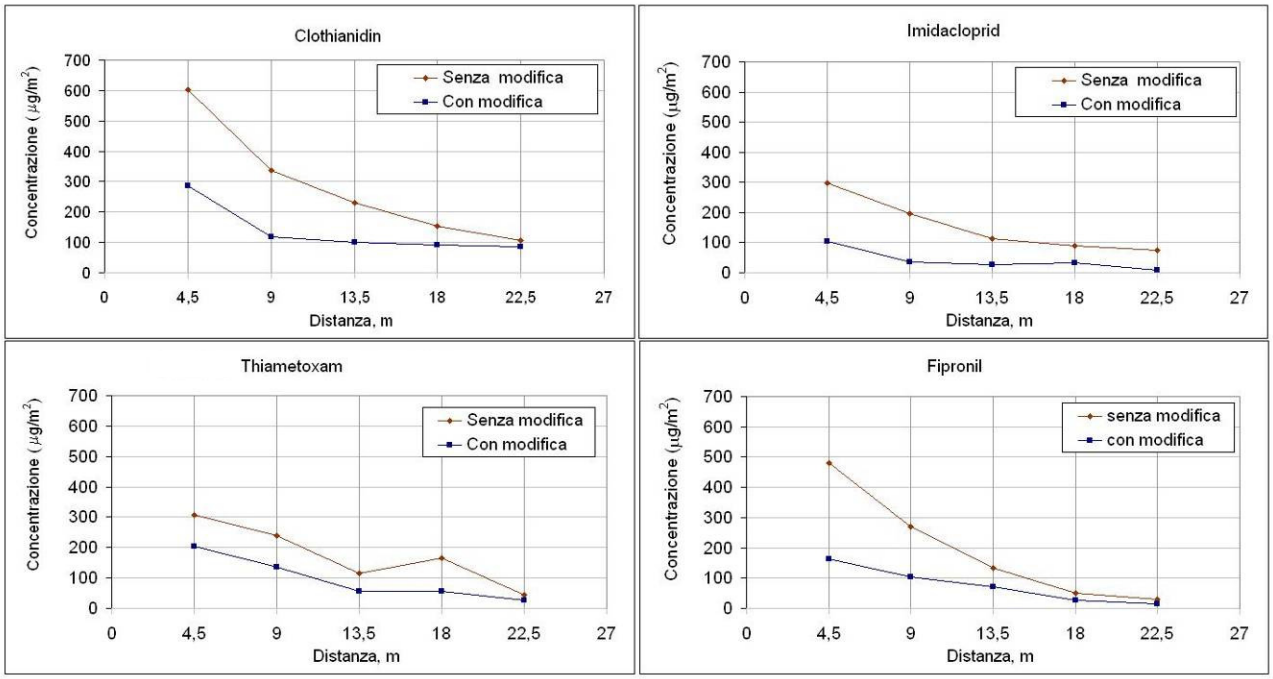


Figure 5 –Results of the fixed point test: quantity of active ingredient in the Petri dishes at the various distances from the seeder, in relation to the surface unit (m^2). Translation of text within the figure: *Senza modifica* = Without modification [red line]; *Con modifica* = With modification [black line]; *Concentrazione* = Concentration.

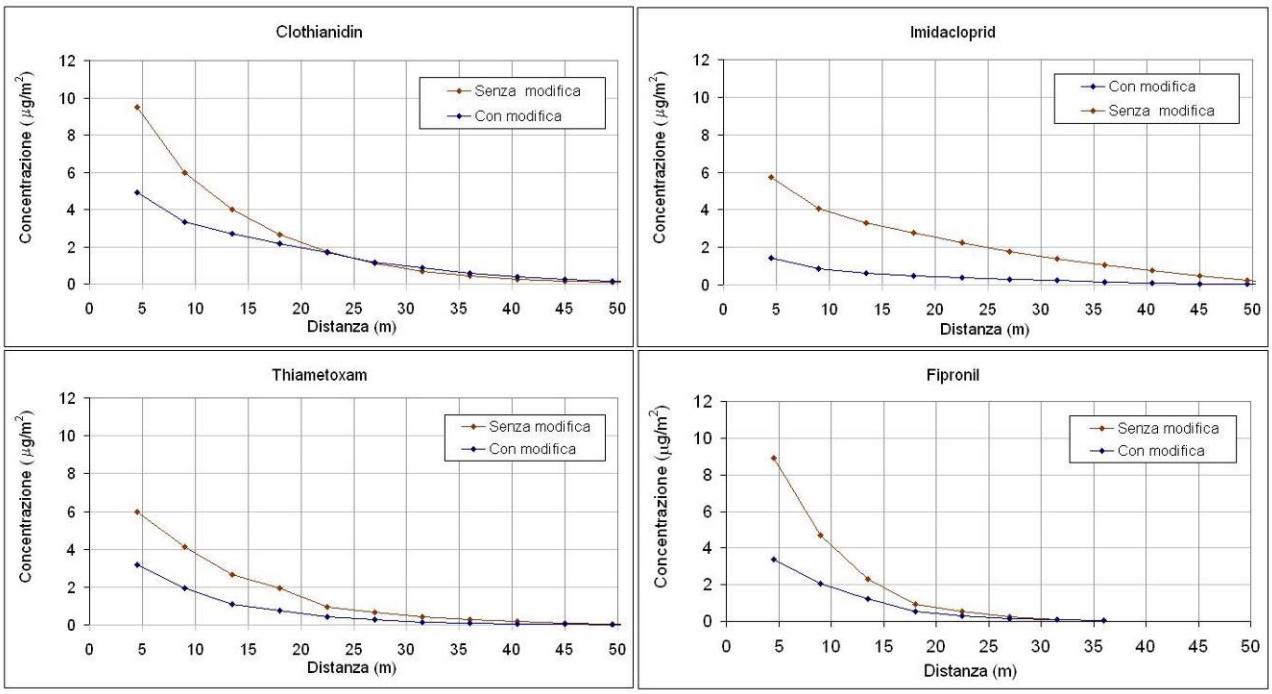


Figure 6 –Results of the fixed point test: expected ground level concentration in field conditions, evaluated on the basis of the data shown in Figure 5 up to a theoretical distance of 50 m from the sowing field. Curves refer to 8 runs of the seeder (corresponding to 36 m sowing width) at 6 km/h sowing speed, with a mean wind speed of 1.5 m/s at ground level and 1.85 m/s at 2 m above the ground. Translation of text within the figure: *Senza modifica* = Without modification [red line]; *Con modifica* = With modification [black line]; *Concentrazione* = Concentration; *Distanza* = Distance.

However, it is reasonable to assume that the concentrations observed in the field trials of the previous years, which were obtained in less favourable circumstances than the fixed point trials in terms of wind speed and direction, may have been underestimated as compared to real concentrations. This is particularly likely to happen if wind conditions change while the field trial is on-going: in such a case, the single side submitted to sampling may effectively not be the side most greatly affected by dust.

The possibility of controlling and repeating the fixed point test conditions not only allows a reliable estimate of ground level concentrations, but also leads to a more objective and more precise comparative evaluation of active ingredient concentrations. In this perspective the results obtained in 2009, which suggested a roughly 50% abatement of the quantity of active ingredient dispersed, were substantially confirmed.

Finally, Figure 7 shows the trend of active ingredient concentration in the air. A reduction in concentration was observed concurrently with utilization of the seeder modification. The extent of reduction was variable, being very marked for clothianidin (80% at 5 m) and roughly 30% for imidacloprid and thiamethoxam. For fipronil a variable pattern was found. These different trends could be attributed to factors such as difference in active ingredient content in dust generated by each active ingredient, and the possible different granulometry of dust generated. (For example, the four active ingredients may interact differently with the seed coating process and this could favour the formation of coarser or finer dust, in which case the different grades of dust could be deposited with a difference in time lag, or closer or further away.) Such factors may influence drift dust behaviour, and a specific study on these aspects should therefore be conducted. The values shown in Figure 7 were on average 10 times higher than air concentrations detected in the 2009 field tests at 5 m and 10 m. In the case of the results of the analyses of the air samplers filters, we did not carry out a computation designed to obtain air concentrations in field conditions, similarly to the procedure described for ground level concentrations in Figure 6, as the presuppositions were different. In this respect, the fixed point test system once again constitutes a sort of amplifier of the effects and differences between the modified and unmodified seeder, and it therefore provides helpful information.

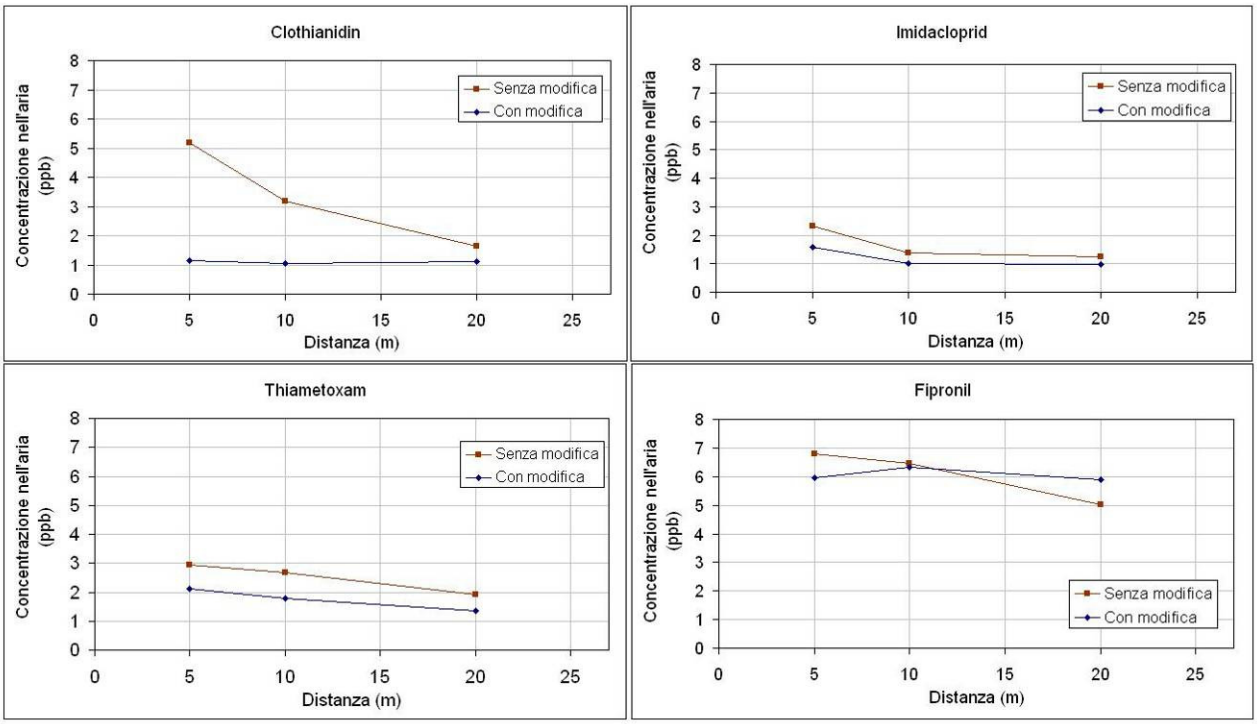


Figure 7 – Results of the fixed point test: pattern of active ingredient concentration in the sampling zone at different distances from the sowing area. Translation of text within the figure: *Senza modifica* = Without

modification [red line]; *Con modifica* = With modification [black line]; *Concentrazione nell'aria* = Air concentration.

2.3 Field sowing trials

The setup used in this series of trials mirrors that applied in trials conducted by the agricultural industry in agrobusiness field trials, which in turn were taken over from a methodology designed to study liquid pesticide drift (*BBA Drift Guideline, Part VII, 2-1.1, 1992, "Measuring direct drift when applying liquid plant protection products outdoors*). The modifications introduced compared to this model were due to the need to minimize the influence of environmental factors and to complete the tests in as short a time as possible, and thus to limit the number of analyses required without compromising the significance of the results, in order to furnish the project details within the deadline specified by the Ministries responsible for this sector (MiPAAF, Ministry of Health).
On the basis of the 2009 experiences, the following considerations can be put forward:

- Rigidity of the test system conducted in a single operation (hives + Petri dishes) , given the potential variability of meteorological conditions. With the hives placed around the trial plots, it is not possible to orient the plots themselves and the sowing direction on the basis of wind direction, and it is not always feasible to postpone the trial.
- *Study of drift without the presence of hives* removes the need to sow in periods and zones that are significant for the presence of flowers. Thus in this case the study can be carried out theoretically at any time provided the temperature conditions are compatible with the biology of the caged and appropriately fed bees.
- All tests on *abrasion dust drift* conducted in 2009 were carried out on small plots (1600 m^2). We believe this size is insufficient for a study of the effects on bees (both bees housed in cages and colony bees), and that to conduct this test the plot size should be significantly increased. The increased surface area may also be helpful for study of drift (better quality of data on active ingredient concentration at ground level and in the air), which would be examined in conditions closer to real sowing circumstances.

2.3.1 Aims

- Evaluation of the extent of dust abatement after application of modifications to the machine, in field conditions (validation of the fixed point test system);
- determination of ground level distribution of the active ingredients at different distances from the sowing field;
- determination of active ingredient uptake by bees restrained in monitoring cages positioned close to the Petri dishes, and the relation between uptake and active ingredient concentration in the vicinity of the dishes.
- individuation of the corresponding active ingredient concentration in the air.

2.3.2 Materials and methods

With regard to the seeder and the seed batches, readers are referred to the description concerning the fixed point tests (row spacing 75 cm, and spacing at 18 cm along the row: 75000 seeds/ha). .

The effects of modifications applied to the seeder were evaluated in field trials, in which ground dispersion of abrasion dust (with the corresponding active ingredient content) was studied after field sowing trials. The effects induced in bees housed in the monitoring cages and exposed to the same substances were also studied.

Since the drift effect is influenced by the presence of wind (speed and direction), the layout shown in Figure 8 was adopted in order to limit these effects. Thus for each active ingredient, a single large field measuring 3-4 ha was used for sowing. This arrangement was chosen because the size in question was felt to give more significant results in determining ground-level active ingredients and possible contamination of the surrounding area than was the case with the previous plots. The

sampling area for determination of ground-level active ingredient concentration was represented by a 20 m band surrounding the large plot. With regard to investigation of active ingredient concentration in the air, only three air samplers were available. These were placed at the same distances as the Petri dishes, on the side which was downwind at the moment of performing the trial. The air flow setting on the samplers was regulated to an air flow of 4.5 l/min. The trials were partly (4 out of 8) conducted on land made available by CRAF-PCM, bordering on CRA-ING of Monterotondo. Maize cropping on the plot in question was carried out by the CRA-PCM farm in accordance with normal operations; the farm operators also ensured that irrigation and normal crop management practices were applied.

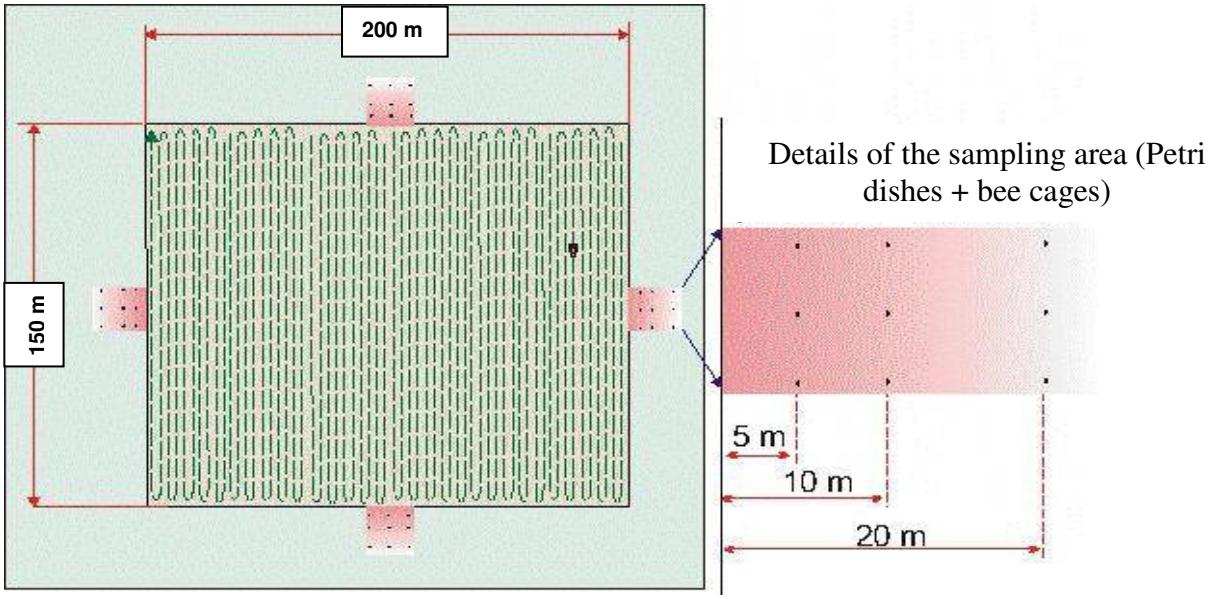


Figure 8 –Example of trial plot. The sampling area was extended to all sides of the seeded plot, the surface area of which was extended in order to observe concentration levels deriving from real sowing operations.

Each trial field had an area of 3 ha and measured 200 m in length by 150 m in width.
Sowing speed was 6 km/h. Data acquisition and sampling began at the start of sowing, which on average took 80 min. Acquisition then continued for a further 20 min after the end of sowing to allow dust deposition /dispersal. During this overall time period of roughly 100 minutes, the samplers filtered an average of 450 l of air. The resulting air flow, equal to 4.5 l/min, was the same as applied in the 2009 trials, in which roughly 100 l of air were sampled over a time span of approximately 22 minutes.

The Petri dishes were positioned on all four sides; three samplers were used for each of the three distances (5 m, 10 m, 20 m) from the sowing field. Another two Petri dishes containing known solutions of active ingredient and maintained at a safe distance from the sowing field were used as controls. Thus a total of 38 Petri dishes were utilized. Management and analysis of the samples was entrusted to CRA-PAV of Rome, similarly to the procedures described for the fixed point tests (section 2.2). During each test, and with due consideration of temperature, wind and the considerable duration of the test, the Petri dishes were carefully topped up with acetonitrile solution in order to prevent them from drying out in consequence of evaporation.

For the analysis of the content of the Petri dishes, readers are referred to the earlier description of the fixed point tests. (section 2.2.3).

To conduct observations on bees, small cages containing adult bees were placed at the four sides of the sowing area. The cages were sampled and arranged according to the following criteria:

- choice of a colony presenting good health and development status;

- for each small cage, insertion of 30-40 adult forager bees of the same age, collected from the frames of the supers;
- the cages were placed on the 4 sides of the trial sowing area at the pre-established distances of 5-10-20 meters, next to each Petri dish. For each of the 4 sides, 3 cages were placed at each distance (for a total of 9 cages per side); during the observation period the bees restrained inside each cage were fed with 1:1 sucrose syrup contained within a small dispenser (Figure 9);
- 9 control cages were placed in the same conditions as described for the test bees in the trial sowing area, but sufficiently distant to protect them from contact with sowing dust;
- roughly 20' after the end of sowing the cages were collected and placed in a climate-controlled environment at 25°C with adequate relative humidity;
- bee mortality inside the cages was evaluated up to 48 h after sowing. Mortality percentage was then calculated and subjected to the Mann-Whitney non-parametric test and, after angular transformation, to analysis of variance using Statistica® Software.

Figure 9 – From the left: cage with feeder dispenser, containing roughly 30 bees; small cage on the ground beside a Petri dish during one of the sowing trials; anemometer recording wind speed and direction, and air sampler capsule containing the filter.

At the end of the 48-h period, the bees were placed in the freezer pending the analysis testing for active ingredients.

For each trial condition (machine with and without modification; four active ingredients) the pattern of active ingredient deposition around the seeded field was investigated, taking into consideration wind speed and direction. The negative effects of wind speed and direction can be partially limited by the availability of data pertaining to all four sides of the sowing field, with particular attention to the downwind side. Ground-level concentration was expressed in micrograms of active ingredient per square meter of land ($\mu g/m^2$).

The starting date for the trials was originally set for the beginning of March. Due to unfavourable climatic conditions, access to the fields was not feasible until April 18, 19 and 20. Subsequently, rain caused a further suspension of activity until June 8. The field stage then terminated on June 16.

2.3.3 Results

Active ingredient concentrations detected at ground level and in the air

Table 8 shows the mean values of the main environmental parameters recorded during the field trials. Similarly to 2009, wind was found to be the most variable factor, both in direction and speed. Thus it was found to be useful to perform ground level sampling on all sides of the trial field.

Table 8 - Main environmental data during the sowing trial involving the four active ingredients. Data were obtained from the central meteorological unit of CRA-ING integrated with field anemometrical measurements. All data refer to the actual conditions in the trial fields.

Active ingredient	Modification (yes/no)	Sowing date	Mean temp. (°C)	R.H.. (%)	Wind speed (m/s)	Wind direction	Note
clothianidin	yes	19-Apr-10	20.8	85	1.4	from N and NW	Wind direction & speed fairly constant
	no	20- Apr -10	21.7	75	1.6	from N and NNW	Wind direction & speed fairly constant
thiamethoxam	no	21- Apr -10	21.4	65	2.24	from S and SSE	Wind direction & speed fairly constant
	yes	07-June-10	24.3	67	1.03	from S and E	Direction fairly constant. low wind
imidacloprid	yes	08- June -10	27.9	63	1.47	from S. SW. NE and NW	Wind direction very variable
	no	14- June -10	29.67	56	2.05	from SSE. S. SSW	Wind direction fairly constant
fipronil	no	15- June -10	25.31	80	2.5	from S and SO	Wind direction & speed constant
	yes	16- June -10	28.4	60	2	from S and SW	Wind direction & speed constant

The results of analyses on the content of the Petri dishes, in $\mu g/m^2$, are shown in Table 9 in terms of means ± standard error of the values observed on each side, and as a general mean ± standard error for each sampling distance. The columns in blue typeface correspond to the downwind sides that are most exposed to drift. The results are partial as some analyses are still in progress.

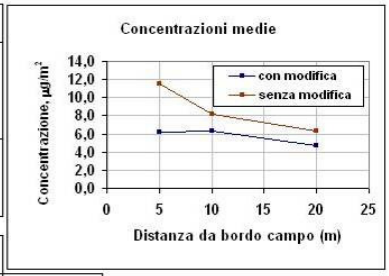
The first noteworthy finding is that the downwind sides did not always receive a greater quantity of active ingredient. For example, in both trials with clothianidin the Northern side, which was facing upwind, received the greatest concentration. This could be explained by the fact that if wind speed is low, and possibly also if certain types of sowing practices are adopted (depending on the shape of the field, the type of manoeuvres to be performed, sowing the side of the field, etc.), the wind may be unable to disperse the dust that has been raised around the sampling area.

In the case of imidacloprid, the drift pattern was more in line with expectations. Although generalizations on distribution in relation to wind direction are unreliable, due to the marked variability of wind variation, general means deriving from observations on the four sides gave useful information. The values shown in Table 9 were also used to plot the diagrams pertaining to each active ingredient, in order to given an immediate idea of the pattern of concentrations in relation to use of the deflector.

According to the data available so far, percent differences associated with the presence or absence of the modification were analogous to those recorded for the fixed point tests performed on the same active ingredients. These results confirm the outcome of the 2009 field tests and of the above described fixed point tests (section 2.4), which showed that the deflector pipe modifications reduced the quality of dispersed active ingredients by an average of 50%, although this value was also dependent on seed quality, which in turn was influenced by the coating treatment.

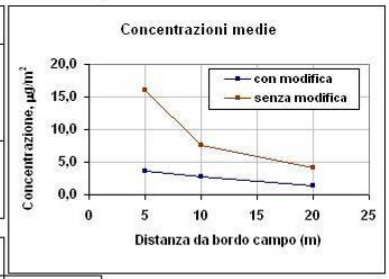
Table 9 - Results of field trials: mean values and corresponding standard errors of concentrations along the four sides, at the three sowing distances, and overall mean and standard error for each distance. The diagrams show the pattern of the means. Columns in blue typeface refer to the downwind sides of the trial field. In the fields sown with thiamethoxam and fipronil-treated seed, some mean values are shown in red typeface: taking into account that the results of certain analyses were lower than LOQ (namely 0.015 and 0.001 µg/dish for thiamethoxam and fipronil respectively), it was decided that merely eliminating such values would not constitute a correct procedure. Instead, they were assigned the value of the LOQ itself. Translation of text within the figure: *direzione vento* = wind direction; *velocità* = speed; *con modifica* = with modification; *Distanza da bordo campo* = Distance from field border; *Concentrazione lato Nord* = concentration on the Northen side; *Concentrazione lato Sud* = concentration on the Southern side; *Concentrazione lato Est* = concentration on the Eastern side; *Concentrazione lato Ovest* = concentration on the Western side; *media generale* = overall average; *media* = mean value; *Err. St.* = Standard Error; *senza modifica* = without modification; *Concentrazioni medie* = average concentrations; *Concentrazione* = concentration. Commas indicate decimal separation.

direzione vento velocità (m/s)	da NO 1-1,5	clothianidin con modifica									
Distanza da bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)		
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.
5	8,979	±	1,828	4,875	±	0,564	6,770	±	0,879	4,384	±	0,672	6,252	±	0,832
10	7,752	±	1,246	5,121	±	0,624	7,313	±	0,215	5,437	±	0,246	6,323	±	0,547
20	4,490	±	0,414	3,788	±	0,438	5,858	±	1,094	4,735	±	0,425	4,718	±	0,416



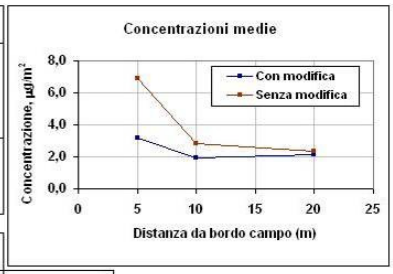
direzione vento velocità (m/s)	da N e NE 1-1,5	clothianidin senza modifica										differenza con /senza mdoifica (%)
Distanza dal bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)			
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	
5	15,223	±	1,875	9,085	±	3,962	10,558	±	0,843	11,435	±	0,335	11,575	±	1,355	45,98
10	7,050	±	1,264	7,752	±	1,281	10,523	±	1,125	7,226	±	0,702	8,138	±	0,735	22,30
20	8,348	±	1,621	6,068	±	0,371	4,490	±	0,351	6,349	±	0,699	6,314	±	0,658	25,28

direzione vento velocità (m/s)	da N e NO 1-1,5	imidacloprid con modifica									
Distanza da bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)		
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.
5	3,367	±	0,530	3,613	±	0,405	3,122	±	0,609	4,525	±	2,648	3,657	±	0,713
10	2,596	±	0,928	2,385	±	0,448	3,788	±	0,701	2,192	±	1,011	2,740	±	0,450
20	1,999	±	0,379	1,894	±	0,182	0,526	±	0,263	1,333	±	1,070	1,438	±	0,355



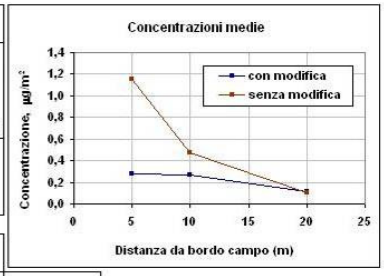
direzione vento velocità (m/s)	da S e SO 2	imidacloprid senza modifica										differenza con /senza mdoifica (%)
Distanza dal bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)			
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	
5	46,685	±	19,767	5,402	±	0,405	3,788	±	1,918	8,173	±	1,231	16,012	±	7,894	77,16
10	11,961	±	5,025	10,277	±	5,473	3,928	±	0,195	4,262	±	2,001	7,607	±	2,265	63,98
20	4,490	±	1,367	6,489	±	1,984	3,964	±	0,456	1,368	±	1,105	4,078	±	0,918	64,73

direzione vento	da S e SW	Thiametoxam con modifica											
velocità (m/s)	1												
Distanza da bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)		
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.
5	7,541	±	3,593	1,824	±	0,153	1,684	±	0,105	1,613	±	0,035	3,166	±	1,249
10	2,701	±	0,873	1,824	±	0,246	1,578	±	0,000	1,578	±	0,000	1,920	±	0,275
20	3,858	±	1,666	1,578	±	0,000	1,578	±	0,000	1,613	±	0,000	2,157	±	0,534



direzione vento	da S e SE	Thiametoxam senza modifica												
velocità (m/s)	2,4													
Distanza dal bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)			differenza con /senza mdoifica (%)
	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	media	±	Err. St.	
5	10,768	±	4,224	5,577	±	1,318	5,016	±	1,703	6,173	±	2,131	6,884	±	1,506	54,01
10	5,472	±	2,137	1,929	±	0,969	1,578	±	0,000	2,385	±	0,153	2,841	±	0,790	32,41
20	3,016	±	0,827	3,087	±	1,508	1,578	±	0,000	1,578	±	0,000	2,315	±	0,495	6,82

direzione vento	da S e SO	Fipronil con modifica											
velocità (m/s)	2,5												
Distanza da bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)		
	media	±	errore standard	media	±	errore standard	media	±	errore standard	media	±	errore standard	media	±	errore standard
5	*0,105*	±	*0,000*	*0,351*	±	*0,246*	*0,105*	±	*0,000*	0,544	±	0,167	0,276	±	0,097
10	0,386	±	0,035	0,140	±	0,035	0,281	±	0,126	0,281	±	0,126	0,272	±	0,055
20	0,105	±	0,000	0,105	±	0,000	0,140	±	0,000	0,105	±	0,000	0,114	±	0,010



direzione vento	da S e SO	Fipronil senza modifica												
velocità (m/s)	2													
Distanza dal bordo campo (m)	concentrazione lato Nord (µg/m²)			concentrazione lato Sud (µg/m²)			concentrazione lato Est (µg/m²)			concentrazione lato Ovest (µg/m²)			media generale (µg/m²)			differenza con /senza mdoifica (%)
	media	±	errore standard	media	±	errore standard	media	±	errore standard	media	±	errore standard	media	±	errore standard	
5	*2,140*	±	*2,034*	0,246	±	0,035	*0,456*	±	*0,300*	1,789	±	0,895	1,157	±	0,622	84,35
10	0,421	±	0,316	0,316	±	0,105	0,421	±	0,161	0,737	±	0,316	0,474	±	0,132	-
20	0,105	±	0,000	0,105	±	0,000	0,105	±	0,000	0,105	±	0,000	0,105	±	0,000	-

The concentration levels observed, independently of side, were on average double as compared to the 2009 values recorded on small sized plots (1600 m^2). This confirms the predictions put forward in the 2010 experimental plan, which suggested that with increasing seeded plot size (3 ha in 2010) there is likely to be an increase in contamination of the surrounding area, albeit less than proportionately.

Finally, the data on imidacloprid show an apparently abnormal value of 46.685 µg/m^2 (printed in red typeface) as the mean of three values recorded in the Petri dishes, two of which were 71 µg/m^2 and 56 µg/m^2. These two values were due to the presence of dust fragments in the corresponding Petri dishes, visible to the naked eye. This testifies to the fact that in the absence of deflectors, even coarse fragments of dust can be dispersed in the area surrounding the sowing field. If the two "accidental" values are disregarded, the final mean would be 6.47 µg/m^2 rather than 16.012 µg/m^2, and the reduction would thus amount to 43.48%.

This finding is confirmed statistically by the analysis of variance conducted on the concentration values recorded (Table 10). With the exception of fipronil, differences attributable to the presence or absence of the modification applied to the seeder were almost always significant, despite the variability due to the field side, which itself was influenced by the wind factor.

The diagrams in Figure 10 show active ingredient concentration in the air at the various distances. Due to the small amount of data available, on account of the type of trial organization and the availability of only three air samplers, statistical analysis could not be carried out. The diagrams are included here purely to show a trend. The trends differed from one another, probably due to the different environmental conditions and the specific characteristics of the dust. Thus for clothianidin no information could be derived on the effect of the deflector, while for the other two active ingredients a reduction clearly emerged from the data, albeit with different trends. It is worth noting that for fipronil, concentration increased with increasing distance when the unmodified machine was used.

Air concentration levels were measured, and were found to be decidedly higher for clothianidin than for the other two products, reflecting the greater dustiness for clothianidin in the Heubach test; thus levels were on the same order of magnitude as in the 2009 tests. This is plausible and can be explained by noting that greater contamination at ground level is not necessarily due to greater air concentration. It may also be caused by contamination from a larger air mass, in which the concentration, in ppb, may actually be the same as in trials that involved more limited surfaces and smaller air masses.

This is exemplified by Table 11, which lists the quantities of active ingredient found in the filters (µg/filtro), instead of listing the concentrations. It can be seen from the Table that the 2010 quantities were much higher than those recorded in 2009. The apparent contradiction can be explained by the fact that, as mentioned earlier (section 2.3.2), in 2010 the air samplers were in function for a longer duration (100 minutes), in connection with the greater duration of sowing: consequently, they trapped a volume of roughly 450 l of air over 3-ha fields; in the 2009 trials, on the other hand, the air volumes sampled were roughly 100 l over a much shorter duration, in which plots with a smaller surface area were seeded.

The values shown in the last columns on the right of Table 11 indicate that: 1) with the unmodified machine the ratios between the concentrations detected in the two trial years at 5 m and at 10 m, respectively 6.43 and 5.24, were close to the value of 4.5 (ratio between the volumes 450 l and 100 l), confirming the statement just above ; 2) with the modified machine, the ratio at 5 m was found to be very elevated (14.12), whereas at 10 m it was reduced to 1.88. This was probably due to the effect of the deflector, which, by deflecting the dust downwards, partly removes it from the sphere of action of the wind, so that the dust remains concentrated close to the sowing field.

An interesting aspect linked to these two points concerns determination of the persistence of the active ingredients in the air after sowing. Air persistence is affected by highly variable and uncontrollable environmental factors. A preliminary investigation was carried out during sowing of thiamethoxam-coated seed, placing the three air samplers at 20 m from the border of the field after more than two hours had elapsed since the end of sowing with the modified seeder, and then sampling for a further 100 minutes (450 l). In all cases, analysis of the filters showed a quantity of active ingredient lower than LOQ. However, this aspect deserves further study given that environmental variability is not the only factor to be taken into consideration: the dustiness characteristics of the different active ingredients and the doses applied in seed coating may lead to divergent levels of concentration in different environments.

Table 10 – Analysis of variance of concentrations detected at ground level in field trials at the various distances and on the four sides or each plot (the data for fipronil are partial). Translation of text within table: *Analisi della varianza – Significatività delle differenze alle varie distanze per i quattro principi attivi* = Analysis of variance – Significance of differences at the various distances for the four active ingredients; *cause di variazione* = causes of variation; *F tavole* = tabular value of F; *Ris*. = Result; *Ripetizioni* = Repetitions; *lato campo* = side of field; *Sem M* = Modified seeder; *Sem NM* = Non modified seeder; *Interaz. Sem / Pos* = Interaction Seeder / Position. Pink: not significant; Light green: significant at p=0.05; Dark green: significant at p=0.01. Commas indicate decimal separation.

Analisi della varianza - Significatività delle differenze alle varie distanze per i quattro principi attivi													
cause di variazione		5 m				10 m				20 m			
		F calc	F tavole		Ris.	F calc	F tavole		Ris.	F calc	F tavole		Ris.
			p = 0,05	p = 0,01			p = 0,05	p = 0,01			p = 0,05	p = 0,01	
Clothianidin	ripetizioni (3)	2,06	19,39	99,40		0,67	19,39	99,40		0,17	19,39	99,40	
	lato campo (4)	3,83	3,29	5,42		0,81	3,29	5,42		1,28	3,29	5,42	
	Sem M / Sem NM	21,75	4,54	8,68		7,29	4,54	8,68		7,63	4,54	8,68	
	int. Sem / Pos.	0,71	4,10	7,56		3,29	4,10	7,56		5,38	4,10	7,56	
imdiacloprid	ripetizioni (3)	1,22	19,39	99,40		0,80	19,39	99,40		0,29	19,39	99,40	
	lato campo (4)	4,49	3,29	5,42		1,01	3,29	5,42		2,73	3,29	5,42	
	Sem M / Sem NM	6,59	4,54	8,68		6,36	4,54	8,68		12,59	4,54	8,68	
	int. Sem / Pos.	6,93	4,10	7,56		2,00	4,10	7,56		2,55	4,10	7,56	
thiametoxam	ripetizioni (3)	2,16	19,39	99,40		0,66	19,39	99,40		4,01	19,39	99,40	
	lato campo (4)	3,69	3,29	5,42		3,40	3,29	5,42		3,07	3,29	5,42	
	Sem M / Sem NM	6,68	4,54	8,68		2,18	4,54	8,68		0,10	4,54	8,68	
	int. Sem / Pos.	0,07	4,10	7,56		1,59	4,10	7,56		1,45	4,10	7,56	
firponil	ripetizioni (3)	0,76	19,39	99,40		0,57	19,39	99,40		1,0714	19,39	99,40	
	lato campo (4)	0,79	3,29	5,42		0,81	3,29	5,42		1,0714	3,29	5,42	
	Sem M / Sem NM	2,51	4,54	8,68		2,43	4,54	8,68		1,0714	4,54	8,68	
	int. Sem / Pos.	1,10	4,10	7,56		0,72	4,10	7,56		1,6071	4,10	7,56	

Legenda
non significativo
significativo per p=0,05
significativo per p=0,01

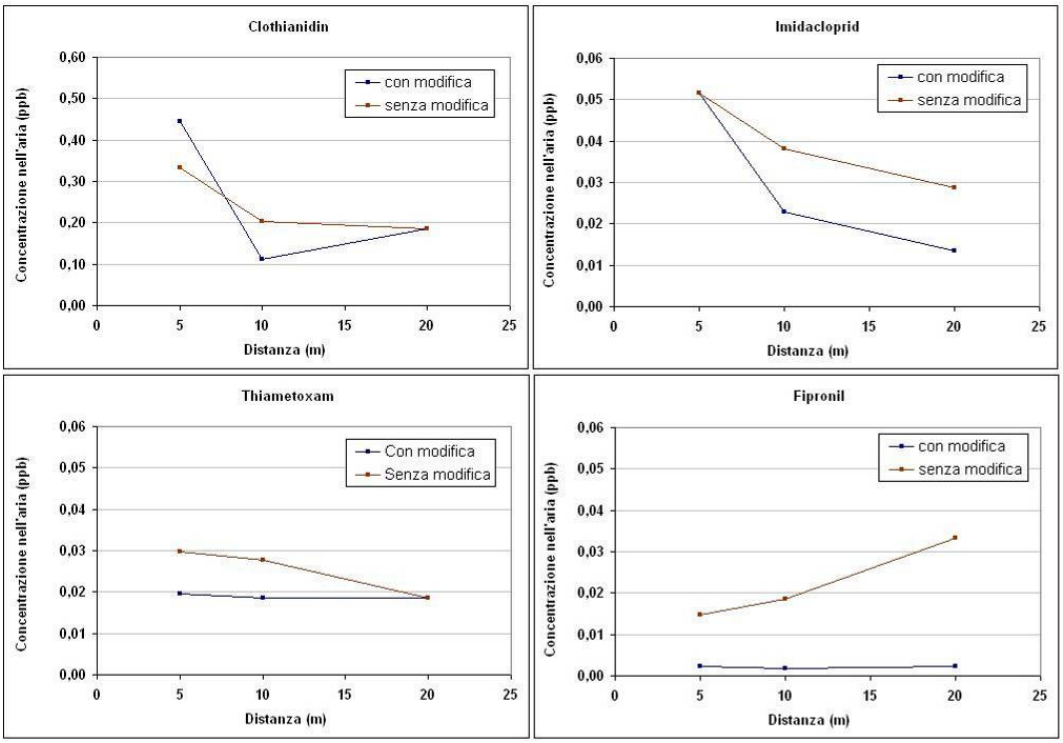


Figure 10 –Trends of active ingredient concentration in the air at 2 m above the ground, at varying distances from the sowing field on the downwind side. Translation of text within the figure: *Con modifica* = With

modification [black line]; *Senza modifica* = Without modification [red line]; *Concentrazione nell'aria* = Air concentration; *Distanza* = Distance. Commas indicate decimal separation.

Table 11 - Quantity of clothianidin intercepted by the air sampler filters in the 2010 trials compared to 2009 findings .

Distance (m)	2009		2010		Ratio 2010/2009	
	With modification	Without modification	With modification	Without modification	With modification	Without modification
	µg/filter	µg/ filter	µg/ filter	µg/ filter		
5	0.017	0.028	0.24	0.18	14.12	6.43
10	0.032	0.021	0.06	0.11	1.88	5.24
20	-	-	0.10	0.10	-	-

Observations on bees

During the sowing operations, wind speed was fairly low (on average 1.8 m/sec) and wind direction was often variable. It was therefore decided not to take the orientation factor into consideration for data processing, and to consider all cages as placed at the same distance from the seeded field,

As stated earlier in point 2.3, the smaller-sized experimental plots were not suitable for study of acute toxic effects on bees. Therefore the cages were placed around the seeded field essentially in order to determine the quantity of dust trapped by the bodies of bees restrained in the cages on the ground next to the Petri dishes (analyses are ongoing). For these reasons, and also because the trial was stopped at 48 h, mortality linked to acute toxicity was not observed (but at 72 h, in the trials described in point 6.1, mortality continued to be observed, with peaks precisely on the 3rd day with clothianidin, Figure 27).

Thus when analyzing the mortality percentage of all 360 cages used in the trials (4 active ingredients, two seeders, 4 orientations, 3 distances and 3 replications, as well as the control cages), it can be noted that in the majority of cases mortality did not exceed 1% (Table 12). If the cases in which mortality was lower than 1% are disregarded, the majority of cages nevertheless showed mortality ranging between 2 and 5%.

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Table 12 – Number of cages for each mortality class, in all trials.

Mortality class	Number of cages
>=20%	1
10.00-19.99%	5
5.00-9.99%	9
2.00-4.99%	77
1.00-1.99%	9
<1.00%	259

However, if an overall assessment of the 4 active ingredients together with the two types of machine (with and without modification) is made, and the mortality percentage at 48 h in the cages placed at the different distances is compared to the control, then some statistically significant differences emerge (Table 13). In particular, at 5 m in 13 cases the mean value for the cages was significantly greater as compared to the control, while in 6 cases the opposite was true, but the difference was neutralized at the greater distances. This suggests that the difference between cages exposed to coated seed versus the control was attenuated with increasing distance from the seeded

field. Therefore the subsequent computations took into consideration only the cages placed at 5 meters.

Table13 – Number of cases in which the mean of the replications placed at the various distances showed a significantly greater or lower mortality rate than the control, as an overall assessment for all 4 active ingredients and the two seeders. ANOVA followed by Tukey's HSD test, on angular transformed data.

Statistical comparison	Distance from field		
	5 m	10 m	20 m
coated > control	13	11	12
coated < control	6	8	11
No significant difference coated vs contr.	13	13	9

Table 14 – Bee mortality percentage in cages placed at 5 m from the seeded field, as compared to control cages. Significant values of p are indicated in red. Student's *t* test on angular transformed data .

Active ingredient	Modified	% mortality		
		5 m	control	*T* test
Clothianidin	seeder	1.41	0.26	p<0.05
	unmodified	0.75	0.73	n.s.
Imidacloprid	modified	4.40	3.93	n.s.
	unmodified	0.87	0.33	p<0.05
Thiamethoxam	modified	1.74	0.25	p<0.05
	unmodified	0.63	1.56	p<0.05
Fipronil	modified	0.26	0.40	n.s.
	unmodified	0.82	0.72	n.s.

Table 14 shows a comparison, made separately for each active ingredient and type of seeder, between mortality (at 48 h) recorded in the cages situated at 5 meters from the trial field and control cage mortality. It is clear from the table that in 3 cases mortality was significantly greater in the treatment as compared to the controls, while in only one case was the opposite observed.

Finally, in a comparative assessment of bee mortality rates, corrected with the Shneider-Orelli formula[1], obtained after sowing with the two types of seeder (modified and unmodified), no generalizable pattern was found (Figure 11). For two active ingredients (clothianidin and thiamethoxam) mortality was greater with the modified seeder, whereas the opposite result was obtained for the other two active ingredients (imidacloprid and fipronil).

[1] %corrected mortality = [(% mortality associated with coated seed- % control mortality) / (100 - % control mortality)]*100.

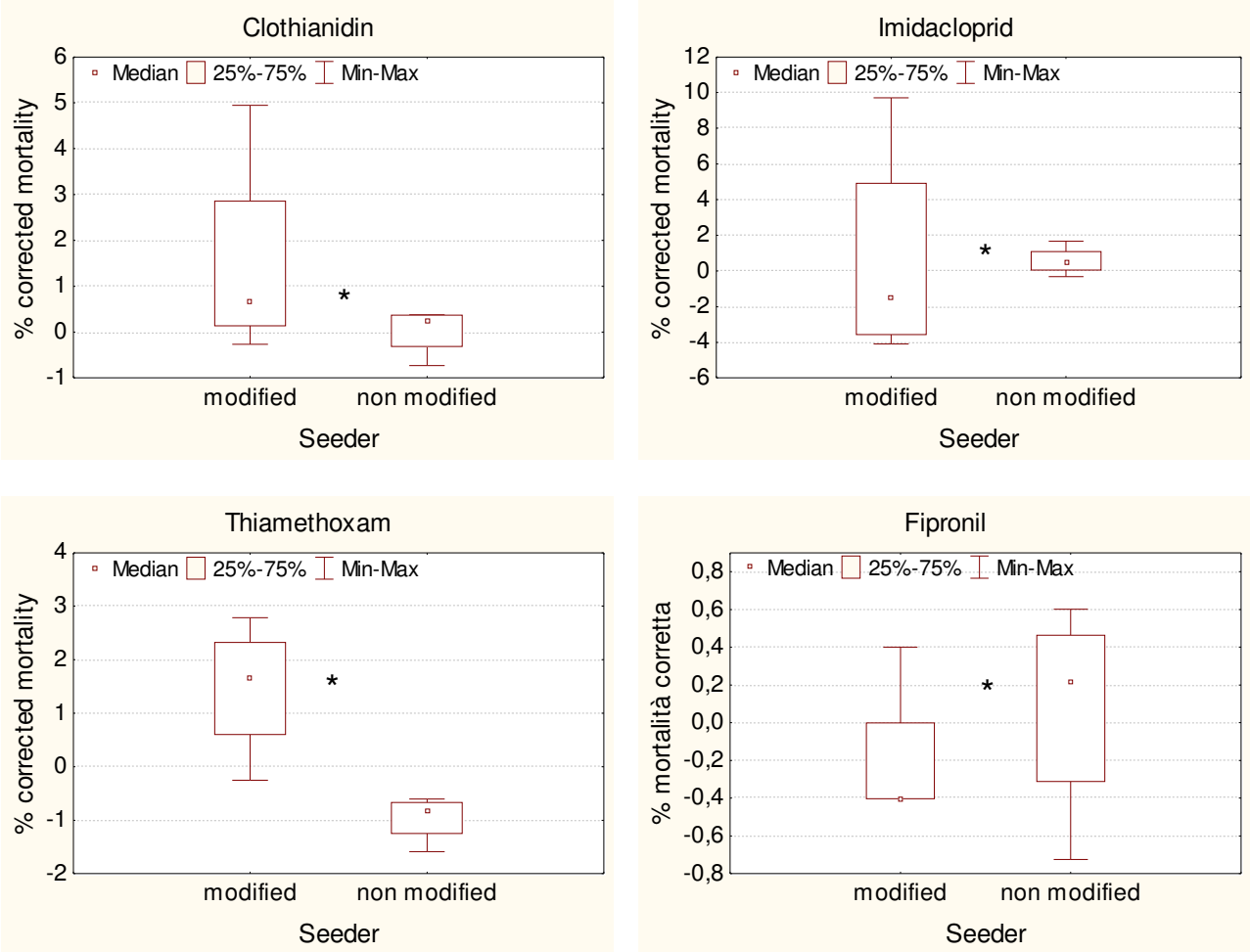


Figure 11 – Mortality percentage, corrected with the Shneider-Orelli formula, recorded in the cages placed at 5 m from the trial field, with a comparative assessment based on use of the two types of seeder. Mann-Whitney's U Test (p=0.05).

These observations in no way solve the problems linked to the effects of sublethal doses, which the active ingredients in question have already been shown to exert on non target insects such as bees. Such effects cannot be evaluated through this kind of trial typology (see Chapter 6).

2.4 Testing for active ingredient residues in soil and in maize plants at different phenologic stages

The 2010 activity also includes a second experimental year for evaluation of residues of imidacloprid in soil and in maize plant tissue. Thus 3 plots measuring 1000 m^2 each were set up. To eliminate variability due to possible divergent interaction between the active ingredient and the metabolism of the different hybrids used in the two-year period, the same seed as in 2009 was used, with a modified seeder (sowing carried out by CRA-ING).

The experiment was conducted to evaluate the soil persistence of imidacloprid during crop development. Additionally, translocation of the active ingredient into plant tissue during the

different phenologic stages was investigated, with particular attention to sampling pollen at tassel emergence, in order to test for the possible presence of imidacloprid.

Activity involved :

- soil characterization (granulometry, texture, pH, etc.);
- quantification of the presence of active ingredient within the seeded field;
- meteorological and climatic measurements;
- sampling of soil, coated seed-grown maize plants and controls at different phenologic stages of the crops, performed in triplicate for each plot;
- analysis of sampled plants (gathering a sufficient number of samples to allow analysis of the different plant tissues: roots, stem, leaves, pollen and grain) in order to assess presence of the product and possibly of its metabolites;
- determination of plant fresh and dry weight.

2.5 Conclusions

Dispersal of the active ingredients was found to depend on a number of factors. Firstly, the seed coating procedure: if the seed coating treatment is of good quality, only limited amounts of dust (and of active ingredient) are released. The tests described in recent years by manufacturers of these substances not only were based on the use of conventional (unmodified) seeders but they also involved less recent and less efficient seed coating procedures compared to the systems currently available. Thus the earlier tests showed much higher concentration values (roughly 10-fold higher) than detected in the most recent trials. From this point of view the 2009 results are substantially confirmed by the findings in 2010: under equal conditions of seed dustiness (Heubach cylinder test), although the 2010 concentrations obtained in the fixed point tests (prediction, Figure 6) and in the field (over a more extensive area, Table 8) were more than twice as high as in 2009, they were of the same order of magnitude as the 2009 findings, both for the conventional and the modified seeder.

The second factor concerns use of the modification (deflector). The deflector used in 2010, which differs both in size and shape from the type utilized in 2009, is based on a similar principle and provided results in line with the 2009 findings, both in the fixed point and the field tests, with a marked reduction in concentration as compared to the conventional machine. Looking at the diagrams of the results, both with regard to the fixed point (Figs. 5 and 6) and the field (Table 8) tests, the reduction for the four active ingredients can be summarily quantified as 50%.

Research into more efficient solutions for abatement of abrasion dust dispersal is currently in progress. It is considered that the target to aim at should be the absence of any abrasion dust dispersal during use of the seeders. This would be possible by trapping the dust in some manner inside the seeder itself, and postponing its disposal to a phase subsequent to sowing, in line with procedures analogous to those adopted for all pesticide residues. This would allow the problem of dust to be transferred from a poorly controllable situation (the field, with all the operational and environmental variables mentioned repeated in this paper) to one in which the possibility of control is maximized (a circumscribed area of the CRA complex), thereby ensuring compliance with all the required precautions for operator health safeguards.

The third factor concerns meteorological and environmental conditions. These are not controllable and it is therefore difficult to obtain perfectly comparable trial conditions. In our view, sampling conducted on all the sides of the trial field contributes to bypassing this difficulty and certainly provides a better description of the phenomenon with regard both to individual tests and also to comparison between the modified and the conventional machine. However, the comparison remains affected by the elevated environmental variability, as also testified by the statistical analysis of the field trials (Table 9). From this perspective, the fixed point system proposed in order to normalize

trial conditions can provide more reliable indications. This system confirmed the findings of the field trial concerning the abatement percentage.

Once the method proposed for predicting field concentrations from data obtained in simulated tests has undergone further verification, fine-tuning and validation, it may become possible to avoid the field phase, thus making operations for the evaluation of a machine or a modification much more practical and faster. Thus if devices designed to reduce dust dispersal are introduced in a generalized manner on pneumatic seeders, it will be necessary to set up a trial system capable of "validating" the performance of the device. This could be carried out by using a system analogous to that described in the present report. In this context, it should be noted that CRA-ING is entrusted with the "ENAMA performance and safety certification tests for seeding and transplanting machines".

All trials have so far been conducted using seed coated with the four active ingredients under study according to the normal seed coating procedures. If the (field or fixed point) trials are broadened into a more systematic monitoring program, it will be fundamental for the tests to be conducted using seed deriving from the normal seed coating procedures, but taking care to replace each active ingredient with a substance having similar behavioural characteristics (in terms of dust production, analytical characteristics) but totally innocuous.

In the field trials on 3-ha plots, concentrations observed in the sampling areas were on average twice as high as those recorded in the 2009 trials on 1600 m^2 plots. This confirms the hypotheses put forward as the basis for the 2010 activity, namely that extension of the seeded area influences the level of contamination of bordering areas. Therefore, in order to obtain reliable data on the level in question, it is necessary to approximate real operating conditions.

Furthermore, it should be borne in mind that during work in 2009 on the 1600 m^2 plots the characteristics of the seeder available at the time made it necessary to adopt a very elevated seed density. If normal seed density had been adopted (75,000 seeds/ha), the area corresponding to the quantity of seed distributed would have measured roughly 2700 m^2. Therefore, with an increase in seeded area ranging between 12 times greater (ratio between 30,000 m^2 and 2700 m^2) and 19 times greater (ratio between 30,000 m^2 and 1600 m^2), a doubling of ground level concentrations was observed. On the basis of these data it is reasonable to assume that the further increase in seeded area may increase the concentration of active ingredients in the surrounding areas. However, in the absence of further experiences, it is not possible at this stage to give a reliable estimate of the extent of the phenomenon.

As far as observations on bees are concerned, it can be said that overall the bees were unaffected – in terms of acute effects – by exposure to the concentrations recorded around each cage, even though such concentrations were twice as high as in the previous year. However, it was pointed out earlier (point 2.3) that when there was an increase in the seeded area, an increase (albeit less than proportional) in concentrations was also observed. At present, no information is available to suggest how and to what extent bees would be affected by extensive sowing of a very large area, a practice frequently adopted in maize-growing zones. Thus further research into this issue is required, taking into account that utilization of these active ingredients has been suspended for the past two years, and that during this two-year ingredient-free period the number of reports of problems affecting bees in maize-growing areas has substantially declined (Table 1). There remains the issue of the effects induced by sub-lethal doses, which cannot be evaluated in the cages. This question will be addressed in chapter 6.

In conclusion, the manner in which seed coating is performed and the type of modification applied to the seeder constitute fundamental elements in active ingredient drift abatement. To date, despite careful attention to these aspects, a certain degree of environmental dispersal of insecticidal coating dust is still observed.

3. Effects induced in bees by contact with dust during flight over a field sown with coated maize seed

3.1 Premise

The purpose of this study was to evaluate the effect of direct exposure of bees, during flight, to the dust emitted by the seeder during the process of sowing coated maize seed. The argument put forward here is that when a bee makes repeated flights towards flowering plants and flies over plots sown with coated maize seed, it may suffer lethal poisoning as a result of the dust it comes into contact with during flight.

Preliminary trials with bees restrained inside tulle netting cages and directly exposed to dust emitted by the seeder showed a toxic effect of this type of exposure. However, in these conditions the bees could not avoid contact with the dust by escaping from the cage. To simulate conditions closer to field conditions, a trial was set up in which bees were trained to visit a feeder and were obliged during the journey between the feeder and the hive to fly over a field sown with coated maize seed.

A number of experiments were carried out in 2009 and 2010. The majority of the data thereby obtained are in press; therefore only the results pertaining to two experiments carried out in spring-summer 2010 are given here.

3.2 Materials and Methods

The trials were performed at the Experimental Farm of the Agricultural Faculty, located in Legnaro (Province of Padua), where 4 beehives supplied by the Bee-Keepers' Association of Padua were made available. The bees of the 4 trial hives were trained to visit a feeder having a diameter of 25cm and containing sucrose solution. The feeder was brown in order to merge with the colour of soil so that it would not attract bees from other apiaries. It was situated at progressively spaced distances from the hives, up to a distance of roughly 100 m. When the bees started out from the apiary (45°20'39.45"N; 11°57'16.05"E) to fly towards the feeder, they had to rise up for at least 2-3 m in order to fly over the top of a screen-house, then fly over a small vineyard, and cross a road and 70 m of ploughed land. During observation of their flight, they could be seen flying at around 2 m of height, and a count of the number of bees in flight to and from the food source gave a figure of roughly 100 bees a minute.

Sowing was carried out on the first portion of a plot measuring 50m x 70m, at a distance between 35 and 65 m from the hives, and at least 35 m from the feeder. A 4-row MONOSEM NG-Plus (Monosem, Largeasse-France) seeder was utilized, as this is the most widely used seeder for maize growing at the University farm. Roughly 73,000-74,000 seed/ha were sown. The working speed of the machine was 6-7 km/ha: at this speed, with an effective sowing width of 3m, the machine would theoretically take 30 minutes to sow 1 ha, although the actual time required was 45 min. The air exhaust vent (150 l/sec), placed on the right-hand side of the seeder, discharged at a height of 1.8 m, at an angle of 45° to the horizon.

The seed was supplied by A.I.S. (Associazione Italiana Sementi); the hybrid utilized was X1180D 964890 produced by Pioneer Hi-bred Italia in 2009 and 2010, and coated with Celest XL®, Poncho® and Gaucho 350FS®.

Whenever the bees, having become accustomed to flying over the plot on their way towards the feeder, caught sight of the shape of the seeder, they avoided it either by flying over it or by moving aside while in flight or passing a few meters away from the machine. Observations on this behaviour were possible by looking at the seeder in action with the sun behind the observer's shoulders.

At the beginning of sowing and subsequently at 15 minute intervals, bees were captured near the feeder with a test-tube, placed individually in 5 x 5 cm tulle netting cages and fed with a drop of

honey, which was placed on the netting of the small cage and periodically replaced. 24 samples of bees were captured for each time interval, beginning with the moment of starting up the tractor and subsequently at 15 minute intervals during the sowing process.

The samples of 24 bees were then transported in the cages to the laboratory in controlled temperature conditions of 22±1.5°C. Subsequently, for each time interval, one half (12 cages) of the samples, randomly chosen, were maintained in laboratory humidity conditions while the remainder (12 cages) were placed in plastic boxes with humidity conditions approaching saturation (> 95%). The elevated relative humidity was obtained by placing the cages inside transparent plastic boxes: these were non hermetically closed with plexiglass lids and the interior bottom was lined with a sheet of damp paper. The interior sides and the lid were sprayed with water, and the cages were raised off the bottom of the box by means of a strip of polystyrene, to avert the risk of the bees getting wet with water that could accumulate at the bottom of the box.

A total of 120 bees were assayed for each a. i. (24 bees at 5 time intervals) apart from thiamethoxam in which 72 bees were assayed (24 bees at 3 time intervals).

Each test had an overall duration of 60 minutes. In all tests, dead bees in front of the hives were counted at one hour after the end of sowing and the day after; additionally, in some tests, samples of bees from in front of the hives and from around the feeder were collected and submitted to chemical analysis.

3.3 Results

Mortality among bees that were captured at the intervals of time after sowing and were maintained in the laboratory in different humidity conditions is shown in Tables 15, 16 and 17.

Bees captured at the beginning of sowing showed no symptom of poisoning and no mortality was recorded in either of the two humidity conditions; bees captured at subsequent intervals and maintained in elevated humidity showed 100% mortality within 24 h, some even within an hour after the end of sowing, whereas those maintained in conditions of laboratory humidity showed a lower mortality rate.

The short-term results are sufficient to show the synergy between exposure to dust and elevated humidity.

Table 15 – Mortality of foraging bees captured in the field need the feeder after flying over the seeder during sowing of fipronil-coated maize seed.

Minutes after beginning of sowing with fipronil-coated seed	**Number of dead bees /Total number of bees in the cage**					
	HUMID			**DRY**		
	1 h after sowing	**2 h after sowing**	**24 h after sowing**	**1 h after sowing**	**2 h after sowing**	**24 h after sowing**
0	0/12	0/12	0/12	0/12	0/12	0/12
15	12/12	12/12	12/12	4/12	9/12	11/12
30	12/12	12/12	12/12	1/12	6/12	10/12
45	10/12	12/12	12/12	8/12	9/12	11/12
60	9/12	12/12	12/12	0/12	3/12	8/12

Table 16 - Mortality of foraging bees captured in the field need the feeder after flying over the seeder during sowing of thiamethoxam -coated maize seed.

Minutes after beginning of sowing with thiamethoxam–coated maize seed	**Number of dead bees/Total number of bees in the cage**			
	HUMID		**DRY**	
	2 h after sowing	**24 h after sowing**	**2 h after sowing**	**24 h after sowing**

0	0/12	0/12	0/12	0/12
15	12/12	12/12	6/12	12/12
30	12/12	12/12	6/12	10/12

Table 17 – Mortality of foraging bees captured in the field near the feeder after flying over the seeder, in the various tests, according to the kind of coating, the moment of capture and humidity conditions.

Sowing date	**Coating agent and year**	**Humidity**	**Time of capture from beginning of sowing (min)**				
			0	**15**	**30**	**45**	**60**
14/07/2009	Clothianidin 2009	70%	0/12	0/12	0/12	0/12	0/12
		>95%	0/12	12/12	12/12	12/12	12/12
23/07/2009	Imidacloprid 2009 (1)	70%	0/12	2/12	0/12	1/12	3/12
		>95%	0/12	12/12	11/12	12/12	12/12
15/10/2009	Imidacloprid 2009 (2)	70%	0/12	0/12	0/12	1/12	4/12
		>95%	0/12	10/12	12/12	12/12	12/12
02/09/2010	Fludioxonil + Metalaxil-M 2010	70%	0/12	0/12	0/12	1/12	0/12
		>95%	0/12	0/12	1/12	0/12	1/12
02/09/2010	Clothianidin 2010	70%	0/12	1/12	1/12	3/12	5/12
		>95%	0/12	7/12	12/12	11/12	12/12

The first results of chemical analyses on dead bees in the laboratory cages indicate mean contamination levels exceeding 500 ng/bee of active ingredient.

In the trials with fipronil and thiamethoxam, several hundred dead or dying bees were observed in front of the hives, expelled during the hours immediately following the test or on the subsequent day, with a maximum of 1000 dead bees in front of some of the hives. This was observed in particular when the tests were conducted on days of elevated relative humidity in the air. An average of more than 100 ng/bee was found in the samples of bees collected in front of the hives on the day after the test. However, evaluations were not performed to determine the effects on colonies, which apparently showed no marked reductions in flights of foraging bees.

In the trial with clothianidin 2009, 400 dead bees were observed in front of the hives 3 hours after the test, while the number rose to 1490 the following day.

In the trial with imidaclorprid 2009 (1) bee mortality was lower (less than 50 dead bees in front of the 4 hives) while in (2) 300 dead bees were observed on the day of the trial and 500 on the following day.

In the trial with clothianidin 2010 about a hundred dead bees were observed in front of the 4 hives on the day following the test.

The results of the chemical analyses on the dead bees collected during the trials with imidacloprid 2009 and clothianidin 2009, from in front of the hives and from around the feeders, are reported in Table 18.

Table 3 – Residues of neonicotinoids in samples of dead bees collected in front of the hives and near the feeder at the end of the two over-flight tests.

Sowing date	**Coating agent and year**	**Collection place**	**Time interval between sowing and sample collection**	**N. of analysed bees**	**Quantity of a. i. (ng/bee)**
14/07/2009	Clothianidin 2009	feeder	30 min	7	674
		hive	3 h	7	161
		hive	24 h	7	118

15/10/2009	Imidacloprid 2009 (2)	feeder	30 min	4	3.661
		feeder	45 min	8	442
		hive	3 h	8	500
		hive	4 h	8	53

3.4 Conclusions

The results of these trials indicate that when a bee travelling towards a food source flies over a seeder that is sowing insecticide-coated maize seed, the bee may be exposed to a lethal dose of active ingredient, probably even in a single flight. The results also demonstrate that the dust emitted by the seeder is sufficient to kill the bees without the poisoning effect being mediated by ingestion of contaminated food. In contrast, previous explanations of bee die-offs following coated maize seed sowing were consistently based on the hypothesis of contaminated food. It was suggested that contaminated dust drifted onto vegetation bordering the seeded field, and since the active ingredients present in the seed treatment process are water soluble and have systemic activity, they can penetrate into vegetation and enter into circulation in plant tissues, thereby affecting nectar and pollen and consequently poisoning the bees that feed on these substances. Although this hypothesis appears credible and is not without foundation, it does not suffice to explain the death of bees just a few hours after sowing operations.

A further interesting aspect to emerge from this trial is the effect of humidity on bee death. Bees maintained in laboratory humidity conditions showed a much lower mortality rate, despite having been subjected to the same dust exposure as bees maintained under elevated humidity, given that the two groups had been randomly divided. This could suggest that even quantities of 500 ng/bee may not necessarily be lethal under low R.H.

The sequence of events can thus be depicted as follows: when bees encounter the seeder, in their attempt to avoid it they become dusted with a potentially lethal dose of neonicotinoid; if R.H. is elevated, the bees die within a few hours, but if the air is dry, they generally survive, so that the association between pneumatic seeder, maize seed coated with neonicotinoids and bee die-off is no longer clearly evident. Once their bodies have been dusted with the product, the bees may die close to the food source (as observed near the feeder), along their flight path or upon their later return to the hive. In the latter case, they are expelled by the other bees, who may in their turn have been contaminated by the dust.

The elevated toxicity of the dust emitted by the seeder, as compared to the toxicity of the active ingredients themselves when delivered in spray formulation, requires an explanation that considers not merely the variations in humidity. Examination of the quantity of active ingredient in the dust fragments collected at the exit from the seeder showed that the active ingredient constituted 20%, in weight, of the particulate. The use of neonicotinoids in spray formuation in orchards requires the active ingredient to be diluted in water for clothianidin, around 75 ppm, (Dantop® 50% clothianidin, utilized at 15 g/hl), which in comparison to 20% is at least 2.600 times lower. And in addition to the extremely elevated concentration of active ingredient detected in the dust in our trial, a further problem concerns the shape of the integument of bees. As the integument is specifically adapted to collecting pollen, it is extremely likely to trap dust. Moreover, the quantity of active ingredient on the seed (1.25 mg of active ingredient per seed, each seed having a weight of 0.3 g) is greater than 3500 ppm, which in itself represents an ecological problem directly correlated with the phenomenon of guttation (Girolami *et al*. 2009).

Other problems arise in connection with the pattern of maize-growing areas. In certain traditional maize-cropping areas, maize occupies vast areas: for example, in the Province of Padua maize is grown on 30% of the total area (> 65.000 ha out of 215.000 ha) and on roughly 50% of the SAU (Superficie Agraria Utile [*Usable Agricultural Area*]), namely 136,000 ha (data from the region of

Veneto Direzione Sistema Statistico Regionale, 2006). But to a considerable extent, maize is also grown on innumerable small plots intermingled with other crops and green belt areas of various kinds, as can easily be observed by consulting the online land use registry for the Veneto Region, where the die-off and related phenomena described and simulated in this experiment were detected more frequently in previous years. It is interesting to note that other countries (eg. Germany) have likewise experienced die-off phenomena in mixed cropping areas (Nikolokis *et al*. 2009), whereas in monoculture maize cropping areas, as in France, the phenomena are less marked, since the bees are less likely to fly over the sown fields, given the absence of blossoms. Therefore the problem of spring mortality affecting bees can be seen as linked to the fragmentation of crops and to the habitual foraging flights by bees, which in all likelihood involve flying over mixed cropping areas that include fields sown with maize.

The results of the trials described here thus contribute to shedding light on the problem of bee mortality in areas sown with maize, with particular reference to the period of sowing between March and mid May, especially in conditions of elevated humidity.

4. Evaluation of the productive and agronomic utility of maize seed treatment and persistence in plant tissues of the active ingredients used for seed coating

4.1 Evaluation of the productive and agronomic utility of maize seed coating

Evaluation of the productive and agronomic utility of maize seed coating was performed by means of several trials, carried out by the CRA-Maize Research Unit of Bergamo (CRA-MAC) (paragraph 4.1.1) with the collaboration of Veneto Agricoltura for entomological aspects; by the DiSTA of the University of Bologna and by the DIVAPRA of the University of Turin (paragraph 4.1.2); by Veneto Agricoltura (paragraph 4.1.3) and partly by the regional network of Lombardy, Piedmont and Veneto in the framework of the project "Major Crops" (paragraph 4.1.4 and 4.1.5).

4.1.1 Agronomic trials

The trials conducted at the CRA-Maize Research Unit of Bergamo (CRA-MAC) aimed to compare the production yield of materials deriving from seed treated only with fungicide (Celest) versus materials deriving from the same seed coated not only with fungicide but also with one of the four active ingredients under study, utilized against ground-dwelling insects and phytomyza species in general (imidacloprid, clothianidin, thiamethoxam and fipronil).
The agronomic trials were set up in 20 localities, mostly situated in traditional maize-growing areas (Lombardy, Piedmont, Veneto, Friuli, Emilia Romagna) and in Tuscany (Table 19).

Table 19 - List of the 20 localities in which the Apenet 2010 agronomic trials were set up.
A list of the symbols of the Italian provinces can be found on the website:
http://www.tuttocamere.it/files/varie/Province_Sigle.pdf

Region	**Locality**
Lombardy	Bergamo
	S.Angelo Lodigiano (LO)
	Luignano (CR)
	Caleppio di Settala (MI)
	Castenedolo (BS)
	Pudiano (BS) * NO HARVEST
	Ostiglia (MN)
Piedmont	Vigone (TO)
	Chivasso (TO)
	Castelceriolo (AL)
Veneto	Lonigo (VI)
	Montagnana (PD)
	Villadose (RO)
	Noventa Vicentina (VI)
Emilia Romagna	Ambrogio (FE)
	Parma
Friuli	Mortegliano (UD)
	Palazzolo della Stella (UD)
	Codroipo (UD)
Tuscany	Marciano della Chiana (AR)

Materials and methods
For the 2010 experiments, as agreed in the framework of the APENET Project, materials supplied by the Italian Seed Association - ASSOSEMENTI were utilized. A different hybrid compared to the

2009 trials was provided, although the same one had been asked for: thereby the commercial Maize hybrid PR32G44- FAO 600 instead of PR31N27- FAO 700 was used. The materials were prepared starting from a batch of homogenous seed, according to the following 5 treatments:

TREATMENT	Fungicide	Insecticide active ingredient
1 - Control	*Celest	none
2 - Cruiser	*Celest	thiamethoxam
3 - Gaucho	*Celest	imidacloprid
4 - Poncho	*Celest	clothianidin
5 - Regent	*Celest	fipronil

* The fungicide Celest contains fludioxonil and metalaxyl.

The 5 treatments under study were assayed in the framework of each agronomic trial, in a randomized block plan with 4 repetitions; 30 sq m plots were used for the trials, in which seed of each treatment was sown at a density of 7 plants/m^2.

For each agronomic trial, standard measurements and agronomic evaluations were performed for each of the 5 treatments under study, as listed here below:

- production (q/ha-15.5% R.H.)
- grain humidity (R.H. %)
- hectolitric weight (kg/hl)
- plant height (cm)
- ear insertion height (cm)
- percentage broken stalks (%)
- percentage lodged plants (%)

The mean data for these parameters, measured in 19 agronomic trials (data from the Pudiano-BS trial was not collected) are reported in Table 20.

Results

Statistical analyses of the data, conducted by analyses of variance to compare the treatments object of the study, assuming treatment as a fixed factor and locality as a random factor, showed that the mean values of the measured parameters do not differ significantly (treated vs non-treated). However, as reported in Table 20, a tendency towards a greater yield in insecticide-coated seed compared to non-coated (control) was evident. More specifically, in the case of clothianidin-treated maize (treatment 4-PONCHO) the average crop yield was about 6 q/ha-(15.5% R.H.) higher compared to control, showing a marked effect of the a.i. on production levels.

As mentioned above the commercial hybrid supplied by Assosementi for the 2010 trials is different compared to the one supplied in 2009. The hybrid used in the 2010 trials (PR32G44- FAO 600) may have determined a different genotype-environment interaction, also in relation to the seed coating with insecticide, not comparable to what was observed in 2009.

Table 20 – Mean values from 19 agronomic trials – APENET 2010

Treatment	Insecticide (active ingredient)	Yield (q/ha 15.5%r.h.)	Grain humidity (r.h. %)	Hectolitric weight (kg/hl)	Plant height (cm)	Ear insertion height (cm))	% plants w. broken stalks	% lodged plants
1 - CONTROL	none	132.15	23.59	73.11	260.06	129.25	8.11	5.12
2 - CRUISER	thiamethoxam	134.90	23.50	73.12	260.64	129.44	6.83	5.92

3 - GAUCHO	imidacloprid	134.60	23.29	72.85	262.19	129.55	7.78	4.14
4 - PONCHO	clothianidin	138.17	23.28	72.96	264.69	131.73	7.05	5.03
5 - REGENT	fipronil	135.99	23.48	72.88	262.72	131.94	8.04	5.25
DMS 0 .05		4.37	0.28	0.44	3.67	2.73	7.56	1.72

4.1.2 Monitoring of harmful soil insects

In some of the localities where the agronomic trials were set up, risk maps for harmful maize soil insects (Wireworms: *Agriotes* spp and Western Corn Rootworm: *Diabrotica virgifera virgifera*) were drawn up in cooperation with Veneto Agricoltura, DiSTA of the University of Bologna and DIVAPRA of the University of Turin.

Monitoring of hypogeal phytophages was carried out according to two methods:

1) determination of larval populations, plant densities, and pest attacks in plots where Wireworms were monitored by pheromone traps in 2009
2) monitoring with YATLORf pheromone traps set to respond to adult forms of the main Wireworm species and Western Corn Rootworm

Materials and methods

1) Determination of larval populations, plant densities and pest attacks in plots where Wireworms were monitored by pheromone traps in 2009

In many of the plots where pheromone traps were placed in 2009, their position was identified and marked with a pole; at that point larval traps were put in place and a portion of the field all round the traps was kept free from geo-insecticide treatment.

The larval traps with natural attractant (Chabert e Blot, 1982) were made with plastic pots (diameter 10 cm) with drainage, filled with vermiculite and 30 ml of maize seed and 30 ml of wheat grain, then filled with more vermiculite. After having been thoroughly wetted, the pots were buried in such a way that the upper border of the pot was 5 cm below ground surface. Then 2 cm of earth were placed on top of the pot, followed by an over-turned flowerpot holder (diam. 18 cm) and more earth up to ground level. The traps were placed when soil temperature was above 9 °C and with relatively high humidity; in the trap locations the soil was free from vegetation. After 7-10 days the pots were collected, codified, and placed in bags. Each pot was analysed by manually breaking up the vermiculite mixed with the seeds and the newly formed roots, and observed Wireworm larvae were counted so as to yield an estimate of average number of larvae per trap. The observed material was positioned on funnels on top of a test tube, to collect the larvae, which move to the lower surface as the material dries out.

Placing of the larval traps was carried out according to the following stages:

a) Exact identification of the position of the 2009 pheromone traps;
b) Placement of the larval traps according to specific diagram (Fig. 12);
c) Collection of traps, maintenance of indications of NON treated plot;

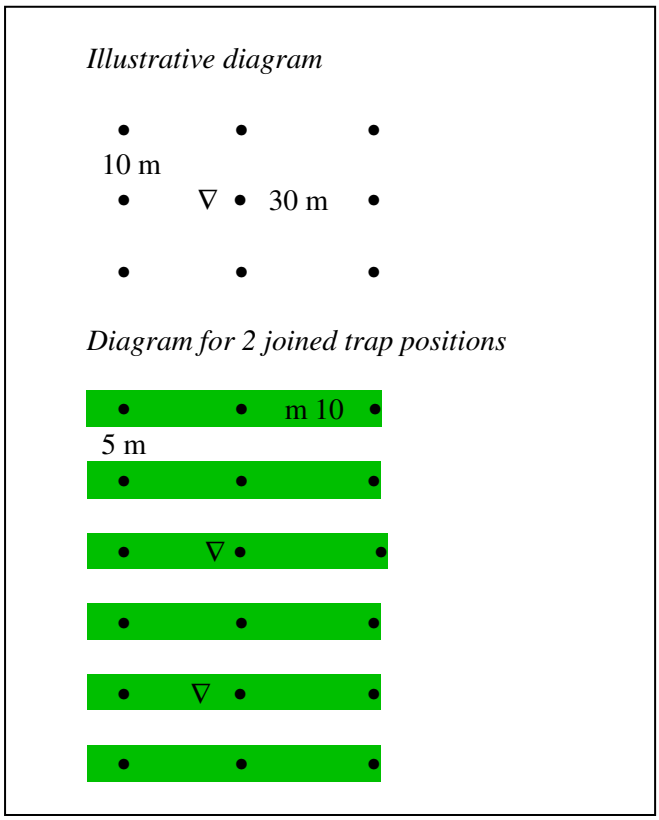


Figure 12 - – Diagram of trap positioning:
∇ = position of 2009 pheromone trap;
• = larval trap.

In the stations which hosted pheromone traps in the previous year (2009) absence of insecticide treatments was guaranteed. In the area around the trap location 5-6 sub-plots, measuring 20 m length and 3-6 rows wide, were randomly chosen and the following parameters measured:

- plant density at emergence
- attack on seed – plantlets
- plant density at 4-8 leaves stage
- attack on 4-8 leaves stage
- other attacks (aphids, viruses, ….) on 4-8 leaves stage
- plant density at harvest

2) Monitoring with YATLORf pheromone traps set to respond to the main Wireworm species and Western Corn Rootworm

As far as possible, traps were placed in the same positions as 2009, or as near as possible so as to obtain information on the temporal dynamics of the insect populations, on the stability of the monitoring indications and to obtain further data to study the correlation between the level of adult and larval populations and pest attack on the crop.
In each station, 1 or 2 pheromone traps were placed in the plot in presence of the crop preceding maize in the rotation. The distance between traps in the same station was at least 50 m. The traps were placed inside the plot or on the edge if the crop was very thick.

The time schedule of operations concerning monitoring with Yf traps was approximately the following:

1 The coordinates of trap positions were identified;
2 The trap was placed at ground level with the terminal basal point completely stuck into the soil, including some soil around the edge;
3 On March the 20th traps were placed in the middle of the experimental area, with the pheromone (Kartel 730) dispenser[a] for *A. brevis* in a low position and the opening towards the ground;
4 On the 10th of April the captured insects were collected[b] and the pheromone dispenser[a] for *A. sordidus* was placed in the middle of the trap and the opening towards the ground;
5 On the 10th of May the captured insects were collected[b] and the pheromone dispenser[a] for *A. sordidus* was replaced with a new one placed in the same position;
6 On May the 20th the captured insects were collected[b] and the dispenser[a] for *A. brevis* was replaced by the dispenser for *A. litigiosus* , placed at the bottom of the trap with the opening towards the ground;
7 On the 15th of June the captured insects were collected[b] and the pheromone dispenser[a] for *A. litigiosus* was replaced with a new one, while at the top of the trap pheromones of *A. ustulatus* and Western Corn Rootworm were placed together;
8 On August the 10th the captured insects were collected[b].

When the trap was located in a thickly sowed crop (forage grass, wheat) and in any case after inserting the sexual pheromone for Western Corn Rootworm, a piece of dog-collar with insecticide was placed at the bottom of the trap.

[a] = Handling of pheromone attractants (Kartel 730 capsules for *A. brevis*, *A. sordidus* and *A. litigiosus, A. ustulatus*):
The attractants must be kept in their sealed packet in the freezer (-18°C) or if not possible in the fridge (0-4°C).
When extracted from the packet the attractants must never be touched but must be handled by the specific plastic strip. The capsules should never be opened.

[b] = Collection of captured insects:
1- remove the trap from the ground;
2- place the trap in a wide and transparent plastic bag before opening the trap. While keeping the bag as closed as possible remove the base of the trap letting the insects fall in the bag;
3- close the bag as soon as the trap has been opened;
4- substitute and readapt the trap;
5- re-position the trap in the ground;
6- count all the insects present in the bag;
7- report on the bag the following data: name, location and code of the trap, date of collection and number of counted insects.

Table 21 - Overview of results of Wireworm monitoring in the Veneto and Friuli plots.

REGION	Monitored plots	With risk factors (*A.brevis, A.sordidus*)	With risk factors (*A.litigiosus, A.ustulatus*)	*A. brevis* mean (s.e., min-max)	*A. sordidus* mean (s.e., min-max)	*A. litigiosus* mean (s.e., min-max)	*A. ustulatus* mean (s.e., min-max)	*A. brevis* mean (s.e., min-max)	*A. sordidus* mean (s.e., min-max)	*A. litigiosus* mean (s.e., min-max)	*A. ustulatus* mean (s.e., min-max)
VENETO	51	6	6	76 (18.3, 0.0-691.00)	523 (53.1, 91.00-2129.00)	ND.	548 (88.4, 0.00-2786.00)	0.03 (0.01, 0.00-0.25)	0.14 (0.03, 0-0.83)	ND.	1.03 (0.35, 0-9.95)
FRIULI	11	2	0	169 (19.70, 86.00-323.00)	335 (66.58, 59.00-763.00)	12 (6.41, 0.00-52.00)	ND.	ND.	ND	ND.	ND
TOTALE	62	8	6								

Table 22 – Overview of the effects of Wireworm attacks in the Veneto and Friuli plots.

REGION	Monitored plots	Plant density: plants/ m^2 (HEALTHY PLANTS): mean (s.e., min, max)	Mean (% healthy on sown)	% emerged plants attacked by Elaterids (*Agriotes sordidus*) mean (s.e., min, max)	Visible symptoms without repercussion on yield (up to stains 10% eroded)	Severe damage (>20%, re-sowing required)
VENETO	51	6.46 (0.07, 5.30-7.38)	90.3	1.14 (0.024, 0.0- 7.0)	2	0
FRIULI	11	6.63 (0.05, 6.35 - 6.90)	90.7	0.059 (0.01, 0.05- 0.1)	0	0
TOTALE	62				2	0
INCIDENCE (%)					3,2	0

Results

1) Determination of larval populations, plant densities and pest attacks in plots where Wireworms were monitored by pheromone traps in 2009

An overview of results is reported in Tables 21 and 22. In the test plots the number of larvae per trap was always below tolerance threshold and no severe damage from soil insects was observed. The obtained results confirm the findings of trials on this issue described in the past decade, including the first year of Apenet experiments.
Severe damage on the maize crop (such that yield is compromised) caused by soil insects was confirmed as a rare event, plant densities were high and the insect attacks lower or only slightly higher than 1% of total plants, including plants with easily reversible symptoms (yellow stripes).
A clear correlation between adult species captured with the pheromone traps and larval populations.

2) Monitoring with YATLORf pheromone traps set to respond to the main Wireworm species and Western Corn Rootworm

The results of the monitoring activity in the Veneto region are summarised in Table 23. In the second year, apart from limited cases, the distribution of adults in the main species corresponds to what was observed in the first year. Variability among locations is high, confirming that an integrated pest management can be applied differentially according to risk levels.

Table 23 – Numbers of adult Wireworms (*Agriotes* spp) and Western Corn Rootworms (WCR) (*Diabrotica virgifera virgifera*) captured with YATLORf traps in the Veneto plots. Prov. = Province.
A list of the symbols of the Italian provinces can be found on the website:
http://www.tuttocamere.it/files/varie/Province_Sigle.pdf

Prov.	**Municipality**	**2010 crop**	**Previous crop in rotation**	**Total *A. brevis***		**Total *A. sordidus***		**Total *A. ustulatus***		**Total WCR year 2010**
				2009	**2010**	**2009**	**2010**	**2009**	**2010**	
VE	San Donà di Piave	maize	maize	30	60	430	677	365	492	0
VE	Caorle	maize	soybean	22	28	655	557	252	750	1
VE	S. Stino	maize	soybean	31	1	786	758	243	n.r.	n.r.
VE	S. Stino	maize	soybean	50	1	1015	486	278	1010	0
VE	S.Stino	maize	soybean	47	9	1170	767	nd	1063	n.r.
VE	Motta di L.			45	7	805	856	90	95	n.r.
TV	Motta di L.	maize	maize	36	no	1080	no	nd	no	n.r.
TV	Chiarano	maize	maize	40	41	422	750	55	50	n.r.
TV	Chiarano	maize	maize	48	40	644	864	150	100	0
TV	Chiarano	sugar beet	maize	4	50	941	1500	9	30	n.r.
TV	Chiarano	sugar beet	maize	4	45	891	920	22	35	n.r.
VE	Caorle	alfa-alfa	alfa-alfa	274	147	361	1920	nd	900	n.r.
VE	Caorle	alfa-alfa	alfa-alfa	145	197	185	1111	24	687	n.r.
VE	Caorle	alfa-alfa	alfa-alfa	30	191	602	1650	10	n.d.	n.r.
VE	Caorle	maize	sugar beet	36	198	2129	1424	27	111	0
VE	Caorle	rye-grass/ soybean II	sugar beet	no	125	no	619	no	1275	n.r.
VE	Caorle	wheat/ soybean II	sugar beet	no	303	no	1188	no	755	n.r.
VE	Torre di Mosto			10	no	570	no	nd	no	n.r.
VE	Torre di Mosto			12	no	729	no	nd	no	n.r.

VE	Concordia Sagittaria			20	no	1871	no	119	no	n.r.
VE	Concordia			49	no	875	no	nd	no	n.r.
VE	Caorle	maize	barley	3	4	206	1084	2786	1421	n.r.
VE	Caorle	maize	oilseed rape	0	17	505	1022	2754	1353	0
VE	Caorle	maize	barley-soybean	8	39	529	887	2541	1100	n.r.
VE	Caorle	maize	barley	1	25	467	1580	1616	1910	n.r.
VE	Caorle	soybean	sugar beet	0	22	225	1435	1340	n.r.	n.r.
VE	Caorle	soybean	sugar beet	no	20	no	757	no	2568	n.r.
VE	Eraclea	maize	sugar beet	36	3	535	438	300	1533	n.r.
VE	Eraclea	maize	sugar beet	30	4	414	460	320	1955	n.r.
VE	Eraclea	soybean	maize	13	5	456	525	974	1878	n.r.
VE	Eraclea	soybean	maize	10	3	324	612	750	1105	n.r.
VE	Eraclea			18	no	344	no	615	no	n.r.
VE	Eraclea			21	no	344	no	512	no	n.r.
TV	Ponte di Piave	maize	soybean	no	745	no	782	no	28	n.r.
TV	Cessalto	soybean	maize	no	78	no	1665	no	n.r.	0
TV	Cessalto	soybean	maize	no	120	no	2562	no	n.r.	n.r.
TV	Cessalto	soybean	soybean	no	110	no	1541	no	n.r.	n.r.
TV	Ceggia	maize	soybean	n.r	100	n.r.	1500	821	n.r.	n.r.
TV	Ponte di Piave			64	no	165	no	720	no	n.r.
TV	Ponte di Piave			22	no	133	no	72	no	n.r.
TV	Ponte di Piave			42	no	148	no	600	no	n.r.
TV	Ponte di Piave			144	no	489	no	410	no	n.r.
TV	Cessalto			35	no	1040	no	177	no	n.r.
TV	Cessalto			40	no	1175	no	n.d.	no	n.r.
VE	Eraclea	maize	maize	78	97	287	1015	1000	n.r.	n.r.
VE	Eraclea	maize	maize	4	2	245	250	620	n.r.	n.r.
VE	Eraclea	maize	maize	0	3	264	619	880	n.r.	n.r.
VE	Eraclea	maize	maize	1	n.r.	235	n.r.	800	n.r.	n.r.
VE	San Donà di Piave	maize	maize, maize	49	50	301	305	284	200	0
VE	San Donà di Piave	maize	maize, maize	56	28	178	389	543	n.r.	0
VE	San Donà di Piave	maize	maize, alfa-alfa	35	4	101	425	518	n.r.	n.r.
VE	San Donà di Piave	maize	soybean	79	361	767	1500	1766	1511	0
VE	San Donà di Piave	soybean	maize	54	72	639	934	1322	990	n.r.
VE	Eraclea	maize	maize, maize	41	10	269	458	393	300	0
VE	Eraclea	maize	maize, maize	33	6	519	743	628	610	n.r.
VE	Eraclea	maize	soybean	36	47	126	348	496	297	n.r.
VE	Eraclea	maize	soybean	39	24	139	320	397	422	n.r.
VE	Eraclea	maize	soybean	15	26	91	208	329	745	0
VE	Torre di Mosto	maize	maize	167	98	471	403	602	n.r.	n.r.
VE	Ceggia	soybean	maize	47	no	273	no	719	no	n.r.
TV	Cessalto	maize	maize	71	196	574	2335	n.r.	230	n.r.
TV	Cessalto	maize	maize	691	234	1464	1520	n.r.	n.r.	3
TV	Cessalto	alfa-alfa		506	96	416	730	n.r.	n.r.	n.r.

The results obtained in the plots in Lombardy, Piedmont and Veneto show high variability of presence of the different sampled adult species (*Agriotes brevis, Agriotes sordidus, Agriotes litigiosus, Diabrotica virgifera*) (Table 24).

Table 24 - Data from Wireworm and Western Corn Rootworm monitoring in 8 stations where the 2010 evaluation of agronomic utility were carried out.

Region	Locality	ADULTS (captures: total/trap per site)					Wireworm larvae captures
		Agriotes brevis	*Agriotes sordidus*	*Agriotes litigiosus*	*Agriotes ustulatus*	*Diabrotica virgifera*	
Lombardy	Bergamo	189	0	131.5	N.D.	52	0
	Ostiglia (MN)	N.D.	131	632	N.D.	0	N.D.
Piedmont	Vigone (TO)	N.D.	453.5	454	N.D.	66.5	N.D.
	Chivasso (TO)	N.D.	395	1026	N.D.	211	N.D.
Friuli	Mortegliano (UD)	0	1012.5	28.5	19	N.D.	N.D.
	Palazzolo della Stella (UD)	0	904.5	17.5	332	N.D.	N.D.
	Codroipo (UD)	0	15	578	0	N.D.	N.D.
Tuscany	Marciano della Chiana (AR)	N.D	1	253.5	0	0	N.D.

N.D.: not determined

4.1.3 Strip-tests using seed coated with the different active ingredients

Materials and methods

In some fields in Veneto the agronomic trials were carried out by sowing large (300-1200 mq) parallel plots with the same commercial hybrid PR32G44 (PIONEER) coated in one of the following ways:

1) Fungicides only: metalaxil+fludioxonil (Celest®) at the dose of 100 ml/q of seed;
2) Cruiser: seed treated with fungicide as in 1) and also with thiametoxam (Cruiser®), at the dose of 0.65 mg of a. i./seed;
3) Regent: seed treated with fungicide as in 1) and also with fipronil (Regent® TS) at the dose of 0.50 mg of a. i./seed.
4) Gaucho: seed treated with fungicide as in 1) and also with imidacloprid (Gaucho®) at the dose of 1.0 mg of a. i./seed.
5) Poncho: seed treated with fungicide as in 1) and also with clothiadinin (Poncho®) at the dose of 1.25 mg of a. i./seed.

The main characteristics of the different fields are reported in Table 25. The experimental set up consisted in 2-4 repetitions per site in 7 localities.

The measured parameters were:
• plant density at emergence
• attack on seed – plantlets
• plant density at 4-8 leaves stage
• attack on 4-8 leaves stage
• other attacks (aphids, viruses, ….) on 4-8 leaves stage
• plant density at harvest

The results are summarised in Table 26. The differences in plant densities among the experimental groups were not statistically significant, although the proportion of attacked plants was significantly higher in the control group.

The small differences in average crop yield were not statistically significant. The average crop production from insecticide-coated seed was 119.85 q/ha, from non-insecticide-coated seed (control group) the average yield was 119.3 q/ha.

Table 25 – Characteristics of the experimental fields used in the 2010 trials.

Farm	**Municipality**	**Prov.**	**Soil**	**Previous crop**	**Wireworm population**		**Size of strip plot (m^2)**	**Sowing date**	**Seed density (seed/m^2)**	**Harvest date**
					larvae/trap *A. sordidus*	**larvae/trap *A.* spp**				
Greggio	Eraclea, Ponte Crepaldo	VE	sand-silt	maize	0.50	0.00	297	18-apr	7.84	23-oct
Parcianello	Eraclea, Coda di Gatto	VE	loam	soybean	0.05	0.00	364	21-apr	7.24	07- oct
San Donà, Isiata	Florian	VE	loam	maize	0.05	9.95 *A.ustulatus*	951	17-apr	7.21	21- oct
Vallevecchia	Caorle	VE	loam	soybean	0.17	0.00	459	20-apr	7.25	20- oct
Zanazzo	Cessalto	TV	clay	maize	0.33	0.83 *A.ustulatus* 0.25 *A.brevis*	390	20-apr	6.87	14- oct
Diana	Mogliano Veneto	TV	loam	wheat	0.08	0.58 *A.ustulatus* 0.25 *A.brevis*	722	15-apr	7.28	11- oct
Sasse Rami	Ceregnano	RO	loam	wheat	0.20	0.06 *A.litigiosus*	1395	16-apr	7.13	15- oct

Table 26 - Effect of seed coating on maize crop. Mean values from 7 fields. Dati medi di 7 campi. Means not follone by a same letter are statistically significant at $p<0.05$.

TREATMENT	**Plant density (healthy plants/ m^2)**		**Attacked plants**		**Yield**
	emergence	***6-8 leaves***	***plants/* m^2**	***emergence***	***6-8 leaves***
Fungicide only	6.56a	6.70a	0.16b	2.33	119.3a
Fungicide + Cruiser	6.34a	6.67a	0.02a	0.29	117.4a
Fungicide + Regent	6.46a	7.03a	0.05a	0.71	119.4a
Fungicide + Gaucho	6.43a	6.69a	0.03a	0.45	119.5a
Fungicide + Poncho	6.46a	6.77a	0.04a	0.59	123.1a
F 4.95 (ANOVA)	0.16	1.73	7.07		0.26
P	0.9583	0.1498	0.0001		0.9047

4.1.4 On-farm experiments with clothianidin

Materials and methods

During the 2010 maize-growing campaign, the Regions of Lombardy, Piedmont and Veneto (Veneto Agricoltura) planned a series of trials jointly with the Apenet Project, in order to assess the extent to which final grain production is influenced by a neonicotinoid insecticide applied to maize seed.

Seed of the hybrid PR32G44 (considered representative of the maize germplasm currently used in Italy), belonging to the same lot (size of grain, year and place of production) was used; the seed was divided in two parts: one (group A) was treated with the fungicide CELEST and then with the insecticide Poncho (a.i. Clothianidin) at the dose of 1.3 mg a.i. per seed, the other was treated only with the fungicide CELEST.

Simple experimental designs were chosen to minimize the effect of environmental variability using experimental strips or large plots placed "side by side".

The study was conducted in 65 localities representative of the whole Italian maize growing area, articulated in different experimental networks:

- 37 localities coinciding with the "On Farm" varietal network, situated mainly in Lombardy, but also in Piedmont and Veneto; the treatments were inserted in large plots (800-1200 m^2) "side by side" within the overall field layout, without replication.[1]
- In 9 localities experimental designs with randomised blocks were applied, still within the "on farm" layout, with 2 to 6 replications. These localities were chosen in Lombardy, in the area between Cremona, Brescia, Bergamo e Lodi, where severe Western Corn Rootworm infestations were noted. In these trials specific surveys on the efficacy of seed coating on corn root protection from Diabrotica larvae attacks were carried out. [2]
- In 19 localities long row plots measuring 30 m^2 were inserted in the split-plot design with 4 replications of the national agronomic-varietal trials, situated in Piedmont, Lombardy, Veneto, Friuli, Emilia Romagna and Tuscany.

[1] The "On Farm" varietal network is made up from the group of regional trials networks belonging to the Regions:

- ***Lombardy**, coordinated by the General Agriculture Directorate and ERSAF and run in collaboration with the Provinces of Pavia, Milano, Bergamo, Brescia, Lodi and Cremona in the framework of the project "Major Crops";*
- ***Piedmont** coordinated by the General Agriculture Directorate and ERSAF and run in collaboration with the Provinces;*
- ***Veneto**, run by Veneto Agricoltura.*

[2] Run in collaboration with the Lombardy Region

Results

The results of the two treatments object of study (T= treated with Poncho; NT= not treated with Poncho) are reported in Table 27. Localities are listed according to decreasing productive differences between the T and NT groups. Le columns on the left of the table indicate the sites and specify the exact location, the first column indicates the sub-network of the trial (OF= "on farm" network; OF+=additional "on farm" network in areas with high Diabrotica infestation; AG=national trials network).

Table 27 - Trial localities and productive results from the treated groups.
A list of the symbols of the Italian provinces can be found on the website:
http://www.tuttocamere.it/files/varie/Province_Sigle.pdf

Trial		Region	Province	Locality	Production of grain 14% (q.l/ha)		Production difference	Replications
					PR32G44	**PR32G44 + Poncho**		
1	AG	Friuli	UD	Palazzolo della Stella	73.18	96.35	**23.17**	4
2	AG	Veneto	PD	Montagnana	139.98	162.68	**22.7**	4
3	OF	Lombardy	CR	Trigolo	130.55	153.19	**22.64**	1
4	AG	Piedmont	AL	Castelceriolo	132.65	148.08	**15.43**	4
5	OF	Lombardy	MN	Medole	144.10	159.38	**15.28**	1
6	OF	Lombardy	CR	Stagno lombardo	131.92	147.15	**15.23**	1
7	OF	Veneto	VE	Vallevecchia	103.90	118.90	**15.00**	1
8	AG	Friuli	UD	Mortegliano	135.15	149.1	**13.95**	4
9	AG	Lombardy	BS	Castenedolo	112.50	125.75	**13.25**	4
10	OF	Lombardy	LO	Ossago Lodigiano	155.44	168.63	**13.19**	1
11	OF	Lombardy	MI	Robecco	135.77	148.32	**12.56**	1
12	OF	Lombardy	BS	Cigole	135.54	147.33	**11.80**	1
13	AG	Lombardy	MN	Ostiglia	114.65	126.33	**11.68**	4
14	OF	Piedmont	CN	Scarnafigi	104.07	115.73	**11.66**	1
15	OF	Lombardy	CR	Castelleone	96.40	107.68	**11.28**	1
16	OF	Lombardy	BG	Pagazzano	144.77	155.90	**11.14**	1
17	AG	Lombardy	MI	Caleppio di Settala	167.83	178.78	**10.95**	4
18	OF	Lombardy	CR	Luignano di Sesto C.	144.33	155.27	**10.93**	1
19	OF+	Lombardy	CR	San Bassano	117.86	128.63	**10.77**	2
20	OF+	Lombardy	LO	Terranova dei Passerini	124.29	134.62	**10.33**	2
21	OF	Lombardy	BS	Orzinuovi	118.76	128.69	**9.93**	1
22	OF+	Lombardy	CR	Castelgabbiano	105.86	114.99	**9.13**	2
23	OF	Veneto	TN	Mogliano Veneto	113.2	122.2	**9**	1
24	OF	Lombardy	BG	Arcene	120.86	129.83	**8.96**	1
25	OF	Lombardy	BS	Travagliato	114.80	123.11	**8.31**	1
26	OF	Veneto	RO	Ceregnano Sasse	121.50	129.80	**8.30**	1
27	AG	Veneto	VI	Noventa V.	133.65	141.48	**7.83**	4
28	OF+	Lombardy	BS	Cigole	108.96	116.6	**7.64**	2
29	OF	Lombardy	MI	Cisliano	131.43	139.04	**7.61**	1
30	OF	Piedmont	TO	Vische	109.56	117.16	**7.60**	1
31	OF+	Lombardy	CR	Soncino	114.26	121.45	**7.19**	2
32	AG	Emilia Romagna	FE	Ambrogio	133.73	140.28	**6.55**	4
33	OF	Lombardy	PV	Bereguardo	108.60	114.93	**6.33**	1
34	OF	Lombardy	CR	Spinadesco	109.73	114.75	**5.03**	1
35	OF	Piedmont	CN	Cuneo	152.68	157.38	**4.70**	1
36	OF	Lombardy	CR	Castelleone	100.95	105.38	**4.44**	1
37	OF	Veneto	TV	Parcianello	121.13	125.54	**4.41**	1
38	AG	Veneto	VI	Lonigo	119.58	123.65	**4.07**	4

39	AG	Lombardy	BG	Bergamo	145.75	149.33	**3.58**	4
40	OF	Lombardy	MI	Albairate	137.92	141.31	**3.39**	1
41	AG	Veneto	RO	Villadose	140.98	143.75	**2.77**	4
42	OF	Veneto	TV	Florian	116.44	119.15	**2.71**	1
43	OF	Piedmont	AL	Alessandria	131.62	134.29	**2.67**	1
44	OF	Piedmont	CN	Savigliano	162.22	164.88	**2.66**	1
45	OF+	Lombardy	CR	Trigolo	129.84	132.36	**2.52**	2
46	OF	Veneto	TV	Florian	119.18	121.1	**1.92**	1
47	OF	Piedmont	CN	Fossano	152.06	153.65	**1.59**	1
48	OF	Lombardy	BS	Carpenedolo	126.66	128.10	**1.44**	1
49	OF	Piedmont	BI	Cavaglia	133.05	134.27	**1.22**	1
50	OF	Lombardy	MI	Cuggiono	125.34	126.20	**0.86**	1
51	AG	Lombardy	CR	Luignano di Sesto C.	107.93	108.65	**0.72**	4
52	AG	Friuli	UD	Codroipo	153.28	152.98	**-0.3**	4
53	AG	Lombardy	LO	S.Angelo Lodigiano	124.80	124.20	**-0.60**	4
54	OF+	Lombardy	BG	Fontanella	127.03	126.16	**-0.87**	6
55	OF+	Lombardy	CR	Palazzo Pignano	128.52	127.3	**-1.22**	2
56	OF	Piedmont	TO	Villareggia	143.31	141.78	**-1.52**	1
57	OF+	Lombardy	CR	Ripalta Arpina	115.51	113.76	**-1.75**	2
58	OF	Lombardy	LO	S.Angelo Lodigiano	143.16	140.41	**-2.75**	1
59	AG	Piedmont	TO	Vigone	157	153.65	**-3.35**	4
60	AG	Emilia Rom.	PR	Parma	143.65	140.1	**-3.55**	4
61	AG	Piedmont	TO	Chivasso	133.95	129.65	**-4.30**	4
62	OF	Piedmont	CN	Cherasco	149.58	144.86	**-4.72**	1
63	OF	Lombardy	BS	Comezzano	164.81	157.66	**-7.15**	1
64	OF	Lombardy	BG	Mapello	156.04	148.29	**-7.75**	1
65	AG	Tuscany	AR	Marciano della Chiana	140.73	130.53	**-10.2**	4
				media	**129.15**	**135.11**		135

The average yield of grain with 14% humidity obtained in the 65 trial locations, with 135 single observations, of the Poncho treated group was 135.11 q/ha while the average yield of the non Poncho treated group was 129.15 q/ha, with an average difference of 5.96 q/ha.
Analyses of variance of the single observations showed statistically significant effects ($p<0,01$) for the factors locality and treatment, the latter with a low variability coefficient and a minimum significant difference of 1.78 q/ha.
In Figure 13 the frequency distribution of the classes of yield difference are reported: in 7 localities the productive advantage of the treatment were higher than 14 q/ha, in 19 localities higher than 8 q/ha, in another 19 localities the productive gain was included between 2 and 8 q/ha. In 12 localities no productive differences were observed, and finally in 8 localities the non treated group had a higher yield than the treated group.
This last result is not easily interpretable and is probably due to "experimental error" (non homogeneous soil, non accurate agronomic management etc) rather than due to specific negative effects of the treatment, already described for example, in the interference with germinability characteristics and germination energy.

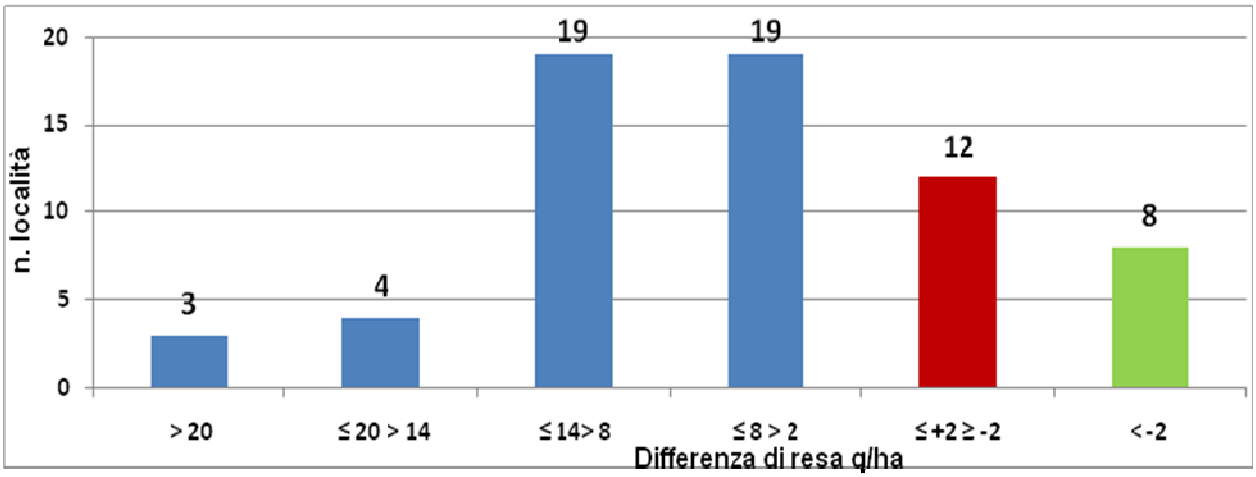


Figure 13 - Frequency distribution of the yield difference classes. Translation of text within the figure: *Differenza di resa* = Yield difference; *n. località* = number of localities.

Figure 14 summarises the partitioning discussed previously: 70% of the trial sites (reactive locations) showed a positive reaction to use of seed coating, 18% (non reactive localities) showed no reaction while in the remaining 12% negative or "questionable" reactions were registered.

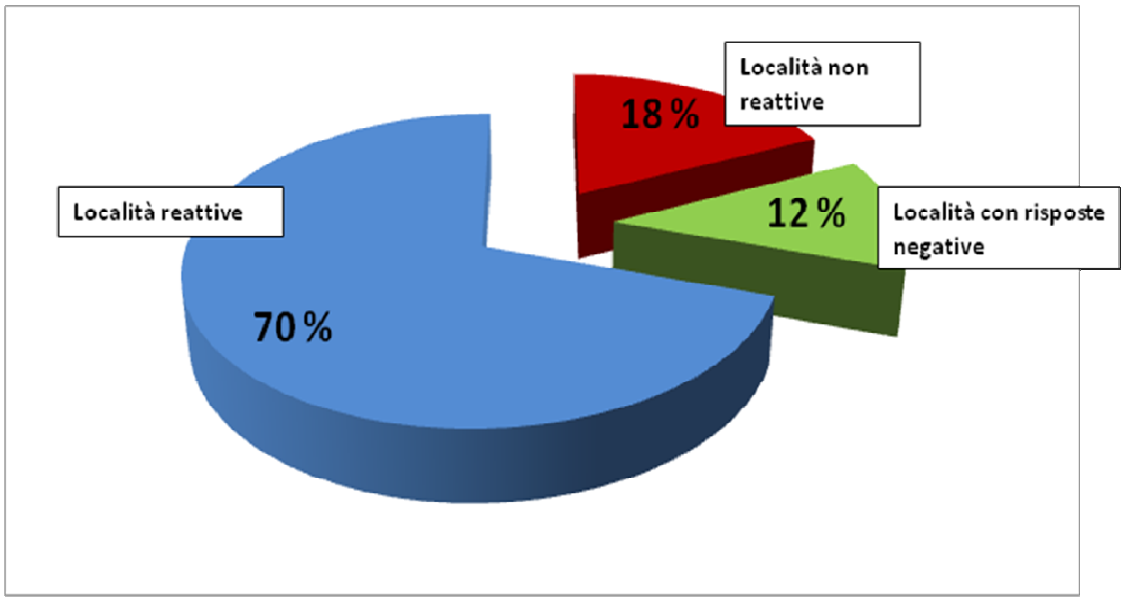


Figure 14 - Percentage of trial sites with positive (*Località reattive*), absent (*Località non reattive*) or negative (*Località con risposte negative*) productive reaction to seed coating with Poncho (clothianidin).

Considering that i) the number and geographic distribution of the trial sites give the farms the status of representative samples of the Italian maize growing area and that ii) the coating technology is specific for the protection of the plant from insects during the first stages of the crop establishment, we could suggest a more practical and likely reading of the data from the trials: in the 2010 productive season the global pressure of harmful insects was "appreciably" evident in 70% of the surveyed sites; in these areas the use of Poncho coated seed allowed a productive gain (or to save a quantity of grain) corresponding to 9.56 q/ha.

4.1.5 Effects of seed coating on the entity of root damage caused by Western Corn Rootworm

In 9 localities of the previous trials, chosen in an area of Lombardy between Cremona, Brescia, Bergamo and Lodi, in which a strong pressure of Western Corn Rootworm was registered, the effect of seed coating on the protection of maize roots from the larvae was specifically studied. In Table 28 the list of surveyed localities and the agronomic practices considered important for the plant-insect interaction dynamics are reported.

Table 28 - Location of the trials and agronomic practices relevant to insect activity.

STRIPS	**Sowing date**	**Date of 1° irrigation**	**Previous crop**			**Treatment on adults**	
			2007	2008	2009	2009	2010
Trigolo (CR)	11/04/2010	12/06/2010	maize	maize	maize	YES	YES
Soncino (CR)	10/04/2010	07/06/2010	maize	maize	maize	YES	YES
Terranova dei Passerini(LO)	13/04/2010	10/06/2010	maize	maize	maize	YES	NO
Ripalta Arpina (CR)	17/04/2010	05/06/2010	maize	maize	maize	YES	YES
Castelgabbiano (CR)	11/04/2010	11/06/2010	maize	maize	maize	YES	YES
Comezzano-Cizzago (BS)	26/05/2010	08/06/2010	maize	maize	maize	YES	YES
San Bassano (CR)	16/04/2010	04/06/2010	maize	maize	maize	YES	YES
Palazzo Pignano (CR)	12/04/2010	12/06/2010	maize	maize	maize	NO	NO
Fontanella (BG)	16/04/2010	11/06/2010	maize	maize	maize	NO	NO

In this survey the same groups as in the other trials were considered: seed treated with Poncho versus non-Poncho treated seed. ;sowing occurred within the "on farm" layout, "side by side" in large plots (800-1200 m^2) with 2-6 replications.
Specific observations were carried out to estimate the presence of larvae on the roots and the entity of root damage. In the month of June, with the plants in the vegetative growth stage, 3 plants for each replication of each group were sampled, yielding a total number of 114 plants. Larvae, mostly belonging to 2^{nd} and 3^{rd} stages, were extracted with the simple method of the "Berlese funnel".
Plants belonging to the non treated group had a much higher number of larvae, compared to plants belonging to the treated group (130 vs 24) in all localities, with the exception of Ripalta Arpina (Figure 15).

In July, when the larval stage is over and the insect has reached the adult stage, root damage was estimated. Five plants from each replication and group were sampled, yielding a total of 220 plants.
Root damage was measured according to the Oleson international classification *(Node-injury Scale, Oleson, J.Econ. Entomol. 98(1): 1-8 2005)* (Fig. 16). The observations revealed greater damage on the non treated plants: the treated plants showed a 50% reduction of root damage compared to non treated plants.
When the two series of data were submitted to regression analysis, a strong association between use of seed coating and root damage reduction, with values of the determination coefficient R^2 close to 1 and regression coefficient b=0.5 (Figure 17).

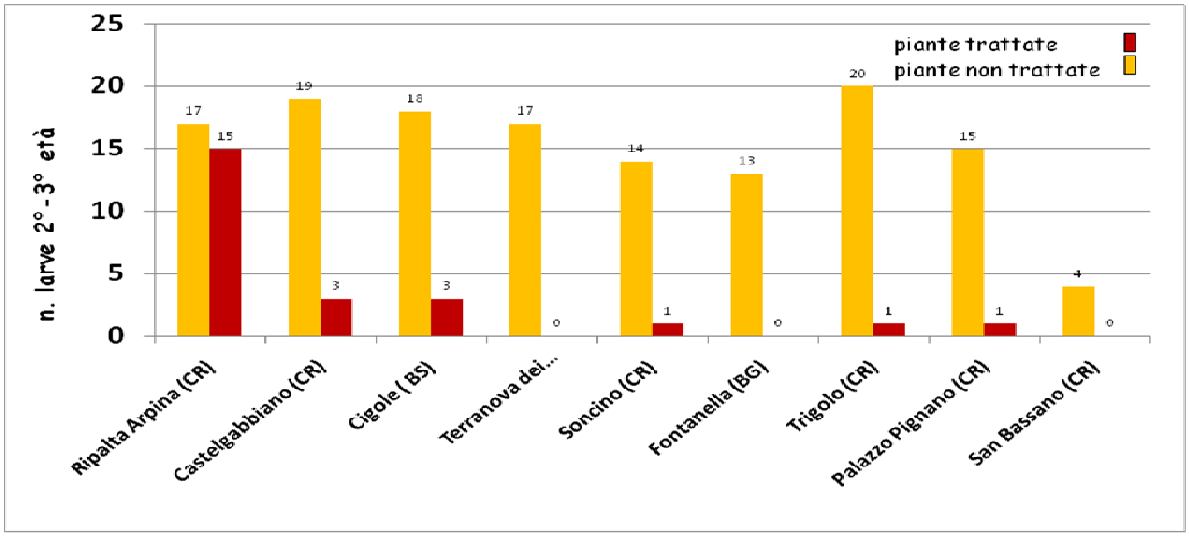


Figure 15 - Observed number of Western Corn Rootworm larvae in maize plants originating from treated and non treated seed. Translation of text within the figure: *n. larve 2°-3° età* = number of 2nd-3rd stage larvae; *piante trattate* = treated plants; *piante non trattate* = non treated plants.

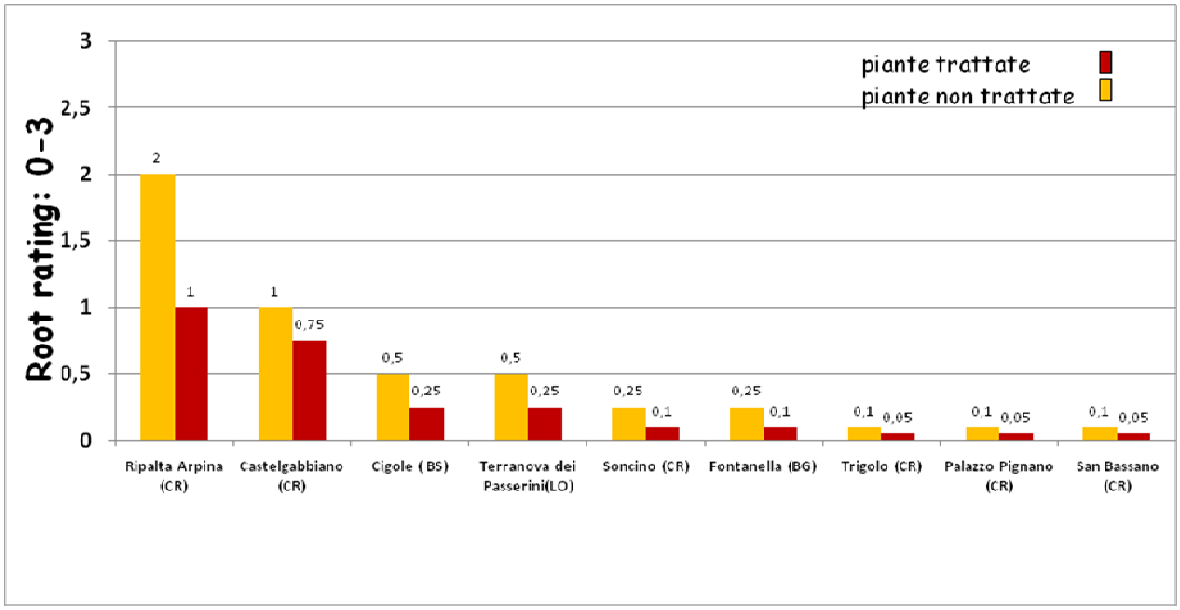


Figure 16 - Root damage (Node-injury Scale) observed in the treated (*piante trattate*) and non treated (*piante non trattate*) groups.

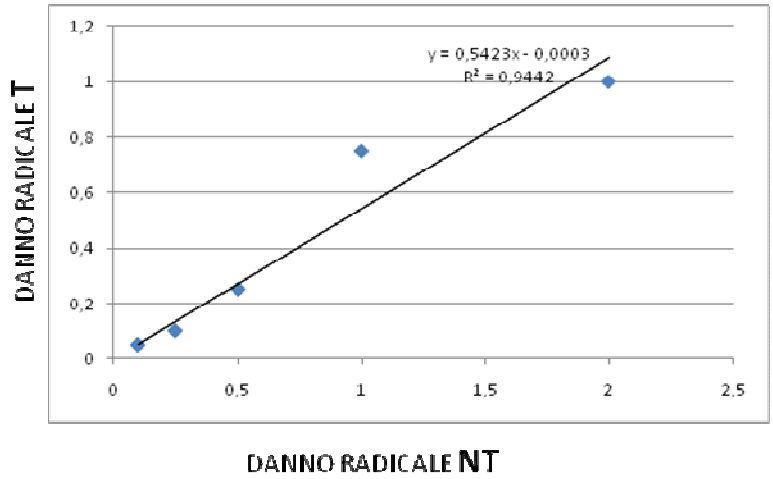


Figure 17 - Regression line of root damage in treated (T) vs non treated (NT). Translation of text within the figure: *Danno radicale* = root damage.

Table 29 summarises the results of observations performed in this trial, as well as the number of larvae present on the roots and damage classification, grain yield of the two groups are reported. In our experimental conditions, with low levels of infestation, no correlation between final grain production and root damage was highlighted in either group.

Table 29 – Relation between root damage caused by diabrotica and grain yield of the crop.

Locality	**N. larvae**		**Root damage**		**Yield q/ha**		**Difference in yield**
	NT	T	NT	T	NT	T	
Ripalta Arpina (CR)	17	15	2	1	115.51	113.76	-1.75
Castelgabbiano (CR)	19	3	1	0.75	105.86	114.99	9.13
Cigole (BS)	18	3	0.5	0.25	108.96	116.60	7.64
Terranova dei P. (LO)	17	0	0.5	0.25	124.29	134.62	10.33
Soncino (CR)	14	1	0.25	0.1	114.26	121.45	7.19
Fontanella (BG)	13	0	0.25	0.1	127.03	126.16	-0.87
Trigolo (CR)	15	1	0.1	0.05	129.84	132.36	2.52
Palazzo P. (CR)	13	1	0.1	0.05	128.52	127.30	-1.22
San Bassano (CR)	4	0	0.1	0.05	117.86	128.63	10.77
Mean ± SD			*0.53 ± 0.62*	*0.29 ± 0.34*	*119.13 ± 8.73*	*123.99 ± 7.64*	

4.2 Study of persistence in plant tissue of the active ingredients used in seed coating

Materials and methods

In order to study the persistence of active ingredients of seed coating in maize plants at diferent stages of development, 50 m long plots were set up at the CRA-MAC Experimental Farm during the 2009 maize growing season. The plots were sown with material sent by the Associazione Italiana Sementi-ASSOSEMENTI for the 2009 agronomic trials, namely a commercial Maize hybrid (PR31N27- FAO 700). Trials involved the follwing 5 treatments:

Treatment	Fungicide	Insecticide active ingredient
1 - Testimone	*Celest	none
2 - Cruiser	*Celest	thiamethoxam
3 - Gaucho	*Celest	imidacloprid
4 - Poncho	*Celest	clothianidin
5 - Regent	*Celest	fipronil

* The fungicide Celest contains fludioxonil and metalaxyl.

For each of the five treatments under study, determinations were performed on organs of maize plants at different phenologic stages, taken from the trial plots.
Evaluation of the persistence of the active ingredient of seed coating in different plant development stages was carried out by adopting the HPLC/MS/MS method, in accordance with Good Laboratory Practices (B.P.L. Prot. CH-012-2010-Test Laboratory of ChemService Prot. CH - 013/2010), adapting the protocol of Bonmatin *et al*. 2003, *Anal. Chem*., 75, 2027-2033.

Results

In Table 30 the dilution factor compared to initial content, brought to 1 (dilution unit) and equivalent to 100% of a. i. contained in a single seed, is reported for each coating a. i. at various maize plant phenologic stages. The results indicate that the four insecticidal active ingredients studied showed a drastic reduction in levels detected in leaves, from the 2^{nd}-3^{rd} to the 7^{th} -8^{th} leaf stage and then declined to to non detectable levels (n.d.: not detected, lower than L.O.D. < 0,5.0,5 µg/kg) by the stage of the 13^{th} – 14^{th} leaf. More specifically, Fipronil showed a drastic reduction in levels from the early plant development stages (2^{nd}-3^{rd} leaf), while the other 3 a. i. persisted, at this stage, at higher concentrations.
Investigations on the persistence of a. i. used for seed coating in pollen are underway, in collaboration with colleagues of CRA-PAV (Research Centre for Plant Pathology).

Table 30 – Persistence of the active ingredients used in seed coating in maize plant tissue at different phenological stages.

Insecticide active ingredient	**Coated seed**	**2^{nd}-3^{rd} leaf**	**7^{th} – 8^{th} leaf**	**13^{th} -14^{th} leaf**
	Initial content*	Dilution factor	Dilution factor	Dilution factor
Thiamethoxam	1*	16500	79260	> 428000
Imidacloprid	1*	7150	155200	> 714000
Clothianidin	1*	5500	71900	> 892000
Fipronil	1*	94200	162700	> 358000

* Initial content brought to 1 (dilution unit) and equivalent to 100% of a. i. contained in a single seed.

5. Effects of maize guttation on bees

Current knowledge on the factors that influence the presence of neonicotinoids in maize guttation is scanty. As neonicotinoid concentration in maize guttation may vary ten-fold without any apparent explanation, careful assessment of the various factors that may interact synergically or contrastively is required.
In the previous trials (2009), it was found that sowing on ordinary agricultural land rather than in containers did not *per se* induce differences in the composition of guttation fluid. In 2010 studies were undertaken to examine whether active ingredient concentration in guttations of greenhouse- or field-grown plants may be influenced by such factors as: 1) different length of exposure of seed and/or plantlets to different irrigation regimes; 2) protection of plants against weather conditions; 3) time of day at which sampling took place; 4) different soil typology.

5.1 Neonicotinoids in guttation fluid of field-grown maize plants derived from seed coated with thiamethoxam, clothianidin and imidacloprid

In April 2010, within an area forming part of the Faculty of Agriculture of the University of Padua, located in Legnaro (PD), eight 80 m-long rows were sown, using seed coated in 2009 for the first 4 rows and seed from 2010 for the remaining 4 rows. For the trials pertaining to each of these two years, the 3 neonicotinoid seed coatings were assayed, plus a further treatment using seed coated with fungicide alone (Celest XL). Sowing layouts followed the standard maize cropping spacing (75 cm inter-row and 19 cm intra-row).

1. A first series of samplings was performed by collecting guttation fluid according to three different procedures:
   - For each row, multiple collections of droplets of guttation liquid were taken from roughly 40 plants between 07.30 h. and 08.30 h. The droplets were stored in vials, using separate multiple-droplet vials in each row for each active ingredient and each seed coating procedure.
   - For each row, except for the two containing Celest only, further multiple collections were taken between 10.00 h. and 11.00 h of the same day, from roughly 40 plants situated in a additional portion of the field; the droplets were likewise stored in vials, using separate multiple-droplet vials in each row for each active ingredient and each seed coating procedure.
   - For each row sown with seed coated in 2010, 4 plants not previously sampled were identified, and from each of these plants 10 µL was collected from a single guttation droplet and then placed in a separate vial (date 30 April 2010, 08.00 h).
2. Additional samples were taken at various dates later than that of point 1.
3. Samples were taken from plants treated with clothianidin and imidacloprid, growing in a part of the same respective row but protected from adverse weather conditions by a sheet of plexiglass placed at a height of 50 cm from the ground.

Plantlet emergence, on 29th April, was regular and homogeneous. The first series of droplets (Point 1 above) was sampled on the next day (30th April 2010). The samplings of point 2 were performed on 7th, 14th, 17th, 18th and 21st May at varying times of the day, due to considerable rainfall during the period in question; each sampling was done on roughly 40 plants for all types of seed coating except for those containing Celest XL alone. The samplings of point 3 were performed

on 6th and 14th May, on plants grown from seed coated in 2010 with only clothianidin or with only imidacloprid.

Analyses of guttation samples collected at different times on the specified day, and analyzed at the Department of Chemistry of the University of Padua, are shown in Tables 31 and 32, and represented graphically in Figures 18 and 19.

Table 31 - Concentrations of different active ingredients in leaf guttation fluid collected from roughly 40 plants on 30th April 2010.

Time of sampling	Thiamethoxam 2009 (mg/L)	Thiamethoxam 2010 (mg/L)	Clothianidin 2009 (mg/L)	Clothianidin 2010 (mg/L)	Imidacloprid 2009 (mg/L)	Imidacloprid 2010 (mg/L)
8:00	79.1	117.3	31.9	44.7	105.7	179.8
11:00	201.3	227.3	46.3	19.2	221.7	76.9

Table 32 - Concentrations of active ingredients detected in samples by collecting one drop on 30/04/10, at 08:00 h, from 4 different plants grown from 2010 coated seed.

Plant	Active ingredient (mg/L)		
	Thiamethoxam*	Clothianidin	Imidacloprid
1	206.6	267.6	nr**
2	145.1	150.7	185.280
3	158.9	156.4	225.480
4	214.3	82.9	nr**

*Presence of Clothianidin in concentrations varying between 10 and 16 mg/L was detected in samples containing Thiamethoxam
**Not detected.

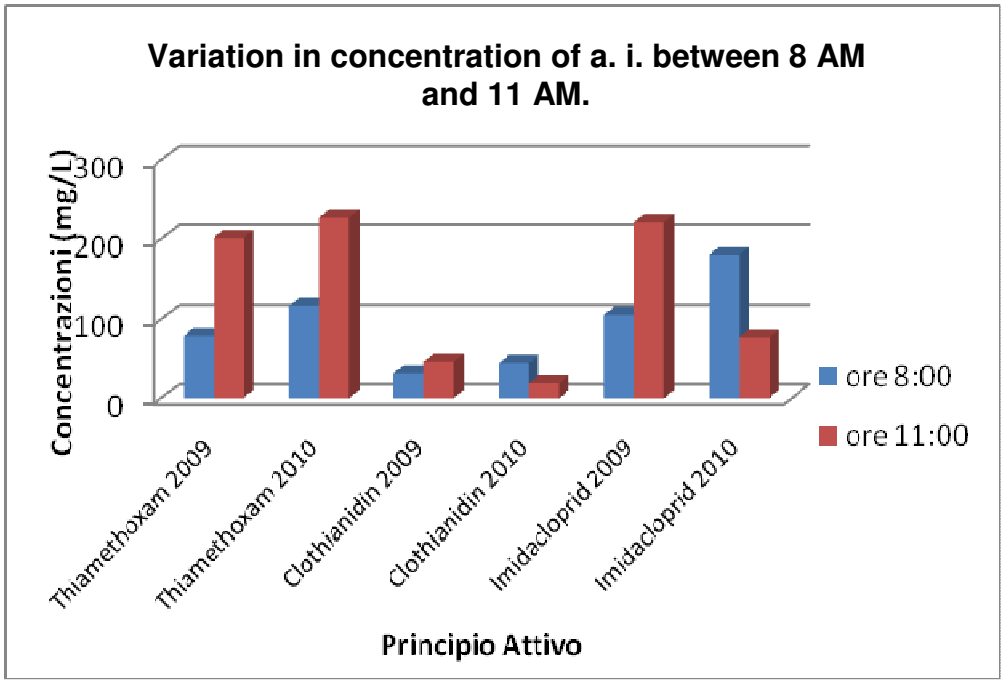


Figure 18 - Variation in concentration of the active ingredients under study between 08.00 h and 11.00 h.

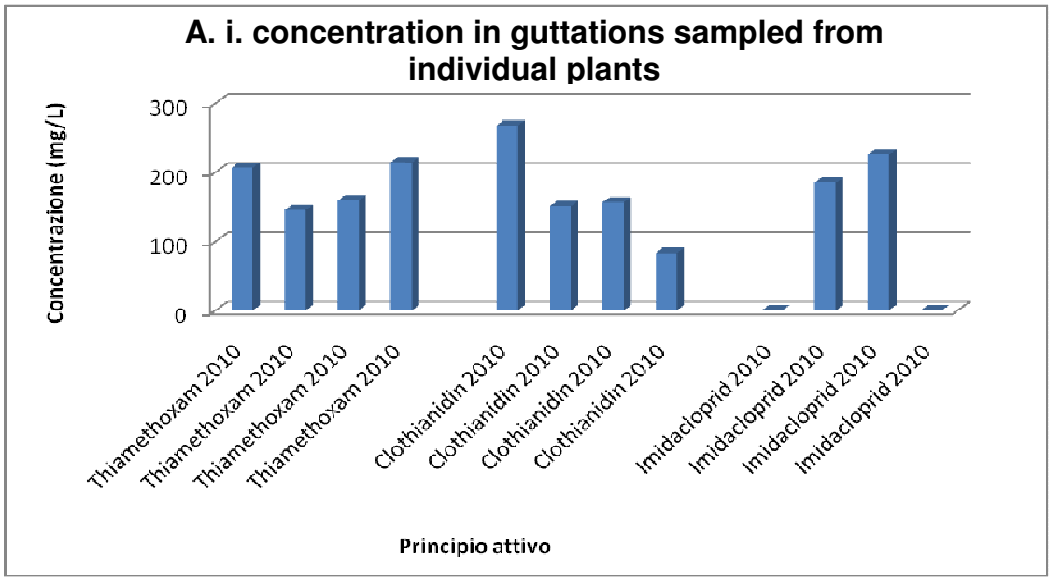


Figure 19 - Distribution of active ingredient concentrations in guttation droplet sampled from individual plants.

The results on "multiple" guttations collected at 08.00 h (Table 31) showed that persistence of the active ingredient used in the seed coating process was similar in the two years. Imidacloprid was dispersed through the plants via the lymph flow to a greater extent than occurred with thiamethoxam, and the latter in turn had a broader distribution pattern than clothianidin. This finding is basically in line with the water solubility of these active ingredients (clothianidin is more hydrophobic), given that the lymph is a fundamentally watery medium.

With regard to guttations collected at 11.00 h, concentration and the distribution pattern of the active ingredient used in the seed coating process did not differ between 2009 and 2010, with the exception of thiamethoxam, for which a twofold greater concentration was detected at 11.00 h than at 08.00 h in 2010. The other two active ingredients exhibited an opposite trend: thus concentration

at 11.00 h compared to 08.00 h dropped by roughly 50% for plants grown from 2010 coated seed, whereas it rose, to an extent ranging between 30% greater and 100% greater.for 2009 seed coating. Since no substantial physical difference can be noted between the two seed coating processes, it is difficult at this time to provide a satisfactory explanation for these results. The expected trend, namely an increase in concentration due to evaporation of the watery component with increasing solar radiation from 08.00 h to 11.00, was observed only in guttations of plants grown from 2009 coated seed.

Analyses of individual droplets (Table 32) gave no clear-cut conclusions, as the results were highly variable from plant to plant. Furthermore, particularly in the case of clothianidin, the results on individual droplets were in strong contrast with the findings for "multiple" guttations. It cannot be ruled out that this variability may have been caused simply by the drop falling prior to sampling, in which case the question of evaporation does not arise at all. However, devising a procedure to test this hypothesis is likely to be very challenging.

The values of guttation samples collected in the field on different dates are shown in Table 33. The results indicate that immediately after emergence the values were elevated; subsequently, probably on account of the heavy and frequent rainfall (which for a week made sampling impossible), active ingredient concentration exhibited a decreasing trend as the days went by.

Finally, Table 34 shows the clothianidin and imidacloprid concentrations detected in plants maintained beneath the plexiglass cover in comparison to concentrations in analogous but field-grown plants (from seed coated with the same active ingredients). A greater presence of active ingredient was noted in the plants that were protected against rain. This was probably not only because the active ingredient had been subjected to a lesser degree of leaching, but also because the different microclimate under the plexiglass resulted in stronger concentration of the guttation droplets.

Table 33 - Variation over time in concentration of active ingredients in guttation droplets

Day	Thiamethoxam 2009 (mg/L)	Thiamethoxam 2010 (mg/L)	Clothianidin 2009 (mg/L)	Clothianidin 2010 (mg/L	Imidacloprid 2009 (mg/L	Imidacloprid 2010 (mg/L)
30 April	140.2	172.3	39.1	31.9	163.7	128.4
7 May	1.3	2.4	1.3	1.0	2.2	1.0
14 May	0.3	0.4	2.4	1.0	0.8	0.8
17 May	0.1	0.2	0.8	1.1	0.4	0.5
18 May	0.2	0.3	1.2	0.8	0.6	0.4
21 May	0.1	0.1	0.7	0.6	0.4	0.2

Table 34 - Concentration of two different active ingredients under varying environmental conditions.

Day	Situation where plants were grown	Clothianidin 2010 (mg/L)	Imidacloprid 2010 (mg/L)
14 May	Open field	1.021	0.825
14 May	Under cover	2.376	1.172

5.2 Insecticide concentration in guttation droplets derived from plants sown and grown in a tunnel under different water regimes.

In order to assess the effect of different water availability on active ingredient concentration in guttations, maize was sown in a tunnel (to protect plants from rain), on agricultural soil. The tunnel was divided into 8 plots (4,5.4,5 x 4 m); each plot was sown with maize seed that had undergone seed coating with one of the 3 neonicotinoids or fipronil. Different quantities of water were applied: 50 litres/22.5 m² (dry), 1.100 litres/22,5.2,5 m² (damp) and 2.500 litres/22,5.2,5 m² (wet). Water was sprinkled on the plots during the three days after sowing, up to the above indicated volumes of water. Sowing spacing was as follows: on the left-hand side of the tunnel, 60 x 20 cm; on the right-hand-side of the tunnel 30 x 20 cm. These contrasting distances were chosen in order to determine whether the different density of the emerged plantlets gave a different insecticide concentration in guttations.

Guttation samples were collected from the day of emergence up to the twenty-seventh day after plantlet emergence. The analyses, performed at the Department of Chemistry of the University of Padua, were conducted first of all on samples deriving from plants sown at the 60 cm interrow spacing, examining the results obtained with the three irrigation regimes for each of the 4 active ingredients and for each sampling day. Subsequently, the corresponding samples deriving from the 30 cm interrow spacing were analyzed according to the same procedures.

For the majority of the series of samples, greater concentration of active ingredient was observed in guttation droplets collected on the eleventh and thirteenth day after emergence. These concentrations were considerably higher than previously observed in container-sown plant guttations. Furthermore, an interesting finding was that guttations from plants in the 30 cm interrow spacing almost always showed lower concentration peaks compared to plants in the 60 cm interrow spacing. Details of the results are shown in Table 35 and Figure 20.

Table 35 - Neonicotinoid concentration in guttation droplets from plants subjected to different water regimes. Translation of text within the figure: *Bagnato* = Wet; *Umido* = Damp; *Secco* = Dry.

Guttazione mg/L									
		Imidacloprid		Clothianidin		Thiamethoxam		Fipronil	
		30 cm	60 cm	30 cm	60 cm	30 cm	60 cm	30 cm	60 cm
Bagnato	02/11/09	66.74	61.9	31.37	36.78	42.97	38.076	0	0
Umido	02/11/09	80.40	66.8	44.43	35.04	97.44	71.967	0	0
Bagnato	04/11/09	196.52	78.3	71.11	77.2	68.59	47.17	0	0
Umido	04/11/09	199.1	130.5	55.72	45.62	190.41	68.03	0	0
Secco	04/11/09	121.1	97.1	50.37	75.56	219.88	520.59	0	0
Bagnato	08-10/11	716.73	412.77	60.3	261.5	67.5	155.0	0	0
Umido	08-10/11	70.91	828.0	68.2	310.4	128.1	253.0	0	0
Secco	08-10/11	54.65	340.1	42.2	185.2	56.6	1153.5	0	0
Bagnato	23-24/11	109.2	213.2	45.7	140.9	14.1	141.7	0	0
Umido	23-24/11	46.5	214.1	28.4	69.1	86.1	26.9	0	0
Secco	23-24/11	57.2	76.1	24.5	11.6	33.9	68.1	0	0

These findings confirm that the water regime affects production and insecticide concentration of guttations. In a dry regimen, the appearance of guttations is delayed. With greater rainfall a diluting effect is observed, whereby the active ingredient is partially washed out. This effect is more marked for thiamethoxam, which is the most water soluble compound among those considered. Accordingly, thiamethoxam was found to be the most concentrated active ingredient in the plants grown in a dry water regime. Thus in a damp/wet regime, the active ingredient concentration appears to be in line with the polarity of the compound. These concentrations, on the basis of acute toxicity data (Girolami *et al.* 2009) are in any case always lethal for bees.

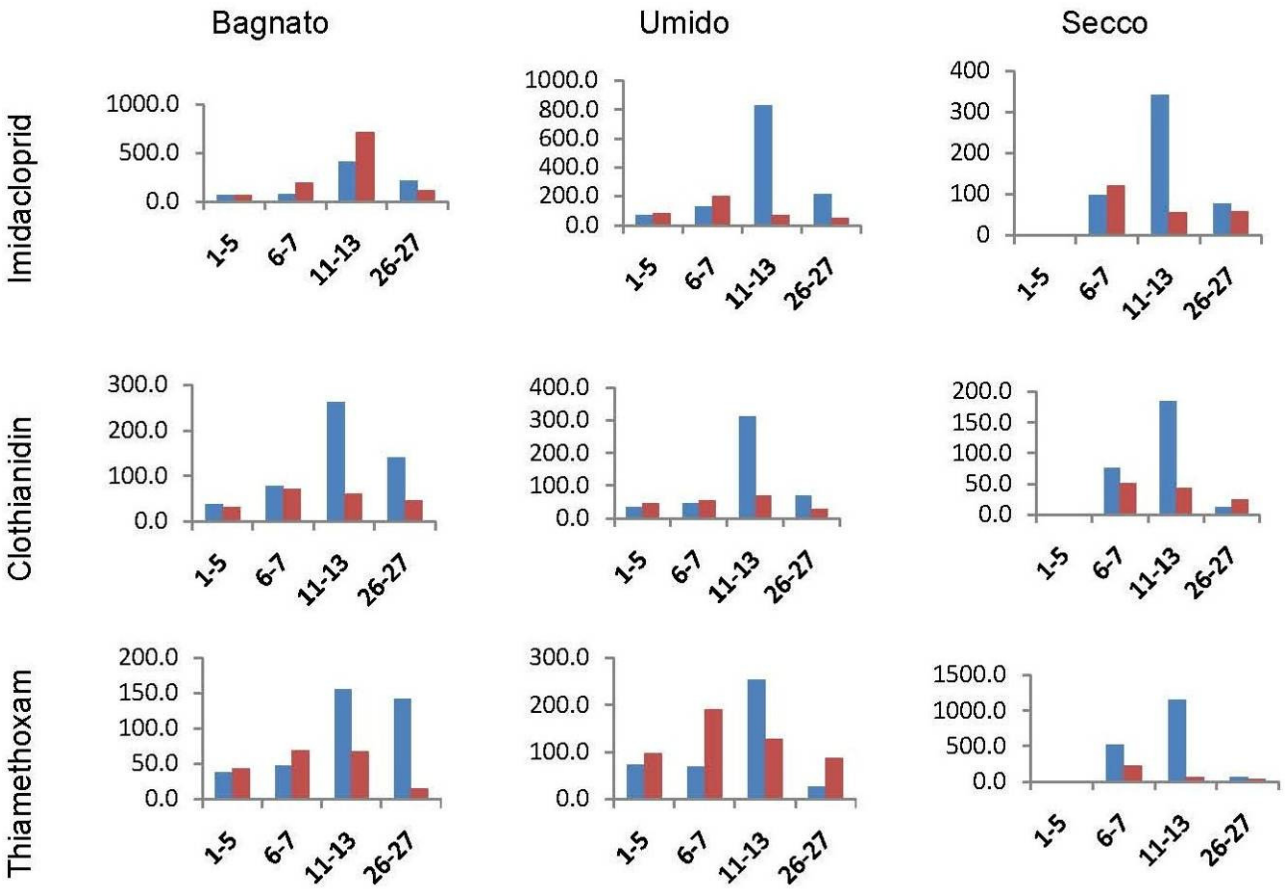


Figure 20 - Pattern of concentrations from the 1st to 27th day after emergence.
Ordinates: mg/L active ingredient.; Abscissae: days after emergence.
Light blue: interrow sowing 60 cm; Red: interrow sowing 30 cm.
Translation of text within the figure: *Bagnato* = Wet; *Umido* = Damp; *Secco* = Dry.

5.3 Insecticide concentration in guttation droplets deriving from plants sown and grown on soils having different texture

In order to study the presence of neonicotinoids in maize plantlet guttation droplets and the variations in concentration with varying soil types, the following experiment was set up. For each of the three different plots utilized, each of which had specific soil characteristics, 4 rows of maize seed coated with Celest (row 1, as control), Celest and Clothianidin (row 2), Celest and Imidacloprid (row 3) and Celest and Thiamethoxam (row 4) were sown.

Soil characteristics in the three plots were as follows:

a) Sandy soil rich in skeleton and strongly draining ("Field S");

b) Medium loam soil ("Field M");

c) Clayey soil rich in organic matter with poor drainage ("Field N");

Sowing was carried out on 30/04/2010 for "Field S" and "Field M" and on 21/05/2010 for "Field N". Complete maize plantlet emergence was recorded after 11 days (11/05/2010) for fields "S" and "M" and after 8 days (29/05/2010) for "Field N".
Guttation droplet sampling was carried out on more than one plant in each row, as shown in Table 36.

The results of the analyses are given in Tables 36 and 37 and in Figures 21, 22 and 23, divided by soil typology, and in Figures 24, 25 and 26, divided by active ingredient.

Table 36 - Neonicotinoid concentrations in leaf guttations of plants grown in three different soil typologies.

Field	Date	Neonicotinoid (mg/L)				
		Row 2	Row 3		Row 4	
		Clothianidin	Imidacloprid	Clothianidin	Thiamethoxam	Clothianidin
S	19 May	143.964	242.070	2.400	53.428	14.278
	21 May	40.418	16.731	-[a]	15.822	4.277
	24 May	13.593	6.527	-[a]	1.048	0.307
	26 May	8.073	1.775	-[a]	0.606	0.150
	31 May	0.725	0.093	-[a]	0.020	0.012
	1 May	0.509	0.137	-[a]	0.015	0.007
	7 May	0.644	0.227	-[a]	-[a]	0.025
M	26 May	2.503	1.826	-[a]	-[b]	-[b]
	26 May	-[b]	-[b]	-[b]	-[b]	-[b]
	31 May	0.367	0.179	-[a]	0.035	0.018
	1 June	0.532	0.238	-[a]	0.089	0.034
	1 June	-[b]	-[b]	-[b]	-[a]	-[a]
	4 June	0.533	0.116	-[a]	-[a]	0.040
	7 June	0.367	0.128	-[a]	-[a]	0.033
N	7 June	20.813	2.970	-[a]	5.434	0.909
	11 June	2.899	0.613	-[a]	1.016	0.408
	12 June	5.213	0.970	-[a]	0.531	0.258
	17 June	0.131	0.048	-[a]	0.022	0.021

[a] Concentrations lower than detectability limit. [b] Sampling not performed.

Table 37 - Neonicotinoid concentrations present in leaf guttation droplets of plants grown in three different soil typologies, as measured at varying times after complete plantlet emergence from soil.

Days after plantlet emergence	Row 2 (Clothianidin)			Row 3 (Imidacloprid)			Row 4 (Thiamethoxam)		
	Concentration (mg/L)			Concentration (mg/L)			Concentration (mg/L)		
	Field S	Field M	Field N	Field S	Field M	Field N	Field S	Field M	Field N
8	143.964			242.070			53.428		
9			20.813			2.970			5.434
10	40.418			16.731			15.822		
13	13.593		2.899	6.527		0.613	1.048		1.016
14			5.213			0.970			0.531
15	8.073	2.503		1.775	1.826		0.606		
19			0.131			0.048			0.022
20	0.725	0.367		0.093	0.179		0.020	0.035	
21	0.509	0.532		0.137	0.238		0.015	0.089	
24		0.533			0.116			< LOD	
27	0.644	0.367		0.227	0.128		< LOD	< LOD	

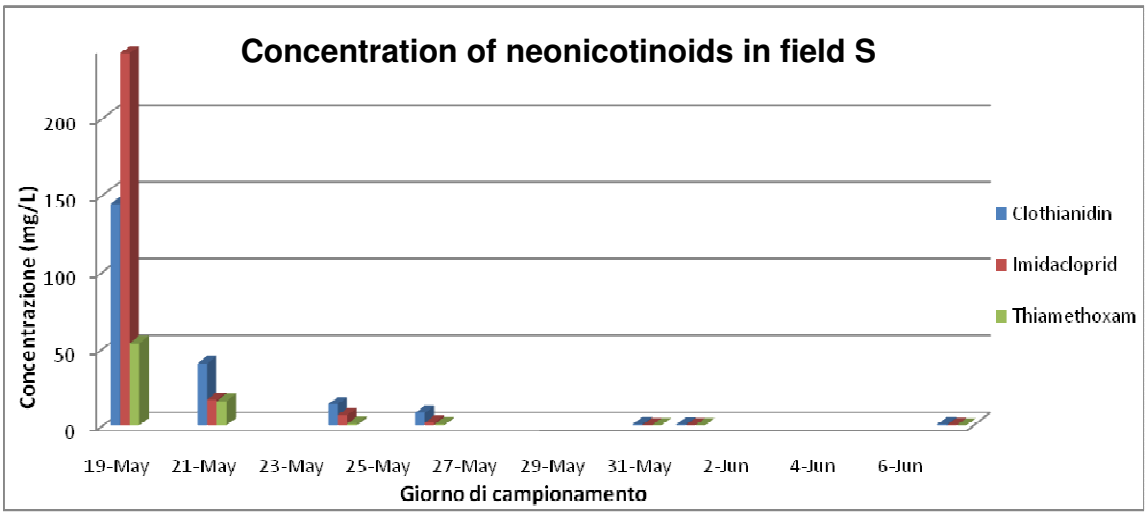


Figure 21 - Concentration of Clothianidin, Imidacloprid and Thiamethoxam in leaf guttation droplets collected from "Field S" maize plants. Translation of text within the figure: *Giorno di campionamento* = Sampling date.

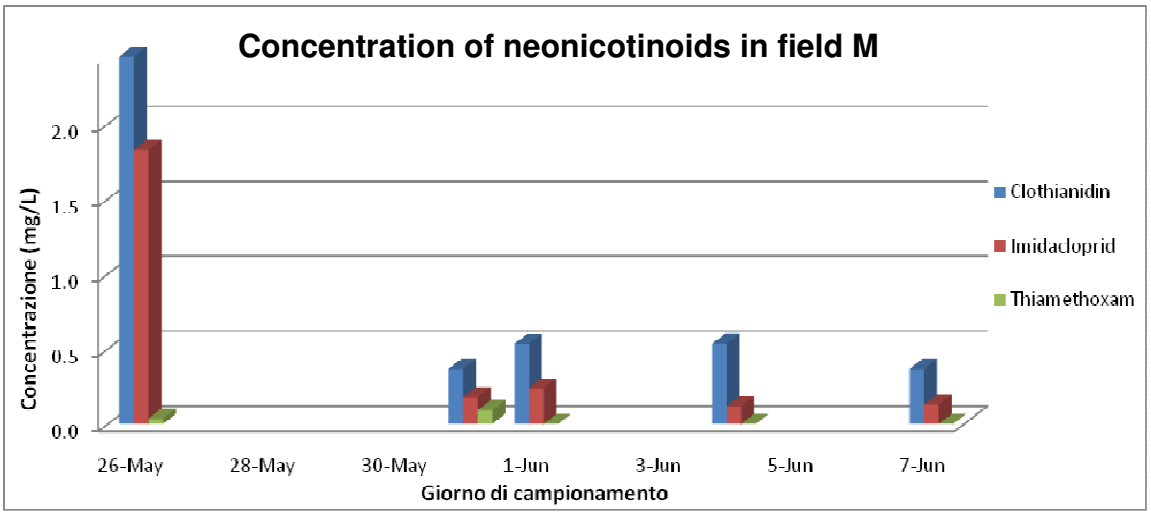


Figure 22 - Concentration of Clothianidin, Imidacloprid and Thiamethoxam in leaf guttation droplets collected from "Field M" maize plants. Translation of text within the figure: *Giorno di campionamento* = Sampling date.

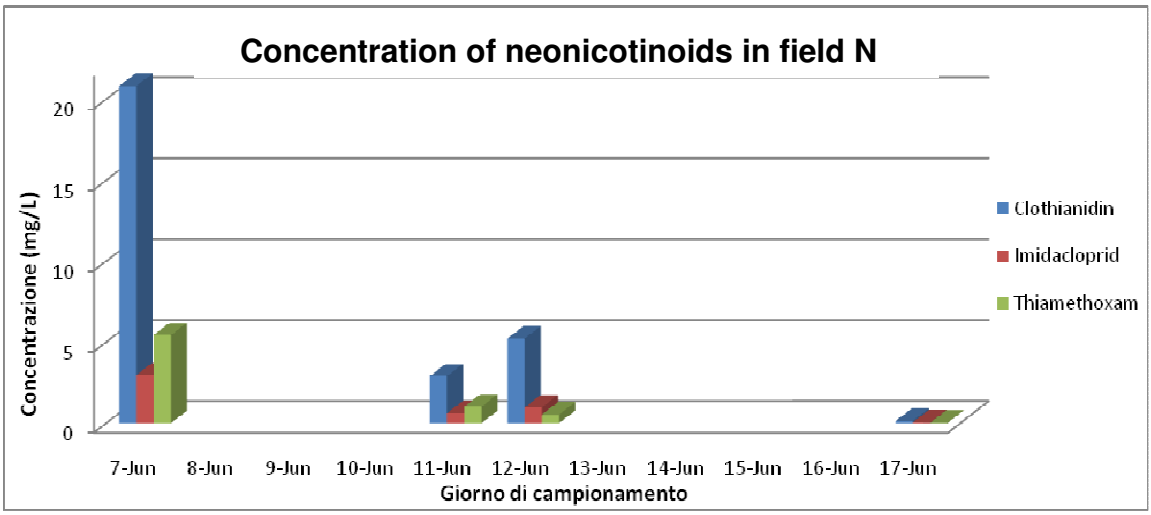


Figure 23 - Concentration of Clothianidin, Imidacloprid and Thiamethoxam in leaf guttation droplets collected from "Field N" maize plants. Translation of text within the figure: *Giorno di campionamento* = Sampling date.

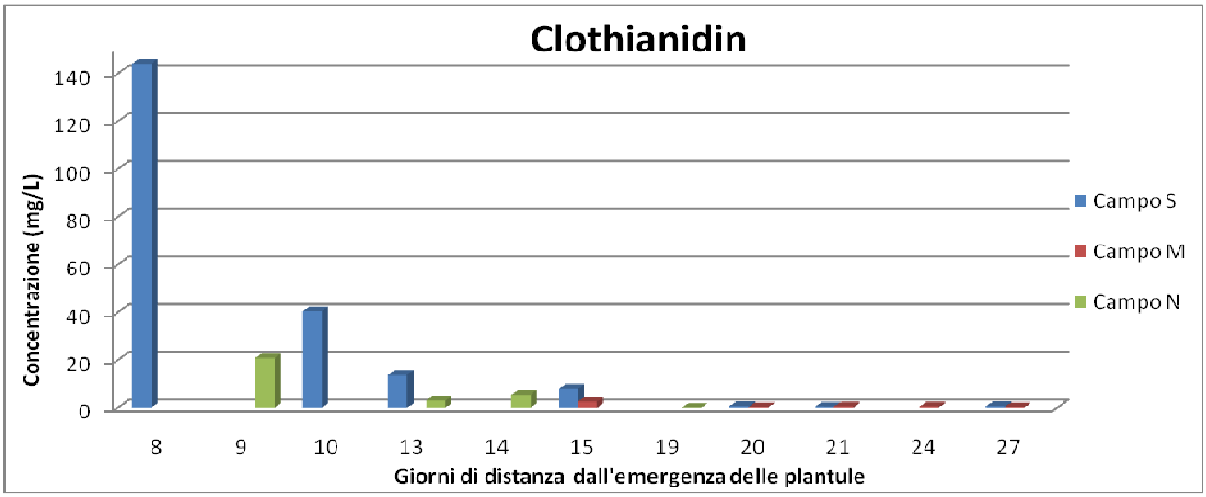


Figure 24 - Comparison between concentrations of Clothianidin in leaf guttation droplets of plantlets grown in three different soil types, as measured at varying times after complete plantlet emergence from soil. Translation of text within the figure: *Giorni di distanza dall'emergenza delle plantue* = Days after plantlet emergence; *Campo* = Field.

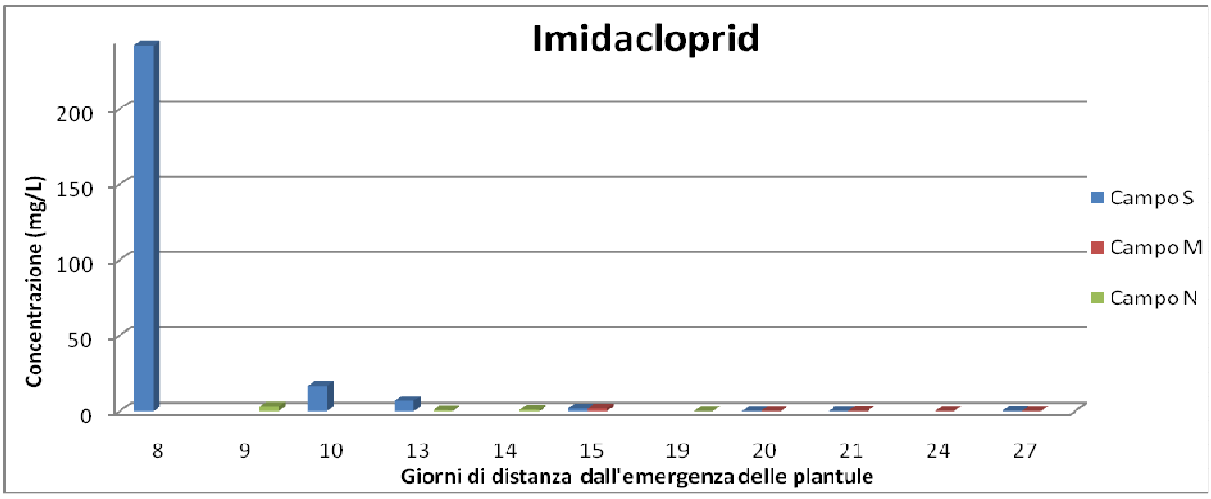


Figura 25 - Comparison between concentrations of Imidacloprid in leaf guttation droplets of plantlets grown in three different soil types, as measured at varying times after complete plantlet emergence from soil. Translation of text within the figure: *Giorni di distanza dall'emergenza delle plantue* = Days after plantlet emergence; *Campo* = Field.

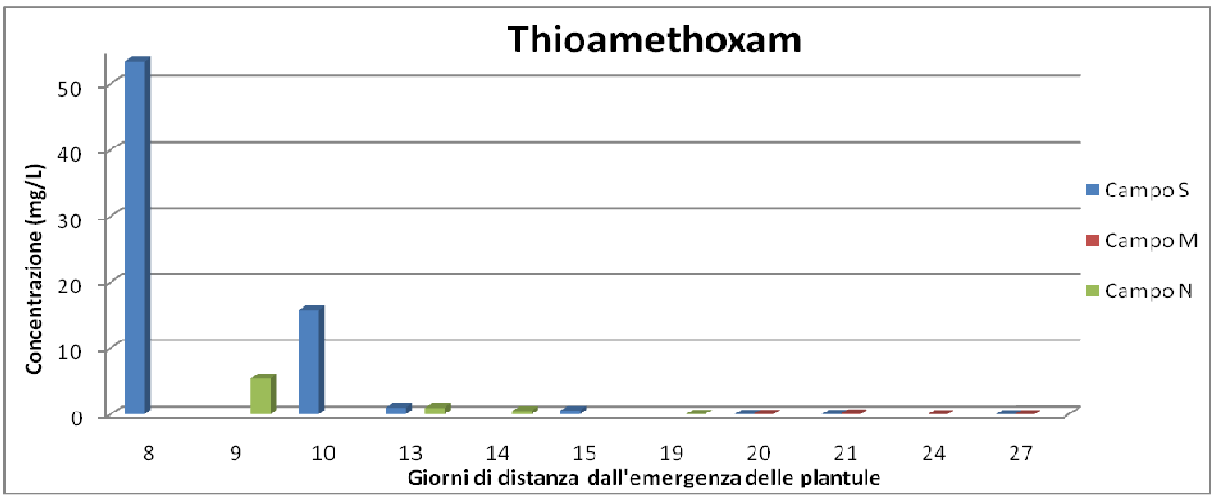


Figure 26 - Comparison between concentrations of Thiamethoxam in leaf guttation droplets of plantlets grown in three different soil types, as measured at varying times after complete plantlet emergence from soil. Translation of text within the figure: *Giorni di distanza dall'emergenza delle plantue* = Days after plantlet emergence; *Campo* = Field.

The results show that in all three soil types, rapid decline in concentration of the three active ingredients was observed with increasing number of days after sowing. The overall trend observed for all three trial fields showed greater concentration of Clothianidin compared to the other two active ingredients (except for the first finding recorded for "Field S"), followed by Imidacloprid and Thiamethoxam for Fields "S" and "M" respectively. Furthermore, in the case of the clayey field (Field "N"), and in contrast to the other two cases, Thiamethoxam concentrations were found to be on average higher compared to Imidacloprid. Assuming similar meteorological conditions among sowings performed at different times, it can be noted that for samplings conducted at the same number of days after plantlet emergence, guttations in "Field S" consistently presented higher concentrations of the three active ingredient compared to the other "Fields". A genuine comparison with the medium loam soil ("Field M") cannot be carried out as samplings were performed at an excessively long time lapse after sowing (on account of the delay in sowing due to adverse meteorological conditions).

5.4. Evaluation of bee foraging activity on guttations

In April and May 2010 assessments of bee foraging on maize guttations were conducted in fields near the apiary of the Experimental Centre of the Agricultural Faculty of the University of Bologna in Cadriano (BO). Observations were carried out along transepts in the early morning hours on three separate maize plots.

1. field measuring roughly 1,300 m^2 situated at about 50 m from the apiaries;
2. field measuring roughly 6,000 m^2 situated at about 300 m from the apiaries
3. field measuring roughly 1,500 m^2 adjacent to the apiaries.

The transepts were effected along the rows, observing the 4 adjacent rows. In the first and third plot, 5 transepts were made within the field, and in the second plot, 8 transepts. For each plot, observations along the access paths bordering on the fields were also carried out. The observations were performed from appearance of the first real leaf up to appearance of the 6^{th} leaf.

The results are shown here below.

- 22 April: flying activity regular; guttations present. No bees recorded along the transepts.
- 26 April: flying activity scanty and guttations present. Swarm affecting 1 or 2 hives. No bees recorded along the transepts.
- 30 April: flying activity regular; guttations present. 1 bee recorded in the 2^{nd} transept (about 08.30 h) in field 1, but no guttation droplets foraged .
- 3 May: flying activity regular; guttations present. No bees recorded along the transepts.
- 7 May: flying activity almost absent; guttations present. Transepts could not be effected due to sudden rainfall.
- 11 May: rainfall.
- 14 May: rainfall.
- 18 May: flying activity regular; guttations present. Between fields 1 and 3 a bee was seen drinking in a puddle on the road.
- 21 May: flying activity regular; guttations absent (sunny with slight breeze).
- 27 May: flying activity regular; guttations present. No bees recorded along the transepts.
- 1 June: flying activity regular; guttations absent (sunny with slight breeze).

The field data recorded during this spring season confirmed the findings of 2009, namely that bee foraging on maize guttation droplets in the environmental conditions of our latitude (Bologna) is nil or negligible.

6. Lethal and sublethal effects induced in bees by the active ingredients used in seed coating

6.1 Effects of clothianidin-coated maize seed dust on bees: laboratory mortality evaluation

A laboratory test was conducted to evaluate the effects of acute toxicity caused by indirect contact with clothianidin (trade name: Poncho®)-coated maize dust emitted by the seeder during sowing operations. A comparison with the same active ingredient sold in liquid formulation (trade name: Dantop®) was made. The test involved allowing bees to walk for 3 h on a substrate (organic apple leaves) treated with the active ingredient to be assayed; the substrate was placed inside plastic cages (13 cm x 66 cm x 11 cm height) on the bottom of each cage. 10 foraging bees were introduced into each cage. The substrate was then removed after the 3rd hour. Each cage was equipped with a dispenser containing water and sugar for the bees.

Prior agreements had specified that the clothianidin quantities to be used in the present study should be those obtained in experiments conducted by CRA-ING of Monterotondo (Rome) during the 2010 spring trials of the Rome unit, which were to focus on detecting active ingredient drift at 5 m from the edge of the field. However, meteorological conditions of the spring of 2010 were highly adverse (constant rainfall in the months of April and May), as documented in a letter sent by the coordinator dated 8/6/2010 nr. Prot. 1474 to the competent authorities of Mipaaf, in addition, a breakdown affected the analytical tool forming part of the equipment of CRA-PAV of Rome (HPLC-MS-MS). Therefore the decision was made to begin the laboratory tests using a quantity amounting to 5.12 µg/m^2 , estimated on the basis of previous field trials. The subsequent trials in Monterotondo recorded a quantity of active ingredient of 6.25 µg/m^2 at the distance of 5m. These data should be taken into account in the overall evaluation of the trials, although the effects highlighted in the trials described here below could only have been of equal or greater intensity since the dose utilized here was lower than that actually obtained.

The clothianidin-containing dust, obtained from maize seed supplied by Assosementi, was prepared with a precision 45 µm mesh sieve and then analyzed. Active ingredient content was found to constitute 81% of the dust. To allow homogeneous dispersal of dust on the cage substrate (with an area of 57.2 cm^2) in proportion to the quantity of active ingredient deposited at 5 m (x 1: 0.029 µg of active ingredient/substrate), it proved necessary to mix the dust (0.036 µg/substrate) with an inert material (talc) through geometric dilutions, starting from a dose that was 1000 times more concentrated. For the liquid treatment (Dantop), each substrate was sprinkled with 200 µl of solution, while in the dust treatment (Poncho) the leaves were sprinkled with 0.01 g of clothianidin-containing dust mixed with talc. For each treatment, the 4 doses utilized for the dilutions (x 1, x 10, x 100, x 1000) as well as the negative control, namely talc for Poncho and water for Dantop, were assayed. Each dose involved 5 repetitions (5 cages with 10 bees each). During the trials, the cages containing the bees were maintained for 3 days in a darkened cell at 25°C ± 1 and with 70-80% R.H.

Mortality counts were taken every 3 hours up to the 12th hour of the first day, then at the 24th hour and subsequently every 24 hours up to the 4th day (3, 6, 9, 12, 24, 48, 72 hours). Figure 27 shows mortality corrected with the Schneider-Orelli formula at the different hours, with the active ingredient dose of Poncho and Dantop recorded at a distance of 5 m during sowing operations (x 1). Up to the 24th hour, mortality induced by the two products was very similar, with both products proving to be “slightly toxic”. During the subsequent hours the number of dead bees increased more substantially in the Poncho dust treatments, up to “moderately toxic” at 48 hours and “notably toxic” at 72 hours.

When a comparison of the CL_{50} values was made (in µg of active ingredient /cage) at 48 hours, calculated with the mortality data of the four concentrations, the results showed greater toxicity of Dantop as compared to Poncho, with 0.149 for Dantop (limits: 0.044-0.372) and 1.872 for Poncho (limits: 0.417-5.165).

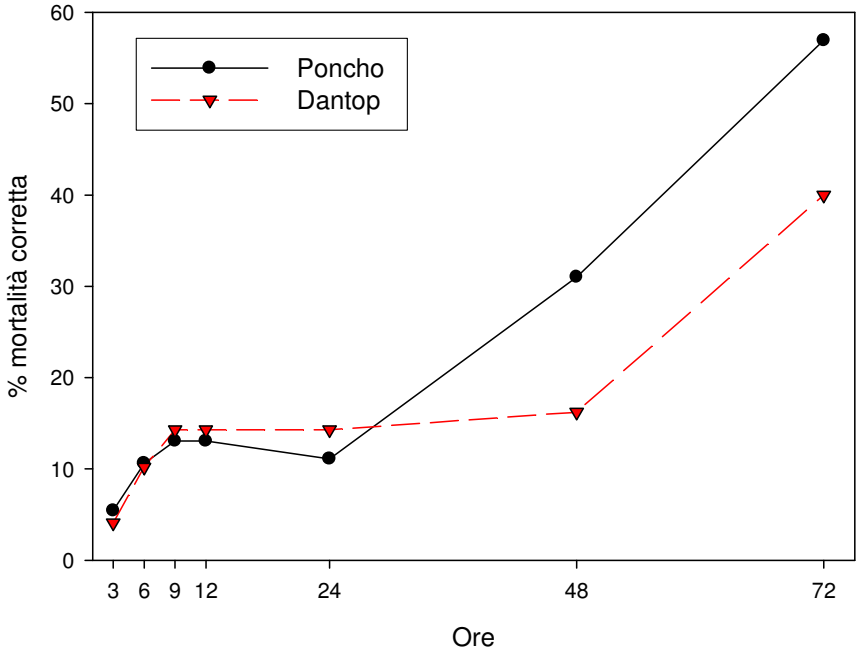


Figure 27 - Corrected mortality at the concentration of 0.029 µg/substrate (x 1) of Poncho® (powder) and of Dantop® (liquid), measured at the various time points. Translation of text within the figure: *mortalità corretta* = corrected mortality; *Ore* = hours.

6.2 Effects induced in bees by dust from maize seed coated with clothianidin, thiamethoxam, imidacloprid and fipronil: laboratory evaluation of mortality due to indirect contact

Experiments were carried out with the same procedures as described in 6.1, with the exception of repetitions and doses assayed. In the present trial, 3 repetitions were carried out instead of 5. The active ingredient quantities were assayed for the various active ingredients at a distance of 5 m (x1) from the seeded field, as previously described for point 6.1, (clothianidin in the genuinely detected quantity of 6.25 µg/m^2) (Table 38), but in the present trial the doses x10, x100 and x1000 were also assayed.

Table 38 - Quantity of active ingredient present in dust drifting to 5 meters (x1) and assayed in the laboratory

Active ingredient	**Q.ty of act.ingr. drifting to 5 m (µg/m^2)**	**% of act. ingr. after sieving**	**Q.ty of act.ing./ cage in µg**	**Q.ty of dust/cage in µg**
clothianidin	6.25	81.00	0.036	0.044
imidacloprid	3.66	60.10	0.0209	0.035
thiamethoxam	2.77	46.30	0.0158	0.034
fipronil	0.28	47.00	0.0016	0.0034

The data available so far, given in Figs. 28, 29, 30 and 31, show a pattern of mortality (corrected with the Schneider-Orelli formula) correlated with the doses assayed, for all active ingredients under study. The only abnormal finding concerned the highest dose of fipronil (FIP x 1000), in which mortality up to the 24th hour was not as high as mortality at the lower doses but then rose drastically at the 48th and 72nd hour, reaching the highest mortality percentage of all doses (Figure 31). Clothianidin, at the dose of 6.25 µg/m^2, caused mortality above 20% even in the first few

hours; in the following hours mortality exceeded 30%, reaching 40% by the 72nd hour. It is interesting to compare these findings for Clothianidin with the previous Clothianidin trial, in which the dose of 5.12 µg/m^2 was used: at the latter dose, mortality was 5% in the early hours, and then rose to 10% and remained at this percentage up to the 24th hour; subsequently, mortality increased to 30% at the 48th hour and to over 50% by the 72nd hour.

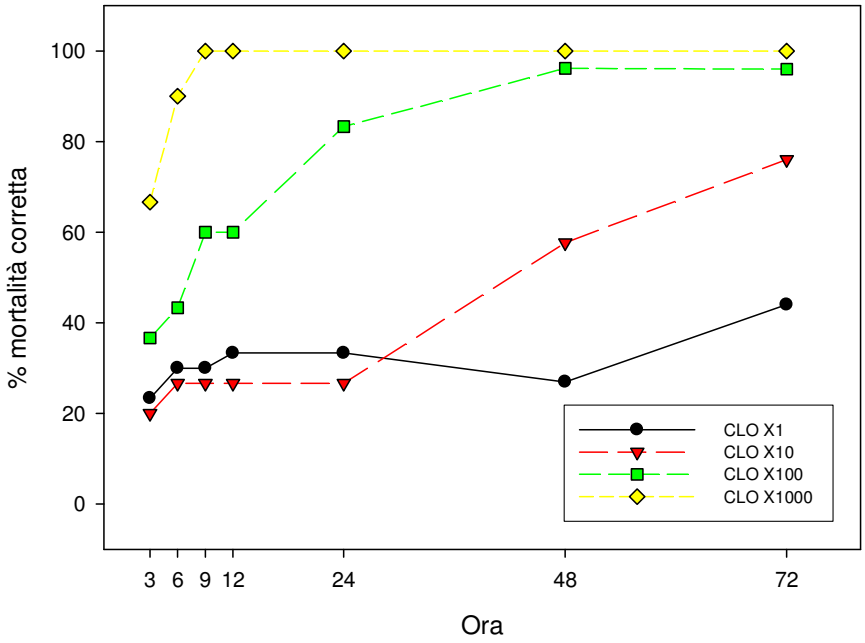


Figure 28 - Corrected mortality percentage of bees poisoned with clothianidin. Translation of text within the figure: *mortalità corretta* = corrected mortality; *Ora* = hour.

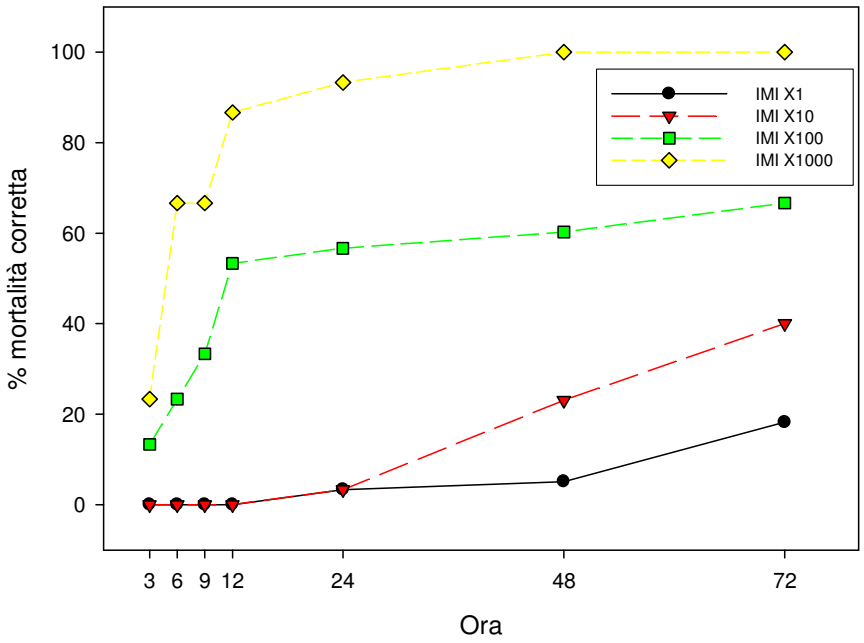


Figure 29 - Corrected mortality percentage of bees poisoned with imidacloprid. Translation of text within the figure: *mortalità corretta* = corrected mortality; *Ora* = hour.

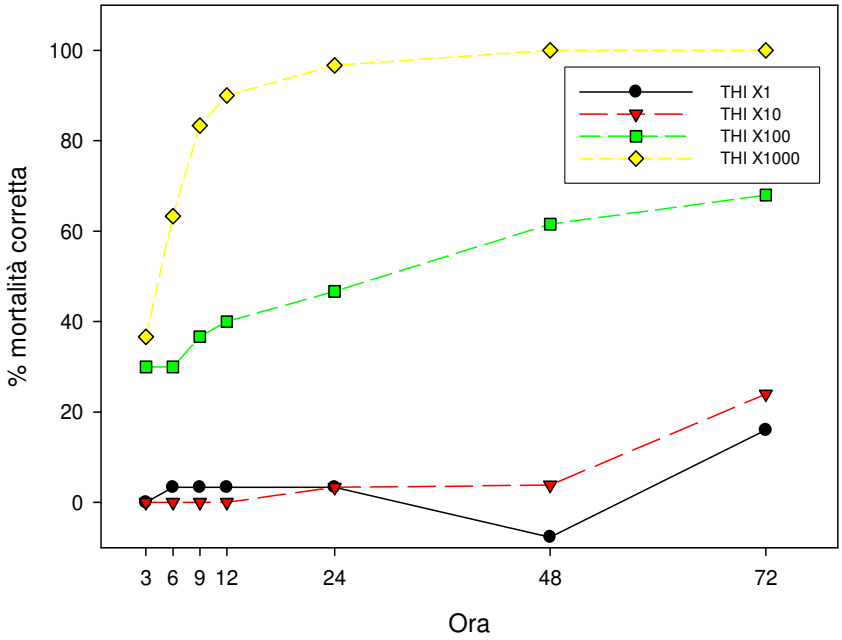


Figure 30 - Corrected mortality percentage of bees poisoned with thiamethoxam. Translation of text within the figure: *mortalità corretta* = corrected mortality; *Ora* = hour.

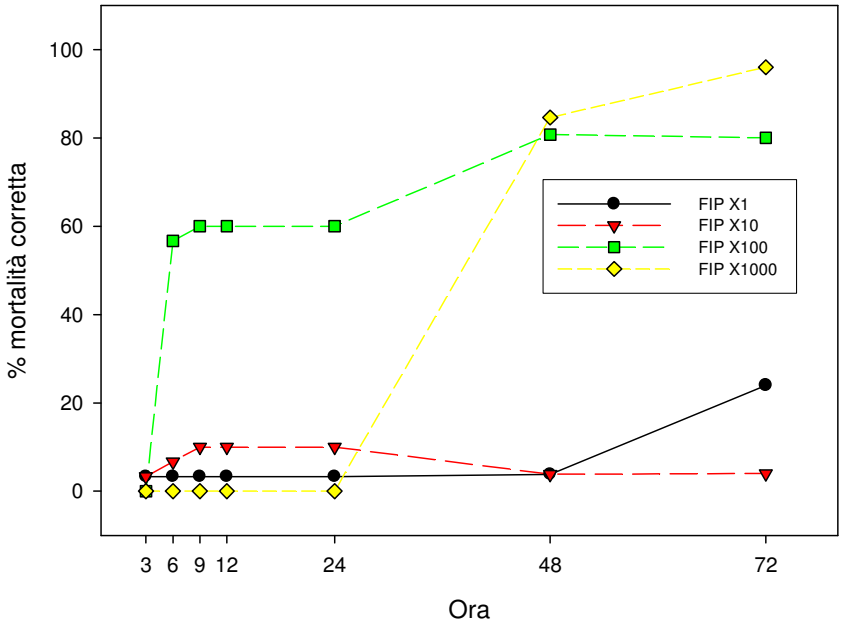


Figure 31 - Corrected mortality percentage of bees poisoned with fipronil. Translation of text within the figure: *mortalità corretta* = corrected mortality; *Ora* = hour.

The data obtained at the different hours for the dose x1 were classified according to the five toxicity classes generally adopted for this type of trials: “non toxic” (<1%), “slightly toxic” (1-25%), “moderately toxic” (26-50%), “notably toxic” (51-75%), “highly toxic” (76-100%). The results showed that all the products (except for imidacloprid In the first hour) constantly maintained a

certain toxicity. Clothianidin was found to be the most toxic active ingredient (also on account of the greater quantity of product deposited at 5 meters) (Table 39).

Table 39 - Bee toxicity of the different active ingredients as measured by dust drift at 5 m (x1) from the edge of the seeded field.

Active ingredient	6th hour	12the hour	24th hour	48th hour	72a ora
clothianidin	Moderately toxic	Moderately toxic	Moderately toxic	Moderately toxic	Moderately toxic
imidacloprid	Non toxic	Non toxic	Slightly toxic	Slightly toxic	Slightly toxic
thiamethoxam	Slightly toxic	Slightly toxic	Slightly toxic	Non toxic (*)	Slightly toxic
fipronil	Slightly toxic	Slightly toxic	Slightly toxic	Slightly toxic	Slightly toxic

*In the calculation of corrected mortality the number of dead bees of the control treatment was higher than in the active ingredient treatments.

As compared to the 2009 trials (in which the plots measured 0.16 ha) conducted by CRA-ING in Monterotondo, in the 2010 trials the increase in sowing area up to 3 ha and the increase in sowing time led to a rise in the quantity of dust drift. In particular, an increase in dust deposited after sowing with the unmodified seeder was observed (see Chapter 2). If the quantities were really 10 times higher, then clothianidin at the 48th hour would shift from the moderately toxic category to notably and highly toxic. But if the dust deposited were 100 times greater than the quantities measured, then the situation would undergo a radical change. From the 12th hour onwards (and clothianidin from as early as the 3rd hour), almost all the products would shift to higher toxicity classes. Therefore, in our view, a better overall evaluation of the data can be obtained by taking into account two important aspects: firstly, the fact of the greater plot sizes in the 2010 trials compared to the 2009 trials, given that an increase in seeded area can influence the level of contamination of the surrounding areas (as also pointed out in Chapter 2 of this report); secondly, the fact that the trial lasted for an overall total of only one hour and three quarters, whereas normally in the maize-growing area of Italy (contained within the regions of Lombardy, Piedmont, Veneto, Friuli Venezia Giulia and Emilia Romagna), all farmers tend to carry out sowing operations during the same period, working from dawn to dusk in order to avoid rainfall.

6.3 Effect induced in bees by dust from clothianidin-coated maize seeds: evaluation of mortality and other semifield parameters

The effect of the clothianidin quantity contained in the dust deposited at 5 meters from the edge of the maize field during the seeding experimental carried out at CRA-ING of Monterotondo (Rome) (see point 6.1 for an estimate of the value) was evaluated in small colonies (‘*nukes’*) of bees placed within tunnels and foraging on rapeseed. The dust utilized, mixed with talc, was prepared as in the laboratory trial (point 6.1). At the end of May 2010, six experimental tunnels were set up (3 for the controls and 3 for the active ingredient treatments), on a field sown with rapeseed at the Experimental Farm of Cadriano (BO). The tunnels, measuring 43 m^2 each, were covered with anti-aphid netting. The tunnels of the two different sets of treatments were arranged in random order. On 31st May, when the rapeseed crop had reached roughly 50% flowering, bees were introduced into the tunnels, using one small colony of bees per tunnel. Each tunnel was then placed on 3 frames (two brood frames and one containing roughly 20-25% honey supply),. Each tunnel was also equipped with a cage for collecting dead bees (of the “underbasket” type) , positioned in front of the small colony. The trial was performed according to EPPO guidelines, and the following data were gathered both before and after the treatment:

1. Daily mortality (number of foraging bees present within the underbasket);

2. Strength of the colony: development of the colony (adult bees, brood) evaluated by the sixths method (Liebefeld method);

3. Flight activity: number of bees exiting from the hive in 30";

4. Foraging activity: number of bees present on rapeseed flowers, as evaluated on three 0.25 m^2 plots homogeneously distributed within each tunnel. Bee counts were made with the instantaneous method. Although bee ethology was often found to be impaired under the tunnel, foraging activity was evaluated not only in terms of the number of bees present on flowers, but also from the qualitative point of view, by recording behaviour that could signal abnormalities: for example bees immobile on leaves or flowers, bees cleaning their legs and/or antennae, or bees that appeared to be dazed (Giffard and Mamet, 2009);

5. Observations in front of the hive: presence of foragers with pollen, and any signs of abnormal behaviour.

6. Analysis of residues on dead bees collected in the underbaskets and on the vegetation treated with clothianidin-containing dust, samples being collected on various days after treatment (-4, 0 (+1h), +1, +3, +7).

7. .Acquisition of data on the sociophysiological status of the bee family, by recording temperature inside the small colony within the brood zone (with iButton DS1923 sensor) and the degree of construction of a honeycomb on an empty frame (without wax sheet), the frame having been inserted into each small clolony on the day of treatment.

Evaluation of behaviour in front of the hive, flying activity and foraging was carried out on days -3, -1, 0, 1, 2, 3, 5 and 7 in relation to treatment day. Observations were performed every two hours during the central part of the day (10.00 h. - 12.00 h. -14.00 h. -16.00 h.) with the exception of day -3, on which only data from the afternoon were acquired. Dead bees were collected on the morning of observation days. Strength of the colony was evaluated prior to treatment (day -4) and 7 and 15 days after treatment . The temperature and humidity sensor was introduced during the first temperature check inside the hives (day -4) and the data were downloaded after the third temperature check (day +15). Construction of the honeycomb on the empty frame was evaluated on day +7 and day +15.

The treatment was performed with a mechanical pulverizer (Cifarelli series M3. two-stroke 77 engine; engine power: 3.6 kw; dust emission: 0 - 6 Kg/min; air speed: 125 m/sec; air volume: 20 m^3/min) on 7 June at 12.30 h, sprinkling 200 kg of contaminated dust containing 204.77 µg of active ingredient mixed with talc over rapeseed plants in full flower, in every tunnel involved in the trial. Pure talc was applied in the tunnels forming part of the control treatment. The anti-aphid netting cover in the tunnels was removed on 15th June (8 days after application of the treatment) in order to allow the bees to forage freely in the remaining zones of the rapeseed plot and in the surrounding fields.

Daily comparison between mortality in the treated groups versus control mortality (data normalized by calculation in relation to pre-treatment daily mortality) showed statistically significant differences in the two days following treatment. In the treated bees, mortality was greater on days +1 and +2, but then tended to stabilize to control levels on subsequent days (Figure 32). Applying the index proposed by Schmidt (*Schmidt H.-W., Brasse D., Künast C., Mühlen W., von der Ohe W., Tornier I., Wallner K. (2003). Introduction of indices for the evaluation of tent tests and field tests with honeybees. Bulletin of Insectology, 56(1): 111-118*) to compare the effects of pesticides in field and semifield tests, it was found that mortality in the treatment group was roughly ten-fold higher than in control bees. This index is the result of the ratio between mortality in the treated group vs mortality in the control group, normalised with pre-treatment mortality of each hive (Table 40).

Despite the peak of mortality in tunnels treated with clothianidin-containing dust, no significant differences emerged with regard to strength of the colony, either in bees or in the brood. Statistically significant differences emerged with regard to days both in the clothianidin treatment

and the control treatment: thus the sixths of bees and of the brood both increased from 14th June (+7) to 22nd June (+15), after removal of the netting that covered the tunnel. The day-treatment interaction proved to be non significant (Table 41, Figure 33).

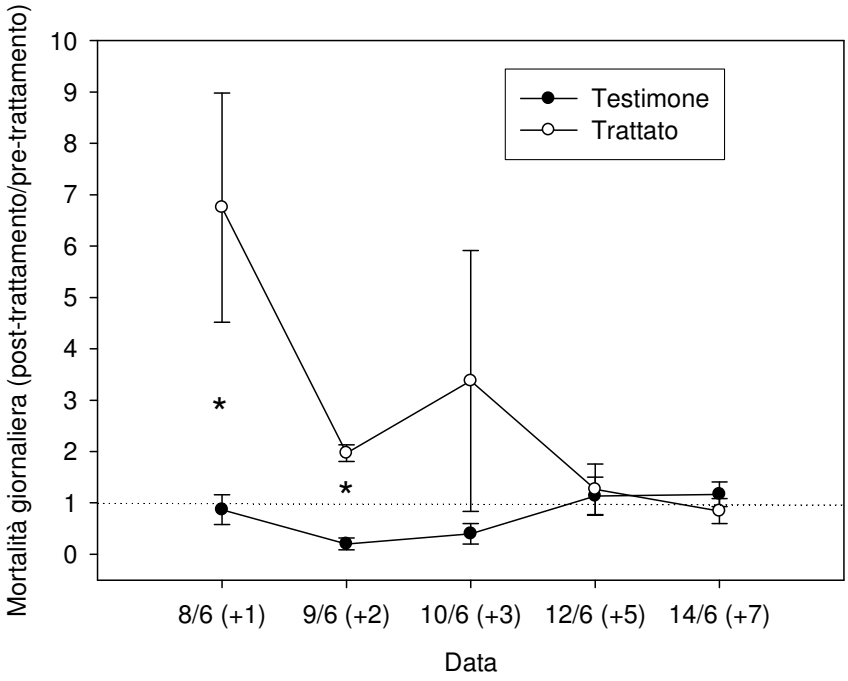


Figure 32 - Ratio of daily mortality on the different post-treatment days to mean mortality on pre-treatment days. The dotted line indicates when post-treatment mortality is equal to pre-treatment mortality (ratio = 1). * = Statistically significant differences in the Mann-Whitney U test (p<0,05) between the treated group and controls. Translation of text within the figure: *mortalità giornaliera (post-trattamento/pre-trattamento)* = daily mortality (post-treatment/pre-treatment); *Data* = date; *Testimone* = control; *Trattato* = treated.

Table 40 - Index defined by the ratio of mean mortality normalized with treated tunnel pre-treatment mortality to control mortality (from Schmidt *et al.* 2003).

Hives	Treatment	Pre-treatment daily mortality (3 days)	Post-treatment daily mortality (5 days)	Mean post-treatment /pre-treatment index
1	Control	9.00	4.80	0.93
3	Control	3.33	1.80	
5	Control	2.67	4.60	
2	Control	0.33	7.80	9.67
4	Control	6.67	6.40	
6	Control	3.67	17.00	
Treatment/control comparison index				**10.67**

Table 41 - Results of repeated measures Anova to test for the effects of treatments, days and interaction on post and pre-treatment of bee and brood quantities (expressed in sixths of frame surface).

Effects	Bee sixths		Brood sixths	
	F	p	F	p
Treatment	0.19	0.69	0.09	0.78
Days	11.04	0.03 *	107.26	< 0.01 *
Interaction	0.01	0.92	1.12	0.35

*Statistically significant differences (p<0,0.0,05).

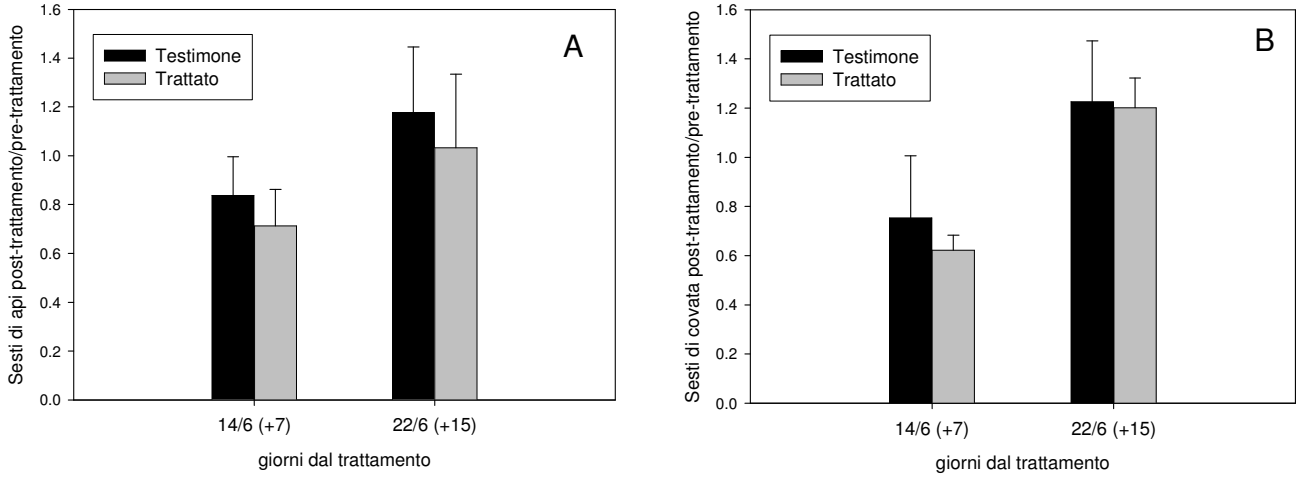


Figure 33 - Ratio of bee (A) to brood (B) sixths on post-treatment days (+7 and +15) to pre-treatment days (-4). Translation of text within the figure: *Sesti di api post-trattamento/pre-trattamento* = Surface of combs (expressed in sixths) covered by bees post-treatment/pre-treatment; *giorni dal trattamento* = days from treatment; *Testimone* = control; *Trattato* = treated; *Sesti di covata post-trattamento/pre-trattamento* = Surface of combs (expressed in sixths) covered by brood post-treatment/pre-treatment.

Statistical elaboration of flight activity and foraging data is currently in progress; however, the general pattern (Figure 34) does not seem to point to negative effects. On average, flight activity after treatment was greater in treated small colonies compared to controls (8.7 vs 7.3 bees flying out every 30"), while the mean number of bees observed on the plots post-treatment was similar in both groups (5.6 and 5.4 for controls and treatment respectively). In addition, the other modes of behaviour observed on the plots showed no obvious symptoms of poisoning. This was demonstrated by the low frequency of behavioural abnormalities in both groups and also by the fact that abnormal behaviour, where observed, was present both before and after treatment (Table 42).

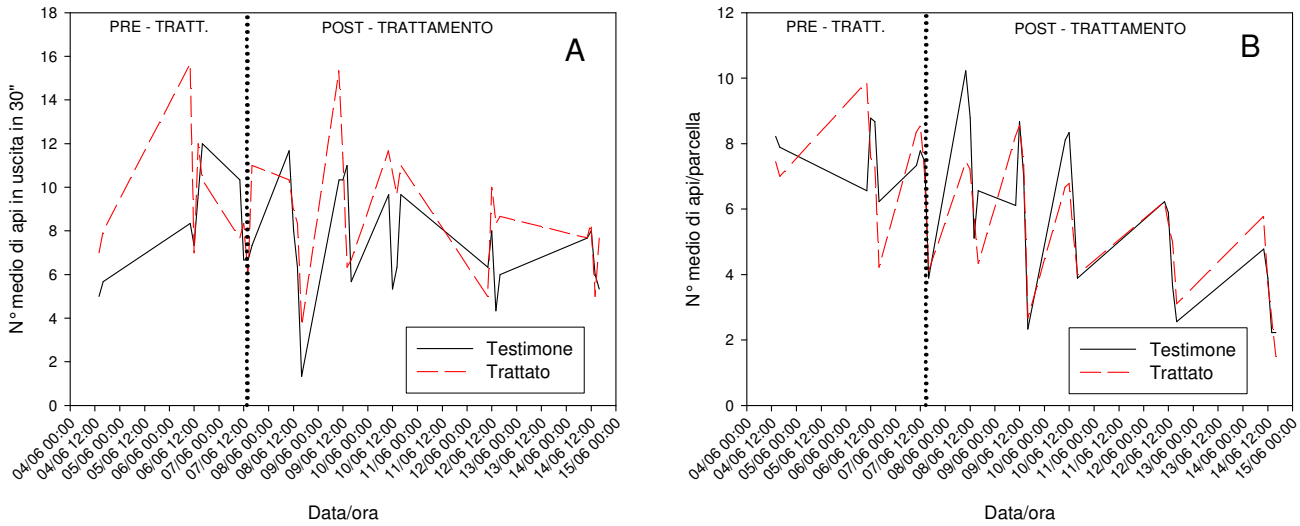


Figure 34 - Mean flight activity (A) and foraging (B) for the three tunnels of each treatment, as recorded during the trial. Translation of text within the figure: *N° medio di api in uscita in 30"* = Average number of bees flying out in 30"; *Pre-tratt.* = before treatment; *Post-trattamento* = after treatment; *Testimone* = control; *Trattato* = treated; *Data/ora* = date and time; *N° medio di api/parcella* = Average number of bees/plot.

Table 42 - Total number of bees observed on rapeseed plots exhibiting abnormal behaviour. Values between parentheses refer to clothianidin-treated bees.

	Bees immobile on leaves or flowers	**Bees engaging in cleaning activity**	**Stunned bees**
Pre-treatment	0 (0)	0 (4)	0 (0)
Post-treatment	3 (10)	0 (2)	0 (0)

The percentage of honeycomb constructed on the empty frame likewise showed no statistically significant differences between control and treated small colonies ($F_{(1.4)} = 0.086$; $p= 0.783$). By 15 days after treatment, bees had on average built roughly 20% of the frame.

Computation of temperature data inside the hives and analyses on residues detected on dead bees and on vegetation are currently in progress.

In conclusion, the results highlight a significant effect of clothianidin (Poncho)-treated maize seed dust on bee mortality. The peak of mortality was observed immediately after bee exposure to the active ingredient, and persisted for 2-3 days. However, although the families affected were effectively weakened, they were not damaged from the point of view of number of bees and brood, at least in the medium period (up to 15 days after treatment).

6.4. Sublethal effects of clothianidin on bee foraging behaviour and homing ability in the field

6.4.1 Effects of clothianidin on foraging and on the bee dance

Bees in a glass-walled hive composed of 6 frames, of which 2 of honey and 1 of brood, were trained to forage for pollen from an artificial dispenser (Figure 30). The bees were marked and the dispenser was gradually shifted to 150 m from the nest. The time taken by bee flights to and from the dispenser as well as flight frequency were measured. Bees that showed assiduous visits to the dispenser were chosen for the trial, and divided into 10-bee groups.

Individuals were captured at the location of the dispenser, after the bee had finished composing the pollen ball, so that the bee would still be motivated to return to the nest and communicate the location of the dispenser to the other bees. After capture, each bee was inserted into the tip of a Gilson pipette whose point had been cut off. After roughly 30' of starvation, treatment consisting of 0.70 ng/bee of clothianidin in 5 microlitres of 50% sucrose solution was applied to one group (N=10); 5 microlitres of 50% sucrose solution was administered to the second group (N=10).

60' after administration of the treatment, the bees were released in small groups near the dispenser. For 3 hours the behaviour of each individual in flights back and forth between the dispenser and the nest was observed, recording flight frequency and time required for return to the hive and filming nest behaviour for 5 minutes once a bee had returned to the nest. Concurrently, the main aspects of behaviour (pollen discharge, dance, trophallaxis, immobility, exit) were recorded. Similar videorecordings and direct observations were performed after 20-24 hours. Finally, the trial was repeated with the same procedures using a lower clothianidin dose of 0.47 ng/bee.

Figure 35 - A: bees during pollen collection from the dispenser; B: marked bees in the nest.

Examination of the videorecordings is currently in progress. Observations conducted at the time of release after application of the treatment and in the nest revealed normal behaviour for untreated bees (direct flight to the nest, pollen discharge, interactions and exchange of food with companions, exit and immediate return to the dispenser to collect more pollen). In contrast, only one of the bees treated with the highest clothianidin dose returned to the nest, where it did not discharge pollen, and remained isolated and immobile for a prolonged period of time. Bees treated with the lower clothianidin dose did return to the nest but they experienced difficulty in discharging pollen, and during the first 3 hours of observation they did not return to the dispenser. Of the clothianidin-treated bees, only 80% reappeared the next day.

The main data from the direct observation are shown in Figure 36.

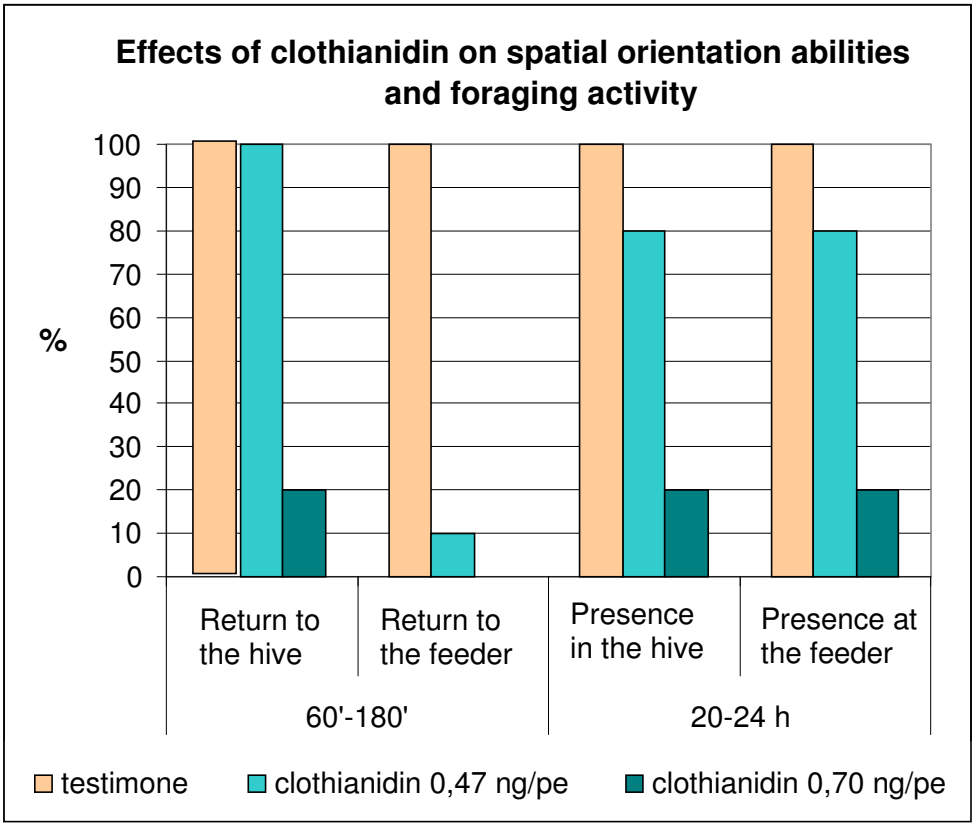


Figure 36 - Main behavioural patterns observed.

6.4.2 Effects of clothianidin-coated maize seed dust on bee homing ability

This trial was set up to assess whether bees' orientation ability was affected by contact with the quantity of dust dispersed to a distance of 5 m during the sowing of Poncho-coated maize seed. The study aimed to test the hypothesis that during foraging activity bees may come into contact with sublethal quantities of clothianidin contained in the dust that drifts into the surrounding atmosphere during maize sowing. Contact may occur in various ways (ingestion, direct contact, indirect contact), on single or multiple occasions.

To perform the trial, bees of a hive were marked individually and then contaminated in the various different ways. Their return to the hive and to the food dispensing point was then evaluated.

The first part of the trial (indirect contact) was carried out in a predominantly maize-growing area near Bolognina (village of Crevalcore – BO) (coordinates: 44° 46' 16" N; 11° 08'53" E) (Figure 37). On-site inspections and interviews with local residents and bee-keepers had established that no apiaries were present within a radius of 2 km.

In preparation for the training phase, a dispenser with water and honey was transported to the location on the evening of 26th July 2010 and placed at roughly 330 m from the hive. Since this was an extremely attractive food source, and the area lacked substantial flowering plants and blossoms during the period in question, the bees were attracted to the dispenser and rapidly became accustomed to visiting it. The dispenser was constituted by a tray filled with 50% water and sugar and was covered with a plexiglass lid in which holes had been punched. Small sponges that absorbed the solution were placed inside the holes.

Once the bees had become accustomed to the route, they were marked using small brushes soaked with drops of Uniposca®. They were then captured (30 bees per treatment) and restrained inside the cages for application of the clothianidin treatment, as follows: 1) treated bees (0.044 µg of dust/cage, a value corresponding to 6.25 µg/m^2 , the quantity of clothianidin detected at 5 m from the field sown with maize; 2) control (talc).

The marked bees captured in the dispenser were allowed to walk for an hour on a substrate (organic apple leaves) dusted with pure talc (or contaminated with Clothianidin dust). The bottom of the experimental plexiglass cages was lined with an apple leaf substrate, and was also equipped with a dispenser (the stopper of an Eppendorf) containing 200 µl of sucrose solution. To restrain the bees on the bottom of the cage and oblige them to walk as much as possible on the treated substrate, a cardboard separator was utilized.

Data collection began immediately after release of the bees, and continued for 3 hours of continuous observation of the hive and the feeding dispenser. The protocol also provided for a further check 24 hours after release, if only a limited percentage of bees, or no bees at all, had returned within that time span.

Data elaboration is still in progress, but the first analyses suggest that the lost bees (i.e. those that neither returned to the hive after their release nor were seen on the food dispenser) amounted to 10% in the clothianidin –treated group, and 3.4% in the control group. In both treatments, bees returned to the hive within a mean time span of roughly 30 minutes after release. In the control treatment, 87.5% of bees returned within the first hour, vs. 80.8% of bees that had come into contact with clothianidin.

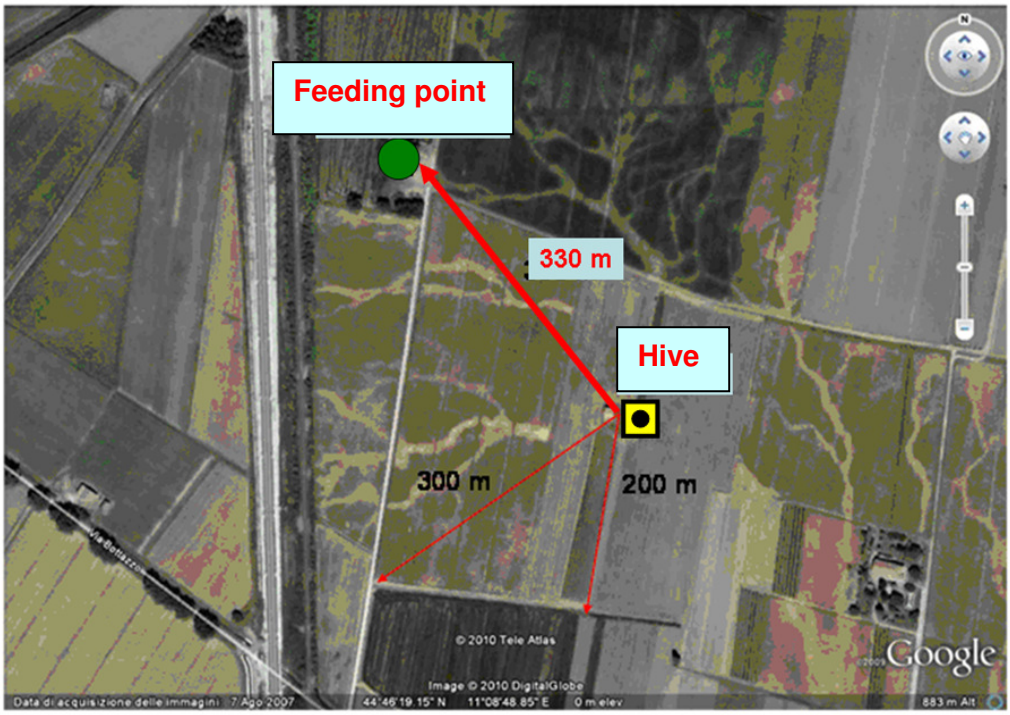


Figure 37 – Map of the trial area.

6.5 Effects induced in bees by dust from seed coated with clothianidin, thiamethoxam, imidacloprid and fipronil: evaluation of effects on learning and memory of odours and colours and spatial orientation

Study of the *Proboscis Extension Reflex* (PER) in the presence of odours associated with administration of sucrose liquids allows an assessment of the impact of pesticides on some cognitive processes such as learning and memorization of different types of environmental stimuli. Impairment of odour-associative learning can be taken as an index of disturbance of cognitive abilities, severely affecting the foraging functions of bees and also leading to dangerous disorientation. The 2009 trials conducted in the framework of the "*Api e Agrofarmaci*" study of the Apenet Project provided evidence that the quantity of dust dispersed by the seeder and deposited on the ground at the distance of 5 m is sufficient to induce an adverse effect in bees that repeatedly come into contact with the substance. Affected bees in the 2009 trials showed reduced ability to recognize odours associated with a reward in the context of specific training.

Since these effects are detectable at sub-lethal doses, our programme planned to repeat the experiment only if the seeder modifications introduced to reduce dust emission were subsequently found to have lowered dust drift to below the quantities we assayed in 2009. In actual fact, the quantities measured appear to be equal to or greater than those detected in 2009. However, partly also in the light of the debate during the round table held on June 4th 2010 in Rome at the head office of CRA, in the presence of European experts on the neuronal mechanisms the underline bee learning and memory, we decided to continue the trials, adopting a partly modified PER Protocol in order to increase the margins of appropriateness of the method.

The odours utilized in this second year of trials involve bee social life. The odours used were linalol, a component of the Nasonov gland with functions of summoning forager bees back to the colony and of aggregation, and a pheromonic mixure (Bee Boost) containing some components of the queen pheromone, composed of scantily volatile molecules with multiple functions that are fundamental for colony cohesion and to stimulate the worker bees to fulfil their tasks. This approach was adopted in order to achieve a better fit between the experimental method and genuine

field conditions, and to favour an interpretation based on the life of the hive rather than the individual bee.

In the same perspective, the Y-maze test on orientation ability was conducted outdoors, using bees accustomed to flying freely. Thus the bees tested were true foragers, at work in connection with their own hive, but trained in this experiment to search for the reward in our special facility.

6.5.1 PER-test: Evaluation of the effects of clothianidin, imidacloprid, thiamethoxam and fipronil (contaminated dust) on recognition of linalol, a component of the Nasonov gland

Materials and methods

Hives, number of bees, repetitions: a single hive was used and 3-4 repetitions were performed (each made up of 9-11 bees).

Capture: Bees were captured and placed in purpose-made plexiglass cages, the bottom of which consisted of an 8.5 cm diameter Petri dish (10 bees per cage). Each cage was equipped with a food dispenser.

Tested active ingredients: clothianidin, imidacloprid, thiamethoxam and fipronil.

Origin of contaminated dust: the dust utilized in this trial was extracted from coated seed batches supplied by Assosementi at CRA-ING in Rome, utilizing the Heubach cylinder.

Tested concentrations: the quantity of active ingredient per surface area utilized in the present trial was equal to that estimated to be deposited at 5 m from the sowing field using a seeder without modification, and concentrations 10, 100 and 1000 times higher (Tab. 1).

Since the experimental cages had a total area of 56.72 cm^2 and an 8.5 cm Ø Petri dish as their bottom, the quantity of dust utilized per cage was calculated in proportion to the area available (Tab. 1).

The dust containing the concentrated active ingredient extracted from the Heubach cylinder was prepared and mixed with talc by the DISTA Unit, in order to obtain the sub-lethal concentrations for this trial. In each container 0.01 g of talc containing the calculated quantity of active ingredient were introduced.

Each cage contained 10 bees.

Table 44 - Quantity of a. i. per tested surface. CLO= clothianidin, IMI= imidacloprid, THI= thiamethoxam; FIP= fipronil.

	CLO	**IMI**	**THI**	**FIP**
% a. i. in the dust	33	31.1	33.5	32
a. i. at 5 m (ug/m^2)	2.25	3.63	2.53	0.91
Total dust at 5 m (ug/m^2)	6.82	11.67	7.55	2.84
Dust in a 8.5 cm Ø Petri dish (=56.72 cm^2)	**0.039**	**0.066**	**0.043**	**0.016**
q.ty a. i. x1	**0.012762**	**0.020589**	**0.014350**	**0.005162**
q.ty a. i. x10	0.12762	0.205894	0.143502	0.051615
q.ty a. i. x100	1.2762	2.058936	1.435016	0.516152
q.ty a. i. x1000	12.762	20.58936	14.35016	5.16152
contact LD50 (ug/bee)	0.0218	0.0179	0.0299	0.006

Manner of contamination with the active ingredient: immediately after the capture of bees flying out from the hive, the bottom of each cage was replaced with a Petri dish containing the pre-established dose of active ingredient. Each cage was maintained for 3 hours (after administration of

the product) in an incubator at 26° C in darkness. Bees had access to sugar syrup immediately after being captured. The feeder was removed 2 hours later to starve the bees in preparation of the PER test.

Preparing bees for the PER test: Each bee, after being submitted to treatment, was placed individually inside Gilson pipette tips.

Training: Training began with some exercises aimed at conditioning bees to an air flow for 15 seconds, followed by:

1. exposure to citronellol for 5 seconds (drop on the tip of an insulin syringe held at 1 cm from the bee's head), follwoed by tapping the bee's antennae with the syringe containing citronellol and offering the reward (sugar syrup) for 1 second;
2. after 6' exposure to peppermint odour (in the same way as above) followed by touching antennae and administrating a saline solution;
3. after 6' new exposure to the rewarded odour (citronellol) for 5 seconds, in the same way described above, followed by the reward.

Test: the PER-based odour recognition test was carried out at 60', 180' and 24 h after the last training test, in order to verify the ability of the bees to recognise the odours, by presenting them with the rewarded or punished odour, and assessing the responses on the basis of the following categories:

1. Correct C+M-: response (proboscis extension) only to the odour which was rewarded during the training (citronellol) and not to the punished odour (peppermint).
2. Partially correct C+M+: response to both odours.
3. Partially wrong C-M-: no response to either odour.
4. Wrong: response only to the punished and not to the rewarded odour.

Each odour recognition test consisted in 10 alternate presentations of the rewarded and punished odour (i.e. 10 presentations for each odour), starting with the punished odour. During these tests the bee was offered neither reward nor punishment.

At the end of the test conducted at 180', the bees were fed a drop of 30 µl sucrose solution.

Viability at the end of the test: After the test conducted at 24 h, bees were released into a free flight cage to monitor viability data linked to motor functionality. The following behavioural modes were recorded: flight (V), walking (C), rale (R).

Data analysis: After checking the robustness of the premises (homogeneity of variance), a one way ANOVA for each a. i. and each time interval was performed, considering treatment (a. i., untreated control) as the main factor.

Results

Bees exposed to the 4 a. i. at the doses registered in the field at 5 m from the seeder without modification

The graphs (Figs. 38-41) show the percentages of correct responses at the different time intervals (60', 180', 24h) for all the a. i. at increasing concentrations, starting from the concentration corresponding to the quantity of a. i. deposited by the seeder at 5 m (indicated with x1) increasing to x10, x100, x1000. The trials herewith described involved bees which had survived exposure: it must be noted that the used concentrations caused noticeable mortality, as confirmed by other trials in the framework of the APENET project. Exposure of groups of bees was repeated several times until a sufficient number of surviving bees to use in the behaviour tests was available.

Results showed a significant effect of treatment with all tested a. i. on odour recognition ability 24 h after exposure. The percentage of fully correct responses (proboscis extension in presence of citronellol but not of mint, C+M-) was significantly lower in treated bees compared to the untreated controls (Figs38-41)

As expected, the differences mainly concerned the ability to recognise odours 24 h after exposure, although, for clothianidin, a significant reduction was already evident 180' after exposure.

These data demonstrate a clear negative effect of sub-lethal doses of the tested a. i. on the ability to shape and / or recover olfactory memory. This is true both when bees were exposed to doses equal to the ones measured in the field, and when bees survived exposure to much higher doses, as may happen in the case of dust drift by wind, or when crossing in flight a dust cloud containing contaminated particles emitted during seeding.
When bees were freed, at the end of the 24 h test, they were all able to walk and to fly, thus excluding the hypothesis that the absence of response was due to motor inability.

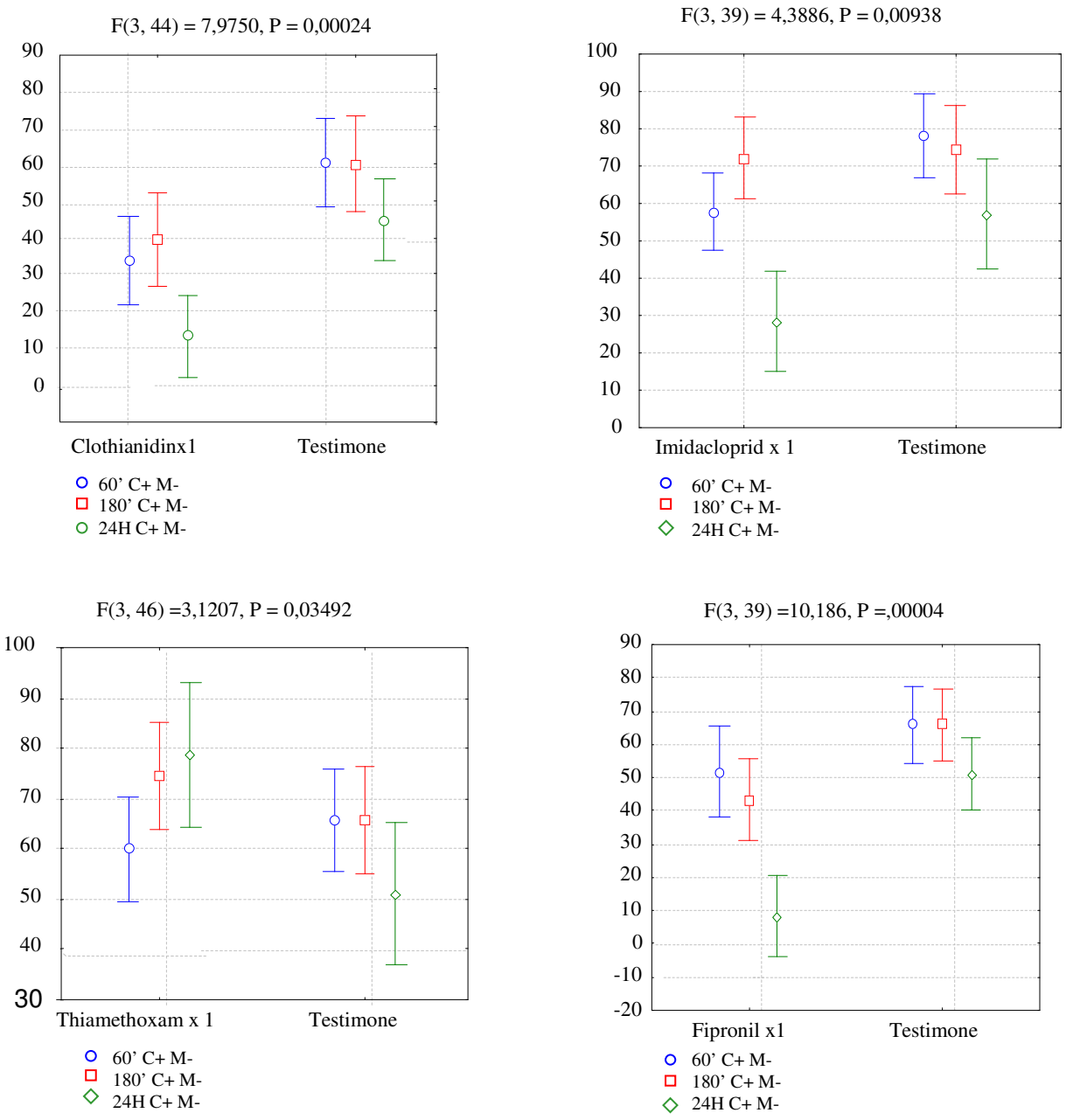


Figure 38 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

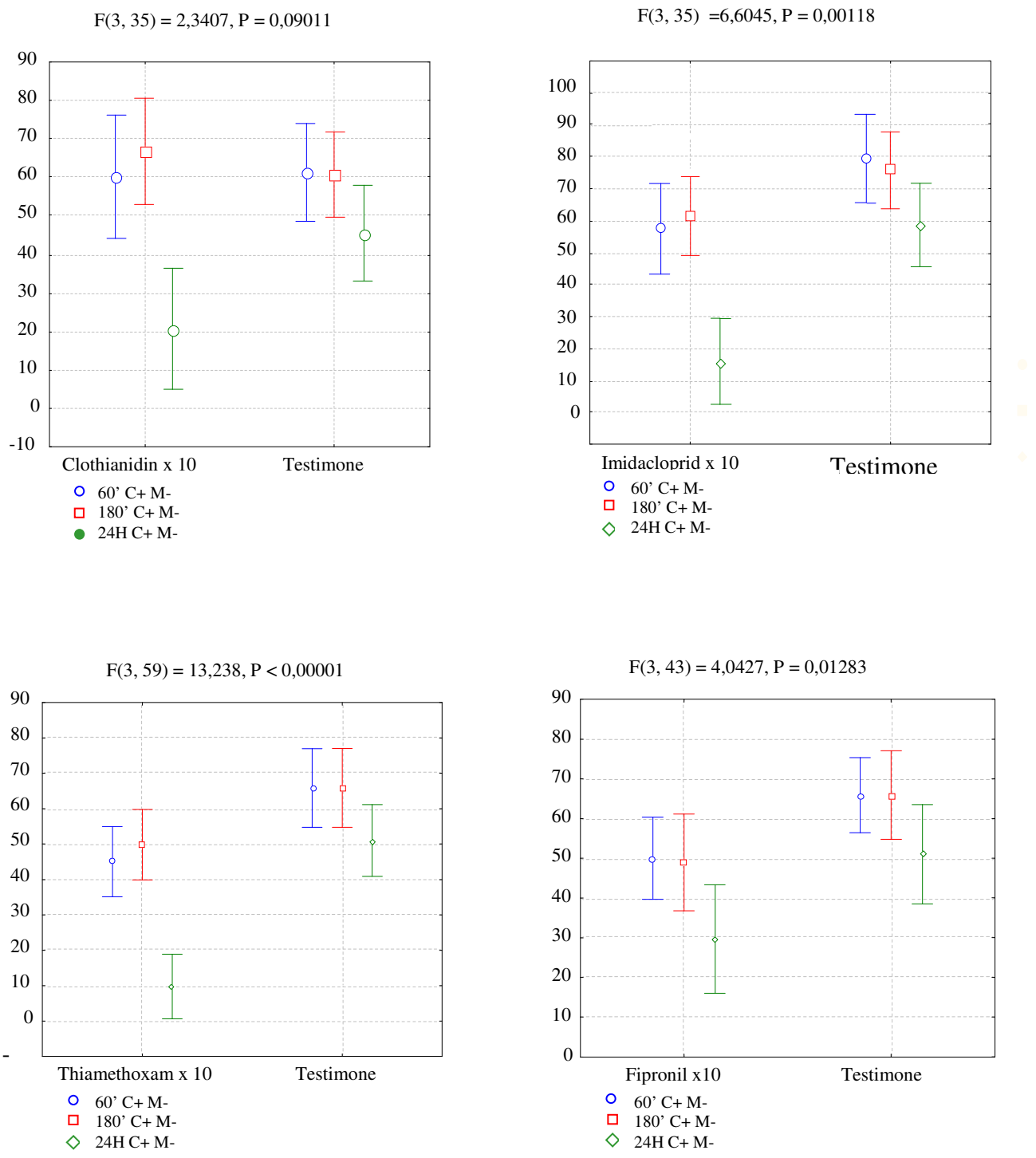


Figure 39 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 10 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

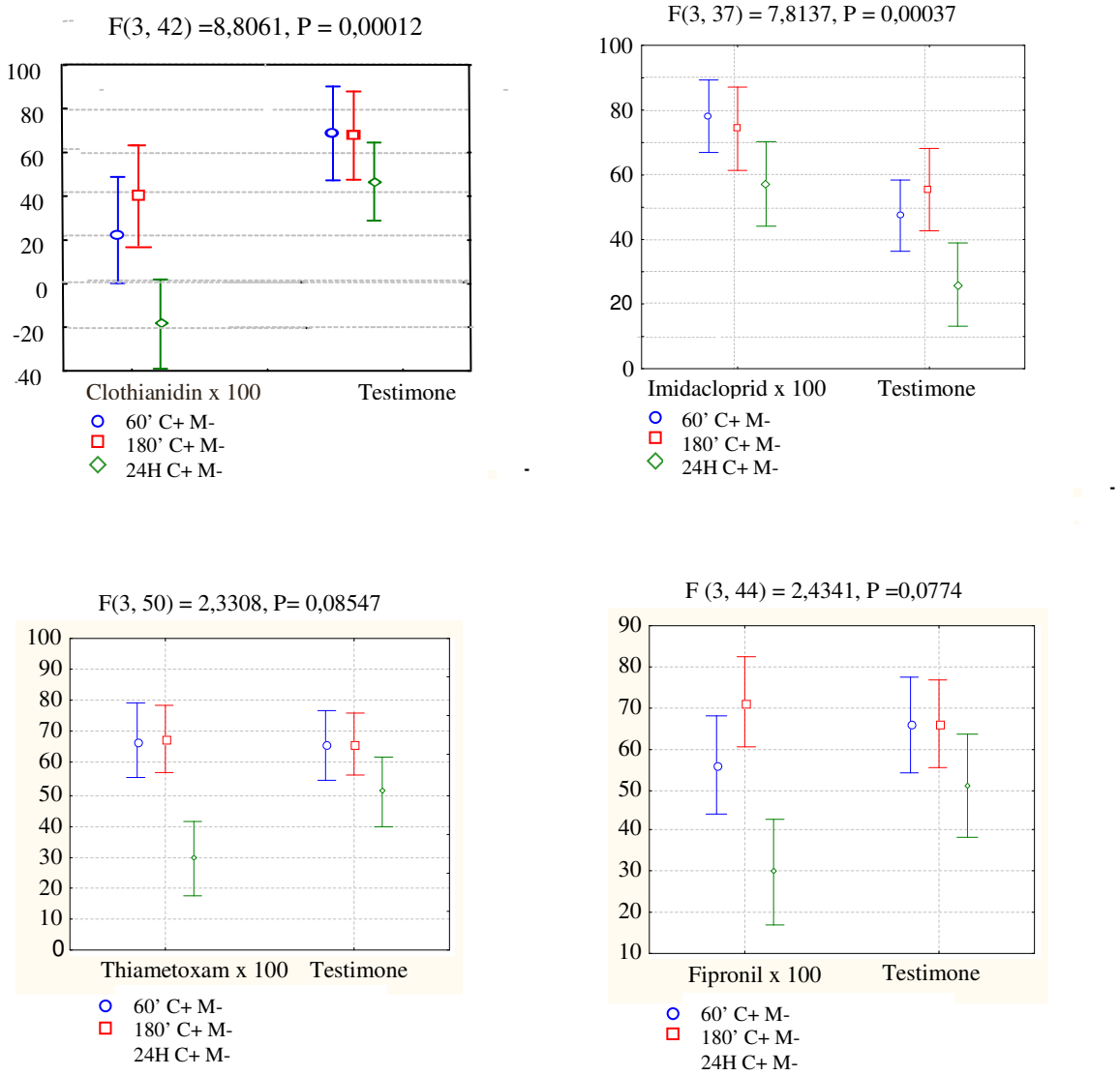


Figure 40 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 100 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

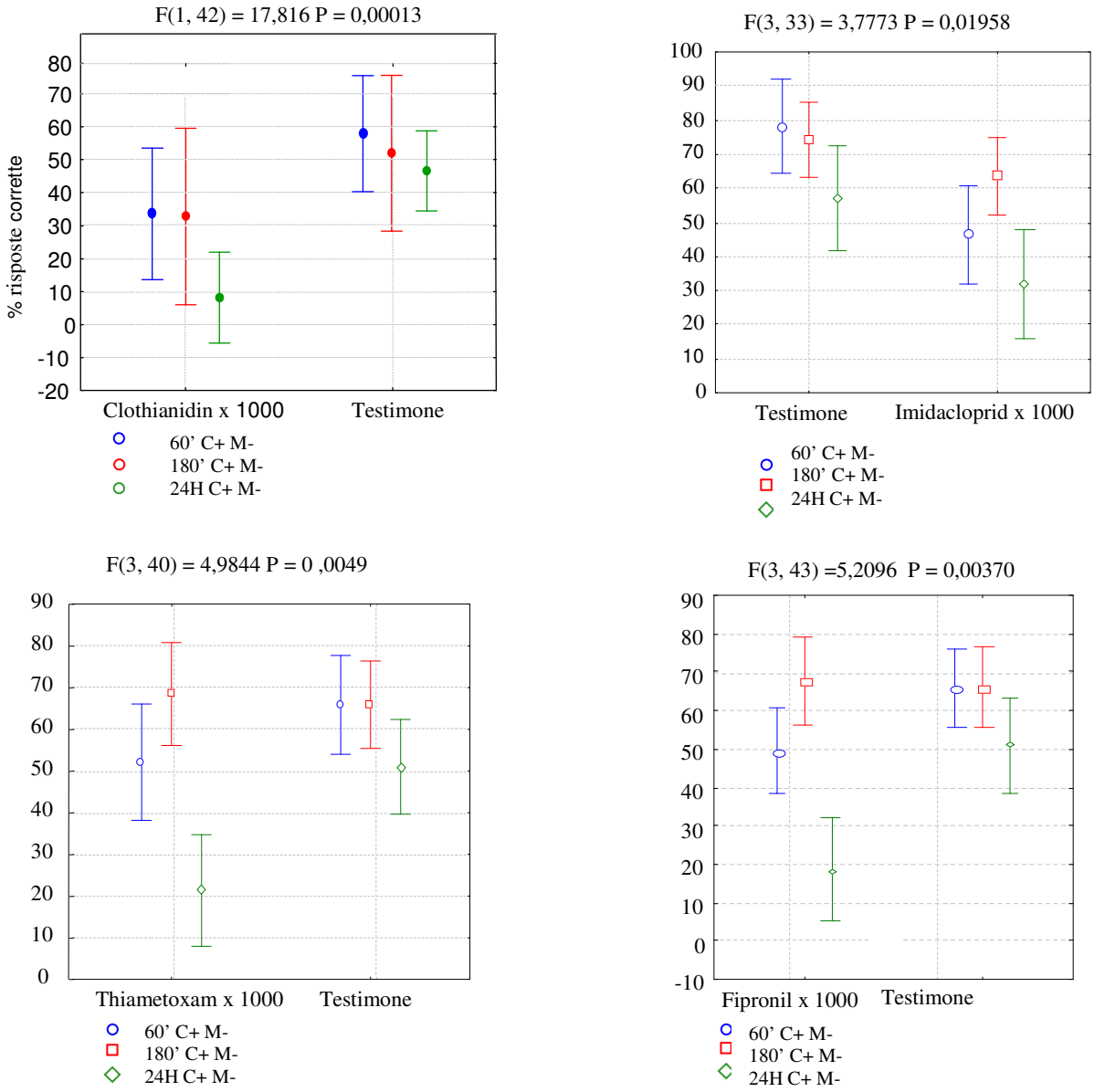


Figure 41 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 1000 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

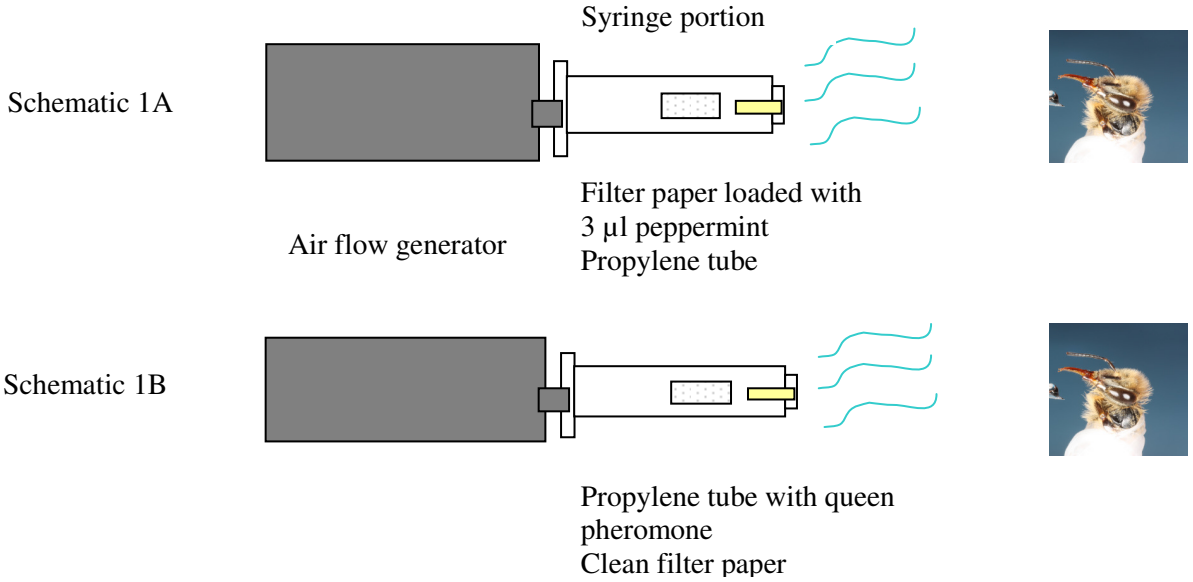
6.5.2 PER-test: Evaluation of the effects of clothianidin (contaminated dust) on queen pheromone recognition ability

The materials and methods utilized were the same as described in the trials of point 6.5.1.

Odours and manner of presentation: Some modifications were introduced as compared to the previous trial (6.5.1) on account of the nature of the odours utilized, which required a different mode of presentation.

For the punished odour, we used a strip of filter paper loaded with 3 microlitres of peppermint (identical to the previous trial), placed in a 5 ml syringe with the piston removed and the hole widened (without needle). For the rewarded odour, a pheromonic mixture containing the main components of queen pheromone was used. Since this pheromonic mixture was constituted by poorly volatile molecules, adsorbed onto a propylene tube, the following procedures were adopted to ensure that the syringes utilized for odour presentation were *visually identical*:

- an odour-free propylene tube was added in the syringe containing the strip of filter paper loaded with mint (schematic 1 A);
- a strip of odour-free filter paper was added in the syringe containing the small tube with the pheromonic mixture (schematic 1 B).



Hives, numbers and repetitions: the trial is still in progress. Two repetitions of 11 bees each have been completed for hive C. Data were analyzed statistically with one-way ANOVA.

The results showed a significant effect of clothianidin treatment on recognition of queen pheromone components. The percentage of fully correct responses (proboscis extension in presence of the pheromone and not of mint, QP+M-) was significantly lower in treated bees as compared to untreated controls for all time intervals considered (Figure 42), while the percentage of partially wrong responses (QP-M-) was significantly higher in treated compared to control bees (Figure 44).

Furthermore, at 24 h after treatment, the percentage of consistently positive (QP+M+, Figure 43) and completely wrong (QP-M+, Figure 45) responses also increased, confirming findings for thiamethoxam detected in 2009.

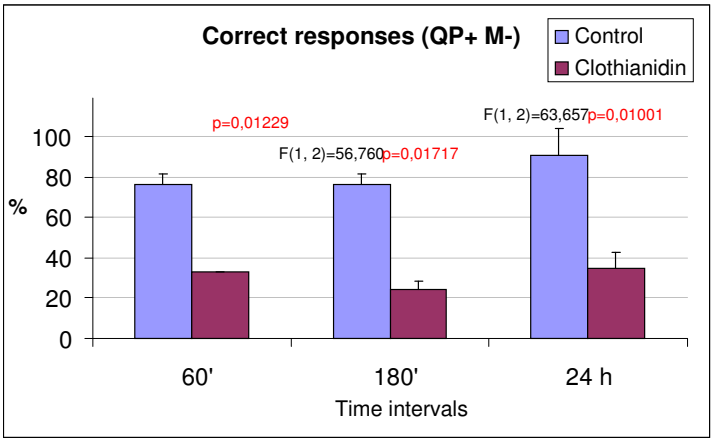


Figure 42 - Explanation in the text.

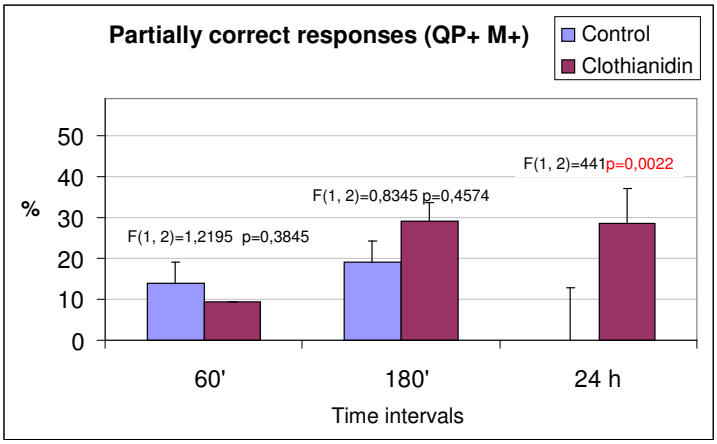


Figure 43 - Explanation in the text.

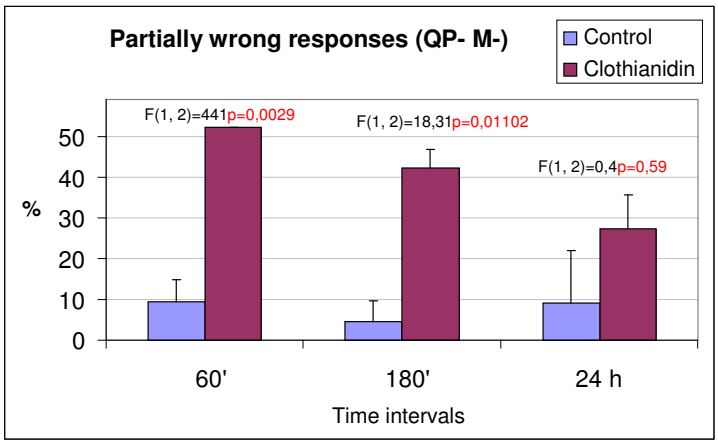
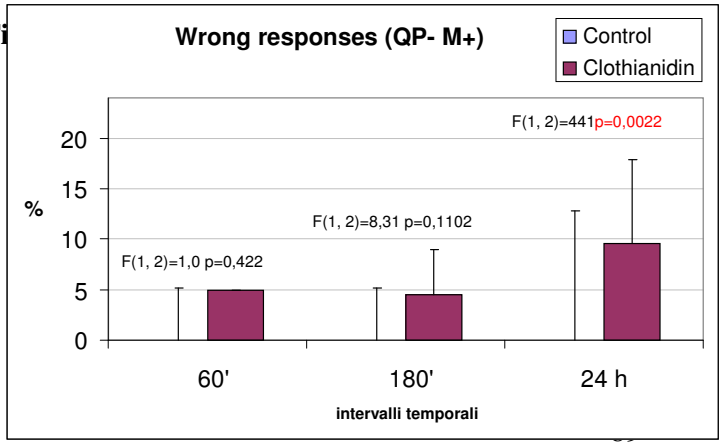


Figure 44 - Explanation in the text.



Ability to overcome the stress of immobilization, viability after release, and the capacity to perform different types of motor functions were evaluated by releasing bees into a free flight cage. Results summarized in Figure 46 show that there were no noteworthy differences between treated and untreated bees. However, this type of assessment is not sufficient to demonstrate the absence of motor effects, as the free flight cage measures only 40 x 30 x 30 cm. On the other hand, it does confirm that cases of failed responses to the *PER* test cannot be attributed to an inability by bees, after immobilization, to engage in simple motor activity such as proboscis extension.

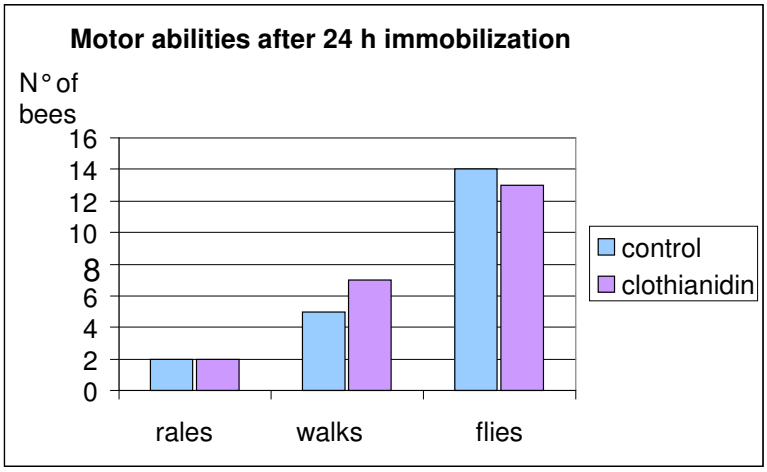


Figure 46 - Explanation in the text.

6.5.3 Learning and memory of colours and spatial orientation in the Y-maze

Spatial and colour-related training – A simple Y-maze was placed outdoors, at roughly 25 m from the hives (Figure 47). Bees were divided into groups for preliminary training in extracting 50% sucrose solution from a white dispenser in the antechamber of the maze (phase 1).

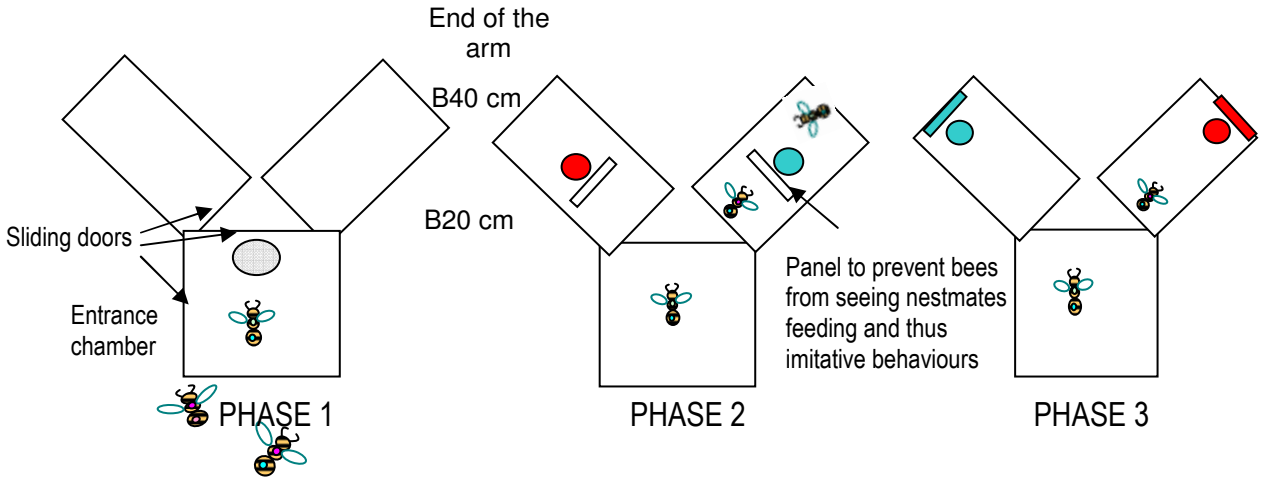


Figure 47 - Training scheme.

A group of 8-10 bees were then marked with water colours (Uniposca) on the thorax and the abdomen, making them individually recognizable (Figure 48 A). The other subjects were excluded from the subsequent phases by means of capture and release into a specific cage. In phase 2 a strip of white cardboard was placed vertically in front of the dispenser so that the dispenser was hidden

to the view of incoming bees (Figure 48 B). Training continued exclusively for the marked individuals, inside the common chamber and subsequently inside the two arms, on dispensers with a coloured lid: blue = reward (50% sucrose solution); red = punishment (saturated saline solution) (phase 3).

Each bee performed:

- 4 visits with dispensers placed half-way between the two arms (B20), with a vertical obstacle in front of the dispenser (Figure 48 B);
- 4 visits inside the labyrinth up to the end of the two arms (B40), with a vertical obstacle;
- 4 visits inside the labyrinth up to the end of the two arms (B40),without vertical obstacle and with the addition of a square having the same colour as the dispenser lid on the vertical wall behind the dispenser itself (Figure 48 C);

As part of the training, only one subject at a time was allowed access. The position of the rewarded colour in one or the other arm was determined according to a semirandom sequence (no more than two successive visits on the same side), and the bees were divided into two groups: one started the training (first reward) on the right, and one on the left.
For each visit, arrival time and first choice arm of the subject was recorded.

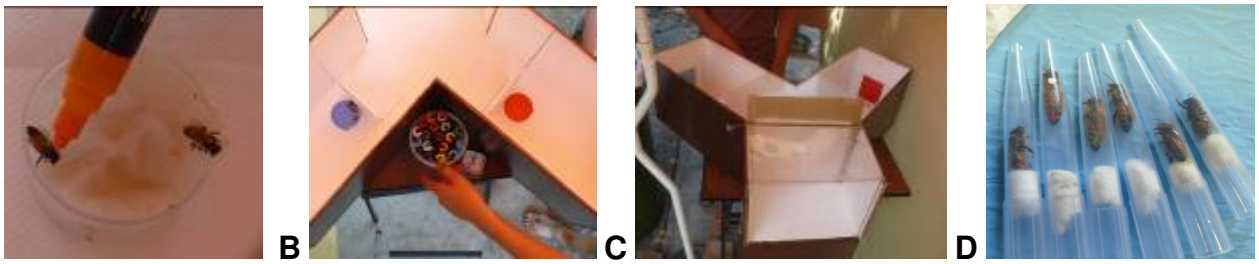


Figure 48 - A: marking; B: training with colours protected by a vertical white barrier; C: final training with colour visible to incoming bees, with a coloured cardboard strip on the rear wall at the end of the arm inside the labyrinth; D: bee treatment in the tip.

Capture and treatment – Capture took place during the final training visit, after the subject had alighted on the correct dispenser, but before the subject began to feed:

- the subject was then inserted into a Gilson pipette tip (200 microlitres), whose point had been cut off (Figure 48 D);
- starvation for 30 minutes;
- administration of treatment (see Table 38) by ingestion, inside the tip itself; control bees were given the same quantity of 50% sucrose solution;
- bees were restrained in the tip for 1 hr in darkness.

Table 45 – Active ingredients, doses and manner of administration utilized in the experiment.

Active ingredient	**DL50 48 h**	**Dose assayed**	**Proportion as compared to DL50 at 48 h (*)**
Clothianidin (Dantop 50 WG®)	4 ng/bee	0.47 ng/bee	1/10
Imidacloprid (Confidor 200 SL®)	3.7 ng/bee	0.036 ng/bee	1/100
Thiamethoxam (Actara 25 WG®)	5 ng/bee	0.05 ng/bee	1/100
Fipronil, pure, powder formulation	4.17 ng/bee	1.2 ng/bee	1/5

(*) Data available on Footprint (http://sitem.herts.ac.uk). The proportion as compared to DL50 for the various active ingredients was chosen on the basis of the 2009 mortality trials conducted after administration of the active ingredient.

Test 60': Each subject was released 60 minutes after administration; release took place near the Y maze, and all subjects were released simultaneously.

The coloured food dispensers (visible to incoming bees, partly also due to the presence of the identically coloured strip of cardboard on the rear wall inside the arm of the labyrinth) did not contain either reward or punishment.

The test consisted in alternate visits to the arms, in which, on the first visit, the rewarded colour was placed on the side where the subject had first begun its training. Access was allowed to only one bee at a time, regulating the entry with movable vertical barriers positioned at the entry to the labyrinth and to the common chamber.

For each bee, the following parameters were recorded: the time of each visit, the first arm explored (on the right or on the left), and behaviour towards the feeding dispenser located on the rear wall inside the arm: V = flight inside the arm without alighting on the feeder; N = feeds, in other words the subject alights on the food dispenser and extends the proboscis at the position of the holes in order to access the solution contained within them (not present); A = alights on the food dispenser without attempting to feed.

The visits of each test were *non corrective*: that is to say, the bee was allowed to move around in the first arm chosen: furthermore, independently of the colour that was present, the bee was not allowed the possibility of moving into the other arm. Instead, the bee was made to exit immediately from the labyrinth through removal of the transparent Plexiglas lid that covers the labyrinth. For each subject, the wait for its return to the labyrinth after release extended for a maximum of 3 h.

At the end of the two visits composing the test, each individual was captured and inserted together with the other subjects of the same group (treated / controls) into a cage containing a food dispenser, and maintained in darkness at 26 °C until the following day.

24 h test: the subjects of the two groups were released 24 h after administration of the active ingredient. The test was performed according to the same procedure as described for the 60' test.

Computations of the data recorded for this test are still in progress. The first results of the statistical analysis are given in Figures 49 and 50.

Figure 49 A-H shows the percentage of bees which, after release, chose the arm which presented the food dispenser with the correct colour on the rear wall inside the arm (the colour that was rewarded with sucrose solution during training).

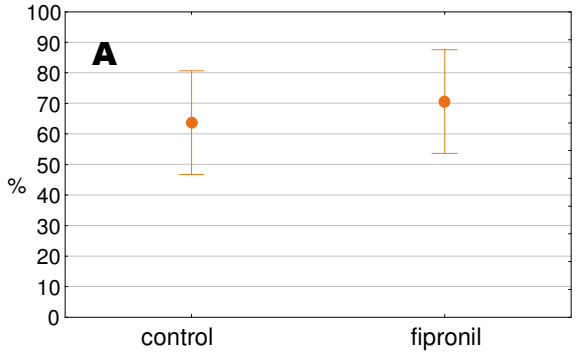
One-way ANOVA carried out for each active ingredient and for each test indicated a statistically significant reduction in the ability to enter the correct side 60 minutes after clothianidin and imidacloprid treatment. In contrast, bees submitted to thiamethoxam and fipronil treatment and tested 60 minutes after administration of the active ingredient did not differ from the untreated controls as regards orientation ability. On the other hand, at 24 h after treatment, all the active ingredients studied led to a significant reduction in orientation ability as compared to the control groups.

Figure 50 shows the percentage of choice made by bees when faced with the feeder they encountered on the rear wall inside the chosen arm. Percentages were calculated as an overall percentage of the total number of bees that constituted the group; statistical analysis of the percentages has not yet been completed.

Although the analyses are still in progress, a general tendency towards a decrease in food searching behaviour can be detected. With imidacloprid, clothianidin and fipronil treatment, a difficulty in recognizing the rewarded colour can also be discerned, as suggested by the non negligible attempts to feed at the red food dispenser, namely the dispenser which, during training, contained the punishment (saturated saline solution).

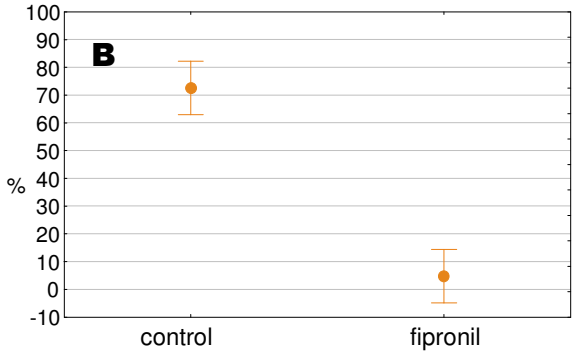
Test 60': Enters the right arm

F(1, 4)=0,64576, p=0,46667



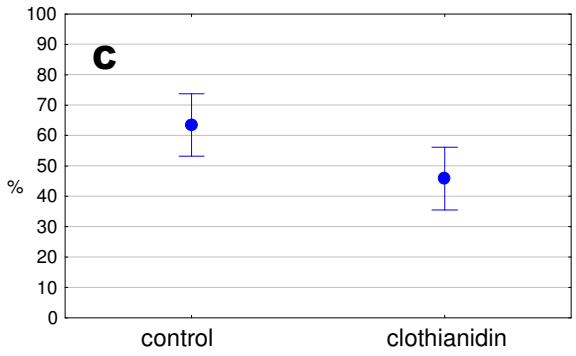
Test 24 h: Enters the right arm

F(1, 4)=191,12, p=0,00016



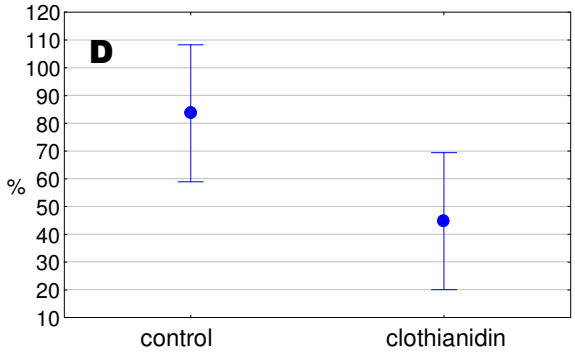
Test 60': Enters the right arm

F(1, 4)=11,364, p=0,02801



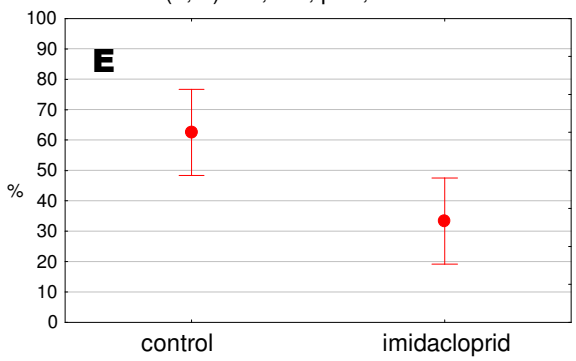
Test 24 h: Enters the right arm

F(1, 4)=9,5526, p=0,03655



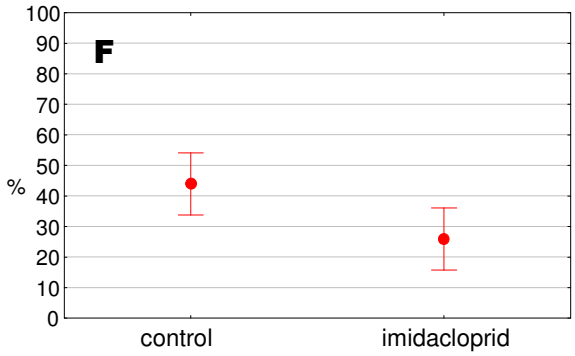
Test 60': Enters the right arm

F(1, 4)=16,333, p=0,01559



Test 24 h: Enters the right arm

F(1, 4)=12,168, p=0,02516



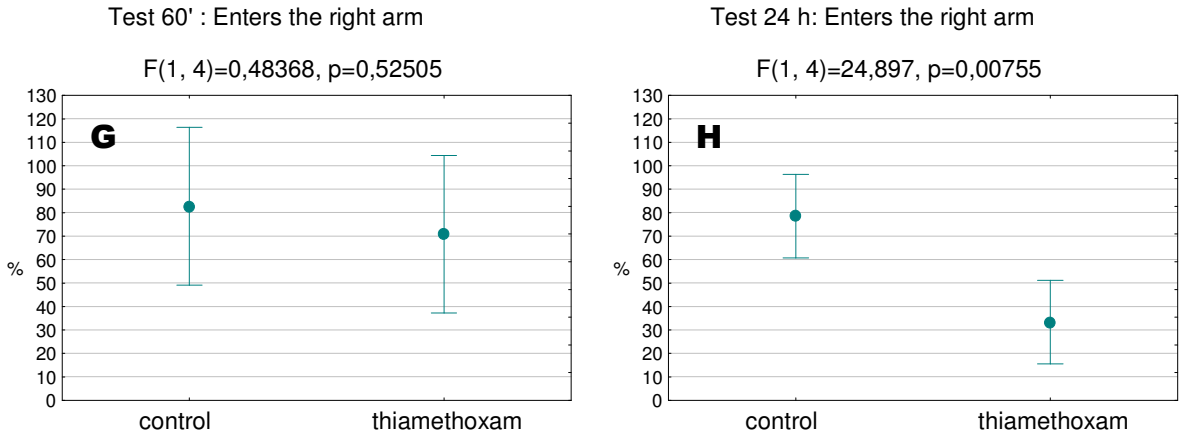
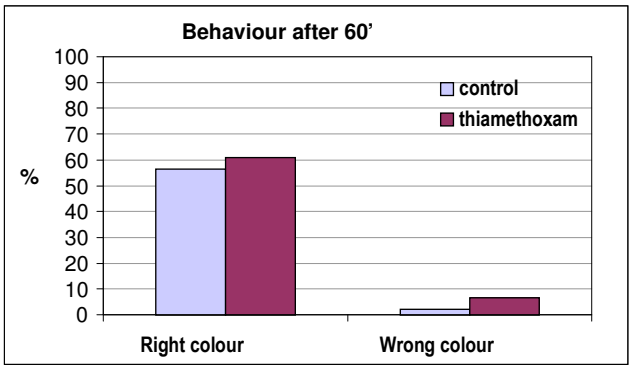
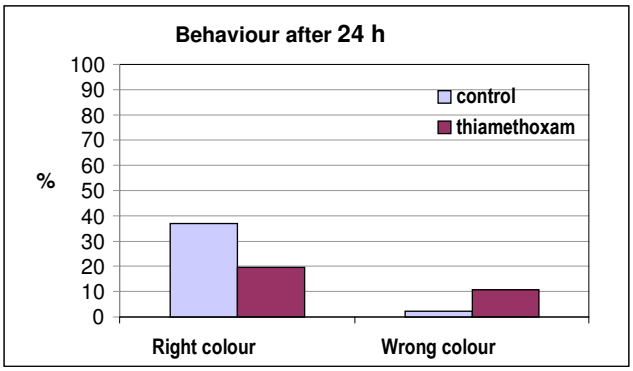
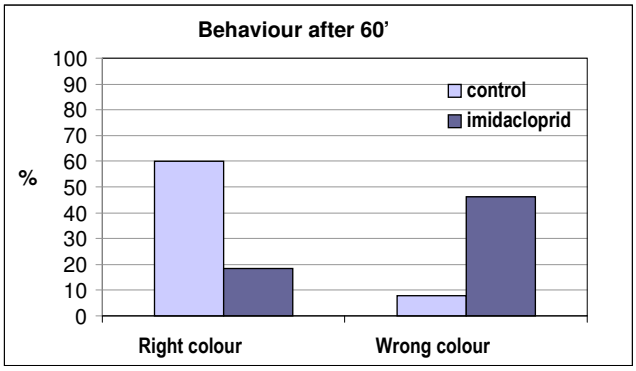
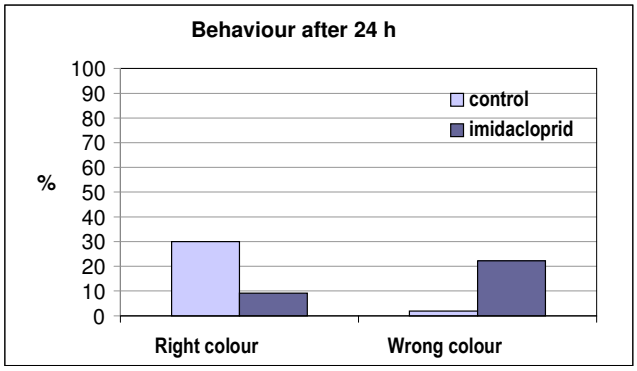


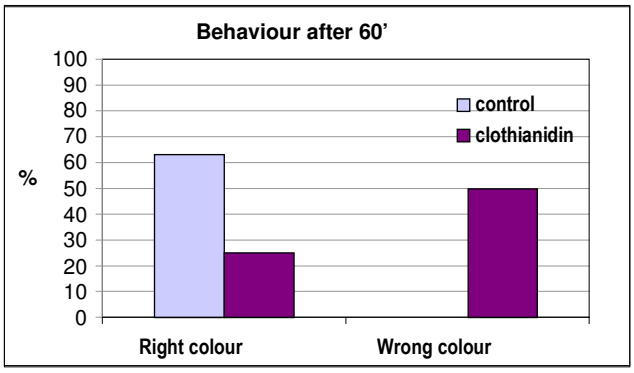
Figure 49 – Explanation in the text.

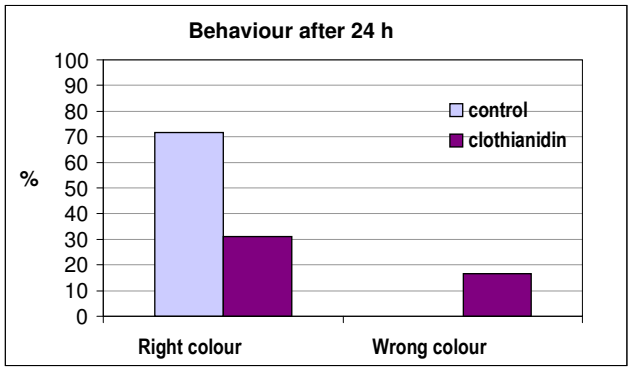


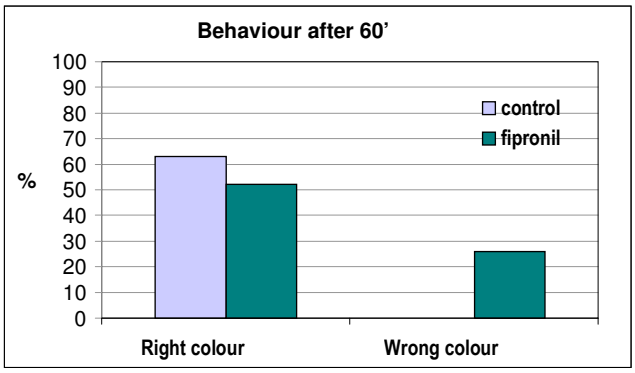












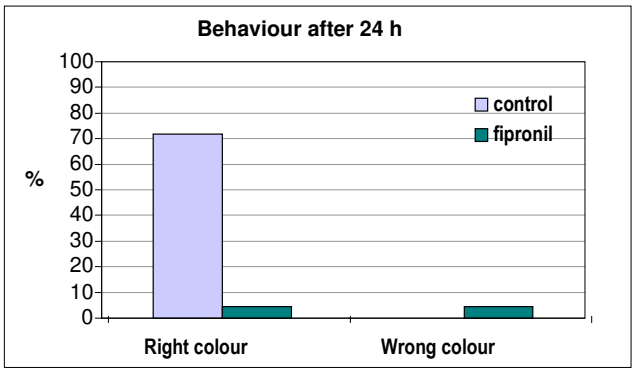


Figure 50 - Percentage of bees that engaged in food searching behaviour at the correct and wrong food dispenser (both empty) during the 60' and 24h tests. Only one of the two was situated on the rear wall inside the arm. Bees that moved towards it were allowed only one exploration and then were made to exit from the tunnel.

6.5.4 Conclusions

The trials are still in progress, but the data acquired so far allow confirmation of the results of experiments conducted in 2009.

The protocol applied in these experiments, which was designed to assess the sub-lethal effects of odours on memory, was partially modified as compared to the 2009 approach. The new element in the 2010 protocol concerns the prolonged training phase (12 presentations) followed by a very short test (1 attempt), whereas the 2009 protocol provided for a short training period (2 presentations) and a prolonged testing period (10 attempts) during which the bee was assayed repeatedly within the same time span.

So far, the two approaches have resulted in the same findings, showing a significant reduction in long-term memory (24 hours) of bees contaminated with dust containing sub-lethal doses of clothianidin.

The rewarded and punished odours utilized in the memory trials were linalol, as the rewarded odour, and a queen pheromone mixture as the punished odour. Choice of these odours was based on the following rationale: linalol is a component of the Nasonov gland, with functions of summoning bees back to the colony and of aggregation, while queen pheromone has multiple functions and is fundamental for maintaining colony cohesion and for stimulating the worker bees to fulfil their appropriate age-dependent tasks. Therefore any disturbance in perception of such odours strongly affects not only the foraging bees themselves and their food gathering function, but also the balance within the colony, inasmuch as the perception of pheromones plays an essential role in relations among companions within the nest and between the overall group of companions, the queen and the larvae. Consequently, pheromone perception is crucial in determining strength of the family.

Experiments were also conducted on bee orientation ability with a simple Y maze, testing the ability of free-flying bees to enter the maze and find the reward on the basis of a visual stimulus, namely a colour. Results on the active ingredients studied showed that at 24 hours after administration of treatment, sub-lethal doses of all active ingredients contained in dust deposited at 5 m from the seeded field were capable of compromising bee ability to visit a known food source and to recognize the colours associated with the sucrose reward. For imidacloprid and clothianidin this effect was visible as early as 60 minutes after administration of the treatment.

Scientists and Institutions in charge of the trials

1. The monitoring network

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2. Dust drift during coated maize seed sowing and estimation of effects on bees

Scientist in charge of the sub-project

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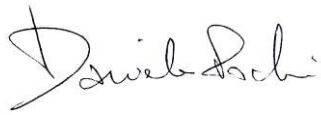

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3. Effects induced in bees by contact with dust during flight over a field sown with coated maize seed

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Scientist in charge of the sub-project
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Dr. Carlotta Balconi

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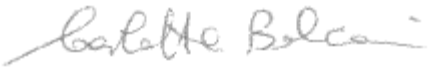

External collaborator

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Veneto Agricoltura

5. Effect of maize guttation on bees

Scientist in charge of the sub-project

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6. Lethal and sublethal effects on bees in the laboratory exerted by the active ingredients used in maize seed coating

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F(1, 2)=79,867

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