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 first year of activity of the APENET project

apeNet

Report refers to results available on 4th September 2009, including data and analyses received by CRA-API at the previous reporting date (30th June 2009).

Contents

With reference to D.M. 19735/7303/08 dated 29th December 2008, which grants the Agricultural Research Council(CRA) a contribution to conduct the research project “APENET monitoring and research in bee-keeping”, and upon the request, expressed in the letter bearing the protocol nr. 611 dated 12th January 2009 in which the Decree itself is transmitted, to award priority to experiments on the effects induced in bees by coated maize, the present survey outlines the first results obtained in the framework of the research lines pertaining to the above-stated project. Such a priority was granted following suspension of use of the active ingredients which are used against harmful soil insects and sap-sucking insects: imidacloprid, clothianidin, thiamethoxam and fipronil for seed coating (Ministerial Decree 17/09/2008).

1. The monitoring network

Figure 1 – Monitoring network: localisation of the Apenet stations (red dots) and of the stations belonging to the regional networks of Lombardy, Friuli Venezia Giulia and Piedmont (pink dots).

The Apenet Project provides for the establishment of a national monitoring network, composed of modules, each of which consists of five 10-hive stations, situated in geographically differentiated areas of each Region of Italy. The function of the monitoring network is to gather information on the health status of the bee colonies that make up the modules, by means of periodic sampling and laboratory analyses performed on different colony matrices (dead bees, live bees, brood, honey, wax, pollen).

In addition to routine analyses, in the case of any abnormal bee die-off there will be supplementary samplings, carried out at times distinct from the pre-established sampling periods, and laboratory analyses will be performed for each case.

The national monitoring network, composed of at least one module for each Region and Autonomous Province (Figure 1), was set up during the first semester of 2009. Other national or local monitoring bodies, either already established or under construction, also work closely with the national network. Such bodies include the Protected Nature Reserve monitoring network, which is funded by the Ministry of the Environment and managed by the Institute for Environmental Protection and Research (ISPRA) and has now been set up in 4 areas (Veneto, Emilia Romagna, Tuscany, Latium), as well as the regional monitoring networks already active in Lombardy, Tuscany, Friuli Venezia

Giulia and Piedmont. These networks may be expanded by the establishment of a second module in the Region of Umbria and by the cofinancing of the modules in the Region of Basilicata.

By the end of the first semester of activity, the initial two of the four planned inspections had been carried out, and the third was ongoing; routine analyses of samples collected during the survey were in progress and partially completed.

During the first semester of activity (March-August 2009) of the monitoring network, no unusual die-offs or population losses were observed, either during the pre-established March-April inspections or in those carried out during the month of June as part of the second round of observations. Likewise, no adverse events were noted between the first and the second sampling, with the exception of station CLB 2 of Rossano Calabro, which borders on the citrus-growing area of the Sibari Plane (Piana di Sibari): in this station a severe bee die-off occurred, due to the utilization of neonicotinoids during citrus tree flowering (monitoring carried out at delayed date in the month of May).

Additionally, inspections of the regional modules in Sicily, Sardinia and Campania were conducted, with similar inspections in Marche, Puglia, Calabria and Bologna set for dates shortly thereafter.

The monitoring network and the field data management database are managed by the Bee Health Reference Centre of the Animal Disease Prevention Institute of North-East Italy (IZSV), in collaboration with the Department of Agroenvironmental Sciences and Technologies (DiSTA) of the University of Bologna.

1.1 The reporting system

The monitoring programme is supported by a highly important tool in the form of the reporting system, which makes it possible to record anomalous events in hives that do not belong to the network. The reporting system asks bee-keepers to report any bee loss episode to the Veterinary Service of the Local Health District (ASL) or to the collection centres specifically entrusted with collecting such reports; the body in question will then perform the necessary inspection and collect samples, to be stored in appropriate conditions (-20°C) and sent to the laboratory of the Animal Disease Prevention Institute for the required analyses.

In previous years, and above all in the spring of 2008, reports of colony loss or depopulation sent in by bee-keepers proved to be of fundamental importance for identification and quantification of honey bee die-offs caused by sowing of coated maize.

The comparison between reports sent in spring 2008 to the Veterinary Services and to the collection centres and those sent in the spring of 2009 in maize-growing areas is summarized in Table 1.

Region	Nr. of reports in **maize-growing** areas		Other reports during spring 2009
	Spring 2008	Spring 2009	
Lombardy	40	1	
Piedmont	8		2
Emilia-Romagna	7	1+1*	
Veneto and Trentino	20		3
Friuli Venezia Giulia	110		1
Calabria			1
TOTAL	**185**	**2+1**	**7**

Table 1 – Number of reports sent to the Veterinary Services and the collection centres in the spring of 2008 and of 2009 in maize-growing and non maize-growing areas (Source IZS).
* non official report

In the spring of 2009 only two official report were sent to the Veterinary Service during the maize-sowing period, plus one non-official report sent directly to the Honey bee and silkworm Research Unit of the Agricultural Research Council (CRA-API). All three were found to be linked to non-authorized use of coated maize seed. The Lombardy case concerned a bee-keeper from the province of Varese who reported an abnormal die-off on 5th May 2009, subsequent to maize sowing on plots close to the beehives. Analyses conducted on dead bees did not reveal the presence of residues, whereas analyses of maize plantlets detected the presence of 6000 ppb of clothianidin. The other two cases will be described in the following sections.

During the spring of 2009 a further 7 samples linked to reports were sent to the Veterinary Services. Of these, 5 were found to be positive for neonicotinoids, and the event itself was found to be caused by improper use of neonicotinoid-based products sprayed on orchards. For the other 2, no presence of residues was detected by the tests.

1.2 The Emergency Action Team

An Emergency Action Team (SPI) was set up in connection with the reporting system. The Team intervenes directly at the location where damage affecting bees is reported by a bee-keeper. The Team then proceeds to gather information and collect samples for the laboratory analyses. It is important to note that emergency action is undertaken whenever the reported event is judged, on the basis of an interview with the bee-keeper, to be of unknown origin. During 2009 particular attention was paid to cases occurring concomitantly with maize sowing.

The Emergency Action Team is composed of an expert from CRA-API or from DiSTA - University of Bologna and a skilled technician with expertise in bee health, together with additional support figures if necessary. Among the emergency interventions conducted in early 2009, the two concerning bee die-off during maize sowing are described in detail here below.

In the first case an official on-site investigation was performed on 22nd March 2009 in Ozzano Emilia (Province of Bologna) where, a few days after maize sowing, a 39-hive apiary situated on the border of a maize field was found to have been severely damaged. The bee population in the hives proved to have been reduced by roughly 50%; many of the bees had presumably died in the field, while others were found dead in front of the hives. The hives showed low or nil levels of flying activity and the live bees displayed nervous spasms, lack of energy, lethargy, disorientation, tremulous and very slow movements, all of which were symptoms consistent with the effects of neonicotinoid poisoning reported in the literature. Such symptoms were still evident at the subsequent on-site inspection, carried out 6 days later at the new station to which the apiary had immediately been moved.

Analyses conducted by the Animal Disease Prevention Institute of North-East Italy on the maize seeds and plantlets gathered in the field detected the presence of residues of 3 different neonicotinoids, at different concentrations (in seeds: imidacloprid 30 ng/g, thiamethoxam 320 ng/g, clothianidin 60 ng/g; in plantlets: clothianidin 2.900 ng/n). Analyses on wildflowers and vegetation bordering around the field, carried out at CRA-API, detected the presence of imidacloprid (470 ng/g) and thiamethoxam (1.060 ng/g) on flowers of *Veronica* sp.. Thus the tests demonstrated that damage inflicted on the hives was caused by non authorized utilization of maize seed coated with neonicotinoids (probably mixed batches of seeds remaining from the previous season, coated with different active ingredients).

In the second case, the report came from a bee-keeper in the province of Reggio Emilia and concerned a permanent apiary of 20 hives located close to a maize field (at a distance of 800 metres). Elevated mortality and low levels of flying activity were observed in the hives, as well as abnormal adult bee behavior such as aggression and nervous spasms. Analysis on dead bees conducted at CRA-API revealed 54 ng/g of clothianidin, suggesting illegal utilization of seed coated with this neonicotinoid.

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

The research line involves:

- dustiness measurement of maize seed coated with the 4 main active ingredients under investigation;
- quantification of dust and active ingredients deposited on soil and dispersed in the air during sowing with a modified or unmodified pneumatic seed drill;
- evaluation of the productive and agronomic utility of maize seed coating;
- evaluation of persistence of active ingredients in soil and their translocation into different plant parts.

Following the meeting held on 9th February 2009 beween the Apenet project researchers and the representatives of the association of crop protection product companies (Agrofarma-Federchimica), of the agricultural machine manufacturers unions, confederations and institutions (UNACOMA, UNIMA, ENAMA, CONFAI) and of the seed production companies, it was decided that:

a) coated seed should respect a dust threshold not exceeding 3g/100 kg, measured according to the Heubach method (ESA 09.125.1 method);
b) seeds drill tests should utilize the most widespread pneumatic seed drill model, subsequently identified by UNACOMA and UNIMA as a six-row Matermacc model;
c) the seed drill should be equipped with a dual pipe deflector dust reduction system, and a comparison between the modified and unmodified seed drill should be conducted.

These decisions were then illustrated and ratified in the subsequent meeting convened by the Ministry of Agriculture (MIPAAF) on the same date, in the presence of representatives of the Ministries of Health and Environment and the Trade Associations.

It is important to note, with regard to the seed drill delivered to the Agricultural Engineering Research Unit of the Agricultural Research Council (CRA-ING) by MaterMacc Srl on 2nd March 2009, that the selected model was set for seeding at an inter-row spacing of 45 cm and was not modifiable.

2.1. Seed dustiness test

The results of the seed dustiness test performed with the Heubach drum method are given in Table 2, where a comparison with the results obtained in the manufacturer's test performed within 48 h after seed coating is also shown.

The quantity of fine dust, i.e. that which is trapped in the Heubach filter and on which the evaluation is performed, was found to be higher than the value declared by the manufacturer, although levels remained below the established limit of 3 g/100 Kg.

In addition to fine dust, emission of elevated quantities of coarse dust was also observed. The coarse dust, which was not intercepted by the filter instrument, constituted roughly 90% of the total extracted dust.

Seed coating	Manufacturer's declared dustiness (g/100 Kg)	Dustiness detected by CRA-ING (g/100 Kg)		
	Fine dust	Fine dust	Coarse dust	Total dust
Gaucho (imidacloprid	0.9600	1.6664	14.9975	16.6639
Poncho (clothianidin)	1.7700	2.1668	33.3358	35.5026
Cruiser (thiamethoxam)	1.3300	2.4999	16.6658	19.1657
Regent (fipronil)	1.1100	1.6663	18.3291	19.9953

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

2.2 Dust drift during sowing

Field tests carried out by CRA-ING in the Monterotondo and Tormancina experimental fields involved sequential seeding of experimental plots using maize coated with the 4 active ingredients; sowing was performed with the modified or unmodified seed drill. Trials were planned to begin in mid March 2009, which is the maize sowing time generally recommended in many Italian regions. However, due to heavy rainfall, access to the fields was not feasible before mid April and sowing began only in early May. Despite the late start, field tests were completed for all 4 active ingredients with the modified seed drill, and for imidacloprid, clothianidin and thiamethoxam with the unmodified seeder. Each trial plot was split into three sub-plots (repetitions), which were seeded on the same day. Sampling of soil-dispersed dust was carried out by means of a series of Petri dishes filled with a 50% acetonitrile/water solution, which fixes the active ingredients present in dust. Dishes were placed in each plot at increasing distances (5, 10, 20, 30, 50 m) from the sowing area, according to the diagram shown in Figure 2. The mean active ingredient concentration per surface unit in the Petri dishes was then calculated. Three Petri dishes were placed at each distance in each of the 3 repetitions; thus the final value of dust dispersion derives from the mean of 9 values for each of the distances. In addition, for each test the following environmental parameters were measured: temperature, relative humidity, wind speed and mean hourly solar radiation. The environmental conditions referring to days in which seeding was carried out with the different active ingredients are shown in Table 3.

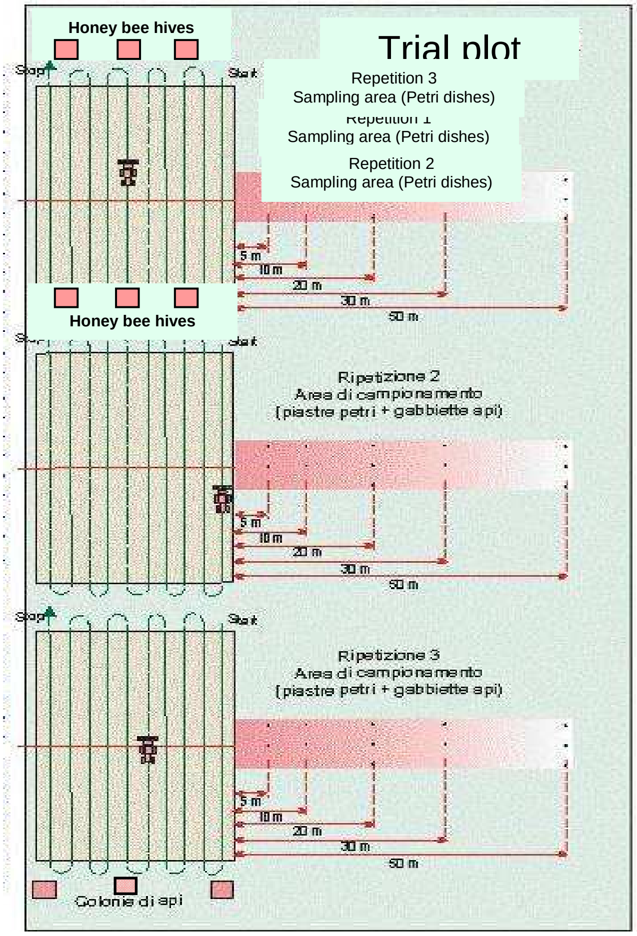

Figure 2 – Diagram of the dust drift field tests. For sowing of each active ingredient, a given plot was split into three plots. For each plot, a series of Petri dishes for dust trapping were placed at increasing distances from the sowing area (5, 10, 20, 30, 50 m). Six hives were placed around the plot, three on each side.

Active ingredient	Deflector (yes/no)	Sowing date	T (°C)	R. H. (%)	Wind speed (m/sec)	Mean hourly solar radiation (KJ/m^2)
imidacloprid	yes	16/04/09	18.74	47.20	3.07	-
clothianidin		04/05/09	15.80	75.20	0.93	2918.50
thiamethoxam		12/05/09	25.36	40.00	1.28	3407.00
fipronil		20/05/09	29.54	36.80	1.60	3606.80
imidacloprid	no	04/06/09	26.74	73.00	3.90	3277.80
clothianidin		11/06/09	27.40	33.00	2.52	3640.40
thiamethoxam		18/06/09	30.90	39.40	1.80	3696.00
fipronil		-	-	-	-	-

Table 3 - Mean environmental data recorded during the days of sowing (source UCEA).

Analysis of residues, performed by the Plant Pathology Research Centre of the Agricultural Research Council (CRA-PAV) in Rome, was carried out by means of liquid chromatography/tandem triple-quadrupole mass spectrometry (tandem HPLC-MS MS) using Waters 4 micro instrumentation, interface ESI POS and ESI NEG in MRM modality. All methods were validated according to standard CLP procedures. Complete results of analyses on Petri dishes were obtained for all active ingredients involved in the tests carried out with the modified seed drill. With regard to the unmodified seed drill tests, data on fipronil were not available at the time of writing. The results of ground-level dispersion of active ingredients are summarized in Figures 3a and 3b.

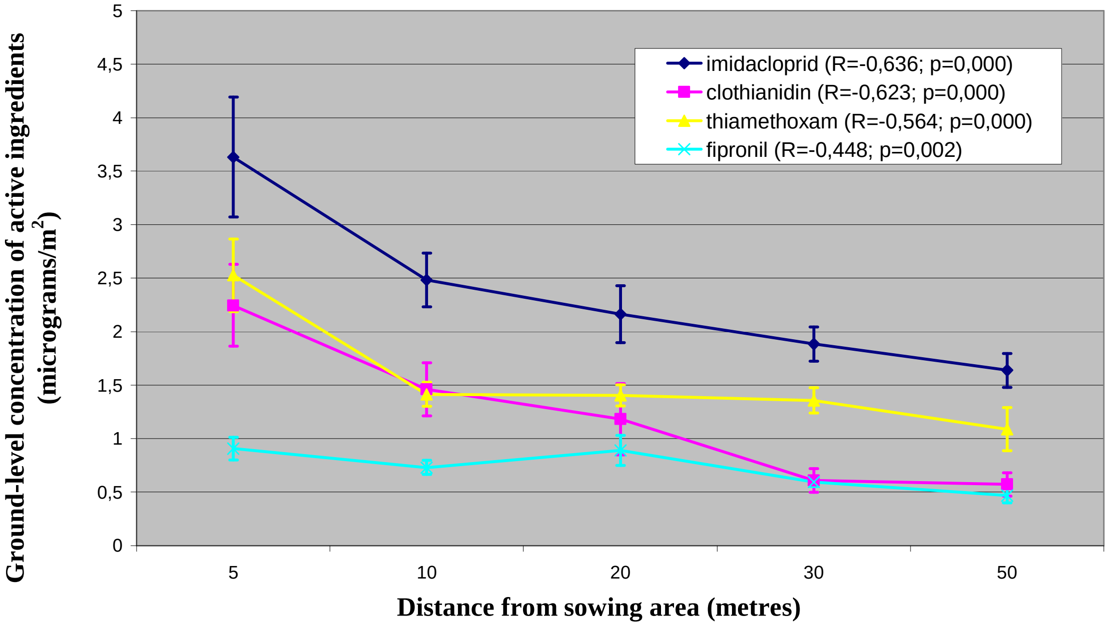

Figure 3a – Ground-level dispersion of the four active ingredients during sowing with the modified seed drill. Each point on the graph represents mean ± standard error for n=9. Differences between distances were statistically significant for all active ingredients (Spearman's rank correlation coefficient).

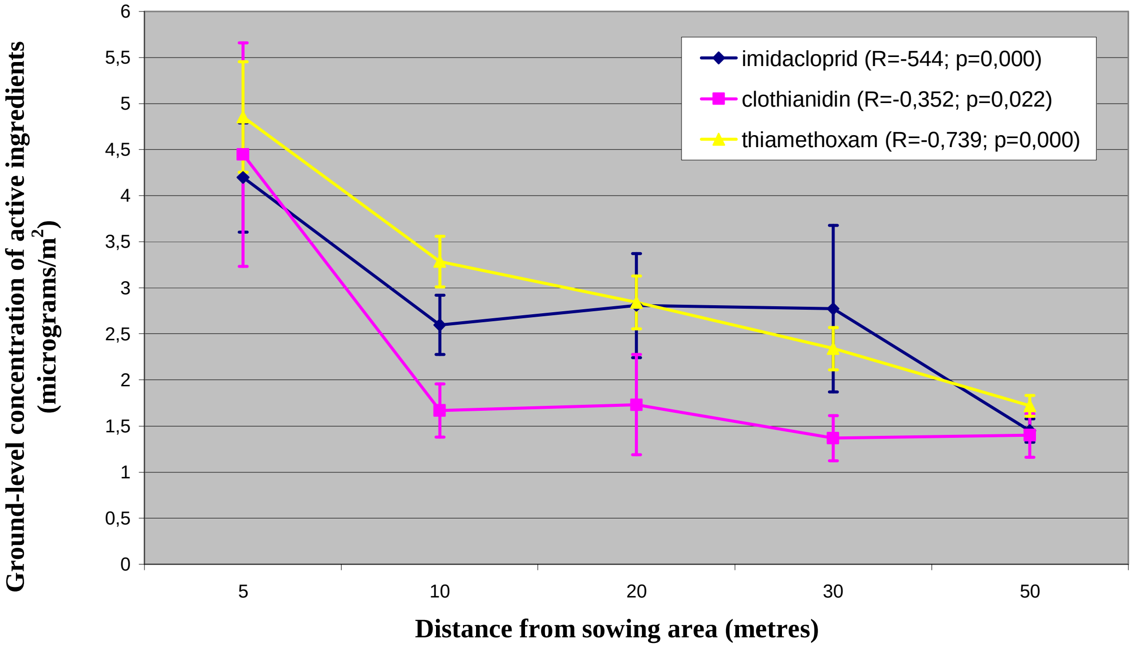

Figure 3b – Ground-level dispersion of the four active ingredients during sowing with the unmodified seed drill. Each point on the graph represents mean ± standard error for n=9. Differences between distances were statistically significant for all active ingredients (Spearman's rank correlation coefficient).

In all trials, both with the modified and unmodified seed drill, ground-level concentration of the active ingredient was found to decrease with increasing distance from the edge of the seeded plot. This decrease was statistically significant for all active ingredients tested (Spearman's rank correlation test).

The values recorded and the abatement percentages obtained with the deflector are listed, for 3 of the 4 active ingredients, in Tables 4a, 4b and 4c. Application of the dual pipe deflector modification to the seed drill led to highly variable abatement percentages, as a function of the active ingredient involved.

Distance from sowing point (metres)	Ground-level concentration of **imidacloprid** (µg/m^2) (mean ± standard error)		Abatement percentage	p (Mann-Whitney's U test)
	Unmodified seed drill	Modified seed drill		
5	4.20±0.59	3.63±0.56	13%	0.344563
10	2.60±0.32	2.48±0.25	4%	0.964784
20	2.81±0.56	2.16±0.27	23%	0.630428
30	2.77±0.90	1.89±0.16	32%	0.791082
50	1.45±0.13	1.64±0.16	-13%	0.452913

Table 4a – Comparison between ground-level concentrations of the active ingredient imidacloprid resulting from use of the modified versus the unmodified seed drill (mean ± standard error, n=9). Differences between the two treatments were not statistically significant for any of the distances (Mann-Whitney's U test).

Distance from sowing point (metres)	Ground-level concentration of **clothianidin** (µg/m^2) (mean ± standard error)		Abatement percentage	p (Mann-Whitney's U test)
	Unmodified seed drill	Modified seed drill		
5	4.45±1.21	2.25±0.38	49%	0.665
10	1.67±0.29	1.46±0.25	12%	0.791
20	1.73±0.54	1.18±0.33	32%	0.402
30	1.37±0.25	0.61±0.11	56%	0.018
50	1.40±0.24	0.57±0.11	59%	0.005

Table 4b – Comparison between ground-level concentrations of the active ingredient clothianidin resulting from use of the modified versus the unmodified seed drill (mean ± standard error, n=9). Differences between the two treatments were statistically significant (p values in red) for the distances of 30 an 50 metres (Mann-Whitney's U test).

Distance from sowing point (metres)	Ground-level concentration of **thiamethoxam** (µg/m^2) (mean ± standard error)		Abatement percentage	p (Mann-Whitney's U test)
	Unmodified seed drill	Modified seed drill		
5	4.85±0.60	2.53±0.34	48%	0.005
10	3.29±0.28	1.42±0.11	57%	0.000
20	2.84±0.29	1.40±0.10	51%	0.000
30	2.34±0.23	1.36±0.12	42%	0.004
50	1.72±0.11	1.09±0.20	37%	0.017

Table 4c – Comparison between ground-level concentrations of the active ingredient thiamethoxam resulting from use of the modified versus the unmodified seed drill (mean ± standard error, n=9). Differences between the two treatments were statistically significant (p values in red) for all distances (Mann-Whitney's U test).

Dust drift during sowing was also measured by samplers composed of aspirator pumps equipped with a 45 mm fluoropore membrane filter. Each of the pumps, positioned at distances of 5 and 10 m, at a height of 1700 mm from the ground, had a sampling capacity of 100 L of air. Calculations based on the air samples thereby obtained showed the concentration of the four active ingredients deriving from sowing with the modified seed drill. The results are shown in Figures 4a and 4b. Differences in concentration of dust trapped by the samplers at the two distances were statistically significant only for imidacloprid in sowing with the modified seed drill (Mann-Whitney's U test, p values in the figure).

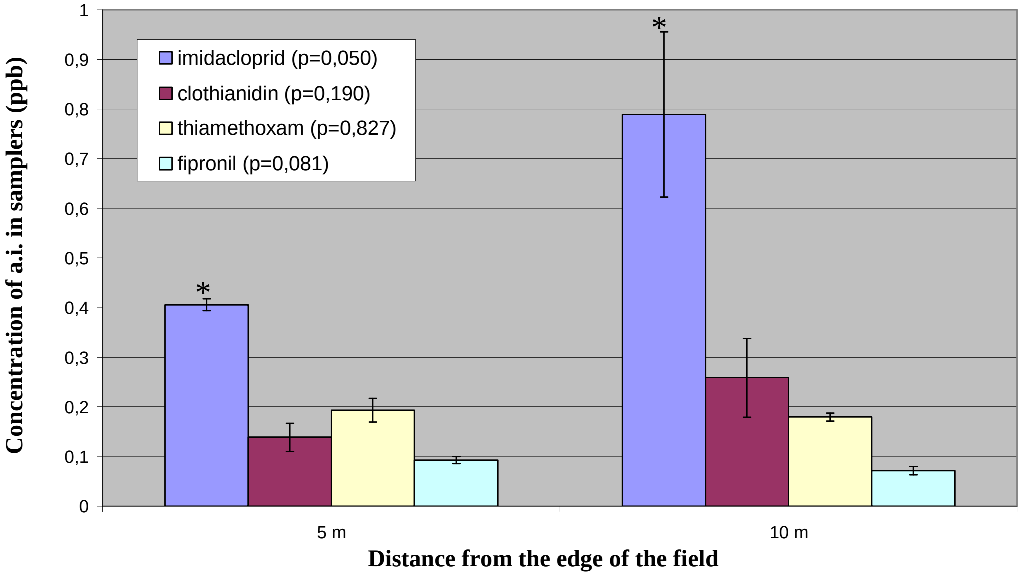

Figure 4a – Air concentration of dust from the 4 active ingredients, as detected by samplers at 5 and 10 metres from the plot seeded with the modified seed drill. Bars represent mean ± standard error, for n=3. Bars marked with an asterisk indicate statistically significant differences between the 2 distances (Mann-Whitney's U test).

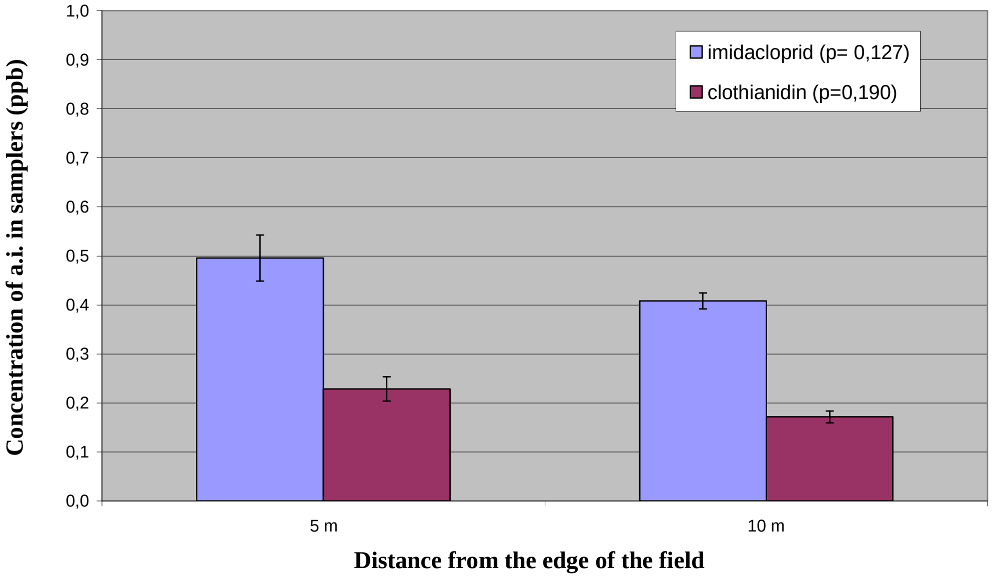

Figure 4b – Air concentration of dust from two of the 4 active ingredients, as detected by samplers at 5 and 10 metres from the plot seeded with the unmodified seed drill. Bars represent mean ± standard error, for n=3. The difference in concentration between the 2 distances was not statistically significant (Mann-Whitney's U test).

For imidacloprid and clothianidin, the comparison of air dust concentration after sowing with the modified or unmodified seed drill is shown in Tables 5a and 5b. For both distances, differences in concentration of dust emitted in sowing with the modified and unmodified seed drill were borderline statistically significant for $p<0.05$ (Mann-Whitney's U test, p values in the table).

Distance from sowing point (metres)	Air concentration of **imidacloprid** (ppb) (mean ± standard error)		Abatement percentage	p (Mann-Whitney's U test)
	Unmodified seed drill	Modified seed drill		
5	0.50±0.05	0.41±0.01	18.13%	0.050
10	0.41±0.02	0.79±0.17	-93.31%	0.050

Table 5a - Comparison of air concentration of imidacloprid after sowing with the modified or unmodified seed drill (mean ± standard error, n=3). Differences between the two treatments were borderline statistically significant for $p<0.05$ (Mann-Whitney's U test).

Distance from sowing point (metres)	Air concentration of **clothianidin** (ppb) (mean ± standard error)		Abatement percentage	p (Mann-Whitney's U test)
	Unmodified seed drill	Modified seed drill		
5	0.2286	0.1388	39.29	0.050
10	0.1714	0.2585	-5.79	0.275

Table 5b - Comparison of air concentration of clothianidin after sowing with the modified or unmodified seed drill (mean ± standard error, n=3). Differences between the two treatments were borderline statistically significant at 5 metres and non significant at 10 metres for $p<0.05$ (Mann-Whitney's U test).

2.3 Assessment of effects on bees caused by dust drift during sowing

To assess the effects induced in bees by dust drift during sowing, six hives were positioned on the edge of the trial field during seeding of the three plots (see diagram in Figure 2). Colony vitality parameters (mortality and colony strength) were evaluated up to three weeks after sowing.

Dead bees were collected in purpose-placed underbasket cages, counted and, in the case of elevated mortality, taken to the laboratory for analysis. Pollen, collected by means of purpose-designed traps mounted on the hives, was submitted to analyses which were in progress at the time of writing. Control hives were maintained in the same conditions and the same environment, but at an appropriate distance from the seeded trial plots.

Mean mortality rates of bees collected in the underbasket cages after sowing are shown in Figures 5, 6, 7 and 8. Statistically significant differences are marked by an asterisk (Mann-Whitney's U test).

Dead bees removed from two hives 4 days after sowing with imidacloprid-coated seed were subjected to analyses. The quantity of active ingredient detected was 0.04 ng/bee and 0.14 ng/bee respectively.

Dead bees removed from two hives the day after sowing with clothianidin-coated seed were found to have active ingredient levels of 0,02 ng/bee and 0,07 ng/bee respectively.

Analyses of dead bees removed from colonies exposed to sowing with thiamethoxam-coated and fipronil-coated seed were in progress at the time of writing.

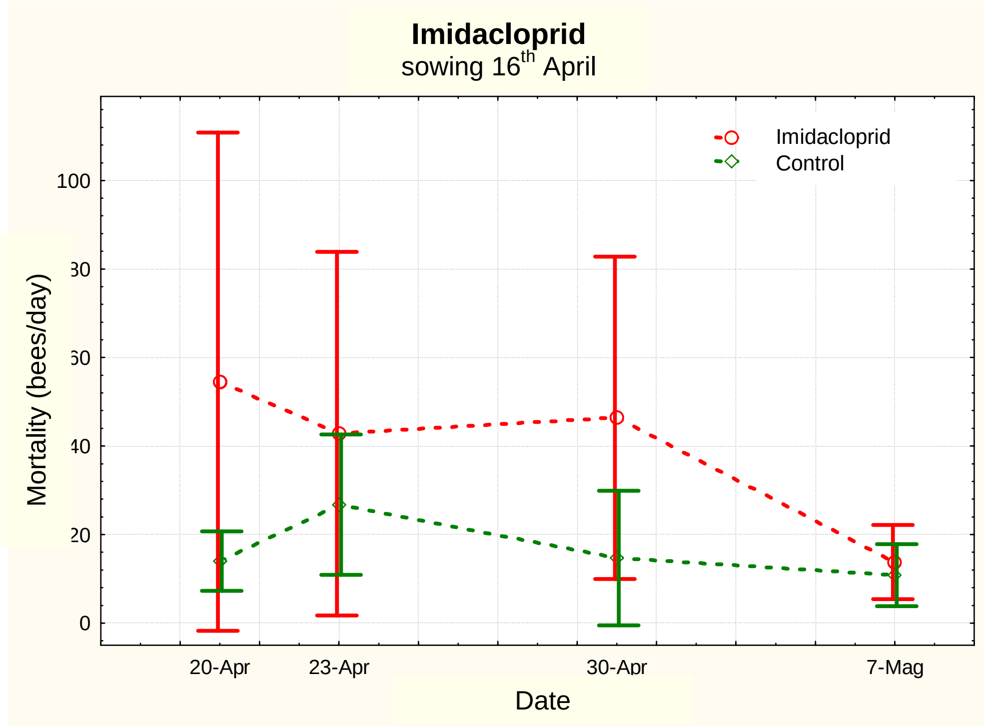

Figure 5 – Mean mortality (± standard error) of bees collected in underbasket cages of hives exposed to sowing of imidacloprid-coated seed versus control hives. No statistically significant differences were observed (Mann-Whitney's U test; $p<0.05$).

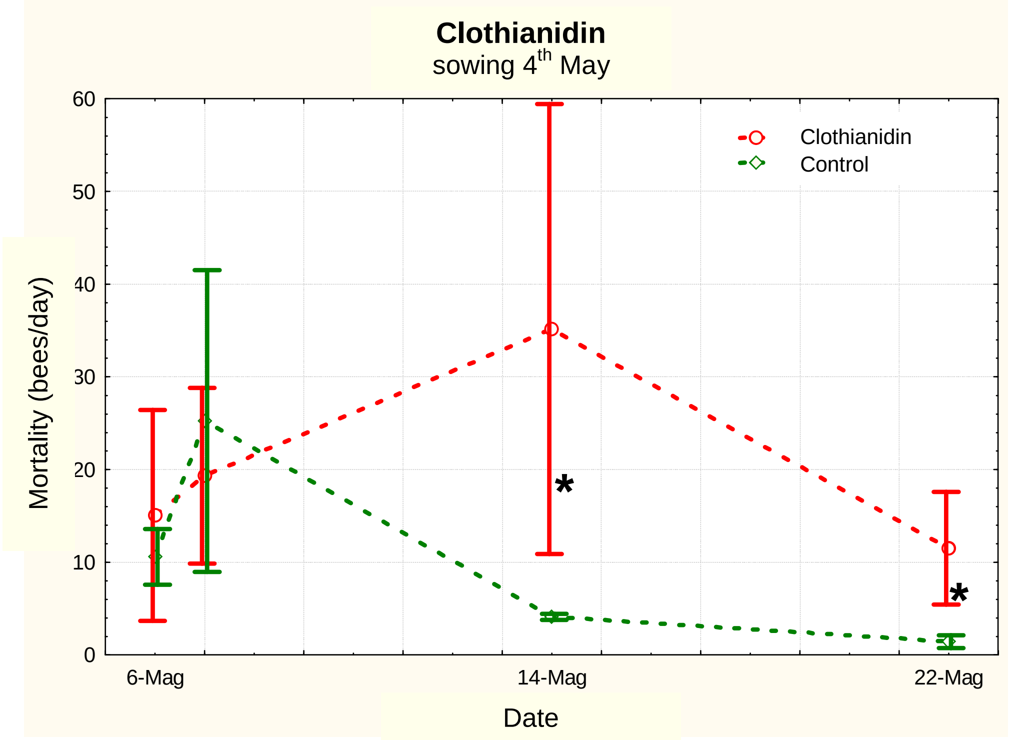

Figure 6 - Mean mortality (± standard error) of bees collected in underbasket cages of hives exposed to sowing of clothianidin-coated seed versus control hives. Values marked with an asterisk indicate statistically significant differences (Mann-Whitney's U test , $p<0.05$).

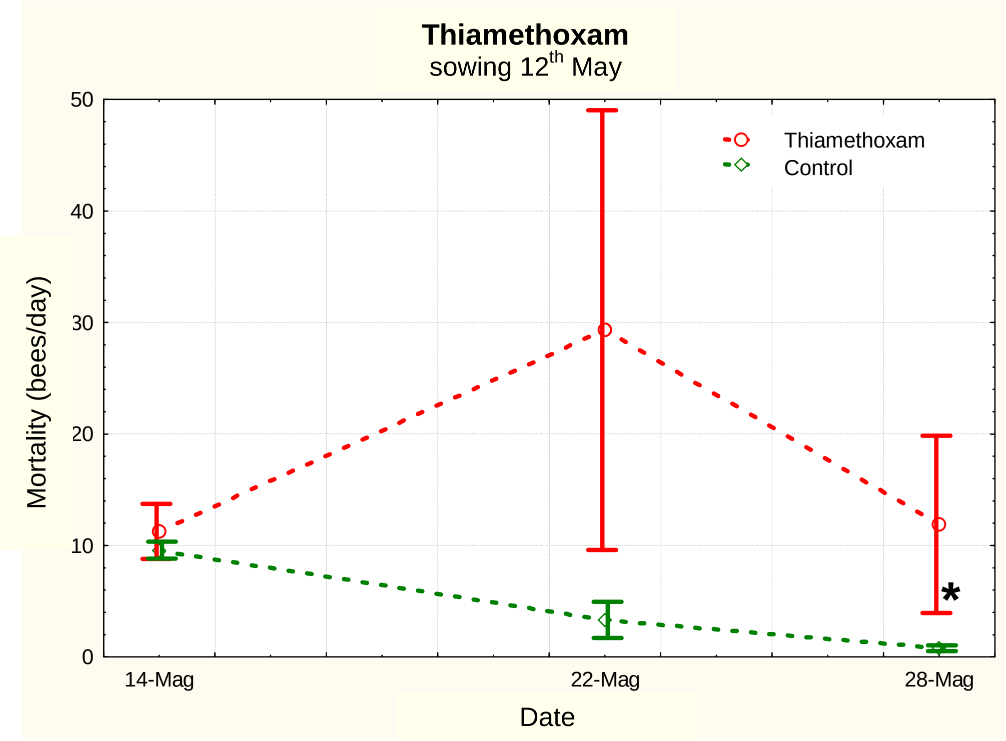

Figure 7 - Mean mortality (± standard error) of bees collected in underbasket cages of hives exposed to sowing of thiamethoxam-coated seed versus control hives. Values marked with an asterisk indicate statistically significant differences (Mann-Whitney's U test; $p<0.05$).

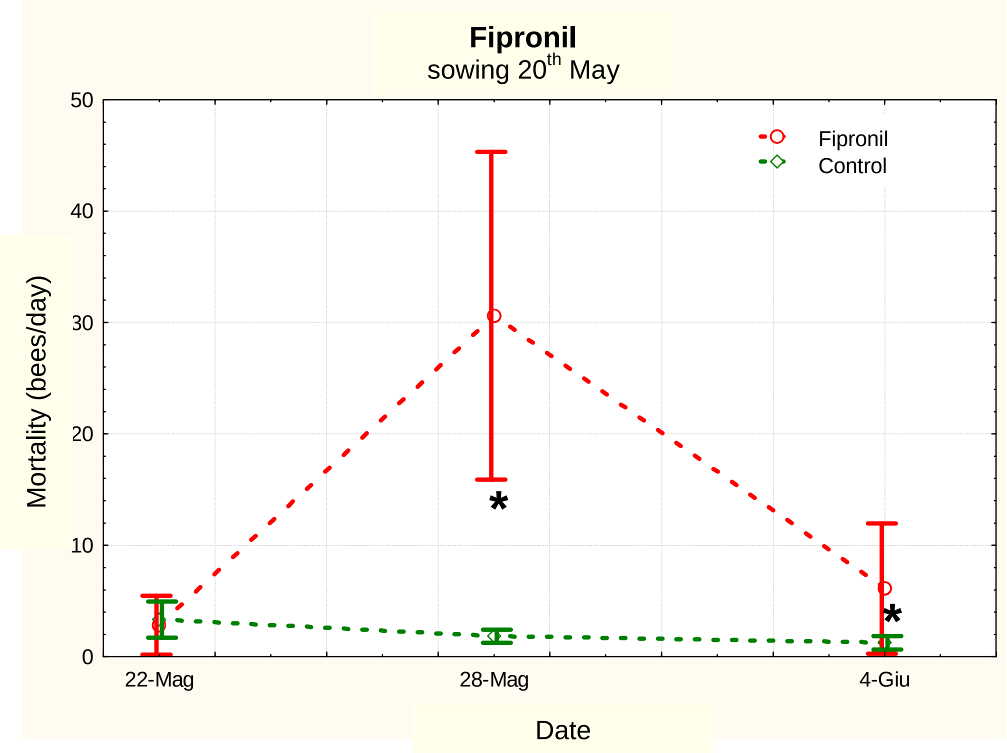

Figure 8 - Mean mortality (± standard error) of bees collected in underbasket cages of hives exposed to sowing of fipronil-coated seed versus control hives. Values marked with an asterisk indicate statistically significant differences (Mann-Whitney's U test, $p<0.05$).

The results on colony vitality, evaluated in terms of thousands of bees and brood cells before and after sowing, are given in Table 6.

The difference between pre-sowing and post-sowing number of brood cells in hives exposed to thiamethoxam drift versus control hives was found to be statistically significant (ANOVA for repeated measures, p<0.05).

	Thousands of bees			Thousands of brood cells		
	Prior to sowing	7 days after sowing	Percent variation	Prior to sowing	7 days after sowing	Percent variation
Imidacloprid	14.63±0.63	15.33±0.69	+4.78	40.53±3.54	42.00±1.33	+3.63
Control	15.94±0.30	16.75±0.31	+5.08	39.20±1.03	41.40±2.45	+5.61
			p=0.276			p=0.961
Clothianidin	19.96±1.43	18.17±1.06	-8.97	42.67±4.10	48.93±3.55	+14.67
Control	16.56±0.60	18.56±0.19	+12.08	45.00±2.52	40.00±2.83	-11.11
			p=0.085			p=0.290
Thiamethoxam	16.06±1.84	18.21±1.49	+13.39	33.47±3.09	33.00±3.28	-1.40
Control	18.56±0.19	23.13±1.20	+24.62	40.00±2.83	58.00±3.83	+45.00
			p=0.052			p=0.008*
Fipronil	22.46±1.37	22.71±1.39	+1.11	51.47±2.15	56.67±2.17	+10.10
Control	23.13±2.15	25.13±2.17	+8.65	58.00±3.83	58.00±1.15	0.00
			p=0.483			p=0.372

Table 6 - Vitality of colonies, evaluated in terms of thousands of bees and thousands of brood cells, prior to sowing and one week after sowing. The table shows mean ± standard error (n=6) and percent variation between before and after sowing. Values marked by an asterisk indicate a significant difference between exposure to the active ingredient versus controls (ANOVA for repeated measures, p values in the table).

2.4 Estimate of the productive and agronomic utility of maize seed coating

Trials were undertaken by the Maize Research Unit of the Agricultural Research Council (CRA-MAC) in Bergamo in order to compare the yield of maize crops deriving from seed treated with fungicide alone (Celest) versus maize crops deriving from fungicide-coated seed that is additionally coated with the 4 active ingredients forming the object of the present study (imidacloprid, clothianidin, thiamethoxam and fipronil).

Accordingly, within the framework of the Network of Agronomic-Varietal Experimentation in the Italian environment, 17 localities were chosen, distributed mainly in traditionally maize-growing areas (Lombardy, Piedmont, Veneto, Friuli, Emilia Romagna) and in Tuscany. The distribution of the localities is shown in Table 7.

In each locality, 30 m^2-plots were set up (length 10-12 m, 4 rows). Plots were sown with seeds prepared from a homogeneous lot of a commercial maize hybrid (PR31N27- FAO 700) provided by AIS (Italian Seed Association) and coated either with the four active ingredients plus the fungicide, or with the fungicide alone (control) (Table 8). The 5 treatments were replicated 4 times in each locality.

Following the infestation of maize crops with the Western Corn Rootworm (*Diabrotica virgifera virgifera*) in various parts of Italy during the 2009 productive season, CRA-MAC carried out specific surveys in the Network of Agronomic-Varietal Experimentation localities to verify the relationship between the lack of seed coating with neonicotinoids and the Western Corn Rootworm infestation. The results of these surveys are reported in Table 9.

Region	Localities	Sowing date	Harvest date
Lombardy	Bergamo	07/04/2009	18/09/2009
	S.Angelo Lodigiano (LO)	15/04/2009	28/09/2009
	Luignano (CR)	12/05/2009	28/09/2009
	Caleppio di Settala (MI)	9/05/2009	29/09/2009
	Castenedolo (BS)	24/04/2009	23/09/2009
	Pudiano (BS)	25/04/2009	24/09/2009
Piedmont	Vigone (TO)	15/04/2009	2/10/2009
	Chivasso (TO)	10/04/2009	28/09/2009
	Castelceriolo (AL)	07/05/2009	7/10/2009
	Cigliano (VC)	8/04/2009	24/09/2009
Veneto	Castelnovo Bariano (RO)	14/04/2009	2/09/2009
	Villadose (RO)	12/05/2009	12/09/2009
Emilia Romagna	Ambrogio (FE)	17/04/2009	10/09/2009
	Fognano (PR)	17/04/2009	24/09/2009
Friuli	Mortegliano (UD)	08/04/2009	11/09/2009
	Palazzolo della Stella (UD)	06/05/2009	9/10/2009
Tuscany	Marciano della Chiana (AR)	12/05/2009	26/10/2009

Table 7 - List of the 17 localities in which trial plots were set up.

Group	Fungicide	Insecticide (active ingredient)
1 - CONTROL	Celest*	none
2 - Cruiser	Celest*	thiamethoxam
3 - Gaucho	Celest*	imidacloprid
4 - Poncho	Celest*	clothianidin
5 - Regent	Celest*	fipronil

Table 8 – Kind of seed treatment in the 5 experimental groups.
* The fungicide Celest contains fludioxonil and metalaxyl.

None of the typical symptoms of Western Corn Rootworm, such as “goose-necking” or lodging of more mature plants (root damage caused by larvae) or incompletely filled ears (damage caused by adults clipping corn silks) were observed for any of the experimental groups in any locality (Table 9). No clear differences in agronomic performance were observed among any of the experimental groups.

The presence of adult beetles caused silk clipping but to an extent that did not appear to hinder development of the ear. Damage to the roots was not measured directly because non of the plants showed signs of lodging. At the moment of survey no noticeable differences in kind and level of damage among the 5 groups were recorded.

It must be noted that Western Corn Rootworm was present in the experimental plots during the surveys, although in vast maize growing areas in the provinces of Cremona and Brescia (Lombardy), including the experimental fields part of the Network of Agronomic-Varietal Experimentation, specific treatments against the adult forms were carried out between mid-June and mid-July. All the agronomic performance trials in Lombardy are monoculture maize out of rotation.

In Piedmont no adulticide treatment was performed for any of the agronomic performance trials.

In Tuscany neither juvenile nor adult forms of Western Corn Rootworm were observed.

Region	Locality	Survey date	Noticeable differences among groups	% lodged plants	% incompletely filled ears
Lombardia	Bergamo	17/07/2009	NONE	0	0
	S.Angelo Lodigiano (LO)	28/07/2009	NONE	0	0
	Luignano (CR)	29/07/2009	NONE	0	0
	Caleppio di Settala (MI)	28/08/2009	NONE	0	0
	Castenedolo (BS)	20/07/2009	NONE	0	0
	Pudiano (BS)	22/07/2009	NONE	0	0
Piemonte	Vigone (TO)	23/07/2009	NONE	0	0
	Chivasso (TO)	23/07/2009	NONE	0	0
	Castelceriolo (AL)	30/07/2009	NONE	0	0
	Cigliano (VC)	30/07/2009	NONE	0	0
Veneto	Castelnovo Bariano (RO)	31/07/2009	NONE	0	0
	Villadose (RO)	10/08/2009	NONE	0	0
Emilia Romagna	Ambrogio (FE)	08/09/2009	NONE	0	0
	Fognano (PR)	06/08/2009	NONE	0	0
Friuli	Mortegliano (UD)	05/08/2009	NONE	0	0
	Palazzolo della Stella (UD)	05/08/2009	NONE	0	0
Toscana	Marciano della Chiana (AR)	11/09/2009	NONE	0	0

Table 9- Symptoms of Western Corn Rootworm infestation in the 17 chosen localities of the Network of Agronomic-Varietal Experimentation.

For each of the experimental groups, the following observations and the standard agronomic measurements were performed on samples of the crop at different phenologic stages:

- grain humidity (%)
- yield (t/ha)
- hectolitric weight
- plant height
- ear height
- percentage plants with split stalk
- percentage lodged plants

The data from the agronomic performance trials are summarised in Table 10. Statistical analysis performed with ANOVA showed that there are no significant differences among groups for the measured parameters.

In some of the network localities maize soil insect (Wireworms) risk maps were drawn up, in collaboration with DiSTA - University of Bologna, the Department for Valorisation and Protection of Agroforestry Resources (Di.Va.P.R.A.) of the University of Turin and the Department of Environmental Agronomy and Plant Production-Entomology of the University of Padua. The collected data show variability between localities for presence of adult forms of *Agriotes brevis, Agriotes sordidus, Agriotes litigiosus* (Table 11).

The average yield data for each experimental group, in the 5 locations where the agronomic performance were accompanied by the Wireworm survey, are reported in Table 12. No significant differences among experimental groups were observed within any of the test locations.

Group	Insecticide	Yield (t/ha-15.5%u.r.)	Grain humidity (u.r. %)	Hectolitric weight (kg/hl)	Plant height (cm)	Ear hight (cm)	% Plants with split stalk	% Lodged plants
1 - Control	none	13.541	22.3	75	268	119	4.44	0.06
2 - Cruiser	thiamethoxam	13.245	22.1	75	269	121	3.80	0.08
3 - Gaucho	imidacloprid	13.373	22.1	75	267	121	5.25	0.19
4 - Poncho	clothianidin	13.667	22.1	75	271	121	5.28	0.06
5 - Regent	fipronil	13.379	22.3	75	268	123	4.19	0.06

Table 10 - Mean data from 17 agronomic performance trials (Network of Agronomic-Varietal Experimentation)

Region	Locality	Adults (survey: total/trap per site)			Captured larvae
		Agriotes brevis	*Agriotes sordidus*	*Agriotes litigiosus*	
Lombardy	Bergamo	54.5	1.5	0	0
Piedmont	Vigone (TO)	N.D.	512.5	524	N.D.
Veneto	Castelnovo Bariano (RO)	0	137	21	N.D.
	Villadose (RO)	120	1613	116	N.D.
Tuscany	Marciano della Chiana (AR)	0	0.5	451	N.D.

Table 11- Data from 2009 Wireworm survey in 5 agronomic performance localities.
N.D.: not determined

Region	Locality	YIELD (t/ha-15.5%U.R.)				
		1 - Control	**2 – Cruiser** thiamethoxam	**3 – Gaucho** imidacloprid	**4 – Poncho** clothianidin	**5 – Regent** fipronil
Lombardia	Bergamo	15.320	15.703	15.508	15.588	16.250
Piemonte	Vigone (TO)	16.845	17.055	17.103	17.380	17.210
Veneto	Castelnovo Bariano (RO)	15.283	15.370	14.648	14.613	15.105
	Villadose (RO)	10.085	10.055	10.423	10.068	10.238
Toscana	Marciano della Chiana (AR)	12.239	11.283	11.876	9.936	12.063

Table 12 – Yield data in the 5 agronomic performance trials (Network of Agronomic-Varietal Experimentation) in which the 2009 Wireworm survey was carried out.

Finally, a study was performed to evaluate the extent to which the active ingredients used for seed coating persist in the various stages of maize plant development. Tests were thus undertaken to analyse residues of the four active ingredients in plots sown with maize and in different plant tissues (leaves, roots, stalk, male and female inflorescences) considered at different plant developmental stages, starting from plants germinated in trial plots or in a controlled environment.

Experimental plots with a length of 50 m were set up at the CRA-MAC experimental farm, in which the same seed used in the agronomic trials was sown, according to the 5 experimental groups described in Table 8. In each experimental plot samples of different plant tissues at different phenological stages were collected, as shown in Table 13.

Sampling date	**Days after sowing**	**Leaf tissue/ phenological stage**
18/06/09	15	1 st-2 nd leaf
18/06/09	15	3 rd leaf
18/06/09	15	4 th leaf
26/06/09	23	5 th leaf
01/07/09	28	6 th leaf
06/07/09	33	7 th leaf
10/07/09	37	8 th leaf
14/07/09	41	9 th leaf
17/07/09	44	10 th leaf
20/07/09	47	11 th leaf
24/07/09	51	12 th leaf
29/07/09	56	13 th leaf
05/08/09	63	14 th leaf
NOTE: 14 th leaf envelops the tassel		
		Male inflorescence
06/08/09	64	Tassel
06/08/09	64	MIX anthers and pollen
07/08/09	65	Only anthers
07/08/09	65	Only pollen
		Female inflorescence
06/08/09	64	Bracts
06/08/09	64	Silks
06/08/09	64	Spikelets
29/09/09	117	Grain

Tabella 13 - Sampling of maize plant tissues.

The information to be obtained from these tests, together with that deriving from the productivity comparisons, is designed to help clarify whether maize seed dressing is genuinely necessary and appropriate in the trial areas.

3. Effect of maize guttation on bees

In plants that are affected by root pressure, the exudation of droplets of fluid from the leaf margins, known as guttation, is frequently noticed. The "dew drops" visible on the leaf tips of grasses in the early morning may actually be guttation droplets. Evidence of guttation is most visible when transpiration is weak and relative humidity is high. Guttations are present in the morning during the spring period; they may be produced and remain on the leaf lamina of maize plantlets for several hours, except in the case of dry wind (bora). In the calyx, guttation may remain throughout the day.

3.1 Active ingredient residues in guttation fluid of container-grown maize plantlets

Analyses were carried out at the Department of Environmental Agronomy and Plant Production-Entomology of the University of Padua, to test for the possible presence of active ingredient residues in leaf guttation fluid and in droplets collected from the calyx of container-sown maize plantlets grown from seed coated with the 4 active ingredients that form the object of the suspension. Results are shown in Tables 14 and 15.

Seed dress	**Active ingredient**	**Concentration a.i. (mg/L)**		
		24 March	26 March	30 March
Gaucho 0.5	imidacloprid	89.16		56.91
Gaucho 1.25	imidacloprid	292.23	345.76	102.91
Poncho	clothianidin	101.72	89.06	76.15
Cruiser	thiamethoxam	16.22	40.85	25.31

Table 14 – Variation in active ingredient concentration in leaf guttation fluid of neonicotinoid-treated maize plantlets, on different days of the week.

Seed dress	**Active ingredient**	**Concentration a.i. (mg/L)**		
		26 March	26 March	30 March
Gaucho 0.5	imidacloprid	134.66		
Gaucho 1.25	imidacloprid	59.17	120.35	8.23
Poncho	clothianidin	46.99	41.50	7.33
Cruiser	thiamethoxam	21.34	25.54	2.93

Table 15 - Variation in active ingredient concentration in guttation droplets collected from the calyx of neonicotinoid-treated maize plantlets, on different days of the week.

Active ingredient concentration in guttation fluid was found to be very elevated for plantlets obtained from seed coated with the 3 neonicotinoids imidacloprid, clothianidin and thiamethoxam. These concentrations were notably above the LD_{50} limit established for these molecules, and also exceeded allowable doses of the same molecules when used as leaf spray. In contrast, presence of the active ingredient fipronil was not detected.

Bees that were submitted to a simple laboratory test in the form of oral ingestion of guttations containing residues of the three above-stated neonicotinoids died within a few seconds. Bees that were given guttation droplets from maize plants grown by sowing fipronil-coated seed remained unaffected.

The complete results concerning tests on active ingredient residues in guttation droplets and their effects on bees have been published in: Girolami V., Mazzon L., Squartini A., Mori N., Marzaro M., Di Bernardo A, Greatti M., Giorio C., Tapparo A,, 2009. Translocation of Neonicotinoid Insecticides From Coated Seeds to Seedling Guttation Drops: A Novel Way of Intoxication for Bees. J. Econ. Entomol. 102 (5): 1808-1815.

3.2 Clothianidin residues in guttation droplets of field-grown maize plants and tests on bee foraging activity

Field trials were conducted at the Experimental Teaching Centre of the Agricultural Faculty of the University of Bologna to test for the presence of residues in guttation fluid of maize grown from clothianidin-coated seed and to assess the extent to which bees may effectively come into contact with the guttation droplets.

Sowing took place on 16th April and guttation droplets were collected from different points of the maize plantlets on 3 dates: 15th May, 21st May and 4th June. Results of analyses of the droplets collected on 15th May are shown in Table 16. Values were found to be markedly lower than those recorded for container-grown plantlet guttations described in the previous section. Analyses of droplets collected on the two following dates, as well as the laboratory tests on bees to determine the effects of guttation, were in progress at the time of writing.

In order to assess bee foraging activity on guttation droplets, the number of bees present on the maize field grown from clothianidin-coated seed was counted, along a transept constituted by a route of three 180 m rows, during the early morning (from 6.30 to 8.00 h) on pre-established days. Bees on the untreated field were analogously counted. The results are listed in Table 17. Along the transepts a total of 3 bees were seen in the coated-seed maize field: one resting on the ground, one in flight and one on the leaves but without collecting guttation droplets.

Origin of guttation droplets	**Clothianidin residues (mg/L)**
Droplets collect from leap tips	0.415
Droplets collected from leaf lateral margins	0.086
Droplets exudated from leaves following breakage	0.128

Table 16 – Clothianidin residues detected in guttation droplets collected from different points of the plant.

Field	**Sampling date**			
	15th May	26th May	29th May	4th June
Coated maize	1	2	0	0
Non coated maize	0	0	0	0

Table 17 – Number of bees observed in the maize field grown from coated and non coated seed.

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

4.1 Sublethal effects: PER test to assay the ability to learn and recognize odors

The Proboscis Extension Reflex (PER) test was utilized to evaluate the extent to which sublethal doses of the molecules utilized for maize seed coating influence learning and memory of odors in adult honey bees.

The test is based on evaluation of the reflex behavior of proboscis (or ligula) extension performed by bees when they perceive environmental stimuli associated with the presence of a sugar source. Neurotoxic insecticides, which bind to the receptors of the neurotransmitter acetylcholine, can negatively affect areas of the brain in charge of learning and memory formation. Imidacloprid and fipronil have already been shown to compromise medium-term and long-term memory respectively.

Trials carried out up to September 2009 in the framework of the Apenet project concerned imidacloprid, clothianidin, fipronil and thiamethoxam, and were designed to assess the effects of the active ingredients both on learning and odor recognition.

After isolating bees in a container in order to leave them free to extend the ligula, each bee was administered the active ingredient diluted at sublethal doses in 3-5 μL of 40% sugar solution.

After 30’ the bees were then trained to extend the ligula in presence of the odor citronellol, subsequently receiving sugar solution as a reward, and not to extend it in presence of the odor of mint, as mint, in contrast to citronellol, was punished by saturated saline solution.

After 60’, 180’ and 24h, the ability to recognize the odor was tested by submitting the bees both to the correct and the incorrect odor (10 times each, with regular alternation of the two odors). The response percentages were recorded, listing % correct responses (extension of the ligula only in presence of the rewarded odor), % partially correct responses (responding to both odors or to neither) and % incorrect responses (extension of the ligula in presence of the wrong odor and failure to respond to the odor rewarded during training).

The sublethal doses assayed up to September 2009 for each active ingredient and the comparison with the corresponding values of LD_{50} are shown in Table 18.

Three hives were used, and each active ingredient and dosage were administered to 10-12 bees per hive, repeating the test 3 times for each hive. Additionally, the same number of untreated bees were assayed for each hive.

Active ingredient	**LD_{50} 48 hours**	**Dose assayed**
clothianidin	4.7 ng/bee	0.92 ng/be
imidacloprid	5 ng/bee	0.2 ng/bee
thiamethoxam	1 ng/bee	0.2 ng/bee
fipronil	6 ng/bee	1.2 ng/bee
		0.03 ng/bee

Table 18 – Active ingredient doses assayed with the PER test

For ease and conciseness of presentation, the data presented below refer to the percentage of correct answers, disregarding the other response categories.

Statistical analysis (two-way ANOVA, considering the active ingredient and the hive as main factors) showed no significant differences in bee behavior between the 3 hives, while the active ingredient induced a significant difference between the 3 treatments under comparison (Fig. 9).

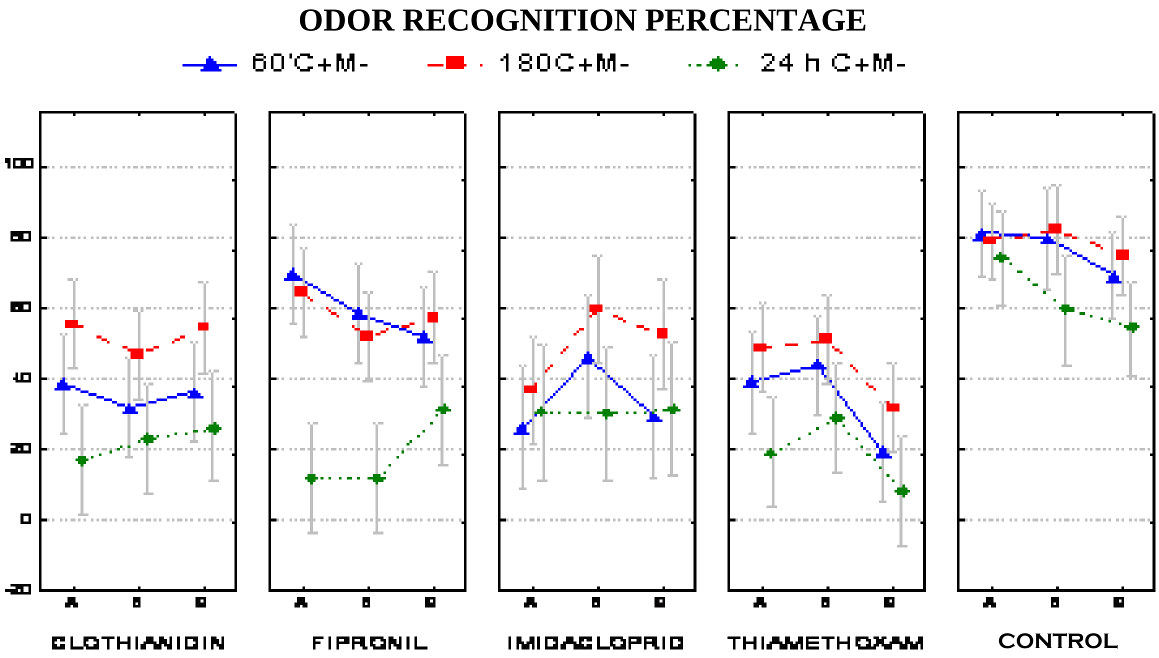

Figure 9 – Effect of interaction between hive and active ingredient on ability to respond correctly to presentation of an odor (citronellol) associated, through preliminary training, with the reward of a sugar solution.

Comparison of the behavioural effects at the different time intervals (two-way ANOVA considering treatment and time as the main factors) in control groups versus each active ingredient-treated group showed a significant reduction, for all active ingredients assayed, in the ability to recognize the odor at all time intervals considered (Figs. 10, 11, 12, 13). For fipronil an additional assay was performed at a much lower dose (0.03 ng/bee corresponding to1/200 of the LD_{50}), which revealed a certain reduction in ability to respond to presentation of the odor, although no clear statistically significant differences were detected due to notable variation among the data obtained (Fig. 14).

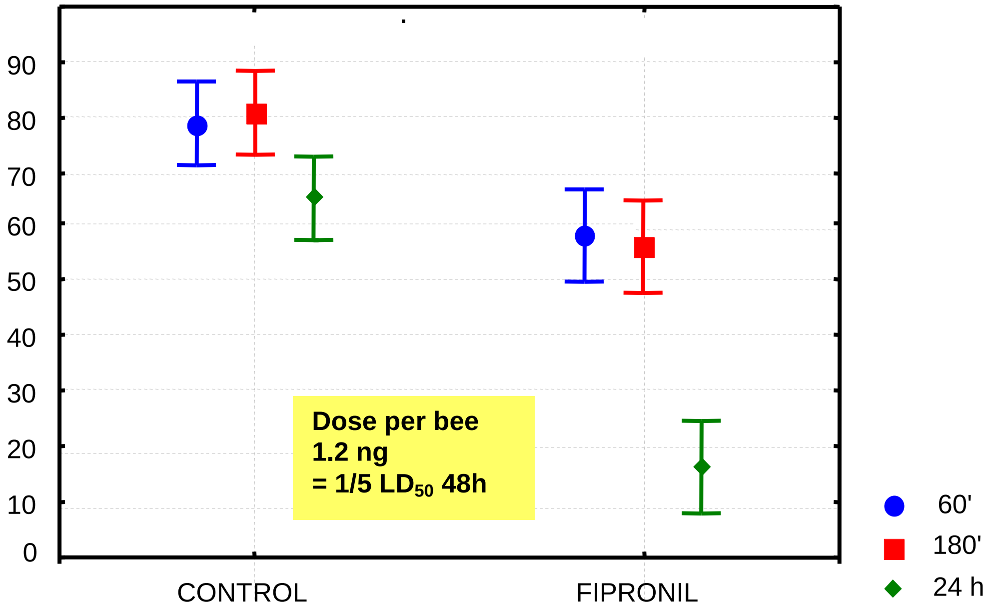

Figure 10 – Percentage of correct responses (extension of ligula in presence of the odor) at different time intervals after treatment (60’-blue, 180’-red, 24h-green), in controls versus the treatment group (fipronil). Pairwise comparison differences were statistically significant for $p<0.001$.

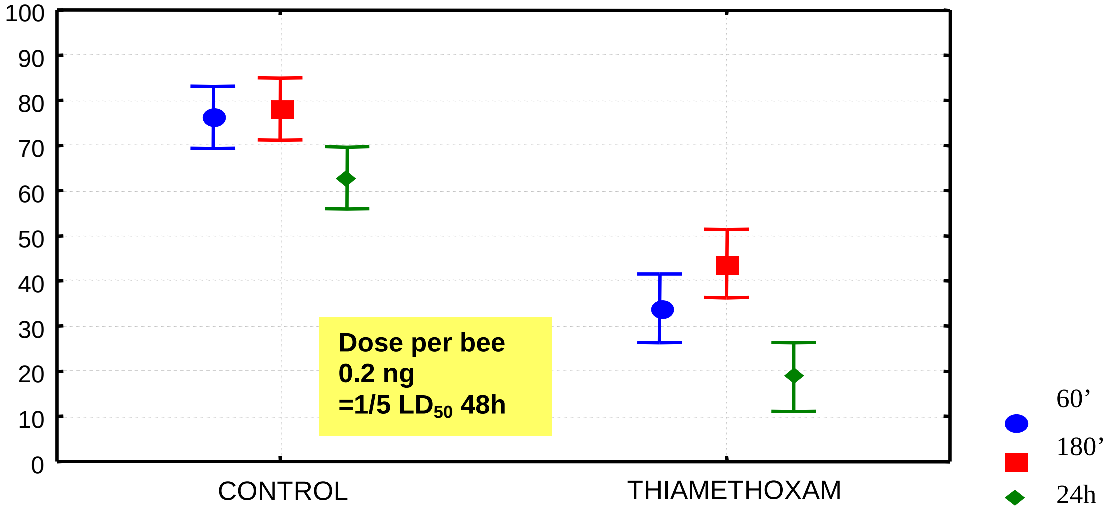

Figure 11 - Percentage of correct responses (extension of ligula in presence of the odor) at different time intervals after treatment (60’-blue, 180’-red, 24h-green), in controls versus the treatment group (thiamethoxam). Pairwise comparison differences were statistically significant for $p<0.001$.

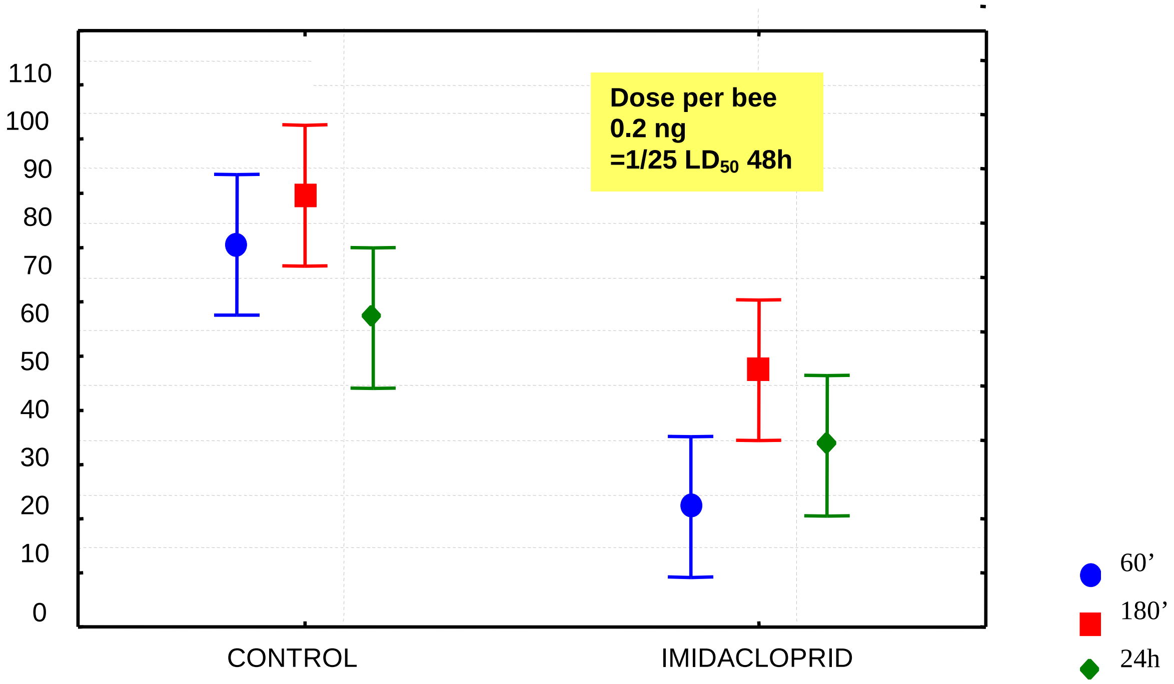

Figure 12 - Percentage of correct responses (extension of ligula in presence of the odor) at different time intervals after treatment (60’-blue, 180’-red, 24h-green), in controls versus the treatment group (imidacloprid). Pairwise comparison differences were statistically significant for $p<0.001$.

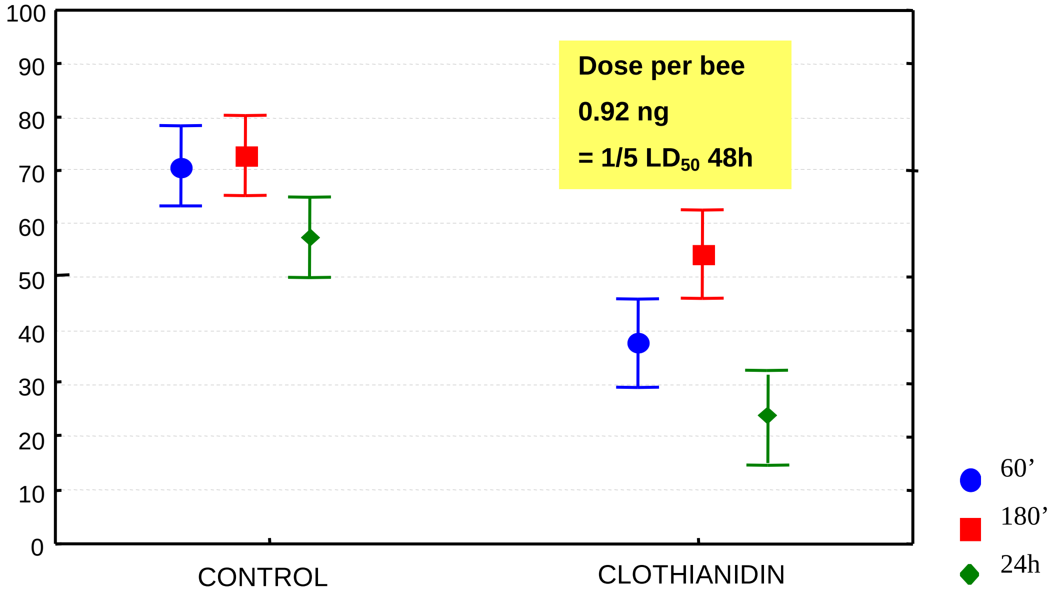

Figure 13 - Percentage of correct responses (extension of ligula in presence of the odor) at different time intervals after treatment (60’-blue, 180’-red, 24h-green), in controls versus the treatment group (clothianidin). Pairwise comparison differences were statistically significant for $p<0.001$.

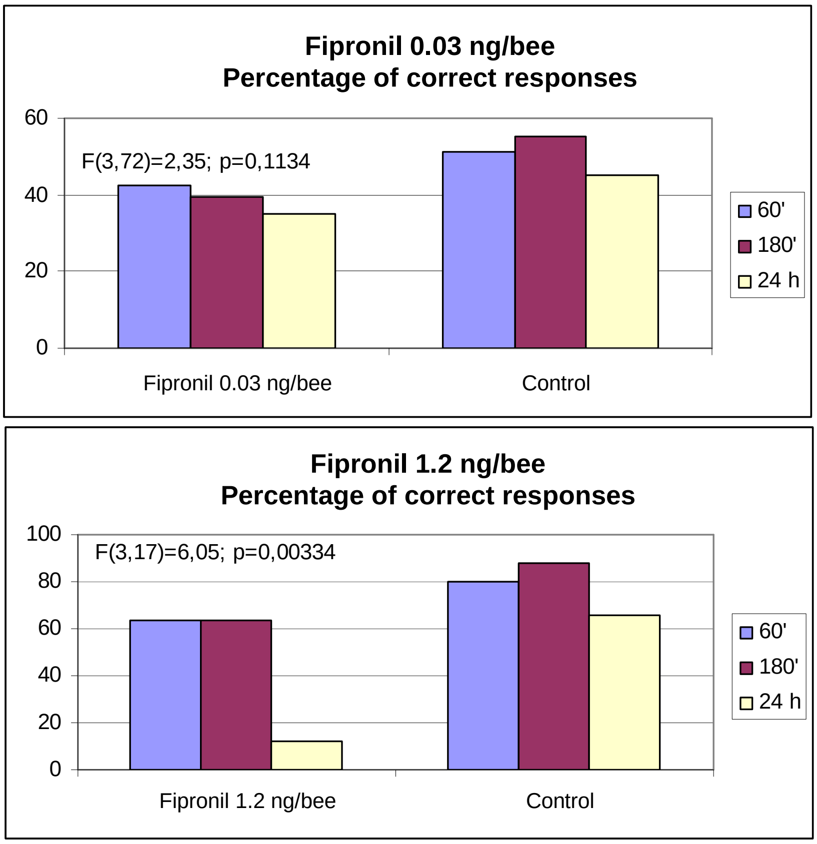

Figure 14 – Ability to recognize the odor used for training (extension of ligula only in presence of the correct odor) at different time intervals (60’, 180’, 24h).

Results of the sublethal effect tests thus showed that the assayed neonicotinoids and fipronil, administered 30 minutes prior to the training session, exerted a marked negative impact on odor recognition ability, with a significant reduction in correct responses to odor presentation at all the chosen time intervals.

Administration of the active ingredient prior to training can influence two behavioural stages, namely learning (ability to form an association between recognizing an odor and obtaining the reward), and memory formation, a process that requires the transition from short-term to medium-term and long-term memory.

All bees treated with imidacloprid, clothianidin and thiamethoxam proceeded correctly throughout the training (for all active ingredients, in 100% of cases bees extended the ligula in presence of the rewarded odor and received the sugar reward, and also “tasted” the mint-flavoured salt, receiving the punishment). For fipronil, only 78% of bees carried out the training correctly.

It can thus be hypothesized that imidacloprid, clothianidin, thiamethoxam and fipronil do not prevent bee memory formation with regard to odors, as these active ingredients did not interfere with training; however, it can further be speculated that the effect is exerted at the moment of recovery of memory, at different time intervals.

Our data highlighted a greater impact of the active ingredients at the 24 h test, showing that the most significant effect is exerted on long-term memory.

In the case of the neonicotinoids, our data only partly confirm data available in the literature on tests conducted with imidacloprid and clothianidin, which were previously reported to induce effects above all on recovery of medium-term memory.

By contrast, our results with regard to fipronil are in agreement with data obtained in previous studies, which highlighted a marked reduction in correct responses after 24 h.

4.2 Sublethal effects: labyrinth test for assessment of impact on orientation

Further investigations by means of the PER test to assess the sublethal effects of the active ingredients and their impact on spatial orientation were conducted in the framework of this study; detailed elaboration of the results was in progress at the time of writing.

Preliminary results showed a reduction in orientation ability, partly due also to overall effects on motor coordination (tremors, twitching, rolling), which are often transitory.

The above-mentioned motor effects were filmed, so that the results can be codified and analysed by means of specific behavioural analysis programmes in order to provide a complete description and quantify the effects. Data elaboration was in progress at the time of writing.

4.3 Lethal effects

As the trials were conducted by administering 1/5 of the LD_{50} 48h calculated for each active ingredient on the basis of data available in the literature, lethality of the doses administered was tested according to the EPPO/Council of Europe risk assessment protocol, in which the dose is administered in 200 μL of sugar syrup to groups composed of 10 bees.

Additionally, tests were conducted to ascertain the lethality of the same quantity of active ingredient administered according to the procedures enacted for the PER test and the labyrinth test, which require the active ingredient to be diluted in 3-5 μL (depending on the active ingredient in question) and submitted to bees.

The lethality tests were still in progress at the time of writing, with only the tests on imidacloprid having been completed.

All tests were carried out on 3 hives, with 3 replications.

Results obtained up to September 2009 indicated that the theoretically sublethal doses used in our tests led to significant bee mortality, with a hive-dependent significant interaction.

In addition, data on mortality recorded for administration of the dose with highest concentration (3-5 μL) compared to mortality with the most diluted dose (20 μL) showed greater toxicity of the 3-5 μL than the 20 μL dose (Fig. 15).

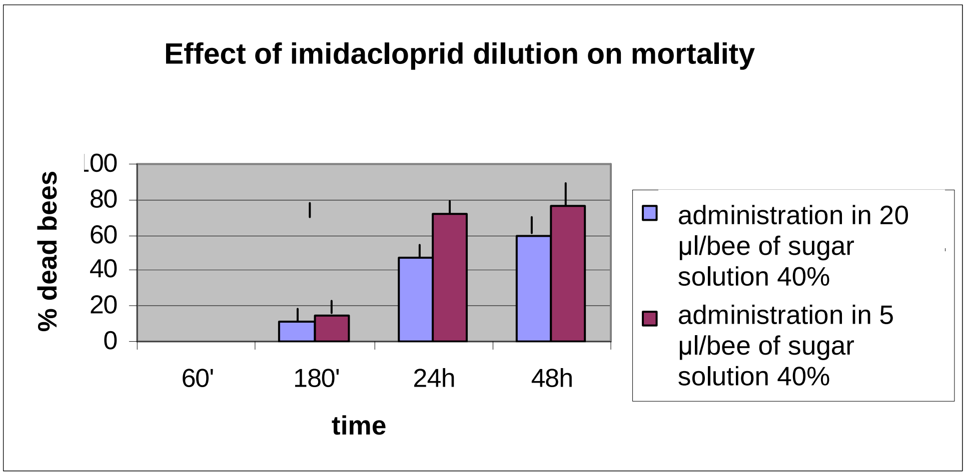

Figure 15 – Mortality recorded at increasing time intervals.

4.4 Effects on the brood

The protocol drawn up by Aupinel *et a*l. at INRA (France) was applied. This protocol allows in vitro bee rearing without the aid of nurse bees and can assess the response to different stress factors.

Toxicity (expressed as LD_{50} at 48h) of the molecules in question on larvae was evaluated through administration of 5 increasing doses of the active ingredient to a sample of 48 larvae and the controls. Preliminary results are illustrated in Table 19.

Results for the first two ingredients assayed, clothianidin and fipronil, suggested that the brood was considerably less susceptible to poisoning compared to adult bees. For clothianidin it was not possible to calculate larval LD_{50}, as the highest dose that could be administered (based on active ingredient solubility), namely 3000ng/larva, led to 11.4% corrected mortality. Larval toxicity tests for the other two active ingredients (imidacloprid and thiamethoxam) were in progress at the time of writing.

Active ingredient	**LD_{50} (24h) adult bees**	**LD_{50} (24h) larval**
clothianidin	4 ng/bee	>>3000 ng/larva
fipronil	4 ng/bee	39 g/larva

Table 19 – Comparison between larval LD_{50} of clothianidin and fipronil and that of adults at 48 h (data obtained from the literature).

5. Conclusions

Data obtained from the trials conducted up to September 2009, albeit incomplete in some parts and requiring repetitions in certain sections, allow some partial conclusions to be drawn.

The Apenet monitoring network, activated throughout Italy, recorded no phenomena of die-off or depopulation linked to maize sowing during the early part of the year. In none of the monitoring stations were serious cases observed, with the exception of apiaries situated in the plane of Sibari, where extensive poisoning occurred, linked to use of neonicotinoids in spray formulation (Actara, active ingredient thiamethoxam) during the flowering period.

The reporting system, which in the spring of 2008 recorded 185 cases of die-off with 132 samples collected during the maize sowing season, roughly half of which tested positive for the active ingredients used in maize seed coating, received a total of 10 reports in the first semester of 2009. Of these, only 3 pertained to the maize sowing period, and the samples revealed unlawful utilisation of coating products that are currently suspended. Of the other 7 reports, 5 were found to be caused by neonicotinoids applied in spray formulation, while the presence of residues was not detected in the remaining 2 cases.

The trials pertaining to sowing of maize coated with the 4 active ingredients investigated gave the following results:

- dustiness of coated maize seed was lower than the established limit of 3g/100 Kg;
- dust emitted in the field during sowing with the pneumatic seed drill varied between 0.5 and 3.5 $\mu g/m^2$ with the modified seed drill and between 1 and 5 $\mu g/m^2$ with the unmodified seed drill. Emissions showed a significant decrease with increasing distance from the sowing area;
- air concentrations of dust emitted during sowing varied between 0.1 and 0.8 ppb, with these values increasing when distances were increased from 5 to 10 m in trials with the modified seed drill, but decreasing in the trials with the unmodified seed drill;
- our data suggest that although the system involving application of the dual pipe deflector for dust abatement allowed a notably variable reduction in ground-level dust concentrations, it contributed to greater dust dispersion in the air, with drift spreading over greater distances and thereby increasing the probability of contact with bees in flight and with hives situated in the surrounding countryside.

In colonies directly exposed to sowing of seed coated with the four active ingredients, use of the seed drill equipped with deflectors was found on certain days to result in higher adult bee mortality rates compared to the control hives. No depopulation phenomena were detected. The analyses conducted up to September 2009 (for only 2 active ingredients) showed that the active ingredient concentrations observed in dead bees remained below the lethal threshold. With regard to sowing of thiamethoxam-coated seed, colony vitality and development was found to be lower in hives exposed to sowing as compared to the control group.

Active ingredient concentration in guttation droplets proved to be highly variable, depending on the plant phenologic stage and the mode of propagation (container- or field-grown), but values were consistently greatly above or close to the bee toxicity threshold. No bee foraging activity on the droplets could be observed during the first field observations.

The first results of tests assessing sublethal effects on adult bees showed that recovery of medium-term and long-term memory was compromised. Such effects were detected at much lower doses (roughly 1/5) than the LD_{50} stated in the literature.

Results of the first brood toxicity tests indicated that the larvae were markedly less sensitive to the active ingredients assayed up to September 2009 (clothianidin and fipronil) than was the case for adults. This is in line with the damage typology reported for phenomena of poisoning during coated seed sowing, in which the main effect is loss of adult bees. Any damage to the brood is usually indirect, due to absence of nurse bees.

In conclusion, the first partial results underscored several elements demonstrating that the active ingredients used in maize seed coating have a certain degree of toxicity towards bees. Although improvements in the coating process have limited the extent of dust emission from coated seed, some damage to hives was still detected during the trials described in this study.

Furthermore, although the quantities of active ingredient emitted during field sowing fell below the lethality threshold for bees, the magnitudes detected were close to those shown by the first results of laboratory tests to be capable of inflicting damage on adult bee learning processes and memory formation.

Finally, it is important to note that the climatic conditions of the spring of 2009, characterized by heavy rainfall, did not favour dust drift into the atmosphere and the environment. Since it is generally believed that climatic factors strongly influence the impact of coated maize seed sowing on bees (and in the past this phenomenon was found to be highly variable across the years), it is considered advisable to replicate the trials in at least one subsequent season and in other localities having different soil and climate characteristics, in order to acquire more clear-cut data.

It can likewise be recommended, before drawing definitive conclusions, to await the final results of the ongoing laboratory tests, both lethal and sublethal, in order to integrate the present data with the results of analyses on the active ingredients that had not been completed up to September 2009, and to allow replication of the tests with coated seed dust obtained by means of the Heubach drum rather than with the pure active ingredient. Trials using the Heubach drum method will allow simulation of a situation closer to real field exposure.

6. 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

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3. Effect of maize guttation on bees

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

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