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 third year (2011) activity of the APENET project

This report refers to results available on 6th October 2011, including 2010 results not described in the Italian 2010 report.

CONTENTS

2010 DATA

(not published in the August 2010 Italian report because obtained after that date – published in part in the 2010 English report)

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

1.1 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) 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 and in part by Veneto Agricoltura .

1.1.1 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 1).

Table 1 - 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 2.

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 2, 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.

Table 2 - 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

Of the 19 total localities, in 6 (31.5%) of them there was significant difference in yield between one or more treatments (seed coating) and the control or other treatments (Table 3). More specifically in 2 of these 6 localities, a negative effect of one of the seed coatings was observed: at the Bergamo location with imidacloprid (Gaucho) and at Ambrogio (FE) with fipronil (Regent). In the remaining 4 (21%) localities: Castenedolo (BS), Castelceriolo (AL), Montagnana (PD), Palazzolo della Stella (UD), clothianidin (Poncho) had a significant positive effect on yield compared to other seed dressings and/or the control.

Table 3 – Yield data from 19 agronomic trials – APENET 2010

		YIELD (q/ha-15.5% R.H.)					
Region	Locality	Control	Thiamethoxam (Cruiser)	Imidacloprid (Gaucho)	Clothianidin (Poncho)	Fipronil (Regent)	Variance analysis
Lombardia	Bergamo	145,75	142,75	128,35	149,33	138,68	LSD (0,05) 13,55
	S.Angelo Lodigiano (LO)	124,80	140,33	134,40	124,20	138,73	N.S.
	Luignano (CR)	107,93	101,70	122,63	108,65	115,43	N.S.
	Caleppio di Settala (MI)	167,83	179,85	170,73	178,78	167,13	N.S.
	Castenedolo (BS)	112,50	120,25	101,50	125,75	115,50	LSD (0,01) 11,52
	Pudiano (BS)	-	-	-	-	-	
	Ostiglia (MN)	114,65	121,55	132,50	126,33	134,58	N.S.
Piemonte	Vigone (TO)	157,00	152,58	154,33	153,65	163,83	N.S.
	Chivasso (TO)	133,95	130,75	126,60	129,65	127,70	N.S.
	Castelceriolo (AL)	132,65	140,53	132,05	148,08	151,10	LSD (0,01) 10,05
Veneto	Lonigo (VI)	119,58	120,50	123,18	123,65	127,13	N.S.
	Montagnana (PD)	139,98	140,68	146,75	162,68	150,43	LSD (0,01) 8,30
	Villadose (RO)	140,98	148,25	138,58	143,75	142,45	N.S.
	Noventa Vicentina (VI)	133,65	142,70	140,20	141,48	131,78	N.S.
Emilia Romagna	Ambrogio (FE)	133,73	129,10	147,75	140,28	124,48	LSD (0,05) 14,49
	Parma	143,65	141,98	147,28	140,10	138,75	N.S.
Friuli	Mortegliano (UD)	135,15	146,38	133,83	149,10	147,00	N.S.
	Palazzolo della Stella (UD)	73,18	83,80	90,33	96,35	94,93	LSD (0,01) 9,39

	Codroipo (UD)	153,28	156,60	157,00	152,98	154,20	N.S.
Toscana	Marciano della Chiana (AR)	140,73	122,83	129,43	130,53	120,08	N.S.

Furthermore, as mentioned before the commercial hybrid supplied by Assosementi for the 2010 trials was 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.

1.1.2 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
3)

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;

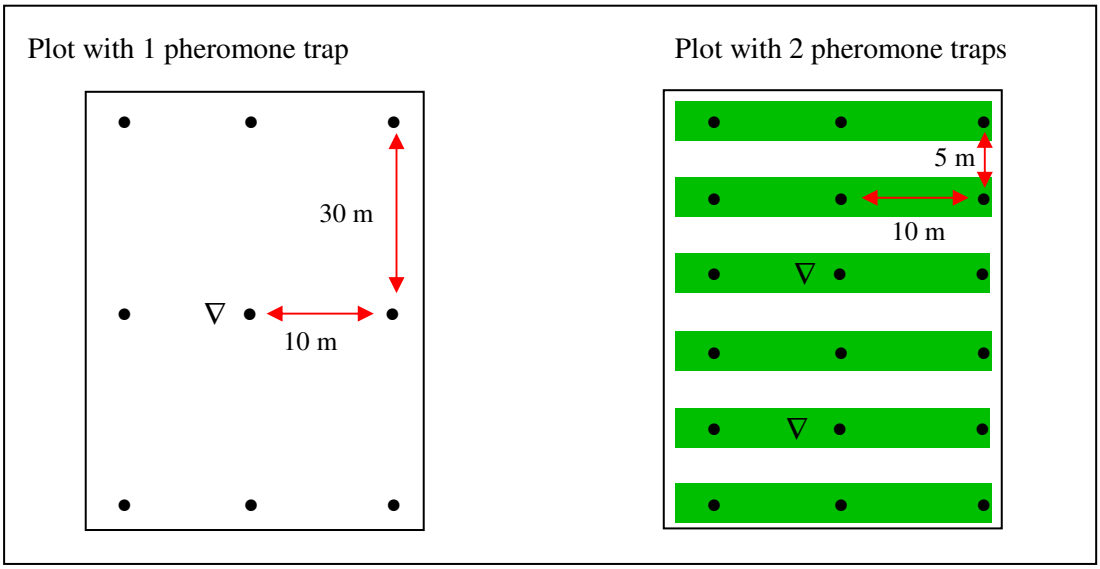

Figure 1 - Diagram of trap positioning: ∇ = position of 2009 pheromone trap; • = larval trap..

The aim of positioning the 2010 larval traps around the positioning of the 2009 pheromone traps was to correlate the larval population with the adults of the previous year, in order to evaluate the forecasting ability of the pheromone 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 4 – Overview of results of Wireworm monitoring and their effects on maize crops on a sample of plots in the main maize-growing regions (n.d.= not detected).

				Adults 2009				**Larvae 2010**				**Maize 2010**				
Region	**Monitored plots**	**With risk factors (*A.brevis*, *A.sordidus*)**	**With risk factors (*A.litigiosus*, *A.ustulatus*)**	***A. brevis media* (*e.s., min-max*)**	***A. sordidus media* (*e.s., min-max*)**	***A. litigiosus media* (*e.s., min-max*)**	***A. ustulatus media* (*e.s., min-max*)**	***A. brevis media* (*e.s., min, max*)**	***A. sordidus media* (*e.s., min, max*)**	***A. litigiosus media* (*e.s., min, max*)**	***A. ustulatus media* (*e.s., min, max*)**	**Plant density healthy p /sqm mean (*s.e. min, max*)**	**Mean (% healthy plants on sown)**	**% attacked plants (*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	6	76 (18.3. 0-691)	523 (53.1. 91-2129)	n.r.	548 (88.4. 0-2786)	0.03 (0.01. 0-0.25)	0.14 (0.03. 0- 0.83)	n.r.	1.03 (0.35. 0-9.95)	6.46 (0.07. 5.30-7.38)	90.3	1.14 (0.024. 0.0-7.0)	2	0
Emilia Romagna	105	7	4	n.r.	245 (26.44. 4-2201)	253 (24.29. 6-1141)	n.r.	n.r.	n.r	n.r.	n.r	n.r.	n.r.	n.r.	1	0
Lombardia	10	2	1	n.r.	983 (243.9. 189-2349)	629 (202.3. 63-2087)	n.r.	n.r.	n.r	n.r.	n.r	6.48 (0.06. 4.80-7.3)	93.2	0.17 (0.071. 0.10-0.81)	1	0
Lombardia (Mn)	4	1	1	n.r.	1259 (254. 814-1973)	1293 (355. 693-2190)	n.r.	0.06 (0.03. 0-0.11)	0.14 (0.05. 0.00-1.44)	0.00	0.00	n.r.	n.r.	n.r.	0	0
Piemonte	6	1	0	n.r.	553 (242. 46-2153)	781 (232. 123-2311)	n.r.	n.r.	0.244 (0.061. 0.00-0.560)	0.044 (0.03. 0.00-0.220)	n.r.	7.00 (0.12. 6.40-7.40)	94.6	5.8 (0.017. 0-12)	0	0
Friuli	11	2	0	169 (19.70. 86-323)	335 (66.58. 59-763)	12 (6.41. 0-52)	n.r.	n.r.	n.r	n.r.	n.r	6.63 (0.05. 6.35-6.90)	90.7	0.059 (0.01. 0.05-0.1)	0	0
Total	187	19	12												4	0
Incidence (%)															2.1	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 Table 4. 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 5. 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 5 - 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 6).

Table 6 - 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)					Wire worm 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 (due to damaged traps, excess water).

1.1.3 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 7. 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

Table 7 - Characteristics of the experimental fields used in the 2010 trials.

Farm	**Municipality**	**Prov.**	**Soil**	**Previous crop**	**Wireworm population**		**Farm**	**Data di semina**	**Densità di semina (semi/mq)**	**Data di raccolta**
					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-ott
Parcianello	Eraclea, Coda di Gatto	VE	loam	soybean	0.05	0.00	364	21-apr	7,24	07-ott
San Donà, Isiata	Florian	VE	loam	maize	0.05	9.95 *A.ustulatus*	951	17-apr	7,21	21-ott
Vallevecchia	Caorle	VE	loam	soybean	0.17	0.00	459	20-apr	7,25	20-ott
Zanazzo	Cessalto	TV	clay	maize	0.33	0.83 *A.ustulatus* 0.25 *A.brevis*	390	20-apr	6,87	14-ott
Diana	Mogliano Veneto	TV	loam	wheat	0.08	0.58 *A.ustulatus* 0.25 *A.brevis*	722	15-apr	7,28	11-ott
Sasse Rami	Ceregnano	RO	loam	wheat	0.20	0.06 *A.litigiosus*	1395	16-apr	7,13	15-set

The results are summarised in Table 8. 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 8 - 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***
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

1.2 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.

Risultati

Results
In Table 9 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 have been completed, in collaboration with colleagues of CRA-PAV (Research Centre for Plant Pathology), and showed that in all groups the 4 insecticide a. i. did not reach detectable levels (n.d., not detected, lower than L.O.D. < 0,2 µg/kg).

Table 9 – 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*	16.500	79.260	> 428.000
Imidacloprid	1*	7.150	155.200	> 714.000
Clothianidin	1*	5.500	71.900	> 892.000
Fipronil	1*	94.200	162.700	> 358.000

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

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

2.1 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. This was tested by using two different protocols: free flying bees (Girolami *et al*. 2001) and bees inside mobile cages (Girolami *et al*., in preparation).
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 (Marzaro *et al*., 2011; Tapparo *et al*., 2011). 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. Most of the experiments were carried out in 2009 and 2010, and in part completed in 2011

2.2 Free flying bees

2.2.1 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.

2.2.2 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 10, 11 and 12.
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 10 - 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 11 - 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	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 12 - 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 + Metalaxyl-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 13

Table 13 - 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**

2.3 Bees in mobile cages

2.3.1 Materials and methods

For these trials 2010 seed batches coated with the following formulations were used: Poncho®, Gaucho® e Cruiser® 350FS.

The influence of a quick dusting, simulating a single bee flight over the seeder, was assessed by using an aluminium bar measuring 4 m length, on which, at 40 cm intervals, 10 cages containing a single bee were hung. The bar was supported by two vertical poles 2.5 m long. Exposure of bees to the dust cloud generated by the seeder was ensured by two operators who moved the bar at a speed of 5-7 Km/h in such a way as to intercept the dust cloud at fixed distances (0-4 m and 4-8 m). Exposure occurred on tbhe right hand side of the seeder and parallel to the direction followed by the tractor, so that there was an extremity of the bar in line with the tractor and the other at 4 m from the tractor. For capturing the dust cloud at 4-8 m from the seeder the bar was positioned so that one extremity was at 4 m from the tractor, and the other at 8 m from the tractor.

For each distance two repetitions were carried out, each of which consisted in one forward run and one return, at the same speed as the seeder. After exposure to the dust cloud the cages with the single bees were placed in the laboratory at high humidity conditions, as described above. Mortality was recorded in the following 24 h.

In a subsequent trial involving only imidacloprid bees were placed in the tulle cages hanging from the mobile bar in order to obtain different distances from the seeder. They were then submitted to a single forward and return run of the seeder, one group on the right and one on the left of the seeder. Of the 20 bees exposed per side it was decided to analyse only 5 of them for detection of insecticide content.

2.3.2 Results

Results of the bee flight test with mobile cages is reported in Table 14. In the trials with imidacloprid and clothianidin, bees placed at 0-4 m showed a higher mortality than those exposed at 4-8 m, while in the trial with thiamethoxam mortality rate was the same at the two exposure distances. For all three a. i. mortality rate was significantly higher compared to bees exposed to only fungicide containing dust.

Table 14 – Mortality of bees exposed to dust produced during seeding with the mobile cage method.

Active ingredient	Exposure distance from seeder	Dead	Alive	N. tested bees
Imidacloprid	0-4	16	4	20
	4-8	11	9	20
Clothianidin	0-4	20	0	20
	4-8	17	3	20
Thiamethoxam	0-4	13	7	20
	4-8	13	7	20
Metalaxyl-fludioxinil	0-4	1	19	20
	4-8	0	20	20

The results of the flight test on either side of the seeder are reported in Table 15. It must be mentioned that discharge of the seeder is placed on the right: bees which were exposed on that side contained much higher levels of a. i. compared to bees exposed on the left-hand side of the seeder.

Table 15 - Imidacloprid content in bees exposed to abrasion dust on either side of the seeder in mobile cages.

Distance from seeder	ng/bee bees exposed on the right of the seeder	ng/bee bees exposed on the left of the seeder
1 m	4786.3 ± 0.6	-
2.25 m	457.3 ± 0.6	410 ± 2
4.5 m	142.3 ± 0.6	110 ± 1
6.75 m	523 ± 3	98 ± 2
9 m	198.7 ± 0.6	33 ± 1

2.4 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 demonstration of the fact that bees are contaminated with doses much higher than LD50 are the results of the chemical analyses of bees exposed to abrasion-dust on the right-hand side of the seeder, which revealed quantities of a. i. ranging between 200 and 4700 ng/bee of a. i.. In free flying bees exposed to abrasion-dust and found dead at the hive and feeders, the average quantity of detected a. i. was 800 ng/bee.

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.

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.

2.4 References

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3. *PER (Proboscis Extension Reflex)* test used to evaluate the effects of clothianidin, imidacloprid, thiamethoxam and fipronil administered as contaminated abrasion-dust

3.1 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 cm diameter Petri dish (10 bees per cage). Each cage was equipped with a food dispenser (syringe inserted on the lid).

Tested active ingredients: clothianidin, imidacloprid, thiamethoxam e 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 the one estimated in the APENET 2009 trials to be deposited at 5 m from the sowing field using a seeder without modification, and concentrations 10, 100 and 1000 times higher (Table 16).

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. 17).
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 16 - Quantity of a. i. per tested surface.

	Clothianidin	**Imidacloprid**	**Thiamethoxam**	**Fipronil**
% a. i. in the dust	33	31.1	33.5	32
A. i. at 5 m (µg/m^2)	2.25	3.63	2.53	0.91
Total dust at 5 m (µg/m^2)	6.82	11.67	7.55	2.84
Dust in a 8.5cm Ø Petri dish (=56,72 cm^2) (ng)	0.039	0.066	0.043	0.016
Quantity of a. i. x 1 (ng)	**0.012762**	**0.020589**	**0.014350**	**0.005162**
Quantity of a. i. x 10	0.12762	0.205894	0.143502	0.051615
Quantity of a. i. x 100	1.2762	2.058936	1.435016	0.516152
Quantity of a. i. x 1000	12.762	20.58936	14.35016	5.16152
Contact LD50 (µg/bee)	0.0218	0.0179	0.0299	0.006

Manner of contamination with the active ingredient: Bees flying out from the hive were captured and placed in cylindrical cages. Immediately after capture, 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.

3.2 Results

The following graphs 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 (Figs. 2-5).

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.

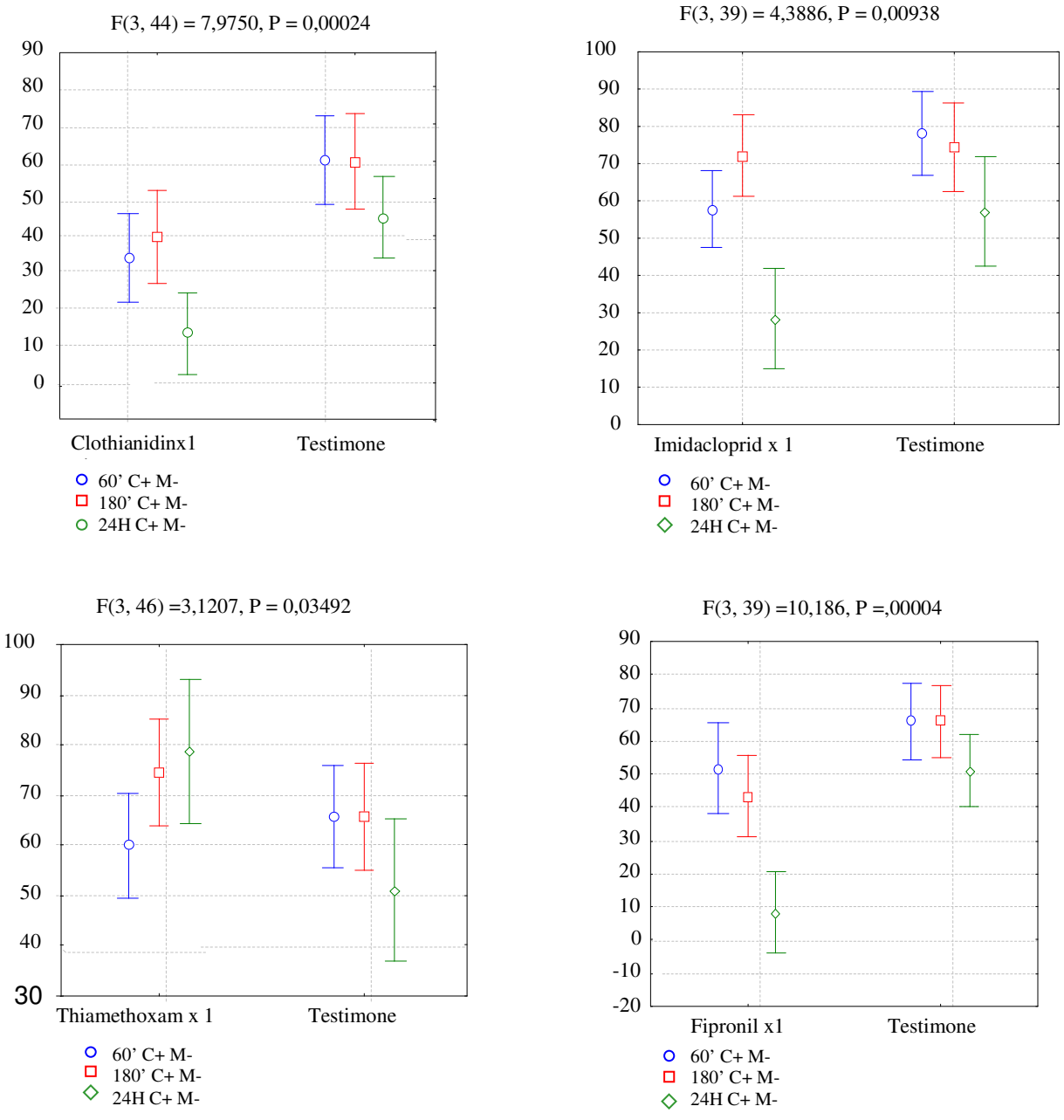


Figure 2 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

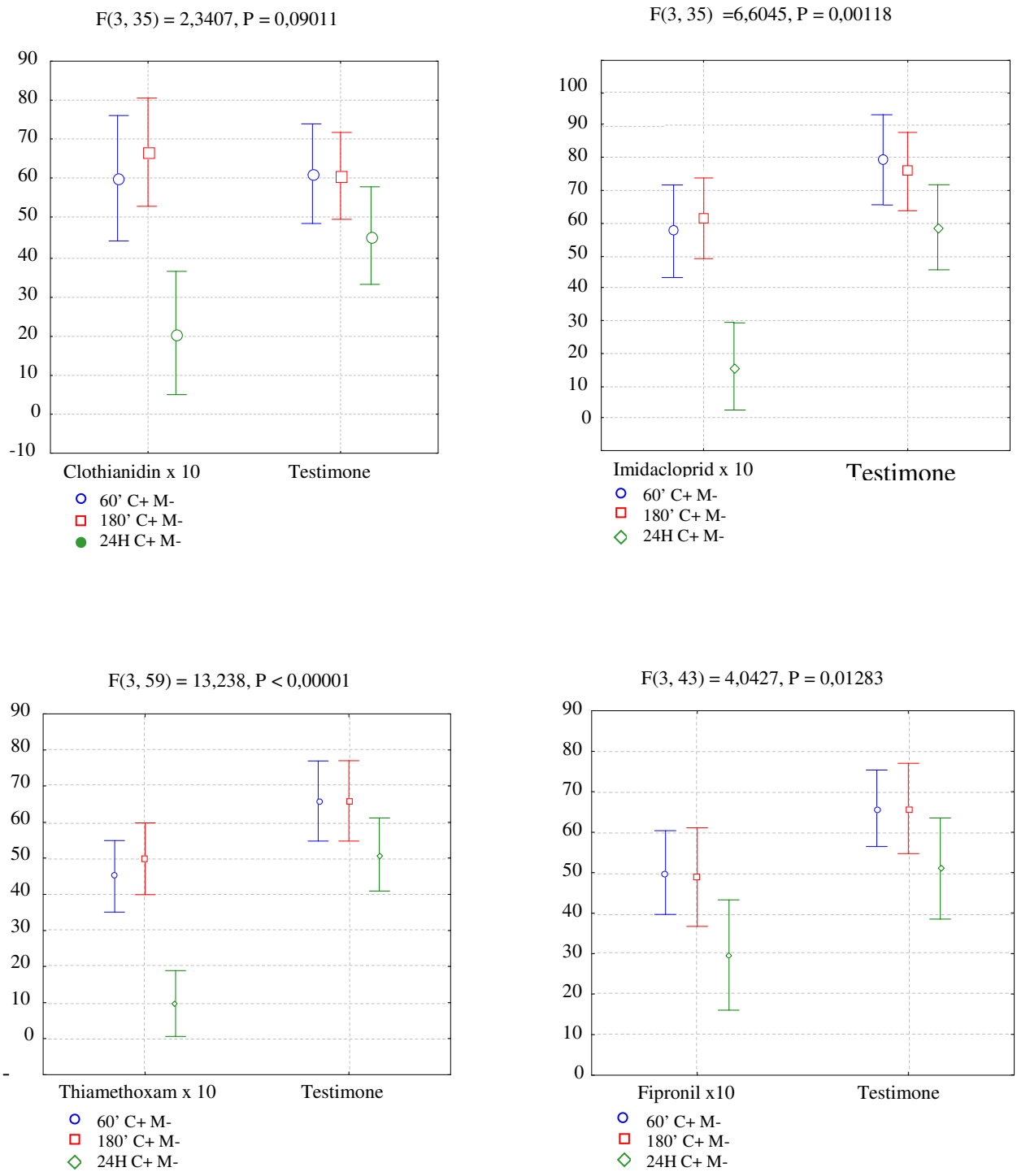


Figure 3 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 10 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

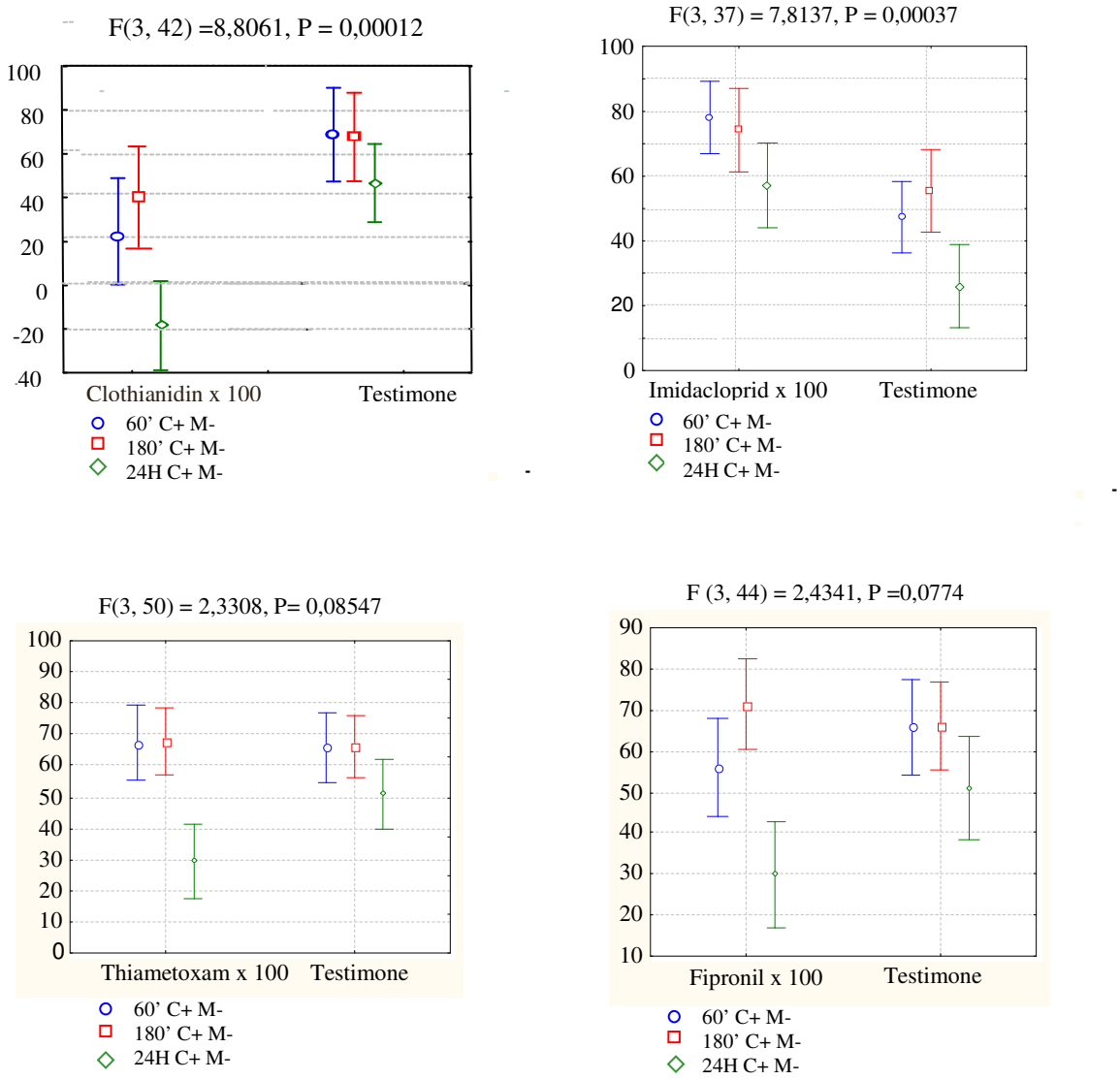


Figure 4 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 100 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

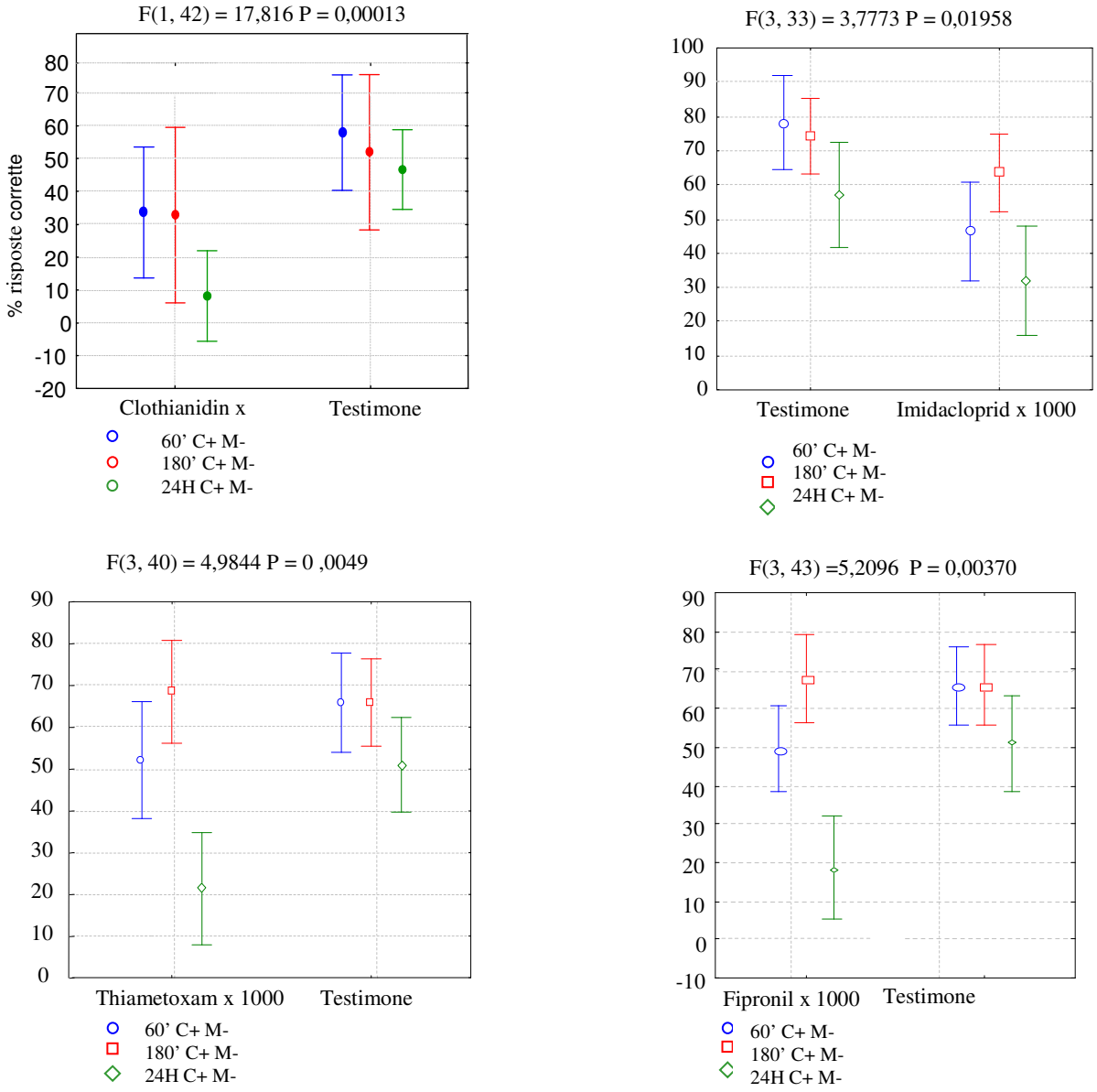


Figure 5 - Effects of the 4 a. i. on olfactory memory: bees exposed to a dose/bee corresponding to 1000 times the one deposited in the field at 5 m by the seeder without modifications. Translation of text within the figure: *Testimone* = control.

2011 DATA

4. The monitoring network

In the framework of the Italian APENET Project, a national monitoring network has been set up, 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 colonies 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.
On the basis of the APENET network winter mortality in 2010/2011 was estimated to be 22.48% (78 dead colonies on 347). Winter colony losses estimated by means of the Coloss European network questionnaire were 13.44% (1850 colonies on 13770).

Analyses conducted to identify 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.
The samplings carried out in 2010 in the APENET network confirmed the presence of *Nosema ceranae* only: *N. apis* was not identified in any sample.

Among **viruses**, in the 2009 sampling 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. 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.
In the samples collected in 2010, the same viruses as the previous year were detected with the addition of KBV and IAPV, the latter found in 3 apiaries in Sardinia, Lazio and Tuscany. Of the 378 samples analysed in 2010, 12 resulted negative, while the prevalence of each virus in the remaining 366 samples was 96% for BQCV, 78% for DWV, 60% for SBV, 29% for ABPV. With the exception of AIV, which was never detected, the prevalence of the other analysed viruses was below 10% (Fig. 6).

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.

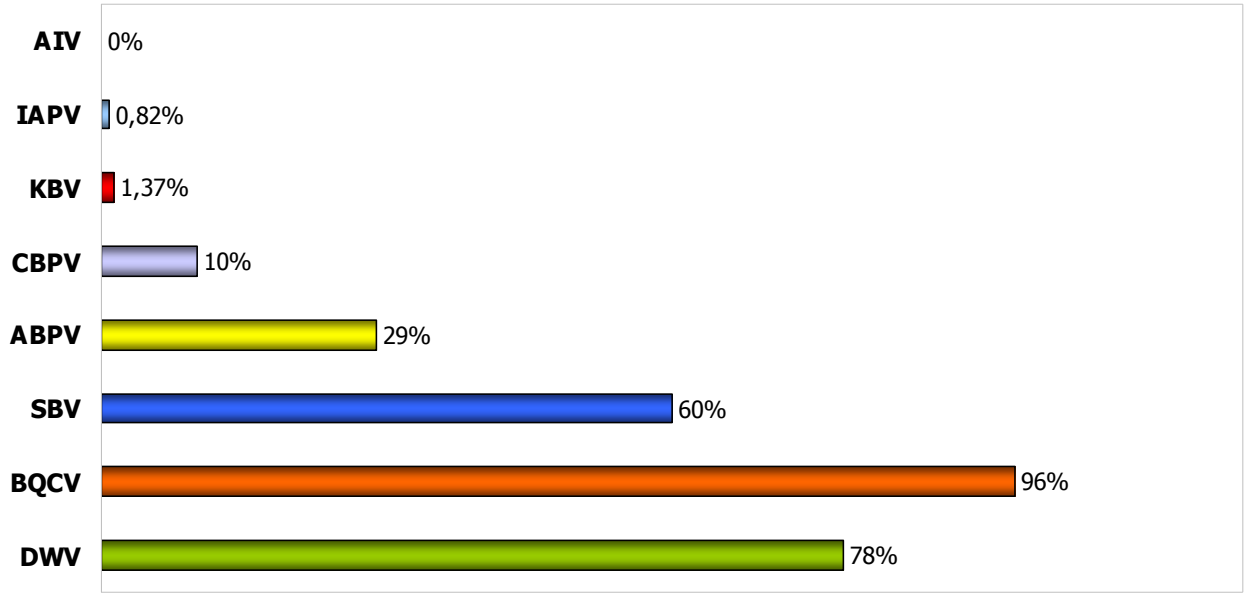


Figure 6 – Prevalence of viruses in samples collected in the APENET network in 2010.

The **chemical analyses** carried out on wax and bee samples to test for residues of organophosphate, organochlorurate, carbamate and neonicotinoid pesticides, produced the results shown in Tables 17 and 18. In both kinds of samples residues of pesticides were revealed quite frequently. In wax, which is known to "accumulate" residues, th incidence was around 43%, while in bees the presence of pesticides was revealed in 12% of the samples. The lower frequency could partially explain the good health status of the observed colonies. It notable that the active ingredients most present are those with an acaricidal activity, used for control of the mite *Varroa destructor* (whether registered or not).
As far as pollen is concerned it is interesting to observe (Table 19) that there is a fair presence of samples containing residues of pesticides (about 27%). Compared to bee and wax samples, in pollen there is a higher percentage of residues of active ingredients not connected to beekeeping activities. More specifically, residues of neonicotinoids were found in several samples.

Table 17 - Results of chemical analyses conducted on bee samples collected in 2009-2010.

Module	Year	Analysed samples	Positive samples	Active ingredients detected	Concentration (ng/g)
Abruzzo	2009	10	2	coumafos - fluvalinate	16 - 103
	2010	4	1	fluvalinate	183
Bolzano	2009	11	3	bitertanol - clothianidin	24 - 90
	2010	3	0		
Basilicata	2009	16	1	flumethrin	103
	2010	15	0		
Calabria	2009	15	0		
	2010	66	7	chlorfenvinphos – flumethrin - coumafos - fluvalinate - acrinathrine	15 - 135
Campania	2009	20	7	coumafos - fluvalinate - rotenone - teflubenzuron	10 - 55
	2010	20	1	teflubenzuron	12
Emilia Romagna	2009	35	6	methomyl - rotenone – flumethrin	10 - 452
	2010	47	5	dithianon – fenbuconazolo - propamocarb	6 - 125

Liguria	2009	20	7	bitertanol - coumafos - imidacloprid	10 - 48
	2010	23	0		
Lazio	2009	20	3	rotenone -fluvalinate	10 - 119
	2010	17	2	coumafos - procimidone - fluquinconazolo - folpet	75 - 255
Molise	2009	20	3	rotenone	11 - 45
	2010	20	0		
Marche	2009	18	5	bitertanol - rotenone	18 - 119
	2010	17	1	coumafos	472
Puglia	2009	20	5	chlorpyriphos ethyl - rotenone	10 - 121
	2010	10	0		
Sicilia	2009	20	4	clothianidin - fluvalinate - coumafos	53 - 103
	2010	8	0		
Sardegna	2009	18	2	imidacloprid - clothianidin - coumafos	16 - 65
	2010	18	2	fluvalinate - flumethrin	15 - 22
Trento	2009	16	1	imidacloprid	16
	2010	12	2	fludioxonil	68 - 71
Toscana	2009	19	2	acrinathrine - imidacloprid	23 - 30
	2010	20	2	fluvalinate	35 - 40
Umbria	2009	16	2	chlorfenvinphos - dimethomorph	82 - 150
	2010	19	0		
Veneto	2009	20	0		
	2010	26	4	fludioxonil – clothianidin - tebuconazole	7 - 55

Table 18 - Results of chemical analyses conducted on wax samples collected in 2009-2010.

Module	Year	Analysed samples	Positive samples	Active ingredients detected	Concentration (ng/g)
Abruzzo	2009	12	11	coumafos - fluvalinate - chlorfenvinphos - bitertanol - metamitron - acrinathrine	11 - 568
	2010	4	2	coumafos - fluvalinate	20 - 121
Bolzano	2009	10	4	fluvalinate	11 - 54
	2010	3	1	fluvalinate	26
Basilicata	2009	16	7	coumafos - imidacloprid - fluvalinate - chlorfenvinphos	10 - 175
	2010	15	4	fluvalinate - coumafos	12 - 677
Calabria	2009	15	14	chlorfenvinphos – coumafos - fluvalinate – flumethrin	6 - 365
	2010	66	42	chlorfenvinphos - fluvalinate - acrinathrine - flumethrin – cyprodinil - coumafos	10 - 2600
Campania	2009	20	8	chlorfenvinphos - coumafos - rotenone	10 - 213
	2010	20	6	flumethrin - fluvalinate - coumafos	10 - 84
Emilia Romagna	2009	40	13	chlorfenvinphos - acrinathrine - coumafos – rotenone -fluvalinate - fludioxonil	12 - 709
	2010	57	14	acrinathrine - fluvalinate – flumethrin – metalaxyl - coumafos	10 - 839
Liguria	2009	20	3	coumafos - fluvalinate	19 - 520
	2010	23	7	coumafos - fluvalinate	14 - 124
Lazio	2009	20	6	acrinathrine - fluvalinate	11 - 504
	2010	20	11	fluvalinate - flumethrin	12 - 1043
Molise	2009	15	4	coumafos - fluvalinate	20 - 870
	2010	20	5	chlorfenvinphos - acrinathrine - coumafos - metalaxyl	10 - 109

Marche	2009	18	6	chlorfenvinphos - coumafos - fluvalinate	11 - 1428
	2010	17	8	coumafos - fluvalinate - propamocarb	12 - 12779
Puglia	2009	20	3	chlorfenvinphos - acrinathrine - coumafos - fluvalinate	15 - 100
	2010	10	5	chlorfenvinphos - coumafos	35 - 76
Sicilia	2009	16	4	chlorfenvinphos - coumafos - fluvalinate - acrinathrine	20 - 102
	2010	4	2	coumafos - fluvalinate	8 - 3000
Sardegna	2009	17	9	chlorfenvinphos - coumafos - fluvalinate	10 - 559
	2010	3	1	flumethrin - fluvalinate - coumafos	10 - 183
Trento	2009	16	6	coumafos - fluvalinate – pirimicarb – cyprodinil	10 - 68
	2010	15	4	coumafos - fluvalinate – pirimicarb – cyprodinil - pyrimethanil	10 - 217
Toscana	2009	19	7	coumafos - fluvalinate - metalaxyl - acrinathrine	11 - 200
	2010	20	6	fluvalinate acrinathrine	25 - 863
Umbria	2009	22	17	chlorfenvinphos - coumafos – imidacloprid – fluvalinate – tiametoxan - cyprodinil	10 - 1157
	2010	27	10	chlorfenvinphos - coumafos - fluvalinate	13 - 450
Veneto	2009	20	17	cyprodinil - piperonil butossido - chlorfenvinphos – coumafos - fluvalinate - acrinathrine	7 - 760
	2010	26	21	chlorfenvinphos - coumafos - fipronil – fluvalinate - acrinathrine	10 - 459

Table 19 - Results of chemical analyses conducted on pollen samples collected in 2009-2010.

Module	Year	Analysed samples	Positive samples	Detected active ingredients	Concentration (ng/g)
Abruzzo	2009	12	6	chlorfenvinphos - coumafos - kresoxim methyl – acrinathrine - coumafos - fludioxonil - fluvalinate	17 - 431
	2010	4	4	fluvalinate - coumafos	20 - 288
Bolzano	2009	6	1	cyprodinil	8
	2010	3	2	fluvalinate - coumafos - imidacloprid	99 - 363
Basilicata	2009	6	0		
	2010	9	3	fluvalinate - coumafos - thiamethoxam	16 - 1619
Calabria	2009	15	6	fluvalinate - coumafos - flumethrin	23 - 476
	2010	55	18	rotenone – flumethrin – dimethoate – fluvalinate – coumafos – fludioxonil – clothianidin – acrinathrine - chlorfenvinphos – flumethrin – dimethoate - fenpiroximate	12 - 1560
Campania	2009	5	2	cyprodinil - fludioxonil - dimethoate	7 - 25
	2010	20	4	dimethoate - coumafos - metalaxyl	9 - 50
Emilia Romagna	2009	2	0		
	2010	25	5	coumafos – fludioxonil - fluvalinate	44 - 295
Liguria	2009	18	9	fluvalinate - coumafos	45 -1508
	2010	23	3	fluvalinate	47 - 367
Lazio	2009	20	7	fluvalinate - rotenone - propamocarb - pyrimethanil	18 - 393
	2010	16	7	propamocarb - fluvalinate	58 - 2328
Molise	2009	12	2	propamocarb - coumafos	68 - 193
	2010	20	5	oxamil - propamocarb - fluvalinate	38 - 153

Marche	2009	17	8	propamocarb - pirimicarb - coumafos	10 - 5616
	2010	15	5	coumafos - propamocarb	22 - 1712
Puglia	2009	14	0		
	2010	10	0		
Sicilia	2009	7	1	acrinathrine	325
	2010	5	1	fluvalinate	67
Sardegna	2009	17	2	imidacloprid - metamitron	9 - 14
	2010	18	2	imidacloprid - fluvalinate	20 - 55
Trento	2009	6	0		
	2010	11	6	pyrimethanil - tiametoxam - dimethomorph – pirimicarb - flumethrin	14 - 584
Toscana	2009	18	4	chlorfenvinphos - fluvalinate – acrinathrine	73 - 215
	2010	20	7	fluvalinate – acrinathrine - thiophanate methyl	76 - 302
Umbria	2009	22	3	benalaxil - fludioxonil - metalaxyl	10 - 44
	2010	27	5	chlorfenvinphos – dimethoate - propamocarb - fenamidone	8 - 1335
Veneto	2009	0	0		
	2010	8	3	kresoxim methyl - tebuconazole - coumafos - fluvalinate	84 - 167

4.1 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 are performed in cooperation with the APENET network.
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.
With regard to the spring of 2010, reports (Table 20) 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.
Analagous to the previous year, in spring 2011 no report came from maize-growing areas, and 14 out of the 16 reports registered until the end of June 2011 were official.
Between May and September 2011 further reports of bee die-offs from various parts of the country were received by the three institutes involved in the APENET project (CRA-API, IZS-Ve and DiSTA-UNIBO). The details of each report are visible in Table 21.

It is important ot note that the APENET project was officially terminated at the end of March 2011, together with the associated reporting system. The subsequent reports are thereby fruit of voluntary service.

Table 20 - 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).

Region	N. of reports in maize-growing areas		Other reports during spring 2009	Spring 2010		Spring 2011	
	Spring 2008	Spring 2009		Maize-growing areas	Other reports	Maize-growing areas	Other reports
Lombardia	40	1		-	nd	-	-
Piemonte	8		2	-	nd	-	1*
Emilia-Romagna	7	1 + 1*		-	2** + 3*	-	2+1*
Veneto e Trentino	20		3	-	8***	-	9
Bolzano				-	2	-	1
Friuli Venezia Giulia	110		1	-	1	-	1
Abruzzo						-	1
Calabria	0		1	-	1 + 2*	-	-
Basilicata				-	1	-	-
Sardegna					1	-	-
TOTAL	**185**	**2 + 1***	**7**	**0**	**16 + 5***	**0**	**16**

* Non-official reports

** One of the reports concerns an APENET network apiary

*** In two cases the presence of the following was revealed:

1. Bees: thiametoxan, penconazole
2. Leaves: acetamiprid, iprodione, tebuconazole; bees: acetamiprid

Analyses of the other samples are under way.

nd = as confirmed by direct contact (2/7/10) with IZSPLV (Asti) and IZSLER (Brescia) no official reports of bee die-offs were received.

Table 21 – Reports received by the APENET network after March 2011.

Date (or period)	Area (Province)	N° hives affected/ Total hives in apiary	Probable cause indicated by beekeeper	Action taken by relevant Health Authority, analyses, etc.
Beginning of May	Lecce	100/100 (in 2 apiaries)	Aphid treatment on citrus and watermelon cultivations	Yes (ASL Lecce), analyses under way
15 June	Bologna	19/19	Pesticide poisoning	Yes (IZS-LER), negative results
30 June	Bologna	18/18	Pesticide poisoning	No, analyses under way at CRA-API
5 July	Cremona	20/20	Treatment against adult stages of Western Corn Rootworm carried out during maize flowering	Yes (ASL Cremona), analyses under way
14 July	Cremona	22/22	Treatment against adult stages of Western Corn Rootworm carried out during maize flowering	Yes (ASL Cremona), analyses under way
20 July	Bologna	24/24	Pesticide poisoning	No, no sample collection
15 August	Cremona	20	None	Yes (ASL Cremona), analyses under way
8 August	Grosseto	48/48	Treatment against olive fly	Yes, analyses under way at IZS-LT
End of August – beginning of September	Lecce	145/175 (in 2 apiaries)	Treatment against grapevine moth	No, analyses under way at CRA-API
Second half of September	Catania	180/200 In the area 90% of 3500 hives were affected	Treatment with organophosphates (chlorpyrifos, phosmet, piriproxifen) against *Protopulvinaria piryformis* in the presence of honeydew (treatment performed according to production protocol)	No action taken by Health Authority despite repeated requests; analyses under way at CRA-API; action taken by Plant Protection Authority and University of Catania

5. Determination of the minimum level of dust dispersal during coated maize seed sowing with modified seeders and estimated effects on bees

PART A: Static trials aimed at establishing a method for evaluating the efficiency of reduction of abrasion-induced dust and experimental assessment of a dust reduction device prototype devised by CRA-ING

5.1 Introduction

The decision to postpone the suspension of use of neonicotinoids and fipronil to coat maize seed, officialised with a Ministerial Decree on 16th September 2010, was taken " with the aim to safeguard the national honey bee population and the safety of people involved" (Agriculture Minister Galan).
The suspension document reports that "the studies (carried out in the frame work of the APENET roject) on the sublethal effects of the active ingredients clothainidin, thiamethoxam, imidacloprid and fipronil on bees, especially as far as learning ability, olfactory memory and spatial orientation are concerned, clearly showed negative effects on bees even at low concentrations of the active ingredients contained in the dust released by the seeding machines when sowing".
The Decree contains the official request of the Minister of Agriculture, Food and Forestry, to the Agricultural Research Council (CRA), "to increase the activity aiming towards the development of new and more efficient prototypes of deflectors to be applied to the seeder machines".
In the framework of the INTRAC project, which has recently received funding by the Agriculture Ministry, among other things, CRA-ING aims to develop appliances for precision maize seeders which will reduce abrasion-induced dust dispersed during sowing of coated seed. Although the main objective of the project is to reduce the operator risk of exposure to chemical agents, the concentrations of a. i. released by the seeders with the modifications developed by the INTRAC project can be used as a baseline fro risk assessment on bees within the APENET project.
The aims and the time-frames proposed for the two projects for the studies concerning coated seed were the following:

1. Determination of the minimum level of dust released by modified seeders during maize sowing technically obtainable by applying modifications to the seeders.

2. Evaluation of sublethal effects on bees of the concentrations determined in point 1, in experiments conducted by CRA-API and University of Bologna.

5.2 Performed activity

- Optimisation of the system for fixed point tests set up in 2010 (artificial wind, simulated soling) to quantify the the amount of dust released by seeders with and without modification.
- Set up of two prototypes of modifications which can be applied to the seeders in order to reduce the amount of dust released into the environment.
- Analyses and data processing for evaluation of the modifications and for the estimation of the dispersal theoretically obtainable in the field with the same wind conditions.
- Communication of the concentration levels to the colleagues in charge of the experiments on the bees.

5.3 Materials

5.3.1 Fixed point test

The first trials with fixed point seeders were 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 in relation to the type of damage inflicted on seed.
The development of a fixed point system and the evaluation of machines and devices designed to abate dispersal of abrasion dust constitute part of the activities specified in the APENET research project for which CRA-ING is responsible.
The main aim was 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 pursued 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 for such machines.

The trial area, represented diagrammatically in Figure 7, 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.

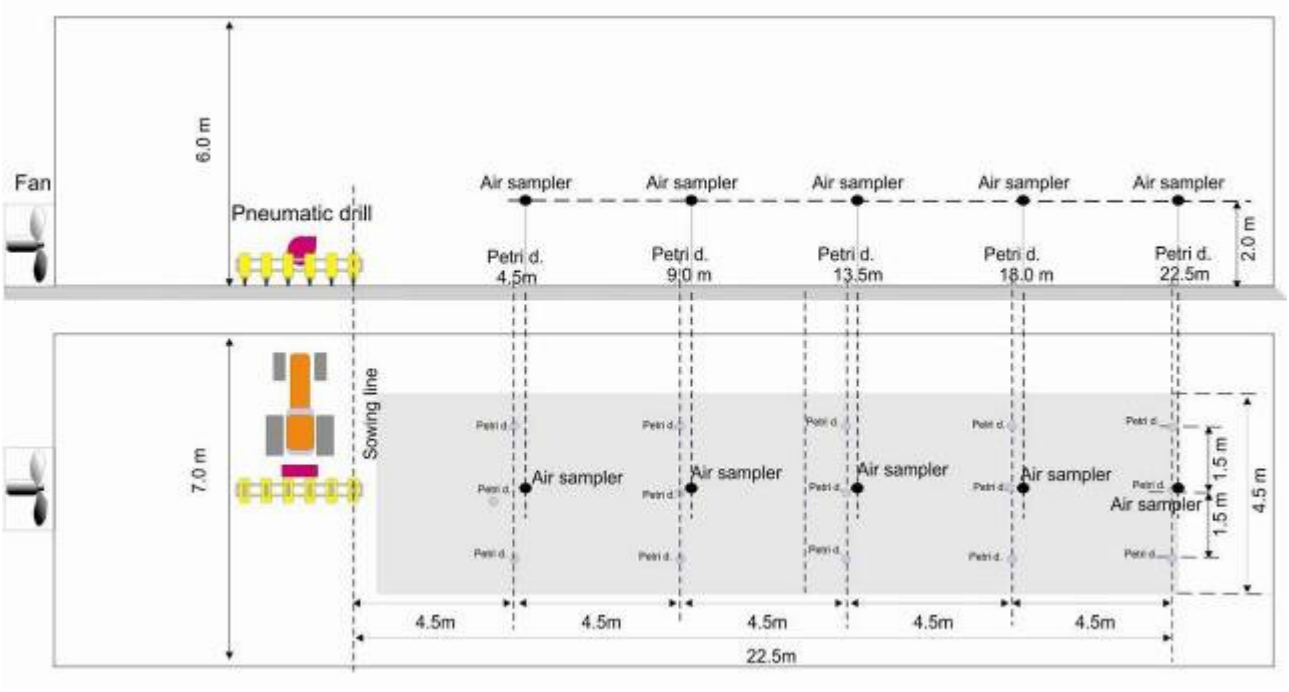


Figure 7 –Schematic of the layout of the fixed point trials. The Petri dishes were placed to the right of the seeder, at distances representing multiples of the sowing width (4.5 m, 9 m, 13.5 m, 18 m 22.5 m) along three rows spaced 1.5 m apart. Artificial wind was generated by an industrial ventilator powered by an electric motor. The rotation regime was regulted by an inverter at 1358 rpm for all trials, in order to obtain conditions of constant wind.

Sampling at ground level paralleled the procedure adopted in field tests conducted both within the APENET project (2009 activity) and 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, some modifications concerning the controlled conditions were introduced. 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 five 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 7.

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 47 mm diameter. For the sampling procedure, the five instruments were set at an air flow of 15 l/min.
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 8). 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) and depression inside the seeder's pneumatic system.

Figure 8 – Left: electric motor and inverter used to power the drive wheel; Right: electric motor and inverter used to power the depressor.

5.3.2 Seeder machine used in the trials

A Gaspardo Magica six-row precision pneumatic seeder (planting layout 0.75m x 0.18m, 75000 seed /ha) (Figure 9-A), was used, as for the 2010 trials.
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 (Figure 9-B). 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.
Prior to use in the experiments performer by CRA-ING (begun in March 2010), the machine was subjected to trials by the Julius Kuhn Institut di Braunschweig (Germany) (JKI method) which

certified that the seeder equipped with deflectors reduced dispersal of a tracking dust (brilliant-sulfoflavina) by at least 90% compared to a MONOSEM seeder. The MONOSEM seeder represents the reference machine for calculation of seeder dust dispersion with the JKI method.
It is useful to note that in the APENET fixed point and field trials, performed in 2010, the dust abatement obtained by applying deflectors was measured to be on average 50%, ranging from 30% to 70% reduction, according to the considered a. i.. Furthermore, the concentrations of a. i. measured at ground level during the field trials with the modified seeder, were found by the Units performing toxicity tests on honey bees, to cause sublethal effects.
It can thus be stated that the 90% dust dispersal abatement level certified with the JKI method is not sufficient to ensure harmlessness to bees.

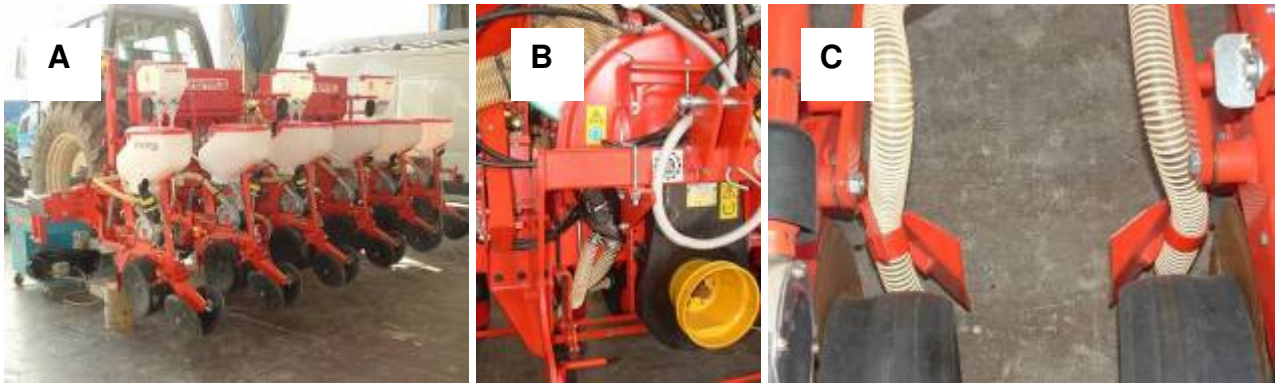


Figure 9 – A) Seeder utilized in the trials; B) detail of ventilator with air vent oriented downwards, on which the modification is applied; two of the four deflector pipes visible on the modification; C) two of the four deflector pipes that terminate behind the coulters.

Following results from the 2010 trials, activity was undertaken to increase the seeder dust dispersal abatement efficiency. To this aim two prototypes were built, applied to the Gaspardo-Magica seeder and tested in the fixed point test (5.3.1.). The description of the prototypes and the results of the trials are reported further on.

5.3.3 Seed

Hybrid maize seed "Lolita" supplied by Pioneer Hi-Breed (weight of 1000 seeds: 340g) was used for the trials. Seed was delivered to CRA-ING between 3rd-30th March 2010, thus causing delay in the beginning of the experimental activity. However, trials were initiated prior to delivery of the 2011 seed using unopened and perfectly conserved batches of imidacloprid coated seed left over from the 2010 trials.
This decision was taken considering the fact that at the beginning of the 2010 experiments, trials were undertaken to assess the dustiness of the 2009 seed (also kept in unopened and perfectly conserved batches): the Heubach test showed that there was no increase of dustiness of the same seed one year later. This trial is described in the 2010 report. For this reason, and to gain time, it was decided, only for imidacloprid, to use seed from the previous year (2010).
Differently from 2009 and 2010, in 2011 seed was not subjected to the Heubach test. As the same hybrid and same coating treatment as in 2010 were used, the dustiness values measured in 2010 were considered valid (Table 22).

Table 22 - Dustiness of seed treated with the 4 a. i., measured with the Huebach cilinder.

Seed coating (a. i.)	Manufacturer data	Data measured by CRA-ING

	Fine dust (Heubach filter) (g/q)	A. i. 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

5.3.4 Seeder modification prototypes devised by CRA-ING

CRA-ING developed two alternatives aimed at increasing the abatement power of the air deflectors described in the 2009 and 2010 reports. One alternative is envisaged for use on seeders already in use, by applying limited modifications. The second is envisaged for new seeders, wherein the modification is applied by the machine manufacturer. Descriptrions are reported in Figures 10 and 11, respectively.

The principle behind the modifications is to maintain the dust containing the a. i. inside the machine by a system of air recycling. To avoid that this may negatively influence the precision of the seeder, by altering the depression created by the centrifugal ventilator, the system was equipped with openings and appropriate filters that allow exit of the air in excess without the a. i. contaminated dust.

Prototype 1 (Figure 10) uses the common air deflectors to recycle air inside the seed hopper. The hopper lids are airtight and equipped with an upper opening that allows excess air to exit (right hand picture). This opening is provided with a filter that catches the dust. In this version the used filter is an air filter normally used for cars. An analogous prototype was applied to the Gaspardo Magica seeder, adapting it to the kind of defectors it is equipped with. On that version an activated carbon anti-pollen filter normally used for car interiors is applied.

Prototype 2 (Figure 11) was developed to optimise application of prototype 1, with the idea that it could be used on new seeders and directly applied by the manufacturers after an engineering study. Air exiting the deflectors is channelled into a collecting tube, and from here sent to the hoppers, equipped with air tight lids. In this case the air excess is channelled out through the lower side of the grey box located between two seeder elements, after having passed an internal filter that catches the dust. The system acts as an “impactor”, decreasing air speed and thereby allowing the dust to be deposited inside the circuit. In the process, the air is slightly heated (4-5°C), while the depression value is reached with a motor rotation speed 20 rpm lower compared to standard condition, thus contributing to energy saving. The filter used in the prototype is an activated carbon anti-pollen filter normally used for car interiors.

The single great air collector tube also has the function of making the recycled air pressure uniform, thus avoiding negative effects on sowing uniformity. The conFiguretion showed in the photographs is provisional and aimed at performing the fixed point tests; for field use the different elements will be placed maintaining the same functions.

Figure 10 – Prototype 1 of CRA-ING applied to MaterMacc seeder used in APENET 2009 trials. An analogous prototype was applied to the Gaspardo MAgica seeder, adapting it to the different air deflectors. This prototype was devised as a modification which can easily be applied to existing seeders.

Figure 11 – Prototype 2 of CRA-ING applied to the Gaspardo Magica seeder described in point 5.3.4. The filter used in this prototype is a common activated carbon anti pollen car filter.

5.4 Methods

5.4.1 Efficacy evaluation of the filtering material and physical characterisation of abrasion dust

This part of the activity was carried out in March and April 2011 and concerned only imidacloprid, as only seed coated with this a. i. was available. The activity was carried out in collaboration with the Institute for Environmental Contamination (IIA) of the CNR in Montelibretti (Rome), which possesses specific expertise in dust sampling.
In point 5.3.4 the two kinds of filters used in the modifications devised by CRA-ING are described. The activated carbon anti-pollen filter used in the most recent tests due to its supposed greater efficiency was tested in order to measure its ability in capturing abrasion dust and the a. i. in it contained. This evaluation was carried out by sampling air exiting from the seeder's pneumatic system, with and without modifications. The sampling was carried out during simulated fixed point seeding. Sampling was carried out in the following conditions (Figure 12):

1. machine without modification – all the air expelled by two of the four deflectors present on the machine, with no filtering system applied, was channelled into a single PVC pipe (diameter 118 mm) in which the sampling occurred (Figure 12 A);
2. all the air expelled by two of the four deflectors present on the machine was channelled into the prototype's grey filter box to be filtered, and from there to the same tube as described above for sampling (Figure 12 B);

3. the prototype was attached to the seeder as devised, and all the air exiting the filter box was channelled into the sampling tube (Figure 12 C). The results obtained from this condition compared to results from condition described in point 1 allowed estimation of efficacy of the whole modification (filter and tubes).

Figure 12 – A) Sampling of exiting air without filters; B) Sampling air directly editing filter; C) Sampling editing air when modification is attached to seeder ready for field use.

In the tested conditions air speed inside the ending tubes, sampling capacity and volume of treated air, were as much as possible constant, to ensure isokinesis (Table 23). The sampling described in the three conditions was performed by use of an isokinetic air sampler provided with PTFE Millipore filters with 0,2 µm pore diameter.

Table 23 – Filtering efficiency trial conditions

Cond.	Sampling condition	Air speed (m/s)	Air capacity (l/s)	Air capacity (m^3/min)	Total volume (m^3)
1	Air directly from two deflectors, without anti-pollen filter (F.A.P.)	3.09	33.79	2.03	22.30
2	Air directly from two deflectors, with F.A.P.	3.60	39.37	2.36	25.98
3	Air from complete modification: four deflectors with F.A.P.	3.10	33.90	2.03	22.37

5.4.2 Observations on dust drift

Estimation of dust drift abatement caused by the prototypes descrive in point 5.3.4. was carried out with the same conventional seeder (with no appliance). Trials were carried out in controlled conditions according to the procedure described in 5.3.1. and observing the dust deposited at ground level and the air concentration.

The seeder was raised from ground to ensure rotation of the traction wheel at a constant speed of 6 km/h. Each trial condition was repeated three times. Two seed doses (50,000 seedes) were distributed for each repetition, corresponding to 6666.67 m^2. Sampling commenced at the start of seeding and continued for 10 minutes after the end of seeding, to allow the drift/deposition of the dust in the air (in the field tests this additional sampling after seeding continued for 15 minutes). After each repetition the trial area was accurately cleaned and dust and a. i. residues removed by suction.

5.4.3 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 in Rome according to the HPLC MS/MS method, applying the methodologies devised by the

manufacturing companies for Imidacloprid and for the other a. i. at the time of product registration. An analogous method was adopted for analysis of the air sampling filters.

5.4.4 Processing of the test results

As a premise it must be stated that the test results refer to a theoretical sown surface of 6666.67 m^2, in which the sampling area is 4.5 m wide and 22.5 m long. The expected amounts of a. i. are very high in these conditions, and this is one of the reasons why tests on bees were not envisaged in these conditions, as they are not comparable to the real field exposure. Analyses conducted on the Petri dishes supplied the amount of a. i. contained in each dish (µg/dish). The quantity was measured firstly in relation to surface unit (µg/m^2). 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 (equal to 4.5 m for the hatched area in Figure 7) 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/m^2 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.

Analyses were also performed on the air samplers, in order to determine the quantity of a. i. 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.

5.5 Results

5.5.1 Fixed point tests

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.

The trial area was monitored prior to use in order to identify the distance between the atomizer and the seeder which would ensure greatest air speed uniformity in the whole test area, necessary to define the most suitable sampling distances from the seeder and to verify trial conditions repeatability. Figure 13 shows the results of these observations. Wind speed was always found to be within the 1-5 m/s range required by the *"BBA Drift Guideline, Part VII, 2-1.1, 1992, "Measuring direct drift when applying liquid plant protection products outdoors"* methodology.
The seeder acts as a wind shield for part of the air to be sampled, as can be noticed in Figure 14 which shows wind maps at the different monitored heights.
These conditions proved to be repeatable throughout tests. It was observed that a certain amount of dust is driven out together with the seed by the seeding elements (the average concentration measured after each test between the two seed-press wheels of each seeding element, by means of the air sampler with filters described in point 5.4.1. was 0.45 ppb). Since the seeder was raised from the ground, real field conditions were simulated by partially covering the seed-press wheels with plastic bags, up to the height that soil would reach in the field, so that the dust that would normally stay in the ground, would not undergo wind drift and bias the trials.

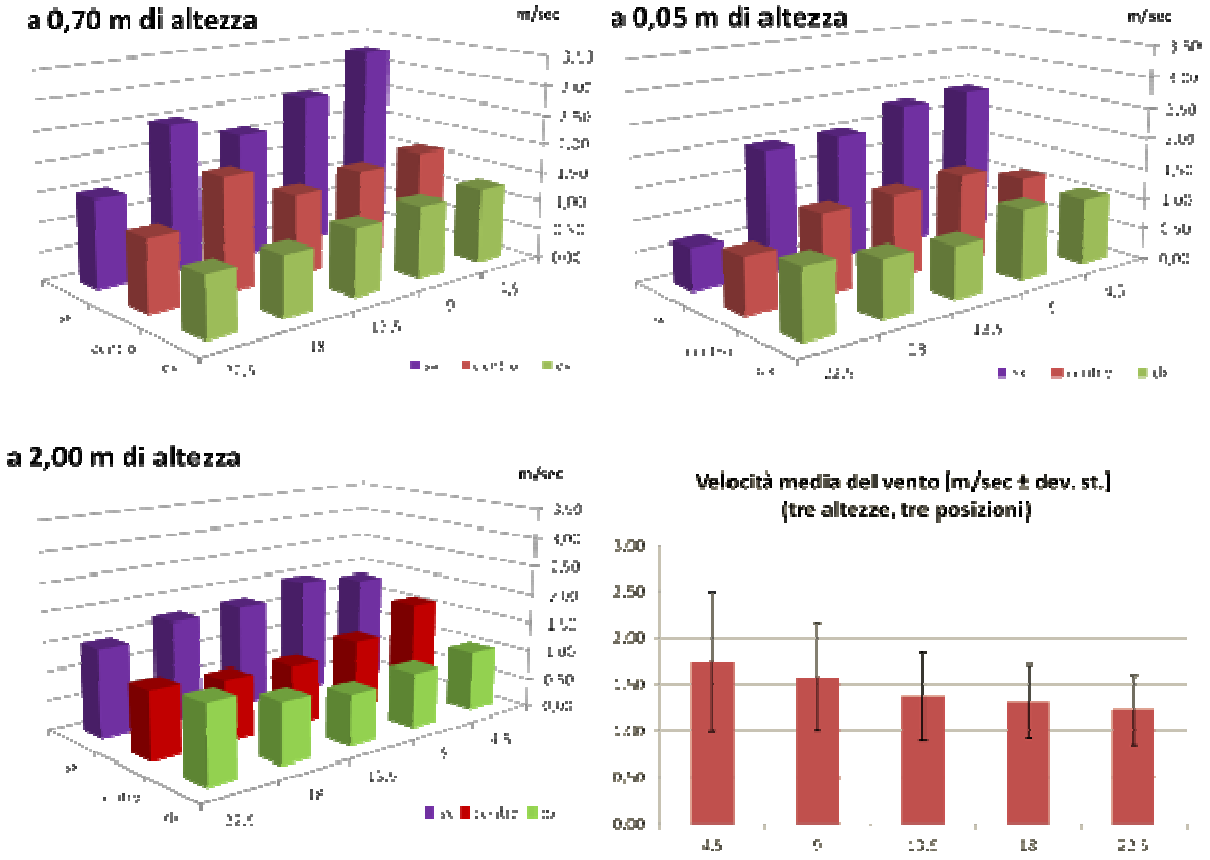


Figure 13 –Wind speed in the trial area, downstream from the seeder. The different monitoring heights and the average value are reported. Translation of text within diagram: *altezza* = height; *Velocità media del vento (tre altezze, tre posizioni)* = Average wind speed (three heights, three positions).

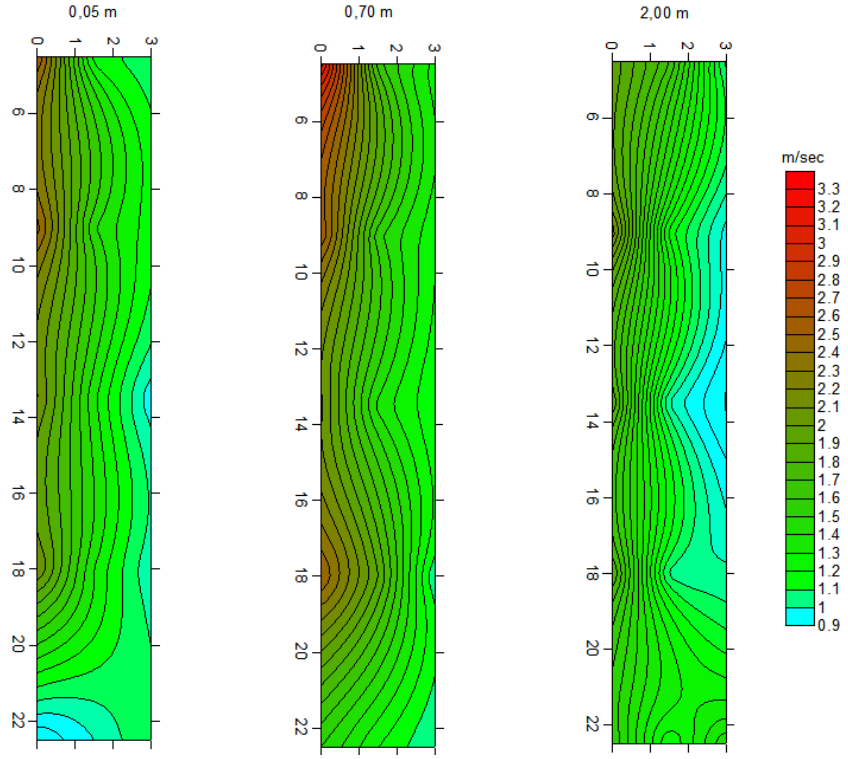


Figure 14 – Wind map in the trial area. On the right hand side of each diagram the wind shield effect of the seeder is noticeable.

5.5.2 Efficacy evaluation of the filtering material and physical characterisation of abrasion dust

Results of the evaluation tsts on the activated carbon anti-pollen filters (FAP) are reported in Table 24. The values resulting from the gravimetric and chemical analysis are coherent and show that the FAP on its own is able to reduce dust emission by 95.87% and a. i. in it contained by 95.20%. When inserted into the modification prototype devised by CRA-ING, abatement rises to 98.01% and 97.57%, for total dust and quantity of imidacloprid respectively, with reference to the volume of air expelled by the seeder during each test (column highlighted in orange in the table).

Table 24 – Results of the anti-pollen filter efficacy tests.

Analisi gravimetrica dei campioni (campionatori TECORA)						Rif a volume totale di aria espulsa dalla seminatrice			
condiz	volume aria, m^3	massa polvere, µg	concentr., µg/m^3	concentr., ppb	riduz., %	massa polvere, µg	riduz., %	polvere/sup seminata, µg/ha	Note
1	0,24	811,00	3337,45	2724,45	-	148867,84	-	225557,33	In entrambi i casi, a parità di concentrazione, trattandosi di 2 tubi su 4, la quantità di polvere espulsa va raddoppiata
2	0,23	31,58	137,90	112,57	95,87	6151,24	95,87	9320,07	
3	0,23	30,50	132,61	108,25	96,03	2957,52	98,01	4481,10	Il campionamento è riferito a 4 tubi su 4
Analisi chimica dei campioni (campionatori TECORA; p.a. imidacloprid)						**Rif a volume totale di aria espulsa dalla seminatrice**			
condiz	volume aria, m^3	massa p.a., µg/filtro	concentr., µg/m^3	concentr., ppb	riduz., %	massa p.a., µg	riduz., %	p.a./sup seminata, µg/ha	Note
1	0,24	80,70	332,10	271,10	-	14813,36	-	22444,48	In entrambi i casi, a parità di concentrazione, trattandosi di 2 tubi su 4, la quantità di p.a. espulso va raddoppiata
2	0,23	3,65	15,94	13,01	95,20	710,96	95,20	1077,21	
3	0,23	3,71	16,13	13,17	95,14	359,75	97,57	545,08	Il campionamento è riferito a 4 tubi su 4 (tutta l'aria espulsa dalla

Translation of text within table: *Analisi gravimetrica dei campioni (campionatori TECORA)* = Gravimetric analyses of samples (TECORA samplers); *Rif a volume totale di aria espulsa dalla seminatrice* = Reference

to total air volume expelled by the seeder; *Analisi chimica dei campioni (campionatori TECORA; p.a. imidacloprid)* = Chemical analyses of samples (TECORA samplers; a. i. imidacloprid); *condiz.* = condition; *volume aria* = air volume; *massa polvere* = dust mass; *riduz.* = reduction; *polvere / sup. seminata* = dust / seeded surface; *Note* = Notes; *In entrambi i casi, a parità di concentrazione, trattandosi di 2 tubi su 4, la quantità di p. a. espulso va raddoppiata* = in both cases, with same concentration, considerino that 2 of 4 pipes were involved, the amount of expelled a. i. must be doubled; *Il campionamento è riferito a 4 tubi su 4 (tutta l'aria espulsa)* = sampling refers to 4 pipes out of 4 (all the expelled air).

As far as the dimensional characterisation of abrasion dust is concerned, the data are still being processed. However, some results from the test with the laser particle counter are available and shown in Figure 15, indicating the filter's ability in capturing dust particles according to thei size. The bars on the left show the number of particles present in non-filtered (blue bars) and filtered air (red bars). The difference between the blue and red bars gives an idea of the amount of particles captured for each dimension class.

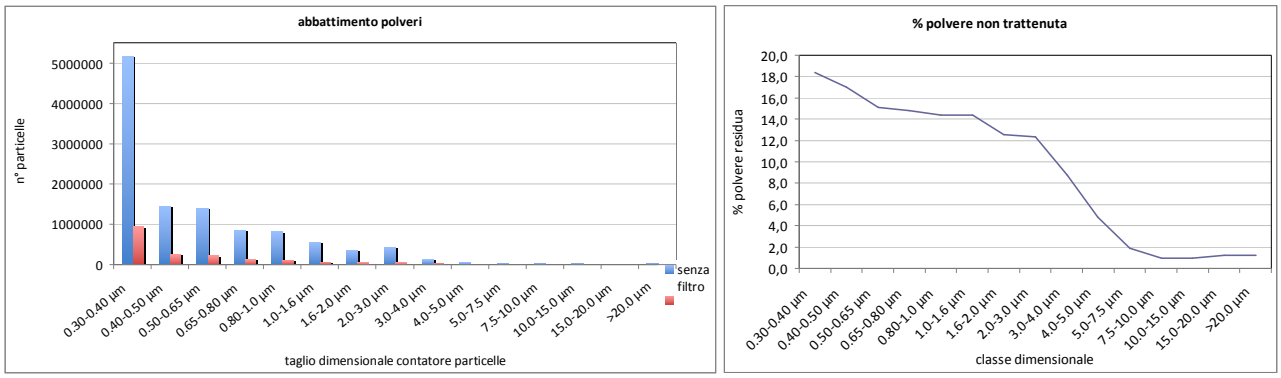


Figure 15 – Results of the test with the laser particle counter. Left: bars with the number of particles per dimension class; right: percentage of dust not captured by filter compared to the amount released by a conventional seeder. Translation of text within diagrams: *Abbattimento polveri* = Dust abatement. *% polvere non trattenuta* = % non captured dust.

The filter proved to be efficient across all dimension ranges. With decreasing size of dust particles the percentage of non-captured dust increases (up to a maximum of 18.5%, diagram on the right). It must be stressed however that the finest fractions are quantitatively (in terms of mass) less relevant. On the other hand, due to their characteristics, they probably persist longer suspended in air and are thus more likey to drift.

5.5.3 Observations on dust drift

Concentrations at ground level and abatement percentage

Despite the delay in arrival of the coated seed batches, the simulted soling trials were completed for all a. i. and for all the seeder conFiguretions described in point 5.3.4. As a result of the delay, the analyses results are, at the time of writing, only partial. They are however sufficient to yield interesting results on the abatement level which can be obtained by applying the modifications developed by CRA-ING to a conventional seeder.

The available results concern tests with all four a. i.. For clothianidin, thiametoxam and fipronil only data from a few repetitions and from trials with prototype 2 are available, while all data from the imidacloprid trials are available (due to the earlier start of the trials). These trials were useful for optimisation of the modifications. The results were variable, starting with lower abatement values and proceeding to values in line with expectations (Figure 16). The reported values are mean values of the concentrations per m^2 measured at each sampling distance. As these are fixed point tests the values are very high and correspond theoretically to the quantity of dust that would have fallen at ground level when sowing a surface of 6666.67 m^2.

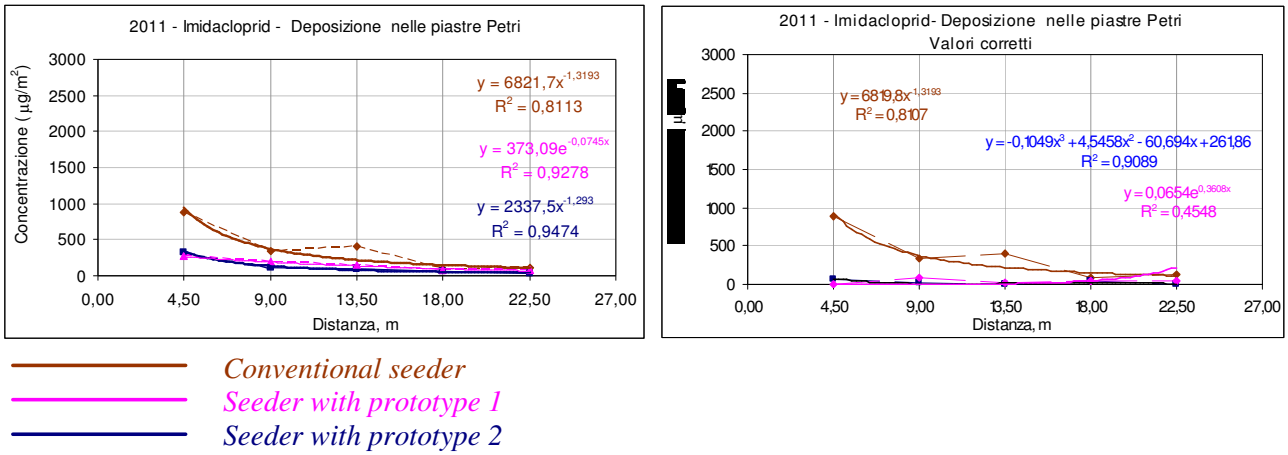


Figure 16 – Concentrations of imidacloprid measure in the fixed point tests. Left quantity of active ingredient in the Petri dishes at the various distances from the seeder, in relation to the surface unit (m^2); right: plots corrected according to optimisation operations carried out on the prototypes. Regression curves are not shown for a clearer reading of the graph. Translation of text within diagrams: *Deposizione nelle piaster Petri* = Deposition in Petri dishes.

The graph on the left shows a rather irregular trend. On the same graph the regression functions related to the three seeder conFiguretions are reported. By integrating the regression curves in function of the distance it was possible to calculate the total amount of dust deposited at ground level in the whole trial area, and to calculate the abatement percentage, which was 64.4% for prototype 2 and 54.4% for prototype 1. The comparison between this result and the high abatemnebt values measured in the estimation of the FAP filter efficacy, induced a specific search of possible dust dispersal from other parts of the seeder. It was thus revealed that significant amounts of dust were dispersed through a gap that formed in the cupling edge between the deflectors and the centrifugal ventilator nozzle, due to deterioration of the original manufacturer's sealer (silicone).
Moreover, a certain amount of dust was dispersed through the inspection windows of the six seeder elements. These points were sealed, and by repeating the tests and analysing the data it was possible to determine the amounts of dust and a. i. previously dispersed through these gaps. The obtained values were used to correct the data in the left hand diagram of Figure 16, thus resulting in the right hand diagram, where the abatement levels, calculated from the regression curves as above, was 89% for prototype 1 and 95.4% fro prototype 2. In Figure 17 the graphs of ground level dust dispersal available at the time of writing for clothianidin, thiamethoxam e fipronil are reported. The trials on these a. i. were carried out in this order, after imidacloprid, and observations and changes in the prototype set up were carried out for the whole duration of the trials.
Data analyses (using the same method descrive for imidacloprid, with integration of regression curves on the distance) showed the following abatement levels: clothianidin: 74.4%; thiamethoxam: 88.6%; fipronil: 94.8%.

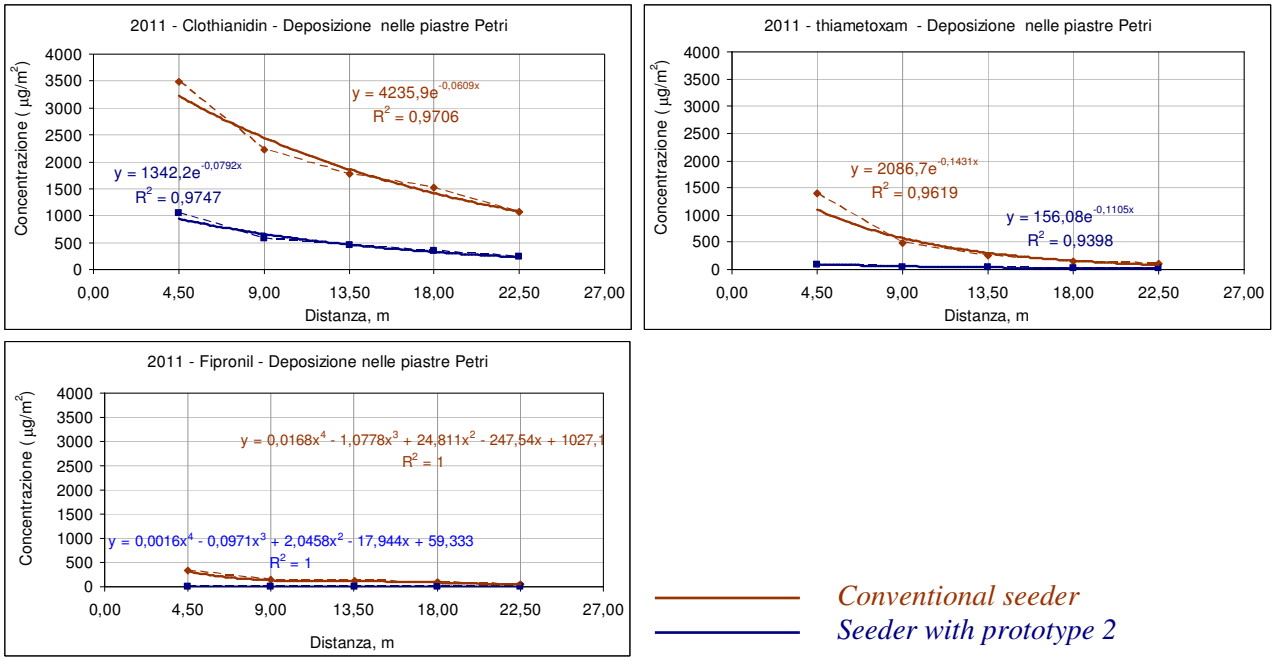


Figure 17 – Concentrations of clothianidin, thiamethoxam e fipronil measured in the fixed point tests. The regression curves were used to calculate the total quantity of dust dispersed by the seeder in the two tested conFiguretions, by integrating on the distance (0-25 m). Translation of text within diagrams: *Deposizione nelle piaster Petri* = Deposition in Petri dishes.

It is interesting to note that, as observed in the 2010 trials, the amount of dispersed clothianidin was much higher compared to the other a. i.. This was visible to the naked eye from the amount of red dust present on the ground in the trial area, even at the greater distances. This phenomenon could be due to the lower dust abatement obtained for clothianidin.

Air concentration

The amounts of a. i. detected on the filter discs applied to the air samplers, related to the volume of sampled air and its density, provided the a. i. air concentration (ppb). For imidacloprid the observations made in point 5.5.3 concerning the necessity of adjusting the prototypes apply, and the recorded data was thus corrected. The results are reported in the graphs in Figure 18. The reduction of air concentration after applying the modifications is clear.

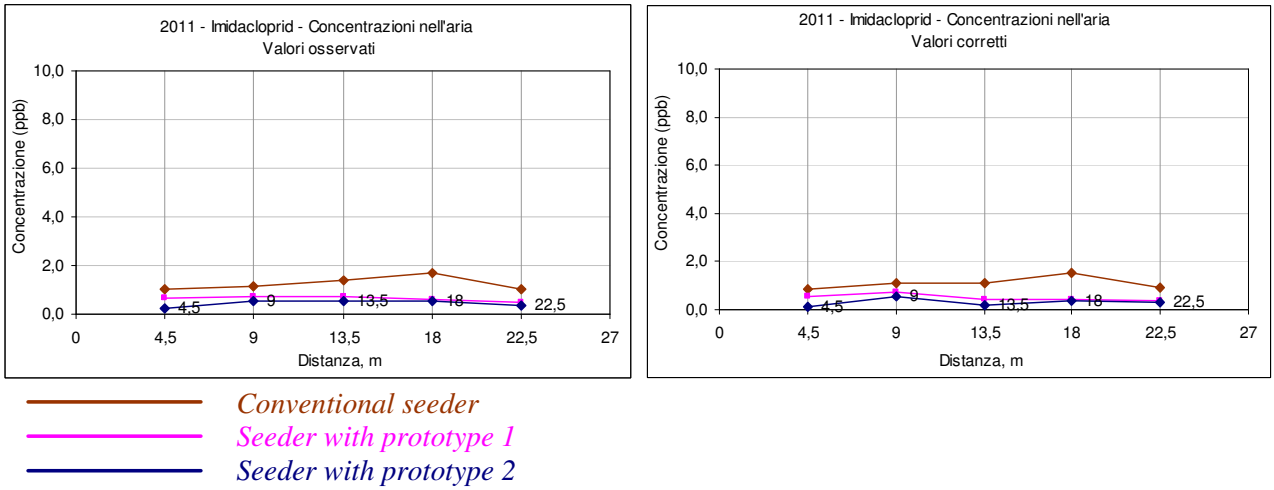


Figure 18 – Air concentration of imidacloprid in the fixed point test. Left: data from the first set of trials; right: corrected plots on the basis of optimisation operations on the prototypes. Translation of text within

diagrams: *Concentrazione nell'aria* = Concentration in air; *Valori osservati* = observed values; *Valori corretti* = corrected values.

The raw data which produced the the plot on the left of Figure 18 showed a reduction of 62.93% and 46.04%, respectively for prototype 2 and prototype 1. When corrected as explained above, the reduction rises to 72.44% and 53.13%. The graphs show that values do not decrease with distance, suggesting a tendency of the dust to persist and drift suspended in air. This is in agreement with results of the dust characterisation tests that show the presence of a very fine fraction, not captured by the used anti-pollen filters. In Figure 19 the observed concentrations of clothianidin, thiamethoxam e fipronil are plotted.

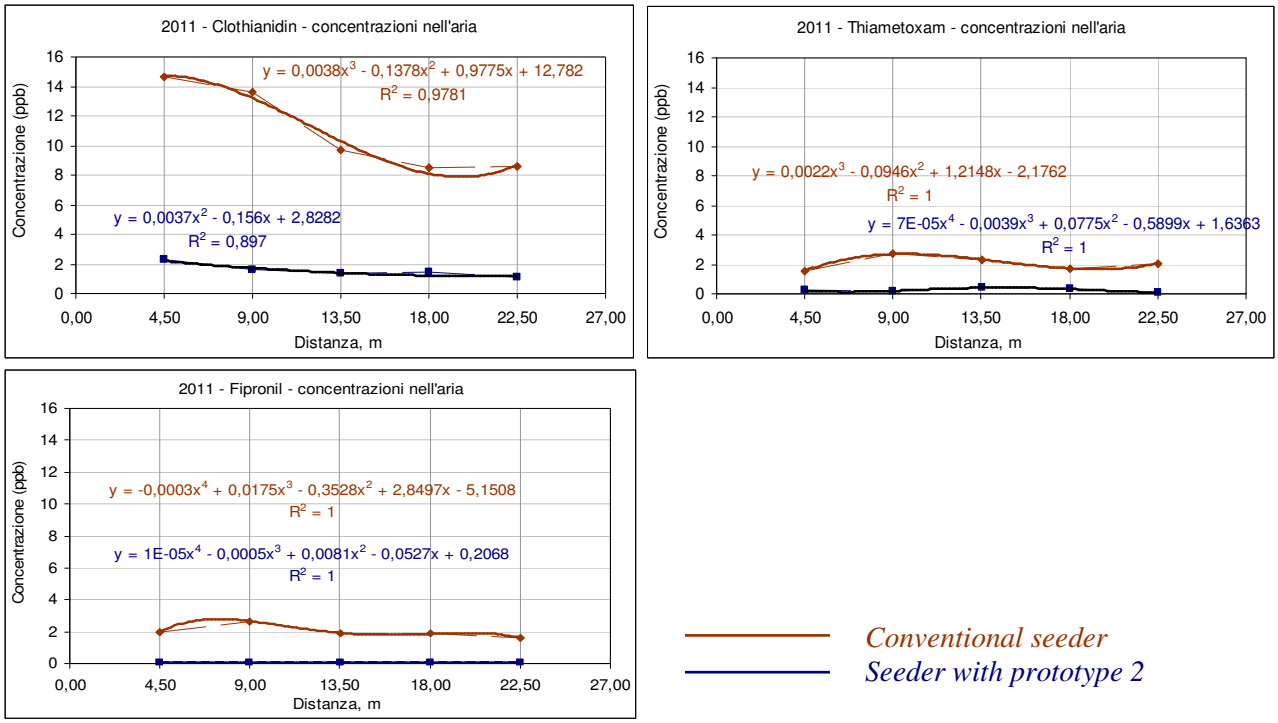


Figure 19 – Air concentration of clothianidin (upper left), thiamethoxam (upper right) and fipronil (lower left) observed in the fixed point tests. Translation of text within diagrams: *Concentrazione nell'aria* = Concentration in air.

Clothianidin air concentration was markedly higher than the other two a. i., analogous to what observed in the ground level trials. However, the concentration distribution in relation to distance, observed in the trials with the conventional seeder, is similar for the three a. i.. The reduction due to use of prototype 2 is measured to be 86%, 90%, 96% respectively for clothianidin, thiamethoxam and fipronil. The concentrations don't seem to be influenced by the distance from the sowing area and confirm the observations made for imidacloprid.

5.5.4 Forecasting field concentrations

Two examples of forecasting ground level concentrations for clothianidin, thiamethoxam and fipronil, calculated from the values measured in the fixed point tests (Figure 20). The calculation method is described in point 5.4.4. The concentration values are compatible with those measured in the field tests in 2009 and 2010. Integrating the regression plots with the distance from the sowing area, the quantity of a. i. that is dispersed on a hectar of ground contiguous to the sowing area can be calculated.

The reduction of dust concentration linked to use of prototype 2, calculated in the same way as above, was similar ro the one indicated in point 5.5.3.: 85.9% for imidacloprid, 78.3% for clothianidin, 88.4% for thiamethoxam, 91.5% for fipronil.

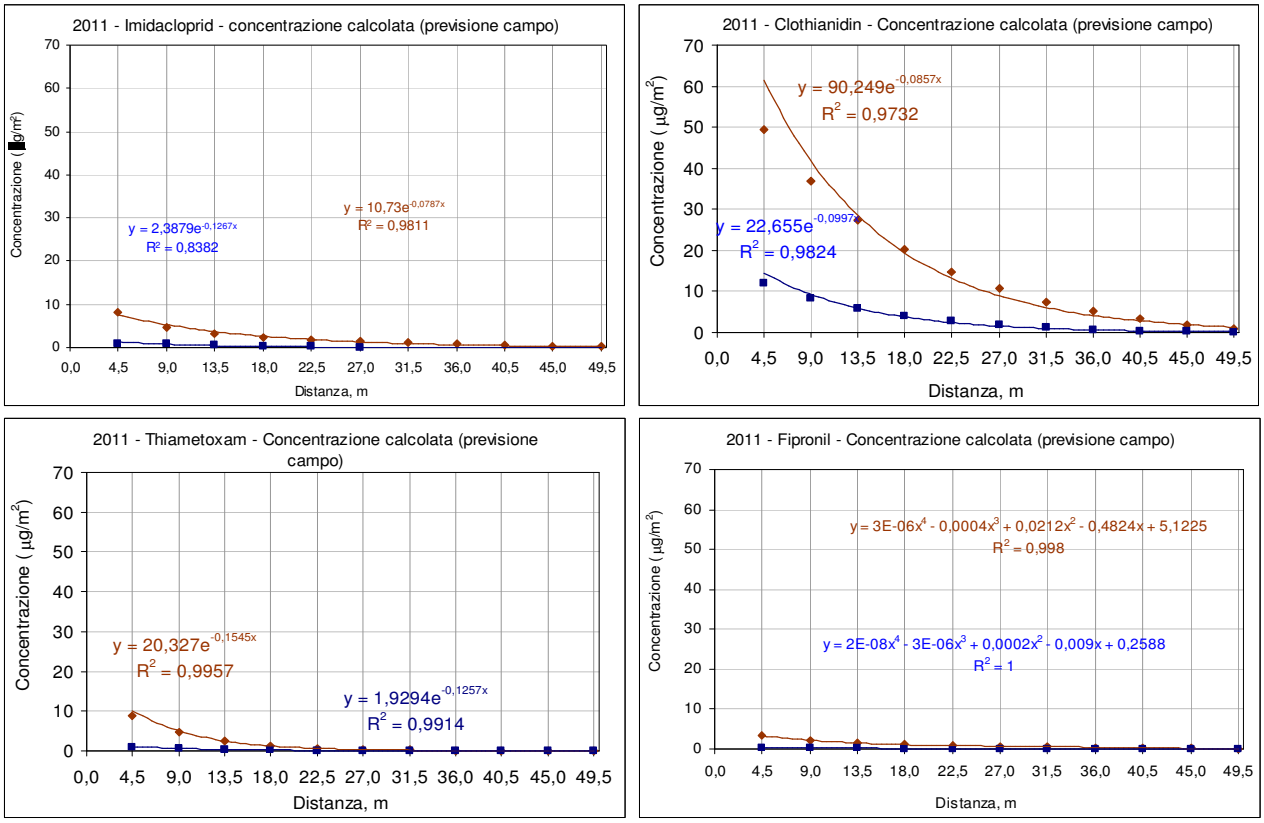


Figure 20 –Forecast of ground level concentrations in the field with the conditions applied in the fixed point tests. Translation of text within diagrams: *Concentrazione calcolata (previsione campo)* = calculated concentration (field forecast).

5.6 Conclusions

1. The filters used in the trials showed a marked efficiency in capturing dust and the substances it contains (up to 97% of the a. i.). The ability to capture the dust decreases with dust size. All dust particles greater than 4-5 μm are captured.
2. The modifications devised by CRA-ING significantly increase dust abatement of the air deflectors. Although complete results are not yet available, the preliminary data show that for prototype 2 the dust abatement at ground level increased from 50% with the air deflectors (2009 and 2010 trials) to 74.8% for clothianidin, 88.6% for thiamethoxam, 95.4% for imidacloprid and 94.8% for fipronil. With the exception of imidacloprid (for these trials considerations made in point 5.5.3 apply), the above values were obtained in the reported order, showing an imporvement of dust abatement linked to ongoing improvements to the prototype. It is therefore likely that in future trials a levelling of dust abatement at the higher levels may be obtained for all a. i. (with special reference to clothianidin).
3. The obtained dust abatement levels, applied to the values of ground level dust dispersal measured in the APENET 2010 trials with the same seeder, without air deflectors, allow calculation of the hypothetical concentrations that prototype 2 would have determined in those conditions. For assessment of risk on bee health of the concentrations obtained by use of prototype 2 (follone by prototype 1), one must refer to results of experiments carried out by the units in charge of these research lines (CRA-API, Università di Bologna).
4. Use of the modifications caused the same level of reduction of dust concentration in the air and at ground level, with the same reduction percentages. It is important to note however that the finest dust fraction is not captured by the filters used in the prototypes. In agreement with what is stated in point 1, and observing the concentration trend according to distance from source, it appears that the dust persists in the air and is able to move across a great distance.The presence of this dust fraction in the air gave rise to the need of measuring the quantity of a. i. met and captured by a bee in flight in an area where abrasion dust is present. To this aim, various Units participating in the project planned an experiment in which a 4 hectar plot was sown using the seeder equipe with prototype 2. . In the course of the trial bees are exposed to the dust cloud formed during the sowing operations. Bees are then analysed to determine the amount of a. i. captured by their bodies, in relation to certain parameters and reference values (lethal and sub-lethal dose). The field sowing test also has the aim of evaluating functionality of the seeder with prototype 2 (uniformity of seeding) in field conditions. Details of these trials are reported in Part B of this chapter.
5. The solutions proposed by CRA-ING are prototypes which are not commonly used in real field conditions. The low dust dispersal concentrations obtained with these prototypes represent the current lowest possible concentration which can be technically reached. If these concentrations prove to be innocuous for bees, thereby gaining the status of threshold levels that must not be exceeded, the next step will be to develop a strategy to equip all seeders with modifications (either the ones here described or others equally efficient), also considering the enourmous number of seeders in use in Italy.
6. Application of the modifications described in these studies entails handling the spent filters which need to be periodically substituted. The result is that the problem of dust dispersion is shifted from a non controllable environment (the field) to a controllable one. The functional duration of a filter will be assessed with specific tests. It is obvious that the duration must be as long as possible. Manipulation and disposal of the spent filters must be enacted according to specific protocols for operator health protection (use of gloves, masks with filters, protective goggles, etc). The disposal will presumably occur with the same procedures used

for pesticide packaging. Following EU law, member states will have to establish specific protocols for disposal of agricultural waste within 2013.

7. In account of the fact that the finest diust fraction is not captured by the filter, and seems to persist in time and space (Figures 18 and 19), CRA-ING has devised solutions to further increase the abatement efficacy of the prototypes. These modifications will be the object of a new project concerning safety and ergonomics, now in the starting stage (INTRAC project, funded by MiPAF), and coordinated by Dr. Carlo Bisaglia of CRA-ING in Treviglio. CRA-ING in Monterotondo will be in charge of the research line focusing on operator exposure to residues of pesticides during their application.
8. The last consideration concerns the fixed point test system. If a decision is made to introduce modifications with the aim of reducing abrasion dust dispersal, i twill be necessary to establish criteria to assess their efficacy, on the basis of reference parameters obtained with the APENET trials (concentrations of a. i. at ground level and in the air compatible with bee health). The tests performed during the APENET project point to a greater repeatability and comparability of results obtained from the fixed point test system rather than from the field tests. If a decision is made to certify the above devices, the assessment procedure must be modified in order to make it faster, more economical and more suitable for routine evaluations, while maintaining the following essential characteristics: 1) it should produce a result that can be expressed as concentration of a. i. which may be compared to reference values; 2) the test should be carried out with coated seed; 3) the substance used for seed coating must be harmless to operators involved and easily revealed (also by non chemical methods). All this requires activities aiming at optimising the method. For this reason, CRA-ING has prepared a draft for a trial methodology that considers the results and knowledge gained during the APENET project and parallel proposals put forward by other research units affiliated with ENTAM (European Network for the Testing of Agricultural Machines), a network which aims at defining a standard European test procedure. Although some parts of the proposal (choice of the tracking substance to be used as a. i. and of the most suitable analytic method) are still under study, this draft represents a concrete basis for future developments.

Part B – Field sowing trial: functional test of seeder with the CRA-ING prototype 2 for abrasion dust abatement; evaluation of ground level distribution and air concentration of residual a. i.; bee flight trials over the sown field

5.7 Aims of the trials

The trials had the following aims:

- observation of field performance of the CRA-ING modification named "prototype 2", with special attention to sowing quality ;
- indications concerning ground level distribution and air concentration obtained by use of prototype 2, in relation to abatement levels observed in the fixed point trials;
- assessment of lethal effects on bees flying over the seeding area where prototype 2 was used.

5.8 Materials and methods

The sowing trials were performed on the 12th July 2011 at CRA-ING in Monterotondo, on an approximately square plot measuring 4 hectares. The Gaspardo Magica seeder described in point 5.3.2, equipped with prototype 2 was used for sowing maize seed coated with clothianidin (belonging to the batches provided in March 2011 and used for the fixed point tests) (Figure 21). The hoppers were loaded on the edge of the field, with 12 seed sacks.

Figure 21 - Seed used in the trials.

5.8.1 Sowing quality

Assessment of sowing performance was carried out immediately after seeding by opening the soil along randomly chosen tracts of the seeding line, so as to uncover the seeds at the bottom of the furrow and to measure the distances between the seeds. The measurements were performed in accordance with the "ENAMA Cat. 04 Protocol – Seeding machines" (July 2002). The parameters used for assessment of performance quality of the seeder in the course of the sowing trials are the following:

- uniformity of transversal seed distribution for all seeder elements;
- efficacy of seed covering, by means of counting uncovered seeds present on soil surface;
- real distance between deposited seeds, in relation to theoretical distance established by the manufacturer in the calibration table and determination of the number of multiple seedings (distance between seeds 0 – 0.5 times the theoretical distance), regular seedings (distance

between seeds 0.5 – 1 times the theoretical distance) and missed seedings (when the distance between seeds is above 1.5 times the theoretical distance).

On the basis of these observations, seeding quality evaluation indexes were calculated as the ratio between the above seeding distance classes and the total number of seeding events observed in the trial.

The same observations were also performed after plant emergence, on the maize plantlets.

5.8.2 Distribution of dust abrasion dust containing an active ingredient

To monitor distribution of dust in the area adjacent to the sowed field, 2 series of 9 Petri disse were positioned at 1 and 5 m from the first seeding line. For this observation the side which was downwind immediately before the beginning of the trial was chosen. Distance between dishes of the same series was 10 m, thereby covering a 40 m strip (samping area) located at the centre of the chosen side (Figure 22). The Petri dishes contained a 50% acetronile solution used to capture the a. i. present in the dust.

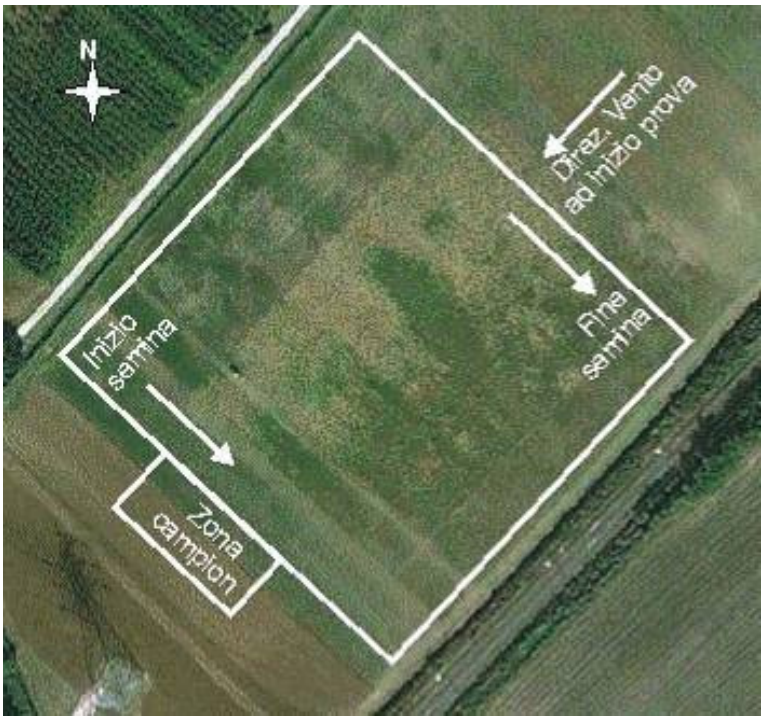


Figure 22 - CRA-ING field used for sowing trials (photo from Google Earth).

Four air samplers, 15 m from each other, were positioned above the series of Petri dishes placed at the 5 m distance. To obtain a greater correspondence with bee flight conditions, sampling height was placed at 2 m. PTFE Millipore filters with 0.2 µm pore diameter, measuring 47 mm, were used for air filtering. Sampling was carried out by setting the three instruments on an air capacity of 15 l/min.

Conservation of the samples and analyses methods are the same as thoise described in point 5.4.

Wind speed and direction were monitored continously with a wind dial ANEMOMETRO placed at 2 m from the ground. For data processing, the mean values of the angles (compared to the North) from which the wind was blowing, and its speed, were calculated with reference to 1 minute intervals. Then the frequency of the mean angle values for frequency classes of 10° (from 0 to 360°) was calculated to produce a “radar” graph. Finally, for each frequency class the average wind speed was obtained and analogous “radar” graphs produced.

5.8.3 Bee flight trials

Honey bees from hives situated in the CRA-PAV farm, close to CRA-ING, were used. Forager bees of uniform age and size were selected and placed in small cages, visible in Figure 23 (one bee per cage), provided with feeders.
In each trial (or replication), 10 cages were hung to an alluminium horizontal bar, measuring 4 m length. According to the kind of trial, the bar was placed in the sowed field as described in Table 25. Bee exposure occurred during two complete runs of the seeder (back and forth). Length of the field was 200 m, speed of the seeder 1.67 m/s, thereby resulting in exposure of the bees to the dust cloud of 270 s on average (including flight time).
After having emptied the hoppers and having left the seeder running for a few minutes without seed to clean it out from dust residues, a control trial was performed in a field some distance away from the sown field. The control trial was performed in such a way that the bar with the cages followed the seeder (running without seed), at a distance of 4 m and 1.8 m height.

Figure 23 – Cages containing one honey bee each, used in the flight trials.

Table 25 - Experimental groups used in bee flight trials. Details of the experimental groups used in the bee flight tests.

Experimental group	Description	Repetions	Time of test
A	Cages are placed immediately after seeder at 2.5 m height, and follow the machine (Figure 24)	1	11:30
		2	11:40
B	Cages follow the seeder at 4 m from the tractor and 0.5 m height, to intercept the dust close to ground level	1	12:00
C	Cages follow the seeder at 4 m from the tractor and 0.5 m height, placed laterally, downwind to tractor, so as to intercept the dust cloud moved by the wind (Figure 25)	1	12:10
D	Cages follow the seeder without CRA-ING modifications (deflectors behind coulters, as suggested by producer)	1	15:00
		2	15:10
Control	Cages placed at 1.8 m height follow the seeder without seed at 4 m from the tractor	1	14:30
		2	14:40

Figure 24 – Experimental group A: the horizontal bar which supports the 10 cages at 2.5 m height is fixed to the posterior part of the seeder. The picture on the right shows the system during the seeding.

Figure 25 – Experimental group C: the bar that supports the 10 cages is kept at 1.8 m height by two operators. They proceed at 4 m from the seeder, laterally, so as to intercept the dust cloud shifted by the wind.

After the sowing test, the cages containing the bees were placed in damp chambers at 25°C for 24 h, provided with honey, and checked for the presence of dead bees. At the end of the monitoring period, dead bees were collected (separately for each experimental group) and kept at -20°C before chemical analysis.

5.9 Results and discussion

5.9.1 Seeding quality

Figures 26 and 27 show some examples of the observations described in point 5.8.1 concerning seed deposition and plantlet emergence.

The observations on seeds and plantlets allowed calculation of parametres which provide indications on performance of the seeder equipped with prototype 2. The results are reported in Table 26, together with a synthesis of the evaluations according to indications of the ENAMA Protocol mentioned in point 5.8.1, and adopted by CRA-ING for certification of seeder performance.

Figure 26 – Evaluation of regularity of seed deposition in the ground.

Figure 27 – Evaluation of plantlet emergence. As the trial was not aimed at obtaining a crop, the field was not subjected to normal agricultural practices such as weed control and irrigation. The plantlets, visible among the weeds, emerged in a uniform way, although they showed differences in development due to lack of water.

Table 26 – Main parametres of seeding quality derived from observation of seed distribution in the ground.

Uniformity and efficacy index	Value	Assessment*
Coefficient of transversal irregularity (%) (from static trials)	3.7	Excellent
Seed covering efficacy (%)	96.4	Excellent
Multiple seeding index (%)	0	-
Regular seeding index (%)	97.7	Excellent
Missed seeding index (%)	2.3	-

* The assessment range is described in the "ENAMA Protocol" (see 5.8.1).

On the basis of the obtained results it can be stated that use of prototype 2 does not cause deterioration of seeding quality.

5.9.2 Dispersal of abrasion dust containing active ingredient

Observations concerning wind conditions are synthesised in the diagrams in Figure 28. From the diagrams it is evident that wind direction was not constant during the trial, and changed compared to the direction which was assessed when choosing where to place the Petri dishes and the air samplers (Figure 22). Thus, these were downwind only in the first stages of sowing.
On the other hand, wind speed was fairly low (total average wind speed: 0.63 m/s), suggesting that dust dispersal far from the sampling area was limited. On this basis, samples were considered suitable for processing and analyses.

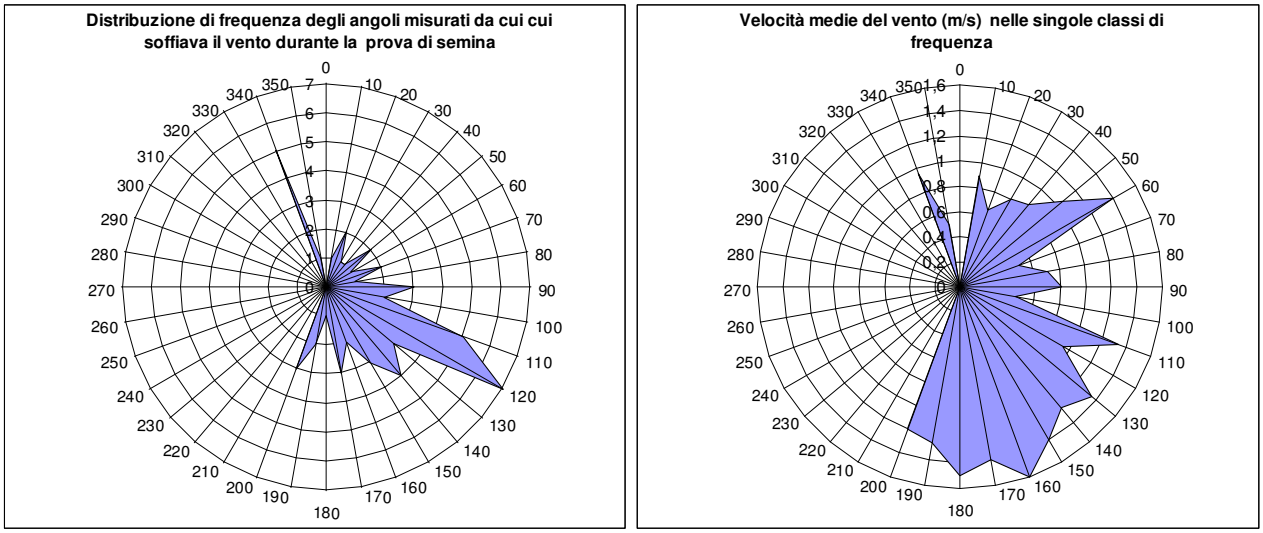


Figure 28 – Wind conditions during trial. Left: frequency of the one-minute intervals according to 10° angle classes. Right: average speed (m/s) values calculated for each angle class. Translation of text within the diagrams: *Distribuzione di frequenza degli angoli misurati da cui soffiava il vento durante la prova di semina* = frequency distribution of angles from which wind was blowing during the sowing trial; *Velocità medie del vento (m/s) nelle single classi di frequenza* = Average wind speed (m/s) in the single frequency classes.

Results of the analyses carried out by CRA-PAV in Rome are reported in Table 27. These consist in a.i. deposition (µg/m^2) observed in the 9 Petri dishes placed at the two distances (1m and 5 m) from the sowing line and the concentration (ppb) of a.i. in the filters of the four air samplers placed at 5 m between the Petri dishes.

The results show that at 1 m from the sowing line dust deposition was lower than the indicated analytic limit, while at 5 m, in 4 cases, the values of dust deposition were fairly high.
Of the four filters, the first two, placed in line with the Petri disse n. 1, 2 and 3, for which the highest values of dust deposition were observed, also had high concentrations of a. i., while the other two were below the LOQ.
The location of the positive samples appears to be concentrated in a single isolated area, as no traces of a. i. detected in any of the samples collected at 1 m from the sowing line. This result, together with the concentration levels observed in the positive samples, suggests a spot contamination with a. i. of a specific area, not connected with the sowing but rather with the seed loading and other preliminary seeding operations.

Table 27 – Results of analyses of Petri dish contents (LOQ <0.010 µg/dish) and air sampler filters (LOQ <0.010 µg/filter) following field sowing trials. Placing of data within the table reproduces location of sampling points in the field.

Petri dishes			**Filters**	
n	**1m**	**5m**		**n**
	µg/m²	**µg/m²**	**ppb**	
1	<LOQ	50.93	283.94	1
2	<LOQ	30.56		
3	<LOQ	30.56	84.38	2
4	<LOQ	<LOQ		
5	<LOQ	<LOQ	0.00	3
6	<LOQ	<LOQ		
7	<LOQ	<LOQ	0.00	4
8	<LOQ	28.01		
9	<LOQ	<LOQ		

5.9.3 Bee flight trials

Dead bee counts for all experimental groups are reported in Table 28, together with time of observation.
Bees which died within 24 h of exposure to abrasion dust produced during sowing were analysed individually by UFLC (Ultra Fast Liquid Cromatography) by the laboratory of the Department of Chemical Science of the University of Padua. The analytic limit of these determinations was LOD < 15 ng/bee. Results shown in Tables 29 and 30 indicate ng/bee detected in the control group, in experimental groups A, B, C (Table 29) and in experimental group D (Table 30).
Data from chemical analyses of dead bee samples show that application of prototype 2 to the seeder determines a consistent reduction of dust emission and consequently of a. i. compared to the same machine equipped with only the air deflectors which expel air behind the plowers. Data in Table 30 show that in all samples from experimental group D the quantities of detected a. i. were higher than LOD and than contact LD50 (equal to 21.8 ng/bee for clothianidin, Iwasa *et al.*, 2004). In 16 out of 24 samples from the other groups (Table 29) the quantity of a. i. was lower than the LOD, and of the 8 samples with levels higher than LOD 5 had levels of a. i. higher than LD50.
To conclude, some degree of bee mortality is observed even with application of the prototype, due to currently unmanageable leakage of residual dust from the seeder.

Table 28 – Dead bee counts in the 24 h following bee exposure to abrasion dust during seeding.

Tesi A - Rip 1 - Ora della prova: 11,30												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00		1		1							2
13/07/2011	8,30	1		1		1			1	1		5
13/07/2011	10,00											0
13/07/2011	12,00											0
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		7

Tesi A - Rip. 2 Ora della prova: 11,40												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00											0
13/07/2011	8,30		1					1				2
13/07/2011	10,00											0
13/07/2011	12,00									1		1
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		3

Tesi B - Ora della prova: 12,00												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00							1				1
13/07/2011	8,30		1	1						1		3
13/07/2011	10,00											0
13/07/2011	12,00					1						1
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		5

Tesi C - Ora della prova: 12,10												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00				1							1
13/07/2011	8,30		1			1	1	1				4
13/07/2011	10,00			1								1
13/07/2011	12,00											0
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		6

controllo 1 - ora della prova: 14,30												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00											0
13/07/2011	8,30	1										1
13/07/2011	10,00											0
13/07/2011	12,00											0
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		1

controllo 2 - ora della prova: 14,40												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00										1	1
13/07/2011	8,30					1						1
13/07/2011	10,00											0
13/07/2011	12,00											0
13/07/2011	14,00											0
13/07/2011	15,00											0
										Totale morte		2

Tesi D - Ripetizione 1 - Ora della prova: 15,00												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00							1			1	2
13/07/2011	8,30	1					1			1		3
13/07/2011	10,00											0
13/07/2011	12,00		1			1						2
13/07/2011	14,00											0
13/07/2011	15,00								1			1
										Totale morte		8

Tesi D - Ripetizione 2 - Ora della prova: 15,10												totale morte
data	ora controllo	1	2	3	4	5	6	7	8	9	10	
12/07/2011	18,00						1		1	1	1	4
13/07/2011	8,30	1		1	1	1						4
13/07/2011	10,00											0
13/07/2011	12,00											0
13/07/2011	14,00							1				1
13/07/2011	15,00											0
										Totale morte		9

Table 29 - Concentration of clothianidin in bees dead after exposure to abrasion dust from modified seeder. Key: *Tesi* = Experimental group; *Controllo* = control group; *Rip / Ripetizione* = Repetition; *Ora della prova* = Time of trial; *total morte* = total dead; *data* = date; *ora controllo* = time of count.

Clothianidin concentration (ng/bee)						
Samples	Control 1	Control 2	Above tractor 1	Above tractor 2	Behind tractor	Behind tractor downwind
1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2		18	30	147.5	11	39
3			22.5	< LOD	55.5	12
4			< LOD		< LOD	< LOD
5			< LOD		< LOD	< LOD
6			< LOD			< LOD
7			< LOD			

Sample number bears no relation with position of bees in cages.

Table 30 - Concentration of clothianidin in bees dead after exposure to abrasion dust from non-modified seeder.

Clothianidin concentration (ng/bee)		
Samples	Behind tractor First trial	Behind tractor Second trial
1	51	23
2	29.5	32
3	44	122
4	50.5	30
5	151	51
6	36.5	26
7	291.5	22
8	39	94.5
9		24.5

Sample number bears no relation with position of bees in cages.

Table 31 contains a synopsis of data reported in Table 28, expressed in percentages of dead bees. Considering that average mortality observed in the control group was 15%, the mortality data of the other groups should be corrected by subtracting this value from the observed mortality.

In experimental group A, a great difference between the two repetitions was observed (70% in the first and 30% in the second). The explanation for this difference consists in the fact that immediately prior to sowing the following operations were carried out:

1. a preliminary trial was carried out on the first sowing line to determine the points in which abrasion dust was expelled from the machine. To this aim the empty hoppers were filled with talc and the hopper lids left open, while the seeder proceeded for some meters. The air recycling system of prototype 2 entails return of the pipes on the hopper lids, thus a large amount of material (talc and abrasion dust already present inside the seeder which was not cleaned prior to use so as to simulate normal working conditions) was expelled by them. Thus the area was probably “contaminated” for the subsequent runs. If this hypothesis is correct,

analyses of dead bees from the first repetition should reveal residues of a. i. used in the previous trials (the last a. i. was fipronil, the penultimate thiamethoxam).

2. loading seed in the hoppers in proximity of the first sowing line: this caused a noticeable deposition of dust on the seeder. This dust, in the first seeding stages of the first repetition, may have drifted into the bee cages suspended on the bar on top of the tractor before being dispersed.

Following these considerations, we decided it was correct to exclude the results of the first repetition of experimental group A, while considering the 30% mortality data of the second repetition as valid. This value can be corrected to 15% (considering the control mortality), while the gain compared to mortality in experimental group D (seeder with normal deflectors that exit behind the coulters) is 78.6% (Table 31.
Mortality measured in experimental groups B and C was higher than group A, and the respective gains compared to experimental group D are consequently lower (respectively 50% and 35.7%). This can be explained by the different movement of the cages. In group B they followed the seeder at a height of 50 cm from ground level, coming into contact with the dust (earth and abrasion dust escaped from the action of prototype 2) raised by the seeding elements. Bee contact with dust is equally probable in group C, in which the cages were placed downwind compared to the sowing line so as to intercept the dust cloud.

Table 31 - Summary of mortality data fro each experimental group in the 24 h following the test.

Tesi	rip.	mortalità dopo 24 h				Note
		Valore (%)	Media (%)	Media corretta (rif. Controllo) (%)	Guadagno (rif tesi D) (%)	
A	1	70	30	15	78,6	Zona di semina inquinata da polvere durante carico seme e messa a punto - prova non valida
	2	30				
B	1	50	50	35	50,0	
C	1	60	60	45	35,7	
D	1	80	85	70	0,0	
	2	90				
Contr	1	10	15	0	100,0	
	2	20				

Key: *tesi* = experimental group; *rip*. = repetition; *mortalità dopo 24 h* = mortality after 24 h; *Note* = Notes; *Valore* = Value; *Media* = Mean; *Media corretta (rif. controllo)* = Corrected mean (with reference to control); *Guadagno (rif. tesi D)* = Gain (compared to group D); *Zona di semina inquinata da polvere durante carico seme e messa a punto – prova non valida* = Seeding area contaminated with dust during seed loading and development procedures – repetition not valid.

From a technical point of view it must be noted that, as described in paragraph 5.5 of part A of this chapter, prototype 2 is able to block up to 97% of the a. i. that hits the filter (point 5.5.2) and even, after several improvements, able to block up to 95% of the a. i. that would be expelled by a seeder

with no other dust abatement system (point 5.5.3). The residual expelled quantity cannot negatively influence the bees.
In our view the observed negative effects (groups A, b and C) are mainly due to the quantity of a. i. that exits, together with the seed, from the six distribution elements. It is useful to remember that in the static trials (part A, paragraph 5.5.2) conducted by placing the air samplers between the two back seed-cover wheels, the observed imidacloprid air concentration was 0.45 ppb, both with the prototype and without (it was not possible to repeat the trial for the other a. i.). This testifies a constant exit of dust from the distribution elements, not currently controllable. Possible solutions to contain these leakages are being devised and await testing.
As far as the bee flight tests are concerned the following conclusions can be drawn: application of the prototype to the seeder determines a considerable improvement compared to the same seeder equipped only with deflectors. However, negative effects on bees still occur, due to the amount of a. i. which escapes action of the prototype (as described a few lines above). A complete abatement (100%) probably represents a utopistic goal, and it is likely that bee mortality will continue to occur even with very low percentages of dispersed dust. On the basis of the set up of the bee flight trials (excluding the first repetition), we can state that:

- bees flying at a height of 2.5 m, following the seeder for 400 m and exposed to the dust cloud for 270 s had 30% mortality rate;
- bees flying at a height of 50 cm, following the seeder for 400 m and exposed to the dust cloud for 270 s had 50% mortality rate;
- bees flying at a height of 1.8 m, following the seeder for 400 m at a distance of 4 m, and placed sideways and downwind compared to the seeder had 60% mortality rate;
- control bees had 15% mortality rate;
- bees flying behind the seeder equipped witht the air deflectors only (without prototype 2) for 400 m and exposed to the dust cloud for 270 s had 85% mortality rate.

We do not think that these data can be generalised, as they represent the worst possible flight conditions for the bees, with respect to the sowing line and wind direction in the seeded field. Bees in these conditions have a mortality rate higher than control , although application of prototype 2 does reduce mortality compared to the condition “without prototype 2”.

6. Sub-lethal effects of neonicotinoids and fipronil on learning and memory of odours and spatial orientation

6.1 Introduction

Neonicotinoids (imidacloprid, thiamethoxam, clothianidin) are acetylcholine antagonists, as they bind to the nicotinic receptors of this neurotransmitter, causing its persistent activation and inducing hyperexcitation, followed by death (Jeschke and Nauen, 2008).

Phenylpyrazols, such as fipronil, bind to the ionic pumps that are activated by gamma-aminobutyric acid, interfering with the functioning of the latter and causing, as above, hyperexcitation and death (Gunasekara et al., 2007) .

LD50 calculated for the active ingredients under study is very low (on the order of nanogrammes per bee). Different values are reported in the various different studies available in the literature: this is due to the variable detoxification capacity of the molecules depending on the colony in question (43.9 ng/bee for clothianidin by contact, Factsheet EPA, 2003; 3.89 ng/bee for clothianidin by ingestion, EPA 2003; Iwasa et al. 2004: 18 ng/bee for imidacloprid, 30 ng per thiamethoxam, and 75 ng for dinotefuran; Schmuck et al. 2001: more than 200 ng/bee for imidacloprid).

In the case of imidacloprid, LD50 acute toxicity of its metabolites is the same (5-hydroxy - metabolite) or sometimes higher (olefin-imidacloprid) (Suchail et al. 2001).

Neonicotinoids and phenylpyrazols are systemic insecticides, which penetrate into the plant and can be found in pollen and nectar produced during the flowering period (EPA 2003).

Some studies found no negative effects on bees because only death from acute toxicity was considered (Nguyen et al., 2009). On the other hand, many other studies examined more suitable methods for evaluating the risk faced by bees that come into contact with neonicotinoids and phenylpyrazols (Desneux et al., 2007).

Such studies include toxicity tests on in-vitro reared larvae (Aupinel et al., 2005), tests based on the proboscis extension reflex (PER) to assay their effects on bee learning ability and memory formation (Decourtye and Pham-Delegue, 2002), various other behavioural tests (Thompson, 2003), and studies on the effects induced by chronic exposure, which is a concrete risk associated with the systemic nature and persistence of the above cited insecticides (Suchail et al., 2001; Decourtye et al., 2005; Ailouane et al., 2009).

Chronic poisoning

In a study on 10-day chronic poisoning, all metabolites of imidacloprid showed equal toxicity towards bees at doses ranging from 3,000 to 100,000 times lower than the dose necessary to produce the same effects by acute poisoning. Ingestion of nanodoses of imidacloprid or of one of its metabolites for 8 days, for a total of barely 0.1 ng/bee, caused the death of 50% of the bees (Suchail et al., 2001). In other studies conducted for 10 days, LD50 by chronic oral poisoning was observed after cumulative ingestion of 0.1-10 ng /bee (Suchail et al., 2000, 2002, 2004; Guez et al. 2001, 2003), in relation to the exposure protocol (Bonmatin et al., 2005a, b)

An in-depth study (Bonmatin et al., 2003a) found that mean concentration of imidacloprid in dressed maize leaves, flowers and pollen was 4.1, 6.6 and 2.1 microgrammes/kg, respectively. Taking into account that maize pollen represents 20-40% of the protein requirements of a beehive, consumption of 6 mg/day of pollen exposes bees to an extremely elevated 10-day chronic poisoning mortality risk, given that the PEC/PNEC (Probable Exposure Concentrations/Predicted No Effect Concentration) ratio reaches a value of 500-600 (in this ratio, the value 1 corresponds to the risk threshold) (Bonmatin et al., 2003b, 2004).

The use of neonicotinoids for maize seed dressing leads to soil contamination (NTPN 1998, Bacey 2001); in addition, it has also been demonstrated that subsequent crops and weed species may in some cases be contaminated for as long as two years after maize sowing (Bonmatin et al. 2002, 2003c)

Studies have also been conducted on fipronil. Its metabolites (the sulfonated derivative and the desulfated product, resulting from photodegradation) have been found to maintain extremely elevated insecticidal efficacy, remaining as effective as the starting molecule (tests performed on the household fly). Consequently, the metabolites of fipronil contribute to the overall efficacy of the insecticide in guaranteeing prolonged crop protection (Hainzl and Casida 1996). Dust dispersion and deposition during sowing allow the process of photodegradation, with the formation of metabolites having insecticidal action.

Sub-lethal effects on cognitive processes (learning and memory of odours and spatial orientation)

As mentioned above, contamination of soil, water and weeds can come about in many ways as a result of the systemic nature and persistence of the insecticides under study; consequently, nectar and pollen products are likewise contaminated. While the concentrations specified in the above cited studies cause mortality at extremely low doses, on account of chronic repeated exposure, intake of even lower doses (or for shorter time periods) of the active ingredients under study here can still lead to effects on bee physiology and behaviour. A vast bibliography with numerous in-depth studies has been built up on these aspects (Erber et al., 1975a, b; Sandoz et al., 1995; Gerber et al. 1998; Lambin et al, 2001; Pahm-Delegue et al., 2002; Decourtye et al, 2004; El Hassani et al. 2008; Maccagnani et al., 2008).

Study of the proboscis extension reflex in presence of odours associated with administration of sugar solutions allows an examination of the impact of insecticides on some cognitive processes, such as learning and memorisation of different types of environmental stimuli (Decourtye and Pham-Delegue, 2002; Maccagnani et al., 2008). It follows that impaired odour-associated learning can be taken as an index of disruption of cognitive processes, which can severely affect bees' capacity to fulfil their foraging functions and can lead to dangerous disorientation. For fipronil, an effect was demonstrated using doses ranging from 0.075 to 0.15 ng/bee/day, which represent, respectively, 1/80 and 1/40 of LD50 according to Chauzat et al. (2006).

The 2009 experiments conducted in the framework of the "Bees and Pesticides" line of research of the APENET project, provided evidence that the quantity of insecticide contained in dust dispersed by the seeder and deposited on the ground at a distance of 5 m was sufficient to induce an adverse effect in bees that repeatedly came into contact with the substance. Affected bees showed reduced ability to recognise odours associated with a reward during a purpose-designed bee training trial; furthermore, they exhibited difficulty in spatial orientation, odour recognition and a reduction in the function linked to foraging activity.

Since, as extensively described above, the systemic nature and persistence of the active ingredients and their metabolites implies that the nectar and pollen produced by flowers may be likewise affected, experiments were conducted in 2010 on the effects that ingestion of very low doses of the above-mentioned active ingredients can cause on learning and odour memory and on orientation in a simple labyrinth.

These experiments demonstrated: 1. for all the active ingredients in question, impaired ability to recognise odours associated with the reward, and this impairment was observed both in the case of typical flower odours (citronellol) or the odour of the Nasonov gland or a component of the queen pheromone (see 2010 report); 2. a significant reduction in colour recognition ability, i.e. in an ability (learned during the training period) that should have facilitated bee orientation in the labyrinth, and should have aided spatial orientation in a simple labyrinth; 3. for clothianidin (so far, the only active ingredient studied on this particular aspect) it was demonstrated that a single administration of the active ingredient at the dose of 0.7 ng/bee impairs the ability to return to the nest, and at 0.47 ng/bee the treated bees succeed in returning to the nest but for a number of hours they are unable to perform the foraging functions satisfactorily.

The research programme to be set up for the spring of 2011 was planned to evaluate the following aspects:

1. The effect of contact contamination due to dust dispersion;
    a. the effect induced in learning and odour memory (PER test) by exposure to 90-95% lower quantities of dust than occurred with the unmodified machine, as a result of the introduction of a new deflector prototype developed by CRA-ING, which significantly reduces dust dispersion (study completed).
    b. the effects of dust-derived contamination on orientation ability in a simple labyrinth (study begun).
2. The effect of ingestion of nanodoses of clothianidin on orientation ability (return to the hive):
    a. on behaviour in the hive and on frequency of nectar forager visits to the feeding dispenser.
    b. on behaviour in the hive displayed by pollen foragers (behavioural analysis of videorecordings performed during the 2010 studies).

Since no data are available on concentrations present in nectar and pollen of weed species contaminated during the sowing procedures, we used increasing sub-lethal doses according to various different protocols, in order to assess their effects on orientation ability (study completed for the clothianidin administration protocol).

Doses per bee to be administered by ingestion were established by starting out from the bibliographical data referring to oral LD50, and by applying successive dilutions until a dosage was reached that did not impair bee viability and motor ability.

The quantities of clothianidin administered to bees by ingestion (from 0.092 ng/bee to 0.552 depending on the treatment protocol) are comparable to the quantities used in acute toxicity studies and, in our view, also comparable (or at least of the same order of magnitude) to quantities taken in through the cuticle, as detected in studies on the effect of contact with dust on odour learning (PER test) (3.31 ng of clothianidin in a small cage containing 10 bees).

6.2 Effects on learning/olfactory memory caused by contact contamination with dust having reduced neonicotinoid and fipronil content - *PER* test

6.2.1 Materials and Methods

Hives, number of bees, repetitions: One single hive was used (the same Hive B as used in 2010). To date, 5 repetitions of untreated controls, and 4 of imidacloprid, thiamethoxam and clothianidin and fipronil have been carried out.

Capture: Bees were captured in small purpose-designed plexiglass cages, each having an 8 cm Ø Petri dish as its base (10 bees per cage). At the moment of capture, each cage was already equipped with a syringe, adapted to act as a dispenser.

Active ingredients utilised: Imidacloprid, clothianidin, thiamethoxam and fipronil.

Concentrations and quantities of active ingredient utilised for contamination: Dust for the experiment was extracted with the aid of the Heubach cyinder at CRA-ING of Rome, using dressed seed supplied by Assosementi. In order to calculate the amount of dust to be tested, reference was made to the active ingredient content of dust that was dispersed by a fixed point seeder equipped with the deflector patented by CRA-ING of Monterotondo. This deflector made it possible to reduce – by different percentages depending on the dressing product – the quantity of active ingredient dispersed, as compared to dispersion resulting from use of the unmodified machine.

The quantities of active ingredient per surface area used in our studies, which were equal to quantities deposited at 5 m from the seeded field sown by a seeder equipped with a modification developed by CRA-ING, are listed in Table 32. Quantities were calculated according to the following procedure:

1) Taking into account the concentration of active ingredient recorded at the 5 m distance in the 2010 Rome CRA-ING tests, which were conducted with an unmodified seeding machine, and also taking into account the results showing 80-90% dust abatement obtained by use of the deflector developed in 2011, it proved possible to calculate the active ingredient concentration per m^2. This concentration was presumed to be equivalent to roughly 10-20% of the dust dispersed by the unmodified machine. It should be noted that when these trials were performed, the definitive dust abatement data referring to the 2011 deflector model, given in section 5.5.3., were not yet available.

2) Knowing that the total area of the cage measured 56.72 cm^2, a proportion was performed in order to determine the quantity of each active ingredient to be introduced into the cage, in such a manner as to obtain the same concentration per unit of area.

3) A quantity of contaminated talc amounting to 0.01 g was spread at the bottom of each cage (as stated, the bottom consisted of a Petri dish).

Table 32 – Calculation of the doses utilised for the contact contamination test in the cage

Active ingredient	Active ingredient deposited at 5 m from the unmodified seeding machine, 2010 trial ($\mu g/m^2$)	Active ingredient to be assayed ($\mu g/m^2$)		Quantity of active ingredient per cage (μg)
		maximum %	quantity	
Clothianidin	11.57	20%	2.314	0.00331
Thiamethoxam	6.88	10%	0.688	0.00197
Imidacloprid	16.01	10%	1.601	0.00458
Fipronil	1.157	10%	0.1157	0.000662

The dust extracted by the Heubach cylinder was mixed with talc, on the premises of DiSTA, in order to obtain the quantities of active ingredient /area to be used..
Ten bees were placed in each cage.
Procedure for contaminating bees with the active ingredient: groups of 10 bees were captured as they flew out of the hive and placed in the cages. The bottom of each cage was replaced by a Petri dish spread with the required quantity of active ingredient. Each experimental unit was maintained for 3 hours (after administering the product) at 26°C in darkness.

Preparing the bees for the *PER* test: at the end of the treatment**,** each bee was induced to enter into a small container made of a Gilson pipette tip with the point cut off; the bee was then immobilised in such a manner that it could move only its head.

Training: the training session began by familiarising the bee with an air flow for 25 seconds; this was followed by 12 tests (spaced 12 minutes apart) in which two odours were presented, namely citronellol and peppermint. The odours, which were associated, respectively, with the reward (40% sucrose solution, "Z") and punishment (saturated saline solution, "S"), were presented in a semirandom sequence ZZSZSSZZSSZS.
The odours (a strip of filter paper loaded with 3 microlitres of the odour) were placed in a 5 ml syringe with the piston removed. The modified syringe was then placed over the nozzle of the air compressor in order to collect the flow of air, which then passed through the syringe and flowed out through the open end of the syringe, thereby conveying the odour.
Presentation of the rewarded stimulus was performed as follows. The rewarded odour was presented in the air flow for 6 seconds; after the first 3 seconds, the bee's antennae were touched with a 40% sucrose solution; when the proboscis was extended, the bee was given the reward for 3 seconds together with the odour. Presentation of the punished stimulus was performed in the same manner.

Test: the PER odour recognition test was performed 60', 180', and 24 h after the final training procedure. Each test consisted of one trial in which the bee was presented with the rewarded and the punished odour in succession. During the presentation the bee was given neither the reward nor the punishment.

Each answer was classified according to the following categories:
5. Correct: response only to the rewarded but not to the punished odour.
6. Partially correct: response to both
7. Partially incorrect: no response
8. Incorrect: response only to the punished and not to the rewarded odour.

At the end of the 180' test, bees were fed with a drop of 30 µl of sucrose solution per bee.

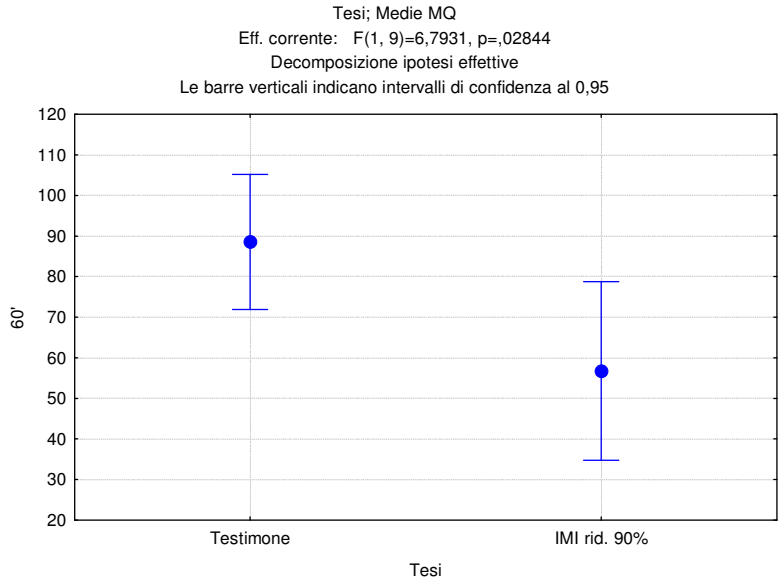
Viability at the end of the test: after the 24h test, bees were released into a free flight cage for determination of viability data in terms of capacity to perform different types of motor functions. Data on the following types of behaviour were recorded: flying (F), walking (W), and raling (R).

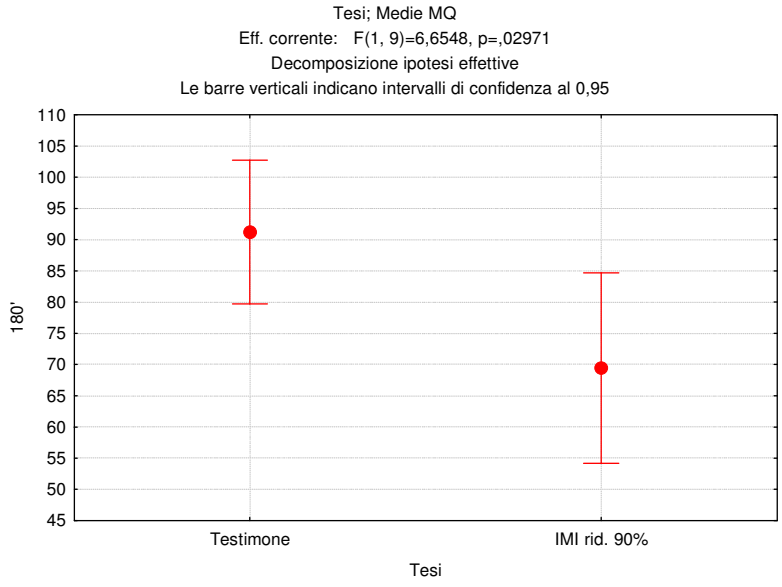
Data analysis: so far, data has been analysed to compare the percentage of bees that responded correctly, at the increasing time intervals (60', 180', 24h), to odour presentation; the analysis was performed by one-way ANOVA, considering the test as a factor of variability.

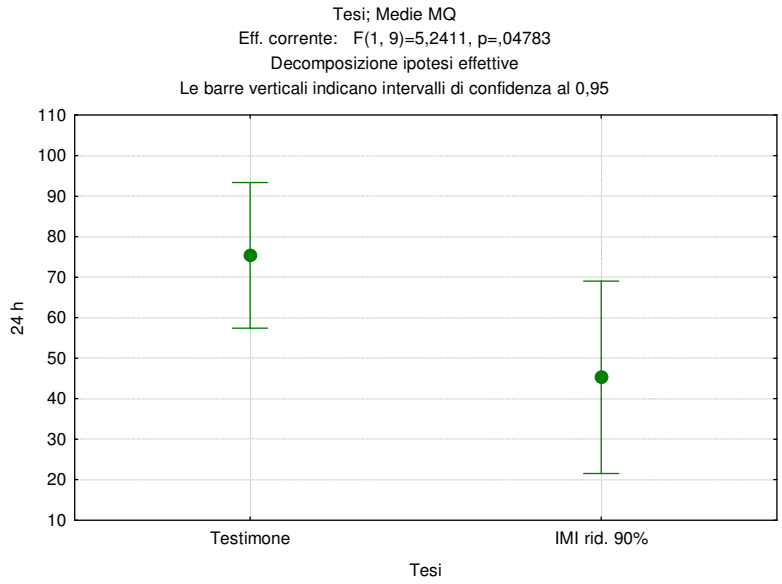
6.2.2 Results

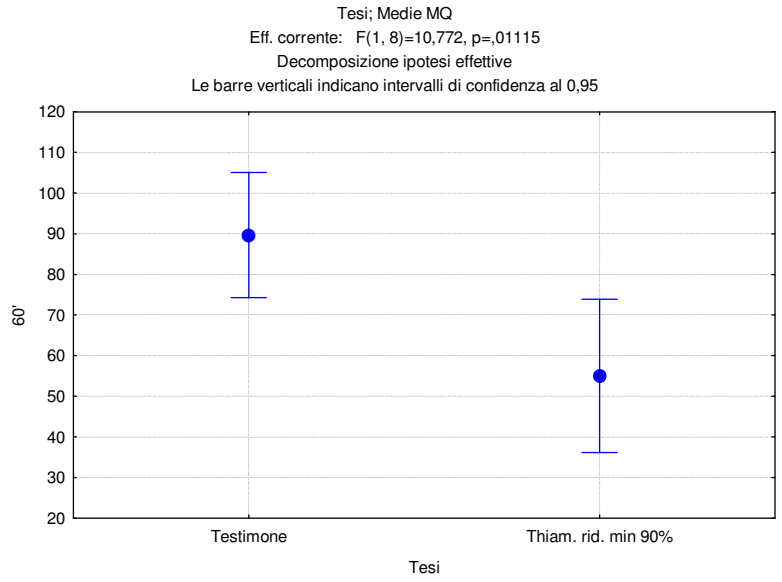
The results of experiments conducted so far are shown in the graphs below, which indicate the percentage of correct answers recorded for bees that had entered into contact with imidacloprid, thiamethoxam, clothianidin and fipronil. (Correct = C+M- proboscis extension only in presence of the citronellol odour, which was rewarded during training, and not in presence of peppermint);
Bees entered into contact with dust containing 10% of imidacloprid, thiamethoxam and fipronil. Although this was a lower percentage compared to the quantity dispersed by the unmodified seeder, it nevertheless impaired odour recognition as early as after 60' (short-term memory) and at 180' (medium-term memory) as well as at 24 h (long-term memory). At the 24 h test, the level of

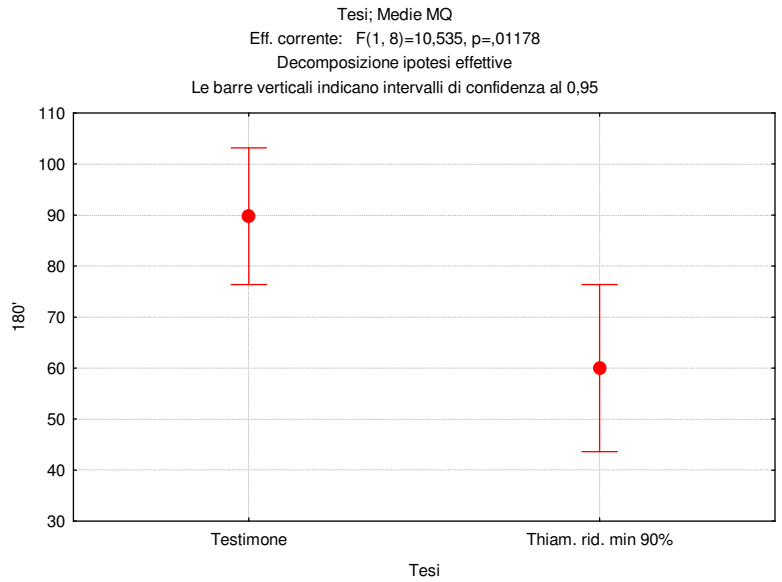
statistical significance for bees treated with fipronil was found at p = 0.055. A similar result was observed for bees that had entered into contact with dust containing a maximum of 20% of clothianidin, compared to the values obtained with the unmodified seeder.

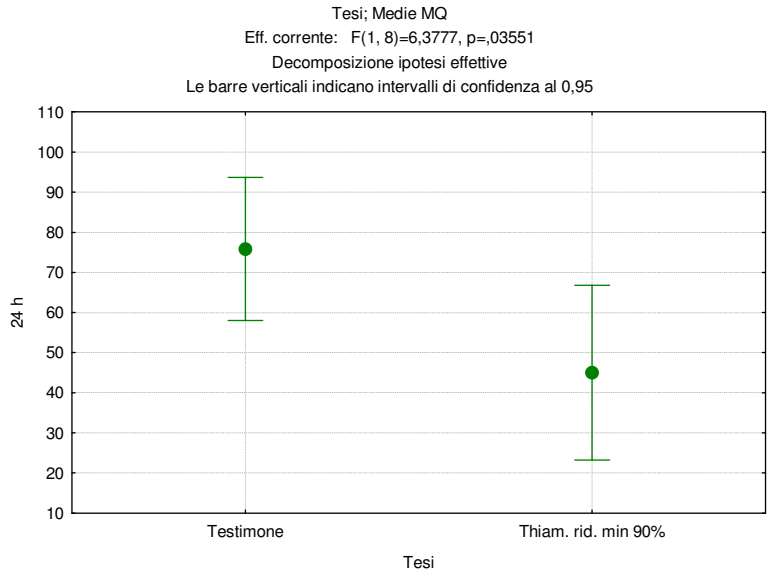





Tesi; Medie MQ
Eff. corrente: F(1, 9)=5,2411, p=,04783
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
24 h
110
100
90
80
70
60
50
40
30
20
10
Testimone
IMI rid. 90%
Tesi


Tesi; Medie MQ
Eff. corrente: F(1, 8)=10,772, p=,01115
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
60'
120
110
100
90
80
70
60
50
40
30
20
Testimone
Thiam. rid. min 90%
Tesi


Tesi; Medie MQ
Eff. corrente: F(1, 8)=10,535, p=,01178
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
180'
110
100
90
80
70
60
50
40
30
Testimone
Thiam. rid. min 90%
Tesi


Tesi; Medie MQ
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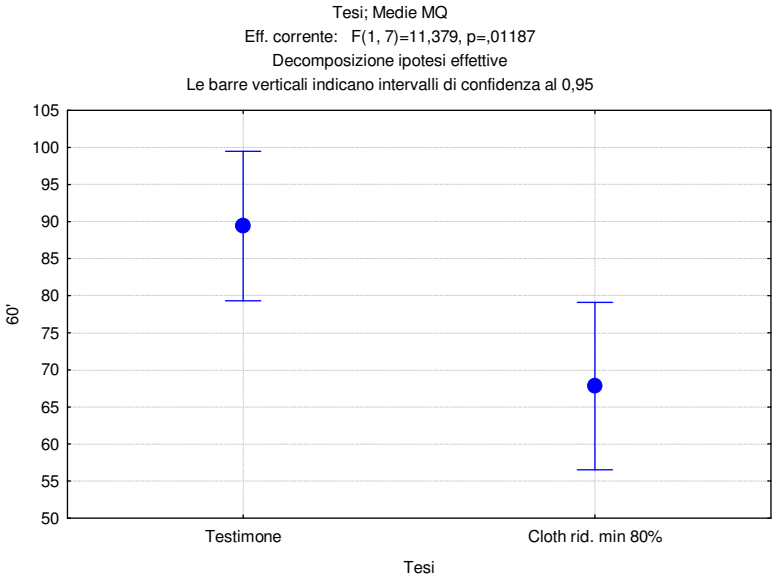
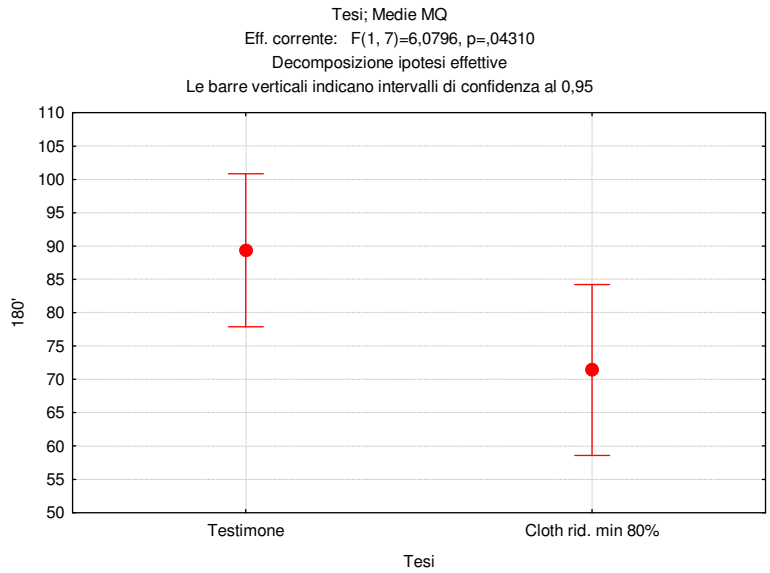
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
24 h
110
100
90
80
70
60
50
40
30
20
10
Testimone
Thiam. rid. min 90%
Tesi


Tesi; Medie MQ
Eff. corrente: F(1, 7)=11,379, p=,01187
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
60'
105
100
95
90
85
80
75
70
65
60
55
50
Testimone
Cloth rid. min 80%
Tesi


Tesi; Medie MQ
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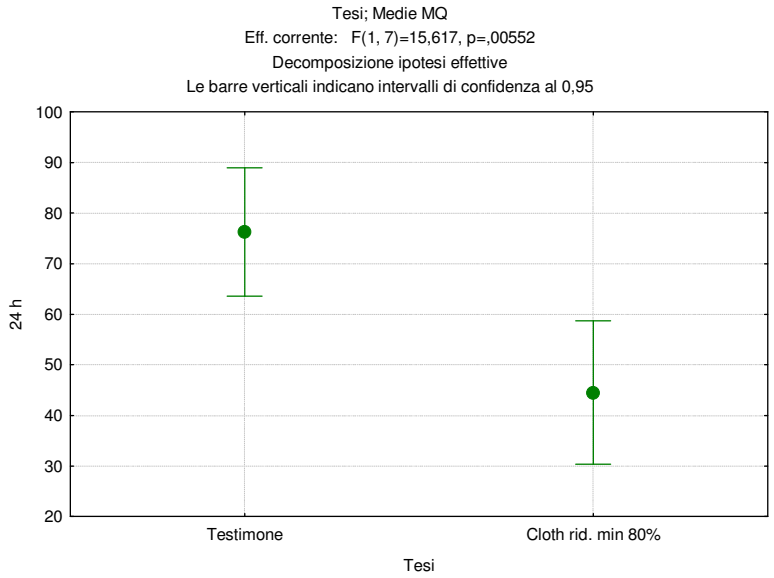
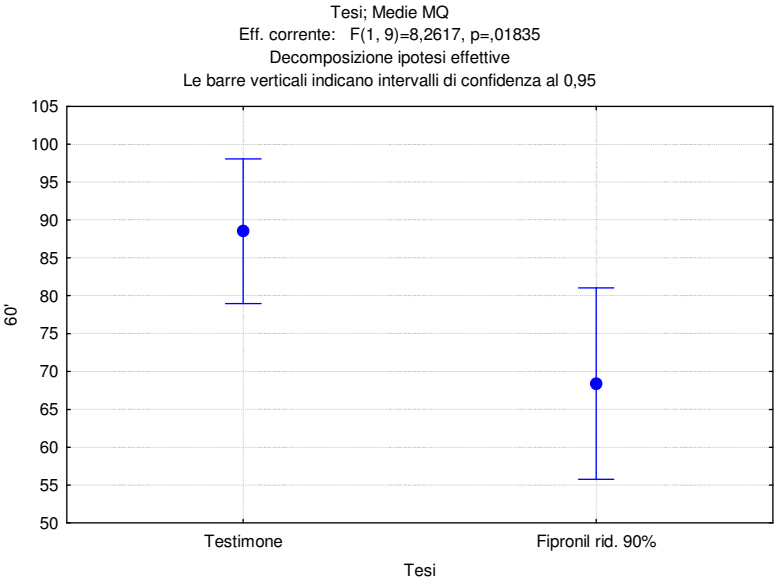
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
180'
110
105
100
95
90
85
80
75
70
65
60
55
50
Testimone
Cloth rid. min 80%
Tesi


Tesi; Medie MQ
Eff. corrente: F(1, 7)=15,617, p=,00552
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
24 h
100
90
80
70
60
50
40
30
20
Testimone
Cloth rid. min 80%
Tesi


Tesi; Medie MQ
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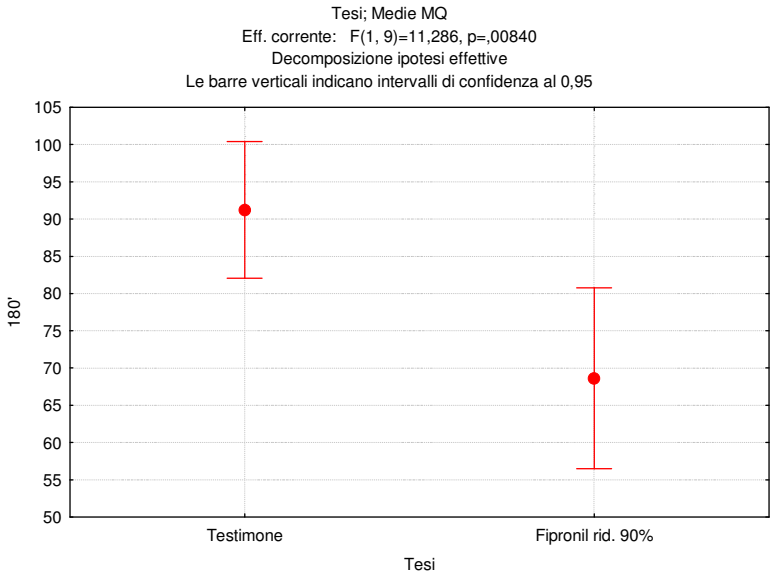
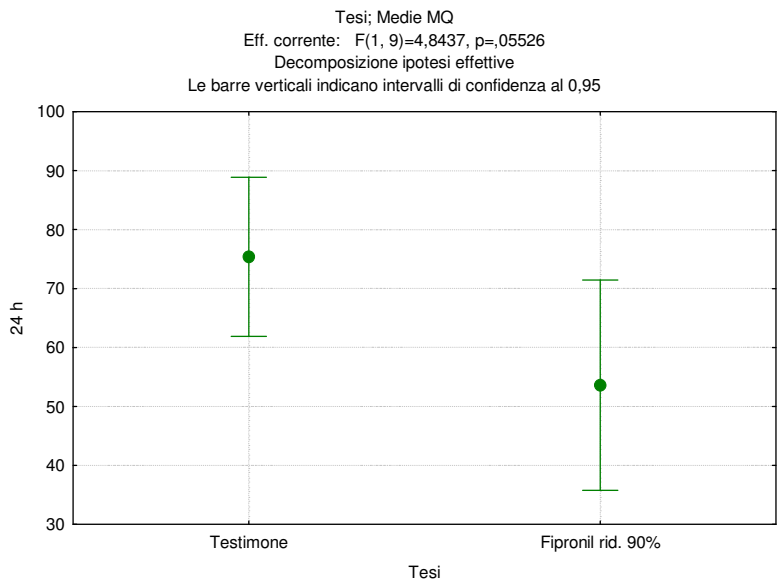
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
60'
105
100
95
90
85
80
75
70
65
60
55
50
Testimone
Fipronil rid. 90%
Tesi


Tesi; Medie MQ
Eff. corrente: F(1, 9)=11,286, p=,00840
Decomposizione ipotesi effettive
Le barre verticali indicano intervalli di confidenza al 0,95
180'
105
100
95
90
85
80
75
70
65
60
55
50
Testimone
Fipronil rid. 90%
Tesi



Key: Vertical bars indicate confidence levels at 95%; *Testimone* = Controls; *Tesi* = Treatments; IMI = Imidacloprid ; *Thiam* = thiamethoxam; *Cloth*. = clothianidin.

6.3 Effects of contamination with thiamethoxam-containing dust on orientation ability in a simple labyrinth and on colour recognition.

6.3.1 Materials and methods

Hives, number of bees, repetitions. The experimental design was intended to involve 3 repetitions of 10 bees each. Due to time restrictions it was not possible to complete the experiment; consequently, the behaviour of only 4 bees was studied.

Investigative procedure: A beehive composed of only one frame with queen and brood (heated in order to maintain an adequate temperature) was linked up to a free flight chamber placed outside the hive. The foragers were prompted to search for a sucrose solution reward in a Y-shaped labyrinth connected to the free flight chamber, the reward being situated at the opposite end compared to the hive.

Stage 1: preliminary training and marking of individuals: free and group training in the antecamera of the Y labyrinth (Figure 29), using 40% sucrose solution placed in a colourless container. Markings were placed on a group of bees, and the remaining subjects were captured in purpose-prepared cages and excluded from further phases of the test.

Stage 2: orientation training inside the labyrinth, using colour. A dispenser containing 40% sucrose solution (= reward) and covered with a blue lid, and a dispenser containing saturated saline solution (= punishment) and covered with a red lid, were used for training. The bees were each allowed 6 visits to the two arms (distance from the decision-making chamber to the end of the arms = 20 cm). The position of the colour associated with the reward (or the punishment) was assigned according to a semirandom sequence (SDSSDD or DSDDSS). At each visit, the subject's time of arrival and first choice arm were recorded.

Capture and contamination with thiamethoxam-containing dust. The bees were captured during their final training visit, after they had alighted on the correct dispenser but before they began feeding. If fewer than 10 bees had completed the training, the number was made up by collecting additional foragers from the free fight chamber in order to reproduce the contamination procedure described in the section devoted to contamination by contact with dust (see above). The subjects were maintained inside the contamination cage for 3 hours at 26°C in darkness.

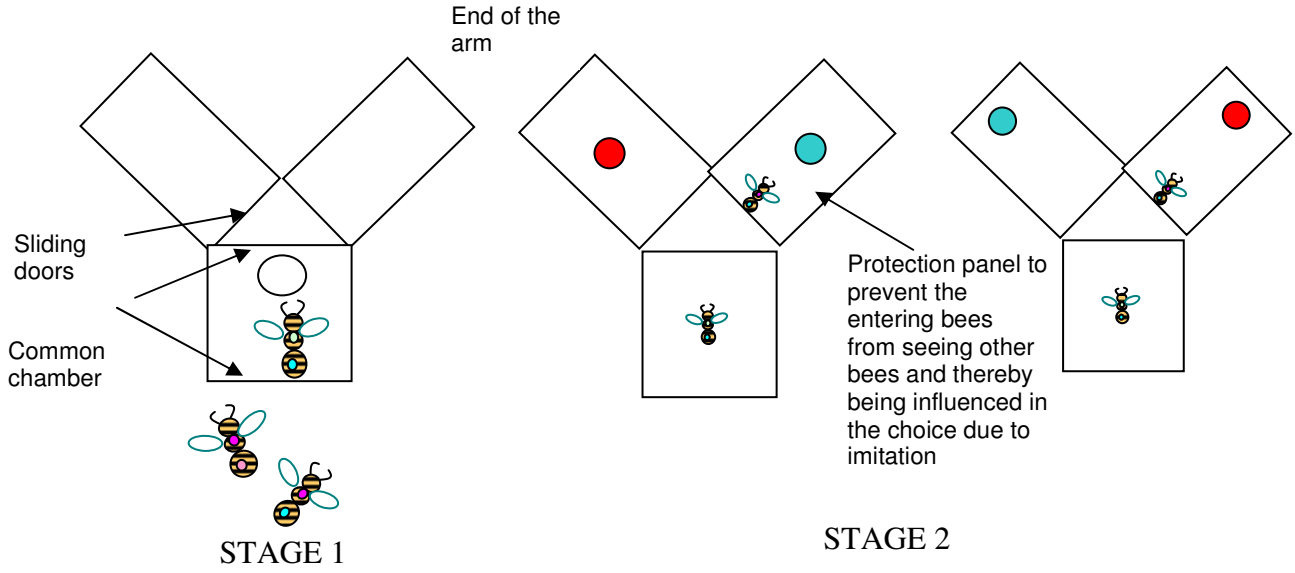


Figure 29 - Diagram of the training procedure designed to associate the reward with the blue colour, namely with the visual stimulus chosen to train the bees to achieve correct orientation inside the Y labyrinth. Bees were marked with colours on the thorax and the abdomen. Stage 1 and 2: see the text.

1 h Test: after 3 hours in the contamination cage, bees were released into the free flight chamber one at a time, and each bee was allowed 5 minutes to enter into the labyrinth. If, after 5 minutes had elapsed, the bee had not entered the labyrinth, it was accompanied into the common chamber, and was allowed a further 5 minutes to choose the arm with the correct colour.
The test consisted in two alternate visits to the arms. For each bee, the time of each visit and behaviour vis-à-vis the dispenser was recorded: F = flight within the arm without alighting on the dispenser; N = Nutrition: the bee feeds, i.e. alights on the dispenser and extends its proboscis towards the holes; A = the bee alights on the dispenser without attempting to feed.
Regardless of the colour (i.e. the arm) chosen, the bee did not have the option of shifting to the other are in the case of an incorrect choice (non-corrective choice). If a bee made an incorrect choice, it was captured in a jar and returned to the free flight chamber, from where it was then prompted to perform a second visit, with the same procedure as in the first visit.
After the two visits, the bee was captured, placed in a Gilson pipette, fed to satiation with a 40% sucrose solution and maintained in a dark cell at 26°C until 24 hours had elapsed from the time of the contamination phase.
24 h test: the subjects were released 24 h after contamination and submitted to test 2 according to the procedure described for the 60' test.

6.3.2 Results

The experiment has only just begun and 4 bees have been submitted to the treatment. The results are shown in Table 33.
The bees had no difficulty in associating the colour with the reward, as this is a task known to be very simple for bees. Thus all the bees had learned how to follow the transfer of the correct dispenser (the one with the blue lid) from one arm of the labyrinth to the other. However, the data listed in the table indicate that bees contaminated with thiamethoxam dust experienced considerable difficulty in recovering the correct memory of the colour associated with the reward.
As can be seen, correct choice percentages were below 50%. This result, although preliminary, given the low number of bees submitted to the test, would appear to suggest that individuals treated with thiamethoxam recover memory of the wrong colour at the moment of making their choice.

Table 33 - Number of correct choices made during the test. Each bee was allowed two attempts.

Bee	Test at 3 h	Test 24 h
Bee 1	2	1
Bee 2	0	0
Bee 3	1	1
Bee 4	0	0
Mean percentage	37.5 %	25.5 %

6.4 Effects of ingestion of nanodoses of clothianidin and fipronil on bee homing ability and on forager behaviour in relation to the hive

The experiment was conducted using clothianidin, to complete the trial begun in 2010 in which two groups of bees were subjected to ingestion treatment involving, respectively, 0.7 and 0.47 ng/bee of clothianidin. the 2010 experiment had shown marked impairment both of ability to return to the nest and also of foraging frequency after only 1 ingestion of the above cited doses.

6.4.1 Materials and methods

The bees of a hive with glass walls, consisting of 6 frames of which 2 were brood frames, underwent training to search for a 40% sucrose solution from an artificial dispenser. Markings were placed on the bees, and the dispenser was gradually moved to 150 m from the nest.
Frequency of bee flights towards and returning from the dispenser was recorded for 40 min prior to treatment. Bees observed to be assiduous visitors to the dispenser were chosen for the experiment, with the aim of achieving the number of 10 bees for each treatment procedure and for the control.
Individuals were captured at the dispenser after they had begun to feed on the sucrose solution, so that the bee's motivation at the moment of capture would be that of returning to the nest and communicating the position of the dispenser to the other bees in the hive.
After capture, each bee was inserted into a Gilson 1000 pipette tip with the point cut off, and was submitted to the treatment. The different active ingredient intake protocols are summarised in Table 34.

Table 34 - Clothianidin ingestion protocols.

	Concen-tration a. i. (ng/µl)	Quantity of administ ered a. i. (ng/bee)	Quantity of sucrose solution utilised for the administration	N. administrat ions	Additional intakeof non contaminated sucrose solution permitted to fill the honey sac	Bee maintained in pipette tip after treatment	N. bees treated/ Control
Protocol 1	0,0184	0,092	5 microlitres	3-12 in successive trips	si	No	12/15
Protocol 2	0,092	0,47	5 microlitres	1	no	Si	9/10
Protocol 3	0,092	0,47	5 microlitres	3 in successive trips	no	Si	4/0
Protocol 4	0,0184	0,552	30 microlitres	3	no	Sì	1/0

Protocol 1
This protocol involved administering a dose of 0.092 ng/bee in 5 microlitres of 40% sucrose solution. The bee was allowed to fill the honey sac with non contaminated sugar, so that what was simulated in this protocol was the very low dose contamination of only a portion of the bee's foraging area. Bees were allowed to empty the collected nectar immediately, thereby limiting

immediate active ingredient intake to a minimum. This was achieved by releasing the bee straightaway after each administration, and at each return to the dispenser the bee was once again captured for a further administration. This procedure was continued in the same manner for as long as the bee succeeded in returning to the feeding point.
In this trial, only the frequencies of visits to the dispenser and presence in the nest were recorded; no videorecordings were performed.
.Control bees were captured the same number of times as the treated bees, and were fed with non contaminated sucrose solution.

Protocol 2 and 3
These protocols involved administration of a dose of 0.47 ng/bee in 5 microlitres as a single procedure (Protocol 2) or repeated up to 3 times (Protocol 3), maintaining the bee in the Gilson pipette tip after the treatment (to simulate a foraging experience of medium duration) and without allowing the bee to complete the filling of the honey sac with non contaminated sucrose solution. Thus Protocol 3 simulated a field situation in which the entire foraging area of the bee in question was contaminated. The behaviour of each individual at the dispenser and in the nest was observed after 1, 3 and 24 h, recording return flight frequency and duration and filming (whenever possible) behaviour vis-à-vis the nest 3 times (duration 5 minutes). At the same time the main behavioural aspects were recorded (discharging the nectar, the dance, trophallaxis, walking, immobility, flying out). Non-treated bees underwent the same number of captures as the treated bees, and were fed with uncontaminated sucrose solution.

Protocol 4
This protocol involved administering a dose of 0.552 ng/bee in 30 microlitres of 40 % sucrose solution, a quantity appropriate for filling the honey sac, and then allowing the bee to empty the honey sac after it had returned to the nest. This procedure simulated an extremely low dose contamination of the entire foraging area and considers a minimum length of time to complete the flight to and from small scattered sources located a short distance from the hive.

6.4.2 Results

Due to the restricted time available for the project, it was not possible to complete all the protocols. Protocols 1 and 2 have been completed. Examination of the videorecordings is in progress.
The observations conducted at the moment of release after treatment and in the nest showed normal behaviour for the untreated bees (direct flight towards the nest, discharge of nectar, interactions and exchange of food with the companion bees, flying out and immediate return to the dispenser for a renewed collection). The results of the experiments are shown in the following tables and graphs.

Table 35 - – Frequency of return to the food source observed in bees treated according to protocol 1.

Active ingredient.	Bee	Frequency of visits to dispenser during 40' prior to treatment	N. administrations of the nanodose to the bee during the day	Presence in the hive after 24 h
CLOT 0,092 prot 1	PinkGreen	2	5	no
CLOT 0,092 prot 1	PinkYellow	3	10	no
CLOT 0,092 prot 1	Multicoloured	5	3	no
CLOT 0,092 prot 1	PinkBlue	3	10	no
CLOT 0,092 prot 1	PinkPurple	4	12	no
CLOT 0,092 prot 1	OrangeOrange	5	8	no
CLOT 0,092 prot 1	PurpleWhite	4	10	Si

CLOT 0,092 prot 1	PinkWhite	5	5	Si
CLOT 0,092 prot 1	PurplePink	3	5	No
CLOT 0,092 prot 1	GreenOrange	1	6	No
CLOT 0,092 prot 1	GreenPink	3	4	No
CLOT 0,092 prot 1	YellowGreen	5	7	No
	Mean	**3.5**	**6.29**	**2/12 (16.7%)**
Control	OrangeBlue	5	16	si
Control	GreenSilver	3	33	si
Control	PurplePurple	5	22	si
Control	PurpleGreen	4	31	si
Control	PurpleYellow	4	17	si
Control	PurpleYellow2	-	20	si
Control	PurpleWhite	3	21	si
Control	PurplePink	2	19	si
Control	BluePink	6	2	sì
Control	GreenBlue	4	14	no
Control	GreenWhite	2	25	si
Control	GreenGreen	3	2	si
Control	GreenPurple	4	34	si
Control	PinkGreen	1	24	si
Control	PurpleGreen	1	27	no
	Mean	**3.13**	**20.5**	**13/15 (86.7%)**

Table 36 - Frequency of return to the food source observed in bees treated according to protocol 2.

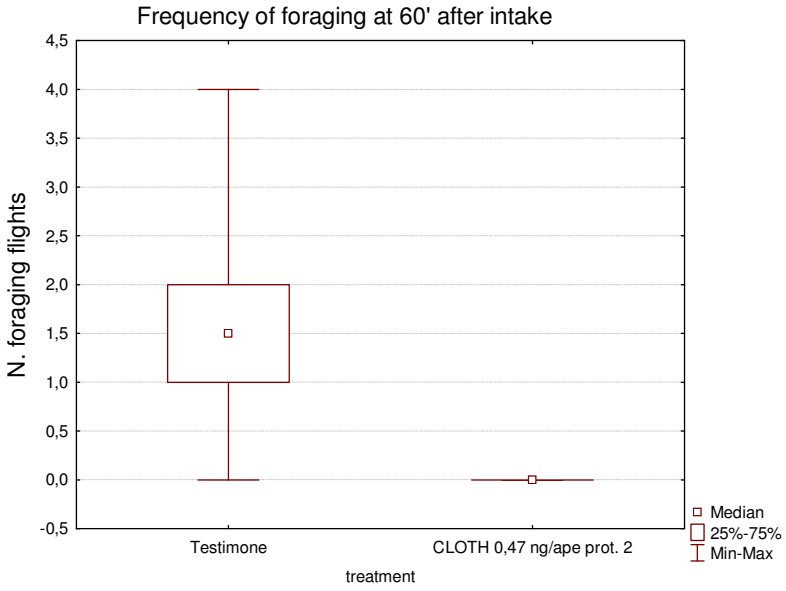
Active ingredient	Bee	Frequency of visits to dispenser in 40' prior to treatment	Administered doses	Freq. 1 h	Freq. 3 h	Freq. 24 h
Control	YellowYellow	4	0	0	2	4
Control	YellowOrange	2	0	1	6	3
Control	FuchsiaPink	4	0	2	3	4
Control	FuchsiaBlue	5	0	1	4	5
Control	FuchsiaYellow	4	0	2	7	7
Control	FuchsiaGreen	5	0	3	5	5
Control	PurpleGreen	3	0	2	2	4
Control	BlueYellow	5	0	1	7	4
Control	BluePinka	4	0	1	7	6
Control	Multicoloured	6	0	4	7	4
	Mean	**4.0**		**1.9**	**5.5**	**5.0**
CLOTH 0,47 ng/ape	WhiteOrange	3	1	0	4	5
CLOTH 0,47 ng/ape	YellowBlue	3	1	0	2	5
CLOTH 0,47 ng/ape	PinkOrange	3	1	0	0	2
CLOTH 0,47 ng/ape	GreenFuchsia	5	1	0	0	1
CLOTH 0,47 ng/ape	PinkFuchsia	4	1	0	0	0
CLOTH 0,47 ng/ape	YellowFuschsia	6	1	0	0	0
CLOTH 0,47 ng/ape	BlueWhite	5	1	0	0	0
CLOTH 0,47 ng/ape	OrangeGreen	4	1	0	5	6
CLOTH 0,47 ng/ape	OrangePurple	4	1	0	6	0

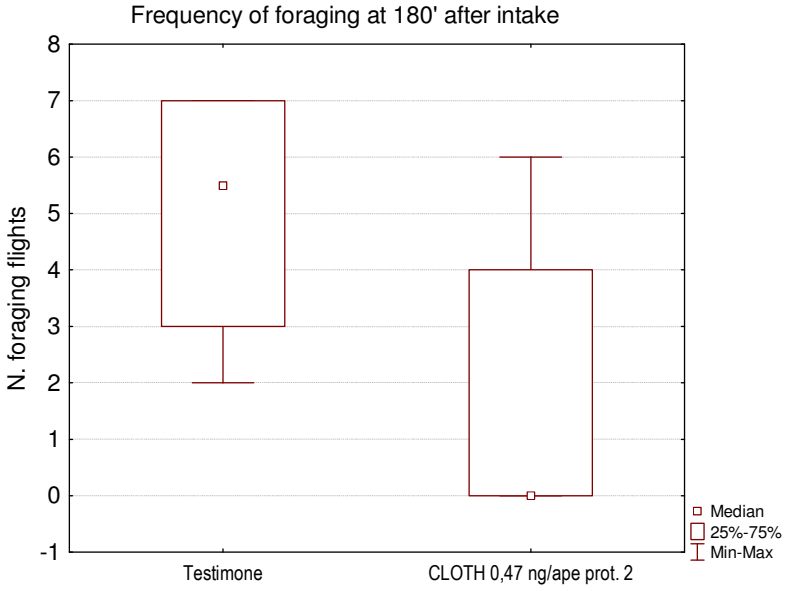
	Mean	**3.7**		**0.0**	**1.9**	**2.1**

Table 37 - Frequency of return to the food source observed in bees treated according to protocol 3.

P.A.	Bee	Frequency of visits to dispenser in 40' prior to treatment	Administered doses	Freq. 1 h	Freq. 3 h	Freq. 24 h
CLOTH 0.47 ng/bee	OrangePurple	8	2	1	1	0
CLOTH 0.47 ng/bee	SilverWhite	5	1	1	0	0
CLOTH 0.47 ng/bee	GreenOrange	6	3	1	1	0
CLOTH 0.47 ng/bee	SilverOrange	3	2	1	0	0
	Media	**5.5**		**1**	**0,5**	**0**

Only one bee was treated according to Protocol 4, which involved submitting the bee to 0.552 ng/bee in 30 microlitres of sucrose solution once, in order to fill the honey sac, and then allowing the bee to empty the sac immediately upon its return to the nest. The bee came back three times to the dispenser, but after the third administration it did not return to the nest again.





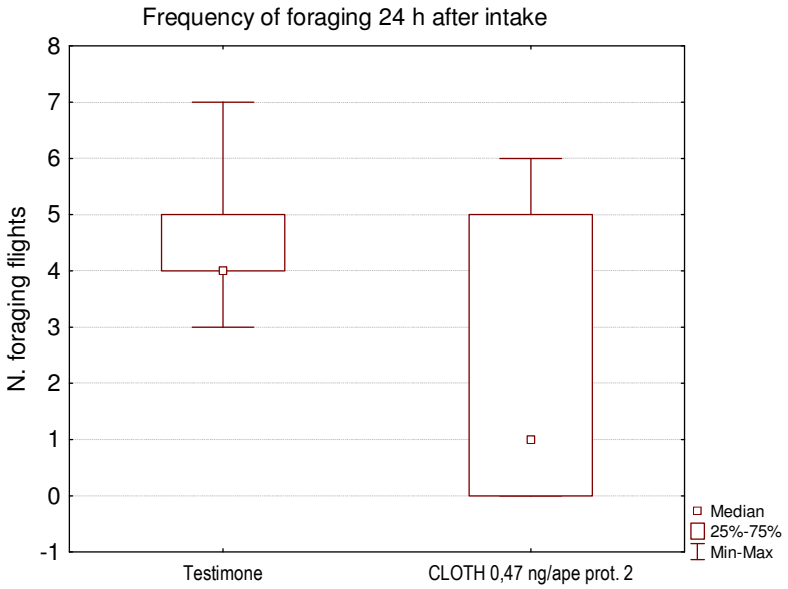


Figure 30 - Frequency of foraging flights in bees treated with 0.47 ng/bee by ingestion in 5 microlitres (Protocol 2) and in untreated bees.
Translation of text within figures: *Testimone* = controls; *Media* = Mean.

Protocol 1: Although the bees had the possibility of feeding after ingesting the treatment, the overwhelming majority of bees left the feeding point. Examination of the frequency data listed in Table 35 showed that the treated bees were capable of visiting the contaminated source for an average of 6.3 times (i.e. they withstood 6.3 assumptions of clothianidin), but then they ceased returning to the food source. In contrast, during the same time period the controls made an average of 20 flights. Thus treatment even at these extremely low doses had a marked impact on bee presence at the feeding point 24 hours after treatment: whereas 86.7% of control bees were present at the dispenser, this percentage decreased to 16.7% in treated bees.
Statistical analysis of frequency of visits to the dispenser by bees submitted to Protocol 3 (Mann-Whitney U test) showed a significant difference between frequency of foraging flights of bees that had ingested a single dose of 0.47/bee (Figure 30) and the foraging frequency of non-treated bees, both at 60' and at 180'. Comparison between mean frequencies at 24 h was not statistically significant, although a disparity in the median between the two treatments should be noted. The interpretation is that bees which overcame the poisoning phase were able to resume foraging with a frequency similar to that of the non-treated controls; however, half of the treated bees were not present at the dispenser at 24 h after treatment.

6.4.3 Analysis of behaviour in the hive displayed by the pollen foragers utilised in studying the effects of ingestion of nanodoses of clothianidin on homing ability in 2010.

Analysis of the videorecordings conducted on the bee nest utilised in studying the effects of ingestion of sub-lethal doses of clothianidin on homing ability and behaviour hasd been completed.
The analysis was carried out by means of a dedicated software program (The Observer), which makes it possible to assign an alphanumeric code to a series of behavioural modes. Visual examination of the videorecordings (in slow motion if necessary) and sequential digitation of the behaviour observed allows calculation of the number of times a form of behaviour is repeated, the duration of such behaviour, etc.
It proved necessary to define a conFiguretion in which the fundamental variables are established, as well as the forms of behaviour to observe, and also their character (whether they are events or a state).
The conFiguretion is shown in figure 31.

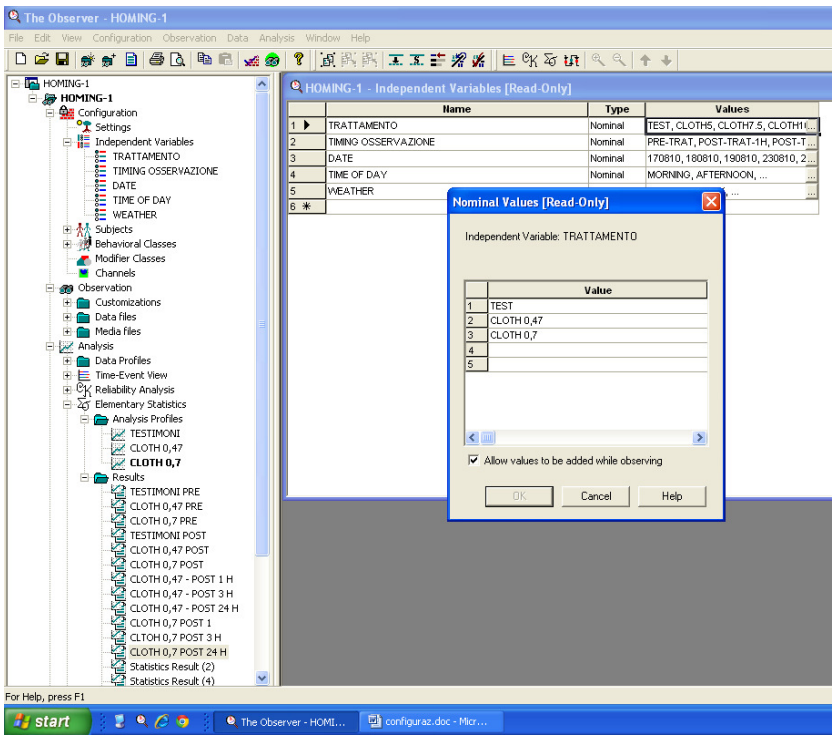


Figure 31 - A part of the conFiguretion and of the structure of the program.

Tables 38, 39 and 40 show the raw data obtained at the end of the analysis. The data refer to forms of behaviour found to be indicative for evaluating the behaviour of the three groups of bees. In all three tables, the penultimate right-hand columns show the sum of the forms of behaviour considered to be indicative of a bee's normal activity and manner of behaviour (walking, exploring the cells, discharging pollen, offering food, emitting pheromones, flying out), while the right-most columns show behaviour regarded as abnormal that can be associated with a situation of poor competence following ingestion of the main active ingredient (wandering around without any particular goal, remaining immobile for a prolonged period of time, staggering unsteadily).
Other types of behaviour, such as the dance, were inserted into the conFiguretion, but were not taken into consideration for comparison of the effects of the active ingredient, as only a few bees, either in the pre-treatment or post-treatment phase, performed recruitment dances.

Table 38 - Number of types of behaviour observed repeatedly during pre- and post-treatment in control bees (5 microlitres of sucrose solution). Translation of text within table: *Trattament i*= treatments; *Timing Osservazione* = observation timing; *Danza* = dance; *Ferma*= stationary; *Gira senza meta* = wanders around with no particular goal; *Cammina* = walks; *Esplora cellette* = explores the cells; *Scarica polline* = discharges pollen; *Offre cibo* = offers food; *Si pulisce* = cleans itself; *Immobile* = immobile; *Barcolla* = staggers unsteadily; *Esce* = flies out; *Comp. Normali* = normal behaviour; *Comp. Anomali* = abnormal behaviour.

Trattamento	Timing osservazione	Danza	Ferma	Gira senza meta	Cammina	Esplora cellette	Scarica polline	Offre cibo	Emette feromone	Si pulisce	Immobile	Barcolla	Esce	Comp. normali	Comp. anomali
Testimone	Pre	0	13	0	14	1	1	5	17	4	0	0	1	43	0
Testimone	Pre	0	6	0	7	1	1	3	9	1	0	0	1	23	0
Testimone	Pre	1	4	0	5	1	1	4	2	0	0	0	1	14	0
Testimone	Pre	0	1	0	2	1	1	1	4	0	0	0	1	10	0
Testimone	Pre	1	1	0	1	0	1	0	1	0	0	0	1	4	0
Testimone	Pre	0	8	0	9	1	1	6	6	1	0	0	1	25	0
Testimone	Pre	0	9	0	10	1	1	3	8	0	0	0	1	24	0
Testimone	Pre	0	4	0	4	1	1	3	3	1	0	0	1	14	0
Testimone	Pre	0	5	0	5	1	1	1	6	1	0	0	1	16	0
Testimone	Pre	0	0	0	1	1	1	2	2	0	0	0	1	8	0
Testimone	Pre	1	1	0	1	0	1	2	1	0	0	0	1	6	0
Testimone	Pre	0	6	0	8	3	1	2	6	0	0	0	1	21	0
Testimone	Pre	0	6	0	7	0	0	4	3	0	0	0	0	14	0
Testimone	Post 3 h	0	7	0	7	0	0	2	8	8	0	0	0	25	0
Testimone	Post 3 h	0	4	0	5	0	1	1	2	1	0	0	1	11	0
Testimone	Post 3 h	0	4	1	5	0	1	0	1	1	0	0	1	9	1
Testimone	Post 3 h	0	10	0	11	0	1	5	10	11	0	0	1	39	0
Testimone	Post 3 h	0	2	0	2	0	0	0	0	2	0	0	0	4	0
Testimone	Post 3 h	0	1	0	2	0	1	2	14	7	0	0	1	27	0
Testimone	Post 3 h	0	1	0	0	1	1	1	5	6	0	0	1	15	0
Testimone	Post 3 h	0	1	0	2	0	0	1	1	0	0	0	1	5	0
Testimone	Post 3 h	0	8	0	7	1	1	5	11	7	0	0	1	33	0
Testimone	Post 3 h	0	8	0	8	0	0	1	4	4	2	0	1	18	2
Testimone	Post 3 h	0	2	0	2	0	0	0	4	1	0	0	1	8	0
Testimone	Post 3 h	0	7	0	8	1	1	1	6	2	0	0	1	20	0
Testimone	Post 3 h	0	12	0	14	2	1	4	9	2	0	0	1	33	0
Testimone	Post 24 h	0	13	0	15	1	1	4	13	2	0	0	1	37	0
Testimone	Post 24 h	0	1	0	2	1	1	2	4	0	0	0	1	11	0
Testimone	Post 24 h	0	1	0	2	1	1	1	0	0	0	0	1	6	0
Testimone	Post 24 h	0	8	0	9	0	1	2	11	6	0	0	1	30	0
Testimone	Post 24 h	0	6	0	8	1	1	6	5	0	0	0	1	22	0
Testimone	Post 24 h	3	3	0	4	0	1	1	6	2	0	0	1	15	0
Testimone	Post 24 h	0	4	0	6	2	1	1	5	1	0	0	1	17	0
Testimone	Post 24 h	0	4	0	4	1	1	5	3	0	0	0	1	15	0
Testimone	Post 24 h	0	7	0	7	1	1	3	9	2	0	0	1	24	0
Testimone	Post 24 h	0	6	0	6	0	0	2	5	4	0	0	0	17	0

Table 39 - Number of types of behaviour observed repeatedly during pre- and post-treatment in the group of bees treated with 0.47 ng/bee of clothianidin. Translation of text within table: *Trattament i*= treatments; *Timing Osservazione* = observation timing; *Danza* = dance; *Ferma*= stationary; *Gira senza meta* = wanders around with no particular goal; *Cammina* = walks; *Esplora cellette* = explores the cells; *Scarica polline* = discharges pollen; *Offre cibo* = offers food; *Si pulisce* = cleans itself; *Immobile* = immobile; *Barcolla* = staggers unsteadily; *Esce* = flies out; *Comp. Normali* = normal behaviour; *Comp. Anomali* = abnormal behaviour.

Trattamento	Timing osservazione	Danza	Ferma	Gira senza meta	Cammina	Esplora cellette	Scarica polline	Offre cibo	Emette feromone	Si pulisce	Immobile	Barcolla	Esce	Comp. normali	Comp. anomali
Cloth 0,47	Pre	0	1	0	4	3	1	4	0	0	0	0	1	13	0
Cloth 0,47	Pre	0	3	0	5	1	1	1	4	1	0	0	1	14	0
Cloth 0,47	Pre	0	5	0	6	1	1	2	5	0	0	0	1	16	0
Cloth 0,47	Pre	0	4	0	5	1	1	1	3	0	0	0	1	12	0
Cloth 0,47	Pre	0	6	0	7	1	1	6	6	2	0	0	1	24	0
Cloth 0,47	Pre	0	3	0	4	1	1	2	3	1	0	0	1	13	0
Cloth 0,47	Pre	0	4	0	5	0	1	4	5	0	0	0	1	16	0
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Cloth 0,47	Pre	0	8	0	9	1	1	2	8	0	0	0	1	22	0
Cloth 0,47	Pre	2	4	0	5	2	1	1	2	0	0	0	1	12	0
Cloth 0,47	Pre	0	3	0	4	1	1	3	1	0	0	0	1	11	0
Cloth 0,47	Pre	0	2	0	1	1	1	1	2	6	0	0	1	13	0
Cloth 0,47	Pre	1	3	0	5	0	1	2	2	0	0	0	1	11	0
Cloth 0,47	Pre	0	3	0	3	1	1	2	5	8	0	0	1	21	0
Cloth 0,47	Pre	0	3	0	2	1	1	2	7	2	0	0	1	16	0
Cloth 0,47	Pre	0	5	0	5	1	1	0	7	5	0	0	1	20	0
Cloth 0,47	Pre	0	6	0	6	1	1	5	8	4	0	0	1	26	0
Cloth 0,47	Pre	0	7	0	8	1	1	2	6	1	0	0	1	20	0
Cloth 0,47	Post 3 h	0	8	0	1	0	0	0	0	7	0	7	0	8	7
Cloth 0,47	Post 3 h	0	2	3	2	0	0	0	0	1	0	3	0	3	6
Cloth 0,47	Post 3 h	0	2	1	2	0	0	0	1	1	1	0	0	4	2
Cloth 0,47	Post 3 h	0	1	1	0	0	0	0	0	0	0	0	0	0	1
Cloth 0,47	Post 3 h	0	4	5	1	0	0	0	0	0	0	0	0	1	5
Cloth 0,47	Post 3 h	0	2	0	0	0	0	0	1	3	1	2	0	4	3
Cloth 0,47	Post 3 h	0	0	1	1	0	0	0	0	0	0	0	0	1	1
Cloth 0,47	Post 3 h	0	3	1	4	2	0	3	0	0	0	0	0	9	1
Cloth 0,47	Post 3 h	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Cloth 0,47	Post 3 h	0	4	0	3	1	1	1	5	7	0	0	0	18	0
Cloth 0,47	Post 3 h	1	5	0	4	2	1	3	1	0	0	0	1	12	0
Cloth 0,47	Post 3 h	0	1	0	2	0	0	1	0	0	0	0	1	4	0
Cloth 0,47	Post 3 h	0	2	0	1	0	0	0	1	4	3	6	0	6	9
Cloth 0,47	Post 3 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cloth 0,47	Post 3 h	0	5	0	2	0	0	1	3	5	2	3	0	11	5
Cloth 0,47	Post 3 h	2	5	0	6	1	1	4	0	0	0	0	1	13	0
Cloth 0,47	Post 3 h	0	7	0	6	2	1	3	7	0	0	0	1	20	0
Cloth 0,47	Post 24 h	0	5	0	6	0	1	2	2	3	0	0	1	15	0
Cloth 0,47	Post 24 h	0	8	0	8	4	1	2	5	3	0	0	0	23	0
Cloth 0,47	Post 24 h	0	3	0	3	1	1	0	4	0	0	0	0	9	0
Cloth 0,47	Post 24 h	0	2	0	3	1	1	2	4	1	0	0	1	13	0
Cloth 0,47	Post 24 h	0	4	0	6	1	1	1	6	0	0	0	1	16	0
Cloth 0,47	Post 24 h	0	0	0	1	0	0	0	0	0	0	0	1	2	0
Cloth 0,47	Post 24 h	0	1	0	3	1	1	4	4	0	0	0	1	14	0
Cloth 0,47	Post 24 h	0	12	0	12	0	0	0	2	5	4	0	0	19	4
Cloth 0,47	Post 24 h	0	2	0	4	1	1	2	1	0	0	0	1	10	0
Cloth 0,47	Post 24 h	0	2	0	3	0	1	2	0	0	0	0	1	7	0
Cloth 0,47	Post 24 h	0	5	0	6	0	1	5	2	0	0	0	1	15	0
Cloth 0,47	Post 24 h	0	1	0	1	0	0	0	1	2	0	0	0	4	0
Cloth 0,47	Post 24 h	0	9	0	9	1	1	1	7	1	0	0	0	20	0

Table 40 - Number of types of behaviour observed repeatedly during pre- and post-treatment in the group of bees treated with 0.7 ng of clothianidin. Number of types of behaviour observed repeatedly during pre- and post-treatment in the group of bees treated with 0.47 ng/bee of clothianidin. Translation of text within table: *Trattament i*= treatments; *Timing Osservazione* = observation timing; *Danza* = dance; *Ferma*= stationary;

Gira senza meta = wanders around with no particular goal; *Cammina* = walks; *Esplora cellette* = explores the cells; *Scarica polline* = discharges pollen; *Offre cibo* = offers food; *Si pulisce* = cleans itself; *Immobile* = immobile; *Barcolla* = staggers unsteadily; *Esce* = flies out; *Comp. Normali* = normal behaviour; *Comp. Anomali* = abnormal behaviour.

Trattamento	Timing osservazione	Danza	Ferma	Gira senza meta	Cammina	Esplora cellette	Scarica polline	Offre cibo	Emette feromone	Si pulisce	Immobile	Barcolla	Esce	Comp. normali	Comp. anomali
Cloth 0,7	Pre	0	3	0	5	1	1	3	3	1	0	0	1	15	0
Cloth 0,7	Pre	0	1	0	2	1	1	0	2	0	0	0	1	7	0
Cloth 0,7	Pre	0	2	0	3	1	1	2	1	1	0	0	1	10	0
Cloth 0,7	Pre	0	9	0	10	1	1	6	9	0	0	0	1	28	0
Cloth 0,7	Pre	0	7	0	8	1	1	3	8	0	0	0	1	22	0
Cloth 0,7	Pre	0	9	0	9	1	1	5	11	0	0	0	1	28	0
Cloth 0,7	Pre	1	2	0	1	1	1	3	3	0	0	0	1	10	0
Cloth 0,7	Pre	0	7	0	8	1	1	6	11	1	0	0	1	29	0
Cloth 0,7	Pre	0	6	0	6	1	1	2	5	0	0	0	1	16	0
Cloth 0,7	Pre	0	11	0	10	2	1	3	10	1	0	0	0	27	0
Cloth 0,7	Pre	0	6	0	6	1	1	2	10	4	0	0	0	24	0
Cloth 0,7	Post 3 h	0	3	1	4	0	0	2	0	0	0	0	0	6	1
Cloth 0,7	Post 3 h	0	1	3	1	0	0	1	0	0	0	2	0	2	5
Cloth 0,7	Post 3 h	0	0	2	1	1	0	0	0	0	0	0	0	2	2
Cloth 0,7	Post 3 h	0	2	0	0	0	0	0	0	0	1	3	0	0	4
Cloth 0,7	Post 24 h	0	0	0	1	0	0	0	0	0	0	1	0	1	1
Cloth 0,7	Post 24 h	0	5	0	6	1	1	3	3	2	0	0	0	16	0
Cloth 0,7	Post 24 h	0	6	0	7	1	1	1	7	1	0	0	0	18	0

It is important to note that the "stationary" condition was a "state" during which the bee could perform activities and adopt behaviour compatible with normal activity (offer food, emit the Nasonov gland). Therefore the behaviour in this condition was kept separate from the condition of immobility, the latter being compatible with the effects of poisoning, in which the bee was often seen in marginal areas of the hive, and did not engage in any other forms of behaviour while it remained immobile, and did not interact with its companions or emit the Nasonov gland. However, the "stationary" condition was not considered as one of the forms of behaviour useful for the comparative analysis, as it was already represented by other more specific forms of behaviour adopted during this state.

The graphs in Figures 32-43 show the behavioural trend observed over time for the three groups of bees prior to capture (pre-treatment), in the subsequent 3 hours (post-treatment 3 h) and after 24 hours (post-treatment 24 h). In order to provide a general overview, the graphs show the trend of all three groups of bees in reference to each of the forms of behaviour observed, but it should be underlined that the statistical analysis was performed by making separate comparisons for each group of bees with regard to the number of times a bee engaged in a certain type of behaviour before and after treatment.

The analysis was carried out by comparing pre- and post- treatment behaviour both in bees submitted to clothianidin administration (0.47 e 0,7 ng/bee) and also in control bees fed with sucrose solution only, as it was not ruled out that capture and introduction of bees into the Gilson pipette could constitute an additional stress factor.

The numerosity of bees in the various groups was not uniform, due to the fact that many bees were lost after administration of the treatment, especially after the 0.7 ng/bee treatment. Furthermore, it was not always possible to record the behaviour of all the bees, and frequently the premises of homogeneity of variance among the data was not respected. Consequently, non-parametric analysis

of variance according to Kruskal-Wallis was performed; the results are shown in the figure captions.
Figures 32-38 show the data pertaining to behaviour considered to be "normal". As can be seen, the number of times the control bees produced forms of behaviour considered as normal (walking, exploring the cells, discharging nectar, offering food, emitting pheromones, cleaning themselves, flying out) was on average not different during pre- and post-treatment observations. The control bees showed no types of behaviour considered as abnormal, either in pre- or post-treatment (Figures 39-41).
In contrast, bees treated with 0.47 ng/bee showed significant differences between observations performed before and after treatment, for all types of behaviour investigated, with the exception of cleaning, with regard to which no significant differences between pre- and post-treatment were observed.

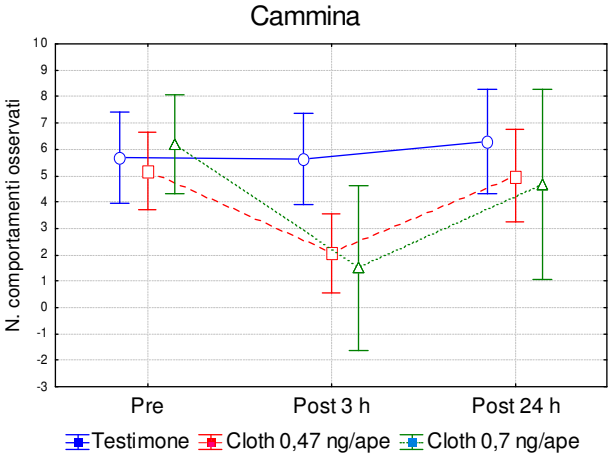

Figure 32 - Mean and. Standard dev. of number of cases of "**walking**" behaviour.
Controls: H(2, N= 36)=0.214 p=0.89
Cloth 0.47 ng/bee: H (2, N= 48) =14.2 p=0.0008
Cloth 0.7 ng/bee: H(2, N= 18)=6.04452 p=0.049

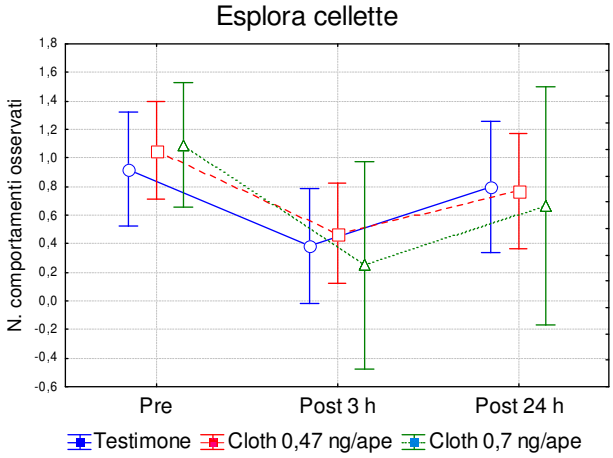

Figure 33 - Mean and. Standard dev. of number of cases of "**exploring cells**" behaviour.
Controls: H(2, N= 36)=5.011 p=0.0817
Cloth 0,47 ng/bee: H(2, N= 48) =7.668 p=0.0216
Cloth 0,7 ng/bee: H(2, N = 18)=8.606 p=0.0135

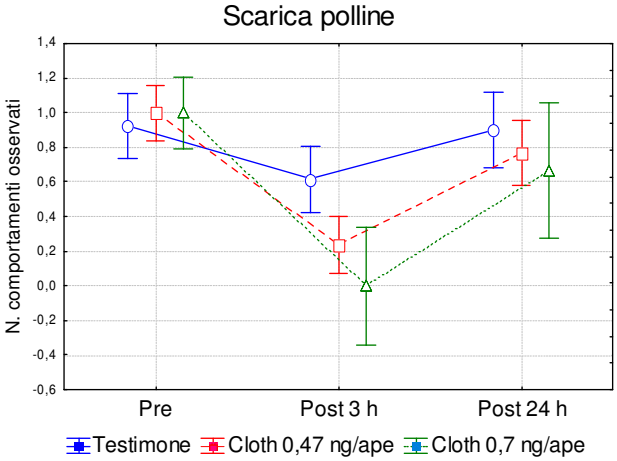

Figure 34 - Mean and. Standard dev. of number of cases of "**discharging pollen**" behaviour.
Controls: H (2, N=36) = 4.59 p = 0.1010

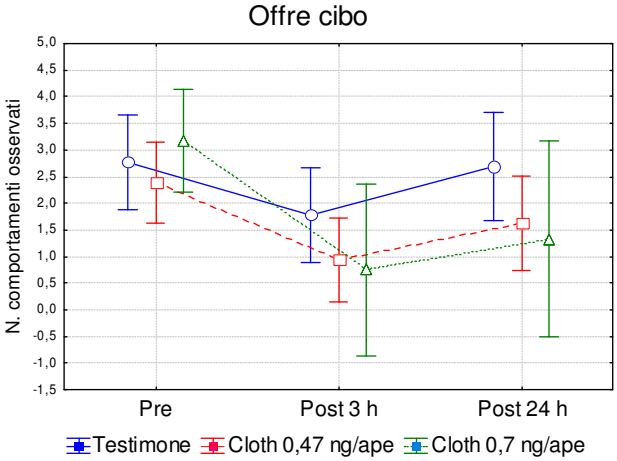

Figure 35 - Mean and. Standard dev. of number of cases of "**offering food**" behaviour
Controls: H(2, N= 36)=3.434 p=0.1796

Cloth 0.47 ng/bee: H(2, N=48)=23.354 p=0.0001
Cloth 0.7 ng/bee: H(2, N=18)=13.86154 p=0.001

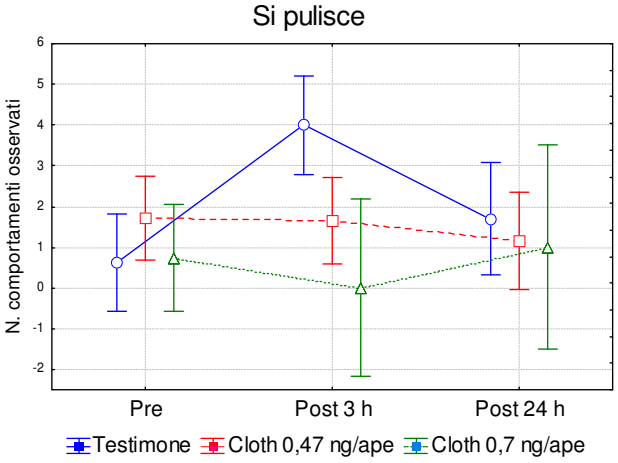

Figure 36 - Mean and. Standard dev. of number of cases of "**cleaning itself**" behaviour.
Control: H (2, N= 36) =11.5595 p=0.0031
Cloth 0.47 ng/bee: H (2, N= 48)=0.40723 p=0.0816
Cloth 0.7 ng/bee: H (2, N= 18)=3.5368 p = 0.171

Cloth 0.47 ng/bee: H(2, N=48)=8.591 p=0.014
Cloth 0.7 ng/bee: H(2, N= 18)=6.3394 p=0.042

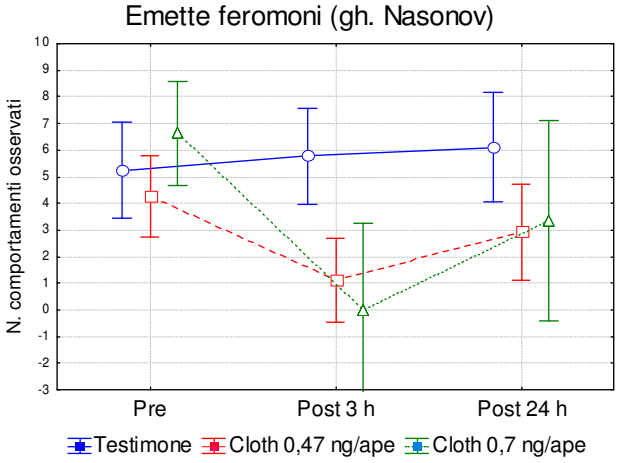

Figure 37 - Mean and. Standard dev. of number of cases of "**emitting pheromones**" behaviour.
Control: H (2, N= 36) = 0.476 p = 0.789
Cloth 0.47 ng/bee: H(2, N=48)=15.3534 p = 0.0005
Cloth 0.7 ng/bee: H (2, N=18)=9.3858 p = 0.009

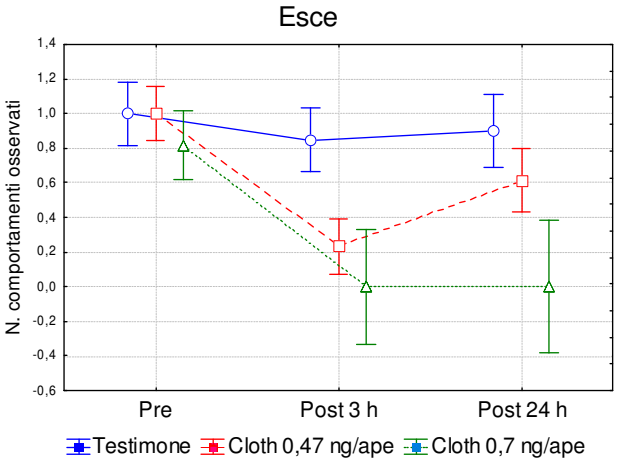

Figure 38 - Mean and. Standard dev. of number of cases of "**flying out**" behaviour.

Control: H (2, N=36)=7.11598 p=0.0285
Cloth 0.47 ng/bee: H (2, N=48)=5.271 p=0.072
Cloth 0.7 ng/bee: H (2, N=18)=10.8182 p=0.0045

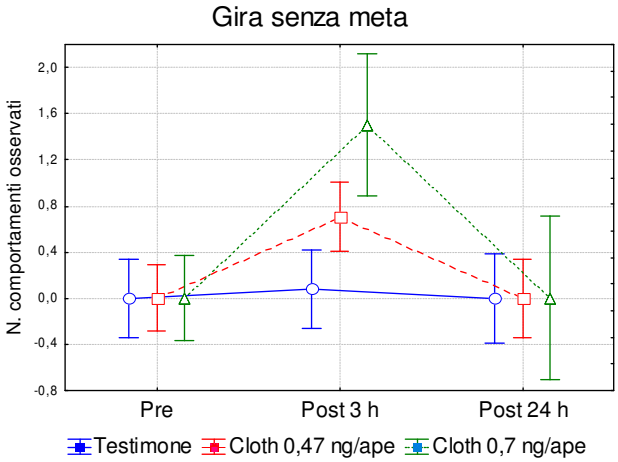

Figure 39 - Mean and. Standard dev. of number of cases of "**wandering around without a particular goal**" behaviour.
Control: H (2, N= 36) =1.769231 p = 0.413
Cloth 0.47 ng/bee: H (2, N= 48) =12.193 p = 0.0023
Cloth 0.7 ng/bee: H (2, N= 18) =11.784 p = 0.0028

If one examines the data pertaining to behaviour considered as abnormal (Figures 39-41), it can be seen that the treated bees showed a significant increase in all forms of abnormal behaviour (wandering around without a particular goal, remaining immobile, staggering unsteadily).
If one considers the two categories taken as a whole, shown in the graphs in Figures 42 and 43, the differences between the controls and the treated bees emerged even more clearly. Thus the control bees showed no difference in behaviour between pre- and post-treatment, either with regard to normal forms of behaviour (all of which were present) or to abnormal forms of behaviour (all of

which were absent). In contrast, for treated bees the sum of behavioural types highlights the difference between pre- and post-treatment behaviour even more strongly.

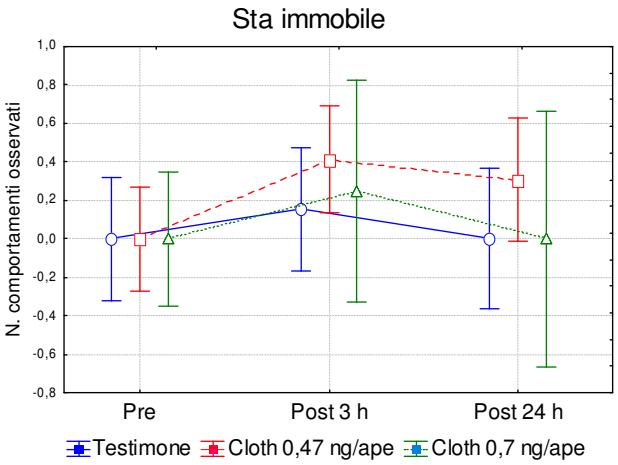

Figure 40 - Mean and Standard dev. of number of cases of "**remaining immobile**" behaviour.
Controls: H (2, N= 36) = 1.769 p = 0.413
Cloth 0,47 ng/bee: H (2, N= 48) = 4.933 p = 0.085
Cloth 0,7 ng/bee: H (2, N= 18) = 3.500 p = 0.174

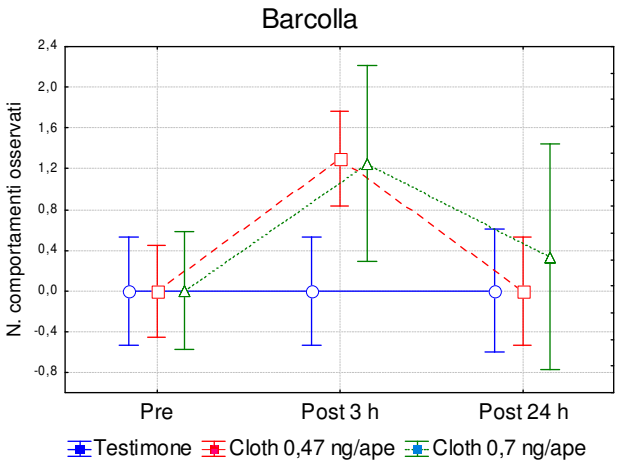

Figure 41 - Mean and Standard dev. of number of cases of "**staggering unsteadily**" behaviour.
Controls: H (2, N= 36) = 0.000000 p =1.000
Cloth 0,47 ng/bee: H (2, N= 48) =12.175 p = 0.0023
Cloth 0,7 ng/bee: H (2, N= 18) = 5.909 p = 0.052

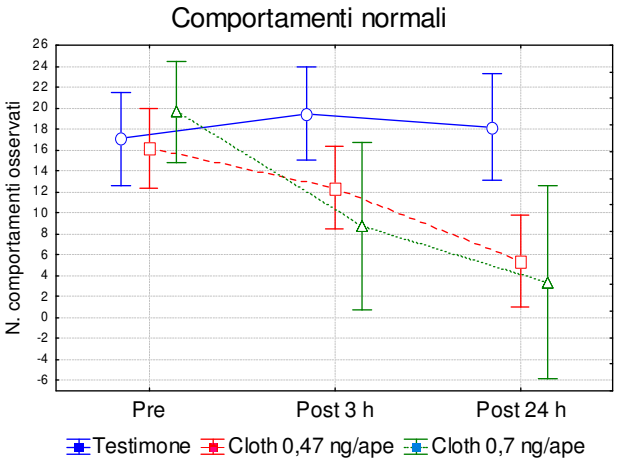

Figure 42 - Mean and Standard dev. of number of cases of behaviour definable as "**normal behaviour**".
Controls: H (2, N= 36) = 1.769 p = 0.413
Cloth 0,47 ng/bee: H (2, N= 48) = 4.933 p = 0.085
Cloth 0,7 ng/bee: H (2, N= 18) = 3.500 p = 0.174

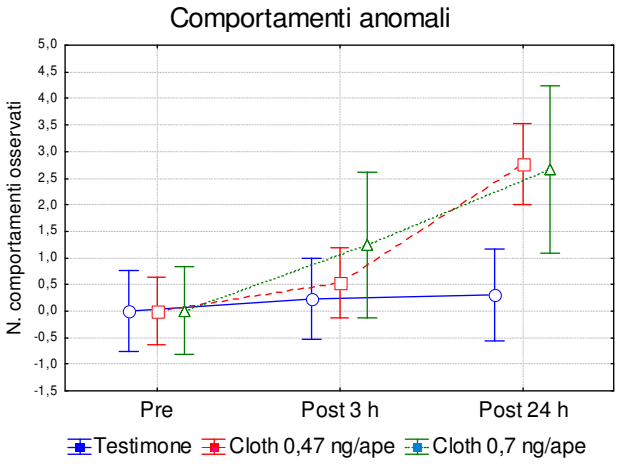

Figure 43 - Mean and Standard dev. of number of cases of behaviour definable as "**abnormal behaviour**".

Controls: H (2, N= 36) = 0.000000 p =1.000
Cloth 0,47 ng/bee: H (2, N= 48) =12.175 p = 0.0023
Cloth 0,7 ng/bee: H (2, N= 18) = 5.909 p = 0.052

6.5 Bee disorientation tests in the complex labyrinth

The experiment consisted in training the bees to move along a route inside a labyrinth in order to gain access to the reward in the form of sugar syrup. A bee's orientation within a complex labyrinth is based on associative learning that links a visual mark with the reward that consists of sugar syrup. The aim of the test was to assess the sublethal effect of clothianidin, an active ingredient used in maize seed dressing, on bee orientation ability within the labyrinth. Together with the PER (Proboscis Extension Reflex) test, this method could be used in future as part of official guidelines (EPPO, OECD) to assay the sublethal effects of pesticides. However, unlike the PER test, on which many publications are available and which has a methodology that is by now fairly standardised, the complex labyrinth test has so far been little used. The only studies available in the literature are those by Zhang et al. (2000) and Decourtye (2009)1.
Therefore the second aim of our experiment was to set up a protocol using the complex labyrinth to assay the sublethal effects of pesticides on bees. Starting out from the method devised by Decourtye et al. (2009), we carried out five trials designed to adapt the protocol proposed by the authors to our environmental conditions and thus to test the procedure both on treated bees and on the controls.
In the study by Decourtye et al., the labyrinth, together with a nuke of bees, was situated in a tunnel made of netting. To make a comparative analysis, on the same colony, of the bee response before and after exposure to the active ingredient, the protocol involved three periods: two non-treated periods separated by a treated interval. However, this method presented a number of disadvantages: 1) it is very lengthy, as several days must elapse between one phase and the next in order to renew and retrain the new bees, because a fresh batch of bees was used for each period; 2) comparison between the treatments could be "affected" by the time factor, as the assays were performed on different days for the bees submitted to treatment versus the controls; 3) the fact of being confined inside the tunnel could exert a negative influence on the bees' performance: in effect, in our preliminary test, performed in late May 2011 (see APENET report 19 June), when both the bees and the labyrinth itself were contained inside a net tunnel, it was found to be impossible, in our environmental conditions. to carry out the experiment with the bees confined as described. The high temperature inside the tunnel, especially in the middle of the day, obliged the bees to stay inside the hive where they could seek ventilation and maintain a lower temperature, or to collect the water in containers located inside the tunnel rather than the sucrose solution inside the labyrinth. As a result, the number of bees available for the disorientation test became drastically reduced. In the work by Decourtye et al. there is no specification of the period in which the trial was carried out, nor is there any mention of the internal temperature, whereas in Zhang et al. (2000) the test was carried out in a temperature-controlled greenhouse set to 24±5 °C during the day and 17±3 °C during the night.

6.5.1 Materials ad methods

Our experiment was conducted at CRA–API (Council for Research and Experimentation in Agriculture – Beekeeping and Silkworm Rearing Research Unit) of Bologna, from June to August 2011. As compared to the above-described preliminary trial, this time the labyrinth was removed from the tunnel and placed under a canopied shed in a shady spot of the CRA-API premises. A sucrose solution dispenser was moved little by little up to the first compartment of the labyrinth, and the bees were thus prompted to proceed along the route (roughly 50 m) from the apiary used for the experiment up to the labyrinth (Figure 31). The dispenser was made of a transparent plastic box with a layer of cotton wool soaked with 50% sucrose solution placed on the bottom, and an entrance

[1] Zhang S., Mizutani A., V. Srinivasan M. (2000). Maze Navigation by Honeybees: Learning Path Regularity. *Learning & Memory* 7: 363-374.

Decourtye A., Lefort S., Devillers J., Gauthier M., Aupinel P., Tisseur M. (2009). Sublethal effects of fipronil on the ability of honeybees (*Apis mellifera* L.) to orientate in a complex maze. *Julius-Kühn-Archiv*, 423: 75-83.

composed of a hole that matched the entry into the compartments. Each of the five trials was conducted in three phases:

1) training: when the bees of the apiary situated on the CRA-API premises reached the first compartment of the labyrinth, they began to learn to associate the correct route (marked by a round green sticker) with a sucrose solution source (reward);
2) treatment: the bees were first marked and then given a sublethal solution of a neonicotinoid (containing the active ingredient clothianidin) at the concentration of 10 µg/L (20 µg/L in trial 5), which they assumed by ingestion;
3) assay: bee performance in reaching the labyrinth and moving through it up to the last compartment, where the reward was located, was recorded in writing. The behaviour of the bees was observed for roughly 3 hours after their release (after the treatment).

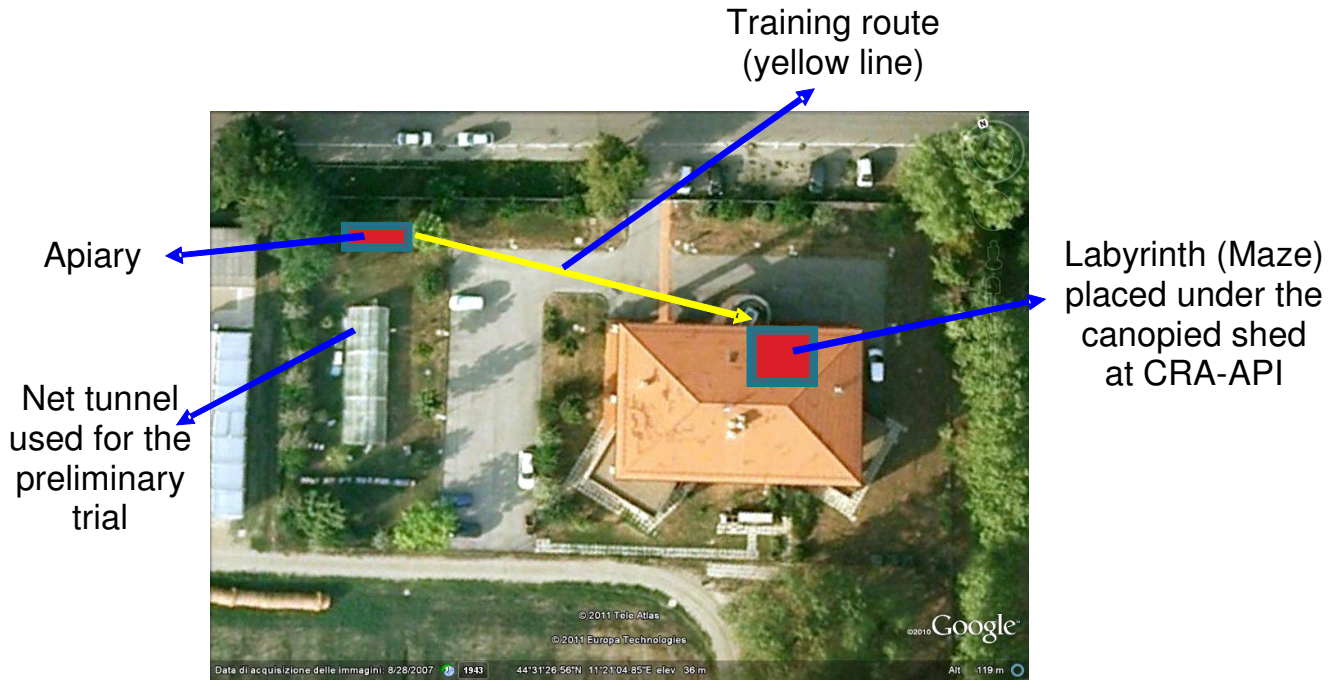

Figure 44 - Satellite view of the CRA-API of Bologna building where the experiment was conducted.

The utilised labyrinth was made entirely of opaque Perspex (Polymethyl methacrylate) and was covered with transparent film. It was composed of a matrix of 4 x 5 identical cubic compartments each with side walls measuring 30cm. Some of the walls had a 4 cm Ø hole to allow the bee to pass on to the next compartment. The structure was composed of 4 "decision compartments" and 5 "non-decision compartments". The "non-decision" compartments had two holes, each in different walls, one being an entry hole and the other an exit hole. In contrast, the "decision compartments" had three holes, which meant that the bee entered through one of the holes and could choose one of the other two holes in order to continue along the route.
The route to be followed up to the dispenser was marked by a round green sticker placed below the hole through which the bee had to pass. The dispenser was composed of a transparent plastic recipient inside which there was a Petri dish containing a wad of cotton wool soaked with 50% sucrose water. The bee could reach the dispenser by passing through a hole that matched that of the compartment (Figure 32 c).
The training consisted in making the bees associate the route indicated by the green sticker with the reward. When the bees, as part of their training, were prompted to move towards the labyrinth, they were attracted into the first compartment because a feed dispenser had been placed in the compartment. After about an hour the dispenser was gradually moved into the subsequent

compartments (up to the fifth compartment), maintaining it for roughly half an hour in each compartment. From the fifth compartment the route led directly to the ninth compartment, and a marking was placed on bees that reached the objective. Once a bee reached the dispenser, it did not receive training to return back into the labyrinth: instead, it was released by opening the cover of the compartment, and the time of release was recorded. The manner of training of the bees up to the ninth compartment was the same in all the trials.
After the treatment phase (which was conducted with different modalities in the various trials in order to identify which manner of proceeding least disturbed the bees), the test to assess the effect of the active ingredient on the bees was performed. The assay consisted in making the bees enter the labyrinth one at a time, recording the time that elapsed from the moment of treatment up to the bee's return to the labyrinth, and also recording the time required for the bees to move through the compartments until they reached the sucrose solution. Once a bee reached the compartment with the reward, it was captured in order to avoid letting it repeat the same course along the route (with the exception of trial 5). Periodically the route within the labyrinth was modified and the structure was cleaned with acetone in order to ensure that the bees would associate the correct route exclusively with the green sticker at the entry to the compartment.

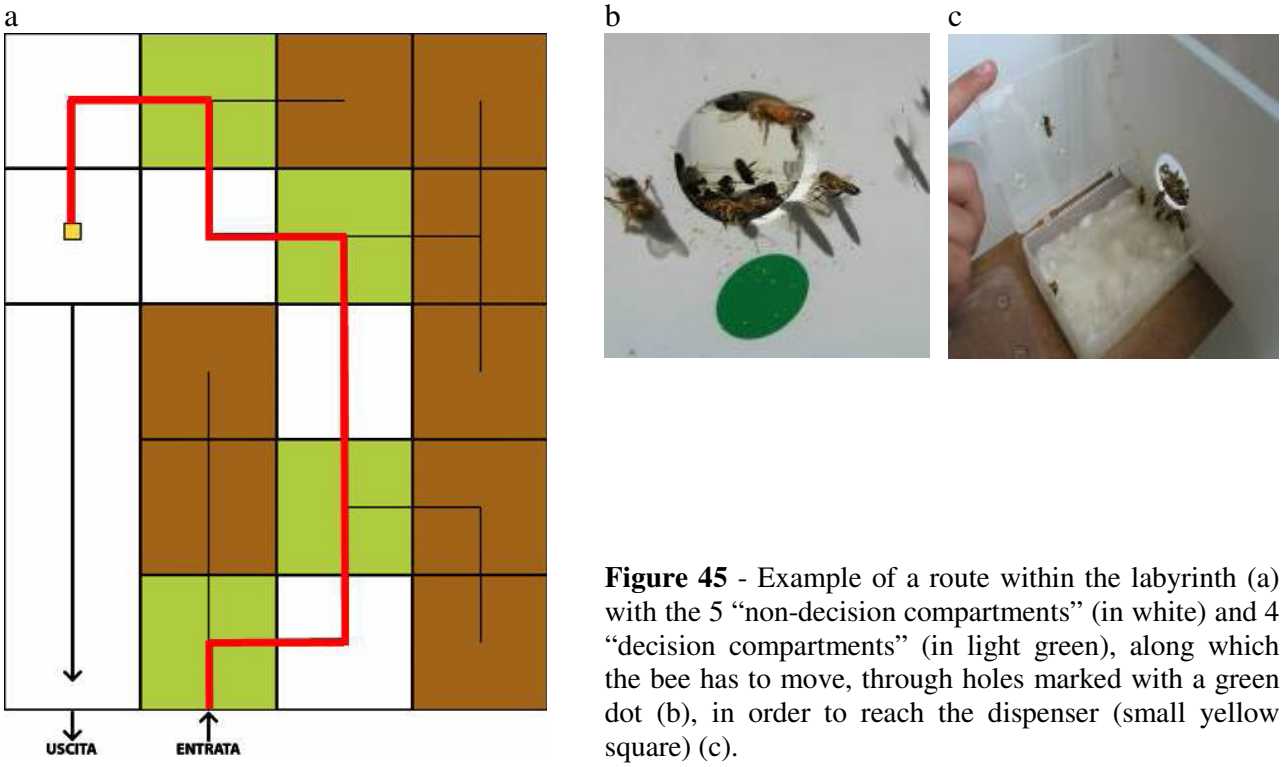

Figure 45 - Example of a route within the labyrinth (a) with the 5 "non-decision compartments" (in white) and 4 "decision compartments" (in light green), along which the bee has to move, through holes marked with a green dot (b), in order to reach the dispenser (small yellow square) (c).

Trial 1
On 16 June, whenever bees reached the ninth compartment a white mark was placed on them to distinguish them, and they were then released to continue their foraging flights from the dispenser to the hive. Subsequently, when a certain number of bees had been reached (N = 70) (the progressive numbering was marked on the dispenser), the dispensing recipient was closed and transferred to the laboratory; the bees inside it were stunned with CO2. At this point the bees were divided into four groups and marked to indicate the treatment: two for the "treated" bees (ingestion of 10 µg/L of clothianidin) and two for the "controls" (Figure 33). The bees were then inserted in groups of 15-20 into cardboard cages (9,5x5x6,5 cm) with a dispenser containing the sucrose solution (pure for the "controls", and supplemented with the active ingredient in the "treated bees". The bees consumed the solution completely within about 3 hours.

Two groups (one "treated" and one "control") of 125 bees each were released immediately after administration of the active ingredient solution in order to evaluate the immediate effect, while the other two groups (again composed of a "treated" and a "control" group), each composed of 20 bees, were released after 24 hours to study a possible delayed effect. These bees, while awaiting release, were maintained in an incubator at 25 °C, in darkness and fed ad libitum with water and sucrose solution (25%). Since not all the bees in the immediate effect trial returned to the labyrinth within the first day of trials – and this non-return was expected – and since the colours utilised for the immediate effect treatment group and for the delayed effect treatment group (and also for the control treatment group) were the same, bees released after 24 hours were then distinguished from those of the day before by submitting them to stunning again and marking them with different colours The test was performed between the apiary and the labyrinth straightaway after release of the bees.

For each group of bees the following parameters were recorded: bees that succeeded in returning, bees that reached the dispenser located in the ninth compartment, time elapsing between release and arrival in the labyrinth, and time taken to travel along the route inside the labyrinth (from entry up to the final compartment). During the treatment and up to the beginning of the test, no other bees were allowed to enter the labyrinth in order to avoid an excessive flow of bees during the time evaluation phase.

On 17 June a second trial was performed with the same procedure as the previous one (with the exception of the 24 h test), but without interrupting the flow of bees to the labyrinth during the treatment phase. 40 bees were marked and divided between the two types of treatment.

Figure 46 - Marking and division of bees stunned with CO_2 (on the left) and division of the bees into 4 groups marked with different colours and divided among the cages (on the right)

Trial 2

Given the possibility that excessive utilisation of CO_2 in the marking phase and during transfer into the small cages had perhaps have induced too much stress in the bees, which could have interfered with evaluation of the effects of the active ingredient, carbon dioxide was not used in this second trial.

On 20 June the bees were trained to reach the labyrinth, and then on the second day they were subjected to the test as described in trial 1. In the absence of CO_2, treatment was administered by replacing the dispenser with pure sucrose solution (controls) and with another dispenser containing a quantity of active ingredient at the dose of 10 µg/L (treated group). Bees were allowed to ingest the active ingredient only once (one single flight), directly on the treatment dispenser. Bees stopped sucking the solution when, presumably, the honey sack was full (40 mg). In order to have two distinct groups, the bees (N = 14) that reached the final compartment and fed on the sucrose solution were marked with white (controls); immediately afterwards, the second group of bees (N =

17) that reached the final compartment and fed at the dispenser containing the contaminated solution were marked with green (treated group). Entry to the final compartment was closed between one group and the other.
In this trial, we realised that some bees did not feed fully because the markings were placed on the bees while they were feeding; therefore it cannot be ruled out that some of the bees may have been disturbed by the marking procedure and may have flown away, with the markings on them but without having completely absorbed the active ingredient. For this reason, CO_2 was re-utilised in the subsequent trials, but we standardised the times.

Trial 3
On 19 July the bees were trained to reach the ninth compartment as described above. Treatment was carried out as in trial No. 2, i.e. administering the active ingredient to the bees only once, directly from the dispenser. When a sufficient number of bees had been reached, the dispenser was closed and immediately transferred to the laboratory in order to place the markings on the bees, stunning them in CO_2 for 30 minutes. Bees that had ingested the contaminated solution (N = 19) were marked with pink, while those that had ingested the control solution (N = 17) were marked with white. The bees in the respective dispensers were placed in a single empty compartment of the labyrinth and released after 90 minutes. During the marking and poisoning phase, a flow of bees to the labyrinth was maintained, leaving the dispenser with the sucrose solution in the ninth compartment. The problem encountered in this trial was the stress induced in the bees by excessive confinement after exposure to CO_2 (from capture to release: 120 minutes). It was therefore decided that this length of time should be reduced.

Trial 4
On 25 July the bees were trained to reach the ninth compartment as described in the previous trials. Poisoning was performed as in trials 2 and 3, with the difference that the bees were left for 30 minutes in contact with the dispenser before the marking procedure, in order to make sure that all the bees had fed on the solution containing the active ingredient. Subsequently the bees were stunned in CO_2 for 10 minutes, and the bees that had ingested the control solution (N = 19) were marked with green while those that had ingested the contaminated solution (N = 20) were marked with orange.
As compared to the previous trial, the length of time of exposure to CO_2 during the marking phase was decreased, for a total of 10 minutes per group in comparison to 30 minutes in trial 3. Subsequently, the bees were placed in the labyrinth, in separate and ventilated compartments, and released after 30 minutes (total length of time from capture to release: 75 minutes). In this trial as well, the flow of bees to the labyrinth was maintained during the treatment and marking phases, with the dispenser placed in the ninth compartment.

Trial 5
On 1 August the bees were trained to reach the ninth compartment, and were then marked and treated with the same procedure as in trial 4. The 34 active ingredient-treated bees were marked with blue, while the 33 control bees were marked with pink. In this trial, as compared to the previous trials, the active ingredient concentration in the solution administered was doubled (20 µg/L). Bees that reached the dispenser were then re-marked (with purple for the treated bees and white for the controls) in order to assess a possible delayed effect in the second flight.

Statistical analyses
The $\chi 2$ test was utilised to analyse the number of bees of the treatment and control groups which: 1) did not return to the labyrinth ("lost"), 2) returned but were unable to reach the final compartment ("disoriented"), 3) returned to the labyrinth and reached the final compartment ("arrived"). With regard to the "arrived" group, the time taken to move along the route inside the labyrinth was

calculated, and the differences among the treatment groups were analysed by means of the t-test (or the Mann-Whitney test in cases in which the presuppositions of normality were not respected). Additionally, the time elapsed between release from the cage (trial 1) or treatment (trial 2) and return of the bees to the labyrinth was compared among the treatments using the t-test (or the Mann-Whitney test in cases in which the presuppositions of normality were not respected).

6.5.2 Results

As compared to the preliminary trial, the flow of bees that arrived at the labyrinth after the latter was moved outside of the tunnel was very intense. This made it easy to train an elevated number of bees to reach the final compartment (the ninth). The training phase from the first to the ninth compartment thus proved to be very simple, requiring roughly 2-3 hours.

Trial 1

On 16 June, over 70 bees succeeded in completing the entire route of the labyrinth and were submitted to treatment (sucrose or contaminated solution). However, both in the immediate effect test and the test performed after 24 hours, many of the bees (60-80%) failed to return to the labyrinth after their release. Among those that did return, only 5-20% reached the final compartment (Figures 34 and 35).

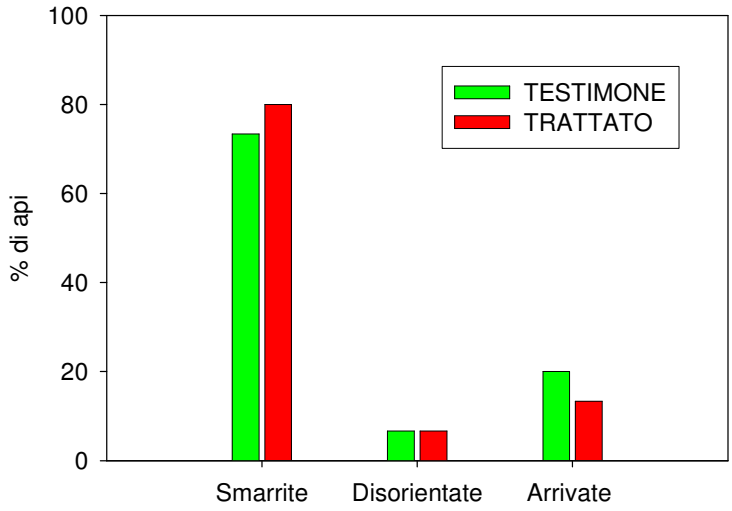

Figure 47 - Behaviour of bees after release (immediate effect). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

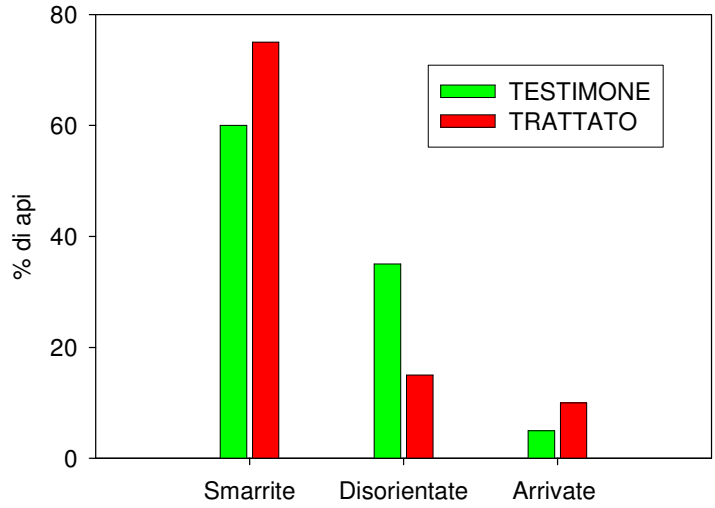

Figure 48 - Behaviour of bees after release (effect after 24 hours). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

Comparison between the active ingredient-treated bees and the controls showed no statistically significant effects, either in the immediate effect ($\chi 2$ = 0,243; gdl = 2; P = 0,885) or the effect after 24 hours ($\chi 2$ = 2,267; gdl = 2; P = 0,322). It is likely that the interruption of the flow of bees to the laboratory discouraged the marked bees from returning; consequently, on 17 June it was decided that the flow of bees to the labyrinth would not be interrupted, and a dispenser was maintained at the entry into the labyrinth during the period in which the marked bees were in the cage. In this case the number of bees that returned to the labyrinth was greater than on the previous day, but still rather low, especially in the active ingredient-treated group (Figure 36). Comparison of the frequencies of bees that returned to the labyrinth and correctly moved along the route up to the final compartment showed no significant differences between treated bees and controls ($\chi 2$ = 4,578; gdl = 2; P = 0,101). Similarly, as far as time taken to return (after release of the bees) was concerned, no statistically significant differences between treated and control bees were observed, either on June 16 or June 17 (Table 41). The difficulty encountered by the bees in trying to return to the labyrinth may have been due to the fact that the stunning with CO_2, performed in order to insert the bees into the small cages, and the stress induced by confinement in the cages, perhaps discouraged the bees from returning to the labyrinth. For this reason, it was decided that in the next trial the bees would not be stunned with CO_2, and that the active ingredient would be administered directly from the dispenser placed in the final compartment.

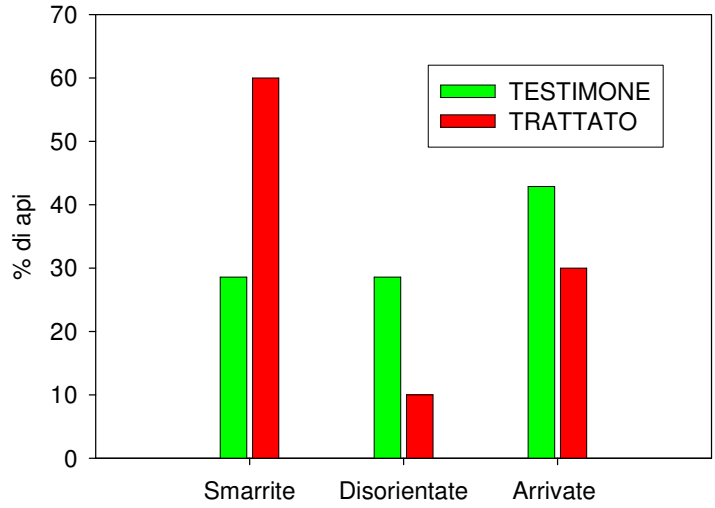

Figure 49 - Behaviour of bees after release (trial dated 17 June)**.** Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

Table 41 - Mean time (± St.E.) of return and travel within the labyrinth and result of the statistical test (t-test or Mann-Whitney).

Data	Time taken for return to the labyrinth (minutes)			Travel time along the labyrinth route (seconds)		
	Control	Treated	Test	Control	Treated	Test
16 June (immediate effect)	45.5±17.7 N = 4	13.3±10.4 N = 3	P = 0.216	96.7±34.2 N = 3	64.0±46.0 N = 2	P = 0.6
16 June (delayed effect, at 24 h)	26.2±7.2 N = 8	34.0±11.9 N = 5	P = 0.564	43.0 N = 1	49.0±1.0 N = 2	
17 June	37.8±12.0 N = 14	76.3±16.5 N = 8	P = 0.562	73.6±15.0 N = 8	40.3±7.04 N = 6	P = 0.097

Trial 2

Therefore in this test, performed on June 21, the bees were not stunned with CO_2 but marked directly on the dispenser, placed in the final compartment, while they were feeding. The control bees were feeding on 25% water and sugar, and the treated bees on the solution contaminated with 10 µg/L of clothianidin. It was observed that the number of bees that returned to the labyrinth and reached the ninth compartment was significantly greater in the control group than in the treated group ($\chi 2$ = 6,457; gdl = 2; P = 0,04; Figure 37). On the other hand, no significant differences emerged between the treated group and the controls with regard to the time required for completing travel through the labyrinth (t = -0,29; P = 0,78; gdl = 17). Mean time taken by the control bees was 52,6 ± 6,6 seconds (N=12), versus 56±10.6 in the treated bees (N=7).

Trial 3

On June 19, the procedure of stunning the bees with CO_2 in order to perform the marking was resumed, for the reasons described above. However, exposure time was excessive and many bees failed to return to the labyrinth within the time allotted for the trial. But after six days some bees (both of the control and the treated group) were seen on the dispenser in the labyrinth, thus

suggesting a transitory effect. Observation of the number of bees that returned to the labyrinth revealed no significant differences between the treated group and the control group ($\chi 2 = 4{,}32$; gdl = 2; P = 0,115; Figure 38).

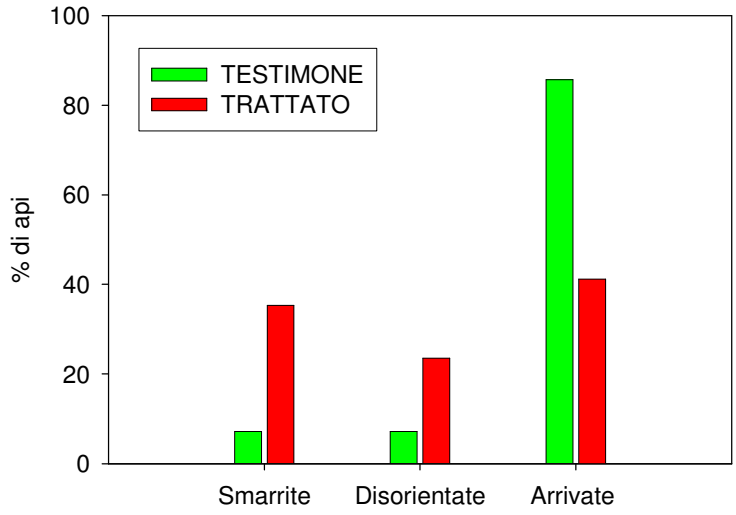

Figure 50 - Behaviour of bees after release (trial dated 21 June). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

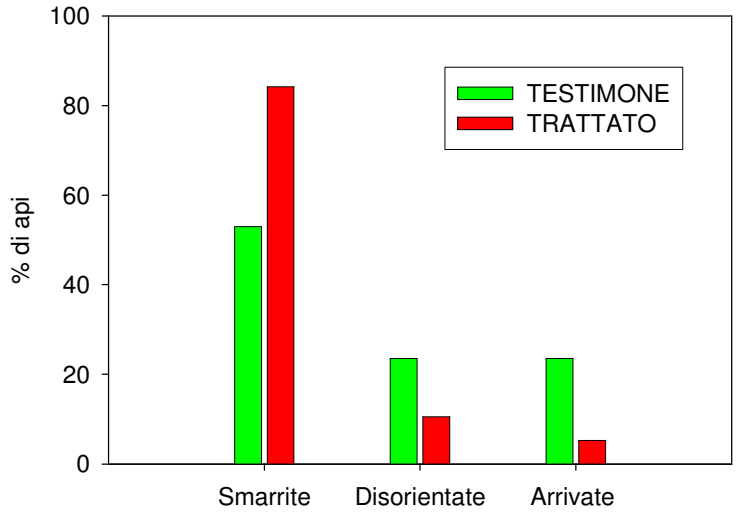

Figure 51 - Behaviour of bees after release (trial dated 19 July). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

With regard to time taken to complete travel along the route in the labyrinth, it was not possible to carry out any statistical test to compare treated bees and the controls, as only one bee among those submitted to the active ingredient treatment reached the dispenser, in a time of 34 seconds. In contrast, it took the control bees 72.75 ± 11.49 (mean ± SE) seconds (N = 4) to reach the dispenser.

Trial 4

In this trial a greater number of bees returned to the labyrinth (62%) as compared to the previous trial (31%). This was probably due to the shorter exposure time to CO_2 during the marking phase, and to the reduced time of confinement within the compartment (30 minutes versus 90 min in trial 3).

Differences in frequency of arrival of active ingredient-treated versus control bees were not significant ($\chi^2 = 0.931$; gdl = 2; P =0.628; Figure 39).
After the bees had ingested the active ingredient, they travelled along the route in the labyrinth in 62.70 ± 11.49 (mean ± SE) seconds (N = 10), while it took the control bees 47.37 ± 5.348 (mean ± SE) seconds (N = 8). No significant differences between active ingredient-treated and control bees were observed (t = 1.09; gdl = 16; P = 0.29)..

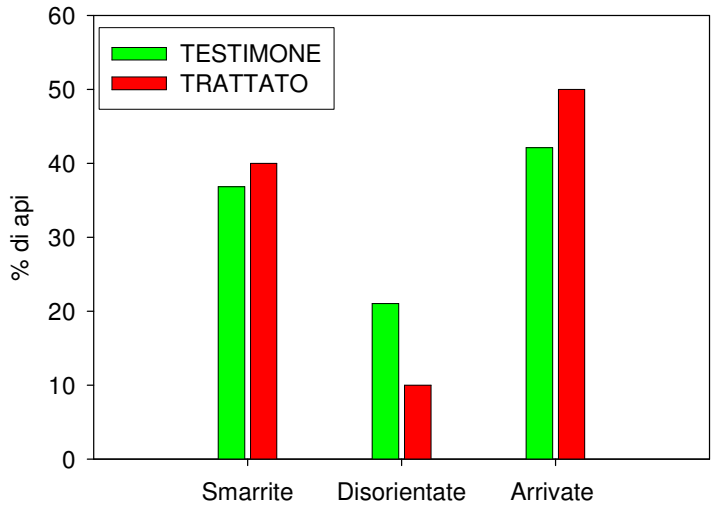

Figure 52 - Behaviour of bees after release (trial dated 25 July). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

Trial 5
In the final trial, the total number of bees that had returned to the labyrinth within three hours after release was similar to that of the previous trial (52%). Both in the first flight and also in bees that made two flights, frequency of arrival showed no significant differences between active ingredient-treated and control bees, either in the first or the second flight (1st flight: $\chi 2 = 1.237$; gdl = 2; P =0.539; Figure 40; 2nd flight: $\chi 2 = 2.165$; gdl = 2; P =0.339; Figure 41).
Duration of travel through the labyrinth likewise showed no significant differences, either in the first flight (t-test = -0.16; gdl = 16; P = 0.87; control: 90.87±15.74 seconds (N = 8); active ingredient-treated: 94.25±14.17 seconds (N = 10)).

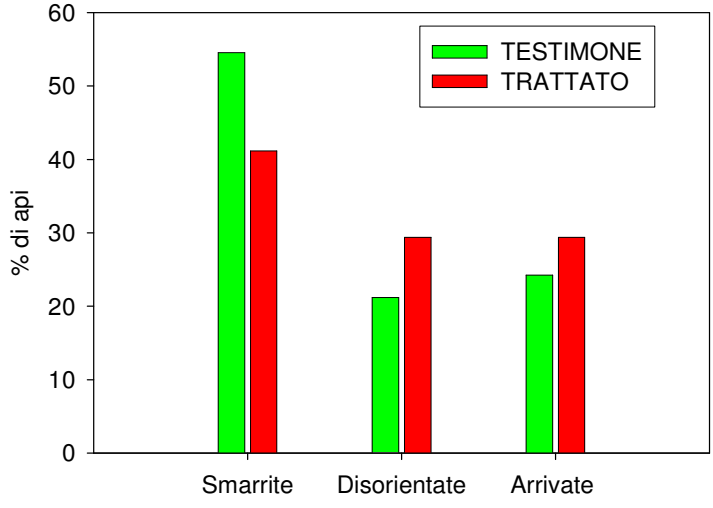

Figure 53 - Behaviour of bees after release – 1st flight (trial dated 1 August).Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

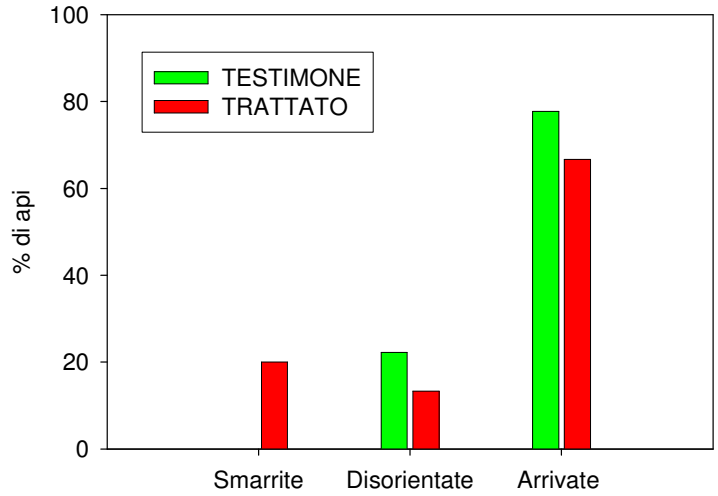

Figure 54 - Behaviour of bees after release – 2nd flight (trial dated 1 August). Translation of text within figure: *Testimone* = Control; *Trattato* = treated; *% di api* = % of bees; *Smarrite* = Lost; *Disorientate* = disoriented; *Arrivate* = arrived.

6.6 Conclusions

Olfactory memory
The experiments confirmed the results of the experiments conducted on dust-derived contamination in 2009. In the 2011 experiments, all the active ingredients assayed, even at these extremely low concentrations, were found to be able to impair olfactory learning and memory. The mean percentage of bees that responded correctly to odour presentation was consistently lower in active ingredient-treated bees than in the controls, for all time intervals.

Colour recognition and orientation in the Y labyrinth
The experiments were conducted to assess the orientation ability of unconfined bees in a simple Y labyrinth, in which the bees faced the task of entering and finding the reward on the basis of a visual stimulus, namely by following a colour. Our results showed that the sub-lethal doses of thiamethoxam contained in dust deposited at 5 m by the modified seeding machine (5 % of active ingredient as compared to the unmodified seeder) were capable, after 24 hours, of impairing the bees' ability to move towards a known food source (only 50% of bees returned to the labyrinth after 24 hours) and to recognise the colours associated with the sugar reward. We even recorded two cases of a bee repeatedly choosing the colour that had been associated with salt during the training.

Homing behaviour in relation to the nest
Research on the effects of sub lethal doses on homing ability and on bee behaviour in the hive is in progress. However, our preliminary results already provide clear evidence that even at extremely low doses, such as that applied in protocol 1 (0.092 ng/bee, equivalent to 1/10 of the dose used in the PER experiments concerning the effects of ingestion contamination on olfactory memory, - 2009 and 2010), contact with the active ingredient by ingestion led, after a number of ingestions

that varied from 3 to 10, to poisoning of the bee with impairment in foraging activity and loss of orientation ability, as tested after 24 hours. .
We also examined how many repeated occurrences of micro-intake of active ingredient were necessary to induce the blocking of bee activity, which in most cases resulted in loss of the bees. The number was variable, with some bees failing to return to the feeding point after 3-5 administrations, and simply stopped at the nest, while others performed 10-12 flights (which effectively meant exposure to 10-12 administrations). Only one bee was present in the nest after 24 hours, whereas none of the control bees were missing.
Administration protocol 2 constitutes the repetition of the experiments conducted during 2010. In the latter trials, the behaviour of 10 treated bees and 10 controls was examined, and overall the results obtained during the two years of study display considerable similarity. Bees submitted to a single administration of a quantity of clothianidin defined as 0.47/bee showed marked immediate effects: the bees returned to the nest but remained immobile for a prolonged period of time, and did not exchange food or discharge the honey sac. Exposure to clothianidin significantly reduced foraging flight frequency during the hours immediately after ingestion, and on the following day the foraging frequency of many bees was nil.
Protocol 3 involved repeated administration of the dose utilised for protocol 2 (0.47 ng/bee). Two bees out of 4 returned to the feeding point a second time, thus receiving administration of a second dose; these bees returned to the nest but did not fly out again, and at the 24 hour inspection they were not present either at the dispenser or in the nest. The only bee that succeeded in returning a third time, thus receiving a third dose, was not able to return to the nest.
The only bee that was given a comparable quantity (0.552 ng/bee in 30 microlites (honey sac full) did not return to the nest.
It is evident that clothianidin, administered according to various protocols that simulated different modes of contact or ingestion in the field, produced in all cases a reduction in foraging frequency, impairment in behaviour of bees that returned to the nest, or loss of orientation with failure to return to the nest when the dose reached roughly 0.4 ng/bee through multiple exposures to very low doses.
Therefore, it can be concluded that exposure to extremely low doses of neonicotinoids and fipronil, either in the form of contact from dust or intake by ingestion (from nectar, pollen or contaminated water) impairs a bee's ability to adopt the appropriate behaviour required for fulfilling its functions. If exposure is repeated (as is obviously the case if the dose is not such as to kill the bee immediately), this leads to disorientation (inability to return to the nest) and/or death.
The logical conclusion to be drawn is that since the bees forage en masse on the same crops, if there is an outbreak of contamination among a significant number of foraging bees then this impairs the equilibrium of the entire colony. The assumption of sub lethal doses of these active ingredients can therefore contribute to creating a state of "chronic weakness" of the colony.

Disorientation trial in the complex labyrinth
One of the aims of this experiment was to set up a protocol for evaluating the effects of neonicotinoids on bee orientation ability in a complex labyrinth, which we adapted to our experimental conditions. Our study was initially based on the research by Decourtye et al. (2009), who pioneered the first experiments in a tunnel were carried out, but our environmental conditions proved unsuitable for training a large number of bees to move along the route in the labyrinth, with the labyrinth located in a tunnel made of netting (a maximum of 6 bees, and only up to the fifth compartment: see 19 June APENET report). In our setting, the bees showed only scanty flying activity, especially during the warmest hours in the middle of the day, and a marked weakening of the family was observed. This problem was attributed to the excessive temperature in the tunnel, and to the fact that the bees suffered far greater stress in the very confined environment. Furthermore, Decourtye and co-workers divided the experimental procedure into three phases (two "untreated" periods separated by a "treated" interval), in which the bee response was evaluated

before and after exposure to the pesticide. In our view, a possible consequence of this method is that the experiment may be subject to the "time" variable. Thus one of the aims of our study was to assay both the active ingredient-treated and the control bees at the same time.
Moving the labyrinth outside of the tunnel facilitated the process of training the bees to reach the labyrinth and to reach the ninth compartment. It thus became possible to use from a minimum of 30 bees up to a maximum of 70 for all the tests. The most delicate phases of the test were represented by the procedure of marking the bees and administering the treatment, with particular regard to making sure that the solutions administered were entirely ingested by the bees. In the tests in which CO2 was used, and the bees were confined in cages to ingest the solution containing the active ingredient, the number of bees that returned to the labyrinth increased with decreasing time of exposure to carbon dioxide and of confinement in the cage (Table 42). In agreement with this observation, the highest percentage of bees returning to the labyrinth was found in trial 2, in which the bees were not treated with CO2, and thus never interrupted their activity. However, it was also found that if markings were placed on the bees directly while they were on the dispenser, and thus without stunning them (marking first the active ingredient-treated bees and then the controls), there was a risk that some control bees would not ingest the full quantity of sucrose solution because their feeding was disrupted by the marking procedure. In this case, the possibility could not be ruled out that some control bees might, once they were released, return into the labyrinth and travel along the entire route up to the dispenser precisely during the phase in which the active ingredient-treatment bees were feeding. This would have implied the risk that the control bees would be exposed to the contaminant. For this reason it was not possible to mark an elevated number of bees. In trials 4 and 5 it was found that by standardising and reducing to a minimum the interruption of flight and foraging activity, the percentage of bees that returned to the labyrinth became substantially acceptable (Table 42).

Table 42 - Percentage of bees returning to the labyrinth to be submitted to the test

	Trial 1 16 June (Immediate effect)	Trial 1 16 June (Delayed effect, at 24 hours)	Trial 1 17 June	Trial 2 21 June (without CO_2)	Trial 3 19 July	Trial 4 25 July	Trial 5 1 August
N	30	40	41	31	36	39	67
CO_2 (minutes)	30	30	30	0	30	10	10
Confinemento (minutes)	180	24 h	180	0	120	75	75
% returning bees	23.3	32.5	56.1	77.4	30.6	61.5	52.2

In all the trials (with the exception of trial 2), no significant differences in the percentages of bees that returned and in the time taken to return and to move along the route in the labyrinth were observed between the controls and the active-ingredient-treated bees. The concentration assayed in the first 4 trials was 10 µg/L, as follows: in trial 1 the quantity of solution administered to each bee was 10 µL, corresponding to 0.1 ng/bee of active ingredient (40 times lower than the LD50); in trials 2, 3 and 4 each bee ingested from the dispenser up to 40 mL of solution (the capacity of the honey sac) containing the active ingredient, again at the concentration of 10 µg/L, corresponding to a dose of 0.4 ng/bee of active ingredient (10 times lower than the LD50); finally, in the last trial, i.e. trial 5, concentration rose to 20 µg/L, corresponding to a maximum active ingredient dose of 0.8 ng/bee (5 times lower than the LD50). The quantities of active ingredient assayed in our study, administered to the bee as a single dose and not ad libitum, are comparable to the active ingredient concentration theoretically observable on drops of dew or nectar on the surrounding vegetation, calculated as roughly 15 µg/L (based on the assumption that a dewdrop amounts to 0.05 µL). The

15 µg/L value was computed on the basis of the quantity of active ingredient (clothianidin) deposited at 5 metres, calculated with the 2011 modified seeding machine.

The effects of these quantities of active ingredient, administered as a single dose, are less evident and less clearly noticeable in terms of immediate effect or effect a few hours after exposure, as compared to administration ad libitum (the latter, however, being the more likely case in nature). Other studies have shown that sublethal effects appear after 3 hours (Bortolotti et al., 2003; Medrzycki et al., 2003), but with higher doses. This notwithstanding, certain consequences were detected even in our extremely low dose study, as pointed out in trial 2, where a significant number of active ingredient-treated bees failed to return to the labyrinth.

6.7 References

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7. Possibility of adopting integrated control for virus control in maize crops

Main aim: assessment of the possibility of integrated control against viruses in maize crops and identifying the possibility of control with reduced application of insecticides.
Intermediate aim: assessment of the incidence of viruses**,** hybrid susceptibility, possibility of vector monitoring and of post-emergence treatment on vectors.

7.1 Materials and methods

7.1.1 Sites

The risk of viral infection, especially Rough Dwarf Virus, tends to increase with increasing presence of weeds and crops ((*Echinocloa crus-galli, Agropyrum repens, Cynodon dactylon, Triticum aestivum, Oryza sativa, Hordeum vulgare*), as this type of vegetation favours the vector populations (in particular *Laodelphax striatellus).* It is known that Rough Dwarf Virus is more widespread in certain types of geographic areas with specific characteristics (high plateau with a marked incidence of grassland, including stable grassland, and uncultivated areas of Friuli, Veneto and Piedmont). The experimental sites were selected from areas where the presence of plants affected by rough dwarf disease had already been ascertained in the past or from zones having numerous uncultivated areas surrounding the planted plots. Due to the marked presence of grassy areas, at times partially forested or intermixed with woodlands , in the vicinity of the experimental plots, 7 out of the 11 selected sites can be considered to have a potential for elevated virus vector presence (in one of the sites, Friuli, Premariacco, a location was chosen in which the presence of rough dwarf virus was ascertained analytically in 2010). The other 4 fields can be considered to have medium or medium-low pressure, linked only to the grassy areas round the edges.
At each site, in order to distinguish the virosis factor from pressure exerted on the crop by other pests, attempts were made, as far as possible, to select plots with low populations of western corn rootworm (*Diabrotica virgifera virgifera*) and wireworms (*Agriotes* spp.). Given the substantial available knowledge allowing determination of population levels of these phytophages which involve a low risk of damage, choice of plot was based on assessment of the risk factors of the various sites (Furlan *et al*., 2011).

7.1.2 Comparative description of the treatments

a) Maize sensitive to DKC 5276 virus, fungicide only;
b) Maize sensitive to DKC 5276 virus, dressed with Poncho®-clothianidin-0.5 mg a.i./seed + fungicide;
c) Maize tolerant of DKC 6666 virus, fungicide only;
d) Maize tolerant of DKC 6666 virus, dressed with Poncho®-clothianidin-0.5 mg a.i./seed + fungicide;
e) Maize tolerant of DKC 6677 virus, fungicide only;
f) Maize tolerant of DKC 6677 virus, dressed with Poncho®-clothianidin-0.5 mg a.i../seed + fungicide;
g) PR32G44, fungicide only;
h) Maize sensitive to DKC 5276 virus + post-emergence treatment with pyrethroid (Karate Zeon 200 cc/hl) in the presence of virus vectors (trap observations).

Fungicide common to all treatments: Metalaxil+fludioxonil (Celest®) at the dose of 1 l/t of seed.

The crop management technique, with conventional ploughing and complementary tillage, was homogeneous in the trial plots. In all plots, treatment against pyralidae was applied between 10 and

25 July in order isolate the main factor under study (virosis) more effectively and to reduce the possible incidence of pyralidae on within-field variability.
The main agronomic characteristics of each trial plot are summarised in Table 43.

Table 43 – Main characteristics of the trial fields and the techniques adopted in 2011.

Regione	PV	COMUNE	Azienda	Pressione vettori virus nanismo ruvido	Terreno	Schema di campo	Epoche di semina	Coltura precedente	Data di semina
Friuli	UD	Orsaria di Premariacco	De Sabbata	alta	medio impasto	parcellare	2	mais	1 apr, 27 a
Friuli	UD	Camino al Tagliamento	Panigutti	alta	medio impasto	strip	1	mais	12-apr
Veneto	VE	S. Donà di Piave	Bogoni	alta	medio impasto	strip	1	mais	9-apr
Veneto	VE	S. Donà di Piave	Florian	medio-bassa	medio-impasto	strip	1	mais	9-apr
Veneto	RO	Ceregnano	Sasse Rami	medio-bassa	medio impasto argilloso	strip	1	frumento	12-apr
Veneto	TV	Mogliano Veneto	Diana	alta	medio impasto argilloso	parcellare	1	colza	14-apr
Veneto	VE	Caorle	Vallevecchia	alta	sabbioso-limoso	parcellare	1	sorgo	14-apr
Veneto	VE	Eraclea	Moizzi	medio-bassa	medio impasto	strip	1	soia	14-apr
Veneto	PD	Legnaro	Corte	alta	medio impasto	strip	1	frumento	29-apr
Lombardia	BG	Bergamo	CRA MAC	medio-bassa	medio impasto	parcellare	2	mais	22 apr, 16 g
Piemonte	TO	Cavour	Bertinetto	alta	medio impasto	strip	1	mais	12-apr

Key: *Regione*: Region; *PV* : Province; Comune: Municipality; *Azienda*: Farm estate; *Pressione vettori virus nanismo ruvido*: Rough dwarf virus vector pressure; *Terreno*: Soil; *Schema di campo*: Experimental design; *Epoche di semina*: Sowing times; *Colture precedente*: Previous crop; *Data di semina*: Sowing date; *Densità di semina*: Sowing density; *Alta*: high; *Medio impasto* : medium loam; *Parcellare*: ordinary-sized plot; *Mais*: maize.

7.1.3 Experimental designs

The treatments were distributed among the selected plots according to randomized blocs of large plots (for the strip test) or ordinary-sized plots (for the plot trials).

Ordinary Plots

The size of the individual plots was 45 – 60 m2 (m 3 X m 15-20). Determinations were performed in the central portion (8 – 10 m) of the 2 central rows, with 4 repetitions.

Strip test (large plots)

The size of the large plots varied from 400 to 1500 m2 (m 3 – 9 wide by the length of the plot under study). Homogeneous seed from taken from the same batch was used throughout.

Repetitions from 2 to 6 per site.

7.1.4 Determinations

A) Agronomic

Ordinary-sized plot trials

At the centre of each plot, on 8-10 m of the two central rows at the stage of 4-6 leaves and 8-12 leaves (areas marked off with boundary stakes that were maintained in place from the beginning to the end of the trial), the following determinations were performed:

A1 First phases

A1.1 number of normal plants (no symptom);
A1.2 number of plants with symptoms of Wireworm attack;
A1.3 number of plants with symptoms of attack by other harmful soil insects;
A1.4 number of plants with symptoms of rough dwarf disease (samples were gathered outside of the trial area for analysis);
A1.5 number of plants with symptoms of other virus diseases (yellowing not attributable to elaterid-induced damage) (gathering of samples outside of the trial area for analysis);
A1.6 number of plants with (green, black) aphids, or leafhoppers (*Cicadellidae*);
A1.7 early vigour.

A2 Pre-harvest assessment

The following determinations were performed on the same trial areas:

A2.1 total plants;
A2.2 plants without ear (not attributable to virus disease);
A2.3 plants with symptoms of virus attack – rough dwarf disease (specific, unmistakable), with gathering of samples to be placed immediately in the freezer ((<-18 C°); plants with symptoms of Barley BYDV [Barley Yellow Dwarf Virus] infection (red stripes between the leaf veins on leaves in different positions, smaller plants, small and deformed cob); plants with symptoms of phytoplasmosis of maize (the most evident symptoms can be seen on the leaves, which show leaf vein reddening that subsequently extends to the leaf and stalk, followed by decline and withering of the plant [these symptoms, according to information available to date, are similar to Barley], gathering of samples to be placed immediately in the freezer (<-18 C°);
A2.4 abnormal plants with other not precisely definable symptoms – gathering of samples to be placed immediately in the freezer (<-18 C°);
A2.5 other phytophages (aphids, red spider mite): observations on sub-plot plants, distinguishing them into 3 “aphid” categories and 3 “red spider mite” categories: 0 = no significant presences; 1 = presence of 1-2 not particularly extensive colonies, 2 = various colonies covering significant surfaces of the leaves and stalk.

A3 Production: regular elimination of the heads, measurement of the length of the residual ordinary-sized plots, harvesting such residual plots with Small Plot combine harvester or

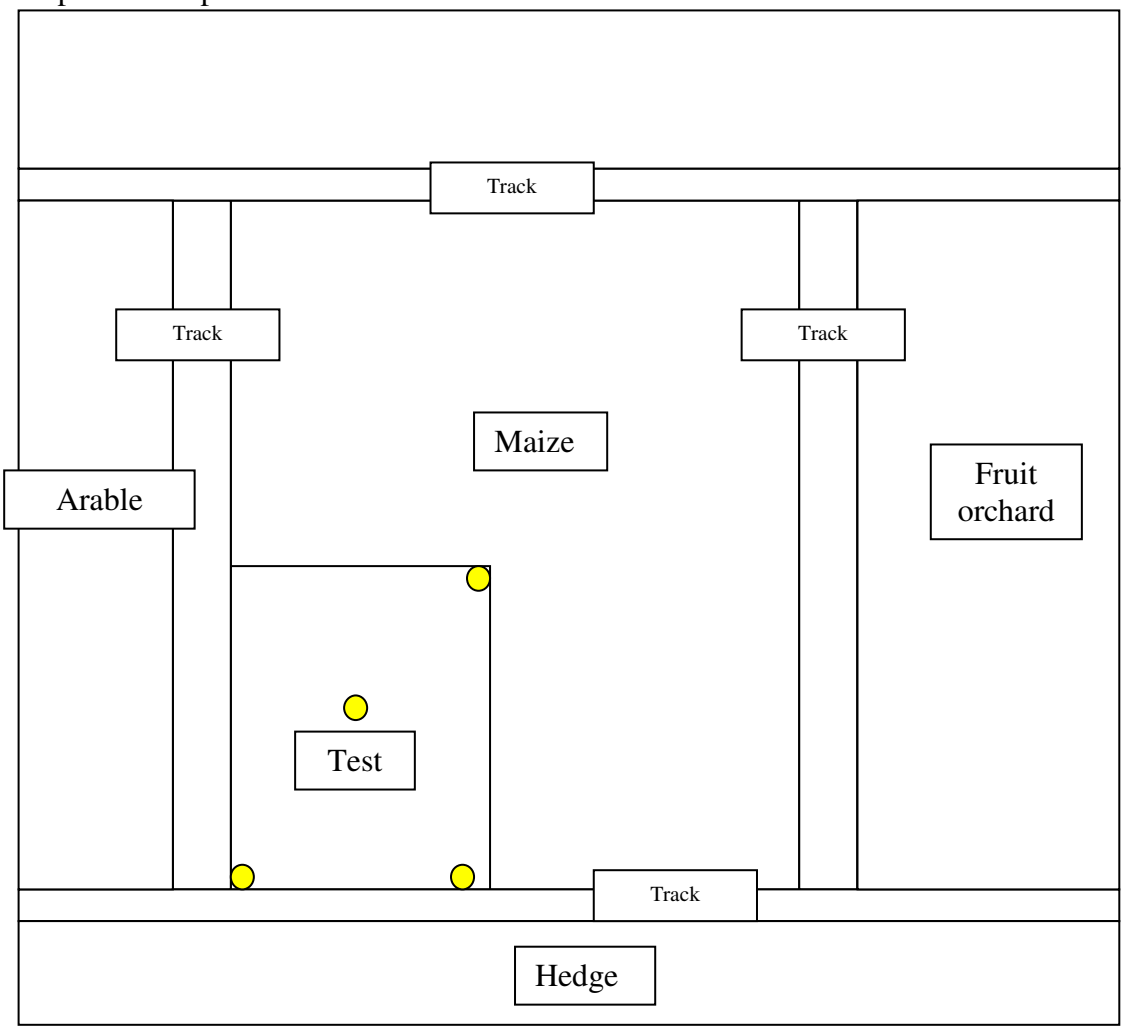
conventional combine harvester, collecting roughly 1 kg grain sample for moisture and hectolitric weight determination.

Strip test (large plots)
As above, but the tests were performed on at least two 20 m x 2 rows, in subplots of the large plots, selected randomly along the large plot, after evaluating the homogeneity of the large plot itself. Harvesting was carried out as described above, but using conventional combine harvesters and weigh wagons.

B) Entomological

The presence of virus vector species (Delphacidae) and in particular, vectors of rough dwarf virus, *L. striatellus*) was monitored by means of chromotropic traps and also with small nets on the grassy areas around and within the trial plots.
For each trial plot, the following data were recorded: geographic coordinates, size of the area sown with maize, distances from the edges of the plots, plants present at the edges for a depth of up to 100-150 m. In particular, if the vegetation did not consist of crops (for example, if the vegetation was formed of hedgerows), then the predominant plant species were indicated.

Example of a map.



After plant emergence, at the stage of 2-3 real leaves, at least 4 Temo-o-cid Kollant yellow sticky traps, with the sticky side facing outwards, were placed at the edges of the trial plot (i.e. in a position corresponding to the outermost row of maize plants), and at the centre of the trial plot. The

traps were placed as follows: two along each of the two longer sides, or at least along the sides bordering onto grassy or uncultivated areas, at a height of roughly 1 m. The traps were replaced every 15-20 days, taking care to cover them with transparent nylon film and marking on one corner the following data: the locality, the acronym of the trap (position – E – exterior or I internal, number from 1 upwards clockwise starting from the head of the route), and date.
After trap replacement, the traps were stored in a cool dry place.
Sampling with the small sweep net
During the months of August and September, sampling was carried out with a small sweep net and an entomological aspirator in the grassy patches around the trial plots as well as in the centre of the plots. For each sampling, outside of or within the plot, 60 sweeps of the vegetation were performed, with observation and capture of the insects every ten sweeps, followed by binocular observation of the captured specimens. The sampled specimens of L. striatellus were soaked in RNAlater solution and then stored in the freezer at -80°C ±1 for subsequent molecular analyses.

7.1.5 Data processing
Data were processed by the t test in the case of two treatments under comparison, and by ANOVA with subsequent Tukey test (HSD) in the case of three or more treatments under comparison. Data from plants with disease symptoms were transformed into the square root of x+0.5 prior to analysis.

7.2 Results

7.2.1 Agronomic results

Investments and attacks
Investments were found to be good (Table 44). Additionally, attacks by hypogeal pytophages were negligible or low at all stations, similarly to results obtained in previous experiments (Balconi et al., 2010a, 2010b, 2011, Furlan et al. 1989, 2001, 2002, 2007, 2009, 2011a, 2011b, APENET reports 2009 and 2010).

Table 44 - Effect of type of hybrid and of seed insecticide dressing on the health status of the maize crop; all trials (ordinary-sized plots trials and strip) and sowing times (early – normal - late) were considered (213 trials, of which eleven in the first period, two in the second period).
Values followed by the same letters were not significantly different at P<0.05 (Tukey).

	Investimento (pp/mq)		piante attaccate da elateridi		piante sintomi con nanismo ruvido 1° rilievo			piante sintomi nanismo ruvido (raccolta)		
	alla raccolta	*con spiga*	*pp/mq*	*%*	*pp/mq*		*%*	*pp/mq*		*%*
sensibile DKC5276	6,338	6,297	0,072	1,13	0,012	a	0,19	0,016	a	0,25
sensibile DKC5276+poncho®	6,502	6,470	0,045	0,69	0,001	b	0,02	0,003	b	0,05
sensibile DKC5276+post emergenza	6,343	6,317	0,066	1,04	0,005	ab	0,07	0,010	ab	0,16
tollerante DKC6666	6,285	6,253	0,045	0,72	0,001	b	0,02	0,004	b	0,06
tollerante DKC6666+poncho®	6,476	6,445	0,041	0,64	0,000	b	0,01	0,001	b	0,02
tollerante DKC6677	6,391	6,338	0,047	0,74	0,001	b	0,02	0,008	ab	0,13
tollerante DKC6677+poncho®	6,469	6,438	0,044	0,68	0,000	b	0,01	0,001	b	0,02
PR32G44 no insetticida	6,232	6,138	0,062	0,99	0,003	b	0,05	0,007	ab	0,11
F (ANOVA)	1,426	1,730	1,331		4,066			3,318		
P	0,193	0,100	0,234		0,000			0,002		

	Investimento (pp/mq)		piante attaccate da elateridi		piante sintomi con nanismo ruvido 1° rilievo			piante sintomi nanismo ruvido (raccolta)		
	alla raccolta	*con spiga*	*pp/mq*	*%*	*pp/mq*		*%*	*pp/mq*		*%*
sensibile DKC5276	6,338	6,297	0,072	1,13	0,012	a	0,19	0,016	a	0,25
sensibile DKC5276+poncho®	6,502	6,470	0,045	0,69	0,001	b	0,02	0,003	b	0,05
sensibile DKC5276+post emergenza	6,343	6,317	0,066	1,04	0,005	ab	0,07	0,010	ab	0,16
tollerante DKC6666	6,285	6,253	0,045	0,72	0,001	b	0,02	0,004	b	0,06
tollerante DKC6666+poncho®	6,476	6,445	0,041	0,64	0,000	b	0,01	0,001	b	0,02
tollerante DKC6677	6,391	6,338	0,047	0,74	0,001	b	0,02	0,008	ab	0,13
tollerante DKC6677+poncho®	6,469	6,438	0,044	0,68	0,000	b	0,01	0,001	b	0,02
PR32G44 no insetticida	6,232	6,138	0,062	0,99	0,003	b	0,05	0,007	ab	0,11
F (ANOVA)	1,426	1,730	1,331		4,066			3,318		
P	0,193	0,100	0,234		0,000			0,002		

Key: *sensibile*: sensitive; *Investimento*: investment*; Piante attaccate da elateridi*: plants attacked by wreworms; *Piante sintomi nanismo ruvio (raccolta)*: plants with symptoms of Rough Dwarf Disease (harvest); *1° rilievo*: first sampling; *Alla raccolta* : at harvest; *Con spiga*: with ear; *Raccolta*: harvest; *.pp/mq*: plants/sqm.

In some trials, there were failed areas, locally with appreciable density, caused by birds during the emergence stage – 3 leaves, but the final investments of hybrids treated with clothianidin were not found to be statistically different from the non-treated trials. Thus plant density did not appear to be a factor capable of exerting a significant influence on the results obtained with the different hybrids.

Epigeal Phytophages

Presence of epigeal phytophages (aphids, leafhoppers, flea beetles) was very low in all experimental fields and never reached a level capable of significantly affecting the results of the different hybrids. European corn borer (*Ostrinia nubilalis*) pressure was found to be low in all plots of every site (percentage of plants broken above and below the ear was under 5%). Therefore this factor is unlikely to have caused appreciable variability in the experiments.

Noctuids

Noctuid attacks were absent or negligible (<0.01 % of plants) in all experimental fields. Consequently, they were unable to induce variability.

Virosis incidence

Incidence of plants with rough dwarf symptoms was found to be very low. Presence of symptoms was detected only in 2 plots out of 11 and was much lower than 1% of plants observed (Table 44), despite the choice of plots with special risk factors for this disease. Incidence was found to be significant, albeit still contained, only in one site (Friuli, Premariacco, Tables 45, 46, and 47), where the presence of the virus disease had also been ascertained analytically in 2010 (Salvador, 2010). In the locality in question, the border effect was significant; in addition, the hybrid regarded as sensitive to the virus did show a significantly higher incidence of plants with symptoms of rough dwarf virus compared to the other hybrids, which were regarded as tolerant. The presence of plants with disease symptoms showed a decreasing trend from the outer rows towards the interior (Table 48), in agreement with observations on the disease reported in the literature (Caciagli, 1991; Furlan et al., 2009).

Clothianidin used as seed dressing significantly reduced disease incidence (Tables 45, 46, and 47); analogous low levels of disease incidence were recorded in tolerant hybrids even if such hybrids had not been dressed with the insecticide.

Post emergence insecticide treatment showed lower levels of damage compared to the control, but damage was never statistically significant.

Disease incidence was found to be lower in the trial sown during the second period (Table 47), but the hybrid and treatment effects remained similar to results found with first period sowing.
No plants with symptoms of other viruses were found.

Table 45 - Effect of hybrid type and seed dressing on maize health status in the first ordinary-sized plot trial (first and second sowing period) in Premariacco, Friuli, where the highest rough dwarf virus pressure was detected.
Values followed by the same letters were not significantly different at P<0.05 (Tukey).

	Investimento (pp/mq)		piante attaccate da elateridi		piante sintomi nanismo ruvido 1° rilievo			piante sintomi nanismo ruvido (racc.ta)	
	alla raccolta	*con spiga*	*pp/mq*	*%*	*pp/mq*		*%*	*pp/mq*	*%*
sensibile DKC5276	**5,992**	**5,992**	**0,042**	0,70	**0,086**	**a**	1,43	**0,095**	1,59
sensibile DKC5276+poncho®	**6,375**	**6,375**	**0,025**	0,39	**0,010**	**b**	0,16	**0,023**	0,37
ensibile DKC5276+post emergenz	**6,017**	**6,017**	**0,058**	0,97	**0,030**	**ab**	0,50	**0,051**	0,84
tollerante DKC6666	**5,808**	**5,808**	**0,092**	1,58	**0,010**	**b**	0,18	**0,027**	0,47
tollerante DKC6666+poncho®	**6,358**	**6,358**	**0,042**	0,66	**0,003**	**b**	0,04	**0,008**	0,12
tollerante DKC6677	**6,150**	**6,150**	**0,050**	0,81	**0,008**	**b**	0,13	**0,059**	0,95
tollerante DKC6677+poncho®	**6,417**	**6,417**	**0,033**	0,52	**0,003**	**b**	0,04	**0,010**	0,16
PR32G44 no insetticida	**6,108**	**6,108**	**0,058**	0,95	**0,018**	**b**	0,30	**0,042**	0,68
F (ANOVA)	1,641	1,641	0,456		2,160				1,407
P	0,132	0,132	0,864		0,044				0,210

Key: *sensibile*: sensitive; *Investimento*: investment*; Piante attaccate da elateridi*: plants attacked by wireworms; *Piante sintomi nanismo ruvio (raccolta)*: plants with symptoms of Rough Dwarf Disease (harvest); *!° rilievo*: first sampling; *Alla raccolta* : at harvest; *Racc.ta*: harvest; *Con spiga*: with ear.; *pp/mq*: plants/sqm.

Table 46 - Effect of hybrid type and seed dressing on maize health status in the first-period (1 April) ordinary-sized plot trial in Premariacco, Friuli, where the highest rough dwarf virus pressure was detected.
Values followed by the same letters were not significantly different at P<0.05 (Tukey).

	Investimento (pp/mq)		piante attaccate da elateridi		piante sintomi nanismo ruvido 1° rilievo			piante sintomi nanismo ruvido (racc.ta)		
	alla raccolta	*con spiga*	*pp/mq*	*%*	*pp/mq*		*%*	*pp/mq*		*%*
sensibile DKC5276	**5,917**	**5,917**	**0,067**	1,13	**0,122**	**a**	2,07	**0,141**	**a**	2,38
sensibile DKC5276+poncho®	**6,617**	**6,617**	**0,050**	0,76	**0,003**	**b**	0,04	**0,029**	**a**	0,43
ensibile DKC5276+post emergenz	**6,067**	**6,067**	**0,083**	1,27	**0,052**	**ab**	0,28	**0,091**	**a**	1,51
tollerante DKC6666	**6,017**	**6,017**	**0,117**	1,93	**0,018**	**b**	0,09	**0,052**	**a**	0,79
tollerante DKC6666+poncho®	**6,583**	**6,583**	**0,067**	1,00	**0,005**	**b**	0,23	**0,016**	**a**	0,26
tollerante DKC6677	**6,033**	**6,033**	**0,067**	1,08	**0,016**	**b**	0,04	**0,117**	**a**	1,76
tollerante DKC6677+poncho®	**6,667**	**6,667**	**0,050**	0,82	**0,003**	**b**	0,47	**0,018**	**a**	0,30
PR32G44 no insetticida	**6,150**	**6,150**	**0,033**	0,55	**0,029**	**ab**	0,87	**0,076**	**a**	1,24
F (ANOVA)	1,992	1,992	0,319		2,478			1,991		
P	0,077	0,077	0,942		0,031			0,077		

Key: *sensibile*: sensitive; *Investimento*: investment*; Piante attaccate da elateridi*: plants attacked by wireworms; *Piante sintomi nanismo ruvio (raccolta)*: plants with symptoms of Rough Dwarf Disease (harvest); *!° rilievo*: first sampling; *Alla raccolta* : at harvest; *Racc.ta*: harvest; *Con spiga*: with ear.; *pp/mq*: plants/sqm.

Table 47 - Effect of hybrid type and seed dressing on maize health status in the second-period (27 April) ordinary-sized plot trial in Premariacco, Friuli, where the highest rough dwarf virus pressure was detected. Values followed by the same letters were not significantly different at P<0.05 (Tukey)

	Investimento (pp/mq)		piante attaccate da elateridi		piante sintomi nanismo ruvido 1° rilievo		piante sintomi nanismo ruvido (racc.ta)	
	alla raccolta	*con spiga*	*pp/mq*	*%*	*pp/mq*	*%*	*pp/mq*	*%*
sensibile DKC5276	**6,067**	**6,067**	**0,017**	0,27	**0,049**	0,82	**0,049**	0,82
sensibile DKC5276+poncho®	**6,133**	**6,133**	**0,000**	0,00	**0,018**	0,30	**0,018**	0,30
ensibile DKC5276+post emergenz	**5,967**	**5,967**	**0,033**	0,54	**0,008**	0,04	**0,010**	0,17
tollerante DKC6666	**5,600**	**5,600**	**0,067**	1,06	**0,003**	0,00	**0,003**	0,05
tollerante DKC6666+poncho®	**6,133**	**6,133**	**0,017**	0,27	**0,000**	0,00	**0,000**	0,00
tollerante DKC6677	**6,267**	**6,267**	**0,033**	0,55	**0,000**	0,04	**0,000**	0,00
tollerante DKC6677+poncho®	**6,167**	**6,167**	**0,017**	0,28	**0,003**	0,13	**0,003**	0,04
PR32G44 no insetticida	**6,067**	**6,067**	**0,083**	1,49	**0,008**	0,14	**0,008**	0,13
F (ANOVA)	1,964	1,964	1,072		0,509		0,4895	
P	0,082	0,082	0,397		0,823		0,8372	

Key: *sensibile*: sensitive; *Investimento*: investment*; Piante attaccate da elateridi*: plants attacked by wireworms; *Piante sintomi nanismo ruvio (raccolta)*: plants with symptoms of Rough Dwarf Disease (harvest); *!° rilievo*: first sampling; *Alla raccolta* : at harvest; *Racc.ta*: harvest; *Con spiga*: with ear.; *pp/mq*: plants/sqm.

Table 48 - Incidence of plants with rough dwarf symptoms at increasing distances from the stable grassy area, in the maize plot located in Premariacco, Friuli.
Values followed by the same letters were not significantly different at P<0.05 (Tukey).

distanza media da prato stabile (m)	piante con sintomi nanismo ruvido per 48 m^2	
1	**25,3**	**a**
4	**15,0**	**ab**
16	**6,5**	**b**
F (ANOVA)	9,138	
P	0,015	

Key: *distanza media da prato stabile*: mean distance from stable grassy area; *piante con sintomi nanismo ruvido:* plants with rough dwarf symptoms.

Yields

At the time of writing, harvesting was nearing completion, and the data refer to over 80% of the trials (Table 49). Only limited differences observed between hybrids that were or were not treated with the insecticide dressing were observed, and these differences were not statistically significant. Such a result is in agreement with data published over the last decade concerning the effect of the dressing treatments on productions under different agronomic conditions and parasite pressure (Table 50). This suggests that dressing is not a "strong" production factor capable of exerting a marked effect on the final outcome of the maize crop, as **in no publication** whatsoever are the production differences (which are limited to no more than a few quintals) between seed that is or is not treated with neonicotinoids or with phenylpirazols statistically significant.

If the dressings are considered as a whole, or the dressings without clothianidin, it can be stated that the increases in production recorded in the past few years are nil or negligible even in presence of strong Western Corn Rootworm pressure. Even clothianidin tends to give negligible production increases in conditions of low phytophage pressure (which is by far the most widespread circumstance), while its effect, although still not statistically significant, is slightly more marked under strong Western Corn Rootworm pressure. However, control of this phytophage could best be addressed with the implementation of integrated control (obligatory as from 2014), based on correct application of rotation sequences without special recourse to insecticides.

Table 49 - – Effects of dressing on maize yield in the 2011 trials. Mean data of 10 trials.
Values with at least one letter in common were not significantly different at P<0.05 (Tukey).

	q/ha 14%	
sensibile DKC5276	114,097	b
sensibile DKC5276+poncho®	118,696	ab
nsibile DKC5276+post emergenz	114,796	b
tollerante DKC6666	117,972	ab
tollerante DKC6666+poncho®	121,293	ab
tollerante DKC6677	124,006	ab
tollerante DKC6677+poncho®	127,077	a
PR32G44 no insetticida	120,868	ab
F (ANOVA)	3,409	
P	0,002	

Key: *sensibile*: sensitive; *tollerante*: tolerant.

7.2.2 Entomological determinations

Based on captures performed with the sweep net (Table 51) and on the first observations of the chromotropic traps (which will still require many days of work for a complete examination), the presence of virus vector species (*Delphacidae)* and the vector of rough dwarf virus (*L. striatellus)* was virtually ubiquitous. The latter was identified in all grassy areas bordering on the trial plots. However, this species appears to have a low tendency to enter into the plots, because very few specimens were captured inside the plots. Usually, the insect temporarily colonises the outmost rows of plants.

Table 50 - Effects of dressing on maize production in the articles published to date including APENET 2009 – 2010 data.

REGIONE	AUTORI	periodo	ibrido	numerosità località/ripetizioni (+bassa, +++++elevata)	Pressione diabrotica (+bassa, +++++elevata)	solo fungicida	fungicida + concianti insetticidi	differenza insetticidi vs solo fungicida	% su test	Significatività statistica	fung. + concianti senza poncho	differenza concianti senza poncho vs solo fungicida	% su test	Significatività statistica	fungicida + poncho	differenza poncho vs solo fungicida	% su test	Significatività statistica
						t/ha 14%	t/ha 14%	t/ha 14%			t/ha 14%	t/ha 14%			t/ha 14%	t/ha 14%		
Veneto	**Furlan et al.,2007**	2003-2006	Tevere DKC, 6530	+++	**no**	**12,43**	**12,17**	**-0,264**	**-2,12**	**no**	**12,166**	**-0,264**	**-2,12**	**no**	**no**	**no**	**no**	**no**
Veneto	**Furlan et al., 2007**	2006	DKC 6530	++	**no**	**11,59**	**11,42**	**-0,170**	**-1,47**	**no**	**11,470**	**-0,120**	**-1,04**	**no**	**11,170**	**-0,420**	**-3,62**	**no**
Pianura Padana	**Furlan et al., 2007**	2006	DKC 6530	+	**si++**	**7,96**	**7,79**	**-0,170**	**-2,14**	**no**	**7,650**	**-0,310**	**-3,89**	**no**	**8,070**	**0,110**	**1,38**	**no**
Pianura Padana, Toscana	**Boicelli, 2007**	2000-2006	Tevere DKC 6530	+++++	**si+**	**n.d**	**n.d.**	**0,300**	**2,00**	**no**	**n.d**	**0,300**	**2,00**	**no**	**n.d**	**n.d**	**n.d.**	**n.d.**
Veneto	**Furlan et al., 2009**	2007-2008	DKC 6530	++	**no**	**10,90**	**10,57**	**-0,332**	**-3,05**	**no**	**10,510**	**-0,390**	**-3,58**	**no**	**10,740**	**-0,160**	**-1,47**	**no**
Veneto	**Furlan et al., 2009**	2007-2008	mess, Kla	++	**no**	**12,68**	**n.d**	**n.d.**	**n-d**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**12,950**	**0,270**	**2,13**	**no**
Lombardia	**Agosti et al., 2009**	2007-2008	PR33A46	+	**si++++**	**12,71**	**13,61**	**0,907**	7,14	**no**	**13,371**	0,665	5,23	no	**13,855**	1,149	**9,04**	**no**
Lombardia	**Agosti et al., 2010**	2009-2010	PR32G44	+	**si++++**	**11,39**	**11,84**	**0,454**	3,99	**no**	**11,487**	0,100	0,88	no	**12,294**	0,907	**7,97**	**no**
Pianura Padana, Toscana	**Balconi et al., 2010; Relazione APENET 2009**	2009	PR31N27	++++	**no**	13,30384	13,18593	-0,12	-0,89	**no**	13,14663	-0,16	-1,18	no	13,43157	0,13	0,96	**no**
Pianura Padana, Toscana	**Balconi et al., 2011; Relazione APENET 2009**	2010	PR32G44	++++	**no**	12,98942	13,35297	0,37	2,8	**no**	13,28419	0,3	2,27	no	13,57895	0,6	4,54	**no**
						MEDIA		**0,108**	**0,696**			**0,013**	**-0,159**			**0,323**	**2,616**	

Key: *Regione*: region; *Lombardia*: Lombardy; *Pianura Padana*: the River Po valley; *Toscana*: Tuscany; *Autori*: authors; *Periodo*: period; *Ibrido*: hybrid; *Numerosità località / ripetizioni (+ bassa, ++++++elevata)* : numerosity of the localities /repetitions (+ low, +++++elevated); *Pressione diabrotica* : Western corn rootworm pressure (+ low, +++++elevated); *Solo fungicida*: only fungicide; *Fungicida + concianti insetticidi*: fungicide + insecticidal dressing; *Differenza insetticidi vs solo fungicida*: difference by comparing insecticide vs fungicide alone; *% su test*: % out of the test; *Significatività statistica*: statistical significance; *Fung-+concianti senza poncho*: fungicide + dressing without poncho; *Differenza concianti senzaponcho vs. solo fungicida*: difference by comparing dressing without poncho vs. fungicide alone; *Fungicida + poncho*: fungicide + poncho; *Differfenza poncho vs. solo fungicida*: Difference by comparing poncho vs. fungicide alone; n.a.: not available.

Table 51 - Individuals of *Laodelphax striatellus,* the vector of rough dwarf disease in maize*,* detected at the different experimental sites by means of sweep nets and entomological aspirator. Total exemplars captured with 60 sweeps of the vegetation, with observation and capture of the insects every 10 sweeps.

Region	**Agricultural estate**	**Sampling date**	**N. of *Laodelphax striatellus***	
			Outside the plot	**Inside the plot**
Friuli	De Sabbata	09/08/2011	19	0
Friuli	Panigutti	09/08/2011	10	0
Veneto	Bogoni	11/08/2011	0	0
Veneto	Florian	11/08/2011	5	0
Veneto	Sasse Rami	22/08/2011	2	0
Veneto	Diana	22/08/2011	7	0
Veneto	Vallevecchia	11/08/2011	4	0
Veneto	Moizzi	11/08/2011	1	0
Piemonte	Cavour-Bertinetti	20/07/2011	4	0
Piemonte	Cavour-Bertinetti	25/07/2011	3	0
Piemonte	Cavour-Bertinetti	05/08/2011	15	0
Piemonte	Cavour-Bertinetti	17/08/2011	6	0
Piemonte	Cavour-Bertinetti	30/08/2011	3	0
Piemonte	Cavour-Bertinetti	26/08/2011	2	0

7.3 Conclusions

Although the trials were limited to only one growth season, the data obtained appear particularly interesting with regard to three aspects: 1) virus incidence seems low and limited to specific geographic areas already found in the past to have significant disease presence; 2) clothianidin used as a dressing on sensitive hybrids succeeds in significantly reducing the incidence of rough dwarf disease even in sensitive hybrids; 3) a similar reduction in disease incidence can be achieved by adopting resistant hybrids without using insecticides. The presence of virus vector species (*Delphacidae*) and, in particular, presence of the vector of rough dwarf disease (*L. striatellus*), is ubiquitous. The latter species was identified in all the grassy areas bordering on the trial plots. However, the species appears to have a low tendency to enter into the plots, as few specimens were captured inside the plots. A scanty, statistically non significant, effect of the insecticide dressing on productions of grain maize was confirmed.

7.4 References

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8. Synergistic interactions between stress agents and bee colony collapse

8.1 Introduction

A number of authors have hypothesised that the recent phenomena of bee die-off also reported in Italy may derive from interactions between pathogens and other stress factors, such as parasites.
To clarify this aspect, a study was conducted on beehives in which collapse was induced as a result of progressive infestation by the parasite mite Varroa destructor. The study was set up by installing two apiaries in an isolated locality, one of which was then treated with conventional acaricides, while the other was left untreated. Monitoring of the colonies was performed throughout the season, by means of periodic observations on size of the family, infestation of the family, bee mortality and prevalence of the main bee pathogens. In parallel, laboratory investigations were carried out in order to confirm the effects observed in the field, as well as transcriptomic investigations focusing on the genes of bee immune defences. It was found that in the trial conditions studied, colony collapse was brought about by a sharp rise in bee mortality recorded at the beginning of autumn, concomitantly with an increase in the number of genomic copies of deformed wing virus (DWV). At the same time, severe effects on the expression of key genes of the bee immune system were recorded.
Overall, these results suggest that Varroa exerts a synergic effect with regard to DWV, in such a manner that its influence on bee immune defences leads to a transition from innocuous latent infections to disastrous viral explosions (report submitted to the journal Science and currently under assessment by the reviewers).
The sensitivity of the immune response factors, identified by the present study, to various environmental stress factors suggests that not only disease but also forms of stress deriving from nutritional deficiencies or from sublethal doses of pesticides can interfere with defences against pathogenic organisms, and can thus have harmful effects on bee health (Desneaux et al., 2007; Mullin et al., 2010).
The trials described here aimed to evaluate, in controlled laboratory conditions, the impact of the neonicotinoid pesticide clothianidin on DWV and on survival of bees exposed to doses up to 15 times lower than the officially reported LD50 values. The data obtained indicate that in our trial conditions clothianidin was capable of inducing proliferation of latent viruses, and this proliferation was associated with mortality rates that were higher than expected.
Since the antiviral immune response, similarly to numerous reactions to biotic and abiotic stress agents, is also modulated by the Toll pathway (Zambon et al., 2005; Sabin et al., 2010), this study aimed to assess the impact of clothianidin on the Toll pathway, using transgenic lines of *Drosophila melanogaster*. The results obtained suggest that the neonicotinoid under examination has a negative influence on this transcriptional activation of antimicrobial molecules.

8.2 Materials and methods

A brood frame with capped cells was taken from a bee colony in good health, and placed in an incubator with controlled temperature and humidity (34 °C, 80% R.H). Within 12 hours the number of newly emerged coetaneous bees required for the experiment was obtained. The bees were divided into 8 groups of 30 bees each, and placed in purpose-designed containers (Figure 42), according to the protocol of Evans *et al.* (2009) after applying to the bee's thorax 1 µl of acetone, as the control, or an acetone solution containing amounts of clothianidin ranging between 3 ng and 50 ng.
Each group of bees was fed with protein-containing sugar syrup, supplied *ad libitum*, and maintained in the incubator under the above specified conditions. For each group, 12, 24 and 48 hours after the beginning of the treatment all dead bees were counted and eliminated; additionally, for each group, 5 live bees were collected and placed at -80 °C for subsequent analyses. This test was repeated twice.

In the live bees, DWV levels were quantified by Real-time RT-PCR, according to a consolidated protocol (Chen *et al.*, 2005). LD50 was calculated from the experimental data by "Probit-analysis" (Finney, 1971).
Drosophila represents an important model system for study of the basic mechanisms and evolution of immunity in insects (Lemaitre & Hoffmann, 2007). In *Drosophila,* transgenic lines expressing the GFP protein under the control of the promoters of various different genes coding for antimicrobial peptides have been produced (AMP) (Tzou *et al*., 2000). Use of these lines allows analysis of the effects exerted on the immune response that is mediated by the Toll and/or IMD pathway: the analysis is achieved by quantifying the fluorescence emitted by GFP, used as an indicator of the level of transcriptional activation of the different AMP genes. To assess the effects of clothianidin (a neonicotinoid pesticide) on the immune response, we used a transgenic line of *Drosophila* expressing the GFP protein fused to the antimicrobial peptide drosomycin under the control of the specific promoter of the drosomycin gene (drs-GFP). Because activation of this chimeric gene is under the control of the Toll pathway, inhibition of the pathway can be visualised by means of the absence or reduction of fluorescence produced by GFP.
Given that the most marked effect on bees was observed at the LD50 concentration, the experimental *Drosophila* larvae were treated with this concentration. Definition of LD50 for *Drosophila melanogaster* was performed on 3rd-instar larvae, appropriately treated with different doses of active ingredient (from 1 to 100 ng/larva), using larvae taken from a wild population reared on an artificial diet under constant temperature (25 °C). Then, 24 hours after exposure, the larvae were isolated and dead individuals counted. Calculation of LD50 was carried out in the manner indicated previously.
The experiment with the transgenic *Drosophila* line was performed as follows: third-instar larvae were treated individually with 1 µl of acetone containing clothianidin at a concentration equal to LD50. The larvae were then individually infected by pricking with a tungsten needle that had been dipped into a concentrated mould solution. After the injection, the larvae were placed in the incubator at 21 °C. Four hours later the larvae were observed by epifluorescence microscopy. As controls, third-instar larvae were treated with acetone only, and subsequently infected and incubated in the same manner and for the same length of time as those treated with clothianidin.

Figure 55 – Isolators utilised for maintaining the treated bees.

8.3 Statistical analysis

The results of DWV quantification were expressed as the mean of viral copies present in each bee, for each pesticide dose, 12, 24 and 48 hours after treatment. The experimental data were analysed with the Kruskal-Wallis and Dunn's Multiple Comparison Test statistical tests. For *Drosophila*, results were expressed as percentage of larvae with intense fluorescence. Differences were analysed with the Chi-square (χ^2) statistical test.
All analyses were performed using the "Prism 5.0c" (GraphPad Software, Inc.) statistical packet.

8.4 Results

Figure 43 shows the quantity of DWV detected at different times after treatment in bees submitted to different clothianidin doses. At 48 hours after treatment, no survival was recorded in bees submitted to the doses of 30 and 40 ng/bee. DWV levels in bees treated with doses of 10 and 20 ng/bee increased significantly with increasing times. At the dose of 3 ng/bee and in the controls, no statistically significant differences were detected.
It is important to note that the quantitative differences observed were not only statistically significant but also, in absolute terms, fairly substantial, although the logarithmic scale adopted in the graphic representation does not permit immediate perception of the circumstance. If one considers the ratio between the viral loads of clothianidin-treated bees versus the controls, the value is roughly 100 for the doses of 10 ng, and roughly 1000 for the 20 ng dose.

On the basis of the survival curves of bees exposed to different quantities of clothianidin (Figure 44), an experimental LD50 of 18.89 ng/bee can be calculated (Figure 45). This value is roughly half the officially reported value (EC working document, 2005).
For Drosophila, as a preliminary procedure, LD50 was estimated on 3rd-instar larvae, purpose-treated with different doses of active ingredient (from 1 to 100 ng/larva). Larvae taken from a wild population of the fly were used. LD50 at 24h was estimated to be 42.53 ng/larva.
In larvae treated with 40 ng of clothianidin, *drs*-GFP expression was noticeably reduced, as only 20% of larvae showed an elevated level of GFP fluorescence, while in the remaining 80% a very low or absent level of GFP fluorescence was found (Figure 46). These values were significantly different from those recorded in the controls (χ^2= 7.500; df=1; p=0.0062), which showed very strong fluorescence in roughly 80% of larvae (Figure 47).
Given that expression of the *drs*-GFP transgene is activated by the Toll pathway, its reduced expression in larvae treated with 40 ng of clothianidin indicates that this pesticide inhibits the function of a component of this pathway.

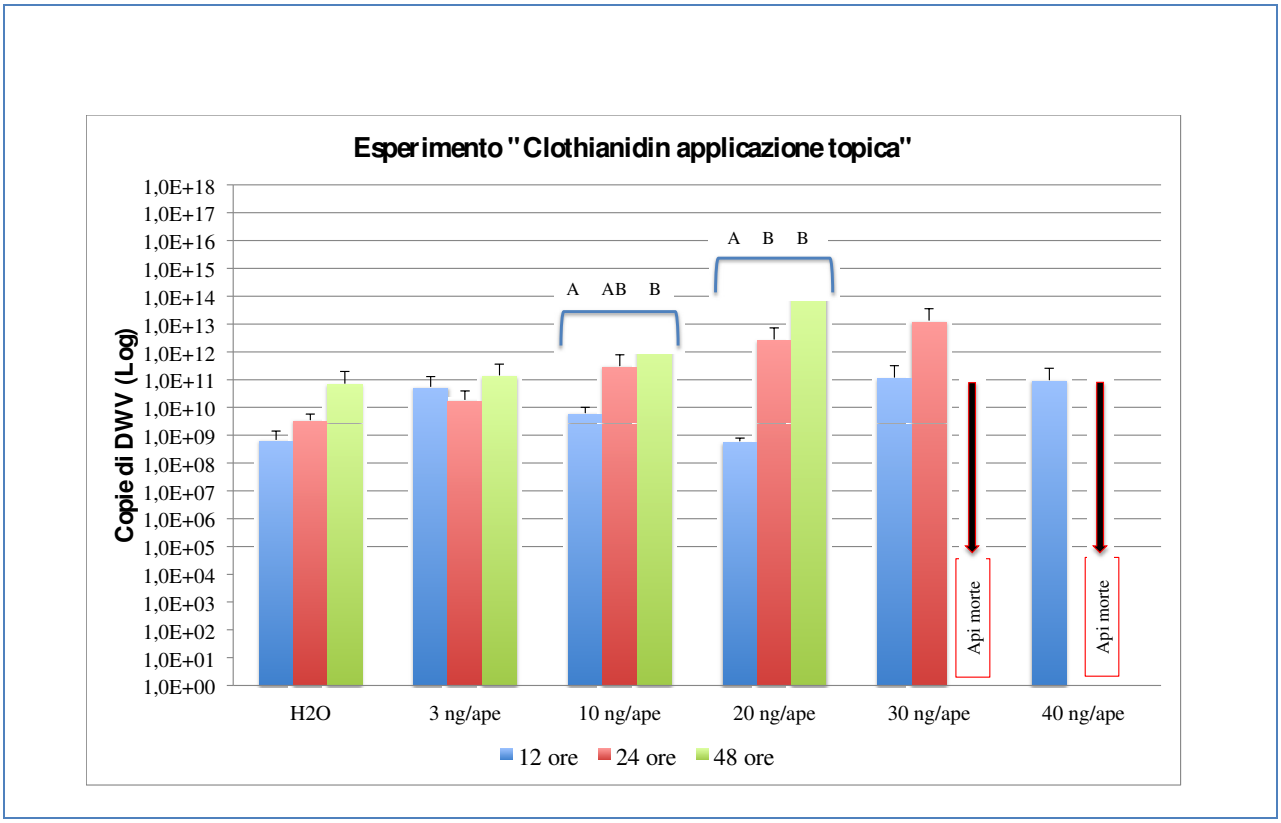


Figure 56 – DWV quantification in clothianidin-treated bees. For the 10 ng/bee dose, p=0.0192 (ANOVA, Kruskal-Wallis Test); for the 20 ng/bee dose p=0.0022 (ANOVA, Kruskal-Wallis Test).
Key: *Esperimento "Clothianidin applicazione topica"*: Experiment "Clothianidin topical application"; *Copie di DWV (Log)*: Copies of DWV (Log); *ng/ape*: ng/bee; *ore*: hours.

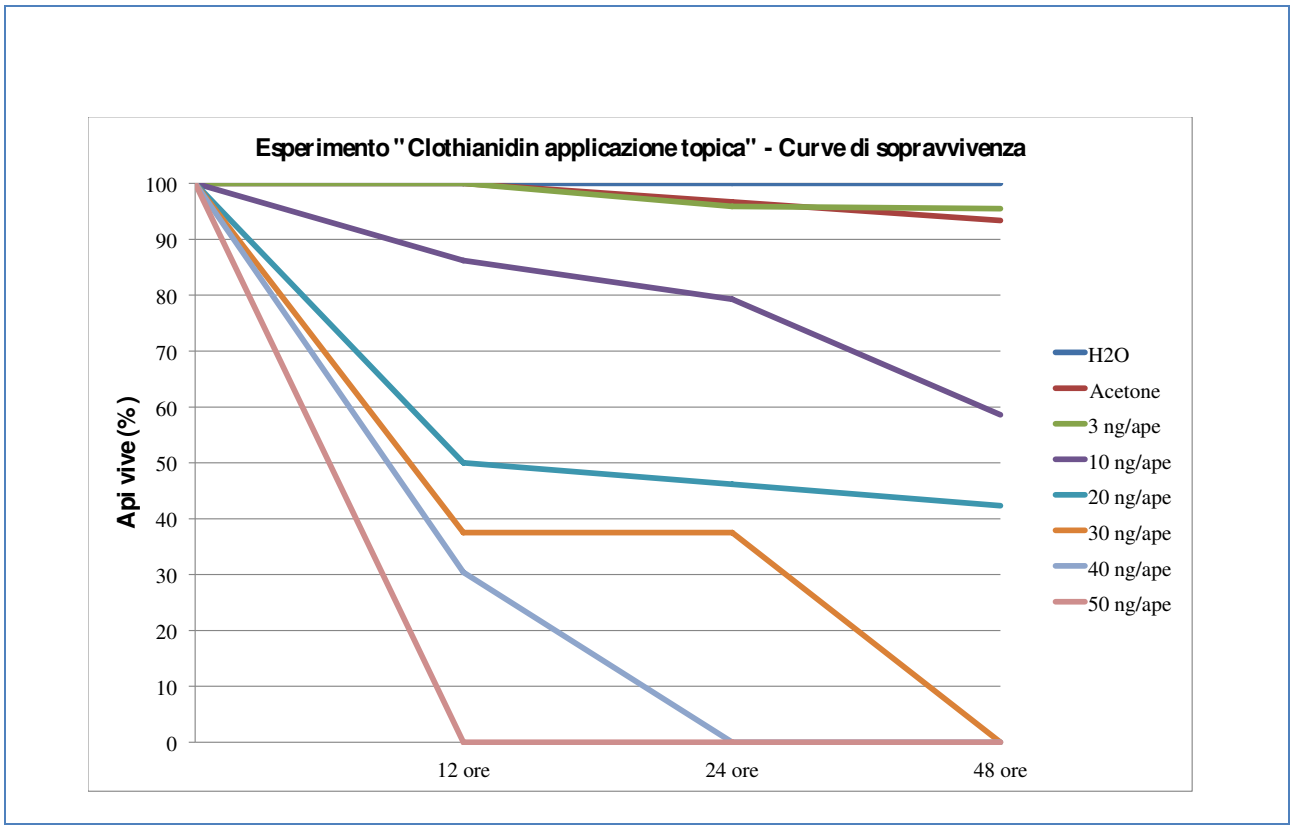


Figure 57 – Mortality curves in clothianidin-treated bees.
Key: *Esperimento "Clothianidin applicazione topica" - Curve di sopravvivenza "*: Experiment "Clothianidin topical application" – survival curves; *Api vive*: live bees; *ng/ape*: ng/bee.

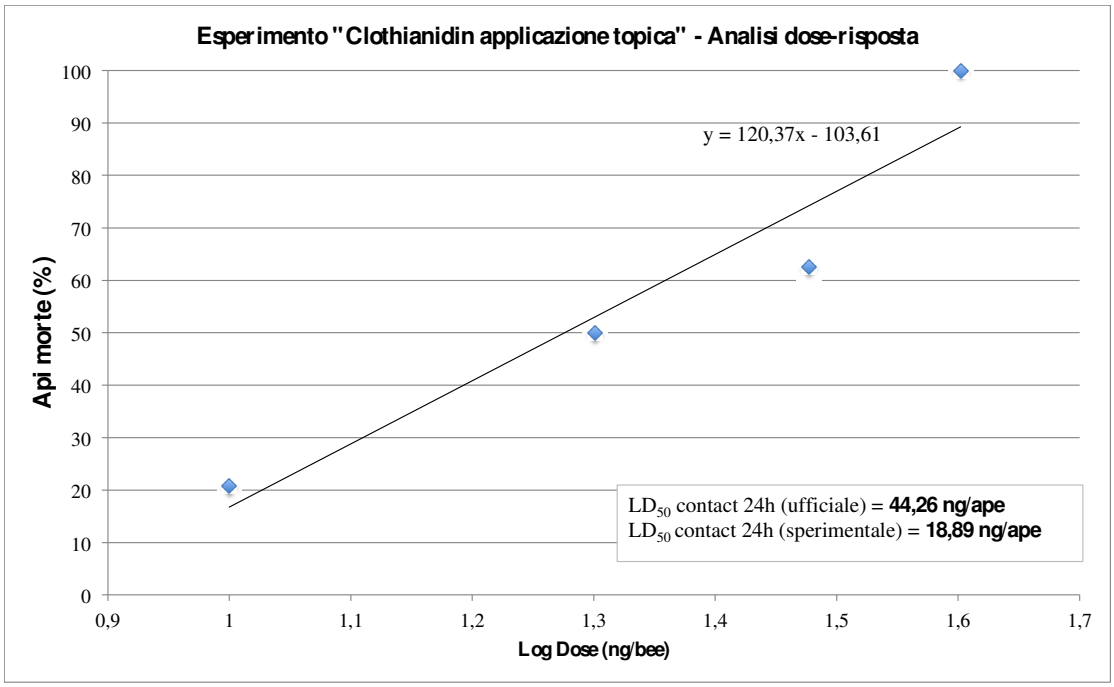


Figure 58 – Dose-response analysis of clothianidin-treated bees.
Key: *Esperimento "Clothianidin applicazione topica" – Analisi dose-risposta "*: Experiment "Clothianidin topical application" – Dose-response analysis; *Api morte*: dead bees.

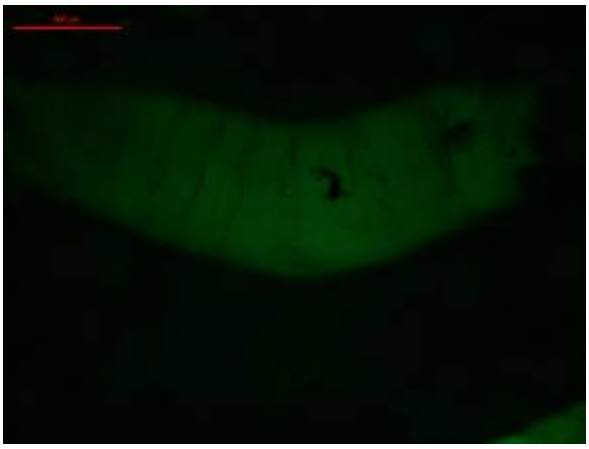
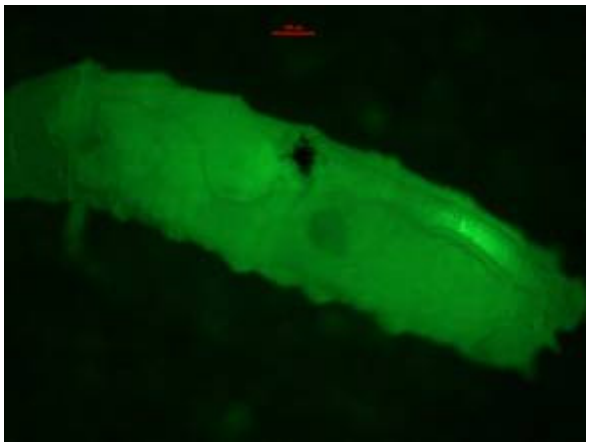
(A)  (B) 

Figure 59 – GFP expression level in *Drosophila* larvae treated with 40 ng of clothianidin (A) and controls (B).

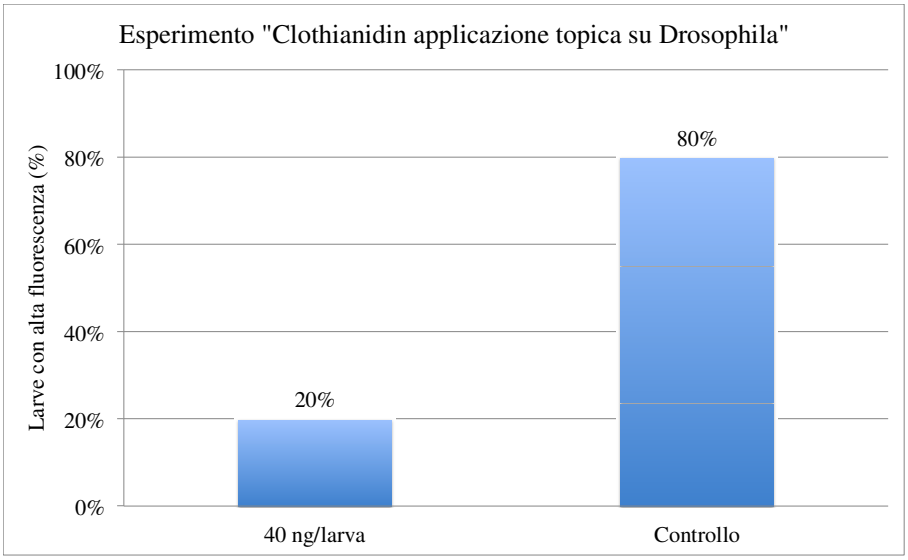


Figure 60 – Relative frequency of *Drosophila* larvae showing elevated GFP expression levels.
Key: Esperimento "Clothianidin applicazione topica su Drosophila": Experiment "Clothianidin topical application on Drosophila*"; Larve con alta fluorescenza*: larvae with elevated fluorescence; *Controllo*: control.

8.5 Conclusions

The obtained results indicate that clothianidin is able to promote DWV proliferation. Although DWV is commonly present in bees as a latent infection, its proliferation has very evident negative consequences for bee survival. The adverse effect of clothianidin on modulation of the Toll pathway–mediated immune response could explain the proliferation of latent DWV observed in bees exposed to this pesticide. The extent of the negative impact deriving from this increment, which in turn is due to the viral load, depends on the state of infection at the moment of exposure to the active ingredient and also on other concomitant stress conditions (parasites, diet, etc.), which influence the level of immune defences. This may also provide a partial explanation of the variability of toxicological data recorded in the various studies performed. Therefore, in evaluating the effects of clothianidin and other pesticides on bees, it is important to consider this additional aspect of indirect toxicity, the final outcome of which can vary as a function of colony health.

8.6 References

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Final conclusions on the different investigated aspects

2010 Data (results not discussed in the previous report)

In the experiments on the **agronomic and production-related utility** of maize dressing, comparison among the various agronomic parameters measured – including grain production – revealed no significant differences between treated (dressed) and control (fungicide only) maize crops with regard to the characters examined, partly also on account of the marked variability of results obtained in the different localities and among the different active ingredients used for dressing. When the data were processed locality by locality, significant (positive or negative) differences in production between one or more dressed treatments as compared to the control or to other pesticides were indeed found in 6 out of the total of 19 localities (31.5% of cases) Overall, however, seed treated with clothianidin showed a mean production increase of almost 5% (at 15.5% R.H.) compared to the control.
It is not possible to make a comparison with results obtained in the previous trials as the commercial hybrid supplied to our Institute by Assosementi for the 2010 trials (the commercial hybrid PR32G44- FAO 600) was different from that supplied for the previous trial (PR31N27-FAO 700). This substitution could have led to a different genotype-environment interaction, making a comparison with the previous year's results impossible; furthermore, the response to the pesticide used for maize dressing may also have been different.
In the agronomic trials conducted in Veneto using the strip-test technique (adjacent large plots), no statistically significant differences between mean productions of the different treatments (controls versus dressed treatments) were observed, even though the percentage of plants attacked by parasites was significantly higher in the control compared to the dressed treatments.

Determinations of **larval populations**, monitored with pheromone traps for Elaterids, showed that severe hypogeal phytophage attacks on maize (such as would influence production) were a rare event. Investments were good and attacks were lower than or only slightly above 1% of plants, including plants with easily reversible symptoms (yellow stripes). Results referring to plots in the regions of Lombardy, Piedmont and Veneto, obtained by means of pheromone trap monitoring for Wireworms and Western Corn Rootworm, indicated notable variability among localities with regard to captures of different species of adults (*Agriotes brevis, Agriotes sordidus, Agriotes litigiosus, Diabrotica virgifera*). This suggests the possibility of applying integrated control, differentiating the areas according to risk levels.

Field evaluations of the effect on bees in flight deriving from direct exposure to dust emitted by the seeder during the sowing of maize dressed with the different active ingredients confirmed the preliminary 2010 results: dust emitted by the seeder was sufficient to kill bees even when the toxic effect was not mediated by ingestion of contaminated food.

2011 Data

On the basis of data collected by the APENET monitoring network, 2010/11 **winter mortality** was 22.48%. This finding was not different from that recorded in the winter of 2009/10 both by the APENET network (17.6%, 113 dead hives out of 753) and as the result of the administration of questionnaires (19.5%, 2.437 dead hives out of 12.933).
With regard to the **reporting system**, in the spring of 2010 no report involved maize-growing areas. In none of the monitoring stations were noticeable phenomena reported.

The additional modifications adopted for the **seeding machines (antipollen filters)** were able to achieve a significant increase in the dust abatement capacity of simple air deflectors. Currently available data show that in the best hypothesis, abatement of the percentage of dust deposited on the ground, which was around 50% for the deflectors (2009 and 2010 trials), has now risen to 89.5% for thiamethoxam, 90% for imidacloprid and 95.2% for fipronil. However, a part of the finest dust fraction (below 4-5 μm) is not retained by the filters utilised: observation of the trend of concentrations as a function of distance shows that the dust tends to persist in the air and can reach considerable distances.

Trials to assess the **effect on bees** induced by the dust emitted from the seeder equipped with filters were conducted using bees closed in net cages: the cages were passed over the operating seeder that was equipped with the antipollen filter, to simulate bee fight over the machine. Results showed that elevated mortality percentages were still found, ranging from 30 to 60% depending on the height of flight. Such values were significantly higher than in controls (which showed 15% mortality), but decidedly lower than that of bees flying over the seeder equipped with deflectors but without filters (85% mortality).

Results obtained so far in **sub lethality** trials, although conducted with a lower number of repetitions and replications and with a lower number of individuals than in previous years, on account of the restricted time available, confirmed the findings of the APENENT 2009 and 2010 trials, which were performed with higher doses. In the 2011 trials it was found, on the basis of the first laboratory results, that the quantities of active ingredient assayed, although below the acute toxicity threshold for bees, are capable of causing damage to the learning processes and memory of adult bees.
Contact with dust containing 10% of imidacloprid, thiamethoxam and fipronil (as compared to the amount dispersed by the unmodified seeder) is capable of adversely affecting the capacity to recognise odours as early as after 60' (short term memory) and at 180' (medium-term memory), as well as at 24 h (long-term memory). In the tests on orientation capacity in a simple labyrinth and on colour recognition, the percentages of correct choice were below 50%. Although these results are preliminary, and the low number of bees submitted to the test should be taken into account, these findings indicate that individuals treated with thiamethoxam recover memory of the wrong colour at the moment of making the choice.
In the orientation tests performed in a complex labyrinth, no significant differences between the controls and the treated bees were observed with regard to the percentage of bees that returned, time taken for return and time required for completing the route within the labyrinth. However, some consequences were noted, as observed in one of the trials, where a significant number of treated bees did not return to the labyrinth.

The results obtained by studying **synergistic interactions** between stress agents and bee colony collapse suggest that clothianidin is capable of promoting deformed wing virus (DWV) proliferation, a virus that is commonly present in bees as a latent infection. Proliferation of DWV has severe adverse consequences on bee survival.

Scientists and Institutions in charge of the trials

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

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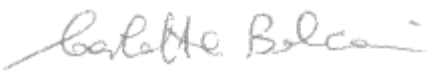

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Veneto Agricoltura

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

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3. PER *(Proboscis Extension Reflex)* test used to evaluate the effects of clothianidin, imidacloprid, thiamethoxam and fipronil administered as contaminated abrasion-dust

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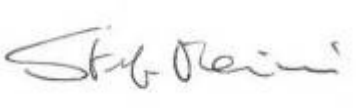

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5. Determination of the minimum level of dust dispersal during coated maize seed sowing with modified seeders and estimated effects on bees

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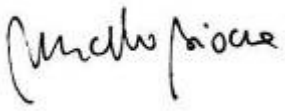

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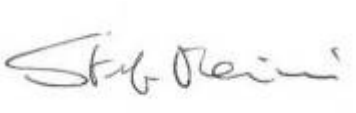

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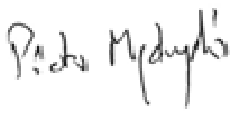

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7. Possibility of adopting integrated control for virus control in maize crops

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