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Lethal aerial powdering of honey bees with neonicotinoids from fragments of maize seed coat

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Abstract

Losses of bees have been reported in Italy concurrent with the sowing of maize coated with neonicotinoids where pneumatic drilling machine were used. Solid particles with systemic insecticide, falling on the vegetation surrounding the sown area, were thought to poison bees foraging on contaminated nectar and pollen. However, bees fed with guttation drops and dew collected from the surrounding vegetation of sown fields showed no acute toxicity. Chemical analysis showed a relatively low content of neonicotinoid in dew and guttation. Thus, the acute poisoning of bees linked to the vegetation contaminated by seed coated fragments containing neonicotinoids was again unproven. For this reason the direct aerial powdering of bees was investigated exposing caged bees around the sown area, not in contact with vegetation. High or low toxicity emerged in different trials. The synergistic effect on bees of high humidity on toxicity of powder containing neonicotinoid was hypothesized. A clear indication that bees were killed by powdering, only if held in high humidity, emerged. Chemical analysis showed high quantities of neonicotinoid insecticide in dead bees earlier exposed to dust in the field.

Key words: *Apis mellifera*, neonicotinoids, seed coating, toxic powder, humidity influence.

Introduction

In the last decades the European and American honey bee heritage has been subjected to heavy and sudden losses (Potts *et al.*, 2010). In Europe colonies decreased from over 22.5 million in 1990 to about 15.5 million in 2008 (FAO, 2009). The main causes of those deaths are attributable to viruses, fungi (*Nosema* spp.) and to the parasitic bee mite *Varroa destructor* Anderson et Trueman (Thompson *et al.*, 2002; Ribiere *et al.*, 2008; vanEngelsdorp and Meixner, 2010). Pesticides were also blamed for colony losses (Barnett *et al.*, 2007; Karise, 2007), in particular neonicotinoid insecticides that are widely used for seed coating in crops such as maize (*Zea mays* L.), sunflower (*Helianthus annuus* L.) and winter rape (*Brassica napus* L.). Neonicotinoids are used in the coat to protect the seeds and young plants from wireworms (*Agriotes* spp.), cutworms (*Agrotis* spp.), western corn rootworm *Diabrotica virgifera* (Le Conte) and from numerous species of aphids and leafhoppers (Altmann, 2003).

In the last few years sudden losses of foraging bees, with accumulations of dead insects in front of the hives, have been observed during maize sowing period, from mid March to May, in the maize growing regions of Italy and Europe (Bortolotti *et al.*, 2009; Pistorius *et al.*, 2009). The death of bees seems to be correlated with the use of seeds coated with neonicotinoids sown using pneumatic drilling machines (Greatti *et al.*, 2003), but the correlation is not always clear so further studies are required (Giffard and Dupont, 2009).

The finding that the pneumatic drilling machine, during the sowing, emits into the atmosphere fragments of seed coat (Greatti *et al.*, 2003), has suggested the hypothesis that dressing fragments containing insecticides falling on the herbaceous vegetation on the margin of the fields, by virtue of the systemic properties of neonicotinoids, penetrate into plants, contaminating nectar and pollen (Greatti *et al.*, 2006). Nevertheless, the amount of insecticides found in vegetation did not seem to justify such rapid losses during, or immediately after the sowing, since the insecticide content is about 50 ppb (Greatti *et al.*, 2006) that is too low a dose to cause poisoning by ingestion according to Yang *et al.* (2008), even if sub-lethal effects over the long period can be considered (Colin *et al.*, 2001; Suchail *et al.*, 2001; Colin *et al.*, 2004; Decourtye *et al.*, 2004; Medrzycki *et al.*, 2003; Maini *et al.*, 2010; Laurino *et al.*, 2011). Chemical analyses of dead bees have also confirmed the presence of neonicotinoids (Sabatini *et al.*, 2008) even if the amount of the insecticide did not, as a rule, seem sufficient to induce acute mortality, considering the oral intake LD_{50} of 40-80 ng/bee (for imidacloprid) reported by Maus *et al.* (2003).

Lethal sources of neonicotinoids in the field during maize sowing have been identified but obviously the mechanism by which bee come into contact with them have not yet been.

Lethal concentrations of neonicotinoids in the field were found in guttation drops of *Z. mays* (Girolami *et al.*, 2009) but the sudden death phenomena that occurred during the sowing cannot be explained since the guttation appears after plant emergence, at least a week after sowing.

This study investigates two hypothetical mechanisms through which honey bee can come into lethal contact with the insecticide used to coat maize seed during the sowing.

The first hypothesis is the direct contamination during sowing, of dews and guttation drops, on the marginal vegetation, by coating fragments containing water-soluble insecticides (before absorption into the plant as previously reported). This was considered as a possible source of poisoning for bees when they collect water for the intensive spring foraging on flowers. The second hypothesis was the possibility that bees could be directly poisoned with the fragments emitted by the drilling machine, that is a possible direct aerial contact of foragers with the dust where there is no contact with the contaminated vegetation.

Bee deaths, however, are not regularly observed during maize sowing, so the possibility was considered, that the toxicity of bee dusting could be influenced by particular environmental conditions.

Materials and methods

Experimental sites and insect origin

Field trials took place at the experimental farm of the Agricultural Faculty (University of Padova) located in Legnaro (Veneto Region - 45°20'29.07"N 11°57'30.03"E).

The Padova Beekeeping Association (A.P.A. Pad) supplied 7 hives. For the trials, the insects were caught with a net in front of the hives. The bees were kept in tulle mesh cages 20 cm x 20 cm x 20 cm and repeatedly fed with honey drops on the top of cages. Bees inside the larger cage, in sunny days (but not in rainy days), were freed in the evening and renewed daily. At the time of the tests, caged bees were collected (from the 20 cm cage) in a test tube and transferred each one in smaller cubic cages of 5 cm in tulle and again fed with drops of honey placed on the top.

Seed employed

For the trials three batches of seed were used: one of 2008, a second of 2009 and another of 2010 hereafter called “2008/2009/2010 coating” respectively. The 2008 seeds, hybrid PR34N84 from Pioneer Hi-bred Italy (Johnston, IA), were coated with the fungicide Celest® XL (Syngenta; Fludioxonil 2.4% and Metalaxyl-M 0.93%) and insecticide Poncho® (Bayer Cropscience AG., Leverkusen, Germany; Clothianidin 1.25 mg/seed) (Andersch and Schwarz, 2003; Altmann, 2003). For the 2009 and 2010 coating the hybrid employed was X1180D 964890 from Pioneer Hi-bred Italy, coated with Celest® XL, Celest® XL + Poncho® and only for 2009 Celest® XL + Cruiser® 350FS (Syngenta International AG, Basel, Switzerland; Thiamethoxam 1 mg/seed) (Robinson, 2001; Maienfisch *et al.*, 2001). The seeds were supplied in 2009 and 2010 by A.I.S. (Italian seed association) courtesy of MiPAAF (Ministry of Agriculture, Food and Forestry) for the research project Apenet.

The 2009 and 2010 seed batches have a quantity of dust abrasion under the limit of 3 g/q. The quantity was tested with the Heubach test, considered the method which best allows standardization of dust abrasion measurements within the seed industry (Apenet, 2009; Nikolakis *et al.*, 2009). The 2008 batch was a common commercial seed.

Drilling machines and sowing

A Monosem NG Plus (Monosem, Largeasse-France) pneumatic drilling machine was used for all the sowing operations. Normally 73,000 to 74,000 seeds per hectare were sown (75 cm between rows, 18 cm between seeds in the row). The drill moves at 6-7 kph with a seeding width of 3 m and, theoretically requires 30 min to sow 1 ha. The air waste pipe is situated on the right hand side of the machine and expels air (and dust) at ≈ 150 l/min, at a height of 1.8 m and an angle of 45° to the horizontal. After the experimental employment the machine and the seeding equipment was cleaned with a current of compressed air and, where possible, with water spray.

Toxicity of dew and guttation on marginal vegetation

In trials 1a, 1b, 2a and 2b (table 1), an area of 3,500 m^2 (70 x 50 m North-South oriented) was sown with seeds treated with both the 2008 and 2009 coating of Clothianidin. In the first instance (trials 1a and 2a) seeds with the 2008 coating were sown and 30 minutes later, seeds with the 2009 coating (trials 1b and 2b) were sown. After the sowing, samples of dew and guttation drops of 5 ml were separately collected from the vegetation on the margins of the sown area, on the East and West side. The first samples were collected before the starting up of the drilling machine as a control, a second at the end of the first sowing (after 30 min) and the third after the second sowing (after 60 min) (for a total of 6 samples, 3 East and 3 West). The day after the trial, repeat samples were collected in the same way (table 1, trial 2a and 2b). In all the trials the drops were collected using a glass Pasteur pipette, put in sealed glass vials and stored in a refrigerator (at 2-4°C). For toxicity test, 15% honey was added to a part of the samples and fed to the bees on the day of collection. Drops of the mixture of 30 µl were placed on the top of the net cage inside a capillary glass tube (Girolami *et al.*, 2009). For each sample at least 6 bees were tested.

Table 1. Details of field trials carried out to evaluate the toxicity of dew and guttation.

No.	Date	Starting time - length (min)	Insecticide and coating year	Meteorological conditions				No.
				T	RH	wind		bees
				(°C)	(%)	direction	speed (m/s)	tested
1a	13/V/09	9.00-30	Clothianidin - 2008	20	73	N	2.1	18
1b	13/V/09	9.30-30	Clothianidin - 2009	20	73	N	2.1	18
2a	14/V/09	9.00-30	Clothianidin - 2008	21	79	ENE	2.6	18
2b	14/V/09	9.30-30	Clothianidin - 2009	21	79	ENE	2.6	18

Samples of dew and guttation on the vegetation of the margin were collected during the trial n. 5b (table 2) of 21/X/2010 (1 h and 24 h after the sowing) for chemical analysis.

Direct field dusting inside cages

The bees were exposed to the dust emitted by the drilling machines for 30 min, inside the small cages (5 × 5 × 5 cm) on the margins of the sowing area and avoiding contact with the vegetation.

Conditions after exposure and influence of relative humidity

After the exposure to the insecticide dust in the field, honey bees were transferred to a room held at a controlled temperature (22 ± 1.5 °C). For trials where influence of humidity was considered (table 2), half of the cages were kept at the relative humidity of the laboratory lower than 70%, with the use of de-humidifier if needed, hereafter called lab humidity. The other half of the cages were kept at a relative humidity close to saturation (> 95%), hereafter designated as high humidity. To obtain conditions of high humidity, caged bees were held in plastic boxes with Plexiglas sprayed with water on the top and a moistened paper on the bottom. The cages were raised above the paper to prevent the bees getting wet. The humidity was repeatedly checked with an electronic hygrometer and also with a traditional hygrometer (with dry and wet bulb). All the bees were fed with drops of honey on the top of the cages.

In trial 2c (table 2), the cages were placed in field on poles at a height of 1.80 m, 20 cages with one bee to a cage were used, 10 were placed on the West side and 10 on the East side of the field. The first was upwind and the second downwind according to the direction of the wind was blowing across N-S orientated plot (table 2). After exposure the cages were taken to the laboratory and held at 22 ± 1.5 °C.

Field exposed honey was taken from the top of cages (in which the bees had died) and was fed to 10 other single caged bees.

In trial 3 (table 2), poles were connected by cords and cages were placed around the plot at differing heights (1.5-2-2.5 m). The cages with a single bee inside, were attached to the cord, at intervals of ≈ 2 m; 72 cages were used, 36 on the West side (upwind) and 36 on the East side of the field (downwind). After exposure the cages were taken to the laboratory and held at 22 ± 1.5 °C.

Trial 4 (table 2) was similar to the experiment no. 2c. 60 bees were exposed on poles at a height of 1.8 m; 30 cages East side and 30 cages West side of the field. At the end of the sowing (after 30 min) 15 cages of each group were put in laboratory humidity and 15 cages in high humidity.

In trial 5a seeds coated with Celest® XL (2010 batch) were sown, for 30 minutes; 60 single caged bees were placed on poles at a height of 1.8 m along both longer sides of the plot.

In trial 5b seed treated with Clothianidin (Poncho® - 2010 batch) was sown for further 30 minutes and other 60 caged bees were exposed at the same height as trial 4.

In trial 5c, during trial 5 b, 60 caged bees were exposed (on poles) not less than 40 meters from the sown area (trial n. 5c). This trial was considered an untreated control.

Chemical analysis

Neonicotinoid content in dew and guttation

Analytical determination standards and analytical methods are reported in Girolami *et al*. (2009) and more specifically in Tapparo *et al.* (2011).

Neonicotinoid content of the maize seed coat

Large fragments taken from the new seed shell coating with Clothianidin (Poncho® Bayer Cropscience AG.- Dormagen – Germany) were collected manually at the air outlet of the drilling machine after sowing experiment. This powder was weighed using an Ohaus AP250D balance (0.01 mg) and dissolved in a known amount of water-methanol (50% v:v) and placed in an ultrasound bath for 20 min.

The solution thus obtained, was diluted and filtered using Millex HV 0.45 μm (Millipore) syringe filter and was then analysed by UFLC - DAD procedure, using the method reported below.

Table 2. Details of field trials carried out to evaluate the toxicity on caged bees.

No.	Date	Starting time - length (min)	Insecticide and coating year	Meteorological conditions				No. bees tested	Humidity conditions[3] after exposure
				T (°C)	RH (%)	wind direction	wind speed (m/s)		
2c	14/V/09	9.30-30	Clothianidin - 2009	21	79	ENE	2.6	20	N.C.
3	26/V/09	9.30-30	Clothianidin - 2009	34	34	SE	3.6	72	N.C.
4	10/VI/09	15.00-30	Thiametoxam - 2009	22	41	ENE	2.4	60	L-H
5a	21/X/10[1]	11.00-30	Celest® XL - 2010	18	69	S	2.1	60	L-H
5b	21/X/10*	11.30-30	Clothianidin - 2010	16	71	N	1.9	60	L-H
5c	21/X/10[2]	11.30-30	Clothianidin - 2010	16	71	N	1.9	60	L-H

* Samples of dew and guttation were collected for chemical analysis
[1] Control
[2] Untreated (bees exposed 40 m distance)
[3] L = lab humidity; H = high humidity; N.C. = not specifically controlled

Table 3. Number of dead bees (groups of 6) within 24 h of being fed with water drops collected from the margins of the sowing area, upwind (East) and downwind (West), at different times from the beginning of sowing on 2 consecutive days. Seeds were coated with Clothianidin.

Time from start of sowing	13/V/09 Upwind	13/V/09 Downwind	14/V/09 Upwind	14/V/09 Downwind
0 min	0	0	0	1
30 min	1	0	0	0
60 min	0	0	1	0

Neonicotinoid content in bee

Samples of honey bees that died after the sowing of maize coated with neonicotinoid insecticides (Poncho® 1.25 mg/seed, 2010), were taken for analysis of the insecticide content to the Department of Chemical Sciences of the University of Padova. Samples were stored at +2°C for few days before the analysis.

The treatment of the samples started with a drying process. The bees were put in a thermostatic oven, at 100 °C for about 2 h. The samples were then ground with a metallic pestle, then put into a solution of methanol-water (50% v:v) and placed in an ultrasonic bath for 20 min. Samples were finally centrifuged, the floatage was separated and filtered with Millex HV 0.45 µm syringe filter (Millipore).

The analyses were performed in a UFLC instrument (Ultra Fast Liquid Cromatography, Shimadzu XR - Prominence) equipped with an UV-Vis diode array detector and a Shimadzu XR - ODS II (2.2 µm, 2 × 100 mm) analytical column and a Phenomenex Security Guard pre-column.

The following instrumental procedure was optimized: eluent flow rate of 0.4 ml/min, gradient elution (0-0.5 min 70:30% water/acetonitrile; 0.5-1.5 min linear gradient to 100% acetonitrile; 1.5-3 min 100% acetonitrile), 45 °C column temperature, multiwavelength acquisition of detector signal and analytes quantification at 269 nm for Clothianidin.

Instrumental calibration (external) was performed by the analysis of standard solutions in the 0.1-100 mg/liter concentration range of analytes, prepared in methanol-water solutions (50% v:v) from pure analytical standards (Sigma-Aldrich Group, Milan, Italy; Pestanal, purity > 99.7%).

Methanol (VWR, International, Milan, Italy) and acetonitrile (Riedel de Haen, Sigma-Aldrich Group) were of HPLC grade. Pure water was produced by Milli-Q equipment (Millipore, Billerica, MA).

Statistical analysis

For comparing different mortalities of bees χ^2 test was used.

Results

Toxicity of dew and guttation on the marginal vegetation

In trials 1a, 1b, 2a and 2b (table 1) bees fed with drops collected from the vegetation on the margin of the sowing area did not demonstrate symptoms of poisoning; only 3 bees out of 72 died (table 3) without specific symptoms of neonicotinoid poisoning such as a jerky inward arching of the abdomen (Girolami *et al.*, 2009). There were no subsequent mortalities detected either in the control (0 minutes) before the starting up of the drilling machine, or in other successive samples within 48 h. The bees were then freed, into the sunlight, and almost all were able to fly away. Even the samples collected after two consecutive days of sowing, despite probable higher quantities of fragments of coating on vegetation on the margins, did not demonstrate any acute toxicity to honey bees.

Direct field dusting inside cages

In trial 2c (table 2), bees contained in tulle cages, were exposed to the dust of the drilling machine at the margins of the sowing area for 30 min and then taken to the laboratory. The 10 bees that were exposed West-upwind were all still alive after 24 h, while bees exposed East-downwind all died (in the laboratory) (table 4) within 5 to 10 h.

To eliminate the doubt that the death of bees could be due to feeding on drops of hypothetically contaminated honey on the top of cages exposed to dust in the field, honey was taken from cages were bees had died and fed to 10 other bees, of which, only one showed symptoms of neonicotinoid poisoning within 24 h.

In trial 3 (table 2) bees inside cages were placed around the plot at differing heights (1.5-2-2.5 m). None of the 72 bees taken to the laboratory, after exposure, showed evident symptoms of poisoning within 24 h (table 4). The honey bees exposed in this trial did not die and the height of exposure seems not to be determinant in bee mortality.

In trial 4 (table 2) the influence of relative humidity was evaluated on 2 groups of bees exposed to dust in the field, (30 East side and 30 West side), by holding them in laboratory humidity or in a semi-saturated condition. In high humidity 73% of bees died (22 out of 30).

Table 4. Number of dead bees (2 groups of 10 or 36) exposed on the margins of the sown area to the dust of the drilling machine for 30 min in two experiments.

No. - date of trial	Insecticide	West Dead	West Survived	East Dead	East Survived	χ^2*	Probability
No. 2c - 14/V/09	Clothianidin-2009	0	10	10	0	20	p < 0.0001
No. 3 - 26/V/09	Clothianidin-2009	0	36	0	36	0	n.s.

* χ^2 was calculated in the same line

Table 5. Number of dead bees (groups of 30) exposed in single cages, to the emissions of the drill, taken to the laboratory and kept in varying conditions of RH.

No. - date of trial	Insecticide	Conditions after exposure	Mortality dead	Mortality survived	χ^2 *	Probability
No. 4 - 10/VI/09	Thiamethoxam - 2009	Lab humidity	5	25	19.46	$p < 0.0001$
		High humidity	22	8		
No. 5a - 21/X/10[1]	Celest XL® - 2010	Lab humidity	3	27	-	n.s.
		High humidity	4	26		
No. 5b - 21/X/10	Clothianidin - 2010	Lab humidity	15	15	13.87	$p < 0.0002$
		High humidity	28	2		
No. 5c - 21/X/10[2]	Clothianidin - 2010	Lab humidity	1	30	0.35	n.s.
		High humidity	2	30		

* χ^2 was calculated in the same column and between two humidity conditions
[1] Control
[2] Untreated (bees exposed 40 m distance)

Of the corresponding bees held in lab humidity, respectively only 16% died (5 out of 30), showing highly significant differences in the χ^2 test between the two humidity conditions (table 5).

In trial 5a (table 2), only seed treated with fungicides (Celest® XL) was used. Of the 60 bees exposed in the field and then taken to the laboratory, 3 out of 30 died in lab-humidity and 4 out of 30 died in high humidity without significant differences between high and low relative humidity conditions.

In trial 5b (table 2), seed treated with Poncho® was used immediately after the sowing with fungicides. High mortality was observed in bees with significant differences between high and low humidity after the exposure. In trial 5c (table 5), bees were exposed at not less than 40 meters from the drilling machine and almost all survived without significant differences between high and low humidity.

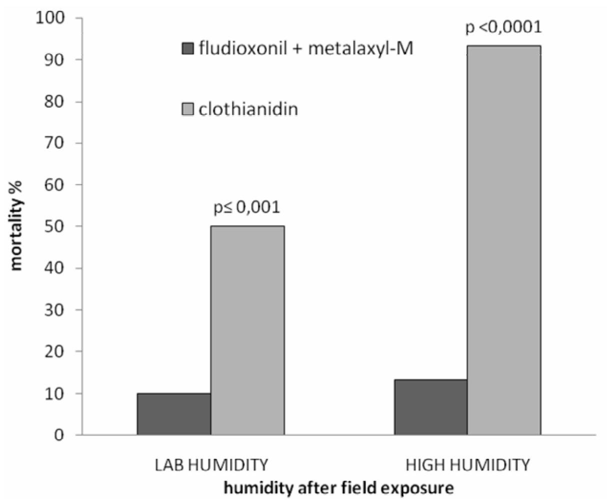

Figure 1. Percent mortality of *A. mellifera* exposed to the dust emissions of the drilling machine using maize treated with only fungicides or fungicides plus insecticide (table 5, trial 5a and 5b). p-values refer to mortalities in the same humidity conditions.

Therefore, highly significant differences emerged between different humidity regimes when seeds treated with insecticides were used whilst, using seeds treated only with fungicides or holding the cages a distance from the drilling machine, no significant differences emerged.

Comparing the mortality between fungicide and insecticide exposed bees (table 5, trials 5a and 5b), separately in high humidity or lab humidity, highly significant differences emerged (figure 1).

Therefore high humidity increases mortality only when insecticide is used and not with fungicide.

Chemical analysis

Dew and guttation analysis

The chemical analysis of the fragments of seed coating showed approximately, or more than, 20% (wt:wt) of Clothianidin a.i. content.

In both samples of dew and guttation drops collected one hour and 24 h after the sowing the insecticide used for seed dressing was found in concentrations lower than 30 ppb, with an overall average of 15.87 ppb (table 6).

Neonicotinoid content in bees

Chemical analysis of dead bees found an average of 279 ± 142 ng/bee of Clothianidin in high humidity while in low humidity the average was 514 ± 174 ng/bee, with an overall mean of 396 ng/bee.

Table 6. Content of Clothianidin in samples of dew and guttation drops collected from vegetation on the field margins after the sowing.

No. - date of trial	Insecticide	Field side	Time of sampling-quantity (ppb) 1 h	24 h
No. 5 - 21/X/10	Clothianidin - 2010	East	27	6.5
		West	17.5	12.5

Discussion

Toxicity of drops of dew and guttation

No acute toxicity was found in bees fed with dew and guttation drops collected on the margins of the seeded area even after two consecutive days of sowing in the same plot. Thus the hypothesis that the bees were acutely poisoned by solid fragments falling on the vegetation was again unproven. In particular, it seems that honey bees cannot be lethally poisoned by drinking dew and guttation on vegetation during, or after sowing of maize coated with neonicotinoids. These observations agree with semi-field trials based on the contamination of flowers sprayed with doses of neonicotinoids (imidacloprid) relatively higher than the quantity that would fall during the sowing (Schnier *et al.*, 2003).

The absence of mortality is congruent to the neonicotinoid content of dew and guttation drops: chemical analysis showed an average content of Clothianidin of 15.87 ppb. Considering that a bee can drink 30 µl of solution in a single session (Beekman *et al.*, 2004), the intake of active ingredient would be 0.5 ng of Clothianidin. That is a dose more than fifty times lower than that required to cause an acute poisoning with a single ingestion (Girolami *et al.*, 2009).

Obviously the absence of acute toxicity of vegetation containing low doses of neonicotinoids cannot exclude other poisoning sources for honey bees that may be present during the sowing with dressed maize. Similarly, the effects of chronic toxicity over a long period, due to sub-lethal doses of neonicotinoids, cannot be excluded (Medrzycki *et al.*, 2003; Aliouane *et al*., 2009).

Direct field dusting inside cages

The data from the first experiment with caged bees (table 4, trial 2c) implied, as a probable contamination, the direct powdering of bees exposed in small cages, for half an hour to the dust of the drill and unable to fly freely. The hypothesis of direct dusting appeared to be contradicted in trial 3 where no mortality was observed (table 4). The weather conditions between the two trials (table 2) corresponded, the first to spring conditions with a low temperature (21 °C) and high humidity (79%), the second to summer conditions with a high temperature (33 °C) and low humidity (34%). It was thought that weather variables could influence mortality, in particular it was suggested that given the water solubility of the neonicotinoids, humidity could play a role in the deaths of bees. This hypothesis was tested in a subsequent trial (table 5, trial 4) where exposed bees were kept in the laboratory at different humidity. The mortality of bees kept in high (semi-saturated) humidity was very high, whereas, in lab humidity (≤ 70%), almost all survived (table 5). The influence of high humidity corresponding to weather conditions that frequently are present in spring, in the first few hours of morning sun, was verified in a further trial (table 5, trial 5b).

In trial no. 5a, where seeds treated only with fungicides were sown, or bees were kept in cages far from the sown area (trial 5c), a low mortality was recorded in bees held in both humidity conditions. For this reason, it is possible to consider these fungicides coating as not toxic to honey bees, and as an acceptable untreated control. Moreover, in these trials (5a and 5c), no significant differences were found between mortalities in the two humidity conditions, this suggest that high humidity, in itself, could not cause mortality. High humidity, on the other hand, seems to have a synergistic influence on the toxicity of insecticides that come into contact with honey bees.

The amount of insecticide found in samples of dead bees (analyzed 24 h after the end of the trial), is sufficient to explain the mortality, because the quantity found are more than 10 times higher (table 7) than the contact LD_{50} for Clothianidin of 21.8 ng/bee (Iwasa *et al.*, 2004).

There are no doubts that the bees tested died because of the high amounts of insecticides that reached them, but the mechanism through which they get contaminated, in particular if the wind has a role, as suggested by the first trial where mortality was observed only downwind, remains to be investigated.

From the data reported it is possible to suppose that honey bees die in spring, throughout the maize sowing period, because they are contaminated by insecticide dust emissions during foraging activity when they fly near a working drilling machine.

As reported, bees were exposed to the dust emitted by the drilling machine for half an hour without the possibility of flying away, therefore other experimentation to demonstrate that the bees can be dusted in flight, are necessary.

The reason why the powder emitted by the drilling machine, independently of the synergistic effects of humidity, had such a dramatic effect on bees may have a rather simple explanation. The fragments expelled during the sowing, contain more than of 20% of neonicotinoid, that is a concentration of insecticide at least 2,600 times greater than that diluted in water for agricultural sprays (for example Dantop®, Clothianidin 50%, is used at 15 g/hl, that is 75 ppm).

Table 7. Quantity of insecticide (Clothianidin) found in dead bees after 30 minutes exposure to the dust emissions of the drilling machine.

No. - date of trial	Insecticide	Conditions after exposure	ng/bee of insecticide East	ng/bee of insecticide West	Average (ng) ± s.e.
No. 5 - 21/X/10	Clothianidin - 2010	High humidity	694	147	279 ± 142
			54	220	
		Lab humidity	264	262	514.25 ± 174.7
			527	1004	

The presence in the field of sources of highly concentrated insecticide, sufficient to kill bees, was previously not considered, probably because the lethal effects are contingent upon the differing humidity in the field.

In any case it seems that acute poisoning of bees can more probably be linked to an aerial contamination rather than to a contact with marginal vegetation. It is important to investigate the possible mechanism through which honey bees come into contact with the dust emitted by the drilling machines. Once this mechanism is clarified, it will be possible to improve drilling machines and to take measures to mitigate risk.

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