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LPE-41/00 / MO-03-005868

FINAL STUDY REPORT

"FIELD EVALUATION IN ARGENTINA OF POSSIBLE RISK FOR HONEY BEES

FROM THE PRODUCT 'GAUCHO' ON SUNFLOWERS"

This study was prepared in accordance with resolution 350/99 for

reevaluation of chemicals in Argentina

STUDY NUMBER

LPE – 41/00

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CONFIDENTIALITY STATEMENT

[redacted]

ABBREVIATIONS

BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft
CONICET	Consejo Nacional de Investigaciones Científicas y Técnicas
FCEyN	Facultad de Ciencias Exactas y Naturales
INTA	Instituto Nacional de Tecnología Agropecuaria
LIBIQUIMA	Laboratorio de Investigaciones Bioquímicas, Químicas y de Medio Ambiente
LPE	Laboratorio de Parasitología y Ecotoxicología
MACN	Museo Argentino de Ciencias Naturales
OEPP/EPPO	Organisation Européenne et Méditerranéenne pour la Protection des Plantes / European and Mediterranean Plant Protection Organization
SOP	Standardized Operation Procedure
SENASA	Servicio Nacional de Sanidad y Calidad Agroalimentaria
UBA	Universidad de Buenos Aires
UNC	Universidad Nacional del Comahue

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SUMMARY

The possible risk that the product Gaucho may present to honey bees is assessed in this paper through a field test. The evolution of hives that were exposed to flowering sunflower from seeds treated with Gaucho was qualitatively and quantitatively evaluated. Variables sensitive to factors that have an impact on bees such as: weight of hive, honey yield, nectar, pollen and brood were recorded; as well as field activity, incoming pollen in hives and mortality.

In order to validate this paper and to extrapolate it to other tests that have been done in various European countries, LPE, MACN, and CONICET drafted a test protocol based on the guidelines of BBA (1980) and OEPP/EPPO (1992), that was approved by the *"Working group for the reevalution of Imidacloprid for possible negative effects on bees"* (SENASA) at the 01/10/2000 meeting. As required by the *Good Laboratory Practices* (GLP), *Standardized Operation Procedures* (SOP) were added for each of the actions related to the test; as well as the *Amendments,* aimed at including the necessary corrections in order to obtain, at the end of the testing, a validated protocol; and the *Deviations*, which permitted to overcome specific features related to the imponderables of this particular test.

Since this study is multidisciplinary, LPE – MACN –CONICET, as scientific coordinator of the study, invited several members of university academic sector in Argentina as well as institutes and researchers of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), to proceed to analytical chemical, statistical, palinologic, and other tests, whose reports support the conclusions in this paper. SENASA -directly or through the appointment of an auditor (INTA)-, BAYER S.A. -manufacturer of the product Gaucho, and LPE – MACN – CONICET, in charge of the scientific coordination, have all been involved in the field work and samplings, from sowing of sites to the last evaluation of the beehives.

The test formally started with the treatment of sunflower seeds with the product Gaucho, according to label recommendation, and with the installation of 32 beehives, 16 of which were randomly selected for the test. From that moment on, a permanent follow up of the sunflower crop and the plant health treatments was made, as well as the follow up of wild flora in adjoining sites. Sunflower test sites were culturally managed based on good agricultural practices in Argentina. Special attention was paid to the assessment of the phenological condition of sunflower in order to adapt the hive exposure to the terms of the usual pollination practices. Basic meteorological data were recorded while hives remained on the sunflower sites. According to apicultural recommendations a program of hive sanitarian treatments was developed with different products to prevent varroa and nosema diseases.

The samples during the test were taken in triplicate and immediately distributed to SENASA, BAYER S.A. and LPE – MACN – CONICET, the latter being used specifically for the test and the rest being kept as counter-samples. Samples were taken of seeds, soil, sunflower inflorescences, wax, honey and pollen to determine Imidacloprid residues; on the other hand, samples of honey and pollen were taken for palinologic tests.

The complete study includes: original protocol, amendments, deviations, study protocol, scope, materials and methods, results and discussion, conclusions and annexes. In order to obtain a picture of the time structure of the study, activities developed at each of the evaluation times are summarized in Table 9 (p. 17). For the same reason, the results of the analytical tests were summarized in Table 11 (p. 48), and those concerning population in Table 10 (p. 47),

where differences between hives on the treated site and those on the control site are highlighted for each of the variables tested.

From the results of this study, it may be concluded that:

Concerning plant density and the phenologic condition of sunflower in test sites, at transfer of hives to sunflower sites (at time T2) plant density of the site treated with Gaucho was higher than that of the control site, a fact that probably has to do with seed treatment with the tested product. The number of flowering plants was similar in both sites. However, towards the middle of flowering and throughout the flowering period a higher proportion of plants without pollen was observed in the control site as compared with the treated site.

As for bees activity and mortality in test sites between dates T2 and T 3, field activity was significantly higher in the treated site as compared with the control. No significant differences were observed on bees with pollen entering hives from both sites. Mortality measured in front of hives of both test sites was not statistically different.

In pollen counts made on honey samples taken in T3 a high percentage of sunflower pollen (> 20%) is observed as it can be expected for a test under field conditions and in conformity with literature information (*Maurizio & Louveaux, 1963; Ricciardelli d'Albore, 1997).* Furthermore, honeys were identified *in situ* according to their origin as "sunflower honey" in accordance to the organoleptic properties of the samples obtained.

On the other hand, when exposure to sunflower (date T2) began, composition and structure of the population in the hives were uniform; weights and frame area percentages filled with honey, nectar, pollen and brood, did not show significant differences. At the end of the exposure period of hives to sunflower (date T3), increases in hives of the control site and hives of the treated site were observed for the following parameters: average weight of hives, amount of honey and nectar in top supers and amount of pollen and brood in bottom supers. However, increases of these parameters were significantly higher for hives in the treated site.

At date T4, 24 days after removing the hives from sunflower, the amount of pollen, nectar and honey stocks in hives that were exposed to the treated site was significantly higher as compared with those of the control site.

In samples of sunflower seeds treated with Gaucho that were obtained before sowing, an average content of 0.2458 mg Imidacloprid /seed was determined. That is in agreement with the treatment of seed that was applied. As for the Imidacloprid residue tests, no quantifiable Imidacloprid residues were found in samples of soil and sunflower heads at date T2. No quantifiable Imidacloprid residues were found in samples of either pollen, honey or wax at dates T3 and T4.

It can be considered that, during the stay of hives in sunflower sites, hives of the site treated with Gaucho developed more rapidly than those in the control site. However, 24 days after their removal from sunflower, both hive groups (control and treated), reached a similar level of population development, even if honey and pollen production was higher for the hives that were in the treated site. Differences in hive development of both sites may be related to differences observed in field activity and with the different proportion of plants with available pollen that were present in both sunflower sites during flowering.

I. INTRODUCTION

Gaucho, an Imidacloprid-based product, is used in sunflower as a seeddressing to control soil insects that affect the normal germination and development of the crop. In contact with soil, Gaucho forms a protection halo around the seed and due to its systemic properties it is transferred to the plant and distributed to the upper plant organs.

The residual activity of the product is difficult to visualize with regard to soil insects. The same use for the control of aphids, however, shows that residuality does not exceed very few weeks. Ten weeks after planting, flowering period of sunflower and collecting of nectar by bees start.

II. SCOPE

The aim of this study is the qualitative and quantitative evaluation of the development of the hives that were exposed to flowering sunflower from seeds treated with the product Gaucho by observing the evolution of the variables: weight, frame covering (by honey, nectar, pollen, brood), and bee activity (bees with field activity and bees carrying pollen into the hives) and mortality.

III. MATERIALS AND METHODS

III. 1. *PRINCIPLE OF THE STUDY*

One of the two scheduled tests (deviation 1, annex VII.3) was done according to added protocol (LPE Pr – 1/00, annexes VII.1 and VII.4) that was prepared in accordance with BBA (1980) and EPPO (1992) guidelines. Hives were transferred to sites with flowering sunflower. Plants of one site grew from seeds that were treated with Gaucho (treated site) and plants on the other site grew from seeds without treatment with Gaucho (control site). In both cases, seeds were treated with fungicide. The agricultural management, from tasks before sowing to the removal of beehives, was similar in both sites and followed the usual standard for the region.

In order to make the observations required by the study, hives were installed in the control and treated site at the time of crop flowering in accordance with the right apicultural practice for crop pollination. The health condition of the beehives was assessed before and after their installation in the middle of the crop.

During the study several moments were defined when measurements of variables were made and data were taken to evaluate hive development (table 9). While hives remained in the sunflower sites, periodical observations were made for field activity on sunflower heads, pollen collection, mortality and other parameters related to hive development.

III.2. *IDENTIFICATION OF THE SUBSTANCE TO BE TESTED*

Trade name: GAUCHO 60 FS.
Active principle (identification and content): Imidacloprid 60% p/v.
Type of formulation: seeddressing in suspension (FS).
Aptitude: seeddressing insecticide.
Manufacturing lot number and origin: 802D00073, Germany.
Manufacturing date: 02/1998. Expiration: 05/01/2000.
Test Certificate: Figure 1.

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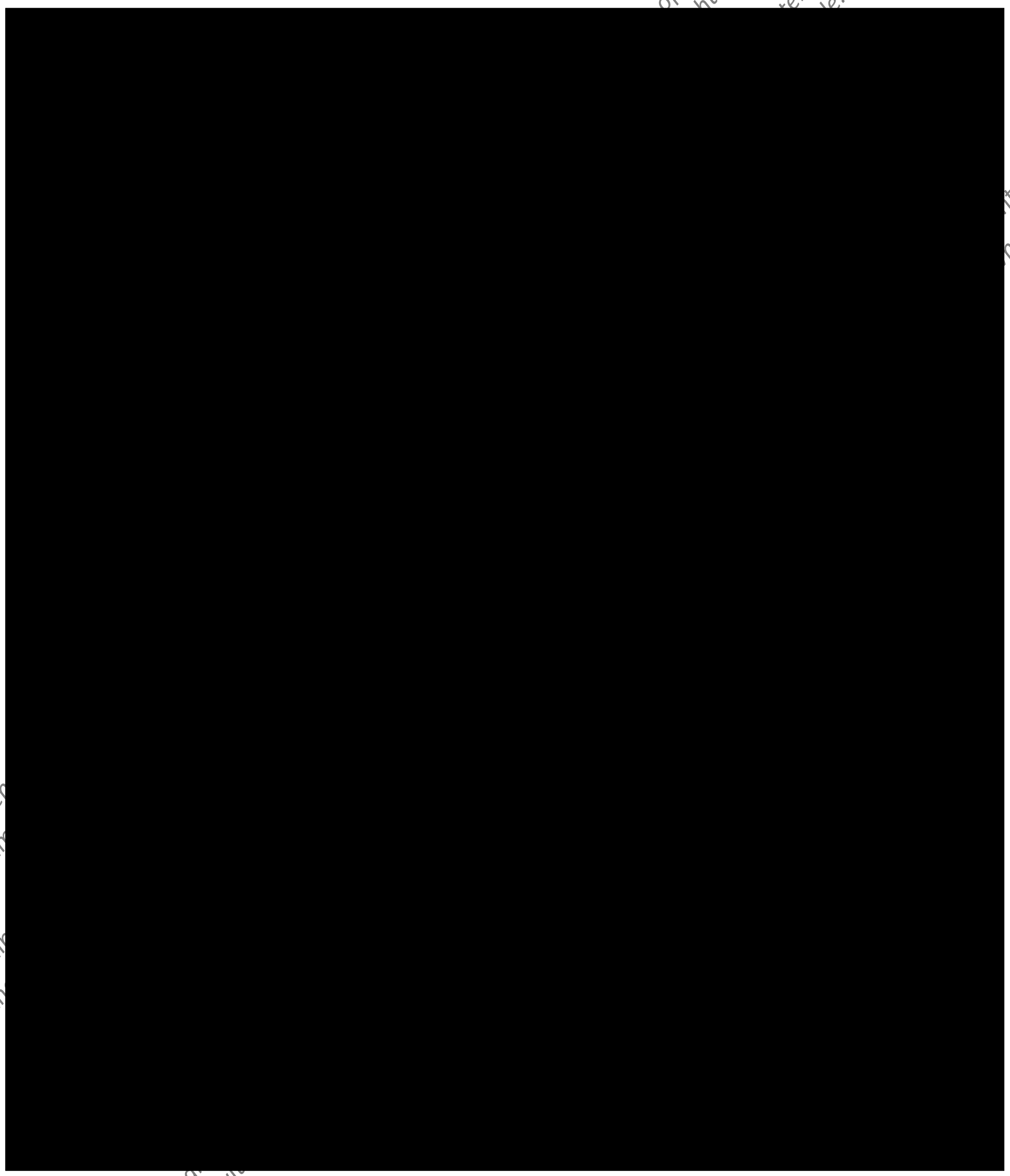


Figure 1. Test certificate of the product Gaucho used for the treatment of sunflower seeds.

II.3. *IDENTIFICATION OF SEEDS*

Species: sunflower (*Helianthus annuus*).
Variety/hybrid: trade hybrid DEKASOL 3915 G3.
Origin of Seed: breeding ground DEKALB (MONSANTO S.A.)
Registration N°: 5124 *Registro Nacional de Cultivares.*
Weight of Seed: 75,3 g.

III.4. *DESCRIPTION OF BEES AND HIVES*

III.4.1. Bee species and origin

Identification of species: Apis mellifera

Origin (producer): "Malka" owned by [redacted] Los Hornos, La Plata, prov. Buenos Aires.

Registration N° (SENASA) 8918, [redacted] import and export of queen bees

Registration N° 3752 –1635, apicultural farmer. Registro de Marcas del Ministerio de Asuntos Agrarios de la prov. de Buenos Aires.

Queens of all hives used for the test were sisters.

III.4.2. *Description of hives*

Number of supers/hive at T0: 1
Number of frames/hive at T0: 7 frames with brood, 1 frame with honey, 1 frame of foundation wax and a feeder (at T2 half super was added with ten more frames and an excluder between supers to avoid oviposition and brood development on the top super)
Estimated number of bees/hive at T0: 20.000.

At T0 a frame by frame general inspection of all hives took place and samples of bees were taken to diagnose nosema disease, in the presence of [redacted] SENASA).

III.5. *DESCRIPTION OF TEST SITES*

III.5.1. *Location and identification*

Geographic location: Estancia "La Catalina" (ruta nacional n° 7, Km. 370). lots Santa Lucía and Santa María. Control and treated sites are at a distance of 7760 m from each other.

The test was laid out as indicated in table 1.

TABLE 1. *Characteristics of test sites*

Test	*Identification*	*Treatment*	*Location*	*Area* (ha)	*Length* (m)	*Width* (m)
1	**1.A**	Control	Estancia La Catalina **Lot Santa Lucía**	22,4	640	350
	1.B	Treated	Estancia La Catalina **Lot Santa María**	22,4	640	350

III.5.2. Characteristics of test sites

- Type of site soil (class on account of use aptitude).

Lot SANTA LUCÍA: Class I_2.
Lot SANTA MARÍA: Class II_w.

Class I_2: deep soils, moderately well drained, in moderately undulating or almost flat areas.

Class II_w: imperfectly drained soils. Due to slow water elimination they are excessively wet during a certain period of the year but not permanently.

- Lots background:

Lot SANTA LUCÍA: consociated grassland
Lot SANTA MARÍA: consociated grassland

III.5.3. Agricultural Management

Treatment of seeds with fungicide

Seeds received a fungicidal treatment, with two products simultaneously. Details of treatment are indicated in table 2.

TABLE 2. *Facts related to fungicide treatment of seeds*

Date of treatment	April/99	
Products (and formulation) used	**RITIRAM PLUS SEMILLERO AC CAPTAN (FS)**	**APRON 35 SD** (WP)
Active ingredient	Captan 37%	Metalaxyl 35%
Dose of formulated product	120cc/100 kg of seed	50 g/1000 kg of seed
Vehicle dose (water)	126cc / 100 Kg seed	
Application equipment	Machine: CIMBRIA HEID; Model: "Centricoater CC 50 Duo:	
Mode of application	Seeds fall into the curing machine rotating cylinder and then the treatment broth is added with cylinder rotating. Seeds are then stored and put into bags.	

Treatment of seeds with Gaucho

Date of treatment: 11/29/99.
Dose of formulated product: 600 cc/100 Kg (= 0.426 cc/1000 seeds)
Vehicle dose (water): 1200 cc/100 Kg of seeds.
Broth dose: 1800 cc/100 Kg of seeds.
Application equipment: standard moto concrete mixer

Mode of application: Broth needed for the treatment of 20 (12.8 Kg) bags of sunflower seeds was prepared by mixing 1526 cc of Gaucho 60 FS with 3064 cc of water (final broth volume of 4600 cc) and shaken to get a homogenous mix. Seed bags were treated one by one, according to the *Standardized Operation Procedure* (SOP) 9 (annex VII.5). With the moto concrete mixer operating, 230 cc of broth were poured into it, and then a bag of seeds was added and mixed during 2 minutes. Then, seeds were deposited on linen cloth to dry in the air. 20 seed bags of 12.8 Kg each were treated, in the presence of Arg. Eng. [redacted] (INTA- UEEA Venado Tuerto).

Taking of seed samples

25 bags of seeds non-treated with Gaucho (control) and 20 bags of seed treated with Gaucho (treated) were available. From each bag of seeds (treated and control) samples were taken (according to SOP 10, annex VII.5), they were mixed and homogenized in order to have a representative sample of treated seed and another one of non treated seed. Each sample was divided in three sub-samples. So, there were three sub-samples of treated seed and three sub-samples of non-treated seed available. Each sub-sample was divided in 4 bags "cerealista"-like that were sealed and identified numerically on the sealing lock. 24 cardboard bags were then available with the following destination:

- 16 bags (8 treated + 8 control) were retired by INTA's auditor, 8 for INTA and 8 bags were sent to SENASA.
- 8 bags (4 treated + 4 control) in the custody of Bayer Argentina S.A.

Sowing

Date of sowing:
Control site (lot "Santa Lucía"): 12/6/99;
Treated site (lot "Santa María): 12/7/99.
Type of sowing: semidirect.
Type of sowing machine used: "Autotrailer Sembrador AgroPla", capacity: 12 individual hoppers.
Distance of ruts: 2.5 cm.
Sowing depth: 2.5 cm
Sowing density: 60.000 seeds/ha.

Initial plant density upon emergency in control lot was 41.500 plants/ ha while in the treated lot it was 50.300 plants/ha.

Fertilization

No fertilization took place.

Plant Health Management

Treatments that were applied with plant protection products, as from the preparation of sites to be sown to hive removal (T3) are indicated in table 3.

TABLE 3. *Applications of plant protection products to test sites*

Time	**Type of product**	**Product** (active ingredient) **and dose**
PRE-EMERGENCE 12/7/99	*Insecticide*	Beta Baythroid 5 EC (Beta Cyfluthrin) 80 cc/ha
	Herbicide	Glifos Bayer + Twin Pack [(Glyphosate + (Fluorochloridone + Acetoclor)] 2500 cc/ha + 1700 cc/ha
POST-EMERGENCE 01/7/00	*Herbicide*	Centurión (Twin Pack of Select + Coadyuvante Tomen) [Clethodim and (Dilauril ester and polyethylenglycol ester)] 1 can / 4 ha: (700 cc/ha of Select + 2000 cc/ha of Coadyuvante Tomen)
01/27/00	*Insecticide*	Galgofan [Endosulfan 35%] 1500 cc/ha + 7 l/ha of gas oil

III.5.4. *Adjoining Lots*

Diagrams of Figures 2 and 3 represent lots adjoining test sites.

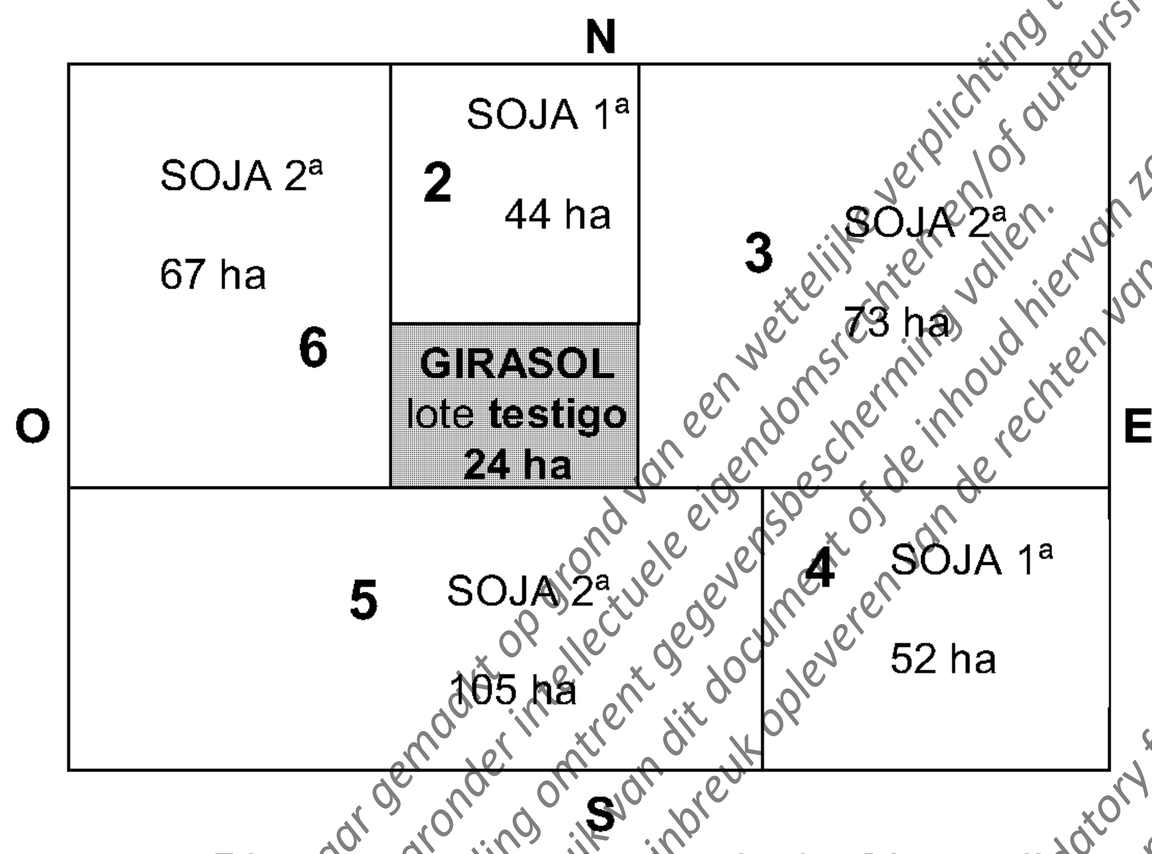


Figure 2. Diagram (not scale drawing) of lots adjacent to control site

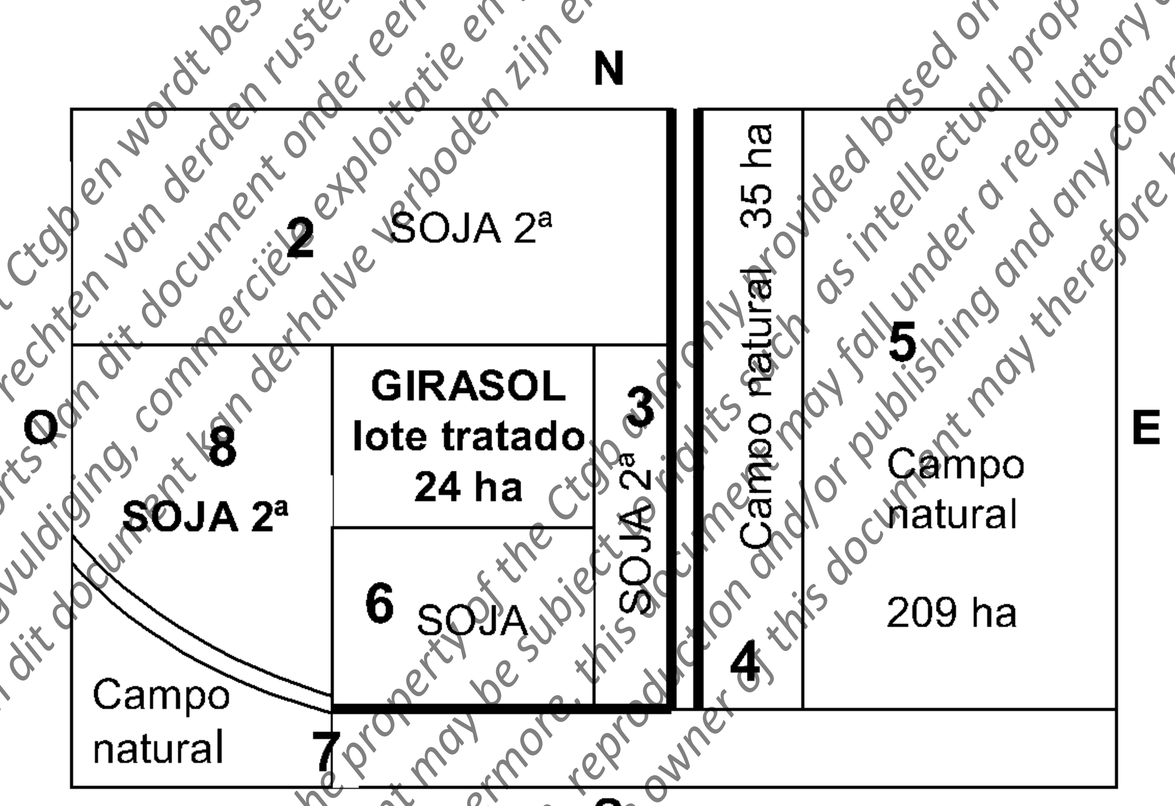


Figure 3. Diagram (not scale drawing) of lots adjacent to control site.

III.5.5. Evaluation of sunflower phenologic condition

Flowering in sunflower is centripetal, that is, from the head perimetrical flowers proceed towards the centre. Typically, a flowering sunflower head shows from outside to the centre, dry flowers, a ring of flowers releasing pollen, a ring of flowers with receptive stigmas and, finally, flowers not opened at the centre (figure 4). The second line of tubulous flowers from the edge of the head towards its centre (considering that each line of flowers includes, in fact, 3 sub-lines of

flowers) must be observed to determine that a sunflower plant has begun to blossom. This phenomenon is an indication that a plant started flowering.

Determination of "10% of flowering"

Since flowering does not begin at the same time in all sunflower crop plants, it is necessary to determine the percentage of plants that are flowering. After the first heads opened, the percentage of flowering plants was determined on a daily basis for the sunflower crop both in control and treated test sites. This parameter was measured at four random spots per site by counting the number of flowering plants out of the total of plants per 14.3 rut meters long from each spot (Agr. [redacted] *com.per.*). Percentages for each spot were calculated, and the average percentage per day and per site was obtained. 10% of flowering was determined when 10% of the sunflower plants of the site were flowering.

Determination of "available pollen" and of "end of flowering"

As with flowering, pollen releasing does not occur in all crop plants at the same time. In sunflower test sites, the percentage of plants with pollen was determined on a daily basis. Four random spots were picked up per site, the number of plants with pollen out of the total of present plants was calculated for 14.3 rut meters long from each spot ([redacted], *com.per.*). Percentage per each spot was calculated and the average percentage of plants with pollen and the average percentage of plants without pollen were calculated per site and per day. The end of flowering was established when 80% of the plants ended their pollen production at the centre of the head.

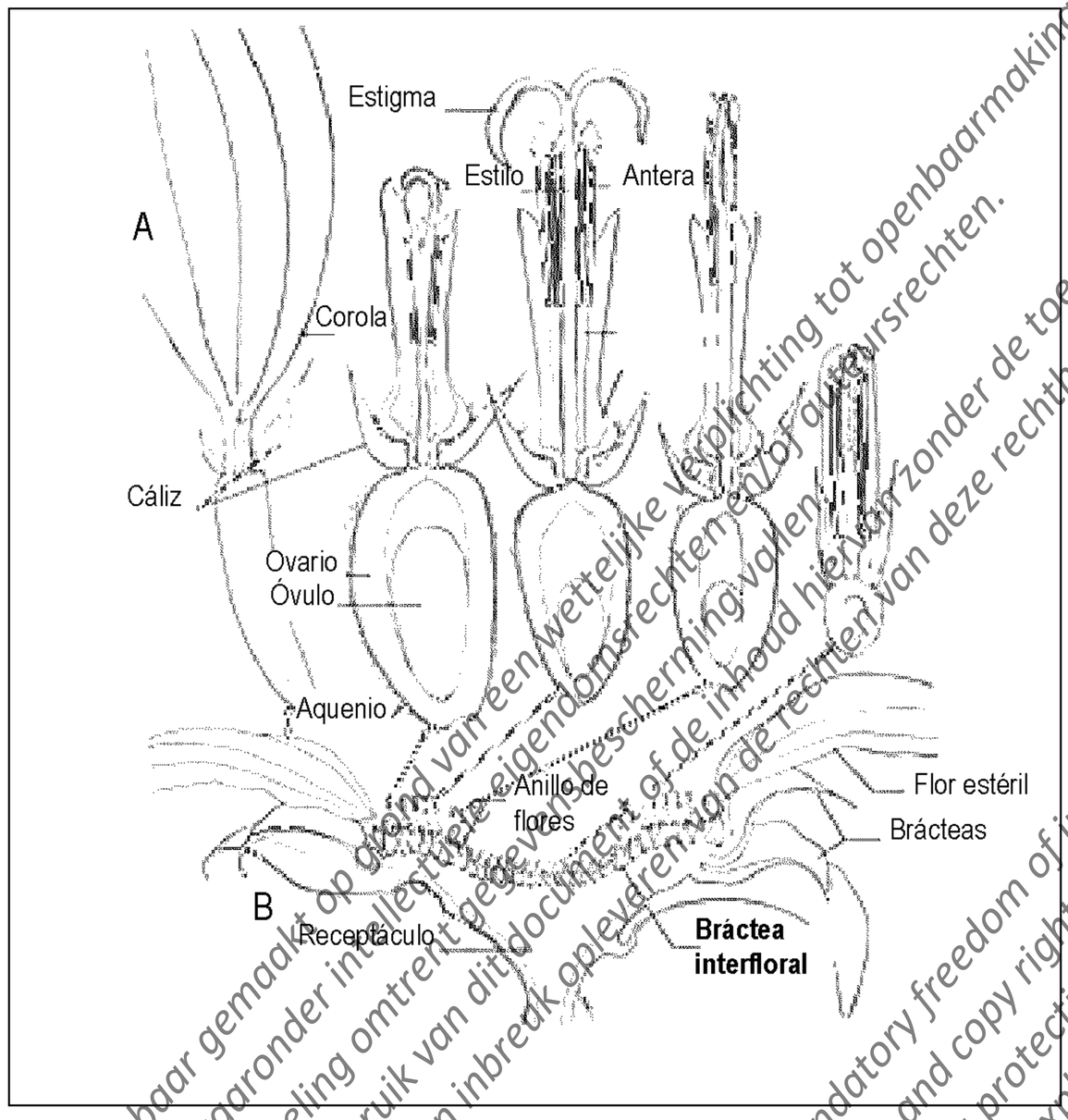


Figure 4. Longitudinal view of a sunflower head, x ½, with individual flowers. A, sterile flower, x5; B, ring of flowers at different development stages, x 5. In *Insect Pollination of Cultivated Crop Plants* by S.E. McGregor, USDA, 1976 updated at:

http://gears.tucson.ars.ag/gov/book/chap/sun.html.

III.6. *CONTROL OF WEEDS IN LOTS ADJOINING TEST SITES*

Before transferring hives to test sites several activities took place to control weeds in adjoining lots. Figures 4 and 5 show these activities.

TABLE 4. *Activities to control weeds of lots adjoining control test site "Lot Santa Lucía".*

Date	Activity
01/26/00	Glyphosate 48% was applied, application dose 8%, on wire fences adjoining control site.
01/31/00 and 02/01/00	*Helianthus annuus* that appeared as weed in soja site N° 2 was controlled (fig. 2).
02/01/00	Cutting and rolling up of alfalfa 1380 m northeast from control lot.

TABLE 5. *Activities to control weeds of lots adjoining treated test site "Lot Santa María"*

Date	Activity
01/19/00	Sides adjoining lots 2 and 3 (fig. 3) were weeded using a tractor propelled weeding machine (cut width 3.2 m) where the presence of *Datura ferox* was observed.
01/25/00	Curing and rolling up of alfalfa 730 m southeast from treated lot.
01/27/00	Glisofato was applied (with hand sprayer) on sides adjoining lots 6 and 8 to control the presence of *Datura ferox, Portulaca oleracea* and *Carduus acanthoides.*
01/28 and 29/00	Cutting and rolling up of alfalfa 770 m northeast from treated lot.
02/01/00	Lot 4 and half lot 5 (fig. 3) were weeded with weeding machine tractor propelled where the presence of *Carduus acanthoides* was observed.
02/02/00	Weeding of lot 5 was completed. Weeds on wire fences of lots 4 and 5 were controlled with machete.

III.7. *INSECTICIDE TREATMENTS OF TEST SITES AND ADJOINING LOTS*

Insecticide applications to test sites and adjoining lots (tables 6 and 7) were recorded.

TABLE 6. *Insecticide treatments of control site and adjoining lots*

Lot N°	**Crop/ vegetation**	**Date of application**	**Product** (active principle and concentration)	**Volume** (l/ha)	**Method**	**Time**
1	Sunflower. Control site.	01/27/00	Endosulfan 35% + 7 l of gas oil	1.5	Aerial	6:30 p.m.
2	Soja. 1st sowing	01/03/00	Chlorpyrifos 48% Cypermethrin 25%	0.6 0.1	Aerial	6 p.m.
4	Soja. 1st sowing	01/03/00	Chlorpyrifos 48% Cypermethrin 25%	0.6 0.1	Aerial	7 p.m.
3	Soja. 2nd sowing	02/05/00	Chlorpyrifos 48% Cypermethrin 25%	0.6 0.1	Aerial and soil on 100 m stripe (lot 3 and 5) or 120 m (lot 6) width limit with test site	5.50 a.m.
5	Soja. 2nd sowing					
6	Soja. 2nd sowing	02/09/00 *	LORSBAN PLUS (chlorpyrifos 50% and cypermethrin 5% + 4 l of water)	0.75		5:55 a.m.

* NOTE: *Rain during 10 min after application began.*

TABLE 7. *Insecticide treatments of treated site and adjoining lots*

Lot N° (fig. 3)	**Crop/ vegetation**	**Date of application**	**Product** (active principle and concentration)	**Volume** (l/ha)	**Method**	**Time**
1	Sunflower. Site treated with Gaucho	01/27/00	Endosulfan 35% + 7 l of gas oil	1.5	Aerial	7 p.m.
2, 3, 6, 8	Soja.2nd sowing	01/27/00	Chlorpyrifos 48% Cypermethrin 25%	0.6 0.1	Aerial	7 a.m.
4,5,7	Wild land	No insecticide treatments were applied.				

III.8. *ARRANGEMENT OF HIVES DURING PERIODS OF NON EXPOSURE TO SUNFLOWER*

Eight days before the first inspection at T0 (table 9) hives were installed by [redacted] in "El Gabi" (calle 600 y 154, Arana, partido de La Plata), the same observation post provided for periods of non exposure to sunflower (before T2 and after T3).

III.9. *ARRANGEMENT OF HIVES IN TEST SITES*

On 02/02/2000, with crop at 5% of flowering (48 hours before opening hives for inspection at T2) (photos 3 and 4), according to pollination practices for production of sunflower seed (Zorzín and Woodward, 1998; Cirnu, 1960; Cirnu and Sanduleac, 1965), hives were transferred to test sites. 8 hives were installed in each one of the test sites (photos 1 and 2). Hives were distributed regularly in the middle of the site according to SOP 4 (annex VII.5). The minimum distance between hives was 1.5 m. Hives were placed on fiber cement blocks at a minimum height of 20 cm from the ground. Considering the location of the opening of the hives, they were arranged to facilitate the orienting behaviour of bees (figure 5). The day before, on 02/01/00, cement blocks had been on the ground and 1 x 1 m fine half shadow screens in front of each hive had been set up to evaluate bees mortality (SOP 4, annex VII.5).

On 01/18/00, using a weeding machine, path towards the centre of the site and a 30 m x 15 m clearing at the centre of the site had been arranged for hives to be installed (SOP 4, annex VII.5).

Other 4 more hives for possible replacements were installed on 02/02/2000 in a rural property of San Gregorio, 20 km from test lots.

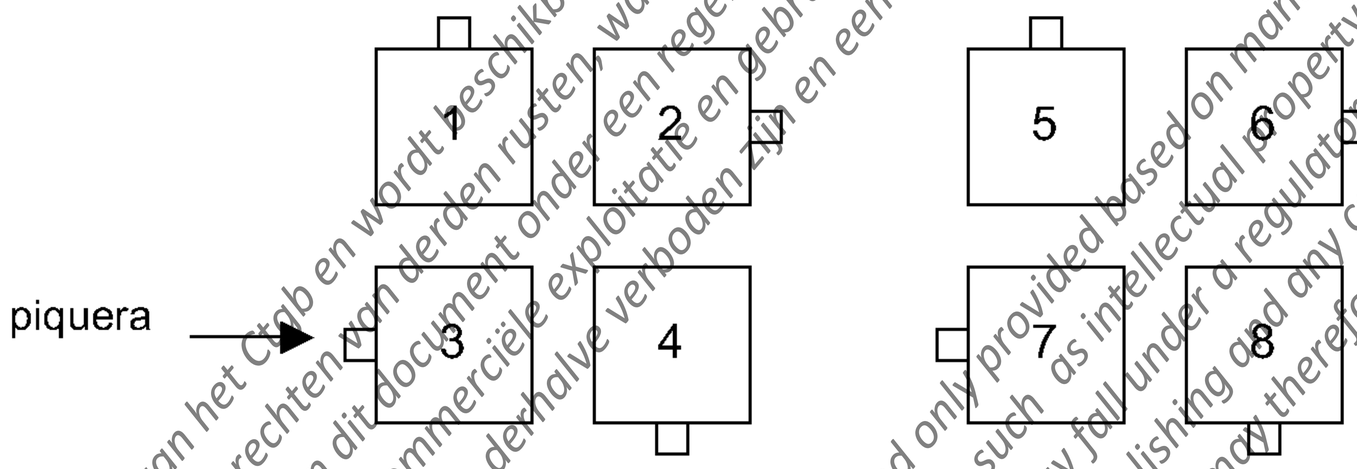


Figure 5. Location of hives in test site.

Photo 1. Hives of control site at the time of inspection T2 (2/3/00).

Photo 2. Hives of treated site at the time of inspection T2 (2/3/00).

III.10. *REMOVAL OF HIVES FROM TEST SITES*

Hives were removed from sunflower sites after inspection T3 (on 02/19/2000), when the production of sunflower pollen ended (photos 5 and 6). Hives transfer was accomplished according to "Guía de tránsito de SENASA". Hives were installed for further observation at "El Gabi" (calle 600 y 154, Arana, partido de La Plata) (photos 7 and 8).

Photos 3 and 4. Sunflower of control site at 5-6% flowering 24 hours after installation of hives (2/3/00).

Photo 5. Sunflower of treated site, 79% of plants without pollen. Time of inspection T3 (02/15/00).

Photo 6. Sunflower of treated site, 79% of plants without pollen. Time of inspection T3 (02/15/00).

Photo 7. Hives of control and treated site at “El Gabi” upon inspection T4 (3/15/00).

Photo 8. Hives of control and treated site at “El Gabi”, upon inspection T4 (3/15/00).

III.10.1. *Sanitarian treatments of hives*

Once removed from test sites sanitarian treatments of hives were recorded (table 8).

TABLE 8. *Sanitarian treatments of hives*

Date	Used product	Type of Treatment	Goal (control of)	Remarks
02/21/00	**COLMESAN LS** (Amitraz 1.25% p/v)	gasified	Varroa disease	
02/26/00				
03/02/00				
03/07/00				
03/12/00				
03/17/00				
04/13/00	**FUMIDIL – B (Bicyclohexylamonium fumagillin, 2.1% p/p)**	Liquid	Nosema disease	Sugar syrup (2 parts of sugar and 1 part of water), 2.25 liters each time.
04/20/00				
04/27/00				
05/04/00				
08/04/00	**COLMESAN LS**	Gasified	Varroa disease	
08/09/00	**FUMIDIL – B**	Liquid	Nosema disease	Sugar syrup (2 parts of sugar and 1 part of water), 2.25 liters of water each time.
08/11/00	**COLMESAN LS**	Gasified	Varroa disease	
08/16/00	**FUMIDIL – B**	Liquid	Nosema disease	Sugar syrup (2 parts of sugar and 1 part of water), 2.25 liters of water each time.
08/18/00	**COLMESAN LS**	Gasified	Varroa disease	
08/23/00	**FUMIDIL – B**	Liquid	Nosema disease	Sugar syrup (2 parts of sugar and 1 part of water), 2.25 liters of water each time.
08/25/00	**COLMESAN LS**	Gasified	Varroa disease	
08/30/00	**FUMIDIL – B**	Liquid	Nosema disease	Sugar syrup (2 parts of sugar and 1 part of water), 2.25 liters of water each time.
09/01/00	**COLMESAN LS**	Gasified	Varroa disease	-
09/08/00	**COLMESAN LS**	Gasified	Varroa disease	-

III.11. *INSPECTION OF HIVES*

III.11.1. *Time of inspection*

Hives were weighed and/or inspected at times described in table 9 as T0, T2, T3, T3', T4 and T4'.

TABLE 9. *Times of inspections of hives and location of hives*

Time	Location of Hives	Date of inspection	Activities
T0	"El Gabi" (La Plata) *(see II.8)*	01/20/00	Opening and general inspection of hives and bee sampling for diagnosis of nosema disease Identification of bottom super and frames of hives with seal of SENASA. Sampling of sealed wax sheets to be used in hives' top super to analyze residues in general.
T1		Cancelled	Deviation **2** (annex VII.3)
TRANSFER		02/02/00	Transfer of hives from La Plata to La Catalina and installation of hives in test sites
T2	**Sunflower** test sites (Estancia La Catalina) *(see II.5.1.)*	02/03/00	Weighing of hives
T3		02/04/00	Opening of hives and evaluation of percentage of frames in bottom super covering. Top super was not evaluated due to its recent installation
		02/14/00 and 02/15/00	Weighing of hives. Opening of hives and evaluation of percentage of frames of bottom and top super. Sampling of honey, pollen and wax for Imidacloprid residues and palinologic tests.
TRANSFER		02/19/00	Removal of hives from test site for their transfer to La Plata.
T3'	"El Gabi" (La Plata) *(see II.8)*	02/22/00	Weighing of hives.
T4		03/14/00 and 03/15/00	Weighing of hives. Opening of hives and evaluation of percentage of frames inbottom and top super. Sampling of honey, pollen and wax for Imidacloprid residues and palinologic tests
T4'		03/21/00	Weighing of hives.
T5		Cancelled	Deviation **3** (annex VII.3).

III.11.2 *Population parameters observed*

A sanitarian inspection of hives was accomplished at T0, bee samples were taken for diagnosis of nosema disease, and samples of sealed wax sheets to be used in the top super of hives were taken for further general residue tests. Also, hives, top super and frames were identified according to SOP 1 (annex VII.5).

At T2, T3 and T4 the following items were observed per hive:

- **Weight of hive**

The device used was a digital scale like an electronic dynamometer (1) ORTELLI of 50 g sensitivity and a 50 g capacity at 150 kg, with connection to battery. The dimensions of indicator head (2) are 230 x 90 x 65 mm. Scissors (3) and a support structure (4) were used to lift hives (photo 9).

- **Frame cover**

  - Estimated number of bees per hive. To quantify this the degree of bee covering on frames was used (percentage estimation). Digital photography of the top of the hive, once open, was also used as hive strength index (photographic record, annex VII.7).

  - Estimated proportion of cells occupied by honey, nectar and pollen, and cells occupied by worker brood (sealed and not sealed), sealed drone brood, empty cells and non constructed area.

Hive opening, frame evaluation and sample taking at T4 were implemented under a tent (photo 10) designed to protect the hive from the effect of pillaging by other bees. The tent consisted of a metal structure 2 m x 2 m and 2.2 m high, and a half-shadow screen cover.

At T3' and T4' hives were weighed to rule out possible effects on weight due to factors non related to the test that were caused by the opening, sampling and transfer of hives after T3.

Every hive inspection was audited by especially appointed staff by SENASA.

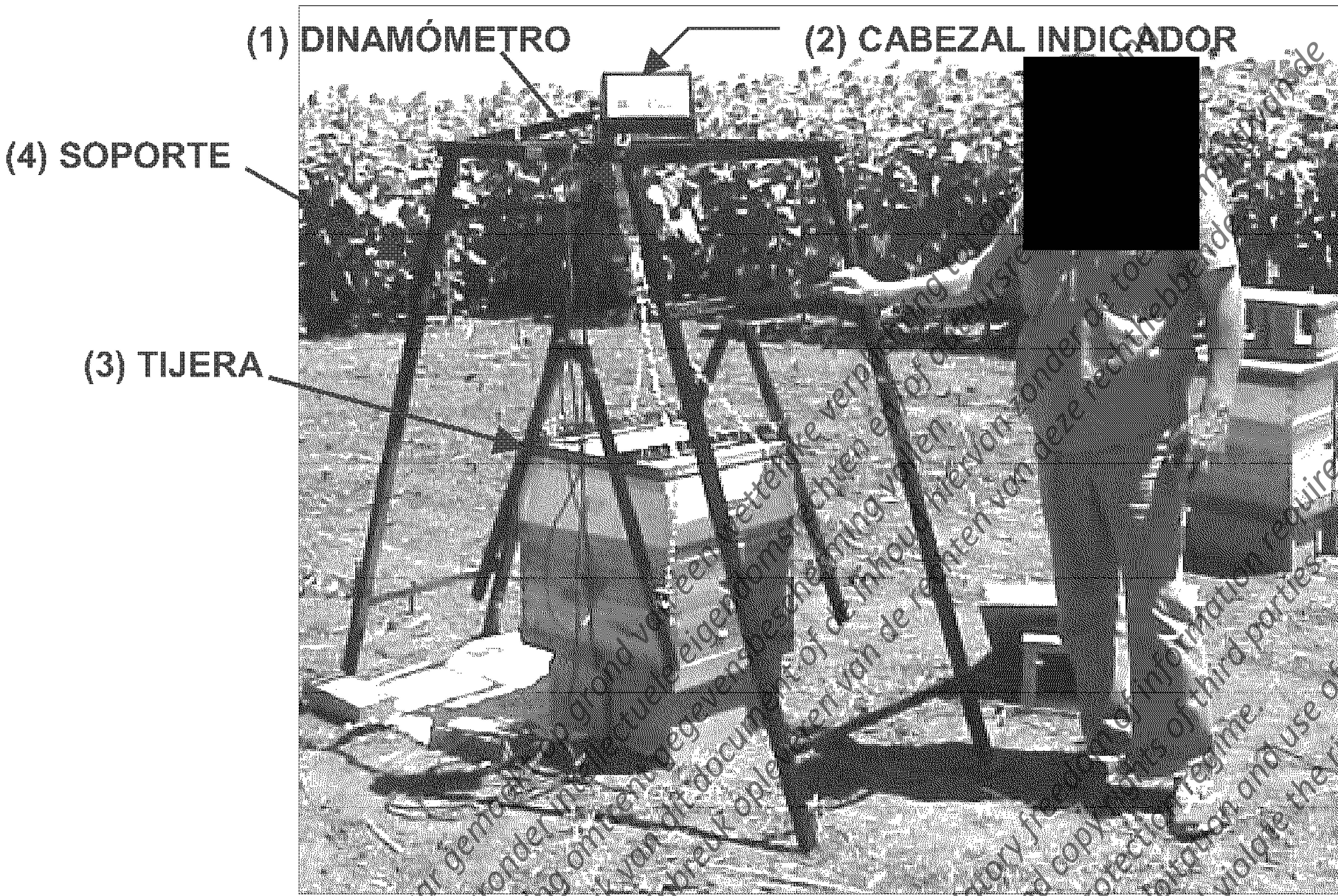


Photo 9. Scale, hive lifting scissors and support structure to weigh hives.

Photo 10. Tent used for hive opening, inspection and sampling at T4 (3/15/00).

III.12. *BEE ACTIVITY AND MORTALITY IN TEST SITES*

III.12.1. *Observation period*

As from T2 (02/05/00) until removal of bees from test sites after T3 (02/15/00) bee field activity in sunflower, pollen incoming in hives and bee mortality measured in front of hives were observed virtually on a daily basis in test sites.

III.12.2. *Parameters observed*

Field activity

Four observation spots were established in each test site. The number of bees on 100 sunflower heads was counted per head in southwest walking direction in accordance with SOP 6 (Annex VII.5) (figure 6). Observations were made during the time slot of bee activity (9 to 12 a.m. and 2 to 5 p.m.)

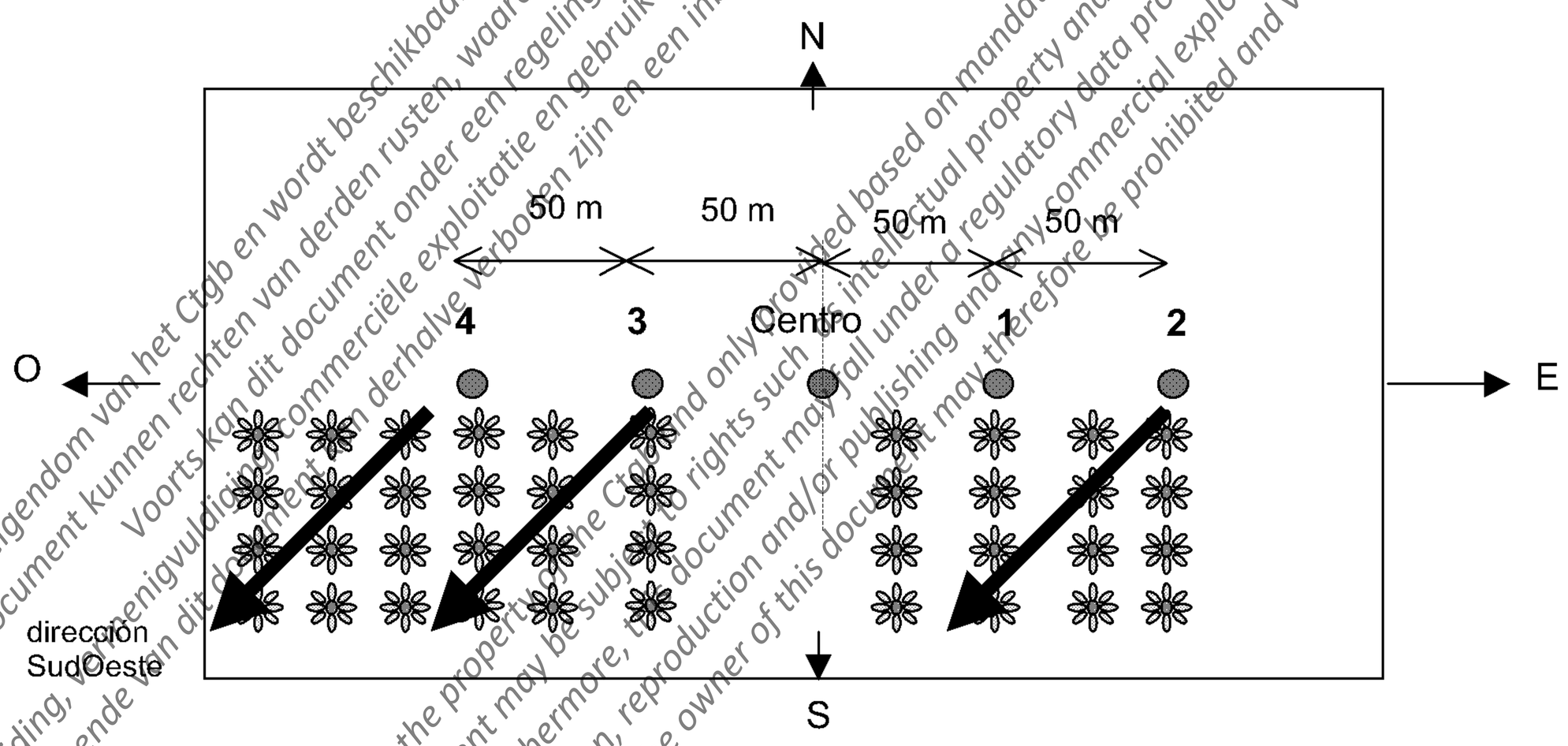


Figure 6. Observation spots established to count the number of bees per sunflower head.

Incoming pollen

For each hive, the number of bees entering the hive with pollen per minute was recorded during a 3 minutes observation interval according with SOP 5 (annex VII.5).

Mortality

Mortality was evaluated by determining the number of dead bees on a fine 1 m x 1 m screen placed on the ground in front of each hive according to SOP 7 (annex VII.5).

III.13. *POLLEN, HONEY, WAX AND SUNFLOWER HEADS SAMPLING FOR ANALYTICAL TESTS*

III.13.1. *Time of sampling*

Specification of the time of hive inspection when samples were taken.

- Taking of **pollen** samples to test:
  - palinologic: at the time of T3.
  - Imidacloprid residues: at the time of T3 and T4.

- Taking of **honey** samples to test:
  - palinologic: at the time of T3 and T4.
  - Imidacloprid residues: at the time of T3 and T4.

- Taking of **wax** samples to test:
  - residues in general: at the time of T0.
  - Imidacloprid residues: at the time of T3 and T4.

- Taking of **sunflower heads** samples to test for Imidacloprid residues: at the time of T2.

- Taking of soil samples to test for Imidacloprid residues: at the time of T2.

III.13.2. *Method to take samples*

The procedure used to take samples of pollen, honey and wax was SOP 3 (amendment to protocol 2, annexes VII.2, VII.4 and VII.5), which invalidated sample splitting as provided in the original protocol (samples were taken separately for each one of the recipients).

Pollen

Pollen samples were taken with an ad hoc extractor (photos 11 and 12). 3 pollen samples (at the time of T3) or 4 (at the time of T4) were taken from every hive with the following destinations:

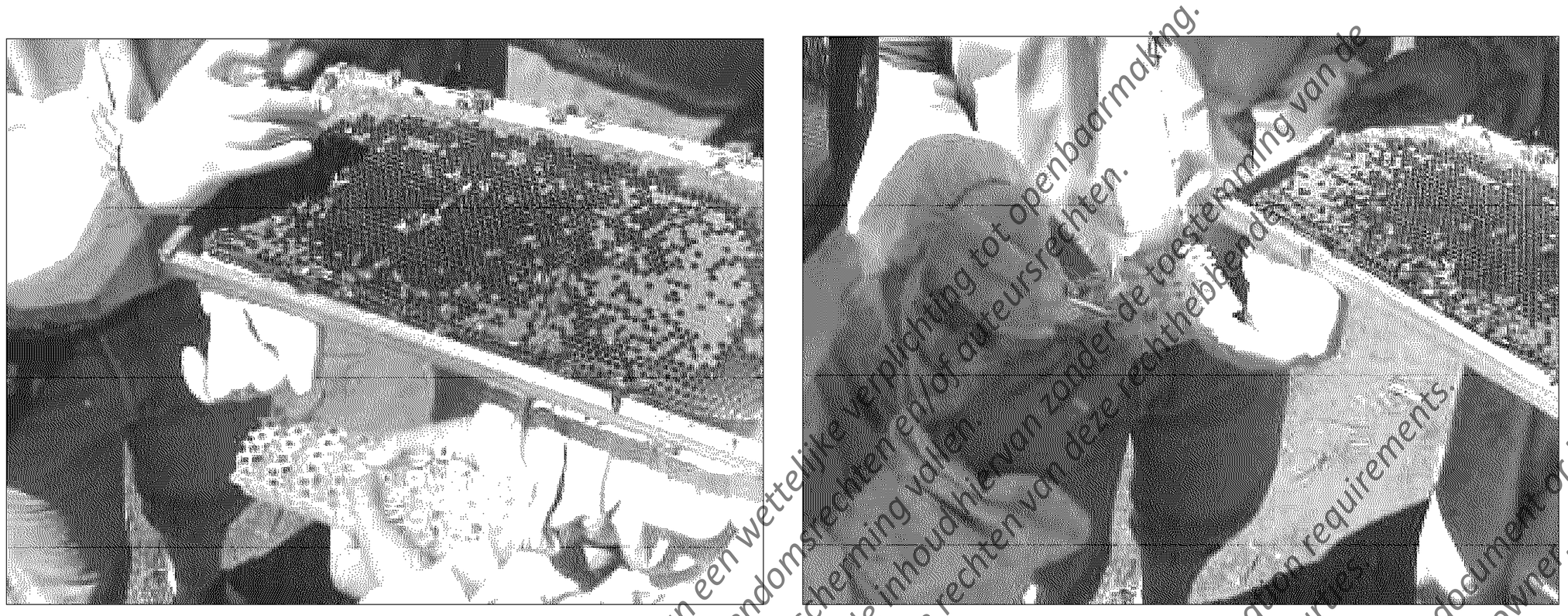
At T3:

- SENASA
- Bayer Argentina S.A.
- (Imidacloprid) residue tests
- Palinologic tests.

At T4:

- SENASA
- Bayer Argentina S.A.
- (Imidacloprid) residue tests

Every recipient received a pollen sample per hive and 8 samples per test site.



Photos 11 and 12. Pollen samples taken from a hive of the treated site. Time of inspection is T3 (2/15/00).

Honey

Honey samples were taken with disposable plastic spoons on 20 ml glass vials (photos 13 and 14). Four honey samples were taken from each hive with the following destinations:

- SENASA
- Bayer Argentina S.A.
- Imidacloprid residue tests
- Palinologic test.

Every recipient received a honey sample per hive and 8 samples per test site.

Photos 13 and 14. Honey samples taken from a hive of the treated site. Time of inspection is T3 (2/15/00).

Wax

At the time of **T0** samples of sealed wax sheets to be used in hive top supers were taken to be tested for residues in general. The procedure used was an adaptation of SOP 3 (annex VII.4). Groups of 4 to 6 wax sheets were sampled. A group of sheets were perforated by using a pollen sample extractor. 3 Eppendorf tubes were available for every group of sheets, each one with the following destination:

- SENASA
- Bayer Argentina S.A.,
- General residue tests.

The amount of wax of two perforations in a group of wax sheets was deposited into each tube. At the end of the sampling, every recipient received 36 Eppendorf tubes containing wax samples.

At the time of **T3** and **T4,** wax samples were taken in 20 ml glass vials, hands being covered with new latex gloves for collection. Three wax samples were taken from each hive, each one with the following destination:

- SENASA,
- Bayer Argentina S.A.
- Imidacloprid residue test.

Every recipient received a wax sample per hive and 8 samples per test site.

Sunflower and soil

At the time of T2 samples of soil and sunflower heads were taken at 8 spots per site to be sent and tested for Imidacloprid residues. The procedure used to take samples was SOP 8 (annex VII.5).

At every sampling spot (figure 7) two soil samples and two sunflower head samples were taken, each one for the following recipients:

- SENASA
- Bayer Argentina S.A.

The soil sample was taken on the rut at a superficial level up to a 5cm depth and 5 cm maximum distance from sunflower stem. Zip poly-ethylene bags were used to collect soil samples. To collect sunflower heads samples waste bags were used, depositing a sunflower head (one sample) per bag.

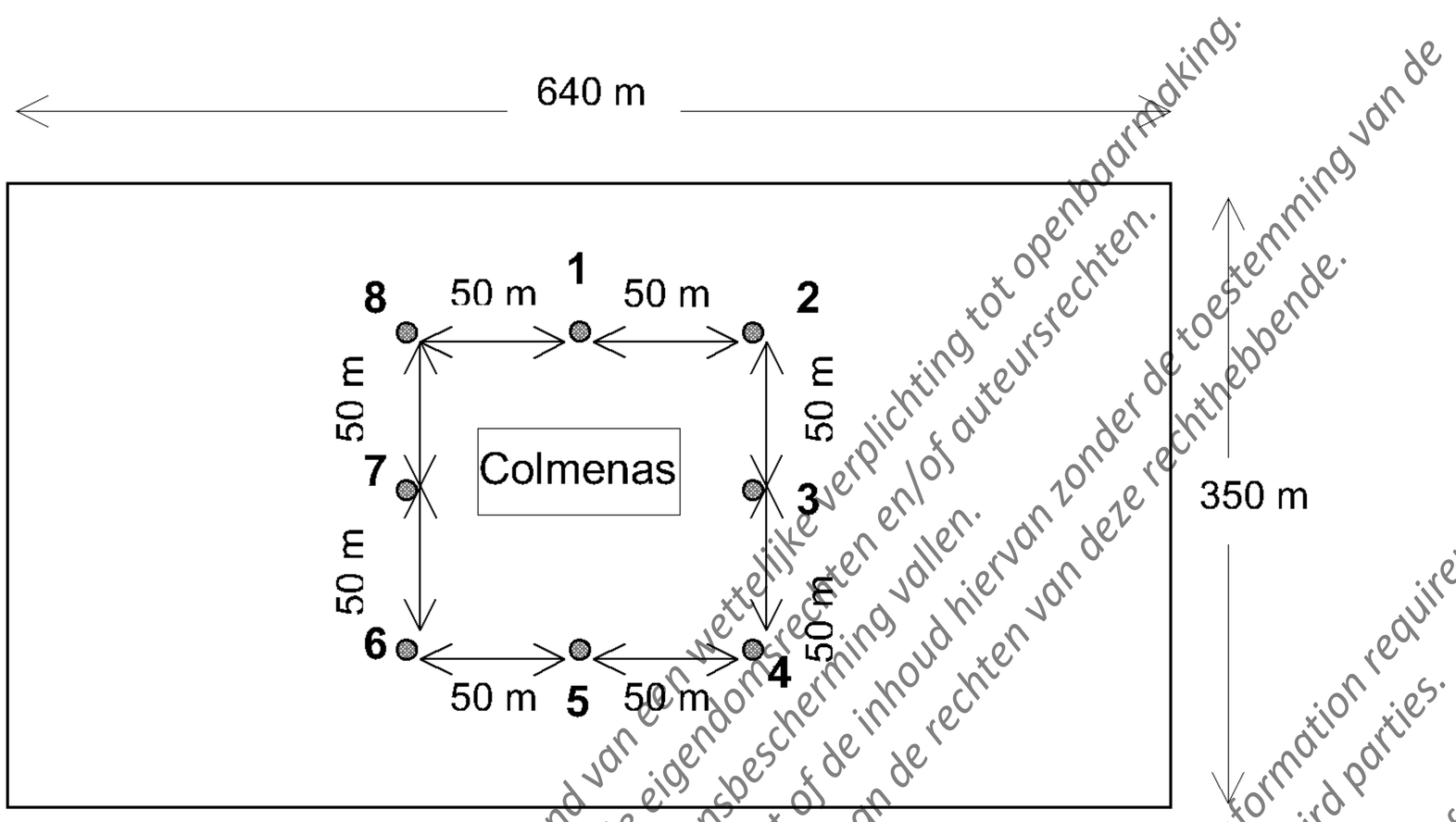


Figure 7. Distribution of eight spots for soil and sunflower heads sampling in control and treated site.

Once taken, every sample was properly identified and cold preserved (+ 2 - + 5°C). Samples were taken in the presence of auditing staff appointed by SENASA.

III.14. *WEATHER INFORMATION*

While hives were on the sunflower sites (T2 and T3) and at the time of observations, the following weather data were recorded:

- temperature (maximum and minimum temperature of the day and temperature upon observations)
- relative humidity
- nebulosity (eighth of sky covered)
- precipitation
- atmospheric pressure
- wind range and direction.

Temperature and relative humidity data were measured with an "AMAREL" digital hygrothermometer. Atmospheric pressure was measured with a barometer and precipitation with a "AGUACERO" millimeter calibration plastic pluviometer. Maximum and minimum temperature data were provided by "La Catalina" weather station. Nebulosity, wind range and direction were estimated through visual appreciation.

III.15. *FLOWERING VEGETATION IN TEST AND ADJOINING SITES*

While hives remained in sunflower sites flowering weeds were observed in test sites and flowering vegetation in the adjoining lots.

III.16. *STATISTICAL ANALYSIS OF RESULTS*

The statistical analysis of results was performed by the Biometry Chair of the FCE y N – UBA Department of Biological Sciences. The related report is attached to annex VII.8 ("STATISTICAL ANALYSIS OF RESULTS").

At every time of inspection, T2, T3 and T4, and for each of the observed parameters, mean values obtained from hives that were exposed to the treated site were as compared with those of control hives, thus determining significant differences. The parameters taken into account were the following:

- Weight of hives.
- Percentage of cells occupied by honey and occupied by nectar.
- Percentage of cells occupied by brood (sealed, not sealed and drones)
- Percentage of empty cells.
- Percentage of area not constructed.
- Field activity.
- Incoming pollen in hive.
- Bee mortality in front of hive. For the statistical analysis, just the total number of worker bees that were found dead in front of the hives was considered, since the number of dead drones and pupae was not relevant enough to be included in the analysis.

III.17. *CHEMICAL ANALYSIS IN SUNFLOWER SEEDS TREATED WITH GAUCHO AND OBTAINED BEFORE SOWING*

Studies were made at INQUIMAE – FCEyN – UBA. The related report is attached to annex VII.9 ("IMIDACLOPRID TEST IN SUNFLOWER SEEDS TREATED WITH GAUCHO AND OBTAINED BEFORE SOWING").

Samples of seeds treated and non-treated with Gaucho were tested. Imidacloprid content was determined in (treated and non-treated) seeds and also its distribution in thirty treated seeds. The test protocol followed was *Bayer Analytical Method 2001-0026606-95E.*

III.18. *RESIDUE TESTS OF POLLEN, HONEY, WAX, SUNFLOWER HEADS AND SOIL SAMPLES*

Studies were developed by Coll. Grad. [redacted] LIBIQUIMA (School of Engineering, Comahue National University) at Bayer laboratories, Centre of Agricultural Research, Monheim (Germany). The related report is attached to annex VII.10 ("IMIDACLOPRID RESIDUE TESTS IN POLLEN, HONEY, WAX, SUNFLOWER HEADS AND SOIL SAMPLES").

For every matrix (pollen, honey, wax, heads and soil) at the various times of sampling (T2, T3, and T4), all the 8 samples per site were homogenized thus producing a composite sample. Tests were done in duplicate on the composite sample

III.18.1. *Soil*

The method used was“ High Performance Liquid Chromatography (HPLC) method for the determination of insecticide Imidacloprid residues in soil” [redacted] method number 00267).

III.18.2. *Honey, wax, pollen and sunflower heads*

The method used was: “Analytical residual method for the determination of residues of insecticide Imidacloprid and its Hydroxylic- and Olefin- metabolites in nectar, honey, flowers and pollen of sunflower and bees through High Performance Liquid Chromatography (HPLC) with Masa-Masa electrospray detection” ([redacted], study number P61284703).

III.19. *PALINOLOGIC TEST OF POLLEN AND HONEY SAMPLES*

Tests were done by [redacted] CICyTTP – CONICET y MACN – CONICET. The related report is attached to annex VII.11 (“PALINOLOGIC TEST OF POLLEN AND HONEY SAMPLES”).

III.19.1. *Pollen*

Aliquots were taken from pollen samples. Pollen aliquots were broken up with 10% (KOH 10%) potassium hydroxide and then homogenized. The related microscopic preparations and counts were made of a 1000 g per preparation minimum.

III.19.2. *Honey*

Microscopic preparations were obtained and counts were made on a 1000 pollen grains minimum or, in pollen barren samples, on the total amount present in the preparation.

IV. RESULTS AND DISCUSSION

IV.1. *PLANT DENSITY AND FENOLOGICAL CONDITION OF SUNFLOWER*

At the time of T2, the relative plant density in the site treated with Gaucho was higher than that of the control site. Initial plant density at the time of emergence in the control site was 41.500 plants/ ha as compared with 50.300 plants/ ha for the treated site. These differences may be ascribed to the initial crop health management with the product under study.

Upon transfer of hives to test sites (2/2/00) sunflower was at 5% of flowering in both sites. In sunflower pollination experiments for the production of seed, hives are introduced into the crop when 5% of plants are flowering (Cirnu, 1960; Cirnu and Sanduleac, 1965; Zorzin and Woodward, 1998).

As from 2/2/00, the number of flowering plants was the same for both sites. However, from 2/9/00 to the end of flowering, a higher proportion of plants without pollen was observed in the control site in comparison with the treated site. In both sites, the end of flowering was virtually simultaneous (80% of plants without pollen, *see III.5.5.*).

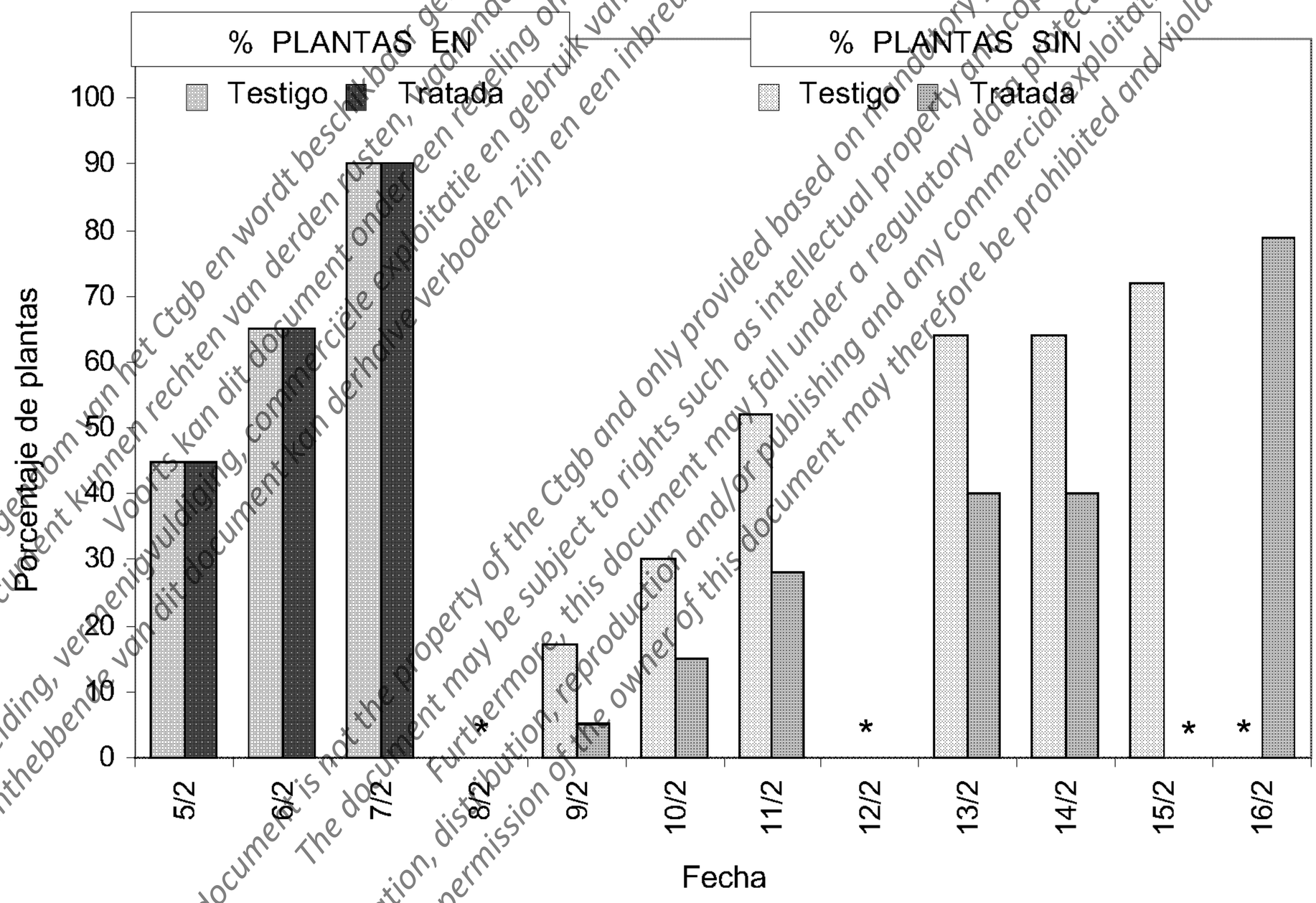


Chart 1. Percentage of flowering sunflower plants and of plants without pollen while hives were placed in test sites. * *Non available datum. Original data in annex VII.6.2.1.*

IV.2. *FLOWERING VEGETATION IN TEST AND ADJOINING SITES*

IV. 2.1. *First observation*

CONTROL SITE (02/07/00) (figure 8)

Observation post 1: Flowering sunflower crop. Some flowering plants of *Amaranthus quitensis* and *Chenopodium album* (quinoa) were observed in very low density. No presence of bees was observed in them.

Observation post 2: First sowing soja crop in phenologic condition R5 (grain growth). The presence of fructifying *Sorghum halepense* (sorgo de alepo) was observed. On the border of test site, some flowering plants of *Brassica ampestris* were observed, as well as some of fructifying *Sonchus oleraceus* and *Digitaria sanguinales*. No presence of bees was observed in them.

Observation post 3: Second sowing soja crop at the beginning of flowering (phenologic condiction R2). A 50 m^2 area, approximately, of *Chenopodium album* (quinoa) and thistle in blossom were observed. No presence of bees was observed.

Observation post 4: First sowing soja crop. Six thistle plants in blossom were observed. No presence of bees was observed.

Observation post 5: Second sowing soja crop at the beginning of flowering. Bitter pumpkin, turnip and *Sorghum halepense* were observed on a 20 m^2 area, approximately. Some thorn apples were also observed. No presence of bees was observed in either case.

Observation post 6: Second sowing soja crop in fenologic condition R5 (grain growth). Some flowering thistles and *Chenopodium album* (quinoa) were observed. No presence of bees was observed.

TREATED SITE (02/05/00) (figure 9)

Observation post 1: Flowering sunflower crop. Some flowering plants of *Amaranthus quitensis* and *Chenopodium album* (quinoa) were observed in very low density. No presence of bees was observed in them.

Observation post 2: *Trifolium repens* (white clover) in blossom, two *Anthemis cotula* (chamomile) in blossom on a 30 m^2 area, approximately, were observed. No presence of bees was observed.

Observation post 3 and 6: Second sowing soja crop. No presence of weeds was observed.

Observation post 4: Dry natural grassland consisting of "agropiron", "festuca" and "gramón". Some plants of thistle in blossom, some plants of flowering bishop's weed and a very few flowering *Lotus corniculatus* were observed. No presence of bees in any of these weeds was observed. This lot was weeded at the day, when hives were installed in test sites to eliminate thistle.

Observation post 5: Dry natural grassland consisting of "agropiron", "festuca" and "gramón". This lot was weeded at the day, when hives were installed in test sites to eliminate thistle.

Observation post 7: White clover in blossom was observed at the sides of the road. Dry natural grassland with the presence of *Agropiron elongatum* already fructified.

Observation post 8: Second sowing soja crop. Some very sparse plants of *Datura ferox* (thorn apple) were observed without the presence of bees.

IV.2.2. ***Second observation*** (02/16/00)

At the moment of observation of February 16, second sowing soja crops adjoining treated test site–lots 3, 6 and 8 were in fenologic condition R2 (in blossom).

As for adventitious flowering vegetation in both test sites and adjoining lots, no differences from the first date were noticed.

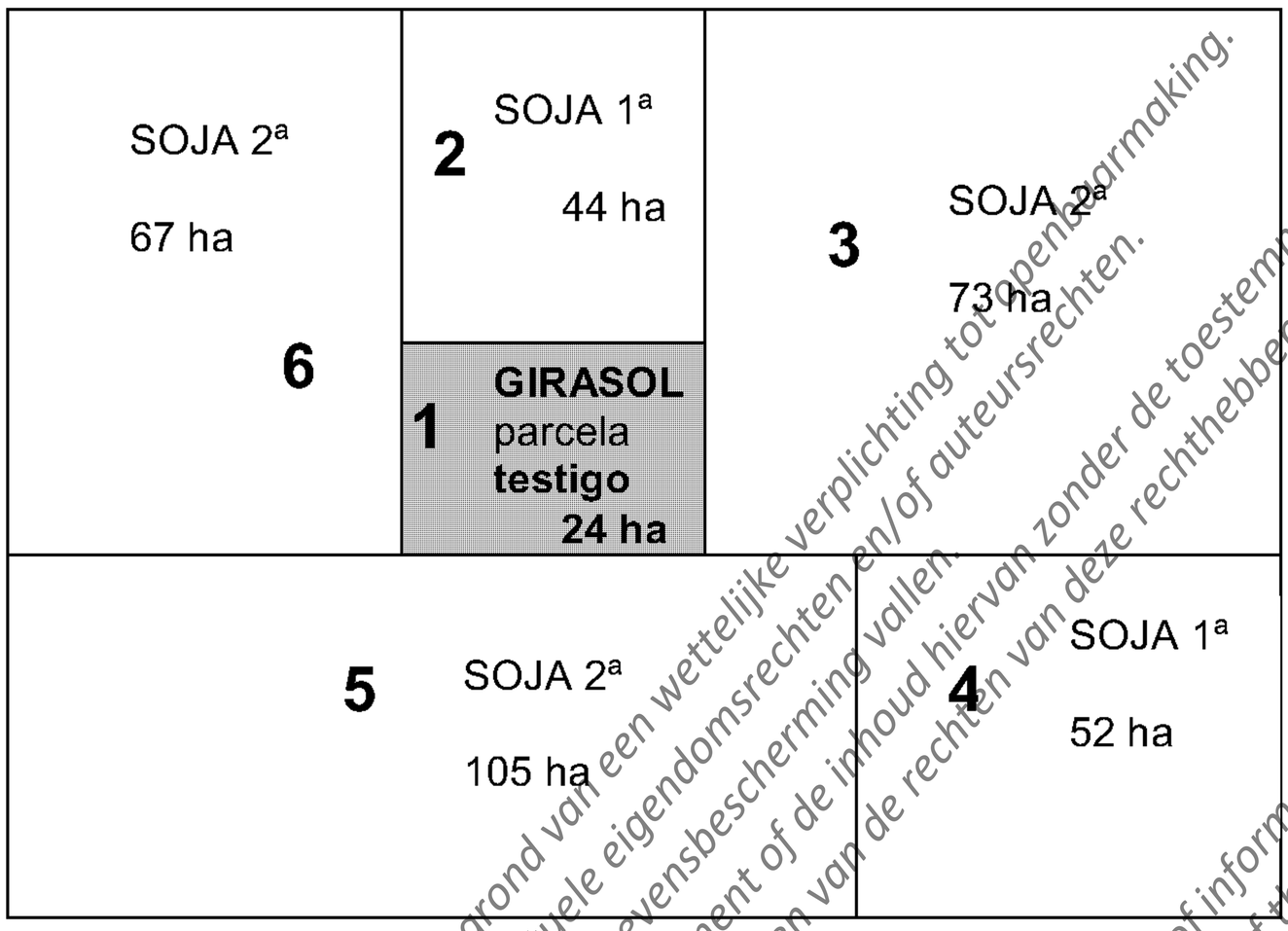


Figure 8. Diagram (not scale drawing) of observation posts of vegetation in lots adjoining control site.

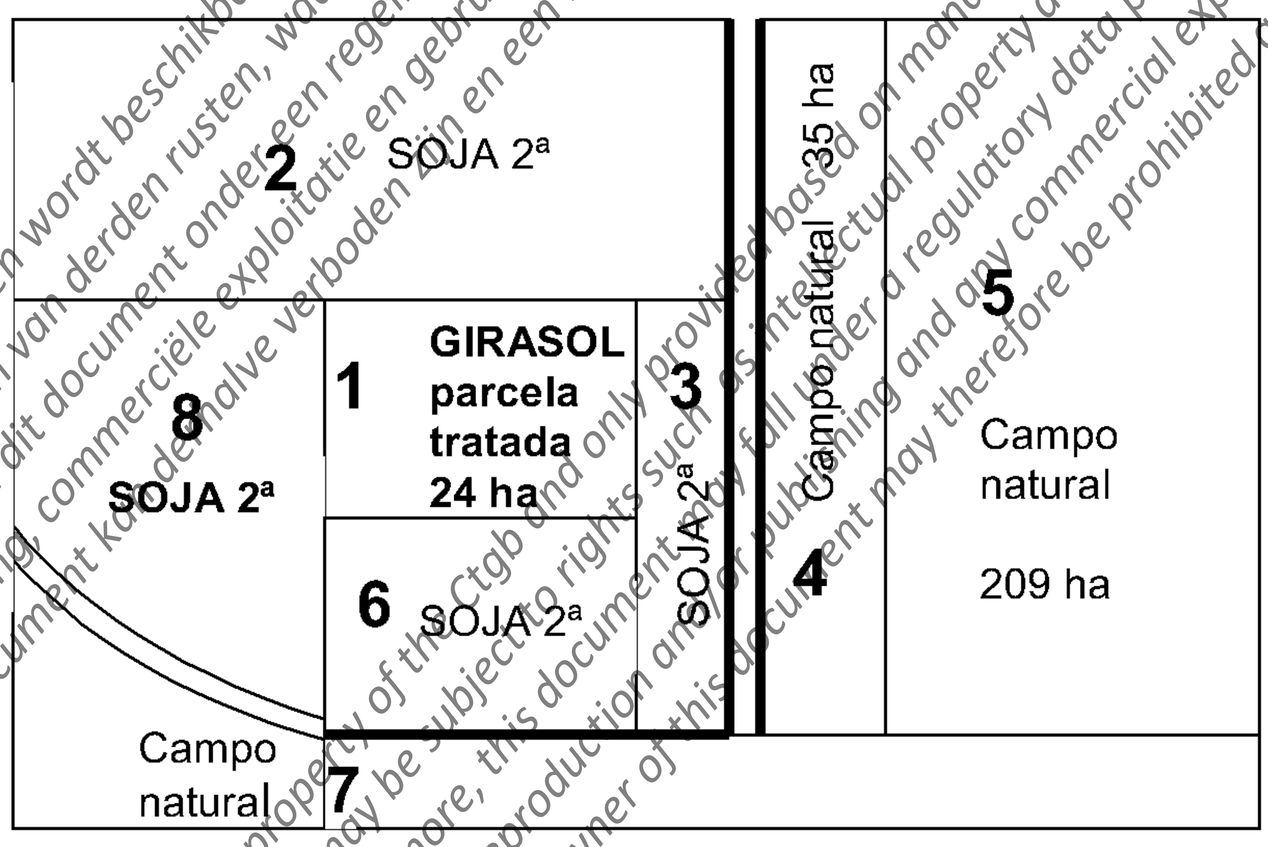


Figure 9. Diagram (not scale drawing) of observation posts of vegetation in lots adjoining treated site.

Influence of wild vegetation and other crops in blossom present in control site and adjoining lots was nil or virtually non-existent, because it was controlled between T2 and T3 (*see III.6, tables 4 and 5*). Due to field conditions related to the test, it is not excluded, however, that bees might have flown to adjoining flora beyond the observation context established for this test (plants, bushes or trees) in search of pollen, nectar, etc.

IV.3. *POPULATION PARAMETERS*

IV.3.1. *Weight of hives*

Upon transfer of hives to both test sites (**T2**) (III.9 and table 9) no significant differences were found between the average weight of hives in treated site and control site hives (*P = 0.9670,* Annex VII.8: tables 1 and 2) (chart 2).

By the end of the period of exposure to sunflower (**T3**) an increase of weight statistically significant with regard to T2 was observed in hives of the control site (2.55 kg, *P = 0.0284*) as well as in hives of the treated site (5.98 Kg, *P = 0.0000*) (Annex VII.8; tables 1 and 2). Average weight of hives in the treated site was higher than that of control hives, this difference being statistically significant (difference = 3.46 kg; *P = 0.0008*; Annex VII.8; tables 1 and 2).

When hives were removed from sunflower sites (**T3'**) a weight reduction was verified due to factors external to the test, such as opening and sampling (T3), and transfer. This average weight reduction was higher for hives of the treated site (2.32 kg, *P = 0.0156*). However, the average weight of hives in the site that was treated with Gaucho was significantly higher than that of control hives (difference 2.06 kg, *P = 0.0000;* Annex VII.8; tables 3 and 4).

Both at T3 and T3', the difference observed for the average weight of hives was statistically significant in both cases and favourable to those from the treated site (Annex VII.8; tables 1, 2, 3 and 4).

Between T3' and T4, an increase of the average weight of hives was observed that was not significant (*P = 0.1099*). On the other hand, a 1.93 kg difference of the average weight favourable to hives from the treated site was observed at T4', but it was not statistically significant (P = 0.0749) (Annex VII.8: tables 5 and 6).

A significant reduction of the average weight of hives (*P = 0.0203)* was observed at **T4'** due to opening and sampling at T4. Moreover, the average weight difference favourable to hives that were exposed to sunflower treated with Gaucho persisted, but it was not statistically significant (*P = 0.1362)* (Annex VII.8; tables 7 and 8).

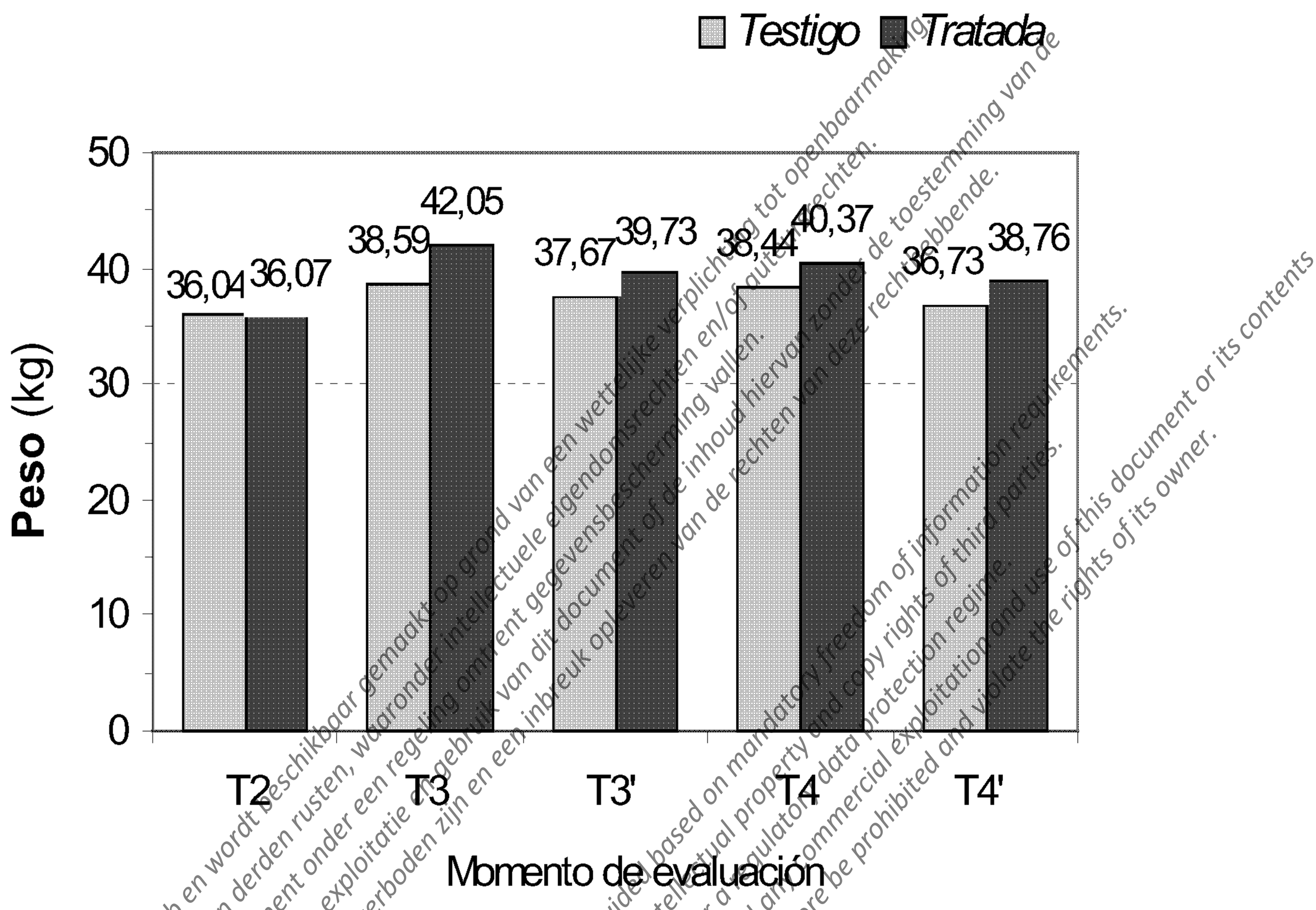


Chart 2. Average weight of hives per test site at the different moments (T2, T3, T3', T4 and T4'). *Original data in annex VII.6.1.1.*

IV.3.2 FRAME *COVERING*

T2

When exposure to sunflower started (T2), the structure and population of hives on control and treated site were similar, no significant differences being detected between the averages of the parameters: percentage of cells occupied by honey and nectar (*P = 0.1044),* pollen *(P = 0.9542)*, brood (sealed, *P = 0.0803,* and non sealed, *P = 0.1140)* and empty cells (*P = 0.1428)* (chart 3) (Annex VII.8: tables: 9, 10, 11, 12, 13, 14, 15 and 16).

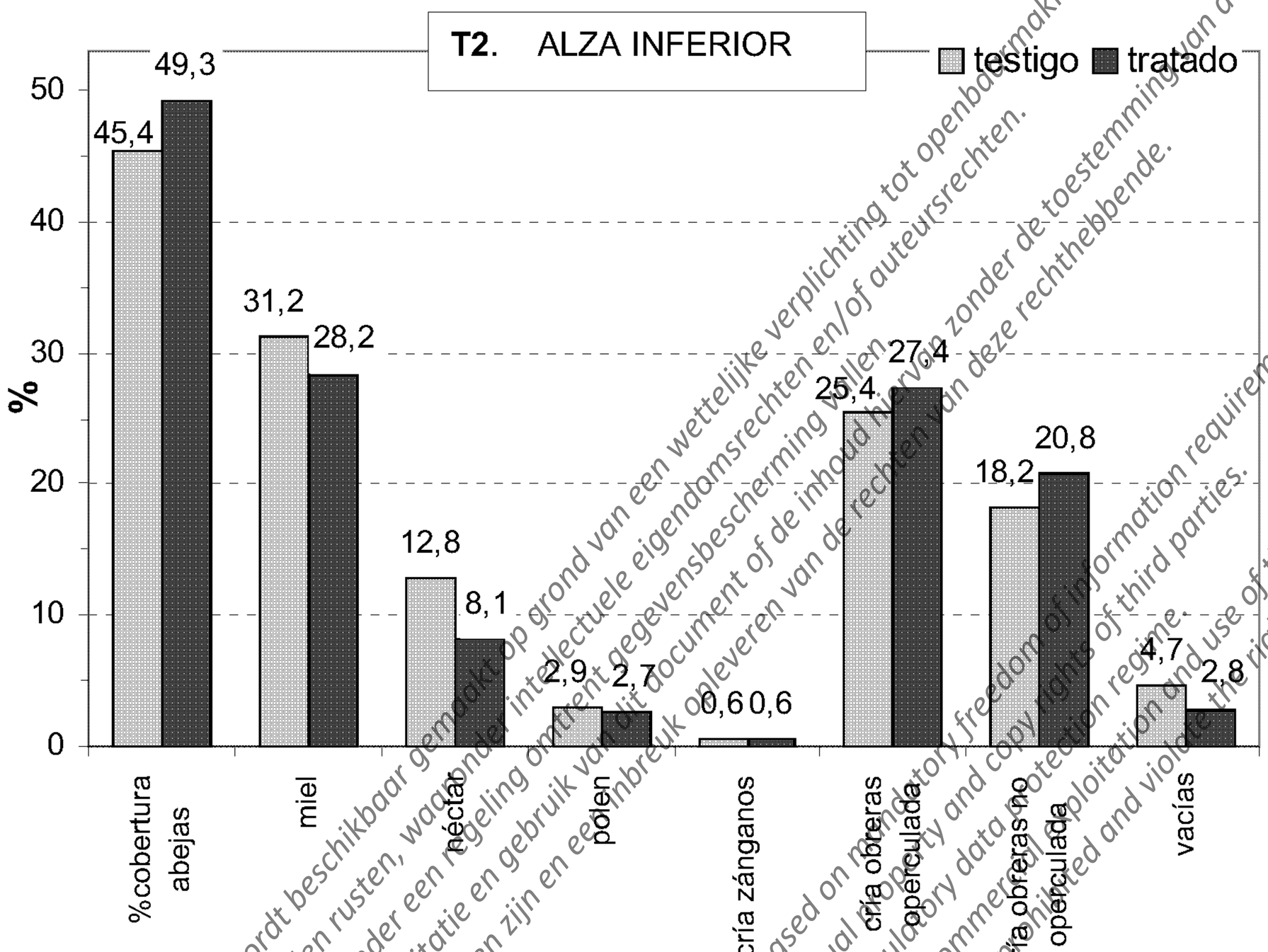


Chart 3. Percentage of frame area occupied by bees and percentage of cells occupied by honey, nectar, pollen, brood and empty cells at **T2** (mean values per hive and per site). *Original data in annex VII.6.1.2.*

T3

At the end of the period of exposure to sunflower, at T3, it was observed in hives on the treated site that virtually the whole area of top super was constructed, as compared with hives on the control site where most of the frame area of top super was sealed wax (*P = 0.0002)* (Annex VII.8; tables 25 and 26) (chart 5). Later, at T4, there was a reduction of the high average percentage of the non-constructed frame area in top supers of control site hives (chart 7).

A higher honey and nectar storage was observed on the top super of the treated site hives (T3) (*P = 0.0000)* (Annex VII: tables 17 and 18). It is worth noting that total honey reserves in hives of both sites (T3) were similar, since from T2 on control site bees kept storing in the bottom super, while treated site bees started storing in the top super (charts 4 and 5).

In frames of bottom super in the treated site (T3), a higher proportion of cells occupied by pollen *(P = 0.0228)* and brood (sealed, *P = 0.0040,* and non-sealed, *P = 0.0019*) was observed, in comparison with hives in control site (chart 4) (Annex VII.8: tables 19, 20, 21 and 22).

No significant differences were found in the average percentage of empty cells between hives in treated and control sites (T3) (*P = 0.3255)* (Annex VII.8: tables 23 and 24).

T4

24 days after hives were removed from sunflower fields at T4 the area proportion of top super frames occupied by pollen was still higher in hives coming from the treated site (*P = 0.0096,* Annex VII.8: tables 29 and 30). However, differences in the amount of brood between hives in control and treated sites were not statistically significant at T4 (sealed, *P = 0.0560*, non-sealed, *P = 0.2500,* Annex VII.8: tables 31 and 32) (chart 6).

In bottom supers, a higher storing of honey and nectar was perceived in hives of the control site but the difference was not statistically significant (*P = 0.0568,* Annex VII.8: tables 27 and 28). In top supers, on the other hand, the proportion of frame area covered with honey and nectar was significantly higher for hives of the treated site (*P = 0.0003,* Annex VII.8: tables 27 and 28) (chart 7). Moreover, total reserves of honey and nectar (both supers considered) in hives exposed to treated sunflower exceeded total reserves of control hives (*P = 0.0134,* Annex VII.8: table 27). The percentage reduction of cells occupied by nectar that was observed at T4 is probably due to its transformation into honey (sealed cells).

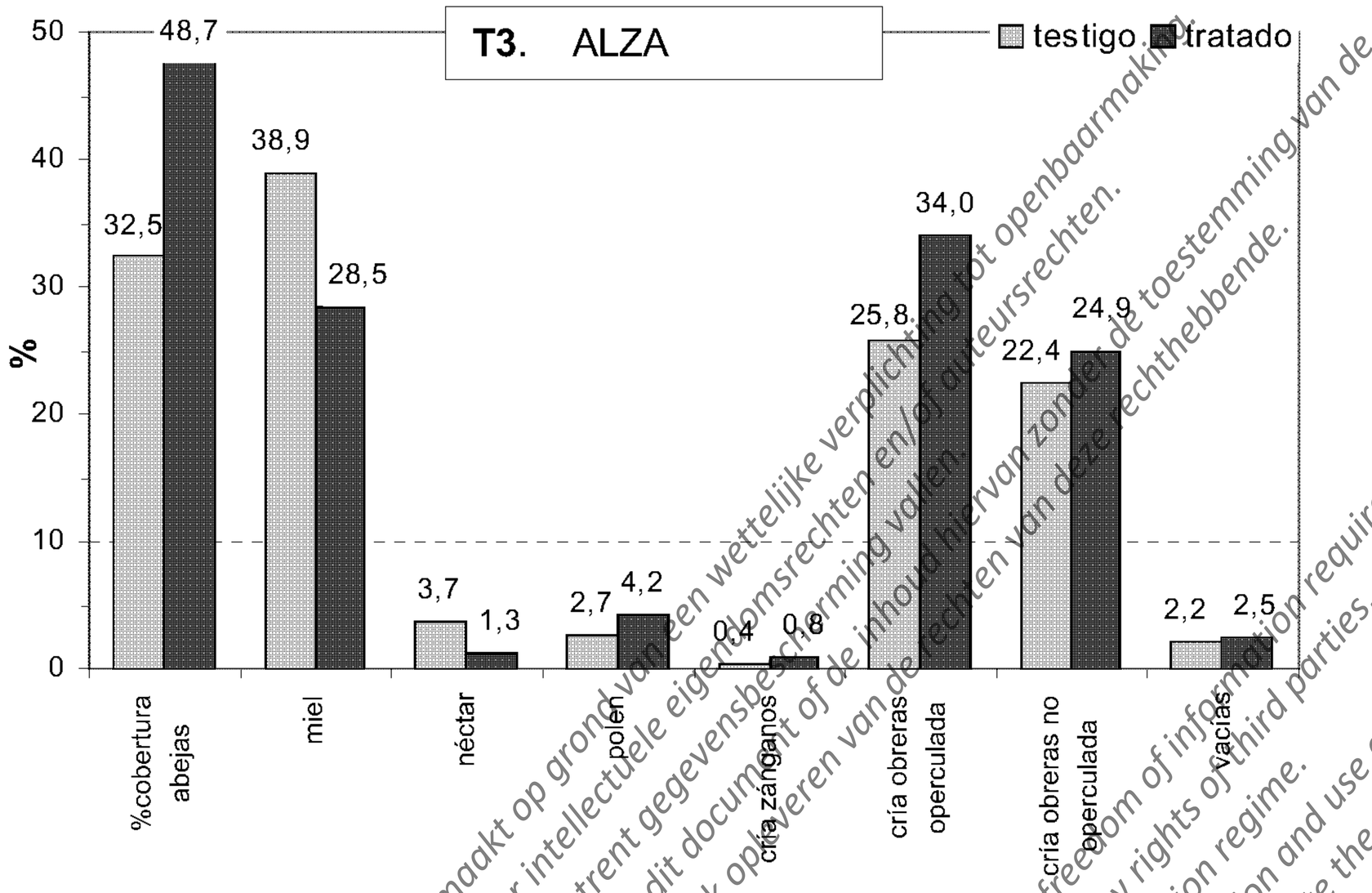


Chart 4. BOTTOM SUPER. Percentage of frame area occupied by bees and percentage of cells occupied by honey, nectar, pollen, brood and empty cells at **T3** (mean values per hive and per site) *Original data in annex VII.6.1.2.*

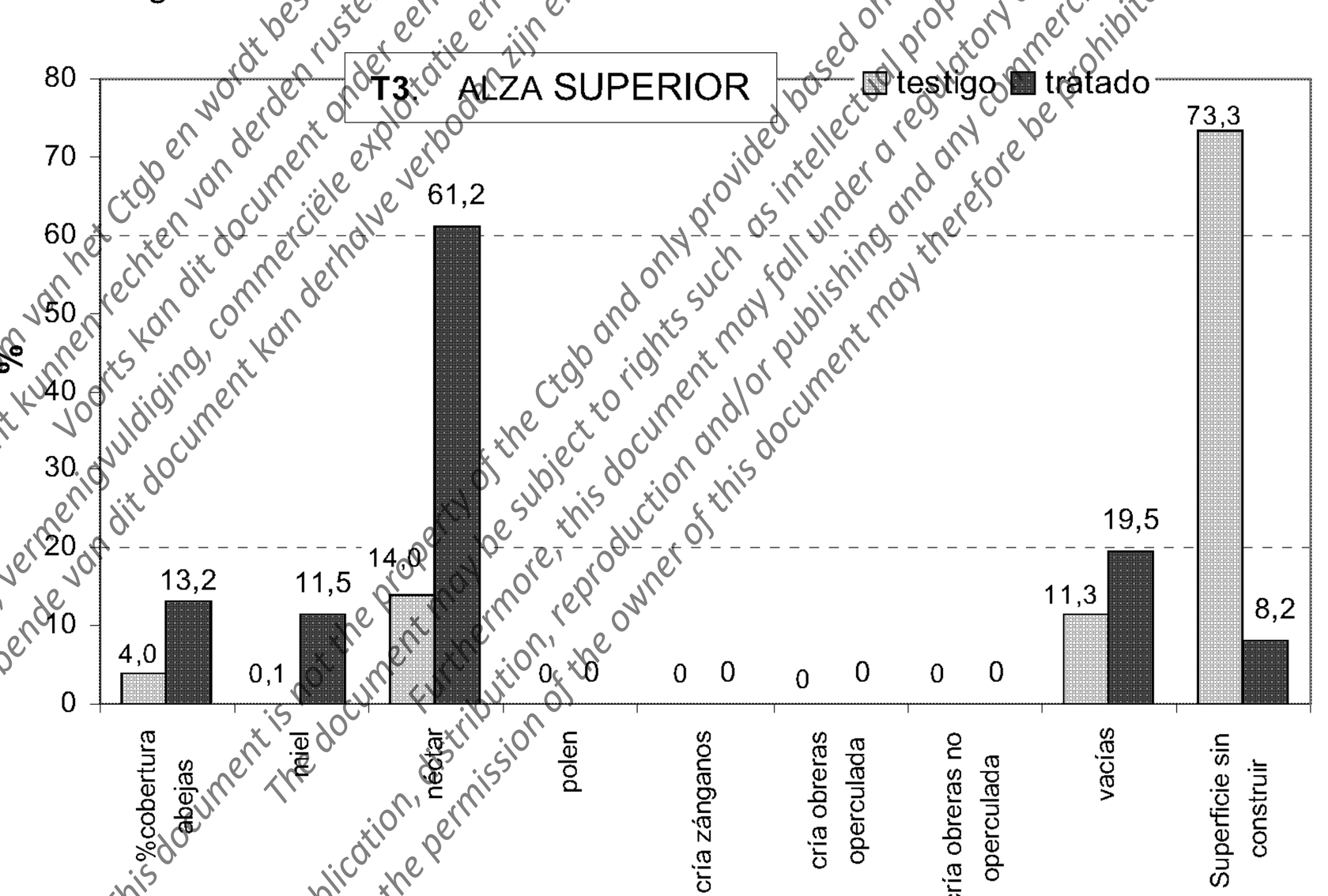


Chart 5. TOP SUPER. Percentage of frame area occupied by bees and percentage of cells occupied by honey, nectar, pollen, brood, empty cells and non-constructed area at **T3** (mean values per hive and per site). *Original data in annex VII.6.1.2.*

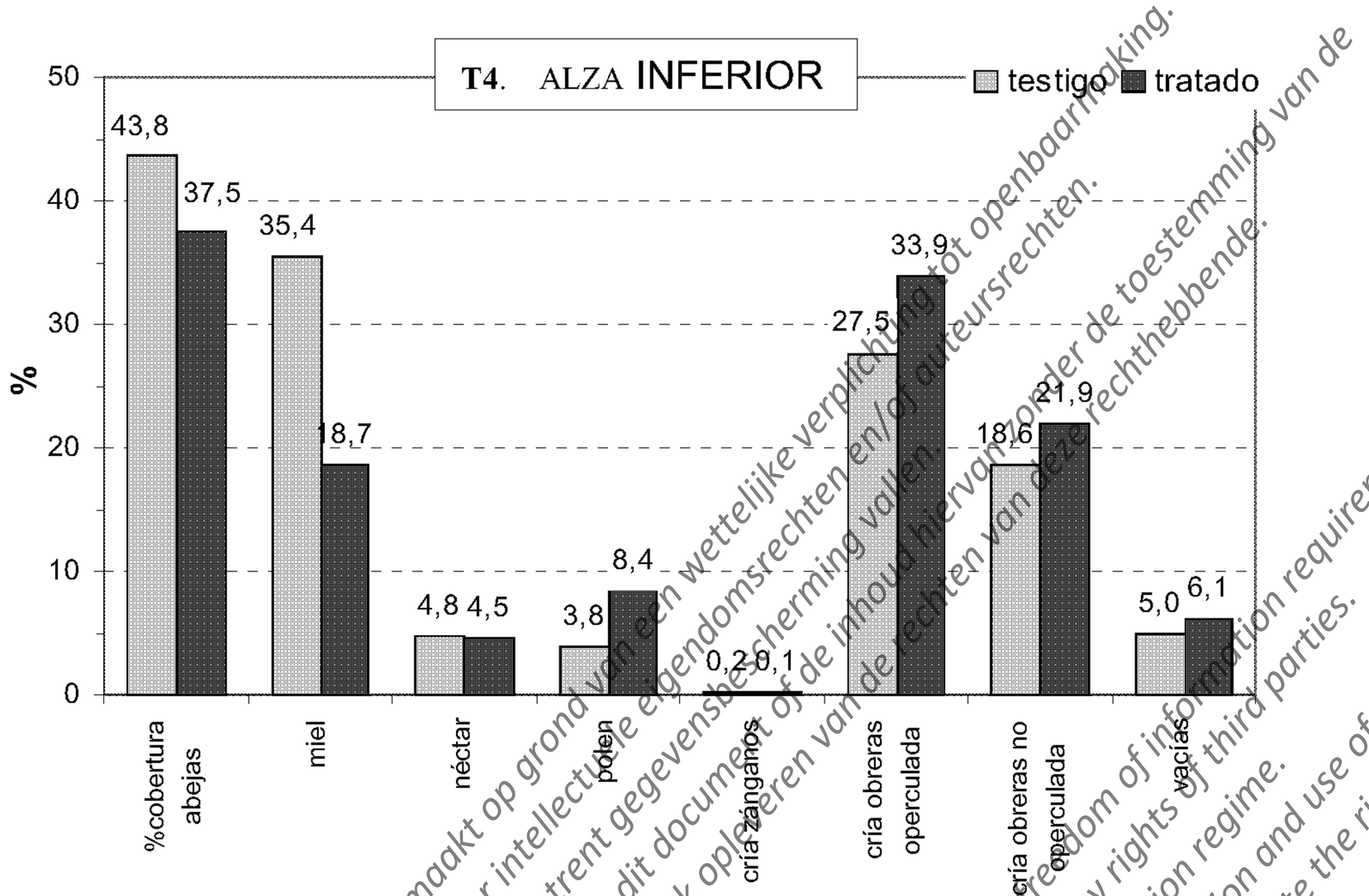


Chart 6. BOTTOM SUPER. Percentage of frame area occupied by bees and percentage of cells occupied by honey, nectar, pollen, brood and empty cells at **T4** (mean values per hive and per site). *Original data in annex VII.6.1.2.*

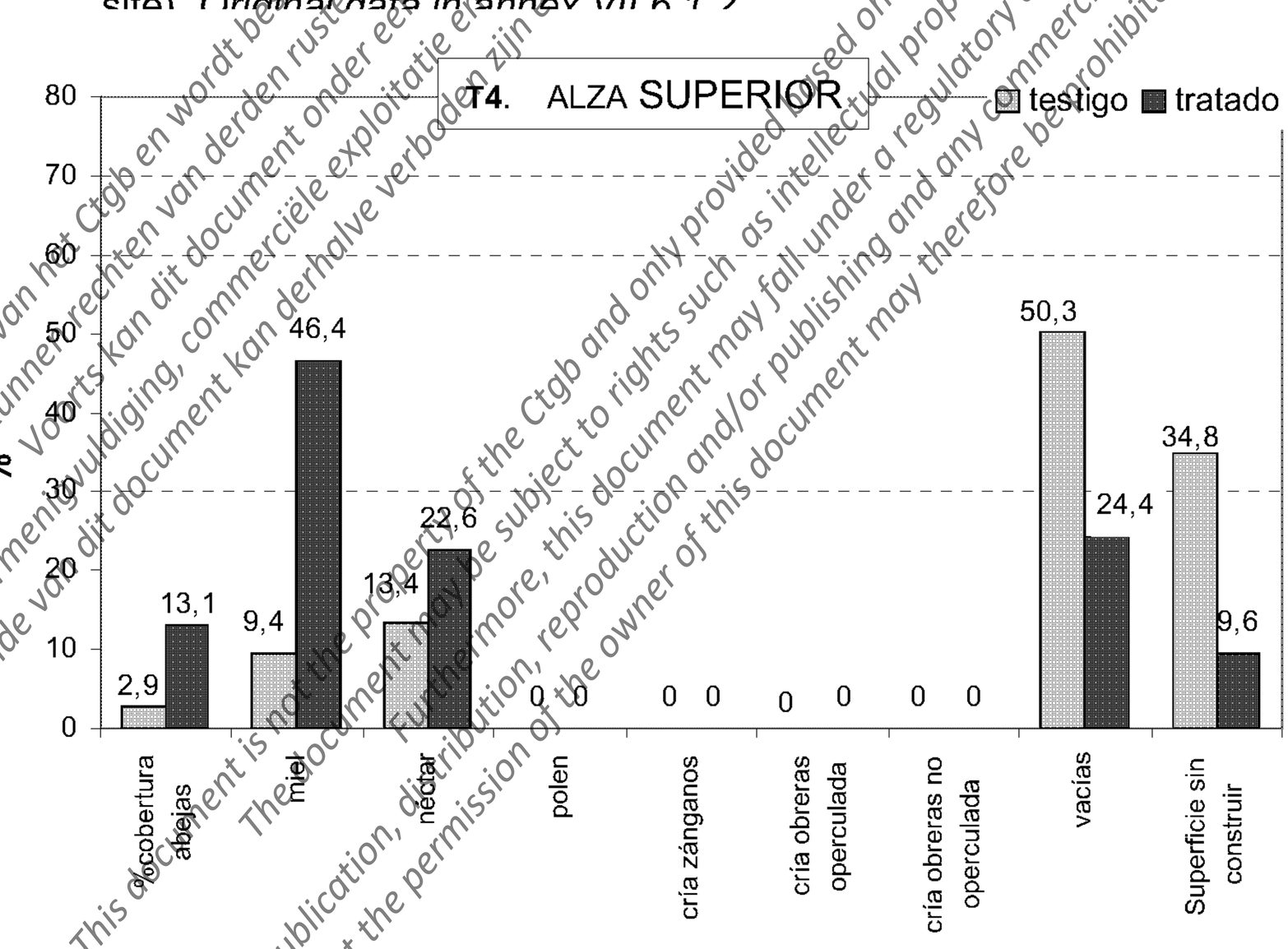


Chart 7. TOP SUPER. Percentage of frame area occupied by bees and percentage of cells occupied by honey, nectar, pollen, brood, empty cells and non-constructed area at **T4** (mean values per hive and per site). *Original data in annex VII.6.1.2.*

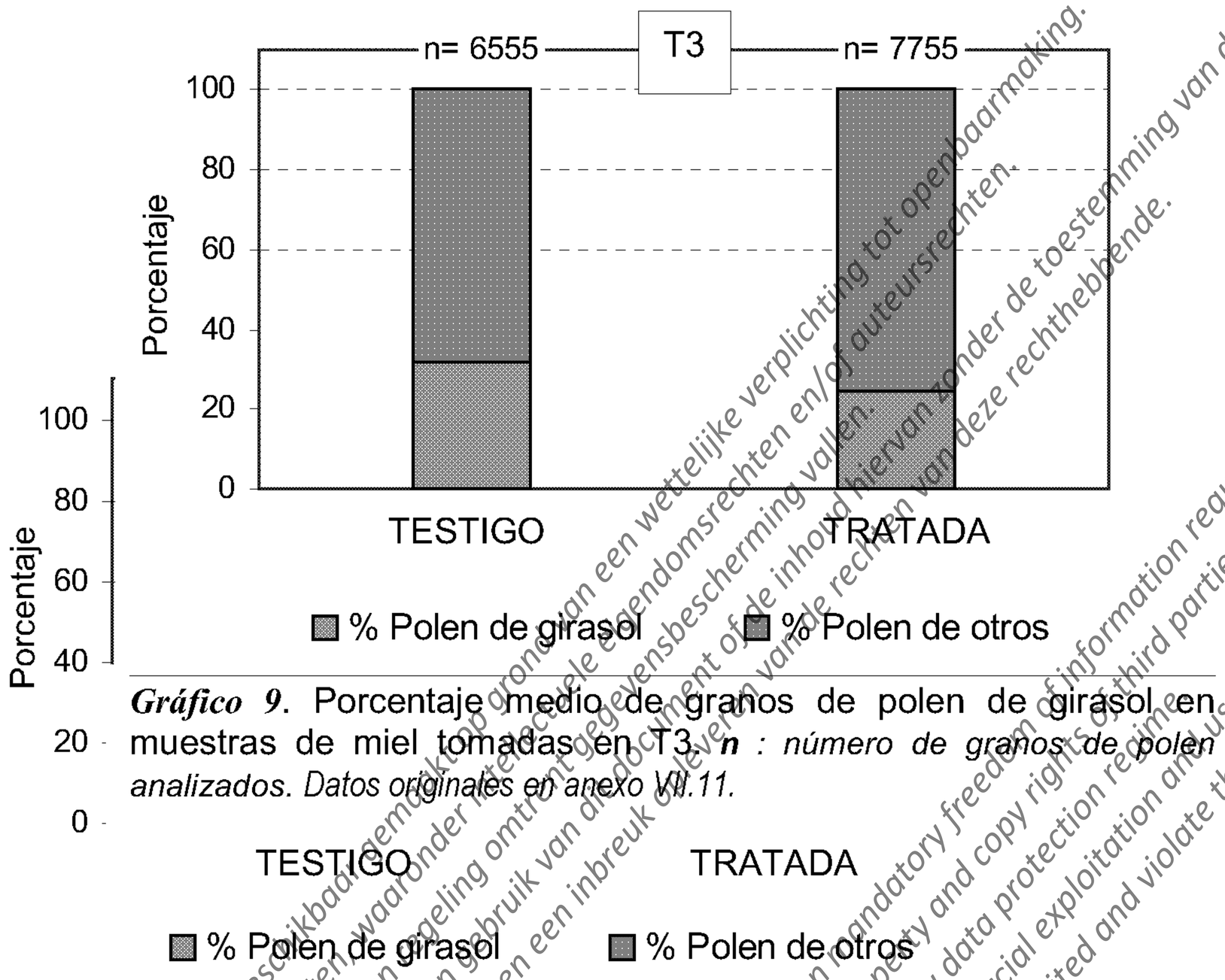


Gráfico 9. Porcentaje medio de granos de polen de girasol en muestras de miel tomadas en T3. ***n*** *: número de granos de polen analizados. Datos originales en anexo VII.11.*

Chart 8. Average percentage of sunflower pollen grains in pollen samples taken at T3. ***n****: number of pollen grains studied. Original data in annex VII.11.*

IV.4. *PALINOLOGIC TESTS OF POLLEN AND HONEY SAMPLES*

IV.4.1. *Pollen samples*

Pollen samples taken from hives of the treated site at **T3** contain a higher proportion of sunflower pollen than samples of control hives (chart 8) (Annex VII.11).

It is important to consider that pollen samples taken at T3 come from frames with brood of the bottom super; so, pollen that came into hives between T2 and T3 is associated with the pollen that these frames had initially at the time of T2. Consequently, it is possible that pollen that was collected before T3 was randomly incorporated into the samples from flora belonging to previous hive location (see III.8).

IV.4.2. *Honey samples*

The percentage of sunflower pollen in honey samples from hives of control site (31,8%) at **T3** was slightly higher than that of honey samples from hives of the treated site (24,6%) (chart 9) (Annex VII.11).

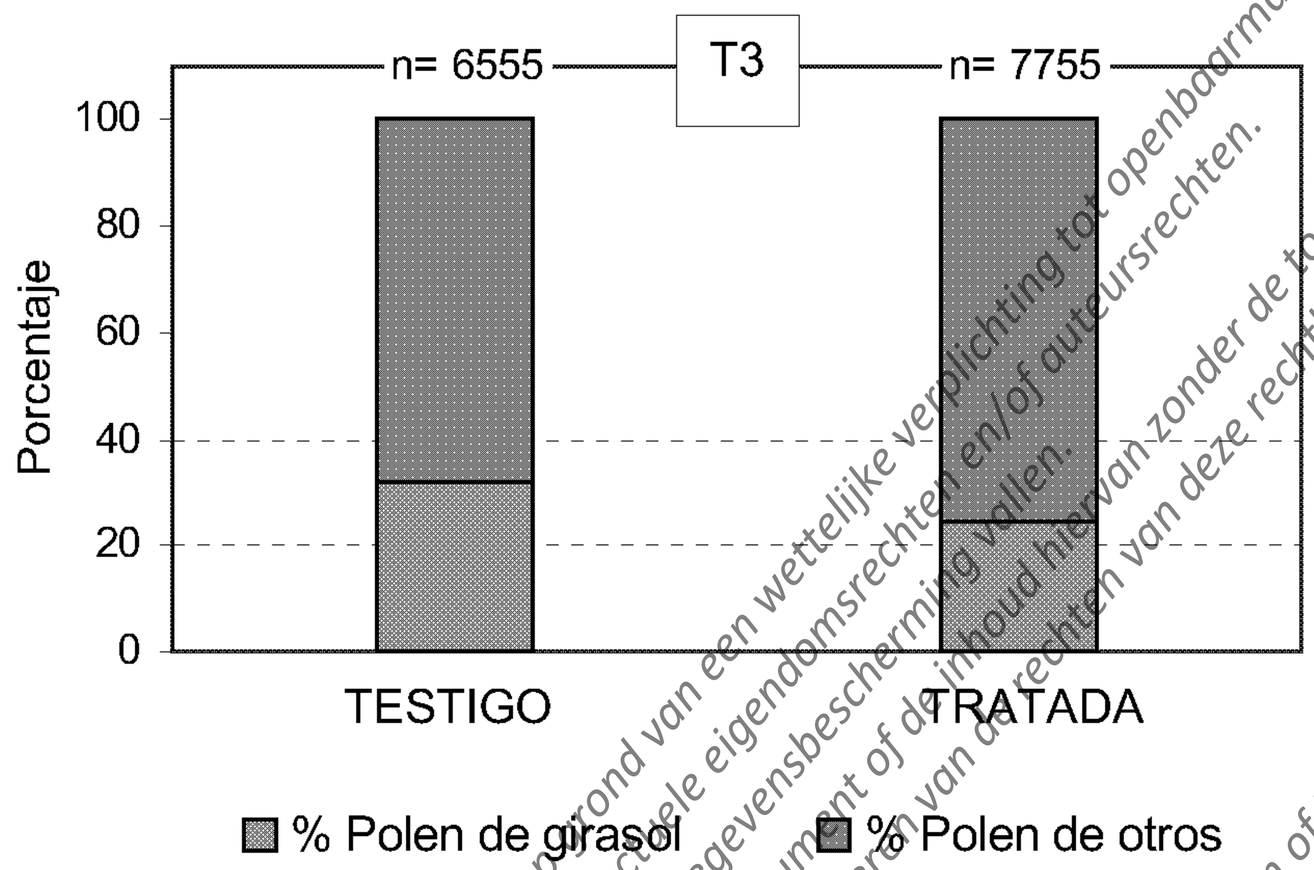


Chart 9. Average percentage of sunflower pollen grains in honey samples taken at T3. ***n:*** *number of pollen grains studied. Original data in annex VII.11.*

Information is very diverse, both on sunflower pollen content, that appears in honeys of sunflower crops, and on honeybee preference for flowers of different plant species. Ricciardelli d'Albore (1997) defines a 20% to 60% content of sunflower pollen as a mellisopalinologic feature of honeys. Maurizio & Louveaux (1963) defines *Helianthum* honeys from 10% to 15% pollen content for this species.

On the other hand, Hedtke (1998), using pollen traps in field condition in a sunflower crop, finds that in samples obtained the content of sunflower pollen hardly reaches 1.5%. Burgstaller (1990) explains this phenomenon by *A. mellifera's* field behaviour in sunflower crops. The bee collects sunflower pollen by early morning hours and late afternoon, but in the meantime, it prefers other pollen sources even when these are more distant.

Since this test was done in field conditions, i.e. not confined, it is possible that bees have flown beyond the observation context set up for the test to other plants, bushes or trees in search of pollen and nectar. This behaviour was already considered and announced at the time the experimental design was conceived and it is in line with the information from the international literature on this matter. Counts made on honey samples that were obtained at T3, showed the high percentage of sunflower pollen that can be expected for a test under field conditions (chart 9) and in agreement with the available literature (Ricciardelli d'Albore, 1997; Maurizio & Louveaux, 1965).

Furthermore, honeys were identified as *"sunflower honey"* in situ at T3 by an apiarist expert according to their origin and the organoleptic properties of samples obtained from different hives (Annex VII.12).

At **T4**, honey samples from hives of the treated site contained a higher average percentage of sunflower pollen (12.2%) than honey samples from hives of the control site (2.4%) (chart 10) (Annex VII.11). A reduction of sunflower pollen content in honey was

observed in comparison with honey samples at T3. This reduction is probably due to the fact that most of the honey of that source has already been consumed, the rest being diluted with new contributions from flora present at "El Gabi" (La Plata) (see III.8). T4 honey samples were combined with honey produced before and after T3 from both sunflower and flora in "El Gabi". It is also worth noting that T4 honey samples are the result of a 24 days exposure period to wild flora (between T3 and T4), which favours processes of mix and dilution of sunflower pollen in honey, in addition to each hives consumption (charts 9 and 10).

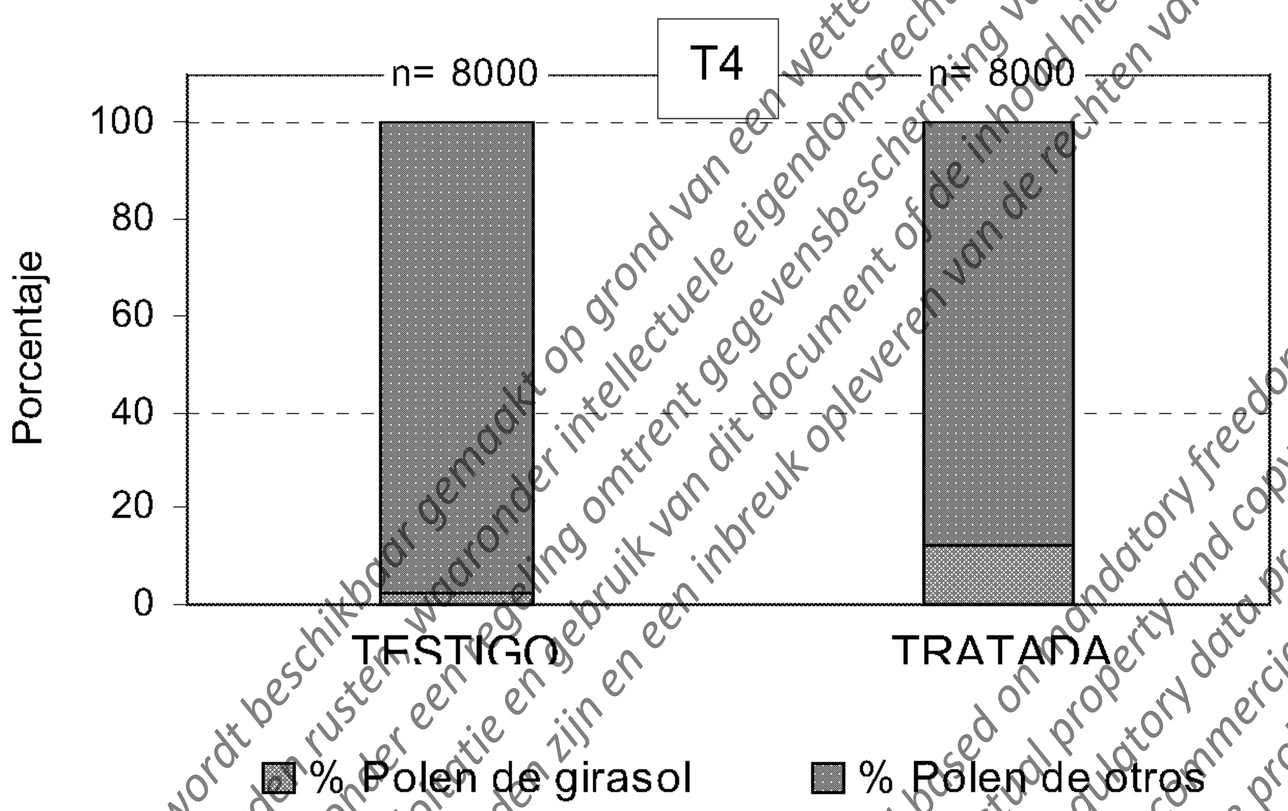


Chart 10. Average percentage of sunflower pollen grains in honey samples taken at T4. ***n:*** *number of pollen grains studied. Original data in annex VII.11.*

IV.5. *BEE ACTIVITY AND MORTALITY IN TEST SITES*

IV.5.1. *Field activity*

During the observation period (T2 – T3), the average daily number of bees foraging on sunflower was higher in the treated sunflower site (*P = 0.0319,* Annex VII.8: tables 37 and 38) except on 2/10, probably due to the greater wind speed in treated site than control site (chart 11) (Annex VII.6.3.).

As it may be observed in chart 11, daily field activity changed significantly (*P = 0.0067,* Annex VII.8: tables 37 and 38). During the first observation days, when the crop in both sites was in the same fenologic condition, field activity in the treated site exceeded that of the control site, probably due to differences of physiological condition or state of crop as previously discussed (*see III.3*). Later, the difference between field activity in one site and the other increased as from 2/8 (chart 11). This could be partly due to the higher proportion of pollen plants observed in the treated site as compared with the control site on that same day (*see IV.1,* chart 1).

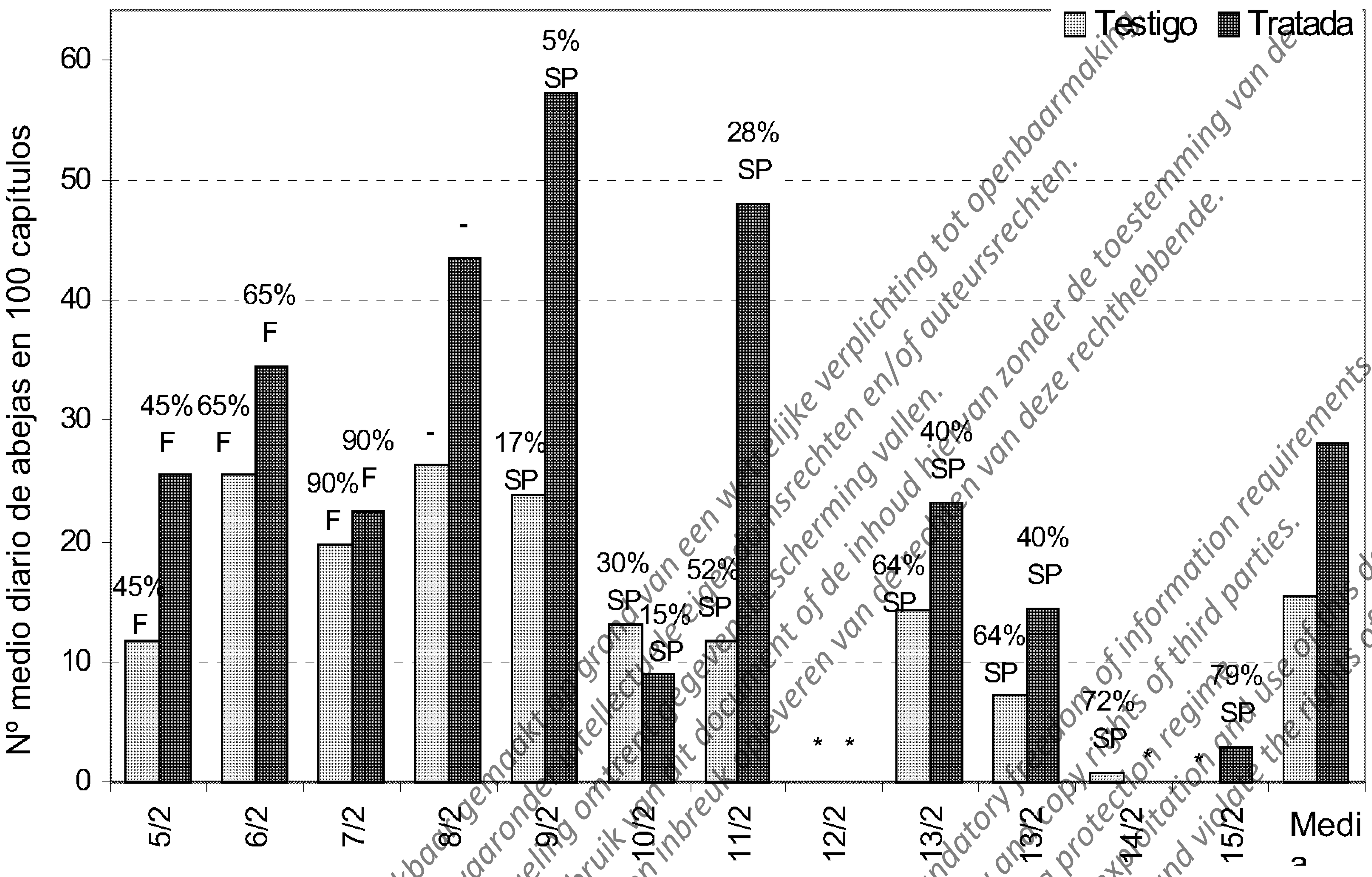


Chart 11. Average daily amount of field activity in one hundred sunflower heads of test sites. *Crop condition is indicated above bars (**F** = percentage of flowering plants, **SP** = percentage of plants without pollen). *Non available datum. Original data in annex VI.6.2.1.*

Field activity observed at T2 and T3 was high considering that, for commercial sunflower pollination practices –where hives charge is much higher (usually, 2 hives/ha) a 25 bees per 100 heads value is considered high (Zorzín and Woodward, 1998). There are unknown factors affecting field activity and bee distribution in a sunflower crop. Investigations by Benedek *et al.* (1972) conclude that the seed production in sunflower depends on bee density on the heads and that other factors still unknown have a dramatic influence on it.

The strongest field activity observed in the treated site is related to the higher percentage of sunflower pollen found in samples of pollen taken at T3 (chart 8) (*see IV.4.1*). Crop in the treated site showed a higher plant density, higher competitivity and probably a better physiological condition than the control site; differences which were previously discussed *(see IV.1)*. Crop condition or state is probably related to the quantity and quality of nectar and pollen of plants, which make them more or less attractive for bees. It is worth noting that there is evidence of a positive relation between saccharose content in sunflower nectar and the visit of bees (Pham Delègue *et al.,* 1994).

IV.5.2. *Incoming pollen*

Differences in the daily counting of incoming pollen (average number of bees entering the hive with pollen per minute) observed in control and treated site (chart 12) were not statistically significant (*P = 0.1233,* Annex VII.8: tables 39 and 40).

During the first observation days (2/5 – 2/10), incoming pollen was higher for the treated site. On subsequent days, pollen carried into the hives of the control site increased, thus exceeding average incoming pollen in the treated site. No differences statistically significant with regard to the analyzed variable (average number of bees entering with pollen in both sites (*P = 0.9950,* Annex VII.8: tables 39 and 40) were found throughout the observation period.

Pollen carried into the hives was higher for the treated site as compared with the control site during the first days (chart 12). Based on that observation, it could be concluded that during the first days of hive exposure to sunflower, bees of the control site visited sunflower in search of pollen less often than bees on the treated site. On subsequent days, pollen entering hives of the control site increased, probably due to pollen been collected on sources other than sunflower, a phenomenon that is reflected by the reduction of field activity in control sunflower (charts 11 and 12). This observation is in agreement with the results from the palinologic test of pollen samples at T3, which showed a lesser percentage of sunflower pollen from hives in the control site as compared with the treated one (chart 8) (*see IV.4.1*). This could be also due to the lesser proportion of plants with available pollen in the control site (chart 1) (*see IV.1*).

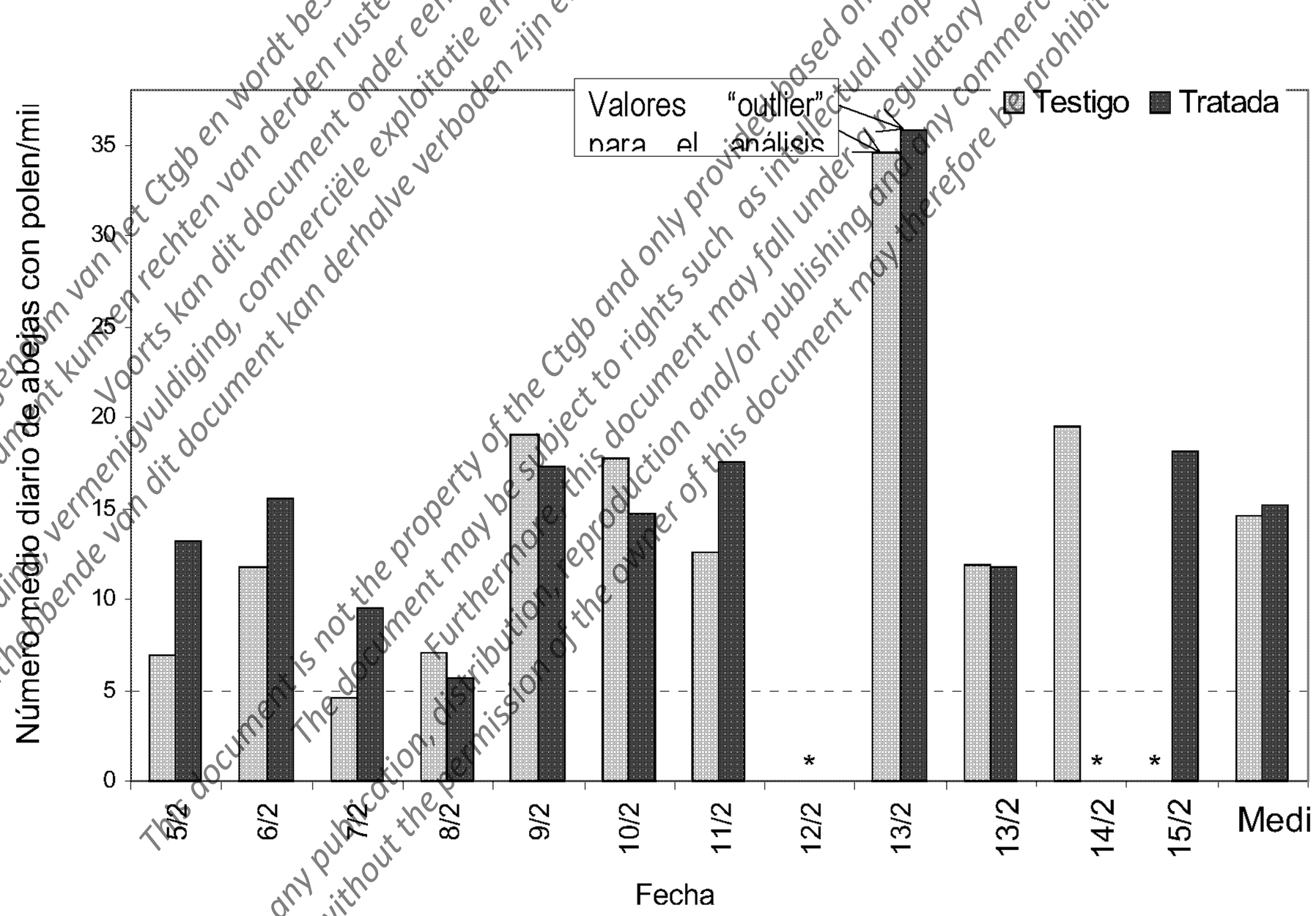


Chart 12. Average daily number of bees per minute entering hive with pollen in test sites. * *Non available datum Original data in annex VI.6.2.2.*

IV.5.3. *Mortality measured in front of hives*

Both in control and treated site, significant differences were found between daily values of the number of dead worker bees (*P = 0.0141,* Annex VII.8: table 41). On 2/5 and 2/6 mortality measured in front of hives in test site was higher than that observed during the remaining observation period (chart 13). This difference could be explained by analyzing factors external to this test, such as the effect of hive transfer (transfer and installation) to sunflower sites (T2).

The average daily number of dead bees in front of hives throughout the observation period was similar for both test sites, no differences statistically significant being observed (*P = 0.1545,* Annex VII.8: tables 41 and 42).

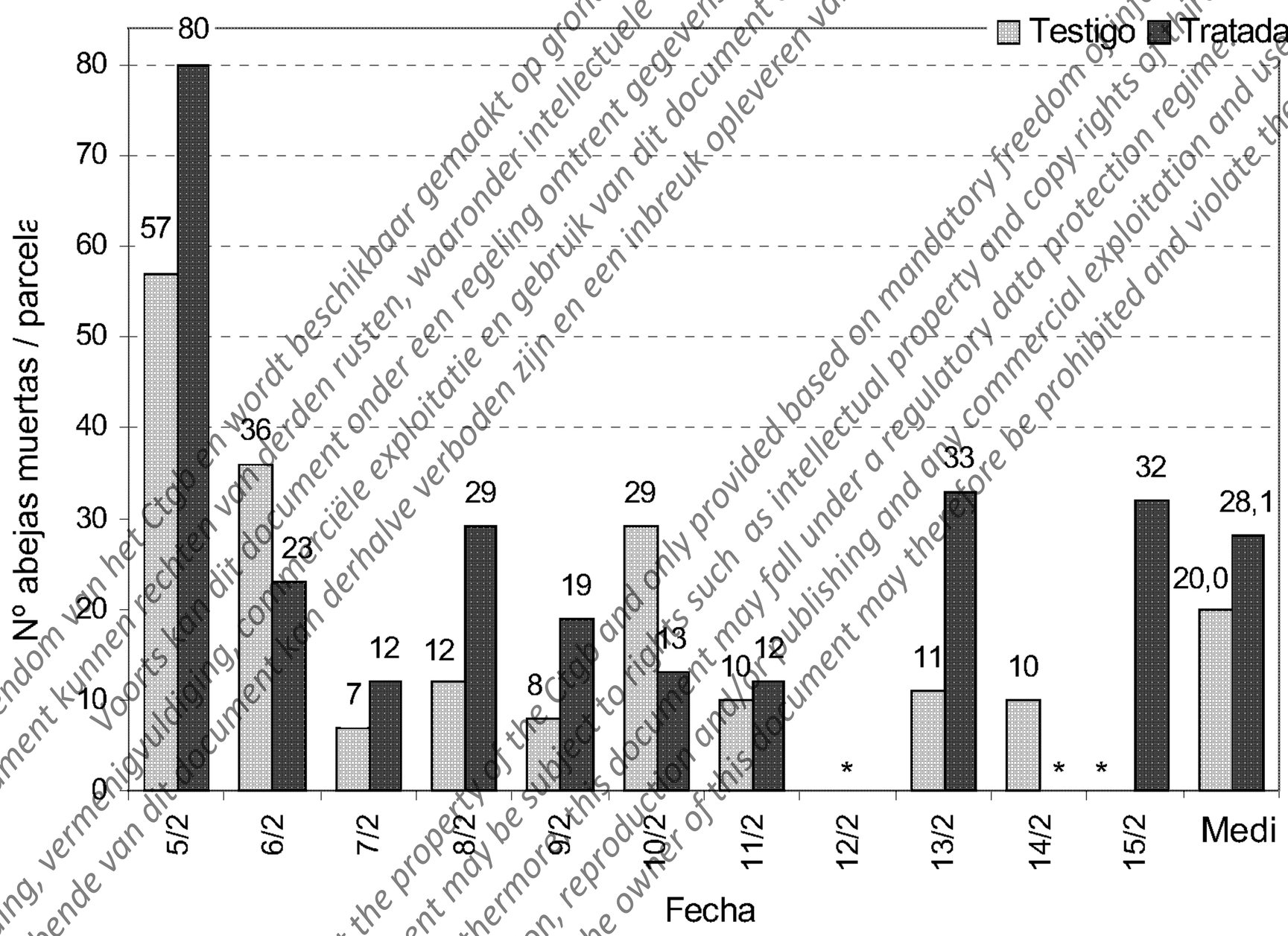


Chart 13. Total daily number of dead bees in front of hives in test sites. * Non available datum. Original data in annex VII.6.2.3.

IV.6. *SANITARIAN CONDITION OF HIVES*

In sanitarian evaluations performed upon opening and inspection of hives at T0, T2, T3 and T4 it was observed that their sanitarian condition was good (see annex VII.13).

IV.7. *CHEMICAL ANALYSIS IN SAMPLES OF SUNFLOWER SEEDS TREATED WITH GAUCHO AND OBTAINED BEFORE SOWING*

IV.7.1. *Determination of Imidacloprid content*

Results obtained for samples of non-treated sunflower seeds were *"non detected"* or lesser than quantification limits. In treated seeds, a mean value of 0.2458 mg of Imidacloprid/seed was obtained, that complies with the expected value in accordance with the dose used for seed treatment (pages 3 and 4 of annex VII.9).

IV.7.2. *Individual seed distribution of Imidacloprid content*

As for Imidacloprid distribution on individual seeds, an average content of 0.2592 mg of Imidacloprid/seed was obtained with a standard deviation of 0.1239 mg/seed and a 47.8% variability coefficient (page 5 of annex VII.9).

IV.8. *RESIDUE TESTS IN SAMPLES OF POLLEN* (T3 and T4), *HONEY* (T3 and T4), *WAX* (T3 and T4), *SUNFLOWER HEADS* (T2) *AND SOIL* (T2)

IV.8.1. *Pollen, honey and sunflower heads*

Results obtained (table 4 of annex VII.10) for all samples of honey, pollen and sunflower heads were *"non detected"*, that is, no residues were detected, either of Imidacloprid or its metabolites (hydroxylated and olefinic) (table 3 of Annex VII.10).

IV.8.2. *Wax*

No residues were detected in T3 wax samples. However, *"non quantifiable"* Imidacloprid residues were detected in T4 wax samples in the treated site (below quantification limit) (concentration between 1.5 and 5.0 µg/kg) (table 4 of annex VII.10).

IV.8.3. *Soil*

In T2 soil samples both from control and treated lot, *"non quantifiable"* Imidacloprid residues were detected (concentration between 2 and 6 µg/kg) (table 4 of annex VII.10).

SUMMARY OF RESULTS FOR POPULATION PARAMETERS OBSERVED IN HIVES

Table 10 shows a summary of hive evolution at times of observation T2, T3 and T4, based on differences observed at evaluated parameters level.

TABLE 10. *Qualitative comparison of mean values obtained for parameters evaluated in hives from treated and control site*

Location of hives		*Sunflower sites*		*"El Gabi" (La Plata)*		
		TIME OF OBSERVATION				
PARAMETER OBSERVED		T2	T3	T3'	T4	T4'
Weight of hives		Control ~ Treated(*)	Control < Treated (*)	Control < Treated (*)	Wtness < Treated (*)	Wtness < Treated
Percentage of cells occupied by:						
Honey + nectar	*Bottom Super*	Control ~ Treated(*)	Control ~ Treated (*)		Control ~ Treated (*)	
	Top Super	--------	Control < Treated (*)		Control < Treated (*)	
	Complete Hive	Control ~ Treated (*)	Control ~ Treated		Control < Treated (*)	
Pollen	*Bottom Super*	Control ~ Treated (*)	Control < Treated (*)		Control < Treated (*)	
Brood	*Bottom Super*	Control ~ Treated (*)	Control < Treated (*)		Control ~ Treated (*)	
Empty Cells	*Complete Hive*	Control ~ Treated (*)	Control ~ Treated (*)		Control ~ Treated (*)	
Constructed Area	*Top Super*	-------	Control < Treated (*)		Control ~ Treated (*)	
Field Activity		Control < Treated (*)				
Pollen Incoming		Control ~ Treated (*)				
Mortality		Control ~ Treated (*)				

(*) Probability of assert > 95%. Statistical analysis of results and values obtained in Annex VII.8.

SUMMARY OF RESULTS OF ANALYTICAL TEST RESULTS

Table 11 shows a summary of the results of Imidacloprid determinations in sunflower seed samples and results of Imidacloprid residue tests and its metabolites in sunflower heads and pollen, honey and wax samples.

TABLE 11. *Results of Imidacloprid residue tests and its hydroxylic -and olefin metabolites in samples of (treated and non treated with Gaucho) sunflower seeds, sunflower heads and samples of pollen, honey and wax taken from hives in treated site and control site.*

Location of hives		*"El Gabi" (La Plata)*	*Sunflower sites*		*"El Gabi" (La Plata)*
		TIME OF SAMPLING			
TESTED SAMPLE		**Before Sowing**	**T2**	**T3**	**T4**
Sunflower seeds	*Control Site*	Non detected			
	Treated Site	0.2458 mg Imidacloprid/ seed			
Soil	*Control site*		Non quantifiable		
	Treated site		Non quantifiable		
Sunflower heads	*Control site*		Non detected		
	Treated site		Non detected		
Honey	*Control site*			Non detected	Non detected
	Treated site			Non detected	Non detected
Pollen	*Control site*			Non detected	Non detected
	Treated site			Non detected	Non detected
Wax	*Control site*			Non detected	Non detected
	Treated site			Non detected	Non quantifiable

Results and values obtained in annexes VII.9 and VII.10.

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V. CONCLUSIONS

1. Plant density and sunflower fenologic condition in test sites

1.1. Upon transfer of hives to sunflower sites (T2), plant density of site treated with Gaucho (50.300 plants/ ha) was higher than that of control site (41.500 plants/ ha), which is probably related to the treatment of seed with the tested product.

1.2. The number of flowering plants was similar in both sites. However, towards mid-flowering and up to its end, a higher proportion of plants without pollen was observed in the control site in comparison with the treated site.

2. Bee activity and mortality in test sites between T2 and T3

2.1. Field activity (average count of bees foraging in sunflower) was significantly higher in the treated site as compared with the control site.

2.2. No significant differences were observed in counts of bees carrying pollen into the hives of both sites.

2.3. Mortality measured in front of hives in both test sites was not statistically different.

3. Setting and population structure of hives

3.1. At the beginning of hive exposure to sunflower (T2) hive setup and population structure were uniform; weights and percentages of frame areas occupied by honey, nectar, pollen and brood did not show significant differences.

3.2. By the end of hive exposure to sunflower (T3), significant differences were observed in the following parameters:

3.2.1. The increase of hive **average weight** in the treated site was higher than that of hives in control site.

3.2.1. The increase of frame area covered with **honey and nectar** in the top super was higher in the hives of treated site as compared with those in control site.

3.2.1. The increase of frame area covered with **pollen and brood** in the bottom super was higher in the hives of treated site as compared with those in control site.

3.3. 24 days after their removal from sunflower sites at T4, the amount of **pollen, nectar** and **honey** reserves in hives exposed to the treated site was significantly higher in comparison with those from the control site.

4. Sunflower pollen content in honey samples

4.1. In pollen counts of honey samples that were taken at T3, a high percentage of sunflower pollen (> 20%) was observed, which can be expected from a test in field conditions and in agreement with international literature (Maurizio & Louveaux, 1963; Ricciardelli

d'Albore, 1997). Moreover, honeys were identified in situ based on their origin as "sunflower honey" in accordance with the organoleptic properties of the samples taken.

Based on the outlined partial conclusions, it can be considered that, during the exposure in sunflower sites, hives in the site treated with Gaucho developed faster than those in the control site. However, 24 days after their removal from sunflower, both groups of hives (control and treated) reached a similar level of population development, even if honey and pollen production was higher for the group of hives in the treated site. Differences of hive development in both sites are probably related to differences observed in field activity and to the different proportion of available plants with pollen present in both sunflower sites during flowering.

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DECLARACIÓN DE CONFIDENCIALIDAD

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4. Contenido de polen de girasol en muestras de miel

4.1. En los recuentos de polen realizados sobre las muestras de miel obtenidas en T3 se observa un alto porcentaje de polen de girasol (> 20%) esperable para un ensayo en condiciones de campo y de acuerdo con la información bibliográfica internacional (Maurizio & Louveaux, 1963; Ricciardelli d'Albore, 1997). Además, las mieles fueron identificadas in situ de acuerdo a su origen como "miel de girasol" según las propiedades organolépticas de las muestras obtenidas.

Sobre la base de las conclusiones parciales expuestas, puede considerarse que, durante la permanencia de las colmenas en las parcelas de girasol, las colmenas de la parcela tratada con Gaucho evolucionaron más rápidamente que las de la parcela testigo. Sin embargo, 24 días después de ser retiradas del girasol, ambos grupos de colmenas (testigo y tratado), alcanzaron un nivel de desarrollo poblacional similar, aunque la producción de miel y de polen fue superior en el grupo de colmenas provenientes de la parcela tratada. Las diferencias en el desarrollo de las colmenas de ambas parcelas se relacionan probablemente con las diferencias observadas en la actividad pecoreadora y con la distinta proporción de plantas con polen disponible presentes en ambas parcelas de girasol durante la floración.

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10/05/2000

Fecha