Mating disruption of the ambrosia beetle *Megaplatypus mutatus* (Coleoptera: Platypodidae) in poplar and hazelnut plantations using reservoir systems for pheromones. Field trials in Argentina and Italy.

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Running title

Mating disruption of the ambrosia beetle *Megaplatypus mutatus*

Key words: *Megaplatypus mutatus*, mating disruption, sex pheromones, controlled release devices, poplar , hazelnut, sulcatol, sulcatone, 3-pentanol.

Abstract

Megaplatypus mutatus is an Ambrosia beetle native to South America, but it has recently been introduced to Italy and represents a serious problem in poplar and fruit trees commercial plantations.

Previous studies by our laboratory showed that male pheromones are composed by (+)-6-methyl-5-hepten-2-ol ((+)-sulcatol), 6-methyl-5-hepten-2-one (sulcatone) and 3-pentanol. Mating disruption is a widely used pest management technique for controlling many lepidopteran pests, but it is seldom exploited for coleopteran species.

We built plastic pheromone reservoir type dispensers for sulcatol, sulcatone and 3-pentanol with zero order kinetics that were deployed in the field during the female flying period. Field trials were performed in poplar plantations in Junín, Province of Buenos Aires, Argentina, and in poplar and hazelnut plantations in Caserta, Campania Region, Italy.

In all the experiments, the number of galleries where effective mating was achieved was significantly higher in control than in treated areas, indicating that female behavior and male localization were effectively interfered by the pheromones applied. Damage reduction for field trials in both countries was greater than 56%. The lower effectiveness observed in Italy can be attributed to the shorter length of the experiment and to the higher initial population density in Italy compared to Argentina. The results reported here show the potential of *M. mutatus* mating disruption in commercial poplar and hazelnut plantations. This is the first trial of this kind, so these results are very promising.

Introduction

Platypodid ambrosia beetles (Platypodinae, Curculionidae) are an important group of forest pests that attack mainly felled or weakened trees. The common name derives from the fungus carried and inoculated by the adult beetles, and on which the larval stages feed. The dark staining of the tunnels caused by the decaying ambrosia mycelium *Raffaelea santoroi* (Guerrero) (Bascialli *et al*., 1996) reduce the quality of the wood required for export.

Megaplatypus mutatus (=*Platypus mutatus*) (Chapuis) is an Ambrosia beetle native to South America. Unlike most ambrosia beetles, it attacks only living trees, penetrating the xylem of its host by boring long tunnels. This weakens the stem causing it to break under extreme stress, representing a serious problem in poplar *Populus deltoides* Marshall commercial plantations (Alfaro *et al*., 2007; Achinelli *et al*., 2005).

The attack is initiated when the male excavates a tunnel through the bark. With the particles of sawdust produced it builds an arrangement surrounding the gallery with the shape of a crown from which it emits volatile emissions to attract individuals of the opposite sex. This wait can last between 2-12 days (Santoro, 1962). If the female detects the male call, lands at the entrance hole of the gallery and the male accepts it, mating soon takes place. After mating, the couple continues extending the gallery and depositing sawdust at the gallery entrance. This sawdust is clearly distinguishable from the pre-mating one (Santoro, 1963), as it is explained in materials section.

Previous studies by our laboratory showed that male volatile emissions are composed mainly by (+)-6-methyl-5-hepten-2-ol ((+)-sulcatol), 6-methyl-5-hepten-2-one (sulcatone) (González Audino *et al*., 2005) and 3-pentanol (Gatti Liguori *et al*. 2008). Our laboratory recently performed the first trial to monitor *M. mutatus* in the province of Buenos Aires, Argentina, using the two first pheromone compounds described above ((+)-sulcatol and sulcatone), and obtained promising results (Funes *et al*. 2009a).

M. mutatus was accidentally introduced to Italy in 1998 (EPPO/OEPP 2004, 2007; Tremblay *et al*. 2000) causing great concern because poplar plantations are a highly important economic resource. In 2000, it was detected in *Populus canadensis* (Mönch) in the Caserta province in the Campania region. Attacks were also reported in *Juglans regia* (L.) and the European hazelnut *Corylus avellana* (L.) (Tremblay *et al*. 2000; Allegro and Della Beffa 2001), and later on in *Malus* spp., *Pyrus* spp., *Castanea* spp., *Prunus* spp., *Quercus* spp. and *Eucalyptus* spp. (Carella and Spigno 2002). However, these last reports do not indicate whether the attacks produced offspring or were abortive in these hosts.

The risk of the dispersion of *M. mutatus* to other regions of Europe and its corresponding potential damage (Alfaro *et al*. 2007) is of great concern to European regulatory authorities, who added it to the EPPO/OEPP Alert List in 2004 and in 2007 recommended treating it as a quarantine pest (Allegro and Griffo 2008; EPPO/OEPP 2004, 2007). The dispersal is facilitated by transportation of infested logs.

Mating disruption (MD) is a widely used pest management technique for controlling many lepidopteran pests, but it is seldom exploited for coleopteran species. Pityol was used to reduce damage by the white cone beetle in seed orchards (Trudel *et al*., 2004) and the sex pheromone of oriental beetles was used for mating disruption in cranberries (Wenninger *et al*., 2006).

Taking into account that *M. mutatus* is relatively immobile, that males are monogamous, and that the pheromones are of low commercial cost, stable in field conditions during the required period and can be formulated in a controlled release system, we evaluated the pest control by disruption. The management of *M. mutatus* populations by disruption of communication between the sexes by applying male pheromone in slow release formulations could be a highly potential tool for infested poplar plantations.

Materials and methods

Pheromone dispensers.

Sulcatone (6-methyl-5-hepten-2-one), (±) sulcatol ((±) 6-methyl-5-hepten-2-ol) and 3-pentanol were analytical grade (Aldrich Co., Saint Louis, MO, USA).

We built plastic pheromone reservoir type dispensers for sulcatol, sulcatone and 3-pentanol with zero order kinetics and high release rates that were deployed in the field during the female flying period. In reservoir systems the pheromone is stored separately from the release rate controlling polymeric membrane. In a previous work we demonstrated that racemic sulcatol can be used in replacement of (+)-sulcatol, as the (-) isomer does not interfere with its attracting capacity (Funes *et al*. 2009b).

For sulcatone and 3-pentanol we used rectangular bags made with a non-permeable side (high density polyethylene of 80 microns) and a semi permeable side (low density polyethylene of 40 microns). For sulcatol we built bags with two semi permeable sides. We studied the correlation between pheromone release rate and effective surface areas of the dispensers. To test their release rate in the lab we monitored the daily weight loss in wind tunnel (Tº C 27-28º C, 0.5 m/s) until they reached constant weight indicating that all the pheromone was gone. Linear regressions of the weight loss values were calculated to determine the release rates and to establish their profile of linearity. Microsoft Office Package 2002 and Microcal (TM) Origins working model (version 6.0) (1999) was used for these calculations.

The devices were homogeneously distributed by hand throughout the plot representing 1.26, 1.82 and 1.58 g/ha for sulcatone, 3-pentanol and (±) sulcatol respectively. The dispensers were attached to the trees with a pin 1.6 m above the ground. Pheromone dispensers were used in sets of three, one for each component.

Field trial locations

Junín, Buenos Aires, Argentina. The field trial was performed during the 2007-2008 summer season, between 19 November 2007 and 24 January 2008 (67 days) in a commercial poplar (*Populus deltoides*, clone Australiano l29/60) plantation located in Morse, Junín, Province of Buenos Aires, Argentina ((S) 34º 43´ 56,3´´, (W) 60º 51´ 11,5´´) at 59 m.o.s.l. The experimental area consisted of 12 ha of a 10 year-old plantation with a tree density of 625 trees/ha (square of plantation of 4 m x 4m) and a mean diameter at breast height (DBH) of 30.76 cm. ± 0.49 (SE). Treatment and control plots were of 1 ha each, located at the extremes of the diagonal line crossing the plantation with a distance of 1,414 m between them.

Caserta, Campania, Italy. Field trials were performed during the 2008 summer season, May-June, in Caserta, Campania Region, Italy. The plantations were located in Falciano del Massico (41° 09' 07" N, 13º 57' 54.3"E and 38 m.o.s.l.).

The poplar plantation (*Populus x euroamericana* (Dode) Guinier Louisa Avanzo clone) consisted of 0.8 hectares of an 11 year-old plantation with a tree density of 493.8 trees/ha (square of plantation of 4.5 X 4.5 m) and a mean diameter at breast height

(DBH) of 26.57 ± 0.95 cm (ES). Treatment and control plots were of 0.3 ha in each area. Trial plots were laid out with a distance of at least 100 m between pheromone-treated and control plots (Sower *et al*. 1982). A building also separated the plots. Although the plot sizes are relatively small, this limitation is hard to avoid, as typical plantations in Italy are very small size. The trial lasted for 24 days.

The hazelnut trees plantation (*Corylus avellana* L. San Giovanni clone) consisted of 4.8 hectares of a 16 year-old plantation, with a tree density of 444.4 tree/ha (square of plantation of 4.5 X 5 m). Treatment and control plots were of 0.5 ha in each area. Trial plots were laid out with 300 m spacing between pheromone treated and control plots. The trial lasted 35 days.

The three experimental areas cannot be considered as replicates.

Detection of insect flying period

To determine the beginning of the female flying period in order to spread the pheromone dispensers, we monitored the population using pheromone-baited traps (Funes *et al.* 2009a). The temporal monitoring was performed in a nearby plantation with the same agro meteorological characteristics and of the same clones. The baits used were rectangular bags with lower release rates than the ones used for mating disruption. The release rates of rectangular bags used for sulcatol and 3-pentanol were 29, 84 ± 1,80 and 11.27 ± 0.51 mg /day respectively. For sulcatone we used glass vials with a plastic semipermeable cap of polyethylene and a daily release rate of 6.68 ± 0.63 mg /day (Funes *et al.* 2009a). The release rates for the different baits were quantified in the lab at 27 - 28° C using a wind tunnel with a linear air velocity of 0.5 m/s. Traps (20 traps/ha) were cross-vane traps made of two acrylic panels in a cross arrangement above a funnel (Funes *et al*. 2009a and Mastrap ®).

Damage assessment

M. mutatus damage in control and pheromone-treated plots was assessed before placing the pheromone devices and after the end of the experiment. The previous assessment was necessary to establish the similarity in the level of attack of both treatment and control zones. Damage assessment was carefully carried out before the trial by examining tree trunks and identifying active galleries (AG), which were quantified and individualized with markers. Into each experimental area (treated and control ones) we sampled 30% of the trees in a random form (Sower *et al.* 1982).

AG are the entrance holes where males initiated the attack, lured the females, mating took place, and offspring was produced. The characteristic sawdust are disaggregated particles of irregular shape and farinaceous aspect and size between 0.13 a 0.15 mm (Santoro, 1963) (Fig. 1) and the length of the gallery is more than 6 cm. The length is measured with a small calibrated wire. Damage was expressed as the mean number of AG per tree. A t-test was carried out to compare treated and control areas before the trial. Only treated and control plots with no significant differences between them were used for Mating Disruption trials. The surveillance of galleries was performed up to 2 m high for each tree. Old galleries (dry) from previous seasons were also marked to avoid any confusion when the galleries were quantified later on.

After the trial, damage assessment was done by identifying and quantifying the galleries where mating had been successfully achieved (MG) and damage was expressed as mean number of MG per tree. MG are galleries where male initiated their attack, lured female, mating took place, and both male and female are extending the gallery length inwards. These galleries are from 5 to 10 cm long in a straight line with no twists and the female is in the pole position (Santoro, 1962).

Although both MG and AG represent galleries that achieved successful mating, we decided to use MG as a parameter to evaluate the success of the mating disruption because to survey AG we had to wait more than two months until the larval development was achieved.

To test the possibility of introducing another damage assessment parameter, in the Campania field trial we also quantified the new galleries with a calling live male inside (CM) that exhibited the characteristic sawdust in a crown arrangement (Fig. 2). In this case the sawdust particles are 2 to 3 mm long and 0.13 to 0.15 mm wide (Santoro, 1963). CM are only detectable in poplar trees as in hazelnuts the crown arrangement of sawdust is not formed on the surface of the tree. Therefore, CM were only evaluated in poplar plantations in Italy.

Damage was expressed as mean number of MG and CM per tree, and the means of treated and control areas were compared by a t-test after the trial.

Results

Pheromone dispensers

All the dispensers showed a linear release rate until complete consumption as expected for diffusion controlled membrane-moderated reservoir systems (Tojo 1985) and as we showed in our previous work (Funes *et al*. 2009a).

We observed for each compound that release rate increased linearly with the surface (Fig. 3). We calculated the respective slopes of the lines obtained and their linear correlation coefficients. According to the results obtained we selected the necessary effective area in order to achieve the desired release rates. Table 1 shows the selected release rates of (+)-sulcatol, sulcatone and 3-pentanol.

Detection of the female flying period

Fig. 4 and 5 show the time pattern of the females caught daily in traps in poplar plantations of Junín and Campania. The dispensers were placed when we detected a rise in the number of females caught in traps each day and withdrawn when the number decreased. According to these results, the MD trial in Morse was launched on November 19 and finished on January 24 and in the poplar plantation at Campania it was launched on May 26 and ended on June 19, while at the hazelnut plantation, located in the vicinity of the last one, it began on May 12 and finished on June 16.

Mating disruption of <u>M. mutatus</u> in the poplar plantation in Junín, Argentina

The number of AG was not significantly different (t-value = -0.7314; P= 0.435; df= 332) in treated and control sites before the application of pheromones (Table 2). This was a necessary condition to launch the MD treatment because if the previous levels of attack were not similar it would be impossible to compare the results of the trial.

Following the treatment, the number of MG galleries per tree was significantly higher in the control areas (t-value = -6.226; P= 1,375E-09; df= 347) (Table 2). This was the expected result as male pheromones affect female behaviour, interfering with the localization of calling males.

Mating disruption of <u>M. mutatus</u> in the poplar plantation in Caserta, Campania Region, Italy

The number of AG was not significantly different in treated and control sites before the pheromone trial (t-value= -0.9515; P= 0.345; df= 58) (Table 3). As explained above this was a necessary condition to launch the MD treatment.

The pheromone treatment did not modify the number of calling males (CM) per tree (t-value= 1.1125; P= 0.27; df=58) (Table 3). This result was as expected, as the males are pioneer beetles that start the attack initiating the construction of galleries and the application of male pheromones applied should not affect their behaviour. However, the number MG per tree found after the trial was significantly higher in control areas (t-value= -2.25; P=0.0277; df= 58) (Table 3). This was also the expected result as male pheromones affect female behaviour by interfering with the localization of calling males.

Mating disruption in the hazelnut plantation in Caserta, Campania Region, Italy

The number of AG was not significantly different in treated and control sites before the application of pheromones for both poplar and hazelnut plantations (t-value= -0,421; P= 0.675; df= 46) (Table 4). As explained above this was a necessary condition to launch the MD treatment. As was observed in the poplar plantation, the pheromone treatment did not modify the number of calling males (CM) per tree (t-value= -1.2; P= 0.233; df=46) (Table 4).

The number of MG per tree after the trial was significantly higher in control than in treated areas (t-value= 2.5; P=0.015; df=46) (Table 4). Once again, this was the expected result as male pheromones affect female behaviour by interfering with the localization of calling males.

Discussion

In order to develop pest monitoring and control systems based on volatile sex pheromones, efficient controlled release systems are essential to deliver behaviorally relevant aerial concentrations of the bioactive compounds. In this work we developed a reservoir system that delivers a constant a high rate of pheromone during the entire females flying period. In these systems, the pheromone is stored separately from the release rate-controlling polymeric membrane.

In all the treatments, the number of MG after the experiment was significantly higher in control than in treated areas, indicating that mating disruption using the pheromone doses reported here could be a potential tool for management of these hazelnut and poplar plantations. However, the number of new male attacks was not different between treated and control areas as male behavior and host localization should not be affected by the presence of sexual male pheromones. Disruption of mating probably resulted from desensitization of female chemoreceptors and disorientation of insect within pheromone treated areas (Cardé and Minks, 1995). Inhibiting female orientation may produce delays finding the male; furthermore, this delay could prove to be fatal as females soon begin to suffer from dehydration in the hot summer temperatures. Even if the pheromone does not prevent mating and only delays finding males, a definitive effect is produced on the population, as males very frequently die while waiting for the female to arrive between days 10 and 38 if this takes too long to arrive (Santoro 1963).

Damage reduction for field trials in both countries was greater than 56%, and the duration of the trial in each country was selected according to the flying female period in each area. In Junín, Argentina, where the trial lasted two months, the reduction was of 77 %; on the other hand in Caserta, where the field trial lasted 24-35 days, the reduction was of 65% in the poplar plantation and 56 % in the hazelnut plantation. The lower effectiveness observed in Italy can be attributed not only not the shorter length of the experiment, but also to the higher initial population density in Italy compared to Argentina. Insect density is a major limiting factor that can affect mating disruption (Howell *et al.* 1992). In *Cydia pomonella* the efficacy of different types of formulations with pheromones is highly dependent on codling moth density (Stelinski L. L. *et al.*, 2008, Vickers and Rothschild, 1991; Trimble, 1995). Also, mating disruption of *Lobesia botrana* was less effective with aggregated populations because of the increased chance of a female entering in the active space of a calling male (Schmitz *et al.* 1995). Several aspects of *M. mutatus* biology contribute to the promising success of mating disruption for controlling this species. First, treatments are effective at low densities; second, the treated area is not reinvaded by egg bedding females as adults are relative immobile; third, the pest species has a cryptic lifestyle which protects it against treatments with conventional insecticides (Jutsum *et al.*, 1989); fourth, adult feeding has not been observed nor inside or outside the host befor or after emergence (Gatti Liguori, unpublished results) and this probably enhances the influence of pheromones more than in species that spend time foraging (Hasewaga *et al.* 1993); lastly, the sex ratio is 1:1 (Santoro, 1963), making the localization of males by females more unlikely. Also, the non destructive nature of damage assessment is positive in the sense that it does not affect the subsequent generation.

The results reported here preliminary show the potential of *M. mutatus* mating disruption in commercial poplar and hazelnut plantations. This is the first trial of this kind, so these results are very promising. The similarity of results between poplar and hazelnut plantations indicates the wide potential of mating disruption for this generalist pest. As this is the first study evaluating the possibility of MD for controlling this ambrosia beetle, it would be worth investigating the minimum application rate and time needed to effectively disrupt mating in poplar and fruit tree plantations. Although synthetic pheromones are not expensive the application is labor intensive. Therefore, decreasing the application rate could have a significant impact on the overall cost of the treatment for forest producers. Also in this sense, it is critical to have an effective monitoring schedule to detect the beginning of the flying period with pheromone baited traps in order to maximize the cost-benefits of the control treatment.

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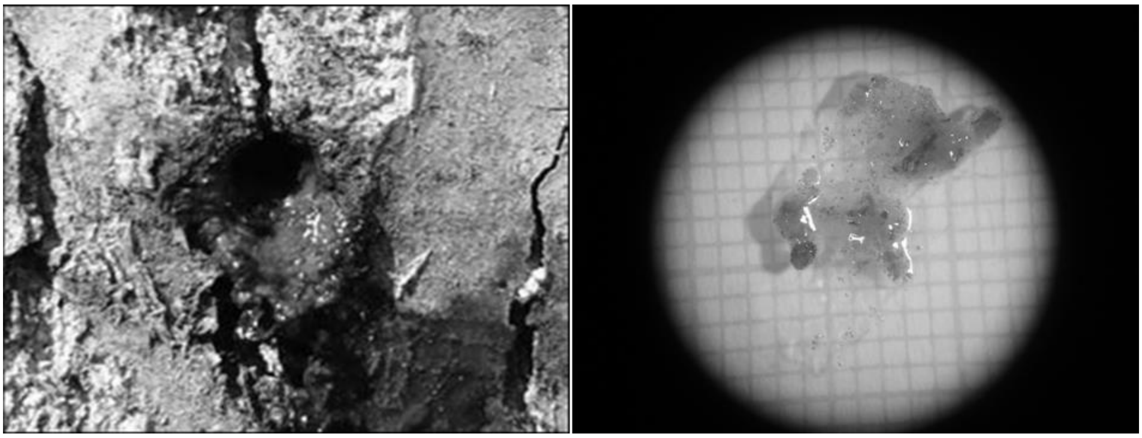
Fig. 1. Left: Active galleries (AG). Right: microscopic view of the sawdust.

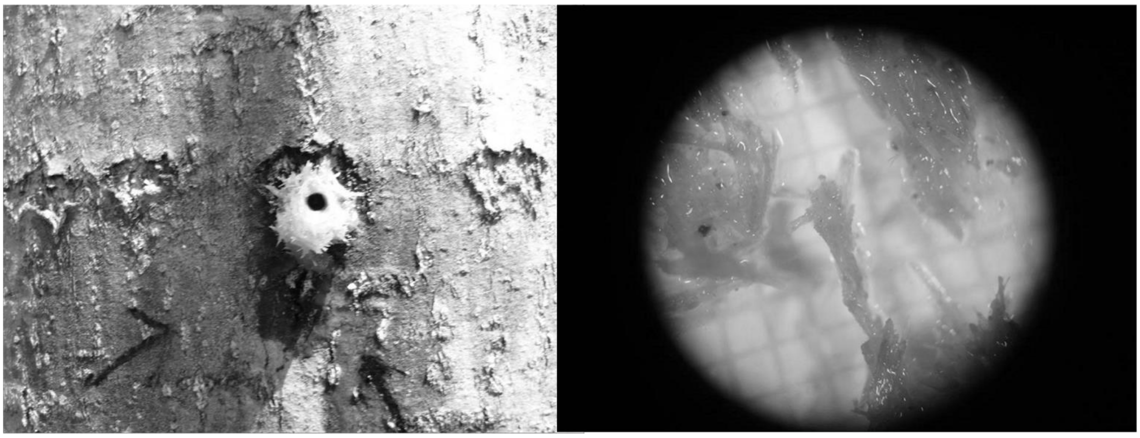
Fig. 2. Left: calling male sawdust crown arrangement (CM). Right: microscopic view of the sawdust.

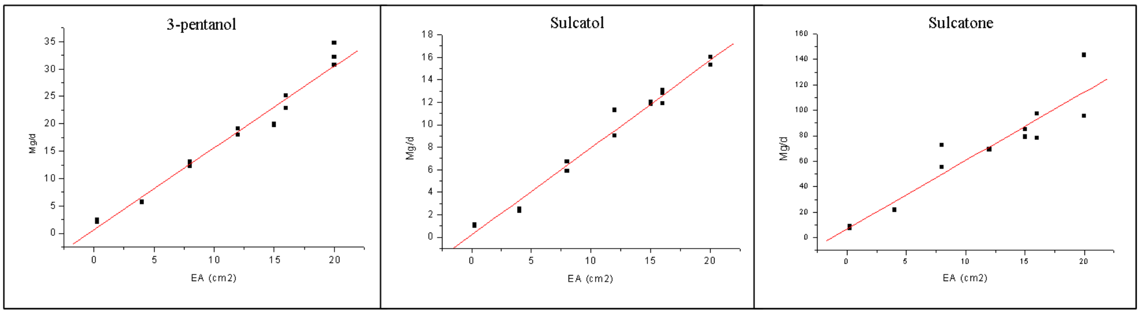
Fig. 3. Correlation between pheromone release rate and effective area in laboratory conditions of plastic pheromone lures. (Y= ax+b, R^2 are means of three replicates. For 3-pentanol, a=1.49, b= 0.69, $R^2 = 0.97$, $p < 0.0001$; sulcatol, a= 0.78, b=0.18, $R^2 = 0.98$, $p < 0.0001$; sulcatone, a= 5.38, b= 0.77, $R^2 = 0.90$, $p < 0.0001$).

Fig. 4. Number of female *Megaplatypus mutatus* captured per day during the 2007-2008 season in Junín, Buenos Aires, using pheromone baited traps. Arrows indicate the duration of the mating disruption trial. ITD: insects per trap per day.

Fig. 5. Number of female *Megaplatypus mutatus* captured per day during the 2008 season in Campania, Italy, using pheromone-baited traps. Arrows indicate the duration of the mating disruption trial. ITD: insects per trap per day.






3-pentanol
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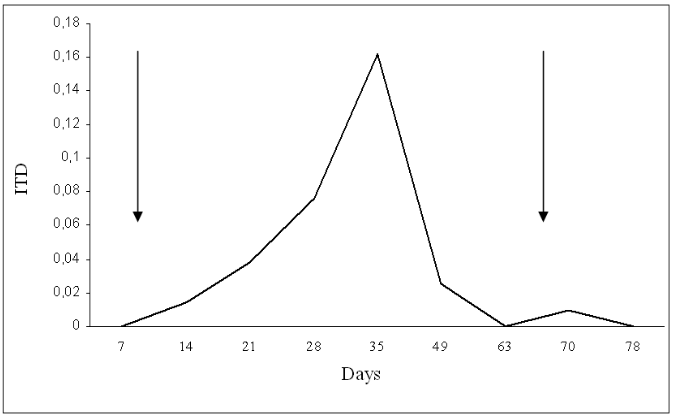
Mg/d
EA (cm2)


ITD
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
7
14
21
28
35
49
63
70
78
Days

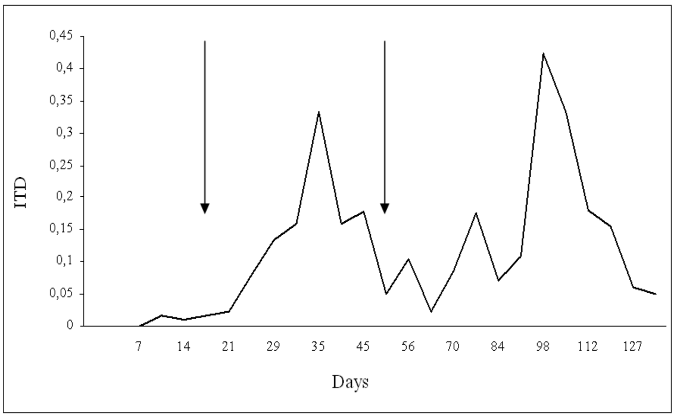

0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
ITD
7
14
21
29
35
45
56
70
84
98
112
127
Days

Table 1. Daily release rate under laboratory conditions of plastic pheromone lures containing (+)-sulcatol, sulcatone or 3-pentanol. Linear correlation coefficients R^2 are calculated from the means of three replicates.

Table 2. Mean number of active galleries (AG) per tree in infested control and pheromone treated plots of poplar plantation before and after the trial in Junín, Buenos Aires, Argentina. Comparisons are valid within the same column. Means followed by the same letter are not significantly different ($P < 0.05$).

Table 3. Mean number of active galleries (AG), live calling males (CM) and effective mating galleries (MG) per tree in infested control and pheromone treated plots of the poplar plantation in Caserta, Italy. Comparisons are valid within the same column. Means followed by the same letter are not significantly different ($P < 0.05$).

Table 4. Mean number of active galleries (AG), calling male galleries (CM) and effective mating galleries (MG) per tree in infested control and pheromone treated plots of hazelnut plantation in Caserta, Italy. Comparisons are valid within the same column. Means followed by the same letter are not significantly different ($P < 0.05$).

Compound	Effective release area (cm^2)	Release rate (mg/day)	R^2
Sulcatone (6–methyl-5-hepten-3-one)	12	63.2	0.98
3- pentanol	64	91.2	0.99
(±) Sulcatol ((±) -6-methyl-5-hepten-3-ol)	128	79.4	0.99

	AG/tree before trial	MG/tree after trial
Control	0,50 ± 0,052^{a}	0,41 ± 0,046^{a}
Treated	0,45 ± 0,051^{a}	0,096 ± 0,023^{b}

	AG/tree before trial	CM/tree after trial	MG/tree after trial
Control	1,96 ± 0,36[a]	1,60 ± 0,28[a]	0,66 ± 0,17[a]
Treated	1,53 ± 0,27[a]	2,06 ± 0,31[a]	0,23 ± 0,07[b]

	AG/tree before trial	CM/tree after trial	MG/tree after trial
Control	4,54 ± 0,89^{a}	2.45 ± 0,67^{a}	2,08 ± 0,35^{a}
Treated	4,08 ± 0,65^{a}	1.58 ± 0,31^{a}	0,92 ± 0,31^{b}