Synthesis and Field Evaluation of the Sex Pheromone of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) in Canola (*Brassica napus* L.)

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Summary

The diamondback moth *Plutella xylostella* (L.) is known to cause economic damage to rapeseed, cabbage, and other cruciferous crops worldwide. Sex pheromone components of *P. xylostella*, including (*Z*)-11-hexadecenal, (*Z*)-11-hexadecenol, and (*Z*)-11-hexadecenyl acetate, were synthesized in a concise and divergent fashion in yields appropriate to develop field tests. Two blends were prepared from these three components (8:18:100 and 10:1:90), and they were used to evaluate the number of *P. xylostella* adult male captures in commercial canola fields. Our results indicate that both blends were effective at attracting the microlepidoptera. Furthermore, they show that this type of pest-specific method could lead to the development of sustainable management strategies to rationalize the use of pesticides.

Keywords: canola, *Plutella xylostella*, sex pheromone, synthesis, (*Z*)-11-hexadecenal

Resumen

Sintesis y evaluación a campo de la feromona sexual de *Plutella xylostella* (L.) (Lepidóptera: Plutellidae) en canola (*Brassica napus* L.)

La polilla dorso de diamante *Plutella xylostella* (L.) causa daños económicos en canola y otras crucíferas. Los componentes de la feromona sexual de *P. xylostella*, (*Z*)-11-hexadecenal, (*Z*) -11-hexadecenol y acetato de (*Z*)-11-hexadecenilo, fueron sintetizados por medio de una estrategia concisa y divergente, obteniéndose cantidades adecuadas para el desarrollo de ensayos de campo. Dos formulaciones fueron preparadas a partir de los tres componentes (8:18:100 e 10:1:90) para evaluar el número de machos adultos de *P. xylostella* capturados en un campo comercial de canola. Nuestros resultados indican que ambas formulaciones fueron eficaces en atraer al microlepidóptero. Los resultados obtenidos son promisorios y muestran que este método específico para plagas permite el desarrollo de estrategias de manejo sustentable para racionalizar el uso de pesticidas.

Palabras clave: canola, *Plutella xylostella*, feromona sexual, síntesis, (*Z*)-11-hexadecenal

Introduction

Canola (*Brassica napus* L.) is a cruciferous oleaginous with high production levels worldwide, being the second most important oleaginous crop after soybean (FAO, 2013). Due to its ability to grow and develop in low temperatures, it is one of the few oleaginous adaptable to wide extensions. It is characterized for possessing oil with excellent quality for human consumption and for the preparation of biofuel, as well as an extraction residue with high protein level for animal feed. In Uruguay, canola appeared as a promising alternative for crop rotation instead of the usual winter cereals. As a matter of fact, this practice has been promoted by Alcoholes del Uruguay (ALUR) for biodiesel, glycerin, and protein wheat production in an effort to achieve high levels of bio-renewable energy (Uruguay. Poder Legislativo, 2007). These types of policies allowed Uruguay to develop an energy matrix in which biomass is represented by 30 %, in line with the Kyoto protocol, by contributing with the sustainable development of the region.

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is one of the most destructive insects of cruciferous plants throughout the world (Talekar and Shelton, 1993), being the major pest of canola in South America and other regions. This microlepidoptera has a wide distribution due to its ability to adapt to a broad range of weathers and to cover large expanses through migrations (Nguyen *et al.*, 2014). The lack of effective natural enemies is considered to be the major cause of the high pest status in most parts of the world (Lim, 1986). During bloom, and until the start of the development of pods, canola field monitoring must be performed in order to check for *P. xylostella* larvae. Early monitoring of adults and larvae, and the rational use of insecticides when populations are above threshold levels, is crucial for pest management and to avoid economic loss by diamondback moth damage. Chemical control in canola is particularly challenging, as insecticides are required in the fructification stage and this coincides with the presence of beneficial insects and pollinators which frequent the crop. For this reason, the choice of selective insecticides is essential. Moreover, the effectiveness of insecticides has been reduced due to the development of resistance to many of these compounds (Lee *et al.*, 1993; Chung *et al.*, 1997). *P. xylostella* is known for having resistance to pyrethroids (Liu *et al.*, 1982), organophosphorus (Miyata *et al.*, 1982), growth regulators (Lin *et al.*, 1989), and *Bacilus thuringensis* (Hama *et al.*, 1992), among others. Indeed, the diamondback moth was the first insect found to have become resistant to *B. thuringensis* toxin in the field (Tabashnik *et al.,* 1997; Safraz *et al.*, 2005; Grzywacz *et al.*, 2010). Therefore, alternative control strategies to the sustained use of synthetic insecticides are urgently needed.

With the use of sex pheromones, the population of insect pests can be reduced, the presence of insects of agricultural interest may be detected, the behavior of pest populations can be learned, and decisions can be made regarding the adequate use of insecticides (Zarbin *et al.*, 2007). The *P. xylostella* sex pheromone can be used for monitoring as well as for mating disruption of this species (Yang *et al.*, 2007). Pheromone traps have been used to monitor adult populations, and can predict the next generation of larval population peaks occurring 11 to 21 after peak adult trap catch (Miluch *et al.*, 2013). The sex pheromone of *P. xylostella* was first isolated from female abdominal preparations (Chow *et al.*, 1974), and later identified (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) as the components in the female pheromone gland extract (Tamaki *et al.*, 1977). The addition of (*Z*)-11-hexadecenol (*Z*11-16:OH) further synergized male response to the first two components (Koshihara and Yamada, 1980), and it was later shown to be a minor pheromone component (Suckling *et al.*, 2002). Analyses of the female gland extract (Yang *et al.*, 2007) demonstrated that the ratio of *Z*11-16:Ald, *Z*11-16:OH, and *Z*11-16:Ac found was 8:18:100, respectively. However, significant geographical variations in sex pheromone production and response have been reported in *P. xylostella* (Suckling *et al.*, 2002; Chisholm *et al.*, 1979). Different research groups have used different ratios of the main pheromone components during tests or in pheromone monitoring. In South Korea (Yang *et al.*, 2007), researchers found that the ternary blend of *Z*11-16:Ald, *Z*11-16:OH, and *Z*11-16:Ac at a ratio 10:1:90 was more effective at caching *P. xylostella* males than the natural ratio and other reported blends (Figure 1).

Due to the worldwide distribution and pest status of the diamondback moth, populations are monitored in brassicaceous cropping systems around the world. Synthetic sex pheromone lures to monitor adult diamondback moth

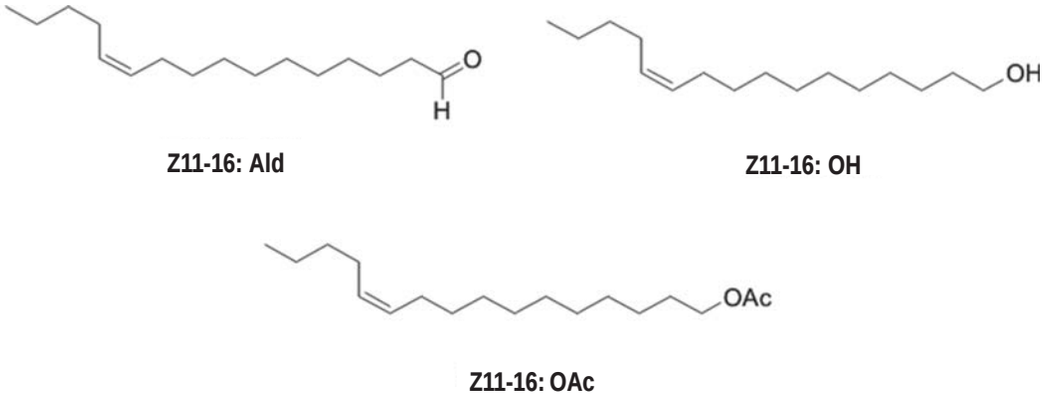

Figure 1. Chemical components of the sex pheromone of *P. xylostella*.

populations are available from many different companies, but the efficacy of these lures is variable (He *et al.*, 2003), and the information regarding the comparison between different available lures in one geographic location is scarce (Evenden and Gries, 2010). The promotion of canola crops in Uruguay for biodiesel production led us to compare the attractiveness of different synthetic pheromone blends to diamondback moth males for their potential use as a monitoring system. In this report we present a concise and divergent synthesis of the three pheromone components, as well as field tests with two different blends, to evaluate the number of *P. xylostella* adult male captures in commercial canola fields.

Materials and Methods

Synthesis

The three components of the sex pheromone of P. xylostella, (Z)-11-hexadecenol, (Z)-11-hexadecenal and (Z)-11-hexadecenyl acetate (Chow *et al*., 1974; Suckling *et al.*, 2002), were prepared according to the below synthetic protocol, in order to perform field experiments.

Preparation of 11-hydroxyundecyltriphenylphosphonium bromide (2): 11-bromo-1-undecanol (1, 24.7 g, 98.3 mmol) and triphenylphosphine (38.6 g, 147.5 mmol) were dissolved in absolute etanol (100mL), and the mixture was heated to reflux for 48 h. Crystallization by addition of hexanes and wash of the solid obtained with hexanes gave pure 11-hydroxyundeciltriphenylphosphonium bromide in 95 % yield.

Preparation of (Z)-11-hexadecenol (3): 11-Hydroxyundeciltriphenylphosphonium bromide (2) (1.5 g, 2.9 mmol) and solid potassium carbonate (0.4 g, 2.9 mmol) were dissolved in the minimum amount of tetrahydrofuran, and a tip of spatula of 18:crown:6 ether was added at 0 ºC. Pentanal (0.2 mL, 2.7 mmol) was added and the reaction mixture was heated to reflux for 12 hours. The solvent was distilled under vacuum and the residue purified by column chromatography on silica gel using hexane: ethyl acetate (1:1) mixture, to obtain (Z)-11-hexadecenol in 82 % yield.

Preparation of (Z)-11-hexadecenal (4): (Z)-11-hexadecenol (3) (0.78 g, 3.24 mmol) and pyridinium chlorochromate (PCC) (1.04 g, 4.86 mmol) were stirred in CH_2Cl_2 (30 mL) at room temperature for 1 hour. The reaction mixture was then filtered through celite, the solvent evaporated, and the residue purified by column chromatography on silica gel using hexane: ethyl acetate (9:1) mixture, affording 4 in 70 % yield.

Preparation of (Z)-11-hexadecenyl acetate (5): (Z)-11-hexadecenol (3) (2.65 g, 11 mmol) was dissolved in dry dichloromethane under a nitrogen atmosphere. Triethylamine (9.2 mL, 66 mmol), acetic anhydride (3.1 mL, 33 mmol) and a spatula tip of 4-dimethyaminopyridine were added while stirring the solution in an ice bath. Upon finished the reaction (1 hour), the crude was washed with a cold aqueous saturated solution of sodium carbonate and with 5 % hydrochloric acid aqueous solution. The product was purified by column chromatography using a hexane: ethyl acetate (9:1) mixture, affording 5 in a 90 % yield.

Once these tree compounds ((*Z*)-11-hexadecenol, (*Z*)-11-hexadecenal and (*Z*)-11-hexadecenyl acetate) were synthesized, two blends were prepared with different composition, for their use as treatments in field experiments.

Field Experiments

Pheromone blends were evaluated in white rubber septa (Sigma-Aldrich, white, 8 mm O.D.) placed in the center of handcrafted plastic Delta traps (15 × 15 × 25 cm). The traps were distributed within a commercial canola field (56 ha) situated in Paysandú, Uruguay (32.5 ° South, 57.8 ° West), planted with the Rivette variety in furrows. No insecticides were applied throughout the crop cycle. The experiment was conducted for seven weeks during spring time. Larvae density before installing the traps was estimated by individual plant examination (Dosdall *et al.*, 2011). The treatments were blends of the synthetic pheromone (*Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac) in 8:18:100 and 10:1:90 composition, with a total septum load of 0.1 mg in 100 µL of hexane. The choice of the septa load was made in agreement with previous reports (Miluch *et al.*, 2014; Yang *et al.*, 2007). Septa loaded with hexane were used as controls. The experiment was arranged in a randomized block design, with seven blocks separated by 60 m, and 30 m trap separation within a block. The traps were hanged just above the height of the foliage. A global positioning system was used for the location of traps. Male captures were checked weekly. The sticky bases of the traps were changed as needed, according to the number of males captured. From the fifth week, new treatments were added to the seven blocks in order to check the effect of volatility or decomposition in the sex pheromone components in field conditions.

Statistical Analysis

For each capture date, a generalized mixed model was fitted. The number of captured males was the response variable, the treatment was considered as a fixed factor and the block as a random factor. Data were analyzed using package «lme4» and provided in the R statistical software. Means

between treatments were compared using «multcomp» package and considered significant if $p < 0.05$ in Tukey´s test.

Results and Discussion

The three components of *P. xylostella* sex pheromone are (Z)-unsaturated long chain related compounds. The general synthetic scheme involved a stereoselective Wittig reaction performed in Boden conditions, and further oxidation or acylation. In this way, the synthesis of (Z)-11-hexadecenol started from commercially available 11-bromoundecanol (1), with the formation of the corresponding phosphonium salt (2). This was followed by Wittig reaction between 2 and valeraldehyde, which yielded the sex pheromone component (Z)-11-hexadecenol in 82 % combined yield for the two steps. The (Z) isomer is here obtained with high stereoselectivity, with a (Z)/(E) ratio of 9/1. The final stage of the synthetic sequence is the oxidation of (Z)-11-hexadecenol to the pheromone component (Z)-11-hexadecenal (Sellanes *et al.,* 2010) using pyridinium chlorochromate as oxidizing agent in 70 % yield. Acetylation of (Z)-11-hexadecenol with acetic anhydride in the presence of triethylamine and 4-dimethylaminopyridine afforded (Z)-11-hexadecenyl acetate in excellent yield (90 %) (Figure 2).

The pheromone components were prepared through a concise synthetic design based on a Wittig olefination, and further functionalization of the oxygenated moiety. This strategy avoids the use of protecting groups and does not afford complex mixtures of stereoisomers. Overall, the desired pheromone components were prepared in a stereoselective fashion, using a concise divergent three step synthesis with overall yields ranging from 54 to 78 % for each component, therefore representing a more efficient approach than those previously reported (Szantay *et al.*, 1981; Zong *et al.*, 2011; Xun *et al.*, 1985).

Field test were performed in an infested commercial canola field, and the treatments were two blends of the synthetic pheromone (*Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac) in 8:18:100 and 10:1:90 ratio (Figure 3).

Field experiments were carried out at the blooming stage when the population of *P. xylostella* was settled and there was an average of 3,2 larvae per plant. This value is higher than the action threshold for chemical control in canola (Western Committee on Crop Pests, 2010). At the initial sampling time, one week old lures baited with synthetic pheromone in the two tested ratios (Z11-16:Ald, Z11-16:OH, Z11-16:Ac), catched a significantly higher number of moths than the control traps baited with hexane (Tukey's test, $p < 0.05$). At this point, the 10:1:90 blend was more attractive than the 8:18:100 blend (Tukey's test, $p< 0.05$). From the second to the fourth week, both pheromone treatments captured more moths than the control, but there were no differences between them (Tukey's test, $p < 0.05$). The efficiency of the 10:1:90 blend has been previously reported with cabbages (Yang *et al.,* 2007). This finding is in line with a previous report (Evenden and Gries, 2010), that studied the release rate of these pheromone components in field tests, showing that there is an initial burst of pheromone release that is diminished over the time under field conditions. Both blends have Z11-16:Ac as the main component, in line with the observation of Miluch *et*

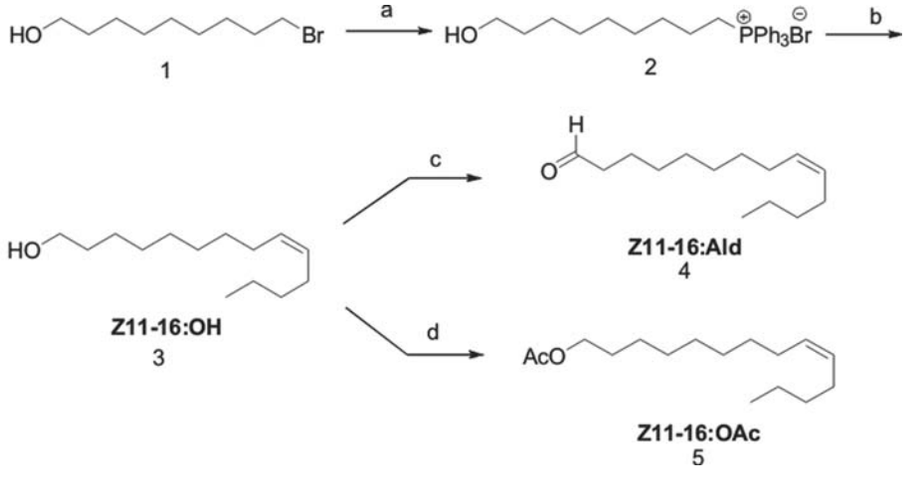

Figure 2. Synthesis of the three components of the sex pheromone of *P. xylostella.*
(a) PPh_3, EtOH, reflux, 48 h. (95 %); (b) valeraldehyde, K_2CO_3, 18-crown-6, THF, reflux, 12 h. (82 %); (c) PCC, CH_2Cl_2, rt, 1h. (70 %); (d) Ac_2O, NEt_3, DMAP, CH_2Cl_2, rt, 1h. (90 %).

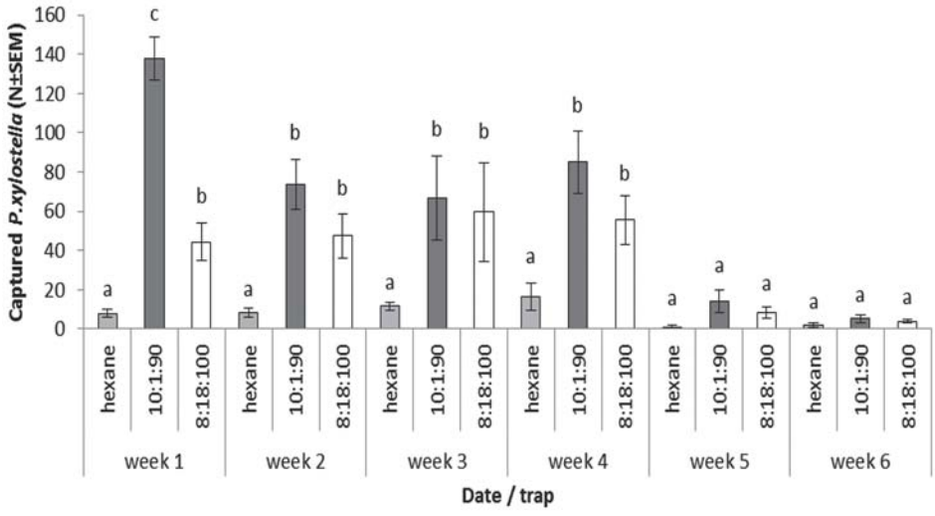

Figure 3. *P. xylostella* adult captures in canola field trial with different pheromone blends. Letters show post-hoc Tukey HSD test at 5 %.

al. (2014), that points that this main component attracts significantly more moths than those in which Z11-16:Ald is the main component. The 8:18:100 blend, which mimicked the ratio found in female gland extracts, has Z11-16:OH as it second major component, while the 10:1.90 blend has Z11-16:Ald as it second major component. As reported earlier (Môttus *et al.*, 1997), the aldehyde component (Z11-16:Ald) is volatilized and decomposed faster than the other components. Therefore, after the second week in field conditions, the blend composition may be altered approaching a similar ratio between the two treatments, showing similar efficacy. Differences between hexane and pheromone treatments were detected until the fourth week of sampling when none of the traps captured a significant number of male moths. This is in agreement with the end of the last generation of *P. xylostella* at the ripening stage of the crop. In order to confirm that this last observation was not due to the loss of pheromone components in field conditions, new treatments were added to the seven blocks at the fifth week. This strategy was chosen over septa replacement because several reports pointed that the traps baited with lures aged under field conditions showed better results in comparison to traps baited with fresh lures (Miluch *et al.*, 2013; Evenden and Gries, 2010). No significant differences were observed between these new set of treatments and those installed earlier (8:18:100 blend: z=-1,77, p=0,39; 10:1:90 blend: z=-1,23 p=0,72).

Monitoring of diamondback moth in canola throughout the crop cycle is usually conducted using sweep net sampling or individual plant examinations. The first method does not provide an idea of larvae density, therefore the most accurate method of estimating this pest population density in canola is to perform counts of diamondback moth specimens per plant (Leoncelli *et al.*, 2013; Miluch *et al.*, 2013). Finding larvae on each plant is a difficult and slow process which involves a high sample effort, especially at the first stage of the larvae when mine within the leaf. Selective chemical intervention is effective until the third larval instar of the pest and pheromone traps are a valuable tool to monitor their development. Miluch *et al.* (2013) determined that when *P. xylostella* populations are established, moth capture from one pheromone-baited trap per field can predict larval populations, and this information can be used to determine the optimal initial sampling date.

Considering that females of *P. xylostella* usually mate and oviposit in the first 24 h of life (Talekar and Shelton, 1993), the first capture in pheromone-baited trap can be used as the date to begin rate summation to apply the stochastic model proposed by Marchioro *et al.* (2016). This model accurately estimates the larval emergence of *P. xylostella* under field conditions in southern Brazil and could offer a promising tool for Integrated Pest Management purposes, although it has to be tested in canola at these latitudes. Moth captures by pheromone traps will allow the implementation of sequential sampling for larval in canola using the model proposed in Argentina by Lietti *et al.* (2014).

Our results suggest than both blends placed on Delta sticky traps have the potential to be developed for commercial use to monitor diamondback moth population. Future

research should determine the optimum timing of insecticide applications using threshold captures in sex pheromone traps, as it was determined for *P. xylostella* in cabbage (*Brassica oleracea* var capitata) and cauliflower (*B. oleracea* var botrytis) (Reddy and Guerrero, 2001).

Conclusions

The three components of the sex pheromone of *P. xylostella* were synthesized through an efficient methodology that allows for future scale-up. The two blends employed in canola field tests were effective. The use of a specific monitoring method for this pest will allow choosing the proper time to use a selective insecticide (i.e. chitin synthesis inhibitor) and the development of a sustainable management strategy in a crop which, as seen by its explosive growth, has gained popularity among producers. Further work is needed in order to develop a monitoring strategy for massive use. Parameters such as septum load and duration, trap height and color, as well as threshold capture levels, need to be optimized for a final monitoring technology (Môttus *et al.*, 1997; Miluch *et al.*, 2013, 2014). It was shown that the two key factors are possible to achieve: a convenient and scalable synthetic route and the capture of males in field traps baited with synthetic pheromone. Field tests performed in this region have an added-value, since significant geographical variations in sex pheromone production and response have been reported for this species (Miluch *et al.*, 2014; Yang *et al.*, 2007). Therefore, population monitoring of *P. xylostella* in canola crops is practical and feasible, and may result in a significant reduction in insecticide use when chemical control is employed preventively.

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