DEPARTAMENTO DE AGRONOMÍA

Escuela Técnica Superior de Ingeniería Agronómica y de Montes

Departamento de Agronomía

TESIS DOCTORAL

**Compatibilidad de Reguladores del Crecimiento de los Insectos (RCI)
con el depredador *Chrysoperla carnea* (Neuroptera: Chrysopidae)
y las interacciones con insecticidas microbianos
para el control de *Spodoptera littoralis* (Lepidoptera: Noctuidae)**

DOCTORANDA: YURANY ANDREA SUÁREZ LÓPEZ

DIRECTOR: PROF. ENRIQUE VARGAS OSUNA

TITULO: *COMPATIBILIDAD DE REGULADORES DEL CRECIMIENTO DE LOS INSECTOS (RCI) CON EL DEPREDADOR CHRYSOPERLA CARNEA (NEUROPTERA: CHRYSOPIDAE) Y LAS INTERACCIONES CON INSECTICIDAS MICROBIANOS PARA EL CONTROL DE SPODOPTERA LITTORALIS (LEPIDOPTERA: NOCTUIDAE)*

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Tesis presentada por Yurany Andrea Suárez López en satisfacción de los requisitos necesarios para optar al grado de Doctor por la Universidad de Córdoba, dirigida por el Profesor D. Enrique Vargas Osuna

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Agricultural, food, forestry and sustainable rural development engineering

**Compatibility of Insect Growth Regulators (IGRs) with the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae)
and interactions with microbial insecticides for the control of *Spodoptera littoralis* (Lepidoptera: Noctuidae)**

Thesis presented by Yurany Andrea Suárez López to qualify for the degree of
Doctor by the University of Cordoba

Prof. Dr. D. Enrique Vargas Osuna   **Yurany Andrea Suárez López**

TÍTULO DE LA TESIS: Compatibilidad de Reguladores del Crecimiento de los Insectos (RCI) con el depredador *Chrysoperla carnea* (Neuroptera: Chrysopidae) y las interacciones con insecticidas microbianos para el control de *Spodoptera littoralis* (Lepidoptera: Noctuidae)

DOCTORANDA: YURANY ANDREA SUÁREZ LÓPEZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Los objetivos de la presente Tesis han sido: 1) determinar los efectos de Reguladores de Crecimiento de Insectos (lufenurón y tebufenocida) sobre los estados de desarrollo del depredador *Chrysoperla carnea*; 2) Estudiar las interacciones de ambos insecticidas químicos con agentes microbianos (NPV, *Bacillus thuringiensis* y *Beauveria bassiana*) en larvas de *Spodoptera littoralis*.

Para ello se han establecido unas metodologías y plan de trabajo que se han desarrollado según los plazos previstos, lo que ha permitido obtener los resultados adecuados para publicar un artículo y que otros dos estén en vías de publicación.

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La Tesis cumple los requisitos de calidad necesarios para su exposición y defensa pública.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 15 de Febrero de 2021

Firma del director

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RESUMEN

En la actualidad se buscan alternativas de control de plagas con menor impacto ambiental y que impliquen un menor riesgo para el entorno, para lo cual se ha de evaluar la compatibilidad entre diferentes agentes de control que puedan ser incorporados en la gestión integrada de plagas.

Spodoptera littoralis (Boisduval) es un lepidóptero de la familia Noctuidae cuyas larvas se desarrollan a expensas de numerosas plantas, causando daños importantes en cultivos del área mediterránea como algodón, alfalfa y hortícolas, entre otros. Con objeto de encontrar nuevas posibilidades para el control eficaz de esta especie, se ha estudiado en primer lugar la compatibilidad de insecticidas de síntesis del grupo de los reguladores del crecimiento de los insectos (RCI) (lufenurón y tebufenocida) con el depredador generalista *Chrysoperla carnea* (Siemens). Para ello, se realizaron ensayos en laboratorio para evaluar los efectos letales y subletales de la aplicación tópica sobre huevos y larvas, el efecto del consumo de presa tratada sobre el desarrollo y mortalidad del depredador, así como la preferencia entre presas tratadas y no tratadas. Además, se estudiaron los efectos en el potencial reproductor de adultos tratados por ingestión. En segundo lugar, se ha estudiado la interacción en larvas de *S. littoralis* de cada uno de los RCI cuando son aplicados a bajas dosis con diferentes agentes de control microbiano (*Bacillus thuringiensis* var. *aizawai*; un aislado nativo de *Beauveria bassiana*; y el nucleopoliedrovirus SlNPV).

Los resultados con lufenurón (inhibidor de la síntesis de la quitina) evidenciaron que el tratamiento de huevos de *C. carnea* no afectó la tasa de eclosión y la supervivencia de las larvas recién nacidas. Por el contrario, presentó alta toxicidad para larvas de segundo estadio, con una Concentración Letal Media (CL_{50}) de 0,0153 ml/L (0,00860-

0,0236 ml/L). Las larvas que sobrevivieron al tratamiento no mostraron alteraciones en el tiempo de desarrollo larval ni el periodo de pupación.

Por otro lado, las larvas que consumieron presa tratada con lufenurón manifestaron una reducción significativa en el tiempo de desarrollo larval a la dosis más alta (1ml/L) y un elevado porcentaje de mortalidad en el estado de pupa (83,3% a la dosis de 0,1 ml/L y 100% a la dosis de 1ml/L). En ensayos de preferencia de presa, las larvas de *C. carnea* tuvieron una significativa tendencia a consumir larvas tratadas frente a no tratadas, tanto en la primera elección como en la segunda. La ingestión de lufenurón por los adultos, a la dosis recomendada en campo, no afectó a la fecundidad de las hembras ni a las longevidades de ambos sexos, pero sí redujo significativamente el porcentaje de viabilidad de los huevos.

En el caso de tebufenocida (análogo de la ecdisona), el tratamiento de huevos no afectó su tasa de eclosión ni la supervivencia de las larvas recién nacidas. La aplicación de este análogo de la ecdisona sobre las larvas de segundo estadio causó muy baja mortalidad, aunque los tiempos de desarrollo de las larvas y pupas supervivientes disminuyeron significativamente. En los bioensayos de elección, el depredador mostró preferencia por las presas tratadas frente a las no tratadas. Además, las larvas del depredador que consumieron presas tratadas con tebufenocida redujeron significativamente el tiempo de desarrollo, aunque una vez en estado adulto no se vieron afectadas la fecundidad, la viabilidad de huevos, ni la longevidad. La ingestión de tebufenocida por los adultos no tuvo efecto significativo en la fecundidad de las hembras, viabilidad de los huevos o longevidad.

En cuanto a la combinación de cada uno de los agentes microbianos con bajas dosis de lufenurón o tebufenocida, los resultados dependieron de los agentes implicados. Los tratamientos simultáneos mostraron efectos aditivos para *B. thuringiensis* y *B. bassiana* con cada uno de los RCI. Se produjo un efecto antagonista cuando se aplicaron simultáneamente el nucleopoliedrovirus SINPV y tebufenocida. Por último,

la combinación de la SINPV y el lufenurón dio lugar a un efecto sinérgico para las dos concentraciones de baculovirus evaluadas.

Los resultados obtenidos constituyen una valiosa contribución para el control de *S. littoralis* en Programas de Gestión Integrada, en los que se incorporen estas materias activas de forma compatible y eficaz, eligiendo las mejores combinaciones y el momento más adecuado de su aplicación.

Palabras clave: Noctuidae, Neuroptera, Entomopatógenos, Tebufenocida, Lufenurón, Nucleopoliedrovirus, *Bacillus thuringiensis*, *Beauveria bassiana,* Efectos letales, Efectos subletales, Compatibilidad.

ABSTRACT

Currently, pest control alternatives with less environmental impact and less risk to the environment are being sought, for which the compatibility between different control agents that can be incorporated in integrated pest management has to be evaluated. *Spodoptera littoralis* (Boisudval) is a lepidopteran of the Noctuidae family whose larvae develop at the expense of numerous plants, causing significant damage to Mediterranean crops such as cotton, alfalfa, and horticultural crops, among others.

In order to find new possibilities for the effective control of this species, the compatibility of synthetic insecticides from the group of insect growth regulators (IGRs) (lufenuron and tebufenozide) with the generalist predator *Chrysoperla carnea* (Siemens) was first studied. For this purpose, laboratory tests were conducted to evaluate the lethal and sublethal effects of topical application on eggs and larvae, the effect of treated prey consumption on predator development and mortality, as well as the preference between treated and untreated prey. In addition, the effects on the reproductive potential of adults treated by ingestion were studied. Secondly, the interaction in *S. littoralis* larvae of each of the IGRs when applied at low doses with different microbial control agents (*Bacillus thuringiensis* var. *aizawai*; a native isolate of *Beauveria bassiana*; and the nucleopolyhedrovirus SINPV) was studied.

The results with lufenuron (chitin synthesis inhibitor) showed that treatment of *C. carnea* eggs did not affect the hatching rate and survival of newly hatched larvae. On the contrary, it presented high toxicity for second instar larvae, with a Mean Lethal Concentration (LC50) of 0.0153 ml/L (0.00860-0.0236 ml/L). The larvae that survived the treatment showed no alterations in larval development time or pupation period. On the other hand, the larvae that consumed prey treated with lufenuron showed a significant reduction in larval development time at the highest dose (1 ml/L) and a high percentage of pupal stage mortality (83.3% at the 0.1 ml/L dose and 100% at the 1ml/L

dose). In prey preference tests, *C. carnea* larvae had a significant tendency to consume treated versus untreated larvae in both the first and second choice. The ingestion of lufenuron by adults, at the recommended field dose, did not affect female fecundity and longevities of both sexes, but significantly reduced the percentage of egg viability.

In the case of tebufenozide (ecdysone analog), egg treatment did not affect egg hatching rate and survival of newly hatched larvae. The application of this ecdysone analog on second instar larvae caused very low mortality although development times of surviving larvae and pupae were significantly decreased. In the bioassays of choice, the predator showed a preference for treated over untreated prey. In addition, predator larvae that consumed tebufenozide-treated prey significantly reduced development time, although once in the adult stage, fecundity, egg, viability and longevity were not affected. Ingestion of tebufenozide by adults had no significant effect on female fecundity, egg viability, or longevity.

As for the combination of each of the microbial agents with low doses of lufenuron or tebufenozide, the results depended on the agents involved. The simultaneous treatments showed additive effects for *B. thuringiensis* and *B. bassiana* with each one of the IGRs. An antagonistic effect occurred when SlNPV and tebufenozide were applied simultaneously. Finally, the combination of SlNPV and lufenuron resulted in a synergistic effect for the two baculovirus concentrations tested.

The results obtained constitute a valuable contribution for the development of Integrated Management Programmes for *S. littoralis*, in which these active substances are incorporated in a compatible and effective way, choosing the best combinations and the most appropriate time for their application.

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Introducción general

INTRODUCCIÓN

Los insectos están naturalmente presentes en los ecosistemas y desempeñan funciones ecológicas vitales, tales como la polinización, la descomposición de materia orgánica y la regulación de las poblaciones de artrópodos equilibrando los sistemas, pero así mismo entran en interacción con las actividades humanas. En este contexto, pueden llegar a ser beneficiosos para el hombre o perjudiciales al ser causantes de pérdidas en la producción de los sistemas agrícolas y forestales, convirtiéndose en plagas (Coscolla, 2004).

Los insectos fitófagos pueden aumentar su densidad e incidencia a niveles tan elevados que afectan los rendimientos de producción de los cultivos, tanto en cantidad como en calidad. Debido a la severidad de los daños causados por las plagas, el hombre ha intensificado cada vez más la búsqueda de métodos de control, siendo la mas generalizada, la aplicación de insecticidas químicos de síntesis.

A principios del siglo XIX, en plena revolución industrial y con una población mundial creciendo de forma exponencial, se buscó replantear la actividad agrícola dándole un enfoque netamente productivista, época conocida como "revolución verde". Un concepto que se debía desarrollar era el uso de insecticidas para disponer de una serie de sustancias tóxicas que ayudaran a la lucha contra las plagas. En los inicios se usaron insecticidas inorgánicos, tales como arseniato de plomo, ácido cianhídrico o fenol (Arata, 1986). Posteriormente, sobre todo después de la II Guerra Mundial, surgió una potente industria dedicada a la síntesis de productos químicos para uso en la agricultura.

Se intensificó el uso de estas sustancias de forma inesperada con el fin de eliminar totalmente a las plagas. Décadas posteriores, se evidenció un sin número de efectos negativos ocasionados por su uso indiscriminado y abusivo: consecuencias en la contaminación del medio ambiente, en la salud de las personas y consumidores

finales de los productos vegetales, así como el desarrollo de poblaciones de insectos resistentes a las materias activas y el surgimiento de nuevas plagas, entre otros. En este periodo de transición los grupos y movimientos ecologistas hacen pública la información sobre los efectos negativos causados por los pesticidas. El final del siglo XX se caracterizó por un fuerte apoyo a los esfuerzos de la protección del ambiente por varios sectores sociales, por lo que las estrategias de control biológico ocuparon cada vez más, lugares destacados. Se pasó luego a una lucha más dirigida, basada en el uso de agentes de control biológico y el manejo de las poblaciones de insectos supervisado por técnicos especializados.

Se crean y generalizan términos como, muestreos poblacionales, umbrales de daño, presencia e incidencia de enemigos naturales y uso de buenas prácticas agrícolas. Al día de hoy han evolucionado muchos de estos conceptos y se han reunido para desarrollar los programas de Gestión Integrada de Plagas (GIP), también denominada Manejo Integrado de plagas (MIP).

La GIP consiste en el examen cuidadoso de todos los métodos de protección vegetal disponibles y la integración de medidas adecuadas. El objetivo es mantener las poblaciones de organismos nocivos por debajo del umbral de tolerancia y hacer un uso sostenible de los productos fitosanitarios y otras formas de intervención, en niveles que estén económica y ecológicamente justificados y que reduzcan o minimicen los riesgos para la salud humana y el medio ambiente.

La GIP pretende el crecimiento de un cultivo sano con la mínima alteración posible de los agroecosistemas, confiando en primer lugar en los mecanismos naturales de control de plagas. Para realizar la GIP es necesaria la utilización racional de una combinación de medidas mecánicas, físicas, culturales, biológicas, biotecnológicas y químicas de modo que la aplicación de productos fitosanitarios se limite al mínimo necesario. Estas medidas de control se deben combinar de forma inteligente con el fin

de mantener los niveles poblacionales de los fitófagos por debajo de sus umbrales económicos de daño.

En la Unión Europea, la gestión integrada de plagas está recogida en la directiva 2009/128/CE y en el Real Decreto 1311/2012 de uso sostenible de productos fitosanitarios, que determinó, el marco de actuación y la obligatoriedad de su uso a partir de enero de 2014, en la cual la producción agrícola en el territorio español se debe regir por criterios de Gestión Integrada de Plagas (GIP) para asegurar la sostenibilidad de la agricultura, la producción suficiente, sin comprometer al medio ambiente. Su enfoque es incentivar el uso de técnicas y prácticas agrícolas, que minimicen el desequilibrio del agroecosistema y preserven la vida de la fauna beneficiosa. Es necesario, para lograr este objetivo, estudiar alternativas que integren, junto al control biológico, opciones más sostenibles de control químico.

Actualmente los programas de protección vegetal procuran aumentar la compatibilidad entre métodos de control, incluso entre los métodos químicos y biológicos. Los plaguicidas que son más seguros para el medio ambiente y tienen baja toxicidad para los enemigos naturales son más útiles en la GIP.

Dentro de las plagas de mayor importancia en el área mediterránea se encuentra, *Spodoptera littoralis* (Boisduval), un lepidóptero de la familia Noctuidae cuyas larvas, extremadamente polífagas, se desarrollan a expensas de numerosas plantas, causando daños de gran importancia en cultivos como algodón, alfalfa y hortícolas, entre otros. Su importancia económica y gravedad de los daños estriba en tres características fundamentales común en numerosas especies de la familia Noctuidae: polifagia, tendencia al gregarismo y comportamiento migratorio.

Entre los agentes de control más usados en los programas de GIP para el caso de *Spodoptera littoralis* destacan enemigos naturales como, *Chrysoperla carnea* depredador generalista y los microorganismos entomopatógenos: la bacteria *Bacillus*

thuringiensis, el Nucleopoliedrovirus (NPV) y el hongo *Beauveria bassiana*. A nivel de insecticidas químicos, destacan por su baja toxicidad para vertebrados, los Reguladores del Crecimiento de los Insectos (RCI), tales como tebufenocida (análogo del receptor de la ecdisona) y lufenurón (inhibidor de la biosíntesis de la quitina), materias activas elegidas para este estudio, por formar parte de los insecticidas con mecanismos de acción exclusivos para artrópodos y bajo la hipótesis de selectividad.

1. EL FITÓFAGO

1.1. *Spodoptera littoralis*, la especie polífaga

1.1.1. Generalidades

El género *Spodoptera* (Lepidoptera: Noctuidae) está formado por más de 25 especies, entre las que destacan por la importancia de sus daños: *S. exigua* (Hübner), *S. mauritia* (Boisduval), *S. litura*, *S. frugiperda* (J.E. Smith), *S. ornithogalli* (Guenee), *S. exempta* (Walker) y *S. littoralis* (Boisduval) (Brown y Dewhurst, 1975).

La especie *S. littoralis* se distribuye por toda la cuenca mediterránea, desde la Península Ibérica hasta el Golfo Pérsico (Líbano, Irán, Irak, Jordania), incluyendo países como Italia, Grecia y Turquía, la zona norte de África, así como gran parte del centro y sureste; posiblemente con el transporte de material vegetal llegó hasta Gran Bretaña y al centro y norte de Europa continental (Figura 1), cuya presencia se ha observado en invernaderos (Carter, 1984). En España se encuentra distribuida por toda la península, Islas Baleares y Canarias, si bien los daños de mayor importancia económica son causados en Levante sobre cultivos hortícolas (Gómez de Aizpúrua y Arroyo Varela, 1994).
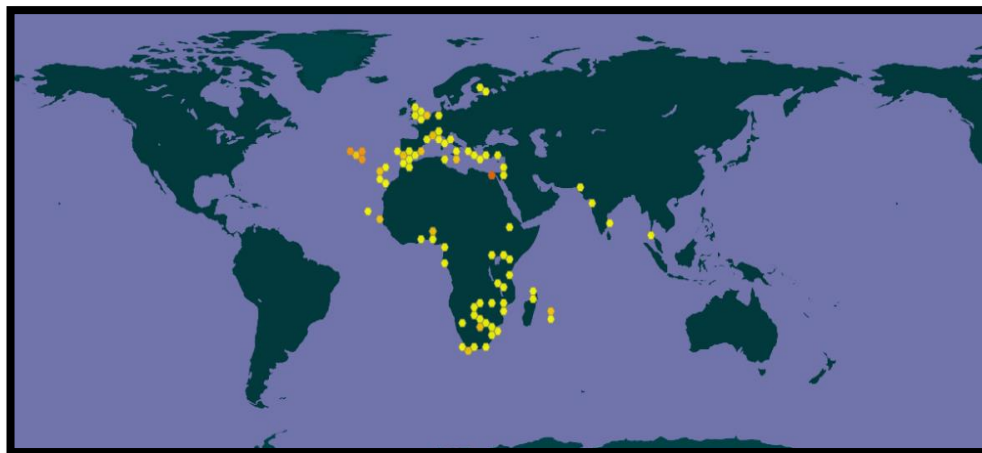
Figura 1. Distribución geográfica de *Spodoptera littoralis* a nivel mundial (GBIF, 2020)

La posición taxonómica de *S. littoralis*, según GBIF (2020), es la siguiente:

Reino	Animal
Filo	Arthropoda
Clase	Insecta
Subclase	Pterygota
Orden	Lepidoptera
Superfamilia	Noctuoidea
Familia	Noctuidae
Subfamilia	Hadeninae
Género	*Spodoptera*
Especie	*Spodoptera littoralis* (Boisduval)

Esta especie es uno de los lepidópteros más dañinos para los cultivos, pues se alimenta de un gran número plantas herbáceas de importancia económica, ya sea en campo abierto o en ambiente protegido. Se le conoce con el nombre común de "rosquilla negra" por el comportamiento de las larvas de enrollarse en espiral al sentirse amenazadas (Gómez de Aizpúrua y Arroyo Varela, 1994).

El adulto es una mariposa de 3 a 4 cm de envergadura que en posición de reposo pliega sus alas hacia atrás protegiendo su cuerpo peludo, robusto y de color pardo. Las alas anteriores, que son estrechas y alargadas, presentan una serie de dibujos ocres, entre los que resalta uno en forma de 4. Las alas posteriores son blanquecinas, translúcidas y con sus bordes anteriores y externos de color marrón (Cayrol, 1972). El abdomen termina en un mechón de pelos, más abundantes en la hembra, que los desprende en el momento de la puesta para proteger los huevos agrupados en plastones. Los huevos son casi esféricos, acentuando en su superficie unos resaltes de conformación reticular (Gómez Clemente y del Rivero, 1951). Las larvas presentan tres pares de patas torácicas y cinco pares de falsas patas abdominales.

El aspecto y coloración del cuerpo es variable según el estadio de desarrollo en que se encuentra. La larva de último estadio tiene el cuerpo de coloración negruzca y recorrido por tres líneas longitudinales amarillentas, una dorsal y dos laterales; tanto en el mesotórax como en cada uno de los segmentos abdominales se aprecian dos manchas negras en forma de media luna situadas en posición dorsal, una a cada lado de la línea longitudinal central. La parte ventral es de coloración más clara (Cayrol, 1972). La pupa es de color terroso-rojizo, ovalada y con dos pequeños ganchos al final del abdomen (Gómez Clemente y del Rivero, 1951). El color de las pupas recién formadas es verdoso, pero vira a un color rojizo en pocas horas (Gómez de Aizpúrua y Arroyo Varela, 1994).

Figura 2. Pupa y adultos (macho y hembra) de *Spodoptera littoralis* (de izquierda a derecha)

1.1.2. Biología de *Spodoptera littoralis*

Las hembras depositan los huevos en plastones alargados de 1 a 1,5 cm, que contienen entre 20 y 500 huevos, recubriéndolos con una capa algodonosa que los protege. Cada hembra pone durante su vida alrededor de 3000 huevos y el periodo de incubación de éstos oscila entre 3 y 26 días, dependiendo de la temperatura (Carter, 1984).

El número de huevos puestos por una hembra adulta está relacionado con la temperatura durante el desarrollo larvario. En laboratorio, se ha comprobado que hembras adultas que se han desarrollado a 22 ºC alcanzan una media de 1200

huevos, en tanto que si se desarrollan a 30 ºC las puestas son de 700 huevos. También se observa, que sólo un 50% de las hembras que se han desarrollado a 30 ºC son capaces de poner huevos fértiles, mientras que este porcentaje asciende al 87,5% en hembras desarrolladas a 22 ºC (Rivnay, 1961).

El desarrollo de las larvas consta de seis estadios, que en condiciones de campo completan en aproximadamente 33 días (Cayrol, 1972). Recién nacidas son gregarias, pero este tipo de comportamiento sólo dura hasta el segundo o tercer estado larvario, en cuyo momento se separan y dispersan (Gómez de Aizpúrua y Arroyo Varela, 1994). Durante las horas de mayor calor permanecen en el envés de las hojas o en la superficie del terreno, entre la hojarasca o restos vegetales, manteniendo así cierta humedad necesaria para su supervivencia. Durante la noche o en los días nublados, trepan por la planta para alimentarse, siendo una especie de extrema voracidad (Domínguez, 1993).
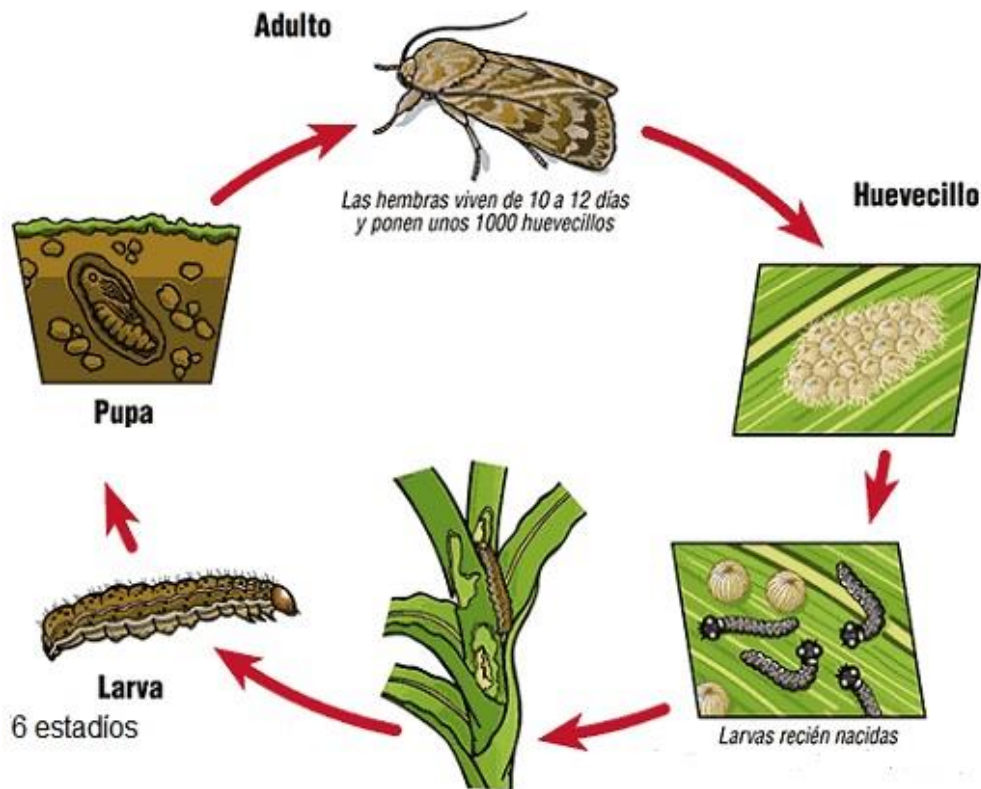
Figura 3. Ciclo biológico de *Spodoptera littoralis*

En climas cálidos, como en los países del mediterráneo, esta especie puede llegar a tener hasta siete generaciones al año. En España se admite que tiene tres generaciones, pudiéndose producir alguna más en zonas cálidas de Levante y Andalucía. Sin embargo, la comprobación del número de generaciones se complica en algunas zonas debido al carácter migratorio de la especie (Gómez de Aizpúrua y Arroyo Varela, 1994). En climas fríos, centro y norte de Europa, sólo pueden sobrevivir en el interior de invernaderos (Carter, 1984).

1.1.3. Importancia económica

Es una especie extremadamente polífaga que se alimenta de más de 87 especies de plantas de 40 familias. Es fitófago habitual en cultivos de remolacha, algodón, girasol, col, melón, pepino, arroz, maíz, alfalfa, espinacas, habas, lentejas, garbanzos, judías, ajos, naranjo, berenjena, pimiento, tabaco, melocotonero, peral, manzana, fresa, gladiolo, tomate, patata y vid. Las larvas jóvenes causan daños al alimentarse de la epidermis del envés de las hojas y las más desarrolladas consumen todas las partes verdes de la planta ya sean hojas tallos o frutos. Su actividad es crepuscular y nocturna, durante el día las larvas permanecen en el suelo entre las hojas secas y hierba (Alford, 2007). En España las larvas de "rosquilla negra" se han identificado en diversidad de cultivos, de tipo herbáceo y leñoso causando daños y pérdidas de consideración (Gómez Clemete y Rivero, 1951; Domínguez, 1993).

1.2. Métodos de control

La lucha tradicional contra *S. littoralis* se ha basado en métodos mecánicos, culturales y químicos. Como métodos mecánicos se recolectaban las larvas y los plastones para su posterior destrucción y como métodos culturales se ha usado principalmente el control de malas hierbas (Gómez Clemente y del Rivero, 1951; Domínguez, 1993). El uso de insecticidas químicos de síntesis poco selectivos ha venido siendo el método más utilizado para el control de esta especie. Sin embargo, actualmente también se dispone de insecticidas biológicos a base de entomopatógenos.

Los productos insecticidas autorizados en este momento en España para el control de especies del género *Spodoptera* son (MAGRAMA, 2020):

- Cyantraniliprol 10% + acibenzolar-s-metil 1,25% [sc] p/v
- Metaflumizona 24% [sc] p/v
- Metoxifenocida 24% [sc] p/v

- Tebufenocida 24% [sc] p/v
- Deltametrin 1,5% [ew] p/v
- *Spodoptera littoralis* nucleopoliedrovirus (SpliNPV) (aislado BV-0005) $5x10^{11}$ OBs/L (SC)
- *Bacillus thuringiensis aizawai* 50% [wg] p/p
- *Bacillus thuringiensis kurstaki* (cepa pb-54) 32% (32 mill. De u.i./g) [wp] p/p
- *Bacillus thuringiensis kurstaki* (cepa pb-54) 8% (16x10e6 u.i./g) [wp] p/p
- *Bacillus thuringiensis kurstaki* (eg 2348) 18,3% (24x106 u.i./g) [sc] p/v

1.2.1. Control químico

La aplicación de insecticidas químicos de síntesis sigue siendo el principal método para el control de esta especie. Sin embargo, la utilización continuada y exclusiva de éstos ha provocado la aparición de poblaciones resistentes a numerosas materias activas y a la limitación de sus propios enemigos naturales (El-Guindy et al., 1983; Wene y Sheets, 1961). En consonancia con el esfuerzo que se está haciendo para el uso de materias activas que además de ser eficaces sean más selectivas, se utilizan insecticidas del grupo de los reguladores del crecimiento de los insectos (RCI) aunque estos productos tampoco están exentos del desarrollo de resistencias (Ishaaya y Klein, 1990). Existe la necesidad de mejorar la eficacia insecticida con nuevas materias activas. Algunas investigaciones se han centrado en el uso de insecticidas de origen natural, como: piretrinas, azadiractina (Gómez de Aizpúrua y Arroyo Varela, 1994), spinosad (Lechuga et al., 2004), o sustancias antiapetitivas procedentes de origen vegetal, como extractos de malas hierbas (Hatem et al., 2006).

1.2.2. Control mecánico y cultural

Para el caso de *Spodoptera* spp. ha sido habitual el laboreo del suelo para exponer las pupas a enemigos naturales, el uso de mallas como cobertura de las plantas impidiendo la oviposición, la erradicación de plantas infestadas, así como de frutos, y

la eliminación de larvas en campo (Dominguez, 1993). Otras prácticas culturales incluyen la modificación en la fecha, densidad y método de siembra, o la rotación de cultivos (Ennis, 1979).

1.2.3. Control biológico

Los ecosistemas agrícolas se componen de elementos biológicos que colaboran en el control natural de las poblaciones de los insectos fitófagos. Estos enemigos naturales se agrupan en entomófagos (depredadores y parasitoides) y entomopatógenos. Los entomófagos ejercen un control natural por ser factores dependientes de la densidad del fitófago. Por su parte, la acción de los entomopatógenos se puede orientar principalmente para su uso como insecticidas microbianos (Hunter- Fujita et al., 1998; Moscardi, 1999).

Los depredadores constituyen, en general, un grupo de entomófagos con espectro de presas relativamente amplio. Sin embargo, se ha ido constatando el importante papel que ejercen en la regulación de las poblaciones de los lepidópteros. Entre ellos destacan especies de Neuroptera (Chrysopidae) y de Hemiptera (Anthocoridae, Nabidae y Reduviidae). En España destacan, en la depredación de huevos y pequeñas larvas, el neuróptero *Chrysoperla carnea* (Stephens) y los antocóridos *Orius* spp. (Gómez de Aizpúrua y Arroyo Varela, 1994), mientras que depredando larvas de mayor desarrollo hay que citar a especies de *Nabidae* (Cabello, 2008).
De la diversidad de depredadores que pueden atacar a *S. littoralis* en su área de distribución (Gómez de Aizpúrua y Arroyo Varela, 1994), algunas especies están comercializadas en España, sobresale el neuróptero *C. carnea*, entre otras de menor eficacia, como *Coccinella septempunctata* (L.), *Orius* spp., *Nesidiocoris tenuis* (Reuter) y *Macrolophus pygmaeus* (Rambur) (MAGRAMA, 2020).

Algunas de los parasitoides asociados a las poblaciones de larvas de *S. littoralis* están siendo desarrollados para su utilización como agentes de control, tal es el caso del

ichneumónido *Microplitis rufiventris* (Kok.) (Abdel-Rahman et al., 2004; Khafagi y Hegazi, 2004). Otras especies de interés son el bracónido *Meteorus pulchricornis* (Wesmael) y el ichneumónido e *Hyposoter didymator* (Thünberg) (Caballero et al., 1990; Oballe et al., 1995).

Los agentes entomopatógenos que afectan a este fitófago pueden ser utilizados con una tecnología de aplicación similar a la de los insecticidas químicos. La bacteria *B. thuringiensis* fue el primer insecticida microbiano que alcanzó la fase comercial, con una eficacia comparable a la de los productos de síntesis (Beegle y Yamamoto, 1992), por lo que existen en el mercado biopreparados a base del complejo espora-cristal de *Bacillus thuringiensis* subsp*. kurstaki* y subsp. *aizawai* para combatir las plagas de *S. littoralis* en cultivo bajo plástico y al aire libre (De Liñan, 2020; MAGRAMA, 2020)

Los virus aislados de poblaciones larvarias de *S. littoralis* pertenecen a la familia Baculoviridae: *Nuclepoliedrovirus* (NPV) y *Granulovirus* (GV) (Martignoni e Iwai, 1986). Varios de estos aislados han sido caracterizados y su actividad insecticida evaluada en ensayos de laboratorio y de campo presentando buenas aptitudes como agentes de control de esta especie *(*Abul-Nasr y El-Nagar, 1980; Jones y McKinley, 1986; Vargas-Osuna y Santiago-Álvarez, 1988; Caballero et al, 1992; Hatem et al., 2011). Actualmente, un formulado insecticida que usa como ingrediente activo un aislado del NPV de *S. littoralis* está comercializado en España para el control de esta especie*.*

2.   EL DEPREDADOR *Chrysoperla carnea*

2.1. La Familia Chrysopidae

Los crisópidos (Neuroptera: Chrysopidae) son uno de los grupos de enemigos naturales más habituales en casi la totalidad de los ecosistemas agrícolas a nivel mundial. Los crisópidos son depredadores generalistas, con distribución cosmopolita,

que se alimentan de fitófagos en numerosos cultivos tropicales y subtropicales. Desde hace tiempo se ha reconocido que las crisopas cumplen la mayoría de los requisitos para ser agentes eficaces de control biológico (New, 1975; Senior y McEwen, 2001): se alimentan de una importante gama de plagas y son depredadores eficientes, la cría en masa es manejable y su desarrollo es rápido, y su acción es compatible con varias estrategias de manejo de plagas (Pappas et al., 2011). Además, su distribución mundial los hace candidatos a ser utilizados en casi cualquier ecosistema agrícola (New, 1984).

La gran mayoría de las investigaciones sobre la aplicación de control biológico aumentativo basado en el uso de crisopas se ha dedicado a las especies del género *Chrysoperla.* En consecuencia, hace tiempo muchos insectarios crían y comercializan estos insectos para su liberación masiva en los cultivos, como parte de una estrategia de aumento de las poblaciones (Rigway y Murphy, 1984; Tulisalo, 1984; Rigway y Murphy, 1984; Szentkiraly, 2001; Henry y Wells, 2007).

La especie *Chysoperla carnea* (Stephens) es el crisópido más abundante y común en toda el área mediterránea (Duelli, 2001) y en España (Carnard and Thierry, 2007), estando distribuida por toda la península ibérica, Islas Baleares y Canarias (Medina, 2001). Su amplia gama de presas y su voracidad, hacen de este enemigo natural un agente idóneo para programas de control integrado de plagas (Tauber et al., 2000). Esta especie se utiliza en sueltas inoculativas en cultivos hortícolas y ornamentales de invernadero en Almería (Van der Blom, 2002) y, además, aparece espontáneamente en numerosos cultivos contribuyendo al control natural de fitófagos, por lo que debe tenerse especialmente en cuenta en los programas de conservación de enemigos naturales.

2.2. Biología de *Chrysoperla carnea*

Es una especie que puede tener varias generaciones al año en función de las condiciones ambientales. Al finalizar el verano, inducida por el fotoperiodo, entra en diapausa en estado adulto, pasando el invierno en lugares secos y oscuros (Nordlund et al., 2010).

El ciclo biológico de la especie se muestra en la figura 4. Los periodos de incubación de huevos varían entre 4 y 13 días a temperaturas desde los 25 ºC a los 15 ºC. Luego las larvas recién emergidas se cuelgan del corion y descienden por medio de pedicelo del huevo. Su fase larval tiene tres estadios, cuya duración varia respecto a factores externos de clima, principalmente a la temperatura, humedad relativa y fotoperiodo, al igual que las condiciones alimentacias que también determinan el tiempo y desarrollo de las larvas (Henry et al., 2002).

Las larvas son más activas en las noches, prefiriendo durante el día ocultarse en las hojas. Cuando han completado su último estado larval comienzan a construir un capullo de seda esférico que la protegerá durante la metamorfosis y que suelen hacer en el envés de las hojas, en ocasiones en las ramas o en el suelo. Las proteínas de las que se compone la seda son muy resistentes, se produce en los tubos de malpigio y la segrega a través del ano (Canard and Volkovich, 2001). Dentro del capullo se transforman en pupa, fase que suele ser más corta en los machos, que normalmente emergerán antes que las hembras. Las pupas tienen un color blanco crema con tonos amarillos (Henry et al., 2002). Los adultos, utilizando sus mandíbulas, cortan en círculo un extremo del capullo a través del cual pueden emerger. Los adultos desarrollan sus gónadas completamente luego de unos días, momento en el cual puede empezar la reproducción. La comunicación durante el cortejo se realiza mediante vibraciones rápidas de su abdomen (Henry and Well, 2015). Las hembras inician las puestas de huevos normalmente a los tres o cuatro días después de su emergencia, en

temperatura de 30º C y fotoperiodo de 16:8 h L/O. Los adultos suelen ser más activos en horas de la tarde y noche (Canard and Volkovich, 2001).

Figura 4. Ciclo biológico de la especie *C. carnea* (huevos, larva, pupa y adulto)

2.3. Relación depredador-presa

Los tres estadios larvales depredan, pero alcanzan su mayor tasa de depredación en el tercero, siendo éste el de mayor duración y en el que puede llegar a consumir el 80% del total de su dieta (Canard et al., 1984; Henry, 2002). En general se alimentan de insectos de cuerpo blando, así como, ácaros, huevos y larvas de lepidópteros. En la interacción depredador-presa, son varios los factores que intervienen, inicialmente se da un reconocimiento por el contacto y los receptores táctiles en su mandíbula y a través de receptores químicos ubicados en las antenas y palpos labiales (Canard y Volkovich, 2001). Estímulos visuales y olfativos también participan en el proceso (Sengonca et al., 1995). Después del contacto físico y reconocimiento, la larva captura la presa con una serie de movimientos característicos para inserterla con sus mandíbulas, que son conductos huecos, con los que inyecta secreciones salivares que paralizan a la presa para luego succionar sus líquidos internos. En el caso de

pulgones puede llegar a devorar entre 15 a 35 (Canard y Duelli, 1984), para el caso de orugas se estima un promedio de 23 larvas y en el caso de huevos del género *Spodoptera*: un total de 453 durante su tercer estadio. La calidad de la presa consumida afecta directamente a la duración y peso de larvas y pupas, así como a la reproducción en adultos.

3. INSECTICIDAS SELECTIVOS

Con el desarrollo y aplicación de los insecticidas selectivos se buscan herramientas que tengan una acción específica y eficaz contra las plagas y que al mismo tiempo se minimicen los efectos adversos en el resto de los organismos (Hul y Beer, 1985).

Como alternativa al uso excesivo de moléculas de síntesis química de amplio espectro, se iniciaron investigaciones dirigidas a métodos "biorracionales", cuya estrategia de acción se basa en el conocimiento de los procesos fisiológicos y bioquímicos a niveles específicos (Vives de Quadras, 1988; Primo-Yúfera, 1991). Los estudios se han enfocado fundamentalmente en tres grupos: Reguladores del crecimiento de insectos (RCI), Insecticidas microbianos y Semioquímicos. Nos centraremos seguidamente en los dos primeros por formar parte de los objetivos de esta Tesis.

3.1. Reguladores de crecimiento de insectos

El crecimiento y la metamorfosis que sufren los insectos desde el estado de huevo al de adulto está controlado por un sistema hormonal complejo en el que intervienen principalmente dos hormonas: la hormona juvenil y la hormona de la muda o ecdisona. Secretadas en una determinada concentración y secuencia permiten el avance del proceso de muda y determinan si la muda va a ser a un nuevo estado juvenil o va a permitir la metamorfosis y la diferenciación sexual. Todo producto que incida sobre

estos procesos alterará de alguna manera las posibilidades de desarrollo y crecimiento. Los reguladores del crecimiento de los insectos actúan imitando o alterando la acción de estas hormonas, o bien perturbando la formación de la cutícula al bloquear la biosíntesis de la quitina (componente principal de la cutícula) (Nation, 2002).

Este grupo de insecticidas son generalmente de acción lenta a moderadamente lenta (IRAC, 2020), pero suelen gozar de un perfil eco-toxicológico más aceptable que los insecticidas tradicionales, debido a que actúan sobre procesos exclusivos de los artrópodos.

3.1.1. Lufenurón

La quitina es un polímero de la N-acetil-D-glucosamina, componente de la cutícula de los insectos, esencial para conferir las características físicas y funcionales del tegumento, cuya síntesis es realizada por el insecto cada vez que muda durante su crecimiento y metamorfosis. La biosíntesis de la quitina puede ser interrumpida en cualquiera de los pasos de la ruta metabólica y algunas sustancias de síntesis, principalmente las benzoilureas interfieren en este proceso y pueden utilizarse como insecticidas en la lucha contra plagas de interés agrícola, forestal o médico-veterinario. (Retnakaran, 1980).

Las benzoilureas originan efectos letales sobre estados inmaduros, principalmente por ingestión y su actividad insecticida es característica para cada producto y especie de insecto (Alaa et al., 1998). Los efectos típicos en las larvas en desarrollo son la ruptura de cutícula malformada o muerte por inanición.

Diferentes benzoilureas se han autorizado para el control de especies de lepidópteros de importancia agrícola (*Spodoptera exigua, Spodoptera littoralis, Spodoptera frugiperda, Helicoverpa armigera*, etc.), tales como diflubenzurón, clorfluazurón,

teflubenzurón, flufenoxurón y hexaflumurón y, más recientemente, lufenurón (Alaa et al., 1998; Viñuela y Marco, 1994). En América Central, las benzoilureas se usaron por primera vez en 1985, para control de poblaciones resistentes del complejo de *Spodoptera* spp. y *Trichoplusia* spp. en el algodón (Sun et al., 2015). Lufenurón es ampliamente utilizado y comercializado en el continente americano, tanto en el centro y sur de América. En la Unión Europea, se contó con el registro para uso comercial hasta el 31/12/2019 (Comisión Europea, 2021).

La molécula del lufenurón (Figura 5) es activa contra las larvas de primeros estadios de desarrollo. Actúa más por ingestión que por contacto y no tiene acción vapor. El producto es muy persistente en las hojas. Tanto el compuesto principal como sus metabolitos pueden ser considerados como poco móviles en el suelo. La adsorción es muy alta y la degradación, en ensayos de laboratorio, rápida. Su vida media en condiciones aerobias en suelo arenoso es de 23,7 días y en suelo arcilloso de 19,4 días para el anillo diclorofenil y de 13 días para el difluorofenil (De Liñan, 2020).

Sus propiedades físicoquímicas y fórmula estructural, son:

Principio Activo: Lufenurón (MoA IRAC Grupo 15)
Familia Química: Benzoylureas
Fórmula Empírica: $C_{17}H_8Cl_2F_8N_2O_3$
Nombre IUPAC: (RS)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy) phenyl]-3-(2,6-difluorobenzoyl) urea
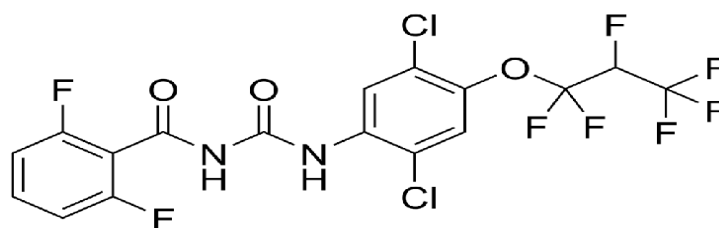
Figura 5. Estructura química de lufenurón

3.1.2. Tebufenocida

Este insecticida es una sustancia capaz de unirse al receptor celular de la hormona de la muda, por lo que pertenece al grupo de los agonistas de la ecdisona aunque su estructura no es esteroidal (Figura 6) ya que pertenece al grupo químico de las dibenzoilhidracinas. Actúa sobre la proteína receptora de la ecdisona, la cual activa y por lo tanto se inicia el proceso de muda larvaria. Como consecuencia se produce un cese en la actividad alimentaria de la larva, la formación de una nueva pero malformada cutícula y posteriormente la muerte de la larva (Smagghe Y Degheele, 1994).

Este insecticida fue descubierto y desarrollado por Rohm and Haas Co. y se caracteriza por su eficacia para el control de lepidópteros y por tener un perfil toxicológico muy favorable. Está catalogado como compuesto no mutagénico, no oncogénico, no teratogénico, en el que no se ha observado ningún tipo de hipersensibilidad, ni toxicidad a embriones o fetos, ni es neurotóxico. Se considera prácticamente no tóxico en mamíferos (LD_{50} > 5000 mg/kg vía oral y dermal en ratas).

Se comercializa bajo los nombres de Confirm® (Tebufenocida 24,7% [SC] P/V) y Mimic® (Tebufenocida 24% [SC] P/V) (Tabla 1), dependiendo de su uso y país. En España, los dos productos cuentan con registro vigente para el género *Spodoptera* y otras orugas defoliadoras en diferentes cultivos y ecosistemas. Mimic® está registrado también en frutales de pepita y viña contra carpocapsa y polilla del racimo respectivamente.

Es un insecticida larvicida que actúa por ingestión, aunque también tiene alguna actividad por contacto. Su actividad ovicida es muy limitada. Las propiedades físico químicas y formula estructural son:

Principio Activo: Tebufenocida (MoA IRAC Grupo 18)

Familia Química: Dihacil-hidracinas

Fórmula Empírica: $C_{22}H_{28}N_2O_2$

Nombre IUPAC: N-ter-butil-N'-(4-etilbenzoil)-3,5-dimetilbenzohidrazida
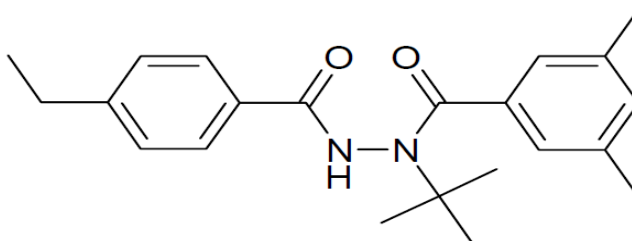
Figura 6. Estructura química de tebufenocida

1 **Tabla 1. Usos y dosis de tebufenocida autorizados España (MAGRAMA, 2020)**

PRODUCTO/NOMBRE COMERCIAL	USO	AGENTE	DOSIS L/HL	Nº APLICACIONES	INTERVALOS	VOL. CALDO (Litros/Hectarea)	FORMA Y ÉPOCA DE APLICACIÓN
TEBUFENOCIDA 24% [SC] P/V. MIMIC®	Lechuga y similares	Spodoptera	0,06 - 0,075	Máx. 3	7 a 14	1000 l/ha	Uso al aire libre para lechuga y escarola. Aplicar durante los primeros estadios larvarios
	Citricos	Phyllocnistis	0,06 - 0,075	1	NA	1466-1833 L/ha	Uso al aire libre. Contra minador, aplicar sobre brotes en crecimiento al inicio del ataque.
	Frutales de pepita	Carpocapsa	0,0625 - 0,075	Máx. 2	10 a 14	1200 - 1400 L/Ha	Aire libre. Aplicar desde inicio de las puestas hasta el inicio de eclosión de huevos, antes de la penetración de las orugas en el fruto. En frutales de pepita excluyendo manzano y peral la dosis será de 0,0625 L/hl.
		Pandemis	0,075	1	NA	1200 L/Ha	Uso al aire libre. Aplicar coincidiendo con la floración.
		Capua	0,075	1	NA		Uso al aire libre. Aplicar coincidiendo con la floración.
	Vid	Piral	0,05 - 0,06	Máx. 3	10	1000 - 1200 L/Ha	Uso al aire libre para vid y parral. Aplicar durante los primeros estadios larvarios.
		Polilla de la Vid					Uso al aire libre para vid y parral. Aplicar desde el inicio de las puestas hasta el inicio de la eclosión de los huevos, antes de la penetración de las orugas en la planta.
	Nogal	Carpocapsa	0,06	Max. 3	14 a 21	1000 l/ha	Uso al aire libre. Aplicar desde el inicio de las puestas hasta el inicio de la eclosión de los huevos, siempre antes de la penetración de las orugas en el fruto.
TEBUFENOCIDA 24,7% [SC] P/V. CONFIRM®	Alcornoque	Orugas defoliadoras	0,3 - 0,4	1	NA	3 a 5 L/Ha	Uso al aire libre. Aplicar el producto antes de la eclosión hasta L4 (primavera / verano).
	Encina	Orugas defoliadoras	0,3 - 0,4	1	NA	3 a 5 L/Ha	Uso al aire libre. Aplicar el producto antes de la eclosión hasta L4 (primavera / verano).
	Pinos	Procesionaria	0,3 - 0,5	1	NA	3 a 5 L/Ha	Tratar desde antes de la eclosión y durante los primeros estados larvarios. Aplicar por medios aéreos mediante técnicas de U.B.V. diluido en 3-5 l de agua o de agua con aceite mineral o vegetal (no más de la mitad del caldo con aceite).
	Robles	Orugas defoliadoras	0,3 - 0,4	1	NA	3 a 5 L/Ha	Uso al aire libre. Aplicar el producto antes de la eclosión hasta L4 (primavera / verano).

3.2. Insecticidas microbianos

La lucha microbiana se refiere al uso de microorganismos entomopatógenos como agentes de control (Van Driesche, 2007). Los entomopatógenos son enemigos naturales de los fitófagos e incluyen un amplio grupo de virus, bacterias, hongos, nematodos y protozoos, que varían en su manera de infectar, el sitio en que se replican y el mecanismo patogénico. Aunque algunos patógenos presentan un amplio espectro de hospedadores, la mayoría prefieren ciertas especies de insectos y también presentan patogenicidad selectiva de acuerdo a los diferentes estados de desarrollo del insecto (Aronson et al., 2001). En las últimas décadas, el mayor interés se ha dirigido hacia su producción masiva y a su aplicación en campo como insecticidas microbianos para el control de plagas agrícolas y forestales. Pueden ser utilizados con una tecnología de aplicación similar a la de los insecticidas químicos y, dada su selectividad y compatibilidad, pueden ser incorporados en programas de Gestión Integrada de Plagas. En 2004, la organización para la cooperación y el desarrollo económico (OCDE) citaba 117 productos registrados basados en 19 patógenos, de los cuales se encontraban seis especies de hongos, cinco de bacterias y siete de baculovirus; 57 de estos productos estaban formulados con *Bacillus thuringiensis* var. *kurstaki* (Kabaluk y Gazdik, 2004).

En la actualidad, los microrganismos entomopatógenos de mayor uso en el control de plagas son las formulaciones basadas en la bacteria *Bacillus thuringiensis*, en el hongo *Beauveria bassiana* y en baculovirus. Las materias activas que se encuentran registrados para el control de plagas de insectos en España, son los siguientes (MAGRAMA, 2020):

- *Bacillus thuringiensis* var. *aizawai*
- *Bacillus thuringiensis* var. *israelensis*
- *Bacillus thuringiensis* var. *kurstaki*
- *Bacillus thuringiensis* var. *tenebrionis*

- *Beauveria bassiana*

- *Verticillum lecanii*

- Virus de la granulosis de la *Carpocapsa*-V15 (CpCV)

- Virus granulosis de la *Carpocapsa* - V22 1% [SC] P/V

- Virus de la poliedrosis nuclear de *Spodoptera exigua*

- Virus de la poliedrosis nuclear de *Helicoverpa armigera* (7,5 x 10E12 OB/L) 50% [SC] P/V.

- Granulovirus de *Cydia pomonella*

- *Isaria fumosorosea Apopka* cepa 97 (antiguamente *Paecilomyces fumosoroseus*)

3.2 1. La bacteria *Bacillus thuringiensis*

Entre las formulaciones biológicas utilizadas para el control de plagas agrícolas destaca una bacteria de la Familia Bacillaceae, *Bacillus thuringiensis* (*Bt*) (Hofte y Whiteley, 1989). Es un ubicuo bacilo Gram positivo, esporulador, aerobio facultativo, nativo del suelo y ampliamente distribuido en el ambiente. Una de las características más importantes de *Bt* es la de sintetizar, durante la fase estacionaria de su ciclo de crecimiento, una inclusión paraesporal de naturaleza proteica (ICP) denominada inclusión cristalina, cristal, δ-endotoxina o proteínas Cry (Schnepf et al., 1998). Desde que ocurrió la primera clonación de un gen cry, que codifica proteínas Cry (Schnepf y Whiteley, 1981) se han identificado muchos otros genes, por lo cual se ha establecido una clasificación basada en el grado de homología de las proteínas (Crickmore et al., 1998).

Bt es un patógeno de insectos y su actividad es atribuida, amplia o completamente (dependiendo del insecto), a las ICP que muestran toxicidad para larvas principalmente de los órdenes Lepidoptera, Coleoptera, Diptera e Hymenoptera. Las proteínas Cry actúan vía ingestión causando la destrucción de las células columnares del mesenterón del insecto por unión de las toxinas con receptores

específicos de membrana, facilitando de esta forma la colonización bacteriana de la hemolinfa, donde las esporas podrán encontrar un lugar idóneo para germinar, lo cual originará una septicemia en el cuerpo del insecto (Hofte y Whiteley, 1989).

La clasificación en subespecies o variedades de *B. thuringiensis,* basada en el análisis serológico de identificación del antígeno flagelar (antígeno H), fue introducida a comienzo de 1960 (de Barjac y Bonnefoi, 1962). Hasta 2005 se habían identificado 84 subespecies (Lecadet et al., 1999; Reyes-Ramírez y Ibarra, 2005).

Los productos basados en esta bacteria son considerados entre los insecticidas más seguros, debido a su nula toxicidad para vertebrados, y han sido ampliamente comercializados para controlar gran variedad de especies de importancia agrícola, forestal y médico-veterinaria (Nester et al., 2002), autorizándose su empleo sobre numerosos cultivos, incluso sin imposición de plazos de seguridad. Las variedades *kurstaki* y *aizawai* son las utilizadas para el control de plagas de lepidópteros, la variedad *israelensis* para el control de larvas de mosquitos y jejenes del orden Diptera, y la variedad *morrisoni* para el control de larvas de especies del orden Coleoptera, entre otras (Gawron y Baum, 1991).

3.2 2. El hongo *Beauveria bassiana*

Los hongos entomopatógenos representan un grupo de importancia ecológica, en parte por el control natural que ejercen en las poblaciones de insectos en diferentes ecosistemas, a menudo dando lugar a infecciones epizóoticas. Así, por ejemplo, *Nomuraea rileyi* causa epizootias naturales en especies del género *Helicoverpa* (Gopalakrishnan y Narayanan, 1988; Teakle, 1991) en cultivos de soja, maíz, sorgo y algodón.

La mayoría de los hongos entomopatógenos con potencial para el control de plagas se encuentra en el reino Eumycota, representado por las principales divisiones

Ascomycota y Entomophtoromycota, donde se sitúan respectivamente los órdenes de mayor importancia: Entomophthorales e Hypocreales (Hibbett et al., 2007; Gryganskyi et al., 2012, 2013; Humber, 2012). Los entomoftorales se caracterizan por ser biotrofos obligados, de difícil multiplicación en medio artificial, lo que limita su empleo al control biológico por conservación (Keller, 2007; Pell et al. 2010). Sin embargo, entre los Hipocreales se encuentran los ascomicetos mitospóricos que son de fácil manejo y producción en masa, lo que facilita su empleo por inundación mediante aplicaciones de micoinsecticidas.

Los hongos entomopatógenos tienen mecanismos de invasión únicos que les permiten atravesar de forma directa la cutícula o la pared del tracto digestivo de los insectos, lo que hace de ellos buenos insecticidas de contacto (Charnley y Collins, 2007). Su complejo modo de acción dificulta el desarrollo de resistencia (Khan et al. 2012). Cuando las esporas asexuales o conidios, que están dispersos en el medio, alcanzan la cutícula de un hospedador, se adhieren fuertemente mediados por fuerzas como la hidrofobicidad de su pared celular, germinan e inician cascadas de reconocimiento y activación enzimática. Seguidamente se forman las estructuras de penetración que, mediante combinación de mecanismos físicos y bioquímicos, atraviesan la cutícula. Una vez en el interior del insecto y superadas las respuestas defensivas, llega a causar la muerte por la utilización de los nutrientes, por invasión de sus tejidos y órganos, por asfixia al desarrollarse en el sistema respiratorio y, en algunos casos, por la producción de metabolitos tóxicos. Al agotarse los nutrientes, el hongo inicia un crecimiento miceliar invadiendo todos los órganos del hospedador y, en condiciones favorables, sus hifas penetran la cutícula desde el interior del insecto y emergen a la superficie de éste para producir y liberar los conidios, que inician un nuevo ciclo contribuyendo a su transmisión horizontal (Goettel et al. 2005; Charnley y Collins, 2007; Quesada-Moraga y Santiago-Alvarez 2008; Vega et al. 2012).

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) es una especie de ascomiceto mitospórico que infecta a un amplio espectro de hospedadores, más de 700 (Inglis et al., 2001), entre las que se encuentran una gran variedad de plagas de insectos, por lo que diferentes aislados están siendo desarrollados como materias activas en insecticidas microbianos.

Como la mayoría de los ascomicetos mitospóricos, *B. bassiana* se localiza de forma natural en el suelo, en las plantas (tanto en el filoplano como endófitos) y en los insectos a los que infectan (Quesada-Moraga et al., 2014). Pueden permanecer en el suelo en forma de micelio, en cadáveres de artrópodos momificados, o en forma de conidios, donde factores edáficos (textura, pH, materia orgánica y humedad) pueden afectar su presencia y distribución (Keller y Zimmerman, 1989; Quesada-Moraga et al., 2007). Su especificidad varía considerablemente y algunos infectan un amplio espectro de hospedadores.

B. bassiana fue clasificada inicialmente dentro de los Deuteromicetos u hongos imperfectos (de los que no se conoce su fase sexual), pero más recientemente ha sido reclasificada como un Ascomiceto a partir del descubrimiento de su teleomorfo (forma sexual). Así, *B. bassiana* representa la fase asexual, mientras que *Cordyceps bassiana* es la fase sexual (Rehner y Buckley, 2005).

Este hongo presenta gran actividad patogénica en varios tipos de insectos incluyendo los órdenes Coleoptera, Lepidoptera, Homoptera y otros artrópodos (Resquín-Romero, 2016). Se han realizado numerosos ensayos de patogenicidad con *B. bassiana* para la búsqueda de aislados que puedan ser utilizados contra plagas agrícolas y forestales (Blanford et al., 2005; Lopez Cruz, 2013; Resquín-Romero, 2016; Scholte et al., 2004).

3.2 3. Insecticida viral: Los baculovirus

Los baculovirus son entomopatógenos de la Familia Baculoviridae que nunca se han encontrado causando enfermedades fuera del Phylum Arthropoda. Por su especificidad, seguridad de empleo, facilidad de aplicación, relativa estabilidad y compatibilidad con otros agentes de control pueden ser usados como materias activas de insecticidas microbianos (Podgwaite, 1985). Morfológicamente se caracterizan por la forma de varilla de las partículas virales que contienen ADN circular en doble hélice (Federici, 1986; Lacey et al., 2002). La replicación tiene lugar en el núcleo de las células infectadas y los viriones resultantes abandonan las células, bien por gemación, o bien por la lisis de éstas una vez incluidos en cuerpos de oclusión (CO) de naturaleza proteica (Granados y Williams, 1986). En esta Familia Baculoviridae se encuentran los Nucleopoliedrovirus (NPV) y los Granulovirus (GV) (Federici, 1997; Caballero et al., 2001; van Oers, 2011), que se diferencian en el tamaño de los CO (Funk et al., 1997).

La principal vía de infección es por ingestión y el período desde el inicio de la infección hasta la muerte del hospedador depende principalmente de la combinación de dosis de inóculo y temperatura, pero también varía con el tipo de virus y la especie hospedadora (Hunter-Fujita et al., 1998), así como con la edad del hospedador, pues en los primeros estadios (L1-L3) la muerte puede ocurrir de 24 a 72 h mientras que en lo últimos el periodo de infección suele oscilar entre 5 y 10 días (Federici, 1997). Además, muchas de las infecciones causadas por baculovirus tienen efectos subletales que se manifiestan durante el desarrollo y reproducción del fitófago (Vargas-Osuna, 2001).

Hasta ahora los baculovirus sólo han sido aislados de artrópodos, principalmente de insectos. De los cientos de aislamientos, la mayor parte se han encontrado en el Orden Lepidoptera y, el resto en Hymenoptera, Diptera, Coleoptera y algunas especies de Crustacea (Volkman et al., 1995). De una gran cantidad de especies

fitófagas se han aislado baculovirus que se están desarrollando como agentes de control. Algunos de ellos están ya registrados (Caballero y Williams, 2008).

En 1975, se comercializó el primer insecticida a base de partículas virales denominado "ElcarTM" (Sandoz Inc.) registrado para controlar al gusano del algodón: *Helicoverpa zea* (Boddie) (Szewezyk et al., 2006). Los primeros casos de control de insectos con baculovirus se reportaron para *Lymantria monacha* (L.) en Alemania y *Lymantria dispar* (L.) en Estados Unidos. Caballero y Williams (2008) reportaron la existencia de 36 bioinsecticidas basados en baculovirus, para controlar plagas agrícolas y forestales. En Brasil, más de un millón de hectáreas de soja son tratadas cada año con el nucleopoliedrovirus de *Anticarsia gemmatalis* (Hübner), que representan el 10% del total del área cultivada (Moscardi y Sosa-Gómez, 1992). Para el control de insectos plaga del género *Spodoptera*, existen varias formulaciones de NPV que son utilizadas en países como Brasil, China, Guatemala, Tailandia, Holanda, Estados Unidos y España (Caballero y Williams, 2008). En España están comercializados el NPV de *S. littoralis* y el NPV de *Helicoverpa armigera* para el control de las respectivas especies en cultivos hortícolas, tanto al aire libre como en invernadero (De Liñan, 2020).

4.   COMPATIBILIDAD ENTRE AGENTES DE CONTROL

Compatibilizar los diferentes tipos de control es una necesidad dentro de la gestión integrada de plagas. Uno de los desafíos es desarrollar estrategias de control biológico que sean compatibles con el control químico.

4.1.   Compatibilidad entre insecticidas RCI y depredadores

Los efectos del control químico sobre los entomófagos pueden ser directos (efectos letales) o diferidos (efectos subletales), los primeros generalmente son a corto plazo

y se deben al contacto directo del enemigo natural con los insecticidas o con sus residuos al moverse por las plantas tratadas, al ingerir fluidos o alimentarse de material contaminado. Por otro lado, los efectos subletales resultan ser esos cambios en la fisiología o comportamiento de los insectos supervivientes (Powell et al., 1985; Desneux et al, 2007; Müller, 2019).

Para evaluar la toxicidad real de un insecticida sobre los entomófagos se deben analizar tanto los efectos letales como los subletales producidos por el mismo, ya que en algunas ocasiones un insecticida que no produce mortalidad directa puede causar efectos subletales, tales como reducción de los parámetros reproductivos, esperanza de vida o cambios en el comportamiento de los insectos (Müller, 2018; Desneux et al., 2007; Mills et al., 2016). A pesar de que los insecticidas de síntesis más modernos generalmente tienen menor impacto en la entomofauna beneficiosa en comparación a los insecticidas tradicionales, es necesario realizar estudios de tipo ecotoxicológico para determinar los efectos de los insecticidas sobre los estados de desarrollo de los principales enemigos naturales (Jacas y Viñuela, 1994; Medina et al., 2001; Schneider et al., 2004).

Rugno et al. (2016) quienes tratando por contacto larvas de *Ceraeochrysa cincta* (Schneider) con 6 isencticidas RCI (diflubenzurón, metoxyfenocida, tebufenocida, lufenurón, buprofecin y pyriproxyfen) encontraron que el diflubenzurón y lufenurón presentaron toxicidad para los cuatro estadios larvales; metoxyfenocida y tebufenocida no afectaron la duración y surpervivencia de los estados inmaduro; y metoxyfenocida redujo la fecundidad y longevidad de los insectos.

Medina et al. (2003) estudiaron la susceptibilidad a aplicaciones tópicas de diflubenzurón en larvas de tercer estadio del depredador generalista *C. carnea*, encontrando efecto letal con una dosis letal media de 6,9 ng/insecto. La susceptibilidad de las larvas de *C. carnea* a las aplicaciones tópicas de lufenurón durante sus primeros estadios se ha demostrado anteriormente (Hussain et al.,

2012; Mohammadi et al. 2014) y la baja mortalidad de larvas tratadas con tebufenocida (Medina, 2001).

Otros resultados encontrados en combinaciones específicas de insecticidas RCI y depredadores se exponen en el apartado de discusión de los capítulos II y III de esta Tesis.

4.2. Uso conjunto de insecticidas RCI y microbianos

El uso de insecticidas con una mezcla de materias activas se considera una forma de manejo de la resistencia de plagas (Georgiou, 1980). Para desarrollar este tipo de estrategias es necesario conocer si hay interacciones entre las sustancias insecticidas involucradas, definiendo interacción como "una respuesta biológica cualitativa o cuantitativamente alterada respecto a la respuesta predicha para la acción individual de esos agentes" (Yang, 2010). Desde la perspectiva de control de plagas, la respuesta se suele medir en términos de mortalidad.

Los estudios sobre interacciones pueden dirigirse a la búsqueda de dos agentes que produzcan una mortalidad total mucho mayor que la esperada, a partir de la suma de mortalidades de cada uno de los dos agentes actuando por separado, es decir, sinergismo verdadero. Por el contrario, se considera antagonismo al caso en que la actividad insecticida simultánea sea menor que la suma de las actividades insecticidas individuales (Figura 7).
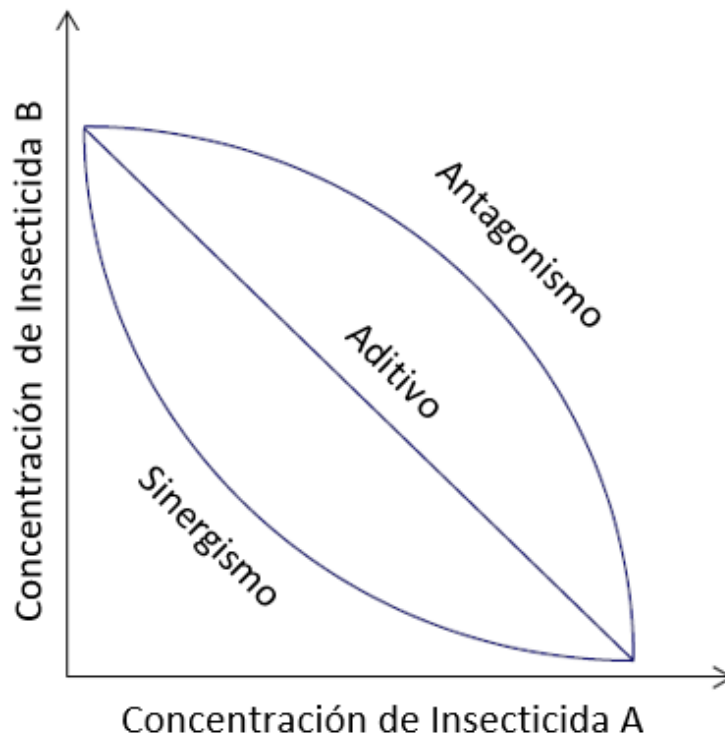
Figura 7. Los ejes X e Y reflejan las tasas de dosis de los componentes individuales de la mezcla binaria. Una línea recta significa que dos sustancias "a" y "b" tienen un efecto neto aditivo. En sinergia, el efecto global de los dos compuestos es mayor de lo que se esperaba de la suma de los efectos, resultando en una curva cóncava. Efecto antagonista el resultado general es menor que el esperado de la suma de los efectos separados generando una curva convexa (Berenbaum, 1989)

Por otro lado, el uso conjunto de dos productos insecticidas se puede buscar, por razones de seguridad medioambiental, como una forma de reducir la concentración de un insecticida químico mediante el uso simultáneo con otro agente compatible. En este caso, el efecto es la suma de las dos mortalidades actuando independientemente (efecto aditivo) puede ser aceptable, mientras que el sinergismo es un efecto extra.

El enfoque para conocer el tipo de interacción es meramente cuantitativo, agrupándose las diferentes respuestas en las categorías de Aditividad (o independencia), Sinergismo y Antagonismo. El método se basa en el descrito por Harper (1986) que consiste en la realización de bioensayos en los que se determina

los porcentajes de mortalidad obtenidos, tanto en tratamientos individuales como en tratamientos conjuntos.

Según Harper (1986), si dos agentes de mortalidad actúan en una misma población del fitófago, cada uno de ellos actúa independientemente y todos los individuos tienen la misma susceptibilidad, la mortalidad global sigue la fórmula probabilística:

$$P_e = p_1 + p_2 - p_1p_2$$

En donde:

P_e: Proporción esperada de población que muere por el tratamiento.

p_1: Proporción de la población que muere por la acción de uno de los agentes.

p_2: Proporción de la población que muere por la acción del otro agente.

Si la mortalidad total observada es significativamente superior a la esperada bajo la hipótesis de independencia, el tipo de interacción es Sinergismo, y si, por el contrario, la mortalidad observada es menor a la esperada, la interacción será considerada Antagonismo.

En la literatura científica hay diversos estudios sobre integración de agentes químicos con microorganismos entomopatógenos, con objeto de maximizar su potencial y disminuir el impacto ambiental que el control químico puede ocasionar (Mendez et al., 2002, Bitsadze et al., 2013). Los resultados encontrados en combinaciones específicas de RCI y entomopatógenos se exponen en el apartado de discusión del Capítulo IV de esta Tesis.

Combinaciones de RCI (metoxifenozida y lufenurón) con el hongo *Beauveria bassiana* resultó en un alto grado de compatibilidad y sinergismo (Pelizza et al., 2015; Vasquez et al., 2004). Efecto aditivo se encontró entre diflubenzurón y lufenurón con *B. bassiana*. (Purwar y Sachan, 2006) Así mismo, algunos estudios han registrado efectos antagónicos como el caso de triflumurón y *B bassiana*. Los

resultados pueden variar en función de la cepa de *B. bassiana* evaluada (Mohan et al., 2007).

Los estudios con bacterias entomopatógenas muestran variabilidad en sus resultados. El lufenurón aumentó significativamente la virulencia de *Pseudomonas aeruginosa* (Schroeter) con un efecto sinérgico en la mortalidad de *Coptotermes formosanus* Shiraki, una termita subterranea. Sin embargo, para esta misma especie la interacción del lufenurón y *Serratia marcescens* Bizio o *Bacillus thuringiensis* Berliner subsp. *israelensis* no fue tan fuerte (Wang et al., 2013).

La aplicación simultánea y secuencial de azadiractina y spinosad con un nucleopoliedrovirus ha demostrado un efecto sinérgico en las larvas de las especies de *Spodoptera* (El-Helaly y El-Bendary, 2015; Méndez et al., 2002; Zamora-Aviles et al., 2013).

La alimentación secuencial de larvas de *Spodoptera exigua* (Hübner) con dos nucleopolyhedrovirus y metoxifenozida dio lugar a efectos aditivos sobre la mortalidad larvaria (Dáder et al., 2020).

5. OBJETIVOS

Actualmente en Europa la norma legal vigente de uso sostenible de plaguicidas busca reducir la cantidad de productos fitosanitarios en la agricultura que pongan en riesgo la salud humana, animal y el medio ambiente. La directiva fomenta la Gestión Integrada de Plagas (GIP) y el uso de técnicas y productos alternativos o complementarios a los plaguicidas químicos convencionales. Ello no implica una sustitución completa del uso de plaguicidas de síntesis, pero sí su rotación, sustitución parcial o combinación con otros agentes de control, entre los que se encuentran los entomófagos y los insecticidas microbianos.

Por esto, el **objetivo general** de este trabajo de investigación ha sido generar conocimiento que pueda ser incluido en los programas de gestión integrada contra el lepidóptero *Spodoptera littoralis*, mediante el estudio del grado de compatibilidad entre agentes de control de la plaga.

Los **objetivos específicos** son los siguientes:

A) Determinar los efectos de la aplicación de los Reguladores de Crecimiento de Insectos (RCI), lufenurón y tebufenocida, sobre los estados de desarrollo del depredador *Chrysoperla carnea* **(Capítulo II y III)**

- Efectos letales en base a la determinación de las concentraciones letales medias y tiempos de supervivencia.
- Efectos subletales que se manifiestan en alteraciones del desarrollo y reproducción.
- Efectos letales y subletales en las larvas del depredador que consumen presas tratadas.
- Preferencia del depredador entre presas tratadas y no tratadas.

B) Estudio de las interacciones de lufenurón y tebufenocida, en bajas dosis, con agentes microbianos (NPV, *Bacillus thuringiensis* y *Beauveria bassiana*) en larvas de *Spodoptera littoralis* **(Capítulo IV)**

- Efecto letal de cada agente insecticida individualmente aplicado.
- Tipo de interacción de tratamientos combinados.

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CAPITULO II

Efectos letales y subletales de lufenurón sobre el
depredaror *Chrysoperla carnea* (Stephens)
(Neuroptera: Chrysopidae)

Lethal and sublethal effects of lufenuron on the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)

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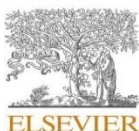
Lethal and sublethal effects of lufenuron on the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)

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ABSTRACT

The neuropteran *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) is a generalist predator present in Mediterranean agroecosystems. It has a wide range of prey including almost all soft-bodied arthropods; its voracity makes it a suitable agent for inclusion in Integrated Pest Management (IPM) programmes. Use of insecticides that inhibit chitin synthesis has increased and includes the use of benzoylureas (e.g. lufenuron) which negatively affect development of the immature stages of phytophagous insects, both by ingestion and by contact. This study evaluated interactions between lufenuron and the predator *C. carnea* in the laboratory. Treatment of eggs with lufenuron 24 or 48 h after oviposition had no effect on the proportion successfully hatching, or in the survival of resulting neonate larvae. Topical application of lufenuron to second instar larvae (L2) of *C. carnea* resulted in high mortality rates; lethal concentration to kill 50% (LC50) was 0.0153 ml/L (0.00860–0.0236 ml/L). Moreover, the development time of third-instar larvae of *C. carnea* that had consumed prey treated with lufenuron 24 h previously (at a dose of 1 ml/L) was significantly truncated and high percentage mortality was observed when these larvae reached the pupal stage. In choice bioassays a high percentage of *C. carnea* larvae chose prey treated with lufenuron in preference to untreated prey. Ingestion of lufenuron by *C. carnea* adults had no effect on female fecundity or adult longevity, but caused a significant reduction in the viability of resulting eggs. In light of these results, the developmental stage of *C. carnea* should be considered when deciding on the timing of lufenuron applications within IPM strategies.

1. Introduction

The green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), is a very effective generalist predator and abundant in the Mediterranean area. Its extended range of prey includes almost all soft-bodied arthropods, and its voracity make it a good control agent for use within integrated pest management (IPM) programmes (Henry et al., 2002; Nordlund et al., 2010). Green lacewings are holometabolous insects and develop to adulthood through egg, larval and pupal stages. All larval stages (three instars) exhibit predatory behaviour but the last instar (L3) is the most voracious, and of the longest duration (Henry et al., 2002). Adults feed on pollen, plant exudates and honeydew and are essential to maintain populations in the field (Desneux et al., 2007; Nordlund et al., 2010).

Effective plant protection programmes seek to increase compatibility between control methods including between chemical and biological methods. Pesticides that are safer for the environment and have low toxicity to natural enemies are more useful in IPM. Insect growth regulators (IGRs) are an option for use in IPM and their use has increased, especially chitin synthesis inhibitors belonging to the benzoylurea group (Agnello et al., 2009; Muthukrishnan et al., 2012; Retnakaran, 1980). Benzoylureas have lethal effects on immature insects, interrupting moulting, which is a critical stage in development. Insecticidal activity depends on the particular active ingredient and on the target pest insect species (Sun et al., 2015). Chitin is a polysaccharide composed of N-acetyl-D-glucosamine units; it is found in the exo-cuticle, trachea, ovarioles and peritrophic membrane of the insect midgut. Alterations in chitin biosynthesis have physiological effects that not only affect insect development, but also reproduction (Mansur et al., 2010; Moreira et al., 2007; Muthukrishnan et al., 2012).

ABSTRACT

The neuropteran *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) is a generalist predator present in Mediterranean agroecosystems. It has a wide range of prey including almost all soft-bodied arthropods; its voracity makes it a suitable agent for inclusion in Integrated Pest Management (IPM) programmes. Use of insecticides that inhibit chitin synthesis has increased and includes the use of benzoylureas (e.g. lufenuron) which negatively affect development of the immature stages of phytophagous insects, both by ingestion and by contact. This study evaluated interactions between lufenuron and the predator *C. carnea* in the laboratory. Treatment of eggs with lufenuron 24 or 48 hours after oviposition had no effect on the proportion successfully hatching, or in the survival of resulting neonate larvae. Topical application of lufenuron to second instar larvae (L2) of *C. carnea* resulted in high mortality rates; lethal concentration to kill 50% (LC50) was 0.0153 ml/L (0.00860-0.0236 ml/L). Moreover, the development time of third-instar larvae of *C. carnea* that had consumed prey treated with lufenuron 24 hours previously (at a dose of 1 ml/L) was significantly truncated and high percentage mortality was observed when these larvae reached the pupal stage. In choice bioassays a high percentage of *C. carnea* larvae chose prey treated with lufenuron in preference to untreated prey. Ingestion of lufenuron by *C. carnea* adults had no effect on female fecundity or adult longevity, but caused a significant reduction in the viability of resulting eggs. In light of these results, the developmental stage of *C. carnea* should be considered when deciding on the timing of lufenuron applications within IPM strategies.

Keywords: Compatibility, Integrated Pest Management, Entomophages, Predator-prey, Benzoilureas, IGR

1. INTRODUCTION

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Effective plant protection programmes seek to increase compatibility between control methods including between chemical and biological methods. Pesticides that are safer for the environment and have low toxicity to natural enemies are more useful in IPM. Insect growth regulators (IGRs) are an option for use in IPM and their use has increased, especially chitin synthesis inhibitors belonging to the benzoylurea group (Agnello et al., 2009; Muthukrishnan et al., 2012; Retnakaran, 1980). Benzoylureas have lethal effects on immature insects, interrupting moulting, which is a critical stage in development. Insecticidal activity depends on the particular active ingredient and on the target pest insect species (Sun et al., 2015). Chitin is a polysaccharide composed of N-acetyl-D-glucosamine units; it is found in the exo-cuticle, trachea, ovarioles and peritrophic membrane of the insect midgut. Alterations in chitin biosynthesis have physiological effects that not only affect insect development, but also reproduction (Mansur et al., 2010; Moreira et al., 2007; Muthukrishnan et al., 2012).

Several benzoylureas have been registered for use against lepidopteran pests: diflubenzuron, chlorfluazuron, teflubenzuron, flufenoxuron, hexaflumuron and

lufenuron (Whalon et al., 2008; Sun et al., 2015). These are considered relatively safe for natural enemies because they act by affecting chitin biosynthesis, especially in immature stages, without directly affecting adults (Cohen, 1987; Muthukrishnan et al., 2012). However, previous studies have shown that IGRs can affect both biological parameters (development times, reproductive capacity, longevity) and behaviour (mobility, orientation, search and mating capacity) of beneficial insect species (Gorri et al., 2015; Mills et al., 2016; Ono et al., 2017; Perez-Guerrero et al., 2014; Soares et al., 2019).

Some authors have shown that new low-risk insecticides have more sublethal effects in populations of natural enemies, than lethal effects (Amarasekare and Shearer, 2013; Desneux et al., 2007). In addition, several natural enemy developmental stages are exposed at the time of crop spraying with insecticides, each of which are vulnerable to different routes of exposure (topical, ingestion and residual) (Amarasekare et al., 2016; Mills, 2014; Müller, 2018). It is necessary, therefore, to elucidate direct and indirect effects of active ingredients on natural enemies.

Lufenuron is a benzoylurea that is more active via ingestion than via contact. It is also long-lasting on leaves and considered to be a product with limited environmental impact (Muthukrishnan et al., 2012; Sun et al., 2015). The susceptibility of *C. carnea* larvae to topical applications of lufenuron during their first instars has been demonstrated previously (Hussain et al., 2012; Mohammadi et al., 2014). Nevertheless, other effects of lufenuron have not been studied in this species including: the effect on predators of feeding on lufenuron-treated prey, predator preferences for lufenuron-treated and untreated prey; and the effects of ingestion of lufenuron on adults. Evaluation of these predator-prey interactions are of great importance to ascertain the real impact of this insecticide on *C. carnea*.

The objective of this study was to elucidate the interactions between lufenuron and the predator, *C. carnea*. The experiments focused on studying the following effects

of lufenuron: 1) lethal effects on eggs (sprayed); 2) lethal effects on larvae (topically treated) of *C. carnea*, and sublethal effects on the development of treated survivors; 3) lethal and sublethal effects on *C. carnea* larvae following consumption of treated prey; 4) predator preferences for treated or untreated prey when given a choice; 5) sublethal effects on the reproduction and longevity of adult *C. carnea* treated via ingestion.

2.   MATERIALS AND METHODS

2.1.   Insects and insecticide

The population of *C. carnea* was supplied regularly as required for experiments, as first and second instars (L1-L2), by the companies Koppert and Agrobio S.L. These larvae were fed *ad libitum* with *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs. Emerging adults were fed a diet rich in carbohydrates and proteins, as described by Hassan (1975).

Larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) used as prey in various bioassays came from a colony maintained by the Agroforestry Entomology Laboratory at the University of Cordoba. During their development *S. littoralis* larvae were fed on artificial diet, as described by Poitout and Bues (1974), with some modifications (Vargas-Osuna and Santiago-Álvarez (1988).

Insect colonies and bioassays were maintained in chambers under standard conditions of 25 ± 2ºC, 65 ± 5% relative humidity (RH) and a photoperiod of 16:8 h (L:D).

The insecticide used was the commercial product (Match®) containing 5 g/L lufenuron formulated as a concentrated emulsion (Syngenta Crop Protection Ltd.).

2.2. Effects of lufenuron on *C. carnea* eggs

Eggs of *C. carnea* were treated in groups of approx. 100 by spraying them with lufenuron at a concentration of 1 ml/L in 0.1 ml/L aqueous Tween 80. The volume sprayed per group was 3 ml using a manual sprayer. One group was comprised of eggs that had been laid 24 hours earlier and a second group was comprised of eggs that had been laid 48 hours earlier. Control groups were treated in the same way but with only 0.1% aqueous Tween 80. After treatment the *C. carnea* eggs were allowed to dry for 3 hours, and were then placed, in their groups, into clean Petri dishes with larval food (*E. kuehniella* eggs) to support hatching larvae. Newly-hatched *C. carnea* larvae were counted and removed daily. Six days after treatment those *C. carnea* eggs that had not hatched (not viable) were counted under a stereoscopic microscope. The assay was repeated on three occasions. Twenty larvae hatching from eggs treated 48 hours after they had been laid, and twenty larvae hatching from the corresponding control group, were incubated individually with *E. kuehniella* eggs for 5 days and larval mortality recorded.

2.3. Lethal and sublethal effects of lufenuron on *C. carnea* larvae

To valuate lethal effects, individual second-instar (L2) *C. carnea* were placed in plastic boxes (30 mm in diameter and 15 mm high, with lids) and treated topically with a drop of 3 µl of lufenuron in 0.1% aqueous Tween 80 using a precision micropipette. Five concentrations were used: 1, 0.2, 0.04, 0.008 and 0.0016 ml/L (1 ml/L is the maximum recommended concentration for use in the field in Spain). Control larvae were treated in the same way but with only 0.1% aqueous Tween 80. There were twenty replicate larvae for each treatment concentration and the control; the bioassay was repeated on three occasions (N = 60 in total for each treatment and control). Cumulative percent mortality was determined up to 8 days after treatment. During the bioassay *C. carnea* larvae were fed with *E. kuehniella* eggs. Daily observations were made to determine larval mortality and development time.

The proportion dead in each treatment was Abbott-corrected with respect to the mortality that occurred in the control treatment (Abbott, 1925).

A second bioassay was done to determine sublethal effects on the development time and pupal duration of second-instar larvae that survived treatment. Forty second-instar larvae per concentration of lufenuron (1, 0.2, 0.04, 0.008 and 0.0016 ml/L), and the control, were treated and then incubated following the same procedure as described previously in this section. Observations were made every 24 hours to determine mortality, larval development time of those surviving treatment (days between treatment and the beginning of cocoon formation) and pupal duration of those surviving treatment (days between cocoon formation and adult emergence).

2.4. Lethal and sublethal effects on *C. carnea* that consume prey previously treated with lufenuron

Third-instar larvae of *S. littoralis* were placed individually in plastic boxes (30 mm in diameter and 15 mm high, with lids) and allowed to feed, for 24 hours, on an alfalfa *Medicago sativa* (L.) (Fabales: Fabaceae) leaf disk onto which 3 µl lufenuron (in 0.1% aqueous Tween 80), had been deposited using a precision micropipette. Two concentrations of lufenuron were evaluated: 1 ml/L and 0.1 ml/L. Control larvae were treated in the same way except that the leaf disks were treated with 3 µl of 0.1% aqueous Tween 80 only. There were thirty replicate larvae per treatment and control, and the entire bioassay was repeated on three occasions. Larvae that completely consumed the alfalfa leaf disk were offered as prey to L3 larvae of *C. carnea,* held individually in plastic boxes (30 mm in diameter and 15 mm high), at a rate of one prey item per larva. After 24 hours, *C. carnea* larvae that had consumed the entire prey item, were maintained on *E. kuehniella* eggs and observed daily for mortality and to determine when surviving insects pupated, and adults emerged.

2.5. Preference of *C. carnea* larvae for untreated vs. treated prey

Third-instar *S. littoralis* larvae were fed leaf disks treated in the same way as described in 2.4. Leaf disks were either treated with lufenuron at a concentration of 1 ml/L in 0.1% aqueous Tween 80, or with 0.1% aqueous Tween 80 (control larvae). Only larvae that had consumed the entire leaf disk within 24 hours were used as prey items. After 24 hours, L3 larvae of *C. carnea* were placed individually in a Petri dish with six *S. littoralis* prey items: three *S. littoralis* larvae treated with lufenuron and three untreated larvae. Marking *S. littoralis* larvae with paint on the thorax, following the methodology of Ortiz-Moreno and Vargas-Osuna, (2009) ensured treated and untreated larvae could be distinguished from each other. Forty larvae of the predator were used and preference and predation sequence data were gathered by means of continued visual observation for 12 hours.

2.6. Effects of lufenuron on the *C. carnea* adults treated by ingestion

Newly-emerged *C. carnea* adults were sexed and pairs (one male and one female) were confined in cylindrical transparent plastic containers (160 mm diameter x 60 mm height). A filter paper was placed in the upper part of each cylinder as an egg-laying substrate. A piece of plastic impregnated with diet was placed at the bottom of each cylinder, alongside a small water container (30 mm in diameter and 15 mm in height).

In the treatment group, the water container was replaced with one filled with lufenuron at a concentration of 1 ml/L, for 24 hours; the control group received only distilled water. After this period all pairs (15 per treatment and control) were transferred to clean cylinders with diet and water, and evaluated every 48 hours for 18 days or until the adult female died. At each evaluation point the eggs laid by each couple were removed, counted and incubated for 6 days in Petri dishes with larval food. Newly hatched larvae were counted and removed daily. After the six days

those eggs that had not hatched (non-viable eggs) were counted under a stereoscopic microscope.

2.7. Statistical analysis

Larval mortality data (section 2.3) were subjected to Probit analysis to produce a dose-mortality regression line. The analysis was based on the maximum likelihood method of Finney, (1971). The goodness of fit was determined using a χ^2 test at 5%. The median lethal concentration (LC_{50}) was calculated with fiducial limits at 95%. Analysis was done using the POLO Program (LeOra Software Inc. Berkeley, CA, USA).

Larval development time, length of pupal period, reproduction and adult longevity data (sections 2.3, 2.4, 2.6) were subjected to ANOVA (after testing the data normality and homoscedasticity). When significant differences were found, means were compared using the Minimum Significant Difference (MDS) test, applying a 0.05 significance level, in the program STATISTIX 10.0. Data expressed as percentages (egg viability, larval mortality, prey preference and adult malformations) (sections 2.2, 2.3, 2.4, 2.5, 2.6) were analysed by χ^2 at 95%.

3. RESULTS

3.1. Effects of lufenuron on *C. carnea* eggs

There was no significant difference in viability (% hatching) of either 24 or 48 hour-old eggs that had been treated with lufenuron and their respective controls (Table 1). Survival of larvae that hatched from 48-hour-old treated eggs was high; only two larvae died which was equivalent to 2.3% mortality N = 85) and there was no mortality in the control (N = 60).

Tabla 1. Viability of *Chrysoperla carnea* eggs treated with lufenuron

Dose	24-hour-old eggs		48-hour-old eggs	
(ml/L)	N	% hatching ± SE	N	% hatching ± SE
0	283	89.8 a ± 5.76	245	83.3 a ± 9.64
1	304	90.8 a ± 9.04	260	85.4 a ± 9.50

N = Number of eggs treated.

Values followed by the same letter in a column do not differ significantly from each other at the 95% level

3.2. Lethal and sublethal effects of lufenuron on *C. carnea* larvae

Mortality of second-instar *C. carnea* larvae directly positively corelated with lufenuron concentration, and ranged between 9.9% (at the concentration of 0.0016 ml/L) and 84.90% (at the concentration of 1 ml/L) (Table 2). Mortality was assessed up to 8 days after treatment and the greatest mortality occurred between days 2 and 3

Tabla 2. Mortality of second-instar *Chrysoperla carnea* larvae following topical application of lufenuron, and sublethal effects of surviving larvae

Dose (ml/L)	N	Mortality		Larval development		Pupation period	
		%	Corrected[1]	n	Mean ± SE (days)	n	Mean ± SE (days)
0	60	10.00	-	32	6.44a ± 0.19	22	9.59a ± 0.11
0.0016	60	20.00	9.90	31	6.74a ± 0.19	14	9.36a ± 0.13
0.008	60	38.33	28.23	17	7.41b ± 0.25	6	9.83a ± 0.20
0.04	60	76.67	66.57	8	6.87ab ± 0.37	6	9.50a ± 0.20
0.2	60	95.00	84.90	-	-	-	-
1	60	95.00	84.90	-	-	-	-

[1]Mortality corrected by Abbott's formula (1925)
N = Number of treated larvae
n = Number of larvae
Means followed by the same letter in a column do not differ significantly at 95%
Mortality was assessed up to 8 days after treatment.
Regression line (x: log dose; y: Probit mortality) y= 1.43x + 1.87, X^2 (1) = 0.495

The Probit regression line was y = 1.43x + 1.87, where 'x' is the log concentration and 'y' the mortality, with a good fit, as deduced from the value of χ^2 (2) = 0.50. The LC_{50}, calculated from the regression line, was 0.0153 ml/L with a confidence interval between 0.0086 and 0.0236 ml/L.

The development time of L2 larvae that survived treatment with different concentrations of lufenuron ranged from 6.74 to 7.41 days. The values was of 6.44 days in control larvae (Table 2); however, the differences were only statistically significant to the control at 0.008 ml/L lufenuron (F = 3.21, df = 3, p=0.0270).

The duration of the pupal period of larvae that survived treatments with different concentrations of lufenuron ranged from 9 to 10 days, with an average value of 9.36 days at the lowest concentration, and 9.83 days at the highest (Table 2); there was no significant differences between pupal durations at different concentrations of lufenuron.

3.3. Lethal and sublethal effects on *C. carnea* larvae consuming prey treated with lufenuron

Mortality rates of L3 *C. carnea* larvae (i.e. the number dying before they could pupate) that had consumed prey treated with lufenuron was significantly greater than for the control group, at both doses: 0.1 ml/L (χ^2 = 7.44, df = 1, p = 0.0076); 1 ml/L (χ^2 = 6.85, df = 1, p = 0.0121) (Table 3). However, treated larvae had very long survival times; average values were 17 and 14 days at 0.1 ml/L and 1 ml/L, respectively.

Tabla 3. Larval and pupal mortality of *Chrysoperla carnea* after fed on prey treated with lufenuron, and development of the survivors

	Lethal effect on larvae				Development of survivors								
Dose (ml/L)	Larval mortality		Larval duration (days)		Larval development (days)		Pupal mortality		Pupal period (days)		Adult malformated	
	N_1	%	N_2	Mean ±SE	N_3	Mean ±SE	N_3	%	N_4	Mean ±SE	N_4	%
0	80	7.5a	6	13.50a ±0.57	74	9.61a ±0.97	74	0a	74	9.72a ±1.29	74	4.05a
0.1	78	23.08b	18	17.16a ±1.92	60	9.42a ±0.74	60	83.33b	10	11.10a ±1.27	10	50.00b
1	76	22.37b	17	14.00a ±0.99	59	8.58b ±0.90	59	100c	-	-	-	-

= Number of treated larvae
N_2 = Number of dead larvae
N_3 = Number of surviving larvae
N_4 = Number of surviving pupae
Means followed by the same letter in a column do not differ significantly at 95%

The larval development time of *C. carnea* larvae that survived after consuming prey treated was shorter than the control group, significantly so for the highest concentration of lufenuron (1 ml/L; F = 4.95, df = 2, p = 0.008). In contrast, of those pupae surviving to emerge as adults, there was no significant difference in pupal duration amongst treatments and control (Table 3).

Of those surviving to the pupal stage, mortality (i.e. the number dying before they could emerge as adults) was very high in the treatment groups (83.33% and 100% at 0.1 ml/L and 1 ml/L, respectively) and significantly higher than the 0% mortality in the control group (χ^2 = 15.88, df = 1, p = 0.0001) (Table 3). The difference in pupal mortality between the two treatments was also statistically significant (0.1 ml/L c.f. 1 ml/L; χ^2 = 10.74, df = 1, p=0.0013). In addition, five of the ten individuals that survived to the pupal stage following lufenuron treatment emerged as adults with malformations in the abdomen and wings with a mortality percentage significantly higher than the control (χ^2 = 21.58, df = 1, p = 0.0004).

3.4. Prey preference of *C. carnea* when given a choice between *S. littoralis* prey treated with lufenuron and untreated prey.

During the 12 hours of the experiment most *C. carnea* larvae predated between two and five larvae of the six larvae they had been offered. Significantly more *C. carnea* larvae selected *S. littoralis* larvae treated with lufenuron as their first (82.05%; $\chi^2 = 8.84$, df = 1, p = 0,0039) and second prey item (82.05%; $\chi^2 = 11.91$, df = 1, p = 0,0008) compared with untreated larvae (Figure 1). In general, there was a marked preference for treated prey (75.65% of the total prey consumed were treated) with significant difference ($\chi^2 = 16.14$, df = 1, p = 0,0001) compared with untreated prey.

Figure 1. Preference of *Chrysoperla carnea* larvae when given a choice between untreated and lufenuron-treated prey
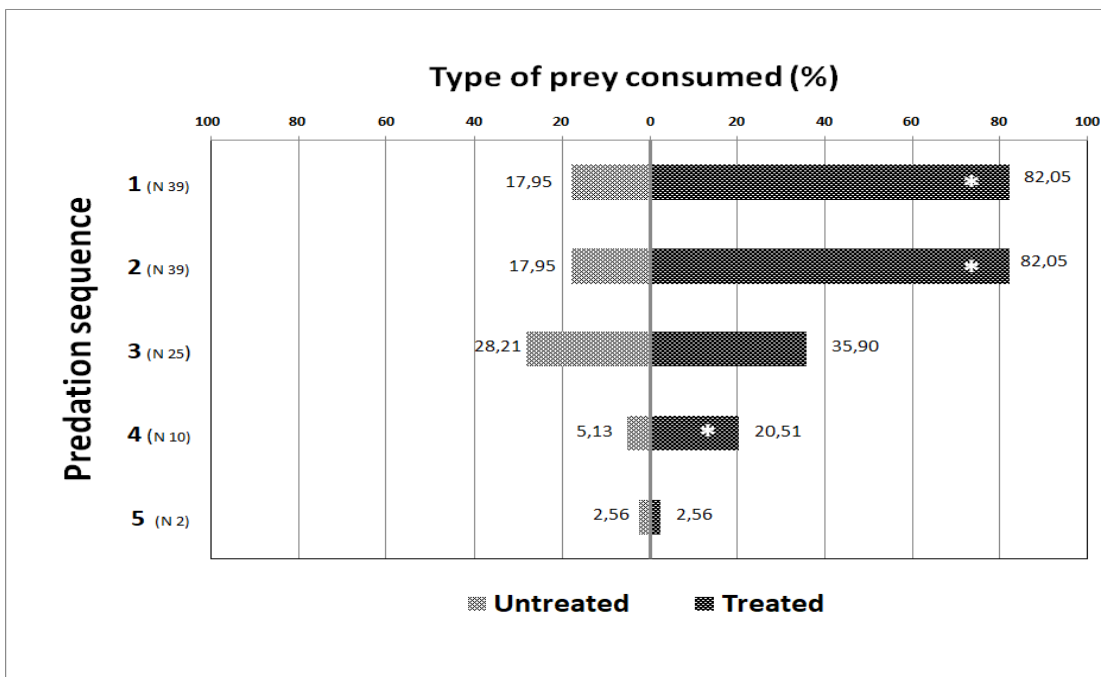
N = Number of *C. carnae* larvae that fed on prey at each sequence point
All the percentage values are expressed on the total of *C. carnea* larvae that showed predation activity during the bioassay (n = 39)

3.5. Reproduction and longevity of adult *C. carnea* treated via ingestion of lufenuron

There was no significant difference in the fecundity and longevity of adults treated via ingestion of lufenuron compared with the control group. However, egg viability was significantly reduced (F = 5.86, df = 1, p = 0.0223); only 53.2% of eggs hatched in the treatment group compared with 75.0% in the control group (Table 4).

Tabla 4. Reproduction and longevity of adults of *Chrysoperla carnea* following ingestion of lufenuron.

Dose (ml/L)	N	Fecundity (nº eggs)	% egg hatchability	Female Longevity (days)	Male Longevity (days)
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0	15	355.07a ± 43.08	75.0a ± 3.25	34.73a ± 3.49	40.23a ± 2.33
1	15	283.67a ± 61.64	53.2b ± 8.40	31.93a ± 3.58	36.69a ± 4.82

N= Number of couples (repetitions)

Means followed by the same letter in a column do not differ significantly at 95%

4.   DISCUSSION

Studies on the effects of lufenuron on *C. carnea* have focused mainly on residual action after insecticide application. The consensus is that this effect is low compared with other chemical insecticide groups, particularly neonicotinoids (Ayubi et al., 2013; Bueno and Freitas, 2001; Rezaei et al., 2007).

In our study, viability of *C. carnea* eggs was not affected by spraying with lufenuron. Similar results were found by Nasreen et al., (2007) for *C. carnea*, and by other authors for *C. externa* (Godoy et al., 2004a; Pasini et al., 2018), and other chrysopids (Rugno et al., 2016). This has been interpreted as a consequence of the protection provided to the embryo by the chorion, that results in greater tolerance to phytosanitary products (Ayubi et al., 2013; Bueno and Freitas, 2004). In addition, we observed that lufenuron did not affect the survival of the larvae that emerged from treated eggs (48 hours old at time of treatment). This confirms results found for *C. externa* (Godoy et al., 2004a), although other authors have reported reductions in *C. externa* larval survival (Bueno and Freitas, 2004).

Direct effects of lufenuron on L2 larvae of *C. carnea* was determined by topical application. The larvae died within the first few days following treatment, demonstrating high susceptibility to lufenuron (LC50 = 0.0153 ml/L). Other studies have also observed susceptibility of lacewing larvae to topical applications of lufenuron including both *C. carnea* (Hussain et al., 2012; Mohammadi et al., 2014), *C. externa* (Bueno and Freitas, 2004; Godoy et al., 2004a; Zotti et al., 2013) and *Ceraeochrysa cincta* (Schneider) (Rugno et al., 2016). As the recommended dose for field applications of lufenuron is 1 ml/L, it follows that foliar sprays must have a serious negative effect on larval populations of this generalist predator. In addition to this direct effect, our results also showed that surviving larvae had longer larval development times; this may have a negative impact on *C. carnea* larva, as they

would be exposed to their natural enemies or adverse environmental factors for longer.

Predators can be exposed to insecticides indirectly through their prey (Müller, 2018). In this study we evaluated sublethal effects on *C. carnea* larvae brought about by feeding on treated prey. Approximately 23% of *C. carnea* larvae died before pupation (doses 0.1 ml/L), and almost all the larvae that pupated died (83%) inside the cocoon without emerging as adults. Malformed adults were detected amongst the few that emerged. At the recommended dose for field use, mortality during the pupal stage was 100%. The mortality we observed in pupae was probably due to inhibition of moulting, which is the typical mode of action of chitin synthesis inhibitors (Oberlander and Silhacek, 1998; Sun et al., 2015). *Chrysoperla carnea* larvae have a low excretion rate for this group of insecticides which could also have contributed (Medina et al., 2002); these authors reported a high mortality in *C. carnea* pupae following topical treatment with diflubenzuron, which was similar to our results (Medina et al., 2003).

The results described above were complemented by those obtained in the preference bioassay where *C. carnea* were offered a choice between untreated and treated prey. It was evident that the majority of *C. carnea* larvae chose prey previously treated with lufenuron as their first and second options (83%). Only few studies investigated responses to insecticide-exposed prey by unexposed predators. In feeding assays after dimethoate treatment, seven-spot ladybirds, *Coccinella septempunctata* (L.) preferred unexposed over exposed aphids to dimethoate (Singh et al., 2004). *Myrmica rubraworker* ants responded more frequently to insecticide-exposed than to unexposed larvae (Müller et al., 2019). If the defensive behaviours of treated larvae were reduced as a result of treatment, then they may be more easily captured by *C. carnea* which may account for the results observed. Generalist predators commonly locate and select their prey using cues associated with the prey (Nesbit et al., 2015) so it may also be speculated that *C. carnea* reacts

to chemical cues produced by the treated prey (Pickett and Glinwood, 2007). Insecticide exposure can alter the cuticular surface (Navarro-Roldán and Gemeno, 2017), or can induce change in synthesis of compounds of the larval secretion which act defensive against generalist predators (Müller et al., 2019). Further studies are required to elucidate the mechanisms involved. The preference by treated prey and the adverse effects on *C. carnea* development after ingestion of treated preys will add to the direct negative contact effects of lufenuron on this predator, which could lead to severe negative impacts on its populations following application of lufenuron in the field.

There have been several studies on different chrysopid (Neuroptera) species that have demonstrated sublethal effects on adults following topical application of lufenuron or following acquisition as a result of residual toxicity (Carvalho et al., 2011; Cordeiro et al., 2010; Rugno et al., 2016). Although the lufenuron molecule is registered as an IGR insecticide, it is known to have adverse effects on the reproductive capacity of Diptera, Coleoptera, Hemiptera and Lepidoptera (Costa et al., 2017; Gangishetti et al., 2009; He et al., 2018; Mansur et al., 2010; Storch et al., 2007). IGRs may have effects on the histological structure of insect gonads, according to studies by El-Bokl et al., (2010). These sublethal effects in adults exposed to insecticide are associated with both female reproductive cells (oogenesis) and spermatogenesis in males (Agüero et al., 2015). Other authors suggest that alterations in reproductive capacity may be associated with disorders of the middle intestine which hamper or delay nutrient absorption and, as a consequence, affect vitellogenesis and egg maturation and production (Engelmann, 1970).

Female fecundity and adult longevity in *C. carnea* were not affected by ingestion of lufenuron, but there was a significant reduction in the viability of their eggs. Similarly, Medina et al., (2002) found that topical applications of adult *C. carnea* with diflubenzuron did not cause mortality or adverse effects on female fecundity, but

completely inhibited egg hatch due to embryo death. Godoy et al., (2004b) also reported reductions in fertility of adult female *C. externa* after topical applications of lufenuron. Ono et al., (2017), found that topical application of *Ceraeochrysa cuban* (Hagen) (Neuroptera, Chrysopidae) adults with diflubenzuron and lufenuron also reduced fertility and longevity. In an ultrastructural study of the effects of bezoylurea on *Drosophila melanogaster* Meigen embryogenesis, Gangishetti et al., (2009) demonstrated that egg hatch was completely inhibited when adult females were treated with a high dose of lufenuron following mating with untreated males; the embryos completed development, but failed to rupture the vitelline membrane. Costa et al., (2017) concluded that lufenuron induced pathological changes in the histological and histochemical structure of the midgut epithelium and gonads of *Anthonomus grandis* (Boheman), which affected spermatogenesis and oogenesis processes. These studies all suggest that lufenuron affects embryogenesis and reduces egg viability.

In conclusion, our results show a significant adverse effect of lufenuron, both by direct contact on lavae and following ingestion by adults. In addition, when *C. carnea* larvae fed on prey treated with lufenuron, which was their preference, high pupal mortality rates ensued. This could have severe negative consequences for natural populations of *C. carnea*. These results must be considered when developing integrated control programmes, especially given that *C. carnea* is one of the key predators used for biological control in economically important crops.

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CAPITULO III
Efectos de tebufenocida sobre huevos, larvas y adultos de *Chrysoperla carnea* (Neuroptera: Chrysopidae)

Effects of tebufenozide on eggs, larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

Este capítulo es una versión del artículo enviado a la revista "*Pest Managment Science*" y que se encuentra en proceso de revisión

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Effects of tebufenozide on eggs, larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

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Effects of tebufenozide on eggs, larvae and adults of *Chrysoperla carnea* (Neuroptera: Chrysopidae)

ABSTRACT

Quantifying compatibility between control agents is essential for development of Integrated Pest Management (IPM). *Chrysoperla carnea* (predator) and Insect Growth Regulator insecticides are widely used in IPM of Lepidoptera. Here we evaluated lethal and sublethal effects of tebufenozide on *C. carnea* under laboratory conditions.

Hatching rate of eggs and survival of resulting neonate larvae was unaffected by treatment with tebufenozide 24 or 48 h after oviposition. Toxic effects of tebufenozide on topically-treated larvae was low; development times of surviving larvae and pupae decreased significantly compared with controls. In choice bioassays, a high percentage of third-instar larvae chose prey (*Spodoptera littoralis*) treated with tebufenozide in preference to untreated prey. Moreover, second-instar larvae that had previously (24 h) consumed tebufenozide-treated prey (0.75 ml/L) had significantly reduced larval development time compared with controls while longevity of surviving adults, fecundity and egg viability were unaffected. Ingestion of tebufenozide by adults at the recommended field dose had no significant effect on female fecundity, egg viability or adult longevity.

Tebufenozide had low toxicity to the developmental stages of *C. carnea* and is therefore a good candidate for inclusion in IPM strategies.

Keywords: Sublethal effects, green lacewing, Integrated Management, prey preference, *Spodoptera littoralis*, IGR.

1. INTRODUCTION

Integrated pest management (IPM) is an approach based on the philosophy of combining the use of natural enemies with chemical pesticides which are the most commonly used conventional pest control products (Flint and Van den Bosch,1981; Van der Blom, 2002). The concept was later expanded to include crop resistance and cultural techniques. Integration of insecticide treatments with conservation biological control (modifying conditions to encourage the activity of natural enemies) is becoming an increasingly important component of IPM (Jonsson et al., 2008). A better understanding of the interactions between insecticides and the most valuable natural enemies in a crop is essential to determine whether they are compatible as pest control agents.

Global use of synthetic pesticides (insecticides, herbicides and fungicides) has increased dramatically leading to several problems. These include toxic effects on wildlife (e.g., birds and beneficial insects e.g. bees) and non-target natural enemies (e.g. predators and parasites). Amarasekare and Shearer (2013) determined the lethal and sublethal effects of various insecticides on two predatory lacewing species (Chrysopidae [Neuroptera]). Under laboratory conditions insecticides have the potential to reduce the efficiency of these predators. Studying only the lethal effects of pesticides does not provide the whole picture; sublethal effects may not be sustainable for crop protection if not investigated and evaluated properly (Bakker et al., 1992).

Insect growth regulators (IGRs), including tebufenozide, have been used for the control of arthropod pests in different crops. Tebufenozide is a nonsteroidal ecdysone antagonist that stimulates the moulting hormone receptor of target insect pests, especially Lepidoptera, inducing premature and lethal moulting (Dhadialla et al., 1998; IRAC, 2020). IGRs are used against a range of pests in agro-ecosystems where *C. carnea* is an important natural enemy of which ecdysone antagonists are

considered the most selective and, therefore, safer (Desneux et al., 2007; Medina et al., 2003a; Medina et al., 2003b; Ono et al., 2017; Zotti et al., 2013). Nevertheless, while effects on natural enemies are less likely to be lethal, sublethal effects on their life history performance and behaviour are possible (Desneux et al., 2007).

Chrysoperla carnea (Stephens) (Neuroptera: Chrysopidae) is an important arthropod predator of insect pests of economically important crops. Commonly called the green lacewing development to adulthood passes through the egg, larval and pupal stages. Its three larval stages are all predatory, but the last stage (L3) is the most voracious and lasts the longest (Canard et al., 1984; Henry et al., 2002). Adults feed on pollen, plant exudates and honeydew and are essential for reproduction and maintenance of populations in the field (Nordlund et al., 2010). It is considered promising as a candidate for pest management programmes worldwide (Tauber et al., 2000; Pineda et al., 2007) due to its wide prey range and geographical distribution, resistance/tolerance to pesticides, voracious larval feeding capacity and commercial availability (New, 1975). Mass (inundative) field releases of *C. carnea* have effectively controlled complexes of pest populations in various crops (Ridgway and Murphy 1984), increasing their widespread usefulness over other predators and parasitoids that are specialists (Bigler, 1984).

The purpose of this study was to determine the effects of tebufenozide on *C. carnea*, specifically: 1) the lethal effects of tebufenozide spray treatments on *C. carnea* eggs; 2) the lethal effects of tebufenozide topical treatments on larvae of *C. carnea* and the sublethal effects on development of treated survivors; 3) predator preferences when offered a choice between treated and untreated prey 4) lethal and sublethal effects of tebufenozide on larvae of *C. carnea*, following consumption of treated prey; 5) effects of tebufenozide on reproduction (fecundity and viability) and longevity of *C. carnea* adults treated with tebufenozide via ingestion.

2. MATERIALS AND METHODS

2.1 Insects and insecticide

The *C. carnea* population was supplied by Koppert S.L. Biological Systems (Almeria, Spain), as first-stage larvae (L1). Larvae were fed *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs (provided by Koppert S.L.) *ad libitum* and adults were fed a nutritious artificial diet made following the methods of Hassan (1975).

The laboratory colony of *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) was established from larvae collected on alfalfa, *Medicago sativa* (L.) (Fabales: Fabaceae) in Cordoba province, southern Spain; the population was renewed annually with individuals taken from the same areas. Larvae were reared following the methodology of Poitout and Bues (1974) and fed on an artificial diet containing alfalfa, as described by Vargas-Osuna (1985).

Insect colonies and bioassays were maintained in chambers at 25 ± 2ºC, 65 ± 5% relative humidity (RH) and a photoperiod of 16:8 h light:dark (L:D) at the Laboratory of Agroforestry Entomology, University of Córdoba.

The insect growth regulator (IGR) tested was Mimic® (tebufenozide 24% SC), formulated as a concentrated emulsion (Certis Europe Ltd., Alicante, Spain). It is registered in Europe for the control of lepidopteran pests of crops and forests.

2.2 Experiments

2.2.1 Effects of tebufenozide on *C. carnea* eggs

Two groups of eggs, (approximately 150 each) were spray-treated with tebufenozide at a concentration of 0.75 ml/L. one group was 24hrs old and the other group was

48hrs old (since oviposition). The treated and control groups of eggs of each age was repeated on three occasions (N = 50 in total for each treatment and control) and the control group were also treated but only with distilled water and a wetting agent (Tween 80 at 0.1%). The volume sprayed per group was 3 ml using a manual sprayer. After treatment, the eggs were allowed to dry for 3 h and then were placed in Petri dishes (200 mm x 20 mm) with food (*E. kuehniella* eggs) for the neonate larvae as they hatched. Dishes were observed daily (at 6 hour intervals) and newly-hatched larvae removed. Six days after treatment those *C. carnea* eggs that had not hatched (not viable) were counted under a stereoscopic microscope. Samples of 30 larvae per replicate that hatched from 48 hr old eggs and its corresponding control group were maintained individually with *E. kuehniella* eggs; 3 and 6 days after treatment larval mortality was recorded.

2.2.2 Effects of tebufenozide on *C. carnea* larval mortality and development times

Newly-moulted second instar *C. carnea* larvae (L2) were placed individually in plastic cups (30mm diameter and 15mm high, with lids) and the dorsal surface of each treated topically with 3µl of tebufenozide in 0.1% Tween 80 using a micropipette. Five concentrations were used 0.75, 0.15, 0.30, 0.06 and 0.012 ml/L (0.75 ml/L is the maximum recommended concentration for field, according to Spanish authorities). Control larvae were treated using the same method but with 0.1% Tween 80 in distilled water and no pesticide. There were twenty replicate larvae for each concentration and the control, and the entire experiment was repeated on three occasions. (N = 60 in total per treatment). During each bioassay larvae were fed *ad libitum* with *E. kuehniella* eggs under laboratory conditions. Larval development stage and mortality were recorded daily from which larval development time of those surviving treatment (days between treatment and the beginning of cocoon formation) and pupation period of those surviving treatment (days between cocoon formation and adult emergence) were determined. The proportion of dead larvae in each

treatment was Abbott-corrected with respect to the mortality that occurred in the control (Abbott, 1925).

2.2.3 Larval *C. carnea* preference for untreated vs. treated prey

Third-instar larvae of *S. littoralis* were placed individually in plastic boxes (30 mm in diameter and 15 mm high, with lids) and allowed to feed for 24 h on a leaf disk of alfalfa *Medicago sativa* (L.) (Fabales: Fabaceae); the leaf disk of 5 mm diameter had either been treated with 3 µl tebufenozide at a concentration of 0.75 ml/L in 0.1% aqueous Tween 80, or with 0.1% aqueous Tween 80 (control larvae). Only larvae that had consumed the entire leaf disk within 24 h were used as prey items. After 24 h, L3 larvae of *C. carnea* were placed individually in clean Petri dishes (60 mm Ø) with six *S. littoralis* prey items: three *S. littoralis* larvae treated with tebufenozide and three control larvae. Treated and control *S. littoralis* larvae were distinguished from each other using paint on the thorax, following the methodology of Ortiz-Moreno and Vargas-Osuna (2009). Forty replicate larvae of the predator were used and preference and predation sequence data were gathered by continuous visual observation for 12 h.

2.2.4 Effects of consumption of tebufenozide-treated prey on *C. carnea*

Second-instar larvae of *S. littoralis* were placed individually in plastic boxes (30 mm in diameter and 15 mm high, with lids) and allowed to feed for 24 h on an alfalfa leaf disk treated with 3 µl tebufenozide (in 0.1% aqueous Tween 80) as described previously. Two concentrations of tebufenozide were evaluated: 0.15 ml/L and 0.75 ml/L. Control larvae were treated in the same way except that the leaf disks were treated with 3 µl of 0.1% aqueous Tween 80 only. There were thirty replicate larvae per treatment and control, and the entire bioassay was repeated on three occasions. Larvae that completely consumed the alfalfa leaf disk were offered as prey to L2 larvae of *C. carnea* held individually in plastic boxes (30 mm in diameter and 15 mm

high), at a rate of one prey item per larva. After 24 h, *C. carnea* larvae that had consumed the entire prey item were maintained on *E. kuehniella* eggs and observed daily for mortality and to determine: larval development time; pupation period; and the fecundity, viability and longevity of adults emerging from larvae that survived the treatment.

2.2.5 Effects of tebufenozide on the *C. carnea* adults treated by ingestion

Newly-emerged *C. carnea* adults were sexed and couples (one male and one female) were confined in cylindrical transparent plastic containers (160 mm diameter x 60 mm height). A filter paper was placed in the upper part of each cylinder as an egg-laying substrate. A piece of plastic impregnated with diet was placed at the bottom of each cylinder, alongside a small water container (30 mm in diameter and 15 mm in height). In the treatment group, the water container was replaced with one filled with tebufenozide at a concentration of 0.75 ml/L, for 24 h; the control group received only distilled water. After this period all pairs (10 per treatment and control) were transferred to clean cylinders with diet and water, and evaluated every 48 h for 16 days or until the adults died. At each evaluation point the eggs laid by each couple were removed, counted and incubated for 6 days in Petri dishes with larval food. Newly hatched larvae were counted and removed daily. After six days those eggs that had not hatched (non-viable eggs) were counted under a stereoscopic microscope.

2.2.6 Statistical análisis

Larval and pupal development times, total fecundity and adult longevity data were analyzed by ANOVA (after testing the data for normality and homoscedasticity); comparisons of means amongst mating combinations was done using Tukey test, applying a 0.05 significance level in the program STATISTIX 10.0. Data expressed

as percentages (egg viability, larval mortality, prey preference) were analysed by χ2 test at 95%

3 RESULTS

3.1 Effects of tebufenozide on *C. carnea* eggs

There was no significant effect of tebufenozide treatment on viability (hatching rate) of either 24 or 48 h old eggs of *C. carnea*. In all cases their percentage viability (% hatching) was high (Table 1). Survival of larvae hatched from 48 hr treated eggs was high and there was no mortality in the corresponding control.

Tabla 1. Viability of 1 and 2-day-old eggs of *Chrysoperla carnea* following spray-treatment with tebufenozide

Dose (ml/L)	Eggs 1 day after oviposition		Eggs 2 days after oviposition	
	N	% Viability ± SE	N	% Viability ± SE
0	130	88.58 a ± 5.27	140	90.68 a ± 2.16
0.75	147	88.58 a ± 3.04	207	88.43 a ± 2.73

N = Number of eggs treated.

Values followed by the same letter in a column do not differ significantly from each other at the 95% level

3.2 Effects of tebufenozide on *C. carnea* larval mortality and development time

The mortality of second instar larvae of *C. carnea* treated topically with five doses was not affected by tebufenozide treatment, with the exception of the highest concentration (0.75 ml/L) where 13.4% of larvae died which was significantly higher

than the control ($x2 = 5.93$, df = 1, p = 0.0295; Table 2). Sublethal effects of tebufenozide were studied on *C. carnea* second instar larvae that survived topical treatment. The larval development period was significantly shorter in the treatment (from 4.6 - 5.3 days) than in the control (5.9 days) ($F_{5,258} = 7.62$, p<0.0001). The pupation period was significantly shorter in the treatment (9.2 - 9.6 days) than the control (9.8 days) $F_{5,258} = 3.46$, p=0.0048. No significant differences were detected at the 0.75 ml/L concentration (Table 2).

Tabla 2. Mortality of second-instar *Chrysoperla carnea* larvae following topical application of tebufenozide and development time of the survivors.

Doses (ml/L)	N	Mortality		n	Larval development time	n	Pupal duration
		%	Corrected[1]		Mean ± SE (days)		Mean ± SE (days)
0	60	3.3	-	46	5.95 a ± 0.234	41	9.80 a ± 0.136
0.012	60	1.7	-	53	4.63 bc ± 0.175	47	9.45 bc ± 0.113
0.06	60	1.7	-	58	4.63 c ± 0.181	51	9.27 c ± 0.063
0.30	60	1.7	-	47	4.54 c ± 0.158	46	9.41bc ± 0.073
0.15	60	6.7	3.4	49	5.30 b ± 0.227	43	9.51 bc ± 0.090
0.75	60	16.7 *	13.4	36	4.68 c ± 0.188	31	9.68 ab ± 0.149

* indicates significant differences with respect to the control at the 5 % level using the $x2$ test
[1]Mortality corrected by Abbott's formula (1925)
N = number of treated larvae.
n = number of surviving larvae.
Means followed by the same letter in a column do not differ significantly from each other at the 95% level.

3.3 Preference in *C. carnea* prey selection when given a choice between tebufenozide-treated *S. littoralis* prey and untreated prey

Experiments on the overall prey preference and the sequence of prey selected showed that third instar *C. carne* larvae selected *S. littoralis* larvae treated with tebufenozide in preference to untreated *S. littoralis* larvae ($x2 = 7.06$, df = 1, p =

0.0104). More *C. carnea* selected tebufenozide-treated *S. littoralis* (67.5%) as their first prey item, than untreated *S. littoralis*. Significantly more *C. carnea* larvae selected tebufenozide-treated *S. littoralis* as their second prey (85%; $\chi 2 = 11.17$, df $= 1$, p $= 0.0016$) and third prey items (76.3%; $\chi 2 = 5.65$, df $= 1$, p $= 0.0315$) compared with untreated *S. littoralis* (Fig 1). Amongst the 40 replicates, there was a marked preference for consumption of tebufenozide-treated prey; 62.8% of the total prey consumed were treated which was significantly than the percentage of untreated prey consumed ($\chi 2 = 7.06$, df $= 1$, p $= 0.0104$).

Figura 1. Preference of *Chrysoperla carnea* larvae when given a choice between untreated and tebufenozide treated prey.

Type of prey consumed (%)
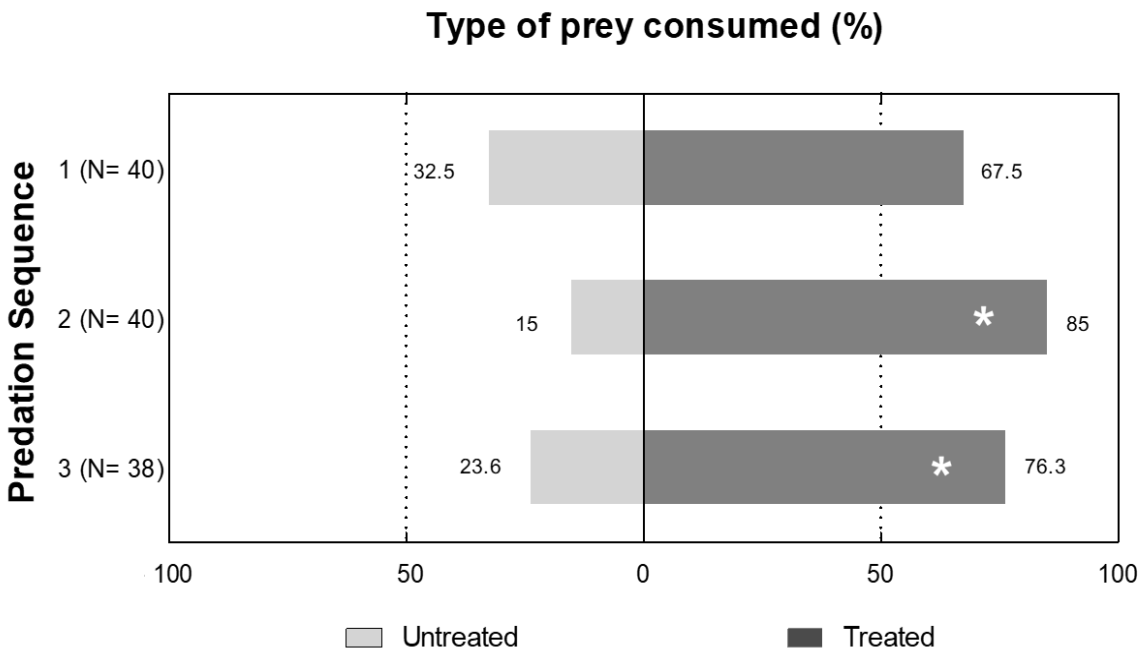
* Indicates differences between treated and untreated prey that are statistically significant at the 5 % level using the $\chi 2$ test
N = Number of *C. carnae* larvae that fed on prey at each sequence point
All the percentage values are expressed on the total of *C. carnea* larvae that showed predation activity during the bioassay (n = 40)

3.4 Lethal and sublethal effects of consuming prey treated with tebufenozide on *C. carnea* larvae

Mortality in *C. carnea* larvae that consumed prey treated with either of the two concentrations of tebufenozide (0.15 ml/L and 0.75 ml/L) was not significantly different to mortality in the untreated control (Table 3). Corrected mortality ranged from 4.7% to 2.1% at concentrations of 0.15 ml/L and 0.75 ml/L, respectively. The larval development time was shorter in *C. carnea* that consumed tebufenozide-treated prey than in the control group and this was significant at the higher tebufenozide concentration ($F_{2,117}=19.49$, p = 0.0205; Table 3). In contrast, pupal duration of those surviving to emerge as adults was not significantly different amongst treatments and control. For adults that survived following consumption (as larvae) of prey treated with 0.15 ml/L tebufenozide, there was a slight, but not statistically significant, reduction in fecundity and viability of eggs (Table 4); at the 0.75 ml/L concentration surviving adults showed a slight, but not statistically significant, increase in fecundity and egg viability. In both sexes, adult longevity was not significantly affected by prior consumption of tebufenozide-treated prey as larvae.

Tabla 3. Mortality of second-instar *Chrysoperla carnea* larvae fed on prey treated with tebufenozide, and development time of the survivors.

Doses (ml/L)	N	Mortality		n	Larval development time	n	Pupal duration
		% *	Corrected[1]		Mean ± SE (days)		Mean ± SE (days)
0	57	7.01	-	53	8.00 a ± 0.47	40	9.58 a ± 024
0.15	59	11.86	4.78	52	6.94 ab ± 0.47	42	9.42 a ± 0.23
0.75	54	9.25	2.17	49	6.16 b ± 0.48	42	9.57 a ± 0.23

* No significant differences were found respect to the control at the 5 % level using the χ2 test
N = number of treated larvae.
n = number of surviving larvae.
[1]Mortality corrected by Abbott's formula (1925)
Means followed by the same letter in a column do not differ significantly from each other at the 95% level. Mortality was assessed up to 8 days after treatment.

Tabla 4. The fecundity, % viability and longevity of surviving adults of *C. carnea* fed on prey treated with tebufenozide as larvae.

Doses (ml/L)	N	Fecundity (number of eggs) Mean ± SE	% Viability Mean ± SE	Female Longevity (days) Mean ± SE	Male Longevity (days) Mean ± SE
0	15	112.53 a ± 19.10	95.17 a ± 0.88	16.53 a ± 1.96	25.80 a ± 1.96
0.150	16	92.56 a ± 18.49	91.58 a ± 0.88	17.56 a ± 1.89	25.06 a ±1.90
0.750	13	142.46 a ± 20.51	96.02 a ± 0.95	18.69 a ± 2.10	27.15 a ±2.11

N = Number of couples (replicates).

3.5 Fecundity, viability and longevity of adult *C. carnea* treated with tebufenozide via ingestion

There was no mortality in adult *C. carnea* treated via ingestion with 0.75 ml/L tebufenozide; fecundity, egg viability and longevity of tebufenozide-treated adult *C. carnea* was not significantly different to the control (Table 5).

Tabla 5. Fecundity and longevity of adult *Chrysoperla carnea* following ingestion of tebufenozide.

| Doses (ml/L)	N	Fecundity (number of eggs) Mean ± SE	% eggs hatchability Mean ± SE	Female Longevity (days) Mean ± SE	Male Longevity (days) Mean ± SE
0	10	356.8 a ± 49.75	78.1 a ± 6.38	38.4 a ± 7.05	40.7 a ± 17,18
0.75	10	313.3 a ± 36.94	77.5 a ± 5.47	35.7 a ± 10.04	43.6 a ± 17,92

N = Number of couples (repetitions).
Means followed by the same letter in a column do not differ significantly from each other at the 95% level.

4 DISCUSSION

New classes of selective pesticides that reduce risks to non-targets are increasingly replacing traditional broad-spectrum pesticides. However, it is important to understand how they can best be integrated with biological control agents to achieve sustainable management as promoted by European regulations. Beneficial organisms can be exposed to pesticides via multiple routes (Symondson et al., 2002). This study evaluated the direct and indirect effects of tebufenozide on developmental stages of the predator through exposure that was topical, via feeding on treated prey, and via ingestion. Our results also demonstrated high predator preference for consumption of prey that had been treated with tebufenozide.

4.1 Effects of tebufenozide on *C. carnea* eggs

Effects of insecticides on eggs and embryo survival vary according to the insect species and the active substance under study. The egg phase is usually the stage most tolerant to the action of pesticides. In this study viability of *C. carnea* eggs was not affected by tebufenozide sprays. This can be interpreted as a consequence of the protection offered to the embryo by the chorion, which is composed of impermeable sclerotized proteins that limit the entry of acqueous pesticides (Nation, 2002). Egg tolerance to some insecticides has already been observed in chrysopids (Passini et al., 2018) and particularly for *C. carnea* (Suarez-Lopez et al., 2020). However, the eggs of *Chrysoperla externa* (Hagen) were more susceptible to conventional insecticides (endosulfan and cypermethrin) than selective pesticides (Rimoldi et al., 2008).

No mortality in embryos or larvae newly hatched from treated eggs was observed in our study; additionally, tebufenozide did not affect the survival of larvae emerging from 48 h old treated eggs. This is similar to results described previously for *C. externa* (Godoy et al., 2004).

4.2 Effects of tebufenozide on mortality and development time of larval *C. carnea*

Our results showed that *C. carnea* second instar larvae were slightly susceptible to topical treated with tebufenozide. Other authors have documented similar results when at maximum field-recommended concentration tebufenozide were harmless to larvae *C. carnea* as a result of low rates of absorption and penetration in the insect integument after application (e.g, <45%, in 24 h); low penetration of tebufenozide helps to explain its nontoxicity to *C. carnea* larvae (Medina et al., 2002). Yu (1988) reported that several mechanisms may be involved in the selectivity of tebufenozide in *C. externa* larvae these included limited penetration through the cuticle, but also alterations in the target site. Using molecular techniques, Zotti et al., (2013) on chrysopids found that subtle differences in architecture of the ecdysone receptor domain may interfere with binding of tebufenozide.

Effect of insect growth regulators on non-targets can be slight compared with other chemical insecticides but this depend on active ingredient and species of insect (Mandour, 2008; Garzon et al., 2015; Ijaz et al., 2017; Maia et al., 2016). For example, wexythiazox and imidacloprid were categorized as causing harmful toxicity in larvae of *C. externa* under laboratory condition (Bueno and Freitas, 2004) while the insect growth regulator, diflubenzuron, caused significant levels of mortality in L3 larvae of *C. carnea* topically treated (Medina et al., 2003a). Amarasekare and Shearer (2013) found that the insect growth regulator novaluron and the pyrethroid lambda-cyhalothrin were both toxic to L2 larvae of *C. carnea* and *Chrysoperla johnsoni* (Henry et al., 2002).

Larval development time and pupal duration were shorter in *C. carnea* L2 larvae treated with tebufenozide than in the control. These sublethal effects could be explained by the timing of the treatment. Subsequent development of surviving larvae may be modified due to the mechanism of action of the insecticide (Smagghe

et al., 2018; Trisyono and Chippendale, 1997). However, no adverse effects were observed on larval and pupal duration and survival of *Ceraeochrysa cincta* (Neuroptera: Chrysopidae) exposed topically to tebufenozide (Rugno et al., 2016).

4.3 Preference of *C. carnea* larvae for untreated vs. treated prey

In the choice bioassay *C. carnea* larvae preferred *S. littoralis* prey treated with tebufenozide compared with untreated larvae. This preference may be because treated prey were weak and slow moving, making them easier to predate than the untreated larvae. It is also possible that the defensive behaviours of treated larvae were reduced which would also make them easier to catch; this has been reported previously (Symondson et al., 2002).

Using the same methodology, previous studies have shown similar results with a variety of active ingredients: preference behaviour was more obvious in *C. carnea* larvae preying on *S. littoralis* treated with lufenuron (76.5% preference for treated prey), a inhibit chitin synthesis (Suarez-Lopez et al., 2020); and also significant when preying on *Xanthogaleruca luteola* (Müller) previously infected with the entomopathogenic fungus *Beauveria bassiana* (Balsamo) (Mena Castillo, 2019). It is likely that the effect is not related to the active ingredient but to the effects on physiological and growth processes inside treated larvae that alters their reactions and defence behaviours. Larvae of *C. carnea* react to chemical cues produced by prey (Pickett et al., 2007) and it is possible that pesticide treatment affects these cues. Navarro-Roldan and Gemeno (2017) reported that the insecticide exposure altered the cuticular surface and may have induced changes in the synthesised compounds present in prey larval secretions that act defensively against generalist predators (Müller et al., 2019).

Huerta et al. (2004) showed that *C. carnea* larvae preferred *S. littoralis* larvae treated with imidacloprid and natural pyrethrins compared with control larvae. In feeding assays after dimethoate treatment, seven-spot ladybirds, *Coccinella septempunctata* (L.) preferred unexposed aphid prey compared with prey treated with dimethoate[47]. Müller et al., (2019) observed that *Myrmica rubra* (Hymenoptera: Formicidae) worker ants preferred more frequently to insecticide-exposed than to unexposed larvae.

Preference for treated prey could have a lethal effect on *C. carnea*, as seen for feeding on prey treated with lufenuron (Suarez-Lopez et al., 2020). Whether feeding on insecticide-treated prey causes mortality or not, predators could also suffer a greater energy expense as a result of consuming treated prey if they have lower nutritional content compared with untreated prey.

4.4 Effects on *C. carnea* larvae of consuming prey treated with tebufenozide

Sublethal effects of insecticide on predators via feeding on treated prey can have an impact on the efficiency and abundance of biological control agents in the field. In this study, we evaluated sublethal effects on *C. carnea* larvae brought about by feeding on prey treated with one of two tebufenozide concentrations. Results showed that larval development time was significantly reduced in both treatments compared with the control. Suarez-Lopez et al. (2020) also found significant reduced development times in *C. carnea* larvae consuming prey treated by lufenuron (1ml/L). Shorter larval development times as a result of exposure to insecticides decreases the overall rate of predation.

No sublethal effects were apparent for the adult *C. carnea* that survived feeding on tebufenozide-treated prey as larvae; fecundity, viability and adult longevity were not significantly different to controls. Mandour (2008) found similar results for *C. carnea* larvae fed on spinosad-treated *Brevicoryne brassicae* L. aphids; there was no

negative impact on mortality, fecundity, fertility of survival adults. Giolo et al. (2009) studied exposure of larvae and adults of *C. carnea* to residues of various pesticides on plant leaves; for residues of methoxyfenozide (other ecdysone agonists) they found no sublethal effects on the reproductive behaviour of adults (fecundity and fertility). Smagghe and Degheele (1994) found that the predator *Podisus sagitta* (Fabricius) remained able to lay eggs when fed on larvae of *Spodoptera exigua* (Hübner) topically treated with 20 μg/larvae of tebufenozide. In our results, the longevity of adult *C. carnea* survivors was not affected at either concentration of tebfenozide. These results confirm those of Amarasekare and Shearer (2013) looking at the sublethal effects on larvae of two chrysopids fed novaluron-contaminated (IGR) eggs of *E. kuehniella*; surviving adults had similar longevity to the control group.

Pesticide mortality and negative effects on behaviour of natural enemy species has been reported, mainly caused by consumption of prey contaminated with neonicotinoids. This includes the coccinellid predator, *Serangium japonicum*, and the predatory mite, *Neoseiulus californicus* (McGregor) (Poletti et al., 2007), *Phytoseiulus macropilis* (Banks) (He et al., 2012) and the hemipteran predator, *Orius insidiosus* (Say) (Camargo et al., 2017).

4.5 Adult bioassay

Several studies suggest that IGRs affect embryogenesis and reduce the egg viability of target pests but these effects differ among insect species. Beneficial arthropods exposed also respond differently, according to the method of application and the active substance used.

The safety of tebufenozide in relation to *C. carnea* adults is attributed to its low rate of absorption and penetration through the adult integument after topical application (Medina et al., 2003b). In our study, no toxicity was found when adults of *C.*

carnea ingested tebufenozide-treated prey; fecundity and egg viability were not altered compared with the control. Similarly, Medina et al., (2003b) found that *C. carnea* adults treated topically with field rates of tebufenozide or receiving inoculum by ingestion of treated prey did not cause mortality or adverse effects on fecundity, or egg hatching. To understand the lack of negative effects on reproduction parameters Medina et al., (2002) made a microscopic study of 8-day old females topically-treated with radioactively-labelled tebufenozide and showed that oocyte growth and process of ovulation was not altered and no radioactivity was recovered in the ovaries and eggs.

Oral ingestion of spinosad in artificial diet rapidly killed *C. carnea* adults and toxic symptoms included tremors, paralysis and swelling; spinosad ingestion also had a profound effect on fecundity of *C. carnea* (Mandour, 2008). Schneider et al. (2008) found that contact-treatment of adult *Hyposoter didymator* (Thunberg) parasitoids with tebufenozide (100, 500 and 1,000 mg/l) had no effect on any life parameter of their offspring and low absorption of tebufenozide.

Neurotoxic insecticides vary in their lethal and sublethal effects on predators. Imidacloprid killed adult *C. carnea* (91.7%) within four days of ingestion without altering reproduction, and triflumuron inhibited fertility of treated adults (Huerta et al., 2004). The same results were reported by Kanna and Chandrasekaran (2006), who found that longevity of *C. carnea* adults fed with emamectin at a low rate (5.0 g a.i. ha-1), was not significantly reduced compared with controls. Garzón et al., (2015) stated that deltamethrin significantly reduced the fecundity and fertility of adult *C. carnea* adults when exposed to dried residues on the glass surfaces.

5. CONCLUSION

In natural habitats, exposure to pesticides can be via several routes, e.g. at the time of spraying and through the food chain. This study showed that tebufenozide is not toxic to eggs of the predator *C. carnea* and of low toxicity to its larvae. Larval development times and pupal duration were both significantly reduced when treated topically. The prey preference test showed that third-instar larvae of *C. carnea* prefer treated larvae to untreated ones. Sublethal effects caused by the consumption of treated prey were mild with low toxicity. Reproductive parameters in adults were not affected, and there were no effects on fertility, fecundity or longevity of adults that ingested tebufenozide. Based on our results we conclude that the use of this compound can be recommended in IPM programmes involving lacewings.

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CAPITULO IV

Interacciones de entomopatógenos con insecticidas
reguladores del crecimiento para el control de
Spodoptera littoralis (Lepidoptera: Noctuidae)

Interaction of entomopathogens with insect growth regulators for the control of *Spodoptera littoralis* (Lepidoptera: Noctuidae)

Este capitulo es una versión del artículo enviado a la revista "Biological Control" y que se encuentra en proceso de revisión

Biological Control

Interactions of entomopathogens with insect growth regulators for the control of Spodoptera littoralis (Lepidoptera: Noctuidae)
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Abstract:	Agriculture is currently expected to establish different control alternatives that have less impact and involve a lower environmental risk, one of which is the integration of microbial insecticides in pest management programmes. This work evaluates the effects of three microbial agents to control Spodoptera littoralis: Bacillus thuringiensis var. aizawai ; an isolated native of Beauveria bassiana ; and the S. littoralis nucleopolyhedrovirus (SINPV). These have been used both in individual treatments and in combination with low doses of lufenuron and tebufenozide, synthetic molecules that act as Insect Growth Regulators (IGRs). The SINPV mortality rate demonstrated its lethal efficacy. The median lethal concentrations (LC $_{50}$) of SINPV, lufenuron, and tebufenozide on third instar S.littoralis larvae were 6.6 x 10 ^{5} OB/ml, 0.0052 ml/L, and 0.058 ml/L, respectively. The results of simultaneous treatments showed additive effects for B. thuringiensis and B. bassiana with each of the synthetic insecticides. An antagonistic effect occurred when SINPV and tebufenozide were administered simultaneously. In contrast, the combination of SINPV and lufenuron resulted in a significantly higher larval mortality rate than treatments using each agent alone. It is concluded that simultaneous applications of these entomopathogens together with low concentrations of the IGRs can be included in Integrated Pest Management (IPM) programmes, with the exception of the combination SINPV and tebufenozide. A product based on lufenuron and the SINPV is presented as a candidate for commercial development.

Interaction of entomopathogens with insect growth regulators for the control of *Spodoptera littoralis* (Lepidoptera: Noctuidae)

ABSTRACT

Agriculture is currently expected to establish different control alternatives that have less impact and involve a lower environmental risk, one of which is the integration of microbial insecticides in pest management programmes. This work evaluates the effects of three microbial agents to control *Spodoptera littoralis*: *Bacillus thuringiensis* var. *aizawai*; an isolated native of *Beauveria bassiana*; and the *S. littoralis* nucleopolyhedrovirus (SlNPV). These have been used both in individual treatments and in combination with low doses of lufenuron and tebufenozide, synthetic molecules that act as Insect Growth Regulators (IGRs). The SlNPV mortality rate demonstrated its lethal efficacy. The median lethal concentrations (LC_{50}) of SlNPV, lufenuron, and tebufenozide on third instar *S.littoralis* larvae were 6.6×10^5 OB/ml, 0.0052 ml/L, and 0.058 ml/L, respectively. The results of simultaneous treatments showed additive effects for *B. thuringiensis* and *B. bassiana* with each of the synthetic insecticides. An antagonistic effect occurred when SlNPV and tebufenozide were administered simultaneously. In contrast, the combination of SlNPV and lufenuron resulted in a significantly higher larval mortality rate than treatments using each agent alone. It is concluded that simultaneous applications of these entomopathogens together with low concentrations of the IGRs can be included in Integrated Pest Management (IPM) programmes, with the exception of the combination SlNPV and tebufenozide. A product based on lufenuron and the SlNPV is presented as a candidate for commercial development.

Keywords: Integrated Pest Management*,* Insecticidal Activity*,* Nucleopolyhedrovirus, *Bacillus thuringiensis, Beauveria bassiana,* Interactions.

1 INTRODUCTION

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) is distributed throughout the Mediterranean basin, as well as much of central and south-eastern Africa. Its larvae are extremely polyphagous and feed on the leaves of a wide diversity of cultivated plants, both herbaceous and woody. Their economic importance and damage severity are associated with three fundamental characteristics: polyphagia; a tendency towards gregariousness; and migratory behaviour (Cabello and Belda, 1994). Synthetic chemical insecticides with a low degree of selectivity (broad-spectrum) remain the main method for controlling this species. However, the continuous and exclusive application of these compounds has led to the development of populations resistant to many active substances (Kranthi et al., 2002). In recent decades, there has been increasing use of Insect Growth Regulator (IGR) insecticides; these are more selective, as they only affect arthropods, but their use may also lead to the development of resistant populations (Ishaaya and Klein, 1990).

Among the options for integrated pest management is biological control with the use of entomopathogenic microorganisms. These include a large group of viruses, bacteria, fungi and nematodes, among others, that vary in their infection pathways, replication sites, and pathogenic mechanisms. Few of these pathogens have a broad spectrum of hosts, most prefer certain species of insects, and some even have a selective pathogenicity according to the different developmental stages of the insect (Lacey et al., 2015). In recent years, a great deal of interest has been focused on their mass production and application in the field as microbial insecticides to control agricultural and forest pests. They can be applied using technology similar to that for chemical insecticides and, given their selectivity and compatibility, hold great promise for integrated pest management programmes. Currently, the most widely used microbial insecticides against lepidopteran pests are formulations based on the bacterium *Bacillus thuringiensis* Berliner, the fungus *Beauveria bassiana* (Balsamo)

Vuillemin (Hyphomycetes), and viruses in the Baculoviridae Family (Vargas-Osuna, and Santiago-Álvarez, 1988; Tanada and Kaya, 1993; Lacey et al., 2015).

IGRs are lethal for immature stages of insects, mainly through ingestion, and the insecticidal activity is specific to each product and species (Aldebis et al, 1988; Graf, 1993). Chitin synthesis inhibitors, such as the insecticide lufenuron, act by inhibiting chitin synthesis in holometabolous larvae (Lepidoptera, Coleoptera and Diptera) (Beeman, 1982; IRAC, 2020). The IGR tebufenozide, a highly selective nonsteroidal ecdysone agonist against Lepidoptera (Heller et al., 1992), has a specific insecticidal mechanism: it mimics the function of natural insect ecdysone, interacts with the ecdysone receptor protein, induces early maturity, and causes insects to moult to death (Lee et al., 2019; IRAC, 2020). It acts mainly through ingestion, but has also been shown to have a residual contact or topical application effect against some species. The compound has been demonstrated to be very effective against species in the genus *Spodoptera* (Gobbi et al., 2000).

The negative effects of the exclusive and unreasonable use of broad-spectrum chemical insecticides have resulted in the need to improve insecticidal efficacy with new active substances that are more ecologically selective and less aggressive, and therefore of greater utility in terms of the integrated protection that is the current trend in pest control. One of these alternatives is microbial control, where more selective active materials, such as naturally occurring pathogenic microorganisms are used.

A combined application of an entomopathogen with low doses of lufenuron and tebufenozide may present benefits such as improved entomopathogen efficacy and a reduced mortality time (compared to the use of either agent alone). Moreover, lower doses of chemical insecticides result in reduced environmental pollution and less harm to natural enemies (Hernandez et al., 2017; Suarez-Lopez et al., 2020). According to Hunter-Fujita (1998), the interaction between two control agents can be defined as the effect of each agent in the presence of the other, usually measured

in terms of host mortality. Interaction studies can involve the search for two agents that together produce a much higher total mortality rate than expected according to the sum of the mortality caused by each of the two agents acting separately, in other words, true synergism. Furthermore, for reasons of environmental safety, means can be sought to reduce the concentration of a chemical insecticide through the simultaneous use of a compatible natural agent. In this case, the overall effect is the sum of the two mortality rates combined (additive effect), while synergism may be an extra effect. Interactions where the total mortality rate is less than the sum of deaths expected from each component acting individually are, of course, unacceptable and referred to as antagonism.

Given the possibility of using microbial insecticides combined with IGRs on *S. littoralis*, we studied the insecticidal effect of entomopathogens and the IGRs lufenuron and tebufenozide, using both individual treatments and the simultaneous application of low concentrations of IGRs with each of the entomopathogens, under laboratory conditions.

2 MATERIALS AND METHODS:

2.1 *Spodoptera littoralis*.

The laboratory colony of *S. littoralis* was established from larvae collected on alfalfa *Medicago sativa* (L.) (Fabales: Fabaceae) in the Cordoba province, southern Spain. The population is renewed annually with individuals taken from the same areas. The larvae were reared following Poitout and Bues (1974), and fed an artificial alfalfa-containing diet (Vargas-Osuna, 1985). All the bioassays used newly moulted 3rd-instar (L3) *S. littoralis* larvae. The insect colonies and bioassays were kept under insect chamber conditions at 25±2ºC, 65±5% relative humidity (RH), and a photoperiod of 16:8 h (L:D).

2.2 Microbial insecticides

2.2.1 *Bacillus thuringiensis* var. *aizawai*

The bioassays were carried out using an aqueous suspension of the spore-crystal complex (S+C) isolated from the commercial biopesticide Xentari® GD: 15% (15 million I.U./g) p/p. WG. Supplied by Kenogard, S.A (Barcelona, Spain) whose active ingredient is *B. thuringiensis* subsp. a*izawai* where Cry protein (Cry1C) is naturally present. The inoculum was obtained by multiplying the isolate in nutrient agar (Cultimed) medium following the method described by Hussien (2012). The spore-crystal suspension obtained was centrifuged twice and the resulting sediment was resuspended with sterile distilled water; the (S+C) richness was determined using a Neubauer chamber.

2.2.2 *Beauveria bassiana*

B. bassiana isolate, from the collection of entomopathogens of the Agroforestry Entomology Group at the University of Cordoba (with reference S-14), was obtained using the "Galleria trap" method from a soil samples from the province of Cordoba (Ortega, 2012), and preserved in a sterile sand tube in cold storage at 4°C. The inoculum was prepared as an aqueous suspension of conidia whose concentration was determined using a Neubauer chamber.

2.2.3 SINPV

The SINPV is a Morocco isolate supplied by the Lutte Biologique de la Minière (INRA) station, France. It was multiplied in *S. littoralis* larvae in the laboratory of the Agroforestry Entomology Group at the University of Cordoba, and obtained as an aqueous suspension of occlusion bodies (OBs). The inoculum concentration was determined under phase-contrast microscopy in a Hawksley counting chamber.

All entomopathogen suspensions were kept at 4°C until their use in the bioassays.

2.3 IGR insecticides

The insect growth regulators (IGRs) tested were: the commercial product Match® (Syngenta Crop Protection Ltda, Spain) containing 5 g/L of lufenuron formulated as an emulsifiable concentrate (EC); and the commercial product Mimic® (supplied by Certis Europe Ltd., Alicante, Spain) containing 240 g/L of tebufenozide formulated as a suspension concentrate (SC). These compounds are currently used as IGR insecticides in horticultural crops.

2.4 Bioassays

2.4.1 Insecticidal activity

Treatments with *B. thuringiensis*, SlNPV, and IGRs were applied via ingestion. Newly moulted 3rd instars of *S. littoralis* were separated into lidded plastic boxes (30 mm in diameter and 15 mm high) and fed, for 24 hours, with 5 mm discs onto which was deposited one drop of 3 µl concentration of the insecticidal product using a precision micropipette. The larvae were kept in the boxes and fed the artificial diet until the end of the experiment (Vargas-Osuna et al., 1988).

The *B. bassiana* treatments involved the topical application of a drop of 3 µl in 0.1% aqueous Tween 80 wetting agent onto the dorsum of *S. littoralis* larvae using a precision micropipette. The control was treated with the same volume of distilled water and Tween 80. These larvae were fed with the artificial diet.

For the bioassays involving the microbial insecticides 15 larvae were used for each concentration and two replicates were made. For IGRs, 20 larvae were used and three replicates were made. The controls were treated in the same way but with

distilled water and a wetting agent, Tween 80 at 0.1%. The mortality rate was recorded daily for up to 12 days.

2.4.2 Interactions of entomopathogenic and IGR agents

The same application method was used in the interaction bioassays as in the individual bioassays of insecticide activity. Two concentrations were used for SlNPV, and one concentration for the other two microbial products, *B. bassiana* and *B. thuringiensis*, at the highest possible concentration given the lower insecticidal activity of these isolates. For lufenuron, a fixed concentration of 0.008 ml/L was used; and for tebufenozide, two concentrations (0.032 and 0.006 ml/L) were tested (Table 1). The bioassay for each entomopathogenic agent consisted of a control, individual applications of the entomopathogen and IGRs, and the simultaneous application of the entomopathogen with each IGR product. Twenty larvae were given each treatment and three replicates were performed. The mortality rate was recorded daily for up to 15 days. The mortality rates of the treatments were corrected according to the control mortality using Abbott's formula (Abbott, 1925).

Tabla 2. Insecticides and doses used in the interaction bioassays

Insecticide	Units	Assay doses	Recommended field rate (Spain)
S. littoralis Nucleopohyedrovirus (SlNPV)	OB/ml	5.2×10^5, 2.6×10^6	-
Beauveria bassiana	Conidia/ml	1.2×10^8, 1.9×10^8	-
Baciillus thuringiensis var. *aizawai*	Spore+crystal /ml	0.5×10^9, 4.4×10^9	-
Lufenurón	ml/L	0.008	1.00 ml/L
Tebufenozide	ml/L	0.006, 0.032	0.75 ml/L

2.4.3 Statistical analysis

The dose-mortality regression line was determined for the larval mortality data in the activity bioassays using Probit analysis. This analysis was based on the maximum likelihood method (Finney, 1971). The goodness of fit was determined using a χ^2 test at 5%. The median Lethal Concentration (LC_{50}) was calculated with fiducial limits at 95% The analysis was performed using the POLO Program (LeOra Software Inc. Berkeley, CA, USA).

The additive, synergistic, or antagonistic effect was determined using a binomial test that compared the expected and observed mortality percentages as described by Harper (1986). If two mortality agents act on the same phytophagous population, and each of these acts independently and all individuals have the same susceptibility, the overall mortality follows the probabilistic formula:

$$Pe= p1 + p2 - p1p2$$

Where:

Pe is the expected mortality expressed in percentage of a combination of the 2 insecticidal agents.
p1 is the mortality percentage after treatment with the insecticide alone.
p2 is the mortality percentage after treatment with the other agent alone.
If the total observed mortality is significantly higher than expected under the independence hypothesis, the interaction is synergetic, whereas if the observed mortality is lower than expected, the interaction is antagonistic.

3 RESULTS

3.1 Insecticidal activity

3.1.1 Entomopathogens

The maximum mortality percentage for *B. thuringiensis* was 43.33% at the highest dose, while the other treatments resulted in relatively low mortality rates, ranging from 3.33% to 22.22%. Larvae treated showed more marked feeding inhibition and growth retardation at the higher doses than the control larvae. The mortality period was between 1 and 3 days after treatment (Figure 1).

The mortality percentage caused by *B. bassiana* did not increase with increased dose. The maximum mortality percentage was 30.00% at a dose of 3.9 x 10^8 conidia/ml. The mortality values did not exceed 30% of the larvae treated. The mortality period was between 4 and 11 days after treatment (Figure 1).

Mortality percentage caused by SlMPV shows a direct relationship between mortality and concentration, with 100% being achieved with the highest dose. The mortality period was between 5 and 11 days after treatmen (Figure 1). Probit regression analysis revealed the following equation: y=1.23x+2.48 (x^2 = 0.2731; 1 df) and a LC$_{50}$ = 6.6 x 10^5 OB/ml with 95% confidence limits of 0.97 x 10^5 and 1.4x10^6 OB/ml

Figure 1. Cumulative mortality on 3rd instar larvae of *Spodoptera littoralis* treated with entomopathogens at different doses

Doses (s+c/ml)	N	Mortality (%)
T 0	30	0.00
A 8.8 x 10^5	29	13.79
B 8.8 x 10^6	30	3.33
C 8.8 x 10^7	27	22.22
D 8.8 x 10^8	30	20.00
E 8.8 x 10^9	30	43.33

Doses (c/ml)	N	Mortality (%)
T 0	30	0.00
A 6.1 x 10^6	30	3.33
B 2.4 x 10^7	30	23.33
C 9.7 x 10^7	30	20.00
D 3.9 x 10^8	30	30.00
E 1.6 x 10^9	29	17.24

Doses (OB/ml)	N	Mortality (%)
T 0	30	0.00
A 5.2 x 10^5	30	43.33
B 2.6 x 10^6	30	80.00
C 1.3 x 10^7	30	93.33
D 6.5 x 10^7	29	96.55
E 3.2 x 10^8	30	100.00
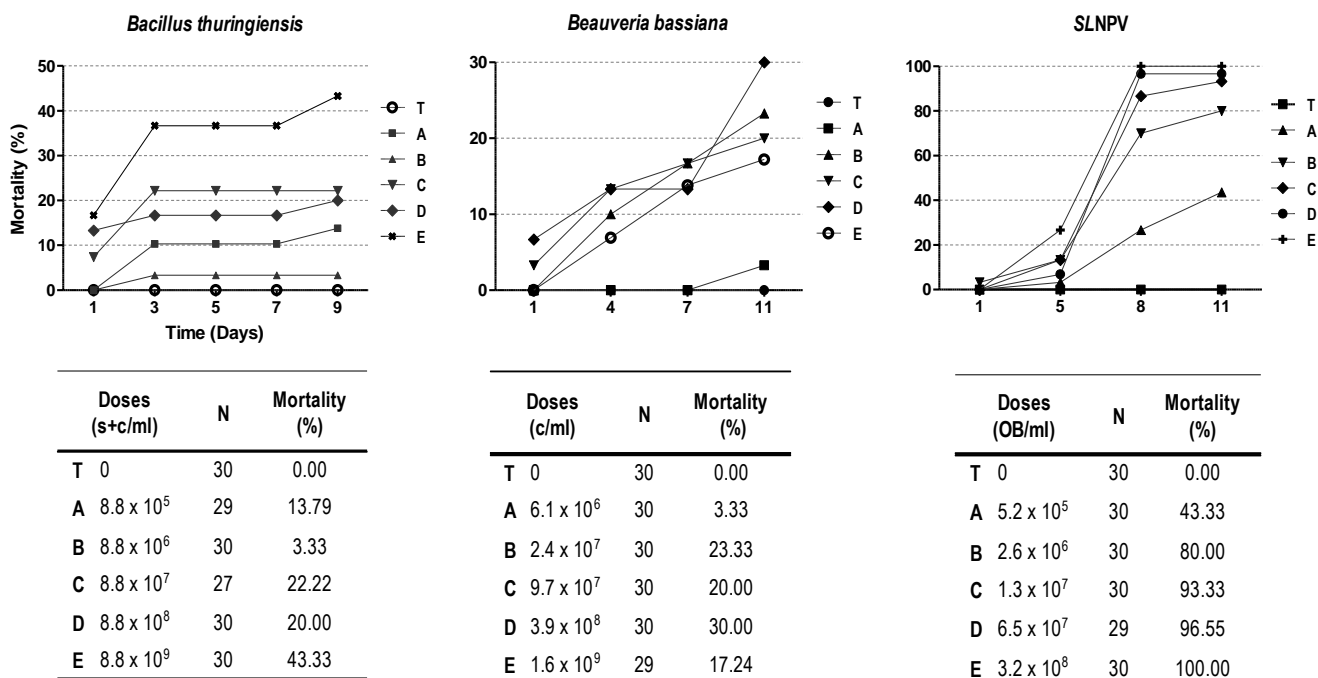
Mortality: % cumulated mortality
N = Number of larvae treated.
The *Spodoptera littoralis* nucleopolyhedrovirus (SINPV) doses are expressed as occlusion bodies per millilitre (OB/ml); *Beauveria bassiana* expressed as conidia/ml and *Bacillus thuringiensis* subsp. *aizawai* (*B.t.*) expressed as spore+crystal/ml.

3.1.2 Insect growth regulators IGRs

The L3 larvae of *S. littoralis* were shown to be highly susceptible to ingested lufenuron; at low doses it caused a mortality rate of 44.9%, and there was a direct relationship between mortality and concentration. Mortality began on day one, with maximum values on day three. Probit regression analysis revealed the following equation: $y=0.77x+5.55$ ($\chi^2=0.0002$; 1 df) and LC$_{50}$=0.0052 ml/L with 95% confidence limits of 0.0009 and 0.0096 m/L.

The mortality percentage caused by tebufenozide increased with increased concentrations. Most of *the S. littoralis* larvae died at the instar in which they were treated. Probit regression analysis revealed the following equation: y=2.07x+1.35, $\chi 2$=0.08955 (1 df) and LC_{50}= 0.058 ml/L with 95% confidence limits of 0.043 and 0.078 ml/L.

3.2 Interactions

The simultaneous application of *B. thuringiensis* with lufenuron caused a mortality rate of 44.97%, higher than the values obtained for the individual treatments (34.97% and 8.30%, respectively). The combination of *B. thuringiensis* and tebufenozide resulted in 50% larval mortality, again higher than the values seen for each product separately (38.28% and 36.62%, respectively) (Table 2). In both cases, applying *B. thuringiensis* together with each of the IGRs produced an additive effect (Table 5).

Tabla 3. Mortality of 3rd instar larvae of *Spodoptera littoralis* treated with *Bacillus thuringiensis* subsp. *aizawai* and IGRs

Treatment	Concentration	N	Mortality	
			(%)	Corrected[1] (%)
Control	0	60	3.33	-
B.t.	4.4×10^9	60	38.33	34.97
Lufenuron	0.008	60	11.67	8.30
B.t. + Lufenuron	4.4×10^9 + 0.008	60	48.33	44.97
Control	0	60	5.00	-
B.t.	0.5×10^9	60	43.33	38.28
Tebufenozide	0.006	60	41.67	36.62
B.t. + Tebufenozide	0.5×10^9 + 0.006	60	55.00	49.95

N = Number of larvae treated.
[1]Mortality corrected using Abbott's formula.
B.t. = *Bacillus thuringiensis* subsp. *aizawai. C*oncentration expressed as spore+crystal/ml.
Lufenuron and tebufenozide concentrations expressed as ml/L

The simultaneous application of *B. bassiana* and lufenuron caused a mortality rate of 44.83%, higher than that of each of the products used separately (13.56% and

30.51%, respectively) (Table 3). For *B. bassiana* and tebufenozide, the mortality rate was 75%, again higher than the individual treatments (66.67% and 34%, respectively). In both cases, applying *B. bassiana* together with each of the IGRs produced an additive effect (Table 5).

Tabla 4. Mortality of 3rd instar larvae of *Spodoptera littoralis* treated with *Beauveria bassiana* insecticides and IGRs

Treatment	Concentration	N	Mortality (%)	Corrected[1] (%)
Control	0	60	0.00	-
B.b.	1.9×10^8	59	13.56	-
Lufenuron	0.008	59	30.51	-
B. b. + Lufenuron	$1.9 \times 10^8 + 0.008$	58	44.83	-
Control	0	60	3.33	-
B.b.	1.2×10^8	55	11.67	8.34
Tebufenozide	0.032	60	70.00	66.67
B. b. + Tebufenozide	$1.2 \times 10^8 + 0.032$	59	78.33	75.00

N = Number of larvae treated.
[1]Mortality corrected using Abbott's formula.
B.b. = *Beauveria bassiana* isolate*. C*oncentration expressed as conidia/ml.
Lufenuron and tebufenozide concentrations expressed as ml/L

The mortality rates for the simultaneous treatments involving the two concentrations of SlNPV (5.2×10^5 and 2.6×10^6 OB/ml) and lufenuron were 71.53% and 93.20%, respectively (Table 4), significantly higher mortalities than those for each of the independently applied agents (26.53% and 57.36%, respectively). The statistical analysis detected that combining SlNPV treatments, at both baculovirus doses, with lufenuron produced a synergist effect (Table 5). In contrast, the same doses of baculovirus combined with tebufenozide resulted in larval mortality values close to 50% (Table 4), values that are lower than the expected mortality rates (73.81% and 75.56%, respectively). The joint action of these two agents, in both combinations produced an antagonistic effect (Table 5).

Tabla 5. Mortality of 3rd instar larvae of *Spodoptera littoralis* treated with Nucleopolyhedrovirus (SINPV) and IGRs

Treatment	Concentration	N	Mortality (%)	Corrected[1] (%)
Control	0	59	5.08	-
SINPV-1	5.2×10^5	60	31.67	26.53
SINPV-2	2.6×10^6	56	62.50	57.36
Lufenuron	0.008	60	35.00	29.87
SINPV-1 + Lufenuron	5.2×10^5 + 0.008	60	76.67	71.53
SINPV-2 + Lufenuron	2.6×10^6 + 0.008	60	98.33	93.20
Control	0	60	3.33	-
SINPV-1	5.2×10^5	60	25.00	21.67
SINPV-2	2.6×10^6	60	30.00	26.67
Tebufenozide	0.032	60	70.00	66.67
SINPV-1 + Tebufenozide	5.2×10^5 + 0.032	60	50.00	46.67
SINPV-2 + Tebufenozide	2.6×10^6 + 0.032	60	55.00	51.67

N = Number of larvae treated.
[1]Mortality corrected using Abbott's formula.
SINPV concentration expressed as OB/ml. Lufenuron and tebufenozide concentrations expressed as ml/L

Tabla 6. Type of interaction between entomopathogens and IGRs (lufenuron and tebufenozide) in 3rd instar larvae of *Spodoptera littoralis*.

Treatment	Concentration	Mortality (%) Observed	Expected	χ2 (1gl)	p	Type interaction
B.t. + Lufenuron	4.4×10^9 : 0.008	44.97	40.20	0.31	0.712	Additivity
B.t. + Tebufenozide	0.5×10^9 : 0.006	49.95	60.94	0.64	0.423	Additivity
B.b. + Lufenuron	1.9×10^8 : 0.008	44.83	40.66	0.14	0.708	Additivity
B.b. + Tebufenozide	1.2×10^8 : 0.032	75.00	69.45	0.38	0.683	Additivity
SINPV-1 + Lufenuron	5.2×10^5 : 0.008	71.53	48.90	6.81	0.009	Synergism
SINPV-2 + Lufenuron	2.6×10^6 : 0.008	93.20	69.90	10.91	0.001	Synergism
SINPV-1 + Tebufenozide	5.2×10^5 : 0.032	46.67	73.87	8.89	0.005	Antagonism
SINPV-2 + Tebufenozide	2.6×10^6 : 0.032	51.67	75.56	7.03	0.013	Antagonism

The type of interaction was determined according to the equation described by Harper (1986).

The *Spodoptera littoralis* nucleopolyhedrovirus (SlNPV) concentrations are expressed as occlusion bodies per millilitre (OB/ml); *Beauveria bassiana* (*B.b.*) expressed as conidia/ml and *Bacillus thuringiensis* subsp. *aizawai* (*B.t.*) expressed as spore+crystal/ml.

IGR concentrations expressed as ml/L

Interaction was based on χ^2 test between expected and observed mortalities.

4 DISCUSSION

Our objective was to study the mortality effect of entomopathogens on third-stage larvae *S. littoralis*, both alone and in combination with low doses of IGRs; the interactions between the agents were explored through simultaneous application.

The effect of *B. thuringiensis* subsp. *aizawai* on *S. littoralis* was characterised as not being directly dose-related, even though this subspecies is the most active for the genus *Spodoptera* (Schnepf et al., 1998). Most larvae died within 1-3 days after treatment, corresponding to the action of Cry toxins on the insects' mesenteron (Schnepf et al., 1998). Feeding inhibition, observed at the highest doses, explains why some larvae died later, probably from starvation (7 to 9 days after treatment), and also explains why the mortality rate was not directly related to the dose.

The native isolate of *B. bassiana* was selected for its good insecticidal activity on lepidopterans, as shown in previous works on *Galleria mellonela*, *Cydia splendana* (Romero, 2013), *Catocala nymphagoga,* and *Dryobotodes monochroma* (Ortega, 2012). However, the third stage larvae of *S. littoralis* were not very susceptible to contact treatment with the fungus, as evidenced by the fact that the deaths occurred over a wide time interval (from 1 to 11 days after treatment) and the low percentage of total mortality obtained (at the highest dose this was only 30%). This low susceptibility of *S. littoralis*, compared to other species, could be related to its cuticle characteristics, in addition to other internal factors involving the defensive response of the larvae to infection and low tolerance to the toxins secreted by these fungi during the infectious process (Tanada and Kaya, 1993).

SlNPV had a slow lethal effect on *S. littoralis* larvae (lethal times ranging from 5 to 11 days after ingestion of the baculovirus), which follows the duration of the infectious process of baculovirus (Granados and Williams, 1986) and corresponds

to that previously determined for this species (Vargas-Osuna and Santiago-Alvarez, 1988).

Lethal effects of lufenuron and tebufenozide occurred within the first three days after treatment, related to the time when the larvae moult into the fourth stage. This is consistent with its mechanism of action (IRAC, 2020). The LC_{50} value for tebufenozide on L3 larvae of *S. littoralis* is of 0.058 ml/L, while for lufenuron this is of 0.0052 ml/L. The lufenuron values correspond to those obtained by other authors: 0.00023 ml/L for L2 larvae of *Spodoptera* sp. (Nascimiento et al., 2016); and 0.0006 ml/L for the L2 larvae of *S. littoralis* (Bakr et al., 2014). These authors also indicate that lufenuron exerts an anti-appetitive effect on larvae that ingest it in high doses.

The results from the previous bioassays allowed us to choose the most appropriate doses for the interaction bioassays, in which low doses of lufenuron and tebufenozide were simultaneously combined with each of the microbial insecticide treatments. The study of interactions between insecticides is based on the search for substances that enhance the final effect, as well as reduce the killing time in comparison with either agent alone, allowing reduced applications on crops and decreased doses of synthetic insecticides without reducing the effectiveness of pest control strategies.

B. thuringiensis and IGRs applied together did produce an additive effect, although this combination registered a slight increase in total mortality with respect to each of the products separately. The additive effect can be explained by the anti-feeding impact of *B. thuringiensis* (Schnepf et al., 1998): this may have decreased larval feeding and, as a result, less quantity of IGRs was ingested. This corresponds with the results of field trials on larval populations of the noctuids *Helicoverpa armigera* (Karim et al., 1999) and *Anticarsia gemmatalis* (Schuster and Rohde, 2012), where the expected increase in mortality and damage reduction when using a mixture of the two insecticides was not obtained. Nouri-Ganbalani et al. (2016) showed that L3

larvae of *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) fed a diet containing *B. thuringiensis* with azadirachtin suffered an antifeedant effect, significantly reducing the activity of digestive enzymes, food consumption, larval weight gain, and nutritional efficiency. In this situation, one possibility would be to apply the bacteria some hours after the chemical insecticide, as studied by Amizadeh et al. (2015), who demonstrated that simultaneous applications of *B. thuringiensis* and low doses of chemical insecticides on 2nd instar lepidopteran larvae produced an antagonistic effect, whereas when the bacteria was applied 12 hours after the larvae had been dosed with dichlorvos and abamectin, the effect was synergistic. Even so, it is necessary to evaluate the financial cost of the technique when transferring it to field conditions.

In addition, Schuster and Rohde (2012) recommended the use of lufenuron at low doses to control species that have several generations per year, due to the deferred effects (pupal mortality and loss of adult reproductive potential) that this chemical insecticide has on individuals that ingest lethal doses, and which has a knock-on effect on the overall population in following generations. Similar outcomes have also been reported for *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), for a mixture of methoxyfenozide (ecdysone receptor agonist) and lufenuron (Chen et al., 2019).

The possible integration of chemical agents with the fungus *B. bassiana* has been previously studied with the aim of maximising its insecticidal potential and reducing the environmental impact that chemical control may cause (Purwar et al., 2006; Bitsadze et al., 2013). A few studies have indicated that chemical insecticides can inhibit the growth of *B. bassiana* (Foster et al., 1996; Oliveira et al., 2003). Combinations of the fungus with various insecticides, including lufenuron, methoxyfenozide carbaryl, fenvalerate, abamectin, and triflumuron, as well as *B. thuringiensis* have also been tested and found to be highly compatible (Vasquez et al., 2004; Wraight et al., 2005; Pelizza et al., 2015).

According to our results, combinations of *B. bassiana* with low doses of lufenuron and tebufenozide result in an additive effect, which would allow simultaneous treatments in the field. However, it would be necessary to improve the activity of the fungus, either by applying it to younger larvae or by selecting an isolate that is more active against *S. littoralis.*

Lufenuron has been successfully applied in combination with *B. bassiana* to larvae of the lepidopteran *Spilarctia obliqua* (Purwar and Sachan, 2006), larvae of the dipteran *Bactrocera carambolae* (Hadi et al., 2013), and nymphs of the orthopteran *Ronderosia bergi* (Pelizza et al., 2015); and methoxyfenozide has been applied together with this fungus to *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae). These last authors found that lufenuron had no adverse effects on the *in vitro* germination of *B. bassiana* conidia and, furthermore, they noted a synergistic effect when lufenuron was used at a dose of 0.5 ml/L (half of the recommended field dose), suggesting that the insecticide favours the action of the fungus by increasing larval stress or affecting the immune system (Quintela et al., 2013). The histological observations of Hassan and Charnley (1989) revealed that the *Manduca sexta* (Lepidoptera: Noctuidae) larval integument was weakened by diflubenzuron, so the fungus more readily penetrated through it into the haemocoel. Furlong et al. (2001) found an additive effect between *B. bassiana* and cyromazine (inhibitory ecdysis) on larvae of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). This effect was explained by the fact that during ecdysis it facilitated the shedding of conidia that had not successfully penetrated the endocuticle.

Treatments combining SlNPV with a low dose of lufenuron had a synergistic effect, which shows the suitability of the two agents for a joint application to control *S. littoralis*. The simultaneous and sequential application of nucleopolyhedrovirus with insecticides such as azadirachtin, has demonstrated a synergist effect on the larvae of *Spodoptera* species (Méndez et al., 2002; Nathan and Kalaivani, 2006; Zamora-Aviles et al., 2013; Shaurub et al., 2014), as well as reducing lethal times in the 3rd

instar of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Kumar et al., 2008).

In this study, the synergism of SlNPV and lufenuron may be due to that this insecticide interferes on the biosynthesis of the chitin that is a polysaccharide found in the peritrophic membrane of the insect midgut (Lehane 1997), the first insect defence mechanism against baculovirus (Wang and Granados, 2000), thus facilitating the entry of the infective NPV particles into the midgut cells. However, the exact mechanism through which this synergism occurs needs to be investigated further.

Some authors (Wilkinson, 1976; Hernandez and Lacasaña, 2017) have mentioned that an antagonistic effect is to be expected in substances that exert their activity on the same mechanism of action. In our study, the mixture of SlNPV and tebufenozide produced an antagonistic effect, probably due to the interference of the two agents in the haemolymph of the larvae. The baculoviruses block insect moult by producing ecdysteroid UDP-glucosyl transferase (O'Reilly and Mille, 1989) which could inhibit the mechanism of action of the ecdysone agonist.

The results of this study are very significant as they demonstrate a high level of compatibility between most of the pest control agents evaluated. The entomopathogenic agents generally act too slowly. This characteristic makes their combination with the faster-acting IGRs, and the greater persistence on leaves, an attractive approach. By acting on different physiological mechanisms of the insect, they have a huge potential to reduce selection pressure and resistance development. In addition to the lethal effects on larvae, sublethal effects on pest populations should also be considered (Vargas-Osuna, 2001).

It is concluded that simultaneously applying low concentrations of IGRs together with entomopathogens, can be included as part of integrated pest management

programmes, with the notable exception of the antagonistic interaction between tebufenozide and SINPV. A product based on lufenuron and SINPV baculovirus is presented as a candidate for commercial development.

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CAPITULO V

Discusión general

1. DISCUSIÓN GENERAL

Las nuevas clases de plaguicidas selectivos que reducen los riesgos para los organismos no diana están sustituyendo cada vez más a los plaguicidas químicos tradicionales de amplio espectro. Sin embargo, es importante entender cuál es la mejor manera de integrarlos con los agentes de control biológico para lograr una gestión sostenible, tal y como promueve la actual normativa europea.

El efecto del grupo de insecticidas de los Reguladores del Crecimiento de los Insectos (RCI) sobre los organismos no diana suele ser leve en comparación con otros insecticidas químicos, según la materia activa y la especie de los insectos (Mandour, 2008; Hussain et al., 2012; Garzon et al., 2015; Ijaz et al., 2017; Maia et al., 2016). Al estar expuestos los organismos beneficiosos a las aplicaciones de plaguicidas a través de múltiples vías, la primera parte de esta investigación evaluó los efectos directos e indirectos de dos RCI, lufenurón y tebufenocida, sobre los estados inmaduros y de desarrollo del depredador *Chrysoperla carnea* a través de la exposición tópica o mediante la alimentación de presas tratadas, así como sobre los adultos tratados por ingestión. En la segunda parte se estudió la interacción de insecticidas microbianos con bajas dosis de los RCI, en aplicaciones individuales y simultáneas sobre larvas del fitófago para conocer su grado de compatibilidad en el control de *Spodoptera littoralis*.

1.1 Efectos de los RCI sobre los huevos de *C. carnea*

Los efectos de los insecticidas sobre los huevos y la supervivencia de las larvas recién nacidas varían según la especie de insecto y la sustancia activa estudiada. La fase de huevo suele ser el estadio más tolerante a la acción de los plaguicidas. En este estudio, la viabilidad de los huevos de *C. carnea* no se vio afectada por las pulverizaciones en laboratorio de lufenurón ni de tebufenocida. Esto puede explicarse como consecuencia de la protección ofrecida al embrión por el corion,

que está compuesto por proteínas esclerotizadas impermeables que limitan la entrada de los insecticidas (Nation, 2002). La tolerancia de los huevos a algunos insecticidas ya se ha observado en crisópidos (Pasini et al., 2018). En algunos estudios sobre huevos de *Chrysoperla externa*, éstos fueron más susceptibles a los insecticidas convencionales (endosulfán y cipermetrina) que a los pesticidas selectivos (Federico et al., 2008; Rimoldi et al., 2008).

Los RCI no afectaron a la supervivencia de las larvas de *C. carnea* que emergieron de los huevos tratados. Esto es similar a los resultados descritos previamente para *C. externa* (Godoy et al., 2004). Por tanto, se puede deducir la nula toxicidad a embriones y a las larvas recién nacidas de *C. carnea* provenientes de huevos tratados. Otros autores han informado de reducciones en la supervivencia de las larvas nacidas de huevos de *C. externa* tratados tópicamente con lufenurón (Bueno y Freitas, 2004).

1.2 Efectos de los RCI en mortalidad y tiempo de desarrollo de las larvas de *C. carnea*

El efecto letal de lufenurón sobre las larvas L2 de *C. carnea* es elevado, demostrando estas larvas una alta susceptibilidad a esta molécula (CL_{50} = 0,0153 ml/L). Otros estudios coinciden con nuestros resultados, tanto para *C. carnea* (Hussain et al., 2012; Mohammadi et al., 2014), como para *C. externa* (Bueno y Freitas, 2004; Godoy et al., 2004; Zotti et al., 2013) y *Ceraeochrysa cincta* (Schneider) (Rugno et al., 2016). Dado que la dosis recomendada para las aplicaciones de campo de lufenurón es de 1 ml/L, las pulverizaciones foliares deben tener un grave efecto negativo sobre las poblaciones larvarias de este depredador generalista. Además de este efecto directo, los resultados también mostraron que las larvas supervivientes tienen tiempos de desarrollo más largos; con un posible impacto negativo ya que estarían más tiempo expuestas a sus enemigos naturales o a factores ambientales adversos.

Por el contrario, las larvas de segundo estadio de *C. carnea* fueron ligeramente susceptibles al tratamiento tópico con tebufenocida. Medina et al. (2003a) obtuvieron resultados similares cuando, a la concentración máxima recomendada en el campo, tebufenocida fue inofensivo para las larvas de *C. carnea*, lo que parece ser consecuencia de las bajas tasas de absorción y penetración en el tegumento del insecto (por ejemplo, <45%, en 24 h) (Medina et al., 2003a).

El desarrollo posterior de las larvas supervivientes puede verse modificado debido al mecanismo de acción del insecticida (Smagghe y Degheele 1994; Trisyono y Chippendale, 1997). En nuetro caso, el tiempo de desarrollo larvario y el periodo de pupación fueron más cortos en las larvas L2 de *C. carnea* tratadas con tebufenocida con respecto al control. Sin embargo, en otros estudios no se han observado efectos adversos en el desarrollo del también crisópido *Ceraeochrysa cincta* tras la exposición tópica a tebufenocida (Rugno et al., 2016).

1.3 Efectos en las larvas de *C. carnea* del consumo de presas tratadas con RCI

Los depredadores también están expuestos a los insecticidas indirectamente a través de sus presas (Müller, 2018). Los efectos del insecticida sobre los depredadores que se alimentan de presas tratadas pueden tener un impacto en el desarrollo de estos agentes de control biológico. Así, hay evidencia de la mortalidad por plaguicidas y de otros efectos negativos en el comportamiento de poblaciones de enemigos naturales, causados principalmente por el consumo de presas contaminadas con neonicotinoides. Esto incluye a depredadores como el coccinélido, *Serangium japonicum* (Chapin), los ácaros *Neoseiulus californicus* (McGregor) (Poletti et al., 2007) y *Phytoseiulus macropilis* (Banks) (He et al., 2012) y al hemíptero *Orius insidiosus* (Say) (Camargo et al., 2017)

En el caso de lufenurón, aproximadamente el 23% de las larvas de *C. carnea* murieron antes de la pupación (a la dosis de 0,1 ml/L), y casi todas las larvas que formaron el capullo para pupar murieron (83%) sin emerger como adultos. Se detectaron adultos malformados entre los pocos que emergieron. Con la dosis recomendada para su uso en el campo (1 ml/L), la mortalidad durante la fase de pupa fue del 100% de las larvas que alcanzaron este estado. La mortalidad se puede explicar por el modo de acción de este inhibidor de la síntesis de quitina (Oberlander y Silhacek, 1998; Sun et al., 2015) teniendo un efecto letal en la muda siguiente al tratamiento. Las larvas de *C. carnea* tienen una baja tasa de excreción para este grupo de insecticidas (Medina et al., 2002), lo que también podría haber contribuido.

Los efectos subletales provocados por la alimentación de las larvas de *C. carnea* con presas tratadas previamente con tebufenocida consistieron en una reducción significativa del periodo de desarrollo larvario, que puede contribuir a disminuir la tasa de depredación, aunque su efecto no es letal.

1.4 Preferencia de larvas de *C. carnea* entre presas tratadas y no tratadas

En los bioensayos de elección, las larvas de *C. carnea* prefirieron las presas de *S. littoralis* tratadas previamente con RCI en comparación con las no tratadas. En el caso del lufenurón, la preferencia fue muy alta (76,5% de las presas consumidas habían sido tratadas). Este efecto puede deberse a que las presas tratadas pueden tener pérdida de movilidad siendo más fáciles de ser depredadas que las presas no tratadas. También es posible que este comportamiento esté relacionado con alteraciones fisiológicas de las presas tratadas que modifican sus reacciones y comportamientos de defensa (Symondson et al., 2002). Como es conocido, las larvas de *C. carnea* reaccionan a las señales químicas producidas por sus presas (Pickett y Glinwood, 2007) por lo que también es posible que el tratamiento puede alterar estos semioquímicos. Navarro-Roldán y Gemeno (2017) informaron que la

aplicación tópica de un insecticida puede alterar la superficie cuticular e inducir cambios en las secreciones que actúan como defensa ante depredadores generalistas (Müller et al., 2019). Se requieren más estudios para dilucidar los mecanismos implicados en la preferencia detectada.

Otros estudios han mostrado este tipo de preferencia, como en el caso de presas de *Xanthogaleruca luteola* (Müller) previamente infectada con el hongo entomopatógeno *Beauveria bassiana* (Balsamo) (Mena Castillo, 2019). Y en otros estudios, las hormigas *Myrmica rubraworker* respondieron más frecuentemente a las presas expuestas a insecticidas que a las no expuestas (Müller et al., 2019).

La alta preferencia por presas tratadas con lufenurón provoca también un importante efecto letal sobre las larvas de *C. carnea*, ya que el consumo de presas tratadas produce la mortalidad de las larvas antes de pupar. Todo ello se suma a los efectos directos de la aplicación del lufenurón sobre este depredador, lo que tendría graves impactos en sus poblaciones.

1.5 Efectos de RCI sobre adultos de *C. carnea*

Varios estudios sugieren que los RCI afectan al potencial reproductor de los insectos adultos, aunque estos efectos difieren entre las especies, método de aplicación y sustancia activa utilizada. Los RCI pueden alterar la estructura histológica de las gónadas de los insectos (El-Bokl et al., 2010) o sus efectos pueden estar asociados con alteraciones en la oogénesis o espermatogénesis (Agüero et al., 2015).

Aunque la molécula de lufenurón está actualmente registrada como insecticida RCI en muchos países del mundo, se sabe que tiene efectos adversos sobre la capacidad reproductiva de dípteros, coleópteros, hemípteros y lepidópteros (Costa et al., 2017; Gangishetti et al., 2009; He et al., 2018; Mansur et al., 2010; Storch et al., 2007). Nuestros resultados con lufenurón indican que la fecundidad de las

hembras y la longevidad de los adultos de *C. carnea* no se ven afectadas por la ingestión de lufenurón, pero se produce una reducción significativa de la viabilidad de huevos. En un estudio ultraestructural de los efectos de esta benzoilurea en la embriogénesis de *Drosophila melanogaster* (Meigen), Gangishetti et al. (2009) demostraron que la eclosión de los huevos se inhibía por completo cuando las hembras adultas eran tratadas con dosis altas de lufenurón (vía ingestión) tras el apareamiento con machos no tratados; los embriones completaban el desarrollo, pero no lograban romper la membrana vitelina. Costa et al. (2017) detectaron que el lufenurón indujo cambios patológicos en la estructura histológica e histoquímica del epitelio del intestino medio y de las gónadas de *Anthonomus grandis* (Boheman), que afectaron a los procesos de espermatogénesis y oogénesis.

En nuestro estudio con tebufenocida, no se encontró toxicidad en adultos de *C. carnea* tratados por ingestión; además, la fecundidad de hembras y la viabilidad de los huevos tampoco se vieron alteradas. Estos resultados complementan los de Medina et al. (2003b), quienes indicaron que los adultos de *C. carnea* tratados tópicamente con la dosis recomendada en campo de tebufenocida no causaron mortalidad ni efectos adversos en fecundidad y viabilidad de huevos, probablemente por la baja tasa de absorción y penetración a través del tegumento del adulto (Medina et al., 2002).

1.6 Compatibilidad de RCI con insecticidas microbianos

Otro de los objetivos de este trabajo fue estudiar la mortalidad de larvas de *S. littoralis* cuando son sometidas a tratamientos simultáneos de un entomopatógeno con dosis bajas de RCI, en comparación con los tratamientos individuales de cada uno de los agentes.

Los efectos letales de la aplicación tópica de lufenurón y de tebufenocida se produjeron en los tres primeros días después del tratamiento, generalmente coincidiendo con la siguiente muda, lo que es coherente con su mecanismo de acción (IRAC, 2020). El valor de la CL_{50} para el lufenurón en las larvas L3 de *S. littoralis* fue de 0,0052 ml/L, mucho más bajo que para tebufenocida que fue de 0,058 ml/L.

El efecto letal de *B. thuringiensis* subsp. *aizawai* sobre *S. littoralis* se caracterizó por no estar directamente relacionado con la dosis, a pesar de que esta subespecie se considera que es la más activa para el género *Spodoptera* (Schnepf et al., 1998). La inhibición de la alimentación, observada en las dosis más altas, explica que algunas larvas murieran más tarde, probablemente por inanición, y también explicaría que la mortalidad no estuviera directamente relacionada con la dosis.

La aplicación de *B. thuringiensis* conjuntamente con cada uno de los RCI tuvo un efecto aditivo, aunque se registró un ligero aumento de la mortalidad total con respecto a cada uno de los productos por separado. Debido a la inhibición de la alimentación causada por *B. thuringiensis* (Schnepf et al., 1998) no cabría esperar un efecto sinérgico ya que disminuye la cantidad ingerida de cada uno de los insecticidas. Esto se corresponde con los resultados de los ensayos de campo en poblaciones de larvas de los noctuidos *Helicoverpa armigera* (Karim et al., 1999) y *Anticarsia gemmatalis* (Schuster y Rohde, 2012), en los que no se obtuvo el aumento esperado de la mortalidad y la reducción de daños al utilizar una mezcla de *Bt* y lufenurón. Para encontrar un posible efecto sinérgico se podría aplicar la bacteria unas horas después del insecticida químico, tal y como estudiaron Amizadeh et al. (2015), quienes demostraron que las aplicaciones simultáneas de *B. thuringiensis* y dosis bajas de insecticidas químicos sobre larvas de lepidópteros de segundo estadio producían un efecto antagonista, mientras que cuando la bacteria se aplicaba 12 horas después de que las larvas hubieran sido tratadas con diclorvos y abamectina, el efecto era sinérgico.

El aislado nativo de *B. bassiana* mostró baja mortalidad en *S. littoralis*, lo que puede deberse a características de la cutícula de este noctuído, o bien a factores internos relacionados con la respuesta defensiva en la hemolinfa y baja tolerancia a las toxinas secretadas por estos hongos durante el proceso infeccioso (Tanada y Kaya, 1993).

En nuestro estudio, las combinaciones de *B. bassiana* con dosis bajas de lufenurón y tebufenocida tuvieron un efecto aditivo; sin embargo, sería necesario mejorar la actividad del inóculo, ya sea aplicándolo a larvas más jóvenes o seleccionando un aislado más activo sobre *S. littoralis*.

La integración de agentes químicos con el hongo *B. bassiana* se ha estudiado previamente (Furlong et al., 2001; Purwar et al., 2006; Bitsadze et al., 2013) y se ha encontrado buena compatibilidad con insecticidas de muchos grupos, incluyendo los RCI (Vasquez et al., 2004; Pelizza et al., 2015). El lufenurón se ha aplicado con éxito en combinación con *B. bassiana* a larvas del lepidóptero *Spilarctia obliqua* (Walker) (Purwar y Sachan, 2006). Pelizza et al. (2015) encontraron que el lufenurón no tenía efectos adversos sobre la germinación in vitro de los conidios de *B. bassiana* y, además, observaron un efecto sinérgico cuando el lufenurón se utilizó a una dosis de 0,5 ml/L (la mitad de la dosis recomendada en campo), lo que sugiere que el insecticida favorece la acción del hongo al aumentar el estrés larvario o al alterar el sistema inmunitario (Quintela et al., 2013). Por otro lado, Schuster y Rohde (2012), recomendaron el uso de lufenurón a dosis bajas para el control de especies que tienen varias generaciones al año, debido a los efectos diferidos (mortalidad de pupas y pérdida de potencial reproductivo de los adultos) que este insecticida químico tiene sobre los individuos que ingieren dosis letales, y que tiene un efecto en cadena sobre el conjunto de la población en las siguientes generaciones.

Los tratamientos en los que se combinó NPVSl con una dosis baja de lufenurón tuvieron un efecto sinérgico, lo que demuestra la idoneidad de ambos agentes en

aplicaciones conjuntas para el control de *S. littoralis*. La aplicación, tanto simultánea como secuencial de nucleopoliedrovirus con otros insecticidas como la azadiractina, ha demostrado también un efecto sinérgico sobre las larvas de especies de *Spodoptera* (Méndez et al., 2002; Nathan y Kalaivani, 2006; Zamora-Aviles et al., 2013; Shaurub et al., 2014), así como la reducción de los tiempos letales en larvas de tercer estadio de *H. armigera* (Kumar et al., 2008).

El sinergismo entre NPVSl y lufenurón puede deberse a que el RCI al interferir en la biosíntesis de la quitina, puede afectar a la membrana peritrófica del intestino medio del insecto que contiene quitina (Lehane 1997). La membrana peritrófica es la primera barrera defensiva del insecto frente a baculovirus (Wang y Granados, 2000) y su alteración facilitaría la entrada de los viriones del NPV en las células del intestino medio. Sin embargo, el mecanismo concreto a través del cual se produce este sinergismo necesita ser investigado más a fondo.

Como señalan algunos autores (Wilkinson, 1976; Hernández y Lacasaña, 2017), es de esperar un efecto antagonista principalmente entre sustancias que tienen el mismo mecanismo de acción. En nuestro estudio, encontramos antagonismo entre NPVSl y tebufenocida. Una hipótesis que puediera explicar este resutado es una supuesta interacción entre el tebufenocida y una enzima (UDP-glucosyl transferasa), producida por los baculovirus durante su infección que interfiere con la ecdisona y retrasa la muda del insecto (O'Reilly y Mille, 1989).

Los resultados de este estudio son muy significativos, ya que demuestran un alto nivel de compatibilidad entre la mayoría de los agentes de control evaluados. Los agentes entomopatógenos actúan en general muy lentamente y esta característica hace que su combinación con los RCI, de acción más rápida, y su mayor persistencia en las hojas, sea un enfoque atractivo. Además, al actuar sobre diferentes mecanismos fisiológicos del insecto, su aplicación conjunta tiene un

enorme potencial para reducir la presión de selección y evitar el desarrollo de resistencia.

2. CONCLUSIONES

1. Los reguladores del crecimiento de insectos lufenurón y tebufenocida no tienen efecto ovicida ni causan mortalidad de las larvas recién nacidas en tratamientos de huevos del depredador *Chrysoperla carnea*.

2. La aplicación tópica de lufenurón sobre larvas de segundo estadio de *Chrysoperla carnea* causa altas mortalidades, con una concentración letal media de 0,0153 ml/L. Por el contrario, tebufenocida tiene leve efecto letal a la dosis recomendada para tratamientos en campo, si bien en las larvas supervivientes se reduce el tiempo de desarrollo larvario y el periodo de pupación.

3. Las larvas de tercer estadio de *Chrysoperla carnea* que se alimentan de presas tratadas con lufenurón sufren elevadas tasas de mortalidad pupal inhibiendo casi totalmente la formación de adultos. Sin embargo, la alimentación de presas tratadas con tebufenocida sólo causó disminución del tiempo de desarrollo larval, sin alterar los parámetros reproductivos de los adultos.

4. Las larvas de tercer estadio de *Chrysoperla carnea* prefieren consumir presas tratadas con lufenurón o con tebufenocida frente a las no tratadas. Este comportamiento es aún más evidente con lufenurón y tiene mayor transcendencia por su efecto adverso sobre la supervivencia del depredador. Son necesarios nuevos estudios para determinar la naturaleza de esta preferencia.

5. Los adultos de *Chrysoperla carnea* tratados por ingestión con lufenurón muestran reducción del porcentaje de viabilidad de huevos, lo que repercutirá negativamente en los niveles poblacionales del crisópido. Por el contrario, la

ingestión de tebufenocida no tiene efecto en los parámetros reproductivos ni en la longevidad de los adultos.

6. Según nuestros resultados, las aplicaciones en campo con lufenurón no deben realizarse con la presencia de poblaciones naturales o con sueltas de *Chrysoperla carnea,* sobre todo en los cultivos en donde el depredador sea un importante agente de control.

7. Tebufenocida puede ser incluido de forma compatible en los programas de gestión integrada de plagas en los que se realice control biológico a través de *Chrysoperla carnea*.

8. Los tratamientos simultáneos de entomopatógenos con bajas concentraciones de los RCI (lufenurón y tebufenocida) sobre larvas de *Spodoptera littoralis* muestran efectos aditivos en los casos de *Bacillus thuringiensis* o *Beauveria bassiana*, por lo que estas combinaciones deben ser evaluadas en ensayos de campo para el control de esta especie.

9. Las combinaciones de NPVSI con lufenurón tiene un claro efecto sinérgico que puede ser de gran interés práctico, mientras que cuando el baculovirus se combina con tebufenocida se produce un efecto antagonista.

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